UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

RAQUEL SOARES DALALBA

DIVERSIDADE GENÉTICA DE LACTOBACILOS ISOLADOS DE DENTINA CARIADA ANTES E APÓS SELAMENTO

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgião-Dentista.

Orientadora: Prof[a]. Dra. Clarissa Cavalcanti Fatturi Parolo

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RESUMO

DALALBA, Raquel Soares. **Diversidade genética de lactobacilos isolados de dentina cariada antes e após selamento.** 2013. 27 f. Trabalho de Conclusão de Curso (Graduação em Odontologia)- Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2013.

Existe hipótese de que haja seleção de bactérias abaixo das restaurações devido a escassez de nutrientes. Por isso o objetivo do trabalho foi avaliar o efeito do selamento de lesões dentinárias de cárie sobre a diversidade genotípica de Lactobacilos utilizando a técnica de AP-PCR. Dezoito pacientes com lesões dentinárias em terço médio de dentina foram submetidos à remoção parcial de tecido cariado e selamento da cavidade por 3 meses. Amostras de dentina foram obtidas antes e após o selamento e cultivadas em Ágar Rogosa, por 72 horas em anaerobiose. Até sete cepas de cada tipo morfológico foram selecionadas e armazenadas em caldo de Brain Heart Infusion (BHI) e glicerol (15%) em -20º C. As amostras foram analisadas quanto à coloração de Gram e morfologia. Somente bacilos e cocobacilos gram-positivos foram mantidos na amostra. Os isolados foram também identificados pelo sequenciamento dos genes *pheS/rpoA*. Do total de 18 pacientes, seis apresentaram crescimento de Lactobacilos antes e após selamento. Assim, a amostra final foi de 93 isolados (44 antes do selamento e 49 depois do selamento). Para a avaliação da diversidade genética, os isolados foram replicados em BHI ágar, cultivadas por 24 horas a 37ºC . Para extração do DNA as colônias foram suspensas em 50µl de água ultrapura estéril. A técnica de AP-PCR foi realizada utilizando o primer OPA3. *Lactobacillus* ATCC (*L. rhamnosus* ATCC 7469) água ultrapura foram utilizadas como controle positivo e negativo, respectivamente. Os produtos de AP-PCR foram analisados por eletroforese em gel de agarose a 1%, corado com SybrGreen 1.6%, a 96 V durante 4 h. As imagens dos géis de AP-PCR foram capturadas por uma câmera digital (Canon Inc., Tóquio, Japão) e armazenadas no formato de arquivo de imagem para análise visual. Na análise dos amplicons, os Lactobacilos do mesmo paciente antes e depois do selamento foram sempre analisados no mesmo gel. Dois examinadores cegos e calibrados realizaram a análise visual. Um total de 43 genótipos distintos foi identificado. Teste t-pareado foi utilizado para comparar a media do número de genótipos encontrados antes e depois do selamento considerando 5% de significância. A média do número de genótipos por paciente foi de 5,17±5,49 e 2,67±2,25 (p=0,344) antes e depois do selamento, respectivamente. O menor número de bandas obtidas pela análise dos géis de AP-PCR foi 2 e o maior foi 15. A média de número de bandas foi 8.72±2.22. Um total de 31 e 16 genótipos diferentes foram encontrados antes e depois do selamento, respectivamente. Houve uma tendência de diminuição da diversidade de Lactobacilos após o período de selamento.

Palavras-chave: Lesão Cariosa. Dentina. Lactobacilos. Diversidade genética. AP-PCR.

ABSTRACT

DALALBA, Raquel Soares. **Genetic Diversity of Lactobacillus isolated from carious dentin before and after sealing.** 2013. 27 f. Final Paper (Graduation in Dentistry) - Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2013.

An hypothesis exists that a selection of bacteria underneath restoration due to a limited access of nutrients. Therefore the aim of this study was to evaluate the effect of carious sealing in genotypic diversity of *Lactobacillus* using the AP-PCR technique. Eighteen patients with dentine lesions, in the middle third of dentin, were submitted to partial caries removal and sealing for 3 months. Dentin samples were obtained before and after sealing and cultured on Rogosa Ágar for 72 hours under anaerobic conditions. Up to seven strains of each morphological type were selected and stored in Brain Heart Infusion (BHI) broth and glycerol (15%) at -20º C. The samples were analyzed for Gram staining and morphology. Only gram-positive *rods* and *coccobacilli* were selected . The isolates were identified by sequencing of genes *pheS/rpoA*. From a total of 18 patients, six showed growth of *Lactobacillus* before and after sealing. The final sample comprised 93 isolates (44 before sealing and 49 after sealing). To evaluate the genotypic diversity, the isolates were replicated in BHI Ágar and cultured for 24 hours at 37º C. For DNA extraction, colonies were suspended in 50µL of sterile ultrapure water. The technique of AP-PCR was performed using primer OPA3. *Lactobacillus* ATCC (*L. rhamnosus* ATCC 7469) and ultrapure water were used as positive and negative controls, respectively. The AP-PCR products were analyzed by gel electrophoresis in 1% agarose, stained with SybrGreen 1.6% at 96 W for 4 h. The images of the AP-PCR gels were captured by a digital camera (Canon Inc., Tokyo, Japan) and stored in the image file format for visual analysis. In the analysis of amplicons, *Lactobacillus* from the same patient before and after the sealing always were resolved on the same gel. Two examiners blind and calibrated performed the visual analysis. A total of 43 different genotypes were identified. Paired t-test was used for compare the mean of number of genotypes found before and after sealing considering 5% of significance. The mean of genotypes found by patient were 5.17±5.49 and 2.67±2.25 (p=0.344) before and after sealing, respectively. The smallest number of bands obtained by the analysis of the gels with AP-PCR was 2 and highest was 15. The average number of bands was 8.72 ± 2.22. A total of 31 and 16 different genotypes were found before and after sealing, respectively. It was found a tendency of decrease genotypic diversity of *Lactobacillus* before sealing showed a tendency of decreased in diversity after sealing.

Keywords: Dental caries. Dentin. *Lactobacillus* spp. Genetic diversity. AP-PCR.

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária é um dos principais agravos de saúde bucal da população brasileira. Ainda que o perfil de cárie como doença tenha sido alterado ao longo das décadas, principalmente em resposta a adição do flúor à agua de abastecimento e ao dentifrício, a população brasileira ainda apresenta uma prevalência e severidade de doença maior do que gostaríamos. Segundo dados do Levantamento de Saúde Bucal (SB Brasil 2010) crianças brasileiras de 12 anos de idade e adolescentes de 15 a 19 anos apresentam, respectivamente, em média 2,07 e 4,25 dentes com experiência de cárie dentária e no que se refere a adultos, o CPO-D médio foi de 16,75 na faixa etária de 35 a 44 anos e 27,53 na de 65 a 74 (BRASIL, 2011). Destaca-se o fato que o componente perdido é responsável por cerca de 44,7% do índice no grupo de 35 a 44 anos e 92% no grupo de 65 a 74 anos, entre adultos e idosos a perda dentária por cárie é o problema mais prevalente. O potencial de destruição das estruturas dentárias revelado pela doença evidencia que lesões dentinárias com necessidade restauradora ainda são rotina na prática clínica diária, as quais requerem tratamentos progressivamente mais invasivos, podendo resultar na extração do elemento dentário, tal como retratam os dados do SB Brasil 2010.

O tratamento destas lesões de cárie cavitadas convencionalmente estabelecido baseia-se na remoção total de dentina cariada (RTTC) segundo critério clínico de dureza. Assim, a dentina estaria livre de bactérias e pronta para receber o material restaurador sem que microrganismos remanescentes sob a restauração pudessem dar continuidade ao processo carioso abaixo da restauração (BLACK, 1908). No entanto, estudos microbiológicos posteriores demonstraram que o critério clínico de dureza não representa necessariamente completa ausência de microrganismos(HENZ, 1997; MALTZ et al., 2012). Microrganismos são rotineiramente selados sob restaurações e isto não resulta fatalmente em insucesso clínico. Entretanto, o tratamento convencional ainda é baseado na remoção total de tecido amolecido com a finalidade de remover o tecido infectado. Quando se opta pela RTTC, pode-se ocasionar uma exposição mecânica da polpa em dentes com lesões profundas de cárie gerando a necessidade de tratamentos mais invasivos como o capeamento pulpar direto ou o tratamento endodôntico (LAKSELL et al., 1996; BJORNDAL; LARSEN; THYLSTRUP, 1997; MALTZ et al., 2002; RICKETTS et al., 2006). Uma vez ocorrida exposição pulpar frente a uma dentina cariada há uma taxa de sobrevida pulpar baixa 32,8% (BJØRNDAL et al., 2010). É importante ressaltar que o acesso aos níveis complexos de atenção (como em casos de necessidade de tratamento endodôntico) no Sistema Único de Saúde é restrito, o que pode, com o passar o tempo, condenar o dente à exodontia.

Tratamentos alternativos tem sido propostos nos casos de lesões profundas de cárie. O tratamento expectante é um deles (BJORNDAL; LARSEN; THYLSTRUP, 1997). Este se caracteriza pela remoção parcial de dentina cariada (RPTC) e manutenção de uma camada de tecido cariado junto à parede pulpar com o objetivo de paralisar a progressão da lesão e permitir a formação de dentina terciária previamente à escavação completa (realizada após período de selamento), reduzindo o risco de exposição (BJØRNDAL; THYLSTRUP, 1998) e melhorando o prognóstico da saúde pulpar.

Diante de evidências da inativação das lesões, com acentuada e significativa redução do número de microrganismos viáveis, da remineralização da dentina remanescente e sucesso clínico (MALTZ et al., 2002; PINTO et al., 2006; LULA et al., 2009) surge o questionamento da necessidade da reabertura da cavidade para realizar a remoção da dentina cariada residual (KIDD, 2004). Um estudo realizado em dentes que receberam RPTC em sessão única demonstra que em 10 anos de acompanhamento há um sucesso da técnica de RPTC semelhante a sobrevivência de restaurações de resina convencionais (RTTC) com mesmo tempo de acompanhamento (MALTZ et al., 2002). Este trabalho foi um ensaio clínico de braço único sem presença de grupo controle. Assim, um novo estudo clínico randomizado controlado foi conduzido pelo mesmo grupo de pesquisa para comparar a efetividade da RPTC e o tratamento expectante. Dados deste trabalho com acompanhamento de 3 anos mostraram taxas de sucesso superiores ao tratamento expectante (MALTZ et al., 2013), além disso, há outras vantagens associadas a RPTC como necessidade de uma sessão clínica apenas, menor desconforto para o paciente, melhor relação custo-efetividade, evitar necessidade de restauração temporária, evitar perda de tecido dentário íntegro pela remoção do material restaurador provisório, evitar eventual exposição pulpar durante a remoção da restauração provisória.

A persistência de bactérias após a RPTC é uma realidade (MALTZ et al., 2002; PINTO et al., 2006; LULA et al., 2009; FIRMINO, 2011); porém, estudos microbiológicos que avaliam a RPTC e selamento demonstram que mesmo com a permanência de uma camada de tecido contaminado no fundo da cavidade, há redução da contaminação bacteriana (MALTZ et al., 2002; LULA et al., 2009). Estudos mostram que há maior contaminação bacteriana logo após a RTTC do que quando se realiza RPTC e selamento (MALTZ et al., 2012). Neste cenário alguns questionamentos surgem. Estes microrganismos que permanecem sob o selamento tem potencial de atuar prejudicando o órgão dental e promover a progressão do processo carioso?

Um dos microrganismos mais prevalentes em lesões de cárie cavitadas e também na dentina cariada após a RPTC e selamento são os Lactobacilos (CAUFIELD et al., 2007; AAS et al., 2008). Os Lactobacilos pertencem ao grupo de bactérias produtoras de ácido láctico, são bactérias acidogências (capazes de produzir ácidos) e acidúricas (capazes de sobreviver em meio ácido), Gram-positivas, associadas à progressão da lesão cariosa (AAS et al., 2008; BADET; THEBAUD, 2008). Através de métodos de cultivo e moleculares comprova-se a presença desse grupo de microrganismos em lesões cariosas (MALTZ et al., 2002; BYUN et al., 2004; FIRMINO, 2011). O gênero Lactobacilos pertence a um grupo extenso de bactérias e algumas destas espécies podem ter maior relação com o desenvolvimento de lesões cariosas (BADET; THEBAUD, 2008). Métodos genotípicos são capazes de identificar cepas de Lactobacilos provenientes de lesões cariosas (CAUFIELD et al., 2007; PAROLO et al., 2011) e discriminar isolados provenientes de uma mesma espécie.

Dentre os diferentes métodos de genotipagem o AP-PCR (ou RAPD) utiliza sequências iniciadoras que arbitrariamente se ligam ao genoma bacteriano em condições de baixa estringência. Esta técnica é bastante vantajosa, pois apresenta baixo custo, não requer equipamentos muito complexos (NAVARRO; JORCANO, 1999) é de fácil aplicação. Segundo Cuenca (2004) as principais vantagens são velocidade dá análise, flexibilidade e facilidade de interpretação. Este método apresenta também limitações como a reprodutibilidade. A técnica é muito sensível às pequenas variações na metodologia como a extração do DNA, tipo de termociclador, concentração de DNA, temperatura de anelamento, concentração de Mg^{+2}, entre outros (TYLER et al., 1997).

Na literatura ainda há escassez de informações a respeito da ecologia dos Lactobacilos na dentina cariada após selamento. Alguns questionamentos necessitam ser respondidos como, por exemplo, se há possibilidade de que ocorra uma seleção de genótipos específicos na dentina cariada após selamento. Além disso, não se sabe qual o perfil de Lactobacilos permanecem após a remoção parcial de dentina cariada. O objetivo deste estudo foi avaliar o efeito do selamento após tratamento conservador envolvendo RPTC, sobre caracterização molecular de Lactobacilos associados à progressão das lesões de cárie dentinária.

2 ARTIGO CIENTÍFICO

Genotypic Diversity of *Lactobacillus* isolated from carious dentin before and after sealing

Keywords:
Dental caries; Dentin; *Lactobacillus* spp.; Genetic diversity; AP-PCR.

Abstract

Aim: The aim of this study was to compare the genotypic diversity of *Lactobacillus* species cultivated from carious dentin before and after sealing

Methods and Results: Carious dentin samples were obtained before and after partial dentin carious removal (PDCR) and sealing. Lactobacillus were isolated from carious dentin samples and cultivated in Rogosa agar. Up to 7 isolates of each morphological type were selected. All isolates were identified by Gram staining. The isolates were identified by sequencing of genes *pheS/rpoA*. Six out of 18 patients harbored Lactobacillus before and after sealing. A total of 93 *Lactobacillus* were genotyping (44 before and 49 after sealing). Genotyping was performed by AP-PCR. The amplicons from the same patient, before and after PCDR and sealing, were always resolved side-by-side for visual comparisons. Paired t-test was used to compare the mean of number of genotypes found before and after sealing considering 5% of significance. A total of 31 and 16 different genotypes were found before and after sealing, respectively. Genotypic diversity of *Lactobacillus* was reduced after PCDR and sealing using AP-PCR fingerprinting analysis. The mean of genotypes found by patient was 5.17±5.49 and 2.67±2.25 (p=0.344) before and after sealing, respectively.

Conclusions: The effect of the nutritional starvation underneath restoration was observed showing a tendency of reducing the genotypic diversity of *Lactobacillus* after 3 months of sealing.

Introduction

The conventional treatment of cavitated caries lesions is the complete carious dentin removal (CCDR), according to the clinical criteria of tissue hardness, and immediate restoration of the cavity (Black 1908). Microbiological studies showed that, even when the treatment of choice is the CCDR, microorganisms remain in dentin (Henz 1997; Maltz et al. 2012). In deep lesions the CCDR creates a high risk of mechanical exposure of the pulp and a

poor dental prognosis (Bjørndal et al. 2010). Stepwise excavation is indicated in cases of deep caries lesions (Bjorndal et al. 1997), and it is characterized by the partial carious dentin removal (PCDR) and temporary sealing. After sealing, the cavity is reopened and the remaining carious tissue is removed and the tooth is restored (Bjørndal et al. 2010). The stepwise excavation treatment therefore reduces the risk of pulp exposure (Bjørndal and Thylstrup 1998) and improves the prognosis of pulp vitality. The PCDR in one session, keeping a layer of carious dentine underneath restoration, has been proposed as an alternative treatment to stepwise excavation. A three years follow-up study showed higher success rate of the alternative treatment in comparison to the stepwise excavation (Maltz et al. 2013).

The persistence of viable bacteria in dentin after PCDR has raised doubts regarding the long-term effectiveness of this treatment (Weerheijm and Groen 1999; Bergenholtz and Spångberg 2004). A limited supply of nutrients is available to the bacteria that survive underneath the restoration. The strains that survive in this microenvironment exhibit specific phenotypic features such as the possibility of degradation of glycoprotein (Paddick et al. 2005). In that way, the source of nutrients underneath the restorations could lead to a modification of the residual microbiota.

Lactobacillus is found in carious lesions and also in the cavitated carious dentin after the PCDR (Caufield et al. 2007; Aas et al. 2008). These genus is aciduric and acidogenic and is associated with caries progression (Aas et al. 2008; Badet and Thebaud 2008). *Lactobacillus* belongs to a broad group of bacteria and some of the species may be more closely related to the development of dental caries (Badet and Thebaud 2008). Genotypic methods are able to identify strains of *Lactobacillus* from carious lesions (Caufield et al. 2007; Parolo et al. 2011) and discriminate isolates from the different sources.

AP-PCR (or RAPD) is a genotyping method that uses random primers for the characterization of the bacterial genome under conditions of low stringency with a good resolving power (Yang et al. 2010). Other genotypic methods had been used to type *Lactobacillus*, chromosomal DNA restriction digest profiles (CDF) (Yang et al. 2010), denaturing gradient gel electrophoresis (DGGE) (Yang et al. 2010), multi locus sequencing typing (MLST) (Parolo et al. 2011), repetitive extragenic palindromic PCR (rep-PCR) (Parolo 2009), ribotyping (Coudeyras et al. 2008), pulse-field gel electrophoresis (PFGE) (Coudeyras et al. 2008). When compared to these others method, AP-PCR is suitable to identify unique genotype, has low cost, does not require specific apparatus and is not laborious (Yang et al. 2010).

There is a lack of evidence in the literature regarding the role of the remaining microorganisms in the progression of carious process underneath restorations after PCDR. It remains unclear if there is a selection of a specific genotype/genotypes best adapted to survive underneath the restoration. Therefore, the aim of this study was to compare the genotypic diversity of *Lactobacillus* species cultivated from carious dentin before and after sealing.

Materials and Methods

Origin of the samples

Patients (n = 18) with permanent molar with carious lesion located in the middle third of dentin were selected for a clinical study trial (Firmino 2011). The patients were submitted to PCDR, by a sterile bur, transferred to and diluted in reduced transport fluid, lining with calcium hydroxide cement and sealing with glass ionomer cement for 3 months. Dentin samples were obtained before and after the sealing period. The *Lactobacillus* species were isolated on Rogosa agar and up to 7 isolates of each morphological type found in the culture were selected and analyzed regarding colony morphology and Gram staining. Gram negative and *cocci* isolates were excluded. After purified, each strain was stored in Brain Heart Infusion (BHI) (HiMedia, Mumbai, India) with 15% (v/v) glycerol at -20°C for further analysis (Firmino 2011).

Extraction of genomic DNA and species-specific identification

Lactobacillus isolates were grown from frozen stocks on BHI agar, incubated for 24 h at 37°C. The genomic DNA was extracted from *Lactobacillus* 1µl colonies resuspending them in 50µl of sterile ultrapure water. To confirm that strains were *Lactobacillus* the genes *pheS* or *rpoA* were amplified (Naser et al. 2007). PCR products were visualized by electrophoresis on a 1% (w/v) agarose gel containing SyBRGreen (Invitrogen), cleaned and sequenced with the same primers as previously described (Parolo et al. 2011).

Genotypic analysis of Lactobacillus isolates by AP-PCR

AP-PCR assays were performed with the arbitrary primer OPA3 (5'-AGTCAGCCAC-3') (Yang et al. 2010). The PCR amplifications were performed with 25µL total volume,

including 2µL of the target DNA, 0,5µL of Taq DNA polymerase (5U/µl) (Invitrogen), 2,5µL of 10x PCR buffer (Invitrogen, SG, Milanese, Italy), 1,75µL of $MgCl_2$ 50mM, 0,5µL of deoxynucleoside triphosphate mix (10mM), 1µL of each primer (20µM) OPA 03 and 16,75µL of ultrapure water . The termocycling conditions consisted of the 95 °C for 5 min, 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. *Lactobacillus* ATCC (*L.rhamnosus* ATCC 7469) and ultrapure water were used as controls. Products of AP-PCR were analyzed by electrophoresis on 1% agarose gel, stained with SybrGreen 1.6%, at 96 V during 4 h, using a 250-pb DNA ladder (Invitrogen). To test the ability of the AP-PCR technique to discriminate different species of *Lactobacillus*, *L. fermentum* (ATCC 9338), *L. delbrueckii* (ATCC 4797), *L. paracasei* (ATCC 335) and *L. plantarum* (ATCC 10012) were resolved for comparison.

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 *Lactobacillus* genotypic profiles from the same patient, AP-PCR products from the isolates obtained before and after the sealing were always resolved in the same gel for visual comparisons. Thus, genotypic diversity was compared among isolates samples before and after the sealing samples. Isolates had the same genotypic identity when they presented identical AP-PCR product-size profiles. Two blinded and calibrated examiners performed the visual analysis without knowing to each period of sealing the strain belonged to. The Cohen's Kappa value was calculated for examiners 1 and 2. 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). Paired t-test was used for compare the mean number of genotypes found before and after sealing considering 5% of significance.

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 n°19218). Informed and written consent was obtained from all individuals. All participants received treatment for basic dental needs.

Results

All patients that showed growth of *Lactobacillus* species before and after the sealing were included in the study (n=6/18) (Figure 1). After Gram staining and typing (data not shown), the final sample comprised 93 isolates (44 before sealing and 49 after sealing). The number of isolates per patients varied from 1 to 26. Except for patients 5 and 6, patients had

*ATCC strains were kindly provided by the Fundação Oswaldo Cruz

examiner 2, suggesting a good level of intra-rater agreement in the evaluation of the AP-PCR patterns. The inter-examiner Cohen's Kappa value was 0,92 showing a very high concordance level between examiners.

The ability of the AP-PCR technique to discriminate different species was tested using ATCC strains. Each of the standard strains produced different profiles of AP-PCR which demonstrates the high capacity of this technique to discriminate different species of *Lactobacillus*. For the ATCC isolates the number of bands varied from 6 to 3. *L. rhamnosus* was used as positive control.

A total of 43 different genotypes were observed. The AP-PCR products with the lower number of bands were 2 and the higher were 15. The average number of bands was 8.72±2.22. Figure 2 shows the example of some of the genotypes found in patient 3.

Overall, a total of 31 and 16 different genotypes were found before and after sealing, respectively. It is possible to observe that before sealing the genetic diversity of *Lactobacillus* was increased in comparison to the period after sealing. The comparison by patient showed that patients 1, 2 and 4 had an increased number of genotypes after sealing and patients 3, 5 and 6 had a decrease number of genotypes. Only in patients 1 and 3 same genotypes were found before and after sealing. In the other patients, the AP-PCR profile was different before and after sealing. Although patient 4 presented the largest number of isolates after sealing (n=26) only 4 different genotypes were identified. On the other hand, the patient 6 that showed the largest number of isolates before sealing (n=16) showed a great diversity of genotypes before sealing (n=15) (Table 1). The mean number of genotypes found by patient was 5.17±5.49 and 2.67±2.25 (p=0.344) before and after sealing, respectively.

Discussion

Genetic-based taxonomic methods are required for the *Lactobacillus* molecular characterization. In this longitudinal study, we analyzed the modification of *Lactobacillus* composition in carious dentin before and after the sealing. The genotyping method of AP-PCR was able to identify a tendency for a decreased genetic diversity in sealed carious dentin.

It seems that a shift occurred in the prevalence of *Lactobacillus* after carious dentin sealing. Microbiological studies that assessed the PCDR and sealing exhibited a low level of bacterial infection when of a layer of carious tissue at the bottom of the cavity is temporary left during the stepwise excavation (Bjorndal et al. 1997; Maltz et al. 2002). In spite of this residual contamination, the remaining carious dentin becomes harder and drier, both characteristics of inactive lesions (Bjorndal et al. 1997).

No difference in the level of contamination between stepwise excavation and CCDR was found when both treatments are compared after sealing (Kneist et al. 2010; Firmino 2011). A reduction of the *Lactobacillus* species occurred after treatment with PCDR (Kneist et al. 2010). This finding is in agreement with the present study where Lactobacillus isolates yielded an average of 5.17±5.49 genotypes among the 44 strains while in the final sample only 2.67±2.25 genotypes were identified among 49 strains. A selection of the *Lactobacillus* underneath restoration takes place after carious dentin sealing.

Our results showed that after PCDR and sealing for 3 months it was still possible to recover *Lactobacillus* by means of cultivation. A longer sealing period could contribute to gradual decrease in population of *Lactobacillus* in dentin. Analysis performed on samples from 10 caries lesions treated with RPTC followed by restoration and reopened after 5 months showed no growth of *Lactobacillus* (Paddick et al. 2005). In a larger sample size study (n=90 permanent teeth), irrespective of the dentin removal technique (a-complete or b-partial and 6-7 months of sealing), no growth of *Lactobacillus* was observed (Maltz et al. 2012). However, viable *Lactobacillus* were found despite of 11 months of sealing (Kneist et al. 2010).

In our study, we identify similar patterns of AP-PCR 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 (Paddick et al. 2005). In that study, no similar REP-PCR profile was found before and after sealing (Paddick et al. 2005). As an explanation for this, the authors mentioned a possible replication of genotypes during the sealing period (Paddick et al. 2005). 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 (Paddick et al. 2005).

Up to 7 strains from different morphological type were selected from Rogosa Agar. From an initial number of 239 strains, 93 presumed Lactobacillus remained in the final sample after they were confirmed as Lactobacillus by gram staining and subtyping by *pheS/rpoA* sequencing (data not shown). Rogosa medium has enjoyed widespread acceptance to selective cultivation of *Lactobacillus* (Caufield et al. 2007). However, Rogosa also permits the growth of oral streptococci as already shown by Yang et al. (2010).

The application of the AP-PCR technique has advantages over other methods of genetic identification for being affordable, easy to apply and interpret, simple and with no need of refined equipment (Navarro and Jorcano 1999). Yang *et al.* (2010) studied *Lactobacillus* from pooled plaque, saliva and dentin using the AP-PCR technique with the same primers selected in our study. They were able to identify 29 genotypes with greater resolving power from 2100 *Lactobacillus* (Yang et al. 2010). AP-PCR was able to identify unique genotypes both inter-and intra-species with adequate standard of reproducibility (Yang et al. 2010). However, this technique is highly sensitive to variations in methodology. Sean and Daniel (2000) observed that some bands were not affected by changes in annealing temperature up to 6° C while other bands ceased to be amplified after changes of only 2° C. Different techniques of DNA extraction, concentration of Mg^{2+}, variations Manufacturer and concentration of Taq DNA polymerase are factors that alter the banding pattern generated by AP-PCR (Tyler et al. 1997). The authors also pointed out the value of implementing the AP-PCR procedure in triplicate (TAP-PCR) for greater reliability of results (Sean and Daniel 2000). We did not perform a triplicated AP-PCR analysis; nevertheless we decided to resolve the strains of interest side-by-side in the same gel and also to standardize the conditions for PCR and electrophoresis to minimize the reproducibility problems. Therefore, the comparison of data obtained from different laboratories is compromised and impractical (Yang et al. 2010).

To our knowledge, this study was the first to compare the genotypic diversity of *Lactobacillus* before and after partial caries removal and sealing for 3 months. Our results suggest that there is tendency of reduction of genetic diversity due to sealing. More studies

should be conducted with similar experimental design and perhaps with a larger number of samples to confirm these results.

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Conflict of Interest Statement

We, Nailê Damé-Teixeira, Raquel Soares Dalalba, Clarissa Cavalcanti Fatturi Parolo and Marisa Maltz declare that we have no proprietary, financial, professional or other personal interest 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 manuscript "Genotypic Diversity of Lactobacillus isolated from carious dentin before and after sealing".

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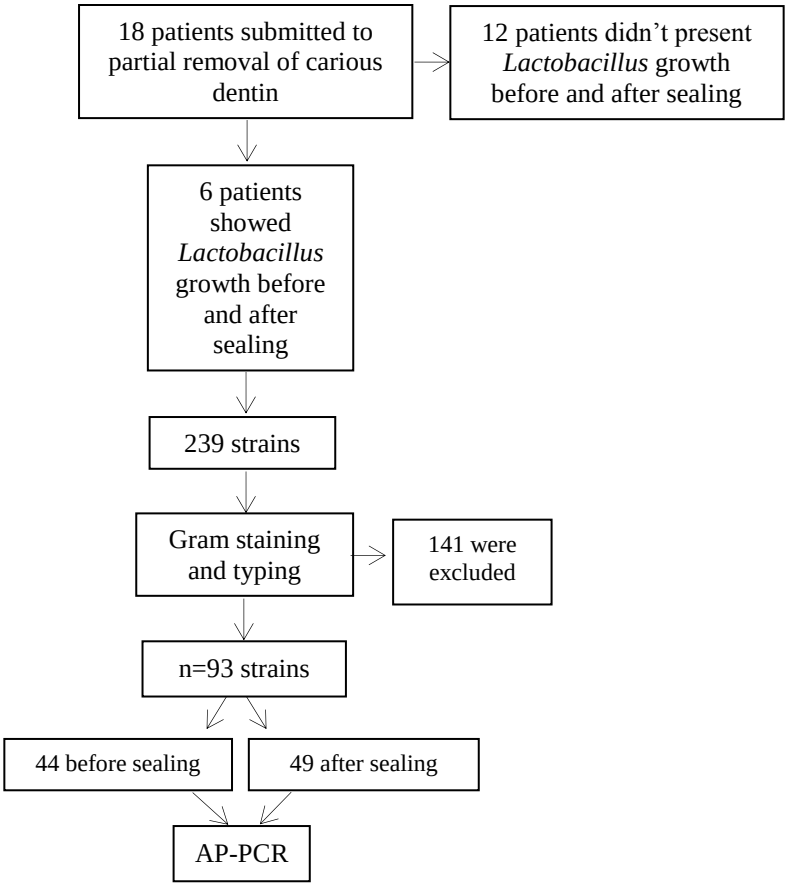

Figure 1. Flowchart showing the total sample of the strains worked.

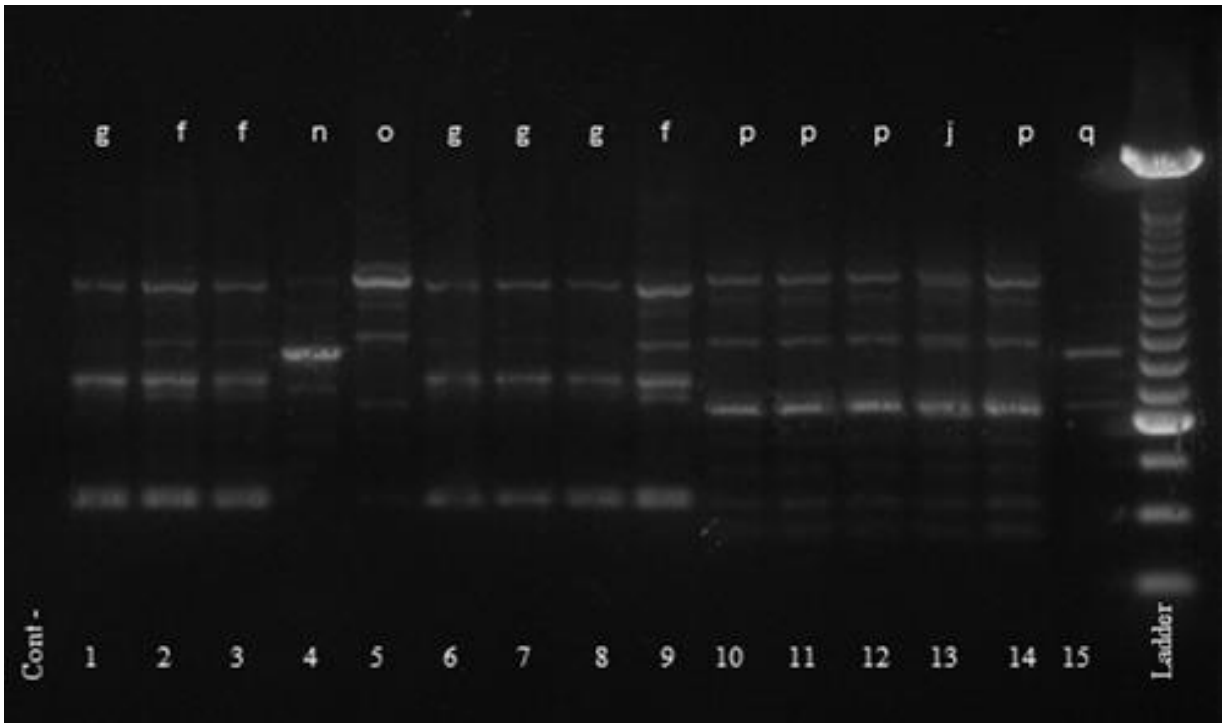

Figure 2. Electrophoresis gel image from AP-PCR-generated fingerprints using primer OPA-03 isolates from carious dentin. Colums 1 to 15 represents different isolates of *Lactobacillus*. Distinct letters show different genotypes. First column is the negative control and the last column is the 250pb Ladder.

Table 1. Number of isolates and genotypes of *Lactobacillus* per patient in dentin before and after sealing

Patient	Before sealing		After sealing	
	Number of isolates	**Genotypes**	**Number of isolates**	**Genotypes**
1	3	a (3)	4	a (3) b (1)
2	1	c (1)	2	d (1) e (1)
3	10	f (1) g (2) h (1) i (1) j (1) k (2) l (1) m (1)	15	f (3) g (4) j (1) n (1) o (1) p (4) q (1)
4	3	r (1) s (2)	26	t (2) u (23) v (1)
5	11	v (3) x (2) z (1) aa (5)	1	ab (1)
6	16	ac (1) ad (1) ae (1) af (1) ag (1) ah (1) ai (1) aj (1) ak (1) al (1) am (1) an (1) ao (2) ap (1) aq (1)	1	ar (1)
Total	44	31	49	16

Distinct letters show different genotypes. The number absolute of the genotypes in relation to the isolates in each condition is represented within the parenthesis.

3 CONCLUSÃO

Os resultados da análise da diversidade genotípica de Lactobacilos provenientes de dentina cariada antes e após o selamento de lesões de cárie de pacientes submetidos à remoção parcial de tecido cariado contribuem para o entendimento das modificações genotípicas dos Lactobacilos na dentina cariada selada. O método de avaliação da diversidade genotípica de Lactobacilos estudado nesse trabalho, AP-PCR, demonstrou que há uma tendência de diminuição da diversidade genética após período de selamento. O efeito da escassez de nutrientes proveniente do selamento da dentina cariada pode ser observado com a seleção das cepas mais adaptadas a sobreviver após RPTC.

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