UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

VANESSA KERN SOARES

# VIRULÊNCIA DE LACTOBACILOS DA MICROBIOTA RESIDUAL DE DENTINA CARIADA

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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.

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Aos meus pais que além da vida que me deram me ensinaram a vivê-la no melhor caminho.

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Colossenses 3.17

## RESUMO

SOARES, Vanessa Kern. **Virulência de lactobacilos da microbiota residual de dentina cariada.** 2012.23f. Trabalho de Conclusão de Curso (Graduação em Odontologia)- Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2012.

O objetivo do estudo foi avaliar o efeito do selamento de lesões dentinárias de cárie sobre a virulência de Lactobacilos através da produção de ácido (P.A) e tolerância ácida (T.A). Pacientes (n=18) com lesões dentinárias 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. As amostras foram 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 em -20º C. As amostras foram analisadas quanto à coloração de Gram e morfologia. Somente bacilos e cocobacilos gram-positivos foram mantidos na amostra. Do total de 18 pacientes, somente seis apresentaram crescimento de Lactobacilos antes e após selamento. Nas análises de P.A e T.A foram avaliadas 66/62 cepas cultivadas antes e 75/74 cepas após o selamento, respectivamente. Para avaliação da produção de ácido, as cepas foram ressuspensas em 5 mL de caldo BHI e incubadas a 37º C, em anaerobiose durante 18 horas. Após lavagem, as cepas foram ressuspensas em caldo BHI, enriquecido com glicose a 1% a uma densidade óptica de 0,03 a 600 nm ($DO_{600}$). As amostras foram incubadas a 37º C e alíquotas foram utilizadas para avaliação de pH em 9, 24, 48 e 72 horas, em triplicata. Um controle negativo foi incubado nas mesmas condições. As análises estatísticas foram realizadas nos diferentes períodos de avaliação (teste t- Student). Para realização do experimento de T.A, os isolados foram ressuspensos em 2 mL de caldo BHI e incubados a 37º C em anaerobiose durante 18 horas. Alíquotas foram ressuspensas em BHI caldo pH 7 e pH 4 a uma densidade óptica de 0,03 a 600 nm ($DO_{600}$). Vinte e cinco microlitros das diluições $10^{-4}$ e $10^{-5}$ foram plaqueadas em BHI ágar no tempo 0 e após 30 minutos de incubação em anaerobiose a 37º C. Foi calculado a média±DP do número de UFC/mL do tempo 0 e após 30 minutos (pH 7 e pH 4). A taxa de crescimento durante 30 minutos foi comparada em cada grupo (pH 7 e pH 4 antes do selamento, pH 7 e pH 4 depois do selamento) (teste ANOVA). A produção de ácido (média±DP) dos Lactobacilos isolados antes e após o selamento, respectivamente, nos diferentes períodos de tempo foram: 9h (6,08±0,70-5,96±0,79 p=0,32), 24h (4,40±0,16- 4,37±0,15 p=0,26), 48h (4,21±0,12-4,21±0,15 p=0,87) e 72h (4,18±0,11-4,19±0,21 p=0,65). A taxa de crescimento antes do selamento no pH 7 foi de 1,77±0,32 $\log_{10}$ UFC/mL e após o selamento foi de 1,73±0,29 $\log_{10}$ UFC/mL (p=1.00). A taxa de crescimento antes do selamento no pH 4 foi de 1,67±0,37 $\log_{10}$ UFC/mL e depois do selamento foi de 1,60±0,34 $\log_{10}$ UFC/mL (p= 0.92). Não foi observado diferença na virulência de Lactobacilos antes e após o período de selamento utilizando a técnica de curva de pH e T.A.



Palavras-chave: Lesão Cariosa. Dentina. Lactobacilos spp. Microbiologia. Fator de Virulência.

## ABSTRACT

SOARES, Vanessa Kern. **Virulence factors of residual Lactobacilli in carious dentin.** 2012. 23f. Final Paper (Graduation in Dentistry)- Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2012.

The aim of this study was to evaluate the effect of sealing in the virulence traits of the residual *Lactobacillus* spp. cultivated from carious dentin using acid production (A.P) and acid tolerance (A.T). Patients (n = 18) with permanent molar with carious lesion were submitted to partial caries removal and cavity sealing for 3 months. Dentin samples were obtained before and after sealing. The samples were cultured on Rogosa agar for 72 hours under anaerobic conditions. Up to seven isolates of each morphological type were selected and stored in Brain Heart Infusion (BHI) broth and glycerol at -20º C. The samples were analyzed for Gram staining and morphology. Only *bacilli* and *coccobacilli* gram-positive were kept in the sample. Of the 18 patients, six showed growth of *Lactobacillus* spp. before and after sealing. For the analysis of A.P and A.T it was evaluated 66/62 isolates cultivated before and 75/74 after sealing, respectively. To perform the A.P, isolates were resuspended in 5 ml of BHI broth and incubated anaerobically at 37º C for 18 hours. After being washed, aliquots were resuspended in BHI broth supplemented with 1% glucose at an optical density of 0.03 at 600nm ($OD_{600}$). The samples were incubated at 37° C for 3 days. In order to perform the pH curve, aliquots were removed and the pH measured at 9, 24, 48 and 72 h. Tubes without inoculums (negative control) were incubated under the same conditions. The samples were performed in triplicate. Statistical analyzes were carried out in different periods (Student´s t test). To perform the experiment of A.T, the isolates were resuspended in 2 ml of BHI broth and incubated anaerobically at 37º C for 18 hours. Aliquots of this culture were resuspended at pH 7.0 and pH 4.0 at an optical density of 0.03 at 600 nm ($OD_{600}$). Twenty-five microliters of the $10^{-4}$ and $10^{-5}$ dilutions were plated in BHI agar at time 0 (Baseline) and after 30 minutes of anaerobic incubation at 37º C. This experiment was performed in triplicate. The mean±SD of the triplicate CFU values was calculated at baseline and after 30 minutes (pH 7.0 and pH 4.00. The growth rate during 30 minutes was compared in each group (pH 7.0 and pH 4.0 before; pH 7.0 e pH 4.0 after cavity sealing) (ANOVA test). The acid production (mean±SD) of *Lactobacillus* spp. cultivated from dentin before and after sealing, respectively, in different time periods were: 9 h (6.08±0.70-5.96±0.79, p = 0.37), 24 h (4.40±0.16-4.37±0.15, p=0.26), 48 h (4.21±0.12-4.21±0.15, p = 0.87) and 72 h (4.18±0.11- 4.19±0.21, p = 0.65). The growth rate before the sealing at pH 7.0 was 1.77 (± 0.32) $\log_{10}$ CFU/ml and after sealing was 1.73 (±0.29) $\log_{10}$ CFU/ml (p = 1.00). The growth rate before the sealing at pH 4.0 was 1.67 (±0.37) $\log_{10}$ CFU/ml and after sealing was 1.60 (±0.34) $\log_{10}$ CFU/ml (p = 0.92). No difference was observed in the virulence of *Lactobacillus* spp. before and after sealing using A.P and A.T.



Keywords: Dental caries. Dentin. *Lactobacillus* spp. Microbiology.Virulence factors.

**SUMÁRIO**

# 1 INTRODUÇÃO

O tratamento restaurador convencional de lesões de cárie é baseado na remoção total de tecido cariado (RTTC) segundo critério clínico de dureza. Estudos microbiológicos demonstram que esse tipo de tratamento não garante remoção total de micro-organismos abaixo das restaurações (IOST, 1995; SHOVELTON, 1972). Diante de lesões profundas de cárie, a RTTC pode levar a uma exposição mecânica da polpa e, caso isso ocorra em presença de tecido cariado, o prognóstico para a polpa é ruim (BARTHEL et al., 2000; BJØRNDAL et al., 2010).

Diante deste problema, tratamentos conservadores têm sido propostos com finalidade de conservar tecido dentinário e melhorar o prognóstico de vitalidade pulpar (BJØRNDAL et al., 1997; BJØRNDAL et al., 2010; MALTZ et al., 2002). Um destes tratamentos é o tratamento expectante, que consiste de uma remoção total de tecido cariado em 2 sessões. Na primeira etapa é realizada remoção completa do tecido cariado das paredes laterais e deixa-se tecido contaminado no fundo da cavidade para evitar a exposição pulpar (KIDD, 2004; LEKSELL et al., 1996; MAGNUSSON, 1977). Após período de selamento, realiza-se a remoção completa do tecido cariado da parede pulpar. O segundo tratamento proposto é a remoção parcial de tecido cariado (RPTC) em apenas uma única sessão (MALTZ et al., 2002; THOMPSON, 2008).

O tratamento de lesões profundas de cárie realizado em única sessão, com a colocação de restauração mesmo sem a remoção completa de dentina cariada tem várias vantagens: (1) evitar contaminação pelo meio externo resultante da perda de selamento temporário, (2) evitar perda de tecido dentário íntegro pela remoção do material restaurador, (3) evitar eventual exposição pulpar durante a remoção da restauração provisória, (4) poupar desconforto ao paciente evitando nova consulta e (5) tornar o tratamento menos oneroso (JARDIM, 2010).

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 (LULA et al., 2009; MALTZ et al., 2002). Estudos mostram que há maior contaminação bacteriana logo após a RTTC do que quando se realiza RPTC e selamento (MALTZ et al., 2012). Porém, ainda torna-se necessário saber a virulência das bactérias remanescentes abaixo de restaurações.

Um dos micro-organismos mais prevalentes na dentina cariada são os Lactobacilos. Pertencem ao grupo de bactérias produtoras de ácido láctico, são bactérias acidogências e acidúricas, associadas à progressão da lesão cariosa (AAS et al., 2008; EDWARDSSON, 1974). Os fatores de virulência dos Lactobacilos relacionados à lesão de cárie cavitada são:

produção de ácido (acidogênese), tolerância ao meio ácido (aciduricidade) e capacidade de degradar matriz protéica. Sendo a acidogenicidade e aciduricidade os fatores de virulência mais associados à cárie dentária (COTTER; HILL, 2003).

O objetivo deste trabalho foi avaliar o efeito da RPTC e selamento na expressão de fatores de virulência dos Lactobacilos identificados na dentina, através da avaliação de produção de ácido e tolerância ácida. Como hipótese, o selamento de dentina cariada não modifica a virulência dos micro-organismos remanescentes.

# ARTIGO CIENTÍFICO

# Virulence factors of residual Lactobacilli in carious dentin after partial caries removal and sealing

Keywords:
Dental caries; dentin; *Lactobacillus* spp.; microbiology; virulence factors.

## Abstract

**Aims:** To evaluate the effect of sealing in the virulence traits of the residual *Lactobacillus* spp. cultivated from the carious dentin through Acid Production (A.P) and Acid Tolerance (A.T).

**Methods and Results:** Dentin samples were obtained after partial caries dentin removal (PCDR) and after 3 months of sealing. *Lactobacillus* spp. was resuspended in BHI broth supplemented with 1% glucose to evaluate the A.P. The pH at 9, 24, 48 and 72 hours was measured. To evaluate the A.T, the strains were incubated in BHI broth during 30 min ($37^{o}$ C anaerobically) at pH 7.0 and pH 4.0. The growth rate was compared at different pH and different treatment times (before and after sealing). It was evaluated 66/62 strains before and 75/74 strains after sealing (A.P/A.T respectively). The A.P by the *Lactobacillus* spp. isolated before and after sealing was not significantly different. These *Lactobacillus* spp. did not differ regarding their ability to tolerate an acidic environment.

**Conclusions:** The sealing of carious dentin did not change the virulence traits of *Lactobacillus* spp.

**Significance and Impact of the Study:** Sealing of carious dentin did not select more cariogenic *Lactobacillus* spp.

## Introduction

Routinely, conventional restorative treatment is based on the complete caries dentin removal (CCDR) and sealing of the cavity. In deep caries lesion, the CCDR can lead to pulp exposure and an alternative treatment is the stepwise excavation. In this technique, a partial caries dentin removal (PCDR) is performed and a layer of carious dentine is left over the pulp, and the tooth is temporarily sealed. After the sealing period, the filling is removed and the CCDR is performed (Leksell et al. 1996; Bjørndal et al. 1997; Kidd 2004). It has been proposed alternative approach to stepwise excavation which is the PCDR in only one session. Clinical studies have demonstrated success over time without the need to reopen the cavity (Maltz et al. 2007; Maltz et al. 2012). 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).

*Lactobacillus* species, *Streptococcus mutans*, *Streptococcus* species, *Actinomyces* species and *Candida albicans* are among the remaining microorganisms from the sealed dentin (Paddick et al. 2005; de Carvalho et al. 2006; Pinto et al. 2006; Wambier et al. 2007; Orhan et al. 2008; Duque et al. 2009; Lula et al. 2009). *Lactobacillus* species are members of lactic acid bacteria group, which colonize the mouth and have been associated with dental caries lesion (Krasse 1954; van Houte et al. 1972; Ikeda et al. 1973; van Houte 1980). This bacteria group appears to be secondary colonizer in some lesions and contributes to the progression of the cavity (van Houte 1980; Tanzer et al. 2001). The acid production and acid tolerance have been proposed as virulence factors in the cariogenicity of *Lactobacillus* species and the latter factor could account for high levels of these bacteria in carious dentin. The progression of the carious lesion is therefore associated with both characteristics of acid production and acid tolerance (Harper and Loesche 1984).

There is a lack of evidence in the literature regarding the role of the remaining microorganisms in the progression of carious process beneath restorations after PCDR. It remains 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 compare the pH-lowering potential and growth capacity of *Lactobacillus* species cultivated from caries dentin before and after sealing.

## Materials and Methods

### *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.

### *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, lining with calcium hydroxide cement and sealing with glass ionomer cement for 3 months. Dentin samples were obtained before and after the sealing period by two sterile

slowly rotating n° 4 round burs. The *Lactobacillus* species were isolated on Rogosa agar and up to 7 strains of each morphological type found in the culture were selected and analyzed for colony morphology and Gram staining. Gram negative and *cocci* strains 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.

*Study design*

*In vitro* analysis of the stored isolates were conducted to determine the virulence of *Lactobacillus* species: the acid production and acid tolerance.

*Acid Production*

The strains were resuspended in 5 ml of BHI broth and incubated anaerobically at 37º C for 18 h. The overnight culture was centrifuged at 3,020 rpm (Centrífuga- CT 5000, Cientec, Brazil) during 5 minutes and the pellet was resuspended in BHI broth supplemented with 1% glucose at an optical density of 0.03 at 600nm ($OD_{600}$) (Spectronic 21D, Milton Roy, USA) in order to standardize the number of cells. Immediately, the samples were incubated at 37° C for 3 days. In order to perform the pH curve, aliquots were removed and the pH measured at 9, 24, 48 and 72 h with a pH meter (pHmetro DM-23, Digimed, Brazil). Tubes without inoculum (negative control) were incubated under the same conditions. The samples were performed in triplicate (van Houte et al. 1996; Napimoga et al. 2004).

*Acid Tolerance*

The strains were resuspended in 2 ml of BHI broth and incubated anaerobically at 37º C for 18 h. Aliquot of this culture was resuspended at pH 7.0 and pH 4.0 at an optical density of 0.03 at 600 nm ($OD_{600}$). Twenty-five microliters of the $10^{-4}$ and $10^{-5}$ dilutions were plated in BHI agar (HiMedia, Mumbai, India) at time 0 (Baseline) and after 30 minutes of anaerobic incubation at 37º C. This experiment was performed in triplicate (Azcarate-Peril et al. 2004; Penaud et al. 2006).

*Calibration and Reproducibility*

A single examiner was calibrated for the counting of the total colony-forming units (CFU) of *Lactobacillus* species. All the counts were performed using colony counter (Hellige, Garden City, USA). Repeated tests were conducted for 12 plates. The time interval between examinations was seven days and the intraclass correlation coefficient was 0.93.

*Data analysis*

For the acid tolerance analysis of *Lactobacillus* species, the mean±SD of the triplicate CFU value were calculated at baseline and after 30 min (pH 7 and pH 4). The growth rate during 30 minutes was compared in each group (pH 7 before cavity sealing, pH 7 after cavity sealing, pH 4 before cavity sealing, pH 4 after cavity sealing). Data was transformed to $\log_{10}$ due to data dispersion and analyzed by ANOVA test, post-hoc analysis–Bonferroni test.

For the acid production analysis, the mean±SD of the triplicate pH values at 9, 24, 48 and 72 h was calculated. The difference of mean between groups was tested using Student's t test.

The level of significance for analysis was considered 5%. The software used was SPSS version 18.0 for Windows.

**Results**

Of the 18 patients from PCDR group only 6 patients showed growth of *Lactobacillus* species before and after sealing. After gram staining and exclusion of *cocci* and Gram-negative, the final sample for the acid production was 141 strains (66 before sealing and 75 after sealing) and the final sample for acid tolerance was 136 strains (62 before sealing and 74 after sealing). The difference occurred because the experiments were not conducted concurrently and some strains were lost (Fig. 1).

The pH (mean±SD) of BHI broth supplemented with 1% glucose at times 9 h, 24 h, 48 h, 72 h after incubation with *Lactobacillus* species before and after cavity sealing is shown in Fig 2. The pH curves showed no significant difference with respect to acid production.

Regarding acid tolerance, no difference in the growth rate of *Lactobacillus* species was observed at pH 7 and pH 4 (Table 1).

**Discussion**

To occur demineralisation of the tooth tissue, microorganisms produce acids from fermentation of carbohydrates. *Lactobacillus* species can produce acids capable to lower the pH below pH 5.0, which is relevant for caries development (Haukioja et al. 2008). In this study, the *Lactobacillus* species before cavity sealing were able to produce acid below 4,2 showing a high acidogenicity and this trait remained in the strains after sealing when submitted to a sugar medium. Other study showed similar low final pH after 2-3 days

incubation of Lactobacillus from advanced root caries lesions in sugar broth medium (<4.2) (van Houte et al. 1996).

Moreover, *Lactobacillus* species still secret acid in significant quantities at low pH (4.0) showing high aciduricity (Klinke et al. 2009). The ability of bacteria to produce acid (acidogenicity) in combination with growth at low pH (aciduricity) are regarded as being two of the most important virulence factors related to dental caries (Cotter and Hill 2003). These abilities of *Lactobacillus* species are essential for the progression of the lesion. In the present study, the growth rate of *Lactobacillus* species in pH 4 was similar to pH 7, means that they grew well both in neutral pH and in acid pH and the cavity sealing did not modified this trait.

It is unclear whether the bacteria beneath restorations represent some danger to the longevity of restorations and if they are more virulent than the bacteria previous to the sealing. It would be relevant to know whether those who survive remain potentially cariogenic. This is the first study to evaluate the phenotypic characteristics of *Lactobacillus* species isolated from sealed caries dentin. In the present study we couldn't find any difference in acid tolerance and in acid production between the *Lactobacillus* species from carious dentin before and after cavity sealing. The *Lactobacillus* species showed the same phenotypic characteristics regarding acid production and tolerance after 3 months of sealing.

Carious dentin of active lesions characterized by a low pH (mean pH= 4.9±0.2) and a lactate-dominant acid profile (Hojo et al. 1994). A variety of bacteria are responsible for this change of pH in the cavity, such as *Lactobacillus* species and *Streptococcus* species (Loesche and Syed 1973; Klinke et al. 2009). On the other hand, the pH and acid profile of carious dentin obtained from lesions beneath a restoration were similar to those of the arrested lesion, a higher pH (mean pH= 5.8±0.7) and an acetate and propionate-dominant acid profile, suggesting that these two types of lesions have less acidogenic and aciduric flora than active lesions. After cavity sealing, a limited supply of nutrients is left for the bacteria that survive beneath restoration (Paddick et al. 2005). The bacteria had access to primarily the serum proteins and glycoproteins, which pass from the pulp through the dentinal tubules of the uninfected dentin to the infected dentin. The results of the present study suggest that the higher pH observed in sealed carious dentin (Hojo et al. 1994) are due to the decrease in the number of theses acidogenic bacteria and the limited access to nutrients (Paddick et al. 2005) and not by their lower capacity of acid production.

Virulence factors that are associated with caries progression in dentin are degradation of hydroxyapatite, biofilm formation, adhesion to hydroxyapatite and collagen type-I, cleavage the terminal sugars from the glycoproteins, acid production and acid tolerance (McGrady et al. 1995; Paddick et al. 2005; Jalasvuori et al. 2012). Acid production and acid tolerance alone do not make bacteria cariogenic, but they are so far an indicative of caries potential. The present study showed that the pH-lowering potential and growth capacity of *Lactobacillus* species from carious dentin before cavity sealing were similar to the isolates cultivated from caries dentin after sealing. In conclusion, the virulence of *Lactobacillus* species through its ability to produce and tolerate acid seems to not modify after cavity sealing.

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## Conflict of Interest Statement

We, Nailê Damé Teixeira, Vanessa Kern Soares, 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 "Virulence factors of residual Lactobacilli in carious dentin after partial caries removal and sealing".

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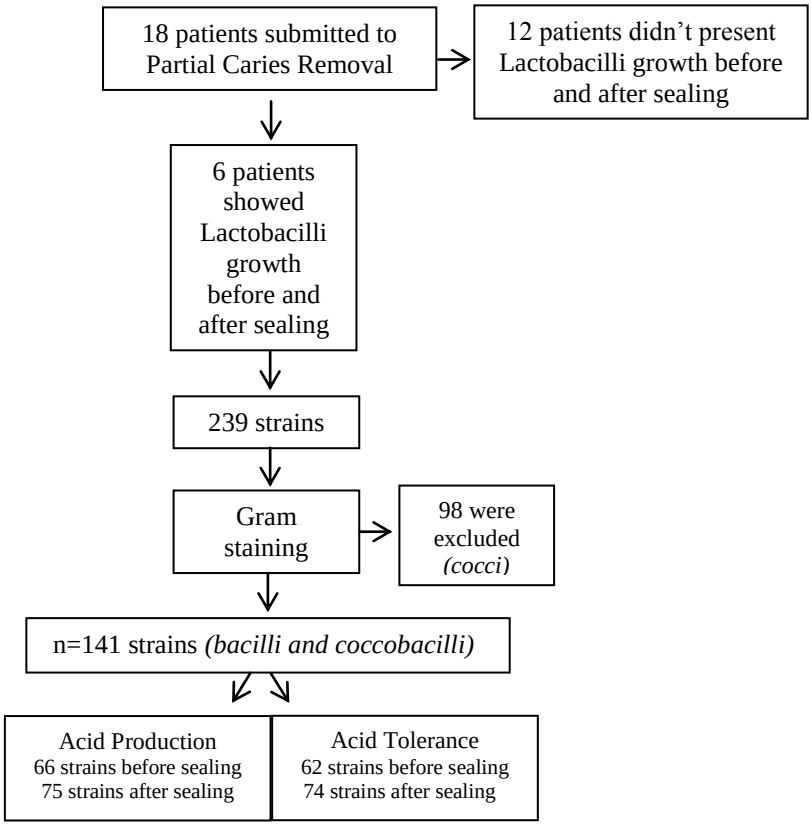


**Figure 1.** Flowchart showing the total sample of the strains worked.

**Figure 2.** pH (mean±SD) of BHI broth supplemented with 1% glucose at times 9 h, 24 h, 48 h, 72 h after incubation with *Lactobacillus* species isolated from carious dentin before (◆) and after (■) cavity sealing.

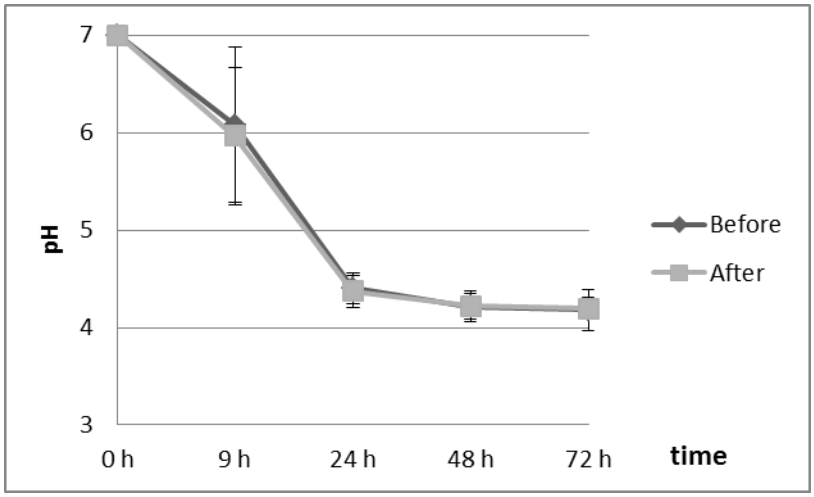

**Table 1.** Mean±SD growth rate of *Lactobacillus* species ($log_{10}$ CFU / ml) at pH 7.0 and pH 4.0 before and after sealing of carious dentin.

| pH | BEFORE CAVITY SEALING | AFTER CAVITY SEALING |
|---|---|---|
| pH 7.0 | $1.77 \pm 0.32^{a}$ | $1.73 \pm 0.29^{ab}$ |
| pH 4.0 | $1.67 \pm 0.37^{ab}$ | $1.60 \pm 0.34^{b}$ |

## 3 CONCLUSÃO

Os resultados da análise da virulência 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 características fenotípicas das bactérias seladas. Os dois métodos de análise de virulência de Lactobacilos estudados nesse trabalho, produção de ácido e tolerância ao ácido, demonstram que não houve uma modificação no potencial patogênico desse micro-organismo após período de selamento. A permanência de bactérias abaixo de restaurações parece não aumentar o potencial cariogênico destas bactérias.

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