Selective stepwise caries removal in primary teeth: a microbiological assessment on surviving microbiota

Remoção seletiva e gradual do tecido cariado em dentição decídua: uma avaliação microbiológica em bactérias sobreviventes

Remoción selectiva de caries por etapas en dientes primarios: una evaluación microbiológica del microbioma sobreviviente

Received: 02/27/2022 | Reviewed: 03/08/2022 | Accept: 03/10/2022 | Published: 03/18/2022

Marília Ferreira Correia
ORCID: https://orcid.org/0000-0003-3748-1621
São Paulo State University, Brazil
E-mail: mariliafcorreia@gmail.com
Gabriel Garcia de Carvalho
ORCID: https://orcid.org/0000-0003-1202-9743
São Paulo State University, Brazil
E-mail: garcia.carvalho@unesp.br
Luis Carlos Spolidorio
ORCID: https://orcid.org/0000-0002-0592-542X
São Paulo State University, Brazil
E-mail: luis-carlos.spolidorio@unesp.br
Denise Madalena Palomari Spolidorio
ORCID: https://orcid.org/0000-0003-2376-1024
São Paulo State University, Brazil
E-mail: denise.mp.spolidorio@unesp.br

Abstract
Partial caries removal performed by the stepwise treatment has a high rate of clinical success and promotes a reduction of microorganisms in carious dentin. However, the adaptive behavior of these cloistered bacteria is not entirely clear. Aim: This study aimed to evaluate the carious dentin and quantify the microorganisms *Streptococcus mutans* and *Lactobacillus acidophilus* at the first intervention and after 90 days, assessing the acidogenicity and aciduricity of these bacteria isolated from the lesions. Methods: Twenty patients presenting deep caries lesion in primary molars eligible to receive the stepwise treatment were selected, dentin samples were collected in two different moments: in the first intervention, just after partial caries removal; and in the second intervention, during the reopening of the cavity (90 days after the temporary sealing of the lesion). The samples were processed for microbiological analyses via culture, identification and quantification. The bacteria isolates were subjected to phenotypic tests of acidogenicity and aciduricity. Dentin consistency and color was also recorded by a calibrated examiner. Data were statistically analyzed. Results: There was a reduction in the number of viable microorganisms while dentin rehardening and browning was noted ($p<.05$), but no change occurred in the acidogenicity and aciduricity properties of *Streptococcus mutans* and *Lactobacillus acidophilus* over time. Conclusion: Thus, the stepwise treatment promoted clinical changes as darkening and hardening of carious dentine and promoted a reduction in the number of viable microorganisms, but no influence was found on the phenotypic characteristics of acidogenicity and aciduricity of the species analyzed after 90 days.
Keywords: Dental caries activity tests; Dental Caries; Microbiota; Microbiology.

Resumo
A remoção parcial do tecido cariado realizada pelo tratamento em duas sessões (stepwise) tem alto índice de sucesso clínico e promove a redução de microrganismos na dentina cariada. No entanto, o comportamento adaptativo dessas bactérias remanescentes enclausuradas não é totalmente claro. Este estudo teve como objetivo avaliar a dentina cariada e quantificar os microrganismos *Streptococcus mutans* e *Lactobacillus acidophilus* na primeira intervenção e após 90 dias, avaliando a acidogenicidade e aciduricidade dessas bactérias isoladas das lesões. Foram selecionados 20 pacientes com lesão de cárie profunda em molares decíduos elegíveis para receber o tratamento "stepwise", amostras de dentina foram coletadas em dois momentos distintos: na primeira intervenção, logo após a remoção parcial da cárie; e na segunda intervenção, durante a reabertura da cavidade (90 dias após o selamento temporário da lesão). As amostras foram processadas para análises microbiológicas via cultura, identificação e quantificação. Os isolados bacterianos foram submetidos a testes fenotípicos de acidogenicidade e aciduricidade. A consistência e a cor da

dentina também foram registradas por um examinador calibrado. Os dados foram analisados estatisticamente. Houve redução no número de microrganismos viáveis enquanto ocorreu o endurecimento e escurecimento da dentina (p<0,05), mas não houve alteração nas propriedades de acidogenicidade e aciduricidade de *S. mutans* e *L. acidophilus* ao longo do tempo. Assim, o tratamento "stepwise" promoveu alterações clínicas como escurecimento e endurecimento da dentina cariada e promoveu redução no número de microrganismos viáveis, mas não foi encontrada influência nas características fenotípicas de acidogenicidade e aciduricidade das espécies analisadas após 90 dias.
Palavras-chave: Testes de Atividade de Cárie Dentária; Cárie Dentária; Microbiota; Microbiologia.

Resumen

La remoción parcial de caries realizada por el tratamiento escalonado tiene una alta tasa de éxito clínico y promueve una reducción de microorganismos en la dentina cariada. Sin embargo, el comportamiento adaptativo de estas bacterias enclaustradas no está del todo claro. Objetivo: Este estudio tuvo como objetivo evaluar la dentina cariada y cuantificar los microorganismos Streptococcus mutans y Lactobacillus acidophilus en la primera intervención y después de 90 días, evaluando la acidogenicidad y aciduricidad de estas bacterias aisladas de las lesiones. Métodos: Se seleccionaron 20 pacientes que presentaban lesión de caries profunda en molares deciduos elegibles para recibir el tratamiento escalonado, se recolectaron muestras de dentina en dos momentos diferentes: en la primera intervención, justo después de la remoción parcial de la caries; y en la segunda intervención, durante la reapertura de la cavidad (90 días después del sellado temporal de la lesión). Las muestras fueron procesadas para análisis microbiológicos vía cultivo, identificación y cuantificación. Los aislados bacterianos fueron sometidos a pruebas fenotípicas de acidogenicidad y aciduricidad. Un examinador calibrado también registró la consistencia y el color de la dentina. Los datos fueron analizados estadísticamente. Resultados: Hubo una reducción en el número de microorganismos viables mientras que se observó endurecimiento y oscurecimiento de la dentina (p<.05), pero no hubo cambios en las propiedades de acidogenicidad y aciduricidad de Streptococcus mutans y Lactobacillus acidophilus con el tiempo. Conclusión: Así, el tratamiento escalonado promovió cambios clínicos como oscurecimiento y endurecimiento de la dentina cariada y promovió una reducción en el número de microorganismos viables, pero no se encontró influencia sobre las características fenotípicas de acidogenicidad y aciduricidad de las especies analizadas después de 90 días.
Palabras clave: Pruebas de Actividad de Caries Dental; Caries Dental; Microbiota; Microbiología.

1. Introduction

It is no longer a novelty that the term "minimally invasive" is almost a synonym of contemporary dentistry. That is why the mechanical removal of carious tissue becomes the "least as possible". The complete removal of the demineralized dentin to reach sound dentine is considered an unwise practice (Schwendicke et al., 2013). Hence, partial removal of decayed tissue through selective excavation might minimize pulp damage, giving it the chance of recovery. Nonselective removal of carious tissue, which consists of removing all softened carious dentin, presents a greater risk of pulp exposure, and it might be considered overtreatment (Casagrande et al., 2017). Pulp exposure may initiate a cascade of re-interventions and numerous complications, resulting in the caries process resolution delay and frequently demanding supplementary longstanding endodontic intervention (Pratiwi et al., 2017).

The principle of the selective stepwise treatment is the certification of the cleanliness of the surrounding walls of the cavity and removal of the real softened dentin from the pulp wall, reducing the risk of pulp exposure and stimulating tissue rehardening (Bjørndal et al., 1997). The later reopening will confirm the dentin mineralization and hardness increase at the bottom of the cavity, allowing treatment conclusion by definitive restoration. Selective stepwise treatment is a widely cited procedure with extensive supporting scientific evidence (Schwendicke et al.,2018; Bitello-Firmino et al., 2018; Maltz et al., 2011). Stepwise excavation is also considered a suitable model to determine the persistent bacteria and their metabolism in clinically excavated lesions (Bjørndal et al., 1997), even though it is considered unnecessary, in some cases, and outdated in view of the selective technique (Elhennawy et al., 2018)

The sealing of carious dentine resulted in the same bacteria count after 3 months compared to the complete removal of the affected dentin (Bitello-Firmino et al. 2018). A decrease in viable anaerobic and aerobic bacteria was observed when this tissue was reassessed after 6 months (Maltz et al., 2012). However, 25.6% of teeth reveal bacterial growth even after complete direct excavation; thus, assumptions that this technique eliminates all bacteria present might not support it (Bitello-Firmino et al. 2018). So, the concept of infected or affected dentin appears to be old-fashioned and restricted for a histopathologic approach; therefore, the visual description based on hardness and color is a lot more tangible, clinically (Barros et al., 2020). Defining the dentin condition, which could differ depending on distinct areas of the cavity, is a difficult task. For practical reasons, checking if the tissue is soft at any spot is of great guidance in the decision-making process "need to be removed or not" (Innes et al., 2016).

The clinical change of carious dentin appearance after the period of cavity sealing is widely noted (Lula et al. 2011; Duque et al., 2009). Although the persistent presence of microorganisms after carious dentin closure has been reported (Maltz et al., 2012; Duque et al., 2009; Lula et al., 2009), the adaptive behavior of these cloistered bacteria is not entirely clear. The sealed cariogenic bacteria, deprived of dietary carbohydrate, are provided with serum proteins from pulp fluids (Ganas et al., 2019; Knutsson et al., 1994). This nutrition seems to be enough to keep producing metabolites and organic acid somehow (Ganas et al., 2019), and that is one of the supporting evidences on the recent bioactive restorative material development, mainly those with antimicrobial additives (Tüzüner et al., 2019). However, the relative simplicity and homogeneity of the nutrient supply significantly affected the surviving microbiota, and the microbiome is less complex regarding phenotypic and genotypic characteristics (Paddick et al., 2005). Just one study has observed the phenotypic characteristics of these bacteria after this fasting period (Paddick et al., 2005). It is intriguing how fasting can affect microbiome, some influence on gut microbiome has already be investigated (He et al., 2019). Therefore, the present study investigated dentin color and hardness alteration, microorganisms' survival, and acidogenicity (acid production) and aciduricity (acid tolerance) properties of mutans streptococci and *Lactobacillus spp.* after selective stepwise removal of carious dentin. The null hypothesis is there is no alternation in microorganisms aciduric and acidogenic properties after 90 days of the temporary sealing of the caries lesion.

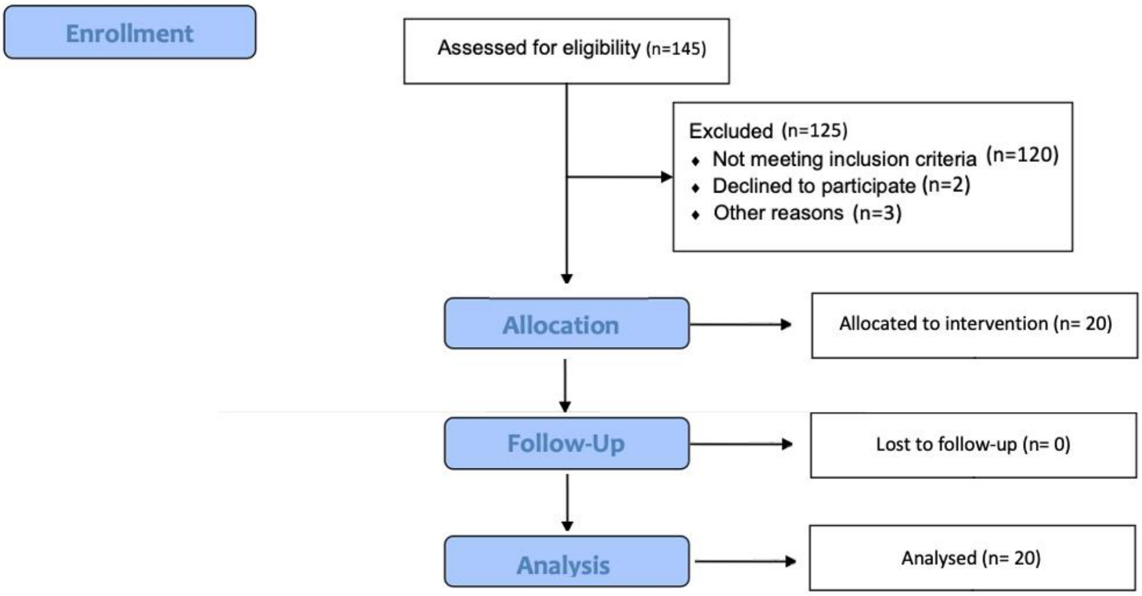
2. Materials and Methods

Sample Selection

After screening 145 patients referred to the Child Dental Care Service at Araraquara School of Dentistry (São Paulo State University, Brazil), twenty patients requiring indirect pulp treatment for primary posterior teeth, diagnosed after clinical and radiographic examination, were included in the present study. The Institutional Ethical Committee approved the study protocol (CAEE: 13823613.5.0000.5416) and informed consent was obtained from all volunteer legal guardianship. All patients (selected and non-selected) were treated at Araraquara School of Dentistry following the standard treatment adopted by the institution, based on the scientific evidence available at the time.

Participants were selected considering the following inclusion and exclusion criteria. The sample calculation was based on the study by Lula et al.; 2011, considering the power of 80%, significance level of 5%, effect size 0.8, with a maximum error of the estimate of 0.6 colony-forming units (CFU, logarithmically transformed) for aciduricity analysis (described later), 16 patients were predicted. Thus, after adjustment for loss to follow-up (20%), 20 patients were included. The conduct of the study is described in the flowchart **(**Figure 1)

Figure 1. Clinical trial flowchart.



Source: Authors.

Inclusion Criteria: Patients with at least one deep caries lesion (active) limited to the occlusal surface and with a risk of pulp exposure during the excavation in the first appointment. Patients aged between 4 to 9 years, without systemic impairment, without report of antibiotic use on the last 3 months previous to screening, without tooth spontaneous pain, tenderness to pressure, mobility, pus formation, and swelling. Radiographically, teeth should have shown radiolucency in the internal half of dentin and absence of internal and/or external dentin resorption and radiolucent area in bi/trifurcation.

Volunteers who showed dental pulp exposure at any step of the study were excluded and those with suspected non-compliance with treatment or where it was not possible to recover the analyzed microorganisms.

Clinical Procedures

Patients who matched inclusion and exclusion criteria underwent indirect pulp treatment with stepwise excavation (not only for the purpose of research). The clinical procedures included local anesthesia (Mepiadre 2% 1:100.000 - Nova DFL, Rio de Janeiro, Brazil), operative isolation with a rubber dam (Sanctuary – Kdent/Quimidrol, Joinville, Brazil), removal of affected enamel (if necessary) to allow access to the dentinal lesion, complete removal of carious dentin on lateral surfaces, and partial removal (only softened tissue) on pulp surface. Curettage was interrupted when tissue was slightly resistant to instrument (Massara et al., 2002). The cavity was washed (0.9% NaCl) and dry (absorbent wipe). Dentin chips were collected from the pulp surface and immediately stored in a microtube filled with phosphate buffer solution (PBS- 1 mL, pH 6.8-7.2), previously weighted. The cavity was filled with restorative glass ionomer (Ketac Molar Easymix – 3M ESPE, Nova Veneza, Sumaré, SP, Brazil) and protected with varnish.

After 3 months, restorative material was removed. The pulp surface was assessed, and another dentin fragment was removed and stored as described previously. Caries lesion was finally sealed with glass ionomer (Vitrebond - 3M ESPE, Nova Veneza, Sumaré, SP, Brazil) followed by resin composite (Z350 - 3M ESPE, Nova Veneza, Sumaré, SP, Brazil). All interventions were performed at Araraquara School of Dentistry (São Paulo State University, Brazil).

Dentin Consistency and Color

All procedures described were carried out by a single investigator who was the same calibrated examiner for dentin texture/hardness and coloration assessment (Kappa - 0.84 and 0.80, respectively). Dentin appearance was evaluated twice, at baseline and 3 months after the baseline. The aspects analyzed were based on Maltz et al., 2002. The dentin color was classified as yellow and light brown. The dentin consistency was classified as soft (little resistance to removal), leathery (greater resistance to removal, being removed with some firmness), and hard (a consistency similar to healthy dentin).

Microbiological analysis

The microtubes containing 1 ml of PBS were weighed before and after placing the dentin chips inside to obtain the weight (Scale AY220, Shimadzu, Barueri, Brazil) of moist dentin in mg. Samples were sonicated for 5 seconds under 20W (UltraSonic Mixing, Unique, Indaiatuba, Brazil). Then, serial dilution was carried out in microtubes with PBS, and 20 µl aliquots were plated on Petri dishes with the following growth medium: Blood agar (BA) (Neogen, Indaiatuba, Brazil) for total microorganism growth; Mitis Salivarius Agar (MSA) (Neogen, Indaiatuba, Brazil) for total streptococci; Mitis Salivarius Bacitracin (MSB) (Neogen, Indaiatuba, Brazil) for mutans streptococci group; and MRS Agar for *Lactobacillus* spp. (Neogen, Indaiatuba, Brazil). Plates were incubated (Kasvi, São José do Pinhais, Brazil) under 5% CO_2 at 37ºC for 24h (BA) and 48h (MSA, MSB, and MRS). Finally, colony-forming units (CFU) were identified and counted by a calibrated investigator (intraclass correlation confidence of 0.99) and express in CFU/mg of moist dentin.

Phenotypic analysis

Two bacteria strains from the American Type Culture Collection (*Streptococcus mutans* ATCC 25175 e *Lactobacillus acidophilus* ATCC 4356) were used as positive controls. Thus, both positive controls and microorganisms isolated after clinical collection were submitted to acidogenicity and aciduricity evaluation, according to Lembo et al., 2007 and Arthur et al., 2011. All strains were incubated in Brain Heart Infusion (BHI) (Neogen, Indaiatuba, Brazil)broth under 5% CO_2 at 37ºC for 18h.

For aciduricity analysis, all strains were adjusted to contain approximately 10^7 CFU/mL through optical density (OD - *Streptococcus mutans* = 0.15 to 0.20 and *Lactobacillus acidophilus* = 0.50 to 0.60) (Biophotometer, Eppendorf, Hamburg, Germany). All microbial suspensions were centrifuged and washed with 0.1 M glycine buffer (pH ~7.0). Next, the pellets were resuspended in 0.1 M glycine buffer at pH 3.0 and 5.0. Serial dilution was carried out immediately after resuspension (T0), and 30 (T1) and 60 (T2) min after incubation at 37ºC. Samples were plated on BHI Agar (Neogen, Indaiatuba, Brazil) Petri dishes and incubated at 37ºC for 48h. Bacterial viability was counted and expressed in CFU/mL. The samples that grew at pH 7.0 were considered as controls.

For acidogenicity evaluation, microorganisms were adjusted as described previously for aciduricity analysis, the samples were resuspended in PBS (pH 7.0), and then glucose at 55.6 mM was added. The pH of the solution was measured four times (baseline, and after 60, 120, 180 min) using a previously calibrated electrode (pH meter 3510 – Jenway, Staffordshire, UK).

Statistical analysis

All data were analyzed regarding assumptions of normality (Shapiro-Wilks test) and homogeneity of variables (Levene test). The CFU/mg values were later transformed in $Log_{10.}$ Wilcoxon nonparametric test was used to identify significant differences in dentin consistency, color, and CFU/mg of moist dentin. For acidogenicity and aciduricity statistical analyses, the two-way ANOVA with repeated measures was applied. Statistical analysis was performed using the SPSS software (SPSS Inc, Chicago, IL), considering a significance level of 5%.

3. Results

Among the 20 patients included in the study, 9 were males and 11 were females. The age ranged from 4 to 9 years old with the mean age of 5.7 ± 1.4. The teeth distribution consisted of 13 primary second molars (6 upper and 7 lower) and 7 primary first molars (3 upper and 4 lower). The average time between the first and the second intervention was 93 ± 7.6 days. No pulp alteration was identified clinically or radiographically, nor pulp exposure or restoration loss.

Alteration regarding color and consistency of the remaining dentin in both moments are shown in Table 1. The selective stepwise removal of carious dentin promoted significant changes in dentin consistency and color after 3 months, reaching a darker and harder aspect.

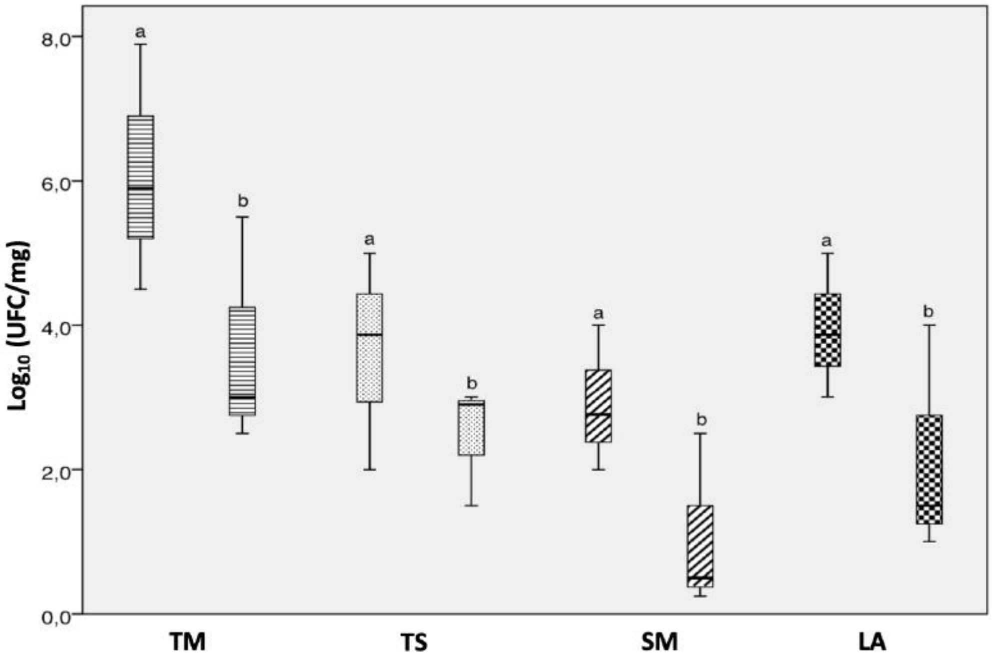
Table 1. Analysis of Dentin color and consistency immediately after selective stepwise removal of carious dentin and three months later.

Time	Consistency	Color		Total
		Yellow	Light Brown	
Immediately after selective stepwise removal of carious dentin	Soft	17	0	17^{C}
	Leathery	3	0	3^{D}
	Hard	0	0	0^{E}
	Total	20^{A}	0^{B}	
Three months after selective stepwise removal of carious dentin	Soft	0	0	0^{C}
	Leathery	13	4	17^{d}
	Hard	0	3	3^{e}
	Total	13^{a}	7^{b}	

* Differences between the period of analysis are noted by different lowercase and uppercase in the total. Wilcoxon test (p <.05). Source: Authors.

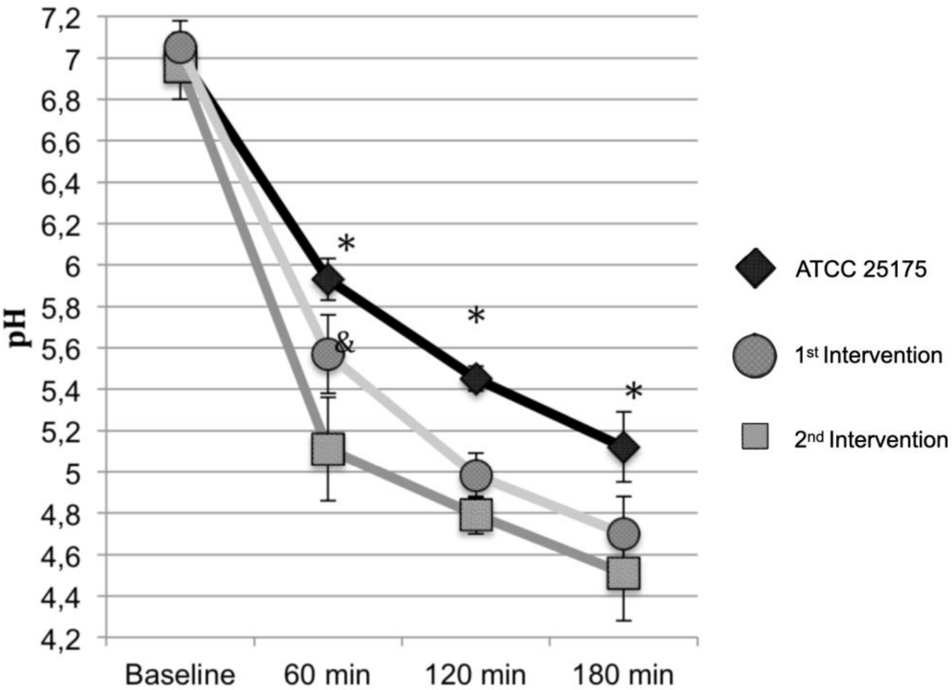
A significant decrease in all microorganisms cultivated between the first intervention and 3 months later was observed (Graph 1). Regarding the phenotypic tests of Streptococcus mutans and Lactobacillus acidophilus, the results of acidogenicity are found in Graph 2 and 3. A similar neutral pH was obtained for all species in the baseline and a significant and consistent reduction was observed later on. Both species showed a significant drop after 60 minutes of measurement. The samples collected from patients presented a lower pH in comparison with ATCC strain in all times set. For Lactobacillus acidophilus, no difference was observed between the samples collected from patients in all moments measured. The patients' samples collected in the first intervention showed a statistical lower pH compared to the sample collected 3-months later, only in the measurement at 60 min, for Streptococcus mutans. This found was not observed afterwards.

Graph 1. Comparison of Total Microorganism (TM), Total Streptococci (TS), *Streptococcus mutans* (SM) and *Lactobacillus acidophilus* (LA) before (a) and 3-months after (b) intervention.



Wilcoxon test (p<.05). Source: Authors.

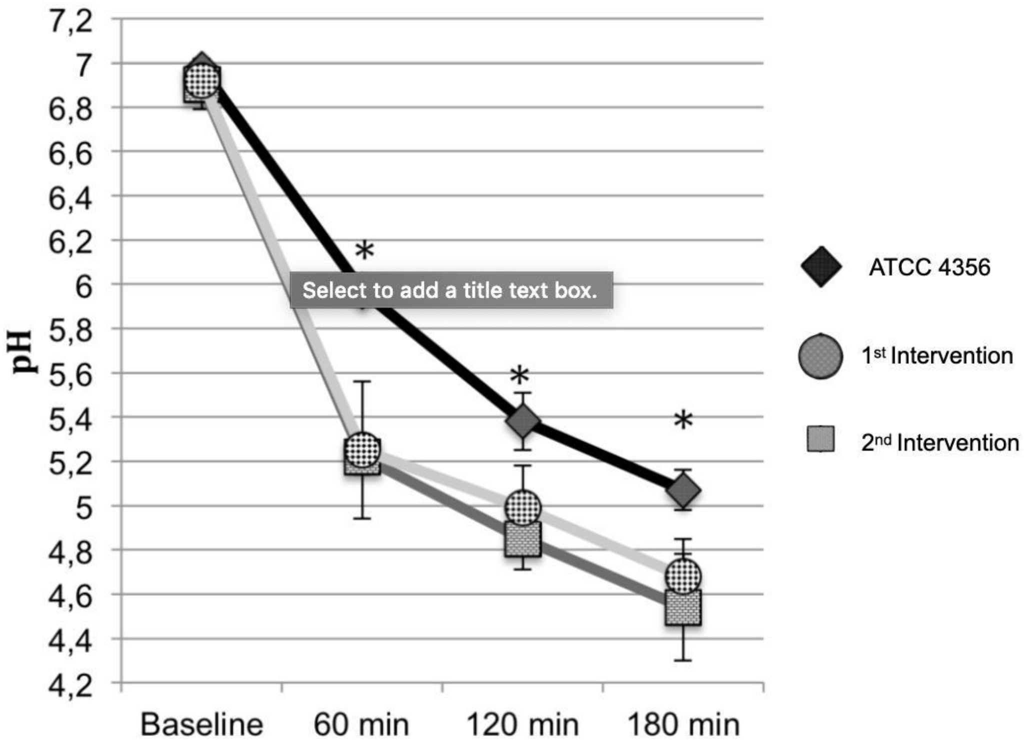
Graph 2. The relationship between the pH measured for *Streptococcus mutans* (mean and standard deviation) and time.



*/& Indicates difference between the period of analysis. One-way repeated measures - ANOVA test (p<.05). Source: Authors.

(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i4.27478

Graph 3. The relationship between the pH measured for *Lactobacillus acidophilus* (mean and standard deviation) and time.



* Indicates difference between the period of analysis. One-way repeated measures - ANOVA test (p<.05). Source: Authors.

The results obtained on aciduricity for Streptococcus mutans and Lactobacillus acidophilus can be seen in Table 2 and 3, respectively. A greater reduction in microbial viability was observed when ATCC strain was tested in both pH (5 and 3), at 30 and 60 minutes in comparison with the samples collected from patients. There was no statistical difference between the viability of the species collected from patients in the first intervention with those collected 3-months later. Streptococcus mutans and Lactobacillus acidophilus viability decreased proportionally as a function of the incubation period. However, for Lactobacillus acidophilus collected from patients, this significant decrease can be observed only after the 60-minute incubation period. For Streptococcus mutans, a significant reduction can be observed even after 30 minutes, but only at pH 3.

Table 2. *Streptococcus mutans* viability according to pH and incubation time

Microorganism	pH	Incubation time (min)		
		0	30	60
ATCC 25175	5	0.94 (0.1) ^{Aa}	0.80 (0.1) ^{Ab}	0.73 (0.2) ^{Ac}
1st intervention		0.97 (0.4) ^{Aa}	0.91 (0.4) ^{Bab}	0.86 (0.3) ^{Bb}
2nd intervention		0.95 (0.2) ^{Aa}	0.89 (0.4) ^{Bab}	0.84 (0.6) ^{Bb}
ATCC 25175	3	0.92 (0.2) ^{Aa}	0.34 (0.1) ^{Cb}	0.13 (0.1) ^{Cc}
1st intervention		0.93 (0.5) ^{Aa}	0.74 (0.8) ^{ABb}	0.58 (0.5) ^{Dc}
2nd intervention		0.95 (0.2) ^{Aa}	0.78 (0.9) ^{ABb}	0.84 (0.7) ^{Dc}

Uppercase letters indicate a statistically significant difference between columns and lowercase letters between rows. Two-way repeated measures - ANOVA test (p<.05). Source: Authors.

Table 3. *Lactobacillus acidophilus* viability according to pH and incubation time.

Microorganism	pH	Incubation time (min)		
		0	30	60
ATCC 4356	5	0.94 (0.1) ^{Aa}	0.80 (0.1) ^{Ab}	0.73 (0.2) ^{Ac}
1st intervention		0.97 (0.4) ^{Aa}	0.91 (0.4) ^{Bab}	0.86 (0.3) ^{Bb}
2nd intervention		0.95 (0.2) ^{Aa}	0.89 (0.4) ^{Bab}	0.84 (0.6) ^{Bb}
ATCC 4356	3	0.92 (0.2) ^{Ba}	0.34 (0.1) ^{Cb}	0.13 (0.1) ^{Cc}
1st intervention		0.93 (0.5) ^{Aa}	0.74 (0.8) ^{Ba}	0.58 (0.5) ^{Bb}
2nd intervention		0.95 (0.2) ^{Aa}	0.78 (0.9) ^{Ba}	0.84 (0.7) ^{Bb}

Uppercase letters indicate a statistically significant difference between columns and lowercase letters between rows. Two-way repeated measures - ANOVA test (p<.05). Source: Authors.

4. Discussion

This study investigated the possible basis for observations that a cloistering and fasting period is able to change phenotypic characteristics of a cariogenic biofilm. The number of viable bacteria was also counted before and after selective stepwise treatment. Dentin consistency and color was assessed as a method of confirming the effectiveness of the treatment protocol, even in the face of the consistent evidence available to support it (Barros et al. 2020). *Streptococcus mutans* and *Lactobacillus acidophilus* showed lower, but not significant, capacity of acid production after 3 months of stepwise treatment. Whereas aciduricity changes was found to be not significant neither, then the hypothesis that selective stepwise removal of carious dentin could impact phenotypic aspects of microorganisms is not accepted.

It is important to note that selective removal of carious dentin may be accomplished in one or two-step protocol, the reason for taking this second (also known as stepwise excavation) in the present study was to allow the phenotypic investigation proposed. The stepwise technique removes caries in stages over two visits some months apart (3 months in the case), permitting the dental pulp time to lay down reparative dentine. There is no sufficient evidence to support one protocol instead of the other (Ricketts et al., 2013), but the cost-effectiveness favors the one-visit treatment (Schwendicke , 2013). It is also important to highlight that the study was carried out in deciduous teeth which have a higher capacity for cell proliferation, response, and regeneration (Kaukua et al, 2015), extrapolation to permanent dentition should be carefully investigated beforehand.

The change in dentin colours and consistency observed in this study is attributed to biochemical reactions, like the Maillard reaction, and the reaction of small aldehydes derived from bacteria with proteins forming polymers with a more brownish color (Kleter et al., 1998). A negative correlation between carious dentin consistency and *S. mutans* has already been suggested (Lula et al., 2011). Once the reduction of viable total bacteria was found in the present study after 3 months, the process of hardening and browning of dentin is expected to be reduced later on.

Several studies have also demonstrated a significant reduction in the quantity and diversity of the collected dentin microbiota after minimally invasive treatment (Duque et al., 2009; Lula et al., 2009; Orhan et al., 2008; Wambier et al., 2007), corroborating the results displayed in Graph 1. Although none of the treatment methods completely eliminates the viable microorganisms, results suggest a higher reduction after reopening in the two-visit treatment (Lula et al., 2009; Ganas et al., 2019; Orhan et al., 2008; Wambier et al., 2007) and no difference between selective and complete removal of carious dentin (Bitello-Firmino, 2018) after 3-months follow-up. The bacterial reduction found is associated to nutritional restriction and antibacterial properties of lining materials, both dependent on adequate marginal sealing of the cavity.

A dysbiotic imbalance within the plaque microbiome is known as the most relevant factor in the dental caries etiology, that leads to an acidic environment as a result of the metabolism of carbohydrates. The ability to survive within this acidic environment should be directly link to the ability to produce acid (Bana et al., 2019). Therefore, a coherence between the acid tolerance and acid production was previously speculated, and later confirmed by the present study.

The permanence of viable microorganisms in the dentin after sealing the cavity, even a smaller amount, is still considered a dilemma. There was no evidence available to define the phenotypic characteristics of cariogenicity of these remaining bacteria. To the best of our knowledge, there is only one study that analyzed the capacity of the remaining microbiota for the production of glycosidic enzymes (Paddick et al., 2005). These enzymes are able to obtain sugars from glycoproteins to keep bacterial metabolism active even with nutritional restriction, conserving their aciduric and acidogenic properties due to the metabolization of sugars from other sources. Thus, the data obtained in this study provide additional evidence to Paddick and collaborators' investigation. The aciduric and acidogenic properties of *Streptococcus mutans* and *Lactobacillus acidophilus* collected before and after the stepwise excavation did not differ statistically from each other, difference was observed only when compared to their standard strains.

Considering that the analyzed bacterial species are reduced after stepwise excavation, but still present, and the phenotypic characteristics of the microorganisms collected at the beginning of the treatment are maintained, in the presence of an eventual marginal microleakage, a dentin recolonization might not be necessary for caries process turnover, only nutrients availability is needed. These results highlight the importance of an appropriate marginal sealing for the success of selective removal of caries.

5. Conclusion

Although a reduction of viable microorganisms was observed after 3-months of selective stepwise excavation, those that still remain have the ability to ferment and survive within an acid medium similar to the microorganisms collected in the initial intervention.

Acknowledgments

PhD scholarships given by Capes (Brazilian Ministry of Education) for the first and second author are greatly acknowledged. This study was supported by the Araraquara School of Dentistry, São Paulo State University – UNESP.

References

Schwendicke, F., Stolpe, M., Meyer-Lueckel, H., Paris, S., & Dorfer, C. (2013). Cost-effectiveness of one-and two-step incomplete and complete excavations. *J Dent Res*. 2013; 92:880–887. doi: 10. 1177/0022034513500792.

Casagrande, L., Seminario, A. T., Correa, M. B., Werle, S. B., Maltz, M., Demarco, F. F., et al. . (2017). Longevity and associated risk fac- tors in adhesive restorations of young permanent teeth after complete and selective caries removal: a retrospective study. *Clin Oral Investig*. 2017; 21:847–855. doi: 10.1007/s00784-016-1832-1

Pratiwi, A. R., Meidyawati, R., & Djauharie, N. . (2017). The effect of MTA application on the affected dentine remineralization after partial caries excavation (in vivo). *Journal of Physics: Conf Series*. 2017; 884012119. doi: 10.1088/1742-6596/884/1/012119

Bjørndal, L., Larsen, T., & Thylstrup, A. . (1997). A clinical and micro- biological study of deep carious lesions during stepwise excavation using long treatment intervals. *Caries Res*. 1997; 31:411–417. doi: 10.1159/000262431

Schwendicke, F., Walsh, T., Fontana, M., Bjørndal, L., Clarkson, J.E., Lamont, T., et al. . (2018). Interventions for treating cavitated or dentine carious lesions. *Cochrane Database Syst Rev*. 2018; 6: CD013039. doi: 10.1002/14651858.CD013039

Schwendicke, F., Dörfer, C. E., & Paris, S. . (2013). Incomplete caries removal: a systematic review and meta-analysis. *J Dent Res*. 2013; 92(4): 306-14. doi: 10.1177/0022034513477425

Maltz, M., Alves, L. S., Jardim, J. J., Moura, M. S., & Oliveira, E. F. . (2011). Incomplete caries removal in deep lesions: a 10-year prospective study. *Am J Dent*. 2011; 24(4): 211-4.

Elhennawy, K., Finke, C., Paris, S., Reda, S., Jost-Brinkmann, P., & Schwendicke, F. . (2018). Selective vs stepwise removal of deep carious lesions in primary molars: 12-Months results of a randomized controlled pilot trial. *J Dent*. 2018 Oct;77:72-77. doi: 10.1016/j.jdent.2018.07.011

Bitello-Firmino, L., Soares, V. K., Damé-Teixeira, N., Parolo, C. C .F., & Maltz, M. . (2018). Microbial load after selective and complete caries removal in permanent molars: a randomized clinical trial. *Braz Dent J*. 2018; 29:290–295. doi: 10.1590/0103-6440201801816

Maltz, M., Henz, S. L., Oliveira, E. F., & Jardim, J .J. . (2012). Conventional caries removal and sealed caries in permanent teeth: a microbiological evaluation. *J Dent*. 2012; 40(9):776-82. doi: 10.1016/j.jdent.2012.05.011

Barros, M. M. A .F., Rodrigues, M. I. Q., Muniz, F. W. M. G., & Rodrigues, L. K. A. . (2020). Selective, stepwise, or nonselective removal of carious tissue: which technique offers lower risk for the treatment of dental caries in permanent teeth? A systematic review and meta-analysis. *Clin Oral Investig*. 2020; 24(2):521-532. doi: 10.1007/s00784-019-03114-5

Innes, N. P., Frencken, J. E., Bjorndal, L., Maltz, M., Manton, D. J., Ricketts, D., et al. . (2016). Managing carious lesions: consensus recommendations on terminology. *Adv Dent Res*. 2016; 28:49–57. doi: 10.1177/0022034516639276

Lula, E. C. O., Almeida Jr, L .J. S., Alves, C. M. C., Monteiro-Neto, V., & Ribeiro, C. C .C. . (2011). Partial caries removal in primary teeth: association of clinical parameters with microbiological status. *Caries Res*. 2011;45(3):275-80. doi: 10.1159/000325854

Duque, C., Negrini, T. C., Sacono, N. T., Spolidorio, D. M. P., Costa, C. A. S., & Hebling, J. . (2009). Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal. *Clin Oral Investig*. 2009;13(4):465-71. doi: 10.1007/s00784-009-0304-2

Lula, E. C .O., Monteiro-Neto, V., Alves, C. M. C., & Ribeiro, C .C. C. . (2009). Microbiological analysis after complete or partial removal of carious dentin in primary teeth: a randomized clinical trial. *Caries Res*. 2009;43(5):354-8. doi: 10.1159/000231572

Ganas, P., & Schwendicke, F. . (2019). Effect of reduced nutritional supply on the metabolic activity and survival of cariogenic bacteria in vitro. *J Oral Microbiol*. 2019;11(1):1605788. doi: 10.1080/20002297.2019.1605788

Knutsson, G., Jontell, M., & Bergenholtz, G. . (1994). Determination of plasma proteins in dentinal fluid from cavities prepared in healthy young human teeth. *Arch. Oral Biol*. 1994; 39:185–190. doi: 10.1016/0003-9969(94)90043-4

Tüzüner, T., Dimkov, A., & Nicholson, J. W. . (2019). The effect of antimicrobial additives on the properties of dental glass-ionomer cements: a review. *Acta Biomater Odontol Scand*. 2019;5(1):9-21. doi: 10.1080/23337931.2018.1539623

Paddick, J. S., Brailsford, S. R., & Kidd, E. A. M., . (2005). Beighton, D. Phenotypic and genotypic selection of microbiota surviving under dental restorations. *Appl Environ Microbiol*. 2005;71(5):2467-72. doi: 10.1128/AEM.71.5.2467-2472.2005

He, Y., Yin, J., Lei, J., Liu, F., Zheng, H., et al. . (2019). Fasting challenges human gut microbiome resilience and reduces Fusobacterium. *Medicine in Microecology*. 2019; 2 (1). doi: 10.1016/j.medmic.2019.100003

Massara, M. L. A., Alves, J. B., & Brandão, P. R .G. . (2002). Atraumatic restorative treatment: clinical, ultrastructural and chemical analysis. *Caries Res*. 2002; 36(6); 430-6. doi: 10.1159/000066534.

Maltz, M., Oliveira, E. F., Fontanella, V., & Bianchi, R. . (2002). A clinical, microbiologic, and radiographic study of deep caries lesions after incomplete caries removal. *Quintessence Int*. 2002; 33(2): 151-9.

Lembo, F .L., Longo, P .L., Ota-Tsuzuki, C., Rodrigues, C. R., & Mayer, M. P. . (2007). Genotypic and phenotypic analysis of Streptococcus mutans from different oral cavity sites of caries-free and caries-active children. *Oral Microbiol Immunol*. 2007; 22(5): 313-9. doi: 10.1111/j.1399-302X.2007.00361.x.

Arthur, R. A., Cury, A. A., Graner, R. O., Rosalen, P. L., Vale, G.C., Paes Leme, A. F., et al. . (2011). Genotypic and phenotypic analysis of S. mutans isolated from dental biofilms formed in vivo under high cariogenic conditions. *Braz Dent J*. 2011; 22(4): 267-74. doi: 10.1590/s0103-64402011000400001.

Ricketts, D., Lamont, T., Innes, N.P., Kidd, E., & Clarkson, J. E. . (2013). Operative caries management in adults and children. *Cochrane Database Syst Rev* 3. 2013 doi: 10.1002/14651858. CD003808.pub3

Kaukua, N., Chen, M., Guarnieri, P., Dahl, M., Lim, M. L., Yucel-Lindberg, T.,et al. . (2015). Molecular differences between stromal cell populations from deciduous and permanent human teeth. *Stem Cell Res Ther*. 2015; 6:59. doi: 10.1186/s13287-015-0056-7

Kleter, G. A. . (1998). Discoloration of dental carious lesion – a review. *Arch Oral Biol*. 1998; 43: 629-32. doi: 10.1016/s0003-9969(98)00048-x.

Orhan, A. I., Oz, F. T., Ozcelik, B., & Orhan, K. . (2008). A clinical and mi- crobiological comparative study of deep carious lesion treatment in deciduous and young permanent molars. *Clin Oral Investig*. 2008; 12: 369–378. doi: 10.1007/s00784-008-0208-6

Wambier, D. S., dos Santos, F. A., Guedes-Pinto, A. C., Jaeger, R. G., & Simionato, M. R. . (20070). Ultrastructural and microbiological analysis of the dentin layers affected by caries lesions in primary molars treated by minimal intervention. *Pediatr Dent*. 2007; 29(3): 228-34.

Bana, J. A., Takanami, E., Hemsley, R.M., Villhauer, A., Zhu, M., Qian, F., et al. (2020). Evaluating the relationship between acidogenicity and acid tolerance for oral streptococci from children with or without a history of caries. *J Oral Microbiol*. 2020; 12(1). doi: 10.1080/20002297.2019.1688449