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Bacterial analysis of biofilms from tooth root surfaces presenting different caries activity

Análise bacteriológica de biofilmes de superfícies dentárias radiculares apresentando diferentes estágios de atividade de cárie

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ABSTRACT

This study evaluated the numbers and determined the proportion of mutans streptococci and *Lactobacillus* spp., which are possible relevant cariogenic organisms, in biofilms recovered from lesions at root surfaces with active caries lesions (ARC), inactive caries lesions, and sound root surfaces (SRS). Samples were cultured in MSB agar for mutans streptococci counts, Rogosa agar for *Lactobacillus* spp. counts, and brain-heart infusion agar for total viable anaerobic counts. After incubation, the number of colony-forming units (CFUs) was determined and compared between groups by the Mann-Whitney U test with a significance level set at 95%. The proportion of counts of mutans streptococci and *Lactobacillus* spp. in the total viable microorganisms was also analyzed by Chi-square test. Ninety samples (30 from each surface) from 37 patients were cultured and analyzed. The CFU was similar between mutans streptococci and *Lactobacillus* spp. These species were present in at least half of the samples and no difference was found in the frequency of isolation of these species. Only 6 samples showed a proportion of more than 10% of mutans streptococci; 4 of the samples were from ARC. Most (93%) SRS samples did not contain viable *Lactobacillus* spp. The data indicate the low counts of mutans streptococci and *Lactobacillus* spp. in root surfaces, regardless of the activity of caries lesions.

Keywords: Root caries. Microbiology. Bacterial Load. *Streptococcus mutans. Lactobacillus.*

RESUMO

O estudo analisou contagens e proporções de mutans streptococci e *Lactobacillus* spp., que podem ser microorganismos importantes em lesões de cárie radicular com diferentes atividades. Biofilmes foram coletados em três locais: ARC – superfície radicular com lesão ativa de cárie; IRC – superfícies radiculares com lesão inativa de cárie; SRS – superfícies de raizes hígidas. As amostras foram cultivadas em agar MSB para contagens de mutans streptococci; agar Rogosa para *Lactobacillus* spp., e agar BHI para contagens de microrganismos viáveis anaeróbicos totais. Após a incubação, o número de unidades formadoras de colônias (UFCs) foi determinado e comparado entre os grupos pelo teste de Mann-Whitney U test. O nível de significância foi estabelecido em 95%. A proporção de contagem de mutans streptococci e *Lactobacillus* spp. no total de microrganismos viáveis também foi analisado através do teste de qui-quadrado. Um total de 90 amostras de 37 pacientes foram cultivadas e analisadas: 30 amostras de ARC, 30 de IRC e 30 de SRS. Números de UFC foram semelhantes entre os grupos para ambos, mutans streptococci e *Lactobacillus* spp. Estas espécies estavam presentes em pelo menos metade de todas as amostras e nenhuma diferença foi encontrada na frequência de isolamento dessas espécies dentro dos grupos. Apenas 6 amostras apresentaram mais de 10% de mutans streptococci e 4 foram de ARC. Em relação aos *Lactobacillus* spp., 93% das amostras não apresentaram proporção dessas bactérias nas SRS. Mutans streptococci e *Lactobacillus* spp. estão presentes em baixa proporção nas superfícies radiculares, independentemente da atividade das lesões de cárie.

Palavras-chave: Cárie radicular. Microbiologia. Carga Bacteriana. *Streptococcus mutans. Lactobacillus.*

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Introduction

Improvements in dental health care globally have led to a decrease in the loss of teeth. In Brazil in 2003, the DMFT (Decayed, Missing, Filled Teeth) index value was 13.2 for individuals 35 to 44 years of age. By 2010, the mean number of missing teeth at the Brazilian population had declined to 7.3[1]. This implies an increased number of exposed root surfaces that are susceptible to caries[2]. The high prevalence and restoration of this type of cavity pose challenges with respect to the lack to restorative materials that bond equally well to both dental tissues (enamel and dentin). Tooth caries have become a significant issue in dentistry. New preventive and efficient treatment strategies are required to avoid tooth extraction and to maintain oral health. In this regard, knowledge of the pathogenicity of the primary etiological factor of root caries, the microbial biofilm, might provide important information for the development of diagnosis and treatment strategies.

Gingival recession, reduced salivary flow rate, and use of xerostomic medications are the major factors contributing to microbiota modification and the risk of root caries, mainly in the elderly[2]. However, the current understanding of the microbial composition of root caries is limited compared to other oral diseases[3,4]. Many studies have tried to identify the pathogenic species that cause root caries[5–11], however there is no consensus on the microbiota associated with root caries. Distinct patterns and individual differences in the microbial composition have been observed, although the observed predominance of *Actinomyces* spp. has linking it as a potential agent of root caries. However, *Actinomyces* spp. were recently demonstrated to be highly metabolically active in carious root surface and sound root surface (SRS) biofilms[12], suggesting that these organisms are related more to the site and not to the dysbiosis. Some studies also suggested a role for mutans streptococci and lactobacilli in root caries. A correlation between these species confirmed the increased risk of root surface caries when they were present together[9]. Most relevant culture-based studies have shown a strong relationship between root caries and mutans streptococci due to the higher isolation frequency and/or higher proportion on carious root surfaces[11,13,14]. *Lactobacillus* spp. are absent or rarely observed in individuals without root caries[15]. However, these studies were developed before the 1990's and no contemporary studies were concerned with looking back at the root caries microbiota.

While there is evidence that the microbiota of root surface caries lesions changes with the activity of the lesion, few studies have differentiated the microbiota in active and inactive root caries lesions[11,14]. Hence, the aim of this study was to evaluate the numbers and determine the proportions of mutans streptococci and *Lactobacillus* spp., which could be relevant cariogenic organisms, in root caries lesions with different caries activity.

Materials and methods

Subjects and sample collection

Patients (n = 122) were recruited at the Hospital de Clínicas of Porto Alegre, according to Declaration of Helsinki. Of these patients, 99 had caries in at least one tooth. Inclusion criteria consisted in presents at least one root caries lesion (active or inactive) and another root surface without caries lesion (sound). As the prevalence of root caries was 37%, the final sample size was 37 patients, who ranged in age 21 to 86 years old (average 54 years; Figure 1). The average age was slightly higher than for the patients who did not fit the inclusion criteria (presence of root caries lesion), who ranged in age from 24 to 75 years old (average 50 years). These patients were not a part of any caries control program and did not control their dietary intake. The patients do not receive any instruction of oral hygiene.

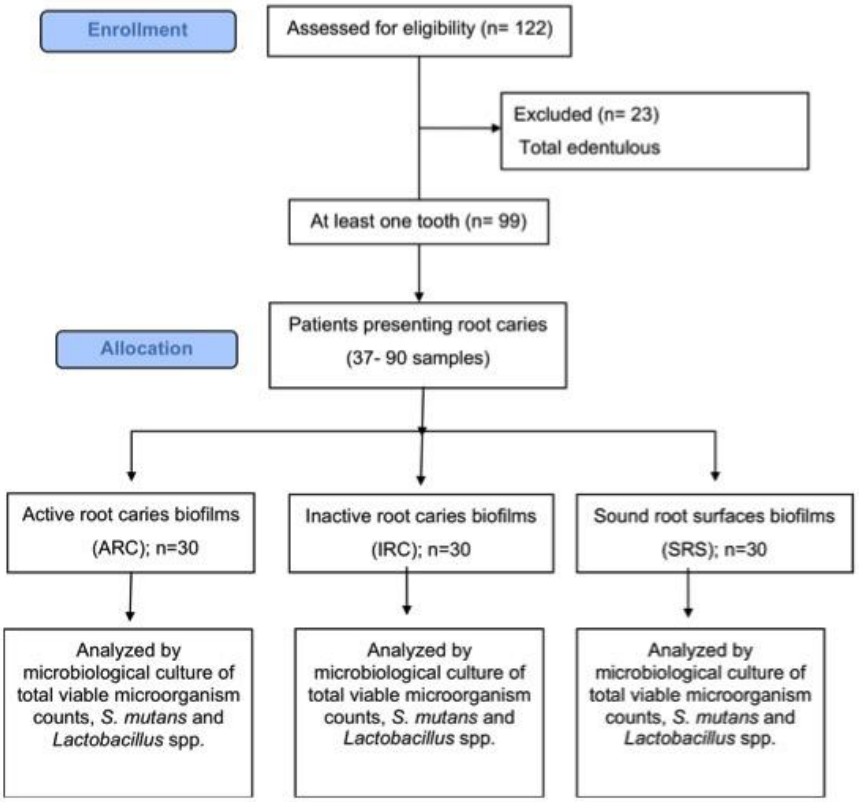

Figure 1: Flow diagram of sampling.

Dental plaque biofilms were collected from different sites from the same patient. The first was root surface with an active caries lesion (ARC; lesions not resistant for probing, light-brown to light-yellow color, cavitated or not cavitated, and opaque). The second was root surface with an inactive caries lesion (IRC; resistant for probing, brown to black in color, and shiny). The third was SRS as a control in the same ARC or IRC patients.

Biofilm samples were collected in the morning, after drying and isolation, with cotton rolls using a no. 17 sterile dentin excavator (Neumar, São Paulo, Brazil) and immediately transferred to a sterile container with 1 mL of reduced transport fluid medium. The amount of biofilm collected corresponded to one full dentin excavator. Samples were maintained on ice and processed within 3 hours. In order to minimize variation in the sampling, the same examiner collected all samples.

Microbiological procedures

Samples were vortexed with glass beads for 60 seconds (PHOENIX AT 56, Piracicaba, Brazil) and 5-fold serially diluted in 0.005 M potassium phosphate buffer (pH 7.3). Subsequently, 25 µL aliquots of 0 to 10^{-3} were cultured in duplicate on the following solid media: MSB agar (Difco; BD, Sparks, USA) supplemented with 20% sucrose, 0.2 units/mL bacitracin, and 1% potassium telurite for mutans streptococci counts; Rogosa selective *Lactobacillus* agar (Difco; BD) for *Lactobacillus* spp. counts; and brain-heart infusion agar (Difco; BD) supplemented with 4% sheep blood and enriched with k-hemin vitamin (BHI) for total viable anaerobic microorganisms counts. BHI agar plates were cultured anaerobically (CO2 95% and

N2 5%), at 37 °C for 120 hours. MSB plates were incubated under microaerophilic conditions at 37 °C for 48 hours and Rogosa plates were incubated under microaerophilic conditions at 37 °C for 72 hours. After incubation, the number of colony-forming units (CFUs) was determined. The counts derived from the selective media included only colonies with the relevant characteristic morphology. In case of doubt, two or three representative colonies from each culture medium were selected for Gram staining.

Statistical analysis

The proportion of count of mutans streptococci and *Lactobacillus* spp. in the total viable microorganisms was analyzed by Chi-square test. The bacterial counts are expressed at log10 and the constant 1 was added to the CFUs. To compare the counts of mutans streptococci and *Lactobacillus* spp. in ARC, inactive root caries, and SRS biofilms, the Mann-Whitney U test was used. The significance level was set at 5% for both tests.

Results

A total of 90 samples from 37 patients were cultured and analyzed; the samples included 30 each of ARC, IRC, and SRS. Counts of mutans streptococci and *Lactobacillus* spp. are shown in Table 1. The CFU log10 was similar between groups, although a tendency towards higher counts was evident for ARC.

Table 1: Counts of mutans streptococci (CFU log10) and ***Lactobacillus*** spp. cultivated in selective media from biofilms from active (ARC) root caries lesions, inactive (IRC) root caries lesions, and sound root surfaces (SRS).

	mutans streptococci			***Lactobacillus* spp.**		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**
ARC, n=30	4.23	10.89	0.00-52.00	1.29	4.92	0.00-1.76
IRC, n=30	1.49	4.20	0.00-21.54	0.24	0.55	0.00-2.33
SRS, n=30	2.14	5.93	0.00-30.83	0.04	0.12	0.00-0.60

Mann-Whitney U Test mutans streptococci: ARC vs IRC - p = 0.42; ARC vs SRS - p = 0.44; IRC vs SRS - p = 0.29; ***Lactobacillus spp.***: ARC vs IRC - p = 0.49; ARC vs SRS - p = 0.23; IRC vs SRS - p = 0.16. SD, standard deviation

These species were present in at least half of all samples and no difference was found in the frequency of isolation of these species within groups. Table 2 summarizes the distribution of the samples in relation to the proportion of studied species in the total viable anaerobic microorganism counts (CFU log10). For most of the samples the proportion of mutans streptococci and *Lactobacillus* spp. were low relative to the viable count of total viable anaerobic microorganisms. Only 6 samples showed more than 10% of mutans streptococci and 4 were from ARC lesions. Most samples (84%) had <0.1% of mutans streptococci. Most (93%) of the SRS samples did not display *Lactobacillus* spp., and only 5 samples showed 1.0% to 10% of the bacteria (3 from active lesions and 2 from inactive lesions).

Table 2: Proportion of mutans streptococci and ***Lactobacillus*** spp. of the total viable anaerobic microorganisms counts (% of total CFU) cultured from biofilms from active (ARC) root caries lesions, inactive (IRC) root caries lesions and sound root surfaces (SRS).

% of total CFU	mutans streptococci			*Lactobacillus* spp.		
	ARC	IRC	SRS	ARC	IRC	SRS
ND	11 (37%)	14 (47%)	12 (40%)	10 (33%)	9 (30%)	14 (47%)
<0,1%	7 (23%)	4 (13%)	10 (33%)	10 (33%)	11 (37%)	13 (43%)
≥0,1%≥10%	12 (40%)	12 (40%)	8 (27%)	10 (33%)	10 (33%)	3 (3%)
TOTAL	**30 (100%)**	**30 (100%)**	**30 (100%)**	**30 (100%)**	**30 (100%)**	**30 (100%)**

* No statistical difference using Chi-square; ND, not determined

Discussion

Dental plaque was collected from root surfaces of three different groups in the same patient (ARC, IRC, SRS) and cultured in media supporting the growth of mutans streptococci and *Lactobacillus sp.*, which are relevant organisms related to root caries. These bacteria, which are the most studied in dental caries, were not highly prevalent in root surfaces with active or inactive caries, as well as sound surfaces. Surprisingly, no differences were found in the microbiota cultivated from active or inactive lesions. This may reflect the collection of samples from ARC, IRC, and SRS sites from the same patient. The findings suggest that the microenvironment could be in dysbiosis in the patient and not only in the lesion.

We expected a higher proportion of mutans streptococci and *Lactobacillus* spp., especially in the active root surfaces. Despite evidence of the relationship of predicted cariogenic species with root caries, the literature shows a very low or very variable percentage of these species in other culture-based studies, with the collective proportion of *S. mutans + S. oralis + A. naeslundii* comprising only 10% of the total cultivable microbiota[9]. Inclusion of *Lactobacillus* and *Veilonella* raises the value only to 20% of the total cultivable microbiota[16]. Total streptococci + enterococci + actinomyces + lactobacilli represented 47% of the total cultivable microbiota in SRS biofilms, 60.1% in non-cavitated root caries lesions, and 63% in cavitated lesions[17]. In the present study, 73% of the samples presented <10% of the total microbiota comprising mutans streptococci and lactobacilli, suggesting a more complex composition of dental plaque in active or inactive root caries lesions. Thus, we can speculate that these organisms are relevant pathogens whose activity is important for the development of the disease, even if they are present as a low proportion of the total population.

The correlation between mutans streptococci and *Lactobacillus* spp. has confirmed the increased risk of root surface caries when they are present together[9]. Emilson et al.[9] showed that subjects with $>5 \times 10^5$ mutans streptococci and $>10^4$ lactobacilli per mL of saliva had approximately five times more root surface lesions than subjects free of these bacteria and subjects who showed either the absence or rare observation of *Lactobacillus* sp. in SRS[9]. The present observation of the low prevalence of *Lactobacillus* sp. in SRS are consistent with

the results reported by Beighton et al.[11], which showed that the higher activity of lesions increased the counts of total anaerobes, gram-positive pleomorphic rods (*Bifidobacteria*), mutans streptococci and lactobacilli[11]. Lactobacilli and *Bifidobacteria* were more abundant in soft dentin than in leathery (remineralizing) root caries lesions[14]. In this study, the counts of mutans streptococci and *Lactobacillus* spp. were higher in ARC than in SRS or IRC, although there was no statistically significant difference in active and inactive root caries lesions.

Many advances in the understanding of dental caries are currently related to the culture-independent methods. These molecular biology tools have contributed significantly to the identification of the microbiota composition and diversity. Among the advantages of molecular techniques to describe oral biofilms, the most important is the assessment of the underestimated non-cultivable microorganisms in oral biofilms. Despite the strong relationship of *S. mutans* and root caries microbiota shown in culture-based studies, some studies that used the culture-independent approach did not show any significant difference in the prevalence of *S. mutans* between healthy and different stages of coronal caries[18,19], suggesting that the virulence of *S. mutans* is tightly controlled by the presence of health-associated competitors[20]. Nevertheless, Preza et al.[3] described a root caries microbiota dominated by *S. mutans*, *Actinomyces* spp., and others. Lactobacilli were absent and mutans streptococci were rarely observed, while *Actinomyces* sp. were present in 50% of the healthy root surfaces samples[3]. A study using 454-pyrosequencing confirmed that *S. mutans* and *Lactobacillus spp.* are more likely to be root caries pathogens than are other species[4].

It is important to acknowledge the limitations of culture-based studies, although we believe that these studies are still reliable to identify viable organisms and to screen species that are already related to diseases. DNA-based studies have explored the complete microbial taxonomy in caries lesion, but they can overestimate some species since DNA from dead or transient cells can be present, and that could be the reason for the disagreement between the results of these studies.

Conclusion

This culture-based analysis showed that mutans streptococci and *Lactobacillus* spp. make up a low proportion of the total populations on root surfaces, despite the activity of caries lesions. Similar counts of these organisms in lesions with different activity from the same patient suggest that dysbiosis occurs in the patient and not only in the lesion.

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