UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

VINÍCIUS TAIOQUI PELÁ

Proteomic analysis of acquired pellicle formed on experimental composite resins containing or not filler and/or protease inhibitors: study *in situ*

Análise proteômica da película adquirida formada sobre resinas compostas experimentais contendo ou não carga e/ou inibidores de proteases: estudo *in situ*

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Dissertation presented to the Bauru School of Dentistry of the University of São Paulo to obtain the degree of Master in Science in the Applied Dental Science Program, Stomatology and Oral Biology concentration area.

Supervisor: Prof. Drª Marília Afonso Rabelo Buzalaf

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Orientadora: Prof. Drª Marília Afonso Rabelo Buzalaf

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FOLHA DE APROVAÇÃO

DEDICATÓRIA

À Deus,

Dedico este trabalho como forma de reconhecimento as inúmeras maravilhas realizadas em minha vida. Através da sua infinita bondade e supremo poder, Ele me permitiu realizar um dos maiores sonhos da vida, o qual, fui capacitado com toda a sua força, bênção e proteção.

"Entrai pelas portas dele com gratidão, e em seus átrios com louvor; louvai-o, e bendizei o seu nome" (Salmos 100:4)

À minha família

À minha mãe Maria Vanderléia Taioqui Pelá, ao meu pai Adailton Pelá e ao meu irmão Adailton Pelá Júnior. Eu agradeço por ter ao meu lado esta incrível família, que sempre está aperfeiçoando as minhas decisões, acolhendo os meus defeitos, demonstrando os afetos familiares e principalmente acreditando no meu futuro.

"A família é a base da sociedade e o lugar onde as pessoas aprendem pela primeira vez os valores que os guiarão durante toda a sua vida"

São João Paulo II

À minha madrinha,

À minha madrinha Aparecida Pellah Monteiro (*in memoriam*), que durante a sua vida me acompanhou, se preocupou, rezou e sonhou com o meu caminho. Hoje, encontra-se na paz, dita como a morada eterna, intercedendo ao lado de Nossa Senhora pela minha felicidade.

"Para o cristão, a morte não é a derrota e sim a vitória"

Santo Alberto Hurtado

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À Deus,

Deus proporcionou várias conquistas em minha vida, dentre elas, profissional, pessoal e espiritual. Foi através da fé, que Ele esteve ao meu lado nos momentos em que eu mais precisei, me oferecendo suporte, discernimento e paz ao meu coração. Dizer “Obrigado meu Deus” não seria suficiente para expressar o quão grato eu sou, já que não existem palavras dignas do tamanho do teu amor e da tua misericórdia. Nesta busca de gratidão, só me resta retribuir de uma forma singela, mais especificamente, abrindo as portas do meu coração e te dando livre acesso para continuar reinando em minha vida.

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Aos meus pais: Adailton e Vanderléia,

Há quase 25 anos, eu recebi o mais preciso presente que existe no Universo: A vida. Para ela, claro, foi fundamental a presença de Deus, do Sol e do amor de duas pessoas iluminadas para permitir a minha entrada ao Mundo. Estas pessoas são chamadas de Pais, mais estritamente, Pai e Mãe, a quem devo todo o meu reconhecimento de amor. Também, a minha gratidão é eterna, por eu ter me tornado um ser humano de caráter, com respeito ao próximo, digno de todas as minhas responsabilidades e, acima de tudo, com a mesma fé que ambos almejam.

De uma forma concomitante, agora justifico-me o porquê de citar o Sol nesta singela homenagem aos meus pais. É pelo fato do Sol ser considerado a estrela central do sistema, assim como os meus pais, que são o centro da minha vida. Através do Sol, é possível gerar vidas e ainda é capaz de influenciar a construção humana. De todas as pessoas que estão neste agradecimento, os meus pais foram os únicos que estiveram ao meu lado por todos estes anos, assim como o Sol. Diante disso, precisaria de infinitas folhas para citar cada bem que eles me proporcionaram, mas neste momento, quero apenas fazer uma retrospectiva do quanto eles contribuíram diretamente para minha vida profissional.

Foi em 2010, quando a distância dos meus pais veio como consequência da minha graduação. Com isso, acabou nos separando, mas os nossos corações foram sempre tão grandes como o Sol, que mesmo longe, sempre foi possível receber luz, calor e amor. Ainda neste ano, ficou marcado pelos meus medos e inseguranças de iniciar uma nova fase. Porém, percebi diante do Sol, que fases são essenciais para gerar mudanças e desenvolver um futuro promissor. Já os meus pais, transbordaram felicidade com a minha escolha, bem como derramaram suores e não mediram esforços para iniciar aquilo que eu mais queria. A Biologia já havia nascido no meu coração e estava fortemente brilhando como o Sol. Foram cinco anos com a presença deles e durante este período, foi intimamente marcado com preocupação, oração e a humildade; muito espelhada ao Sol, que apesar de sua grandeza se põe e deixa a Lua brilhar.

Meus pais lutaram, foram centenas de idas e vindas à Bauru, além de toda ajuda financeira que eu recebi. Dizer que este caminho foi fácil e totalmente retilíneo seria hipocrisia da minha parte, mas foi por conta de cada lado do meu coração (Pai e Mãe), que fez diminuir as dores e o desânimo que em alguns momentos batia na porta. Foi neste período de escuridão, onde o Sol não aparecia, que eu me tornei mais corajoso com os conselhos dos meus pais. Eles me ensinaram que na vida, era certo que o sol voltaria a brilhar, trazendo luz para a temível escuridão para posteriormente, iluminar os caminhos de um belo futuro que estava próximo. Os anos se passaram e para eles eu me tornei Biólogo. Mais uma vez, com todo apoio materno e paterno, eu ingressei ao mais profundo sonho do Mestrado. Isso me fez refletir que o amor familiar é sempre intacto, sem nenhum retrocesso. Ainda, o mesmo tem a capacidade de se

tornar mais nobre, puro e maior. Assim como o sol, que a bilhões de anos tem a mesma essência, mas dizem estar cada vez mais quente.

Hoje, eu finalizo mais uma fase e agradeço do fundo do meu coração, com todas as minhas forças, tudo o que vocês fizeram e continuam fazendo por mim. Obrigado pelos sonhos que construímos juntos e principalmente por transformar estes sonhos em realidades. Serei fiel a vocês, cuidarei e me preocuparei até quando o sol se apagar e não houver mais o brilho e o calor. Que nossa relação possa ser iluminada por Deus com os raios do Sol. E, que nós possamos sempre aprender com Ele, a dar voltas para superar obstáculos, sem perder a esperança de que o mesmo é capaz de voltar nos momentos sem luzes. Que o nosso calor, dito como o calor familiar, possa sempre nos manter como o sistema solar, numa eterna harmonia. Amo vocês e mais uma vez, obrigado por eu ter “três Sois” na minha vida. Quero para sempre refletir com as mesmas luzes que eu recebi de vocês!

Ao meu irmão Adailton Pelá Júnior e à minha cunhada Maria Thereza,

Com uma diferença de sete anos de idade, tenho ao meu lado um grande irmão chamado Júnior. Durante toda a sua vida demonstrou afeto, carinho e consideração. Sou eternamente grato e feliz por ter crescido ao teu lado, por ter me segurado no colo e me protegido do perigo, bem como, ter dividido o quarto até os meus 12 anos, já que eu tinha medo de dormir sozinho.

A nossa infância tornou-se uma epopeia, marcada de sorrisos, brincadeiras, alegrias e momentos únicos que permanecem guardados no meu coração. Você teve parte em todas as minhas conquistas, assim como os nossos pais. Quero também lhe homenagear, mostrando a minha estável admiração, que há muito tempo se tornou um grande exemplo. Um excelente profissional, que carrega firme os maiores valores da vida, com uma luta incansável de apetecer os seus objetivos.

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Aos meus avós,

Aos meus avós maternos: Maria José Taioqui (*in memoriam*) e Ângelo Taioqui (*in memoriam*). Com eles eu vivi intensamente toda a minha infância e parte da minha adolescência. Foi uma linda trajetória preenchida de carinho, respeito e amor. Para eles, eu fui o querido e travesso “Gui, Guiguizinho ou Guigo” numa eterna bagunça. Tive o privilégio de aproveitar aquilo de mais simples que existe na vida, como por exemplo, uma ida ao sítio logo pela manhã, pulando dentro da saudosa Brasília Verde – 1978. Nesta mesma cena nostálgica, próximo ao local, diante de uma “demorada buzinada” descendo a ladeira, fazia com que o gado se aproximasse para receber os cuidados.

Também, como não esquecer dos cheiros e sabores vindo daquele pãozinho quentinho, daquele doce de leite e da coxinha de batata feita pelas mãos da minha Avó Maria, que infelizmente desde 2009, nunca mais encontrei algo parecido. Isso tudo, tornaram-se momentos únicos e que me compromete ser sempre humilde. A saudade bate forte, mas o que conforta é a esperança de um dia reencontrar em uma outra dimensão. Obrigado Vô e Vó por estar neste momento me acompanhando daí de cima.

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À minha querida amiga Aline Silva Braga,

Eu me lembro como se fosse ontem, quando eu a vi pela primeira vez. O primeiro olhar tímido com um sorriso de meio termo já iniciava um contato. De fato, achei que seria só mais uma pequena conhecida, mas Deus me surpreendeu. Ele achou que não deveria ser só mais uma pessoa, mas uma grande amiga e até uma grande irmã. Então eu percebi, que esta história não teria mais fim e que muitos momentos marcantes estavam por vir. E vieram, bem mais forte do que eu esperava!

Os anos se passaram e hoje eu fecho os meus olhos e faço um download só de boas lembranças. Junto a isso, muitas perguntas chegam em minha mente: Qual seria a outra pessoa que poderia me aguentar por seis dias dentro de um ônibus? Quem poderia me aproximar de Deus e por consequência encontrar a minha paz? A reposta disso tudo, gera um livro que só nós dois sabemos! Lí, eu só tenho a te agradecer pelos ombros onde muitas vezes chorei, pelos braços que foram estendidos quando eu mais precisei e pelos os teus sorrisos que fizeram esquecer todos os meus problemas. Com você eu vivi uma das melhores fases da minha vida. “Nossa relação não é estranho, é muuuuuuitooo estranho”. E agora só temos boas e divertidas

gargalhadas, além de uma necessidade de todos os dias fazer aquela mesma ligação. Pode ter certeza, que se você estiver com o barco eu também vou estar. Este é um segredo só nosso […] O meu muito obrigado é pouco, diante de tudo o que já vivemos!

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"Deus nos surpreende sempre, rompe os nossos esquemas, põe em crise os nossos projetos e nos diz: confia em Mim, não tenhas medo, deixa-te surpreender, sai de ti mesmo e segue-Me!".

Papa Francisco

AGRADECIMENTO ESPECIAL

À minha orientadora Profª Drª Marília Afonso Rabelo Buzalaf,

Hoje encerro mais uma etapa, dita como a mais turbulenta de todas. Diante disso, carrego em meu coração um profundo agradecimento e uma imensa satisfação. Sou feliz e honrado ao ver que durante todo o meu Mestrado fui orientado pela pessoa mais iluminada que possa existir no âmbito da Odontologia. Esta minha afirmação fundamenta-se não apenas em artigos científicos encontrados no PubMed, que por sinal existem centenas com o seu nome, mas os parâmetros estão relacionados com aqueles que a Senhora carrega na alma, sobretudo, no coração. Uma mulher alta, bonita, elegante e com características únicas e tão doce, quanto a tua voz que me remete a um anjo, trazendo paz.

Pude acompanhar neste período, uma das mais belas evoluções. Em 2010, no meu primeiro ano de estágio no Laboratório de Bioquímica, acreditava que “a Marília Buzalaf” jamais iria dar atenção a um aluno de 17 anos. Ainda, minha maior vontade era ter uma conversar pessoalmente, mas a timidez e os medos eram maiores. Confesso que o meu coração disparou, quando recebi pela primeira vez um bom dia da Senhora subindo as escadas da faculdade.

Os anos se passaram, os contatos aumentaram e percebi que com esta aproximação, a Senhora era muito mais especial do que eu imaginava. É notável que as qualidades são infinitas, mas vale a pena destacar algumas: A atenção que a Senhora tem por todos, quando ouvimos aquele simpático “Aham”. Também a humildade que reina na sua sala, de tal forma que sempre permanece com a porta aberta, o que é muito raro nos dias de hoje. Outra qualidade marcante, foi quando a Senhora percebeu que eu não estava bem, logo se preocupou e veio oferecer ajuda, trazendo palavras de conforto e de esperanças que os fatos iriam melhorar.

Agradeço por estar na minha vida desde a graduação, já são 8 anos de convívio profissional e pessoal. Em uma breve retrospectiva, quero destacar e agradecer por eu ter desenvolvido uma Iniciação científica. Também pela tua presença na minha banca de TCC e principalmente por aceitar ser minha orientadora durante o mestrado. Quanto ao futuro, não posso deixar de registrar e já adianto os meus agradecimentos pelo doutorado que está chegando.

Hoje, quero-lhe contar um segredo, o qual me deixa muito emocionado. Na nossa última reunião, enquanto a Senhora terminava de corrigir a minha conclusão, de forma totalmente concentrada na tela do computador, eu já respirava com alívio porque esta longa e árdua jornada estava se encerrado. Sem a Senhora perceber, diante desta emoção, uma lágrima caiu dos meus olhos e molhou a minha folha que estava sobre a mesa. Parece algo simples, poderia ter jogado a folha no lixo, mas hoje ela está guardada em uma pasta, para mostrar e dizer aos meus filhos e netos que aquele “borrão” aconteceu por dois motivos. Primeiro, pela emoção de estar ao lado da pessoa que eu sempre admirei e que sempre foi a minha maior inspiração profissional. Segundo, pelo fato de ter tido uma orientadora, que foi capaz de mudar completamente a vida daquele imaturo garoto, que se apresentou com 17 anos e que hoje se torna Mestre com 25 anos.

Na última semana de março, eu estava deitado, prestes a dormir quando muitas lembranças vieram em minha mente. Eu constatei que as mesmas geravam um “efeito cascata” só de bons momentos. Foi então, que eu fechei os meus olhos e agradeci à Deus pelo o teu profissionalismo, que através disso, foi possível a concessão da minha Bolsa FAPESP e a realização daquilo que eu mais sonhava: Ir para o exterior levando a minha pesquisa. Foi um grande desafio e um sonho que eu não queria acordar, pois viajar para fora do país era o que eu mais almejava, ainda Deus me surpreendeu e eu realizei o sonho de rolar na neve. Além disso, com a Senhora eu tive grandes oportunidades, conheci pessoas incríveis e recebi todo o apoio para participar do Projeto Rondônia 2017. As boas lembranças não têm fim, juntos comemoramos prêmios, mas também nos estressamos com a novela dos dentes bovinos (Risos). São incontáveis recordações que eu agradeço e carrego no meu coração e na minha memória.

Para finalizar, quero lhe homenagear por todas as suas conquistas, prêmios e por ser reconhecida em vários países com o teu incrível profissionalismo. Isso me motiva e me faz acreditar que sonhos podem se tornar realidades. A Senhora estará para sempre nas minhas orações. Peço à Deus, que Ele nunca deixe de iluminar os teus caminhos. Por fim, o meu maior desejo, é que no futuro outras folhas sejam borradas pelas minhas lágrimas, diante da minha maior inspiração profissional.

Eu te agradeço do fundo do meu coração!

ABSTRACT

Proteomic analysis of acquired pellicle formed on experimental composite resins containing or not filler and/or protease inhibitors: study *in situ*

In the oral cavity, any exposed surface is prone to the formation of the acquired pellicle (AP), an organic film, free of bacteria, which is formed *in vivo* as a result of the selective adsorption of salivary proteins and glycoproteins to the solid surfaces exposed to the oral environment. The objective of this work was to evaluate the influence of the addition or not of filler (Barium glass alumina silicate and silica) and/or protease inhibitors [*epigallocatechin-3-gallate* (EGCG) or chlorhexidine (CHX)] to experimental composite resins in the protein profile of the AP formed on these specimens, using quantitative label-free proteomic analysis. Three-hundred and twenty-four samples of bovine enamel (6x6x2mm) were prepared. A cavity (4x4mm) was made, filled with experimental resins and divided into 6 groups of 54 specimens each, according to the experimental groups: no filler, no inhibitor (NF-NI); filler, no inhibitor (F-NI); no filler plus CHX (NF-CHX); filler plus CHX (F-CHX); no filler plus EGCG (NF-EGCG); filler plus EGCG (F-EGCG). Nine young adults of both genders participated using a removable jaw appliance (BISPM - Bauru *in situ* pellicle model)) with 2 slabs of each group. The experiment was carried out in 9 consecutive days, during the morning for 120 minutes. The pellicle was obtained through the aid of electrodes filter paper moistened in 3% citric acid. The pellicles collected were processed for analysis by LC-ESI-MS/MS. The obtained MS/MS spectra were searched against human protein database (SWISS–PROT). The proteomic data related to protein quantification were analyzed using the PLGS software. Difference in expression among the groups was expressed as $p<0.05$ for down-regulated proteins and $1-p>0.95$ for up-regulated proteins. A total of 140 proteins were identified in the AP. From these, 16 were found in all the groups, among which are many proteins typically found in the AP, such as two isoforms of *Basic salivary proline-rich protein*, *Cystatin-S*, *Cystatin-AS*, *Cystatin-SN*, *Histatin-1*, *Ig alpha-1 chain C region*, *Lysozyme C*, *Mucin-7*, *Proline-rich protein 4*, *Protein S100-A9*, *Salivary acidic proline-rich phosphoprotein ½*, *Statherin* and *Submaxillary gland androgen-regulated protein 3B*. The total number of proteins identified in each group was 31, 51, 18, 38, 106 and 54

for NF-NI, F-NI, NF-CHX, F-CHX, NF-EGCG and F-EGCG, respectively. The respective amount of proteins exclusively in each group was 6, 14, 1, 6, 51 and 5. Most of the proteins that are not commonly described in the AP that have distinct functions in the organism, being involved in metabolism, cell signaling, cell adhesion, cell division, transport, protein synthesis and degradation were found most prominently in the NF-EGCG group. These results demonstrate that there was a difference in the protein profile of the AP due to the composition of the experimental resins, beyond offering important information on the development of restorative materials with components that can increase the protection in the oral cavity.

Keywords: Acquired Pellicle. Protease inhibitors. composite resins.

RESUMO

Na cavidade oral, qualquer superfície exposta é propensa à formação da película adquirida (PA), sendo considerada um filme orgânico, livre de bactérias que se forma *in vivo* como resultado da adsorção seletiva de proteínas e glicoproteínas salivares às superfícies solidas que estão expostas ao meio bucal. O objetivo deste trabalho será avaliar a influência da adição ou não de carga (vidro de bário alumina silicato e sílica) e/ou de inibidores de proteases (EGCG ou CHX) a resinas compostas experimentais no perfil proteico da PA formada sobre estes espécimes, utilizando estratégias proteômicas quantitativas livres de marcadores. Foram preparadas 324 amostras de esmalte bovino (6x6x2mm), foi feita uma cavidade no centro de 4x4mm, a qual foi preenchida com resinas experimentais. As amostras foram divididas em 6 grupos de 54 espécimes cada, de acordo com os grupos experimentais: Sem carga, sem inibidor (NF-NI); carga, sem inibidor (F-NI); sem carga e CHX (NF-CHX); carga e CHX (F-CHX); sem carga e EGCG (NF-EGCG); carga e EGCG (F-EGCG). Nove adultos jovens de ambos os gêneros paticiparam, usando um aparelho mandibular removível (BISPM - Bauru in situpellicle model) com duas amostras de cada grupo. O experimento foi conduzido por 9 dias consecutivos, durante a manhã por 120 minutos. A PA foi obtida através da ajuda do papel filtro de eletrotodo, umidecido em 3% de ácido cítrico. A película coletada, foi processada por LC-ESI-MS/MS. Os espectros MS/MS obtidos foram confrontados com bases de dados de proteínas humanas (SWISS-PROT). A quantificação livre de marcadores foi feita utilizando o software PLGS. A diferença de expressão entre os grupos foi expressa como $p<0.05$ para as proteínas down-regulated e $1-p>0.95$ para as proteínas up-regulated. Um total de 140 proteínas foram identificadas na PA. Destas, 16 foram encontradas em comum em todos os grupos, dentre elas muitas proteínas típicas da PA, tais como, duas isoformas de *Basic salivary proline-rich protein, Cystatin-S, Cystatin-AS, Cystatin-SN, Histatin-1, Ig alpha-1 chain C region, Lysozyme C, Mucin-7, Proline-rich protein 4, Protein S100-A9, Salivary acidic proline-rich phosphoprotein ½, Statherin* e *Submaxillary gland androgen-regulated protein 3B*. O número total de proteínas identificadas em cada grupo foi 31, 51, 18, 38, 106 and 54 para NF-NI, F-NI, NF-CHX, F-CHX, NF-EGCG e F-EGCG, respectivamente. A respectiva quantidade de proteínas exclusivas de cada

grupo foi 6, 14, 1, 6, 51 e 5. A maioria das proteínas que não são comumente descritas na PA e que tem funções distintas no organismos, estando envolvidas no metabolismo, sinalização celular, adesão celular, divisão celular, transporte, síntese proteica e degradação foram encontradas no grupo NF-EGCG. Estes resultados demonstram que houve uma diferença no perfil preteico da PA, devido à composição das resinas experimentais, além de oferecer informações importantes sobre o desenvolvimento de materiais restauradores com componentes que podem aumentar a proteção na cavidade oral.

Palavras-chave: Película adquirida. Inibidores de protease. Resinas compostas.

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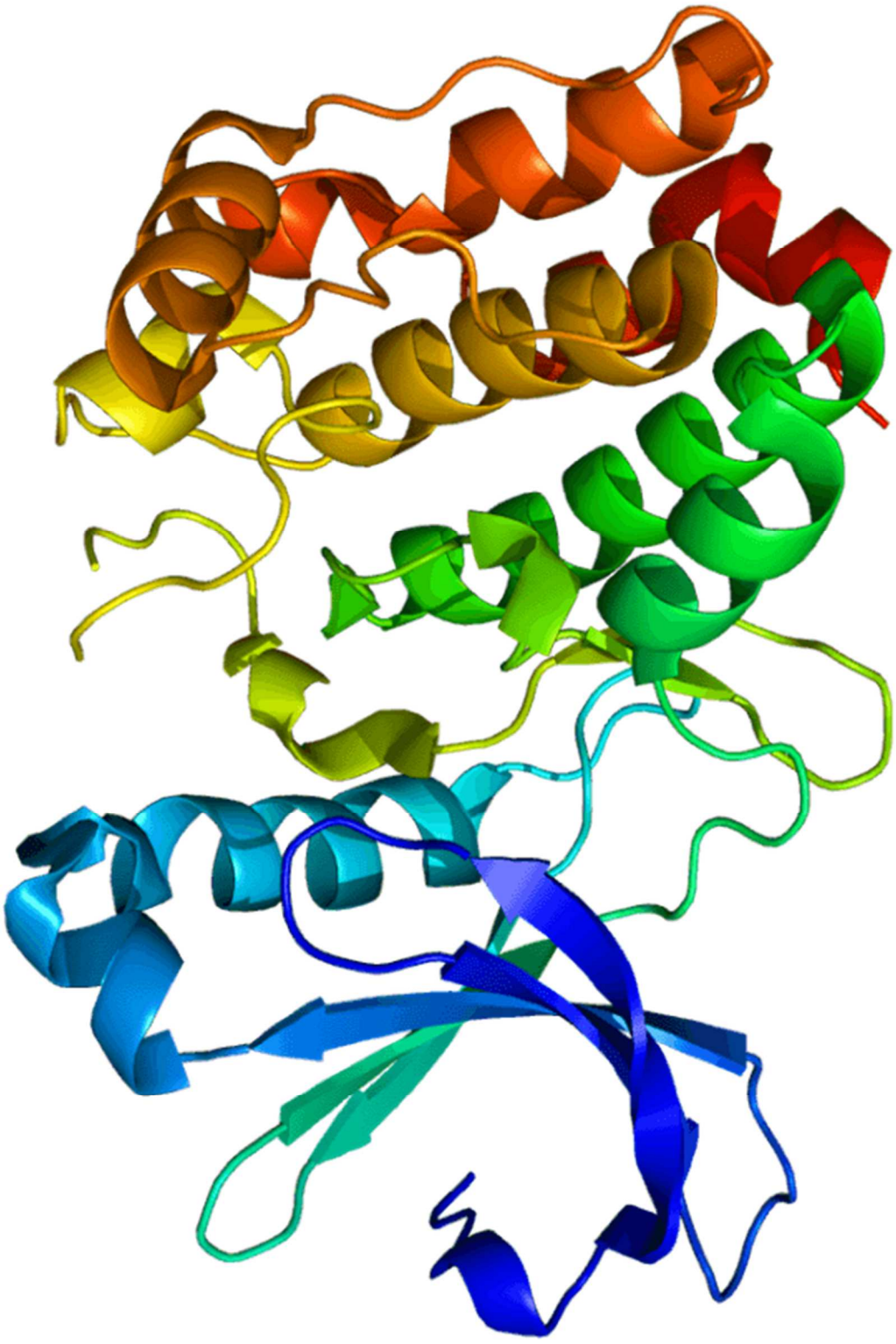


1-Introduction

1 INTRODUCTION

In the oral cavity, any surface exposed is prone to the formation of the acquired pellicle (AP), an organic film, free of bacteria, which is formed *in vivo* as a result of the selective adsorption of salivary proteins and glycoproteins to the solid surfaces exposed to the oral cavity (DAWES et al., 1963). Its formation is a dynamic process, influenced by several factors, such as composition of the buccal microbiota, circadian cycle, proteolytic capacity of the oral environment and physical-chemical properties of dental surfaces, as well as its location in the oral cavity (LENDENMANN et al., 2000; VENTURA et al., 2017).

The formation of the AP in the first stage is characterized by the spontaneous adsorption of salivary proteins on the teeth surfaces. Calcium ions from the enamel crystals, when in contact with saliva, tend to dissolve. As a result, the remaining phosphate ions give a negative charge to this surface, which is then coated with a layer of positively charged calcium ions dissolved. Thus, this electropositive surface interacts with the electronegative proteins (HANNIG; JOINER, 2006). At this stage, phosphoproteins with high affinity such PRP-3, PRP-4 as well as Statherin, instantly adsorb. These proteins are capable of interacting with the calcium and phosphate ions from enamel via ionic interactions, van der Waals and hydrophobic interactions. In the next stage, proteins such as α-amylase, PRPs and cystatins adsorb (HANNIG; JOINER, 2006; LAMKIN; ARANCILLO; OPPENHEIM, 1996). In the last step, there is a continuous adsorption of saliva biopolymers. It also occurs interaction between the salivary proteins and precursor proteins of the primary pellicle, which is a more complex phase of rapid adsorption, followed by a slower phase. The proteins that participate in that last step are PRP-1, PRP-2 and Histatin (HANNIG; JOINER, 2006; LAMKIN et al., 1996). Studies suggest that this phase does not reach its peak at 2 hours, showing that the formation of AP is not complete at this time (HANNIG; JOINER, 2006; LAMKIN et al., 1996).

The AP may also suffer intrinsic and extrinsic maturation, which may affect its solubility. The intrinsic maturation may be caused by the presence of transglutaminase, derived from buccal epithelial cells, which can make cross-links between the basic PRPs and Statherin (HANNIG et al., 2008; YAO et al., 2000), as well as the presence

of alkaline phosphatase. On the other hand, salivary proteolysis, that can occur before (HELMERHORST et al., 2006) or after adsorption to hydroxyapatite (MCDONALD et al., 2011), plays an important role in the extrinsic maturation of the pellicle (SIQUEIRA; OPPENHEIM, 2009; VITORINO et al., 2007). The formation and maturation of the AP can also be influenced by extrinsic factors, such as, tooth whitening products, abrasive dentifrices and ingestion of acidic foods and beverages (HARA; ZERO, 2010).

The composite resins are composed by organic material, mainly constituted by Bis-GMA (*Bisphenol Aglyceratedimethacrylate*) with mixtures of diluents in different proportions and inorganic material or filler, represented by silica and various other combinations, which vary in shape, size and distribution (FERRACANE, 2011; JANDT AND SIGUSCH, 2009; RUEGGEBERG, 2011). The incorporation of inorganic filler particles, such as barium glass, alumina silicate and silica in dental resins is important because it favors mechanical properties and gives stability to the material in the oral cavity.

Major changes are aimed at improving the mechanical strength of the materials, also improving the optical properties. Currently, nanoparticle resins represent the technology in which the two properties are satisfactorily achieved. However, it is observed the effect of increase in surface roughness of the material, which may be related to increased protein adhesion, altering the formation of the acquired pellicle and also bacterial adhesion, facilitating the formation of the dental biofilm, thus leading to caries and periodontal disease (BOLLEN et al., 1997).

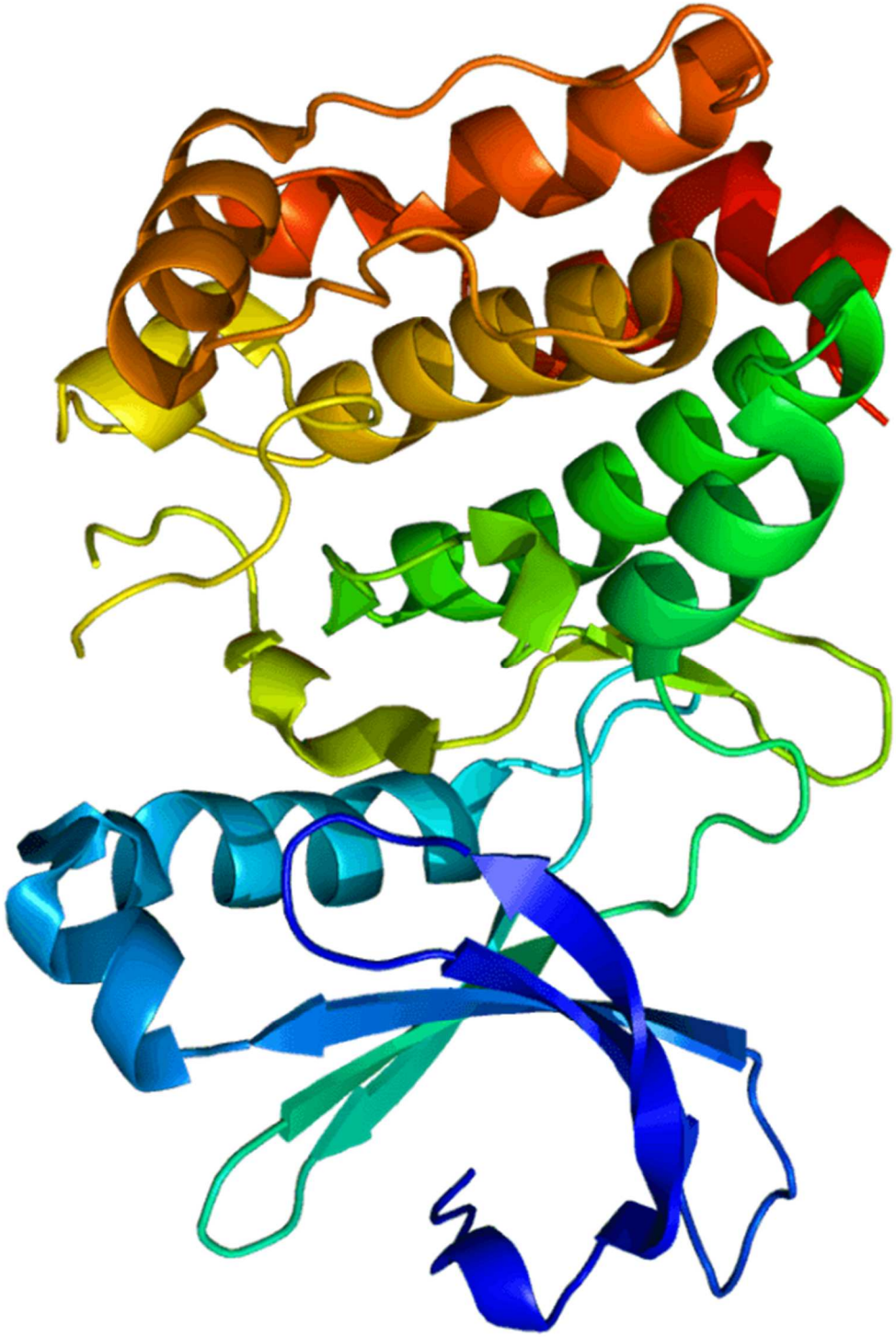
In addition, in an attempt to explore the potential of these composites, the rationale of their use as vehicle to incorporate modifying elements has been tested in several ways, such as antimicrobial products, especially fluoride and chlorhexidine (CHEN et al., 2012). The association with antimicrobials and the incorporation of proteolytic inhibitors have been investigated and may form an interesting association from the biological point of view. The composite resin could be used to intensify the role of these inhibitors, since these enzymes are present in the saliva, where their activity is related to the occurrence of dental caries (HEDENBJORK-LAGER et al., 2015).

Due to the great importance of proteases in the degradation of the demineralized organic matrix of dentin, inhibitors have been fairly recently tested. The

release of protease inhibitors such as Epigallocatechin gallate (EGCG) and Chlorhexidine (CHX) in resinous bases was able to promote the reduction of the gelatinolytic activity of matrix metalloproteinases (MMP) 2 and 9, as well as the bacterial collagenase activity (ZARELLA et al., 2016). In addition, the release of polyphenolic compounds incorporated into composites may interfere with the structure and function of the AP formed. Components of black tea and purified polyphenols *epicatechin3-gallate* (ECG), *epigallocatechin-3-gallate* (EGCG) and theaflavin, found in wine, are adsorbed in the AP. This modification prevents the AP to be eluted with mouthwashes of phosphate buffer and sodium dodecyl sulfate (JOINER et al., 2004; JOINER et al., 2003).

Considering that various proteases are present in the oral environment and in the dentin tissue and that they accelerate the progression of caries and dentin erosion (TJADERHANE et al., 2015), the incorporation of protease inhibitors into composites could become interesting to delay the advance of hydrolytic degradation in the surrounding dental tissue and to improve the quality of the AP formed on the surface, since the incorporation of polyphenols and CHX in the AP is able to stabilize it (JOINER et al., 2006; JOINER et al., 2004; JOINER et al., 2003). The stabilized AP would act as a barrier, providing greater protection to the restoration and adjacent dental tissue against the action of bacterial acids (which would lead to caries) or non-bacterial acids (which would lead to erosion).

Thus, the knowledge of the protein profile of the AP formed on the surface of composites and how this profile can be altered by changes in the formulation of these composites, is extremely important and might help to increase the longevity of the restorations. Thus, the aim of this work was to evaluate the influence of the addition or not of filler (Barium glass alumina silicate and silica) and/or protease inhibitors (EGCG or CHX) to experimental composite resins in the protein profile of the AP formed on these specimens, using quantitative label-free proteomic analysis.



2-Article

2 ARTICLE

Article formatted according to Journal of Dentistry

Proteomic analysis of acquired pellicle formed on experimental composite resins containing or not filler and/or protease inhibitors: study *in situ*

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Running title: Proteomics of acquired enamel with filler and/or protease inhibitors.
Keywords: Acquired Pellicle. Protease inhibitors. composite resins.

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Abstract

Objective: This study evaluated the influence of the addition of filler or not and/or protease inhibitors [*epigallocatechin-3-gallate* (EGCG) or chlorhexidine (CHX)] in experimental composite resins in the protein profile of the acquired pellicle (AP) formed *in situ* on enamel specimens. Methods: 324 samples of bovine enamel were prepared (6x6x2mm). The center of each sample was removed (4x4mm) and filled with experimental resins, divided into 6 groups: no filler, no inhibitor (NF-NI); filler, no inhibitor (F-NI); no filler plus CHX (NF-CHX); filler plus CHX (F-CHX); no filler plus EGCG (NF-EGCG); filler plus EGCG (F-EGCG). Nine healthy subjects with good oral conditions used a removable jaw appliance (BISPM - Bauru *in situ* pellicle model) with 2 pre-sonicated slabs from each group. The AP was formed during the morning, for 120 minutes, in 9 days and collected with electrode filter paper soaked in 3% citric acid. The pellicles collected were processed for analysis by LC-ESI-MS/MS. The obtained MS/MS spectra were searched against human protein database (SWISS–PROT). The proteomic data related to protein quantification were analyzed using the PLGS software. Results: A total of 140 proteins were found in the AP collected from all the substrates. Among them, 16 proteins were found in all the groups, such as 2 isoforms of *Basic salivary proline-rich protein, Cystatin-S, Cystatin-AS, Cystatin-SN, Histatin-1, Ig alpha-1 chain C region, Lysozyme C, Mucin-7, Proline-rich protein 4, Protein S100-A9, Salivary acidic proline-rich phosphoprotein ½, Statherin* e *Submaxillary gland androgen-regulated protein 3B.* Proteins with other functions, such as cell signaling, metabolism and transport, were also identified. Conclusion: The composition of the experimental resins influenced the protein profile of the AP. This opens a new avenue for the development of new materials able to guide AP engineering, thus conferring protection to the teeth.

Introduction

In the oral cavity, any exposed surface is prone to the formation of the acquired pellicle (AP), an organic film, free of bacteria, which is formed *in vivo* as a result of the selective adsorption of salivary proteins and glycoproteins to the solid surfaces exposed to the oral environment[1]. Its formation is a dynamic process, influenced by several factors, such as circadian cycle, composition of the buccal microbiota, proteolytic capacity of the oral environment and physical-chemical properties of the dental surfaces [2], as well as the location in the dental arches [3].

The AP forms on any exposed surface in the oral cavity, including dental materials. Thus, it seems plausible that the surface characteristics (such as rugosity) and composition of these materials might guide the formation of the AP, interfering in the type of proteins that will adsorb onto the surface. Due to the increased prevalence of non-carious cervical lesions, their restoration with distinct materials is commonly performed [4, 5]. The polymeric materials, especially composite resins have been applied clinically as restorative materials in most of these procedures for appropriate functional and aesthetic properties [6]. In an attempt to thoroughly explore the potential of these composites, the rationale of their use as vehicle to incorporate modifying elements has been tested in several ways, such as antimicrobial products, especially fluoride and chlorhexidine (CHX) [7], as well as protease inhibitors, such as CHX and *epigallocatechin gallate* (EGCG) [8]. The role of these enzymatic inhibitors to reduce or postpone hydrolytic degradation of the adhesive layer that links the composite resin to the dentinal tissue has been extensively emphasized, especially when this substrate was previously affected by caries [9, 10, 11, 12]. Thus, the composite resin could be used to intensify the action of these inhibitors, since proteases are present in saliva, where their activity is related to the occurrence of dental caries [13].

Saliva contains several matrix metalloproteinases (MMPs), which effectively degrade the demineralized organic matrix of dentin, being MMP-9 probably the most abundant [14]. The release of protease inhibitors, such as EGCG and CHX from resinous bases was able to promote the reduction of the gelatinolytic activity of MMPs 2 and 9, as well as of bacterial collagenase [15]. In addition, the release of polyphenolic compounds incorporated into composites could interfere in the structure and function of the AP formed on the surface of these resins.

A plethora of proteases is present in the oral environment and in the dentin tissue. These proteases accelerate the rate of progression of dentin caries and erosion [17, 18]. Thus, the incorporation of protease inhibitors into composites could delay the advance of hydrolytic degradation in the surrounding dental tissue and improve the quality of the AP formed on the surface, since the incorporation of polyphenols and CHX in the AP is able to stabilize it [19, 20, 21]. This stabilized AP would act as a barrier, providing greater protection to the restoration and adjacent dental tissue against the action of bacterial (which would lead to caries) or not bacterial acid (which would lead to erosion). Thus, the knowledge of the protein profile of the AP formed on the surface of composites and how this profile can be altered by changes in the formulation of these composites, is extremely important and might help to increase the longevity of the restorations. The obtained results might guide the development of materials that can favorably act in the formation of the AP, which, in turn, may protect the restoration and adjacent dental structure. Thus, the aim of this work was to evaluate the influence of the addition or not of filler (Barium glass alumina silicate and silica) and/or protease inhibitors (EGCG or CHX) to experimental composite resins in the protein profile of the AP formed on these specimens, using quantitative label-free proteomic analysis. The hyphotheses to be tested are that the addition of a) filler and/or b) protease inhibitors in experimental composite resins does not alter the protein composition of the AP formed *in situ.*

Materials and Methods

Experimental design

The protocol of this work was approved by the Research Ethics Committee of Bauru School of Dentistry, University of São Paulo (CAAE 60563516.4.0000.5417). Nine individuals used a removable mandibular apparatus (BISPM - Bauru *in situ* pellicle model) after signing a informed consent. The *in situ* design comprised two factors. One of them was the addition of inorganic filler to the experimental composite resin at two levels (no filler-NF or filler-F) and the other one was the addition of protease inhibitors at three levels (no inhibitor, EGCG or CHX). The main response variable was the quantitative proteomic analysis of the acquired pellicle formed *in situ* in the samples of experimental composite resin.

Formulation of the experimental composite resins

In total, 324 specimens of composite resin were prepared. They were divided into 6 groups of 54 specimens each, according to the experimental groups: no filler, no inhibitor (NF-NI); filler, no inhibitor (F-NI); no filler plus CHX (NF-CHX); filler plus CHX (F-CHX); no filler plus EGCG (NF-EGCG); filler plus EGCG (F-EGCG). All groups had the same basic formulation composed of BisGMA (Bisphenol A glycerate dimethacrylate) and TEGDMA (Triethylene glycol dimethacrylate) (70 and 30% in mol, respectively) (Sigma-Aldrich, St. Louis, MO, EUA). The groups with filler were manually prepared using silanized vitreous filler (barium glass alumina silicate and silica) of average size of 0.7 μm and content of 70% (in weight). EGCG (Epigallocatechin gallate) ≥97.0% (HPLC) (Sigma Aldrich, St. Louis, MO, EUA) and CHX (Chlorhexidine diacetate hydrate salt) (Sigma Aldrich, St. Louis) were added at 1% (in weight) added in the form of salt to 1% in weight [15]. In order that the resin could be photopolimerized 0.2% w/w camphorquinone (CQ, Kerr Corp., Orange, CA, EUA) and 0.2% w/w 2-dimethylamino ethyl methacrylate) (DMAEMA) (Sigma-Aldrich, St. Louis, MO, EUA) were added. The preparation of the experimental resins was performed using a digital scale always starting from the most viscous to the less viscous component to allow more homogeneous mixing, which was performed manually for 5 minutes. The resins were individually refrigerated in amber vials without exposure to light. The entire procedure was performed under temperature 21±2 °C.

To validate the experimental, the degree of conversion (DC) of each mixture was assessed with Fourier transform infrared spectroscopy (FTIR-Shimadzu Corporation, Model IR Prestige 21, Kyoto, Japan) with attenuated total reflectance (ATR- Smart Miracle ^{TM} with diamond plate - Pike Technologies). Standardized uncured amount was dropped on dispositive for individual scanning. After this running, light cured was processed for 40s and new reading was run for DC calculation after 5 minutes. The absorption spectra of uncured and cured DBS were obtained from the region between 4000 and 650 cm^{-1} with 32 scans at 4 cm^{-1} and all assessments were read in triplicate[16]

Preparation of bovine enamel specimens

Three-hundred twenty-four bovine enamel specimens (6x6x2mm) were prepared, using an ISOMET Low Speed Saw (Isomet, Buehler, Lake Bluff, IL, EUA) and two diamond discs (Extec Corporation, XL-12205, Enfield, CT, EUA). The samples were placed for 7 minutes in an Ultrasonic bath (T7 Thornton, Unique Ind. e Com. Ltda., São Paulo, SP, Brasil) for cleaning purposes. Then the center of each specimen was removed with low speed motor (KAVO, Joinville, Santa Catarina, Brazil) and a diamond bur $^{10}/_{14}$ to create a window of 4x4x1mm that was filled with the resin corresponding to each of the 6 groups mentioned above. The resin was polymerized for 20 seconds each increment and 40 seconds for the last increment, with LED Radi Cal (1,100 mW/cm^2, SDI, Victoria, Bayswater, Australia). Then, the samples were stored in an oven at 37°C for one week to allow completion of polymerization.

Selection of individuals

Nine young adults of both genders participated in the intra-oral phase. The exclusion criteria were: smoker, presenting carious lesions, gingivitis, periodontitis and/or low salivary flow (unstimulated and stimulated flow had to be higher than 0.1 and 1.0 ml/minute, respectively) and taking medications that can change the salivary flow. The sample size was chosen based on a previous *in situ* study [22]**.**

Preparation of the removable mandibular apparatus (Bauru *in situ* pellicle model - BISPM)

Alginate impressions were used to make plaster models, which were employed to prepare silicon devices from the mandibular arches of each volunteer. Three silicon devices were prepared for each volunteer. Twelve recessed sites (6X6 mm) were carefully added to each silicon device in the vestibular area. Twelve enamel/resin specimens (2 from each group) were placed in the recessed sites and fixed with cyanoacrylate glue (Super Bonder, São Paulo, Knauff Ltda). The experiment was carried out in 9 consecutive days. Every three days, a silicon device containing new specimens repositioned in different places was used in order that all groups had pellicle collected from all the mandibular vestibular regions. In addition, a number 0.8 orthodontics wire (Morelli Ortodontia, Sorocaba, SP, Brazil) was suspended above the specimens, to avoid direct contact of the mucosa with them, thus making easier the formation

of the AP. This wire was fixed on the silicon devices with godiva in the center of the apparatus and also in the posterior regions (Figure 1).

Formation of the acquired pellicle *in situ*

The formation of the AP occurred in the morning to avoid the circadian effects on the composition of pellicle [23]. The experiment was conducted in 9 consecutive days. Each day, the specimens were submitted to prophylaxis with pumice (Pedra Pomes, S.S. While Artigos Dentários Ltda, Rio de Janeiro, RJ, Brazil) and the pellicle was allowed to form during 120 minutes. During this period, the volunteers were deprived from food and beverage consumption.

Acquired pellicle collection

The pellicle was obtained through the aid of 5X10 mm electrodes filter paper (filter paper wick electrode, Bio-Rad, Hercules, CA) moistened in 3% citric acid (pH 2.5; Sigma-Aldrich, USA). The filter paper was scrubbed (without pressure) on the surfaces of the specimens with tweezers [24]. The wick filters were placed in 2 mL cryotubes and stored at -80°C until used for proteomic analysis. In total 162 wick filters were obtained for each group (2 specimens per day X 9 days X 9 volunteers).

Preparation of the acquired pellicle samples for proteomic analysis

The acquired pellicle samples were prepared following a recently standardized protocol [3]. Briefly, protein extraction was performed twice using a solution containing 6 M urea, 2 M thiourea in NH_4HCO_3 50 mM pH 7.8 and the supernatants were stored. To increase protein recovery, the wick papers were transferred to filter tubes (Corning Costar®Spin-X® Plastic Centrifuge Tube Filters, SigmaAldrich, New York, USA), centrifuged and the supernatant was collected. The supernatants were pooled, centrifuged again and transferred to a falcon tube. Then, 50 mM NH_4HCO_3 was added to dilute the urea and thiourea and samples were placed in Falcon Amicon tubes (Amicon Ultra - 15 Centrifugal Filter Units - Merck Millipore, Tallagreen, Ireland), centrifuged and concentrated to approximately 150 µL. Reduction (5 mM dithiothreitol (DTT) for 40 minutes at 37ºC) and alkylation (10 mM iodoacetamide (IAA) in

the absence of light for 30 minutes) were performed. Samples were then digested using 2% (w/w) trypsin (Promega, Madison, USA). Then 10 µL of 5% formic acid was placed to stop the action of trypsin. C18 Spin columns (Thermo Scientific, United States) were used to desalt and purify the samples and protein was quantified using the Bradford method (Bio-Rad Bradford Assays, United States). The amount of protein obtained ranged between 58.1 µg (F-NI) and 103.7 µg (NF-NI). The samples were resuspended in a solution containing 3% acetonitrile and 0.1% formic acid to be submitted to nano LC-ESI-MS / MS.

Shotgun label-free quantitative proteomic analysis

Nano LC-ESI-MS / MS was performed exactly as previously described [3], using a nanoACQUITY UPLC-Xevo QTof MS system (Waters, Manchester, UK) equipped with a nanoACQUITY HSS T3, analytical reverse phase column (75 µm X 150 mm, 1.8 µm particle size, Waters).

ProteinLynx Global Server (PLGS) version 3.0 (Waters Co., Manchester, UK) was employed to process and search the continuum LC-MSE data. Proteins were identified with the embedded ion accounting algorithm in the software and a search of the *Homo sapiens* database (reviewed only, UniProtKB/Swiss-Prot) downloaded on December 2016 from UniProtKB (http://www.uniprot.org/). The use of the human database excludes the identification of bacterial proteins that could be present in the AP. The identified proteins were classified and assigned by biological function [25, 23], origin and molecular interaction (http://www.uniprot.org/).

For label-free quantitative proteome, three MS raw files from each pooled group were analyzed using the PLGS. All the proteins identified with a score with confidence greater than that 95% were included in the quantitative statistical analysis embedded in the PLGS software. Identical peptides from each triplicate by sample were grouped based on mass accuracy (<10 ppm) and on time of retention tolerance <0.25 min, using the clustering software embedded in the PLGS. Difference in expression among the groups was expressed as $p<0.05$ for down-regulated proteins and $1\text{-}p>0.95$ for up-regulated proteins. The following relevant comparisons were made: NF-NI x F-NI, NF-CHX x F-CHX, NF-EGCG x F-EGCG, NF-NI x NF-CHX, NF-NI x NF-EGCG, NF-CHX x NF-EGCG, F-NI x F-CHX, F-NI x F-EGCG e F-CHX x F-EGCG.

Results

The amount of protein obtained in the different groups ranged between 58.1 μg (F-NI) and 103.7 (NF-NI) μg. A total of 140 proteins were identified in the AP. From these, 16 were found in all the groups, among which are many proteins typically found in the AP, such as two isoforms of *Basic salivary proline-rich protein*, *Cystatin-S*, *Cystatin-AS*, *Cystatin-SN*, *Histatin-1*, *Ig alpha-1 chain C region*, *Lysozyme C*, *Mucin-7*, *Proline-rich protein 4*, *Protein S100-A9*, *Salivary acidic proline-rich phosphoprotein ½*, *Statherin*, *Submaxillary gland androgen-regulated protein 3B*. The proteins *Annexin A1*, 2 isoforms of *Alpha-amylase*, *Pancreatic alpha-amylase*, *Proline-rich protein 27* e *Zinc-alpha-2-glycoprotein* were identified in all groups, except in the NF-CHX, while *Ig alpha-2 chain C region* was not found in the F-NI group. In addition, *Neutrophil defensin 1*, *Neutrophil defensin 3* and *Protein S100-A8* were not identified in the groups NF-NI and NF-CHX, while *Serum albumin* was only identified in the groups F-NI, NF-EGCG and F-EGCG. Similarly, *Antileukoproteinase* was only found in the groups F-NI and F-EGCG, while *Histatin-3* was only found in the F-EGCG group. Finally, among the four isoforms of Annexin that were identified, two appeared only in the NF-EGCG group (Table S1).

The total number of proteins identified in each group were 31, 51, 18, 38, 106 and 54 for NF-NI, F-NI, NF-CHX, F-CHX, NF-EGCG and F-EGCG, respectively (Table 1). The respective figures for proteins found exclusively in one of the groups was 6, 14, 1, 6, 51 and 5. Most of the proteins that are not commonly described in the AP that have distinct functions in the organism, being involved in metabolism, cell signaling, cell adhesion, cell division, transport, protein synthesis and degradation were found most prominently in the NF-EGCG group (Table 2).

Regarding quantitative analysis, 9 comparisons among the 6 groups were made (3 comparisons between groups without and with filler, 3 within groups without filler and another 3 within groups with filler) (Table 3). The addition of filler in the the placebo and CHX groups (comparisons F-NI X NF-NI and F-CXH X NF-CHX) in general increased proteins such as *Statherin*, *Protein S100-A9, Mucin-7*, isoforms of *Cystatin* and *Submaxillary gland androgen-regulated protein 3B*, while *Proline-rich protein 4* was decreased (Table 3).

When the groups without filler are compared to each other (Table 3), the presence of CHX compared to NI (comparison NF-CHX X NF-NI) increased 3 isoforms of Cystatin, *Lysozyme C* and *Mucin-7* and decreased 2 isoforms of Ig alpha, *Submaxillary gland androgen-*

regulated protein 3B and *Protein S100-A9*. On the other hand, the presence of EGCG compared to NI (comparison NF-EGCG X NF-NI) increased several proteins, including 2 isoforms of Basic salivary proline-rich protein, 3 isoforms of Cystatin, *Lysozyme C*, 2 isoforms of Alpha-amylase, *Zinc-alpha-2-glycoprotein, Protein S100-A9*, *Annexin A1* and *Submaxillary gland androgen-regulated protein 3B*, while *Salivary acidic proline-rich phosphoprotein ½*, *Proline-rich protein 4* and *Statherin* were reduced. When the EGCG-containing group was compared with the CHX-containing one (comparison NF-EGCG X NF-CHX), there was an increase in 11 proteins, including 2 isoforms of Basic salivary proline-rich protein, 3 isoforms of Cystatin, 2 isoforms of Ig alpha, *Protein S100-A9* and *Submaxillary gland androgen-regulated protein 3B* and a decrease in 5 proteins, including *Statherin*, *Mucin-7*, *Proline-rich protein 4* and *Salivary acidic proline-rich phosphoprotein ½*.

Finally, when the groups with filler are compared to each other (Table 3), the presence of CHX compared to NI (comparison F-CHX X F-NI) increased 2 proteins (*Basic salivary proline-rich protein 2* and *Mucin-7*) and decreased 10 proteins, including 3 isoforms of Cystatin, *Protein S100-A9*, *Submaxillary gland androgen-regulated protein 3B*, *Proline-rich protein* 4 and *Salivary acidic proline-rich phosphoprotein ½*. On the other hand, the presence of EGCG compared to NI (comparison F-EGCG X F-NI) increased 8 proteins (isoforms of Basic salivary proline-rich protein, *Protein S100-A9*, *Lysozyme C*, Cystatin-B, 2 isoforms of Annexin and *Submaxillary gland androgen-regulated protein 3B*) and decreased another 9 proteins, including *Antileukoproteinase*, *Histatin-1*, *Salivary acidic proline-rich phosphoprotein ½*, *Proline-rich protein 4*, *Mucin-7*, 2 isoforms of Ig alpha and *Statherin*. When the EGCG-containing group was compared with the CHX-containing one (comparison F-EGCG X F-CHX), 11 proteins were increased, including *Submaxillary gland androgen-regulated protein 3B*, *Protein S100-A9*, isoforms of Annexin, *Zinc-alpha-2-glycoprotein*, *Alpha-amylase 2B*, *Basic salivary proline-rich protein 1* and 2 isoforms of Cystatin, while 9 were decreased, including while 2 isoforms of Ig alpha, 2 isoforms of Proline-rich protein, *Salivary acidic proline-rich phosphoprotein ½*, *Statherin*, *Proline-rich protein 27*, *Mucin-7* and *Histatin-1*.

Discussion

The main challenge in the present study was to obtain an appropriate amount of proteins in the AP to allow proper proteomic analysis. Since our main aim was to evaluate the effect of the addition or not of filler and/or protease inhibitors (EGCG or CHX) to experimental composite resins in the protein profile of the acquired pellicle formed on these specimens, we had to conduct the experiment *in situ*, since it would be virtually impossible to find patients with necessity of restorations large enough to allow AP collection *in vivo*. The collection of AP in *in situ* experiments is challenging due to the amount of proteins that can be recovered from the AP. The device developed for this purpose (BISPM; Figure 1) had a special design in order to optimize the collection of enough amount of proteins from the AP. The main responsible for this was the placement of number 0.8 orthodontics wire that was suspended above the specimens, to avoid direct contact of the mucosa with them. In addition to this, the experiment was carried out in 9 consecutive days, and the samples collected from the same treatments were pooled. Furthermore, we worked with a mandibular apparatus instead of a palatal one because more saliva is expected to bath the specimens in the first condition. These strategies were effective to allow enough amount of proteins in the AP to be analyzed. In fact, the amount of proteins obtained was higher than necessary. This implies that future experiments could have less days of collection and/or employ less specimens each day. In the present study we had 9 days of collection to allow proper repositioning of the specimens each day, in order to have specimens from each group distributed into 3 different regions (posterior, medium and central) of the mandibular arches, since variations in the protein profile have been reported for the AP collected from different regions of the dental arches [3]. It is worth mentioning that the removable apparatus developed was well tolerated by the volunteers, without discomfort.

In total, 140 proteins were identified, including those typically found in the AP. This amount is in-line with *in vivo* studies in the literature [22, 23, 24, 26] and the presence of proteins typical from the AP identified in all the groups suggests that our protocol is appropriate to simulate the *in vivo* formed AP. One of our hypotheses was that the addition of filler would increase the surface roughness of the resins, thus allowing more proteins to adsorb onto the surface, which would augment the number of proteins in the AP. This was the case for the NI and CHX groups only. Thus, one of the hyphothesis was partially accepted. For the EGCG group, in fact the addition of filler reduced the number of identified proteins, suggesting that the addition of filler impaired EGCG release from the resin, thus reducing protein adsorption. In the case of CHX, this might have not occurred due to its great affinity by enamel. It is

noteworthy that in the NF-EGCG group that had the greatest number of identified proteins, there was a huge number of intracellular proteins, such as Histones and Keratin, whose functions in the AP are not known so far. These proteins are found together with other proteins with defined function in the AP. The addition of filler to the EGCG resin markedly reduced binding of intracellular proteins (Table 1). The impact of the presence of intracellular proteins in the protective role of the AP should be investigated in future studies. Furthermore, the addition of filler in the NI and CHX groups increased proteins with important protective functions in the teeth and oral cavity, such as *Statherin* [27], *Protein S100-A9* [22], *Mucin-7* [28] and isoforms of *Cystatin* [22].

In the present study, one of the reasons to include the groups without filler is that materials without filler have been proposed to restore erosive lesions [29]. When the groups without filler were compared to each other, the addition of inhibitors in general increased several protective proteins in respect to control, such as Cystatin (antimicrobial and acid resistant) [22], Lysozyme (antyimicrobial) and Mucin (lubrication) [28], regardless of the inhibitor used. For the NF-EGCG group, additional proteins were increased, including isoforms of Alpha-amylase (digestion) [28] and *Protein S100-A9 (*calcium and zinc binding and antibacterial function). However, several other protective proteins were reduced in respect to control, especially in the NI-EGCG group, such as proline-rich proteins and *Statherin* [27]. However, these findings are not so relevant from the clinical point of view, since commercial composites contain fillers. Thus, the comparisons among the groups containing distinct inhibitors and filler is more important. In this case, the best performance in terms of increasing protective proteins was achieved by the F-EGCG group that showed significant increases of isoforms of Basic salivary proline-rich proteins, *Protein S100-A9, Lysozyme C, Cystatin-B, 2 isoforms of Annexin and Submaxillary gland androgen-regulated protein 3B in respect* to control (F-NI). Proline-rich proteins (PRPs) maintain a state of saturation with respect to Ca and phosphate in the oral cavity by inhibiting their precipitation at neutral pH and releasing these ions following acidic attack and during demineralization. These proteins are increased in the pellicle of caries-free individuals [30] and reduced in saliva of caries-susceptible individuals [31]. Protein *S100-A8* binds calcium and zinc, participates in the regulation of inflammatory processes and immune response and also has antibacterial function (UNIPROT). This protein is predominantly found as calprotectin (S100-A8/A9). Due to its ability to bind calcium, this protein can deposit on enamel [32], which, combined to its antimicrobial function, can have important implications for caries prevention. Another protein with antimicrobial activity is lysozyme, since it is able to degrade

bacterial peptidoglycans. In addition, lysozyme inhibits the adherence of *S. mutans* to saliva-treated hydroxyapatite in vitro [33] and reduced amounts of it have been found in unstimulated saliva of children with early childhood caries [34], which highlights its anticariogenic potential. A classical antimicrobial protein that was recently categorized as acid-resistant that was also increased in the AP formed on F-EGCG specimens was *Cystatin B*. This protein was shown to be increased 20 fold in the AP formed in vivo after challenge with 1% citric acid. In addition, it was also increased 13 fold when the AP was challenged with 0.1 M lactic acid [22], which highlights its potential to protect against caries and erosion. In fact, purified polyphenols, such as EGCG promptly adsorb onto the pellicle. This reduces the degree of pellicle elution by different solutions [20]. It is possible that the greater resistance of the AP treated with EGCG might be due to increase in protective proteins, such as those identified in the present study and specially the acid-resistant ones.

In conclusion, the addition of filler was important mainly for the CHX-containing composite, where an increase in *Statherin* was observed in the AP. For the EGCG-containing composite, the addition of filler in fact reduced the number of identifies proteins in the AP. On the other hand, the F-EGCG composite had increase in proteins with important functions in the AP, which might help to explain why pellicles treated with EGCG are more resistant to elution. These results open a new avenue for the addition of compounds to composites in order to improve their therapeutic functions in the restorations.

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Figure legend

Figure 1. The removable mandibular apparatus (Bauru *in situ* pellicle model - BISPM)

Table 1. Classification of total proteins of each group identified in the acquired pellicle.

NF-NI	Accession	Description
	P04745	Alpha-amylase 1 [a, g, o, u]
	P19961	Alpha-amylase 2B [a, g, o, u]
	P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]
	P04280	Basic salivary proline-rich protein 1 [b, l, o, u]
	P02812	Basic salivary proline-rich protein 2 [b, l, o, u]
	P01036	Cystatin-S [a, b, g, o, u]
	P09228	Cystatin-SA [a, b, g, o, u]
	P01037	Cystatin-SN [a, b, g, o, u]
	P04792	Heat shock protein beta-1 [b, d, m, n, p, q, u, w]
	P15515	Histatin-1 [b, i, l, o, u]
	P56915	Homeobox protein goosecoid [b, d, e, m, p, u]
	P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]
	P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]

Q8N9Z9	Lamin tail domain-containing protein 1 [b, d, m, n, p, u]
O95970	Leucine-rich glioma-inactivated protein 1 [e, m, s, w]
P61626	Lysozyme C [a, b, g, i, j, o, u, w]
Q8TAX7	Mucin-7 [b, i, k, o, u]
P18615	Negative elongation factor E [b, m, t, u]
P04746	Pancreatic alpha-amylase [a, g, o, u]
P12273	Prolactin-inducible protein [b, d, m, o, u]
Q6MZM9	Proline-rich protein 27 [b, l, o, x]
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]
P02810	Salivary acidic proline-rich phosphoprotein 1/2 [b, d, h, l, o, u, v]
C9JXZ1	Sodium/potassium-transporting ATPase subunit beta-3 (Fragment) [b, c, m, s, u]
P02808	Statherin [b, e, i, l, o, u]
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]
F2Z2L1	Transmembrane 9 superfamily member [b, m, s, x]
Q92544	Transmembrane 9 superfamily member 4 [b, e, m, r, s, u]

	A0A087WZY1	Uncharacterized protein
	P25311	Zinc-alpha-2-glycoprotein [a, b, g, o, u, w]

F-NI	**Accession**	**Description**
	Q9NQ66	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1 [f, m, n, p, u]
	P60709	Actin, cytoplasmic 1 [b, m, n, q, u, w]
	P6321	Actin, cytoplasmic 2 [a, d, g, j, n, q, u, w]
	P04745	Alpha-amylase 1 [a, g, o, u]
	P19961	Alpha-amylase 2B [a, g, o, u]
	Q5T3N0	Annexin (Fragment) [b, l, n, p, s, u]
	P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]
	P03973	Antileukoproteinase [a, b, g, i, j, o, u]
	P04280	Basic salivary proline-rich protein 1 [b, l, o, u]
	P02812	Basic salivary proline-rich protein 2 [b, l, o, u]
	P27482	Calmodulin-like protein 3 [a, g, o, u]
	P23280	Carbonic anhydrase 6 [a, g, o, u]

P16070	CD44 antigen [b, e, m, s, u, w]
E7EM64	COP9 signalosome complex subunit 6 [b, m, p, u]
P04080	Cystatin-B [a, g, n, p, u]
P01036	Cystatin-S [a, b, g, o, u]
P09228	Cystatin-SA [a, b, g, o, u]
P01037	Cystatin-SN [a, b, g, o, u]
Q9NPB8	Glycerophosphocholine phosphodiesterase GPCPD1 [b, d, m, n, u]
P15515	Histatin-1 [b, i, l, o, u]
C9J7L0	Histone-binding protein RBBP7 (Fragment) [b, m, t, x]
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]
Q8WTQ8	Kallikrein 3, (Prostate specific antigen), isoform CRA_k [b, m, t, x]
P13646	Keratin, type I cytoskeletal 13 [d, m, o, p, q, u]
P61626	Lysozyme C [a, b, g, i, j, o, u, w]
Q8TAX7	Mucin-7 [b, i, k, o, u]
P18615	Negative elongation factor E [b, d, e, m, p, u]
P59665	Neutrophil defensin 1 [b, i, j, o, u]

P59666	Neutrophil defensin 3 (b, i, j, o, u)
Q9GZM8	Nuclear distribution protein nudE-like 1 (f, m, n, u, w)
P04746	Pancreatic alpha-amylase (a, g, o, u)
A0A087WT80	Phosphoinositide phospholipase C (a, e, m, n, p, v)
P78364	Polyhomeotic-like protein 1 (a, b, d, m, p, u, w)
P12273	Prolactin-inducible protein (b, d, m, o, u)
Q6MZM9	Proline-rich protein 27 (b, l, o, x)
A0A0A0MT31	Proline-rich protein 4 (b, l, p, u)
Q15435	Protein phosphatase 1 regulatory subunit 7 (a, b, m, p, u, w)
P05109	Protein S100-A8 (b, e, i, j, l, n, o, q, s, u, w)
P06702	Protein S100-A9 (a, b, g, i, j, n, o, q, s, u, w)
K7EQ96	RING finger protein 165 (Fragment) (b, m, t, x)
P02810	Salivary acidic proline-rich phosphoprotein ½ (b, d, h, l, o, u, v)
Q9NTN9	Semaphorin-4G (a, d, m, s, u)

	P02768	Serum albumin [a, b, c, g, o, u, w]
	Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1 [b, m, p, u, w]
	P02808	Statherin [b, e, i, l, o, u]
	P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]
	B5MCJ1	Tetratricopeptide repeat protein 32 [b, m, t, x]
	Q07011	Tumor necrosis factor receptor superfamily member 9 [f, m, s, u]
	A0A087WZY1	Uncharacterized protein
	Q8TAF7	Zinc finger protein 461 [a, c, d, m, p, u]
	P25311	Zinc-alpha-2-glycoprotein [a, b, g, o, u, w]

NF-CHX	**Accession**	**Description**
	P04280	Basic salivary proline-rich protein 1 [b, l, o, u]
	P02812	Basic salivary proline-rich protein 2 [b, l, o, u]
	P01036	Cystatin-S [a, b, g, o, u]
	P09228	Cystatin-SA [a, b, g, o, u]
	P01037	Cystatin-SN [a, b, g, o, u]

	P04792	Heat shock protein beta-1 (b, d, m, n, p, q, u, w)
	P15515	Histatin-1 (b, i, l, o, u)
	P01876	Ig alpha-1 chain C region (b, e, i, j, o, u)
	P01877	Ig alpha-2 chain C region (b, e, i, j, o, u)
	P61626	Lysozyme C (a, b, g, i, j, o, u, w)
	Q8TAX7	Mucin-7 (b, i, k, o, u)
	P12273	Prolactin-inducible protein (b, d, m, o, u)
	A0A0A0MT31	Proline-rich protein 4 (b, l, p, u)
	P06702	Protein S100-A9 (a, b, g, i, j, n, o, q, s, u, w)
	P02810	Salivary acidic proline-rich phosphoprotein ½ (b, d, h, l, o, u, v)
	P02808	Statherin (b, e, i, l, o, u)
	P02814	Submaxillary gland androgen-regulated protein 3B (a, g, o, u, w)
	A0A087WZY1	Uncharacterized protein
	M0R258	Zinc finger protein 432 (Fragment) (b, e, m, r, x)

F-CHX	**Accession**	**Description**

P04745	Alpha-amylase 1 [a, g, o, u]
P19961	Alpha-amylase 2B [a, g, o, u]
Q5T3N0	Annexin (Fragment) [b, l, n, p, s, u]
P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]
P04280	Basic salivary proline-rich protein 1 [b, l, o, u]
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]
U3KPZ4	BLOC-1-related complex subunit 8 [b, m, t, x]
Q96HY3	CALM1 protein [m, m, t, v]
P62158	Calmodulin [f, m, n, u, v]
P01036	Cystatin-S [a, b, g, o, u]
P09228	Cystatin-SA [a, b, g, o, u]
P01037	Cystatin-SN [a, b, g, o, u]
M0QZ93	Galectin (Fragment) [a, m, t, x]
P15515	Histatin-1 [b, i, l, o, u]
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]

P61626	Lysozyme C [a, b, g, i, j, o, u, w]
Q8TAX7	Mucin-7 [b, i, k, o, u]
P18615	Negative elongation factor E [b, d, e, m, p, u]
P59665	Neutrophil defensin 1 [b, i, j, o, u]
P59666	Neutrophil defensin 3 [b, i, j, o, u]
P04746	Pancreatic alpha-amylase [a, g, o, u]
P12273	Prolactin-inducible protein [b, d, m, o, u]
Q6MZM9	Proline-rich protein 27 [b, l, o, x]
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]
P05109	Protein S100-A8 [b, e, i, j, l, n, o, q, s, u, w]
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]
Q9NSD7	Relaxin-3 receptor 1 [d, m, s, u]
Q9BQY4	Rhox homeobox family member 2 [b, d, e, m, p, u]
P02810	Salivary acidic proline-rich phosphoprotein 1/2 [b, d, h, l, o, u, v]
Q8NBW4	Sodium-coupled neutral amino acid transporter 9 [f, m, r, u, w]
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1 [b, m, p, u, w]

	P02808	Statherin [b, e, i, l, o, u]
	P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]
	I3NI44	TOM1-like protein 1 [b, m, r, x]
	A0A087WZY1	Uncharacterized protein
	A0A0C4DGR5	Zinc finger protein 496 (Fragment) [a, b, e, m, p, x]
	P25311	Zinc-alpha-2-glycoprotein [a, b, g, o, u, w]

NF-EGCG	**Accession**	**Description**
	P31947	14-3-3 protein sigma [f, m, n, p, u, w]
	M0R2R3	17-beta-hydroxysteroid dehydrogenase 14 [b, m, t, x]
	P68032	Actin, alpha cardiac muscle 1 [d, m, n, q, u, w]
	P68133	Actin, alpha skeletal muscle [b, d, m, n, q, u, w]
	P62736	Actin, aortic smooth muscle [b, d, m, n, q, u]
	P60709	Actin, cytoplasmic 1 [b, m, n, q, u, w]
	P63261	Actin, cytoplasmic 2 [a, d, g, j, n, q, u, w]
	P63267	Actin, gamma-enteric smooth muscle [b, m, n, q, u, w]

C9JKR2	Albumin, isoform CRA_k [c, m, o, u]
P04745	Alpha-amylase 1 [a, g, o, u]
P19961	Alpha-amylase 2B [a, g, o, u]
H0YMU9	Annexin [a, b, m, t, v]
Q5T3N0	Annexin (Fragment) [b, l, n, p, s, u]
P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]
P07355	Annexin A2 [a, b, g, j, o, u, v]
P04280	Basic salivary proline-rich protein 1 [b, l, o, u]
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]
Q562R1	Beta-actin-like protein 2 [b, m, n, u, w]
U3KPZ4	BLOC-1-related complex subunit 8 [b, m, t, x]
Q96HY3	CALM1 protein [m, m, t, v]
P62158	Calmodulin [f, m, n, u, v]
P27482	Calmodulin-like protein 3 [a, g, o, u]
P23280	Carbonic anhydrase 6 [a, g, o, u]
Q9BYD5	Cornifelin [a, d, m, n, w]

P04080	Cystatin-B [a, g, n, p, u]
P01036	Cystatin-S [a, g, n, p, u]
P09228	Cystatin-SA [a, g, n, p, u]
P01037	Cystatin-SN [a, g, n, p, u]
Q02928	Cytochrome P450 4A11 [a, b, m, r, u]
Q92608	Dedicator of cytokinesis protein 2 [a, d, e, m, n, q, u, w]
Q9UGM3	Deleted in malignant brain tumors 1 protein [f, m, n, o, v, w]
P61803	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit DAD1 [a, b, m, r, w]
P15515	Histatin-1 [b, i, l, o, u]
P56524	Histone deacetylase 4 [f, m, n, p, u, w]
U3KQK0	Histone H2B [b, m, p, u]
Q96A08	Histone H2B type 1-A [b, m, p, u]
P33778	Histone H2B type 1-B [b, m, p, u]
P62807	Histone H2B type 1-C/E/F/G/I [b, i, j, p, u, w]
P58876	Histone H2B type 1-D [b, m, p, u, w]
Q93079	Histone H2B type 1-H [b, m, p, u, w]

P06899	Histone H2B type 1-J [b, i, j, p, u, w]
O60814	Histone H2B type 1-K [b, i, j, p, u, w]
Q99880	Histone H2B type 1-L [b, m, p, u, w]
Q99879	Histone H2B type 1-M [b, m, p, u]
Q99877	Histone H2B type 1-N [b, m, p, u, w]
P23527	Histone H2B type 1-O [b, m, p, u, w]
Q16778	Histone H2B type 2-E [b, m, p, u, w]
Q5QNW6	Histone H2B type 2-F [b, m, p, u, w]
Q8N257	Histone H2B type 3-B [b, m, p, u, w]
P57053	Histone H2B type F-S [b, i, j, p, u, w]
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]
Q5FC05	IL6ST nirs variant 3 [b, m, t, x]
P01591	Immunoglobulin J chain [a, b, m, o, w]
P40189	Interleukin-6 receptor subunit beta [f, m, s, u, w]
P13645	Keratin, type I cytoskeletal 10 [d, m, n, o, p, s, u]

P13646	Keratin, type I cytoskeletal 13 [d, m, o, u, w]
P02533	Keratin, type I cytoskeletal 14 [d, m, o, u, w]
P19012	Keratin, type I cytoskeletal 15 [b, m, o, u, w]
P08779	Keratin, type I cytoskeletal 16 [b, m, o, u, w]
P08727	Keratin, type I cytoskeletal 19 [b, m, o, u, w]
P19013	Keratin, type II cytoskeletal 4 [d, m, q, u]
P13647	Keratin, type II cytoskeletal 5 [d, m, n, o, q, u]
P02538	Keratin, type II cytoskeletal 6A [b, d, m, o, u, w]
P04259	Keratin, type II cytoskeletal 6B [b, i, o, u, w]
P48668	Keratin, type II cytoskeletal 6C [d, m, o, u]
P31025	Lipocalin-1 [a, b, m, o, w]
P61626	Lysozyme C [a, b, g, i, j, o, u, w]
Q8TAX7	Mucin-7 [b, i, k, o, u]
H7C0Z8	MYCBP-associated protein (Fragment) [b, m, t, x]
P18615	Negative elongation factor E [b, d, e, m, p, u]
P59665	Neutrophil defensin 1 [b, i, j, o, u]

P59666	Neutrophil defensin 3 [b, i, j, o, u]
Q8WX93	Palladin [a, b, d, m, n, w]
P04746	Pancreatic alpha-amylase [a, g, o, u]
P01833	Polymeric immunoglobulin receptor [d, e, m, s, x]
Q92989	Polyribonucleotide 5'-hydroxyl-kinase Clp1 [a, b, m, p, w]
Q6S8J3	POTE ankyrin domain family member E [b, m, o, u]
A5A3E0	POTE ankyrin domain family member F [b, m, o, u]
P0CG38	POTE ankyrin domain family member I [b, m, o, u]
O94913	Pre-mRNA cleavage complex 2 protein Pcf11 [b, m, p, w]
P12273	Prolactin-inducible protein [b, d, m, o, u]
Q6MZM9	Proline-rich protein 27 [b, l, o, x]
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]
A0A1B0GVC5	Protein ABHD14A-ACY1 (Fragment) [b, m, t, x]
Q6P5S2	Protein LEG1 homolog [b, m, o, x]
Q13123	Protein Red [b, m, p, u]
P05109	Protein S100-A8 [b, e, i, j, l, n, o, q, s, u, w]

P06702	Protein S100-A9 (a, b, g, i, j, n, o, q, s, u, w)
Q5VSP4	Putative lipocalin 1-like protein 1 (b, m, o, x)
Q49AS3	Putative protein LRRC37A5P (b, m, t, x)
O94955	Rho-related BTB domain-containing protein 3 (c, m, r, u, w)
P02810	Salivary acidic proline-rich phosphoprotein ½ (b, d, h, l, o, u, v)
P02768	Serum albumin (a, b, c, g, o, u, w)
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1 (b, m, p, u, w)
P02808	Statherin (b, e, i, l, o, u)
P02814	Submaxillary gland androgen-regulated protein 3B (a, g, o, u, w)
Q9UI10	Translation initiation factor eIF-2B subunit delta (b, m, n, w)
P30408	Transmembrane 4 L6 family member 1 (b, m, s, w)
A0A087WZY1	Uncharacterized protein
Q8NA42	Zinc finger protein 383 (b, e, m, n, p, x)
Q96IQ9	Zinc finger protein 414 (b, e, m , p, u, w)
Q8TAF7	Zinc finger protein 461 (b, c, d, m, p, u)
Q8NB42	Zinc finger protein 527 (b, m, p, u)

	P25311	Zinc-alpha-2-glycoprotein [a, b, g, o, u, w]
	Q0P6G1	ZNF527 protein [b, m, r, x]

F-EGCG	**Accession**	**Description**
	P31947	14-3-3 protein sigma [f, m, n, p, u, w]
	P68032	Actin, alpha cardiac muscle 1 [d, m, n, q, u, w]
	P68133	Actin, alpha skeletal muscle [b, d, m, n, q, u, w]
	P60709	Actin, cytoplasmic 1 [b, m, n, q, u, w]
	P63261	Actin, cytoplasmic 2 [a, d, g, j, n, q, u, w]
	P63267	Actin, gamma-enteric smooth muscle [b, m, n, q, u, w]
	C9JKR2	Albumin, isoform CRA_k [c, m, o, u]
	P04745	Alpha-amylase 1 [a, g, o, u]
	P19961	Alpha-amylase 2B [a, g, o, u]
	Q5T3N0	Annexin (Fragment) [b, l, n, p, s, u]
	P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]
	P03973	Antileukoproteinase [a, b, g, i, j, o, u]

P04280	Basic salivary proline-rich protein 1 [b, l, o, u]
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]
P04080	Cystatin-B [a, g, n, p, u]
P01036	Cystatin-S [a, g, n, p, u]
P09228	Cystatin-SA [a, g, n, p, u]
P01037	Cystatin-SN [a, g, n, p, u]
P15515	Histatin-1 [b, i, l, o, u]
P15516	Histatin-3 [b, i, l, o, u]
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]
P01834	Ig kappa chain C region [b, j, o, u]
P01591	Immunoglobulin J chain [a, b, m, o, w]
P06870	Kallikrein-1 [b, m, o, p, x]
P13646	Keratin, type I cytoskeletal 13 [d, m, o, u, w]
P19012	Keratin, type I cytoskeletal 15 [b, m, o, u, w]
P08727	Keratin, type I cytoskeletal 19 [b, m, o, u, w]

P13647	Keratin, type II cytoskeletal 5 (d, m, n, o, q, u)
P02538	Keratin, type II cytoskeletal 6A (b, d, m, o, u, w)
P48668	Keratin, type II cytoskeletal 6C (d, m, o, u)
P61626	Lysozyme C (a, b, g, i, j, o, u, w)
V9GYY6	Malonyl-CoA-acyl carrier protein transacylase, mitochondrial (Fragment) (b, m, t, x)
Q8TAX7	Mucin-7 (b, i, k, o, u)
P18615	Negative elongation factor E (b, d, e, m, p, u)
P59665	Neutrophil defensin 1 (b, i, j, o, u)
P59666	Neutrophil defensin 3 (b, i, j, o, u)
E9PNR1	Oxysterol-binding protein-related protein 9 (Fragment) (b, m, n, x)
P04746	Pancreatic alpha-amylase (a, g, o, u)
P01833	Polymeric immunoglobulin receptor (d, e, m, s, x)
Q6S8J3	POTE ankyrin domain family member E (b, m, o, u)
A5A3E0	POTE ankyrin domain family member F (b, m, o, u)
P12273	Prolactin-inducible protein (b, d, m, o, u)
Q6MZM9	Proline-rich protein 27 (b, l, o, x)

A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]
P05109	Protein S100-A8 [b, e, i, j, l, n, o, q, s, u, w]
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]
P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]
P02768	Serum albumin [a, b, c, g, o, u, w]
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1 [b, m, p, u, w]
P02808	Statherin [b, e, i, l, o, u]
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]
A0A087WZY1	Uncharacterized protein
P25311	Zinc-alpha-2-glycoprotein [a, b, g, o, u, w]

Proteins were classified according to: **General Function:** a) metabolism; b) biological process; c) transport; d) structure and structural organization; e) information pathways; f) miscellanea; **Function in AP:** g) metabolism; h) tissue regeneration; i) antimicrobial; j) immune response; k) lubrication; l) biomineralization; m) unknown biological function; **Origin:** n) cytoplasm origin; o) extracellular origin; p) nucleus origin; q) cytoskeleton origin; r) intracellular origin; s) membrane origin; t) unknown protein origin; **Interaction:** u) protein/protein interaction; v) calcium/phosphate binding; w) other molecular interaction; x) unknown molecular interaction.

Table 2. Classification of protein exclusively each group in the acquired pellicle.

NF-NI	Accession	Description
	P56915	Homeobox protein goosecoid [b, d, e, m, p, u]
	Q8N9Z9	Lamin tail domain-containing protein 1 [b, d, m, n, p, u]
	O95970	Leucine-rich glioma-inactivated protein 1 [e, m, s, w]
	C9JXZ1	Sodium/potassium-transporting ATPase subunit beta-3 (Fragment) [b, c, m, s, u]
	Q92544	Transmembrane 9 superfamily member 4 [b, e, m, r, s, u]
	F2Z2L1	Transmembrane 9 superfamily member [b, m, s, x]
F-NI	**Accession**	**Description**
	Q9NQ66	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1 [f, m, n, p, u]
	P16070	CD44 antigen [b, e, m, s, u, w]
	E7EM64	COP9 signalosome complex subunit 6 [b, m, p, u]
	Q9NPB8	Glycerophosphocholine phosphodiesterase GPCPD1 [b, d, m, n, u]
	C9J7L0	Histone-binding protein RBBP7 (Fragment) [b, m, t, x]
	Q8WTQ8	Kallikrein 3, (Prostate specific antigen), isoform CRA_k [b, m, t, x]

	Q9GZM8	Nuclear distribution protein nudE-like 1 [f, m, n, u, w]
	A0A087WT80	Phosphoinositide phospholipase C [a, e, m, n, p, v]
	P78364	Polyhomeotic-like protein 1 [a, b, d, m, p, u, w]
	Q15435	Protein phosphatase 1 regulatory subunit 7 [a, b, m, p, u, w]
	K7EQ96	RING finger protein 165 (Fragment) [b, m, t, x]
	Q9NTN9	Semaphorin-4G [a, d, m, s, u]
	B5MCJ1	Tetratricopeptide repeat protein 32 [b, m, t, x]
	Q07011	Tumor necrosis factor receptor superfamily member 9 [f, m, s, u]
NF-CHX	**Accession**	**Description**
	M0R258	Zinc finger protein 432 (Fragment) [b, e, m, r, x]
F-CHX	**Accession**	**Description**
	M0QZ93	Galectin (Fragment) [a, m, t, x]
	Q9NSD7	Relaxin-3 receptor 1 [d, m, s, u]
	Q9BQY4	Rhox homeobox family member 2 [b, d, e, m, p, u]
	Q8NBW4	Sodium-coupled neutral amino acid transporter 9 [f, m, r, u, w]
	I3NI44	TOM1-like protein 1 [b, m, r, x]

	A0A0C4DGR5	Zinc finger protein 496 (Fragment) [a, b, e, m, p, x]

NF-EGCG	Accession	Description
	M0R2R3	17-beta-hydroxysteroid dehydrogenase 14 [b, m, t, x]
	P07355	Annexin A2 [a, b, g, j, o, u, v]
	H0YMU9	Annexin [a, b, m, t, v]
	Q562R1	Beta-actin-like protein 2 [b, m, n, u, w]
	Q9BYD5	Cornifelin [a, d, m, n, w]
	Q02928	Cytochrome P450 4A11 [a, b, m, r, u]
	Q92608	Dedicator of cytokinesis protein 2 [a, d, e, m, n, q, u, w]
	Q9UGM3	Deleted in malignant brain tumors 1 protein [f, m, n, o, v, w]
	P61803	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1 [a, b, m, r, w]
	P56524	Histone deacetylase 4 [f, m, n, p, u, w]
	U3KQK0	Histone H2B [b, m, p, u]
	Q96A08	Histone H2B type 1-A [b, m, p, u]
	P33778	Histone H2B type 1-B [b, m, p, u]
	P62807	Histone H2B type 1-C/E/F/G/I [b, i, j, p, u, w]

P58876	Histone H2B type 1-D [b, m, p, u, w]
Q93079	Histone H2B type 1-H [b, m, p, u, w]
P06899	Histone H2B type 1-J [b, i, j, p, u, w]
O60814	Histone H2B type 1-K [b, i, j, p, u, w]
Q99880	Histone H2B type 1-L [b, m, p, u, w]
Q99879	Histone H2B type 1-M [b, m, p, u]
Q99877	Histone H2B type 1-N [b, m, p, u, w]
P23527	Histone H2B type 1-O [b, m, p, u, w]
Q16778	Histone H2B type 2-E [b, m, p, u, w]
Q5QNW6	Histone H2B type 2-F [b, m, p, u, w]
Q8N257	Histone H2B type 3-B [b, m, p, u, w]
P57053	Histone H2B type F-S [b, i, j, p, u, w]
Q5FC05	IL6ST nirs variant 3 [b, m, t, x]
P40189	Interleukin-6 receptor subunit beta [f, m, s, u, w]
P13645	Keratin, type I cytoskeletal 10 [d, m, n, o, p, s, u]
P02533	Keratin, type I cytoskeletal 14 [d, m, o, u, w]

P08779	Keratin, type I cytoskeletal 16 [d, m, o, u, w]
P19013	Keratin, type II cytoskeletal 4 [d, m, q, u]
P04259	Keratin, type II cytoskeletal 6B [b, i, o, u, w]
P31025	Lipocalin-1 [a, b, m, o, w]
H7C0Z8	MYCBP-associated protein (Fragment) [b, m, t, x]
Q8WX93	Palladin [a, b, d, m, n, w]
Q92989	Polyribonucleotide 5'-hydroxyl-kinase Clp1 [a, b, m, p, w]
P0CG38	POTE ankyrin domain family member I [b, m, o, u]
O94913	Pre-mRNA cleavage complex 2 protein Pcf11 [b, m, p, w]
A0A1B0GVC5	Protein ABHD14A-ACY1 (Fragment) [b, m, t, x]
Q6P5S2	Protein LEG1 homolog [b, m, o, x]
Q13123	Protein Red [b, m, p, u]
Q5VSP4	Putative lipocalin 1-like protein 1 [b, m, o, x]
Q49AS3	Putative protein LRRC37A5P [b, m, t, x]
O94955	Rho-related BTB domain-containing protein 3 [c, m, r, u, w]
Q9UI10	Translation initiation factor eIF-2B subunit delta [b, m, n, w]

	P30408	Transmembrane 4 L6 family member 1 [b, m, s, w]
	Q8NA42	Zinc finger protein 383 [b, e, m, n, p, x]
	Q96IQ9	Zinc finger protein 414 [b, e, m , p, u, w]
	Q8NB42	Zinc finger protein 527 [b, m, p, u]
	Q0P6G1	ZNF527 protein [b, m, r, x]
F-EGCG	Accession	Description
	P15516	Histatin-3 [b, i, l, o, u]
	P01834	Ig kappa chain C region [b, j, o, u]
	P06870	Kallikrein-1 [b, m, o, p, x]
	V9GYY6	Malonyl-CoA-acyl carrier protein transacylase, mitochondrial (Fragment) [b, m, t, x]
	E9PNR1	Oxysterol-binding protein-related protein 9 (Fragment) [b, m, n, x]

Proteins were classified according to: **General Function:** a) metabolism; b) biological process; c) transport; d) structure and structural organization; e) information pathways; f) miscellanea; **Function in AP:** g) metabolism; h) tissue regeneration; i) antimicrobial; j) immune response; k) lubrication; l) biomineralization; m) unknown biological function; **Origin:** n) cytoplasm origin; o) extracellular origin; p) nucleus origin; q) cytoskeleton origin; r) intracellular origin; s) membrane origin; t) unknown protein origin; **Interaction:** u) protein/protein interaction; v) calcium/phosphate binding; w) other molecular interaction; x) unknown molecular interaction.

Table 3. Classification and relative quantification of proteins identified in the acquired pellicle collected from different resin.

Accession number	**Protein name: NF-NI / F-NI**	**Ratio**	**P**
A0A087WZY1	Uncharacterized protein	0,94	0,01
A0A0A0MT31	Proline-rich protein 4 (b, l, p, u)	0,94	0,03
P02808	Statherin (b, e, i, l, o, u)	1,21	0,99
P15515	Histatin-1 (b, i, l, o, u)	1,36	1,00
P01876	Ig alpha-1 chain C region (b, e, i, j, o, u)	1,42	1,00
P01877	Ig alpha-2 chain C region (b, e, i, j, o, u)	1,43	1,00
P06702	Protein S100-A9 (a, b, g, i, j, n, o, q, s, u, w)	1,51	1,00
P61626	Lysozyme C (a, b, g, i, j, o, u, w)	1,55	0,96
P25311	Zinc-alpha-2-glycoprotein (a, b, g, o, u, w)	1,62	0,97
P04745	Alpha-amylase 1 (a, g, o, u)	1,63	1,00
Q8TAX7	Mucin-7 (b, i, k, o, u)	1,79	1,00
P01037	Cystatin-SN (a, b, g, o, u)	1,80	1,00
P02814	Submaxillary gland androgen-regulated protein 3B (a,g,o,u,w)	1,80	1,00

P01036	Cystatin-S [a, b, g, o, u]	1,92	1,00
P09228	Cystatin-SA [a, b, g, o, u]	1,93	1,00
P12273	Prolactin-inducible protein [b, d, m, o, u]	2,18	1,00

Accession number	**Protein name: NF-CHX / F-CHX**	**Ratio**	**P**
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]	0,92	0,00
P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]	0,92	0,01
A0A087WZY1	Uncharacterized protein	0,92	0,01
P01037	Cystatin-SN [a, b, g, o, u]	1,12	0,98
P09228	Cystatin-SA [a, b, g, o, u]	1,16	0,98
P01036	Cystatin-S [a, g, n, p, u]	1,20	0,99
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]	1,28	1,00
P04792	Heat shock protein beta-1 [b, d, m, n, p, q, u, w]	1,32	0,96
Q8TAX7	Mucin-7 [b, i, k, o, u]	1,34	1,00
P02808	Statherin [b, e, i, l, o, u]	1,54	1,00
P12273	Prolactin-inducible protein [b, d, m, o, u]	1,55	1,00

P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	1,57	1,00
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]	1,88	1,00
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]	1,88	1,00

Accession number	**Protein name: NF-EGCG/F-EGCG**	**Ratio**	**P**
P04792	Heat shock protein beta-1 [b, d, m, n, p, q, u, w]	0,59	0,00
P15515	Histatin-1 [b, i, l, o, u]	0,66	0,00
P13646	Keratin, type I cytoskeletal 13 [d, m, o, p, q, u]	0,64	0,00
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]	0,74	0,00
P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]	0,74	0,00
A0A087WZY1	Uncharacterized protein	0,73	0,00
P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]	0,79	0,01
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]	0,88	0,01
P04080	Cystatin-B [a, g, n, p, u]	0,81	0,04
P02808	Statherin [b, e, i, l, o, u]	1,23	1,00
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	1,32	1,00

Accession number	Protein name: NF-NI/NF-CHX	Ratio	P
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]	0,77	0
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]	0,78	0
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	0,62	0
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]	0,90	0,02
P09228	Cystatin-SA [a, b, g, o, u]	1,20	0,97
P61626	Lysozyme C [a, b, g, i, j, o, u, w]	1,52	0,97
P01037	Cystatin-SN [a, b, g, o, u]	1,13	0,98
P01036	Cystatin-S [a, b, g, o, u]	1,17	0,99
Q8TAX7	Mucin-7 [b, i, k, o, u]	1,68	1
Accession number	**Protein name: NF-NI/NF-EGCG**	**Ratio**	**P**
P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]	0,82	0,00
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]	0,83	0,00
A0A087WZY1	Uncharacterized protein	0,83	0,00

P02808	Statherin [b, e, i, l, o, u]	0,85	0,01
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]	1,19	0,99
P01037	Cystatin-SN [a, b, g, o, u]	1,65	1,00
P12273	Prolactin-inducible protein [b, d, m, o, u]	1,75	1,00
P61626	Lysozyme C [a, b, g, i, j, o, u, w]	1,77	0,97
P04280	Basic salivary proline-rich protein 1 [b, l, o, u]	1,88	1,00
P19961	Alpha-amylase 2B [a, g, o, u]	1,92	0,99
P25311	Zinc-alpha-2-glycoprotein [a, b, g, o, u, w]	1,92	0,99
P01036	Cystatin-S [a, b, g, o, u]	1,92	1,00
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]	1,97	1,00
P09228	Cystatin-SA [a, b, g, o, u]	1,99	1,00
P04745	Alpha-amylase 1 [a, g, o, u]	2,08	1,00
P04792	Heat shock protein beta-1 [b, d, m, n, p, q, u, w]	2,29	1,00
P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]	3,03	1,00
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	3,16	1,00

Accession number	Protein name: NF-EGCG/NF-CHX	Ratio	P
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]	1,17	1
P01036	Cystatin-S [a, g, n, p, u]	1,63	1
P09228	Cystatin-SA [a, g, n, p, u]	1,64	1
P01037	Cystatin-SN [a, g, n, p, u]	1,46	1
P04792	Heat shock protein beta-1 [b, d, m, n, p, q, u, w]	1,95	1
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]	1,40	1
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]	1,43	1
P12273	Prolactin-inducible protein [b, d, m, o, u]	2,31	1
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]	2,20	1
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	5,10	1
P04280	Basic salivary proline-rich protein 1 [b, l, o, u]	1,93	0,98
P02808	Statherin [b, e, i, l, o, u]	0,86	0,01
Q8TAX7	Mucin-7 [b, i, k, o, u]	0,60	0
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]	0,86	0

P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]	0,86	0
A0A087WZY1	Uncharacterized protein	0,86	0

Accession number	**Protein name: F-NI/F-CHX**	**Ratio**	**P**
P01036	Cystatin-S [a, b, g, o, u]	0,73	0
P09228	Cystatin-SA [a, b, g, o, u]	0,73	0
P01037	Cystatin-SN [a, b, g, o, u]	0,70	0
P12273	Prolactin-inducible protein [b, d, m, o, u]	0,54	0
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]	0,76	0
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	0,54	0
A0A087WZY1	Uncharacterized protein	0,93	0
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]	0,93	0,02
P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]	0,93	0,02
P18615	Negative elongation factor E [b, d, e, m, p, u]	0,23	0,03
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]	1,11	1
Q8TAX7	Mucin-7 [b, i, k, o, u]	1,26	1

Accession number	**Protein name: F-NI/F-EGCG**	**Ratio**	**P**
P03973	Antileukoproteinase [a, b, g, i, j, o, u]	0,41	0,03
P15515	Histatin-1 [b, i, l, o, u]	0,54	0,00
P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]	0,64	0,00
A0A087WZY1	Uncharacterized protein	0,64	0,00
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]	0,65	0,00
Q8TAX7	Mucin-7 [b, i, k, o, u]	0,73	0,00
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]	0,81	0,00
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]	0,85	0,00
P02808	Statherin [b, e, i, l, o, u]	0,87	0,04
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]	1,09	0,99
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]	1,35	1,00
P61626	Lysozyme C [a, b, g, i, j, o, u, w]	1,45	0,96
P04280	Basic salivary proline-rich protein 1 [b, l, o, u]	1,48	1,00
P04080	Cystatin-B [a, g, n, p, u]	1,52	0,96

Q5T3N0	Annexin (Fragment) [b, l, n, p, s, u]	1,73	1,00
P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]	1,77	1,00
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	2,32	1,00

Accession number	**Protein name: F-EGCG/F-CHX**	**Ratio**	**P**
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	4,35	1,00
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]	1,75	1,00
Q5T3N0	Annexin (Fragment) [b, l, n, p, s, u]	1,65	0,99
P12273	Prolactin-inducible protein [b, d, m, o, u]	1,57	1,00
P25311	Zinc-alpha-2-glycoprotein [a, b, g, o, u, w]	1,48	1,00
P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]	1,48	0,98
P09228	Cystatin-SA [a, b, g, o, u]	1,43	1,00
P19961	Alpha-amylase 2B [a, g, o, u]	1,43	0,98
P04280	Basic salivary proline-rich protein 1 [b, l, o, u]	1,42	0,99
P01036	Cystatin-S [a, g, n, p, u]	1,35	1,00
P01037	Cystatin-SN [a, g, n, p, u]	1,35	1,00

P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]	0,84	0,01
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]	0,79	0,00
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]	0,69	0,00
P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]	0,69	0,00
P02808	Statherin [b, e, i, l, o, u]	0,69	0,00
A0A087WZY1	Uncharacterized protein	0,69	0,00
Q8TAX7	Mucin-7 [b, i, k, o, u]	0,58	0,00
P15515	Histatin-1 [b, i, l, o, u]	0,54	0,00
Q6MZM9	Proline-rich protein 27 [b, l, o, x]	0,39	0,00

Proteins were classified according to: **General Function:** a) metabolism; b) biological process; c) transport; d) structure and structural organization; e) information pathways; f) miscellanea; **Function in AP:** g) metabolism; h) tissue regeneration; i) antimicrobial; j) immune response; k) lubrication; l) biomineralization; m) unknown biological function; **Origin:** n) cytoplasm origin; o) extracellular origin; p) nucleus origin; q) cytoskeleton origin; r) intracellular origin; s) membrane origin; t) unknown protein origin; **Interaction:** u) protein/protein interaction; v) calcium/phosphate binding; w) other molecular interaction; x) unknown molecular interaction.

Table S1. Classification of identified proteins from the acquired pellicle collected *in situ* from represented in each group. (1) **NF-NI**, (2) **F-NI**, (3) **NF-CHX**, (4) **F-CHX**, (5) **NF-EGCG** and (6) **F-EGCG**.

Accession number	Protein Name	1	2	3	4	5	6
P31947	14-3-3 protein sigma [f, m, n, p, u, w]	-	-	-	-	Yes	Yes
M0R2R3	17-beta-hydroxysteroid dehydrogenase 14 [b, m, t, x]	-	-	-	-	Yes	-
Q9NQ66	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1 [f, m, n, p, u]	-	Yes	-	-	-	-
P68032	Actin, alpha cardiac muscle 1 [d, m, n, q, u, w]	-	-	-	-	Yes	Yes
P68133	Actin, alpha skeletal muscle [b, d, m, n, q, u, w]	-	-	-	-	Yes	Yes
P62736	Actin, aortic smooth muscle [b, d, m, n, q, u]	-	-	-	-	Yes	-
P60709	Actin, cytoplasmic 1 [b, d, m, n, q, u]	-	Yes	-	-	Yes	Yes
P63261	Actin, cytoplasmic 2 [a, d, g, j, n, q, u, w]	-	Yes	-	-	Yes	Yes
P63267	Actin, gamma-enteric smooth muscle [b, m, n, q, u, w]	-	-	-	-	Yes	Yes
C9JKR2	Albumin, isoform CRA_k [c, m, o, u]	-	-	-	-	Yes	Yes
P04745	Alpha-amylase 1 [a, g, o, u]	Yes	Yes	-	Yes	Yes	Yes
P19961	Alpha-amylase 2B [a, g, o, u]	Yes	Yes	-	Yes	Yes	Yes
H0YMU9	Annexin [a, b, m, t, v]	-	-	-	-	Yes	-
Q5T3N0	Annexin (Fragment) [b, l, n, p, s, u]	-	Yes	-	Yes	Yes	Yes
P04083	Annexin A1 [a, b, g, j, n, o, p, s, u, w]	Yes	Yes	-	Yes	Yes	Yes

P07355	Annexin A2 [a, b, g, j, o, u, v]	-	-	-	-	Yes	-
P03973	Antileukoproteinase [a, b, g, i, j, o, u]	-	Yes	-	-	-	Yes
P04280	Basic salivary proline-rich protein 1 [b, l, o, u]	Yes	Yes	Yes	Yes	Yes	Yes
P02812	Basic salivary proline-rich protein 2 [b, l, o, u]	Yes	Yes	Yes	Yes	Yes	Yes
Q562R1	Beta-actin-like protein 2 [b, m, n, u, w]	-	-	-	-	Yes	-
U3KPZ4	BLOC-1-related complex subunit 8 [b, m, t, x]	-	-	-	Yes	Yes	-
Q96HY3	CALM1 protein [m, m, t, v]	-	-	-	Yes	Yes	-
P62158	Calmodulin [f, m, n, u, v]	-	-	-	Yes	Yes	-
P27482	Calmodulin-like protein 3 [a, g, o, u]	-	Yes	-	-	Yes	-
P23280	Carbonic anhydrase 6 [a, g, o, u]	-	Yes	-	-	Yes	-
P16070	CD44 antigen [b, e, m, s, u, w]	-	Yes	-	-	-	-
E7EM64	COP9 signalosome complex subunit 6 [b, m, p, u]	-	Yes	-	-	-	-
Q9BYD5	Cornifelin [a, d, m, n, w]	-	-	-	-	Yes	-
P04080	Cystatin-B [a, g, n, p, u]	-	Yes	-	-	Yes	Yes
P01036	Cystatin-S [a, g, n, p, u]	Yes	Yes	Yes	Yes	Yes	Yes
P09228	Cystatin-AS [a, g, n, p, u]	Yes	Yes	Yes	Yes	Yes	Yes
P01037	Cystatin-SN [a, g, n, p, u]	Yes	Yes	Yes	Yes	Yes	Yes
Q02928	Cytochrome P450 4A11 [a, b, m, r, u]	-	-	-	-	Yes	-
Q92608	Dedicator of cytokinesis protein 2 [a, d, e, m, n, q, u, w]	-	-	-	-	Yes	-

Q9UGM3	Deleted in malignant brain tumors 1 protein [f, m, n, o, v, w]	-	-	-	-	Yes	-
P61803	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1 [a, b, m, r, w]	-	-	-	-	Yes	-
M0QZ93	Galectin (Fragment) [a, m, t, x]	-	-	-	Yes	-	-
Q9NPB8	Glycerophosphocholine phosphodiesterase GPCPD1 [b, d, m, n, u]	-	Yes	-	-	-	-
P04792	Heat shock protein beta-1 [b, d, m, n, p, q, u, w]	Yes	-	Yes	-	-	-
P15515	Histatin-1 [b, i, l, o, u]	Yes	Yes	Yes	Yes	Yes	Yes
P15516	Histatin-3 [b, i, l, o, u]	-	-	-	-	-	Yes
P56524	Histone deacetylase 4 [f, m, n, p, u, w]	-	-	-	-	Yes	-
U3KQK0	Histone H2B [b, m, p, u]	-	-	-	-	Yes	-
Q96A08	Histone H2B type 1-A [b, m, p, u]	-	-	-	-	Yes	-
P33778	Histone H2B type 1-B [b, m, p, u]	-	-	-	-	Yes	-
P62807	Histone H2B type 1-C/E/F/G/I [b, i, j, p, u, w]	-	-	-	-	Yes	-
P58876	Histone H2B type 1-D [b, i, j, p, u, w]	-	-	-	-	Yes	-
Q93079	Histone H2B type 1-H [b, i, j, p, u, w]	-	-	-	-	Yes	-
P06899	Histone H2B type 1-J [b, i, j, p, u, w]	-	-	-	-	Yes	-
O60814	Histone H2B type 1-K [b, i, j, p, u, w]	-	-	-	-	Yes	-
Q99880	Histone H2B type 1-L [b, i, j, p, u, w]	-	-	-	-	Yes	-

Q99879	Histone H2B type 1-M [b, m, p, u]	-	-	-	-	Yes	-
Q99877	Histone H2B type 1-N [b, m, p, u, w]	-	-	-	-	Yes	-
P23527	Histone H2B type 1-O [b, m, p, u, w]	-	-	-	-	Yes	-
Q16778	Histone H2B type 2-E [b, m, p, u, w]	-	-	-	-	Yes	-
Q5QNW6	Histone H2B type 2-F [b, m, p, u, w]	-	-	-	-	Yes	-
Q8N257	Histone H2B type 3-B [b, m, p, u, w]	-	-	-	-	Yes	-
P57053	Histone H2B type F-S [b, i, j, p, u, w]	-	-	-	-	Yes	-
C9J7L0	Histone-binding protein RBBP7 (Fragment) [b, m, t, x]	-	Yes	-	-	-	-
P56915	Homeobox protein goosecoid [b, d, e, m, p, u]	Yes	-	-	-	-	-
P01876	Ig alpha-1 chain C region [b, e, i, j, o, u]	Yes	Yes	Yes	Yes	Yes	Yes
P01877	Ig alpha-2 chain C region [b, e, i, j, o, u]	Yes	-	Yes	Yes	Yes	Yes
P01834	Ig kappa chain C region [b, j, o, u]	-	-	-	-	-	Yes
Q5FC05	IL6ST nirs variant 3 [b, m, t, x]	-	-	-	-	Yes	-
P01591	Immunoglobulin J chain [a, b, m, o, w]	-	-	-	-	Yes	Yes
P40189	Interleukin-6 receptor subunit beta [f, m, s, u, w]	-	-	-	-	Yes	-
Q8WTQ8	Kallikrein 3, (Prostate specific antigen), isoform CRA_k [b, m, t, x]	-	Yes	-	-	-	-
P06870	Kallikrein-1 [b, m, o, p, x]	-	-	-	-	-	Yes
P13645	Keratin, type I cytoskeletal 10 [d, m, n, o, p, s, u]	-	-	-	-	Yes	-
P13646	Keratin, type I cytoskeletal 13 [d, m, o, u, w]	-	Yes	-	-	Yes	Yes

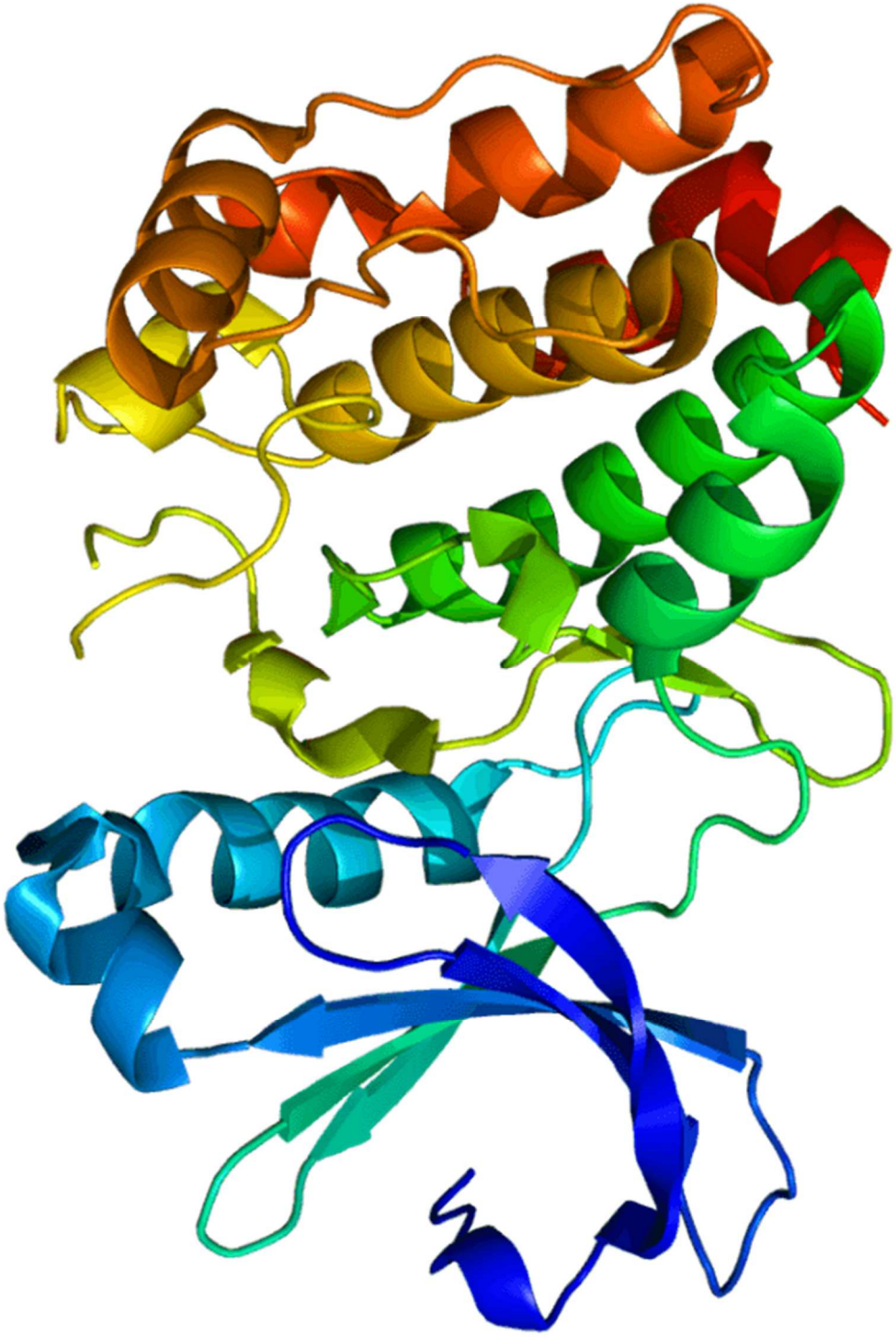
P02533	Keratin, type I cytoskeletal 14 [d, m, o, u, w]	-	-	-	-	Yes	-
P19012	Keratin, type I cytoskeletal 15 [d, m, o, u, w]	-	-	-	-	Yes	Yes
P08779	Keratin, type I cytoskeletal 16 [d, m, o, u, w]	-	-	-	-	Yes	-
P08727	Keratin, type I cytoskeletal 19 [d, m, o, u, w]	-	-	-	-	Yes	Yes
P19013	Keratin, type II cytoskeletal 4 [d, m, q, u]	-	-	-	-	Yes	-
P13647	Keratin, type II cytoskeletal 5 [d, m, n, o, q, u]	-	-	-	-	Yes	Yes
P02538	Keratin, type II cytoskeletal 6A [b, d, m, o, u, w]	-	-	-	-	Yes	Yes
P04259	Keratin, type II cytoskeletal 6B [b, i, o, u, w]	-	-	-	-	Yes	-
P48668	Keratin, type II cytoskeletal 6C [d, m, o, u]	-	-	-	-	Yes	Yes
Q8N9Z9	Lamin tail domain-containing protein 1 [b, d, m, n, p, u]	Yes	-	-	-	-	-
O95970	Leucine-rich glioma-inactivated protein 1 [e, m, s, w]	Yes	-	-	-	-	-
P31025	Lipocalin-1 [a, b, m, o, w]	-	-	-	-	Yes	-
P61626	Lysozyme C [a, b, g, i, j, o, u, w]	Yes	Yes	Yes	Yes	Yes	Yes
V9GYY6	Malonyl-CoA-acyl carrier protein transacylase, mitochondrial (Fragment) [b, m, t, x]	-	-	-	-	-	Yes
Q8TAX7	Mucin-7 [b, i, k, o, u]	Yes	Yes	Yes	Yes	Yes	Yes
H7C0Z8	MYCBP-associated protein (Fragment) [b, m, t, x]	-	-	-		Yes	-
P18615	Negative elongation factor E [b, d, e, m, p, u]	Yes	Yes	-	Yes	Yes	Yes
P59665	Neutrophil defensin 1 [b, i, j, o, u]	-	Yes	-	Yes	Yes	Yes

P59666	Neutrophil defensin 3 [b, i, j, o, u]	-	Yes	-	Yes	Yes	Yes
Q9GZM8	Nuclear distribution protein nudE-like 1 [f, m, n, u, w]	-	Yes	-	-	-	-
E9PNR1	Oxysterol-binding protein-related protein 9 (Fragment) [b, m, n, x]	-	-	-	-	-	Yes
Q8WX93	Palladin [a, b, d, m, n, w]	-	-	-	-	Yes	-
P04746	Pancreatic alpha-amylase [a, g, o, u]	Yes	Yes	-	Yes	Yes	Yes
A0A087WT80	Phosphoinositide phospholipase C [a, e, m, n, p, v]	-	Yes	-	-	-	-
P78364	Polyhomeotic-like protein 1 [a, b, d, m, p, u, w]	-	Yes	-	-	-	-
P01833	Polymeric immunoglobulin receptor [d, e, m, s, x]	-	-	-	-	Yes	Yes
Q92989	Polyribonucleotide 5'-hydroxyl-kinase Clp1 [a, b, m, p, w]	-	-	-	-	Yes	-
Q6S8J3	POTE ankyrin domain family member E [b, m, o, u]	-	-	-	-	Yes	Yes
A5A3E0	POTE ankyrin domain family member F [b, m, o, u]	-	-	-	-	Yes	Yes
P0CG38	POTE ankyrin domain family member I [b, m, o, u]	-	-	-	-	Yes	-
O94913	Pre-mRNA cleavage complex 2 protein Pcf11 [b, m, p, w]	-	-	-	-	Yes	-
P12273	Prolactin-inducible protein [b, d, m, o, u]	Yes	Yes	Yes	Yes	Yes	Yes
Q6MZM9	Proline-rich protein 27 [b, l, o, x]	Yes	Yes		Yes	Yes	Yes
A0A0A0MT31	Proline-rich protein 4 [b, l, p, u]	Yes	Yes	Yes	Yes	Yes	Yes
A0A1B0GVC5	Protein ABHD14A-ACY1 (Fragment) [b, m, t, x]	-	-	-	-	Yes	-
Q6P5S2	Protein LEG1 homolog [b, m, o, x]	-	-	-	-	Yes	-
Q15435	Protein phosphatase 1 regulatory subunit 7 [a, b, m, p, u, w]	-	Yes	-	-	-	-

Q13123	Protein Red [b, m, p, u]	-	-	-	-	Yes	
P05109	Protein S100-A8 [b, e, i, j, l, n, o, q, s, u, w]	-	Yes	-	Yes	Yes	Yes
P06702	Protein S100-A9 [a, b, g, i, j, n, o, q, s, u, w]	Yes	Yes	Yes	Yes	Yes	Yes
Q5VSP4	Putative lipocalin 1-like protein 1 [b, m, o, x]	-	-	-	-	Yes	-
Q49AS3	Putative protein LRRC37A5P [b, m, t, x]	-	-	-	-	Yes	-
Q9NSD7	Relaxin-3 receptor 1 [d, m, s, u]	-	-	-	Yes	-	-
O94955	Rho-related BTB domain-containing protein 3 [c, m, r, u, w]	-	-	-	-	Yes	-
Q9BQY4	Rhox homeobox family member 2 [b, d, e, m, p, u]	-	-	-	Yes	-	-
K7EQ96	RING finger protein 165 (Fragment) [b, m, t, x]	-	Yes		-	-	-
P02810	Salivary acidic proline-rich phosphoprotein ½ [b, d, h, l, o, u, v]	Yes	Yes	Yes	Yes	Yes	Yes
Q9NTN9	Semaphorin-4G [a, d, m, s, u]	-	Yes	-	-	-	-
P02768	Serum albumin [a, b, c, g, o, u, w]	-	Yes	-	-	Yes	Yes
C9JXZ1	Sodium/potassium-transporting ATPase subunit beta-3 (Fragment) [b, c, m, s, u]	Yes	-	-	-	-	-
Q8NBW4	Sodium-coupled neutral amino acid transporter 9 [f, m, r, u, w]	-	-	-	Yes	-	-
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1 [b, m, p, u, w]	-	Yes	-	Yes	Yes	Yes
P02808	Statherin [b, e, i, l, o, u]	Yes	Yes	Yes	Yes	Yes	Yes
P02814	Submaxillary gland androgen-regulated protein 3B [a, g, o, u, w]	Yes	Yes	Yes	Yes	Yes	Yes
B5MCJ1	Tetratricopeptide repeat protein 32 [b, m, t, x]	-	Yes	-	-	-	-

I3NI44	TOM1-like protein 1 [b, m, r, x]	-	-	-	Yes	-	-
Q9UI10	Translation initiation factor eIF-2B subunit delta [b, m, n, w]	-	-	-	-	Yes	-
P30408	Transmembrane 4 L6 family member 1 [b, m, s, w]	-	-	-	-	Yes	-
F2Z2L1	Transmembrane 9 superfamily member [b, m, s, x]	Yes	-	-	-	-	-
Q92544	Transmembrane 9 superfamily member 4 [b, e, m, r, s, u]	Yes	-	-	-	-	-
Q07011	Tumor necrosis factor receptor superfamily member 9 [f, m, s, u]	-	Yes	-	-	-	-
A0A087WZY1	Uncharacterized protein	Yes	Yes	Yes	Yes	Yes	Yes
Q8NA42	Zinc finger protein 383 [b, e, m, n, p, x]	-	-	-	-	Yes	-
Q96IQ9	Zinc finger protein 414 [b, e, m , p, u, w]	-	-	-	-	Yes	-
M0R258	Zinc finger protein 432 (Fragment) [b, e, m, r, x]	-	-	Yes	-	-	-
Q8TAF7	Zinc finger protein 461 [b, c, d, m, p, u]	-	Yes	-	-	Yes	-
A0A0C4DGR5	Zinc finger protein 496 (Fragment) [a, b, e, m, p, x]	-	-	-	Yes	-	-
Q8NB42	Zinc finger protein 527 [b, m, p, u]	-	-	-	-	Yes	-
P25311	Zinc-alpha-2-glycoprotein [a, b, g, o, u, w]	Yes	Yes	-	Yes	Yes	Yes
Q0P6G1	ZNF527 protein [b, m, r, x]	-	-	-	-	Yes	-

Proteins were classified according to: **General Function:** a) metabolism; b) biological process; c) transport; d) structure and structural organization; e) information pathways; f) miscellanea; **Function in AP:** g) metabolism; h) tissue regeneration; i) antimicrobial; j) immune response; k) lubrication; l) biomineralization; m) unknown biological function; **Origin:** n) cytoplasm origin; o) extracellular origin; p) nucleus origin; q) cytoskeleton origin; r) intracellular origin; s) membrane origin; t) unknown protein origin; **Interaction:** u) protein/protein interaction; v) calcium/phosphate binding; w) other molecular interaction; x) unknown molecular interaction.



3-Discussion

3 DISCUSSION

One of the challenges of this study was to obtain an appropriate amount of proteins in the AP to allow proper identification and quantitation by mass spectrometry, since in *in situ* studies the quantity found is typically lower. In order to overcome this problem, we developed a customized removable mandibular apparatus (called Bauru *in situ* pellicle model - BISPM) with a special design in order to optimize the collection of enough amount of proteins from the AP. The most important characteristic of this apparatus was the existence of an orthodontics wire that was suspended above the specimens, to avoid direct contact of the mucosa with them thus allowing proper space for AP formation. In addition, the volunteers used the device for 9 consecutive days and the samples collected from the same treatments were pooled. We opted for not using a palatal apparatus because in the mandibular area there is a greater amount of saliva due to the location of the salivary glands, favoring the formation of the AP. In fact, the amount of protein obtained was greater than expected and needed. Considering the results, we can suggest the use of a lower number of specimens and/or less collection days, since this might be enough to obtain sufficient amount of proteins to be analyzed by mass spectrometry and will make easier the execution of future studies.

The volunteers had a very good rate of acceptance of the removable device, since there was no kind of complaint or discomfort. Concerning the number of days, the choice of nine days was due to the necessity of repositioning the specimens on the three regions of the apparatus (posterior, medium and anterior). It was already reported that there are differences in the formation of the AP in function of its location in the oral cavity (VENTURA et al., 2017). The choice to conduct the study *in situ*, was because it would be practically impossible to find patients with necessity of restorations large enough to allow AP collection in vivo. It is worth mentioning that the resins were placed on the bovine enamel specimens to simulate as close as possible the effects *in vivo*. The AP was collected from the experimental resin specimens and from the enamel borders surrounding the resin.

We expected that the addition of filler would increase the surface roughness of the resins, thus allowing more proteins to adsorb onto the surface, which would augment the number of proteins in the AP. This happened exclusively for NI and CHX

groups. For the EGCG group, the addition of filler in fact reduced the number of identified proteins, which was greatest for the NF-EGCG group among all the groups evaluated. In the NF-EGCG group, several proteins with intracellular functions were identified, such as Histones and Keratin, with functions still unknown in the AP. On the other hand, the addition of filler in the NI and CHX groups increased proteins with important protective functions in the teeth and oral cavity, such as Statherin (CARPENTER et al., 2014), Protein S100-A9 (DELECRODE et al., 2015b), Mucin-7 (DAWES et al., 2015) and isoforms of Cystatin (DELECRODE et al., 2015b).

A total of 140 proteins were identified in the AP. From these 16 were found in all the groups, among which are many proteins typically found in the AP. The total number of proteins identified in each group were 31, 51, 18, 38, 106 and 54 for NF-NI, F-NI, NF-CHX, F-CHX, NF-EGCG and F-EGCG, respectively (Table 1). This amount is in-line with *in vivo* studies in the literature (DELECRODE et al., 2015b; LEE et al., 2013; SIQUEIRA et al., 2007; ZIMMERMAN et al., 2013). These results demonstrate that our protein extraction protocol is appropriate to simulate the *in vivo* formed AP.

A very interesting result was the presence of the protein *Histatin-3* that was identified only in the F-EGCG group. His3-(20-43)-peptide is a potent inhibitor of metalloproteinases (MMP-2 and MMP-9). In addition, this protein presents other functions, such as t antibacterial and antifungal activities. His3-(20-43)-peptide (*histatin-5*) is especially effective against *C.albicans* and *C.neoformans*, and inhibits *Lys-gingipain* and *Arg-gingipain* (rgpB) from *P.gingivalis* (UNIPROT).

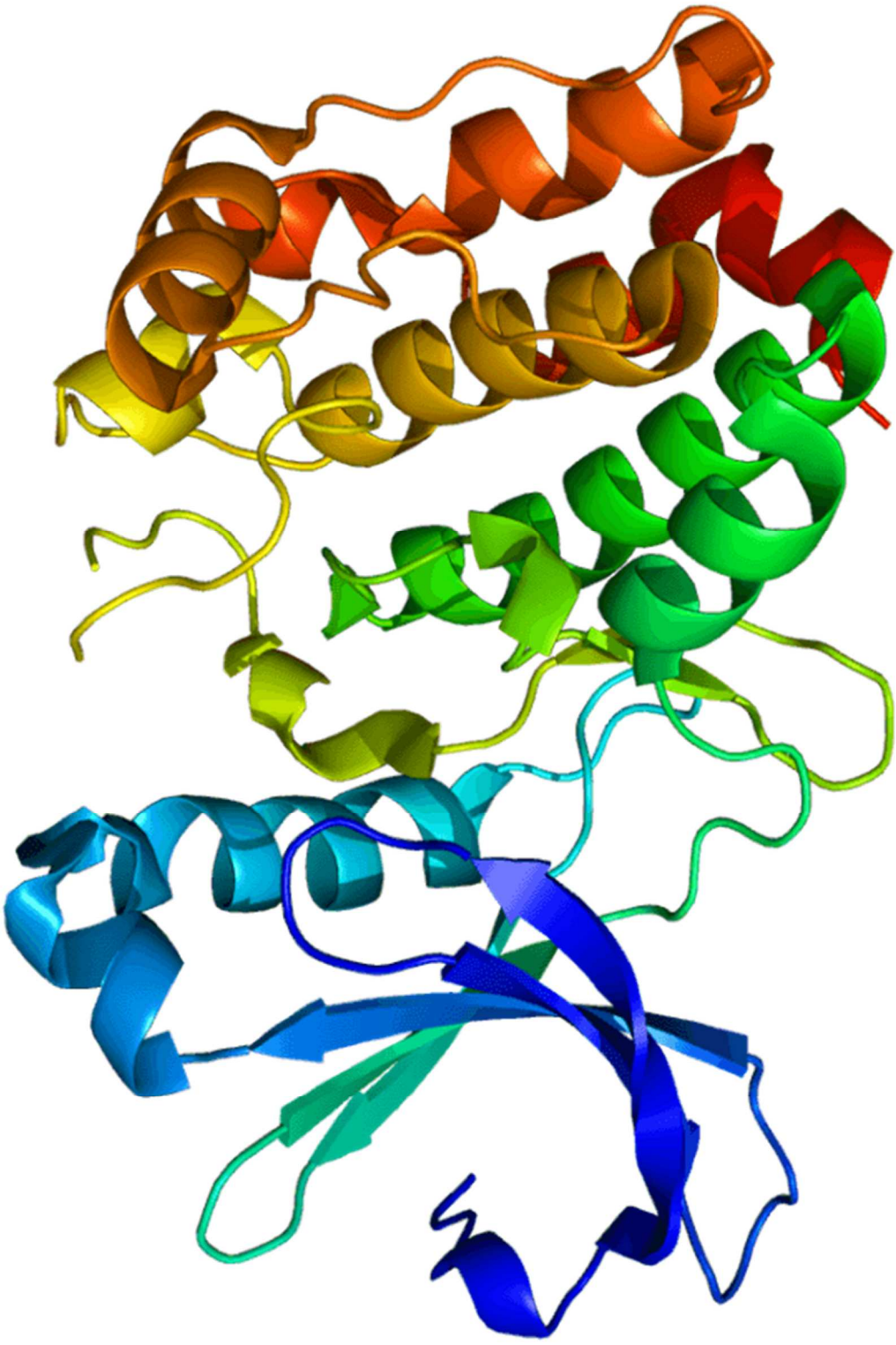
In the comparison between the groups without filler, it was possible to observe that the addition of inhibitors in general increased several protective proteins in respect to control, such as Cystatin (antimicrobial and acid resistant) (DELECRODE et al., 2015), Lysozyme (antyimicrobial) and Mucin (lubrication) (DAWES et al., 2015), regardless of the inhibitor used.

The comparisons containing filler are more relevant from the clinical point of view. The F-EGCG group was the one that presented the greatest increase of protective proteins, such as isoforms of Basic salivary proline-rich proteins, *Protein S100-A9, Lysozyme C, Cystatin-B, 2 isoforms of Annexin and Submaxillary gland androgen-regulated protein 3B in respect* to control (F-NI).

A classical antimicrobial protein that was recently categorized as acid-resistant and was also increased in the AP formed on F-EGCG specimens was *Cystatin B*. This protein was shown to be increased 20 fold in the AP formed in vivo after challenge with 1% citric acid. In addition, it was also increased 13 fold when the AP was challenged with 0.1 M lactic acid (DELECRODE et al., 2015), which highlights its potential to protect against caries and erosion. In fact, purified polyphenols, such as EGCG promptly adsorb onto the pellicle. This reduces the degree of pellicle elution by different solutions (JOINER et al., 2004). It is possible that the greater resistance of the AP treated with EGCG might be due to increase in protective proteins, such as those identified in the present studies and specially the acid-resistant ones.

Several proteins identified deserve highlighting, such as *S100A8,* a calcium- and zinc-binding protein, which plays a prominent role in the regulation of inflammatory processes and immune response. *Lysozyme* has primarily a bacteriolytic function. The isoforms present in tissues and body fluids are associated with the monocyte-macrophage system and enhance the activity of immune agents. *Mucin-7* plays a protective role, since it promotes the clearance of bacteria in the oral cavity. In addition, it helps in mastication, speech, and swallowing due to its lubrication capacity. It is also a n intracellular thiol proteinase inhibitor. (UNIPROT).

In conclusion, this study demonstrated that the addition of filler was important mainly for the CHX-containing composite, where an increase in *Statherin* was observed in the AP. In addition, the BISPM revealed to be a good device to be used in studies involving proteomic analysis of the AP. These results demonstrate that there was a difference in the protein profile of the acquired pellicle due to the composition of the experimental resins, offering important information for the development of restorative materials with components that can increase the protection in the oral cavity.



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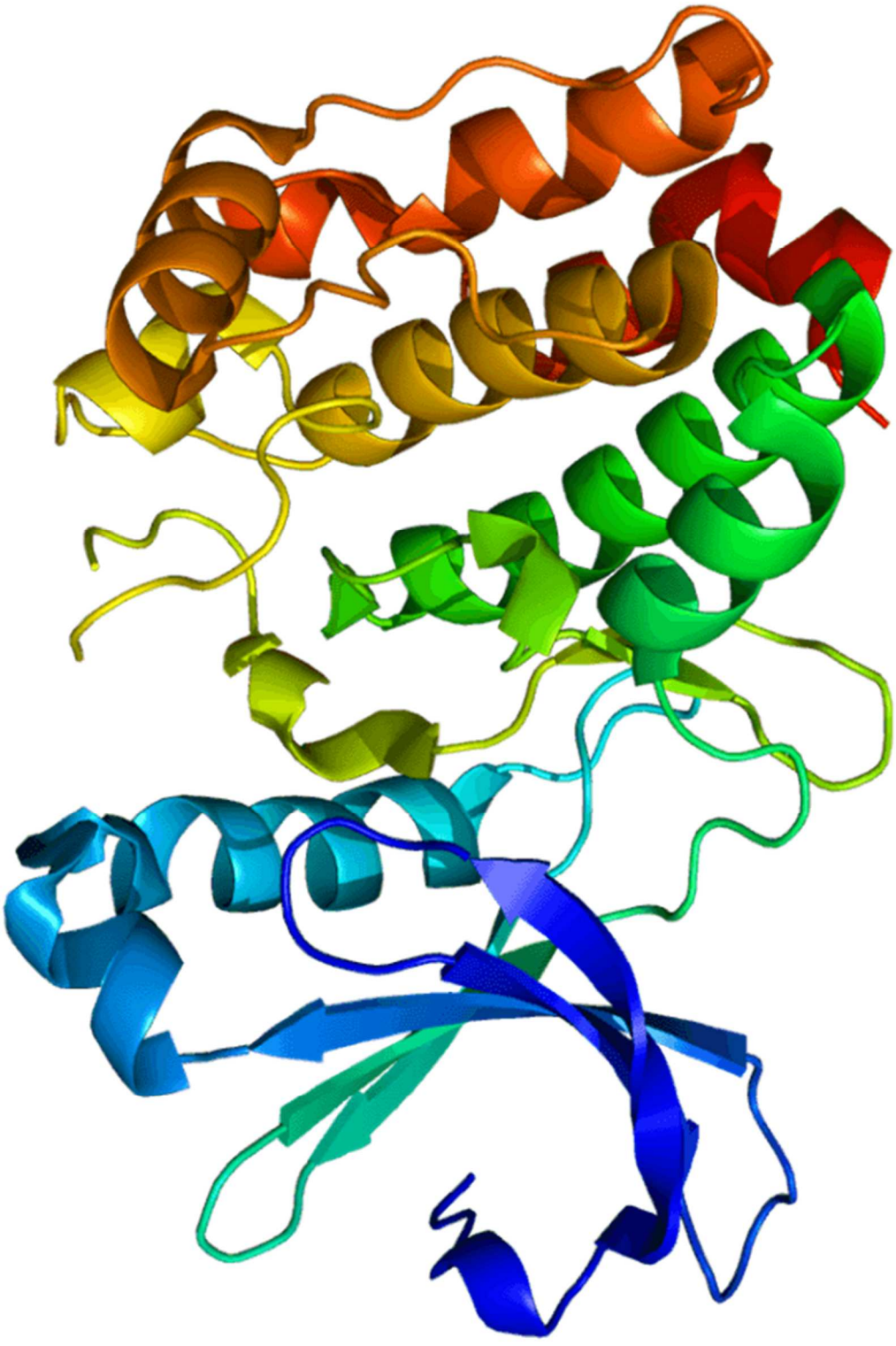

Annex

ANNEX 1

Universidade de São Paulo
Faculdade de Odontologia de Bauru
Departamento de Ciências Biológicas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Convido você estudante da Pós-Graduação desta Faculdade a participar do trabalho experimental intitulado "Análise proteômica da película adquirida formada sobre resinas compostas experimentais contendo ou não carga e/ou inibidores de proteases: estudo in situ". Você deverá comparecer ao laboratório de Bioquímica da Faculdade de Odontologia de Bauru – USP, no dia que será decidido pelo pesquisador que entrará em contato com você um dia antes da coleta da película adquirida. Além disso, o pesquisador estará disponível a qualquer momento para sanar possíveis dúvidas.

O objetivo deste trabalho será avaliar a influência da adição ou não de carga (vidro de bário alumina silicato e sílica) e/ou de inibidores de proteases (EGCG ou CHX) a resinas compostas experimentais no perfil proteico da película adquirida formada sobre estes espécimes de resinas experimentais, utilizando estratégias proteômicas quantitativas livres de marcadores.

Para sua participação na pesquisa, você receberá uma limpeza nos dentes e terá que utilizar uma moldeira de silicone sobre seus dentes inferiores por um período de 2 horas (pela manhã), durante 9 dias consecutivos. Essa moldeira conterá 12 blocos de resinas sobre amostras de esmalte bovino. Ainda será colocado um fio de ortodontia sobre os blocos, para evitar o contato da bochecha com os espécimes, ficando um pequeno espaço.

Este projeto traz como benefício à importância de uma nova análise em favor a ciência, através da análise da película adquirida (camada de proteína proveniente da saliva) coletada sobre resinas experimentais, porém não há benefícios imediatos para os participantes da pesquisa. A participação será voluntária e entende-se que você poderá fazer qualquer pergunta sobre os procedimentos, sendo livre para desistir de participar a qualquer momento da pesquisa, sem nenhum prejuízo de sua parte. Em adição, você terá, também, por parte dos pesquisadores, a garantia do sigilo que assegura a sua privacidade e ainda receberá antes da pesquisa uma via, igualmente válida deste Termo de Consentimento Livre e Esclarecido assinado por ambas as partes (pesquisador e participante), no qual deixa claro seus direitos. Concordando em participar, você entende que este estudo será realizado em benefício das ciências médica e odontológica, e desta forma concorda com a divulgação dos dados obtidos por meio de publicações científicas.

A sua participação neste trabalho acarretará em risco mínimo, que acontecerá no caso de você ter alergia ao fio de ortodontia ou do aparelho de silicone utilizado para o experimento e/ou apresentar enjoos na hora da coleta, alguns participantes podem eventualmente apresentar. Nestes casos, você será acompanhado até um local apropriado e esperado até o enjoo passar, ou então, o pesquisador irá acompanhá-lo até um médico. E você será liberado da participação na pesquisa.

Importante ressaltar que não está sendo considerado nenhum pagamento ou recompensa material pela sua participação neste estudo. Em relação a incômodos, podemos afirmar que nada será acarretado, já que o tempo do uso do aparelho é curto, pois a sua participação será apenas no uso do aparelho. Fica claro que você poderá, a qualquer momento, retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar do estudo alvo da pesquisa e ciente que todo trabalho realizado torna-se informação confidencial guardada por força do sigilo profissional (Art. 9o do Código de Ética Odontológica).

Ainda, se caso houver qualquer tipo de despesas tidas pelos participantes da pesquisa e dela decorrentes, serão de responsabilidade do pesquisador os gastos provenientes e/ou o ressarcimento aos participantes. Por fim, você terá garantido o direito à indenização compensatória caso fique comprovado que a sua participação acarretou algum problema a você.

A liberdade do consentimento deverá ser particularmente garantida para aqueles participantes de pesquisa que, embora plenamente capazes, estejam expostos a condicionamentos específicos, ou à influência de autoridade, caracterizando situações passíveis de limitação da autonomia, como estudantes, assegurando-lhes inteira liberdade de participar, ou não, da pesquisa, sem quaisquer represálias.

Qualquer dúvida ou maiores esclarecimentos o sujeito da pesquisa poderá recorrer a qualquer um dos membros da equipe do projeto (Laboratório de Bioquímica 14-3235-8247).

Rubrica do Participante	Rubrica do Pesquisador

Universidade de São Paulo
Faculdade de Odontologia de Bauru
Departamento de Ciências Biológicas

ou o pesquisador responsável Vinícius Taioqui Pelá (telefone 14-99753-5291). Caso queira apresentar reclamações em relação a sua participação na pesquisa ou denúncias, poderá entrar em contato com o Comitê de Ética em Pesquisa em Seres Humanos, da FOB-USP, pelo endereço da Al. Dr. Octávio Pinheiro Brisolla, 9-75 (sala no prédio da Pós-Graduação FOB/USP) ou pelo telefone (14)3235-8356.

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____, portador da cédula de identidade _____, após leitura minuciosa das informações constantes neste TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicada pelos profissionais em seus mínimos detalhes, DECLARA e FIRMA seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em participar da pesquisa proposta. Fica claro que o participante da pesquisa, pode a qualquer momento retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar desta pesquisa e ciente de que todas as informações prestadas tornar-se-ão confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica)

Por fim, como pesquisador(a) responsável pela pesquisa, DECLARO o cumprimento do disposto na Resolução CNS nº 466 de 2012, contidos nos itens IV.3 e IV.4, este último se pertinente, item IV.5.a e na íntegra com a resolução CNS nº 466 de dezembro de 2012.

Por estarmos de acordo com o presente termo o firmamos em duas vias igualmente válidas (uma via para o participante da pesquisa e outra para o pesquisador) que serão rubricadas em todas as suas páginas e assinadas ao seu término, conforme o disposto pela Resolução CNS nº 466 de 2012, itens IV.3.f e IV.5.d.

Bauru, SP, _____ de _____ de _____.

_____ _____

Assinatura do Participante da Pesquisa Vinícius Taioqui Pelá

O **Comitê de Ética em Pesquisa – CEP**, organizado e criado pela **FOB-USP**, em 29/06/98 (**Portaria GD/0698/FOB**), previsto no item VII da Resolução CNS nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.
Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:

Horário e local de funcionamento:

Comitê de Ética em Pesquisa

Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira, no horário das **14hs às 17 horas**, em dias úteis.

Alameda Dr. Octávio Pinheiro Brisolla, 9-75

Vila Universitária – Bauru – SP – CEP 17012-901

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