UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS GRADUAÇÃO EM ODONTOLOGIA
NÍVEL DOUTORADO
ÁREA DE CONCENTRAÇÃO CLÍNICA ODONTOLÓGICA PERIODONTIA

Tese de Doutorado

IDENTIFICAÇÃO DO PERFIL E DIVERSIDADE MICROBIOLÓGICA PERI-IMPLANTAR ATRAVÉS DO SEQUENCIAMENTO DO GENE 16S rRNA

Fabiana de Melo

Porto Alegre
2020

FABIANA DE MELO

IDENTIFICAÇÃO DO PERFIL E DIVERSIDADE MICROBIOLÓGICA PERI-IMPLANTAR ATRAVÉS DO SEQUENCIAMENTO DO GENE 16S rRNA

Tese apresentada ao Programa de Pós-graduação em Odontologia, nível Doutorado, da Universidade Federal do Rio Grande do Sul, como pré-requisito final para a obtenção do título de Doutor em Odontologia, Clínica Odontológica, ênfase em Periodontia.

Orientador: *Prof. Dr. Rui Vicente Oppermann*
Linha de pesquisa: *Epidemiologia, etiopatogenia e repercussão das doenças da cavidade bucal e estruturas anexas.*

Porto Alegre

2020

The role of the infinitely small in nature is infinitely great
Louis Pasteur

Aos meus pais e irmã pelo carinho e suporte incondicional. E ao meu amor Eduardo pelo incentivo , amor à ciência e à vida. Vocês foram a força e a razão para eu enfrentar este desafio.

AGRADECIMENTOS

Ao meu orientador, Professor Rui Vicente Oppermann, um grande pesquisador e exemplo de dedicação e competência. Obrigada por aceitar me guiar neste mundo científico.

Aos Professores que me ajudaram e compartilharam tantos conhecimentos: Alex Nogueira Haas, Francisco Montagner, Patrícia Angst e Rubens Albuquerque. Vocês foram muito importantes nesta caminhada. Obrigada pelos ensinamentos e apoio durante todos estes anos.

Ao Professor Diogo Onofre de Souza. Querido Mestre, obrigada por me fazer enxergar o amor pela ciência em cada palavra e em cada gesto. Tu és um grande incentivador nesta jornada.

Aos colegas, amigos e colaboradores da McGill, da UFRGS, da USP, do Zimmer Lab e do LABIM. Obrigada pela amizade, pela ajuda nos experimentos em laboratório e pelos incontáveis momentos compartilhando desafios e conhecimentos.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos.

Ao Eduardo, meu noivo, meu amor, meu amigo. Tu és sem dúvidas minha maior inspiração, tanto na ciência quanto na vida. Obrigada por fazer parte da minha vida, por todo amor, companheirismo e por conseguir como ninguém acalmar meu coração e me fazer rir nas piores crises.

Aos meus pais Cirene e Jorge e à minha irmã Vanessa pelo apoio constante, amor incondicional e força nas horas difíceis. Obrigada por serem meu porto seguro. Esta tese também é de vocês.

APRESENTAÇÃO

Esta tese está organizada em três **Partes**, cada uma sendo constituída dos seguintes itens:

Parte I: Resumo, Resumo em inglês (*abstract*), Lista de abreviações, Introdução e Objetivos;

Parte II: Resultados, que estão divididos em capítulos, sendo que cada capítulo contém um breve prefácio seguido de um artigo científico;

Parte III: Considerações finais, anexos e Referências bibliográficas citadas na Introdução da Parte I e Considerações finais da Parte III.

Os trabalhos elaborados nesta tese foram desenvolvidos no laboratório de Bioquímica e Microbiologia da Universidade Federal do Rio Grande do Sul (UFRGS), sob a orientação do professor Dr. Rui Vicente Oppermann, como também no departamento de genética da Universidade McGill (Génome Québec - Canadá), sob a orientação do professor Dr. Rubens Albuquerque Jr e do Dr. Pierre Lepage.

SUMÁRIO

PARTE I

Resumo

Com o aumento do número de implantes dentários para substituir dentes faltantes, houve também um aumento do número de doenças peri-implantares diagnosticadas após a sua instalação. Com isso, o conhecimento da microbiota envolvida nestes sítios se faz necessário e tem sido cada vez mais estudado através de tecnologias de sequenciamento de alto rendimento do gene 16S rRNA. Nesta tese investigamos a microbiota em sua fase de adesão inicial à superfície de titânio, estabelecida em saúde, estabelecida em doença e a sua evolução após a peri-implantite ser tratada por diferentes abordagens (cirúrgica e não cirúrgica). Além disso, comparamos o perfil microbiológico peri-implantar dos pacientes que atingiram sucesso após tratamento com o dos pacientes que continuaram com a doença. Paralelo a isso, realizamos uma comparação de diferentes primers universais utilizados para determinar como estas variáveis afetam as métricas de diversidade bacteriana em amostras de peri-implantite. Através dos índices de diversidade, pudemos observar que a diversidade microbiana se mostrou similar: I) Entre diferentes superfícies de titânio que foram inseridas no meio bucal por 24 horas; II) Entre diferentes primers e regiões variáveis de amostras de peri-implantite; III) Ao comparar os tratamentos cirúrgicos e não cirúrgicos da peri-implantite; IV) Ao comparar os implantes que obtiveram sucesso com os que continuaram com a doença após o tratamento. No entanto, a diversidade bacteriana parece aumentar com a evolução da doença, e as espécies detectadas em baixa abundância são provavelmente as responsáveis para este aumento e contribuem para uma variabilidade interindividual da microbiota oral. Surpredentemente, via análisde de rede, identificamos que as bacterias da microbiota peri-implantar dos pacientes que obtiveram sucesso após o tratamento da peri-implantite estão interagindo mais antes do tratamento, e esse padrão se manteve ao longo de doze meses. Este achado pode sugerir que o grau de interação e sua força podem ser um indicador de como os pacientes responderão ao tratamento, abrindo novas perspectivas para o entendimento da falha dos implantes. Com isso, nesta tese avançamos em termos de conhecimento sobre a microbiota peri-implantar. A percepção das mudanças dinâmicas no microbioma em diferentes estados clínicos pode ser altamente útil no diagnóstico e prognóstico da peri-implantite na prática clínica, porém, ainda precisa ser vastamente estudada.

Palavras-chaves: Implate dentário; Peri-implantite; Microbioma oral; Sequenciamento de alto rendimento

Abstract

With the increase in the number of dental implants to replace missing teeth, there was also an increase in the number of peri-implant diseases diagnosed after their placement. Thus, the knowledge of the microbiota involved in these sites is necessary and has been increasingly studied through high-throughput sequencing technologies of the 16S rRNA gene. In this thesis we investigate the peri-implant microbiota in its initial adherence phase, established in health, established in disease and its evolution after different treatment approaches (surgical and non-surgical). In addition, we compared the peri-implant microbiological profile of patients who achieved success after treatment with those who continued with the disease. Parallel to this, we performed a comparison of different universal primers used to determine how these variables affect the metrics of bacterial diversity in peri-implantitis samples. Through the diversity indices, we could observe that the microbial diversity was similar: I) Between different surfaces of titanium that were inserted in the oral environment for 24 hours; II) Between different primers and variable regions of peri-implantitis samples; III) When comparing the surgical and non-surgical treatments of peri-implantitis; IV) When comparing the implants that were successful with those that continued with the disease after treatment. However, bacterial diversity seems to increase with the evolution of the disease, and the species detected in low abundance are probably responsible for this increase and contribute to an inter-individual variability of the oral microbiota. Surprisingly, via network analysis, we identified that the bacterial interactions in the peri implant microbiota were higher before the treatment in patients were the treatment of peri-implantitis succeed and this pattern remains within the twelve months. This finding may suggest that the degree of interaction and its strength may be an indicator of how patients will respond to treatment, opening new perspectives for understanding the failure of the implants. With this, in this thesis we advance in terms of knowledge about the peri-implant microbiota. The perception of dynamic changes in the microbiome in different clinical states can be highly useful in the diagnosis and prognosis of periimplantitis in clinical practice, however, it still needs to be widely studied.

Keywords: Dental Implate; Peri-implantitis; Oral microbiome; High throughput sequencing

Lista De Abreviaturas

Dpi	Doença peri-implamtar
HI	Implante saudável (do inglês health implant)
OTU	Unidades taxonômicas operacionais
P	Periodontite
PI	Peri-implantite
PM	Mucosite
PS	Profundidade de sondagem
SLA	Superfícies jateadas com areia e tratadas com ácido
SS	Sangramento à sondagem
Ti	Titânio

Introdução

As superfícies dos implantes

Há mais de 40 anos, o implante dentário têm sido utilizado com a finalidade de substituir dentes faltantes, sendo considerado nos dias de hoje um tratamento de escolha para uma reabilitação oral bem sucedida (Gbadebo, Lawal, Sulaiman, & Ajayi, 2014). A biocompatibilidade favorável encontrada no titânio comercialmente puro e nas ligas de titânio que os compõem é considerada um fator importante para o sucesso da osseointegração dos implantes dentários. Além disso, propriedades mecânicas apropriadas, como baixa gravidade específica e baixo módulo de elasticidade também são observados nestes materiais (Brama et al., 2007). No entanto, a formação de biofilme bacteriano em biomateriais susceptíveis a colonização microbiana, como os implantes de titânio, favorecem o desenvolvimento de infecções que, cada vez mais, têm impactado no desfecho dos casos (Arciola, Campoccia, Speziale, Montanaro, & Costerton, 2012; Lim et al., 2013).

Neste sentido, alguns estudos têm mostrado que variações na estrutura, topografia e no processo de fabricação de implantes dentários de diferentes materiais podem influenciar a adesão microbiana e levar a diferenças significativas na formação do biofilme peri-implantar (Rimondini et al., 1997; Schmidlin P.R., Müller P., Attin T., Wieland M., Hofer D. et al., 2013; B. Zhao et al., 2014). O uso de estratégias como modificação da superfície por antibióticos e polímeros antibacterianos por exemplo, tem sido bastante discutido em estudos atuais (H. et al., 2019). Através de um estudo *in vitro*, foi confirmado que o revestimento de polidopamina e prata na superfície do titânio retarda efetivamente o crescimento microbiano (Choi et al., 2019). Além disso, embora

superfícies rugosas sejam benéficas para a formação óssea inicial e osseointegração, elas também foram responsáveis por aumentar a adesão inicial e a subsequente colonização de bactérias orais, acumulando e retendo mais biofilme do que superfícies lisas (Marc Quirynen et al., 2006; Teughels, Van Assche, Sliepen, & Quirynen, 2006). Até o momento, os resultados da literatura demonstram dificuldades em decidir o valor ideal da rugosidade da superfície para melhor osseointegração, e menor adesão bacteriana (A. Jemat,1 M. J. Ghazali,1 M. Razali, 2015).

O biofilme e as doenças Peri-implantares

Devido ao seu elevado fator de virulência, como as endotoxinas e exotoxinas, o biofilme bacteriano é um dos principais desafios enfrentados pela clínica médica (Hall-Stoodley et al., 2012). Após a inserção do implante em boca, moléculas de água e fluidos contendo proteínas da saliva e do fluido crevicular gengival se ligam à superfície do implante, incorporando íons e formando uma película rica em proteínas, que serve de base para o desenvolvimento do biofilme oral (Dorkhan et al., 2012). À medida que o biofilme amadurece, novas espécies se aderem à superfície do biomaterial, aumentando o potencial de inflamação dos tecidos peri-implantares e destruição progressiva do osso de suporte (Dunne, 2002). Sabe-se que espécies de *Streptococcus* ou *Actinomyces* criam as condições prévias para a adesão de microorganismos anaeróbios Gram-negativos de colonização tardia, tais como espécies de *Fusobacterium* ou *Prevotella* (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005). Estes microorganismos, bem como *Aggregatibacter actinomycetemcomitans* ou *Porphyromonas gingivalis*, têm sido freqüentemente isoladas de sítios periodontais ou peri-implantares doentes e têm sido designadas como altamente relevantes para o desenvolvimento de processos inflamatórios crônicos nestes sítios

(Lafaurie et al., 2017; A. Leonhardt, Berglundh, Ericsson, & Dahlén, 1992). A contribuição de diferentes características da superfície do implante para o acúmulo de biofilmes e suas conseqüências clínicas têm sido discutidas (Größner-Schreiber et al., 2009), porém, o padrão de colonização dos implantes dentários parece ser diferente do que nos dentes naturais devido as superfícies imaculadas do implante e a falta da microbiota inicial desejada (M. Quirynen et al., 2005). No entanto, uma vez que o biofilme é estabelecido, ele age como um desafio microbiano causando reações nos tecidos moles e duros ao redor dos implantes. Desta maneira, um desequilíbrio entre o desafio bacteriano e a defesa do hospedeiro pode evoluir para uma doença peri-implantar, mesmo após uma adequada osseointegração do implante (Charalampakis, Ramberg, Dahlén, Berglundh, & Abrahamsson, 2015).

As doenças peri-implantares são divididas em mucosite peri-implantar (PM) e peri-implantite (PI). A PM afeta a mucosa peri-implantar apenas e, desta forma, é considerada uma reação inflamatória reversível. Esta condição pode ser clinicamente detectada por meio da presença de sangramento marginal da mucosa em implantes sem sinais radiográficos de perda óssea, podendo haver sinais adicionais como eritema, edema e supuração. Um aumento de profundidade de sondagem (PS) pode estar presente devido ao edema ou a diminuição da resistência dos tecidos à sondagem (Nicola U. Zitzmann & Berglundh, 2008). Por outro lado, a PI ocorre quando o processo inflamatório progride e envolve os tecidos de suporte do implante, causando sangramento à sondagem e/ou supuração, aumento da PS e/ou recessão da margem da mucosa, além da perda óssea radiográfica em comparação aos exames passados (Berglundh et al., 2018; Charalampakis et al., 2015). No artigo de consenso de 2017, foi estabelecido que na

ausência de radiografias iniciais e medidas de PS inicial, seria necessário perda óssea radiográfica ≥3 mm e PSi ≥6 mm com sangramento para o diagnóstico de perimplantite (Renvert, Persson, Pirih, & Camargo, 2018). Quando temos uma radiografia inicial a remodelação óssea de no máximo 2 mm deve ser levada em consideração durante o primeiro ano que o implante foi colocado em função (Renvert et al., 2018). Qualquer perda óssea vertical maior do que a descrita, foi sugerida como ponto de corte apropriado para indicação de PI e deve ser considerada patológica (Sanz & Chapple, 2012).

Estudos recentes mostraram que tanto a PM quanto a PI são prevalentes. Na revisão sistemática de Atieh et al. (2013), foi constatado que 63,4% dos pacientes e 30,7% dos implantes apresentavam PM. Já a PI foi encontrada em 18,8% dos indivíduos e 9,6% dos implantes (Atieh, Alsabeeha, Faggion, & Duncan, 2012). Em uma outra revisão, foi constatado que a PI ocorre em cerca de 22% dos pacientes e 60% dos implantes, enquanto a mucosite ocorre em cerca de 43% dos pacientes e 66% dos implantes. No entanto, o estudo reporta alta variabilidade dos critérios de diagnóstico utilizados e diferentes tempos de acompanhamento, o que impõe limitações aos dados gerados pela metanálise (Derks & Tomasi, 2015). Curiosamente, do ponto de vista etiopatogênico, as doenças perimplantares (DPi) guardam bastante semelhança com as doenças periodontais. No entanto, diferem no que diz respeito às características anatômicas e à composição celular da lesão e, por isso, não devem ser comparadas diretamente em seus aspectos de diagnóstico e tratamento. Sítios com implantes apresentam ausência de ligamento periodontal, disposição das fibras colágenas paralelas ao implante, menor estrutura vascular, e tecidos mais desorganizados do que os sítios periodontais. Além disso, a PI difere em alguns eventos biológicos como maior

destruição tecidual nos parâmetros clínicos, radiográficos e histomorfométricos, apresentando uma taxa de progressão da doença significativamente maior do que na periodontite (Berglundh et al., 2018; G. E. Salvi, Cosgarea, & Sculean, 2017).

Quanto aos fatores de risco para as doenças peri-implantares, há fortes evidências de que existe um risco aumentado de desenvolvimento de PI em pacientes com histórico de periodontite, controle inadequado da placa e ausência de manutenção regular após a terapia com implantes. Tabagismo e diabetes apresentam dados inconclusivos na literatura como indicadores potenciais de risco para a PI. Adicionalmente, existe evidências limitadas sobre presença de mucosa queratinizada, excesso de cimento, fatores genéticos, sobrecarga oclusal e tipos de superfície implantar como indicadores de risco da PI (Berglundh et al., 2018). Pelo fato de o controle de placa assumir primordial importância para as condições peri-implantares compatíveis com saúde, uma manutenção de um padrão de controle do biofilme adequado é essencial (N. U. Zitzmann, Berglundh, Marinello, & Lindhe, 2001). Com isso, o desenho protético das próteses implanto suportadas é de extrema importância, visto que um perfil de emergência com sobrecontorno mais de 30 graus no ângulo de emergência, dificultam a remoção da placa e consequentemente levam a maiores riscos de desenvolver PI (Katafuchi, Weinstein, Leroux, Chen, & Daubert, 2018).

As terapias propostas para o tratamento das doenças peri-implantares são baseadas no diagnóstico e podem variar de acordo com a severidade da lesão perimplantar (Lindhe & Meyle, 2008). O objetivo principal é controlar a inflamação, reduzindo a carga de microrganismos patogênicos, permitindo a recuperação da saúde dos tecidos perimplantares, possibilitando o ganho ósseo, bem como prevenir a

progressão da doença (L. J. A. Heitz-Mayfield, 2008). Atualmente, diferentes técnicas e protocolos são propostos na literatura como as terapias não cirúrgicas e terapias cirúrgicas, onde é feito um retalho para exposição da superfície do implante infectado facilitando o acesso para descontaminação. As terapias cirúrgicas podem incluir utilização de biomateriais para preenchimento e regeneração dos tecidos lesados pela PI (L. Heitz-Mayfield & Mombelli, 2014; Ramanauskaite, Daugela, Faria de Almeida, & Saulacic, 2016). No entanto, nenhuma metodologia foi estabelecida até hoje como padrão ouro no tratamento dessa doença (Schwarz, Schmucker, & Becker, 2015).

Entre as modalidades de terapias não cirúrgicas existentes estão a raspagem mecânica do local afetado, jato de ar abrasivo, e uso de adjuntos com antissépticos locais, laserterapia, antibioticoterapia local ou sistêmica e terapia fotodinâmica antimicrobiana (Esposito, Grusovin, & Worthington, 2012). As técnicas cirúrgicas podem ser divididas em cirurgia ressectiva e regenerativa (Roccuzzo, Gaudioso, Lungo, & Dalmasso, 2016). O tratamento cirúrgico ressectivo tenta eliminar os fatores etiológicos e manter uma boa condição para o implante, principalmente pela limpeza das superfícies através do debridamento em campo aberto, nivelamento dos defeitos ósseos peri-implantares e também o posicionamento apical do retalho quando necessário. A cirurgia ressectiva visa reduzir a profundidade de sondagem e obter uma morfologia tecidual favorável à higiene. Já a terapia cirúrgica regenerativa tem como objetivo regenerar o defeito ósseo peri-implantar e reconstruir a unidade peri-implantar aos limites fisiológicos normais previamente existentes utilizando enxertos ósseos, membranas e fatores de crescimento (Lang, Wilson, & Corbet, 2000). O tratamento cirúrgico da perimplantite é indicado em casos em que existe uma dificuldade de acesso ao implante através da técnica não

cirúrgica (Figuero, Graziani, Sanz, Herrera, & Sanz, 2014), ou após abordagens não cirúrgicas apresentarem recorrência de sangramento e supuração (Khoury et al., 2019). Além disso, a abordagem cirúrgica vem sendo indicada como a mais apropriada na descontaminação em casos de perimplantite de grau moderado e severo (Mahato, Wu, & Wang, 2016). Por outro lado, as modalidades de tratamento não cirúrgico não regenerativas parecem reduzir a inflamação à curto prazo, mas ainda faltam estudos com acompanhamentos maiores (Khoury et al., 2019). O sucesso do tratamento deve incluir resolução dos sinais inflamatórios como ausência de SSi e redução da PSi em conjunto com preservação do osso de suporte (Sanz & Chapple, 2012). Adicionalmente, o bom controle de biofilme pelo paciente aumenta o sucesso da terapia perimplantar a longo prazo, com isso, após o tratamento, também é essencial reeducar e motivar o paciente em relação à instrução da higiene bucal (Ramanauskaite et al., 2016).

O perfil microbiano e as metodologias de detecção

Para melhor compreender as respostas ao tratamento e a patogênese da doença peri-implantar, é necessário conhecer detalhadamente a microbiota nela envolvida. A doença peri-implantar tem sido descrita como uma infecção anaeróbica colonizada predominantemente por bactérias Gram negativas. Após 30 minutos da colocação do implante, a colonização da superfície por espécies microbianas se inicia e durante a primeira semana a quantidade de bactérias permanece inalterada. Entre a primeira e a décima segunda semana após a cirurgia de colocação do implante, a quantidade de bactérias aumenta significativamente para várias espécies como *P. gingivalis*, *T. forsythia* e *T. denticola* (Fürst, Salvi, Lang, & Persson, 2007). Já aos 12 meses, a carga bacteriana parece ser significativamente maior para algumas espécies, em particular *T. forsythia* e,

em menor grau, *P. gingivalis* (G. E. G. E. Salvi, Fürst, Lang, & Persson, 2008). De acordo com estes estudos citados, a composição microbiana peri-implantar se assemelha à dos dentes vizinhos, o que implica que os dentes podem servir como um reservatório microbiano para a formação do biofilme em torno dos implantes em um mesmo indivíduo tanto na saúde, quanto na doença peri-implantar (Kočar et al., 2010; A. Leonhardt et al., 1999). No entanto, estes estudos tem a limitação de usar métodos dependentes de cultura ou moleculares que identificam apenas bactérias específicas em torno dos implantes.

Atualmente, graças as novas tecnologias, têm sido encontrado um número crescente de microorganismos em ambiente peri-implantar os quais não são encontrados com frequência em torno dos dentes, mostrando esta ser uma infecção mais complexa e diferente da doença periodontal (Belibasakis, Charalampakis, Bostanci, & Stadlinger, 2015; Renvert & Quirynen, 2015). Por outro lado, a microbiota presente nas diferentes condições peri-implantares permanece incerta e levando em conta sua natureza infecciosa, faz sentido tentar traçar o perfil microbiano dessa doença, assim, muitos estudos têm avaliado a microbiota peri-implantar em implantes saudáveis e com doença usando diferentes técnicas de detecção (Padial-Molina, López-Martínez, O'Valle, & Galindo-Moreno, 2016; Rakic, Grusovin, & Canullo, 2016; Renvert & Quirynen, 2015).

Nos últimos anos, ganhou destaque o uso de métodos independentes de cultura, como o sequenciamento de genes do RNA ribossômico 16S (rRNA), onde uma comunidade bacteriana pode ser detectada independentemente de qualquer bactéria conhecida (Größner-Schreiber et al., 2009). O gene 16S rRNA é um marcador versátil e importante para o perfil de populações bacterianas. Geralmente é composto por nove regiões hipervariáveis (V1 a V9), e por nove regiões altamente conservadas (C1-C9).

Essa metodologia de alto rendimento pode fornecer uma riqueza de informações, uma vez que aumenta significativamente a quantidade de espécies detectáveis, pois possibilita a detecção de bactérias incultiváveis e gera uma grande quantidade de informações que podem permitir a identificação de altos níveis de diversidade microbiana (Ziganshina et al., 2015), como por exemplo a detecção dos membros do filo TM7, que eram anteriormente desconhecidos e atualmente tem seu papel no processo saúde-doença sendo investigado (Charalampakis & Belibasakis, 2015). Na área da odontologia, o sequenciamento é provavelmente a técnica que oferece uma análise mais abrangente da taxonomia da comunidade associada a saúde e a doença peri-implantar (da Silva et al., 2014). No entanto, a maioria dos estudos têm analisado a microbiota peri-implantar em estudos observacionais e descritivos, mostrando que existem poucas informações sobre as mudanças da microbiota em acompanhamentos longitudinais (Laksmana et al., 2013).

Objetivo

Investigar a composição bacteriana e diversidade relacionada com implantes de titânio em condições de saúde e de doença peri-implantar por meio de sequenciamento do gene 16S rRNA.

Objetivos Específicos

- Caracterizar o perfil das comunidades microbianas primitivas que colonizam diferentes tratamentos de superfície de titânio após exposição ao ambiente oral.

- Analisar sistematicamente a literatura, a fim de descrever a microbiota apresentada em diferentes condições peri-implantares.

- Avaliar o impacto de diferentes regiões do gene 16S rRNA (V3-V4, V4 e V5-V6) nas métricas de diversidade bacteriana em amostras de peri-implantite.

- Examinar a microbiota submucosa em pacientes com peri-implantite antes e após o debridamento cirúrgico e não-cirúrgico como tratamento da peri-implantite.

- Examinar a microbiota submucosa em pacientes que obtiveram sucesso e em pacientes que continuaram com peri-implantite após o tratamento.

PARTE II

Nesta seção os resultados serão apresentados em capítulos. Os capítulos são compostos por um breve prefácio seguido de um artigo científico publicado ou em preparação. O capítulo I apresenta um estudo *in* situ comparando a adesão bacteriana inicial em diferentes superfícies de titânio. O capítulo II apresenta uma revisão sistemática visando um maior entendimento da microbiota envolvida nas doenças peri-implantares. Por fim, os capítulos III e IV demonstram os estudos experimentais com sequenciamento do gene 16S rRNA com a finalidade de avançar na compreensão da microbiota envolvida na peri-implantite estabelecida e após diferentes tratamentos da doença.

Capítulo I. Identification of oral bacteria on titanium implant surfaces by 16S rDNA sequencing

No **capítulo I** apresentamos o artigo publicado no periódico *Clinical Oral Implants Research.*

Sabe-se que as doenças peri-implantares estão intimamente relacionadas à formação de biofilme bacteriano na superfície do implante dentário. Com isso, cada vez mais tem sido fabricadas opções de superfícies de implantes modificadas e tratadas com o intiuto de reduzir a adesão bacteriana e melhorar a osseointegração. Neste trabalho, queríamos caracterizar o perfil das comunidades microbianas que colonizam os implantes com superfícies diferentes. Para isso, realizamos o sequenciamento do gene 16S rDNA em amostras de biofilme de discos de titânio submetidos em cavidade oral por 24 horas. Nosso trabalho mostrou não haver diferenças em relação às unidades taxonômicas operacionais (OTUs, do inglês *operational taxonomic unit*) e diversidade microbiana, quando comparadas as superfícies jateadas com areia e tratadas com ácido (SLA, do inglês *sandblasted large-grit and acid-etched*) com usinadas. Com estes resultados, sugerimos que uma maior complexidade da topografia da superfície do titânio na fase inicial da maturação do biofilme não parece influenciar significativamente a microbiota colonizadora.

CLINICAL ORAL IMPLANTS RESEARCH

Fabiana de Melo
Cássio do Nascimento
Diogo Onofre Souza
Rubens F. de Albuquerque Jr

Identification of oral bacteria on titanium implant surfaces by 16S rDNA sequencing

Authors' affiliations:
Fabiana de Melo, Diogo Onofre Souza, Department of Biochemistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
Fabiana de Melo, Faculty of Dentistry, McGill University, Montreal, Quebec, Canada
Fabiana de Melo, Department of Periodontology, School of Dentistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
Cássio do Nascimento, Rubens F. de Albuquerque Jr, Department of Dental Materials and Prosthodontics, Faculty of Dentistry of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, Brazil

Corresponding author:
Rubens F. de Albuquerque Jr
Faculty of Dentistry of Ribeirão Preto, University of São Paulo, Av. Café s/n, Ribeirão Preto 14040-904, SP, Brazil
Tel.: +55 16 33154152
Fax: +55 16 33154090
e-mail: rubens.albuquerque@usp.br

Key words: bacteria, dental implant, implant, implant surface, Miseq, oral microbiome, sequencing, titanium

Abstract

Objective: To characterize the profile of microbial communities colonizing titanium implants with different surface treatments after exposure to the oral environment at the genus or higher taxonomic level.

Material and methods: Sixteen titanium disks, machined or sandblasted large-grit and acid-etched (SLA), were mounted on removable intraoral splints worn by four patients. After 24 h of intraoral exposure, biofilm samples were collected from disks and supra/subgingival teeth areas. The 16S rDNA genes from each sample were amplified, sequenced with the Miseq Illumina instrument and analyzed.

Results: A total of 29 genera and seven more inclusive taxa, representing the phyla *Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, Actinobacteria* and candidate division *TM7* were identified in both titanium surfaces and teeth. No differences were found in relation to the operational taxonomic units (OTUs) and microbial diversity, assessed by Chao 1 and Shannon indices, when comparing SLA and machined titanium surfaces.

Conclusions: Machined and SLA surfaces are colonized by similar numbers of prokaryotic OTUs after 24 h of exposure to the oral environment. Higher complexity of the titanium surface topography in the initial phase of biofilm maturation does not seem to significantly influence the colonizing microbiota.

Commercially, pure titanium (cpTi) and the Ti-6Al-4V alloy have been largely used in dental and orthopedic implants and have developed into a successful option for tooth replacement due to their favorable biocompatibility, associated with appropriate mechanical properties such as low specific gravity and low elastic modulus (Brama et al. 2007). However, late implant failures still occur and are frequently associated with inflammation of soft and hard tissues around the implants, which results in bone reabsorption and ultimately leads to implant loss (Lindhe et al. 1992; Esposito et al. 1998). Recent studies showed that peri-implantitis occurs in 20% of the implants with an average time in function varying between 5 and 11 years (Mombelli et al. 2012) and is closely related to bacterial biofilm formation on the implant surface (Teughels et al. 2006). In addition, studies on the literature reported implant failure rates ranging from 4 up to 34% (Chrcanovic et al. 2015). Briefly, after the implant insertion, water molecules and fluids containing protein from saliva, gingival crevicular fluid and serum bind to the implant surface incorporating ions and forming a pellicle rich in protein, which serves as basis for the development of the oral biofilm (Dorkhan et al. 2012). As the biofilm matures, new species become attached to the biomaterial surface, increasing the potential for peri-implant tissue inflammation and progressive destruction of the supporting bone (Dunne 2002).

Structural and topographical variations of different materials and manufacturing processes employed to fabricate dental implants may influence the microbial adhesion and can lead to significant differences in the formed microbiome (Rimondini et al. 1997; Schmidlin et al. 2013; Zhao et al. 2014). Although rough surfaces are beneficial for initial bone formation and osseointegration, they have been also considered to increase initial adhesion and the subsequent colonization of oral bacteria accumulating and retaining more biofilm than surfaces comparatively

Date:
Accepted 20 April 2016

To cite this article:
de Melo F, Nascimento Cd, Souza DO, Albuquerque Jr RF. Identification of oral bacteria on titanium implant surfaces by 16S rDNA sequencing.
Clin. Oral Impl. Res. **00**, 2016, 1–7
doi: 10.1111/clr.12865

smoother (Quirynen et al. 2006; Teughels et al. 2006). Furthermore, modification of titanium (Ti) implants' surface can improve the long-term success by reducing the adhesion of oral biofilm and thereby the risk of infection (Scarano et al. 2004; Grossner-Schreiber et al. 2009).

On the other hand, identifying the oral microbiota associated with specific surfaces is essential to understand the mechanisms involved in the initial bacterial colonization of implants and potential development of diseases. It is well known that some of the periodontal disease-related bacteria were found to be present on dental implant surfaces as early as 30 min after its insertion (Furst et al. 2007). Sequencing technologies have provided new insights into the diversity of microbial communities residing in the human body and also associated with implants and teeth (Li et al. 2010; Shchipkova et al. 2010; Caporaso et al. 2011). Recently, 16S rDNA gene-based techniques have been used to identify potential pathogens from the oral environment (Li et al. 2010; Shchipkova et al. 2010). This strategy has the advantage to potentially detect the complete genome of bacterial communities irrespective of their previous assessment (Grossner-Schreiber et al. 2009; Heuer et al. 2011).

The purpose of this *in situ* investigation was to characterize – via next-generation Miseq Illumina sequencing platform – the profile of early microbial communities colonizing titanium implants with different surface treatments after exposure to the oral environment at genus or higher taxonomic levels.

Material and methods

Four healthy individuals (mean age = 32.5 - years, SD = 11.7) with no history of antibiotic use in the past 6 months, caries free, and without clinical signs of oral mucosal disease or periodontal disease participated in this investigation, which was approved by the McGill Faculty of Medicine Institutional Review Board (IRB). Periodontal health has been defined as the absence of periodontal pocket deeper than 3 mm and no bleeding on probing in more than 10% of the gingival sites (Barendregt et al. 2002). Participants were asked to wear a removable device made of ethylene-vinyl acetate and polypropylene (PP/EVA) with four titanium disks attached on the maxillary arch for 24 h. A total of 16 titanium grade-2 disks (15 mm × 1 mm), eight machined and eight sandblasted large-grit and acid-etched (SLA), were used in this study. One SLA and one machined disk were fixed to the buccal right and left sides of each device. Patients were requested to remove their devices from the mouth and stored them in phosphate-buffered saline (PBS) only during meals and maintain their regular hygiene routine. After 24 h, the disks were separated from the removable device, washed with PBS for removing the non-adherent microorganisms (planktonic biofilm), stored in a TE buffer and frozen immediately at −70°C for nucleic acid extraction. Additionally, supra and subgingival biofilm samples were collected from the upper and lower molars and premolars for characterization of the patients' mature biofilm. Samples from teeth of each participant were collected with individual sterile Gracey curettes and pooled in separate polypropylene tubes containing 50 μl TE (10 mM Tris-HCL and 1 mm ethylenediaminetetraacetic acid [EDTA], pH 7.6).

Nucleic acid extraction

Total DNA was obtained using DNeasy blood and tissue kit, according to the manufacturer's instructions (DNeasy kit; Qiagen, Germantown, MD, USA). DNA extraction was performed for both Gram-positive and Gram-negative bacteria, using lysozyme to break the bacterial wall in the initial steps. Elution was performed by applying two times 100 μl of ultrapure water at 50°C, for a final volume of 200 μl. The samples were concentrated by SpeedVac (evaporation) and then resuspended in 30 μl of Tris-HCl buffer (10 mM, pH = 7.6). DNA concentration was determined with PicoGreen® dsDNA quantitation assay kit (Life Technologies, Sunnyvale, CA, USA), using an U.V. reader SpectraMax Gemini XS (Life Technologies, Molecular Devices LLC, Sunnyvale, CA, USA).

Illumina Miseq sequencing of 16S rDNA genes

Polymerase chain reactions (PCR) and samples barcoding were performed in one step: in a single reaction, we amplify the V4 region of the 16S rDNA gene with universal primers: forward primer 347F (GGAGGCAG CAGTRRGGAAT) and reverse primer 803R (CTACCRGGGTATCTAATCC) (Nossa et al. 2010) with CS1 and CS2 tag and use these tags as anchors in a second PCR performed concomitantly in the same tube to add a barcode and Illumina adapters. 16S rDNA amplification was performed in 25 μl reactions using the Fast Start High Fidelity PCR System from Roche (USA) and 12.5 ng of microbial DNA. PCR cycles were as follows: initial denaturation step of 95°C, for 3 min; 25 cycles of 95°C, for 30 s, 55°C, for 30 s and 72°C, for 30 s; final extension, at 72°C, for 5 min. DNA sequences of 2 × 250 bp were generated with a MiSeq (Illumina, Inc., San Diego, CA, USA) instrument. A total of 6,321,326 paired-end reads (i.e., 3,160,663 forward reads and 3,160,663 reverse reads) were obtained, of which 2,741,186 successfully assembled using shared overlapping parts of read pairs (FLASH 1.2.7, Fast Length Adjustment of Short Reads Software) (Magoc & Salzberg 2011). All reads were trimmed to a default fix length of 165 bp. Primer sequences were removed from assembled reads, which were then filtered for quality; all reads having average quality below 30 or having more than two ambiguous bases (Ns) and three nucleotides below quality 20 were discarded. This gave a total of 920,608 filtered reads that were clustered using an in-house clustering algorithm. Briefly, reads were clustered at 100% identity and then clustered/denoized at 99% identity (dnaclust 3). Clusters having abundance lower than 3 were discarded. Remaining clusters were then scanned for chimeras with UCHIME denovo and UCHIME reference (Edgar et al. 2011) (using the gold reference from the Microbiome Utilities Portal of the Broad Institute) and clustered at 97% (dnaclust 3) to form the final clusters/operational taxonomic units (OTUs). OTUs were then analyzed for taxonomic assignment of 16S rDNA metabarcoding sequences using a combination of in-house programs and scripts from the Qiime (qiime.org) software suite. Briefly, OTUs were classified with the Ribosomal Database Project (RDP) classifier tool (Wang et al. 2007) using an in-house training set containing the complete Greengenes database supplemented with eukaryotic sequences from the Silva databases (http://www.arb-silva.de) and a customized set of mitochondria and chloroplasts 16S sequences. Diversity metrics were obtained by aligning OTU sequences on a Greengenes core reference alignment (DeSantis et al. 2006) using the PyNAST aligner (Caporaso et al. 2010a,b). Alignments were filtered to keep only the hypervariable region part of the alignment. Phylogenetics trees were generated and visualized using FastTree (Price et al. 2009). Alpha (observed species) and beta (weighted, unweighted UniFrac and Bray–Curtis dissimilarity) diversity metrics, taxonomic classifications, UPGMA clustering and OTUs heat map were then computed using the QIIME software suite (Caporaso et al. 2010a,b).

Statistical analysis

Considering "patient" as the independent experimental unit, the outcome variables (OTUs levels and relative abundance of microbial phyla/genera on titanium disks) were summarized as medians and quartiles and compared using Wilcoxon's signed-rank test with Benjamini–Hochberg false discovery rate (FDR) adjustment for multiple comparisons. Means were also calculated and indicated in the figures by the plus sign (+). Chao 1 and Shannon's indices of diversity were determined for titanium disks with machined and SLA surface treatments and compared using Wilcoxon's signed-rank test. Microbial diversity data from teeth were summarized and displayed as descriptive data only. Statistical significance was set at 0.05 probability level. Analyses were performed with the aid of GraphPad Prism 5.0 Software (GraphPad Software Inc., La Jolla, CA, USA) and R Statistical Software Package with the Stats library 3.4.0 (http://www.r-project.org/).

Results

General sequence data

A total of 663.943 sequences were used in the analysis. All reads were trimmed to a fix length (default: 165 bp) and clustering these sequences into OTUs at a 3% genetic distance resulted in 541 different OTUs. Machined and SLA surfaces showed similar number of OTUs (Fig. 1).

Diversity and taxonomy

Overall, OTUs were grouped in 36 higher taxa, consisting of 29 genera and seven more inclusive taxa (e.g., family, order, class), representing the phyla *Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, Actinobacteria, Synergistetes* and the candidate division *TM7,* with *Firmicutes* accounting for 48.4% of all sequences. OTU numbers for specific genera or as a whole did not significantly differ between the titanium surfaces evaluated (Fig. 2). The phylum *Synergistetes* was only found on SLA surfaces.

The total number of bacterial taxa identified in samples collected from teeth, SLA and machined surfaces was 36, 27 and 26, respectively. Percentage of the most abundant bacterial genera from each examined surface is shown in Fig. 3 (percentages lower than 1% were not included). Most of the identified genera were found in higher levels on the SLA surfaces while *Streptococcus* and *Neisseria* were more frequently found on machined surfaces, although the differences were not statistically significant. (Fig. 4). Genera *Kingella, Anaeroglobus, Tannerella* and TG5 were found on SLA surfaces but not on machined surfaces, whereas genera *Selenomonas* and *Eikenella* were found on machined surfaces but not on SLA surfaces. On the other hand, the genera *Jhonsonella, Dialister, Corynebacterium, Cardiobacterium* and the order *EW055* were only found in samples from tooth surfaces. The Fig. 5 represents a phylogenetic tree of the identified OTUs.

Microbial richness of the SLA and machined surfaces, as assessed with the Chao 1 index, was similar. Shannon's diversity index was higher for SLA than machined surfaces, although the difference did not reach statistical significance (Fig. 6).

Discussion

The influence of the diversity and the quantity of biofilm adhesion on the long-term success of dental implants has been approached

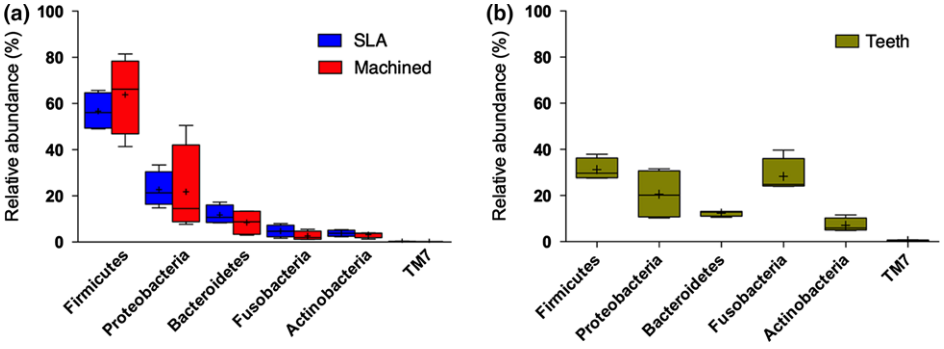


Fig. 2. Relative abundance of the main phyla identified in (a) sandblasted large-grit and acid-etched (SLA) and machined titanium surfaces and (b) teeth surfaces (descriptive data). Only phyla with a relative abundance >0.3% are shown. Together, the phyla depicted account for >99% of all the identified sequences. Data presented as median and quartiles. Mean is also presented as +.

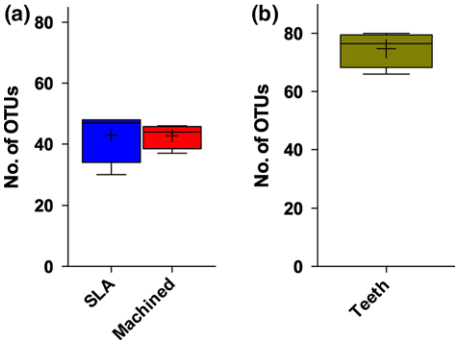


Fig. 1. Number of different operational taxonomic units (OTUs) in (a) sandblasted large-grit and acid-etched (SLA) and machined titanium and (b) teeth surfaces (descriptive data). Data presented as median and quartiles. Mean is also presented as +.

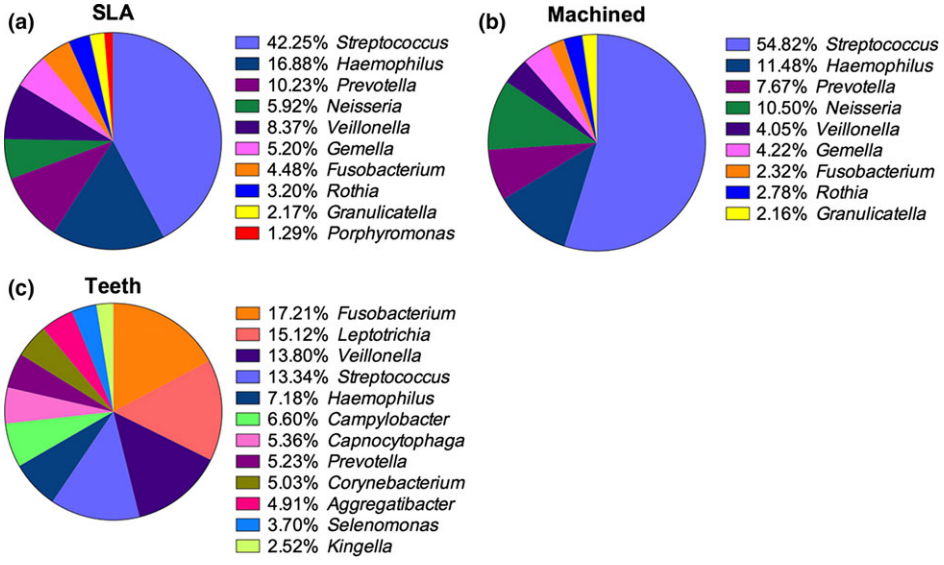


Fig. 3. Percentages of the most abundant bacterial genera on sandblasted large-grit and acid-etched (SLA) titanium (a), machined titanium (b) and teeth surfaces (c). Percentages lower than 1% were not included.

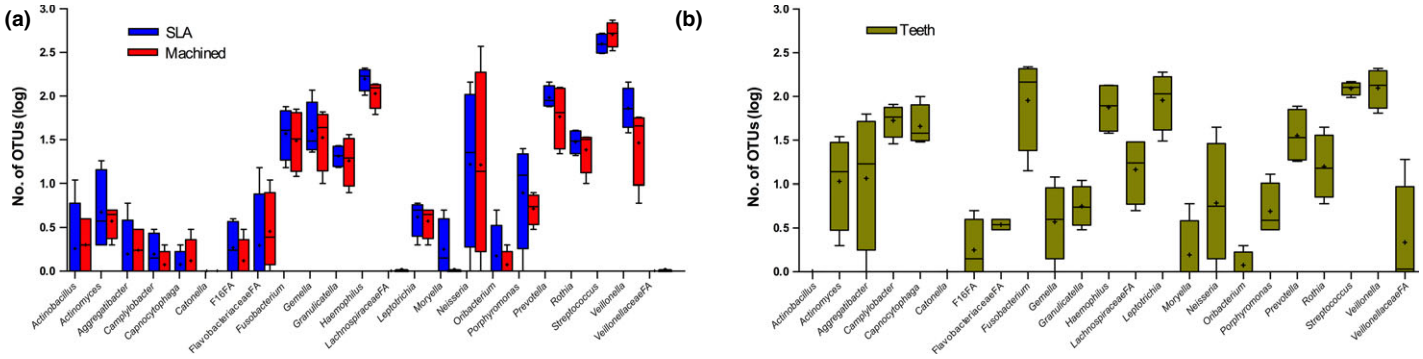


Fig. 4. Log-transformed operational taxonomic units (OTUs) per 1000 reads by genus are shown in (a) sandblasted large-grit and acid-etched (SLA) and machined titanium and (b) teeth surfaces (descriptive data). Data presented as median and quartiles. Mean is also presented as +.

in several studies (Ong et al. 1992; Kolenbrander 2000). These investigations have fairly documented differences between the microbial profile in peri-implantitis and healthy peri-implant tissues, but little is known regarding the microbiota profile of the initial colonization of titanium implant surfaces, and how different surface topography and structure may affect the profile of the adhered biofilm. Recent advances on DNA sequencing and bioinformatics technology has allowed the investigation of dental- and implant-related biofilms under a new perspective, generating a breakthrough in the field of the oral microbiology (Zaura et al. 2009; Griffen et al. 2012). Using this novel approach, we characterized the specific bacterial profiles – as indexed by 16S rDNA sequencing – related to SLA and machined titanium surfaces, as well as teeth, after exposure to the oral environment for 24 h. Despite this relatively short exposure time, tooth surfaces exhibit 60% more OTU numbers than titanium surfaces from 29 genera belonging to seven different phyla. Moreover, this study suggests that SLA and machined titanium surfaces are promptly colonized by a similar prokaryote community. Further studies using an increased number of patients will potentially confirm our early findings. Surprisingly, our findings showed that *Synergistetes* (recognized phylum of anaerobic bacteria) and other Gram-negative anaerobic bacteria represented by *Kingella*, *Anaeroglobus*, *Tanerella* and TG5 genera were restricted to SLA surfaces. On the other hand, *Selenomonas* and *Eikenella* genera were found colonizing only machined surfaces. This is an interesting finding since Gram-negative and anaerobic organisms emerge as the biofilms matures. All of these species are commonly found in advanced stages of biofilm maturation (Jhajharia et al. 2015). These data may be of relevance since *Tanerella* spp. and *Eikenella* spp. may act as a potential risk factor in the etiology of peri-implantitis (Chan et al. 2011; Persson & Renvert 2014).

In our study, we found that the phylum *Firmicutes* predominates in titanium samples. According to Griffen et al. (2012), within the phylum *Firmicutes*, the class *Clostridia* was associated with disease, whereas the class *Bacilli* seems related to oral health (Griffen et al. 2012). Curiously, in our study, the class *Clostridia* was prevalent (*Catonella, Johnsonella, Moryella, Oribacterium Anaeroglubus, Dialister, Selenomonas, Veillonella,* order *clostridiales* and family *Veillonellaceae* and *Lachnospiraceae*), while *Bacilli* (*Streptococcus, Granulicatella* and *Gemela*) was more abundant. In other words, after 24 h exposed to the oral environment, the titanium samples were colonized by a health-related microbiota. Both titanium surfaces presented higher proportions of *Streptococcus*, which is in agreement with previous studies (Grossner-Schreiber et al. 2009; Koyanagi et al. 2010; Kumar et al. 2012; Sanchez et al. 2014; da Silva et al. 2014), although comparisons between the dental biofilm and the early microbial colonization of the titanium surfaces should be interpreted with caution due to their distinct formation time frame. *Streptococci* are considered to be early colonizers, and regarded as pioneers for later colonizers that demand more challenging growth conditions (Socransky et al. 1998). Several studies have shown an improvement in knowledge about the mechanisms that occur during dental biofilm maturation (Ritz 1967; Nyvad & Kilian 1987; Nakazato et al. 1989). In 1987, Nyvad (Nyvad & Kilian 1987) described that in a clean tooth surface, *Streptococci* were the first dominant colonizers, and within 12 h of their adherence, the microbiota diversified appearing strains of *Actinomyces, Capnocytophagae, Haemophili, Prevotellae, Propionibacteria* and *Veillonellae. Proteobacteria* phylum was the second predominant taxa found in our study, with higher counts for *Haemophilus* found on SLA than machined surfaces. This is in accordance with a previous study from Rehman et al. (2012), who evaluated different implant materials exposed to the oral environment of one adult for 24 h and 14 days and identified *Streptococcus* and *Haemophilus* as dominant early colonizers.

Leonhardt et al. (1999) reported that 60% of the patients with peri-implantitis harbored putative periodontal pathogens, such as *Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens* and *Aggregatibacter* actinomycetemcomitans. Furthermore, *Prevotella* spp., *Actinobacillus* spp. and *Porphyromonas* spp. are well known as potential periodontal pathogens (Leonhardt et al. 1999; Mombelli et al. 2000; Romeo et al. 2004). In our study, *Prevotella* and *Actinobacillus* genera were observed in higher counts in SLA followed by machined titanium and tooth surfaces. Interestingly, the *Porphyromonas* genus was more frequently seen in the SLA than the other analyzed surfaces. Despite the overall lack of statistical differences related to bacteria from these taxa, their early adhesion on titanium surfaces, especially in the SLA, was a surprising finding since *Porphyromonas* is considered a late colonizer in the oral flora (Periasamy & Kolenbrander 2009; Rehman et al. 2012).

The results of this study support the hypothesis that topography of surface does not interfere with bacterial adhesion. Frojd et al. (2011) reported in a recent *in situ* investigation that the influence of surface characteristics on adhesion is compensated for by the biofilm development (Frojd et al. 2011). In their study, after 2 h, the biofilm adherence was significantly lower on smooth turned titanium surfaces but after 14 h, the biofilm volume on all surfaces was similar.

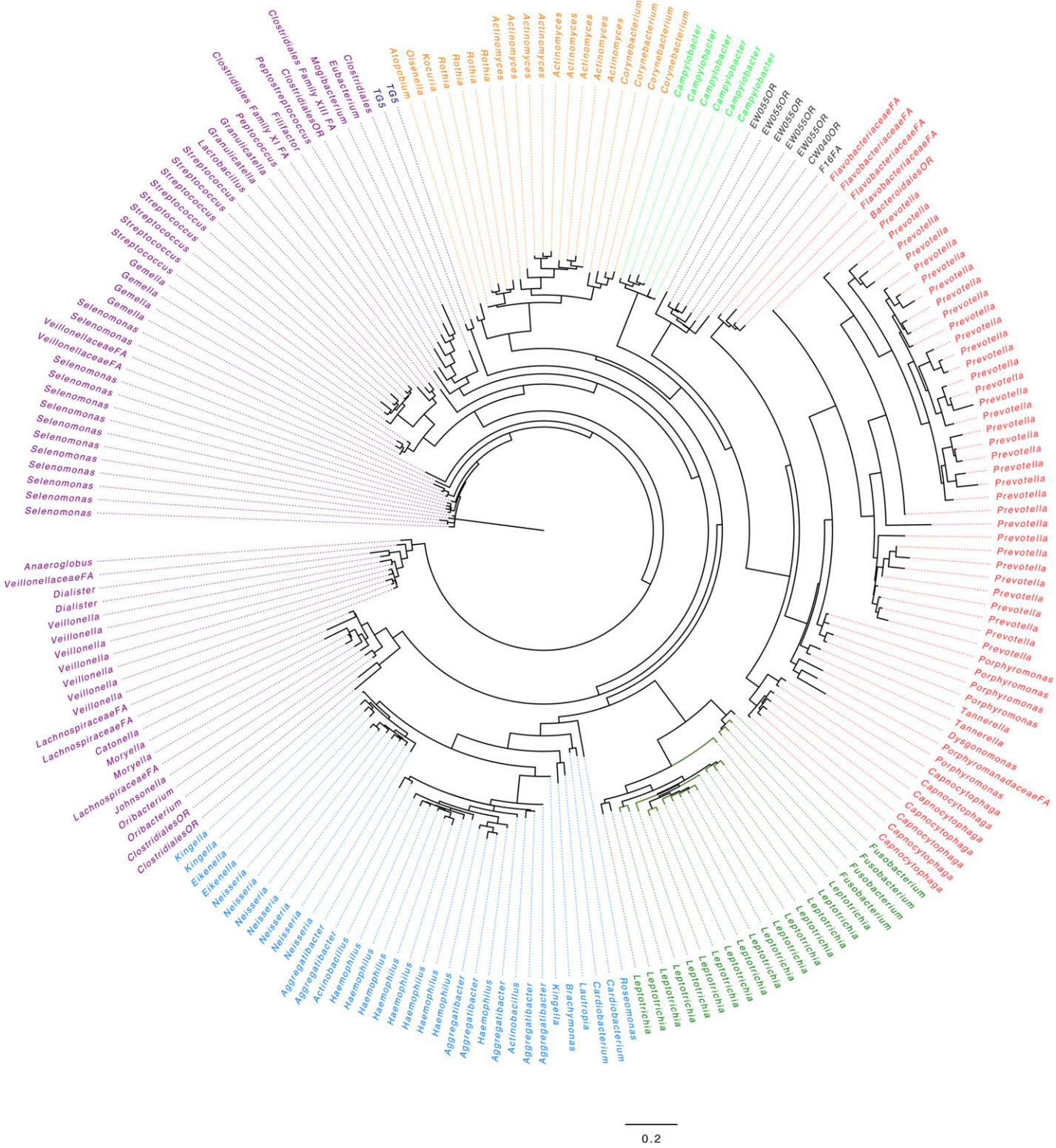


Fig. 5. Circular phylogenetic tree, representing the evolutionary relationships of the identified operational taxonomic units (OTUs). Different colors of OTUs represent different phyla.

Also, in another investigation, it has been shown that regardless of the titanium's surface roughness, bacterial colonization of different implant materials is quite similar over time (Schmidlin et al. 2013). However, we must take into account that these previous studies used culture techniques and that more than 70% of bacteria associated with peri-implant tissue health is uncultivated (Kumar et al. 2012). In our study, we applied a distinct and highly sensitive procedure, pyrosequencing of 16S rDNA genes, which is capable of providing a wider spectrum of microorganisms, revealing in more detail differences between both health- and disease-related oral microbiomes. Moreover, other methods, such as real-time PCR and DNA hybridization, only detect expected bacteria

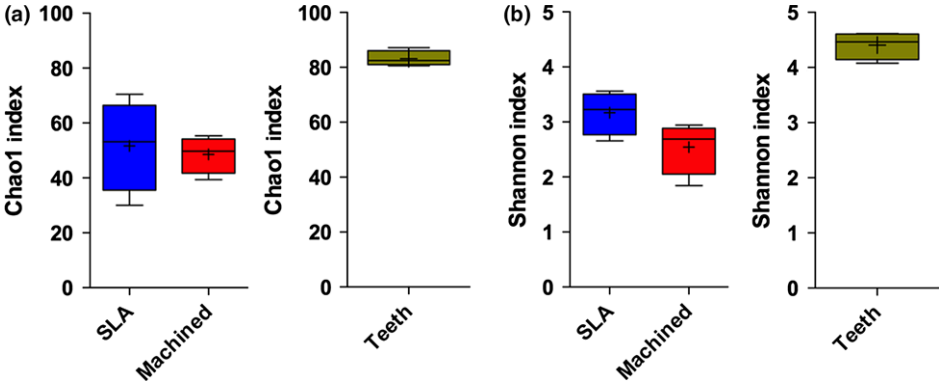


Fig. 6. Chao1 (a) and Shannon (b) index diversity of sandblasted large-grit and acid-etched (SLA) titanium surfaces, machined titanium surfaces and periodontal surfaces (descriptive data). Data presented as median and quartiles. Mean is also presented as +.

using specific primers and probes, while the Miseq sequencing platform does not have this disadvantage. Next-generation methods of detection have provided new insights on the complex microbiota colonizing different sites of oral cavity. In this investigation, we have assessed biofilm from in a small number of periodontally healthy individuals and our results did not indicate statistical differences in the diversity of communities colonizing SLA and machined titanium surfaces. However, due to the small sample size, more investigation is need to reach definitive conclusions on this outcome. Further studies investigating the impact of chemical composition and surface free energy associated with topography surface could add important new information on the bacterial adhesion to dental implants.

In summary, the 16S DNA analysis demonstrated a microbial profile in titanium surfaces consistent with an oral healthy microbiota. Interestingly, we identified species commonly related to late biofilm formation in early stages of colonization. However, despite SLA and machined surfaces have shown a similar diversity, based on the small sample size, we cannot rule out that titanium surface topography potentially affects early colonization. This preliminary study provides comprehensive and reliable data for future study designs involving 16S rDNA sequencing and implant surfaces.

Acknowledgements: This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq. Brazil). We thank Dr. Pierre Lepage (McGill University and Genome Quebec Innovation Centre, Montreal, Canada) for his support in the DNA sequencing and Pascale Marquis and Julien Tremblay (McGill University and Genome Quebec Innovation Centre, Bioinformatics sector, Montreal, Canada) for their assistance in the bioinformatics analyses. We also gratefully acknowledge the support of Dr. Christoph Appert from Straumann Institut for donating the titanium disks.

Conflict of interest

The authors declared that they have no conflict of interest.

References

Barendregt, D.S., Timmerman, M.F., van der Velden, U. & van der Weijden, G.A. (2002) Comparison of the bleeding on marginal probing index and the eastman interdental bleeding index as indicators of gingivitis. *Journal of Clinical Periodontology* **29**: 195–200.

Brama, M., Rhodes, N., Hunt, J., Ricci, A., Teghil, R., Migliaccio, S., Rocca, C.D., Leccisotti, S., Lioi, A., Scandurra, M., De Maria, G., Ferro, D., Pu, F., Panzini, G., Politi, L. & Scandurra, R. (2007) Effect of titanium carbide coating on the osseointegration response in vitro and in vivo. *Biomaterials* **28**: 595–608.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L. & Knight, R. (2010a) Pynast: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**: 266–267.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J. & Knight, R. (2010b) Qiime allows analysis of high-throughput community sequencing data. *Nature Methods* **7**: 335–336.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N. & Knight, R. (2011) Global patterns of 16s rrna diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America* **108**(Suppl 1): 4516–4522.

Chan, H.L., Wang, H.L., Bashutski, J.D., Edwards, P.C., Fu, J.H. & Oh, T.J. (2011) Retrograde periimplantitis: a case report introducing an approach to its management. *Journal of Periodontology* **82**: 1080–1088.

Chrcanovic, B.R., Albrektsson, T. & Wennerberg, A. (2015) Dental implants inserted in male versus female patients: a systematic review and meta-analysis. *Journal of Oral Rehabilitation* **42**: 709–722.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P. & Andersen, G.L. (2006) Greengenes, a chimera-checked 16s rrna gene database and workbench compatible with arb. *Applied and Environmental Microbiology* **72**: 5069–5072.

Dorkhan, M., Chavez de Paz, L.E., Skepo, M., Svensater, G. & Davies, J.R. (2012) Effects of saliva or serum coating on adherence of streptococcus oralis strains to titanium. *Microbiology* **158**: 390–397.

Dunne, W.M., Jr. (2002) Bacterial adhesion: seen any good biofilms lately? *Clinical Microbiology Reviews* **15**: 155–166.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. & Knight, R. (2011) Uchime improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194–2200.

Esposito, M., Hirsch, J.M., Lekholm, U. & Thomsen, P. (1998) Biological factors contributing to failures of osseointegrated oral implants. (ii). Etiopathogenesis. *European Journal of Oral Sciences* **106**: 721–764.

Frojd, V., Chavez de Paz, L., Andersson, M., Wennerberg, A., Davies, J.R. & Svensater, G. (2011) In situ analysis of multispecies biofilm formation on customized titanium surfaces. *Molecular Oral Microbiology* **26**: 241–252.

Furst, M.M., Salvi, G.E., Lang, N.P. & Persson, G.R. (2007) Bacterial colonization immediately after installation on oral titanium implants. *Clinical Oral Implants Research* **18**: 501–508.

Griffen, A.L., Beall, C.J., Campbell, J.H., Firestone, N.D., Kumar, P.S., Yang, Z.K., Podar, M. & Leys, E.J. (2012) Distinct and complex bacterial profiles in human periodontitis and health revealed by 16s pyrosequencing. *The ISME Journal* **6**: 1176–1185.

Grossner-Schreiber, B., Teichmann, J., Hannig, M., Dorfer, C., Wenderoth, D.F. & Ott, S.J. (2009) Modified implant surfaces show different biofilm compositions under in vivo conditions. *Clinical Oral Implants Research* **20**: 817–826.

Heuer, W., Stiesch, M. & Abraham, W.R. (2011) Microbial diversity of supra- and subgingival biofilms on freshly colonized titanium implant

abutments in the human mouth. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology* **30**: 193–200.

Jhajharia, K., Parolia, A., Shetty, K.V. & Mehta, L.K. (2015) Biofilm in endodontics: a review. *Journal of International Society of Preventive & Community Dentistry* **5**: 1–12.

Kolenbrander, P.E. (2000) Oral microbial communities: biofilms, interactions, and genetic systems. *Annual Review of Microbiology* **54**: 413–437.

Koyanagi, T., Sakamoto, M., Takeuchi, Y., Ohkuma, M. & Izumi, Y. (2010) Analysis of microbiota associated with peri-implantitis using 16s rrna gene clone library. *Journal of Oral Microbiology* **2**. doi: 10.3402/jom.v2i0.5104

Kumar, P.S., Mason, M.R., Brooker, M.R. & O'Brien, K. (2012) Pyrosequencing reveals unique microbial signatures associated with healthy and failing dental implants. *Journal of Clinical Periodontology* **39**: 425–433.

Leonhardt, A., Renvert, S. & Dahlen, G. (1999) Microbial findings at failing implants. *Clinical Oral Implants Research* **10**: 339–345.

Li, L., Hsiao, W.W., Nandakumar, R., Barbuto, S.M., Mongodin, E.F., Paster, B.J., Fraser-Liggett, C.M. & Fouad, A.F. (2010) Analyzing endodontic infections by deep coverage pyrosequencing. *Journal of Dental Research* **89**: 980–984.

Lindhe, J., Berglundh, T., Ericsson, I., Liljenberg, B. & Marinello, C. (1992) Experimental breakdown of peri-implant and periodontal tissues. A study in the beagle dog. *Clinical oral Implants Research* **3**: 9–16.

Magoc, T. & Salzberg, S.L. (2011) Flash: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**: 2957–2963.

Mombelli, A., Muller, N. & Cionca, N. (2012) The epidemiology of peri-implantitis. *Clinical Oral Implants Research* **23**(Suppl 6): 67–76.

Mombelli, A., Schmid, B., Rutar, A. & Lang, N.P. (2000) Persistence patterns of *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, and *Actinobacillus actinomycetemcomitans* after mechanical therapy of periodontal disease. *Journal of Periodontology* **71**: 14–21.

Nakazato, G., Tsuchiya, H., Sato, M. & Yamauchi, M. (1989) In vivo plaque formation on implant materials. *The International Journal of Oral & Maxillofacial Implants* **4**: 321–326.

Nossa, C.W., Oberdorf, W.E., Yang, L., Aas, J.A., Paster, B.J., Desantis, T.Z., Brodie, E.L., Malamud, D., Poles, M.A. & Pei, Z. (2010) Design of 16s rrna gene primers for 454 pyrosequencing of the human foregut microbiome. *World Journal of Gastroenterology* **16**: 4135–4144.

Nyvad, B. & Kilian, M. (1987) Microbiology of the early colonization of human enamel and root surfaces in vivo. *Scandinavian Journal of Dental Research* **95**: 369–380.

Ong, E.S., Newman, H.N., Wilson, M. & Bulman, J.S. (1992) The occurrence of periodontitis-related microorganisms in relation to titanium implants. *Journal of Periodontology* **63**: 200–205.

Periasamy, S. & Kolenbrander, P.E. (2009) Mutualistic biofilm communities develop with porphyromonas gingivalis and initial, early, and late colonizers of enamel. *Journal of Bacteriology* **191**: 6804–6811.

Persson, G.R. & Renvert, S. (2014) Cluster of bacteria associated with peri-implantitis. *Clinical Implant Dentistry & Related Research* **16**: 783–793.

Price, M.N., Dehal, P.S. & Arkin, A.P. (2009) Fasttree: computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular Biology and Evolution* **26**: 1641–1650.

Quirynen, M., Vogels, R., Peeters, W., van Steenberghe, D., Naert, I. & Haffajee, A. (2006) Dynamics of initial subgingival colonization of 'pristine' peri-implant pockets. *Clinical Oral Implants Research* **17**: 25–37.

Rehman, A., Hu, J., Ott, S.J. & Grossner-Schreiber, B. (2012) Microbial community composition on modified dental implant surfaces: an in vivo study. *The International Journal of Oral & Maxillofacial Implants* **27**: 811–819.

Rimondini, L., Fare, S., Brambilla, E., Felloni, A., Consonni, C., Brossa, F. & Carrassi, A. (1997) The effect of surface roughness on early in vivo plaque colonization on titanium. *Journal of Periodontology* **68**: 556–562.

Ritz, H.L. (1967) Microbial population shifts in developing human dental plaque. *Archives of Oral Biology* **12**: 1561–1568.

Romeo, E., Ghisolfi, M. & Carmagnola, D. (2004) Peri-implant diseases. A systematic review of the literature. *Minerva Stomatologica* **53**: 215–230.

Sanchez, M.C., Llama-Palacios, A., Fernandez, E., Figuero, E., Marin, M.J., Leon, R., Blanc, V., Herrera, D. & Sanz, M. (2014) An in vitro biofilm model associated to dental implants: structural and quantitative analysis of in vitro biofilm formation on different dental implant surfaces. *Dental Materials: Official Publication of the Academy of Dental Materials* **30**: 1161–1171.

Scarano, A., Piattelli, M., Caputi, S., Favero, G.A. & Piattelli, A. (2004) Bacterial adhesion on commercially pure titanium and zirconium oxide disks: an in vivo human study. *Journal of Periodontology* **75**: 292–296.

Schmidlin, P.R., Muller, P., Attin, T., Wieland, M., Hofer, D. & Guggenheim, B. (2013) Polyspecies biofilm formation on implant surfaces with different surface characteristics. *Journal of Applied Oral Science: Revista FOB* **21**: 48–55.

Shchipkova, A.Y., Nagaraja, H.N. & Kumar, P.S. (2010) Subgingival microbial profiles of smokers with periodontitis. *Journal of Dental Research* **89**: 1247–1253.

da Silva, E.S., Feres, M., Figueiredo, L.C., Shibli, J.A., Ramiro, F.S. & Faveri, M. (2014) Microbiological diversity of peri-implantitis biofilm by sanger sequencing. *Clinical Oral Implants Research* **25**: 1192–1199.

Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C. & Kent, R.L., Jr. (1998) Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology* **25**: 134–144.

Teughels, W., Van Assche, N., Sliepen, I. & Quirynen, M. (2006) Effect of material characteristics and/or surface topography on biofilm development. *Clinical Oral Implants Research* **17**(Suppl 2): 68–81.

Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R. (2007) Naive bayesian classifier for rapid assignment of rrna sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**: 5261–5267.

Zaura, E., Keijser, B.J., Huse, S.M. & Crielaard, W. (2009) Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiology* **9**: 259.

Zhao, B., van der Mei, H.C., Subbiahdoss, G., de Vries, J., Rustema-Abbing, M., Kuijer, R., Busscher, H.J. & Ren, Y. (2014) Soft tissue integration versus early biofilm formation on different dental implant materials. *Dental Materials: Official Publication of the Academy of Dental Materials* **30**: 716–727.

Capítulo II. A systematic review of the microbiota composition in various peri-implant conditions: data from 16S rRNA gene sequencing

No **capítulo II** apresentamos o artigo publicado no periódico *Archives of Oral Biology*.

No capítulo anterior (Capítulo I) avaliamos a microbiota inicial que adere à diferentes superfícies de titânio. Neste estudo, objetivamos investigar o que a literatura nos mostra sobre a composição da microbiota peri-implantar tanto no estado de saúde quanto no estado de doença. Para isso, realizamos uma revisão sistemática através de diversas bases de dados eletrônicas à procura de artigos publicados até abril de 2020 que apresentassem uma descrição da composição microbiana da peri-implantite (PI), mucosite (PM) e/ou de implantes saudáveis (HI) usando análise de sequenciamento do gene 16S rRNA. A partir desta revisão, considerando os achados na maioria dos estudos, pôde-se sugerir que a abundância relativa da microbiota e a diversidade bacteriana aumenta com o progresso da doença peri-implantar.

Archives of Oral Biology 117 (2020) 104776

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

A systematic review of the microbiota composition in various peri-implant conditions: data from 16S rRNA gene sequencing

Fabiana de Melo[a,*], Fernanda Carpes Milanesi[a], Patrícia Daniela Melchiors Angst[b], Rui Vicente Oppermann[b]

[a] Graduate Program in Dentistry, Periodontics Unit, School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil
[b] Department of Conservative Dentistry, Periodontics Unit, School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

ARTICLE INFO

Keywords:
Dental Implants
Microbial
high-throughput
sequencing

ABSTRACT

Objective: To systematically review the literature regarding the microbiota composition in various peri-implant conditions as analyzed by 16S rRNA gene sequencing methods.
Methods: Electronic searches were conducted at MEDLINE/PubMed, Scopus, Embase, ScienceDirect and Web of Science databases looking for articles published up to April 2020. Observational prospective investigations were considered with systemically healthy patients and that had presented the description of the microbiota composition of peri-implantitis (PI), peri-implant mucositis (PM) and/or health implants (HI) by using 16S rRNA gene sequencing analysis were considered eligible.
Results: From 1,380 titles found, 8 studies were considered for qualitative analysis. One article was excluded due to high risk of bias, remaining 7 studies for descriptive analysis. In 6 out of 7 studies the PI microbiota was reported as being in relative abundance and variety though with a different composition from those with HI. There was no consensus regarding which condition had more diversity. The main observed phyla among PI were *Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria* and *Spirochaetes*, while the genera were mainly *Actinomyces, Eubacterium, Fusobacterium, Mogibacterium, Moraxella, Treponema* and *Porphyromonas*. Comparisons between PI and PM microbiota showed conflicting results: one study suggested that PI has greater bacterial diversity; another study reported the opposite result, while another investigation found similar variety for both conditions.
Conclusions: The microbiota of peri-implant conditions have been reported as distinct, although the available literature presents discrepancies. Nonetheless, considering the findings in most studies, it can be suggested that the relative abundance of microbiota and bacterial diversity increased with the progress of peri-implant disease.

1. Introduction

Dental implants have been widely studied and have become a treatment option for missing teeth replacement (Gbadebo, Lawal, Sulaiman, & Ajayi, 2014). Despite the high success rate, infections around dental implants have also been observed. Peri-implant mucositis (PM) is a reversible inflammatory reaction in peri-implant marginal mucosa induced by a bacterial biofilm accumulated around the implant. This condition can be clinically detected by means of the presence of marginal mucosa bleeding in implants without radiographic signs of bone loss (Berglundh et al., 2018). When left untreated, the infecto-inflammatory process can progress and involve the implant supporting tissues, causing bleeding on probing (BOP) and bone loss, with or without concomitant deepening of peri-implant pockets, resulting in characteristic peri-implantitis (PI) (Berglundh et al., 2018). Recent studies have shown that both conditions are prevalent, where PI occurs in ~22% of patients, whereas PM occurs in ~43% (Derks & Tomasi, 2015).

Interestingly, it is recognized that when peri-implantitis is established, it progresses faster than the destructive infecto-inflammatory process in teeth, e.g. periodontitis (P) (Berglundh et al., 2018). Moreover, studies have shown that peri-implantitis treatment is complex, with no one therapy being significantly superior to another (Esposito, Grusovin, & Worthington, 2012; Faggion, Listl, Frühauf, Chang, & Tu, 2014). In this scenario, to better understand the treatment responses and the pathogenesis of peri-implant diseases, it is necessary to know, in detail, the peri-implant microbiota and microbiome. So far, studies have shown that in both health and disease, peri-implant biofilms have

* Corresponding author at: School of Dentistry, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos, 2492 – Rio Branco, Porto Alegre, RS, 90035-004, Brazil.
E-mail address: fabiana.melo@ufrgs.br (F. de Melo).

https://doi.org/10.1016/j.archoralbio.2020.104776
Received 31 January 2020; Received in revised form 6 May 2020; Accepted 17 May 2020

significantly lower bacterial diversity than subgingival biofilms (Dabdoub, Tsigarida, & Kumar, 2013; Heuer et al., 2012; Kumar, Mason, Brooker, & O'Brien, 2012; Zhang et al., 2015). Nonetheless, several species, including previously unsuspected and unknown organisms, are being found in the peri-implant environment but not around teeth, suggesting peri-implant diseases to be a different infection than periodontal diseases. On the other hand, the microbiota present in the different peri-implant conditions remain unclear (Rakic, Grusovin, & Canullo, 2016; Renvert & Quirynen, 2015).

Nowadays, many studies have started to look for the peri-implant microbiota by using multiple detection techniques (Padial-Molina, López-Martínez, O'Valle, & Galindo-Moreno, 2016). Among these, the use of culture-independent methods, such as 16S ribosomal RNA (rRNA) gene sequencing, where a bacterial community can be detected independently of any known bacteria, has gained prominence (Größner-Schreiber et al., 2009). This high-throughput methodology can give a wealth of information once it significantly increases the amount of detectable phylotypes, and by generating a large amount of information that can allow for the identification of high levels of microbial diversity (Ziganshina et al., 2015).

Although the number of studies using such methodologies is growing, the crude and isolated data are complex and difficult to interpret. Thus, the combined knowledge from many studies may reveal microbial patterns whose contribution to health and disease might subsequently be proven in hypothesis-driven studies. What's more, the objective of this review is to systematically analyze the literature in order to describe the microbiota presented in different peri-implant conditions as analyzed by 16S rRNA gene sequencing methods.

2. Materials and methods

2.1. Protocol Registration

This systematic review followed the guidelines of the PRISMA checklist (Moher, Liberati, Tetzlaff, Altman, & PRISMA Group, 2010), and its protocol was registered in PROSPERO database (CRD42018093500).

2.2. Focus Question

The study question was: "Are there differences in microbiota composition between inflammatory peri-implant conditions and healthy implants?"

2.3. Data Sources

Electronic search was performed in the MEDLINE/PubMed, Embase, Scopus, ScienceDirect and Web of Science databases. A manual search was also performed on the website of the most important journals in the field and in the reference lists of the retrieved articles, as well as in previously published review articles on similar topics. The search included articles accepted for publication up to April 2020. The following terms were used in our search strategy: (((Dental Implants) AND Microbial)) OR ((Dental Implants) AND Metagenomic) (Appendix 1). No restriction of language or year of publication was established.

2.4. Selection of Studies

Two independent reviewers (FM; FCM) analyzed the articles at all stages. Reviewers compared decisions and resolved differences through discussion. When consensus could not be reached, a third author (PDMA) was consulted. The search results in each database were loaded in Mendeley® reference editor (version 1.19.4, ©2019 Mendeley Ltda., Amsterdam, NL) where the duplicates were removed, and the titles and abstracts were selected for inclusion. After this screening, eligibility was assessed by full-text reading.

For study selection, the definitions of PECO question were considered. Accordingly, the included studies were to have: 1) the description of peri-implant microbiota as reported by 16S rRNA gene sequencing analysis (main outcome); 2) PI and/or PM diseases as exposure; 3) at least one of the following comparison scenarios: a) differences between PM and/or PI against HI; b) differences along the course of peri-implant disease (e.g. PM versus PI); and 4) prospective investigations in systemically healthy patients. In vitro, in situ or animal studies, and/or investigations reporting other microbial techniques were excluded.

2.5. Quality Assessment

Methodological quality and risk of bias of eligible studies were assessed by the same two reviewers (FM; FCM) by using a quality measurement table based on the Newcastle-Ottawa scale (Wells et al., 2001). Reviewers independently scored the papers that adequately fulfilled each methodological criterion, with a maximum score of 14 points. Then, studies that presented 11-14 points were considered to be of high quality/low risk of bias; those with 8-10 points were of medium quality, and those with < 8 points had low methodological quality/high risk of bias. Studies presenting high risk of bias were excluded from further analysis.

2.6. Data Extraction and Synthesis

The two reviewers independently extracted the data from included articles. Data extracted were: authorship, year of publication, country where the study was conducted, study design, type of peri-implant condition investigated, number of cases, sequencing technology employed, gene fragment used, implant type, sampling method used, number of filtered reads, taxonomic assignment database, detection level and main microbiological findings. Data were compiled into evidence tables and expressed in chronological order according to the publication date.

2.7. Statistical Analysis

Inter-reviewer agreement regarding the process of study selection was calculated by using the Kappa coefficient. A high heterogeneity between the included articles was observed. Consequently, it was not possible to conduct a meta-analysis, limiting the presentation to a descriptive analysis of findings.

3. Results

3.1. Study Selection

Electronic and manual search retrieved 1,380 citations after removing duplicates. Among these citations, 36 were eligible for full-text assessment. Of these, 8 studies were eligible and submitted to qualitative analysis (Fig. 1). The kappa value for inter-reviewer agreement for study inclusion was 0.805 and 0.870 for analysis of titles and abstracts, respectively.

3.2. Quality Assessment

The results of quality assessment of eligible studies are presented in Table 1. Two studies showed high quality (Sousa et al., 2017; Tsigarida, Dabdoub, Nagaraja, & Kumar, 2015), while five studies showed moderate quality (Gao, Zhou, Sun, Li, & Zhou, 2018; Kumar et al., 2012; Sanz-Martin et al., 2017; Yu et al., 2019; Zheng et al., 2015). One study (Vankov et al., 2016) presented low methodological quality, being not included in descriptive analysis.

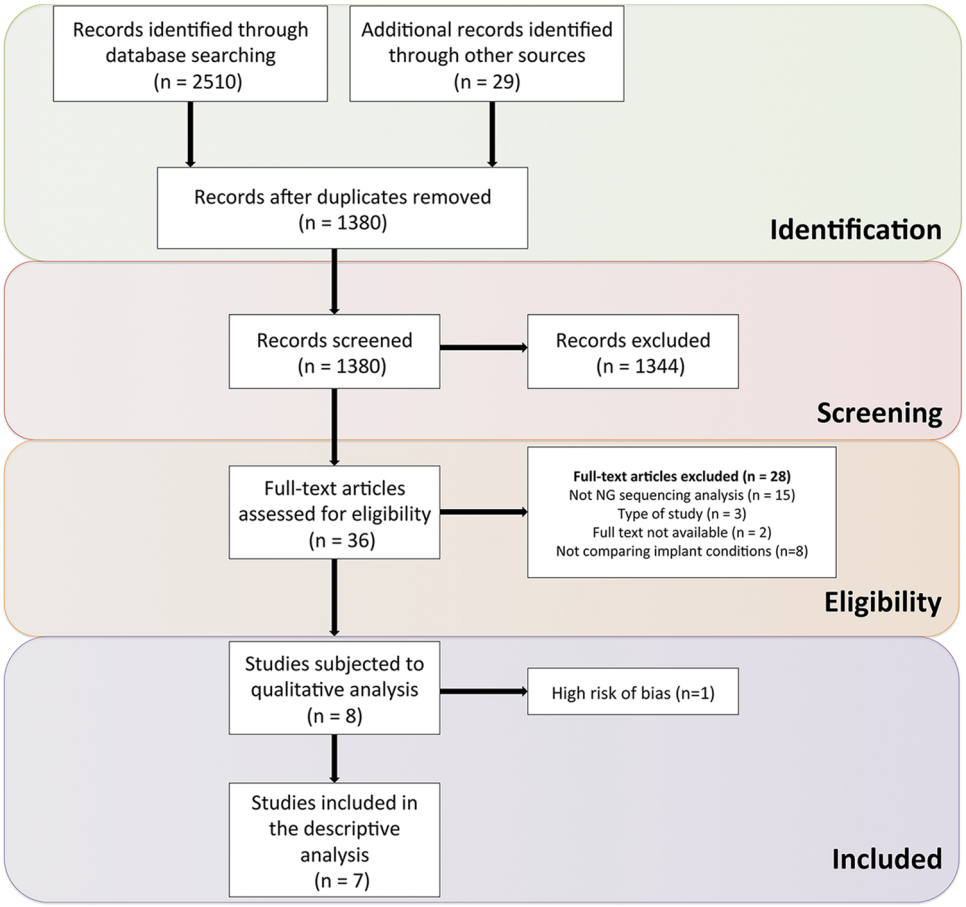


Fig. 1. PRISMA Flow Diagram.

3.3. Descriptive Analysis

3.3.1. Methodological Aspects

Table 2 presents the main characteristics from the 7 included studies. All investigations were cross-sectional and conducted in the past 8 years. In fact, the majority of them were published in the last 5 years (Gao et al., 2018; Sanz-Martin et al., 2017; Sousa et al., 2017; Tsigarida et al., 2015; Yu et al., 2019; Zheng et al., 2015).

Among the included studies, all of them compared PI and HI (Gao et al., 2018; Kumar et al., 2012; Sanz-Martin et al., 2017; Sousa et al., 2017; Tsigarida et al., 2015; Yu et al., 2019; Zheng et al., 2015), while 3 studies also compared PM against HI, and PM against PI (Sousa et al., 2017; Tsigarida et al., 2015; Zheng et al., 2015). Regarding the sample, the size ranged from 18 (Sousa et al., 2017; Yu et al., 2019) up to 80 (Tsigarida et al., 2015) patients. The mean age of patients in the experimental groups ranged between 39 (Kumar et al., 2012) and 59 (Sanz-Martin et al., 2017) years.

The method used to collect the microbiological samples was paper points in 4 studies (Gao et al., 2018; Kumar et al., 2012; Tsigarida et al., 2015; Yu et al., 2019), while 2 studies used sterile Gracey curettes (Sanz-Martin et al., 2017; Sousa et al., 2017), and 1 study used sterile periodontal probes (Zheng et al., 2015). The sequencing technology that was used and the 16S gene variable region that was amplified varied among the included studies. The 454 Roche Pyrosequencing platform (Roche Applied Science, Indianapolis, IN, USA) was used in 3 articles. Among those 3 articles, 1 study amplified the V1-V3 region (Zheng et al., 2015) and 2 studies amplified both the V1-V3 and V7-V9 regions (Kumar et al., 2012; Tsigarida et al., 2015). The remaining 4 articles used Illumina Miseq sequencing technology. In these investigations, the V3-V4 region was amplified in 2 studies (Sanz-Martin et al., 2017; Yu et al., 2019), while the V4-V5 region was amplified in another study (Gao et al., 2018), and the V5-V7 region was amplified in another investigation (Sousa et al., 2017).

3.3.2. Microbiota Composition

Table 3 shows the main findings for species and genus levels of the included studies.

3.3.2.1. Healthy Implants Versus Peri-implantitis Implants. Only one study showed a similar diversity of bacteria when comparing HI and PI (Yu et al., 2019), while the remaining studies observed that both conditions presented differences in bacterial diversity as well as in the relative abundance of the associated microbiota (Gao et al., 2018; Kumar et al., 2012; Sanz-Martin et al., 2017; Sousa et al., 2017; Tsigarida et al., 2015; Zheng et al., 2015) (Table 3). Nonetheless, there is no agreement between these studies on which condition had greater bacterial richness and relative abundance. In particular, Yu et al. (2019) presented *Streptococcus infantis/mitis/oralis* and *Fusobacterium sp.* as the dominant species in both conditions, HI and PI. However, there was noticeable variation in the microbiota among the individuals.

On the other hand, Kumar et al. (2012) and Sousa et al. (2017) reported greater diversity in microbiota in HI than in PI. In fact, despite presenting higher levels of *Actinomyces, Peptococcus, Campylobacter, non- mutans Streptococcus, Butyrivibrio* and *Streptococcus mutans,* PI showed lower levels of *Prevotella* and *Leptotrichia* compared to HI (Kumar et al., 2012). Additionally, *Staphylococcus, TG5* and *Corynebacterium* differed significantly between HI and PI, with *Corynebacterium* presenting higher abundance in HI sites (Sousa et al., 2017).

Table 1
Summary of the quality assessment of eligible studies.

Authors	Selection of patients							Comparability*		Outcome			Statistic		Final Score
	Sample size	Peri-implantitis, peri-implant mucositis, or healthy implants sites characteristics	Control sites characteristics	Assessment of peri-implant condition	Clear definitions of outcomes	Calibration of the examiners	Eligibility criteria	Comparability of patients on the basis of the study design	Management of confounders	Blinding of the examiners	Index for outcome assessment	Adequacy of follow-up *	Statistical analysis	Unit of analysis	
Kumar et al., 2012	0	1	1	1	1	0	1	1	1	0	1	1	1	0	10
Tsigarida et al., 2015	1	1	1	1	1	1	1	1	1	0	1	1	1	0	12
Zheng et al., 2015	0	1	0	1	1	0	1	1	1	0	1	1	1	0	9
Vankov et al., 2016	0	0	0	0	1	0	1	1	1	0	1	1	0	0	**6**
Sousa et al., 2017	1	1	1	1	1	1	1	1	1	0	1	1	1	0	12
Sanz-Martin et al., 2017	0	1	1	1	1	0	1	1	1	0	1	1	1	0	10
Gao et al., 2018	0	1	1	1	1	0	1	1	1	0	1	1	1	0	10
Yu et al., 2019	0	1	1	1	1	0	1	1	1	0	1	1	1	0	10

* Comparability data are not applicable to the cross-sectional and longitudinal observational studies; thus, all articles received scores, as well as the parameter “adequacy of follow-up of the patients”.

Table 2
Characteristics of the included studies.

Study/ Country	Study	Type of patients/sites	No. of cases	Controls	Implant type	Technology	Gene fragment used	Sampling method	Filtered reads	Taxonomic assignment Database	Detection level
Kumar et al., 2012; USA	Cross-sectional	Peri-implant health and peri-implantitis	40 patients: - 10 healthy implants - 10 peri-implantitis	Health implants	Astra Tech, Zimmer, Nobel Biocare	454 Roche Pyrosequencing	16S rRNA, V1-V3 and V7-V9 regions	Paper points	397,286	Greengenes	Genus/Species
Tsigarida et al., 2015; USA	Cross-sectional	Peri-implant health, peri-implant mucositis, and peri-implantitis (smokers and non-smokers)	80 patients: * Smokers: - 20 peri-implant health - 10 peri-implant mucositis, - 10 peri-implantitis. * Non-smokers: - 20 peri-implant health - 10 peri-implant mucositis - 10 peri-implantitis	Health implants	Astra Zimmer Nobel Straumann 3i Calcitek	454 Roche Pyrosequencing	16S rRNA, V1-V3 and V7-V9 regions	Paper points	790,008	HOMD	Species
Zheng et al., 2015; China	Cross-sectional	Peri-implant health, peri-implant mucositis, and peri-implantitis	24 patients: - 10 healthy implants - 8 peri-implant mucositis - 6 peri-implantitis	Health implants	Paragon Straumann Dental Implant System	454 Roche Pyrosequencing	16S rRNA, V1-V3 region	Sterile periodontal probes	424,579	RDP	Genus/Species
Sousa et al., 2017; UK	Cross-sectional	Peri-implant health, peri-implant mucositis, and peri-implantitis	18 patients: - 2 healthy implant - 2 peri-implant mucositis - 2 peri-implantitis	Health implants	No data	Illumina Miseq	16S rRNA, V5-V7 region	Sterile Gracey curettes	Not indicated	Greengenes	Genus
Sanz-Martin et al., 2017; Switzerland	Cross-sectional	Peri-implant health, and peri-implantitis	67 patients: - 32 healthy implants - 35 peri-implantitis	Health implants	Strauman. Branemark Other	Illumina Miseq	16S rRNA, V3-V4 region	Sterile Gracey curettes	7,297,772	RDP	Genus/Species
Gao et al., 2018; China	Cross-sectional	Peri-implant health, and peri-implantitis	40 patients: - 20 healthy implants - 20 peri-implantitis,	Health implants	No data	Illumina Miseq	16S rRNA, V4-V5 region	Paper points	733,759	HOMD	Genus
Yu et al., 2019; China	Cross-sectional	Peri-implant health, and peri-implant disease	18 patients: - 18 healthy implants - 18 peri-implantitis	Health implants	No data	Illumina Miseq	16S rRNA, V3-V4 region	Paper points	4,425,705	HOMD	Genus/Species

PI = Peri-implantitis; PM = Peri-implant Mucositis; HI = Health Implants; RDP = Ribosomal Database Project; HOMD = Human Oral Microbiome Database.

Table 3
Main results and predominant microbial taxa found in statistically significantly higher relative counts/ proportions/ abundance/ prevalence at genus and species levels as reported by the included studies.

Study/ Country	Type of patients/sites	No. of cases	Genus	Species	Microbiologic findings
Kumar et al., 2012; USA	Peri-implant health and peri-implantitis	40 patients: - 10 healthy implants - 10 peri-implantitis	Significantly increased in PI: *Actinomyces, Peptococcus, Campylobacter, non- mutans Streptococcus* and *Butyrivibrio.* Significantly different detection frequencies between the groups: *Pseudoramibacter, Enterococcus, Moraxella, Desulfobulbus, Xanthomonas, Arthrobacter, Anaerococcus, Anaerovorax, Butyri- vibrio, Exiguobacterium, Kingella, Fusobacterium, Pseudomonas, Burkholderia, Bifidobacterium, Clostridium* and *Johnsonella.*	Significantly increased in PI: *Streptococcus mutans.*	84 OTUs. PI and HI presented qualitatively and quantitatively bacterial differences, with greater diversity on microbial profile at HI than at PI. PI was suggested to be a microbial heterogeneous infection with predominantly gram-negative species compared to HI.
Tsigarida et al., 2015; USA	Peri-implant health, peri-implant mucositis, and peri-implantitis (smokers and non-smokers)	80 patients: * Smokers: - 20 peri-implant health - 10 peri-implant mucositis, - 10 peri-implantitis. * Non-smokers: - 20 peri-implant health - 10 peri-implant mucositis - 10 peri-implantitis	NR	Higher abundances in health-associated microbiomes in smokers: 79 species, notably those belonging to the genera *Lactobacillus, Prevotella, Treponema, Propionibacterium*, and *Pseudomonas.* Higher abundances in health-associated microbiomes in nonsmokers: 77 species, especially those belonging to *Streptococcus, Selenomonas*, and *Porphyromonas* (for detailed information see table in Tsigarida et al., 2015).	515 OTUs. HI and PI presented qualitatively and quantitatively bacterial differences, with PI harboring significantly more diverse bacterial communities than HI. PM and PI presented no difference in terms of diversity. HI and PM presented no difference in terms of diversity. However, while HI was associated with higher abundances of fewer bacteria species, PM was characterized by a decrease in abundances and loss of a few "health-associated" species, along with acquisition of several new members (for detailed information see table in Tsigarida et al., 2015). Smokers had a significantly lower diversity and shared greater numbers of species in both HI and PM, than nonsmokers.

(*continued on next page*)

Table 3 (*continued*)

Study/ Country	Type of patients/sites	No. of cases	Genus	Species	Microbiologic findings
Zheng et al., 2015; China	Peri-implant health, peri-implant mucositis, and peri-implantitis	24 patients:	Significantly increased in PI: *Fusobacterium* and *Selenomonas*.	Significantly increased in PI: *Eubacterium infirmum, Kingella denitrificans, Actinomyces israelii, Corynebacterium matruchotii, Treponema medium* and *Gemella sanguinis*.	311-1028 OTUs. HI and PI presented qualitatively and quantitatively bacterial differences, with PI presenting significantly more diverse bacterial communities than HI. PM seems to have greater bacterial diversity than HI profile. *Neisseria* and *Actinomyces* were presented as the numerically dominant OTUs at HI and PM, respectively.
		- 10 healthy implants - 8 peri-implant mucositis - 6 peri-implantitis			PM harbored lower diversity than PI, and was referred to as intermediate in terms of relative abundance and prevalence in relation to HI and PI with known putative periodontal pathogens, such as, *Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia and Capnocytophaga ochracea,* being clustered together in PM.
Sousa et al., 2017; UK	Peri-implant health, peri-implant mucositis, and peri-implantitis	18 patients: - 2 healthy implant - 2 peri-implant mucositis - 2 peri-implantitis	Significantly increased in HI: *Corynebacterium* and *Staphylococcus.*	NR	51 OTUs. HI and PI presented qualitatively and quantitatively bacterial differences, with greater diversity on microbial profile at HI than at PI.
			Significantly increased in PM: *TG5*.		PM presented higher diversity than PI and HI.
Sanz-Martin et al., 2017; Switzerland	Peri-implant health, and peri-implantitis	67 patients: - 32 healthy implants - 35 peri-implantitis	Significantly increased in PI: *Porphyromonas, Treponema, Filifactor, Fretibacterium* and *Tannerella.*	Significantly increased in PI: Red complex species.	94 OTUs. HI and PI presented qualitatively and quantitatively bacterial differences, with greater diversity on microbial profile at PI than at HI.
			Significantly increased in HI: *Streptococcus, Veillonella, Rothia* and *Haemophilus.*	Significantly increased in HI: *R. dentocariosa, Streptococcus sanguinis* and *Veillonella dispar.*	
Gao et al., 2018; China	Peri-implant health, and peri-implantitis	40 patients: - 20 healthy implants - 20 peri-implantitis,	Significantly increased in PI: *Moraxella,Campylobacter Micrococcus* and *Acinetobacter*	NR	263 OTUs. HI and PI presented qualitatively and quantitatively bacterial differences, with greater diversity on microbial profile at PI than at HI.
			Significantly increased in HI: *Prevotella, Vibrio,*		Regarding the richness, the dominant genera (> 1%) in HI included *Neisseria, Haemophilus, Prevotella, Streptococcus, Vibrio, Porphyromonas, Fusobacterium, Capnocytophaga, Leptotrichia, Actinomyces, Treponema, Campylobacter, Rothia*, and *Granulicatella*.
			Granulicatella, Neisseria and *Haemophilus.*		For PI, the dominant genera (> 1%) included *Prevotella, Streptococcus, Acinetobacter, Fusobacterium, Neisseria, Porphyromonas, Treponema, Leptothrix, Capnocytophaga. Micrococcus, Rothia, Moraxella, Haemophilus, Actinomyces,* and *Actinomyces.*

(*continued on next page*)

Table 3 (*continued*)

Study/ Country	Type of patients/sites	No. of cases	Genus	Species	Microbiologic findings
Yu et al., 2019; China	Peri-implant health, and peri-implant disease	18 patients: - 18 healthy implants - 18 peri-implantitis	Significantly increased in PI: *Prevotella, Mogibacterium, Moraxella* and *Johnsonella* Significantly increased in HI: *Leptotrichia, Prevotella, Porphyromonas, Tannerella, Selenomonas* and *Cardiobacterium.*	Significantly increased in PI: *Prevotella multiformis* and *Prevotella fusca.* Significantly increased in HI: *Streptococcus salivarius. Actinobaculum sp., Lachnoanaerobaculum orale* and *Neisseria oralis.*	358 OTUs. HI and PI showed similar bacterial diversity. Streptococcus infantis/mitis/oralis and Fusobacterium sp. were particularly prevalent and abundant in both conditions. However, significant variation among individuals was notable in the core microbiota.

PI = Peri-implantitis; PM = Peri-implant Mucositis; HI = Health Implants, NR = not reported.

On the contrary, other studies found that the PI harbored significantly more diverse bacterial communities than HI (Gao et al., 2018; Sanz-Martin et al., 2017; Tsigarida et al., 2015; Zheng et al., 2015) (Table 3). For example, Gao et al. (2018) indicated that the genera *Moraxella, Micrococcus* and *Acinetobacter* were more abundant in PI compared to HI, while the genera *Prevotella, Neisseria* and *Haemophilus* were more abundant in HI compared to PI. Considering the diversity, the dominant genera (> 1%) in HI included *Neisseria, Haemophilus, Prevotella, Streptococcus, Vibrio, Porphyromonas, Fusobacterium, Capnocytophaga, Leptotrichia, Actinomyces, Treponema, Campylobacter, Rothia*, and *Granulicatella*. On the other hand, among PI, the dominant genera (> 1%) included *Prevotella, Streptococcus, Acinetobacter, Fusobacterium, Neisseria, Porphyromonas, Treponema, Leptothrix, Capnocytophaga. Micrococcus, Rothia, Moraxella, Haemophilus, Actinomyces,* and *Actinomyces.* Additionally, Sanz-Martin et al. (2017) showed that the genera *Porphyromonas, Treponema*, *Filifactor*, *Fretibacterium* and *Tannerella* were abundant in PI, presenting higher relative counts than in the HI group. On the contrary, the *Streptococcus*, *Veillonella, Rothia* and *Haemophilus* genera showed higher relative abundance in HI. Furthermore, in HI species *R. dentocariosa*, *Streptococcus sanguinis* and *Veillonella dispar* were found in high relative proportions when compared to PI. On the other hand, these authors also demonstrated that in PI, the red complex species were more abundant than in HI.

Fig. 2 presents the pooled results from primary studies considering the significant differences at the genus level between healthy implants and those with peri-implantitis.

3.3.2.2. Peri-implant Mucositis Versus Peri-implantitis Implants. A greater variation was found in the results regarding the comparisons between PM and PI microbiota (Sousa et al., 2017; Tsigarida et al., 2015; Zheng et al., 2015) (Table 3).

One study showed PM to have lower diversity than PI, and appeared to be an intermediate condition in terms of relative abundance and prevalence as compared to HI and PI, with known putative periodontal pathogens, such as, *Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia* and *Capnocytophaga ochracea* being clustered together in PM (Zheng et al., 2015). In contrast, Sousa et al. (2017) graphically presented higher diversity in PM than in PI, with *TG5* presenting significantly higher relative abundance in PM than in PI sites. Lastly, Tsigarida et al. (2015) suggested that there is no difference in terms of diversity between these diseases, with a high prevalence of *Fusobacterium* and *Streptococcus* in both conditions.

3.3.2.3. Healthy Versus Peri-implant Mucositis Implants. Of 3 studies, 2 demonstrated that the PM microbiota seems to have greater bacterial diversity than the HI microbiota (Sousa et al., 2017; Zheng et al., 2015) (Table 3). Zheng et al. (2015) showed *Neisseria* and *Actinomyces* to be the numerically dominant OTUs with a mean relative abundance of > 0.5% in HI and PM, respectively. On the other hand, for Sousa et al. (2007) *Staphylococcus, TG5* and *Corynebacterium* differed significantly between healthy and diseased implants, with *Corynebacterium* being the most abundant genus in HI, and *TG5* the most abundant taxa in PM. Although, Tsigarida et al. (2015) showed that diversity was not significantly different between HI and PM. However, the authors compared relative amounts of community members (community structure) as well as the loss and gain of species (community membership). Accordingly, while HI was associated with higher abundances of fewer bacteria species, PM was characterized by a decrease in amounts and the loss of a few "health-associated" species, along with acquisition of several new members (for detailed information see table in Tsigarida et al., 2015). This study also demonstrated that smokers had a significantly lower diversity and shared greater numbers of species in both HI and PM than nonsmokers. Among 486 health-associated s-OTUs, 166 were different among smokers and nonsmokers with HI. The genera *Lactobacillus, Prevotella, Treponema, Propionibacterium*, and *Pseudomonas* were observed in

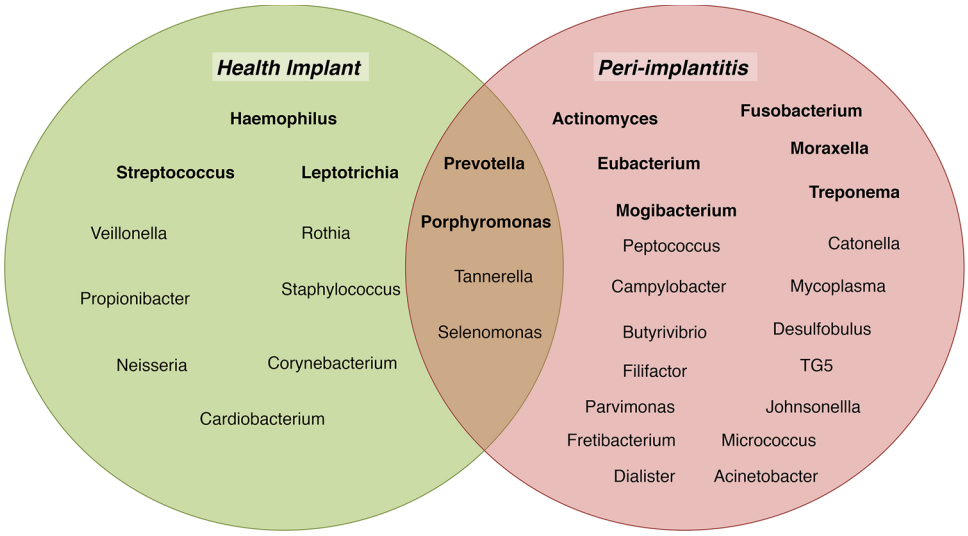


Fig. 2. Venn diagram of the microbiota associated with healthy implants and peri-implantitis sites considering the genus level. Green circle represents healthy implants while the red circle represents peri-implantitis sites. Only species that were statistically different ($p < 0.05$) are presented, and those genus found in more than one article are presented in bold.

higher amounts among health-associated microbiota in smokers, while *Streptococcus, Selenomonas* and *Porphyromonas* were elevated in nonsmokers.

4. Discussion

This systematic review aimed to gather the current evidence available regarding the studies that used 16S rRNA gene sequencing in high-throughput methodologies to analyze the microbiota of different peri-implant conditions. Accordingly, among most included studies, peri-implantitis and healthy implants can be reported as having significant differences in diversity and relative abundance of associated microbiota, where the microbiota of PI tend to be more diverse and abundant. Nonetheless, when compared to the bacterial composition of PI and PM, the studies showed inconclusive results regarding bacterial richness and relative abundance.

In high-throughput sequencing studies, diversity indexes (e.g. Shannon, Chao1, etc.) take into account not only the number of species found (richness), but also the evenness of specie distribution (Magurran, 2004). In this systematic review, most studies showed that healthy implants and peri-implantitis microbiota are different from each other. Regarding the quantitative differences, meaning the relative abundance, *Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria* and *Spirochaetes* represented the main observed phyla in PI, and *Actinomyces, Eubacterium, Fusobacterium, Mogibacterium, Moraxella, Treponema* and *Porphyromonas* the most common genera. In contrast, the *Streptococcus*, *Leptotrichia, Prevotella* and *Haemophilus* genera were the most prevalent in HI (Fig. 2). Nonetheless, considering the bacterial diversity, the results reported by the Shannon or Chao1 indexes in some studies showed that peri-implantitis sites present more diversity than healthy implant sites. These findings propose that beyond a higher level of evenness, the PI microbiota is also characterized by a greater variety in composition, as other bacteria species/genus/phylum start to become part of the submucosal peri-implant biofilm. Regarding the results, the knowledge already available can be understood as a basis of microbiological analysis of the periodontal diseases, where the presence (and deepening) of the periodontal pocket seems to be a suitable niche both in terms of physical and nutritional conditions, and leads to an increase in the number of bacteria. Over time, as the maturation of the biofilm progresses, it develops environmental conditions that allow the most varied bacteria to become residents (Kawada et al., 2004; Marsh, Moter, & Devine, 2011; Marsh, 2012).

In this context, as PM is the first manifestation of an inflammatory process in a previously healthy implant, this condition may be the intermediate pathway to PI (Berglundh et al., 2018). Consequently, despite having some bacteria in common, PM also seems to be microbiologically distinct from both HI and PI. Nevertheless, very few studies are available in the literature that consider the PM microbiota by using 16S rRNA gene sequencing methodologies (Sousa et al., 2017; Tsigarida et al., 2015; Zheng et al., 2015). Specifically among those investigations that compared the PM and HI microbiota, results were inconclusive. Nevertheless, as already reported in the study by Zheng et al., it is reasonable that microbial diversity increases with the progress of peri-implant disease. Thus, PM has greater bacterial diversity than HI, just as PI has greater diversity than PM. The presence of non-abundant OTUs was probably the reason why microbial diversity increased. These results are consistent with previous findings of microbial enrichment in implant sites with disease (Maximo et al., 2009; Tabanella, Nowzari, & Slots, 2009). And, once again, based on microbiology findings in periodontal diseases (Marsh et al., 2011; Marsh, 2012), together with knowledge regarding the pathogenesis of peri-implant diseases (Salvi, Cosgarea, & Sculean, 2017), it is well-understood that as soon as the inflammatory process is established, there are local nutritional, respiratory and immunological shifts that can cause changes in bacterial diversity. Nonetheless, more studies are needed to assess the relationship between healthy implants and peri-implant diseases, and how disease progression occurs.

Regarding the quality of available evidence, a huge heterogeneity in study designs, methods and bacterial composition can be noticed across the included studies. Starting with the number of included subjects ranging from 18 to 80 patients, it is not clear if all authors conducted a priori calculations of sample sizes. Another observation was that no included studies, except for (Sousa et al., 2017), mentioned the use of a negative control during the microbiological analysis. This methodological element is relevant in assessing bacterial DNA contamination, which may arise in manipulation of the extraction kits, laboratory reagents or even on sample preparation. Regarding the microbial sampling technique, most of the included studies opted for paper points instead of sterile instruments in collecting samples around implants. It is still controversial whether or not paper points used for sampling could contain bacterial DNA contamination (van der Horst et al., 2013). Nevertheless, average bacterial counts are relatively in agreement among the results obtained using both sampling methods (Belibasakis, Schmidlin, & Sahrmann, 2014; Jervøe-Storm, Alahdab, Koltzscher, Fimmers, & Jepsen, 2007). Thus, bacterial DNA contamination still needs to be better addressed when using paper points. Regarding amount of bacteria, both paper points and sterile instruments seem reliable tools for biofilm sampling.

Two other important discrepancies among the included studies were the different technologies used and the amplification of distinct 16S

gene variable regions. Regarding the technologies, all the techniques used here employed second generation sequencing methods, and no first or third generation sequencing methods (e.g. Sanger sequencing). Those techniques present advantages as well as limitations. Briefly, the 454 Roche Pyrosequencing platform offers high throughput and long read length in a short run time; however, the reagent cost and error rate are both high, and homopolymer sequences are also sometimes observed. On the other hand, Illumina Miseq technology, despite providing shorter read length, offers high throughput, is cost-effective and sample preparation is easy. Also, it offers greater overall sequencing capacity and greater flexibility in computation as compared to the other methodologies (Koboldt, Steinberg, Larson, Wilson, & Mardis, 2013; Varoni et al., 2019; Zhang, Chiodini, Badr, & Zhang, 2011). These reasons might explain why this last technology was used by the four newest studies included in the review.

The 16S rRNA gene contains regions that are highly conserved in major groups of bacteria as well as nine hypervariable regions (V1–V9). Each variable (V) region ranges from 30 to 100 base pairs in length and has sufficient variability to identify and classify the bacteria of a sample, and in several cases also offers for identification bacteria at the specie level (Varoni et al., 2019). The utilities of V regions and specific primer sets used in the methodologies for bacterial community analysis have been assessed several times in the past few years. Results demonstrated that primer selections and targeted regions could have a significant impact on the determination of bacterial diversity (Liu, Lozupone, Hamady, Bushman, & Knight, 2007; Nossa et al., 2010; Wang & Qian, 2009). For example, in a recent study the relative abundance of bacterial taxa presented low reproducibility when the same sample was processed in different laboratories. The differences were mainly due to the use of different PCR primers (Hiergeist & Reischl, 2016). In fact, the decision about which region to target for amplification varies according to what is known about the bacterial taxa of the sample's microbial community; however, the use of a suboptimal primer pair can cause an under- or over-estimation of certain species (Di Bella, Bao, Gloor, Burton, & Reid, 2013; Wang & Qian, 2009). To date, there has been a lack of standardization and no consensus in the literature about the ideal region for best coverage and specificity in identifying different microbial communities (Klindworth et al., 2013; Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014; Vasileiadis et al., 2012). This could be observed in the studies included in the present systematic review, where a great variability in the amplified regions could be observed ranging from V1 to V9. Additionally, all included studies assigned the sequences to OTUs using a sequence similarity cutoff of 97%. In fact, 97% has been traditionally used as the clustering level since it was thought that this equated to species level. However, it is now observed that distinct species can be more highly related, and thus, OTUs are typically formed at 98.5%–99% sequence similarity (Hussain Bhat & Prabhu, 2017).

The inability to identify differences at the strain level is a limitation addressed by 16S rRNA gene sequencing methods. Nevertheless, this limitation can be overcome with shotgun sequencing of the whole genome. To date, no studies have used this technique to evaluate the microbiota of PI (Padial-Molina et al., 2016). On the other hand, metatranscriptomic analysis is an important tool in studying the functional activity of the microbial community present in a given environment. In genomic and metagenomic studies the microorganisms, or microbial community, are the target, while in the metatranscriptomic studies a set of genes can be transcribed in a given environmental condition. This new technique has been very important for understanding transient expression patterns of genes essential for the survival of microorganisms in a particular niche (Warnecke & Hess, 2009). 16S rRNA gene sequencing can provide a large proportion of microorganisms detected in low abundance and thus contribute to the high diversity and inter-individual variability of the oral microbiota. These species can play a protective role, providing functional redundancy in the niche in which they are inserted (Zaura, Keijser, Huse, & Crielaard, 2009). With the wide variety of microorganisms present in biofilms, interpretation of microbial data can be difficult, and for this reason continuing to study the function of the microbiota with metatranscriptomic and metabolomic analyses is important. Another limitation observed in available studies was the absence of a temporal evaluation. Change in bacterial count seems to be a factor in the establishment of peri-implant disease; however, all included studies were cross-sectional. Therefore, prospective studies should be considered in this field in future research.

In this final part of the present review, the similarities and differences of this investigation in relation to the systematic reviews already existing in the literature should be addressed (Lafaurie et al., 2017; Pérez-Chaparro et al., 2016; Rakic et al., 2016). Among the 7 studies included in this systematic review, 4 articles (57,1%), published between 2017 and 2019, have never been used in a systematic review before. Also, existing reviews contained fewer articles using 16S rRNA gene sequencing techniques compared to the present review due to the fact that this is a more recent technique. In addition, none of these aforementioned reviews compared the microbiota composition of the PM and PI as was done in the present review. Therefore, it is believed that this present review contributes new and unique information regarding the microbiota of peri-implant conditions. Moreover, by pointing out the discrepancies across the primary studies that prevented meta-analyses from being conducted, the present review can be used to improve future investigations into this issue.

5. Conclusions

Peri-implantitis is a complex disease in which associated microorganisms vary widely between studies. The associated microbiota in both healthy implants and peri-implantitis implants are qualitatively and quantitatively different, although there was no unanimity among the articles demonstrating in which peri-implant condition the greatest microbial diversity could be observed. Nevertheless, taking most of the studies into account, the microbiota composition has been shown to increase with the progression of peri-implant diseases.

Sources of Funding

The authors supported this study together with the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES), which provided a scholarship for FM (Finance Code 001).

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

For this type of study, formal consent is not required.

Author's contribution

All authors contributed to the study conception and design. The idea for the article came from Melo F. Material preparation, data collection and analysis were performed by Melo F, Milanesi F, and Angst PDM. The first draft of the manuscript was written by Melo F and critically revised by Angst PDM. Also, all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Fabiana de Melo: Conceptualization, Methodology, Investigation, Writing - original draft. **Fernanda Carpes Milanesi:** Methodology, Investigation, Writing - review & editing. **Patrícia Daniela Melchiors Angst:** Formal analysis, Writing - review & editing, Supervision. **Rui Vicente Oppermann:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix 1 Search strategies

Source	Strategy
MEDLINE/Pubmed	(((dental implants) AND microbial)) OR ((dental implants) AND metagenomic)
Scopus	(dental AND implants) AND (microbial) OR (metagenomic)
Science Direct	(dental implants AND microbial) OR (dental implants AND metagenomic) * only research articles
Embase	"tooth implant" AND “microbial”
Web of Science	#1 - TS = (dental AND implants) #2 - TS = (microbial) #3 - TS = (metagenomic) #4 - TS = (microbial) OR (metagenomic) #1 AND #4 * only articles
Manual search	Reference list of the retrieved articles; Reference lists of previously published review articles; Website of the most important journals in the field: - Clinical Oral Implants Research, - Journal of Clinical Periodontology, - Journal of Dental Research, - Journal of Periodontology, - European Journal of Oral Implantology, - - International Journal of Implant Dentistry.

References

Belibasakis, G. N., Schmidlin, P. R., & Sahrmann, P. (2014). Molecular microbiological evaluation of subgingival biofilm sampling by paper point and curette. *APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica, 122*(4), 347–352. https://doi.org/10.1111/apm.12151.

Berglundh, T., Armitage, G., Araujo, M. G., Avila-Ortiz, G., Blanco, J., Camargo, P. M., ... Zitzmann, N. (2018). Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *Journal of Periodontology, 89*, S313–S318. https://doi.org/10.1002/JPER.17-0739.

Dabdoub, S. M. M., Tsigarida, A. A. A., & Kumar, P. S. S. (2013). Patient-specific analysis of periodontal and peri-implant microbiomes. *Journal of Dental Research, 92*(12 Suppl), 168S–175S. https://doi.org/10.1177/0022034513504950.

Derks, J., & Tomasi, C. (2015). Peri-implant health and disease. A systematic review of current epidemiology. *Journal of Clinical Periodontology, 42*, S158–S171. https://doi.org/10.1111/jcpe.12334.

Di Bella, J. M., Bao, Y., Gloor, G. B., Burton, J. P., & Reid, G. (2013). High throughput sequencing methods and analysis for microbiome research. *Journal of Microbiological Methods, 95*(3), 401–414. https://doi.org/10.1016/j.mimet.2013.08.011.

Esposito, M., Grusovin, M. G., & Worthington, H. V. (2012). Treatment of peri-implantitis: What interventions are effective? A Cochrane systematic review. *European Journal of Oral Implantology* Quintessence Publishing Co., Ltd.

Faggion, C. M., Listl, S., Frühauf, N., Chang, H.-J., & Tu, Y.-K. (2014). A systematic review and Bayesian network meta-analysis of randomized clinical trials on non-surgical treatments for peri-implantitis. *Journal of Clinical Periodontology, 41*(10), 1015–1025. https://doi.org/10.1111/jcpe.12292.

Wells, G. A., Shea, B., O'Connell, D., Peterson, J., Welch, V., & Losos, M. (2001). *The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses.* Retrieved August 5, 2018, from http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp.

Gao, X., Zhou, J., Sun, X., Li, X., & Zhou, Y. (2018). Diversity analysis of subgingival microbial bacteria in peri-implantitis in Uygur population. *Medicine, 97*(5), e9774. https://doi.org/10.1097/MD.0000000000009774.

Gbadebo, O., Lawal, F., Sulaiman, A., & Ajayi, D. (2014). Dental implant as an option for tooth replacement: The awareness of patients at a tertiary hospital in a developing country. *Contemporary Clinical Dentistry, 5*(3), 302. https://doi.org/10.4103/0976-237X.137914.

Größner-Schreiber, B., Teichmann, J., Hannig, M., Dörfer, C., Wenderoth, D. F., Ott, S. J. S. J., ... Ott, S. J. S. J. (2009). Modified implant surfaces show different biofilm compositions under in vivo conditions. *Clinical Oral Implants Research, 20*(8), 817–826. https://doi.org/10.1111/j.1600-0501.2009.01729.x.

Heuer, W., Kettenring, A., Stumpp, S. N., Eberhard, J., Gellermann, E., Winkel, A., ... Stiesch, M. (2012). Metagenomic analysis of the peri-implant and periodontal microflora in patients with clinical signs of gingivitis or mucositis. *Clinical Oral Investigations, 16*(3), 843–850. https://doi.org/10.1007/s00784-011-0561-8.

Hiergeist, A., & Reischl, U. (2016). Multicenter quality assessment of 16S ribosomal DNA-sequencing for microbiome analyses reveals high inter-center variability. *International Journal of Medical Microbiology, 306*(5), 334–342. https://doi.org/10.1016/j.ijmm.2016.03.005.

Hussain Bhat, A., & Prabhu, P. (2017). OTU Clustering: A Window to Analyse Uncultured Microbial World. *International Journal of Advance Engineering and Research Development, 5*(6), 62–68. Retrieved from www.isroset.org.

Jervøe-Storm, P.-M., Alahdab, H., Koltzscher, M., Fimmers, R., & Jepsen, S. (2007). Comparison of curet and paper point sampling of subgingival bacteria as analyzed by real-time polymerase chain reaction. *Journal of Periodontology, 78*(5), 909–917. https://doi.org/10.1902/jop.2007.060218.

Kawada, M., Yoshida, A., Suzuki, N., Nakano, Y., Saito, T., Oho, T., ... Koga, T. (2004). Prevalence of Porphyromonas gingivalis in relation to periodontal status assessed by real-time PCR. *Oral Microbiology and Immunology, 19*(5), 289–292. https://doi.org/10.1111/j.1399-302X.2004.00154.x.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., ... Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*(1), e1. https://doi.org/10.1093/nar/gks808.

Koboldt, D. C., Steinberg, K. M., Larson, D. E., Wilson, R. K., & Mardis, E. R. (2013). XThe next-generation sequencing revolution and its impact on genomics. *Cell.* https://doi.org/10.1016/j.cell.2013.09.006 Cell Press.

Kumar, P. S., Mason, M. R., Brooker, M. R., & O'Brien, K. (2012). Pyrosequencing reveals unique microbial signatures associated with healthy and failing dental implants. *Journal of Clinical Periodontology, 39*(5), 425–433. https://doi.org/10.1111/j.1600-051X.2012.01856.x.

Lafaurie, G. I., Sabogal, M. A., Castillo, D. M., Rincón, M. V., Gómez, L. A., Lesmes, Y. A., ... Chambrone, L. (2017). Microbiome and Microbial Biofilm Profiles of Peri-Implantitis: A Systematic Review. *Journal of Periodontology, 88*(10), 1066–1089. https://doi.org/10.1902/jop.2017.170123.

Liu, Z., Lozupone, C., Hamady, M., Bushman, F. D., & Knight, R. (2007). Short pyrosequencing reads suffice for accurate microbial community analysis. *Nucleic Acids Research, 35*(18), e120. https://doi.org/10.1093/nar/gkm541.

Magurran, A. (2004). *Measuring Biological Diversity.* Retrieved fromOxfordhttps://www2.ib.unicamp.br/profs/thomas/NE002_2011/maio10/Magurran2004c2-4.pdf.

Marsh, P. D. (2012). Contemporary perspective on plaque control. *British Dental Journal, 212*(12), 601–606. https://doi.org/10.1038/sj.bdj.2012.524.

Marsh, P. D., Moter, A., & Devine, D. A. (2011). Dental plaque biofilms: communities, conflict and control. *Periodontology 2000, 55*(1), 16–35. https://doi.org/10.1111/j.1600-0757.2009.00339.x.

Maximo, M. B., de Mendonca, A. C., Santos, V. R., Figueiredo, L. C., Feres, M., & Duarte, P. M. (2009). Short-term clinical and microbiological evaluations of peri-implant diseases before and after mechanical anti-infective therapies. *Clinical Oral Implants Research, 20*(1), 99–108. https://doi.org/10.1111/j.1600-0501.2008.01618.x.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G., & PRISMA Group (2010). Preferred

reporting items for systematic reviews and meta-analyses: the PRISMA statement. *International Journal of Surgery (London, England), 8*(5), 336–341. https://doi.org/10.1016/j.ijsu.2010.02.007.

Nossa, C. W., Oberdorf, W. E., Yang, L., Aas, J. A., Paster, B. J., Desantis, T. Z., ... Pei, Z. (2010). Design of 16S rRNA gene primers for 454 pyrosequencing of the human foregut microbiome. *World Journal of Gastroenterology, 16*(33), 4135–4144. https://doi.org/10.3748/wjg.v16.i33.4135.

Padial-Molina, M., López-Martínez, J., O'Valle, F., & Galindo-Moreno, P. (2016). Microbial Profiles and Detection Techniques in Peri-Implant Diseases: a Systematic Review. *Journal of Oral & Maxillofacial Research, 7*(3), e10. https://doi.org/10.5037/jomr.2016.7310.

Pérez-Chaparro, P. J., Duarte, P. M., Shibli, J. A., Montenegro, S., Lacerda Heluy, S., Figueiredo, L. C., ... Feres, M. (2016). The Current Weight of Evidence of the Microbiologic Profile Associated With Peri-Implantitis: A Systematic Review. *Journal of Periodontology, 87*(11), 1295–1304. https://doi.org/10.1902/jop.2016.160184.

Rakic, M., Grusovin, M. G., & Canullo, L. (2016). The Microbiologic Profile Associated with Peri-Implantitis in Humans: A Systematic Review. *The International Journal of Oral & Maxillofacial Implants, 31*(2), 359–368. https://doi.org/10.11607/jomi.4150.

Renvert, S., & Quirynen, M. (2015). Risk indicators for peri-implantitis. A narrative review. *Clinical Oral Implants Research, 26*, 15–44. https://doi.org/10.1111/clr.12636.

Salvi, G. E., Cosgarea, R., & Sculean, A. (2017). Prevalence and Mechanisms of Peri-implant Diseases. *Journal of Dental Research, 96*(1), 31–37. https://doi.org/10.1177/0022034516667484.

Sanz-Martin, I., Doolittle-Hall, J., Teles, R. P., Patel, M., Belibasakis, G. N., Hämmerle, C. H. F., ... Teles, F. R. F. (2017). Exploring the microbiome of healthy and diseased peri-implant sites using Illumina sequencing. *Journal of Clinical Periodontology, 44*(12), 1274–1284. https://doi.org/10.1111/jcpe.12788.

Sousa, V., Nibali, L., Spratt, D., Dopico, J., Mardas, N., Petrie, A., ... Donos, N. (2017). Peri-implant and periodontal microbiome diversity in aggressive periodontitis patients: a pilot study. *Clinical Oral Implants Research, 28*(5), 558–570. https://doi.org/10.1111/clr.12834.

Tabanella, G., Nowzari, H., & Slots, J. (2009). Clinical and microbiological determinants of ailing dental implants. *Clinical Implant Dentistry and Related Research, 11*(1), 24–36. https://doi.org/10.1111/j.1708-8208.2008.00088.x.

Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., & Nishijima, M. (2014). Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing. *PloS One, 9*(8), e105592. https://doi.org/10.1371/journal.pone.0105592.

Tsigarida, A. A., Dabdoub, S. M., Nagaraja, H. N., & Kumar, P. S. (2015). The Influence of Smoking on the Peri-Implant Microbiome. *Journal of Dental Research, 94*(9), 1202–1217. https://doi.org/10.1177/0022034515590581.

van der Horst, J., Buijs, M. J., Laine, M. L., Wismeijer, D., Loos, B. G., Crielaard, W., ... Zaura, E. (2013). Sterile paper points as a bacterial DNA-contamination source in microbiome profiles of clinical samples. *Journal of Dentistry, 41*(12), 1297–1301. https://doi.org/10.1016/j.jdent.2013.10.008.

Vankov, P. Y., Ziganshina, E. E., Ilinskaya, O. N., Khafizova, F. A., Khafizov, R. G., & Ziganshin, A. M. (2016). Comparative Analysis of Bacterial Communities Associated with Healthy and Inflamed Peri-implant Tissues. *BioNanoScience, 6*(4), 490–495. https://doi.org/10.1007/s12668-016-0270-5.

Varoni, E. M., Bavarian, R., Robledo-Sierra, J., Porat Ben-Amy, D., Wade, W. G., Paster, B., ... Frandsen Lau, E. (2019). World Workshop on Oral Medicine VII: Targeting the microbiome for oral medicine specialists—Part 1. A methodological guide. *Oral Diseases*. https://doi.org/10.1111/odi.13063 Blackwell Publishing Ltd.

Vasileiadis, S., Puglisi, E., Arena, M., Cappa, F., Cocconcelli, P. S., & Trevisan, M. (2012). Soil bacterial diversity screening using single 16S rRNA gene V regions coupled with multi-million read generating sequencing technologies. *PloS One, 7*(8), e42671. https://doi.org/10.1371/journal.pone.0042671.

Wang, Y., & Qian, P. Y. (2009). Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS ONE, 4*(10), https://doi.org/10.1371/journal.pone.0007401.

Warnecke, F., & Hess, M. (2009). A perspective: Metatranscriptomics as a tool for the discovery of novel biocatalysts. *Journal of Biotechnology, 142*(1), 91–95. https://doi.org/10.1016/j.jbiotec.2009.03.022.

Yu, X.-L., Chan, Y., Zhuang, L., Lai, H.-C., Lang, N. P., Keung Leung, W., ... Watt, R. M. (2019). Intra-oral single-site comparisons of periodontal and peri-implant microbiota in health and disease. *Clinical Oral Implants Research, 30*(8), 760–776. https://doi.org/10.1111/clr.13459.

Zaura, E., Keijser, B. J. F., Huse, S. M., & Crielaard, W. (2009). Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiology, 9*(1), 259. https://doi.org/10.1186/1471-2180-9-259.

Zhang, J., Chiodini, R., Badr, A., & Zhang, G. (2011). The impact of next-generation sequencing on genomics. *Journal of Genetics and Genomics*. https://doi.org/10.1016/j.jgg.2011.02.003.

Zhang, Q., Qin, X. Y., Jiang, W. P., Zheng, H., Xu, X. L., & Chen, F. (2015). Comparison of Subgingival and Peri-implant Microbiome in Chronic Periodontitis. *Chinese Journal of Dental Research, 18*(3), 155–162. https://doi.org/10.3290/j.cjdr.a34779.

Zheng, H., Xu, L., Wang, Z., Li, L., Zhang, J., Zhang, Q., ... Chen, F. (2015). Subgingival microbiome in patients with healthy and ailing dental implants. *Scientific Reports, 5*(1), 10948. https://doi.org/10.1038/srep10948.

Ziganshina, E. E., Belostotskiy, D. E., Ilinskaya, O. N., Boulygina, E. A., Grigoryeva, T. V., & Ziganshin, A. M. (2015). Effect of the Organic Loading Rate Increase and the Presence of Zeolite on Microbial Community Composition and Process Stability During Anaerobic Digestion of Chicken Wastes. *Microbial Ecology, 70*(4), 948–960. https://doi.org/10.1007/s00248-015-0635-2.

Capítulo III. Comparison of different 16S rRNA gene V regions for assessing peri-implantitis bacterial divertity

No **Capítulo III** apresentamos o manuscrito preparado para submissão no periódico Journal of Dental Research (JDR).

No capítulo anterior (capítulo II) apresentamos o que a literatura tem nos mostrado até agora em relação a microbiota envolvida nas diferentes condições peri-implantares em estudos que utilizaram técnicas de sequenciamento de alto rendimento. Como foi verificado uma alta heterogeneidade nos protocolos de amplificação e sequenciamento utilizados pelos estudos, e, sabendo que estas mudanças podem ter um impacto significativo na determinação da diversidade bacteriana, propusemos, um estudo para comparar diferentes regiões do gene 16S e o uso de diferentes primers. Neste estudo demonstramos que a microbiota de amostras de peri-implantite foram similares entre diferentes primers e regiões, porém, diferentes entre pacientes, sugerindo que as amostras e não os primers determinaram a composição microbiana.

Comparison of different 16S rRNA gene V regions for assessing peri-implantitis bacterial divertity

Fabiana de Melo[1], Marco Antônio De Bastiani[2], Francisco Montagner[3], Ricardo dos Santos Araujo Costa[1] , Tassiane Panta Wagner[1], Fernando Silva Rios[1], Alex Haas[1], Rui Vicente Oppermann[1]

[1] Periodontology, School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

[2] Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

[3] Department of Conservative Dentistry, Endodontic unit, School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

Corresponding author:
Dr. Rui Vicente Oppermann
Rua Ramiro Barcelos, 2492 – Rio Branco, Porto Alegre - RS,
Zip-code: 90035-004 Brazil
Phone: +55 51 3308 5509
Email: ruioppermann@gmail.com

Conflict of interest and Sources of Funding Statement

The authors financially supported the study, and declare no conflict of interest.

Comparison of different 16S rRNA gene V regions for assessing peri-implantitis bacterial divertity

ABSTRACT

This study compared different 16S rRNA gene V regions (V3-V4, V4 and V5-V6), using 4 primer sets, aiming at evaluating bacterial diversity metrics at peri-implantitis sites.
Microbiological samples were obtained from the deepest site of the dental implant of three subjects diagnosed with peri-implantitis. The V3-V4, V4 or V5-V6 regions of the 16S rDNA gene were amplified with selected sets of universal primers (341F-805R, 347F-803R, 515F-806R and 609D-699R), sequenced with the Miseq Illumina instrument. Alpha and beta diversity metrics, taxonomic classifications and clustering were computed using the Microbiome Analyst tool. A sum total of 89 different OTUs at the genus level were retrieved. 341F-805R, 347F-803R, 515F-806R and 609D-699R primer pairs presented 55, 51, 55 and 80 OTUs, respectively. In general, the genera that corresponded to the most abundant in the communities were detected in a similar way among the studied primer-sets. However, a considerable part of the retrieved species was in low abundance in all primer sets. The abundance of most species was similar within each sample but when analyzed within each primer pair, more dissimilarities were be observed suggesting that the samples and not the primers determined the microbial composition.
Understanding microbial community is necessary not only from the perspective of the most abundant but also from the least abundant. These may have important roles in biofilms such as modifying pathogenicity or virulence factors expression of the most abundant.

Introduction

Etiology of various oral and systemic diseases, such as periodontal diseases, dental caries, obesity and cancer, may directly or indirectly involves the oral microbiota (Teng et al., 2015; Yang et al., 2012). Consequently, it is of great biomedical interest to find relevant associations between microbial profiles and systemic conditions, which may lead to better clinical diagnosis and treatment (Teng et al., 2015). Accordingly, recent breakthroughs allowed by the development of next-generation sequencing (NGS) technologies lead to in-depth sequencing and data analyses of hundreds of microbial communities in different types of samples (SHOKRALLA et al., 2012). In fact, a large number of microorganisms not yet cultured were revealed by molecular approaches, creating a paradigm shift in our knowledge about oral health and disease (Diaz et al., 2006; Gomes et al., 2006; Lillo et al., 2006).

The small subunit ribosomal RNA molecule encoded by bacterial 16S rRNA gene, which usually contains nine hypervariable regions (V1–V9) flanked by nine highly conserved regions (C1–C9), is a versatile and important marker gene for profiling bacterial populations (Baker et al., 2003). Additionally, *in silico* predictions using 16S rRNA gene databases showed that there is different coverage of conserved regions of this gene for distinct bacterial strains. Therefore, all the 16S rRNA gene universal primers commonly used are designed in these regions. Because of these features, the effect of V regions and specific primers sets on commonly applied technologies in bacterial community studies have been assessed over the past years (Liu et al., 2007; Nossa et al., 2010; Wang and Qian, 2009). These investigations showed that primer selections and targeted regions could have a significant impact on the determination of bacterial diversity. In fact, the use of a suboptimal primer pair can cause an under or over estimation of certain species (Wang and Qian, 2009). Besides "coverage", other criteria, such as "spectrum" and "specificity", were also assessed by those studies. However, despite the variety of studies on 16S V regions, the question whether sequence length and depth causes bias in identifying different microbial communities is still an open question in the field (Klindworth et al., 2013; Takahashi et al., 2014; Vasileiadis et al., 2012).

In spite of its issues, high-throughput sequencing technology is the current and future trend to study complex bacterial communities in any sample of interest. Therefore, in this study we compared different 16S rRNA gene V regions (V3-V4, V4 and V5-V6) and examined 4 sets of primers, exploring how the 16S rRNA gene primer sets and covered regions affect bacterial diversity metrics in peri-implantitis sites. The results show useful information to improve current sequencing practices and also provide an important reference regarding which set of primers and region to choose when working on bacterial diversity.

Methods

Subject Selection

Three non-smoker subjects (A1, A1, F2), presenting peri-implantitis (PI) defined as probing pocket depth (PPD) ≥ 5 mm with bleeding on probing (BOP) and radiographic evidence of bone loss ≥ 2 mm were selected (Mombelli et al.,2012). Moreover, subjects had good general health conditions in addition to negative medical history of any systemic condition that indicated the use of antimicrobial chemoprophylaxis. Inclusion criteria included no antibiotic therapy within the last six months.

This study had the approval from the Research Ethics Committee of the Federal University of Rio Grande do Sul (666.930). Written informed consent was obtained from all participants.

Bacterial Sampling and DNA Isolation

Microbiological samples were obtained from the deepest site of the dental implant. Four sterile paper points (Dentsply®) were inserted into the peri-implant pocket for 30 seconds after drying the coronary surface and careful removal of supramucosal biofilm. The collected samples were pooled and stored dry in a 1.5 ml single Eppendorf tube at -80ºC until further analysis. Bacterial DNA was isolated with a Qiagen DNA MiniAmp kit (Qiagen, Valencia, CA) using the tissue protocol according to the manufacturer's instructions. DNA concentration was determined with NanoDrop (Thermo Scientific).

Primer selection and Miseq Sequencing

Polymerase chain reactions (PCR) and samples barcoding were performed by amplifying the V3-V4, V4 or V5-V6 region of the 16S rDNA gene with four selected sets of universal primers: forward primer 347F (GGAGGCAGCAGTRRGGAAT) and reverse primer 803R (CTACCRGGGTATCTAATCC) (Nossa et al. 2010); forward primer 515F (GTGCCAGCMGCCGCGGTAA) and reverse primer 806R (GGACTACHVGGGTAT CTAAT) (Maruyama et al., 2014); forward primer 609D (GGMTTAGATACCCBDGTA) and reverse primer 699R (GGGTYKCGCTCGTTR) (Alm et al., 1996); forward primer 341F (CCTACGGGRSGCAGCAG) and reverse primer 805R (GACTACCAGGGTATCTAAT) (Sanz-Martin et al., 2017) (Table 1). Briefly, a first step PCR with broad-range universal primers and 23 cycles of amplification was used to amplify the 16S rRNA gene. Then, a second step PCR was performed to introduce Illumina adaptor sequences Fluidigm (INDEX) into the DNA. The same barcodes were utilized for each primer set. Quantification was determined using the Picogreen dsDNA quantification assay (Biomek FX, Kit: Quant-iT™ PicoGreen® dsDNA Assay Kit). PCR samples were pooled and then purified using AMPure beads. For quality control, LibQ was performed to measure the size and concentration of the pool to be sequenced. DNA sequences of 2 X 250 bp (PE250) were generated with a MiSeq (Illumina, Inc., San Diego, CA, USA) instrument. Assembled reads were filtered for quality; all reads having average quality below 30 or having more than two ambiguous bases (Ns) and three nucleotides below quality 20 were discarded. Afterwards, reads were clustered using an in-house clustering algorithm. Briefly, reads were clustered at 100% identity and, then, clustered/denoized at 99% identity (dnaclust 3). Clusters having abundance lower than 3 were discarded. Remaining clusters were then scanned for chimeras with UCHIME denovo and UCHIME reference (Edgar et al. 2011) (using the gold reference from the Microbiome Utilities Portal of the Broad Institute) and clustered at 97% (dnaclust 3) to form the final clusters/operational taxonomic units (OTUs). OTUs were classified with the Ribosomal Database Project (RDP) classifier tool (Wang et al. 2007) using an in-house training set containing the complete Greengenes database supplemented with eukaryotic sequences from the Silva databases

(http://www.arb-silva.de). Diversity metrics were obtained by aligning OTU sequences on a Greengenes core reference alignment (DeSantis et al. 2006) using the PyNAST aligner (Caporaso et al. 2010a,b). Alignments were filtered to keep only the hypervariable region part of the alignment. Alpha (observed species) and beta (weighted, unweighted UniFrac and Bray–Curtis dissimilarity) diversity metrics, taxonomic classifications and clustering were then computed using the Microbiome Analyst tool (Chong et al., 2020; Dhariwal et al., 2017). The primer pairs were also evaluated using SILVA TestPrime 1.0 (www.arb-silva.de/search/testprime), which evaluates primer pair coverage by running an *in silico* PCR on the SILVA SSU Ref 138NR data (Klindworth et al., 2013).

Statistical Analysis

OTU data was evaluated using the Microbiome Analyst web tool and package for R statistical environment (Chong et al., 2020; Dhariwal et al., 2017), which was designed for analysis of 16S rRNA marker gene survey data. Firstly, OTU count data, sample metadata and taxonomy tables were supplied with SILVA taxonomy labels. Afterwards, the data was submitted to centered log ratio (CLR) normalization prior to further marker gene analyses. The data was compared using Kruskal-Wallis test. Statistical significance was set as $p < 0.05$. Analyses were performed with the aid of GraphPad Prism 5.0 Software (GraphPad Software Inc., La Jolla, CA, USA) and R Statistical Software Package with the Stats library 3.4.0 (http://www.r-project.org/).

Results

TestPrime 1.0 evaluated coverage of the 4 primer pairs. Overall, V4 and V5-V6 obtained the higher coverage of the Bacteria domain. Coverage by the V3-V4 (341F-805R) primer pair was 0.0% for Archaea, 71.7% for Bacteria, and 0.0% for Eukaryota. Additionally, coverage by the V3-V4 (347F-803R) primer pair was 0.0% for Archaea, 71.8% for Bacteria, and 0.0% for Eukaryota. In contrast, the coverage for V4 (515F-806R) was 51.4% for Archaea, 82.5% for Bacteria, and 0.1% for Eukaryota. Notably, V5-V6 (609D-699R) primer pair showed the higher coverage for Bacteria domain

(92.9%), while 43.8% for Archaea and 0.0% for Eukaryota (Table 1). It is well known that *in silico* evaluation does not always guarantee good laboratory results (Klindworth et al., 2013), for this reason, verification samples were used for experimental validation. A total of 121.938 sequences were used in the analysis. Average of data quality control of sequences generated by Miseq sequencer after data trimming and quality filtering is presented in Table 1. All reads were trimmed to a fix length (V4 and V5-V6: 250 pb; V3-V4: 300 pb) and clustering these sequences into OTUs at a 3% genetic distance resulted in 89 different OTUs at the genus level. 341F-805R, 347F-803R, 515F-806R and 609D-699R primer pairs presented 55, 51, 55 and 80 OTUs, respectively. Figure 1 show the number of read counts of each sample, where we can observe that the primers 515F-806R and 609D-699R returned a larger number of reads in general.

Afterwards, OTUs were grouped in 49 genera and 2 more inclusive taxa (e.g., family, order, class) representing the phyla *Actinobacteria, Bacteroidetes, Cloroflexi, Epsilonbacteraeota, Firmicutes, Fusobacteria, Patescibacteria, Proteobacteria, Spirochaetes, Synergistetes* and *Tenericutes.* Among these*, Firmicutes* accounted for 36% of all sequences. The Phylum *Patescibacteria* was not detectable by 347F-803R and 515F-806R pairs, and the Phylum *Spirochaetes* was not detectable by 347F-803R primer pairs. Actual abundance by phylum in each primer pair is represented at Supplementary figure 1.

Taxa with less than 20 overall sequences were designated as rare. Actual abundance and distribution of genera in each primer pair is represented in figure 2A. 341F-805R, 347F-803R, 515F-806R and 609D-699R primer pairs presented 11, 10, 5 and 20 rare OTUs, respectively. 609D-699R demonstrated higher coverage of rare as well as abundant species than 341F-805R, 347F-803R and 515F-806R. Most of identified taxonomic groups were detected with all studied regions; however, the genus *Oribacterium* was detected with V4 and V5-V6 regions but not with V3-V4 regions, and *Pseudopropionibacterium* was detected only with V5-V6 region. The genera *Veillonella, Fusobacterium, Leptotrichia, Neisseria* and *Fretibacterium* constitute 53% of the community when 341F-805R primer pair is used and 69% when 347F-803R is used. Additionally, the genera *Veillonella, Fusobacterium, Neisseria, Treponema* and *Fretibacterium* constitute 58% of the community when the primer pair 515F-806R is

used. Lastly, when the primer pair 609D-699R is used, the genera *Porphyromonas, Veillonella, Fusobacterium, Leptotrichia* and *Neisseria* constitute 52% of the community. We observed that a considerable part of the retrieved species was in low abundance (less than 3%) in all primer sets (Figure 2B)

Figure 3 shows the Shannon diversity (Median/SD: 341F-805R = 2.4/0.3, 347F-803R = 2.08/0.4, 515F-806R = 2.5/0.3 and 609D-699R = 2.3/0.1) and Chao1 indices (Median/SD: 341F-805R = 29/5.1, 347F-803R = 32/6.6, 515F-806R = 33/5.0 and 609D-699R = 37/4.0) for all primer sets. No statistical differences in the microbial richness metrics were observed (Shannon - Kruskal-Wallis p-value = 0.4965; Chao 1 - Kruskal-Wallis p-value = 0.10085). However, a large effect size was observed on Chao 1 (Eta-squared: 0.45) and Shannon (Eta-squared: 0.29) metrics. Additionally, Shannon and Chao1 were statistically different among samples (Shannon - Kruskal-Wallis p-value = 0.0076 Chao 1 - Kruskal-Wallis p-value = 0.0442). Species richness can be observed using rarefaction curves at Supplementary file 2.

Based on the clustering results of the samples at the family level, the Heatmap plot was obtained considering the most abundant OTUs. This showed that the abundance of most taxa was similar within each sample but when we analyze within each primer pair, more dissimilarities were observed (Figure 4). The differences in the bacterial community between the primers sets were analyzed with the PCoA analysis on genus, using Bray-Curtis distance. Our results showed higher distances between samples than between primers in the first two axis of variation (the contribution of PC1 was 43.4%, while the contribution of PC2 was 37.3%) (Figure 5). In accordance with the two previous results, similarity analysis (ANOSIM) showed that samples are different (P = 0.0002) but primers are similar (P = 0.49). These results suggest that there were little differences in the bacterial community structures within primers of the same samples.

Discussion

The literature has shown that the microbiota involved in peri-implant diseases is quite complex and diverse (Koyanagi et al., 2013, 2010; Shiba et al., 2016; Zheng et al., 2015). Thus, we collected peri-implant samples from patients diagnosed with PI to assess

the extent to which primer design affects the detection of microorganisms in this community. Previous studies reported different hypervariable regions as the most appropriated for better coverage in deep sequencing (Chakravorty et al., 2007; Trotha et al., n.d.; Youssef et al., 2009). In fact, despite the lack of information about a golden standard, V3-V4 regions are frequently used in oral microbiological studies (Apatzidou et al., 2017; Maruyama et al., 2014; Sanz-Martin et al., 2017). Furthermore, previous study used the geodesic distance of phylogenetic trees to quantify discrepancies of the different hypervariable regions and demonstrated that the pairwise distance of V4-VT (combination of all sub-regions) was the smallest distance, which indicated superior phylogenetic resolution for bacterial phyla (Yang et al., 2016). In the present study we examined the performance of 4 different universal primer pairs for the amplification of bacterial 16S rDNA gene in V3-V4, V4 or V5-V6 regions, using an Illumina MiSeq platform sequencer.

Despite the frequency of several taxonomic groups markedly differed between the four primer sets, in general, most abundant genera were similar among the studied primer-sets. Additionally, although samples were collected from a disease environment, bacteria belonging to the genus *Veillonella,* genera that have been previously associated with periodontal health (Kumar et al., 2006), were the most dominant taxonomic group in all primers sets except 609D-699R. This primer pair, however, revealed the genus *Fusobacterium,* a disease-associated genus, as the most abundant taxon. This finding is in accordance with previous analysis of PI based on 16S rDNA gene sequencing that found a high prevalence of *Fusobacterium* on samples (Koyanagi et al., 2013, 2010; Maruyama et al., 2014). Finally, *Prevotella, Porphyromonas* and *Treponema*, also disease-associated genera, were found in high numbers, especially on V4 and V5-V6 regions. In fact, the presence of high-level *Treponema* is in agreement with several previous findings that used different bacterial detection techniques (Apatzidou et al., 2017; Belibasakis et al., 2016; Koyanagi et al., 2013, 2010; Kumar et al., 2012; Maruyama et al., 2014; Papaioannou et al., 1995; Sanz-Martin et al., 2017), suggesting that the phylum *Spirochaetes* and, more specifically, the genus *Treponema* play an important role in peri-implant pathogenesis. Surprisingly, this specific genus was not found when using the

347F-803R primers, suggesting that this may not be the ideal primer pair when evaluating peri-implant samples.

An interesting result is the amount of bacteria in low abundance observed in all tested primers, ranging from 16% to 27%. To date, there are no studies showing the importance of rare species in PI, however, previous studies on periodontal disease have shown that certain microbial pathogens in low abundance can cause inflammatory diseases by increasing the amount of the normal microbiota and changing its composition and it is suggested that other species may be equally or even more active in the process that leads from periodontal health to disease and should be investigated (Hajishengallis et al., 2012). The sequencing data allowed evaluating the alpha diversity through the Shannon and Chao1 indices. The Shannon index is widely used and it is sensitive to rare species and abundance variations and indicates the diversity of a community based on its distribution in a given sample and the number of species found in it. Thus, the higher the index, the greater the number of species reported. Chao1 is a nonparametric richness estimator that takes into account also the number of rare OTUs based on abundance (relative representation of species in the ecosystem). Thus, it favors the analysis of species that are not very abundant within a sample. In both metrics, the samples grouped in 609D-699R primers (V5-V6 region) showed higher values compared to all other primers sets, albeit this difference was not statistically significant, possibly due to sample size. In fact, effect size was considered large in both diversity metrics. Corroborating with this result, some studies have found a correlation between these variables indicating that very rich communities may actually have higher phylogenetic diversity (Koyanagi et al., 2013, 2010; Sousa et al., 2017; Zheng et al., 2015). In contrast, although fragments covering the V3-V4 regions have been widely adopted for NGS in many previous studies with PI samples and several primers have been used to amplify this region (Apatzidou et al., 2017; Maruyama et al., 2014; Sanz-Martin et al., 2017), our results indicate that V3-V4 may underestimate bacterial diversity, corroborating with the SILVA TestPrime result presented. Another result that should be highlighted is that although all samples were retrieved from PI condition, statistically significant difference was observed in the microbial communities, suggesting that the composition of the microbiome varies between individuals. This result is in line with previous studies that reported the

composition of human microbiome is highly variable within and between individuals. In fact it has been suggested that a community of microbes that live in the body could be distinct enough from the rest of the population to be used as a unique microbial 'fingerprint' (Costello et al., 2009; Franzosa et al., 2015). However, it is important to note that while the ability to retrieve as much information (species) as possible is crucial in microbial diversity studies, the lack of gold standard or knowledge of the actual number of species in the sample does not allow us to determine the level detection error (Kumar et al., 2006).

Previous studies showed that PCR conditions could be a source of sequencing bias. In this sense, our study carefully set all primer to receive identical PCR cycling conditions with the intention of reducing the possibility of bias from this source. The evolution of new sequencing platforms and new software tools is improving data acquisition and analysis, thereby, changing the way we see the microbiome (Sultan 2018). As NGS sequencing technology improves in accuracy and cost, the full-length 16S rRNA sequencing should fast become a usual method for evaluate bacteria in different samples. Higher resolution and more comprehensive information can be provided by Metagenomic sequencing than amplicon sequencing technologies for microbial profiling and, within a short time, with sequencing prices continuing to fall, the knowledge of the taxonomic and functional diversity of microbial communities will advance at unprecedented resolution.

Within the limitation of this study, we believe that there were little differences in the bacterial community structures within primers of the same samples. In other words, the abundance of most taxa was similar within each sample but when we analyze within each primer pair, more dissimilarity was observed, which we can suggest that the samples and not the primers determined the microbial composition. Thus, the results presented a useful guide to improve current knowledge about sequencing practices and also provide an important reference regarding which set of primers and region to choose when working on PI bacterial diversity.

References

Alm EW, Oerther DB, Larsen N, Stahl DA, Raskin L. The oligonucleotide probe database. Appl Environ Microbiol 1996;62:3557–9.

Apatzidou D, Lappin DF, Hamilton G, Papadopoulos CA, Konstantinidis A, Riggio MP. Microbiome of peri-implantitis affected and healthy dental sites in patients with a history of chronic periodontitis. Arch Oral Biol 2017;83:145–52. doi:10.1016/j.archoralbio.2017.07.007.

Belibasakis GN, Mir-Mari J, Sahrmann P, Sanz-Martin I, Schmidlin PR, Jung RE. Clinical association of *Spirochaetes* and *Synergistetes* with peri-implantitis. Clin Oral Implants Res 2016;27:656–61. doi:10.1111/clr.12690.

Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 2007;69:330–9. doi:10.1016/j.mimet.2007.02.005.

Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc 2020;15:799–821. doi:10.1038/s41596-019-0264-1.

Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. Bacterial community variation in human body habitats across space and time. Science (80-) 2009;326:1694–7. doi:10.1126/science.1177486.

Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res 2017;45:W180–8. doi:10.1093/nar/gkx295.

Franzosa EA, Huang K, Meadow JF, Gevers D, Lemon KP, Bohannan BJM, et al. Identifying personal microbiomes using metagenomic codes. Proc Natl Acad Sci U S A 2015;112:E2930–8. doi:10.1073/pnas.1423854112.

Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. Nat Rev Microbiol 2012;10:717–25. doi:10.1038/nrmicro2873.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013;41:e1.

doi:10.1093/nar/gks808.

Koyanagi T, Sakamoto M, Takeuchi Y, Maruyama N, Ohkuma M, Izumi Y. Comprehensive microbiological findings in peri-implantitis and periodontitis. J Clin Periodontol 2013;40:218–26. doi:10.1111/jcpe.12047.

Koyanagi T, Sakamoto M, Takeuchi Y, Ohkuma M, Izumi Y. Analysis of microbiota associated with peri-implantitis using 16S rRNA gene clone library. 2010;2:5104. doi:10.3402/jom.v2i0.5104.

Kumar PS, Leys EJ, Bryk JM, Martinez FJ, Moeschberger ML, Griffen AL. Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. J Clin Microbiol 2006;44:3665–73. doi:10.1128/JCM.00317-06.

Kumar PS, Mason MR, Brooker MR, O'Brien K. Pyrosequencing reveals unique microbial signatures associated with healthy and failing dental implants. J Clin Periodontol 2012;39:425–33. doi:10.1111/j.1600-051X.2012.01856.x.

Liu Z, Lozupone C, Hamady M, Bushman FD, Knight R. Short pyrosequencing reads suffice for accurate microbial community analysis. Nucleic Acids Res 2007;35:e120. doi:10.1093/nar/gkm541.

Maruyama N, Maruyama F, Takeuchi Y, Aikawa C, Izumi Y, Nakagawa I. Intraindividual variation in core microbiota in peri-implantitis and periodontitis. Sci Rep 2014;4. doi:10.1038/srep06602.

Nossa CW, Oberdorf WE, Yang L, Aas JA, Paster BJ, Desantis TZ, et al. Design of 16S rRNA gene primers for 454 pyrosequencing of the human foregut microbiome. World J Gastroenterol 2010;16:4135–44. doi:10.3748/wjg.v16.i33.4135.

Papaioannou W, Quirynen M, Nys M, van Steenberghe D. The effect of periodontal parameters on the subgingival microbiota around implants. Clin Oral Implants Res 1995;6:197–204.

Sanz-Martin I, Doolittle-Hall J, Teles RP, Patel M, Belibasakis GN, Hämmerle CHF, et al. Exploring the microbiome of healthy and diseased peri-implant sites using Illumina sequencing. J Clin Periodontol 2017;44:1274–84. doi:10.1111/jcpe.12788.

Shiba T, Watanabe T, Kachi H, Koyanagi T, Maruyama N, Murase K, et al. Distinct interacting core taxa in co-occurrence networks enable discrimination of polymicrobial

oral diseases with similar symptoms. Sci Rep 2016;6:30997. doi:10.1038/srep30997.
Sousa V, Nibali L, Spratt D, Dopico J, Mardas N, Petrie A, et al. Peri-implant and periodontal microbiome diversity in aggressive periodontitis patients: a pilot study. Clin Oral Implants Res 2017;28:558–70. doi:10.1111/clr.12834.
Trotha R, Hanck T, König W, König B. Rapid ribosequencing--an effective diagnostic tool for detecting microbial infection. Infection n.d.;29:12–6.
Wang Y, Qian P-Y. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS One 2009;4:e7401. doi:10.1371/journal.pone.0007401.
Yang B, Wang Y, Qian P-Y. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinformatics 2016;17:135. doi:10.1186/s12859-016-0992-y.
Youssef N, Sheik CS, Krumholz LR, Najar FZ, Roe BA, Elshahed MS. Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. Appl Environ Microbiol 2009;75:5227–36. doi:10.1128/AEM.00592-09.
Zheng H, Xu L, Wang Z, Li L, Zhang J, Zhang Q, et al. Subgingival microbiome in patients with healthy and ailing dental implants. Sci Rep 2015;5:10948. doi:10.1038/srep10948.

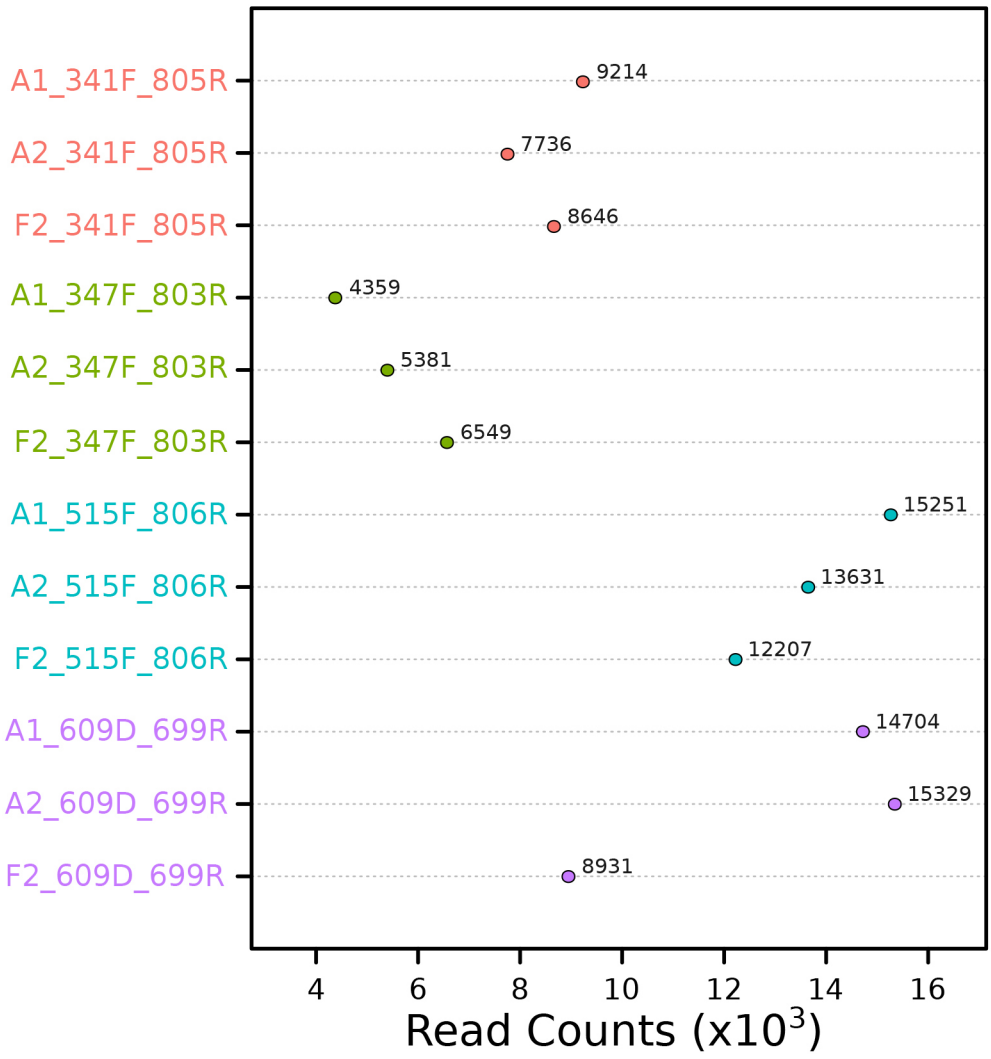


Figure 1. Number of read counts per sample. The samples were grouped in different colors representing each primer pair.

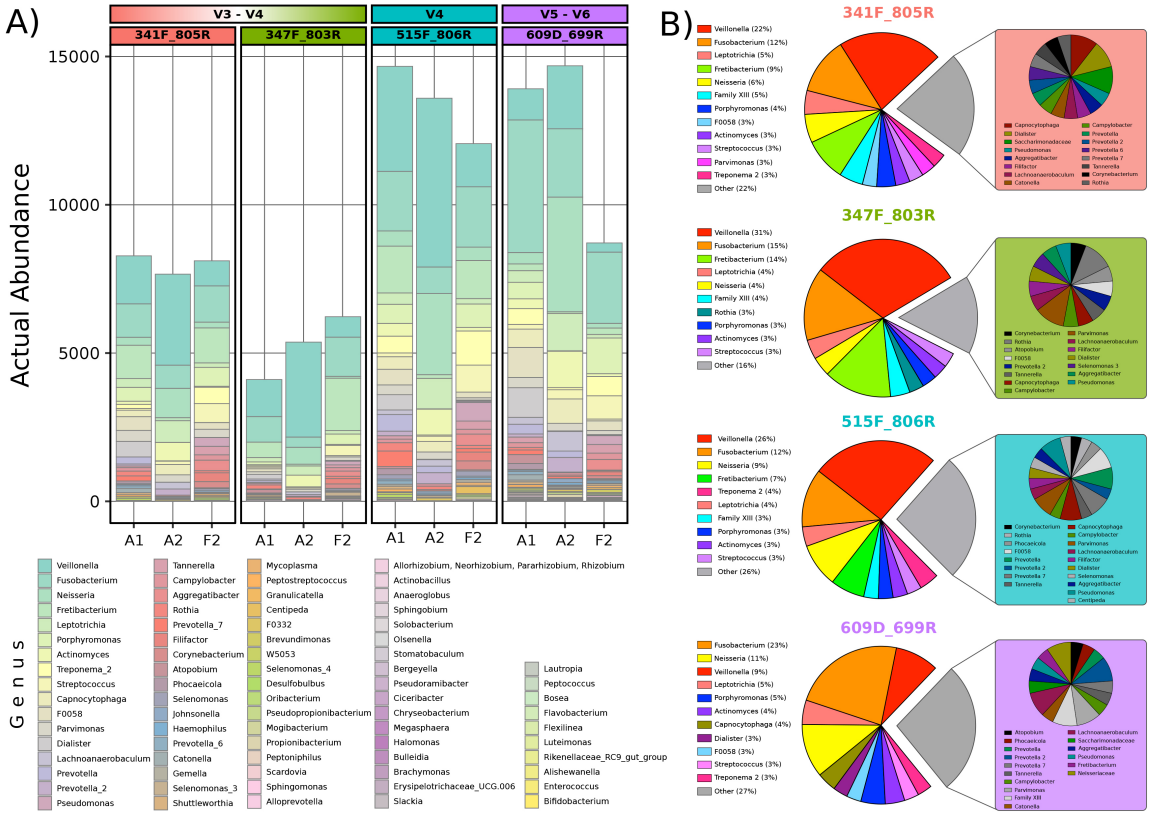


Figure 2. Actual abundance and distribution of genera in each primer pair and V region (2A). Bacterial community structure distribution. Differences in the bacterial community structure distribution in each primer pair were shown at the genus level. Percentage lower than 1% was not included (2B).

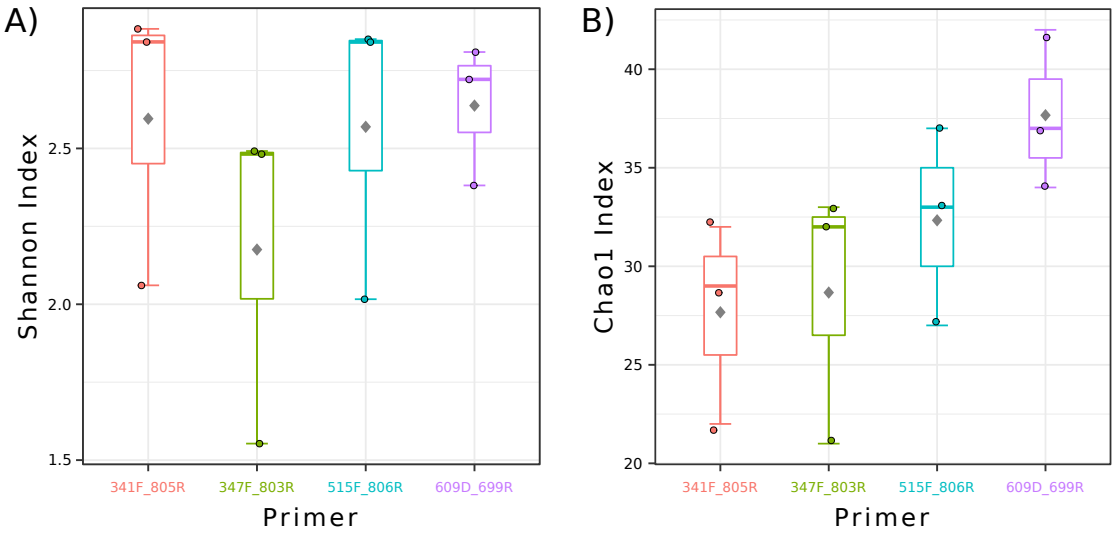


Figure 3. Shannon diversity (A) and Chao1 (B) indices of all primer sets. Data presented as median and quartiles.

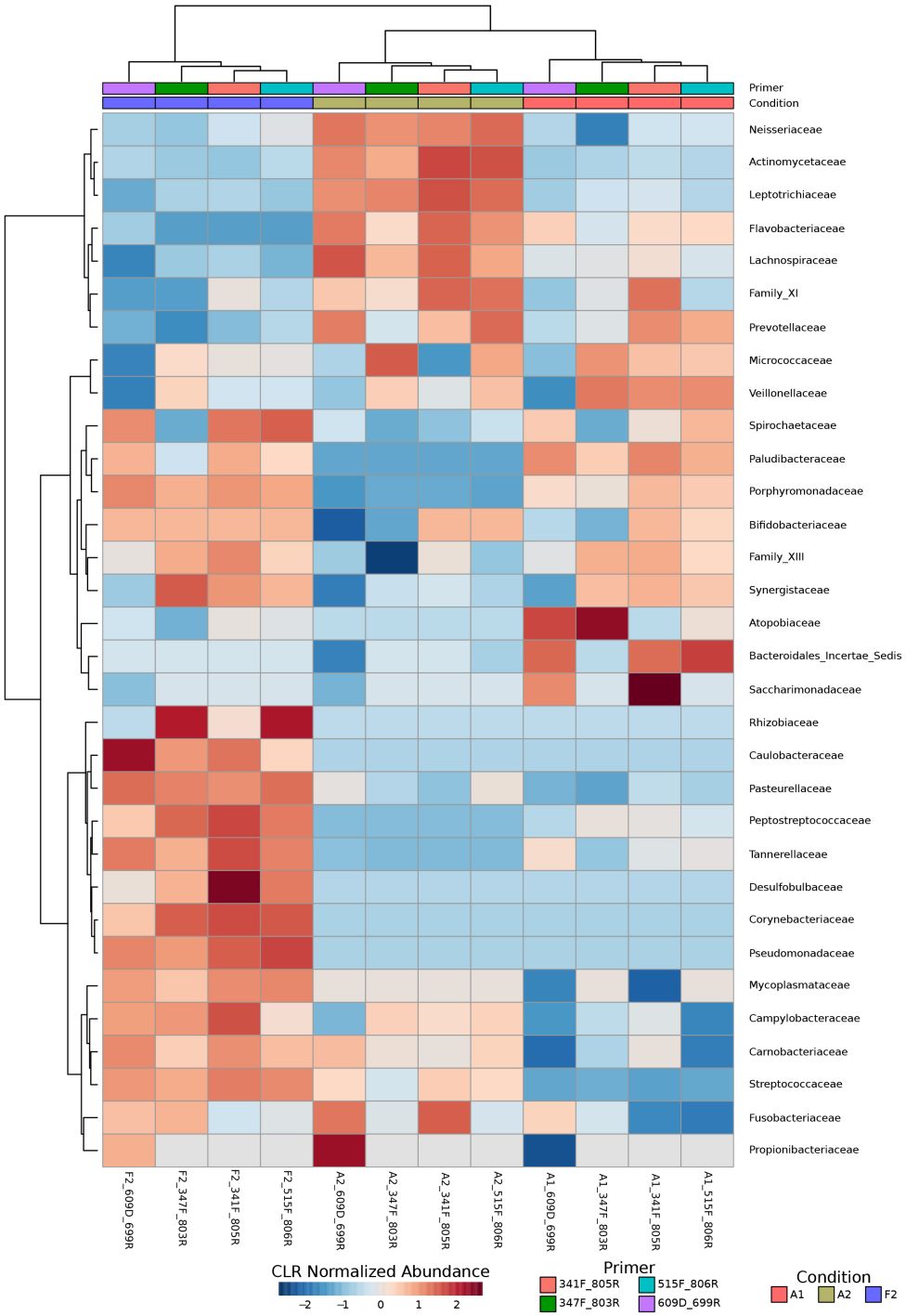


Figure 4. Heatmap showing abundances for each primer and sample at family level, as indicated by the color gradient. Similarity analysis (ANOSIM) showed that samples are different ($P = 0.0002$) but primers are similar (P = 0.49).

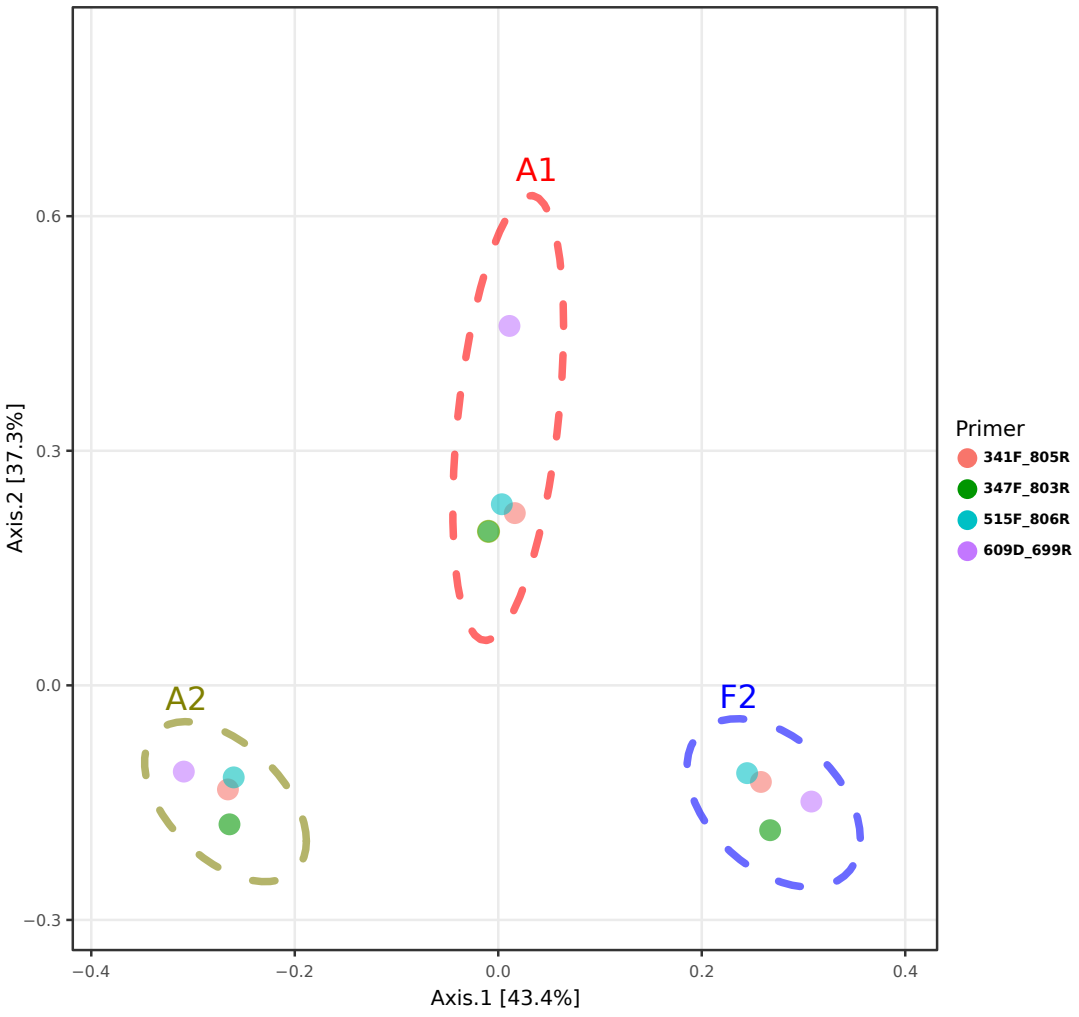
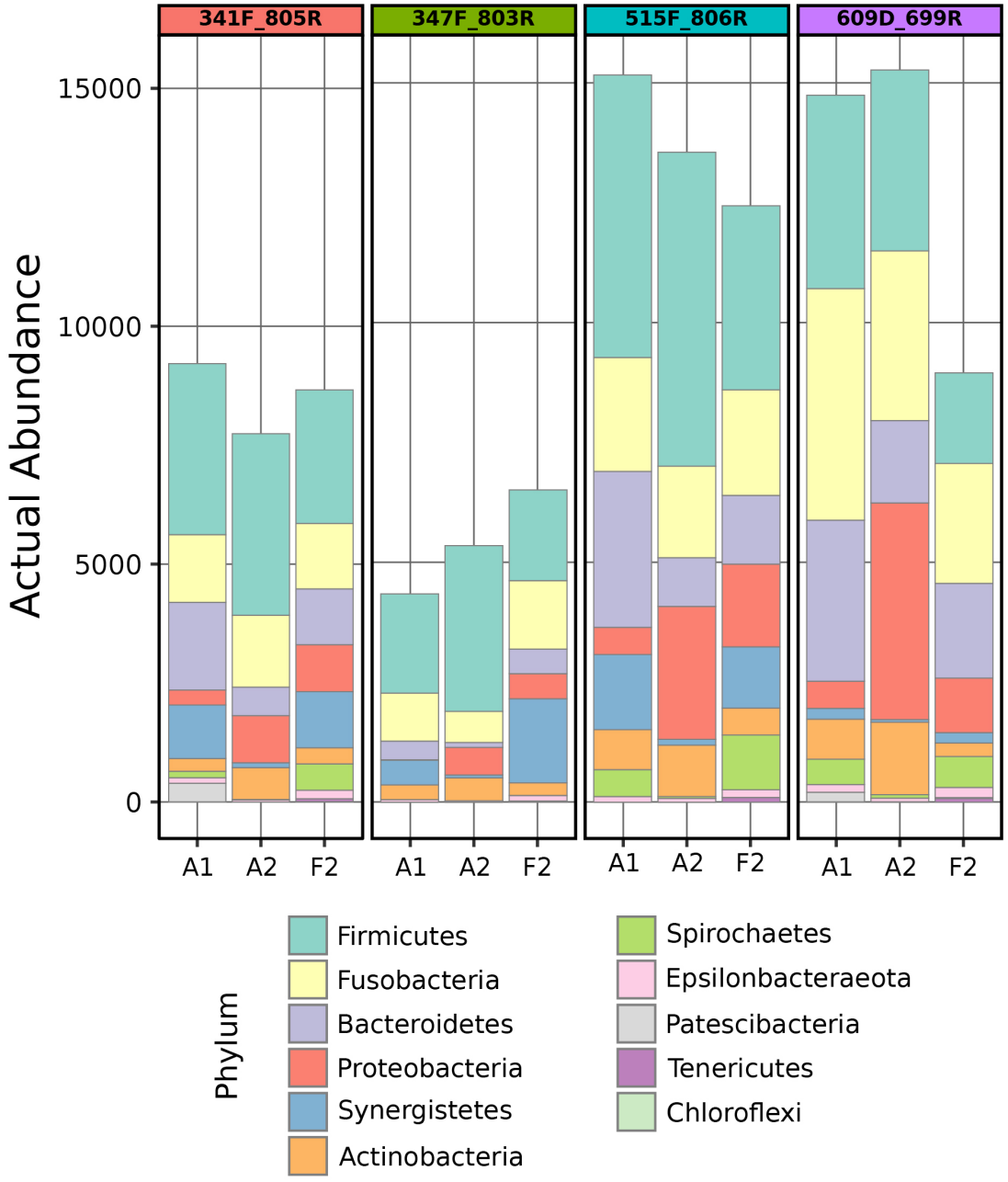
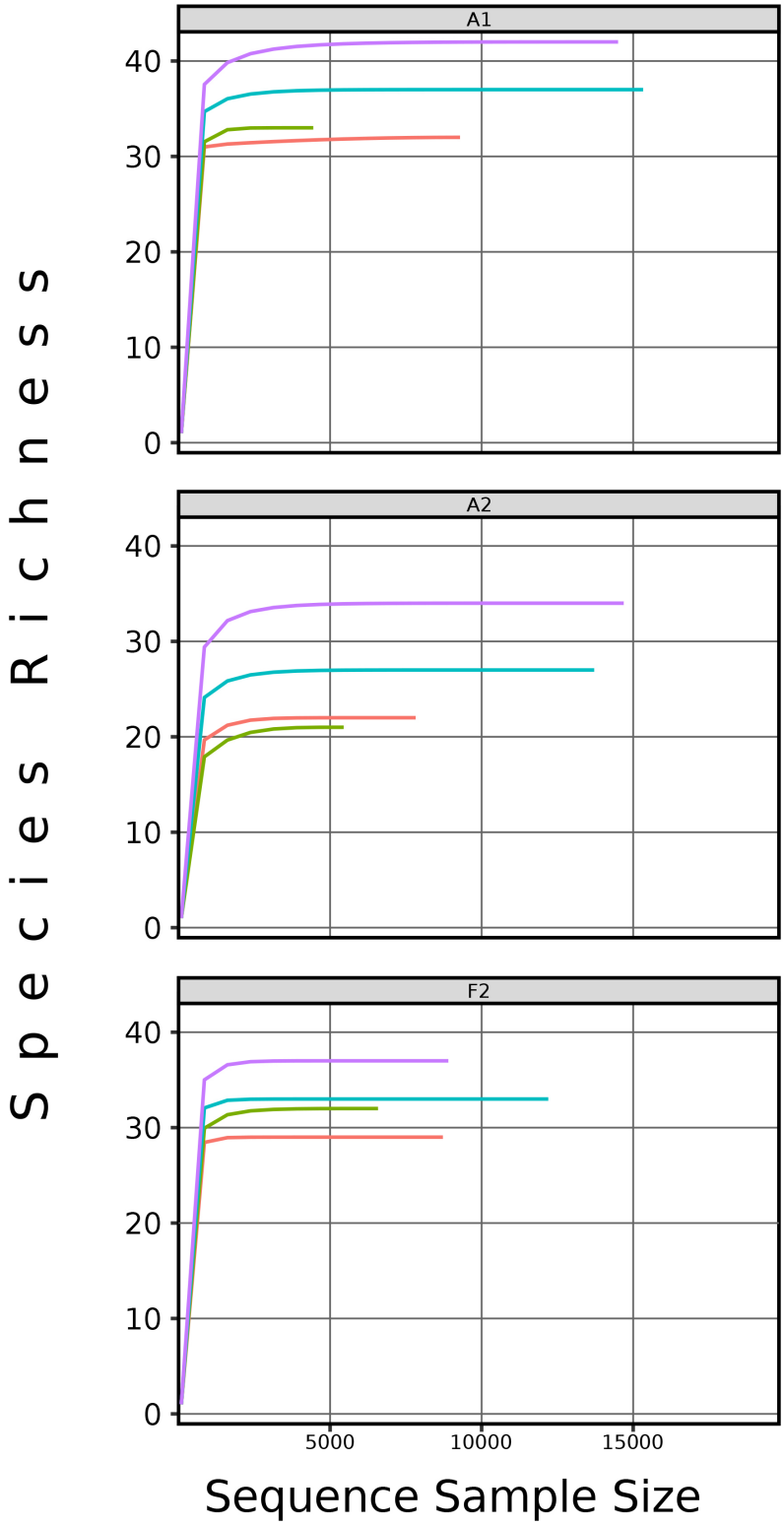


Figure 5. Principal Co-ordinate Analysis (PCoA) on genus, using Bray-Curtis distance. Higher distances between samples than between primers are observed in the first two axis of variation (PC1 - 43.4% and PC2 - 37.3%)



Supplementary figure 1. Actual abundance by phylum in each primer pair



Supplementary figure 2. Rarefaction curves of species richness

Table 1. Average of data quality control of sequences generated by Miseq sequencer after data trimming and quality filtering by a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data (Microbiome Analyst).

Target Region	Primer Pair (common name[a])	Primer sequence	Bacteria Domain Coverage[a]	Base pair counts	Sequence cout	Sequence passed QC pipeline
V3-V4	341 F	5'- CCTACGGGRSGCAGCAG-3	71,7%	31,584,000	52,640	25617
	805 R	5'-GACTACCAGGGTATCTAAT-3				
V3-V4	347 F	5'-GGAGGCAGCAGTRRGGAAT-3	71,8%	24,784,800	41,308	16299
	803 R	5'-CTACCRGGGTATCTAATCC-3				
V4	515 F	5'-GTGCCAGCMGCCGCGGTAA-3'	82,5%	36,132,000	72,264	41390
	806 R	5'- GGACTACHVGGGTAT CTAAT- 3				
V5_V6	609 D	5'-GGMTTAGATACCCBDGTA- 3	92,9%	31,413,500	62,827	39190
	699 R	5'- GGGTYKCGCTCGTTR- 3				

[a] In the SILVA SSU Ref 138 NR database

Capítulo IV. The microbiome of peri-implantitis after treatment: a 12-month randomized trial

No **Capítulo IV** apresentamos o manuscrito preparado para submissão no periódico *The Isme Journal*.

Embora vários estudos tenham demonstrado que a microbiota peri-implantar é complexa e distinta em saúde ou doença, pouco se sabe sobre os processos ecológicos envolvidos na composição da peri-implantite e a mudança que ocorre após a terapia peri-implantar. Além disso, a diferença microbiológica na desinfecção mecânica dos implantes com peri-implantite, com ou sem cirurgia, ainda é pouco estudada. Em vista a estes questionamentos, realizamos um ensaio clínico randomizado para investigar as mudanças dinâmicas longitudinais na microbiota submucosa de pacientes com peri-implantite antes e após o tratamento cirúrgico e não-cirúrgico. Adicionalmente, comparamos o perfil microbiológico peri-implantar dos pacientes que atingiram sucesso após tratamento com o dos pacientes que continuaram com a doença. A diversidade microbiana se mostrou similar tanto ao comparar os tratamentos cirúrgicos e não cirúrgicos da peri-implantite quanto ao comparar os implantes que obtiveram sucesso com os que continuaram com a doença após o tratamento. No entanto, identificamos que as bacterias da microbiota peri-implantar dos pacientes que obtiveram sucesso após o tratamento da peri-implantite estão interagindo mais antes do tratamento, e esse padrão se manteve ao longo de doze meses.

The microbiome of peri-implantitis after treatment: a 12-month randomized trial

Fabiana de Melo[1], Marco Antônio de Bastiani[2], Francisco Montagner[3], Ricardo dos Santos Araujo Costa[1], Tassiane Panta Wagner[1], João Augusto Peixoto de Oliveira[1] Fernando Silva Rios[1], Alex Nogueira Haas[1], Rui Vicente Oppermann[1]

1 Department of Conservative Dentistry, Periodontics unit, School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil
2 Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil
3 Department of Conservative Dentistry, Endodontic unit, School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

*Corresponding author:

Dr. Rui Vicente Oppermann

Rua Ramiro Barcelos, 2492 – Rio Branco, Porto Alegre - RS,

Zip-code: 90035-004 Brazil

Phone: +55 51 3308 5509

Email: ruioppermann@gmail.com

ABSTRACT

Aim: to characterize changes in the peri-implant microbiota after surgical (ST) and non-surgical treatments (NST) of peri-implantitis (PI) and to compare implants that were successful (SU) and those that were unsuccessful (UN) after treatment.

Material and Methods: 21 individuals with one implant with probing depth (PPD) ≥5mm, bleeding on probing (BOP) and radiographic bone loss (BL) ≥2mm were included. In the NST (10 implants), submucosal debridement was performed under local anesthesia. In the ST (11 implants), a mucoperiosteal flap was raised and the surfaces were decontaminated also only by debridement with curettes as made in the NST. Submucosal samples were obtained from the deepest site of each implant at baseline, 3, 6 and 12 months. 16S rDNA genes were amplified and sequenced with the Miseq Illumina instrument.

Results: There were no significant differences between NST and ST groups in clinical parameters and microbial diversity in any of the time points. More than 50% of the genera found were considered rare (<0.1%). Alfa and beta diversity indexes in successful (BOP-) and unsuccessful (BOP+) implants after treatment showed a similar pattern. Hierarchical cluster dendrogram showed that the majority of individuals presented their samples clustered close together. Patients that achieved success presented stronger and higher number of co-occurrence network correlations than patients that remained with disease, and this pattern was maintained throughout the study.

Conclusions: The submucosal microbial communities' profile and diversity were similar before and after NST or ST. No treatment showed superior over the other. Microbial composition and diversity were also similar between successful and unsuccessful PI post treatment. The degree of interaction and the strength in which the bacteria are interacting before treatment can suggest how patients will respond to treatment, opening new perspectives for understanding the failure of implants.

Keywords: Peri-implant diseases; Peri-implantitis treatment; Surgical treatment; Non-surgical treatment; Microbial; High-throughput Sequencing

INTRODUCTION

Dental implant rehabilitation has shown a high degree of patient satisfaction, with the use of predictable surgical techniques and long-term clinical results. However, complications involving implants have also been reported (Balshi, Wolfinger, Stein, & Balshi, 2015; Donati, Ekestubbe, Lindhe, & Wennström, 2016). Peri-implantitis is a bacterial infection that occurs in tissues surrounding dental implants, characterized by inflammation and loss of supporting bone (Berglundh et al., 2018). Thereby, with the prevalence occurring in ~22% of patients, the interest in evaluating the composition of the submucosal microbiota associated with health and disease has increased (Derks & Tomasi, 2015).

Although several studies have shown that the peri-implant microbiota is complex and distinct in health or disease (Koyanagi, Sakamoto, Takeuchi, Ohkuma, & Izumi, 2010; Kumar, Mason, Brooker, & O'Brien, 2012; Sanz-Martin et al., 2017; Sousa et al., 2017; Tsigarida, Dabdoub, Nagaraja, & Kumar, 2015; Zheng et al., 2015), little is known about the ecological processes that govern the composition of the microbiota in peri-implantitis and the shift that occurs after its treatment. In addition, it is still unknown whether different treatment approaches of peri-implantitis will present microbiological differences and diversity in the short and long term based on 16S rDNA gene sequencing.

Monitoring the changes in the microbiome is a promising potential new application in disease diagnosis and prognosis since changes in the microbiome contribute to the pathogenesis of many diseases and reflect the health or disease state of the host (Cho & Blaser, 2012; Huang et al., 2014; Pflughoeft & Versalovic, 2012). Therefore, information on the composition and formation processes of the peri-implant microbiota can be used to develop effective strategies and monitoring protocols for peri-implant therapy. In this sense, randomized clinical trials (RCT) comparing two simple treatments of peri-implantitis including only mechanical debridement evaluating the shift in the peri-implant microbiota overtime are of outmost importance. Therefore, to better understand the role of bacterial communities in peri-implantitis, this study aimed to compare dynamic changes in the submucosal microbiota of implants with peri-implantitis treated with surgical and non-surgical debridement. Also, the submucosal microbiota of

those implants that were successful and those that were unsuccessful after treatment were compared.

MATERIALS AND METHODS

This study was designed as a two-center, parallel-design, single-blinded, randomized controlled trial. The research protocol was registered in www.clinicaltrials.gov (NCT02241577). Although participants were recruited in two centers, microbiological samples were available only at the main research facility (Faculty of Dentistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil). Clinical and radiographic variables comprised the primary outcomes of the trial and were previously reported (Wagner et al. 2020, *submitted*). This study is a report of the peri-implant microbiome outcome after treatment.

Individuals presenting one implant with peri-implantitis defined as probing pocket depth (PPD) ≥5 mm with bleeding on probing (BOP) and radiographic bone loss ≥2 mm (Mombelli, Müller, & Cionca, 2012) were considered eligible. If an individual had more than one implant with peri-implantitis, the implant with the deepest pocket depth (PPD) was included in the analyses of this study. In addition to the diagnosis of peri-implantitis, the participant should present good general health, negative medical history of any systemic condition that would require the use of antimicrobial prophylaxis, negative history of antibiotic therapy in the last six months preceding the study, and do not be pregnant. Patients with past history of periodontitis should have received periodontal treatment at least 3 months before being included in the study.

During the course of the study, the following exclusion criteria were applied: use of antibiotics for other infections, and development of any systemic condition that could interfere with peri-implantitis treatment.

Interventions

Two experienced periodontists (RSAC, JAPO) treated the individuals included in this study. All patients received an initial phase of up to 4 sessions comprising

supragingival scaling, professional supragingival biofilm removal and personalized oral hygiene instructions and motivation.

Before the start of non-surgical and surgical interventions, all crowns that were screw-retained were removed to facilitate the access. Crowns that were cemented were maintained during the intervention, Interventions were performed under local anesthesia. Non-surgical treatment comprised the removal of submucosal biofilm and/or calculus adhered to the implant with Teflon* curettes. When the operator judged that calculus could remain at the implant surface due to hardness or lack of cutting ability of the Teflon curette, stainless-steel Mini-Five curettes† were used to complement debridement. Surgical treatment consisted of biofilm and/or calculus removal, following the raising of a full-thickness flap with relaxing incisions for a complete view of all implant surfaces preserving soft tissue. No ressective bone surgery or implantoplasty were performed. Removal of calculus and/or submucosal biofilm adhered to the implant was also performed manually with curettes as described previously. After removal of the submucosal biofilm, the implant surfaces were irrigated during 1 minute with saline solution. The flap was repositioned with single sutures with 4-0 silk suture threads.

Patients were instructed to rinse with 0.12% chlorhexidine mouthwashes, twice daily, during seven days after the intervention in both groups. Acetaminophen 750mg, every 4 hours, was prescribed in case of pain.

After the interventions, individuals were followed by weekly sessions over the first month. Thereafter, maintenance sessions were made each month during the first 3 months and from there on every 3 months. In these sessions, supragingival biofilm control was checked, and professional biofilm removal at the implant and teeth sites was performed, together with oral hygiene reinforcement if necessary.

Interviews and clinical examinations

At baseline, participants were interviewed using a structured questionnaire containing questions regarding demographic variables, oral hygiene habits, dental

* HuFriedy, Chicago, USA
† HuFriedy, Chicago, USA

treatments and behavioral factors. Also, all present teeth were examined to register visible plaque, PPD, clinical attachment loss (CAL) and BOP.

Implants were examined at six sites (distobuccal, buccal, mesiobuccal, distolingual, lingual, mesiolingual) longitudinally at baseline, 3, 6 and 12 months after treatment. A manual periodontal probe[‡] was used to register PPD (from the mucosal margin to the bottom of the deepest portion of the peri-implant sulcus) and BOP (present if bleeding was evident within 30 sec after probing). Moreover, visible plaque was also recorded.

Biofilm Sampling and DNA Isolation

Prior to sampling, the supramucosal areas of the implant and suprastructure were isolated using cotton rolls, air-dried and the supramucosal biofilm removed. At baseline, submucosal peri-implant biofilm samples were obtained with four sterile paper points (Dentsply®) inserted for 30 seconds into the deepest PPD of each implant. The collected samples were pooled and stored dry in a 1.5 ml single Eppendorf tube at -80ºC until analysis.

DNA was isolated with a Qiagen DNA MiniAmp kit[§] using the tissue protocol according to the manufacturer's instructions. DNA concentration was determined with NanoDrop[**].

16S rRNA gene Sequencing

Samples were sent to McGill University and Genome Quebec Innovation Center (Montreal, Quebec, Canada) were polymerase chain reaction (PCR) and sample barcoding were performed by amplifying the V5-V6 region of the 16S rDNA gene with selected sets of universal primers: forward primer 609D (GGMTTAGATACCCBDGTA) and reverse primer 699R (GGGTYKCGCTCGTTR) (Alm, Oerther, Larsen, Stahl, & Raskin, 1996). Briefly, a first step PCR with broad-range universal primers and 23 cycles of amplification was used to amplify the 16S rRNA gene. Then, a second step PCR was performed to introduce Illumina adaptor sequences Fluidigm (INDEX) into the DNA.

[‡] HuFriedy, PCP15-SE, Chicago, USA
[§] Qiagen, Valencia, CA
[**] Thermo Scientific

Quantification was determined using the Picogreen dsDNA quantification assay (Biomek FX, Kit: Quant-iT™ PicoGreen® dsDNA Assay Kit). PCR samples were pooled and then purified using AMPure beads. For quality control, LibQ was performed to measure the size and concentration of the pool to be sequenced. DNA sequences of 2 X 250 bp (PE250) were generated with a MiSeq (Illumina, Inc., San Diego, CA, USA) instrument. Assembled reads were filtered for quality; all reads having average quality below 30 or having more than two ambiguous bases (Ns) and three nucleotides below quality 20 were discarded. Afterwards, reads were clustered using an in-house clustering algorithm. Briefly, reads were clustered at 100% identity and, then, clustered/denoized at 99% identity (dnaclust 3). Clusters having abundance lower than 3 were discarded. Remaining clusters were then scanned for chimeras with UCHIME denovo and UCHIME reference (Edgar et al. 2011) (using the gold reference from the Microbiome Utilities Portal of the Broad Institute) and clustered at 97% (dnaclust 3) to form the final clusters/operational taxonomic units (OTUs). OTUs were classified with the Ribosomal Database Project (RDP) classifier tool (Wang et al. 2007) using an in-house training set containing the complete Greengenes database supplemented with eukaryotic sequences from the Silva databases (http://www.arb-silva.de). Diversity metrics were obtained by aligning OTU sequences on a Greengenes core reference alignment (DeSantis et al. 2006) using the PyNAST aligner (Caporaso et al. 2010a,b). Alignments were filtered to keep only the hypervariable region part of the alignment. Alpha (Chao1, Shannon, observed species) and beta (weighted, unweighted UniFrac and Bray–Curtis dissimilarity) diversity metrics, taxonomic classifications and clustering were then computed using the Microbiome Analyst tool (Chong, Liu, Zhou, & Xia, 2020; Dhariwal et al., 2017).

Randomization, allocation concealment and blinding

Individuals were randomly assigned into the two treatment groups. Stratified randomization according to smoking status was conducted. Participants were stratified in smokers and non-smokers for at least one year. Those who quit less than a year before the start of the study were considered smokers.

A computerized random sequence of numbers was generated for randomization. A researcher not involved in the treatments and outcomes assessment performed the

entire sample randomization procedure. Individuals were randomized immediately prior to the start of peri-implant interventions (NST and ST).

A researcher not involved in the study was responsible for the concealment of allocation. Subjects were identified by numbers, which were stored in opaque envelopes and sealed with the randomization sequence. The periodontists who performed the periodontal examination and the researcher reading the bacterial DNA counts were blinded to the experimental group of which the participants were allocated.

Reliability

Two calibrated examiners (FSR, TPW) performed the clinical examinations. The same initial examiner continued to evaluate the same patient throughout the whole study time-points. Previously to the start of the study, the examiners performed intra- and inter-examiner duplicate examinations in a total of 16 patients. They previously participated in other studies as main examiners having large experience in periodontal assessment (Wagner et al., 2016). Weighted (±1mm) kappa values for PPD ranged between 0.78 and 0.89.

Ethical aspects

This study was approved by the Research Ethics Committee from the Federal University of Rio Grande do Sul (666.930) and was conducted in accordance with the guidelines of the world Medical Association Declaration of Helsinki. All participants provided written informed consent before entering the study.

Data analysis

The primary outcome variable was the difference in peri-implant bacterial composition before and after NST or ST. OTU data was evaluated using the Microbiome Analyst web tool and package for R statistical environment (Chong et al., 2020; Dhariwal et al., 2017), which was designed for analysis of 16S rRNA marker gene survey data. Firstly, OTU count data, sample metadata and taxonomy tables were supplied with SILVA taxonomy labels. Afterwards, the data was submitted to centered log ratio (CLR) normalization prior to further marker gene analyses. The data was firstly submitted to log

transformation. Then, two-way analysis of variance with repeated measures was conducted (mixed-effect analysis correcting for absent data), where variable one was defined as the "group" or "outcome" and variable two was defined as "time". Interaction was also evaluated (variable one *vs* variable two). Multiple comparisons correction was performed using Tukey's method. Statistical significance was set as $p < 0.05$.

Co-occurrence networks were constructed using CLR normalized taxonomic abundance values and Pearson's product moment correlation test of pairwise taxa. Final networks contain only significant correlations (FDR adjusted pvalue < 0.05) above an absolute correlation coefficient threshold of 0.65 ($|R| > 0.65$). Network visualization was built using RedeR package [PMID22531049].

A secondary strategy was also applied after analyses of clinical outcomes, which revealed no significant differences between groups (Wagner et al 2020, *submitted*). Therefore, the whole sample was analyzed independently of the treatment performed, dividing implants into those with a clinically successful outcome and those unsuccessful. Successful implants were defined as those with negative BOP and unsuccessful implants as those with positive BOP after 12 months.

RESULTS

Surgical versus non-surgical treatments

Clinical outcomes

Twenty-one patients were enrolled in this longitudinal study (NST=10 and ST=11). All patients were collected at baseline and 3 months (21 samples in each time point). Four patients were not collected at 6 months and 12 months (17 samples in each time point) due to logistical issues. Thus, a total of 76 samples were obtained from these individuals. There were no significant differences between groups in clinical and demographics characteristics at baseline. The periodontal status of participants was stable, with low levels of plaque and bleeding, and with shallow probing depths. Clinical peri-implant parameters presented no significant difference between groups at baseline. After treatment, PPD reduced significantly in both groups over time. The percentage of sites with visible plaque reduced in both groups after the first 3 months and remained low (NST=0% and ST=9.09%) until 12 months. The percentage of sites with BOP also

reduced significantly in both groups. There were no significant differences between NST and ST groups in PPD, plaque and BOP in any of the time points. The percentage of implants successfully treated (negative BOP) after 12 months was 40% (95%CI 15.21-64.7) and 45.4% (95%CI 16.3-63.6) in the non-surgical and surgical groups (p=0.98), respectively. All the information presented above is depicted in table 1.

Microbial composition

A total of 8415844 sequences were used in the analysis. Average of data quality control of sequences generated by Miseq sequencer after data trimming and quality filtering is presented in Table S1. All reads were trimmed to a fix length (250 pb) and clustering these sequences into OTUs at a 3% genetic distance resulted in 2496 different OTUs at the genus level. A summary of Operational Taxonomic Units (OTUs) identified in peri-implant sites considering all taxonomic levels are represented in Table S2 (SILVA SSU database, 97% sequence identity). The relative abundance of OTUs for Phylum considering at least 1% of microbiome presence (abundant phylotype) is detailed in Table 2. Of 18 phyla recovered, seven were considered predominant taxa (98.15% of the total reads detected), belonging to Actinobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Proteobacteria, Spirochaetes and Patescibacteria (Figure S1). *Firmicutes* presented higher counts in all samples except for 6M and 12M of NS group, which presented *Proteobacteria* with higher counts. There was no statistical difference considering the predominant Phyla relative abundance between groups.

A total of 236 different genera were originated from all PI biofilm samples analyzed (Table S3). The summary of total genus abundance observed on PI microbial communities from all groups are shown in Table S4. Table 3 presents the relative abundance of OTUs for abundant genera in each time point. Considering abundant genera contributing with at least 0.1% of the microbiome, 87 genera could be recovered. The higher relative abundance (>10%) was found for *Fusobacterium* and*, Streptococcus*. Ten genera were higher than 1% relative abundance for all groups over time (*Fusobacterium, Haemophilus, Streptococcus, Porphyromonas, Veillonella, Parvimonas, Neisseria, Prevotella, Prevotella_7,* and *Leptotrichia*). After treatment, most taxa decreased proportion over the course of the study, including well-recognized pathogenic bacteria

such as *Porphyromonas, Tannerella*, or *Filifactor*. *Prevotella* and *Mogibacteria*, two common genus found in PI sites, decreased after NST and increased after ST. No interaction was found considering the genus relative abundance. However, Two-way ANOVA revealed differences (time or groups effects) in few genera. More specifically, Time effect was observed in *Fretibacterium* (F (2.130, 29.82) = 4.412, p = 0.0192) and group effect was observed in *Atopobium* (F (1, 52) = 7.114, p = 0.0102), *Veillonella* (F (1, 19) = 5.824, p = 0.0261), *Actinomyces* (F (1, 19) = 5.315, p = 0.0326) and *Selenomonas_3* (F (1, 19) = 9.465, p = 0.0062). Two-way ANOVA also revealed time effect (F (2.002, 18.68) = 3.691, p = 0.0445) and group effect (F (1, 28) = 8.627, p = 0.0066) differences in *Pseudomonas*. Figure 1 shows differences in relative abundance and distribution of genera in each group over time only for those showing higher or equal than 1% of abundance.

Of all 236 genera, 172 were less abundant than 0.1%. Unclassified sequences contributed with 4.85% of genera of all samples. The proportion of genus richness, unclassified OTUs, shared and exclusive genera observed into each site and time point are shown in Table 4. The exclusive genera identified by sites from peri-implant biofilm presented relative abundance lower than 0.08%.

Alpha Diversity Analysis

Richness estimator and alpha diversity index (Chao1 and Shannon) and the number of observed OTUs were calculated before and after each treatment at genus level and are shown in Figure 2. Neither metrics showed statistical difference between NST and ST groups in time effect, group effect or interaction using Two way ANOVA. Table S5 presents a summary of observed genus richness, considering the total number of OTUs observed.

Successful versus unsuccessful implants

Clinical outcomes

Significant differences were observed between successful and unsuccessful implants for PPD and BOP (Table 5). The overall number of implants that showed a successful treatment was 9, representing 42.8% (95%CI 22.33-63.39).

Shift in the levels of disease- and health-associated taxa after treatment

Shared genera present in all subjects (100% core threshold) were defined as the core microbiome (Xiao, Ran, Huang, & Liang, 2016). A total of 9 (3.81%) genera were shared among PI biofilm microbiome at baseline in all subjects included in the study, representing a common core microbiome of PI plaques and accounted for an average of 45.89% of reads per sample. One year after treatment, 14 genera were found in all patients who have successfully treated implant (absent BOP), and 9 genera were found in all patients who were unsuccessful after treatment (persistent BOP) (Figure 3). Most abundant genera found in PI baseline, successful and unsuccessful post treatment is represented in Figure 4, where we can observe differences in relative abundance and distribution in the levels of disease- and health-associated genera in each group over time only for those showing higher or equal than 1% of abundance. No interaction was found considering the genus relative abundance. However, Two-way ANOVA revealed differences (time or outcome effects) in few genera. More specifically, Time effect was observed in *Fretibacterium* ($F(2.696, 21.57) = 4.632$, $p = 0.0140$) and outcome effect was observed in *Fusobacterium* ($F(1, 19) = 4.578$, $p = 0.0456$), *Prevotella* ($F(1, 19) = 4.852$, $p = 0.0402$), and *Parvimonas* ($F(1, 19) = 4.763$, $p = 0.0418$) (Table 6). However, very abundant taxa (≥1%) represented less than 20% of total genus found in each sample. While, more than 49% of the genera found were considered rare (<0.1%) (Table S6) (Sogin et al., 2006). Exclusive genera identified in PI baseline, successful and unsuccessful post treatment biofilms can be observed in Table S7.

Figure 5 presents the further alpha diversity analysis that was made comparing post treatment successful versus unsuccessful patients through Chao 1 and Shannon index and number of observed OTUs for genus taxonomic level. Neither metrics showed statistical difference between groups in time effect, group effect or interaction (Two way ANOVA; $p > 0.05$).

Beta diversity analysis

Principal Coordinate Analysis (PCoA) was performed using Weighted Unifrac distance to evaluate the similarities in the bacterial community structures between the implants that were successfully treated (yellow) and implants that continued with disease (purple) (Figure 6). Both groups do not seem to generate clustering of samples, suggesting that the bacterial structures in all individuals were similar. The percentage placed next to each coordinate on its respective axis represents the contribution to variation conferred by each of the main coordinates in the spatial positioning of each sample. The ANOSIM non-parametric test for similarity analysis showed that successful and unsuccessful post treatment samples are similar ($p = 0.13$).

Hierarchical cluster dendrogram by pairwise Bray-Curtis dissimilarity was conducted to observe the dissimilarities or similarities between submucosal biofilm communities of each sample (Figure 7). Three major clusters were observed and more than 60% of samples were grouped by subject. Phylogenetic tree at taxonomic family level can be observed in supplementary figure (S2).

Co-Occurrence Network Analysis

At the taxonomic genus level, successful implants showed an increased number of significant positively and negatively correlations among genus in all time points than unsuccessful implants (Figure 8). Interestingly, this increased connectivity is already present before treatment was performed (Baseline). Successful group presented a decrease in correlations (edges) at 3M (356 to 236), increasing at 6M (236 to 356) and decreasing again at 12M (356 to 284). While, unsuccessful group presented a decrease in correlations (edges) at 3M (44 to 16), and increased at 6M (16 to 84) and at 12M (84 to 98).

DISCUSSION

Assessing the relationships between microbial communities is essential for a better understanding of the etiology of infectious-inflammatory diseases; therefore, there has been a continuing interest in evaluating the composition and assembly of the submucosal microbiota associated with peri-implant health and disease (de Melo, Milanesi, Angst, & Oppermann, 2020; Padial-Molina, López-Martínez, O'Valle, &

Galindo-Moreno, 2016; Rakic, Grusovin, & Canullo, 2016). In this sense, the emergence of advanced techniques such as high-throughput sequencing has provided new insights into the compositions and structures of microbial communities. In the present investigation, using 16S rRNA gene sequencing, we compared the host-microbiome composition profile and diversity of peri-implantitis before and after NST or ST and compared those implants that achieved treatment success to those remaining with peri-implantitis after treatment.

Previous studies that sought to evaluate and compare the composition of the microbiota in patients diagnosed with PI before and after different treatments used analysis strategies based on culture or PCR methodologies revealing a decrease in periodontal pathogenic species after treatment (Y C M de Waal, Raghoebar, Meijer, Winkel, & van Winkelhoff, 2015; Yvonne C.M. De Waal et al., 2013; Galofré, Palao, Vicario, Nart, & Violant, 2018; Isehed et al., 2016). A recent study, using pyrosequencing technique to evaluate the microbial composition of PI before and after 1 month of non-surgical treatment, revealed that NST does not significantly affect the microbial community (Nie et al., 2020). However, to the best of authors' knowledge, this is the first randomized controlled trial comparing microbial profile of surgical and non-surgical mechanical disinfection without adjuvants for the treatment of peri-implantitis and also comparing microbial profile of successful and unsuccessful post treatment.

Regarding the clinical measures, this study demonstrated that NST and ST without additional adjuvant approaches for implant disinfection were able to reduce clinical inflammation, i.e. PPD and BOP, around implants with peri-implantitis in a similar way. Despite that, the percentage of implants becoming healthy (absent BOP) after 12 months still may be considered small (<50%). Previous studies demonstrated similar findings for clinical outcomes. For instance, Machtei *et al.,* using multiple applications of chlorhexidine chips for treating peri-implantitis, presented PPD reduction up to 2.19 after 6 months (Machtei et al., 2012). In the present investigation PPD changed from 6.30mm to 4 mm (D=2.3mm) in NST and 5.72mm to 3.09mm (D=1.82mm) in ST. As early as 3 months a significant reduction in PPD has already been seen in both NST and ST groups. Additionally, previous studies demonstrated BOP reductions averaging from 25% (Y C M de Waal et al., 2015; Yvonne C.M. De Waal et al., 2013; Hallström,

Persson, Lindgren, Olofsson, & Renvert, 2012; John, Sahm, Becker, & Schwarz, 2015) to 50-60% (Bassetti et al., 2014; Machtei et al., 2012; Ramanauskaite, Daugela, Faria de Almeida, & Saulacic, 2016), corroborating our findings that reduced to 60% and 54.5% (NST and ST respectively). Since patients who have poor plaque control and do not attend regular maintenance therapy are at higher risk of developing peri-implantitis (Costa et al., 2012; Dreyer et al., 2018; Monje et al., 2016), we performed a maintenance sessions with professional biofilm removal and oral hygiene instructions for participants over the 12 months follow-up and achieved low levels of visible plaque during the study. Similarly as clinical parameters, microbiological parameters, measured through alpha and beta (data not shown) diversity indices, did not show significant statistical differences between NST and ST. In addition, there was also no difference in diversity and richness at different time points after treatments. Thereby, our analysis focused on mean differences between successful and unsuccessful outcome after treatment, evaluating the clinical and microbiological parameters before and after 3, 6 and 12 months of peri-implantitis treatments.

Distribution patterns of phyla and genera in PI sites in this study were similar to the findings in other studies (Kumar et al., 2012; Sanz-Martin et al., 2017; Sousa et al., 2017; Yu et al., 2019; Zheng et al., 2015). All aforementioned studies compared peri-implant health and peri-implantitis, whereas our study focused on peri-implantitis lesions before and after treatment. Interestingly, the taxa identified in our analyses as associated with 12 months post treatment success, do overlap with those identified as associated with health in case-control studies, e.g., *Streptococcus, Haemophilus, Neisseria* and Rothia. Despite the presence of 18 phyla, only *Firmicutes, Proteobacteria, Fusobacteria* and *Bacteroidetes* were identified with a considerable abundance (higher than 10%) in peri-implant niches, suggesting a highly selective environment. Taken together, the following genera were found to be associated with submucosal plaque of PI: *Fusobacterium, Streptococcus, Haemophilus, Porphyromonas, Veillonella, Neisseria, Parvimonas, Prevotella, Prevotella_7, Atopobium, Dialister, Leptotrichia, Tannerella, Rothia, Aggregatibacter, Treponema_2, Fretibacterium, Selenomonas,* and *Actinomyces.* The genera that were found associated with submucosal plaque in post treatment health implant included: *Streptococcus, Neisseria, Haemophilus, Rothia, Lautropia, Veillonella,*

Fusobacterium, Granulicatella, Actinomyces, Prevotella_7, Parvimonas, Atopobium, Prevotella, Acinetobacter, Alloprevotella, Dialister, Capnocytophaga, Leptotrichia, Porphyromonas, and *Gemella.* We identify that several species were associated with both PI and post treatment health, suggesting distinct pathogenic potentials of bacteria of the same genera.

Comparing PI implants at baseline with successful and unsuccessful post treatment implants, remarkable variations of microbiome compositions in the levels of disease- and health-associated genera were observed and may correlate with treatment outcomes. For example, the level of the disease-associated taxa such as *Fusobacterium, Porphyromonas,* and *Prevotella* decreased in post treatment success subjects and was lower than in post treatment unsuccessful subjects. On the other hand, the levels of health- associated taxa such as *Haemophilus, Neisseria, Rothia,* and *Streptococcus* was higher in post treatment successful than in post treatment unsuccessful or PI baseline, as expected for a site with clinical improvement after treatment. In fact, *Fusobacterium* and *Prevotella* presented a significant outcome effect.

Previous studies commonly focused on the presence or absence of the bacteria that were implicated in the disease, the so-called 'red complex' bacteria (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*) or only on those taxa present in high abundance (Lafaurie et al., 2017). However, recent studies, using new gene-based approaches, have shown that the emphasis on specific bacteria or only on those in high abundance does not capture all the variability in the diseased sites (Griffen et al., 2012; Liu et al., 2012). Interactions between bacteria and hosts have been identified on a large scale and virulence has been described as a response to this interaction (Casadevall & Pirofski, 2003). In addition, less resilient microorganisms, frequently detected in low abundance, contribute to the high diversity and inter-individual variability of the oral microbiome (Zaura, Keijser, Huse, & Crielaard, 2009). In fact, in our study, we observed few genera with high abundance while most genera were present in very low abundance. The significance of this long tail distribution is still unknown, however, it is suggested to be a reservoir of species that can modulate its abundance over time, playing a protective role and providing functional redundancy where multiple species of a variety of taxonomic groups can share similar or identical roles in ecosystem functionality (Zaura et

al., 2009). This functional redundancy may explain the variability observed from individual to individual in human microbiomes, however the extent of this interpersonal variability in microbial composition in the same niche or in varied niches is largely unknown, but these factors probably contribute directly to the disparate results obtained by several association studies that examine the peri-implant microbiota (Gao, Zhou, Sun, Li, & Zhou, 2018; Kumar et al., 2012; Sanz-Martin et al., 2017; Sousa et al., 2017; Yu et al., 2019; Zheng et al., 2015).

The compositions of the peri-implant disease microbiota at baseline were reasonably similar among samples. A common set of core oral microbiota was found consistently across individuals. The respective compositions of the core species identified in this cohort share considerable similarity with those previously identified in several other high-throughput sequencing-based investigations of peri-implant diseases, which included especially *Streptococcus*, *Fusobacterium* and *Veillonella* (Shiba et al., 2016; Tsigarida et al., 2015; Yu et al., 2019; Zheng et al., 2015). These species may or may not be related to pathogenic processes, however, we suggest that an important role is reflected by these similarities within submucosal biofilms.

It is important to note that although sites that achieved success after treatment showed changes in the abundance of taxa, the samples after treatment maintained greater similarity with the pre-treatment samples from the same individual. A recent study of periodontal health and disease microbiota and their changes after nonsurgical periodontal therapy showed, through principal component analysis and classical multidimensional scaling analysis, a clear separation between plaque and saliva samples without obvious separation among saliva of periodontally healthy, saliva of periodontally diseased/ pre-treatment, and saliva of periodontally diseased/ post-treatment or between subgingival plaque of periodontally diseased/pre-treatment and subgingival plaque of periodontally diseased/post-treatment (Chen et al., 2018). Our results corroborate this finding since community-level analyses (Figure 6) showed no clustering among samples suggesting a similar microbiome, even when comparing post treatment success with post treatment failure. However, an interesting result could be observed through the hierarchical cluster dendrogram where the majority of individuals presented their samples clustered close together, suggesting that the submucosal microbiota from different time point of the same

individual were more similar than submucosal microbiota from different individuals. It is well know that different sites of same individual present distinct ecosystems (Chen et al., 2018; Schwarzberg et al., 2014; Zaura et al., 2009). In the present investigation, all samples were from the same site (peri-implant site), which could be suggested that the site exerts a higher pressure on the microbial composition than a successful or unsuccessful outcome, the kind of treatment performed or even the post treatment disease evolution over time.

Network analysis - i.e., how bacteria are interacting in the community - can indicate synergistic or antagonistic interactions and may provide useful information about clinical outcomes (Chen et al., 2018). In our study, alfa and beta diversity indexes of post treatment success and failure showed a similar pattern from the compositional point of view, however, when we evaluate from the point of view of interactions, both groups seems to show a different pattern. Already at baseline, patients that achieved success presented stronger correlations, both positive and negative, and in greater number than patients that remained with disease, and this pattern was maintained throughout the study. These observations may indicate that the degree of interactions and their strength can be an indicator of how patients will respond to treatment. In patients with unsuccessful treatments, bacteria appear to be more self-sufficient, not needing to interact consistently positively or negatively with other species. When we consider submucosal biofilm as a community, we can hypothesize that in patients that remained with disease, the components of this community were more resistant and independent than the components of a community present in patients that improved clinically.

It can be concluded that the submucosal microbial communities profile and diversity are similar before and after NST or ST. In fact, no treatment showed superiority over the other. Additionally, when comparing successful and unsuccessful PI post treatment, microbial composition and diversity were also similar, however, a larger number and stronger interactions were observed among microorganisms of patients who were successful treated, even before treatment was done. This pattern should be pursued further as it may be associated with differences in treatment response. Further studies are urgently needed to improve our understanding of metabolic interactions and gene expression through metabolomic and metatranscriptomic analysis. Therefore, knowing

the dynamic changes in the microbiome within each site for different clinical states could be highly useful in diagnosis and prognosis of individual peri-implant sites in clinical practice.

References

Alm, E. W., Oerther, D. B., Larsen, N., Stahl, D. A., & Raskin, L. (1996). The oligonucleotide probe database. *Applied and Environmental Microbiology*, *62*(10), 3557–3559. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8837410

Balshi, T., Wolfinger, G., Stein, B., & Balshi, S. (2015). A Long-term Retrospective Analysis of Survival Rates of Implants in the Mandible. *The International Journal of Oral & Maxillofacial Implants*, *30*(6), 1348–1354. https://doi.org/10.11607/jomi.3910

Bassetti, M., Schär, D., Wicki, B., Eick, S., Ramseier, C. A., Arweiler, N. B., … Salvi, G. E. (2014). Anti-infective therapy of peri-implantitis with adjunctive local drug delivery or photodynamic therapy: 12-month outcomes of a randomized controlled clinical trial. *Clinical Oral Implants Research*, *25*(3), 279–287. https://doi.org/10.1111/clr.12155

Berglundh, T., Armitage, G., Araujo, M. G., Avila-Ortiz, G., Blanco, J., Camargo, P. M., … Zitzmann, N. (2018). Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *Journal of Periodontology*, *89*, S313–S318. https://doi.org/10.1002/JPER.17-0739

Casadevall, A., & Pirofski, L. A. (2003). The damage-response framework of microbial pathogenesis. *Nature Reviews Microbiology*, *1*(1), 17–24. https://doi.org/10.1038/nrmicro732

Chen, C., Hemme, C., Beleno, J., Shi, Z. J., Ning, D., Qin, Y., … Zhou, J. (2018). Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *ISME Journal*, *12*(5), 1210–1224. https://doi.org/10.1038/s41396-017-0037-1

Cho, I., & Blaser, M. J. (2012, April). The human microbiome: At the interface of health and disease. *Nature Reviews Genetics*. Nat Rev Genet.

https://doi.org/10.1038/nrg3182

Chong, J., Liu, P., Zhou, G., & Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nature Protocols*, *15*(3), 799–821. https://doi.org/10.1038/s41596-019-0264-1

Costa, F. O., Takenaka-Martinez, S., Cota, L. O. M., Ferreira, S. D., Silva, G. L. M., & Costa, J. E. (2012). Peri-implant disease in subjects with and without preventive maintenance: A 5-year follow-up. *Journal of Clinical Periodontology*, *39*(2), 173–181. https://doi.org/10.1111/j.1600-051X.2011.01819.x

de Melo, F., Milanesi, F. C., Angst, P. D. M., & Oppermann, R. V. (2020, September 1). A systematic review of the microbiota composition in various peri-implant conditions: data from 16S rRNA gene sequencing. *Archives of Oral Biology*. Elsevier Ltd. https://doi.org/10.1016/j.archoralbio.2020.104776

De Waal, Y. C. M., Raghoebar, G. M., Huddleston Slater, J. J. R., Meijer, H. J. A., Winkel, E. G., & Van Winkelhoff, A. J. (2013). Implant decontamination during surgical peri-implantitis treatment: A randomized, double-blind, placebo-controlled trial. *Journal of Clinical Periodontology*, *40*(2), 186–195. https://doi.org/10.1111/jcpe.12034

de Waal, Y. C. M., Raghoebar, G. M., Meijer, H. J. A., Winkel, E. G., & van Winkelhoff, A. J. (2015). Implant decontamination with 2% chlorhexidine during surgical peri-implantitis treatment: a randomized, double-blind, controlled trial. *Clinical Oral Implants Research*, *26*(9), 1015–1023. https://doi.org/10.1111/clr.12419

Derks, J., & Tomasi, C. (2015). Peri-implant health and disease. A systematic review of current epidemiology. *Journal of Clinical Periodontology*, *42*, S158–S171. https://doi.org/10.1111/jcpe.12334

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., & Xia, J. (2017). MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, *45*(W1), W180–W188. https://doi.org/10.1093/nar/gkx295

Donati, M., Ekestubbe, A., Lindhe, J., & Wennström, J. L. (2016). Implant-supported single-tooth restorations. A 12-year prospective study. *Clinical Oral Implants Research*, *27*(10), 1207–1211. https://doi.org/10.1111/clr.12726

Dreyer, H., Grischke, J., Tiede, C., Eberhard, J., Schweitzer, A., Toikkanen, S. E., … Stiesch, M. (2018, October 1). Epidemiology and risk factors of peri-implantitis: A systematic review. *Journal of Periodontal Research*. Blackwell Munksgaard. https://doi.org/10.1111/jre.12562

Galofré, M., Palao, D., Vicario, M., Nart, J., & Violant, D. (2018). Clinical and microbiological evaluation of the effect of Lactobacillus reuteri in the treatment of mucositis and peri-implantitis: A triple-blind randomized clinical trial. *Journal of Periodontal Research*, *53*(3), 378–390. https://doi.org/10.1111/jre.12523

Gao, X., Zhou, J., Sun, X., Li, X., & Zhou, Y. (2018). Diversity analysis of subgingival microbial bacteria in peri-implantitis in Uygur population. *Medicine*, *97*(5), e9774. https://doi.org/10.1097/MD.0000000000009774

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., … Leys, E. J. (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *The ISME Journal*, *6*(6), 1176–1185. https://doi.org/10.1038/ismej.2011.191

Hallström, H., Persson, G. R., Lindgren, S., Olofsson, M., & Renvert, S. (2012). Systemic antibiotics and debridement of peri-implant mucositis. A randomized clinical trial. *Journal of Clinical Periodontology*, *39*(6), 574–581. https://doi.org/10.1111/j.1600-051X.2012.01884.x

Huang, S., Li, R., Zeng, X., He, T., Zhao, H., Chang, A., … Xu, J. (2014). Predictive modeling of gingivitis severity and susceptibility via oral microbiota. *ISME Journal*, *8*(9), 1768–1780. https://doi.org/10.1038/ismej.2014.32

Isehed, C., Holmlund, A., Renvert, S., Svenson, B., Johansson, I., & Lundberg, P. (2016). Effectiveness of enamel matrix derivative on the clinical and microbiological outcomes following surgical regenerative treatment of peri-implantitis. A randomized controlled trial. *Journal of Clinical Periodontology*, *43*(10), 863–873. https://doi.org/10.1111/jcpe.12583

John, G., Sahm, N., Becker, J., & Schwarz, F. (2015). Nonsurgical treatment of peri-implantitis using an air-abrasive device or mechanical debridement and local application of chlorhexidine. Twelve-month follow-up of a prospective, randomized, controlled clinical study. *Clinical Oral Investigations*, *19*(8), 1807–1814.

https://doi.org/10.1007/s00784-015-1406-7

Koyanagi, T., Sakamoto, M., Takeuchi, Y., Ohkuma, M., & Izumi, Y. (2010). Analysis of microbiota associated with peri-implantitis using 16S rRNA gene clone library., *2*, 5104. https://doi.org/10.3402/jom.v2i0.5104

Kumar, P. S., Mason, M. R., Brooker, M. R., & O'Brien, K. (2012). Pyrosequencing reveals unique microbial signatures associated with healthy and failing dental implants. *Journal of Clinical Periodontology*, *39*(5), 425–433. https://doi.org/10.1111/j.1600-051X.2012.01856.x

Lafaurie, G. I., Sabogal, M. A., Castillo, D. M., Rincón, M. V., Gómez, L. A., Lesmes, Y. A., & Chambrone, L. (2017). Microbiome and Microbial Biofilm Profiles of Peri-Implantitis: A Systematic Review. *Journal of Periodontology*, *88*(10), 1066–1089. https://doi.org/10.1902/jop.2017.170123

Liu, B., Faller, L. L., Klitgord, N., Mazumdar, V., Ghodsi, M., Sommer, D. D., … Amar, S. (2012). Deep sequencing of the oral microbiome reveals signatures of periodontal disease. *PloS One*, *7*(6), e37919. https://doi.org/10.1371/journal.pone.0037919

Machtei, E. E., Frankenthal, S., Levi, G., Elimelech, R., Shoshani, E., Rosenfeld, O., … Shlomi, B. (2012). Treatment of peri-implantitis using multiple applications of chlorhexidine chips: A double-blind, randomized multi-centre clinical trial. *Journal of Clinical Periodontology*, *39*(12), 1198–1205. https://doi.org/10.1111/jcpe.12006

Mombelli, A., Müller, N., & Cionca, N. (2012). The epidemiology of peri-implantitis. *Clinical Oral Implants Research*, *23*(SUPPL.6), 67–76. https://doi.org/10.1111/j.1600-0501.2012.02541.x

Monje, A., Aranda, L., Diaz, K. T., Alarcón, M. A., Bagramian, R. A., Wang, H. L., & Catena, A. (2016). Impact of maintenance therapy for the prevention of peri-implant diseases. *Journal of Dental Research*, *95*(4), 372–379. https://doi.org/10.1177/0022034515622432

Nie, J., Zhang, Q., Zheng, H., Xu, L. X., Wang, X. Y., & Chen, F. (2020). Pyrosequencing of the subgingival microbiome in peri-implantitis after non-surgical mechanical debridement therapy. *Journal of Periodontal Research*, *55*(2), 238–246. https://doi.org/10.1111/jre.12708

Padial-Molina, M., López-Martínez, J., O'Valle, F., & Galindo-Moreno, P. (2016).

Microbial Profiles and Detection Techniques in Peri-Implant Diseases: a Systematic Review. *Journal of Oral & Maxillofacial Research*, *7*(3), e10. https://doi.org/10.5037/jomr.2016.7310

Pflughoeft, K. J., & Versalovic, J. (2012). Human Microbiome in Health and Disease. *Annual Review of Pathology: Mechanisms of Disease*, *7*(1), 99–122. https://doi.org/10.1146/annurev-pathol-011811-132421

Rakic, M., Grusovin, M. G., & Canullo, L. (2016). The Microbiologic Profile Associated with Peri-Implantitis in Humans: A Systematic Review. *The International Journal of Oral & Maxillofacial Implants*, *31*(2), 359–368. https://doi.org/10.11607/jomi.4150

Ramanauskaite, A., Daugela, P., Faria de Almeida, R., & Saulacic, N. (2016). Surgical Non-Regenerative Treatments for Peri-Implantitis: a Systematic Review. *Journal of Oral & Maxillofacial Research*, *7*(3), e14. https://doi.org/10.5037/jomr.2016.7314

Sanz-Martin, I., Doolittle-Hall, J., Teles, R. P., Patel, M., Belibasakis, G. N., Hämmerle, C. H. F., … Teles, F. R. F. (2017). Exploring the microbiome of healthy and diseased peri-implant sites using Illumina sequencing. *Journal of Clinical Periodontology*, *44*(12), 1274–1284. https://doi.org/10.1111/jcpe.12788

Schwarzberg, K., Le, R., Bharti, B., Lindsay, S., Casaburi, G., Salvatore, F., … Kelley, S. T. (2014). The personal human oral microbiome obscures the effects of treatment on periodontal disease. *PLoS ONE*, *9*(1). https://doi.org/10.1371/journal.pone.0086708

Shiba, T., Watanabe, T., Kachi, H., Koyanagi, T., Maruyama, N., Murase, K., … Nakagawa, I. (2016). Distinct interacting core taxa in co-occurrence networks enable discrimination of polymicrobial oral diseases with similar symptoms. *Scientific Reports*, *6*(1), 30997. https://doi.org/10.1038/srep30997

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., … Herndl, G. J. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere." *Proceedings of the National Academy of Sciences of the United States of America*, *103*(32), 12115–12120. https://doi.org/10.1073/pnas.0605127103

Sousa, V., Nibali, L., Spratt, D., Dopico, J., Mardas, N., Petrie, A., & Donos, N. (2017). Peri-implant and periodontal microbiome diversity in aggressive periodontitis patients: a pilot study. *Clinical Oral Implants Research*, *28*(5), 558–570.

https://doi.org/10.1111/clr.12834

Tsigarida, A. A., Dabdoub, S. M., Nagaraja, H. N., & Kumar, P. S. (2015). The Influence of Smoking on the Peri-Implant Microbiome. *Journal of Dental Research*, *94*(9), 1202–1217. https://doi.org/10.1177/0022034515590581

Wagner, T. P., Costa, R. S. A., Rios, F. S., Moura, M. S., Maltz, M., Jardim, J. J., & Haas, A. N. (2016). Gingival recession and oral health-related quality of life: a population-based cross-sectional study in Brazil. *Community Dentistry and Oral Epidemiology*, 390–399. https://doi.org/10.1111/cdoe.12226

Xiao, C., Ran, S., Huang, Z., & Liang, J. (2016). Bacterial diversity and community structure of supragingival plaques in adults with dental health or caries revealed by 16S pyrosequencing. *Frontiers in Microbiology*, *7*(JUL). https://doi.org/10.3389/fmicb.2016.01145

Yu, X.-L., Chan, Y., Zhuang, L., Lai, H.-C., Lang, N. P., Keung Leung, W., & Watt, R. M. (2019). Intra-oral single-site comparisons of periodontal and peri-implant microbiota in health and disease. *Clinical Oral Implants Research*, *30*(8), 760–776. https://doi.org/10.1111/clr.13459

Zaura, E., Keijser, B. J. F., Huse, S. M., & Crielaard, W. (2009). Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiology*, *9*(1), 259. https://doi.org/10.1186/1471-2180-9-259

Zheng, H., Xu, L., Wang, Z., Li, L., Zhang, J., Zhang, Q., … Chen, F. (2015). Subgingival microbiome in patients with healthy and ailing dental implants. *Scientific Reports*, *5*(1), 10948. https://doi.org/10.1038/srep10948

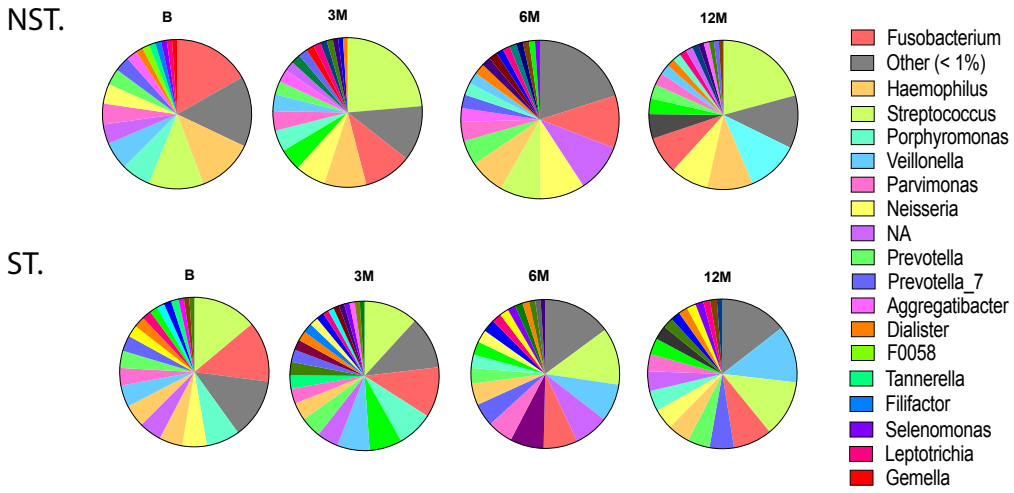


Figure 1. Bacterial community structure distribution. Differences in the percentages of most abundant bacterial genera (≥1%) on PI biofilm for each treatment group and time point. NST. Non-surgical treatment; ST. Surgical treatment. B. Baseline; 3M. Three months; 6M. Six months; 12M. Twelve months.

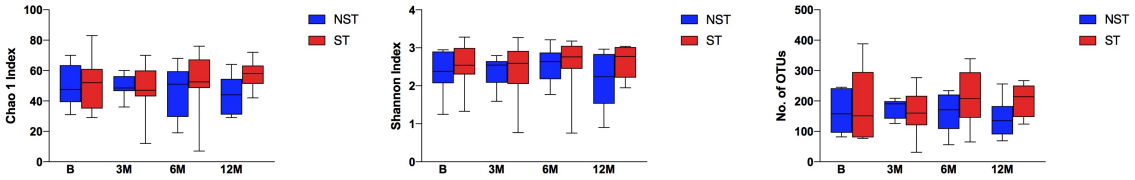


Figure 2. Chao1 (a) and Shannon (b) index diversity and total number of operational taxonomic units (OTUs) observed (richness) for genus taxonomic level on PI biofilm for each treatment group and time point. Data presented as median and quartiles. Mean is also presented as +. NST. Non-surgical treatment; ST. Surgical treatment. B. Baseline; 3M. Three months; 6M. Six months; 12M. Twelve months.

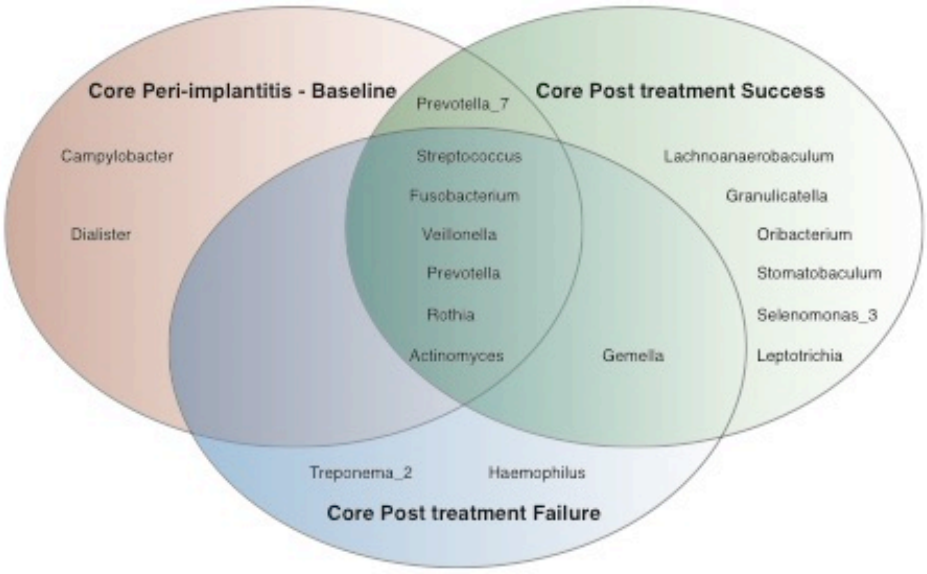


Figure 3. Core microbiome. Genera presented in all subjects of PI baseline, post treatment success and post treatment failure is represented by a red, green and blue circles, respectively.

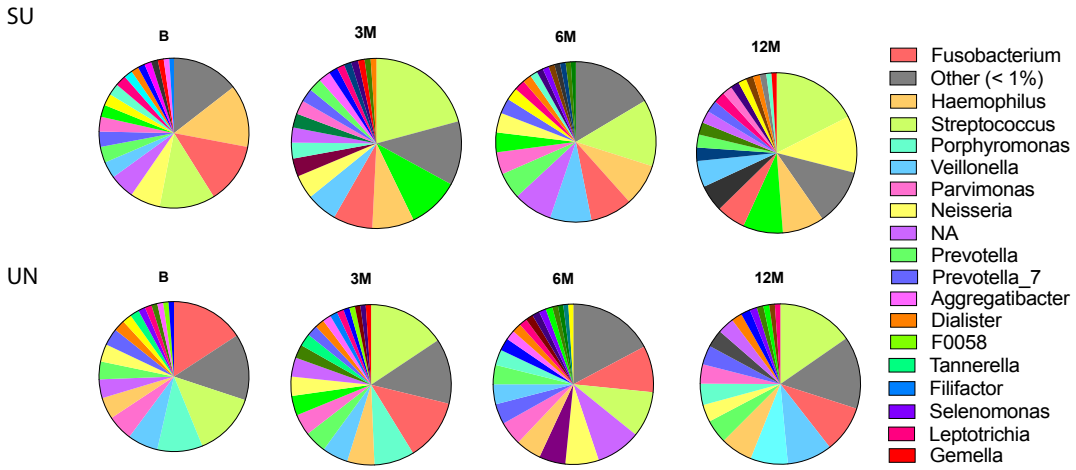


Figure 4. Bacterial community structure distribution. Differences in the percentages of most abundant bacterial genera (≥1%) on PI biofilm for each outcome group and time point. SU. Successful; UN. Unsuccessful. B. Baseline; 3M. Three months; 6M. Six months; 12M. Twelve months.

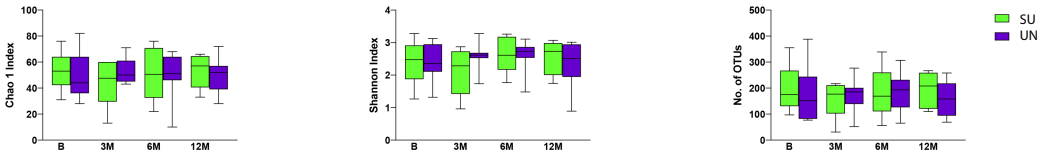


Figure 5. Chao1 (a) and Shannon (b) index diversity and total number of operational taxonomic units (OTUs) observed (richness) for genus taxonomic level on PI biofilm for each outcome group and time point. Data presented as median and quartiles. Mean is also presented as +. SU. Successful; UN. Unsuccessful. B. Baseline; 3M. Three months; 6M. Six months; 12M. Twelve months.

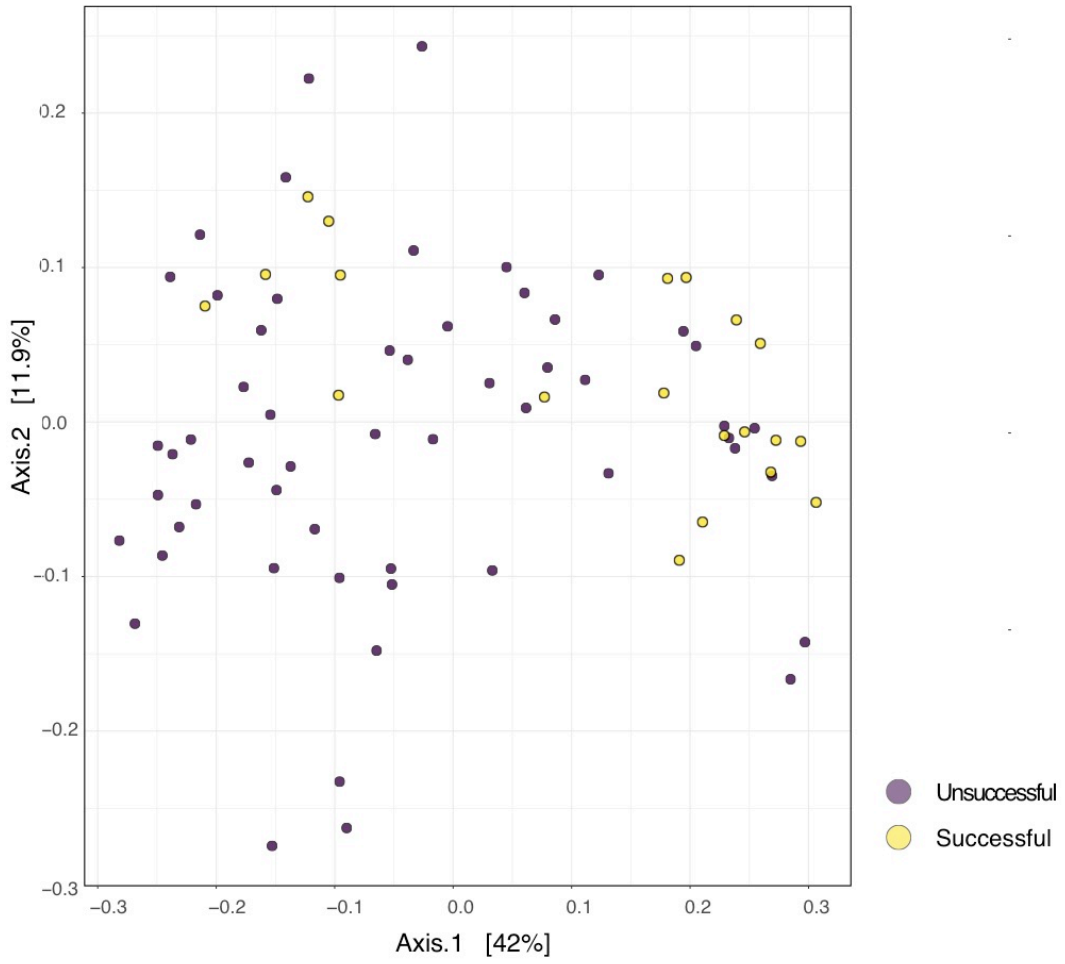


Figure 6. Principal Coordinate Analysis (PCoA) plot on genus, obtained by weighted Unifrac distance of submucosal peri-implant biofilm communities from all subjects at each time point. Each sample is represented by a circle. Yellow circles represents before and after successful treatment. Purple circles represents before and after unsuccessful treatment.

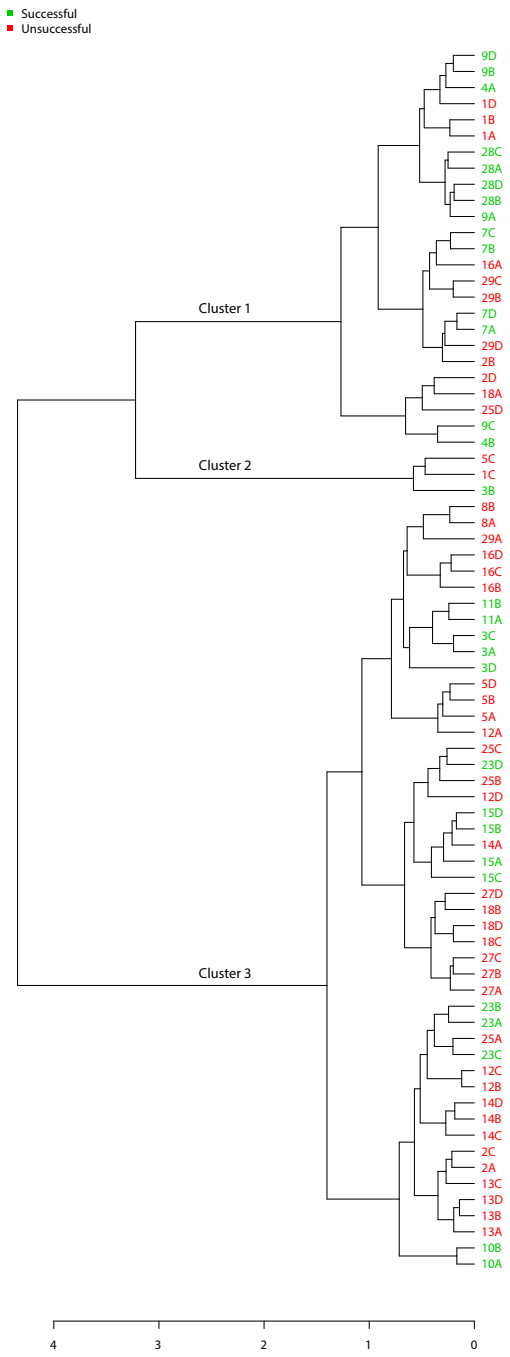


Figure 7. Hierarchical dendrogram (bray-curtis dissimilarity) representing each subject sample and time point (A. baseline, B. 3 months, C. 6 months and D. 12 months after treatment). Green samples represent successful and red samples represents unsuccessful before and after treatment.

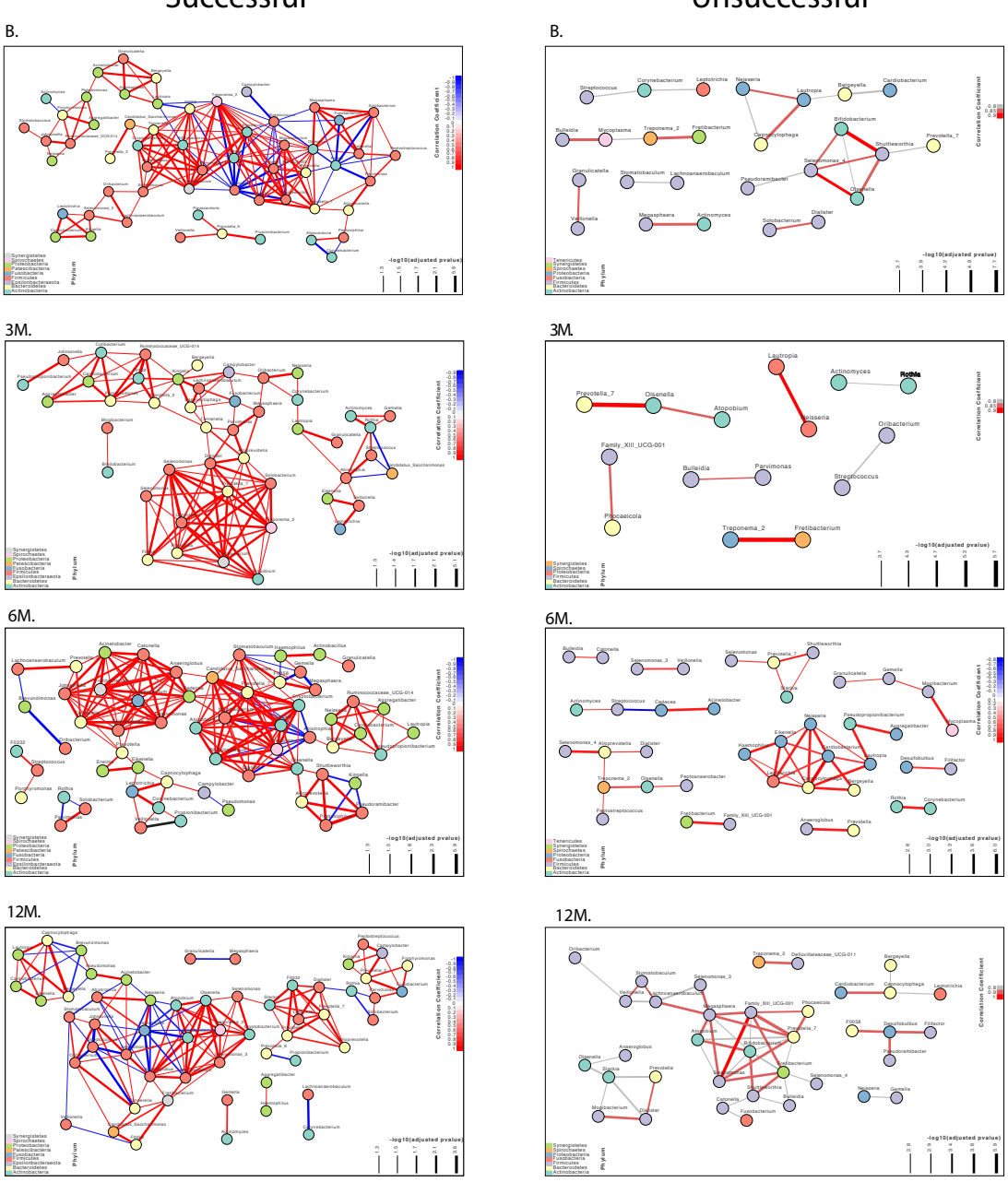
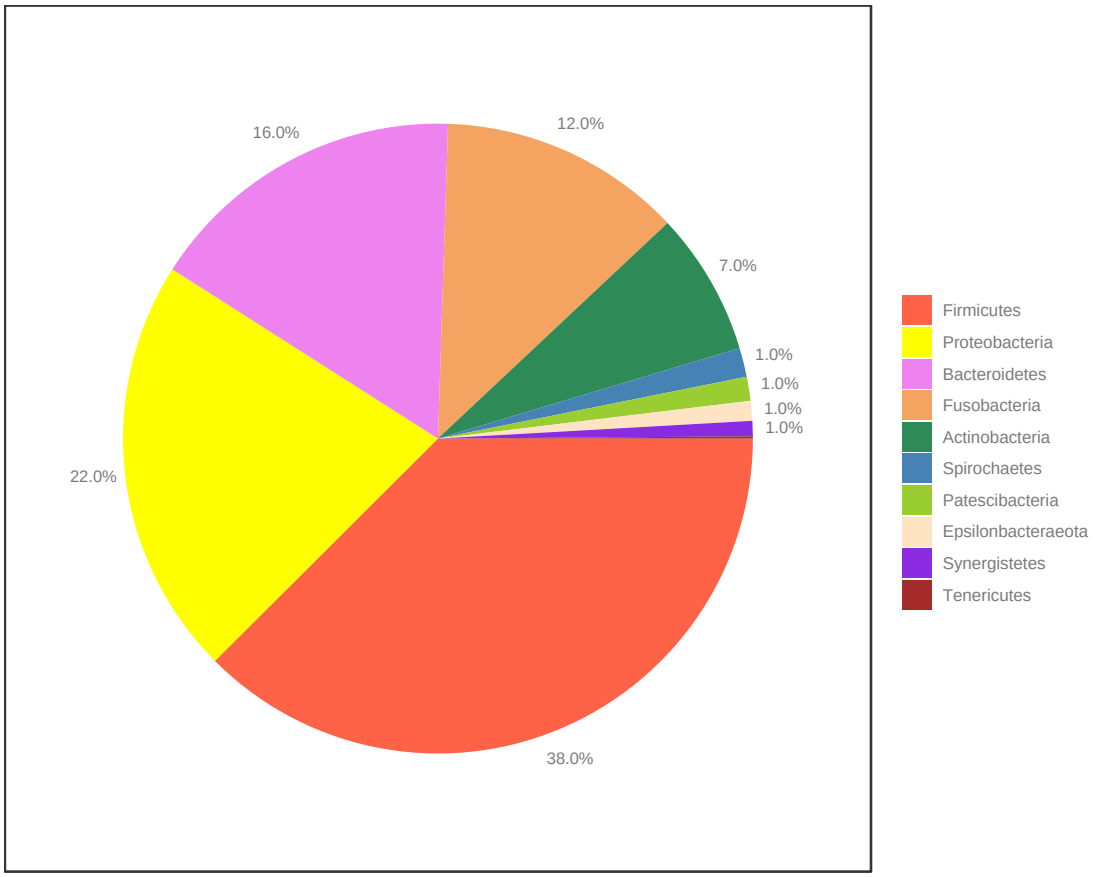
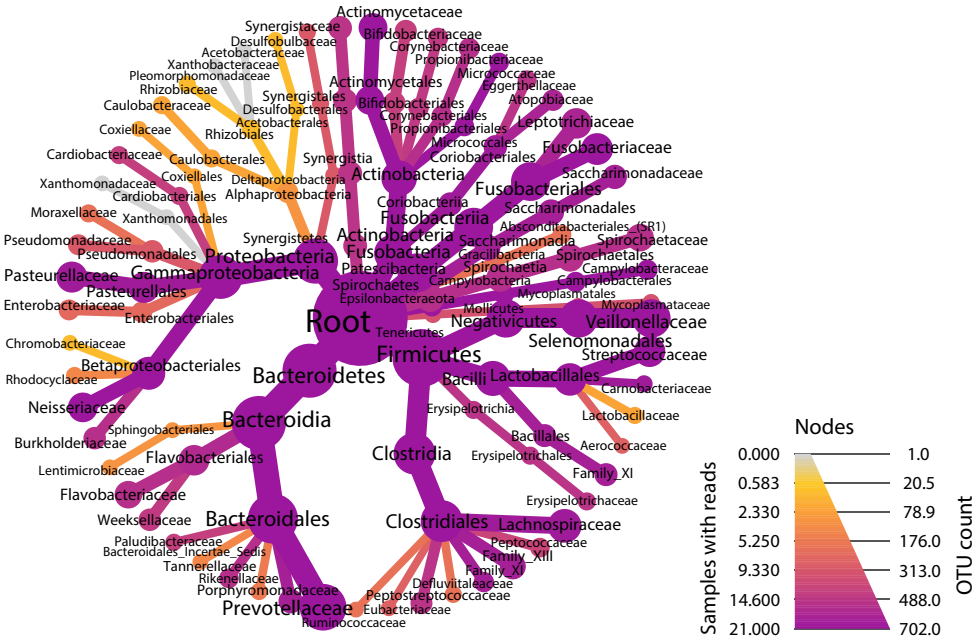


Figure 8. Co-occurrence networks for genus taxonomic level on PI biofilm for each outcome group and time point. B. Baseline; 3M. Three months; 6M. Six months; 12M. Twelve months.



S1. Percentages of most abundant bacterial phyla recovered in all samples.



S2. Phylogenetic tree at taxonomic family level in all samples. The size of the node represents the OTU counts.

Table 1. Patient , periodontal and peri-implant characteristics for the two groups

Characteristic	Non-surgical	Surgical	p value (intergroup)
PATIENTS	10	11	
Age (mean ± SD)	56.7±8.7	63.5±6.1	0.28
Sex (n/%)			
Male	2 (20.0)	2 (18,1)	
Female	8 (80.0)	9 (81.8)	0.91
Tobaco Smoking			
Never Smoker	6 (60.0)	5 (45.4)	
Former Smoker	4 (40.0)	4 (36.3)	
Smoker	0 (0.0)	2 (18.1)	0.47
PERIODONTAL PARAMETERS **			
Number of present teeth	20.8 (1.1)	18.5 (1.6)	0.28
Plaque (% sites)	37.9 (5.3)	43.1 (6.9)	0.56
Bleeding on probing (% sites)	25.8 (15.5)	28.4 (15.0)	0.71
Pocket depth (mm)	1.98 (0.8)	2.32 (0.92)	0.49
Clinical attachment loss (mm)	1.14 (0.13)	1.33 (0.16)	0.39
PERI-IMPLANT PARAMETERS ***			
Plaque (% sites)			
Baseline	20.0±0.1	9.1±0.1	0.80
3M	10.0±0.1	0.0±0.0	0.85
6M	10.1±0.1	0.0±0.0	0.85
12M	0.0±0.0	9.1±0.1	0.89
p-value within groups	0.31	1.00	
Bleeding on probing (% sites)			
Baseline	100	100	0.99
3M	60.0±1.6	45.4±1.5*	0.88
6M	70.0±1.5	54.5±1.5	0.99
12M	60.0±1.6	54.5±1.5	0.99
p-value (within group)	<0.001	<0.001	
Pocket depth (mm)			
Baseline	6.3±0.5	5.7±0.3	0.92
3M	4.9±0.8	4.6±0.5	0.98
6M	5.0±0.7	4.0±0.6*	0.78
12M	4.0±0.4*	3.9±0.7*	0.99
p-value within groups	0.001	0.02	

* p< 0.05 compared to baseline; ** Mean±standard deviation; ***Mean±standard error

Table 2. Relative abundance of operational taxonomic units (OTUs) from most abundant recovered Phylum (more than 1%) from PI biofilm. NST = Non-surgical treatment; ST = Surgical treatment; B = Baseline; 3M = Three months; 6M = Six months; 12M = Twelve months.

Phylum	NST				ST				Time (p)	Group (p)	Interaction (p)
	B	3M	6M	12M	B	3M	6M	12M			
Firmicutes	346038	466739	246511	286571	482859	471343	374142	446326	0.32	0.23	0.54
Proteobacteria	230946	265076	346766	338034	162578	161773	183330	162977	0.27	0.41	0.52
Fusobacteria	181243	136743	117995	82248	199928	157403	89273	104850	0.28	0.96	0.81
Bacteroidetes	185038	157151	169888	82175	250847	272209	133914	195605	0.67	0.81	0.25
Actinobacteria	33544	79888	37119	47294	109942	151630	74905	98267	0.52	0.07	0.86
Patescibacteria	6751*	3507*	10684	6339*	29429	14956	17756	14109	0.88	0.11	0.89
Spirochaetes	8970*	11890	14321	4875*	21119	21798	24860	21792	0.98	0.41	0.72

Font: SILVA database/MicrobiomeAnalyst

Cut off point of 1% of the microbiome.
* Lower than 1% at specific site
p reprents Two Way ANOVA analysis (factor 1 = time and factor 2 = group)

Table 3. Relative abundance of all genera observed more than 0.1% in at least one time point from per-implantitis biofilm for each time point. NST: Non-surgical treatment; ST: Surgical treatment; B: Baseline; 3M: Three months; 6M: Six months; 12M: Twelve months.

Phylum	Genus	NST				ST			
		B	3M	6M	12M	B	3M	6M	12M
Actinobacteria	Actinomyces**	0.963	1.387	0.946	1.114	1.206	2.875	1.380	2.263
	Atopobium**	0.735	0.218	0.876	0.069	3.328	0.851	1.906	1.950
	Bifidobacterium	0.019	0.014	0.191	0.012	0.729	0.079	0.768	0.506
	Corynebacterium	0.159	0.219	0.123	0.098	0.232	0.363	0.462	0.207
	Pseudopropionibacterium	0.117	0.197	0.258	0.250	0.053	0.179	0.021	0.021
	Rothia	0.903	4.729	1.190	3.296	2.010	6.924	2.871	3.477
	Slackia	0.104	0.094	0.171	0.068	0.338	0.135	0.113	0.267
	Olsenella	0.107	0.030	0.057	0.030	0.373	0.153	0.453	0.334
	Microbacterium	0.147	0.003	0.004	0.004	0.003	0.005	0.003	0.003
	Cutibacterium	0.014	0.012	0.017	0.463	0.035	0.008	0.017	0.008
Bacteroidetes	Alloprevotella	0.651	0.865	1.303	1.050	1.207	0.736	0.690	1.469
	Bergeyella	0.127	0.125	0.211	0.254	0.056	0.065	0.073	0.085
	Capnocytophaga	0.899	1.212	1.905	1.243	0.589	1.208	1.041	0.918
	Chryseobacterium	0.00	0.050	0.571	0.014	0.010	0.011	0.023	0.110
	Cloacibacterium	0.005	0.007	1.428	0.005	0.010	0.004	0.057	0.096
	F0058	1.496	0.891	0.422	0.365	0.386	0.639	0.142	0.066
	Porphyromonas	6.298	4.583	2.508	1.718	7.125	7.681	2.893	4.390
	Prevotella	3.545	2.950	5.080	3.155	3.807	4.544	3.399	4.815
	Prevotella_1	0.005	0.104	0.065	0.005	0.015	0.010	0.034	0.120
	Prevotella_2	0.273	0.496	0.649	0.333	0.396	0.257	0.262	0.241
	Prevotella_6	0.041	0.131	0.095	0.133	0.166	0.113	0.319	0.344
	Prevotella_7	3.300	2.003	2.788	1.122	3.723	2.697	4.956	5.245
	Tannerella	1.372	0.515	0.354	0.443	1.633	2.886	0.680	0.413
	Phocaeicola	0.015	0.015	0.015	0.003	0.068	0.109	0.019	0.032

Epsilonbacteraeota	Campylobacter	0.838	0.596	1.719	0.582	0.831	1.236	0.894	0.929
Firmicutes	Anaeroglobus	0.178	0.201	0.458	0.684	1.593	0.758	0.287	0.450
	Catonella	0.353	0.218	0.309	0.151	0.429	0.448	0.533	0.490
	Dialister	1.529	0.989	2.188	1.737	2.515	2.079	1.431	1.961
	Abiotrophia	0.132	0.135	0.067	0.042	0.013	0.095	0.043	0.130
	Filifactor	1.273	0.121	0.816	0.764	0.603	2.035	0.462	0.057
	Gemella	1.074	1.741	0.840	0.769	0.919	0.588	0.430	0.356
	Granulicatella	0.696	1.412	0.814	1.366	0.575	0.806	0.695	1.186
	Johnsonella	0.172	0.157	0.182	0.189	0.264	0.169	0.363	0.228
	Lachnoanaerobaculum	0.235	0.262	0.263	0.256	0.302	0.295	0.348	0.281
	Mogibacterium	0.145	0.073	0.242	0.123	0.143	0.325	1.150	0.410
	Oribacterium	0.422	0.452	0.217	0.197	0.416	0.216	0.324	0.605
	Parvimonas	4.418	4.159	3.831	2.643	4.575	3.174	5.448	3.639
	Peptoanaerobacter	0.174	0.053	0.226	0.097	0.090	0.160	0.080	0.014
	Peptostreptococcus	0.218	0.385	0.227	0.251	0.253	0.142	0.383	0.544
	Peptococcus	0.174	0.106	0.081	0.050	0.094	0.119	0.091	0.026
	Selenomonas	1.253	0.250	1.030	0.255	1.101	1.170	1.755	1.740
	Selenomonas_3**	0.326	0.350	0.514	0.323	0.430	1.037	1.685	1.001
	Selenomonas_4	0.131	0.068	0.149	0.092	0.334	0.163	0.267	0.418
	Shuttleworthia	0.133	0.160	0.112	0.009	0.496	0.122	0.109	0.080
	Stomatobaculum	0.068	0.077	0.098	0.059	0.249	0.369	0.068	0.287
	Streptococcus	11.705	23.447	8.206	20.344	13.711	11.624	12.241	12.108
	Veillonella**	5.867	3.606	2.268	2.152	4.808	6.907	8.007	12.149
	Megasphaera	0.056	0.015	0.020	0.007	0.230	0.107	0.327	0.679
	Lactobacillus	0.025	0.459	0.018	0.020	0.035	0.052	0.035	0.036
	Family_XIII_UCG-001	0.037	0.028	0.103	0.007	0.074	0.128	0.034	0.045
	Defluviitaleaceae_UCG-011	0.025	0.013	0.014	0.012	0.024	0.103	0.121	0.093
	Peptoniphilus	0.004	0.004	0.063	0.026	0.035	0.003	0.200	0.025
	Centipeda	0.012	0.015	0.034	0.026	0.026	0.064	0.161	0.013
	Solobacterium	0.089	0.037	0.092	0.038	0.084	0.044	0.140	0.083
Fusobacteria	Fusobacterium	16.481	10.345	10.604	7.983	12.919	10.737	7.313	8.308

	Leptotrichia	1.203	1.627	1.491	1.579	2.384	1.279	2.088	1.515
Proteobacteria	Neisseria	4.227	6.558	8.970	8.062	5.258	1.828	2.715	4.394
	Haemophilus	11.957	9.076	7.158	9.737	4.960	3.792	4.625	4.765
	Aggregatibacter	2.323	2.744	2.755	1.138	0.382	1.121	0.171	0.056
	Desulfobulbus	0.290	0.006	0.029	0.211	0.210	0.318	0.085	0.005
	Cedecea	0.832	0.050	0.989	0.102	0.045	0.141	7.218	0.340
	Acinetobacter	0.316	0.058	0.924	0.015	0.010	0.021	0.331	0.784
	Lautropia	0.931	0.914	0.853	0.622	0.405	0.152	0.169	3.018
	Pleomorphomonas	0.002	0.002	0.852	0.002	0.002	0.004	0.002	0.001
	Simonsiella	0.00	0.014	0.818	5.187	0.002	0.002	0.002	0.001
	Klebsiella	0.003	0.002	0.682	0.002	0.002	0.005	0.005	0.004
	Pseudomonas***	0.064	2.089	0.600	0.337	0.025	0.051	0.117	0.241
	Xanthobacter	0.00	0.006	0.407	0.007	0.006	2.226	0.143	0.005
	Cardiobacterium	0.115	0.269	0.317	0.305	0.157	1.087	0.208	0.125
	Azospira	0.002	0.007	0.306	0.002	0.007	0.002	0.002	0.001
	Actinobacillus	0.673	0.225	0.288	0.470	0.012	0.008	0.008	0.151
	Roseomonas	0.008	0.010	0.240	0.007	0.006	0.361	0.927	0.114
	Azorhizobium	0.00	0.003	0.198	0.004	0.003	0.028	0.003	0.003
	Raoultella	0.006	0.001	0.196	11.059	0.002	0.008	0.059	0.007
	Eikenella	0.170	0.205	0.144	0.107	0.290	0.366	0.086	0.099
	Acidovorax	0.001	0.026	0.136	0.001	0.006	0.004	0.001	0.008
	Kingella	0.110	0.348	0.125	0.517	0.606	0.348	0.302	0.230
	Brevundimonas	0.017	0.134	0.015	0.021	0.005	0.058	0.010	0.026
	Enhydrobacter	0.003	0.124	0.028	0.575	0.003	0.003	0.003	0.002
	Dechlorosoma	0.002	0.030	1.336	0.002	0.003	0.002	0.010	0.007
	Erwinia	0.002	0.001	0.001	0.001	0.001	0.013	0.537	0.002
	Curvibacter	0.003	0.001	0.001	0.001	0.001	0.001	0.006	0.118
Patescibacteria	Candidatus_Saccharimonas	0.030	0.064	0.065	0.068	0.182	0.054	0.073	0.022
Spirochaetes	Treponema_2	0.923	1.077	1.499	0.618	1.650	1.710	2.714	2.059
Synergistetes	Fretibacterium*	0.777	0.282	0.512	0.041	1.695	1.247	0.489	0.531
Tenericutes	Mycoplasma	0.104	0.056	0.043	0.053	0.100	0.089	0.309	0.164

Unclassified sequences	Derived from Bacteria	4.506	2.466	10.053	1.885	5.095	4.900	7.660	4.477

Font: SILVA database/MicrobiomeAnalyst

*p<0.05 on factor 1 (time); **p<0.05 on factor 2 (group); ***p<0.05 on factors 1 and 2 (time and group).

Table 4. Mean genus richness and proportion of richness, unclassified, shared and exclusive genera identified in PI biofilm microbiome for each group and time point. NST = Non-surgical treatment; ST = Surgical treatment; B = Baseline; 3M = Three months; 6M = Six months; 12M = Twelve months.

Groups	NST				ST			
Time Point / Genus	B	3M	6M	12M	B	3M	6M	12M
% richness (n)	51.69 (122)	55.08 (130)	62.71 (148)	43.64 (103)	96.61 (228)	52.54 (124)	57.62 (136)	59.74 (141)
% unclassified OTUs	4.23	2.18	9.92	1.53	4.87	4.67	7.49	4.27
% shared richness among all groups (75)	61.4	57.6	50.67	72.8	32.8	60.4	55.1	53.1
% uniques (n)	2.12 (5)	3.81 (9)	6.36 (15)	2.97 (7)	2.97 (7)	2.12 (5)	4.24 (10)	6.78 (16)

Font: SILVA database/MicrobiomeAnalyst

Table 5. Clinical outcome in PI baseline, successful and unsuccessful post treatment biofilms. B: Baseline; 3M: Three months; 6M: Six months; 12M: Twelve months.

	Success (n=9)				Failure (n=12)			
	B	3M	6M	12M	B	3M	6M	12M
Pocket depth (mm)	5.5±0.3	4.3±0.4	3.2±0.6*	3.2±0.4*	6.3±0.4	5.0±0.7	5.4±0.5	4.5±0.5*
Plaque (% sites)	0	0	0	0	25±1.3	8.0±0.8	8.0±0.8	8.0±0.8
Bleeding on probing (% sites)	100.0	44.0±1.7*	33.0±1.6*	0*	100.0	58.3±1.4*	83.3±1.1	100.0

Mean±standard error

* $p < 0.05$ compared to baseline

Table 6. Relative abundance of all genera observed in submucosal biofilms communities from PI baseline (before treatment), successful and unsuccessful post treatment groups. B: Baseline; 3M: Three months; 6M: Six months; 12M: Twelve months.

Genus	Successsful				Unsuccessful			
	B	3M	6M	12M	B	3M	6M	12M
NA	5.42	2.87	7.88	2.47	4.05	3.85	9.04	3.34
Fusobacterium**	13.14	7.49	8.47	5.86	15.79	12.43	9.36	9.46
Atopobium	2.55	0.68	2.54	1.66	2.01	0.49	1	0.85
Anaeroglobus	1.51	0.05	0.86	0.16	0.63	0.74	0.21	0.76
Streptococcus	11.98	20.8	13.46	17.51	13.74	15.65	9.22	15.35
Prevotella**	3.44	2.5	5.34	2.62	3.84	4.48	3.89	4.78
Leptotrichia	2.4	1.44	2.17	2.32	1.46	1.38	1.6	1.1
Dialister	1.62	1.09	1.78	1.35	2.44	1.85	1.87	2.15
Fretibacterium*	1.83	0.72	0.4	0.07	0.95	0.83	0.52	0.41
Neisseria	6.52	4.52	3.97	11.46	3.79	3.88	6.75	3.5
Prevotella_7	3.3	2.56	3.07	2.39	3.68	2.22	4.11	3.88
Bifidobacterium	0.55	0.09	0.92	0.46	0.32	0.01	0.31	0.19
Selenomonas	0.58	0.38	1.25	0.25	1.53	0.89	1.41	1.45
Selenomonas_4	0.33	0.06	0.33	0.17	0.18	0.14	0.15	0.32
Slackia	0.36	0.12	0.17	0.32	0.16	0.11	0.14	0.11
Olsenella	0.37	0.02	0.64	0.4	0.18	0.13	0.11	0.09
Tannerella	0.89	0.46	0.42	0.2	1.97	2.52	0.54	0.53
Veillonella	3.58	5.89	8.35	5.3	6.56	5.22	4.02	9.07
Selenomonas_3	0.53	0.47	1.02	0.49	0.23	0.81	1.08	0.77
Candidatus_Saccharimonas	0.22	0.08	0.19	0.08	0.04	0.04	0.02	0.02
Actinomyces	0.74	1.15	1.03	2.49	1.27	2.71	1.15	1.34
Oribacterium	0.35	0.09	0.48	0.35	0.42	0.41	0.15	0.42
F0058	0.46	0.11	0.04	0.09	1.17	1.13	0.37	0.26

Campylobacter	0.8	0.67	0.85	0.58	0.87	1.1	1.51	0.88
Corynebacterium	0.34	0.61	0.33	0.14	0.1	0.13	0.27	0.16
Cardiobacterium	0.27	0.22	0.48	0.28	0.04	0.97	0.18	0.16
Treponema_2	1.52	1.69	0.84	0.37	1.16	1.23	2.52	1.9
Filifactor	1.01	0.51	0.22	0.07	0.85	1.51	0.81	0.53
Haemophilus	13.47	7.97	8.5	8.42	4.66	5.5	5.15	6.45
Porphyromonas	2.51	3.03	1.49	1.06	9.78	8.1	3.15	4.31
Lachnoanaerobaculum	0.23	0.19	0.27	0.22	0.17	0.22	0.21	0.18
Aggregatibacter	1.21	2.28	0.26	0.29	1.27	1.7	1.95	0.66
Megasphaera	0.11	0.05	0.08	0.23	0.19	0.07	0.2	0.46
Rothia	2.6	9.83	3.99	8.05	0.85	3.96	1.36	1.19
Kingella	0.88	0.91	0.69	0.64	0.06	0.05	0.04	0.22
Capnocytophaga	0.99	1.34	1.3	1.73	0.46	1.08	1.5	0.67
Gemella	1.28	1.24	0.86	1.03	0.78	1.07	0.55	0.28
Cryptobacterium	0.05	0.01	0.05	0.04	0	0.01	0	0
Phocaeicola	0.04	0	0	0	0.04	0.1	0.02	0.03
Granulicatella	0.68	1.39	1.12	2.71	0.61	0.95	0.64	0.58
Catonella	0.25	0.26	0.33	0.1	0.45	0.34	0.42	0.42
Alloprevotella	0.94	0.85	1.2	1.49	0.92	0.71	0.89	1.13
Parvimonas**	3.18	2.63	4.48	1.97	5.54	4.28	4.79	3.89
Stomatobaculum	0.05	0.03	0.06	0.08	0.23	0.33	0.07	0.22
Mogibacterium	0.09	0.1	0.45	0.2	0.18	0.26	0.78	0.32
Johnsonella	0.33	0.09	0.26	0.31	0.12	0.17	0.25	0.13
Solobacterium	0.08	0.02	0.17	0.09	0.09	0.05	0.1	0.05
Prevotella_2	0.25	0.29	0.59	0.31	0.35	0.37	0.37	0.22
Prevotella_6	0.11	0.04	0.02	0.2	0.07	0.13	0.24	0.25
Eikenella	0.45	0.31	0.11	0.13	0.09	0.28	0.11	0.08
Family_XIII_UCG-001	0.07	0.02	0.05	0.01	0.04	0.11	0.07	0.03
Shuttleworthia	0.05	0.03	0.09	0.02	0.53	0.19	0.11	0.06
DNF00809	0.01	0	0	0	0	0	0	0
Erysipelotrichaceae_UCG-006	0.03	0	0.01	0	0.01	0.01	0.01	0

Pyramidobacter	0.01	0	0	0	0	0.01	0.02	0
Howardella	0.01	0.02	0.04	0.01	0.03	0.01	0.02	0.02
Ruminococcaceae_UCG-014	0.04	0.04	0.02	0.01	0.08	0.02	0	0.02
Scardovia	0.01	0.02	0.03	0.02	0.07	0.05	0	0.02
Eggerthia	0.01	0	0	0	0.02	0	0	0
Peptococcus	0.15	0.1	0.04	0.05	0.1	0.11	0.09	0.01
Pseudopropionibacterium	0.03	0.1	0.22	0.09	0.11	0.23	0.12	0.14
Prevotella_1	0.01	0.01	0	0	0	0.07	0.06	0.1
Rikenellaceae_RC9_gut_group	0.09	0.05	0.06	0.01	0.04	0.06	0.04	0.06
Centipeda	0.02	0.08	0.14	0.02	0	0.01	0.07	0
Butyrivibrio_2	0.01	0	0.01	0	0.01	0.07	0.06	0.09
Defluviitaleaceae_UCG-011	0.01	0	0.02	0.01	0.02	0.09	0.08	0.07
Peptostreptococcus	0.17	0.18	0.32	0.66	0.28	0.3	0.3	0.3
Propionibacterium	0.05	0.1	0.09	0.03	0	0	0	0
Peptoanaerobacter	0.16	0.08	0.12	0.06	0.1	0.12	0.16	0.04
Propionivibrio	0	0	0	0	0.03	0.01	0.02	0
Lautropia	1.44	0.81	1.16	5.4	0.12	0.36	0.31	0.29
Mycoplasma	0.1	0.03	0.04	0.01	0.1	0.09	0.22	0.16
Ottowia	0	0	0.01	0	0	0	0	0
Bergeyella	0.06	0.11	0.18	0.15	0.07	0.06	0.11	0.15
Pseudomonas	0.02	2.66	0.39	0.55	0.02	0.11	0.34	0.13
Acinetobacter	0.01	0.06	0.94	1.32	0.22	0.01	0.53	0
Mitsuaria	0	0	0	0.01	0	0	0	0.01
Peptoniphilus	0.05	0	0.38	0.04	0	0	0.04	0.02
Actinobacillus	0.34	0	0.55	0.25	0.28	0.16	0.01	0.32
Abiotrophia	0.12	0.24	0.13	0.27	0.03	0.05	0.03	0.01
Chryseobacterium	0	0.05	0.02	0.17	0.01	0	0.4	0
Cutibacterium	0.03	0.01	0	0.1	0.01	0	0.01	0.27
Desemzia	0	0	0	0	0	0	0	0
Tepidiphilus	0	0	0	0	0	0	0	0
Desulfobulbus	0.31	0.06	0	0	0.21	0.23	0.07	0.14

Pseudoramibacter	0.02	0	0.01	0	0.05	0.01	0.01	0.01
Bulleidia	0.06	0.03	0	0	0.06	0.01	0.07	0.03
Moryella	0	0	0	0	0.01	0.02	0.02	0
Lawsonella	0	0	0	0	0	0	0	0
Sphaerochaeta	0	0	0	0	0	0	0	0
Lactobacillus	0.02	0.05	0.02	0.04	0.01	0.33	0.01	0
Parascardovia	0.02	0.02	0.07	0	0	0	0	0
Dolosigranulum	0	0	0	0	0	0	0	0
Alloscardovia	0.01	0	0	0	0.01	0	0.01	0.03
Acidovorax	0	0.04	0.01	0	0	0	0.09	0
Corynebacterium_1	0	0	0	0	0	0	0	0
Methylibium	0	0	0	0	0	0	0	0
Vibrio	0	0	0	0	0	0	0	0
Staphylococcus	0	0	0	0	0	0	0	0.01
Enterococcus	0	0	0	0.05	0	0	0	0
Raoultella	0	0.01	0.01	0.01	0	0	0.17	7.55
Hydrogenophilus	0	0	0	0	0	0	0	0
Aeromonas	0	0	0	0	0	0	0.01	0
Anaerococcus	0	0	0	0	0	0	0	0
F0332	0	0.03	0.06	0.06	0.03	0	0	0.05
Brevundimonas	0	0.24	0.01	0.05	0.01	0.01	0.01	0
Mobiluncus	0	0	0	0	0	0	0	0
Cedecea	0	0.16	0.45	0.51	0.6	0.01	5.32	0.04
Microbacterium	0	0	0	0	0.11	0	0	0
Novosphingobium	0	0.01	0	0.05	0.01	0	0	0
Stenotrophomonas	0	0	0.01	0.02	0	0	0.01	0
Flavobacterium	0	0	0.01	0	0	0	0	0
Sphingobium	0	0.03	0	0.01	0	0	0	0
Sphingomonas	0	0.01	0	0.01	0	0	0	0
Luteimonas	0	0	0	0	0	0	0	0
Phenylobacterium	0	0.01	0	0	0	0	0	0

Roseateles	0	0	0	0	0	0	0	0
Aquabacterium	0	0	0	0	0	0	0	0
Curvibacter	0	0	0	0.01	0	0	0	0.09
Roseomonas	0	0.55	0.01	0.19	0	0	0.78	0
Terriglobus	0	0	0	0	0	0	0	0
Paracoccus	0	0.01	0	0	0	0	0	0
Wolinella	0.01	0	0	0	0	0	0	0
Gardnerella	0	0	0	0	0	0	0	0
Methylobacterium	0	0	0	0	0	0	0.01	0
Aestuariimicrobium	0	0.01	0	0.02	0	0	0	0
Rubrobacter	0	0	0	0.01	0	0	0	0
Cloacibacterium	0	0	0.1	0.16	0.01	0	1.01	0
Methyloversatilis	0	0	0	0.15	0	0	0	0
Coxiella	0	0	0	0	0	0.01	0.03	0.06
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0	0.01	0	0.06	0	0	0.03	0
Roseburia	0.01	0	0	0	0.02	0.07	0.04	0.03
Pelomonas	0	0.08	0	0.04	0	0	0	0
Blvii28_wastewater-sludge_group	0	0	0	0	0.02	0.07	0	0.02
Bacteroides	0	0	0	0	0.02	0	0	0.01
Brachymonas	0	0	0	0	0.01	0.03	0	0.01
Dechlorosoma	0	0.04	0	0.01	0	0	0.95	0
Xanthobacter	0	3.44	0	0	0	0	0.38	0
Diaphorobacter	0	0	0	0.01	0	0	0	0
Klebsiella	0	0	0.01	0	0	0	0.48	0
Dechloromonas	0	0	0	0	0.01	0	0	0
Reyranella	0	0	0	0	0	0	0	0
Variovorax	0	0	0	0	0	0	0	0
Azorhizobium	0	0.04	0	0	0	0	0.14	0
Bradyrhizobium	0	0	0	0	0	0	0	0
Chromobacterium	0	0	0.1	0	0.03	0	0	0

Ciceribacter	0	0.07	0.01	0	0	0	0	0
Lachnospiraceae_NK3A20_group	0	0	0	0	0	0.03	0	0
Alysiella	0	0	0	0.01	0	0	0	0.01
Enhydrobacter	0	0	0.05	0	0	0.09	0	0.39
Burkholderia-Caballeronia-Paraburkholderia	0	0	0	0	0	0	0	0.03
Desulfovibrio	0	0	0	0	0.01	0	0.04	0
Bosea	0	0.01	0	0	0	0	0.02	0
Legionella	0	0.01	0.02	0	0	0	0	0
Kaistia	0	0	0	0	0	0	0	0
H1	0	0	0	0	0	0	0	0
Achromobacter	0	0	0	0	0	0	0	0
Devosia	0	0	0	0	0	0	0	0
Flavitalea	0	0	0	0	0	0	0	0
Afipia	0	0.03	0	0	0	0	0.01	0
Erwinia	0	0	0.01	0	0	0.01	0.35	0
Ruminococcaceae_UCG-005	0	0	0	0	0	0	0	0
Ruminiclostridium_6	0	0	0	0	0	0	0	0
Simonsiella	0	0	0	0	0	0.01	0.58	3.54
Pleomorphomonas	0	0	0	0	0	0	0.6	0
Azospira	0	0.01	0	0	0	0	0.22	0
Family_XIII_AD3011_group	0	0	0	0	0	0.02	0.02	0
Massilia	0	0	0	0	0	0	0	0.03
Magnetospirillum	0	0.01	0	0	0.01	0	0.01	0
Ezakiella	0	0	0	0	0	0.03	0	0
Bacillus	0	0	0	0	0	0.01	0	0
Shinella	0	0	0	0	0	0	0.01	0.01
Paenibacillus	0	0	0.01	0	0	0	0	0
Atopostipes	0	0	0	0	0	0	0	0
Bdellovibrio	0	0	0	0	0	0	0	0
Schlegelella	0	0	0	0	0	0	0	0
Lelliottia	0	0	0	0	0	0	0	0

Hydrocarboniphaga	0	0	0	0	0	0	0	0
Piscinibacter	0	0	0	0	0	0	0	0
Rhodovarius	0	0	0	0	0	0	0	0
Cellvibrio	0	0	0	0	0	0	0	0
Chitinimonas	0	0	0	0	0	0	0	0
Prosthecobacter	0	0	0	0	0	0	0	0
Rhizorhapis	0	0	0	0	0	0	0	0
Erysipelotrichaceae_UCG-008	0	0	0	0	0	0	0	0
Marvinbryantia	0	0	0	0	0	0	0	0
Thermomonas	0	0	0.16	0	0	0	0	0
Ochrobactrum	0	0	0.01	0	0	0	0.03	0
Atlantibacter	0	0	0	0	0	0	0.03	0
Ornithinimicrobium	0	0	0	0	0	0	0	0.02
Rhizobacter	0	0	0	0	0	0	0	0.02
W5053	0	0	0	0	0.01	0	0	0
Acetobacter	0	0	0	0	0	0.01	0	0
Methanobrevibacter	0	0	0	0	0	0.01	0	0
Nitrospirillum	0	0	0	0	0	0	0	0
Anaerosporomusa	0	0	0	0	0	0	0.01	0
Exiguobacterium	0	0.01	0	0	0	0	0	0
Tessaracoccus	0	0	0.01	0	0	0	0	0
Pseudoxanthomonas	0	0.01	0	0	0	0	0	0
Angulomicrobium	0	0	0	0	0	0	0	0
Brevibacillus	0	0	0	0	0	0	0	0
Rhodopseudomonas	0	0	0	0	0	0	0	0
Flexilinea	0	0	0	0	0	0	0	0
Enterobacillus	0	0	0	0	0	0	0	0
Sphingopyxis	0	0	0	0	0	0	0	0
Acholeplasma	0	0	0	0	0	0	0	0
Dermacoccus	0	0	0	0	0	0	0	0
Aquitalea	0	0	0	0	0	0	0	0

Aquicella	0	0	0	0	0	0	0	0
Alishewanella	0	0	0	0	0	0	0	0
Kosakonia	0	0	0	0	0	0	0	0
Amaricoccus	0	0	0	0	0	0	0	0
Dysgonomonas	0	0	0	0	0	0	0	0
Arcanobacterium	0	0	0	0	0	0	0	0
Photobacterium	0	0	0	0	0	0	0	0
Rodentibacter	0	0	0	0	0	0	0	0
Pseudolabrys	0	0	0	0	0	0	0	0
Comamonas	0	0	0	0	0	0	0	0
Romboutsia	0	0	0	0	0	0	0	0
Leucobacter	0	0	0	0	0	0	0	0
Hydrotalea	0	0	0	0	0	0	0	0
Pseudacidovorax	0	0	0	0	0	0	0	0
Conchiformibius	0	0	0	0	0	0	0	0
Microbacter	0	0	0	0	0	0	0	0
Chryseolinea	0	0	0	0	0	0	0	0
Pirellula	0	0	0	0	0	0	0	0
Bilophila	0	0	0	0	0	0	0	0
Leminorella	0	0	0	0	0	0	0	0
Prevotellaceae_NK3B31_group	0	0	0	0	0	0	0	0
Anaerocolumna	0	0	0	0	0	0	0	0
Lactococcus	0	0	0	0	0	0	0	0
Atopobacter	0	0	0	0	0	0	0	0
Sebaldella	0	0	0	0	0	0	0	0
Micrococcus	0	0	0	0	0	0	0	0
Pedomicrobium	0	0	0	0	0	0	0	0
Paludibacterium	0	0	0	0	0	0	0	0

Font: SILVA database/MicrobiomeAnalyst

*p<0.05 on factor 1 (time); **p<0.05 on factor 2 (outcome).

Table S1. Average of data quality control of sequences generated by Miseq sequencer after data trimming and quality filtering. NST = Non-surgical treatment; ST = Surgical treatment; B = Baseline; 3M = Three months; 6M = Six months; 12M = Twelve months.

Groups	Time point	Base pair counts	Sequence cout	Sequence passed QC pipeline
NST	B	77,477,250	154,955	101011.7
	3M	88,317,750	176,636	113167.9
	6M	86,710,833	173,422	107325
	12M	74,621,611	149,243	94801
ST	B	95,575,045	191,150	117374.9091
	3M	94,081,818	188,164	116786.6364
	6M	89,100,500	178,201	114216.25
	12M	107,291,875	214,584	132660.125

Font: SILVA database/MicrobiomeAnalyst

Table S2. Summary of operational taxonomic units (OTUs) identified in PI samples sites, considering all taxonomic levels. NST: Non-surgical treatment; ST: Surgical treatment; B: Baseline; 3M: Three months; 6M: Six months; 12M: Twelve months; Med: Median, Q1: first quartile; Q3: third quartile.

Taxonomic Level	NST												ST											
	B			3M			6M			12M			B			3M			6M			12M		
	Med	Q1	Q3	Med	Q1	Q3	Med	Q1	Q3	Med	Q1	Q3	Med	Q1	Q3	Med	Q1	Q3	Med	Q1	Q3	Med	Q1	Q3
Domain	1	1	1.2	1	1	2	1	1	2	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1
Phylum	9.5	7.7	10	9	8	10	9	6.5	10	9	7.5	9.5	9	9	10	10	9	10	10	9	10	9.5	9	10
Class	14.5	12	16	14	12	15	15	9.5	15	13	11.5	15	15	13	16	15	13	15	15	13.2	15.7	15	14	16.5
Order	23.5	18.2	26.5	23.5	21.7	25.5	21	18.5	24	20	18.5	23.5	22	20	26	23	20	25	24.5	23.2	28.5	24.5	22.5	28.2
Family	34.5	28.7	42.25	37	33.7	39.2	34	25.5	38.5	31	27	35.5	35	30	41	36	32	39	39	35.5	45.5	38.5	34.2	43.2
Genus	53.5	40.7	66.7	56	50.7	64.7	54	42.5	66.5	48	37.5	57.5	55	35	67	53	44	63	63	54.7	73.7	66	54.2	71
Specie	46.5	30	55	47	37.7	54.7	45	25	58.5	35	25	45.5	45	26	68	42	33	61	54	42.7	68.5	49.5	44.2	66.2

Font: SILVA database/MicrobiomeAnalyst

Table S3: Abundance of all genera observed in submucosal biofilms communities from groups included in the study. NST: Non-surgical treatment; ST: Surgical treatment; B: Baseline; 3M: Three months; 6M: Six months; 12M: Twelve months.

Genus	NST				ST			
	B_NS	3M_NS	6M_NS	12M_NS	B_S	3M_S	6M_S	12M_S
Fusobacterium	169600	118745	104000	69150	169308	139889	67586	89128
Haemophilus	123215	104484	70332	84843	64922	49296	42823	51161
Streptococcus	120309	270152	80394	177306	179780	151605	113590	130215
Porphyromonas	64763	52611	24458	14727	93451	100288	26703	47131
Veillonella**	60456	41475	22227	18659	63108	90310	74487	131024
NA	42769	24677	95818	13044	62963	60038	68396	45329
Parvimonas	45615	47988	37767	23069	60175	41536	50737	39255
Neisseria	43405	75476	88288	70253	68922	23637	25088	47205
Prevotella	35646	33069	49233	26684	49033	58438	30892	51185
Prevotella_7	33199	22203	26678	8953	48019	34333	45463	55912
Aggregatibacter	23838	31522	27036	9801	4839	14483	1451	461
Dialister	15670	11287	21479	15064	32963	27097	13237	21072
F0058	15421	10261	4137	3163	5044	8332	1294	683
Tannerella	14040	5805	3371	3748	21344	37659	6235	4360
Filifactor	13130	1375	8026	6660	7903	26631	4289	596
Selenomonas	12458	2381	9728	1771	13975	14825	15994	18421
Leptotrichia	11643	17998	13988	13098	30530	15877	18830	15713
Gemella	10818	19841	8032	6476	11786	7387	3778	3615
Actinomyces**	9248	15328	8707	9115	15127	36919	12326	23898
Lautropia	9574	10514	8374	5400	5277	1933	1536	32545

Treponema_2	8970	11873	14280	4875	21117	21798	24860	21792
Rothia	9274	54555	11693	28776	26403	90669	26715	37494
Capnocytophaga	8569	13279	18131	10216	6940	15017	9134	9309
Campylobacter	8586	6806	16896	5016	10854	16117	8267	9978
Cedecea	8147	125	9350	479	90	1347	66946	3303
Fretibacterium*	7817	3035	4862	147	22087	16118	4397	5574
Atopobium**	7491	2408	8552	501	43704	11039	17688	20977
Granulicatella	7102	16220	7951	11865	7478	10457	6406	12731
Actinobacillus	6888	2530	2781	4051	84	24	16	1573
Alloprevotella	6106	9385	12296	8623	15214	8951	5936	15361
Oribacterium	3969	4814	1772	1350	5052	2382	2701	6230
Catonella	3275	2144	2717	973	5233	5458	4674	4998
Selenomonas_3**	2931	3609	4679	2423	5198	13121	15398	10489
Acinetobacter	3156	554	9016	26	6	146	2995	8374
Desulfobulbus	2970	44	266	1824	2745	4146	770	34
Prevotella_2	2377	5302	6014	2512	4730	2886	2087	2239
Lachnoanaerobaculum	1528	2119	1773	1421	2987	2873	2528	2318
Peptostreptococcus	2206	4405	2202	2159	3282	1818	3535	5842
Anaeroglobus	1741	2230	4434	5901	20883	9833	2607	4793
Peptoanaerobacter	1715	528	2157	774	1095	2011	674	82
Peptococcus	1670	1106	693	319	1102	1425	744	183
Johnsonella	1525	1554	1567	1421	3196	1925	3183	2256
Eikenella	1670	2276	1332	853	3725	4692	733	992
Corynebacterium	1572	2465	1156	793	2975	4677	4263	2190
Microbacterium	1476	0	0	0	0	23	0	0
Mogibacterium	1424	769	2316	1003	1796	4182	10662	4377
Shuttleworthia	1297	1771	1042	4	6452	1512	956	810
Abiotrophia	1356	1555	661	360	168	1239	397	1397

Selenomonas_4	1216	650	1345	672	4247	1995	2390	4412
Bergeyella	1104	1249	1893	2038	500	625	510	748
Pseudopropionibacterium	1157	2222	2503	2141	644	2289	152	188
Cardiobacterium	1076	3005	3031	2572	1955	14123	1853	1265
Kingella	1050	3929	1145	4447	7875	4466	2747	2412
Olsenella	1037	278	495	191	4829	1933	4166	3556
Mycoplasma	1036	596	382	421	1266	1122	2849	1736
Slackia	1052	1062	1664	567	4422	1750	1035	2871
Solobacterium	902	415	901	321	1097	568	1291	889
Bulleidia	808	177	246	28	542	251	764	300
Stomatobaculum	542	732	820	369	3100	4656	509	2978
Scardovia	658	835	67	11	452	140	91	381
Pseudomonas***	412	23880	5701	2720	49	383	892	2406
Rikenellaceae_RC9_gut_group	466	403	439	102	805	895	410	759
Megasphaera	562	164	186	45	3016	1389	3033	7321
Ruminococcaceae_UCG-014	441	384	79	59	1020	180	90	202
Prevotella_6	146	1250	686	918	1905	1191	2760	3514
Chromobacterium	390	0	472	0	0	0	10	13
Howardella	353	202	232	54	205	161	220	226
Family_XIII_UCG-001	313	258	957	0	906	1605	269	440
F0332	331	199	29	672	49	50	298	387
Pseudoramibacter	338	111	177	172	484	38	50	30
Bacteroides	317	0	3	0	0	0	2	191
Candidatus_Saccharimonas	240	677	583	543	2332	632	635	192
Chryseobacterium	154	465	5530	12	0	18	126	1100
Defluviitaleaceae_UCG-011	198	78	82	43	240	1277	1079	950
Lactobacillus	74	5127	7	10	264	483	179	242
Propionivibrio	191	69	203	45	316	125	99	0

Eggerthia	211	39	29	0	123	2	3	70
Novosphingobium	124	2	19	0	14	91	0	376
Bifidobacterium	77	46	1785	0	9477	909	7068	5378
Brevundimonas	120	1498	97	131	0	693	45	231
Phocaeicola	141	153	128	8	877	1407	160	329
Cutibacterium	79	74	109	3995	389	28	110	33
Moryella	88	27	61	0	83	260	275	10
Desulfovibrio	105	0	422	0	0	0	89	0
Centipeda	26	73	252	141	242	734	1427	62
Butyrivibrio_2	90	363	162	771	121	808	744	398
Roseburia	108	0	0	0	299	1133	513	434
Sphingomonas	34	89	5	78	0	0	5	3
Flavobacterium	50	21	0	4	0	26	47	0
Stenotrophomonas	57	3	95	0	0	25	13	119
Phenylobacterium	37	40	17	0	0	7	34	13
Staphylococcus	42	16	34	79	35	0	0	0
Alloscardovia	68	15	105	0	139	38	52	424
Roseomonas	17	42	2308	0	0	4659	8584	1176
Erysipelotrichaceae_UCG-006	36	81	165	0	288	66	20	27
Methylobacterium	13	5	121	0	3	0	0	24
Xanthobacter	0	0	3961	0	0	29099	1282	0
Brachymonas	32	12	21	0	174	522	43	77
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0	21	364	24	0	57	100	405
Raoultella	50	0	1925	96692	14	91	549	72
Prevotella_1	7	1147	597	0	151	74	283	1258
Sphingobium	33	236	9	33	0	3	8	7
Cloacibacterium	0	29	14052	0	84	0	496	1000

Luteimonas	29	0	0	0	0	0	8	0
Aeromonas	3	4	99	0	26	0	0	0
Ciceribacter	12	652	28	21	0	0	37	5
Aquabacterium	22	0	0	0	0	0	0	18
Azorhizobium	0	0	1916	0	0	321	0	0
Paracoccus	12	79	0	8	0	14	9	0
Peptoniphilus	8	19	599	200	428	0	1846	251
H1	9	0	0	0	5	0	0	0
Klebsiella	17	6	6718	0	0	46	33	27
Roseateles	27	0	0	0	0	0	0	0
Corynebacterium_1	5	9	9	0	6	0	0	3
Curvibacter	23	0	0	0	0	0	51	1265
Acholeplasma	22	0	0	0	0	0	0	0
Enhydrobacter	0	1404	256	5005	0	0	0	0
Propionibacterium	0	0	3	124	459	883	428	84
Cryptobacterium	0	272	131	0	465	33	117	288
Burkholderia-Caballeronia-Paraburkholderia	0	0	6	448	0	0	30	0
Bosea	0	93	231	0	0	7	0	0
Alysiella	0	0	11	141	13	0	0	4
Legionella	0	0	4	0	0	117	80	0
Kaistia	0	0	59	0	0	0	0	0
Bradyrhizobium	0	0	21	0	0	0	8	6
Wolinella	11	0	0	0	56	0	0	0
Enterococcus	9	0	0	0	0	0	0	331
Coxiella	6	0	2	0	36	178	370	729
Terriglobus	14	0	0	0	0	0	0	0
Massilia	3	0	0	357	0	0	0	0
Desemzia	13	0	0	0	0	0	0	0

Magnetospirillum	2	59	145	0	137	0	0	0
Simonsiella	0	145	8058	45335	0	0	0	0
Dechlorosoma	0	328	13172	0	15	0	80	59
Pleomorphomonas	0	0	8393	0	5	32	8	0
Azospira	0	57	3002	0	65	0	0	0
Pelomonas	0	0	4	0	0	672	0	261
Parascardovia	12	16	0	0	181	209	337	29
Family_XIII_AD3011_group	0	0	0	0	45	298	317	0
Lachnospiraceae_NK3A20_group	0	0	0	0	15	525	56	13
Pyramidobacter	0	142	269	0	89	0	2	0
Ezakiella	0	423	0	0	0	0	0	0
Dechloromonas	0	0	0	0	163	0	0	21
Bacillus	0	0	0	0	0	134	66	0
Lawsonella	0	8	20	8	35	0	0	43
DNF00809	0	0	0	0	156	31	0	13
Shinella	0	0	106	107	0	0	0	0
Paenibacillus	0	0	10	0	0	0	65	0
Rubrobacter	0	9	5	38	0	0	0	10
Atopostipes	0	0	0	0	0	0	58	0
Achromobacter	0	0	0	0	0	0	28	7
Bdellovibrio	0	0	0	0	0	0	45	0
Variovorax	0	0	0	0	0	0	0	19
Devosia	0	0	0	0	0	4	0	7
Tepidiphilus	9	0	0	0	0	0	0	0
Erwinia	8	0	0	0	0	159	5002	10
Mobiluncus	7	6	4	0	0	0	0	0
Acidovorax	0	291	1334	0	69	42	0	82
Blvii28_wastewater-sludge_group	0	97	0	0	337	937	6	221

Methyloversatilis	0	0	6	0	0	0	0	948
Thermomonas	0	0	776	0	0	0	0	0
Ochrobactrum	0	0	371	0	0	0	22	0
Atlantibacter	0	0	354	0	0	0	0	0
Afipia	0	2	74	0	0	245	0	11
Ornithinimicrobium	0	0	0	252	0	0	0	0
Rhizobacter	0	0	0	243	0	0	0	0
Aestuariimicrobium	0	76	0	102	0	0	0	0
W5053	0	0	0	21	116	0	18	0
Acetobacter	0	148	0	0	0	0	0	0
Methanobrevibacter	0	15	15	0	0	115	0	0
Mitsuaria	0	0	0	0	3	0	0	117
Nitrospirillum	0	0	0	0	0	39	36	0
Anaerosporomusa	0	0	75	0	0	0	0	0
Ottowia	0	7	0	0	17	15	34	0
Exiguobacterium	0	72	0	0	0	0	0	0
Tessaracoccus	0	0	70	0	0	0	0	0
Pseudoxanthomonas	0	68	0	0	0	0	0	0
Angulomicrobium	0	0	64	0	0	0	0	0
Sphaerochaeta	0	17	41	0	2	0	0	0
Brevibacillus	0	0	0	0	0	59	0	0
Diaphorobacter	0	0	0	0	0	0	0	44
Rhodopseudomonas	0	0	42	0	0	0	0	0
Flexilinea	0	0	0	0	0	37	0	0
Reyranella	0	5	0	0	0	0	0	26
Dolosigranulum	0	0	0	0	28	0	0	0
Enterobacillus	0	0	27	0	0	0	0	0
Sphingopyxis	0	25	0	0	0	0	0	0

Hydrogenophilus	0	0	4	0	13	0	0	0
Dermacoccus	0	0	0	0	0	0	15	0
Aquitalea	0	0	0	0	0	8	7	0
Flavitalea	0	0	0	0	0	0	0	14
Aquicella	0	0	0	0	0	0	13	0
Anaerococcus	0	0	0	0	12	0	0	0
Alishewanella	0	0	0	0	0	10	0	0
Ruminococcaceae_UCG-005	0	0	0	0	0	0	0	10
Ruminiclostridium_6	0	0	0	0	0	0	0	10
Schlegelella	0	0	0	0	0	0	0	9
Kosakonia	0	0	0	0	0	0	9	0
Amaricoccus	0	9	0	0	0	0	0	0
Dysgonomonas	0	0	0	0	8	0	0	0
Lelliottia	0	0	0	0	0	0	0	8
Arcanobacterium	0	0	0	8	0	0	0	0
Hydrocarboniphaga	0	0	0	0	0	0	0	7
Piscinibacter	0	0	0	0	0	0	0	7
Photobacterium	0	0	0	0	0	0	6	0
Rodentibacter	0	0	0	0	6	0	0	0
Pseudolabrys	0	0	6	0	0	0	0	0
Comamonas	0	0	6	0	0	0	0	0
Rhodovarius	0	0	0	0	0	0	0	6
Methylibium	0	0	0	0	6	0	0	0
Romboutsia	0	0	0	5	0	0	0	0
Leucobacter	0	0	0	0	0	0	5	0
Hydrotalea	0	0	5	0	0	0	0	0
Pseudacidovorax	0	5	0	0	0	0	0	0
Conchiformibius	0	0	0	0	0	0	5	0

Vibrio	0	0	0	0	5	0	0	0
Microbacter	0	5	0	0	0	0	0	0
Gardnerella	0	0	0	0	4	0	0	0
Cellvibrio	0	0	0	0	0	0	0	4
Chryseolinea	0	0	0	0	0	4	0	0
Pirellula	0	0	0	0	0	0	4	0
Chitinimonas	0	0	0	0	0	0	0	4
Bilophila	0	4	0	0	0	0	0	0
Leminorella	0	0	0	3	0	0	0	0
Prevotellaceae_NK3B31_group	0	0	0	0	0	0	3	0
Prosthecobacter	0	0	0	0	0	0	0	3
Anaerocolumna	0	0	3	0	0	0	0	0
Lactococcus	0	0	3	0	0	0	0	0
Atopobacter	0	0	0	3	0	0	0	0
Sebaldella	0	0	0	0	0	3	0	0
Micrococcus	0	0	3	0	0	0	0	0
Rhizorhapis	0	0	0	0	0	0	0	3
Pedomicrobium	0	0	0	2	0	0	0	0
Erysipelotrichaceae_UCG-008	0	0	0	0	0	0	0	2
Marvinbryantia	0	0	0	0	0	0	0	2
Paludibacterium	0	0	2	0	0	0	0	0

Font: SILVA database/MicrobiomeAnalyst

*p<0.05 on factor 1 (time); **p<0.05 on factor 2 (group); ***p<0.05 on factors 1 and 2 (time and group).

Table S4. Summary of total genus abundance (total reads) observed in PI biofilm microbiome. NST: Non-surgical treatment; ST: Surgical treatment; B: Baseline; 3M: Three months; 6M: Six months; 12M: Twelve months; Min: minimum; Max: maximum Med: Median, Q1: first quartile; Q3: third quartile.

	NST				ST			
	B	3M	6M	12M	B	3M	6M	12M
Min	0	0	0	0	0	0	0	0
Q1	0	0	0	0	0	0	0	0
Med	6.5	8.5	28.5	0	5.0	7.5	13.0	10.5
Q3	634	636.5	1342	408	764.8	1177	768.5	869.3
Max	169600	270152	104000	177306	179780	151605	113590	131024

Font: SILVA database/MicrobiomeAnalyst

Table S5. Summary for observed genus richness in PI biofilm microbiota. NST: Non-surgical treatment; ST: Surgical treatment; B: Baseline; 3M: Three months; 6M: Six months; 12M: Twelve months; Med: Median, Q1: first quartile; Q3: third quartile; Min: minimum; Max: maximum.

	NST				ST			
	B	3M	6M	12M	B	3M	6M	12M
Min	82	126	56	69	77	31	65	124
Q1	95.5	142	108	90	80	120	144.8	147.3
Med	157.5	190.5	171	135	151	160	207.5	214
Q3	241.8	199.5	221	183	295	217	294.3	250.8
Max	245	209	234	256	388	277	339	267
Mean	169.5	177.7	160.1	141.6	190.5	161.3	211.6	204.1

Font: SILVA database/MicrobiomeAnalyst

Table S6. OTUs relative abundance identified in PI baseline (before treatment), successful and unsuccessful post treatment biofilms (%(n)). B: Baseline; 3M: Three months; 6M: Six months; 12M: Twelve months.

	Successful				Unsuccessful			
	B (108)	3M (120)	6M (114)	12M (124)	B (138)	3M (132)	6M (165)	12M (133)
Very abundant (≥1%)	19.44 (21)	17.5 (21)	18.42 (21)	16.93 (21)	13.76 (19)	16.66 (22)	13.33 (22)	14.28 (19)
Moderately abundant (≥0.1%; <1%)	28.70 (31)	20.83 (25)	32.45 (37)	25.80 (32)	23.91 (33)	24.24 (32)	26.66 (44)	27.81 (37)
Rare (<0.1%)	51.85 (56)	61.66 (74)	49.12 (56)	57.25 (71)	62.31 (86)	59.09 (78)	60.0(99)	57.89 (77)

Font: SILVA database/MicrobiomeAnalyst

Table S7. Exclusive genera identified in PI baseline, successful and unsuccessful post treatment biofilms.

PI Baseline	%	Successful	%	Unsuccessful	%
Microbacterium	0.0676%	Methyloversatilis	0.1778%	Simonsiella	3.2822%
Magnetospirillum	0.0058%	Pelomonas	0.0489%	Enhydrobacter	0.3624%
Desulfovibrio	0.0048%	Aestuariimicrobium	0.0191%	Ornithinimicrobium	0.0182%
Pyramidobacter	0.0037%	Diaphorobacter	0.0083%	Rhizobacter	0.0176%
Wolinella	0.0028%	Flavitalea	0.0026%	Shinella	0.0077%
Azospira	0.0027%	Ruminococcaceae_UCG-005	0.0019%	Reyranella	0.0019%
Family_XIII_AD3011_group	0.0019%	Ruminiclostridium_6	0.0019%	Variovorax	0.0014%
Luteimonas	0.0013%	Achromobacter	0.0013%	Afipia	0.0008%
Roseateles	0.0012%	Hydrocarboniphaga	0.0013%	Schlegelella	0.0007%
Aeromonas	0.0012%	Piscinibacter	0.0013%	Lelliottia	0.0006%
Dolosigranulum	0.0012%	Erysipelotrichaceae_UCG-008	0.0004%	Arcanobacterium	0.0006%
Acholeplasma	0.0010%			Devosia	0.0005%
Ottowia	0.0007%			Bradyrhizobium	0.0004%
Terriglobus	0.0006%			Rhodovarius	0.0004%
H1	0.0006%			Romboutsia	0.0004%
Desemzia	0.0006%			Cellvibrio	0.0003%
Hydrogenophilus	0.0005%			Chitinimonas	0.0003%
Anaerococcus	0.0005%			Prosthecobacter	0.0002%
Tepidiphilus	0.0004%			Rhizorhapis	0.0002%
Dysgonomonas	0.0003%			Leminorella	0.0002%
Mobiluncus	0.0003%			Atopobacter	0.0002%
Methylibium	0.0002%			Marvinbryantia	0.0001%
Rodentibacter	0.0002%			Pedomicrobium	0.0001%
Vibrio	0.0002%				
Pleomorphomonas	0.0002%				
Gardnerella	0.0002%				
Sphaerochaeta	0.0001%				

PARTE III

Considerações finais

Avanços recentes na tecnologia de sequenciamento de DNA e bioinformática têm permitido aprofundar ainda mais estudos de biofilmes relacionados a dentes e implantes dentários, levando o campo da microbiologia oral à uma nova perspectiva (Griffen et al., 2012; Zaura, Keijser, Huse, & Crielaard, 2009). Nesta tese, usando a tecnologia de sequenciamento de alto rendimento do gene 16S rRNA, objetivamos avançar no conhecimento sobre as comunidades microbianas que se aderem à superfícies de implantes e ao meio peri-implantar e que podem, muitas vezes levar à perda do implante. Com o crescimento do número de implantes dentários instalados, diferentes superfícies de titânio têm sido estudadas nos últimos anos para buscar uma melhor biocompatibilidade juntamente com a redução da adesão bacteriana (Qin et al., 2015; Schmidlin P.R., Müller P., Attin T., Wieland M., Hofer D. et al., 2013; X. Zhao, Liu, Zheng, Cao, & Liu, 2014). Em virtude disso, o propósito do **Capítulo I** desta tese foi comparar o perfil das comunidades microbianas que colonizam os implantes com superfícies jateadas com areia e tratadas com ácido (SLA) com superfícies usinadas. De acordo com os resultados encontrados, as duas superfícies estudadas são colonizadas por microorganismos semelhantes, suportando, desta forma, a hipótese de que a topografia da superfície não interfere na adesão bacteriana inicial, corroborando com estudos prévios (Frojd et al., 2011; Schmidlin P.R., Müller P., Attin T., Wieland M., Hofer D. et al., 2013). Um achado interessante foi a presença dos gêneros de bactérias anaeróbias Gram negativas *Porphyromonas, Kingella, Anaeroglobus, Tanerella,* TG5, *Selenomonas* e *Eikenella* nas amostras de superfícies de titânio, visto que estas espécies são mais comumente encontradas em estágios avançados de maturação do biofilme (Rehman et al.,

2012). No entanto, diversos estudos já demonstraram que pode existir transmissão de microorganismos de um nicho para outro em um mesmo indivíduo, o que explica nichos periodontais e peri-implantares serem muitas vezes semelhantes (Agerbaek, Lang, & Persson, 2006; Kohavi, Klinger, Steinberg, & Sela, 1995; Å. Leonhardt et al., 1993; Mombelli, Nyman, Brägger, Wennström, & Lang, 1995; Papaioannou, Quirynen, & Van Steenberghe, 1996; M Quirynen, Papaioannou, & van Steenberghe, 1996; Marc Quirynen et al., 2006; Sumida, Ishihara, Kishi, & Okuda, 2002; Takanashi, Kishi, Okuda, & Ishihara, 2004). O fato de termos encontrado espécies que não são comumente vistas em estágios iniciais da formação do biofilme e elas estarem em baixa abundância pode ser explicado por um estudo publicado recentemente por Welch e colaboradores onde é sugerido a hipótese do especialista do local (Site-Specialist Hypothesis) que pressupõe que a maioria dos microorganismos são encontrados primeiramente em apenas um dos principais tipos de habitats da boca. Foi observado que um conjunto bem definido e consistente de espécies bacterianas é rotineiramente observado compondo a maior parte do microbioma oral e que esse conjunto de espécies difere de um local para outro na boca, mas não de pessoa para pessoa. A hipótese prevê que um microorganismo se localize ativamente em seu local preferido e cresça e se divida lá; fora dele, ele será detectado em abundância muito menor e exibirá metabolismo, expressão gênica e organização espacial alteradas. Apesar de não existir um ponto de corte absoluto de abundância ou prevalência que identifique um residente, a distinção é importante para o desenvolvimento de uma compreensão da função da comunidade microbiana (Mark Welch, Dewhirst, & Borisy, 2019). Ainda que numerosos estudos tenham mostrado que indivíduos carregam microbiomas orais estatisticamente distintos uns dos outros (Hall et

al., 2017; Lloyd-Price et al., 2017; Utter, Mark Welch, & Borisy, 2016; Zaura et al., 2009), estudos realizados com resolução a nível de espécie também demonstraram que as espécies mais abundantes são amplamente compartilhadas entre os indivíduos e que os indivíduos são diferenciados por conjuntos de linhagens dentro dessas espécies, bem como por proporções dos principais táxons (Aas et al., 2005; Al-Hebshi, Abdulhaq, Albarrag, Basode, & Chen, 2016; Bik et al., 2010; Hall et al., 2017; Lloyd-Price et al., 2017; Mark Welch et al., 2014; Sato et al., 2015; Utter et al., 2016; Zaura et al., 2009).

Sabendo que nichos diferentes possuem comunidades microbiológicas distintas e que a PI estabelecida progride mais rapidamente do que a P e, com isso, seu tratamento é mais complexo (Berglundh et al., 2018), achamos plausível e necessário conhecer melhor a microbiota peri-implantar, visto que esta permanece incerta (Rakic et al., 2016; Renvert & Quirynen, 2015). Embora o número de estudos usando metodologias de sequenciamento do gene 16S estejam crescendo, os dados brutos e isolados são complexos e difíceis de interpretar. Assim, no **capítulo II** decidimos fazer uma revisão sistemática da literatura para verificar o conhecimento combinado de estudos que utilizaram métodos de sequenciamento do gene 16S e revelar padrões microbianos cuja contribuição à saúde e à doença peri-implantar poderia auxiliar em investigações futuras sobre estas doenças. Nesta revisão mostramos que os gêneros mais comumente encontrados na PI foram *Actinomyces, Eubacterium, Fusobacterium, Mogibacterium, Moraxella, Treponema* e *Porphyromonas*. Já na saúde peri-implantar os gêneros mais encontrados foram *Streptococcus, Prevotella, Haemophilus* e *Leptotrichia*. Através dos índices de diversidade utilizados pelos estudos, pudemos observar que, na maioria deles, a diversidade microbiana aumenta com o progresso da doença. As espécies detectadas em

baixa abundância são provavelmente as responsáveis para este aumento de diversidade e contribuem para uma variabilidade interindividual da microbiota oral (Zaura et al., 2009).

Com os resultados obtidos na revisão sistemática do capítulo II, pudemos observar uma alta heterogeneidade nos estudos com relação às diferentes plataformas de sequenciamento utilizadas. Além disso, as regiões amplificadas do gene 16S variaram de V1 a V9, não fornecendo, desta forma, um padrão ou consenso sobre a região ideal para melhor cobertura e especificidade na identificação de diferentes comunidades microbianas. Desta forma, no **capítulo III** desta tese avaliamos as regiões V3-V4, V4 e V5-V6 através dos pares de primers 341F-805R, 347F-803R, 515F-806R e 609D-699R, com o objetivo de explorar de que forma diferentes regiões e primers afetam a métrica de diversidade bacteriana em locais com peri-implantite. Apesar da abundância de vários grupos taxonômicos terem diferido marcadamente entre os pares de primers estudados, em geral, os gêneros mais abundantes foram semelhantes. Neste estudo demonstramos que a microbiota de amostras de peri-implantite foram similares entre diferentes primers e regiões variáveis, porém, diferentes entre pacientes, sugerindo que as amostras e não os primers determinaram a composição microbiana. Através dos índices de diversidade e riqueza, verificamos que, apesar de não ter significância estatística, foi detectado um tamanho de efeito grande nestas métricas de diversidade. Desta forma, o par de primer 609D-699R (região V5-V6) parece ser mais adequado para estudos de microbioma oral em estado de doença. No entanto, é importante notar que embora a capacidade de detectar o máximo de espécies possível seja crucial em estudos de diversidade microbiana, a falta de padrão ouro ou conhecimento do número real de espécies na amostra não nos permite determinar o grau do erro de detecção. Os resultados apresentam um guia útil para

melhorar o conhecimento atual sobre as práticas de sequenciamento e também fornecem uma referência importante sobre qual conjunto de primers e região escolher ao trabalhar com diversidade bacteriana de PI.

O monitoramento das alterações no microbioma é uma aplicação promissora no diagnóstico e prognóstico da doença, pois estas mudanças podem contribuir para a patogênese de muitas doenças e refletem a saúde ou o estado da doença do hospedeiro (Cho & Blaser, 2012; Huang et al., 2014; Pflughoeft & Versalovic, 2012). Desta forma, no **capítulo IV** realizamos um ensaio clínico randomizado para melhor compreender o mecanismo polimicrobiano da PI antes e após o tratamento cirúrgico ou não cirúrgico da doença. Adicionalmente, comparamos a microbiota submucosa dos implantes que obtiveram sucesso (-SS) com aqueles que continuaram com doença (+SS) após os tratamentos. Novamente utilizamos o sequenciamento do gene 16S rRNA e optamos por utilizar o par de primer 609D-699R por ter apresentado no estudo anterior (capítulo III) um resultado ligeiramente mais acurado com relação à diversidade encontrada. Ao comparar o tratamento cirúrgico com o tratamento não cirúrgico não houve diferença entre eles com relação aos parâmetros clínicos. Embora tenha havido mudanças na abundância de alguns táxons, os índices de diversidade não mostraram diferenças significativas entre os implantes que obtiveram sucesso e os que falharam após o tratamento. Tendo em vista que todas as amostras eram do mesmo local (peri-implantar), sugerimos que o local influencia mais a composição microbiana do que um resultado bem ou malsucedido, o tipo de tratamento realizado ou mesmo a evolução ao longo do tempo após o tratamento. Apesar de sucesso e fracasso pós-tratamento terem apresentado um padrão semelhante do ponto de vista composicional, os dois grupos parecem apresentar

um padrão diferente do ponto de vista das interações bacterianas da mucosa peri-implantar. Desde antes do tratamento, os pacientes que obtiveram sucesso apresentaram interacões mais fortes, tanto positivas quanto negativas, e em maior número do que os pacientes que permaneceram com a doença, e esse padrão se manteve ao longo do estudo. Esse resultado pode sugerir que, em pacientes que não tiveram sucesso após o tratamento, as bactérias podem ser mais autossuficientes, não precisando interagir de forma positiva ou negativamente com outras espécies, sendo mais resistentes e independentes do que os componentes de uma comunidade que melhorou clinicamente.

Estudos recentes, utilizando novas abordagens baseadas em genes, mostraram que a ênfase em bactérias específicas ou apenas naquelas em grande abundância não captura toda a variabilidade nos locais doentes (Griffen et al., 2012; Liu et al., 2012). Além disso, as bactérias não são as únicas responsáveis pela virulência encontrada em locais com doença, mas sim a resposta à interação entre elas e os hospedeiros (Casadevall & Pirofski, 2003). Os microrganismos menos resilientes, frequentemente detectados em baixa abundância, contribuem para a alta diversidade e variabilidade interindividual do microbioma oral (Zaura et al., 2009). De fato, nos estudos apresentados nesta tese, observamos poucos gêneros em grande abundância e a maioria dos gêneros estava presente em abundância muito baixa. O significado desta distribuição de cauda longa ainda é desconhecido, no entanto, é sugerido ser um reservatório de espécies que pode modular sua abundância ao longo do tempo, desempenhando um papel protetor e fornecendo redundância funcional onde várias espécies de uma variedade de grupos taxonômicos podem desempenhar papéis idênticos ou semelhantes na funcionalidade do ecossistema (Zaura et al., 2009). Esta redundância funcional pode explicar a variabilidade

observada de indivíduo para indivíduo em microbiomas humanos, no entanto, ainda não entendemos por que os indivíduos diferem entre si nas proporções de taxa microbiana maior ou menor, nem em que grau essas diferenças podem ser causadas por dieta, ambiente, fatores culturais, genética ou acidente histórico, porém, sabemos que esses fatores provavelmente contribuem diretamente para os resultados díspares obtidos por vários estudos de associação que examinam a microbiota peri-implantar.

A evolução de novas plataformas de sequenciamento e novas ferramentas computacionais está melhorando a aquisição e análise de dados, mudando assim a forma como vemos o microbioma (Sultan, Kong, Rizk, & Jabra-Rizk, 2018). Com isso, em um curto espaço de tempo, o sequenciamento completo do gene 16S rRNA deve rapidamente se tornar um método comum para avaliar bactérias em diferentes amostras e o conhecimento da diversidade taxonômica e funcional das comunidades microbianas avançará em resolução sem precedentes. Além disso, com a grande variedade de microrganismos presentes nos biofilmes, a interpretação dos dados microbianos pode ser difícil e, por esse motivo, mais estudos são necessários para melhorar nossa compreensão das interações metabólicas e expressão gênica por meio de análises metatranscriptômicas e metabolômicas, além de adaptações dos nossos métodos estatísticos devido a grande variabilidade e extensão dos dados do microbioma.

Por fim, nesta tese foi possível constatar que: 1) As duas superfícies de titânio estudadas são colonizadas por microorganismos semelhantes, suportando, desta forma, a hipótese de que a topografia da superfície não interfere na adesão bacteriana inicial. 2) os gêneros mais comumente encontrados na PI foram *Actinomyces, Eubacterium, Fusobacterium, Mogibacterium, Moraxella, Treponema* e *Porphyromonas*. Já na saúde

peri-implantar os gêneros mais encontrados foram *Streptococcus, Prevotella, Haemophilus* e *Leptotrichia.* Através dos índices de diversidade utilizados pelos estudos, pudemos observar que, na maioria deles, a diversidade microbiana aumenta com o progresso da doença. 3) A microbiota de amostras de peri-implantite foram similares entre diferentes primers e regiões variáveis, porém, diferentes entre pacientes, sugerindo que as amostras e não os primers determinaram a composição microbiana. 4) O perfil e a diversidade das comunidades microbianas submucosas foram semelhantes antes e depois dos tratamentos cirúrgico ou não cirúrgico da peri-implantite. Nenhum tratamento mostrou-se superior ao outro. 5) A composição e diversidade microbiana também se mostraram semelhantes entre os pacientes que obtiveram sucesso e os que não tiveram sucesso após o tratamento. Entretanto, interações positivas e negativas maiores e mais fortes foram observadas entre os microrganismos de pacientes do grupo sucesso, mesmo antes do tratamento ter sido feito. Essas observações podem indicar que o grau de interação e sua força podem ser um indicador de como os pacientes responderão ao tratamento. Com isso, o conhecimento das mudanças dinâmicas no microbioma e, principalmente, como as bactérias estão interagindo na comunidade peri-implantar em diferentes estados clínicos pode ser altamente útil no diagnóstico e prognóstico da PI em estudos clínicos e, em breve, potencialmente na prática clínica.

REFERÊNCIAS

A. Jemat,1 M. J. Ghazali,1 M. Razali, 2 and Y. Otsuka3. (2015). Surface Modifications and Their Effects on Titanium Dental Implants - PubMed. *BioMed Research International*. Retrieved from https://pubmed.ncbi.nlm.nih.gov/26436097/?from_term=Surface+Modifications+and+Their+Effects+on+Titanium+Dental+Implants&from_pos=1

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, *43*(11), 5721–5732. https://doi.org/10.1128/JCM.43.11.5721-5732.2005

Agerbaek, M. R., Lang, N. P., & Persson, G. R. (2006). Comparisons of bacterial patterns present at implant and tooth sites in subjects on supportive periodontal therapy: I. Impact of clinical variables, gender and smoking. *Clinical Oral Implants Research*, *17*(1), 18–24. https://doi.org/10.1111/j.1600-0501.2005.01190.x

Al-Hebshi, N. N., Abdulhaq, A., Albarrag, A., Basode, V. K., & Chen, T. (2016). Species-level core oral bacteriome identified by 16S rRNA pyrosequencing in a healthy young Arab population. *Journal of Oral Microbiology*, *8*(1). https://doi.org/10.3402/jom.v8.31444

Arciola, C. R., Campoccia, D., Speziale, P., Montanaro, L., & Costerton, J. W. (2012, September). Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. *Biomaterials*. Biomaterials. https://doi.org/10.1016/j.biomaterials.2012.05.031

Atieh, M. A., Alsabeeha, N. H. M., Faggion, C. M., & Duncan, W. J. (2012). The Frequency of Peri-Implant Diseases: A Systematic Review and Meta-Analysis. *Journal of Periodontology*, *84*(11), 1–15. https://doi.org/10.1902/jop.2012.120592

Belibasakis, G. N., Charalampakis, G., Bostanci, N., & Stadlinger, B. (2015). Peri-implant infections of oral biofilm etiology. *Advances in Experimental Medicine and Biology*, *830*, 69–84. https://doi.org/10.1007/978-3-319-11038-7_4

Berglundh, T., Armitage, G., Araujo, M. G., Avila-Ortiz, G., Blanco, J., Camargo, P. M., … Zitzmann, N. (2018). Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *Journal of Periodontology*, *89*, S313–S318. https://doi.org/10.1002/JPER.17-0739

Bik, E. M., Long, C. D., Armitage, G. C., Loomer, P., Emerson, J., Mongodin, E. F., … Relman, D. A. (2010). Bacterial diversity in the oral cavity of 10 healthy individuals. *ISME Journal*, *4*(8), 962–974. https://doi.org/10.1038/ismej.2010.30

Brama, M., Rhodes, N., Hunt, J., Ricci, A., Teghil, R., Migliaccio, S., … Scandurra, R. (2007). Effect of titanium carbide coating on the osseointegration response in vitro and in vivo. *Biomaterials*, *28*(4), 595–608. https://doi.org/10.1016/j.biomaterials.2006.08.018

Casadevall, A., & Pirofski, L. A. (2003). The damage-response framework of microbial pathogenesis. *Nature Reviews Microbiology*, *1*(1), 17–24. https://doi.org/10.1038/nrmicro732

Charalampakis, G., & Belibasakis, G. N. (2015). Microbiome of peri-implant infections: lessons from conventional, molecular and metagenomic analyses. *Virulence*, *6*(3), 183–187. https://doi.org/10.4161/21505594.2014.980661

Charalampakis, G., Ramberg, P., Dahlén, G., Berglundh, T., & Abrahamsson, I. (2015). Effect of cleansing of biofilm formed on titanium discs. *Clinical Oral Implants Research*, *26*(8), 931–936. https://doi.org/10.1111/clr.12397

Cho, I., & Blaser, M. J. (2012, April). The human microbiome: At the interface of health and disease. *Nature Reviews Genetics*. Nat Rev Genet. https://doi.org/10.1038/nrg3182

Choi, S. H., Jang, Y. S., Jang, J. H., Bae, T. S., Lee, S. J., & Lee, M. H. (2019). Enhanced antibacterial activity of titanium by surface modification with polydopamine and silver for dental implant application. *Journal of Applied Biomaterials and Functional Materials*, *17*(3). https://doi.org/10.1177/2280800019847067

da Silva, E. S. C., Feres, M., Figueiredo, L. C., Shibli, J. A., Ramiro, F. S., & Faveri, M. (2014). Microbiological diversity of peri-implantitis biofilm by Sanger sequencing. *Clinical Oral Implants Research*, *25*(10), 1192–1199. https://doi.org/10.1111/clr.12231

Derks, J., & Tomasi, C. (2015). Peri-implant health and disease. A systematic review of current epidemiology. *Journal of Clinical Periodontology*, *42*, S158–S171. https://doi.org/10.1111/jcpe.12334

Dorkhan, M., Chavez de Paz, L. E., Skepo, M., Svensater, G., Davies, J. R., Chávez de Paz, L. E., … Davies, J. R. (2012). Effects of saliva or serum coating on adherence of Streptococcus oralis strains to titanium. *Microbiology*, *158*(2), 390–397. https://doi.org/10.1099/mic.0.054536-0

Dunne, W. M. (2002). Bacterial adhesion: Seen any good biofilms lately? *Clinical Microbiology Reviews*. Clin Microbiol Rev. https://doi.org/10.1128/CMR.15.2.155-166.2002

Esposito, M., Grusovin, M. G., & Worthington, H. V. (2012). Treatment of peri-implantitis: What interventions are effective? A Cochrane systematic review. *European Journal of Oral Implantology*. Quintessence Publishing Co., Ltd.

Figuero, E., Graziani, F., Sanz, I., Herrera, D., & Sanz, M. (2014). Management of peri-implant mucositis and peri-implantitis. *Periodontology 2000*, *66*(1), 255–273. https://doi.org/10.1111/prd.12049

Frojd, V., de Paz, L. C., Andersson, M., Wennerberg, A., Davies, J. R. R., Svensater, G., … Svensater, G. (2011). In situ analysis of multispecies biofilm formation on customized titanium surfaces. *Molecular Oral Microbiology*, *26*(4), 241–252. https://doi.org/10.1111/j.2041-1014.2011.00610.x

Fürst, M. M., Salvi, G. E., Lang, N. P., & Persson, G. R. (2007). Bacterial colonization immediately after installation on oral titanium implants. *Clinical Oral Implants Research*, *18*(4), 501–508. https://doi.org/10.1111/j.1600-0501.2007.01381.x

Gbadebo, O., Lawal, F., Sulaiman, A., & Ajayi, D. (2014). Dental implant as an option for tooth replacement: The awareness of patients at a tertiary hospital in a developing country. *Contemporary Clinical Dentistry*, *5*(3), 302. https://doi.org/10.4103/0976-237X.137914

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., … Leys, E. J. (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *The ISME Journal*, *6*(6), 1176–1185. https://doi.org/10.1038/ismej.2011.191

Größner-Schreiber, B., Teichmann, J., Hannig, M., Dörfer, C., Wenderoth, D. F. D. F. F.,

Ott, S. J. S. J., … Ott, S. J. S. J. (2009). Modified implant surfaces show different biofilm compositions under in vivo conditions. *Clinical Oral Implants Research*, *20*(8), 817–826. https://doi.org/10.1111/j.1600-0501.2009.01729.x

H., C., H., B., V., M., C., F.-D., Chouirfa, H., Bouloussa, H., … Falentin-Daudre, C. (2019). Review of titanium surface modification techniques and coatings for antibacterial applications. *Acta Biomaterialia*, *83*, 37–54. https://doi.org/10.1016/j.actbio.2018.10.036

Hall-Stoodley, L., Stoodley, P., Kathju, S., Høiby, N., Moser, C., William Costerton, J., … Bjarnsholt, T. (2012). Towards diagnostic guidelines for biofilm-associated infections. *FEMS Immunology and Medical Microbiology*, *65*(2), 127–145. https://doi.org/10.1111/j.1574-695X.2012.00968.x

Hall, M. W., Singh, N., Ng, K. F., Lam, D. K., Goldberg, M. B., Tenenbaum, H. C., … Senadheera, D. B. (2017). Inter-personal diversity and temporal dynamics of dental, tongue, and salivary microbiota in the healthy oral cavity. *Npj Biofilms and Microbiomes*, *3*(1). https://doi.org/10.1038/s41522-016-0011-0

Heitz-Mayfield, L. J. A. (2008). Peri-implant diseases: Diagnosis and risk indicators. In *Journal of Clinical Periodontology* (Vol. 35, pp. 292–304). J Clin Periodontol. https://doi.org/10.1111/j.1600-051X.2008.01275.x

Heitz-Mayfield, L., & Mombelli, A. (2014). The Therapy of Peri-implantitis: A Systematic Review. *The International Journal of Oral & Maxillofacial Implants*, *29*(Supplement), 325–345. https://doi.org/10.11607/jomi.2014suppl.g5.3

Huang, S., Li, R., Zeng, X., He, T., Zhao, H., Chang, A., … Xu, J. (2014). Predictive modeling of gingivitis severity and susceptibility via oral microbiota. *ISME Journal*, *8*(9), 1768–1780. https://doi.org/10.1038/ismej.2014.32

Katafuchi, M., Weinstein, B. F., Leroux, B. G., Chen, Y. W., & Daubert, D. M. (2018). Restoration contour is a risk indicator for peri-implantitis: A cross-sectional radiographic analysis. *Journal of Clinical Periodontology*, *45*(2), 225–232. https://doi.org/10.1111/jcpe.12829

Khoury, F., Keeve, P. L., Ramanauskaite, A., Schwarz, F., Koo, K. T., Sculean, A., & Romanos, G. (2019). Surgical treatment of peri-implantitis – Consensus report of working group 4. *International Dental Journal*, *69*(S2), 18–22. https://doi.org/10.1111/idj.12505

Kočar, M., Seme, K., Ihan Hren, N., Kocar, M., Seme, K., Hren, N. I., … Hren, N. I. (2010). Characterization of the normal bacterial flora in peri-implant sulci of partially and completely edentulous patients. *International Journal of Oral and Maxillofacial Implants*, *25*(4), 690–698. Retrieved from https://www.scopus.com/inward/record.uri?eid=2-s2.0-85018693764&partnerID=40&md5=4faac7b600439735248e21004e552004

Kohavi, D., Klinger, A., Steinberg, D., & Sela, M. N. (1995). Adsorption of salivary proteins onto prosthetic titanium components. *The Journal of Prosthetic Dentistry*, *74*(5), 531–534. https://doi.org/10.1016/S0022-3913(05)80357-9

Lafaurie, G. I., Sabogal, M. A., Castillo, D. M., Rincón, M. V., Gómez, L. A., Lesmes, Y. A., & Chambrone, L. (2017). Microbiome and Microbial Biofilm Profiles of Peri-Implantitis: A Systematic Review. *Journal of Periodontology*, *88*(10), 1066–1089. https://doi.org/10.1902/jop.2017.170123

Laksmana, T., Kittichotirat, W., Huang, Y., Chen, W., Jorgensen, M., Bumgarner, R., &

Chen, C. (2013). Metagenomic Analysis of Subgingival Microbiota Following Non-Surgical Periodontal Therapy: a Pilot Study. *The Open Dentistry Journal*, *6*(1), 255–261. https://doi.org/10.2174/1874210601206010255

Lang, N. P., Wilson, T. G., & Corbet, E. F. (2000). Biological complications with dental implants: their prevention, diagnosis and treatment. In *Clinical oral implants research* (Vol. 11 Suppl 1, pp. 146–155). Clin Oral Implants Res. https://doi.org/10.1034/j.1600-0501.2000.011S1146.x

Leonhardt, Å., Adolfsson, B., Lekholm, U., Wikström, M., Dahlén, G., Leonhardt, A., … Dahlén, G. (1993). A longitudinal microbiological study on osseointegrated titanium implants in partially edentulotis patients. *Clinical Oral Implants Research*, *4*(3), 113–120. https://doi.org/10.1034/j.1600-0501.1993.040301.x

Leonhardt, A., Berglundh, T., Ericsson, I., & Dahlén, G. (1992). Putative periodontal pathogens on titanium implants and teeth in experimental gingivitis and periodontitis in beagle dogs. *Clinical Oral Implants Research*, *3*(3), 112–119. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1290791

Leonhardt, A., Renvert, S., Dahlén, G., Leonhardt, Å., Renvert, S., & Dahlén, G. (1999). Microbial findings at failing implants. *Clinical Oral Implants Research*, *10*(5), 339–345. https://doi.org/10.1034/j.1600-0501.1999.100501.x

Lim, K., Chua, R. R. Y., Saravanan, R., Basu, A., Mishra, B., Tambyah, P. A., … Leong, S. S. J. (2013). Immobilization studies of an engineered arginine-tryptophan-rich peptide on a silicone surface with antimicrobial and antibiofilm activity. *ACS Applied Materials and Interfaces*, *5*(13), 6412–6422. https://doi.org/10.1021/am401629p

Lindhe, J., & Meyle, J. (2008). Peri-implant diseases: Consensus Report of the Sixth European Workshop on Periodontology. In *Journal of Clinical Periodontology* (Vol. 35, pp. 282–285). J Clin Periodontol. https://doi.org/10.1111/j.1600-051X.2008.01283.x

Liu, B., Faller, L. L., Klitgord, N., Mazumdar, V., Ghodsi, M., Sommer, D. D., … Amar, S. (2012). Deep sequencing of the oral microbiome reveals signatures of periodontal disease. *PloS One*, *7*(6), e37919. https://doi.org/10.1371/journal.pone.0037919

Lloyd-Price, J., Mahurkar, A., Rahnavard, G., Crabtree, J., Orvis, J., Hall, A. B., … Huttenhower, C. (2017). Strains, functions and dynamics in the expanded Human Microbiome Project. *Nature*, *550*(7674), 61–66. https://doi.org/10.1038/nature23889

Mahato, N., Wu, X., & Wang, L. (2016, December 1). Management of peri-implantitis: a systematic review, 2010–2015. *SpringerPlus*. SpringerOpen. https://doi.org/10.1186/s40064-016-1735-2

Mark Welch, J. L., Dewhirst, F. E., & Borisy, G. G. (2019). Biogeography of the Oral Microbiome: The Site-Specialist Hypothesis. *Annual Review of Microbiology*, *73*(1), 335–358. https://doi.org/10.1146/annurev-micro-090817-062503

Mark Welch, J. L., Utter, D. R., Rossetti, B. J., Mark Welch, D. B., Eren, A. M., & Borisy, G. G. (2014). Dynamics of tongue microbial communities with single-nucleotide resolution using oligotyping. *Frontiers in Microbiology*, *5*(OCT). https://doi.org/10.3389/fmicb.2014.00568

Mombelli, A., Nyman, S., Brägger, U., Wennström, J., & Lang, N. P. (1995). Clinical and microbiological changes associated with an altered subgingival environment induced by periodontal pocket reduction. *Journal of Clinical Periodontology*,

22(10), 780–787. https://doi.org/10.1111/j.1600-051X.1995.tb00261.x

Padial-Molina, M., López-Martínez, J., O'Valle, F., & Galindo-Moreno, P. (2016). Microbial Profiles and Detection Techniques in Peri-Implant Diseases: a Systematic Review. *Journal of Oral & Maxillofacial Research*, *7*(3), e10. https://doi.org/10.5037/jomr.2016.7310

Papaioannou, W., Quirynen, M., & Van Steenberghe, D. (1996). The influence of periodontitis on the subgingival flora around implants in partially edentulous patients., *7*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9151609

Pflughoeft, K. J., & Versalovic, J. (2012). Human Microbiome in Health and Disease. *Annual Review of Pathology: Mechanisms of Disease*, *7*(1), 99–122. https://doi.org/10.1146/annurev-pathol-011811-132421

Qin, H., Cao, H., Zhao, Y., Jin, G., Cheng, M., Wang, J., … Liu, X. (2015). Antimicrobial and Osteogenic Properties of Silver-Ion-Implanted Stainless Steel. *ACS Applied Materials & Interfaces*, *7*(20), 10785–10794. https://doi.org/10.1021/acsami.5b01310

Quirynen, M., Papaioannou, W., & van Steenberghe, D. (1996). Intraoral transmission and the colonization of oral hard surfaces. *Journal of Periodontology*, *67*(10), 986–993. https://doi.org/10.1902/jop.1996.67.10.986

Quirynen, M., Vogels, R., Pauwels, M., Haffajee, A. D. D., Socransky, S. S. S., Uzel, N. G. G., & van Steenberghe, D. (2005). Initial subgingival colonization of "pristine" pockets. *Journal of Dental Research*, *84*(4), 340–344. https://doi.org/10.1177/154405910508400409

Quirynen, M., Vogels, R., Peeters, W., Van Steenberghe, D., Naert, I., & Haffajee, A. (2006). Dynamics of initial subgingival colonization of "pristine" peri-implant pockets. *Clinical Oral Implants Research*, *17*(1), 25–37. https://doi.org/10.1111/j.1600-0501.2005.01194.x

Rakic, M., Grusovin, M. G., & Canullo, L. (2016). The Microbiologic Profile Associated with Peri-Implantitis in Humans: A Systematic Review. *The International Journal of Oral & Maxillofacial Implants*, *31*(2), 359–368. https://doi.org/10.11607/jomi.4150

Ramanauskaite, A., Daugela, P., Faria de Almeida, R., & Saulacic, N. (2016). Surgical Non-Regenerative Treatments for Peri-Implantitis: a Systematic Review. *Journal of Oral & Maxillofacial Research*, *7*(3), e14. https://doi.org/10.5037/jomr.2016.7314

Rehman, A., Hu, J., Ott, S. J., Grössner-Schreiber, B., Rehman A., Hu J., Ott S.J., G.-S. B., Rehman, A., … Grössner-Schreiber, B. (2012). Microbial community composition on modified dental implant surfaces: an in vivo study. *The International Journal of Oral & Maxillofacial Implants*, *27*(4), 811–819. Retrieved from https://www.scopus.com/record/display.uri?eid=2-s2.0-84924849011&origin=resultslist&sort=plf-f&src=s&st1=dental+implants+AND+microbial&st2=dental+implants+AND+metagenomic&nlo=&nlr=&nls=&sid=b91c361426d4be92fca17f216ca37758&sot=b&sdt=b&sl=96&s=%28TITLE-ABS

Renvert, S., Persson, G. R., Pirih, F. Q., & Camargo, P. M. (2018, June 1). Peri-implant health, peri-implant mucositis, and peri-implantitis: Case definitions and diagnostic considerations. *Journal of Periodontology*. NLM (Medline). https://doi.org/10.1002/JPER.17-0588

Renvert, S., & Quirynen, M. (2015). Risk indicators for peri-implantitis. A narrative review. *Clinical Oral Implants Research*, *26*, 15–44. https://doi.org/10.1111/clr.12636

Rimondini, L., Farè, S., Brambilla, E., Felloni, A., Consonni, C., Brossa, F., … Carrassi, A. (1997). The effect of surface roughness on early in vivo plaque colonization on titanium. *Journal of Periodontology*, *68*(6), 556–562. https://doi.org/10.1902/jop.1997.68.6.556

Roccuzzo, M., Gaudioso, L., Lungo, M., & Dalmasso, P. (2016). Surgical therapy of single peri-implantitis intrabony defects, by means of deproteinized bovine bone mineral with 10% collagen. *Journal of Clinical Periodontology*, *43*(3), 311–318. https://doi.org/10.1111/jcpe.12516

Salvi, G. E., Cosgarea, R., & Sculean, A. (2017). Prevalence and Mechanisms of Peri-implant Diseases. *Journal of Dental Research*, *96*(1), 31–37. https://doi.org/10.1177/0022034516667484

Salvi, G. E. G. E., Fürst, M. M. M. M. M., Lang, N. P. N. P., & Persson, G. R. R. (2008). One-year bacterial colonization patterns of Staphylococcus aureus and other bacteria at implants and adjacent teeth. *Clinical Oral Implants Research*, *19*(3), 242–248. https://doi.org/10.1111/j.1600-0501.2007.01470.x

Sanz, M., & Chapple, I. L. (2012, February). Clinical research on peri-implant diseases: Consensus report of Working Group 4. *Journal of Clinical Periodontology*. J Clin Periodontol. https://doi.org/10.1111/j.1600-051X.2011.01837.x

Sato, Y., Yamagishi, J., Yamashita, R., Shinozaki, N., Ye, B., Yamada, T., … Tsuboi, A. (2015). Inter-individual differences in the oral bacteriome are greater than intra-day fluctuations in individuals. *PLoS ONE*, *10*(6). https://doi.org/10.1371/journal.pone.0131607

Schmidlin P.R., Müller P., Attin T., Wieland M., Hofer D., G. B., Schmidlin, P. R., MÜLLER, P., ATTIN, T., WIELAND, M., Hofer, D., & Guggenheim, B. (2013). Polyspecies biofilm formation on implant surfaces with different surface characteristics. *Journal of Applied Oral Science*, *21*(1), 48–55. https://doi.org/10.1590/1678-7757201302312

Schwarz, F., Schmucker, A., & Becker, J. (2015). Efficacy of alternative or adjunctive measures to conventional treatment of peri-implant mucositis and peri-implantitis: a systematic review and meta-analysis. *International Journal of Implant Dentistry*, *1*(1), 22. https://doi.org/10.1186/s40729-015-0023-1

Sultan, A. S., Kong, E. F., Rizk, A. M., & Jabra-Rizk, M. A. (2018). The oral microbiome: A Lesson in coexistence. *PLoS Pathogens*, *14*(1), e1006719. https://doi.org/10.1371/journal.ppat.1006719

Sumida, S., Ishihara, K., Kishi, M., & Okuda, K. (2002). Transmission of periodontal disease-associated bacteria from teeth to osseointegrated implant regions. *The International Journal of Oral & Maxillofacial Implants*, *17*(5), 696–702. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12381070

Takanashi, K., Kishi, M., Okuda, K., & Ishihara, K. (2004). Colonization by Porphyromonas gingivalis and Prevotella intermedia from teeth to osseointegrated implant regions. *The Bulletin of Tokyo Dental College*, *45*(2), 77–85. https://doi.org/10.2209/tdcpublication.45.77

Teughels, W., Van Assche, N., Sliepen, I., & Quirynen, M. (2006). Effect of material

characteristics and/or surface topography on biofilm development. *Clinical Oral Implants Research*, *17*(SUPPL. 2), 68–81. https://doi.org/10.1111/j.1600-0501.2006.01353.x

Utter, D. R., Mark Welch, J. L., & Borisy, G. G. (2016). Individuality, stability, and variability of the plaque microbiome. *Frontiers in Microbiology*, *7*(APR). https://doi.org/10.3389/fmicb.2016.00564

Zaura, E., Keijser, B. J. F., Huse, S. M., & Crielaard, W. (2009). Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiology*, *9*(1), 259. https://doi.org/10.1186/1471-2180-9-259

Zhao, B., van der Mei, H. C., Subbiahdoss, G., de Vries, J., Rustema-Abbing, M., Kuijer, R., … Ren, Y. (2014). Soft tissue integration versus early biofilm formation on different dental implant materials. *Dental Materials*, *30*(7), 716–727. https://doi.org/10.1016/j.dental.2014.04.001

Zhao, X., Liu, G., Zheng, H., Cao, H., & Liu, X. (2014). Dose-Dependent Effects of CeO2 on Microstructure and Antibacterial Property of Plasma-Sprayed TiO2 Coatings for Orthopedic Application. *Journal of Thermal Spray Technology*, *24*(3), 401–409. https://doi.org/10.1007/s11666-014-0179-x

Ziganshina, E. E., Belostotskiy, D. E., Ilinskaya, O. N., Boulygina, E. A., Grigoryeva, T. V., & Ziganshin, A. M. (2015). Effect of the Organic Loading Rate Increase and the Presence of Zeolite on Microbial Community Composition and Process Stability During Anaerobic Digestion of Chicken Wastes. *Microbial Ecology*, *70*(4), 948–960. https://doi.org/10.1007/s00248-015-0635-2

Zitzmann, N. U., & Berglundh, T. (2008). Definition and prevalence of peri-implant diseases. *Journal of Clinical Periodontology*, *35*(8 Suppl), 286–291. https://doi.org/10.1111/j.1600-051X.2008.01274.x

Zitzmann, N. U., Berglundh, T., Marinello, C. P., & Lindhe, J. (2001). Experimental peri-implant mucositis in man. *Journal of Clinical Periodontology*, *28*(6), 517–523. https://doi.org/10.1034/j.1600-051x.2001.028006517.x

ANEXOS

Anexo A – Termo de consentimento livre e esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Caro participante,

Estamos rea izando um estudo para ava iar dois tipos de tratamento da inf amação que ocorre na gengiva dos imp antes dentários Este será um dos primeiros estudos sobre esse assunto e tem a possibi idade de contribuir para manter a saúde nos imp antes O estudo faz parte dos traba hos de conc usão de curso de a unos de Pós Graduação em Odonto ogia da UFRGS

Os tratamentos propostos não são diferentes daque es tradiciona mente rea izados para o tratamento das doenças de gengiva, porém agora serão comparados em imp antes Um tratamento será composto por impeza dos imp antes abaixo da gengiva Outro tratamento será rea izado com uma cirurgia para enxergar o imp ante e fazer a sua impeza Os tratamentos serão rea izados por dentistas especia istas no tratamento das doenças gengivais, sempre com anestesia para evitar a dor O tratamento será rea izado num período de no máximo quatro semanas sendo que os participantes serão ava iados a cada três meses por um período de um ano após o tratamento

Juntamente com os exames c ínicos dos dentes e imp antes, serão rea izados exames aboratoriais comumente uti izados Será feita a co eta de 15mL de sangue no braço para a ava iação de componentes sanguíneos Essa co eta de sangue será rea izada no início e três vezes ao ongo de um ano (3, 6 e 12 meses após o início)

Os possíveis desconfortos associados à participação neste estudo são aque es decorrentes da rea ização de um tratamento das doenças da gengiva e da co eta de sangue Todas as medidas de biossegurança necessárias tais como uso de materiais descartáveis e instrumentais esteri izados, serão adotadas

Os benefícios re acionados à participação neste estudo são o tratamento da inf amação nos imp antes, bem como encaminhamento para o tratamento de outras condições bucais, quando necessário Fica ainda assegurado o direito ao sigi o de todas as informações co etadas, não sendo permitido acesso por outra pessoa que não o próprio participante ou responsáve O tratamento será gratuito Tratamentos outros, como próteses, quando so icitados pe o paciente, terão o custo que norma mente é cobrado pe a Facu dade de Odonto ogia

Fica, ainda, assegurada a iberdade dos participantes de recusarem se a participar ou retirarem se do estudo a qua quer momento que desejarem, sem que isso traga prejuízos na assistência A continuidade do tratamento da doença gengiva será garantida mesmo que os participantes desejem se retirar do estudo

Toda e qua quer dúvida no decorrer do estudo poderá ser esc arecida pe os envo vidos nesta pesquisa através dos te efones (51) 3308 5318, e (51) 9122 2377 Os pesquisadores A ex Nogueira Haas e Cassiano Rösing estarão sempre a disposição para esc arecimentos Possíveis prob emas podem ser reportados diretamente ao Comitê de Ética em Pesquisa da Facu dade de Odonto ogia da UFRGS 3308 3738

Eu, (participante), dec aro que fui informado dos objetivos e procedimentos que serão rea izados nesta pesquisa, bem como sei dos meus direitos e dos deveres dos pesquisadores Dec aro, ainda, que recebi uma cópia deste Termo

, de de 201

Participante:

R.G.:

Anexo B – Aprovação do Comitê de Ética UFRGS

UFRGS
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: COMPARAÇÃO DOS EFEITOS DOS TRATAMENTOS CIRÚRGICO E NÃO-CIRÚRGICO DA PERIMPLANTITE SOBRE DESFECHOS LOCAIS E SISTÊMICOS: ENSAIO CONTROLADO RANDOMIZADO DE 12 MESES

Pesquisador: Alex Nogueira Haas

Área Temática:

Versão: 3

CAAE: 27156514.1.0000.5347

Instituição Proponente: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL/COMITÊ DE ÉTICA EM

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 666.930

Data da Relatoria: 08/05/2014

Apresentação do Projeto:

O objetivo do presente estudo será comparar os efeitos dos tratamentos cirúrgico e não-cirúrgico da perimplantite sobre desfechos locais e sistêmicos. Utilizará um desenho experimental do tipo ensaio controlado randomizado em paralelo. A amostra será de 108 indivíduos, sendo composta por indivíduos parcialmente desdentados que apresentem implantes reabilitados com próteses fixas unitárias ou múltiplas, com pelo menos um implante com diagnóstico de perimplantite. Os participantes deste estudo serão alocados aleatoriamente em grupos controle e teste que consistirão de tratamento não-cirúrgico e cirúrgico da perimplantite, respectivamente. Será utilizado um questionário estruturado para entrevistar os participantes. O tratamento perimplantar será dividido em duas fases: a primeira objetivando o controle do biofilme supramucoso, e a segunda, o tratamento propriamente dito da perimplantite. Após o término do tratamento, os pacientes serão acompanhados através de consultas periódicas para manutenção do tratamento perimplantar, mensalmente nos 3 primeiros meses, e a cada 3 meses até o término do estudo aos 12 meses. O exame clínico será realizado no início, e após 3,6 e 12 meses. Os parâmetros clínicos periodontais avaliados serão índice de placa visível, índice de sangramento gengival, fatores retentivos de placa, profundidade de sondagem, sangramento à sondagem, nível de inserção clínico dentário e nível de inserção clínico dos implantes. Serão realizadas avaliações clínicas do

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

UFRGS
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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

Plataforma Brasil

Continuação do Parecer: 666.930

grau de estabilidade do implante utilizando o Periotest (Medizintechnik Gulden, Alemanha) nos mesmos tempos de acompanhamento em que serão realizados os exames clínicos. Avaliações radiográficas serão realizadas no início do estudo e 12 meses após o término do tratamento, a análise será conduzida para avaliar a presença de progressão ou ganho no nível ósseo ao redor dos implantes. Amostras microbiológicas submucosas e de fluido crevicular perimplantar serão obtidas no início do estudo, 3, 6 e 12 meses após o término do tratamento. Após, será feita coleta sanguínea dos participantes, para análise dos marcadores imuno-inflamatórios
sistêmicos PCR, 1ß, IL-6, IL-8, IL-10, IFN-¿, TGF-¿ e TNF¿.

Objetivo da Pesquisa:
Comparar os efeitos dos tratamentos cirúrgico e não-cirúrgico da perimplantite sobre desfechos locais e sistêmicos.

Avaliação dos Riscos e Benefícios:
Benefícios estavam informados de forma adequada e na nova versão o pesquisador explicita os riscos e quais as medidas da equipe de pesquisa para minimiza-los.

Comentários e Considerações sobre a Pesquisa:
O estudo possui aprovação da Compesq Odontologia, possui portanto mérito científico. Apresenta de forma consistente o objeto de estudo e a justificativa para sua realização. Cronograma e orçamento estão adequados.

Considerações sobre os Termos de apresentação obrigatória:
O estudo possui aprovação da Compesq Odontologia, possui portanto mérito científico. Apresenta de forma consistente o objeto de estudo e a justificativa para sua realização. Cronograma e orçamento estão adequados.

Recomendações:
O pesquisador atendeu a diligência de forma que:
A nova versão do TCLE expressa que:
-os participantes estão sendo convidados para o estudo,
- há a descrição do número de consultas necessárias para participação no estudo,
- o pesquisador explica que o tratamento odontológico será realizado pela equipe de pesquisadores e em função disso o mesmo será gratuito,

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

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

Continuação do Parecer: 666.930

- está descrito no TCLE o ressarcimento ao participante do gasto efetuado no deslocamento (passagens) para sua participação na pesquisa, de acordo com a resolução 466/2012.

Conclusões ou Pendências e Lista de Inadequações:

O projeto de pesquisa está em condições de aprovação.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Aprovado

PORTO ALEGRE, 29 de Maio de 2014

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

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

Anexo C – Ficha de exame de seleção inicial

Número: ☐☐☐

Nome:_____

Gênero: ☐ masculino ☐ feminino Data de nascimento: ___/___/_____

Endereço_____Contato:_____

_____

1. Você já possui implantes? ☐ sim ☐ não

1.a. Há quanto tempo? _____ anos

1.b. Sente algum incômodo nos implantes? ☐ sim ☐ não

Qual? ☐ dor ☐ sangramento ☐ mobilidade

2. Tem algum doença? ☐ sim ☐ não

2.a. Qual (is)? _____

3. Toma alguma medicação? ☐ sim ☐ não

3.a. Qual(is)? _____

3.b. Tomou antibiótico nos últimos 6 meses? ☐ sim ☐ não

4. Está grávida? ☐ sim ☐ não

_____

Exame clínico

1. Profundidade de Sondagem e Sangramento a sondagem

LOCALIZAÇÃO			LOCALIZAÇÃO			LOCALIZAÇÃO			LOCALIZAÇÃO			LOCALIZAÇÃO			LOCALIZAÇÃO			LOCALIZAÇÃO		
DV	D	DM	DV	D	DM	DV	D	DM	DV	D	DM	DV	D	DM	DV	D	DM	DV	D	DM
MV	M	MD	MV	M	MD	MV	M	MD	MV	M	MD	MV	M	MD	MV	M	MD	MV	M	MD

2. Perda Óssea Radiográfica (mm) _____

3. Possui mais de 10 dentes? ☐ Sim ☐ Não

4. Dentes naturais necessitam de tratamento periodontal prévio? ☐ Sim ☐ Não

Anexo D –Ficha da entrevista

R Data ☐☐/☐☐/20☐☐ REGISTRO Nº ☐☐☐☐

Entrevistador [1] Fernando [2] Tassiane [3] Outro.....

DADOS PESSOAIS

1.1. Nome..... **1.2. Endereço**.....

1.3. Telefone res. ( )..... **1.4. Telefone cel.** ( )..... **1.5. E mail**.....

1.6. Contato familiar..... **1.7. Telefone res.** ( )..... **1.8. Telefone cel.** ( )..... **1.9. Sexo** [1] Masculino [2] Feminino **1.10. Data de nascimento** ☐☐/☐☐/19☐☐

1.11. Idade ☐☐ anos **1.12. Raça** [1] Branca [2] Não branca

1.13. Estado civil [1] Solteiro(a) [2] Casado(a)/União estável [3] Divorciado(a) [4] Viúvo(a) [5] Outro.....

HÁBITOS DE HIGIENE BUCAL

2.1. Com que frequência você escova seus dentes?

[1] Nunca escova [2] Menos de uma vez por dia [3] Uma vez por dia [4] Duas vezes por dia [5] Três vezes ou mais por dia

2.2. Com que frequência você limpa entre seus dentes?

[1] Nunca limpa [2] Menos de uma vez por dia [3] Uma vez por dia [4] Duas vezes por dia [5] Três vezes ou mais por dia

2.3. O que você usa para limpar entre seus dentes? [1] Nada [2] Palito de dentes [3] Fio dental [4] Outro.....

HALITOSE

3.1. Você sente mau hálito, mau cheiro ou gosto ruim na boca?

[1] Nunca [2] Raramente [3] Algumas vezes [4] Repetidamente [5] Sempre

3.2. Em que momento do dia? [1] Não sente [2] Manhã [3] Tarde [4] Noite [5] Todo o dia [6] Não sabe

3.3. Você foi avisado por alguma pessoa que você tem mau hálito? [1] Sim [2] Não

3.4. Por quem? [1] Ninguém [2] Companheiro(a) [3] Familiar [4] Amigo(a) [5] Dentista

IMPLANTES

4.1. Quantos implantes você tem? ☐☐

4.2. Quanto tempo se passou desde a colocação do seu último implante? ☐☐ **meses**

4.3. Você já perdeu algum implante? [1] Sim [2] Não

AUTOPERCEPÇÃO EM SAÚDE BUCAL

Nos últimos seis meses, por causa de problemas com seus dentes, boca ou dentadura:
(nunca=0; raramente=1; algumas vezes=2; repetidamente=3; sempre=4)

5.1. Você teve problemas para falar alguma palavra?		**5.8. Você teve que parar suas refeições?**	
5.2. Você sentiu que o sabor dos alimentos tem piorado?		**5.9. Você encontrou dificuldade para relaxar?**	
5.3. Você sentiu dores na sua boca ou nos seus dentes?		**5.10. Você se sentiu envergonhado(a)?**	
5.4. Você se sentiu incomodado(a) ao comer algum alimento?		**5.11. Você ficou irritado(a) com outras pessoas?**	
5.5. Você ficou preocupado(a)?		**5.12. Você teve dificuldade de realizar atividades diárias?**	
5.6. Você se sentiu estressado(a)?		**5.13. Você sentiu que a vida, em geral, ficou pior?**	
5.7. Sua alimentação ficou prejudicada?		5.14. Você ficou totalmente incapaz de fazer suas atividades diárias?	

ACESSO A SERVIÇOS ODONTOLÓGICOS

6.1. Você tem ido ao dentista nos últimos 3 anos:

[1] Não tem ido [2] Quando tem dor, um dente quebrado ou outra urgência [3] Para revisar e evitar problemas futuros

6.2. De quanto em quanto tempo? ☐☐ meses

6.3. Onde foi a sua última consulta?

[1] Serviço público [2] Serviço particular [3] Plano de saúde ou convênio [4] Não sabe [5] Outro.....

6.4. Você já fez tratamento gengival/periodontal? [1] Sim [2] Não [3] Não sabe

6.5. Há quanto tempo? [ ][ ] meses

HISTÓRIA MÉDICA

Você apresenta/apresentou alguma das seguintes doenças? (sim=1; não=2; não sabe=3)

7.1. Artrite reumatóide		**7.3. Osteoporose**	
7.2. Diabetes		**7.4. Doença Cardíaca ou arterial**	

7.5. Você está usando alguma medicação? [1] Sim [2] Não

7.6. Qual?.....

FATORES COMPORTAMENTAIS

8.1. Você fuma atualmente? [1] Sim [2] Não **8.2. Quantos cigarros por dia?** [ ][ ] **8.3. Há quantos anos?** [ ][ ]

8.4. Você fumou anteriormente? [1] Sim [2] Não **8.5. Quantos cigarros por dia?** [ ][ ] **8.6. Por quantos anos?** [ ][ ]

8.7. Há quantos anos você parou de fumar? [ ][ ]

CARACTERIZAÇÃO SOCIOECONÔMICA

9.1. Você é alfabetizado(a)? [1] Sim [2] Não

9.2. Você estudou até: [1] Nunca estudou [2] 1ª a 4ª série do 1º grau [3] 5ª a 8ª série do 1º grau [4] 2º grau incompleto [5] 2º grau completo [6] 3º grau incompleto [7] 3º grau completo [8] Pós-graduação

Quantos...você possui? (não possui=0; 1; 2; 3; 4 ou mais=4)

9.3. Aspiradores de pó		**9.7. Empregadas** (pagamento mensal)		**9.11. Rádios**	
9.4. Automóveis		**9.8. Freezer** (considerar refrigerador duplex)		**9.12. Refrigeradores**	
9.5. Banheiros		**9.9. Máquinas de lavar roupas**		**9.13. Televisores coloridos**	
9.6. Computadores		**9.10. Microondas**		**9.14. Videocassetes/DVDs**	

9.15. No mês passado, quanto receberam, em reais, juntas, todas as pessoas que moram na sua casa?

[1] Até 250 [2] 251 a 540 [3] 541 a 1500 [4] 1501 a 2500 [5] 2501 a 4500 [6] 4501 a 9500 [7] Mais de 9500 [8] Não sabe [9] Não respondeu

FICHA CLÍNICA

REGISTRO Nº ☐☐☐☐ **EXAME:** _____

Data ☐☐ / ☐☐ / 2 0 ☐☐

Examinador..... **Anotador(a)**.....

Medidas antropométricas: 11.1. Altura 1 m ☐☐ cm **11.2. Peso** ☐☐☐☐ kg

Uso de próteses: (0=ausente; 1=prótese total; 2=removível com estrutura metálica; 3=removível provisória; 4=desdentado sem prótese total)

12.1. Arcada superior ☐ **12.2. Arcada Inferior** ☐

Localização dos implantes:_____ **Periotron:**_____ **Periotest:** _____

IPV (0=ausente; 1=presente)

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D

ISG (0=ausente; 1=presente)

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D

Cálculo supragengival (0=ausente; 1=presente)

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D
D V M	D V M	D V M	D V M	D V M	D V M	D V M	D V M	M V D	M V D	M V D	M V D	M V D	M V D	M V D	M V D

Profundidade de Sondagem

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**

Perda de Inserção Clínica

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**

Sangramento a Sondagem

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**

Tipo de prótese:
13.1. Unitária ☐ **13.2. Múltipla** ☐ **14.1 Cimentada** ☐ **14.2. Parafusada** ☐

Tipo de plataforma:
15.1 Hexágono externo ☐ **15.2 Hexágona interno** ☐ **15.3 Plataforma switch** ☐

Faixa de gengiva ceratinizada (0=ausente; 1=presente 1mm)
16.1 Ausente ☐ **16.2 Presente** ☐