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EFEITO DA REDUÇÃO DO APORTE NUTRICIONAL

NA DIVERSIDADE GENOTÍPICA E

NOS FATORES DE VIRULÊNCIA DE

BACTÉRIAS CARIOGÊNICAS ISOLADAS DE

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NAILÊ DAMÉ-TEIXEIRA

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EFEITO DA REDUÇÃO DO APORTE NUTRICIONAL NA DIVERSIDADE GENOTÍPICA E NOS FATORES DE VIRULÊNCIA DE BACTÉRIAS CARIOGÊNICAS ISOLADAS DE DENTINA CARIADA SELADA

Tese apresentada ao Programa de Pós-Graduação em Odontologia como parte dos requisitos obrigatórios para a obtenção do título de doutora em Clínica Odontológica com ênfase em Dentística/Cariologia.

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Linha de pesquisa: Biomateriais e Técnicas Terapêuticas em Odontologia.

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DAMÉ-TEIXEIRA, Nailê. **Efeito da redução do aporte nutricional na diversidade genotípica e nos fatores de virulência de bactérias cariogênicas isoladas de dentina cariada selada.** 2015. 63 f. Tese de Doutorado. Programa de Pós-Graduação em Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

No tratamento de lesões profundas de cárie, a Remoção Parcial de Dentina Cariada (RPDC) e restauração tem sido proposta como alternativa conservadora para evitar perda de tecido dentário e exposição pulpar. Existe uma hipótese de que uma seleção de bactérias ocorre abaixo de restaurações devido a um acesso limitado de nutrientes. No entanto, há falta de conhecimento sobre a diversidade e potencial de virulência das bactérias cariogênicas residuais seladas abaixo de restaurações sobre dentina cariada. O objetivo deste estudo foi caracterizar *Streptococcus mutans* e lactobacilos isolados de dentina cariada antes e após o estresse nutricional por selamento da cavidade. *S. mutans* e lactobacilos foram obtidos por cultivo da dentina cariada de lesões cavitadas de quatro e seis pacientes, respectivamente. Duas amostras de dentina cariada foram coletadas e cultivadas por paciente: uma antes e outra após três meses de selamento da cavidade. Colônias de *S. mutans* e lactobacilos preditos foram selecionadas, isoladas e analisadas por coloração de Gram. Genes "*housekeeping*" foram utilizados na identificação da espécie (*gtfB* para *S. mutans* e *pheS*/rpoA/groEL/16SrRNA para lactobacilos) e a técnica de AP-PCR foi utilizada para genotipagem. A análise fenotípica (produção de ácido e de tolerância ao ácido) foi realizada. Um total de 48 isolados representativos de *S. mutans* foram analisados (31 antes e 17 após estresse nutricional por meio do selamento). O número de genótipos diferentes de *S. mutans* encontrado foi de nove e seis antes e após selamento, respectivamente. Pelo menos um dos genótipos encontrados antes do selamento foi também encontrado na dentina após o estresse nutricional por meio do selamento. Em relação aos lactobacilos, analisou-se 86 cepas, 41 antes e 45 após estresse nutricional. *L. paracasei* e *L. rhamnosus* prevaleceram e apenas quatro isolados não pertenciam a estas espécies. Um total de 27 e 15 genótipos de lactobacilos diferentes foram encontrados antes e após selamento, respectivamente. Não houve diferença entre isolados de *S. mutans* e lactobacilos de dentina cariada antes e após o estresse nutricional na produção de ácido ou tolerância ao ácido. *L. paracasei* apresentaram menor valor de pH em 48h de crescimento. Em conclusão, a diversidade genotípica diminuiu após o estresse nutricional, mas a virulência de *S. mutans* e lactobacilos permaneceu a mesma. *L. rhamnosus* foi selecionado após o estresse nutricional. Algumas espécies de lactobacilos apareceram após o selamento, sugerindo pressão seletiva no sítio antes do selamento pela alta disponibilidade de nutrientes. Mais estudos avaliando outras características de virulência são necessários para entender melhor a cariogenicidade das cepas residuais seladas abaixo de restaurações e para compreender a resistência de *L. rhamnosus* a este sítio.

DAMÉ-TEIXEIRA, Nailê. **Stress starvation effect on the genotypic diversity and virulence factors of cariogenic bacteria isolated from sealed carious dentin.** 2015. 63 p. PhD Thesis. Post-Graduation Program in Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, 2015.

A hypothesis exists that a selection of bacteria occurs underneath restoration due to a limited access of nutrients. However, there is a lack of evidence regarding their role in the progression of carious process beneath restorations after Partial Dentin Caries Removal. It still unclear if the diversity and the virulence potential of the sealed bacteria remain the same after sealing. The aim of this study was to characterize *Streptococcus mutans* and *Lactobacillus* species isolated from caries dentin before and after starvation stress by cavity sealing. *S. mutans* and lactobacilli were obtained by culture of carious dentin from four and six patients, respectively. Two carious dentin samples were collected and cultured per patient: 1st before and 2nd after three months of cavity sealing. Presumptive *S. mutans* and lactobacilli were selected, isolated and analyzed by Gram staining. Housekeeping genes sequencing were used to the species identification (*gtf*B for *S. mutans* and *pheS*/*rpo*A/*gro*EL/16SrRNA for lactobacilli) and Arbitrary Primer-PCR (AP-PCR) was used for genotyping. Phenotypic analysis (acid production and acid tolerance) was performed. A total of 48 representative *S. mutans* isolates were genotyped (31 before and 17 after the sealing). The number of different genotypes identified was nine and six before and after sealing, respectively. At least one of the genotypes found before the sealing was also found on dentin after the sealing in all patients. Regarding lactobacilli, it was analyzed 86 strains, 41 before and 45 after starvation stress by sealing. *L. paracasei* and L. rhamnosus prevailed and only four isolates did not belong to these species. A total of 27 and 15 different genotypes were found before and after sealing, respectively. There was no difference between isolates from carious dentin before and after starvation stress, neither regarding acid production or acid tolerance, although L. paracasei showed lower pH value in 48 h of growth. In conclusion, genotypic diversity of *S. mutans* decreased after starvation stress, but the virulence traits of *S. mutans* remained unchangeable. *L. paracasei* and *L. rhamnosus* were the most prevalent species of this genus, besides *L. rhamnosus* was selected after starvation stress. There was a decreased genotypic diversity of lactobacilli at the strains level and an increased diversity of species in sealed carious dentin. More studies evaluating other virulence traits are necessary to better understand the cariogenicity of the residual strains underneath restorations and to understand the resistance of *L. rhamnosus* to this site.

Tratamento da Cárie Dentária

A cárie dentária é uma das doenças crônicas mais comuns em todo o mundo: 90% das pessoas tiveram problemas dentários ou dor causados por cárie (Oral health: prevention is key, 2009). Nas últimas décadas, observou-se uma importante redução na ocorrência da cárie devido ao conhecimento dos fatores etiológicos e métodos de controle da doença. Uma grande variedade de inovações tecnológicas e de métodos de diagnóstico e tratamento vem se incorporando a uma nova odontologia. Busca-se atuar no modelo de prevenção e promoção de saúde, que propõe práticas odontológicas com fundamentos básicos centrados na compreensão do fenômeno saúde-doença. De acordo com uma recente crítica publicada na *The Lancet*, a prevenção desta doença bucal é importante e passível de realização, pois existem muitas abordagens terapêuticas e preventivas simples, rentáveis e baseadas em evidências, as quais precisam ser promovidas e implementadas (Oral health: prevention is key, 2009). Contudo, a cárie estabelecida com o desenvolvimento de lesões é a doença sem tratamento mais comum do mundo (Marcenes et al., 2013), sendo que a principal razão é a falta de acesso ao tratamento.

Um problema enfrentado na prática odontológica no tratamento tradicional de lesões em dentina, quando profundas, é uma possível exposição mecânica da polpa, através da escavação completa de dentina amolecida, permitindo a invasão de bactérias e dificultando a manutenção da vitalidade pulpar (Leksell et al., 1996; Mjor, 2002). O tratamento restaurador convencional pode, então, resultar em mau prognóstico e a provável necessidade de tratamento endodôntico (Barthel et al., 2000; Bjorndal et al., 1997). A nível nacional, o serviço

público forma uma rede hierarquizada, onde os problemas mais simples são resolvidos no nível primário de atenção à saúde, que é amplamente disponibilizado nas Unidades Básicas de Saúde e nas Unidades de Saúde da Família. Já no nível secundário, são atendidos problemas de saúde mais complexos, onde se enquadra o tratamento endodôntico: oneroso, de execução difícil, muitas vezes exigindo a intervenção de um especialista da área e com muito pouca disponibilidade de acesso.

Devido à restrita oferta desta terapia no serviço e seu alto custo, esta situação é precursora da perda dentária de um expressivo contingente populacional. Em 2010, o componente de dentes perdidos do índice CPOD para adultos foi de 7,3 (Brasil, 2012). Já aos 12 anos, a prevalência de dentes perdidos por cárie tem sido alta. Em um estudo representativo da cidade de Porto Alegre/RS, foi encontrada prevalência de 5,81% de escolares de 12 anos com pelo menos um dente extraído por cárie ou com indicação de extração, sendo que quase sempre os dentes afetados eram primeiros molares (Alves et al., 2014). Além disso, os escolares sem acesso ao tratamento privado apresentaram a maior probabilidade de apresentar perda dentária. Este achado é provavelmente relacionados com a falta de disponibilidade de tratamentos em nível de atenção secundária no setor público. Dessa forma, assume-se urgência na implementação de alternativas de tecnologias terapêuticas avançadas exequíveis em nível de atenção primária para lesões profundas de cárie. Como consequência, espera-se uma redução de necessidade de tratamento endodôntico, impedindo que a lesão de cárie dentária progrida na sua evolução natural determinando a perda do dente.

A preocupação com a manutenção da vitalidade pulpar em lesões profundas de cárie incentivou o desenvolvimento de técnicas operatórias mais conservadoras, baseadas nos conceitos da Odontologia de mínima intervenção, as quais realizam Remoção Parcial da Dentina Cariada (RPDC). Apesar de resultados promissores resultantes da utilização destas

técnicas, ainda encontra-se resistência no ensino e disseminação de seu uso para dentes permanentes. Um exemplo disso é o resultado de um estudo realizado com dentistas da rede pública de Porto Alegre, RS, que avaliaram imagens fotográficas e radiográficas de lesões profundas de cárie e o tratamento mais comumente indicado foi a remoção total de tecido cariado (71%). As terapias pulpares tiveram indicação direta por 2,5% dos dentistas. Os autores concluíram que o tratamento mais comumente indicado pelos dentistas ofereceria alto risco de exposição pulpar e consequentemente pior prognóstico (Weber et al., 2011).

Lesões profundas de cárie e a aplicação da Remoção Parcial de Dentina Cariada

Convencionalmente, o tratamento restaurador de cárie em dentina pressupõe Remoção Total da Dentina Cariada (RTDC), baseada nos critérios clínicos de dureza. Entretanto, a partir de uma escavação completa de dentina amolecida em lesões cariosas profundas há uma alta probabilidade de exposição da polpa dentária (Bjorndal et al., 1997; Bjorndal et al., 2010; Leksell et al., 1996; Maltz et al., 2002; Ricketts et al., 2006). Diante disto, tem-se um prognóstico sombrio da saúde pulpar (Al-Zayer et al., 2003; Bjorndal et al., 2010; Farooq et al., 2000; Leksell et al., 1996).

No entanto, uma forma alternativa de evitar a exposição pulpar é a realização do tratamento expectante (Barthel et al., 2000; Bjorndal et al., 1997; Dumsha and Hovland, 1985; Jordan and Suzuki, 1971; Massler, 1967). Tratamentos expectantes consistem na RPDC e manutenção de uma camada de tecido cariado sobre a polpa, que será recoberta pela aplicação de um ou mais agentes protetores provisórios. O objetivo desta técnica é paralisar a progressão da lesão e permitir a formação de dentina terciária previamente à escavação completa (realizada após período de 45 dias a nove meses). O tratamento expectante nada mais é do que a RTDC em duas sessões, reduzindo o risco de exposição pulpar (Bjorndal and

Thylstrup, 1998; Bjorndal et al., 2010) e consequentemente melhorando o prognóstico da saúde pulpar pela manutenção da integridade do órgão pulpar (Hayashi et al., 2011). Esta integridade é de vital importância para a produção de dentina reacional, que ocorre devido à contínua atividade odontoblástica (Mjor, 2002; Pashley, 1996). A manutenção da atividade pulpar proporciona a capacidade de resposta dentinogênica aos estímulos biológicos e patológicos, através da formação de dentina secundária, peritubular, reparativa e reacional pelas células odontoblásticas (Tziafas, 2000). Uma revisão sistemática, através da análise dos resultados de 13 estudos que compararam o tratamento expectante com RTDC, publicados entre 1970 e 2008, conclui que o tratamento expectante pode ser considerado o mais eficaz para a preservação da polpa em lesões de cárie muito profundas (Hayashi et al., 2011). Este procedimento é possível visto que ocorre uma inativação da lesão, redução significativa do número de microrganismos viáveis e remineralização da dentina remanescente (Alves et al., 2010; Franzon et al., 2007; Lula et al., 2009; Maltz et al., 2002).

A partir dessas observações, sugeriu-se que é possível tratar lesões profundas de cárie com RPDC e restauração definitiva em uma mesma sessão, mesmo com a manutenção da dentina afetada. Esta forma de tratamento visa evitar contaminação resultante de perda da restauração temporária do tratamento expectante, reduzir o tempo clínico, evitar eventual exposição pulpar durante a remoção da restauração provisória e tornar o tratamento menos oneroso (Alves et al., 2010; Jardim, 2010; Maltz et al., 2002). Além disso, a partir de uma nova intervenção, ocorrerá perda de mais tecido dentário hígido pela remoção do material restaurador. Um ensaio clínico de braço único tratou com RPDC lesões profundas de cárie e demonstrou uma alta taxa de sucesso deste tratamento (97%, 90%, 82% e 63% em 1,5-, 3-, 5- e 10- anos de acompanhamento, respectivamente) (Maltz et al., 2011). Um ensaio clínico randomizado controlado multicêntrico comparou a taxa de sucesso de dentes após tratamento expectante e após RPDC seguido de restauração. Após dois anos de acompanhamento, as

taxas de sucesso da RPDC e do tratamento expectante foram de 93,7% e 73,3%, respectivamente (Jardim, 2010). Após três anos de acompanhamento, a taxa de sucesso foi 91% para tratamento com RPDC enquanto o tratamento expectante apresentou uma taxa sucesso de 69%, com diferença estatisticamente significativas após três anos (Maltz et al., 2012a; Maltz et al., 2013). Esta diferença pode ser explicada pelo elevado número de tratamentos expectantes incompletos e pelo tipo de análise realizada no estudo (intenção de tratar). Insucessos no tratamento expectante foram explicados porque os pacientes que não retornaram para a realização da restauração definitiva tiveram falha nas restaurações provisórias e, como consequência, danos aos tecidos pulpares. O tratamento expectante completado apresentou uma taxa de sobrevivência de 88% enquanto o tratamento expectante incompleto uma taxa de 13%. Além disso, o tratamento com RPDC e restauração na mesma sessão ocasionou uma economia de 67,78% por tratamento comparado ao tratamento expectante, e 2,39% no conjunto da economia do custo anual da Unidade Básica de Saúde em Porto Alegre, RS (Jardim, 2010).

Embora haja fortes evidências de sucesso clínico, discute-se a possibilidade das bactérias que permanecem viáveis após o selamento das lesões de cárie serem prejudiciais ao órgão dentário, o que faz com que este tratamento não seja aceito universalmente. Desta maneira, ainda é necessário elucidar o potencial patogênico das baterias remanescentes após selamento de lesões de carie.

Considerações microbiológicas dos tratamentos restauradores

O entendimento dos princípios dos sistemas ecológicos dos biofilmes orais tem sido foco atual de pesquisa que vem gerando mudanças nos conceitos etiológicos da cárie dentária. A microbiota de biofilmes orais é muito complexa e a diversidade de bactérias vem

sendo relacionada à manutenção da homeostase do biofilme (Marsh, 1999; 2003). Tem sido estabelecido que uma lesão de cárie possa desenvolver-se na presença de um amplo espectro de espécies microbianas (hipótese da placa ecológica). Esta hipótese para o desenvolvimento de cárie afirma que o desenvolvimento e progressão da doença ocorrem devido à ruptura da homeostase do biofilme no sítio doente (Marsh, 1999). Tal hipótese baseia-se no fato de que as bactérias patogênicas também estão presentes em saúde, mas em níveis baixos. A exposição prolongada a acidez pela maior disponibilidade de carboidratos fermentáveis inibiria o crescimento de bactérias não-acidúricas e favoreceria bactérias acidogênicas (Beighton et al., 1993; Brailsford et al., 1998), empurrando a "balança" no sentido da doença. A aciduricidade da bactéria permite a sua sobrevivência em condições ácidas, e contribui para o seu potencial cariogênico e progressão da cárie.

Quando a ruptura da homeostase (disbiose) é mantida por certo tempo, uma lesão de cárie se estabelece pela perda mineral da superfície dentária ocasionada pelo baixo pH proporcionado pelo metabolismo do biofilme (Marsh, 2003). É possível controlar a progressão da lesão pela desorganização e controle do biofilme. Em lesões cavitadas, porém, ocorre impossibilidade de acessar o biofilme que está protegido dentro da cavidade. A microbiota instalada é continuamente exposta a uma vasta variedade de carboidratos advindos da dieta, que fornece nutrientes para o metabolismo e crescimento desses microrganismos. Por isso, uma restauração para selamento da cavidade é necessária.

Lesões dentinárias apresentam gradiente de contaminação e quanto mais profunda, menor a contaminação encontrada na dentina em termos quantitativos e de composição da microbiota. Em restaurações convencionais, mesmo a RTDC seguindo critérios clínicos de dureza e coloração não garante a ausência de bactérias, as quais estão presentes em pequena quantidade, são rotineiramente seladas sob restaurações e isto não resulta em insucesso clínico (Fisher, 1977; Fisher, 1972; Henz, 1997; Iost HI, 1995; Lopes

CMN, 1987; MacGregor A, 1956; Munson et al., 2004; Shovelton, 1970; 1972; Whitehead FI, 1960). Quando lesões profundas de cárie são tratadas com RPDC, uma camada mais superficial de dentina e, portanto, mais contaminada é mantida sob a restauração. Uma grande quantidade de bactérias residuais permanece viável.

Um estudo observou que não houve diferença na composição da microbiota na dentina cariada que seria mantida com tratamentos de RPDC (zona afetada) e na dentina ao final da RTDC. A análise molecular detectou maior prevalência de *Streptococcus mutans* (16%), *Lactobacillus gasseri/johnsonii* (13%), e *Lactobacillus rhamnosus* (8%) em ambas as microbiotas (após RPDC e após RTDC) (Munson et al., 2004). Além disso, apenas três táxons foram encontrados em todas as amostras: *Streptococcus mutans*, *Rothia dentocariosa* e *Propionibacterium* sp. Em outro estudo, foram encontradas 70% de bactérias gram-positivas, das quais 50% eram *Lactobacilos* do total de unidades formadoras de colônias após RPDC (Bjorndal and Thylstrup, 1998). Com relação à quantidade, há maior contaminação bacteriana logo após a RTDC do que quando se avalia a dentina cariada residual após RPDC e selamento temporário (Maltz et al., 2012b).

Acredita-se que através desse selamento da cavidade de cárie, corta-se o substrato proveniente da dieta e do meio bucal. A redução da disponibilidade de carboidratos fermentáveis reduziria a acidificação do meio e, por conseguinte, haveria redução da infecção, redução da pressão seletiva e paralisação da progressão da lesão. Além da reduzida disponibilidade de nutrientes durante o selamento, há simplicidade e relativa homogeneidade dos nutrientes disponíveis (Kneist et al., 2010; Paddick et al., 2005). Porém, as bactérias são capazes de adaptar sua fisiologia aos ambientes em alteração e algumas bactérias podem prevalecer e seguir metabolicamente ativas abaixo das restaurações. Uma pressão seletiva pelo estresse nutricional é exercida sobre as bactérias residuais (Paddick et al., 2005). Neste cenário, uma mudança de metabolismo tem sido sugerida em bactérias isoladas de dentina

cariada após selamento. É provável que os componentes de bactérias inviáveis (que não resistiram à pressão seletiva pelo estresse nutricional) e também glicoproteínas fornecidas pela polpa possam contribuir como fonte de nutrientes para as bactérias que sobreviveram (Paddick et al., 2005).

Alguns estudos mostraram uma modificação da composição da microbiota e uma drástica redução de bactérias depois da RPDC e selamento da cavidade (Bjorndal et al., 1997; Bjorndal and Thylstrup, 1998; Maltz et al., 2002). Uma quantidade massiva de dados enfatiza um papel de estreptococos (principalmente *S. mutans*) e lactobacilos na cárie, sugerindo que estes deveriam compreender a maior carga microbiana total em lesões dentinárias. Um foco particular tem sido a capacidade das espécies dentro desses gêneros de produzir ácido e tolerar ambientes extremamente ácidos (Kianoush et al., 2014). Estes microrganismos constituem parte da microbiota normal da cavidade bucal, desempenhando um importante papel na patogenicidade da cárie dentária (Loesche, 1986). Entretanto, quando a lesão é selada, há redução substancial de lactobacilos (Kneist et al., 2010) e de *S. mutans,* enquanto os microrganismos predominantes são de *Actinomyces naeslundi* e vários estreptococos, não sendo a microbiota tipicamente cariogênica que parece ser resistente (Bjorndal and Thylstrup, 1998; Paddick et al., 2005) .

Com relação à análises genotípicas, alguns estudos fizeram análises pós-cultivo de espécies relacionadas com cárie, com objetivo de observar a presença de genótipos mais cariogênicos. Em geral, os resultados dos estudos mostram uma grande diversidade de genótipos em dentina (dificilmente o mesmo genótipo é identificado em pacientes diferentes, a não ser familiares) e, em geral, uma menor diversidade genotípica em amostras coletadas de sítios hígidos quando comparadas com amostras coletadas de sítios com cárie (Alaluusua et al., 1996; Grönroos and Alaluusua, 2000; Lembo et al., 2007; Li and Caufield, 1998; Napimoga et al., 2004; Paddick et al., 2005; Saarela et al., 1996). Entretanto, a modificação da

diversidade genotípica das bactérias da dentina cariada após o selamento da cavidade só foi observada por um estudo para *S. oralis* e *Actinomyces naeslundii*. Neste estudo, apenas 18% dos isolados genotipados foram observados antes e após restauração. Também foi mostrada uma redução muito significativa da diversidade genotípica após o selamento para ambas as espécies: *A. naeslundii* reduziu a média de genótipos de 13,4 para 1,6 ($p=0,002$), enquanto *S. oralis* reduziu a média de genótipos de 13,6 para 3,4 ($p=0,001$) (Paddick et al., 2005) (Paddick et al., 2005). Não há dados de modificação da diversidade genotípica para *S. mutans* e lactobacilos em dentina cariada selada.

Diante de evidências da inativação das lesões com redução significativa do número de microrganismos viáveis e da remineralização da dentina remanescente (Alves et al., 2010; Lula et al., 2009; Maltz et al., 2002; Pinto et al., 2006), surge o questionamento da necessidade da reabertura da cavidade para realizar a remoção da dentina cariada residual (Dumsha and Hovland, 1985; Kidd, 2004). A persistência de bactérias após a RPDC é uma realidade (Firmino, 2011; Lula et al., 2009; Maltz et al., 2002; Maltz et al., 2012b; Pinto et al., 2006), mas não há dúvidas que há redução gradual da contaminação bacteriana após o selamento da dentina cariada, principalmente da microbiota tipicamente cariogênica (Kneist et al., 2010; Lula et al., 2009; Maltz et al., 2002). Neste cenário, alguns questionamentos surgem. Estes microrganismos que permanecem sob o selamento tem potencial de atuar na progressão da lesão de cárie e consequente comprometimento do órgão pulpar? Há possibilidade de que ocorra uma seleção de espécies na dentina cariada após selamento pela modificação da disponibilidade de nutrientes? Essas bactérias remanescentes seriam potencialmente mais cariogênicas? Há modificação da diversidade genotípica das bactérias cariogênicas após selamento da dentina cariada?

Métodos moleculares para identificação e genotipagem de microrganismos na dentina cariada

As técnicas de biologia molecular têm contribuído significativamente na identificação da microbiota residente em diferentes sítios do corpo humano (Peterson et al., 2008) e na identificação de vários patógenos antes desconhecidos (Faveri et al., 2008; Paster et al., 2001; Saito et al., 2006). As tecnologias baseadas em análises moleculares são de evolução muito rápida. Dentre as vantagens das técnicas moleculares, destacam-se especificidade e sensibilidade na detecção e caracterização dos patógenos.

A análise precisa e individualizada das cepas é possível e importante do ponto de vista científico. Com o avanço dos estudos sobre a etiologia das doenças, a análise qualitativa de microrganismos patogênicos passou a ser importante na identificação de espécies e cepas associadas com doença. Se constatada a implicação de determinadas espécies na doença, a etiologia da doença poderia ser mais bem caracterizada. Esse conhecimento tem o potencial de melhorar o diagnóstico, a prevenção e o tratamento da doença. Apesar da importância da identificação das espécies na etiopatogenia das doenças, os estudos da doença cárie, na sua maioria, analisam os microrganismos apenas de maneira quantitativa (Badet and Thebaud, 2008). É importante a identificação e caracterização (genotipagem) dos microrganismos relacionados com a progressão das lesões cariosas.

Para identificação de espécies bacterianas, o sequenciamento parcial do gene 16SrRNA vem sendo amplamente utilizado para todos os campos da microbiologia, apesar da possibilidade de uso de outros marcadores filogenéticos. Para identificação em nível de espécie de isolados presuntivos de *Streptococcus mutans,* a amplificação do gene *gtfB* já é suficiente (Arthur et al., 2011). Para o gênero lactobacilos, é bem esclarecido que o sequenciamento do gene 16S rRNA não é ideal para a identificação de lactobacilos em nível

de espécie. Múltiplas cópias do gene 16S rRNA com diferentes polimorfismos já foram descritas para *L. paracasei/casei*, *L. rhamnosus* e *L. Zeae*, causando baixo poder discriminatório do sequenciamento desse gene (Vásquez et al., 2005). Também já foi demonstrado que a técnica de sequenciamento de 16SrRNA não consegue diferenciar *Lactobacillus gasseri* de *Lactobacillus johnsonii*, *Lactobacillus pentosus* de *Lactobacillus plantarum*, e *Streptococcus mitis* de *Streptococcus oralis*, além de não identificar em nível de espécies uma série de Pseudomonas (Munson et al., 2004). A utilização de genes constitutivos (*housekeeping genes* - responsáveis pelo metabolismo bacteriano) surge como alternativa para superar esta limitação. A análise simultânea dos genes *pheS* (subunidade α da fenilalanina) e *rpoA* (subunidade α da RNA polimerase) proporciona uma alternativa na identificação confiável e rápida de diferentes espécies de lactobacilos, apresentando um poder discriminatório maior do que o da análise com 16S rRNA (Parolo, 2009; Parolo et al., 2011). A análise do gene *groEL* (chaperona hsp60) também mostra-se como um marcador confiável para a identificação de qualquer espécie de lactobacilos, permitindo uma identificação rápida e confiável de lactobacilos, além da discriminação de espécies com similaridades (Blaiotta et al., 2008).

Dentre os diferentes métodos de genotipagem, o método baseado na amplificação por *primers* arbitrários AP-PCR (ou RAPD) utiliza sequências iniciadoras randômicas para caracterização do genoma bacteriano. Esta técnica é bastante vantajosa, pois apresenta baixo custo, não requer equipamentos muito complexos, é de fácil de aplicação mesmo em amostras grandes, velocidade, flexibilidade e facilidade de interpretação dos dados gerados. Algumas limitações são inerentes ao método, como a baixa reprodutibilidade e a sensibilidade a pequenas variações na metodologia (extração do DNA, tipo de termociclador, concentração DNA, temperatura de anelamento, concentração de Mg^{+2}, entre outros). A validade da técnica de genotipagem de microrganismos por AP-PCR foi garantida por comparações com diversas

outras técnicas (Li and Caufield, 1998; Li et al., 2001; Saarela et al., 1996). Foi mostrado que genótipos diferentes de *S. mutans* podem apresentar potencial cariogênico distinto (Lembo et al., 2007; Mattos-Graner et al., 2004). O AP-PCR tem sido usado também para avaliar o perfil genotípico de *S. mutans* isolados de saliva, biofilme, língua e dentina (Arthur et al., 2011; Baca et al., 2012; Bönecker et al., 2003; Grönroos and Alaluusua, 2000; Lembo et al., 2007; Li and Caufield, 1998; Mitchell et al., 2009; Napimoga et al., 2004; Toi et al., 2003).

Através de metodologias de biologia molecular é possível identificar os perfil da microbiota cariogênica remanescente, aprofundando o conhecimento sobre a função da microbiota residual após a RPDC e selamento da cavidade (estresse nutricional).

Fatores de virulência de bactérias cariogênicas

Alguns fatores de virulência vem sendo estudados e associados com a progressão da cárie em dentina, como degradação de hidroxiapatita, a formação de biofilme, adesão à hidroxiapatita e ao colágeno do tipo I, clivagem dos açúcares terminais das glicoproteínas, produção de ácido e tolerância ao ácido (Jalasvuori et al., 2012; McGrady et al., 1995; Paddick et al., 2005). A capacidade das bactérias para produzir ácido (acidogênese) em combinação com um crescimento a um pH baixo (aciduricidade) são considerados como sendo dois dos fatores de virulência importantes relacionadas com a cárie dentária (Cotter and Hill, 2003). Para entender se as bactérias seladas abaixo de restaurações podem seguir o processo carioso, é interessante observar essas características e saber se as mesmas permanecem potencialmente cariogênicas.

Para ocorrer a desmineralização do esmalte, os microrganismos produzem ácidos a partir da fermentação de carboidratos. A dentina cariada de lesões ativas caracterizadas por

um pH baixo e um perfil de ácidos lactato-dominantes (Hojo et al., 1994). Uma variedade de bactérias é responsável por esta alteração de pH na cavidade, tal como a espécie de lactobacilos e alguns estreptococos, especialmente o *S. mutans* (Klinke et al., 2009; Loesche and Syed, 1973). Estes microrganismos podem produzir ácido capazes de reduzir o pH do meio abaixo de 4,0, o que é relevante para a progressão da cárie dentária (Haukioja et al., 2008). Além disso, algumas espécies de lactobacilos são capazes de secretar ácido em quantidades tão significativas que podem produzir um pH ainda mais baixo (Klinke et al., 2009).

O estresse nutricional causado pela restauração da cavidade poderia reduzir a quantidade de ácido produzido e modificar o padrão de ácidos do meio. Menos ácido lático é esperado pela escassez de carboidratos (Paddick et al. 2005) (Paddick et al., 2005). Através da regulação da enzima piruvato formato-liase (*pfl*) em algumas bactérias, como em *S. mutans*, poderá haver alteração dos padrões de fermentação e as bactérias poderiam seguir produzindo ácidos menos cariogênicos, principalmente formato e o acetato (Abbe et al., 1982; Takahashi-Abbe et al., 2003). Este sistema de regulação já é bem conhecido: a ativação do sistema PFL por *S. mutans* ocorre na escassez de nutrientes (Takahashi-Abbe et al., 2003). *S. mutans* é capaz de obter energia por meio da ativação do *pfl* em condições anaeróbias, através do metabolismo de polissacarídeos intracelulares, como nas lesões seladas. Por esta razão, a acidogênese é considerada como um importante aspecto de virulência das bactérias abaixo das restaurações e isso pode explicar como as bactérias permanecem viáveis, por tempo limitado, abaixo das restaurações.

Também foi descrito que bactérias abaixo de restaurações tem acesso principalmente à proteínas e glicoproteínas advindas da polpa através dos túbulos dentinários (Paddick et al., 2005). Foi observado um pH mais elevado na dentina cariada selada, que

poderia estar associado ao decréscimo do número de bactérias acidogênicas e ao acesso limitado aos nutrientes (Hojo et al., 1994).

Bactérias relacionadas à cárie dentária devem ter a capacidade de crescimento ou de sobreviver a várias condições de estresse ambiental, incluindo alterações na fonte de carboidrato, mudanças entre ambientes aeróbios e anaeróbios e choque ácido. As bactérias em cárie dentária empregam uma série de estratégias para resistir aos efeitos do estresse ácido, como efluxo ativo de prótons ou ativação de chaperoninas, responsáveis por evitar a agregação e enrolamento incorreto de proteínas, além de enrolar novamente proteínas danificadas pelo ácido para evitar degradação (Lemos et al., 2005). Outros mecanismos de regulação de pH importantes são o metabolismo do lactato, que facilita a neutralização do meio, a utilização de nitrogênio por algumas espécies e a liberação de substâncias básicas para diminuir o pH (amônia e ureia) (Gross et al., 2010).

Assim, a caracterização de bactérias cariogênicas da dentina cariada selada pode trazer informação sobre o papel dos microrganismos remanescentes e sua importância na progressão da lesão cariosa após tratamento com RPDC.

Objetivo geral

Avaliar o efeito da redução do aporte nutricional, promovida pelo selamento da cavidade de cárie, nas bactérias cariogênicas remanescentes após tratamento com remoção parcial de dentina cariada.

Objetivos específicos

- Avaliar a diversidade de espécies dos lactobacilos cultivados na dentina cariada antes e após três meses de selamento;

- Avaliar a diversidade genotípica dos *Streptococcus mutans* e de lactobacilos cultivados na dentina cariada antes e após três meses de selamento;

- Avaliar os fatores de virulência (aciduricidade e acidogenicidade) das bactérias cariogênicas (lactobacilos e *Streptococcus mutans*) identificadas na dentina cariada antes e após três meses de selamento.

Esta tese foi baseada nos seguintes artigos científicos:

I. "Genotypic diversity and virulence traits of *Streptococcus mutans* isolated from carious dentin after partial caries removal and sealing."

Nailê Damé-Teixeira, Rodrigo Alex Arthur, Clarissa Cavalcanti Fatturi Parolo, Marisa Maltz.
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II. "Genotypic and phenotypic characterization of lactobacilli isolated from carious dentin after starvation stress."

Nailê Damé-Teixeira, Marisa Maltz, Raquel Soares Dalalba, Vanessa Kern Soares, Laís Daniela Ev, Ariel Goulart Rup, Clarissa Cavalcanti Fatturi Parolo.

Genotypic diversity and virulence traits of *Streptococcus mutans* isolated from carious dentin after partial caries removal and sealing

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Running title: Genotypes of *S. mutans* from carious dentin

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Research Article

Genotypic Diversity and Virulence Traits of *Streptococcus mutans* Isolated from Carious Dentin after Partial Caries Removal and Sealing

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The aim of this study was to compare the genotypic diversity and virulence traits of *Streptococcus mutans* isolated from carious dentin before and after partial dentin caries removal (PDR) and sealing. Carious dentin samples were obtained three months before and after the PDR and cavity sealing. Up to seven isolates of each morphological type of *S. mutans* were selected and strain identity was confirmed using gtfB primer. Genotyping was performed by arbitrary primer-PCR (AP-PCR). Acidogenesis and acidurance of the genotypes were evaluated as virulence traits. A paired *t*-test and a Wilcoxon test were used to compare the virulence of genotypes. A total of 68 representative *S. mutans* isolates were genotyped (31 before and 17 after the sealing). At least one of the genotypes found before the sealing was also found on dentin after the sealing. The number of genotypes found before the sealing ranged from 2 to 3 and after the sealing from 1 to 2 genotypes. No difference was observed in the acidogenesis and acidurance between genotypes isolated before and after the sealing. In conclusion, genotypic diversity of *S. mutans* decreased after the PDR and sealing, but the virulence traits of *S. mutans* remained unchangeable.

1. Introduction

Stepwise excavation has been an alternative treatment for deep caries lesions since the conventional treatment based on complete dentin caries removal could generate pulp exposure and poor dental prognosis [1]. The stepwise excavation involves the partial removal of the decayed tissue, temporary sealing, reopening of the cavity, and the complete removal of the carious tissue followed by restoration [1–3]. After the sealing period, the filling is removed and complete caries removal is performed [2, 4, 5]. Partial carious dentin removal (PDR) in one session, keeping a layer of carious dentin beneath restoration, has been proposed as an alternative approach to the stepwise excavation, avoiding failures due to the loss of temporary filling, additional costs, discomfort to the patient, and the possibility of pulp exposure during the second reopening procedure [6, 7]. This treatment has shown success in clinical studies over time without the need of cavity reentry [6–8]. However, the persistence of viable bacteria in dentin after PDR has raised doubts regarding the long-term effectiveness of this treatment [9].

After the sealing period, a limited supply of nutrients is left for the bacteria that survive underneath the restoration. Strains that are fit for physical, chemical, biological, and environmental changes might dominate and get established after the sealing. Paddick et al. [10] showed that only those bacteria capable of producing the enzymes required for the cleavage of the terminal sugars from the glycoprotein were recovered from the dentin after the cavity sealing. The low nutrient supply underneath the restorations could lead to a modification of the residual biofilm.

Although a limited number of microorganisms persist under restorations a few months after the PDR and sealing [2, 3, 11, 12], some cariogenic bacteria may be found within the remaining microorganisms, such as mutans streptococci, which is currently found on sealed carious dentin [13–17].

These bacteria are capable of producing acids due to fermentation of dietary carbohydrates (acidogenesis) and surviving in that low-pH environment (acidurance), which makes them an important cariogenic microorganism related to caries initiation and progression [18]. However, the relationship between the residual S. mutans found on carious dentin beneath restoration after the PDR and caries progression is still unclear. Thus, it is important to better understand how the environmental changes induced by the dentin sealing affect the diversity and virulence traits of the remaining mutans streptococci.

In this context, genotyping methods based on arbitrarily primed PCR (AP-PCR) have revealed that the community of S. mutans isolated from saliva and dental plaque is diverse [19, 20]. Additionally, it has been shown that distinct S. mutans genotypes may exhibit distinct cariogenic potential [21, 22]. Therefore, the aim of this study was to compare the genotypic diversity of S. mutans isolated from carious dentin before and after the PDR and sealing. We hypothesize that S. mutans genotypes are selectively found after the PDR and sealing and exhibit low cariogenic potential.

2. Methods

2.1. Origin of the Samples. The samples were derived from a previous clinical trial. Briefly, patients ($n = 18$) with permanent molars with carious lesions located in the middle third of the dentin were selected [23]. The patients were submitted to the PDR and sealing with a biocompatible material for 3 months. Dentin samples were obtained after the PDR (before and after the sealing), by a sterile bur, transferred to and diluted in reduced transport fluid, and plated on Mitis Salivarius Bacitracin (MSB) agar (Difco Laboratories, Detroit, MI, USA). Up to 7 isolates of each morphological type found in these cultures were selected and analyzed based on colony morphology. After subculture, each isolate was stored in Brain-Heart Infusion (BHI) (HiMedia, Mumbai, India) with 15% (v/v) glycerol at $-20°C$ for further analysis. From 18 patients, only 4 patients presented S. mutans isolates before and after the sealing.

2.2. Extraction of Genomic DNA. Streptococcus mutans were grown from frozen stocks on BHI agar and incubated for 24 h at 37°C in microaerophilic conditions. The genomic DNA was extracted from colonies resuspending them in 50 μL of sterile ultrapure water [24]. PCR with species-specific primers to gtfB (5′-ACTACACTTTCGGGTGGCTTGG-3′ and 5′-CAGTATAAGCGCCAGTTTCATC-3′) was performed to confirm the identity of S. mutans isolates [19] (Invitrogen, SG, Milanese, Italy). The PCR amplifications were performed with 50 μL total volume, including 1 μL of the target DNA, 0.25 μL of Taq DNA polymerase (5 U/μL), 5 μL of 10 × PCR buffer, 2.5 μL of 50 mM MgCl$_2$, 1 μL of deoxynucleoside triphosphate mix (10 mM), and 1 μL of each primer (10 mM). The amplifications were performed under the following conditions: 30 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 1 min, followed by 1 cycle of 95°C for 30 s, 59°C for 30 s, and a final extension of 72°C for 5 min. Genomic DNA

of S. mutans strain UA159 (provided by FIOCRUZ, Rio de Janeiro, Brazil) and ultrapure water were applied in all the PCR baths, as positive and negative controls. The PCR products were analyzed by electrophoresis on 2% agarose gel and stained with SYBR Green 1.6%, at 100 V for 45 min. Bands were visualized under UV illumination. All the chemicals were provided by Invitrogen (SG, Milanese, Italy).

2.3. Genotypic Analysis of S. mutans Isolates by AP-PCR. AP-PCR assays were performed with the arbitrary primer OPA 02 (5′-TGCCGAGCTG-3′) [19]. The DNA amplification occurred under the following conditions: 95°C for 2 min, for initial denaturation, and 45 cycles of 94°C for 30 s, 36°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. Streptococcus mutans strain UA159 and ultrapure water were used as controls. Products of AP-PCR were analyzed by electrophoresis on 1% agarose gel and stained with SYBR Green 1.6%, at 96 V for 4 hours.

2.4. Virulence Factors Analysis. The virulence traits of genotypes were evaluated as described elsewhere [19]. Frozen stocks of each S. mutans genotype were regrown on BHI agar plates and incubated at 37°C for 24 h. Two loops of 1 μL were inoculated into 30 mL of BHI broth supplemented with 1% glucose and incubated at 37°C for 18 h. In order to evaluate the ability of S. mutans genotypes to lower the suspension pH through glycolysis, 10 mL of the microbial suspension was centrifuged and resuspended in 50 mM KCl/1 mM MgCl$_2$ (Synth, São Paulo, Brazil). The pH of the solution was adjusted to around 7.0, and glucose was added to a final concentration of 55.5 mM. The pH was monitored for 180 min using a glass electrode previously calibrated with pH standards (pH 4.0 and 6.8). The area above the curve (AAC) was determined considering pH 6.5 as a cutoff point. The experiment was performed in duplicate and S. mutans UA 159 was used as a control in all the tests.

The ability of S. mutans genotypes to tolerate acidic environments was evaluated using 10 mL of the overnight growth suspension (described above) for 18 h in BHI broth/glucose. This suspension was diluted to 1 : 20 in BHI/glucose, and the growth was monitored until $OD_{550} = 0.5$. The suspensions were then centrifuged, and the pellets were washed once with 0.1 M glycine buffer (pH 7.0) (Nuclear, São Paulo, Brazil). The washed pellets were then resuspended in 0.1 M glycine buffer pH 2.8, 5.0, and 7.0. Immediately after the resuspension (time zero) and after 30 min and 60 min of incubation at 37°C, aliquots were serially diluted in phosphate buffer (pH 7.2), plated on BHI agar, and incubated at 37°C for 48 h. Cell viability at each time point was expressed as the percent of growth in relation to time zero.

2.5. Data Analysis. Images of AP-PCR fingerprints were captured by a digital camera (Canon Inc., Tokyo, Japan) and stored in Image File Format for visual analysis. For analysis of the S. mutans genotypic profiles from the same patient, AP-PCR products from the isolates obtained before and after the sealing were always resolved side by side in the same gel for visual comparisons. Thus, genotypic diversity was compared

TABLE 1: Number of isolates and genotypes (%) of S. *mutans* isolated per patient from carious dentin before and after sealing.

Patients	Before sealing		After sealing	
	Number of isolates	Genotypes (%)	Number of isolates	Genotypes (%)
1	15	A (26.7) B (60.0) C (13.3)	5	A (100.0) — —
2	11	D (54.5) E (45.4)	3	D (66.6) E (33.3)
3	3	F (66.6) G (33.3) —	8	— G (87.5) H (12.5)
4	2	I (50.0) J (50.0)	1	I (100.0) —
Total	31	9	17	6

Distinct letters show different genotypes in each patient. The designation of genotypes by letters (A, B, C, etc.) is only valid within each patient.

among isolates before and after the sealing samples. Isolates were considered as having the same genotypic identity when they presented identical AP-PCR product-size profiles. Later, a side-by-side analysis on the same gel was performed using each genotype (duplicate) from all the patients to verify the similarities between S. *mutans* isolates from different patients. Two blinded and calibrated examiners performed the visual analysis. Cohen's Kappa value was 0.76. Double cases were discussed and a consensus was reached. Genotypes (number and proportion) were described in each patient before and after the sealing (descriptive analysis). Data were transformed to $\log_{10}$ due to data dispersion, and the normal distribution was confirmed using a histogram and Kolmogorov-Smirnov test, except for the pH 2.8 analysis. A paired t-test and a Wilcoxon test were used to compare the virulence of genotypes (acidogenesis and acidurance) found before and after the sealing. The significance level was set at 5%. The virulence-traits data were analyzed by (1) the paired comparison between genotypes found before the sealing with the same genotypes found after the sealing (genotypes A, D, E, G, and I) and by (2) the comparison between the means of acidogenesis and acidurance of all the genotypes found before the sealing with all the genotypes found after the sealing. Statistical tests were performed in SPSS 18.0 for Windows (IBM SPSS Statistics).

2.6. *Ethical Considerations.* The protocol of the clinical trial was approved by the ethics committee of the Faculty of Dentistry from the Federal University of Rio Grande do Sul (process no. 19218). Informed and written consent was obtained from all the individuals. All the participants received the treatment for basic dental needs.

3. Results

A total of 48 S. *mutans* isolates were obtained from carious dentin corresponding to 31 found before and 17 found after the sealing (Table 1). All of them were identified as being
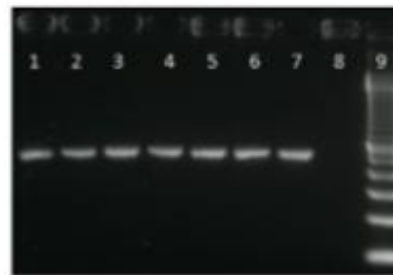

FIGURE 1: S. *mutans* identification by species-specific gtfB primer. Lanes 1 to 6: isolates of S. *mutans*; lanes 7 and 8 correspond to the positive control (S. *mutans* UA 159) and negative control (water), respectively; lane 9: 100-bp DNA ladder.
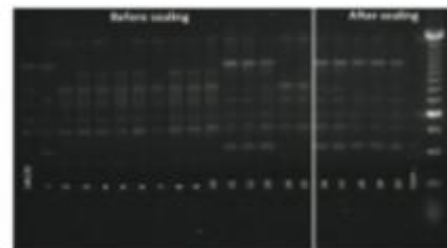
FIGURE 2: Electrophoresis gel image from AP-PCR-generated fingerprints using primer OPA-02 of S. *mutans* reference strain (UA159) and isolates from carious dentin before and after sealing. Lanes 1 to 15 correspond to the S. *mutans* isolates before sealing; lanes 16 to 20 correspond to the S. *mutans* isolates after sealing; lane 21: negative control (water); lane 22: 250-bp DNA ladder.

S. *mutans* by species-specific PCR (Figure 1). The molecular weight fragments generated by AP-PCR from the studied patients range between 490 and 5000 bp generating 9–12 DNA bands. The genotypic diversity of S. *mutans* found in one patient (patient 1) is shown in Figure 2. The number of genotypes found per patient varies between 2-3 and 1-2 before and after the sealing, respectively. At least one of the genotypes found before the sealing was also found on dentin after the sealing in all the patients. Only one patient presented one new genotype after the sealing (patient 3). Two patients (1 and 4) showed reduction in the number of genotypes before and after the sealing. In another patient (patient 2), the same genotypes were found before and after the sealing, although their proportion was different, with predominance of genotype D (Table 1). For patient 3, only one of the genotypes identified before the sealing was found after the sealing. That genotype was the most prevalent, and it colonized the dentin in addition to a new genotype exclusively found after the sealing (Table 1). No similarities were found between S. *mutans* isolates from different patients.

TABLE 2: Acidogenesis (AAC; pH 6.5 as cutoff point) of *S. mutans* genotypes isolated from carious dentin before and after sealing.

	Before sealing Mean (SD)	After sealing Mean (SD)	P
Paired genotypes	87.86 (10.31) ($n = 5$)	93.75 (9.34) ($n = 5$)	0.2
All genotypes	86.25 (3.40) ($n = 9$)	95.01 (4.34) ($n = 6$)	0.08

AAC: area above the curve.

The results of the analysis of the virulence traits are described in Table 2 (acidogenesis) and Table 3 (acidurance). In the acidogenesis analysis, no difference was observed in the AAC neither when the comparisons were made considering genotypes found before and after the sealing within the same patient ($P = 0.2$) nor when the comparisons were made considering all genotypes found before and after the sealing ($P = 0.08$). Regarding the acidurance, no difference was observed in the growth of the genotypes at different pH when the analysis was performed considering paired genotypes found before and after the sealing.

4. Discussion

This study aimed to compare the genotypic diversity and the virulence traits of *S. mutans* in carious dentin after PDR before and after the sealing, using AP-PCR fingerprinting analysis. We observed a reduction in the number of *S. mutans* isolates found after the sealing (Table 1). This is consistent with a reduction in counts of viable cells on carious dentin found after the cavity sealing [23]. AP-PCR has been used to evaluate the genotypic profile of *S. mutans* from saliva, biofilm, tongue, and dentin [19–21, 25–30]. The validity of the AP-PCR technique in the genotypic identification of microorganisms is assured by several comparisons made with other genotyping techniques [20, 31, 32].

Previous studies have shown a decrease in bacterial diversity [10, 33] after the PDR and sealing. A shift in the bacterial genotypes has also been observed [10], but no studies evaluating the genotypic diversity of *S. mutans* were found. In the present study, an altered genotypic diversity was observed after sealing the carious dentin.

It has been discussed that the residual bacteria found after the sealing have limited access to external nutrients. They are fed mainly by glycoproteins provided by the pulp and that nutrient restriction exerts a selective pressure over the residual bacteria [10]. In that scenario, a metabolism shift has been found in bacteria isolated from carious dentin after the sealing [10]. It is also likely that components of dead bacteria, which did not survive after the sealing, may contribute as the source of nutrients to those bacteria that survived below the restorations [10]. Considering that our dentin samples were collected 3 months after the sealing, these findings might explain the altered genotype prevalence found within different patients after the dentin sealing (Table 1). Some genotypes (B and F) were the most prevalent in dentin before

the sealing; however, they were not detected after the dentin sealing (Table 1). The undetected levels of these genotypes after the sealing might have enhanced the competitiveness of other genotypes (A and G) that were most prevalent after the sealing. In patient 3 (Table 1), there was a shift in the prevalence of genotypes found before and after the sealing. We believe that the reduction in the proportion of genotypes D and J has created a better condition for the growth of genotypes E and I. Interestingly, only genotype H was found on dentin samples after the sealing. It means that the genotype was below the detection limit of the microbiological method used [34] on dentin samples before the sealing. Moreover, some rare or transient genotypes in a single sample might be missed in a complex microbiota, which could explain the appearance of a new genotype only after the sealing [35]. The sealing of the cavity reduced the prevalence of genotype F, which might have created better conditions for the growth of genotypes G and H. In an open environment, such as a dental caries cavity, the microbiota of the biofilm above the dentin is continuously exposed to the dietary carbohydrate that provides nutrients for the growth of the diverse oral flora. However, the sealing of carious cavity was responsible for the selection of genotypes that were capable of surviving in the presence of low nutrient availability. Besides reduced availability of nutrients during the sealing, the relative simplicity and homogeneity of these nutrients significantly affect the microbiota surviving under the restorations [10, 33].

Additionally, microbiological studies have reported a significant decrease in the bacterial infection in the residual carious dentin after the cavity sealing [2, 3, 11, 12]. However, despite this microbial residual contamination found after the sealing, the carious dentin becomes harder and drier, both characteristics of inactive lesions [2]. Therefore, the reduction in the number of *S. mutans* genotypes found after the cavity sealing represents to some extent the low number of genotypes frequently found in caries-free individuals [25, 27, 28].

It remains unclear, though, whether the bacteria underneath restorations represent some danger to the longevity of restorations and if they are more virulent than the bacteria prior to the sealing. In this study, no statistical difference was found regarding the virulence traits of *S. mutans* isolated before and after the sealing. Even under a low-nutrient-availability condition, the role of specific phenotypic traits on the prevalence of these genotypes remains the same. That means those genotypes found after the sealing might be metabolically active if a source of external carbohydrates is provided, and, in addition to that, those genotypes are still capable of developing an acid-tolerance response to the acidic condition. Thus, it is important to point out the need for a perfectly sealed restoration.

Moreover, acidurance and acidogenesis of genotypes found before and after the sealing (A, D, E, G, and I) were not different which means that equal genotype presented equal phenotype. In contrast, several studies have shown that distinct *S. mutans* genotypes might show distinct acidurance [21, 22]. The results of the acidurance analysis in the present study suggest that the higher pH observed in sealed carious

TABLE 3: Acidurance of S. *mutans* genotypes isolated from carious dentin before and after sealing.

Time/pH	Paired genotypes			All genotypes		
	Before sealing ($n = 5$) % grown (min–max)	After sealing ($n = 5$) % grown (min–max)	p	Before sealing ($n = 9$) % grown (min–max)	After sealing ($n = 6$) % grown (min–max)	p
30 min						
*pH 7.2	96.1 (82.9–103.0)	91.5 (86.5–97.8)	0.3	95.4 (83.5–100.4)	92.2 (86.5–95.7)	0.5
*pH 5.0	85.3 (35.6–100.9)	98.2 (93.9–101.8)	0.3	91.1 (62.6–102.2)	96.5 (91.9–101.1)	0.6
†pH 2.8	63.4 (35.6–145.7)	38.2 (10.1–51.7)	0.1	48.7 (38.8–72.7)	34.6 (10.1–47.0)	0.3
60 min						
*pH 7.2	89.7 (64.9–103.9)	93.4 (90.1–97.4)	0.6	93.3 (83.5–102.6)	94.2 (90.6–96.9)	0.9
*pH 5.0	90.1 (62.4–105.2)	77.1 (45.5–98.5)	0.4	80.8 (52.9–99.1)	84.5 (71.1–98.5)	0.8
†pH 2.8	35.6 (00.0–93.3)	22.6 (00.0–58.3)	0.5	36.7 (19.3–67.8)	20.3 (00.0–44.9)	0.3

Percent of bacterial growth in relation to time zero (100%) in pH 7.2, pH 5.0, and pH 2.8.
* Paired t-test.
† Wilcoxon nonparametric test.

dentin [36] is due to the decrease in the number of these acidogenic bacteria and the limited access to nutrients [10] and not by their lower capacity of acid production.

Therefore, it is important to know if the biofilm that survived underneath the restorations remains potentially cariogenic [10, 37]. According to Takahashi and Nyvad [38], it is not only important to describe which bacteria are involved in caries, but it is also important to know what is their function [38]. Although S. *mutans* is one important cariogenic microorganism related to caries, only 4 out of 18 patients showed this microorganism after the sealing. Besides this, the S. *mutans* that survived presents similar virulence in comparison to the initial S. *mutans*.

In conclusion, genotypic diversity of S. *mutans* was reduced after PDR and sealing using AP-PCR fingerprinting analysis. Additionally, there was not any difference in acidurance and acidogenesis between genotypes found before and after the sealing. Genotypes found after the PDR and sealing have the same cariogenic potential of those found before the sealing.

Conflict of Interests

The authors declare that they have no proprietary, financial, professional, or other personal interests of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the paper.

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Original Research

Genotypic and phenotypic characterization of lactobacilli isolated from carious dentin after starvation stress

*Research field: Cariology

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ABSTRACT

The aim of this study was to characterize *Lactobacillus* species isolated from caries dentin before and after starvation stress by cavity sealing. Lactobacilli were obtained by culture of carious dentin from six patients. Two carious dentin samples were collected and cultured per patient: 1st before and 2nd after three months of cavity sealing. Presumptive lactobacilli were selected, isolated and analyzed by Gram staining. Housekeeping genes sequencing was used to the species identification and AP-PCR was used for genotyping. Phenotypic analysis (acid production and acid tolerance) was performed. It was analyzed 86 lactobacilli, 41 before and 45 after starvation stress by sealing. The sequence of *gro*EL gene was the greatest option to identify *Lactobacillus* species isolated from carious dentin. *L. paracasei* and *L. rhamnosus* prevailed and only four isolates did not belong to these species. A total of 27 and 15 different genotypes were found before and after sealing, respectively. There was no difference between isolates from carious dentin before and after starvation stress, neither regarding acid production or acid tolerance, although *L. paracasei* showed lower pH value in 48 h of growth. In conclusion, *L. paracasei* and *L. rhamnosus* were the most prevalent. *L. rhamnosus* was selected after starvation stress. There was a decreased genotypic diversity at the strains level and an increased diversity of species in sealed carious dentin. More studies evaluating other virulence traits are necessary to better understand the cariogenicity of the residual bacteria underneath restorations and to understand the resistance of *L. rhamnosus* to this site.

Keywords: Dental caries; *Lactobacillus* spp.; Sanger sequencing; housekeeping genes; genotyping.

1. INTRODUCTION

During an operative treatment, the persistence of a reduced number of bacteria in dentin after carious removal and sealing (1-4) has raised questions about the pathogenicity of these remaining bacteria (2). After the sealing of the carious lesion, a limited supply of nutrients is left for the bacteria that survive underneath the restoration (starvation stress). Adapted bacteria for an environmental change can dominate and become established, while other bacteria, which are not suited to the site, will disappear from sealed dentin (5). Furthermore, the low nutrient supply underneath restorations could lead to a modification of the residual biofilm pathogenicity. *Lactobacillus* species, *Streptococcus* species, *Actinomyces* species and *Candida albicans* are among the remaining microorganisms from the sealed dentin (3-9), although the microbiota in dentin samples is dominated by the genus lactobacilli (10). Lactobacilli could contribute to the progression of caries lesion due to its high acidogenicity and lactic acid production. Lactobacilli identification at species level is important to characterize its pathogenicity and genetic-based taxonomic methods are required for that purpose. Many studies has been conducted using partial sequencing of the 16S rRNA gene for phenotypic identification of lactobacilli (11, 12). Multiple copies of the 16S rRNA gene with different polymorphisms have been described in lactobacilli, leading to a low discriminatory power (12). The use of constitutive genes (housekeeping genes - responsible for bacterial metabolism) is an alternative to overcome this limitation. The simultaneous analysis of *phe*S (α-fenilalanin subunit), and *rpo*A (α-subunity of RNA polymerase) genes provides an alternative for reliable and rapid identification of different species of lactobacilli, with a greater discriminatory power than the 16S rRNA sequencing (13). Another possible housekeeping gene for *Lactobacillus* species identification is the *gro*EL gene (hsp60 heat shock proteins). The analysis of the *gro*EL gene also shows up as a reliable marker for the identification of closely related species of lactobacilli, enabling quick and reliable identification (14).

Besides the species identification, it is important to discriminate lactobacilli at a strain heterogeneity level. Genotypic diversity can be evidenced by AP-PCR that is a method that uses random primers for the characterization of the

bacterial genome under conditions of low stringency with a good resolving power (15). AP-PCR is suitable to identify unique genotype, it has a good reproducibility, low cost, does not require specific apparatus and it is not laborious (15). This approach was chosen to verify if the strains that remain after the cavity sealing are genetically related to the initial unsealed carious dentin. Furthermore, it is important to investigate if a more virulent genotype persists after starvation stress. In a previous study analyzing *S. mutans*, different AP-PCR genotypes were related to different virulence (16), although it was not observed for *S. mutans* isolated before and after cavity sealing (17). There is a lack of evidence in the literature regarding the role of the remaining lactobacilli in the progression of carious process beneath restorations after partial caries removal. It is unclear if the virulence potential of the sealed bacteria remains the same after sealing or if they change, either phenotypically or genotypically. Therefore, the aim of this study was to characterize *Lactobacillus* species isolated from caries dentin before and after starvation stress by cavity sealing, comparing the diversity at the species, genotypic and phenotypic levels.

2. METODOLOGY

Origin of the samples

Lactobacilli analyzed in this study were obtained by culture of carious dentin from patients, who had participated in a clinical trial (18) in the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. The clinical trial was performed from August 2010 to August 2011 (19). Patients (n = 18) with permanent molar with carious lesion located in the middle third of dentin were submitted to partial caries removal. After, the cavity was sealed for three months with calcium hydroxide-containing base material (Dycal®, Dentisply) and glass ionomer cement (Vitromolar®, DFL) as a temporary filling. Dentin samples were obtained before sealing (just after partial caries removal) and after removing the temporary filling (20). The dentin sample collected were immediately transferred to a sterile container with 1.2 mL of RTF (Reduced

transport fluid medium) and glass beads (21). Samples were twice dispersed by sonification for 10 seconds, with a 30 seconds interval, in a high-density ultrasonic processor (Vibra cell TM®, Sonic & Material Inc., Connecticut-USA). Samples were vortexed for 30 seconds and 10-fold serial diluted in RTF. Subsequently, 25 µL aliquots from the appropriated dilution (22) were cultured in duplicate on Rogosa selective *Lactobacillus* agar (HiMedia, Mumbai, India) anaerobically at 37 ºC for 72 hours. Colonies of presumptive lactobacilli were then selected and isolated (up to 7 isolates of each morphological type). All isolates were analyzed for Gram staining. Gram-positive bacilli were stored in Brain Heart Infusion (BHI) (HiMedia, Mumbai, India) with 15% (v/v) glycerol at -20°C for further species identification, genotypic and phenotypic analysis.

Extraction of genomic DNA

Presumptive lactobacilli were grown from frozen stocks on BHI agar, incubated for 24 h at 37°C in anaerophilic conditions. The genomic DNA was extracted by mechanical breakage of cell wall from colonies suspending them in 50 µl of sterile ultrapure water and shaking (23).

Amplification and Sequencing of housekeeping genes

The extracted DNA was used as template in standard Polimerase Chain Reaction (PCR) for amplification of housekeeping genes (Table 1). Primers to amplification of the α-subunit of phenilanyl-tRNA syntases (*phe*S*)* gene; or α-subunit of RNA polymerase (*rpo*A) gene; or Hsp60 chaperonins gene (*gro*EL); or 16S rRNA region (using the universal primers for the domain Bacteria prokaryotic organisms) were used. The *phe*S primers were used as the first choice and if no amplicon was obtained, the *rpo*A*/gro*EL were used as further options respectively (13, 24). In cases of no amplification with any of the mentioned housekeeping genes, a last option was the 16S rRNA amplification.

PCR amplifications were performed with a 25 µL total volume including 1 µL of the target DNA, 0.5 µL of Taq DNA polymerase (5 U/µl) (Invitrogen), 1.8 µL buffer (Invitrogen, SG, Milanese, Italy), 2.5 µL of 50mM $MgCl_2$, 1 µL of a deoxynucleoside triphosphate mix (10mM), 1 µL of each primer (10 µM). The

termocycling conditions consisted of 30 cycles (30 s at 94 °C, 30 s at the annealing temperature for each primer, described in table 1, and 1 min at 72 °C) and one additional, final cycle at 72 °C for 10 min. PCR amplicons were resolved by electrophoresis on a 1% (w/v) agarose gel electrophoresis at 100 V for 30 min. The gel was stained with 1 µL of SybrGreen (Invitrogen, SG, Milanese, Italy). Bands were visualized under UV illumination. PCR products were purified using the DNA purification kit (PureLink® Genomic DNA Mini Kit, Invitrogen), following the supplier recommendation. Fragments of genes were sequenced using an ABI 3730xl DNA Analyser (Applied Biosystems – carried out by ACTgene, Porto Alegre, Brazil). Data obtained by gene sequencing were manually aligned, corrected and edited using BioEdit 7.1.3 software. The identification of species level was performed by comparing the sequences with its homology in the NCBI (BLAST; http://www.ncbi.nlm.nih.gov/) and in the United States and cpnDB database (chaperonin Sequence Database; http://www.cpndb.ca/cpnDB/home.php - for *gro*EL sequences). Similarities higher or equal to 98% were considered for classification of lactobacilli at the species level. Phylogenetic trees were drawn using the software MEGA 6 (Molecular Evolutionary Genetics Analysis).

Genotypic analysis of Lactobacillus species by AP-PCR

AP-PCR assay was performed using the arbitrary primer OPA 03 (5′-TGCCGAGCTG-3′′) (25). DNA amplification was done under the following conditions: 2 min at 95 °C, 45 cycles (30 s at 94 °C, 30 s at 36 °C, 1 min at 72 °C) and one additional, final cycle at 72 °C for 5 min. *L. rhamnosus* ATCC 7469 and ultrapure water were used as a positive and negative control, respectively. Products of AP-PCR were analyzed by electrophoresis on 1% agarose gel, stained with SybrGreen 1.6%, at 96 V during 4 h. Images of AP-PCR fingerprints were captured by a digital camera (Canon Inc., Tokyo, Japan) and stored in Image File Format for visual analysis. In order to analyze lactobacilli genotypic profiles by visual comparisons, AP-PCR products from isolates obtained from the same patient (before and after temporary sealing) were always resolved side by side in the same gel. Isolates were considered as genotypic equal when identical AP-PCR product-size profiles were obtained (no different bands at all). A side-by-side analysis was performed

using each genotype (in duplicates) from all patients to verify similarities between isolates from different patients. Two blinded and calibrated examiners performed the visual analysis, as previously described (16, 17). The Cohen's Kappa value was 0.76. Double cases were discussed and a consensus was reached.

Phenotypic analysis

The acid production (26, 27) was evaluated by suspending one loop of the culture into 5 ml of BHI broth and cultivating at 37° C for 18 h. The overnight culture was centrifuged at 3,020 rpm for 5 min (Centrífuga-CT 5000, Cientec, Brazil) and the pellet was suspended into BHI broth supplemented with 1% glucose. Immediately, it was standardized at an optical density of 0.03 at 600nm (OD$_{600}$) (Spectronic 21D, Milton Roy, USA) and incubated at 37° C for 3 days. In order to perform the pH curve, aliquots were removed and the pH measured at 9 h, 24 h, 48 h and 72 h by a pHmeter (pHmetro DM-23, Digimed, Brazil). Negative controls were always incubated under the same condition. The experiment was performed in triplicates. Mean±SD pH values at each time point were calculated for the triplicate. For the acid tolerance analysis, isolates were suspended into 2 mL of BHI broth and cultivated at 37° C for 18 h. An aliquot of this culture was then suspended in BHI broth at pH 7.0 and pH 4.0 (acidified by chloridric acid) at an optical density of 0.03 at 600 nm (OD$_{600}$) and incubated at 37° C. Serial dilutions were performed at 0 (baseline) and 30 min of incubation and aliquots of 25 µL were plated in BHI agar. This experiment was performed in triplicates. Total Colony-Forming Units (CFU) were counted by a single examiner. Mean±SD of the triplicate CFU value were calculated at baseline and after 30 min incubation (pH 7 and pH 4). The growth rate during 30 minutes was calculated using the formula: "growth rate = CFU at 30 min – CFU at 0 min". Data was transformed to log$_{10}$ due to the dispersion and compared in each group by pH and also by species. Differences between groups (before and after starvation stress by temporary sealing) for both experiments were tested using *t* test, after testing the data normalization. Differences between species were tested using ANOVA. The level of significance was set at 5%. The data were analyzed with SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

Ethical considerations

The ethic committee of the Federal University of Rio Grande do Sul has approved the present study (process n°19218). Informed and written consent was signed from all individuals. All participants received treatment for basic dental needs.

3. RESULTS

All patients that showed growth of presumptive *Lactobacillus* species before and after the sealing were included in the study (n = 6/18). We isolated 239 strains and, after Gram-staining and genetic identification, 86 lactobacilli comprised the final sample (41 before and 45 after starvation stress by sealing) (Figure 1). The number of isolates per patients ranged from 1 to 16. Figure 2 shows three different morphological types and its corresponding Gram stain.

Composition of microbiota in carious dentin

Few isolates could only be amplified by 16SrRNA. The *phe*S gene could not identify most of *L. rhamnosus*. The number of isolates identified by sequencing *phe*S, *gro*EL and 16S rRNA genes were n = 39, n = 37 and n = 8, respectively.

Six *Lactobacillus* species were identified in this study. After starvation stress, a greater diversity of *Lactobacillus* species was found. Before the temporary sealing, *L. paracasei* (n = 24), *L. rhamnosus* (n = 16) and *L. parabuchneri* (n = 1) were detected. After the sealing, *L. paracasei* (n = 19), *L. rhamnosus* (n = 23), *L. plantarum* (n = 1), *L. casei* (n = 1) and *L. vaginalis* (n = 1) were detected. The most frequently species was *L. paracasei* and *L. rhamnosus* (n = 24 and 16 before and n = 19 and 23 after temporary sealing, respectively) (Table 2).

Phylogenetic and genotypic analysis

Figure 3 shows the phylogenetic tree of *Lactobacillus* species from partial sequencing of *phe*S gene. The strains identified with the letters "phes"

followed by numbers represent lactobacilli isolated from carious dentin. It could be observed the formation of clusters. The first group has the largest number of isolates, with 38 isolates belonging to the species *L. paracasei*. The second cluster shows a group of *L. casei* ATCC and *L. zeae*. The third group consists of samples of *L. rhamnosus*. A sample was identified as belonging to the species *L. plantarum* (strain 110). Other sample (strain 49), that was identified as *L. paracasei* by BLAST did not grouped with any other strain. This strain also presented a different AP-PCR pattern. The other species of lactobacilli could be separated into different groups by phylogenetic analysis with the exception of the species *L. gasseri* and *L. johnsonii*. Distinct letters show different genotypes within the parenthesis and strains with the same genotype tended to group.

Figure 4 shows the phylogenetic tree of lactobacilli analyzed by the partial sequencing of *gro*EL gene. The strains identified with the letters "groel" followed by numbers represent lactobacilli isolated from carious dentin. Some samples also were clustered. The first large cluster represents *L. rhamnosus*. The second consists of *L. casei* and *L. zeae*, as in the dendrogram of *phe*S gene. The third group is composed of *L. paracasei*. In general, all species could be differentiated from each other with exception for *L. gasseri*/*L. johnsonii* and *L. acidophilus*/*L. crispatus*. The result of homology with *gro*EL gene nucleotide sequences by the National Center database of Biotechnology Information (NCBI) of the United States and by the cpnDB database (chaperonin Sequence Database) were similar.

The genotypic analysis of the strains is shown in Table 2. Each patient produced different profiles of AP-PCR. A total of 27 and 15 different genotypes were found before and after sealing, respectively. The AP-PCR products with the lower number of bands were 2 and the higher were 15. In general, AP-PCR could distinguish species. The exception happened in the genotypes (p), (u) and (ao), where different species shared the same genotype. Only four genotypes, (a), (f), (g) and (j), remained after starvation stress.

Phenotypic analysis

The pH (mean±SD) of BHI broth supplemented with 1% glucose at times 9h, 24h, 48h, 72h after incubation with *Lactobacillus* species before and after temporary sealing is shown in Figure 5. Table 3 shows the acid tolerance by group by the measurement of growth rate at pH 7.0 and pH 4.0. There was no statistically significant difference between isolates from carious dentin before and after starvation stress, neither regarding acid production or acid tolerance. For the phenotypic analysis by the species level, the pH curves showed no significant difference with respect to acid production at times 9h, 24h and 72h. However, we could observe that *L. paracasei* showed significant lower pH value (mean of pH = 4.1) in 48 h of growth in comparison to the other species. Regarding acid tolerance, no difference in the growth rate of *Lactobacillus* species was observed at pH 7 or pH 4.

4. DISCUSSION

Lactobacilli are widely studied in the food industry, although there are only few studies identificating oral lactobacilli by molecular methods. Considering the lactobacilli higher relationship to caries progression, this study can help the understanding of the residual microbiota underneath restoration. In this longitudinal study, we analyzed the modification of *Lactobacillus* species composition in carious dentin after starvation stress by cavity sealing. Up to 7 isolates that had different morphological type were selected from Rogosa agar, which is a widely used selective media for lactobacilli isolation (28). However, Rogosa also permits the growth of oral streptococci as already shown by Yang et al. (2010). In our study, 41.84% of the initial predictive lactobacilli isolated were excluded for being cocci after Gram staining (Figure 1).

It is already know that genetic-based taxonomic methods are required for the *Lactobacillus* species molecular characterization (13, 24). It was indicated that the sequencing analysis of *phe*S and *rpo*A genes has proven to be a robust system for the identification of lactobacilli (24). A previous study that sequenced 222 oral lactobacilli using these genes showed good identification of *L. paracasei, L. fermentum, L. rhamnosus* and *L. gasseri* (13). In the

present study, the most prevalent *Lactobacillus* species were *L. paracasei* and *L. rhamnosus*. Interestingly, *L. rhamnosus* were selected after starvation stress and this could be associated with a possible advantage of this species to exploit this niche. In the present study, the gene *rpoA* was ineffective for identifying *Lactobacillus* species in carious dentin once it could not be amplified in many cases. The discriminatory power for *L. casei* group, which has been a major problem in the *Lactobacillus* species identification, was similar to *phe*S and *gro*EL genes. Many *L. rhamnosus* and some *L. paracasei* could not be amplified with *pheS* gene. The sequencing of the gene *gro*EL was the alternative found to identify the lactobacilli that were not sequenced by *pheS* gene. Previous studies confirm the superiority of *gro*EL gene as phylogenetic marker relative to 16S rRNA (11). Moreover, another advantage of *gro*EL gene is the possibility of using a specific database for blasting (29), once the homology using the NCBI often wrongly identify *L. paracasei* as *L. casei*. This impasse could be clarified through the construction of phylogenetic trees including strains ATCC 393 (*L. casei*) and ATCC 334 (*L. "casei"*). If the strain approaches phylogenetically to ATCC 393 strain it is a real *L. casei*. If it approaches phylogenetically to ATCC 334 strain, it can be considered as *L. paracasei*. In this study, even with the use of three distinct genes (*phe*S, *gro*EL, *rpo*A), 8 isolates could not be identified. Although the 16SrRNA posses limitations in *Lactobacillus* species identification, it was the only amplified gene for these isolates.

It was desired for the present research to analyze the lactobacilli diversity at the strain level, in order to observe if the genotypes were the same after starvation stress or if a selection of genotypes can occur. We were able to identify a decreased genotypic diversity in sealed carious dentin, showing a shift in the prevalence of lactobacilli after the stress of restoration that sealed carious dentin. Microbiological studies that assessed the partial caries removal and cavity sealing exhibited a low level of bacterial infection (4, 30). In spite of this residual contamination, the remaining carious dentin becomes harder and drier, both characteristics of inactive lesions (30). Paddick *et al.* suggested that the starvation stress may therefore exert its effects by modulating the diversity of niches that can be populated by the resident microbiota: the more homogeneous the niches, the more pronounced the

observed reduction in genotypic diversity (5). In our study, we identify similar patterns of genotypes before and after sealing in only 2 out of 6 patients. Coincident genotypes are expected to be found once the residual microbiota from the dentin after sealing is derived from the initial sample. In the other 4 patients, the genotypes found after sealing showed no banding pattern similar to the initial period. Other study also found the presence of new genotypes of *A. naeslundii* and *S. oralis* after sealing and no similar pattern in genotypes profile before and after sealing (5). As an explanation for this, the authors mentioned a possible replication of genotypes during the sealing period (5). Another possible explanation could be a bias of selection. The most prevalent strains would predominate in the culture media reducing the chance of other less abundant genotypes to be selected. It is also possible to observe a selection process in this inhospitable environment allowing new genotypes to be picked up after the sealing period due to increased ability to exploit the environment underneath restoration. This could also be an explanation to the fact that other species that were not found before sealing were present after starvation generating a higher diversity at species level. Furthermore, more diversity in the bacterial community is expected in healthy biofilms (characteristics of inactivity in the tissue left after temporary sealing) due to the strict condition of the carious niche where there are pressure for species selection (31).

The ability of bacteria to produce acid (acidogenicity) and to growth at low pH (aciduricity) are regarded as being two of the most important virulence factors related to dental caries (32). These abilities of *Lactobacillus* species are essential for the progression of the lesion underneath restorations. It is unclear whether the bacteria beneath restorations, or under starvation stress, represent some danger to the longevity of restorations and if they are more virulent than the bacteria previous to the sealing. The present results suggest that *Lactobacillus* species that survive remain potentially cariogenic, as any difference was found in acid tolerance and in acid production between the *Lactobacillus* species from carious dentin before and after starvation stress by cavity sealing. An exception was observed in *L. paracasei* were a higher acid production occurred at 48h in comparison to the other lactobacillus species

(Figure 5). Fortunately, *L. paracasei* was not the most prevalent *Lactobacillus* species remaining after starvation stress.

The limitations of this study should be taken into account in the interpretation of the results. It should be considered that Rogosa agar was not effective to select lactobacilli and thereat the final number of analyzed isolates decreased more than 40% of the initial isolates. The phenotypic analysis have no direct clinical relevance and it should be considered that lactobacilli were isolated from their natural environment where they lived in a microbial biofilm community and the same situation could not be detected *in vivo.*

In conclusion, the most prevalent *Lactobacillus* species were *L. paracasei* and *L. rhamnosus. L. rhamnosus* were selected after starvation stress. There was a decreased genotypic diversity at the strains level and an increased diversity of species in sealed carious dentin. A secondary finding was that the partial sequence of *groEL* gene is the greatest option to identify *Lactobacillus* species isolated from carious dentin. The *in vitro* tested virulence traits through *Lactobacillus* species ability to produce and tolerate acid did not change after cavity sealing, in exception for *L. paracasei* were a higher acid production occurred at 48h in comparison to the other lactobacillus species. More studies evaluating other virulence traits are necessary to better understand the cariogenicity of the residual bacteria underneath restorations and to understand the resistance of *L. rhamnosus* to this site.

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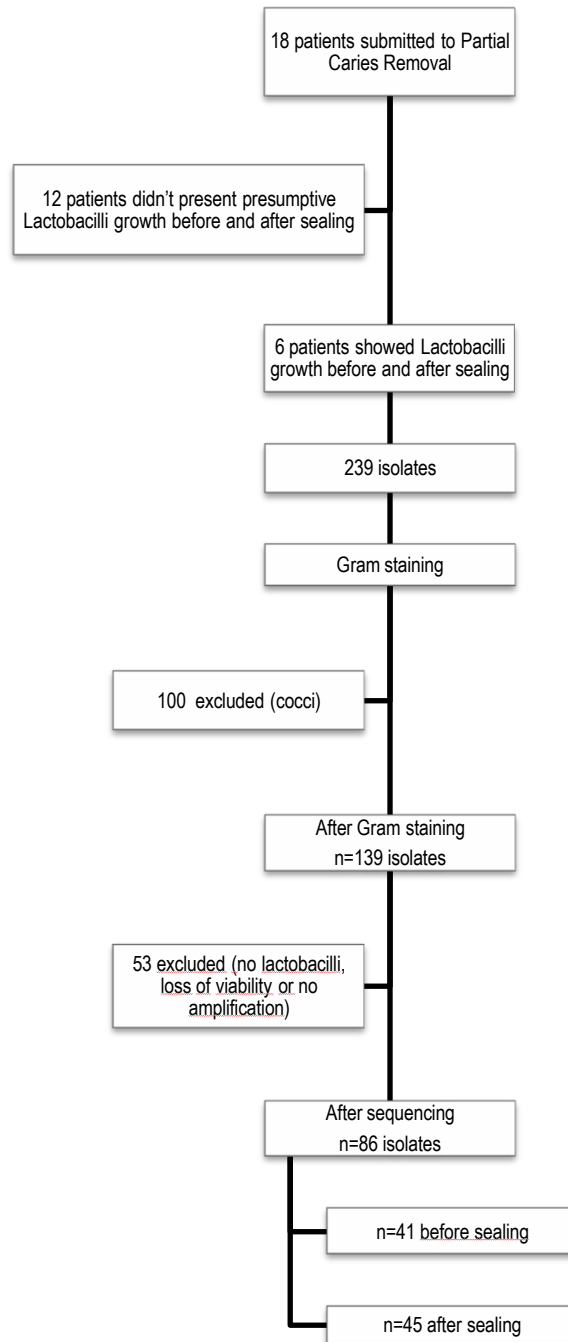
Figure 1. Flowchart of the sampling.

Table 1. Nucleotide sequences of primers used for *Lactobacillus* species identification by housekeeping genes amplification.

| Primer name	Sequence (5'→ 3')	Gene	Gene description	Annealing temperature	Reference
pheS-21F	CAYCCNGCHCGYGAYATGC	pheS	α-subunit of phenilanyl-tRNAsyntases	57°C	(Naser et al., 2005)
pheS-23R	GGRTGRACCATVCCNGCHCC			56°C	
pheS-22R	CCWARVCCRAARGCAAARCC			55°C	
rpoA-21F	ATGATYGARTTTGAAAAACC	rpoA	α-subunit of RNA polymerase	50°C	(Naser et al., 2005)
rpoA-23R	ACHGTRTTRATDCCDGCRCG				
groEL1F	GAAGGNATGAAGAAYGTBAC	groEL	Hsp60 chaperonins gene	47°C	(Podlesny et al., 2011)
groEL1R	AATGTHCCACGVATCTTG				
16S rRNA	TCCTACGGGAGGCAGCAGT	16S rRNA	16S region in the ribosomal RNA (universal primers for the domain Bacteria prokaryotic organisms)	58°C	(Nadkarni et al., 2002)
16S rRNA	GGACTACCAGGGTATCTAATCCTGTT				
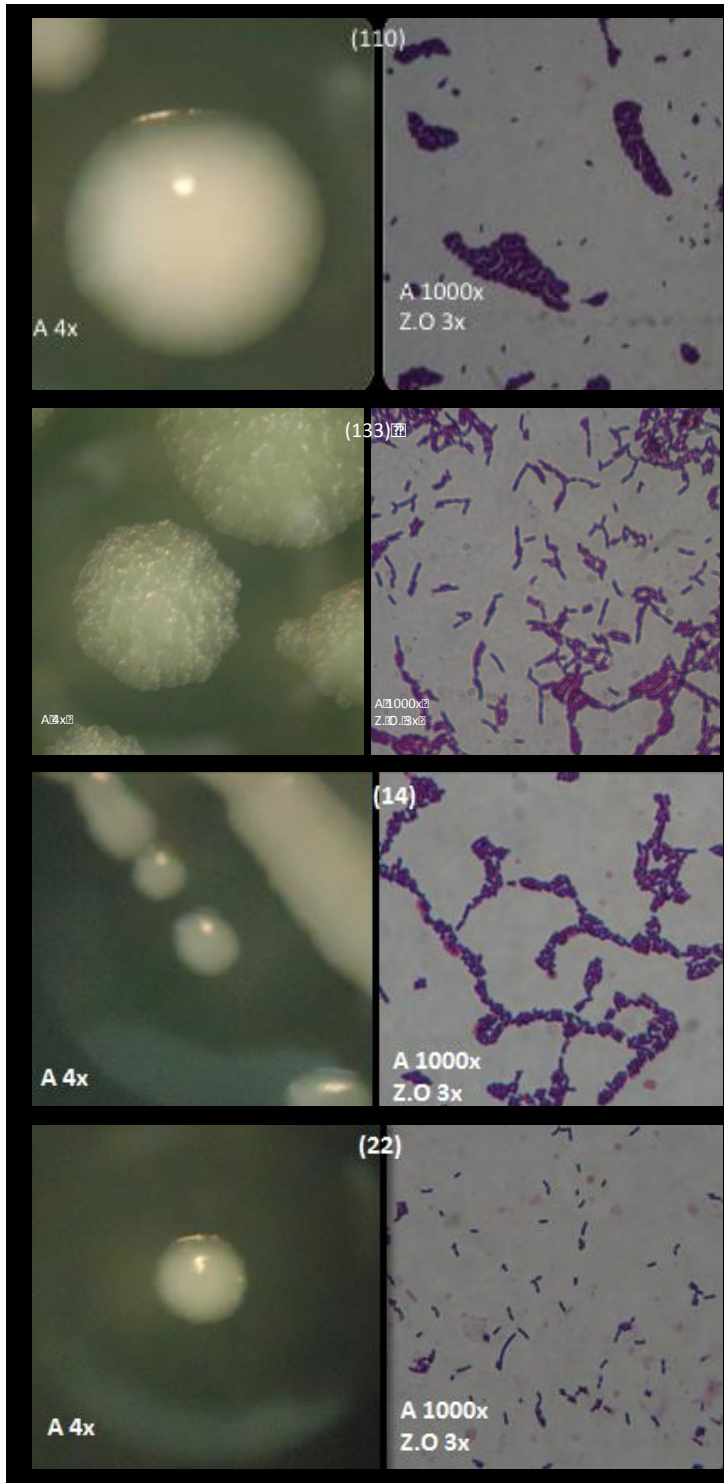
Figure 2. Examples of morphological type (left) and Gram staining (right) of the *Lactobacillus* species isolated from carious dentin before and after 3 months starvation stress by cavity sealing in Rogosa; (strain 110) = *L. plantarum*; (strain 133) = *L. rhamnosus*; (strain 14) and (strain 22) = *L. paracasei.*

Figure 3. Similarity tree of *Lactobacillus* species isolates identified by sequencing *phe*S gene (n=40). The strains identified with the letters "phes" followed by numbers represent lactobacilli isolated from carious dentin. Strains identified by letters AM followed by numbers were obtained from PubMed databases. Distinct letters show different genotypes within the parenthesis. The number after parenthesis represents the group: 1=before sealing; 2=after sealing.
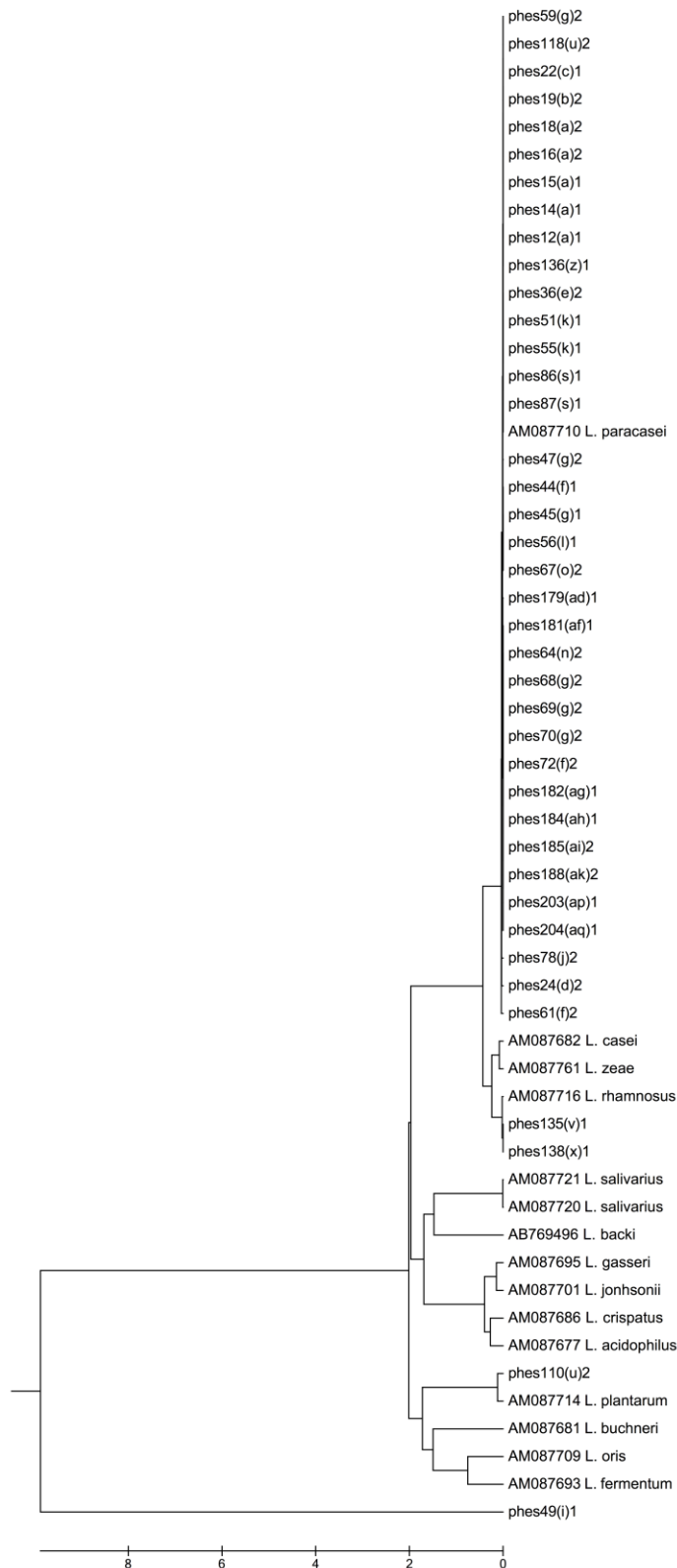
Figure 4. Similarity tree of *Lactobacillus* species isolates identified by sequencing *gro*EL gene (n=39). The strains identified with the letters "groel" followed by numbers represent lactobacilli isolated from carious dentin. Other strains were obtained from PubMed databases. Distinct letters show different genotypes within the parenthesis. The number after parenthesis represents the group: 1=before sealing; 2=after sealing.
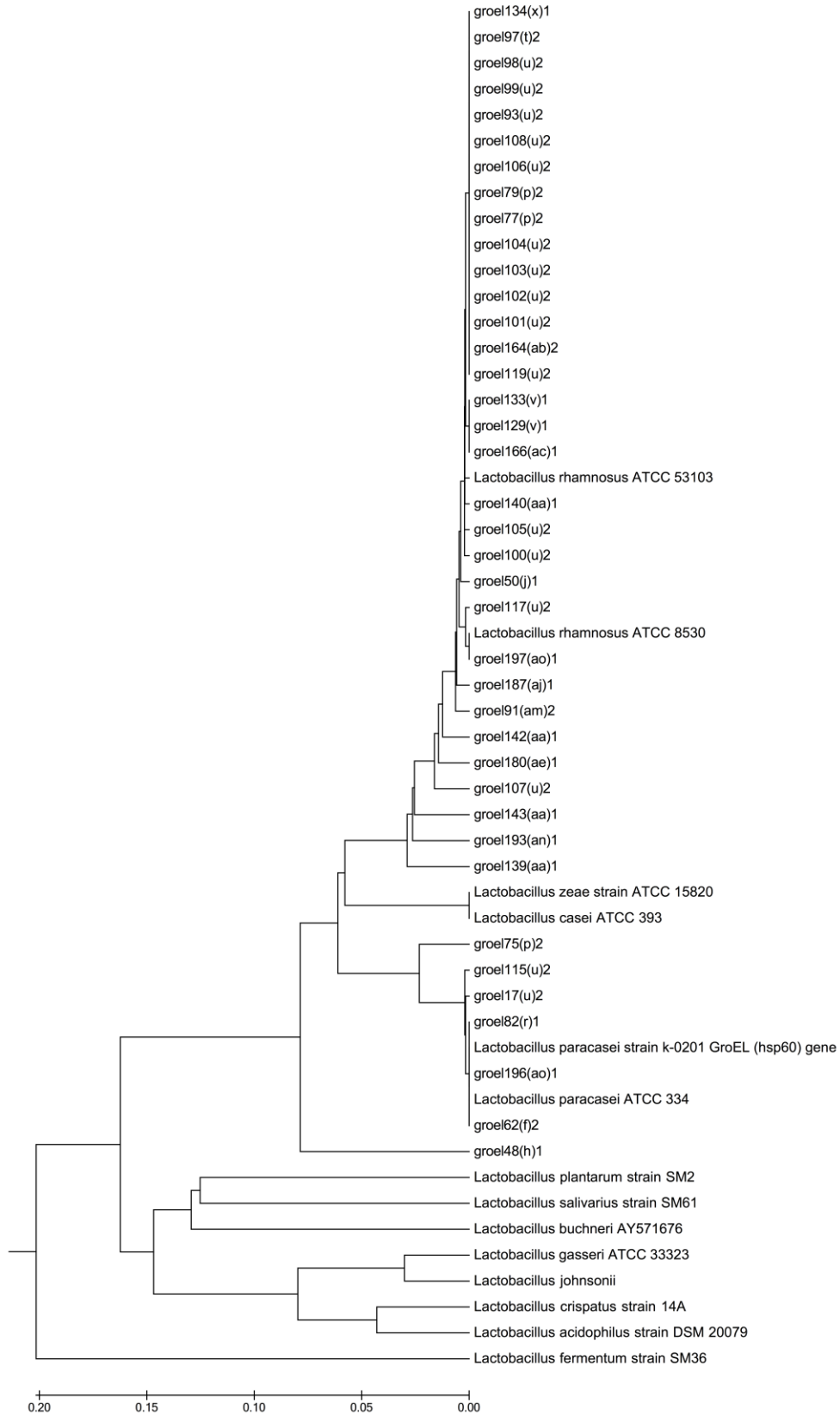
Table 2. Number of patients, isolates and genotypes of Lactobacillus species isolated from carious dentin before and after 3 months starvation stress by cavity sealing. Distinct letters show different genotypes. The number absolute of the isolates in relation to their genotypes in each condition is represented within the parenthesis.

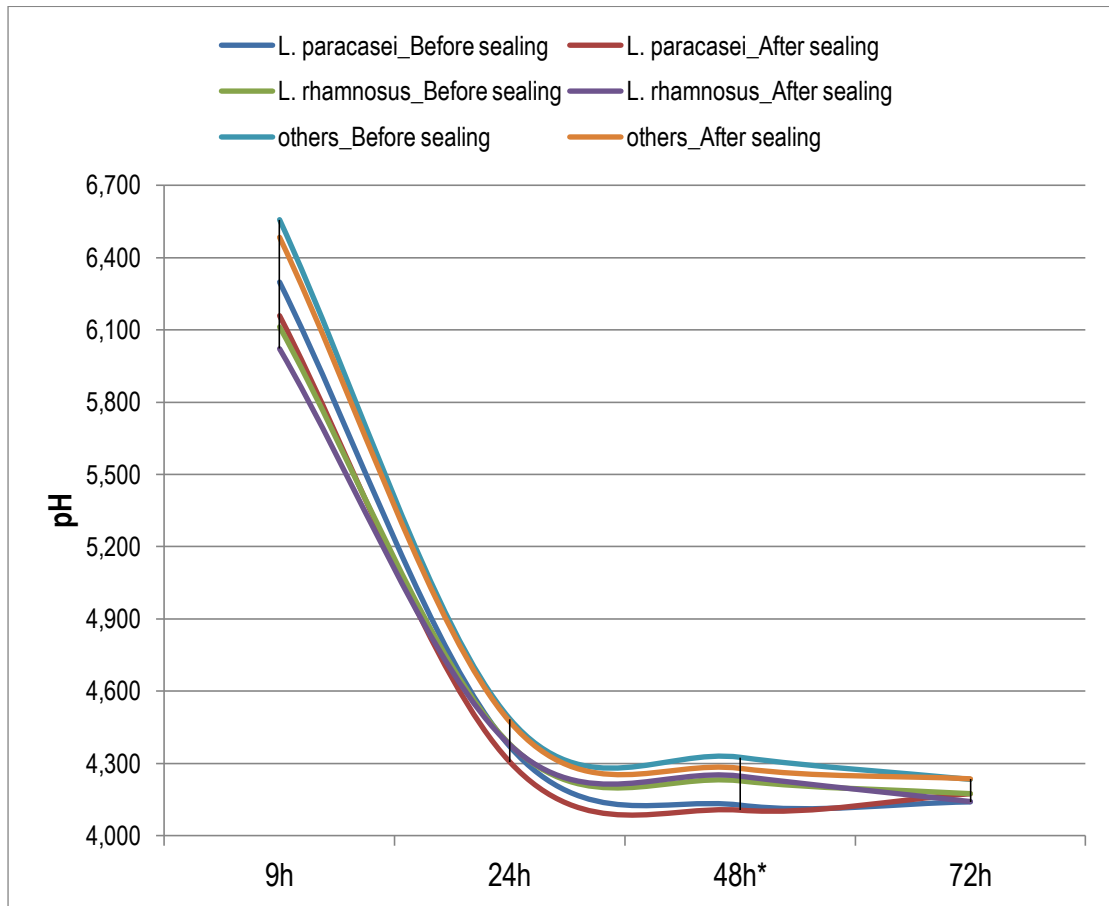|	BEFORE STARVATION STRESS			AFTER STARVATION STRESS		
Patient	Number of isolates	Species	Genotype (n)	Number of isolates	Species	Genotype (n)
1	3	*L. paracasei*	a (3)	4	*L. paracasei*	a (3)
					L. paracasei	b (1)
2	1	*L. paracasei*	c (1)	2	*L. paracasei*	d (1)
					L. paracasei	e (1)
3	9	*L. paracasei*	f (1)	14	*L. paracasei*	f (3)
		L. paracasei	g (2)		*L. paracasei*	g (4)
		L. rhamnosus	h (1)		*L. paracasei*	j (1)
		L. paracasei	i (1)		*L. paracasei*	n (1)
		L. rhamnosus	j (1)		*L. paracasei*	o (1)
		L. paracasei	k (2)		*L. rhamnosus*	p (3)
		L. paracasei	l (1)		*L. paracasei*	p (1)
4	3	*L. paracasei*	r (1)	23	*L. rhamnosus*	t (2)
		L. paracasei	s (2)		*L. plantarum*	u (1)
					L. casei	u (1)
					L. paracasei	u (2)
					L. rhamnosus	u (17)
5	10	*L. rhamnosus*	v (3)	1	*L. rhamnosus*	ab (1)
		L. rhamnosus	x (2)			
		L. paracasei	z (1)			
		L. rhamnosus	aa (4)			
6	15	*L. rhamnosus*	ac (1)	1	*L. vaginalis*	ar (1)
		L. paracasei	ad (1)			
		L. rhamnosus	ae (1)			
		L. paracasei	af (1)			
		L. paracasei	ag (1)			
		L. paracasei	ah (1)			
		L. paracesei	ai (1)			
		L. rhamnosus	aj (1)			
		L. paracasei	ak (1)			
		L. parabuchneri	am (1)			
		L. rhamnosus	an (1)			
		L. paracasei	ao (1)			
		L. rhamnosus	ao (1)			
		L. paracasei	ap (1)			

Figure 5. pH mean of BHI broth supplemented with 1% glucose at 9 h, 24 h, 48 h, 72 h after suspension of *Lactobacillus* species isolated from carious dentin before and after starvation stress by cavity sealing (*ANOVA test; p<0.05)

Table 3. Mean±SD of growth rate of *Lactobacillus* species (log10 CFU/ml) at pH 7.0 and pH 4.0 by species isolated before and after 3 months starvation stress by cavity sealing.

Growth rate	Species	Before starvation stress (Mean±SD)		After starvation stress (Mean±SD)		Mean±SD	
ph 7.0	*L. paracasei*	1.784±	0.250	1.821±	0.305	**1.803±**	**0.018**
	L. rhamnosus	1.726±	0.427	1.692±	0.275	**1.709±**	**0.017**
	Others	1.634±	0.000	1.714±	0.042	**1.674±**	**0.040**
ph 4.0	*L. paracasei*	0.265±	0.298	0.130±	0.356	**0.198±**	**0.068**
	L. rhamnosus	0.169±	0.356	0.235±	0.486	**0.202±**	**0.033**
	Others	0.189±	0.000	0.149±	0.208	**0.169±**	**0.020**

ANOVA to compare species: p>0.05.
t test to compare groups: p>0.05.

Este estudo teve como objetivo avaliar a diversidade de espécies/genotípica e as características mais importantes de virulência dos microrganismos cariogênicos residuais na dentina cariada após RPDC, os quais sofreram estresse nutricional durante três meses de selamento provisório.

Um dos questionamentos desta tese de doutorado era saber se havia possibilidade de que ocorra uma seleção de espécies na dentina cariada após selamento pela modificação da disponibilidade de nutrientes. Houve mudança na prevalência de espécies cariogênicas após o estresse nutricional que exerceu efeito através na modulação da diversidade deste nicho. Foi observada uma maior diversidade de espécies de lactobacilos após estresse nutricional (3 espécies x 5 espécies). As espécies de lactobacilos mais prevalentes em dentina cariada foram *L. paracasei* e *L. rhamnosus* e estes últimos foram selecionados após o estresse nutricional, demonstrando vantagem ecológica.

Também foi objetivo saber se havia modificação da diversidade genotípica das bactérias cariogênicas após selamento da dentina cariada e foi demonstrado que a diversidade genotípica reduziu após estresse nutricional. Alguns genótipos de *S. mutans* foram os mais prevalentes antes do selamento, mas não foram detectados ou detectados em menores níveis após o selamento, enquanto um genótipo de *S. mutans* e a maioria de genótipos de lactobacilos foram encontrados somente após o selamento. Isso demonstra vantagem ecológica de algumas cepas. Pode-se observar uma riqueza e diversidade grande dentro da mesma espécie.

Mesmo sob estresse nutricional, o fenótipo sobre a prevalência destes genótipos permaneceu o mesmo: não houve diferença na aciduricidade e acidogênese entre os isolados de *S. mutans* e lactobacilos, em exceção para *L. paracasei,* que tiveram produção de ácido ligeiramente maior em 48 horas após contato com açúcar em comparação com as outras espécies de lactobacilos. Entretanto, *L. paracasei* não foi a espécie de lactobacilos que prevaleceu após o selamento. Estes resultados sugerem que *S. mutans* e lactobacilos que sobrevivem ao estresse nutricional abaixo das restaurações permanecem potencialmente cariogênicos. Bactérias residuais podem ser metabolicamente ativas se uma fonte externa de carboidratos for fornecida (infiltração da restauração) evidenciando a necessidade de uma restauração perfeitamente selada.

As perspectivas para esta linha de pesquisa consistem em avaliar as características fenotípicas de *L. rhamnosus* e dos genótipos mais resistentes, observando suas funções dentro de comunidades microbianas em ambientes naturais onde se desenvolve a doença.

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Anexos

Universidade Federal do Rio Grande do Sul
Faculdade de Odontologia
Termo de Consentimento Livre e Esclarecido

Via do
pesquisador

Estudo da viabilidade, diversidade e virulência de bactérias após remoção parcial de dentina cariada selada

1.Objetivo do estudo: Avaliar o efeito de duas técnicas de restauração do dente (remoção parcial e total de tecido cariado), sobre a viabilidade (se permanecem vivas ou não), diversidade (identificação de diferentes bactérias) e virulência (o quanto elas agridem os tecidos dentários) de bactérias associadas à progressão das lesões de cárie em dentina (porção mais interna do dente).

2. Seleção dos indivíduos: Para participar desta pesquisa, você deverá apresentar lesões de cárie em metade de dentina. Os participantes serão divididos aleatoriamente (através de uma tabela) para receber um dos dois tipos de tratamento descritos abaixo.

3. Duração: A participação na pesquisa consiste em duas consultas, com duração de cerca de uma hora. A segunda consulta será realizada após três meses.

4. Procedimentos: O tratamento restaurador será executado em duas etapas. Na primeira consulta, será realizada em um grupo a remoção total de dentina cariada (procedimento convencional) e no outro grupo remoção parcial de dentina cariada (procedimento experimental) e coleta de uma amostra para análise das bactérias presentes na lesão de cárie. Na segunda consulta, será coletada uma amostra de tecido cariado, e então o dente será restaurado definitivamente. Se o dente estiver incluído no grupo de remoção parcial, na segunda consulta será feita a remoção de todo tecido cariado (tratamento padrão) com posterior restauração do dente. Isto significa que todos os dentes receberão tratamento padrão após a segunda coleta de dentina cariada.

5. Importância do estudo: Trata-se de um trabalho de finalidade terapêutica (de tratamento). O tema escolhido se justifica pela importância destas abordagens em lesões de cárie em dentina.

6. Danos: Não existem danos previstos. As duas abordagens têm indicação em odontologia. O material coletado será em pequena quantidade, o que não causará qualquer dano ao remanescente dentário (o que tem de tecido dentário após a remoção da cárie).

7. Benefícios: Participando desta pesquisa você contribui para o conhecimento quanto a técnicas conservadoras de remoção de tecido cariado, o que influenciará na realização das mesmas para o tratamento de lesões profundas de cárie. Além disso, qualquer necessidade clínica básica adicional poderá ser sanada pelos pesquisadores.

8. Confidencialidade: Os dados de identificação serão confidenciais e os nomes reservados. Os dados obtidos serão utilizados somente para este estudo, sendo os mesmos armazenados pelo (a) pesquisador(a) principal durante 5 (cinco) anos e após totalmente destruídos (Resolução 196/96).

A participação na pesquisa é totalmente voluntária e o indivíduo tem a liberdade de se recusar a participar ou retirar seu consentimento em qualquer momento do estudo sem nenhum tipo de penalidade.

No caso de dúvidas ou acontecimentos associados à pesquisa, o participante poderá entrar em contato com a pesquisadora Luciana Bitello Firmino, através do telefone 3308 5193/81368193 ou com a orientadora deste projeto e pesquisadora principal, Profª. Drª. Marisa Maltz (3308 5247), e terá a garantia de resposta a qualquer pergunta ou informação extra.

Confirmo que entendi a natureza da pesquisa e me disponho a participar voluntariamente.
   Assinatura sujeito ou responsável:_____ _____

   Menor_____

Pesquisadora principal: _____
         Porto Alegre,_____ de _____ de 20___.
         Comitê de Ética em Pesquisa da Faculdade de Odontologia da UFRGS: 3308 5187.

U F R G S   **PRÓ-REITORIA DE PESQUISA**

UNIVERSIDADE FEDERAL   Comitê De Ética Em Pesquisa Da Ufrgs
DO RIO GRANDE DO SUL

CARTA DE APROVAÇÃO

Comitê De Ética Em Pesquisa Da Ufrgs analisou o projeto:

Número: 19218

Título: Estudo da Viabilidade, diversidade e virulência de bactérias após remoção parcial de dentina cariada

Pesquisadores:

Equipe UFRGS:

MARISA MALTZ TURKIENICZ - coordenador de 15/01/2011 até 15/01/2013
CLARISSA CAVALCANTI FATTURI PAROLO - pesquisador de 15/01/2011 até 15/01/2013
Luciana Bitello Firmino - pesquisador de 15/01/2011 até 15/01/2013

Equipe Externa:

Márcia Mayer - pesquisador de 15/01/2011 até 15/01/2013

Comitê De Ética Em Pesquisa Da Ufrgs aprovou o mesmo, em reunião realizada em 30/09/2010 - Sala de Reuniões do Gabinete do Reitor (Ex Salão Vermelho) - Prédio Reitoria, 6° andar, por estar adequado ética e metodologicamente e de acordo com a Resolução 196/96 e complementares do Conselho Nacional de Saúde.

Porto Alegre, Quinta-Feira, 30 de Setembro de 2010

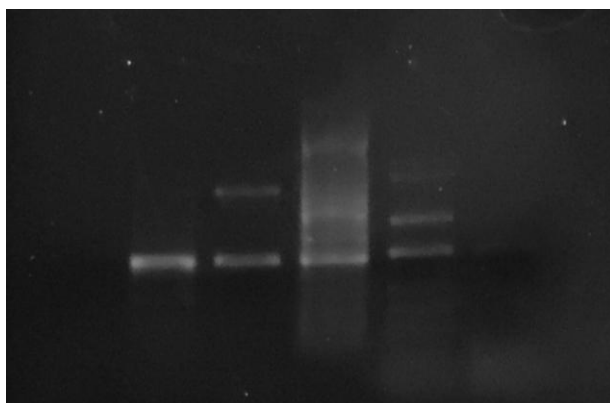JOSÉ ARTUR BOGO CHIES
Coordenador da comissão de ética

Imagem 1. Análise do resultado da amplificação de genes "*housekeeping*" para identificação de lactobacilos a nível de espécies. Na coluna 1, exemplo de banda considerada adequada para purificação do produto de PCR e sequenciamento. Nas colunas 2, 3 e 4, exemplos de bandas dupla ou com arraste, descartadas para sequenciamento por falta de especificidade do PCR. Na coluna 5, exemplo de PCR negativo.
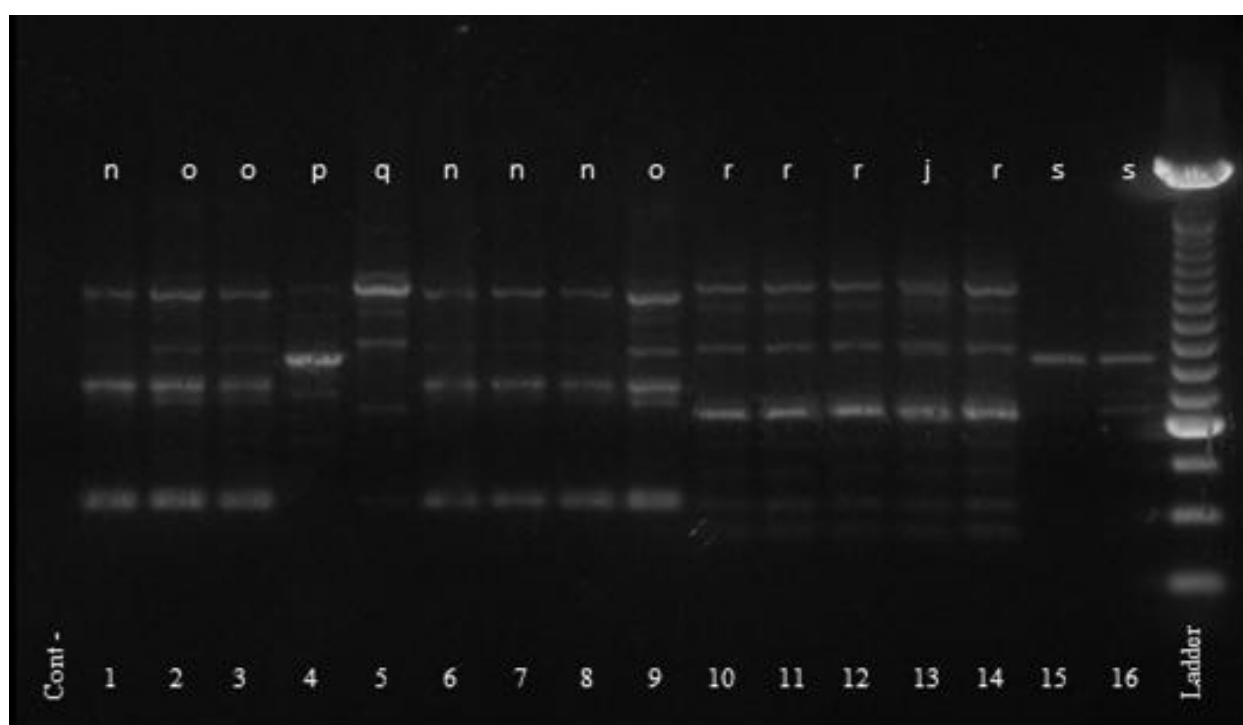

Imagem 2. Exemplo de gel de eletroforese gerado pelo AP-PCR usando *primer* OPA-03 para genotipagem de lactobacilos isolados de dentina cariada. Colunas 1 a 16 representam diferentes isolados de lactobacilos dos pacientes 3 e 4. Primeira coluna contém o controle negativo e a última coluna contém a *ladder* 250pb.