INDIRA MORAES GOMES CAVALCANTI

"INFLUÊNCIA DA NITRETAÇÃO DE SUPERFÍCIE DE TITÂNIO SOBRE A FORMAÇÃO E DESENVOLVIMENTO DE BIOFILMES MULTIESPÉCIES"

"INFLUENCE OF TITANIUM NITRIDE BY COLD PLASMA ON FORMATION AND DEVELOPMENT OF MULTISPECIES BIOFILMS"

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UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Orientadora: Profa. Dra. Altair Antoninha Del Bel Cury

Dissertação de Mestrado apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do título de Mestra em Clínica Odontológica na área de Prótese Dental.

Master thesis presented to Piracicaba Dental School of the University of Campinas to obtain the MS grade in Dental Clinic, concentration area of Dental Prosthesis.

Este exemplar corresponde à versão final da Dissertação defendida pela aluna Indira Moraes Gomes Cavalcanti, e orientada pela Profa. Dra. Altair A. Del Bel Cury

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RESUMO

Tratamentos que alteram propriedades de superfície de titânio são realizados em implantes odontológicos visando uma melhor sinalização celular e a neoformação óssea. Porém, em determinadas situações clínicas, as superfícies de titânio expostas ao meio oral recobertas por película adquirida (PA) podem se tornar substratos para o desenvolvimento de biofilmes associados às doenças inflamatórias, como a peri-implantite. Diante desta observação, os objetivos deste estudo foram (i) caracterizar a superfície de titânio nitretada por plasma a frio quanto as propriedades de rugosidade, topografia, composição química e energia livre de superfície (ELS); (ii) determinar o perfil protéico da PA adsorvida às superfícies (iii) avaliar a influência do tratamento de superfície na formação e desenvolvimento de biofilmes multiespécies. Para o estudo, discos de titânio grau IV receberam polimento e acabamento e foram divididos aleatoriamente no grupo controle (Ti) e experimental TiN (nitretado por plasma a frio). As superfícies foram caracterizadas através da microscopia eletrônica de varredura (MEV) e a rugosidade e topografia determinadas pela Microscopia de Força Atômica (n = 4). A ELS foi avaliada pela técnica ácido-base e leitura em goniômetro (n = 9) e a composição química da superfície foi determinada por Espectroscopia Fotoeletrônica de Raios-X (XPS) (n = 4). Em seguida, os discos foram imersos em saliva para formação de PA por 2 horas e novamente a ELS foi determinada (n = 6). O perfil protéico da PA foi determinado por espectrometria de massas LC-MS/MS (n = 18). Um biofilme composto por um fungo e seis bactérias (*Candida albicans, Veillonella dispar, Streptococcus mutans, Streptococcus oralis, Fusubacterium nucleatum e Actinomyces naeslundii*), foi desenvolvido durante 64,5 horas sobre os discos com película. Após este período, os micro-organismos e o biofilme total foram quantificados em células viáveis (n = 12). A topografia e a organização dos biofilmes foram analisadas por MEV e pela microscopia confocal a laser. Os dados de células viáveis e perfil protéico foram avaliados estatisticamente pelo Teste t de Student e os dados de ELS pela análise de

variância (ANOVA) de dois fatores seguida pelo teste de Tukey com nível de significância de 5 %. Os resultados demonstraram não haver diferença entre as propriedades de rugosidade e topografia entre os grupos e um maior pico de nitrogênio foi detectado na composição química da superfície nitretada. O tratamento não alterou a ELS, que aumentou apenas na presença de PA ($p < 0.001$). Diferentes proteínas se adsorveram à superfície nitretada. A quantidade de células viáveis do biofilme formado nas duas superfícies foi semelhante ($p = 0.416$), confirmado pelas microscopias, porém *Fusobacterium nucleatum* e *Streptococcus oralis* foram quantificados em maior número nos biofilmes do grupo TiN. Conclui-se que a nitretação por plasma a frio não alterou as propriedades de superfície de titânio e não influenciou a quantidade de biofilme formado. Porém, a superfície nitretada aumentou as contagens de *Fusobacterium nucleatum* e *Streptococcus oralis* e selecionou diferentes proteínas na PA.

Palavras chave: Saliva, tratamento, implantodontia, prótese.

ABSTRACT

Surface treatments that alter titanium properties titanium are performed on dental implants in order to improve the cell signalization and bone formation. Although, in certain clinical situations, the surfaces exposed to oral cavity covered by acquired-pellicle (AP) may become substrates for development of biofilms associated with inflammatory diseases as periimplantitis. Given this observation, the aims of this study were (i) to characterize the surface properties of titanium nitride by cold plasma as roughness, topography, chemical composition and surface free energy (SFE), (ii) determine the protein profile of AP adsorbed to the surfaces (iii) evaluate the influence of surface treatment on formation and development of multispecies biofilms. For the study, titanium discs grade IV received polish and finish and were randomly allocated to control (Ti) and experimental TiN (nitride by cold-plasma) groups. The surfaces were characterized by scanning electron microscopy (SEM) and roughness and topography determined by Atomic Force Microscopy (n = 4). The SFE was evaluated using the acid-base technique and read in goniometer (n = 9). The surface chemical composition was determined by x-ray photo-electron spectroscopy (XPS) (n = 4). Then, the discs were immersed in saliva for AP formation per 2 hours and again the SFE was determined (n = 6). The AP protein profile was determined by mass spectrometry LC-MS/MS (n = 18). A biofilm composed by five bacteria and one fugal (*Candida albicans, Veillonella dispar, Streptococcus mutans, Streptococcus oralis, Actinomyces naeslundii and Fusobacterium nucleatum*) was conducted for 64.5 hours on coated-discs. After this period, the viable cells of biofilms were determined (n = 12). The biofilms topography and organization were analyzed by SEM and by confocal laser microscopy. The data of viable cells and protein profile were evaluated statistically by Student's t test and SFE data by two-way ANOVA followed by Tukey's test with a significance level of 5%. The results showed no difference between the properties of roughness and topography in groups. A high peak of nitrogen was detected in the chemical composition of the nitride surface. The treatment did not

alter the SFE, that increased in the presence of AP ($p < 0.001$). Different protein adsorbed to nitride surface. The amount of viable cells in the biofilm formed on both surfaces was similar ($p = 0.416$), confirmed by microscopies. The number of viable cells of *Streptococcus oralis* and *Fusobacterium nucleatum* were higher in TiN. It was concluded that the cold plasma nitriding did not alter the titanium surface properties and did not affect the amount of biofilm. However, it increased the counts of *Fusobacterium nucleatum* and *Streptococcus oralis* and selected different proteins in AP.

Keywords: Saliva, treatment, implantology, prosthesis.

SUMÁRIO

INTRODUÇÃO

Através do advento dos implantes osseointegrados e das próteses sobre implante, o titânio está cada vez mais presente nas reabilitações devido à propriedade de biocompatibilidade com os tecidos orais, resultante da baixa reatividade com o meio e da estabilidade da camada de óxidos presente em sua superfície. Esta propriedade está relacionada à sinalização de células promotoras da osseointegração (Wennerber & Albrektson, 2009).

Visando uma osseointegração mais rápida e eficaz, modificações nas propriedades da superfície de titânio são realizadas por meio dos tratamentos de superfície. Esses tratamentos alteram propriedades como rugosidade (Ra), energia de superfície (ELS) e topografia, bem como a composição química da superfície também pode ser alterada por meio da incorporação de íons ou de agentes de união. A Ra corresponde à média das irregularidades da superfície, que juntamente com a quantidade de flancos, depressões e picos, determinam a topografia de cada superfície (Bolen *et al*., 1997; Wennerberg & Albrektson, 2009). A ELS indica a capacidade de molhamento de cada material, ou seja, a reatividade de sua superfície com líquidos, moléculas e células (Minagi *et al*., 1985).

Dentre os tratamentos de superfície disponíveis incluem-se os jateamentos com partículas, o ataque ácido e a adição ou deposição de substâncias ou íons (Wennerber & Albrektson, 2009), a exemplo da nitretação por plasma a frio. A deposição iônica por plasma a frio vem sendo utilizada em estudos experimentais relacionados à osseointegração e tem-se observado resultados satisfatórios quanto à resposta óssea em alguns estudos (da Silva *et al*., 2011; Meirelles *et al*., 2013). Este tratamento de superfície consiste na deposição iônica por um plasma composto por variados íons, como o nitrogênio (nitretação) sobre a superfície de titânio (Meirelles *et al*., 2013). A nitretação ocorre em alto vácuo, o que gera uma baixa temperatura durante a deposição iônica sobre a superfície (da Silva *et al*., 2011; Meirelles *et al*., 2013).

Em situações clínicas a exemplo do pobre recobrimento dos implantes pelos tecidos moles e na destruição óssea ao redor dos mesmos, é formada sobre a superfície do implante uma película-adquirida (PA) protéica e acelular, composta por glicoproteínas e fosfoproteínas salivares. A composição da PA está diretamente relacionada com as propriedades de rugosidade e energia de superfície do material, bem como de sua composição química de sua superfície (Al-Hashimi *et al*., 1989).

Assim que é formada, a PA torna as superfícies substratos passíveis de colonização microbiana e consequente formação de biofilmes orais, que são comunidades formadas por micro-organismos envoltos em uma matriz extracelular de polissacarídeos, que se adere a um substrato recoberto por película adquirida, os quais podem ser dentes, mucosas ou materiais restauradores diretos e indiretos, como o titânio (Rompen *et al*., 2006).

A formação de biofilmes é um processo que sofre influência das propriedades da superfície do material exposto ao meio oral, a exemplo as superfícies com maiores rugosidades e maiores ELS aumentam a quantidade de biofilme formado (Rompen *et al*., 2006). Além das propriedades de superfície, as proteínas salivares presentes na película adquirida também influenciam a formação de biofilmes. A adesão microbiana ocorre por meio de ligações não-covalentes entre os micro-organismos e o substrato, entre os micro-organismos e as proteínas da PA e entre espécies. Essas interações não-covalentes são ligações mais fracas e reversíveis, sendo ligações do tipo Van Der Waals e interações hidrofóbicas (Rompen *et al*., 2006; Siqueira *et al.,* 2012).

No entanto, a fim de colonizar permanentemente, os micro-organismos estabelecem ligações irreversíveis com as superfícies expostas e com as glicoproteínas da PA. Essas ligações envolvem proteínas da parede bacteriana denominadas adesinas (Jenkinson, 2002; Siqueira *et al.,* 2012). Geralmente as adesinas microbianas estão associadas às fibrilas da parece celular dos micro-organismos, que são responsáveis pela adesão. As adesinas interagem com os constituintes da superfície de outras bactérias, podendo ser com os grupos de carboidratos ou com outras adesinas (Siqueira *et al.,* 2012).

Além da formação de biofilmes sofrer influência da Ra, ELS e da PA, a topografia e a composição química da superfície também podem influenciar a quantidade e a variedade de micro-organismos presentes nos biofilmes (Al-Hashimi *et al*., 1989; Bollen *et al*., 1997). Micro-organismos como *Streptococcus oralis*, *Actinomyces naeslundii, Fusobacterium nucleatum, Veillonella dispar e Candida albicans* que são comensais na cavidade oral, podem prevalecer em determinadas superfícies devido às condições do meio e das características topográficas e químicas do material (Quirynen *et al*., 1995).

Então, essas superfícies expostas em situações da quebra de homeostasia ou na deficiência de higienização das próteses sobre implantes pelo paciente, podem favorecer o acúmulo de biofilme e o desenvolvimento de inflamação nos tecidos adjacentes, como a peri-implantite. A peri-implantite é uma agressão aos tecidos de suporte que pode ocasionar a perda do implante, se tornando uma das causas das falhas do tratamento reabilitador (Koyanagi *et al.,* 2013).

Um estudo prévio utilizando implantes com tratamentos de superfície, cujas propriedades de RA e ELS já são conhecidas, avaliou a influência dessas superfícies na formação de biofilmes. Foi observada uma maior formação nas superfícies cujos tratamentos resultaram em maiores rugosidades e ELS (Teughels *et al*., 2006). Porém, estudos relacionando a deposição iônica por nitretação plasma a frio com biofilmes orais ainda não são relatados na literatura.

Frente a este fato, os objetivos deste estudo foram avaliar propriedades de superfície do titânio após a nitretação por plasma a frio, além de avaliar a influência deste tratamento no perfil protéico da película-adquirida e na formação e desenvolvimento de biofilmes multiespécies.

CAPÍTULO 1*

Effects of titanium nitride by cold-plasma on acquired-pellicle proteome and on multispecies biofilm.

CAVALCANTI, IMG[1]; RICOMINNI FILHO, AP[1]; LUCENA, SC[1]; da SILVA, WJ[1]; PAES LEME, AF[2]; SENNA, PM[1]; DEL BEL CURY, AA[1*]

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ABSTRACT

Objective: The objective of this study was to evaluate the effect of titanium nitride by cold-plasma on saliva proteome profile and on multispecies biofilm formation. Methods: Titanium discs were allocated into control (Ti) and experimental group (nitride by cold plasma- TiN). The discs topography was measured and characterized by Atomic Force (AFM) and Scanning Electron Microscopies (SEM). The surface chemistry composition was determined by x-ray photo-electron spectroscopy (XPS). For acquired-pellicle (AP) formation and its proteome profile analysis, the discs were incubated with saliva and the adsorbed proteins were subjected to liquid chromatography– mass spectrometry. The surface free energy (SFE) was evaluated before and after the AP formation. A multispecies biofilm compound by *Actinomyces naeslundii*, *Veillonella dispar*, *Fusobacterium nucleatum*, *Streptococcus mutans*, *Streptococcus oralis* and *Candida albicans* was

developed on coated discs. The micro-organisms viability was expressed by colony-forming units per milliliter (CFU/mL) and their biomass and average thickness were analyzed in Confocal Laser Scanning Microscopy (CLSM) images using COMSTAT software. The Scanning electron microscopy also analyzed the biofilm topography and organization. Results: Both surfaces showed similar topography. The TiN SFE did not differ from Ti ($p < 0.001$), although the adsorption of salivary proteins increased the SFE in Ti and TiN coated-groups ($p < 0.001$). There is a higher pick of nitrogen in TiN group. Different AP proteins were identified onto Ti and TiN surfaces. Moreover, the viable cells of biofilms was similar for both groups ($p = 0.416$). In contrast, the counts of *F. nucleatum* and *S. oralis* were higher in TiN ($p < 0.001$). The COMSTAT data, CLSM and SEM images showed equivalent biofilms. Conclusion: The titanium nitride by cold-plasma affected the AP proteome profile, selected certain microorganisms although had no effect on total multispecies biofilm.

Keywords: Titanium; biofilm; surface; saliva; proteome.

1. Introduction

Since the advent of osseointegrated implants, titanium has been the preferred material used in screws and prosthetic components in oral rehabilitation due to its biocompatibility with oral tissues and its surface properties, such as roughness (Ra), chemistry composition and topography.[1,2] As it is known, those properties can be adapted and treatments on titanium surface have been proposed to improve protein adsorption, cell signalization and new bone formation. These treatments include turn/ machines, oxidize and add/ implanting substances[3], as cold-plasma nitriding, that insert ions on titanium surfaces.[4,5]

As exposed to oral environment, all surfaces are immediately coated by saliva, forming an acquired-pellicle (AP). Its formation involves non-covalent interactions such as hydrogen bonding, electrostatic and Van der Waals forces between proteins present in saliva and the surface, resulting in the selective

adsorption of those proteins.[6,7] The surfaces Ra and Surface Free Energy (SFE) are known as two of the most important properties that influence the AP formation.[8] It is known that the increased surface area on rougher surfaces facilitate the proteins adsorption[9,10] and the SFE enhances the interaction between the titanium and the biologic fluids.[11] In situations of poor tissue recovery and in bone destruction, those coated-surfaces expose sites for micro-organism attachment.[13]

So, the attractive function present on those modified surfaces to facilitate the protein adsorption, could also attract micro-organisms to colonize them and form biofilms.[13] Oral biofilms are structured communities of bacteria and fungi developed on different substratum[14] and the biofilm formation on titanium is similar to that on enamel.[15] Generally, it is initiated by species of bacteria known as first colonizer, as *Streptococcus oralis* that are directly in contact with coated-surfaces and by co-aggregation, that bacteria attract the second colonizers, as *Fusobacterium nucleatum* and *Candida albicans*, structuring a mature multispecies biofilm.[15] If this colonization is not intercepted, it is able to develop oral biofilms that causes implant-associated disease, the peri-implantite.[16]

In an investigation about surface treatments, as turned, machine and acid etched regarding biofilms concluded that some of those treatments can improve the biofilm formation and precisely when two methods are associated, this observation occurs in a higher frequency.[17] Studies regarding biofilms formation on nitride surface are still scarce. Thus, the aim of this study was to evaluate the effect of titanium cold-plasma nitriding on saliva proteome profile and on multispecies biofilm.

2. Materials & methods

Experimental design

This *in vitro* study had a randomized and blinded design. Titanium discs were submitted to cold plasma nitriding treatment (TiN - experimental group) and the control group (Ti) did not receive it. The topography and Ra of all discs were measured by Atomic Force Microscopy (AFM) and characterized by Scanning

Electron Microscopy (SEM). The surfaces chemistry was determined by x-ray photo-electron spectroscopy (XPS). For AP formation, the discs were exposed to saliva for 2 hours. SFE was measured before and after the AP formation. The AP proteins were subjected to liquid chromatography–mass spectrometry (LC-MS/MS) analysis. For biofilm assay, the multispecies biofilm composed by five bacteria and one fungal was developed for 64.5 hours on coated discs. In sequence, each disc was washed, sonicated and the resultant suspension was serially diluted and plated for viable cells analysis, expressed by colony-forming units per milliliter (CFU/mL). The biomass and average thickness of biofilms were analyzed by Confocal Laser Scanning Microscopy (CLSM) images using COMSTAT software and their topography was analyzed by SEM.

2.1 Preparation of titanium discs and surface treatment

The titanium discs (12.5 mm X 2 mm) (Sandinox; Sorocaba, São Paulo, Brazil) grade IV had their surface polished progressively with smoother aluminum oxide papers (200, 320, 400, 600 and 1200 µm grid) (Carbimet; Buehler, Lake Bluff, IL, USA) in a horizontal polisher (model APL-4; Arotec, Cotia, São Paulo, Brazil). Then, they were thoroughly rinsed and ultrasonically cleaned (Thornton T740; Thornton-Inpec Eletronica Ltda, Vinhedo, São Paulo, Brazil) for 10 min twice in acetone 98% followed by 100% ethanol and distillated water to remove the surface contaminants.[5] After, they were let to dry aseptically and then randomly allocated in control (Ti) and experimental (TiN) groups. The discs from TiN group were submitted to a surface treatment with cold-plasma, previously described. The Air plasma sputtered the titanium surface to remove the contaminants and after, a second ion bombardment with air and noble gases, including nitrogen, at a high vacuum, was applied for 150 seconds on one side of discs surface. The nitriding machine was fabricated by Physical Institute of University of Campinas (UNICAMP).[4,5] The discs from Ti and TiN groups were sterilized by gamma radiation (25 Gy) (Embrarad; Cotia, São Paulo, Brazil).

2.2 Surface analysis

The topography of Ti and TiN surfaces were analyzed by Atomic Force Microscopy (AFM) and (SEM). The AF microscope (Nanosurf EasyScan2 FlexAFM, Liestal, Switzerland) was set at 0.5 seconds for reading at 256 points. Four images of each two discs were obtained and evaluated by SPIP 5.1.8 software (Image Metrology, Hørsholm, *Denmark*).[18] The parameters of surface topography were numerically expressed in roughness parameters: *Sa* the arithmetic mean of the roughness area from the mean plane; *Sds* density of summits, that is the number of peaks per area unit; *Sdr* the ratio between the developed surface area and a flat reference area. The discs were also scanned at 15 kV in SEM (LEO 435 VP, Carl Zeiss SMT, Oberkochen, Germany) (n = 4).

The surface chemistry was determined by x-ray photo-electron spectroscopy (XPS). The spectrometer (Vacuum Scientific Workshop, VSW HA100, Manchester, UK) with hemispherical analyzer operate at constant transmission mode, resulting in line width for Au 4f7 / 2 of 1.6 eV. The electron radiation for excitation was Al Kα, 1486.6 eV. The pressure during the measurements was less than 2 x 10^{-8} mbar. Loading effects were corrected by C1s line and the bonding energy was set at 284.6 eV. The normal to the sample surface was parallel to the axis of the analyzer for all analysis (n = 2).[19]

The SFE was determined by using a goniometer (Ramé-hart Instrument Co., Succasunna, USA) that automatically measured the contact angle formed between the sessile drop (15 μL) of dispensed probe-liquids (purified water, formamide* (polar liquids) and bromonaphthalene* (apolar liquid) (*Sigma-Aldrich Corp., St. Louis, MO, USA) and the surfaces, before and after AP coating. A total of 6 discs/ group/ probe-liquid was used. Based on the achieved data regarding the 3 probe-liquids above, the polar, apolar and the total SFE was automatically calculated by the DROPimage Advanced software (Ramé-hart Instrument co., Succasunna, NJ, USA).[20]

2.3 Acquired pellicle formation

This study was approved by the Local Research and Ethics Committee (075/2011). Eight volunteers, who agreed and signed for it, donated their

stimulated saliva. They were not using antibiotics, mouth rinses or medications known to affect salivary composition and flow. The collection was done during the masticatory stimulation with a flexible film (Parafilm M; American Can Co, Neenah, WI, USA). The saliva pool was clarified in a polypropylene tube by centrifugation at 10.000 g for 5 min at 4°C. The supernatant was filtered through a 0.22-µm membrane filter (Corning Inc, Corning, NY, USA) and used immediately. At all time, three tubes containing each 20 mL of saliva pool was maintained in ice and 1 mL of EDTA and PMSF was added to each one to avoid the protein degradation. For each experiment, the saliva sample was collected at the same volume and time of day to standardize the circadian rhythm. Under aseptic conditions, each disc from TiN and Ti groups were placed inside a 24-well plate with 2 mL of saliva. The plates were incubated for 2 hours at 35 °C in an orbital shaker (Lab-line Incubator Shaker, Elliott Bay Laboratory Services, Seattle, WA, USA).[21]

2.4 Proteome analysis

The adsorbed proteins from the acquired-pellicles were eluted from the titanium discs according to an established methodology.[17] Briefly, eighteen coated-discs from Ti or TiN groups were pooled into a polypropylene tube containing 20 mL of purified water and vortexed for 1 min, then sonicated for 5 min (7 W/4 °C) and again vortex for 1 min. The samples were lyophilized and then subjected to total protein quantification by Bradford method with BSA standard.[22,23] After the quantification, 30 µg of the extracted proteins were subjected to LC-MS/MS. For this, the proteins were reduced, alkylated and so digested by trypsin (1:50, w/w).[24] The resulting peptides were resuspended in 12 µL of 0.1% formic acid and then separated by C18 (100 µm x 100 mm; RPnanoUPLC, nanoAcquity[§]) coupled with a mass spectrometer (Q-Tof Ultima[§]). The instrument was operated in 'top three' mode, in which one MS spectrum is acquired followed by MS/MS of the top three most intense peaks detected. Three independent experiments were performed. The spectra were acquired by the MassLynx software[§] and the raw data files were converted to a peak list format (mgf) by using Proteome Discoverer (Thermo Scientific). The identified amino acids sequences were matched against Human

International Protein Database (IPI).[25] The biological function was accessed in protein knowledge base UniProt (Geneva, Switzerland) and the isoelectric point, net charge and water solubility of each protein were obtained using a peptide calculator tool (Innovagen, Lund, Sweden). § (Waters Corp., Milford, MA, USA)

2.5 Biofilm assay

On pellicle-coated discs, a multispecies biofilm was developed based on Zurich model. [26,27] The biofilm was composed by six microorganisms, five bacteria– *A. naeslundii* OMZ 745, *V. dispar* OMZ 493, *F. nucleatum* OMZ 598, *S. mutans* OMZ 918 and *S. oralis* OMZ 607 and one yeast *C. albicans* OMZ 110.[26,27]

All strains were reactivated on blood agar cultures and their colonies were individually transferred to a tube with 9 mL of modified universal fluid medium (mFUM) supplemented with 0.3% of glucose for inoculum preparation. After 15 hours of incubation, aliquots from each tube were transferred to a new one containing 5 mL of mFUM 0.3% glucose and incubated at 37°C for 7 hours. Then, the optical density of each culture was independently adjusted to 1.0 ± 0.02 (550 nm) and a mix with all strains was prepared with equal volumes of each density-adjusted culture. The pellicle-coated discs were transferred to wells containing 1.8 mL of medium mixture of saliva (0.7%) and mFUM (0.3% glucose) and 0.225 mL of mixed-species inoculum. The 24-well plate was incubated anaerobically at 37 °C for 64.5 hours, with washes in saline solution three times per day. The medium was substitute at 16.5 and 40.5 hours for a fresh mixture of saliva (0.7%) and mFUM (0.15% glucose and 0.15% sucrose).[26,27]

2.6 Biofilm Analysis

For viability, four independent experiments were performed. Ti and TiN disc with 64.5 hours biofilm were transferred to cryogenic tubes containing 3 mL of saline solution. The tube was sonicated (7 W for 30 seconds) and from the suspension, an aliquot of 0.1 mL was 6-fold serially diluted in saline solution and plated in the following culture media: Columbia Blood Agar§ supplemented with 5%

(v/v) defibrinate sheep's blood for total microorganisms count and plus norfloxacin (1 mg/mL), erythromycin (1 mg/mL) and vancomycin (4 mg/mL) for *F. nucleatum*; Mitis Salivarius Agar[§] for *S. mutans* and *S. oralis*; Veillonella Agar for *V. dispar*; Cadmium Sulfate Fluoride Acridine Trypticase Agar for *A. naeslundii*; and Biggy Agar[§] for *C. albicans* ([§] DIFCO BD, Franklin Lakes, NJ, USA).[26,27]

Two microscopies assays were performed in this study, aiming to well describe the biofilm topography and organization. The biofilm topography was analyzed by SEM (LEO 435 VP, Carl Zeiss SMT, Oberkochen, Germany) (n = 4). The discs were fixed in Karnovsky (PBS; pH 7.2) solution overnight, followed by dehydration in a series of ethanol washes (60%, 70%, 80%, 90% for 5 min and 100% for 10 min) and then were allowed to dry aseptically. Afterward, they were gold-metallized for observing in SEM scanned at 15 kV.

The biofilm organization was evaluated by CLSM (Leica Microsystems CMS, Mannheim, Germany) (n = 6). Discs were submitted to fluorescent *in situ* hybridization using rRNA-specific oligonucleotide probes (Invitrogen, Carlsbad, USA) for total biofilm (EUB 388), *F. nucleatum* (FUS664) and *S. oralis* (MIT588) as described previously.[28] The discs with biofilm were incubated in a solution containing the probes at 5 µg/mL for 180 min at 46 °C. COMSTAT software was used to determine the biomass ($\mu m^3/\mu m^2$) and average thickness (µm) of formed biofilms.[28]

2.7 Statistical analysis

The data of viability and the profile of AP proteins were analyzed by Student's t-test; the SFE data were analyzed by two-way ANOVA followed by Tukey's test and designed by SAS software (SAS Institute Inc., version 9.0, Cary, NC, USA) with the significance level set at 5%.

3. Results

The surface topography of Ti and TiN groups were similar as showed in figure 1. Moreover, the 3D reconstruction from Ti surfaces suggested a similar

variation of tops, valleys and flanks (figure 1), expressed numerically by the roughness parameters in table 1.

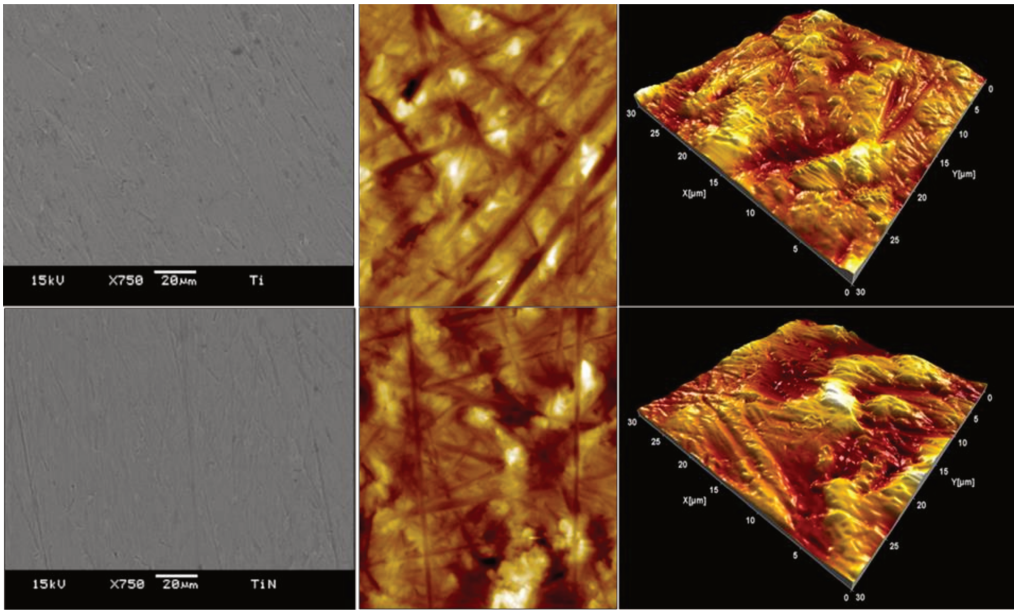

Figure 1: SEM and AFM images followed by 3D reconstructions from Ti and TiN surfaces.

Table 1: The mean of *Sa, Sdr* and *Sds* roughness parameters for Ti (control) and TiN (experimental) surfaces.

Groups	Roughness Parameters		
	Sa (µm)	*Sdr (%)*	*Sds (1/µm²)*
Ti (Cont)	0.078	3.689	1.599
TiN(Exp)	0.098	4.147	1.161

The surface chemistry analyses determined the presence of a high pick of nitrogen after the surface treatment. The higher elements detected in both surfaces were oxygen (O1s) and titanium (Ti2p, Ti 3s e Ti3p) and the carbon (C1s) was also detected (Figure 2).

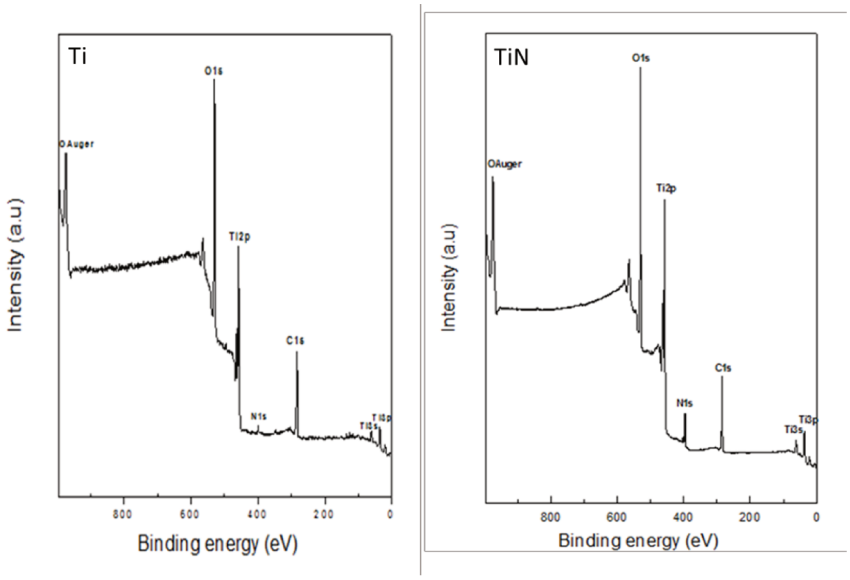

Figure 2: XPS analysis of the representative elements detected on the Ti (control) and TiN (experimental) surfaces. a.u = arbitrary unit. Oxygen (O1s), Titanium (Ti2p, Ti 3s e Ti3p), Carbon (C1s) and Nitrogen (N1s).

There was no difference in SFE after the surface treatment ($p = 0.093$). Although, the polar and apolar components TiN were different from the control group ($p < 0.001$). Also, the presence of AP improved the SFE in both coated-groups ($p < 0.001$) (Table 2).

Table 2: Surface free energy (SFE), polar and apolar (mJ/m^2) components of Ti (control) and TiN (experimental) surfaces before and after AP. (Mean ± S.D; n = 9)

		Polar	Apolar	SFE
No pellicle	Ti	7.13 ± 1.2^{a}	27.5 ± 0.1^{a}	34.6 ± 1.2^{a}
	TiN	12.5 ± 0.6^{b}	22.4 ± 0.2^{b}	34.9 ± 0.5^{a}
+pellicle	Ti	36.7 ± 6.3^{c}	19.3 ± 3.3^{c}	56.1 ± 3.0^{b}
	TiN	31.4 ± 1.7^{c}	17.9 ± 0.1^{c}	49.4 ± 1.8^{b}

Different letters indicate statistical differences between groups. (Two-way ANOVA followed by Tukey's test; $p < 0.001$).

The saliva-pellicle's proteins with their theoretical molecular weight (MW) and label-free relative quantification are presented in Table 3 and 4. Based on the

molecular weight and spectral counts, the most abundant proteins identified in Ti and TiN groups were alpha-amylase 1, cystatin-S, cystatin-SA, mucin 5AC oligomeric mucus/gel-forming, alpha-2-macroglobulin and complement C3 (table 4). The surface treatment selected different proteins to its AP composition. They were zinc-alpha-2-glycoprotein, protein S100 A9 and isoform alpha-enolase of alpha-enolase and the control group had isoform 1 of serum albumin, prolactin-inducible protein, filaggrin and desmoglein-1. The isoeletric point (pl) of adsorbed proteins onto Ti and TiN ranged from 4.44 to 9.85 in and the most proteins were positively charged. In addition, the biological function analysis of the achieved proteome varies from antibacterial activity, bacteriolitic function and transport (Tables 3 and 4).

The viability of each specie and of total microorganisms on the biofilm formed on titanium discs from control (Ti) and experimental groups (TiN) are shown in table 5. There was a significant difference for *S. oralis (So) and F. nucleatum (Fn)* formed on TiN surfaces ($p < 0.001$), although there is no difference between total biofilm formed on both surfaces ($p = 0.416$).

The Confocal images and 3D reconstructions are in accordance with the counts of total biofilm developed on Ti and TiN surfaces (Figure 4). The surface treatment gave an yellow color to titanium discs and the reflect appeared on biofilm images (Figure 4).

Table 3: Proteins identified onto pellicle coated on Ti and TiN surfaces by LC-MS/MS. (* Student's t-test. $p < 0.001$)

	Proteins	MW (kDa)	SC	pI	Net charge	Water solubility	Biological Function
TiN*	Zinc-alpha-2-glycoprotein	34	10.9	5.64	-5.5	+	Lipid degradation
	Protein S100-A9	13	7.5	5.68	-5.3	+	Antimicrobial activity
	Lipocalin-1	19	6.0	5.25	-6.6	+	Play a role in taste perception
	Isoform alpha-enolase of Alpha-enolase	47	5.1	7.17	0.3	+	Stimulate imunoglobulin production
	Alpha-1-acid glycoprotein 1	24	1.9	4.74	-8.9	+	Transport
	Peptidyl-prolyl cis-trans isomerase B	18	2.3	9.85	8.2	+	Catalize isomeration
	Isoform 1 of Acyl-CoA-binding protein	10	1.1	6.14	-0.8	+	Intracellular carrier
	Neutrophil defensin 1	10	0.8	6.77	-0.2	-	Antimicrobial activity
	Cystatin-C	16	0.6	4.98	4.1	-	Regulator of enzimatic activity
	Rho GDP-dissociation inhibitor 2	23	0.5	4.9	-9.6	+	Regulation of proteins
Ti	Isoform 1 of Serum albumin	69	164.3	5.92	7	+	Metabolic process
	Prolactin-inducible protein	17	92.7	8.1	1.8	-	Catalytic activity
	IGL@ protein	25	12.2	5.91	-2.8	-	-
	Putative uncharacterized protein	25	11.8	7.64	1.1	-	-
	Lysozyme C	17	1.8	9.33	8.7	-	Bacteriolitic function
	Fructose-bisphosphate aldolase A	39	0.9	8.25	4.5	+	Scalffolding protein
	Desmoglein-1	114	0.6	4.95	-9.9	+	Cell-cell adhesion
	Filaggrin	435	0.4	9.47	56.9	+	Aggregation
	Isoform H17 of Myeloperoxidase	84	0.3	8.45	15.9	+	Microbicidal activity

The theoretical data of Molecular Weight (MW), isoelectric point (pI), spectral counts* (SC), net charge at pH 7 and water solubility (+ good, - poor water solubility) from Ti (experimental) and Ti (control) groups were obtained using a peptide calculator tool (Innovagen, Lund, Sweden). Spectral counts (SC) - number of MS/MS spectra obtained for a protein.

Table 4: Proteins identified onto pellicle coated on TiN surfaces by LC-MS/MS without significant difference with Ti group (Student's t-test).

P value	Proteins	MW(kDa)	SC	pI	Net charge	Water solubility	Biological Function
0.126	Alpha-amylase 1	58	555.8	6.48	-2.4	-	Carbohydrate metabolism
0.957	Cystatin-S	16	48.8	4.76	-6	+	Enzimatic inhibitor
0.195	Cystatin-SA	16	17.7	4.65	-7	+	Protease inhibitor
0.534	Mucin 5AC, oligomeric mucus/gel-forming	649	18.1	6.3	-48.6	-	Digestion
0.485	Alpha-2-macroglobulin-like protein 1	161	8.7	5.07	-9.7	-	Proteinase inhibitor
0.818	Alpha-2-macroglobulin	163	6.7	6.91	-0.3	+	Proteinase inhibitor
0.866	Complement C3	187	4.7	5.97	-16.4	+	Activation of complement system
0.567	Bactericidal/permeability-increasing protein-like 1	49	6.0	9.29	4.9	-	-
0.166	Immunoglobulin J chain	18	6.1	4.91	-6.1	+	Linker agent
0.093	Isoform 1 of 14-3-3 protein sigma	28	3.3	4.44	-18.8	+	Regulation and signaling
0.196	Isoform 1 of Interleukin-1 receptor antagonist	20	0.7	6.08	-1.9	+	Inhibits the activity of interleukin

The theoretical data of Molecular Weight (MW), isoelectric point (pI), spectral counts* (SC), net charge at pH 7 and water solubility (+ good, - poor water solubility) from both Ti (experimental) and Ti (control) groups were obtained using a peptide calculator tool (Innovagen, Lund, Sweden). Spectral counts (SC) - number of MS/MS spectra obtained for a protein.

Table 5: Colony-forming Units (CFU/mL) of each microorganism and of total multispecies biofilms formed on Ti and TiN surfaces (mean ± SD; n = 12).

	Ca x 10^4	*Sm* x10^7	*So* x10^8	*Vd* x 10^7	*An* x 10^7	*Fn* x10^7	Total x10^8
Ti (Control)	9.8 ± 4.5	8.4 ± 1.2	2.7 ± 1.2	2.8 ± 1.6	5.1 ± 3.2	1.5 ± 1.2	11.2 ± 4.6
TiN (Experimental)	8.5 ± 1.7	10.1 ± 4.8	4.4 ± 1.5*	2.7 ± 0.4	3.3 ± 2.6	4.3 ± 3.3*	13.1 ± 6.0

C. albicans (Ca), S. mutans (Sm), S. oralis (So), V. dispar (Vd), A. naeslundii (An), F. nucleatum (Fn) and total microorganisms (Total). *(Student's t-test; $p < 0.001$)

The SEM images characterized the topography and organization of multispecies biofilm, also in accordance with total counts (Figure 3).

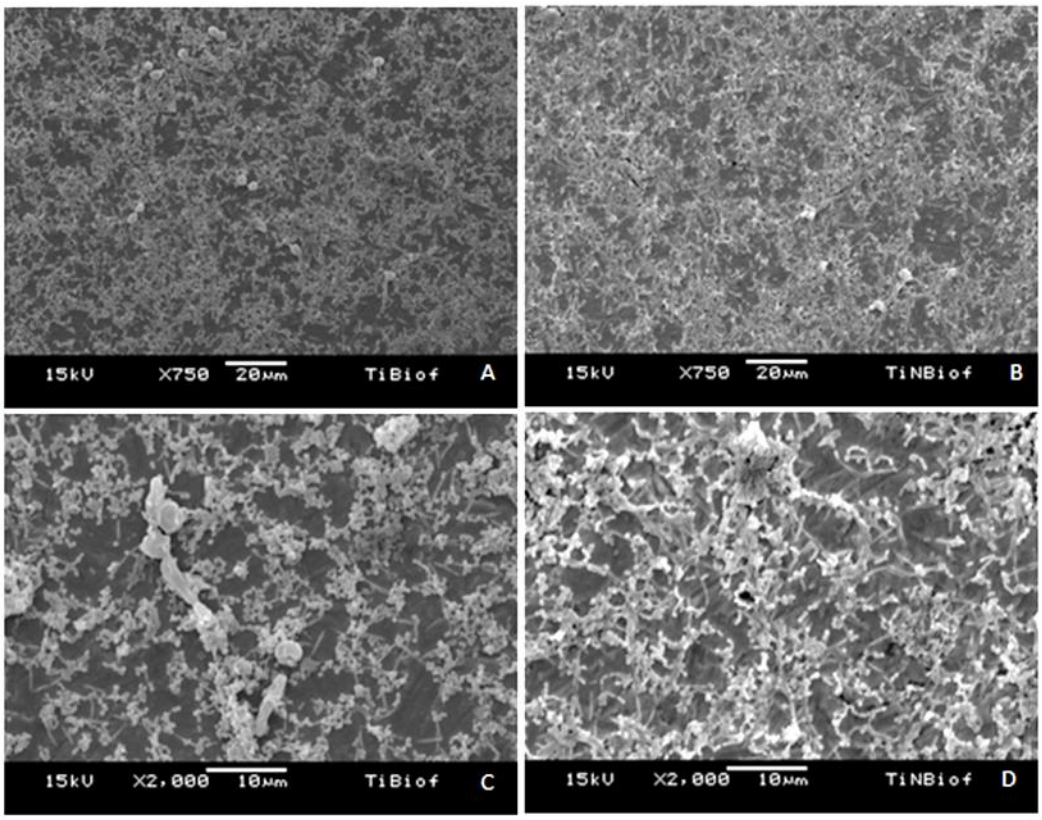

Figure 3: SEM images of biofilms formed on Ti and TiN surfaces. Note the equivalent amount of total microorganisms on both surfaces in different amplitudes. (A) Biofilm formed on Ti surface x 750 and x 2000 (C); biofilms formed on TiN surfaces x 750 (B) and x 2000 (D).

The COMSTAT expressed the data of biomass and average thickness based on CLSM images. Those parameters were similar for biofilms developed on both Ti and TiN surfaces (table 6).

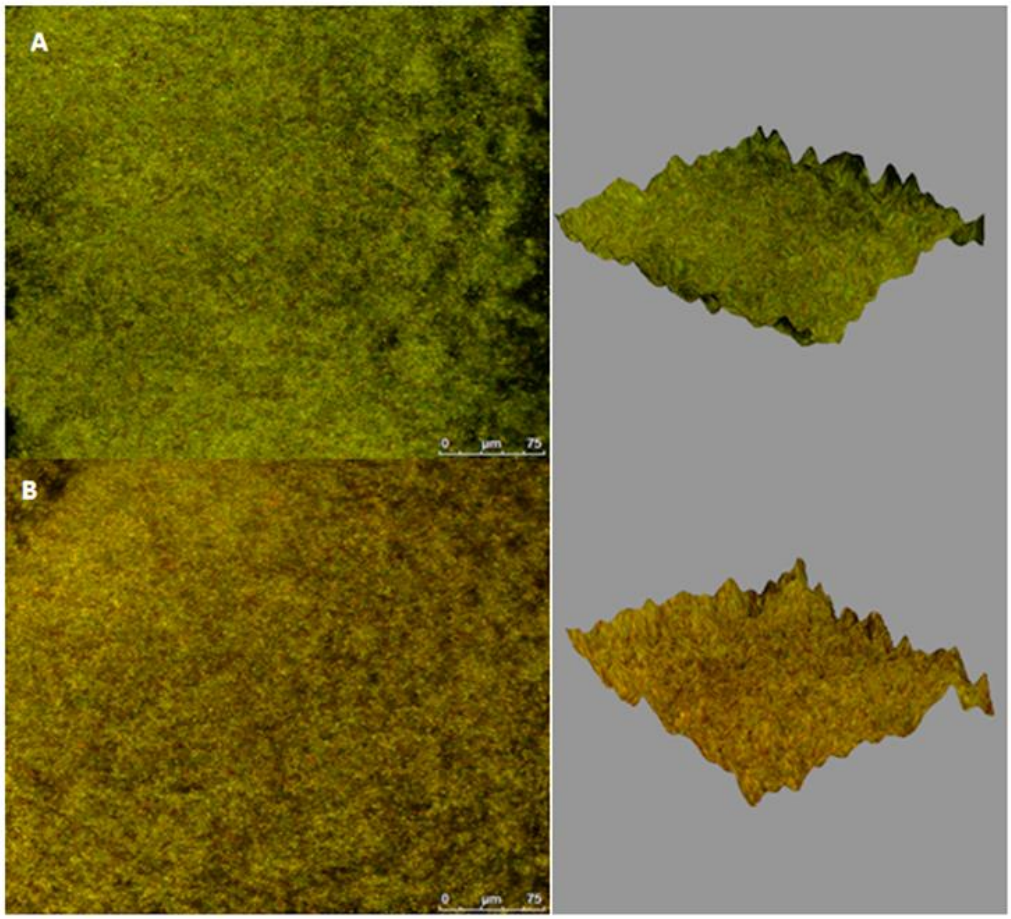

Figure 4: Confocal z-slices images and 3D reconstruction of multispecies biofilms formed on Ti (A- control) and TiN (B- experimental) surfaces. Total biofilms (green), *Fn* (red) and *So* (yellow).

Table 6: Biomass ($\mu m^3/\mu m^2$) and Average thickness (µm) of biofilms formed on Ti (control) and TiN (experimental) surfaces. Data designed by COMSTAT.

Groups	Biomass ($\mu m^3/\mu m^2$)	Average tickness (µm)
Ti	14.77	14.80
TiN	14.62	14.99

4. Discussion

The present study investigated the effects of titanium surface nitride by cold-plasma on saliva proteome profile and on multispecies biofilm formation. Althoug in

previous studies, the effects of titanium cold-plasma nitriding on osseointegration was evaluated,[4,5] there are no studies evaluating this surface treatment regarding the saliva proteome or biofilm formation.

In this study, the roughness and topography (*Sa, Sds, Sdr* parameters) were similar before and after the surface treatment as showed by AFM and SEM. This fact is attributed to the same polish method with the same grid papers for all discs before the surface treatment. Additionally, the ion implantation for surfaces treatment do not alter those properties as others treatments, for example electrochemist, blasting particles and turning.[29,30] Our results are in accordance with a previous evaluation of titanium nitride by cold-plasma.[4] Moreover, as it is known that roughness and SFE influences the adsorption of saliva proteins and also play a role in biofilm formation[6,7] it could be suggested that if surface roughness was similar, other factors, as SFE and surface chemistry composition may have influenced the selective adsorption of salivary proteins and so, the biofilm formation.

The SFE also did not differ before and after the surface treatment, although the polar and apolar components differed in Ti and TiN groups. The surface energy of a material can be affected by several surface characteristics, such as chemical composition[19], which was different in this study. The XPS analysis showed a higher level of nitrogen in TiN group in comparison to Ti. The surface chemistry analysis performed after the surface treated by cold-plasma nitriding showed no alteration on the level of components as Oxygen and Titanium was observed. It has been previously reported that some surface treatments usually do not change the main compositional elements from titanium surface, which consist mainly of titanium and oxygen.[21] Based on the results, the chemical composition seems to contribute to differences in polar and apolar components of Ti and TiN surfaces. Additionally, the adsorption of salivary proteins on both Ti and TiN discs altered their SFE. It was previously reported that SFE is altered after coated by pellicle[31] because the adsorbed proteins changed the surface reactivity. Such differences generate bridges between AP proteins and micro-organisms, initializing the microbial

adhesion. The absence of difference in SFE of Ti and TiN after coated by pellicle can be justified by the protein adsorption that tends to level out this property.[28,29]

The proteome profile exhibited different proteins with the LC-MS/MS analysis of Ti and TiN pellicles. Support comes from findings that the pellicle composition varies from surface to surface, depending on the underlying substratum's chemical properties and SFE.[31] In this study, the characterization of pellicle's proteome adsorbed on titanium surfaces contributed to explain the AP influence on biofilm formation.

In our results, *S. oralis* and *F. nucleatum*, a first and a second colonizer, respectively, were more prevalent in biofilms formed on nitride surface. In the sequence, the influence of TiN chemical on AP proteome profile and such different proteins may be influencing the prevalence of *So* and by co-aggregation, *F. nucleatum* appeared in a high number. It is related to anchoring microorganisms from the red-complex (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*), main prevalent in peri-implantite.[32]

As previously explained, the absence in difference of Sa and SFE reflected the same amount of total biofilm on both surfaces. The SEM and CSLM images are in accordance with the total counts values (11.2 and 13.1 x 10^8). With those results, it can be speculated that the nitride surface maintaining the titanium properties parameters had a positive "non-effect" on total biofilms and the vastly related influence of AP on biofilm formation was not confirmed by our results.

Studies regarding available titanium treatments are needed not only to advance our understanding of the acquired-pellicle formation and biofilm phenomena, but also to aid the development of the next generation of implant surfaces that could enhance osseointegration but not the biofilm formation, because biofilms play a significant role in determining the outcome and success of an implant rehabilitation.

In this *in vitro* study, the surface treatment did not affected the total biofilm, although it selected *F. nucleatum.* So, it has to have more caution with the clinical use of this surface by the fact of selecting bacteria that plays a role in periimplantitis.

5. Conclusion

Based on the results, it can be concluded that titanium surface nitride by cold-plasma altered the profile of AP, selected certain bacteria although there is no effect on total biofilm.

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CONCLUSÃO

Dentro das condições estudadas, pode-se concluir que a nitretação por plasma a frio não alterou as características de superfície de titânio e não influenciou a quantidade de biofilme formado. Porém, a superfície nitretada aumentou as contagens de *Fusobacterium nucleatum* e *Streptococcus oralis* e selecionou diferentes proteínas na PA.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Commitee of Medical Journal Editors - Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

APÊNDICE 1:

Figura 1: Barra de titânio (A) e discos de titânio com superfície oxidada por carbeto de titânio decorrente do processo de usinagem (B).

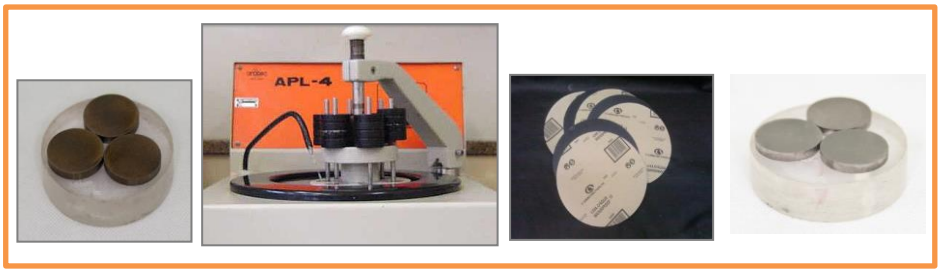

Figura 2: Sequência de polimento e acabamento em politriz com lixas de diferentes granulações.

APÊNDICE 2:

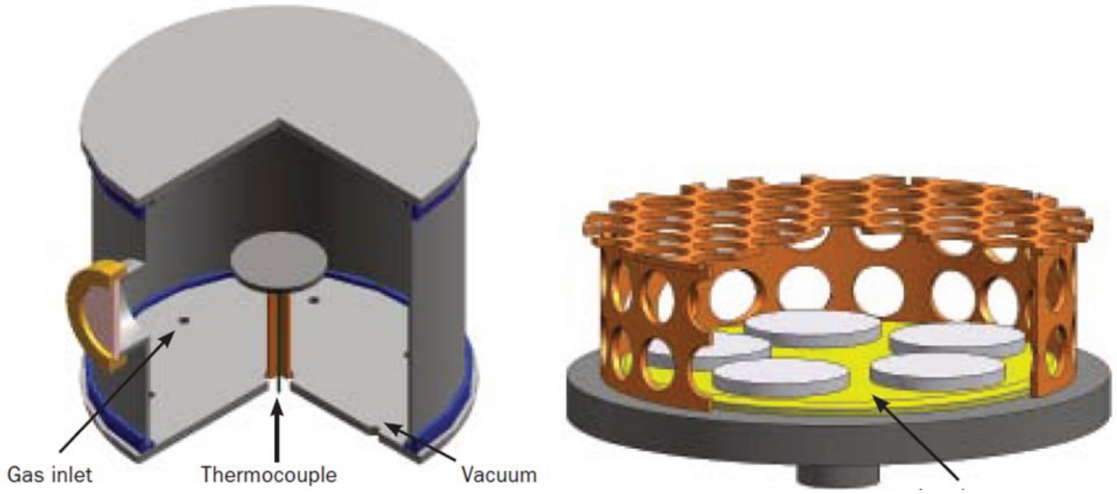

Figura 3: Esquema ilustrativo da deposição iônica/ nitretação por plasma a frio (da Silva *et al.*, 2011).

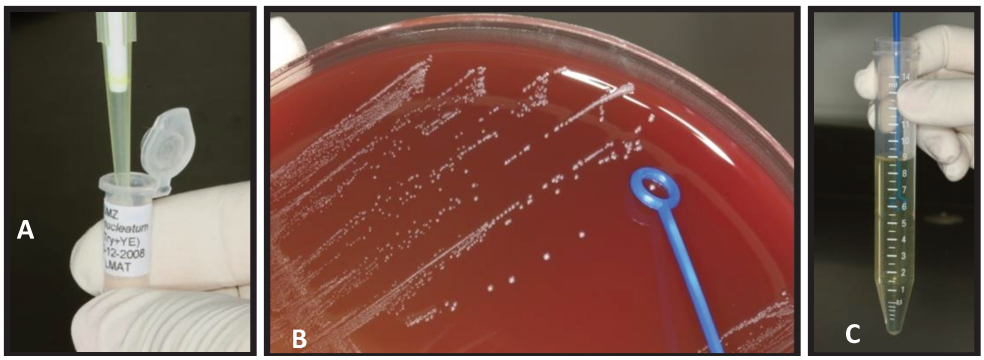

Figura 4. Reativação dos microorganismos para preparo do biofilme. A - Coleta de uma alíquota da cultura de estoque. B - Coleta de colônias em meio sólido (Columbia Blood Agar suplementado com Sangue de carneiro) e C - Transferência das colônias para meio de cultura líquido (FUM 0,3% de glicose).

ANEXO 1:

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Influencia da alteração de superfície de titânio utilizado na reabilitação oral com próteses sobre implantes sobre a formação e desenvolvimento de biofilmes multiespécie"**, protocolo nº 075/2011, dos pesquisadores Altair Antoninha Del Bel Cury, Andréa Araújo de Vasconcellos, Indira Moraes Gomes Cavalcanti e Wander José da Silva, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 03/08/2011.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Development of a multispecie biofilm on surface modified titanium used in oral rehabilitation with implants"**, register number 075/2011, of Altair Antoninha Del Bel Cury, Andréa Araújo de Vasconcellos, Indira Moraes Gomes Cavalcanti and Wander José da Silva, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 08/03/2011.

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

ANEXO 2:

Author's Decision

Thank you for approving "Effects of titanium nitride by cold-plasma on saliva proteome and on multispecies biofilm". An email has been sent to you confirming that the journal has received this submission. Your Co-Author(s) may also receive this email, depending on the journal policy.

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Main Menu

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Article Type: Original Paper

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