UNIVERSIDADE DE UBERABA
TACIANO DOS REIS CARDOSO

A ATIVIDADE METABÓLICA DOS BIOFILMES DE STREPTOCOCCUS MUTANS APÓS O TRATAMENTO COM ENXAGUATÓRIOS BUCAIS COM DIFERENTES COMPOSIÇÕES

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Universidade de Uberaba, área de concentração: Biopatologia, como requisito parcial para a obtenção do título de Mestre.

Orientador: Profº. Dr. Geraldo Thedei Jr.

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Ata da Sessão Pública de defesa de dissertação para obtenção do título de Mestrado, área de Biopatologia, a que se submeteu o(a) aluno(a) Taciano dos Reis Cardoso – matrícula 5013822-2, orientada pelo(a) Prof.(a) Geraldo Thedei Júnior.

Aos trinta dias do mês de setembro do ano de dois mil e nove, às 13:30, no Anfiteatro da Biblioteca Central da Universidade de Uberaba, reuniu-se a Comissão Julgadora da defesa em epígrafe indicada pelo curso de Mestrado em Odontologia da Universidade de Uberaba, composta pelos Professores: Geraldo Thedei Júnior - **Presidente**, Sanívia Aparecida Lima Pereira e Tony de Paiva Paulino, para julgar o trabalho do candidato **Taciano dos Reis Cardoso,** apresentado sob o título: **"A atividade metabólica dos biofilmes de *Streptococcus mutans* após o tratamento com enxaguatórios bucais com diferentes composições"**. O Presidente declara abertos os trabalhos e agradece a presença de todos os Membros da Comissão Julgadora. A seguir o candidato dissertou sobre o seu trabalho e foi argüido pela Comissão Julgadora, tendo a todos respondido às respectivas argüições. Terminada a exposição, a Comissão reuniu-se e deliberou pelo seguinte resultado:

APROVADO X
REPROVADO (anexar parecer circunstanciado elaborado pela Comissão Julgadora)

Para fazer jus ao título de MESTRE EM ODONTOLOGIA ÁREA DE CONCENTRAÇÃO BIOPATOLOGIA, a versão final da tese, considerada Aprovada devidamente conferida pela Secretaria do Mestrado em Odontologia, deverá ser entregue à Secretaria dentro do prazo de 30 dias, a partir da data da defesa. O aluno Aprovado que não atender a esse prazo será considerado Reprovado. Após a entrega do exemplar definitivo, o resultado será homologado pela Universidade de Uberaba, conferindo título de validade nacional aos aprovados. Nada mais havendo a tratar, O Senhor Presidente declara a sessão encerrada, cujos trabalhos são objeto desta ata, lavrada por mim, que segue assinada pelos Senhores Membros da Comissão Julgadora, pelo Coordenador do Programa de Mestrado em Odontologia da UNIUBE, com ciência do aluno. Uberaba, aos 30 dias do mês de setembro de dois mil e nove.

Prof. Dr. Geraldo Thedei Júnior _____

Profa. Dra. Sanívia Aparecida Lima Pereira _____

Prof. Dr. Tony de Paiva Paulino _____

Prof. Dr. José Bento Alves _____
Coordenador do Programa de Mestrado em Odontologia da UNIUBE

Graciela Argenta Nicodemos da Silva _____
Secretária de Pós-Graduação

Ciência do Aluno: Taciano Cardoso

DEDICATÓRIA

Aos meus avós, Francisco (in memorian) e Maria Carmelita, e minha madrinha Maria Wilma, pelo seu amor maternal que muito contribuiu para minha formação como profissional e, acima de tudo, como ser humano.

A minha esposa Desireé Clary, pelo seu amor e companheirismo, sempre acreditando em meu potencial, independente das minhas dificuldades.

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Aos meus pais, Edmo e Vilma, por estarem sempre me mandando bons pensamentos, força e incentivo nesta caminhada.

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À Secretária do Núcleo de Gestão em Pesquisa, Alessandra Scoda, por sua grande ajuda na elaboração desta dissertação, com conselhos sobre as normas de apresentação deste trabalho, vale apena relatar sua competência sobre o assunto.

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Mais um degrau conquistado, nos muitos que estão por vir.

RESUMO

Objetivos: O foco principal desta pesquisa foi investigar a atividade metabólica dos biofilmes de *Streptococcus mutans* após o tratamento com enxaguatórios bucais com diferentes composições. Métodos: Biofilmes de *S. mutans* foram crescidos em placas de poliestireno durante 18 horas, lavados com solução salina estéril, tratados com diferentes enxaguatórios bucais (1min) e incubados com meio completo estéril contendo sacarose, durante 3 horas. Após 60, 120 e 180 min, amostras foram retiradas para mensuração do pH. Além disso, os biofilmes foram cultivados em lamínulas de microscópio, tratados como descrito acima, seguido de coloração com Iodeto de Propídio e Fluoresceína para visualização em microscópio confocal de varredura a laser. Resultados: Observou-se que o tratamento com os enxaguatórios bucais foram deletérios para o metabolismo celular, uma vez que foi observada pouca ou nenhuma acidificação no período de 60 min após o tratamento. Observamos também que os enxaguatórios contendo clorexidina a 0,2% (v/v) ou óleo essencial foram mais eficazes do que o fluoreto ou os enxaguatórios bucais contendo clorexidina a 0,12% (v/v), uma vez que a redução da atividade metabólica induzida por esses enxaguatórios teve a mesma extensão do controlo positivo com etanol 70% (v/v). A análise confocal confirmou, de maneira geral, os resultados observados através da atividade metabólica. Conclusões: O tratamento de biofilmes com enxaguatórios contendo clorexidina a 0,2% (v/v) ou óleo essencial foram mais eficazes do que o fluoreto ou os enxaguatórios bucais contendo clorexidina a 0,12% (v/v) na indução de lesão na membrana e em abolir o metabolismo de *S. mutans*.

Palavras-chave: *Streptococcus mutans*, enxaguatório, acidogenia, cloreto de cetilpiridínio, clorexidina, óleo essencial, biofilme, Microscopia confocal por varredura a laser

ABSTRACT

The main focus of this research was to investigate the metabolic activity of *Streptococcus mutans* biofilms after treatment with mouthwashes containing different composition. *S. mutans* biofilms were grown on polystyrene plates during 18 hours, washed with sterile saline, treated with different mouthwashes (1 min) and incubated with sterile complete medium containing sucrose during 3 hours. After 60, 120 and 180 min, samples were removed for pH measurements. Besides, biofilms were grown in microscope coverslips treated as described above followed by staining with Propidium Iodide and Fluoresceine for visualization into a confocal laser scanning microscopy. It was observed that mouthwashes treatment was deleterious to cell metabolism, since little or no acidification was observed at least 60 min. after treatment. We also observed that mouthwashes containing 0.2% (v/v) chlorexidine or essential oil were more effective than fluoride or 0.12% (v/v) chlorexidine-containing mouthwashes, since the reduction in the metabolic activity induced by those mouthwashes had the same extension than positive control 70% (v/v) ethanol. The confocal analysis overall confirmed the results observed trough metabolic activity. The treatment of biofilms with mouthwashes containing 0.2% (v/v) chlorexidine or essential oil were more effective than fluoride- or 0.12% (v/v) chlorexidine-containing mouthwashes to induce membrane damage and to abolish *S. mutans* metabolism.

Key words: *Streptococcus mutans,* mouthwashes, acidogeny, cetylpyridinium chloride, chlorexidine, biofilm, Confocal Laser Scanning Microscopy

De acordo com Capítulo IV – Da defesa, artigo 46, do Regimento Geral - Mestrado em Odontologia, essa dissertação será apresentada sob forma de artigo científico, segundo as normas da Revista Archives of Oral Biology.

SUMÁRIO

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Metabolic activity of *Streptococcus mutans* biofilms after treatment with different mouthwashes composition

Taciano R. Cardoso[a], Alexandre S. Carvalho[a], Hilara N. Ruas[a], Marcelo E. Beletti[b], Marcelo H. Napimoga[c] and Geraldo Thedei Jr [a*]

[a]Laboratory of Biochemistry of Microorganisms, University of Uberaba, 38.055-500 Uberaba, MG, Brazil.

[b]College of Veterinary Medicine, Federal University of Uberlandia, 38400-902, Uberlândia, MG, Brazil

[c]Laboratory of Biopathology and Molecular Biology, University of Uberaba, 38.055-500, Uberaba, MG, Brazil

*Corresponding author: Thedei Jr., G.

Tel.: +55-34-3319-8959

Fax: +55-34-3314-8910

E-mail: geraldo.thedei@uniube.br

Abstract

Objectives: The main focus of this research was to investigate the metabolic activity of *Streptococcus mutans* biofilms after treatment with mouthwashes containing different composition. **Methods**: *S. mutans* biofilms were growth on polystyrene plates during 18 hours, washed with sterile saline, treated with different mouthwashes (1 min) and incubated with sterile complete medium containing sucrose during 3 hours. After 60, 120 and 180 min, samples were removed for pH measurements. Besides, biofilms were grown in microscope coverslips treated as described above followed by staining with Propidium Iodide and Fluoresceine for visualization into a confocal laser scanning microscopy. **Results**: It was observed that mouthwashes treatment was deleterious to cell metabolism, since little or no acidification was observed at least 60 min. after treatment. We also observed that mouthwashes containing 0.2% (v/v) chlorhexidine or essential oil were more effective than fluoride or 0.12% (v/v) chlorhexidine-containing mouthwashes, since the reduction in the metabolic activity induced by those mouthwashes had the same extension than positive control 70% (v/v) ethanol. The confocal analysis overall confirmed the results observed trough metabolic activity. **Conclusions**: The treatment of biofilms with mouthwashes containing 0.2% (v/v) chlorhexidine or essential oil were more effective than fluoride- or 0.12% (v/v) chlorhexidine-containing mouthwashes to induce membrane damage and to abolish *S. mutans* metabolism.

Key words: *Streptococcus mutans,* mouthwashes, acidogeny, cetylpyridinium chloride, chlorhexidine, essential oil, biofilm, Confocal Laser Scanning Microscopy

Running title: Metabolism of mouthwash-treated *S. mutans*.

1. Introduction

Dental caries is a chronicle contagious diseases caused by several interacting factors which results in the irreversible destruction of the mineralized structures of teeth, compromising dental vitality and dental fixation in the maxillo-mandibular complex.[1, 2]

The Gram positive bacteria *Streptococcus mutans* is a substantial part of the oral microbiota and its importance in the dental caries etiology is unquestionable.[3] This bacteria use carbohydrates present in the diet as an energy source, in an anaerobic process (mainly lactic fermentation) resulting in the production of organic acids. These acids lower the pH to around 4.5 on the tooth surface,[4] inducing its demineralization.

One important characteristic of *S. mutans* in promoting caries development is the ability to adhere firmly to the tooth surface in the presence of sucrose and this adherence is mediated mainly by the enzymatic action of the GTF enzymes.[5-7] These enzymes are considered fundamental for the virulence of *S. mutans* in the pathogenesis of dental caries.

Biofilm formation occurs as a result of a sequence of events: microbial surface attachment, cell proliferation, matrix production and detachment.[8] This process is partially controlled by quorum sensing, an interbacterial communication mechanism that is dependent on population density and is associated with radical changes in protein expression patterns.[8] Mature biofilms demonstrate a complex 3-dimensional structure with numerous microenvironments differing with respect to osmolarity, nutritional supply and cell density. Many antimicrobial agents that are effective against planktonic cells turn out to be ineffective against the same bacteria growing in a biofilm state.[9,10] Planktonic and biofilm cells also exhibit different susceptibilities

to a certain antimicrobial concentration.

Many studies focusing the efficacy of mouthwashes with diverse chemical composition demonstrate that combination of sodium fluoride and sodium lauril sulfate as well as essential oil are able to diminish the metabolic activity of microorganisms present in the dental biofilm.[11-13]

Foster et al.[14] studied the effects of mouthwashes containing essential oil, triclosan, cetylpyridinium chloride and chlorhexidine against *Streptococcus gordonii* biofilms. The confocal laser scanning microscopy analysis demonstrated that all mouthwashes except cetylpyridinium chloride, were able to cause membrane damage after 60 seconds incubation with *S. gordonii* biofilms.

Zhang et al.[15] evaluated the effect of a mouthwash with and without fluoride over metabolic activity of *S.mutans* biofilms and demonstrated that essential oil containing mouthwashes, with or without 100 mg/Kg of fluoride reduces the metabolic activity and the consequent acid production about 36-44%. A significant reduction on total colony forming units (CFU) was observed in saliva of healthy volunteers after a single mouthwash with 0.2% (v/v) or 0.12% (v/v) chlorhexidine, but only the higher concentration showed bactericidal activity against salivary obligate anaerobes.[16] Furthermore, an *in vivo* study showed that both essential oil and alcohol-free chlorhexidine mouthwashes were able to reduce plaque acidogenicity after a sucrose challenge, with no difference between both solutions.[17]

Although several studies have been done, few data about the action of mouthwashes with different active principles focusing on bacterial biofilm metabolism, especially *S. mutans* biofilms, as well as the effects of those mouthwashes in three-dimensional structure of biofilms are available. Thus, in the present study we evaluated the metabolic activity of *S. mutans* biofilms after treatment with 5 different

mouthwashes, employing acidogenic capacity and confocal laser scanning microscopy.

2. Material and methods

2.1 Mouthwashes

Were used the mouthwashes Parodontax® (SmithKline Beecham Consumer Healthcare, United Kingdom), Listerine Cool Mint® (Johnson & Johnson, SP, Brazil), Oral-B® (Rety Laboratories, Barranquilla, Colombia) and Periogard® with and without alcohol (Colgate-Palmolive, SP, Brazil). Positive control used 70% ethanol and negative control was made with sterile 0.9% (w/v) NaCl.

2.2. Streptococcus mutans *growth conditions*

The ATCC 25175 strain of *S. mutans* was purchased from the André Tosello Foundation, Campinas-SP (Brazil). The lineage was kept stored in –20ºC in 40% (v/v) glycerol (Sigma, St. Louis, MO, USA) medium and checked for purity before being grown in broth.

The frozen *S. mutans* cultures were reactivated in 5 mL of Triptic Soy Broth (TSB- Soybean-casein digest medium) from Difco, Sparks, MD, USA, and incubated at 37°C, under microaerophilic conditions for 18h. The cultures were adjusted to $A620_{nm}$=0.2 using a photocolorimeter (Analyser Com & Ind. Brazil) and 750 μL of this suspension was transferred to a tube containing 30 mL of previously autoclaved Complete medium[18] supplemented with 50 mMol/L sucrose as carbon source. Then, 600 μL of this suspension was inoculated in a 24-well cell culture plate (Corning Costar 3524, flat bottom). The plate was then incubated as described above, during 18 hs.

2.3 Effects of mouthwashes on S. mutans *metabolism.*

All procedures were carried out in a blind fashion. After growth as described above, the culture medium of each well was removed and the pH was measured using a PG 1800 pHmeter (Gehaka, São Paulo, Brazil). The formed biofilms were washed 3 times with sterile 0.9% (w/v) NaCl and the mouthwashes were added to each well. After 1 min of incubation, the mouthwashes were removed and the wells washed with abundant sterile 0.9% (w/v) NaCl. To each well was finally added 1 mL of sterile complete medium supplied with 50 mMol/L sucrose as carbon source. The cell culture plate was incubated at 37ºC under microaerophilic conditions and samples were taken at 60, 120 and 180 min for further pH analysis.

The positive control used was ethanol 70% and the negative control sterile 0.9% (w/v) NaCl.

2.4. Confocal Laser Scanning Microscopy (CLSM)

For the CLSM study, glass coverslips were inserted in Falcon Tubes with 30 mL of previously autoclaved Complete medium[18] supplemented with 50 mMol/L sucrose as carbon source. Suspension of 5 x 107 CFU of *S. mutans* were added and cultivated for 18h. The *S. mutans* biofilm formed in the coverslips were washed and treated with different mouthwashes during 1 minute. After that, the coverslips were extensively washed with sterile saline and treated with 1 mMol/L propidium iodide followed by 0.1% fluoresceine. The coverslips were mounted on individual slides and the images was captured for an emission wavelength at 500-530 nm or at 600-675 nm respectively at 63X magnification with a Confocal Laser Scanning Microscope (Carl Zeiss LSM 510 META). The two color images obtained by a CLSM, i.e. a green-filtered emission image and a red-filtered emission image, were converted to digital

image and merged together using the Zeiss LSM Image Browser.

2.5 Statistical analysis:

Data are reported as the mean of triplicate measurement of three independent assays. One-way analysis of variance (ANOVA) was used to determine the significance between treatments. To determine whether the means were statistically different from each other we used the Bonferroni's multiple comparison test, considered to be statistically significant at $P<0.05$.

3. Results

In Figure 1 it is possible to observe that biofilm without treatment was able to continuously acidify the medium in all three time-point measured. Also it was demonstrated that at 60, 120 and up to 180min after essential oils treatment (Listerine®), the acidification of biofilms was significantly smaller than saline-treated biofilms ($P<0.001$) and showed no statistical difference ($P>0.05$) as compared with positive control (70% (v/v) ethanol) suggesting an efficacy against the *S. mutans* biofilm.

We also evaluated 3 different chlorhexidine-containing mouthwashes (0.2% (v/v) and 0.12% (v/v) of chlorhexidine with or without alcohol). All mouthwashes containing this active principle reduced biofilm's acidogenicity as compared with negative control during the 180 min of measurements ($P<0.001$). However, among the three mouthwashes, only that one containing 0.2% (v/v) chlorhexidine (Parodontax) abolished the metabolic activity in a similar fashion than positive control along the studied period ($P>0.05$). Interestingly, between 0.12% (v/v) chlorhexidine-containing

mouthwashes, the one containing alcohol in its composition (Periogard plus alcohol®) was more effective to reduce the *S. mutans* acidogenicity ability, similar to positive control until 60 min after treatment. For subsequent time of this mouthwash and during all the time monitored after treatment with 0.12% (v/v) chlorhexidine mouthwash without alcohol in its composition (Periogard without alcohol®), there was significative statistical difference as compared with positive control, showing that 0.12% (v/v) chlorhexidine fails to reduce the metabolic activity as compared with positive control 70% (v/v) ethanol, in despite of the significative ($P<0.001$) reduction of metabolic activity as compared to negative control.

In Figure 3 we demonstrated that treatment with cetylpyridinium chloride (CPC) plus fluoride mouthwash (OralB®) reduced the biofilm acidogenicity during all time analyzed as compared with negative control ($P<0.001$), but its acidification capacity was significantly higher than positive control at 120min ($P<0.01$) and 180 min ($P<0.001$) after treatment.

To ascertain the viability of bacteria in the biofilm after mouthwashes treatment, we employed a confocal laser scanning microscopy (CLSM). *S. mutans* biofilm without any treatment revealed great viability of the cells (Fig 4A), contrasting with a higher level of membrane damage induced by 70% (v/v) ethanol (Fig. 4B). Furthermore, biofilm treated with essential oil or 0.2% (v/v) chlorhexidine-containing mouthwashes causes extensive damage to biofilms (Fig. 4C and D, respectively), comparable or more extensive than lesions induced by ethanol. Important, it is possible to observe that both antimicrobial agents used effectively penetrated the biofilm. In a smaller extent, treatment of biofilms with 0.12% (v/v) chlorhexidine plus alcohol (Fig 4E) also was able to cause membrane damage, whereas 0.12% (v/v) chlorhexidine without alcohol (Fig 4F) and alcohol-free cetylpyridinium chloride plus

fluoride mouthrinse (Fig 4G) caused a low level of membrane damage, restricted to spots on biofilm and not throughout the biofilm. These results, in a greater extent, are corroborative with pH measurements after treatment of biofilms with mouthwashes.

4. Discussion

The formation of dental biofilm is instantly initiated after tooth cleaning by the adsorption of salivary components to the enamel surface, followed by addition of initial colonizers, to which eventually, the climax community of matured dental biofilm will adhere.[19,11] Bacteria present in dental biofilm are involved in a matrix of salivary proteins and microbial products.[20] This type of growth protects the bacteria from external agents such as antibiotics,[11] and mouthwash components.[21]

In the present study, the mouthwashes with essential oil and 0.2% chlorhexidine showed efficacy similar to 70% (v/v) ethanol to reduce the acidogeny from *S. mutans* biofilms (Figures 1 and 2). These results are in agreement with Albertsson et al. [17], who demonstrated, *in vivo*, that using essential oil or alcohol-free chlorhexidine mouthwashes during a 16-days period reduced plaque acidogenicity after a sucrose challenge.

Kocak et al.[22] showed that a 0.12% (v/v) chlorhexidine without alcohol was effective against oral microorganisms. Our results suggest that a mouthwash containing 0.12% (v/v) chlorhexidine and no alcohol is able to reduce the bacterial metabolism as compared with negative control, but fails, at any time evaluated, to reduce the metabolism in a significative fashion as compared with positive control. The *in vivo* based study of those authors evaluated the efficacy of mouthwashes measuring the number of colony-forming units (CFU) of *S. mutans* present in saliva

after usage of mouthwash probably reflecting only cells that detached from biofilm and not the whole dental biofilm. In our study, the whole biofilm was analyzed and the results clearly showed that 0.12% (v/v) chlorhexidine fails to abolish metabolic activity and also to induce extensive membrane damage to biofilm growing *S. mutans*. Thus, this result indicates that the concentration of chlorhexidine is determinant to its penetrability into the biofilm. Nevertheless, Tomás et al.[16] observed a reduction of total bacterial population after usage of both 0.2 and 0.12% (v/v) chlorhexidine mouthwashes, but these authors also related that only the highest concentration showed bactericidal activity, in agreement with our results of both acidogeny and CLSM assays.

Comparison between 0.12% (v/v) chlorhexidine with alcohol and 0.12% (v/v) chlorhexidine without alcohol, showed a small advantage of alcohol-containing mouthwash, since it causes a 60 min delay in acidogeny as compared with alcohol-free (Figure 2). A similar result was found by Arweiler et al.[23] in which they compare two chlorhexidine solutions against plaque re-growth and bacterial viability, showing that ethanol may significantly contribute to reduce bacterial vitality. Interestingly, in our study the worst results were obtained from mouthwashes without alcohol suggesting that the alcohol may contribute to a better penetrability of the active principle into the biofilm.

Witt et al.[24] observed no difference between an alcohol-free CPC mouthwashes as compared with one containing essential oil, using a Modified Quigley-Hein Plaque Index. On the other hand, in our experiments, the CPC plus fluoride mouthwash had the worst capacity to reduce *S. mutans* metabolism (Figure 3), showed in both acidogeny and CLSM experiments. Among the reasons to explain these results, we can arise: (1) that the penetrability of CPC may not have been able

enough to entirely permeate the biofilms; (2) that the molecule could penetrate but the contact period between CPC and bacterial cells was insufficient to cause membrane damage; or (3) the CPC concentration present in the mouthwash used was below of the necessary to cause extensive membrane damage.

Our data from CSLM strongly suggests that reduction of metabolic activity is due to cell damage as a result of mouthwash treatment. In our study, among 5 mouthwashes tested, only 2 showed efficient penetration of the agents throughout the biofilm as observed in the positive control experiment, visualized by CLSM. Evidence of membrane damage extended from the bottom of coverslips to the surface of biofilms induced by 0.2% (v/v) chlorhexidine and essential oil containing mouthwashes suggests an effective penetration of these molecules through the biofilm. Interestingly, 0.12% (v/v) chlorhexidine showed poor efficacy when compared with 0.2% (v/v), indicating that a small variation in concentration may compromise the penetrability and, consequently, the bacterial inactivation.

Many previous studies measured the efficacy of antimicrobials on *in vivo* dental plaque[17,22,23] and some of these studies had high interindividual variations of the results[17]. The methodology employed in this study was highly reproducible and, also, is low cost and easy to perform. Besides, in this study, we attempted to mimic exposure times often used *in vivo* clinical studies (60 s).[25-27] Thus, in conclusion the mouthwashes containing essential oil or 0.2% (v/v) chlorhexidine presented high efficacy then CPC plus fluoride or 0.12% (v/v) chlorhexidine.

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Figure Legends:

Figure 1: Acidogeny of *S. mutans* biofilms after essential oil mouthrinse (∇), 0.9% (w/v) NaCl (□) and 70% (v/v) ethanol (○) treatment. Acidogeny from mouthrinse-treated biofilms was similar (P>0.05) to positive control at 60, 120 and 180 min after treatment. Negative control showed higher acidogeny than mouthrinse or alcohol-treated biofilms (P<0.001).

Figure 2: Acidogeny of *S. mutans* biofilms after treatment with mouthrinses containing chlorhexidine at 0.2% (v/v) (▲), 0.12% (v/v) plus alcohol (♦) or 0.12% (v/v) without alcohol (■), compared to 0.9% (w/v) NaCl (□) and 70% (v/v) ethanol (○) treatment. Acidogeny from mouthrinse-treated biofilms was similar (P>0.05) to positive control at 60, 120 and 180 min after treatment. Acidogeny from both 0.12% (v/v) chlorhexidine mouthrinses were higher than positive control in all times analyzed (P<0.05), except at 60 min after treatment with mouthrinse plus alcohol. Negative control showed higher acidogeny than mouthrinses or alcohol-treated biofilms (P<0.001).

Figure 3: Acidogeny of *S. mutans* biofilms after cetylpyridinium-chloride plus fluoride mouthrinse (●), 0.9% (w/v) NaCl (□) and 70% (v/v) ethanol (○) treatment. Acidogeny from mouthrinse-treated biofilms was similar (P>0.05) to positive control at 60min after treatment but not at 120 and 180 min, when mouthrinse-treated biofilms showed higher acidogeny (P<0.01 and 0.001, respectively). Negative control showed higher acidogeny than mouthrinse or alcohol-treated biofilms (P<0.001).

Figure 4: Confocal Laser Scanning Microscopy of saline-treated biofilms (A), after treatment with 70% (v/v) ethanol (B) essential oil (C), 0.2% (v/v) chlorhexidine (D), 0.12% (v/v) chlorhexidine plus alcohol (E), 0.12% (v/v) chlorhexidine without alcohol (F) and alcohol-free cetylpyridinium chloride plus fluoride (G) mouthrinses. All images show a three-dimensional reconstruction rotated 90º in the y-z direction (above) and in the x-z direction (right side).

Figure 1 – Cardoso et al

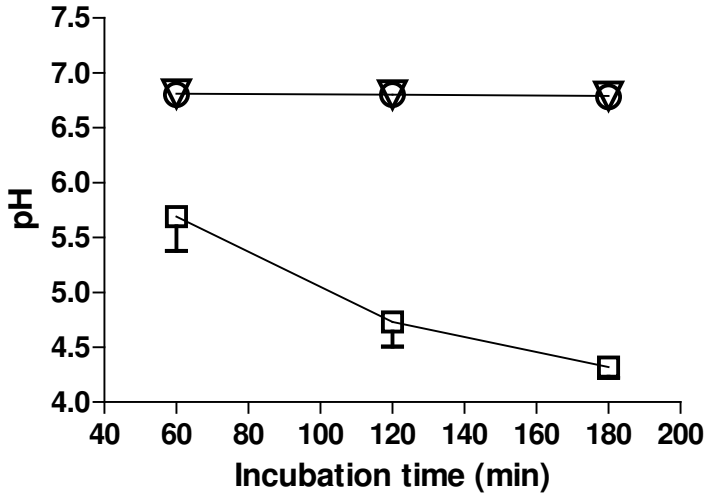
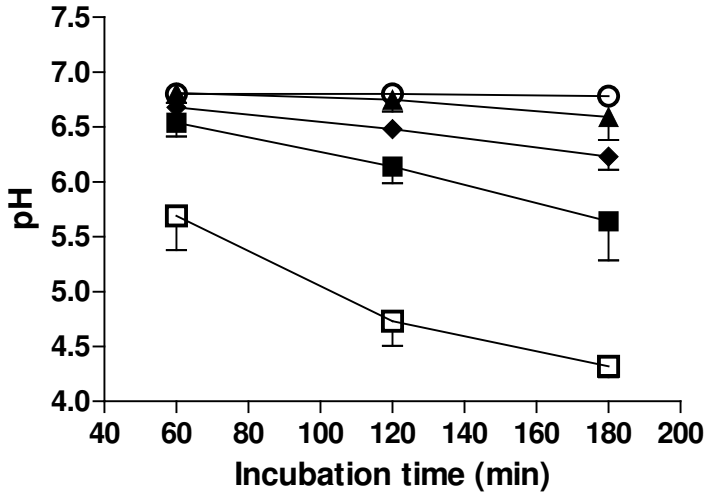


Figure 2 – Cardoso et al



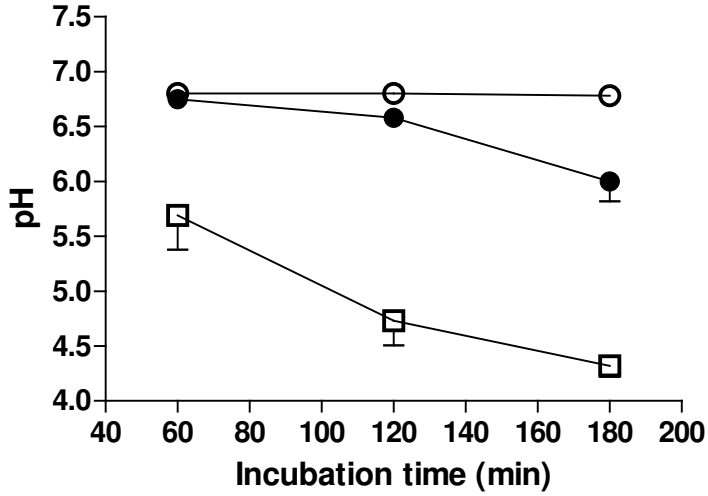
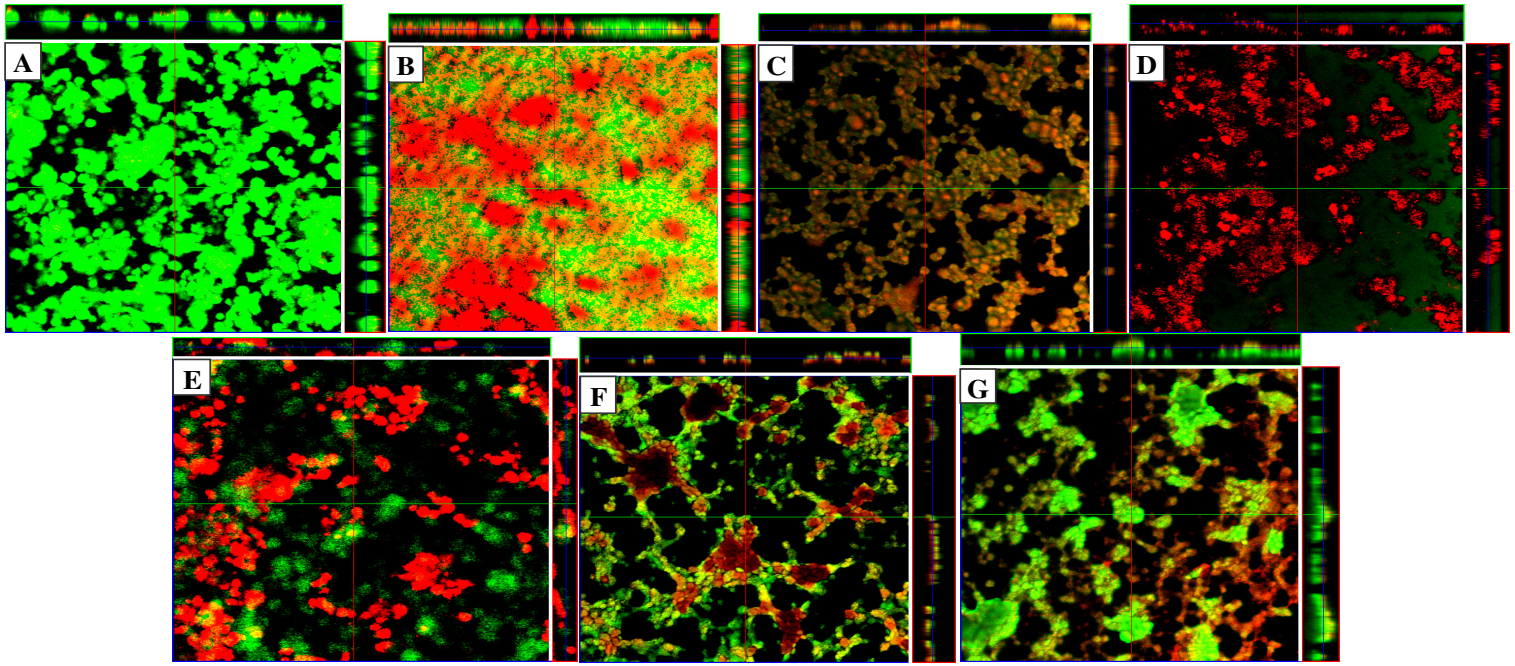


Figure 3 – Cardoso et al

Figure(s)

Figure 4 – Cardoso et al



APÊNDICE A – Análise estatística

Análise estatística enxaguatório Listerine x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos biofilmes crescidos 18 horas				
One-way analysis of variance				
P value	0,0018			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	21,51			
R squared	0,8776			
ANOVA Table	SS	df	MS	
Treatment (between columns)	7,094	2	3,547	
Residual (within columns)	0,9893	6	0,1649	
Total	8,083	8		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs vs Listerine 18 hs	-1,887	5,69	P < 0.01	-2.977 to -0.7967
Controle 18 hs vs Àlcool 70%	-1,88	5,67	P < 0.01	-2.970 to -0.7900
Listerine 18 hs vs Àlcool 70%	0,006667	0,02011	P > 0.05	-1.083 to 1.097

Análise estatística enxaguatório Listerine x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs e 60 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	147			
R squared	0,8672			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	87,44			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	14,81	2	7,404	
Residual (within columns)	2,267	45	0,05037	
Total	17,07	47		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs 60 vs Listerine 18 hs 60	-1,114	14,04	P < 0.001	-1.311 to -0.9164
Controle 18 hs 60 vs Àlcool 70%	-1,108	13,96	P < 0.001	-1.305 to -0.9106
Listerine 18 hs 60 vs Àlcool 70%	0,005834	0,06367	P > 0.05	-0.2220 to 0.2337

Análise estatística enxaguatório Listerine x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs e 120 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	989,8			
R squared	0,9778			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	93,79			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	51,17	2	25,59	
Residual (within columns)	1,163	45	0,02585	
Total	52,33	47		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
CONTROLE 18 hs 120 vs Listerine 18hs 120	-2,067	36,37	P < 0.001	-2.208 to -1.926
CONTROLE 18 hs 120 vs Àlcool 70%	-2,063	36,29	P < 0.001	-2.204 to -1.922
Listerine 18hs 120 vs Àlcool 70%	0,004167	0,06349	P > 0.05	-0.1591 to 0.1674

Análise estatística enxaguatório Listerine x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs 180 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	7140			
R squared	0,9969			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	53,97			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	72,89	2	36,45	
Residual (within columns)	0,2297	45	0,005105	
Total	73,12	47		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs 180 min vs Listerine 18 hs 180 min	-2,466	97,62	P < 0.001	-2.529 to -2.403
Controle 18 hs 180 min vs Àlcool 70%	-2,463	97,52	P < 0.001	-2.526 to -2.401
Listerine 18 hs 180 min vs Àlcool 70%	0,0025	0,08571	P > 0.05	-0.07003 to 0.07503

Análise estatística enxaguatório Parodontax x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos biofilmes crescidos 18 horas				
One-way analysis of variance				
P value	0,0009			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	11,58			
R squared	0,8225			
ANOVA Table	SS	df	MS	
Treatment (between columns)	7,058	4	1,764	
Residual (within columns)	1,523	10	0,1523	
Total	8,581	14		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs vs Parodontax 18 hs	-1,803	5,659	P < 0.01	-2.945 to -0.6620
Controle 18 hs vs Periogard (S/Àlcool)	-1,193	3,745	P < 0.05	-2.335 to -0.05202
Controle 18 hs vs Periogard (C/Àlcool)	-1,55	4,864	P < 0.01	-2.691 to -0.4087
Controle 18 hs vs Àlcool 70%	-1,88	5,899	P < 0.01	-3.021 to -0.7387
Parodontax 18 hs vs Periogard (S/Àlcool)	0,61	1,914	P > 0.05	-0.5313 to 1.751
Parodontax 18 hs vs Periogard (C/Àlcool)	0,2533	0,795	P > 0.05	-0.8880 to 1.395
Parodontax 18 hs vs Àlcool 70%	-0,07667	0,2406	P > 0.05	-1.218 to 1.065
Periogard (S/Àlcool) vs Periogard (C/Àlcool)	-0,3567	1,119	P > 0.05	-1.498 to 0.7846
Periogard (S/Àlcool) vs Àlcool 70%	-0,6867	2,155	P > 0.05	-1.828 to 0.4546
Periogard (C/Àlcool) vs Àlcool 70%	-0,33	1,036	P > 0.05	-1.471 to 0.8113

Análise estatística enxaguatório Parodontax x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs e 60 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	110,1			
R squared	0,8679			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	71,43			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	16,97	4	4,242	
Residual (within columns)	2,582	67	0,03853	
Total	19,55	71		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs 60 vs paradontox 18 hs 60	-1,112	16,02	P < 0.001	-1.314 to -0.9106
Controle 18 hs 60 vs Periogard (S/Àlcool)	-0,8438	12,16	P < 0.001	-1.045 to -0.6423
Controle 18 hs 60 vs Periogard (C/Àlcool)	-0,9846	14,19	P < 0.001	-1.186 to -0.7831
Controle 18 hs 60 vs Àlcool 70%	-1,108	15,96	P < 0.001	-1.309 to -0.9064
paradontox 18 hs 60 vs Periogard (S/Àlcool)	0,2683	3,348	P < 0.05	0.03569 to 0.5010
paradontox 18 hs 60 vs Periogard (C/Àlcool)	0,1275	1,591	P > 0.05	-0.1051 to 0.3601
paradontox 18 hs 60 vs Àlcool 70%	0,004167	0,052	P > 0.05	-0.2285 to 0.2368
Periogard (S/Àlcool) vs Periogard (C/Àlcool)	-0,1408	1,757	P > 0.05	-0.3735 to 0.09181
Periogard (S/Àlcool) vs Àlcool 70%	-0,2642	3,296	P < 0.05	-0.4968 to -0.03152
Periogard (C/Àlcool) vs Àlcool 70%	-0,1233	1,539	P > 0.05	-0.3560 to 0.1093

Análise estatística enxaguatório Parodontax x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs e 120 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	572,3			
R squared	0,9716			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	55,33			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	55,6	4	13,9	
Residual (within columns)	1,627	67	0,02429	
Total	57,22	71		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
CONTROLE 18 hs 120 vs Paradontax 18 hs 120	-2,016	36,59	P < 0.001	-2.176 to -1.856
CONTROLE 18 hs 120 vs Periogard (S/Àlcool)	-1,403	25,46	P < 0.001	-1.563 to -1.243
CONTROLE 18 hs 120 vs Periogard (C/Àlcool)	-1,749	31,74	P < 0.001	-1.909 to -1.589
CONTROLE 18 hs 120 vs Àlcool 70%	-2,063	37,44	P < 0.001	-2.223 to -1.903
Paradontax 18 hs 120 vs Periogard (S/Àlcool)	0,6133	9,64	P < 0.001	0.4286 to 0.7980
Paradontax 18 hs 120 vs Periogard (C/Àlcool)	0,2675	4,204	P < 0.001	0.08280 to 0.4522
Paradontax 18 hs 120 vs Àlcool 70%	-0,04667	0,7335	P > 0.05	-0.2314 to 0.1380
Periogard (S/Àlcool) vs Periogard (C/Àlcool)	-0,3458	5,436	P < 0.001	-0.5305 to -0.1611
Periogard (S/Àlcool) vs Àlcool 70%	-0,66	10,37	P < 0.001	-0.8447 to -0.4753
Periogard (C/Àlcool) vs Àlcool 70%	-0,3142	4,938	P < 0.001	-0.4989 to -0.1295

Análise estatística enxaguatório Parodontax x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs 180 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	528,1			
R squared	0,9693			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	74,08			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	72,39	4	18,1	
Residual (within columns)	2,296	67	0,03427	
Total	74,69	71		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs 180 min vs Parodontax 18 hs 180 min	-2,268	34,64	P < 0.001	-2.458 to -2.077
Controle 18 hs 180 min vs Periogard (S/Àlcool)	-1,316	20,1	P < 0.001	-1.506 to -1.126
Controle 18 hs 180 min vs Periogard (C/Àlcool)	-1,906	29,12	P < 0.001	-2.096 to -1.716
Controle 18 hs 180 min vs Àlcool 70%	-2,463	37,64	P < 0.001	-2.653 to -2.273
Parodontax 18 hs 180 min vs Periogard (S/Àlcool)	0,9517	12,59	P < 0.001	0.7323 to 1.171
Parodontax 18 hs 180 min vs Periogard (C/Àlcool)	0,3617	4,786	P < 0.001	0.1423 to 0.5811
Parodontax 18 hs 180 min vs Àlcool 70%	-0,1958	2,591	P > 0.05	-0.4152 to 0.02356
Periogard (S/Àlcool) vs Periogard (C/Àlcool)	-0,59	7,807	P < 0.001	-0.8094 to -0.3706
Periogard (S/Àlcool) vs Àlcool 70%	-1,148	15,18	P < 0.001	-1.367 to -0.9281
Periogard (C/Àlcool) vs Àlcool 70%	-0,5575	7,377	P < 0.001	-0.7769 to -0.3381

Análise estatística enxaguatório Oral-B x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos biofilmes crescidos 18 horas				
One-way analysis of variance				
P value	0,0056			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	13,86			
R squared	0,822			
ANOVA Table	SS	df	MS	
Treatment (between columns)	5,998	2	2,999	
Residual (within columns)	1,298	6	0,2164	
Total	7,296	8		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs vs Oral - B 18 hs	-1,53	4,028	P < 0.05	-2.779 to -0.2813
Controle 18 hs vs Àlcool 70%	-1,88	4,95	P < 0.01	-3.129 to -0.6313
Oral - B 18 hs vs Àlcool 70%	-0,35	0,9215	P > 0.05	-1.599 to 0.8987

Análise estatística enxaguatório Oral-B x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs e 60 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	139			
R squared	0,8607			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	72,33			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	14,07	2	7,033	
Residual (within columns)	2,277	45	0,0506	
Total	16,34	47		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs 60 vs Oral-B18 hs 60	-1,056	13,28	P < 0.001	-1.254 to -0.8585
Controle 18 hs 60 vs Àlcool 70%	-1,108	13,93	P < 0.001	-1.306 to -0.9101
Oral-B18 hs 60 vs Àlcool 70%	-0,05167	0,5626	P > 0.05	-0.2800 to 0.1767

Análise estatística enxaguatório Oral-B x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs e 120 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	868,5			
R squared	0,9747			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	60,59			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	46,11	2	23,05	
Residual (within columns)	1,195	45	0,02654	
Total	47,3	47		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
CONTROLE 18 hs 120 vs Oral - B 18 hs 120	-1,845	32,04	P < 0.001	-1.989 to -1.702
CONTROLE 18 hs 120 vs Àlcool 70%	-2,063	35,81	P < 0.001	-2.206 to -1.920
Oral - B 18 hs 120 vs Àlcool 70%	-0,2175	3,27	P < 0.01	-0.3829 to -0.05209

Análise estatística enxaguatório Oral-B x álcool 70% e Listerine x salina				
Parameter	Value			
Table Analyzed				
todos 18 hs 180 min				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	2098			
R squared	0,9894			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	42,15			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	55,17	2	27,59	
Residual (within columns)	0,5918	45	0,01315	
Total	55,76	47		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Controle 18 hs 180 min vs Oral B 18 hs 180 min	-1,679	41,42	P < 0.001	-1.780 to -1.578
Controle 18 hs 180 min vs Àlcool 70%	-2,463	60,76	P < 0.001	-2.564 to -2.363
Oral B 18 hs 180 min vs Àlcool 70%	-0,7842	16,75	P < 0.001	-0.9006 to -0.6677

ANEXO A – Confirmação de envio do artigo

UNIUBE

GERALDO THEDEI JUNIOR 6093 <geraldo.thedei@uniube.br>

Submission Confirmation for Metabolic activity of Streptococcus mutans biofilms after treatment with different mouthwashes composition

Archives of Oral Biology <AOB@elsevier.com> **24 de setembro de 2009 17:29**
Para: geraldo.thedei@uniube.br, gthedei@hotmail.com

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Title: Metabolic activity of Streptococcus mutans biofilms after treatment with different mouthwashes composition
Authors: Taciano R Cardoso, graduate student; Alexandre S Carvalho, undergraduate student; Hilara N Ruas, technical assistant; Marcelo E Beletti, Dr; Marcelo H Napimoga, Dr; Geraldo Thedei Jr
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