

Laís Salomão Arias

**EFEITO DO TIROSOL SOBRE BIOFILMES DE
*STREPTOCOCCUS MUTANS***

ARAÇATUBA - SP

2016

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**EFEITO DO TIROSOL SOBRE BIOFILMES DE
*STREPTOCOCCUS MUTANS***

Dissertação apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP, como parte dos requisitos para a obtenção do título de Mestre em Ciência Odontológica – Área Saúde Bucal da Criança.

Orientador: Prof. Dr. Douglas Roberto Monteiro

Coorientador: Prof. Tit. Alberto Carlos Botazzo Delbem

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Dedicatória

Dedico este trabalho

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Arthur Schopenhauer

Resumo

ARIAS, L.S. **Efeito do tirosol sobre biofilmes de *Streptococcus mutans***. 2016 96f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2016.

O tirosol é uma molécula de *quorum-sensing* (QS) que participa do controle da morfogênese em *Candida albicans*. Contudo, seu efeito como agente antimicrobiano sobre biofilmes de *Streptococcus mutans* permanece desconhecido. Assim, o objetivo deste estudo foi avaliar o efeito do tirosol em diferentes concentrações sobre a produção de ácido e formação de biofilmes por *S. mutans* ATCC 25175, bem como quantificar biofilmes pré-formados desta espécie desenvolvidos sobre espécimes de hidroxiapatita (HA) e tratados com tirosol. A concentração inibitória mínima (CIM) de tirosol sobre células no estado planctônico foi determinada de acordo com o método da microdiluição. Na sequência, biofilmes de *S. mutans* foram formados durante 48 horas sobre espécimes de HA na presença de diferentes concentrações de tirosol (11,25, 22,5, 50, 100 e 200 mmol l⁻¹), usando saliva artificial como meio de cultura. Após, a produção de ácido foi avaliada através da mensuração do pH do meio, enquanto a formação de biofilmes foi determinada através da quantificação da biomassa total (BT), atividade metabólica (AM) e número de unidades formadoras de colônias (UFCs). Ainda, biofilmes pré-formados (24 horas) de *S. mutans* foram tratados com tirosol a 100 e 200 mmol l⁻¹ por 1 minuto, duas vezes ao dia, durante três dias, totalizando biofilmes de 96 horas. Em seguida, a atividade antimicrobiana foi avaliada por meio da quantificação da BT, AM, número de UFCs e composição da matriz extracelular (proteínas e carboidratos). Gluconato de clorexidina (490 µmol l⁻¹) foi usado como controle positivo. Os dados foram analisados por ANOVA a um critério, seguido pelos testes de Tukey e Holm-Sidak ($\alpha = 0,05$). Microscopia eletrônica de varredura (MEV) foi utilizada na análise da estrutura dos biofilmes. A CIM de tirosol foi 90 mmol l⁻¹. Tirosol em concentrações subinibitórias (11,25 e 22,5 mmol l⁻¹) não reduziu a produção de ácido dos biofilmes de *S. mutans*. Entretanto, biofilmes formados na presença de tirosol a 50, 100 e 200 mmol l⁻¹ mostraram reduções significativas na AM e no número de UFCs, variando de 23,4 a 85,5 % e 1,19 a 4,54-log₁₀, respectivamente. Para os biofilmes pré-formados, os tratamentos

com tirosol não promoveram reduções significativas, exceto para a AM do biofilme tratado com tirosol a 200 mmol l^{-1} , a qual mostrou uma redução de 40 % ($p = 0.015$) quando comparada ao controle negativo. Ainda, o tratamento com tirosol a 200 mmol l^{-1} resultou em aumento significativo no conteúdo proteico da matriz extracelular do biofilme pré-formado de *S. mutans*. As imagens de MEV confirmaram os resultados de quantificação das UFCs. Portanto, foi possível concluir que o tirosol mostrou melhores efeitos sobre a formação de biofilmes do que sobre biofilmes pré-formados, e esta molécula de QS não foi capaz de reduzir a produção de ácido dos biofilmes de *S. mutans*. Esses resultados podem ser úteis no desenvolvimento de terapias tópicas voltadas para a prevenção de doenças orais associadas aos biofilmes, como a cárie dentária.

Palavras-chave: Percepção de quorum; Anti-infecciosos; Biofilmes; *Streptococcus mutans*; Tirosol.

Abstract

ARIAS, L.S. **Effect of tyrosol on *Streptococcus mutans* biofilms.** 2016 96f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2016.

Tyrosol is a quorum-sensing molecule (QS) that participates in the control of *Candida albicans* morphogenesis. However, its effect as an antimicrobial agent on *Streptococcus mutans* biofilms remains unknown. Thus, the aim of this study was to evaluate the effect of tyrosol at different concentrations on the acid production and biofilm formation by *S. mutans* ATCC 25175, as well as to quantify pre-formed biofilms of this species developed on hydroxyapatite (HA) specimens and treated with tyrosol. Minimum inhibitory concentration (MIC) of tyrosol against planktonic cells was determined in accordance with the microdilution method. Subsequently, *S. mutans* biofilms were formed during 48 hours on HA specimens in the presence of different concentrations of tyrosol (11.25, 22.5, 50, 100 and 200 mmol l⁻¹), using artificial saliva as culture medium. Next, the acid production was assessed by pH determination of the medium, while the biofilm formation was determined through quantification of total biomass (TB), metabolic activity (MA) and number of colony-forming units (CFUs). Further, *S. mutans* pre-formed biofilms (24 h) were treated with tyrosol at 100 and 200 mmol l⁻¹ twice a day for 1 min, during 3 days, totaling 96-h biofilms. Then, the antimicrobial activity was evaluated through quantification of TB, MA, number of CFUs and composition of biofilms' extracellular matrix (proteins and carbohydrates). Chlorhexidine gluconate (490 µmol l⁻¹) was used as positive control. Data were analyzed by one-way ANOVA, followed by Tukey's and Holm-Sidak's tests ($\alpha = 0.05$). Scanning electron microscopy (SEM) observations were performed in order to analyze biofilms' structure. MIC of tyrosol was 90 mmol l⁻¹. Tyrosol at sub-inhibitory concentrations (11.25 and 22.5 mmol l⁻¹) was not able to significantly reduce acid production by *S. mutans* biofilms. However, biofilms formed in the presence of tyrosol at 50, 100 and 200 mmol l⁻¹ showed significant decreases in MA and number of CFUs, ranging from 23.4 to 85.5 % and 1.19 to 4.54-log₁₀, respectively. For pre-formed biofilms, the treatments with tyrosol did not promote significant reductions, except for MA of

biofilm treated with 200 mmol l⁻¹ tyrosol, which showed a 40 % reduction (p = 0.015) compared to the negative control. Moreover, treatment with 200 mmol l⁻¹ tyrosol resulted in a significant increase in the protein content of the extracellular matrix of *S. mutans* pre-formed biofilm. SEM observations confirmed the results of CFU enumeration. In conclusion, tyrosol showed better effects on biofilm formation than on pre-formed biofilm, and this QS molecule was not able to reduce acid production by *S. mutans* biofilms. These results may be useful in the development of topical therapies focused on preventing biofilm-associated oral diseases, such as dental caries.

Keywords: Quorum Sensing; Antimicrobials; Biofilms; *Streptococcus mutans*; Tyrosol.

Lista de Figuras

LISTA DE FIGURAS

- FIGURE 1 -** Mean absorbance values per cm² obtained with crystal violet staining assay for single biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite specimens in the presence of different concentrations of tyrosol. NC: negative control (biofilm formed without tyrosol); PC: positive control (490 μmol l⁻¹ chlorhexidine gluconate); 50T, 100T and 200T: tyrosol at 50, 100 and 200 mmol l⁻¹, respectively. Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups (p < 0.05, using one-way ANOVA)..... **65**
- FIGURE 2-** Mean absorbance values per cm² obtained with XTT reduction assay for single biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite specimens in the presence of different concentrations of tyrosol. NC: negative control (biofilm formed without tyrosol); PC: positive control (490 μmol l⁻¹ chlorhexidine gluconate); 50T, 100T and 200T: tyrosol at 50, 100 and 200 mmol l⁻¹, respectively. Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups (p < 0.05, using one-way ANOVA with Holm-Sidak post-hoc test)..... **66**
- FIGURE 3 -** Mean values of the logarithm of colony forming units per cm² (log₁₀ CFU cm⁻²) obtained for single biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite specimens in the presence of different concentrations of tyrosol. NC: negative control (biofilm formed without tyrosol); PC: positive control (490 μmol l⁻¹ chlorhexidine gluconate); 50T, 100T and 200T: tyrosol at 50, 100 and 200 mmol l⁻¹, respectively. Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups (p < 0.05, using one-way ANOVA with Holm-Sidak post-hoc test)..... **67**

-
- FIGURE 4 -** Mean absorbance values per cm² (crystal violet) for pre-formed biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite, and treated with tyrosol at 100 mmol l⁻¹ (100T) and 200 mmol l⁻¹ (200T). NC: negative control (non-treated biofilm); PC: positive control (490 µmol l⁻¹ chlorhexidine gluconate). Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups (p < 0.05, using one-way ANOVA)..... **68**
- FIGURE 5 -** Mean absorbance values per cm² (XTT reduction) for pre-formed biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite, and treated with tyrosol at 100 mmol l⁻¹ (100T) and 200 mmol l⁻¹ (200T). NC: negative control (non-treated biofilm); PC: positive control (490 µmol l⁻¹ chlorhexidine gluconate). Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups (p < 0.05, using one-way ANOVA with Holm-Sidak post hoc test)..... **69**
- FIGURE 6 -** Mean values of the logarithm of colony forming units per cm² (log₁₀ CFU cm⁻²) obtained for pre-formed biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite, and treated with tyrosol at 100 mmol l⁻¹ (100T) and 200 mmol l⁻¹ (200T). NC: negative control (non-treated biofilm); PC: positive control (490 µmol l⁻¹ chlorhexidine gluconate). Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups (p < 0.05, using one-way ANOVA with Holm-Sidak post hoc test)..... **70**
- FIGURE 7 -** Scanning electron micrographs of *S. mutans* ATCC 25175 biofilms formed on hydroxyapatite in the presence of different concentrations of tyrosol. NC (a) = negative control; PC (b) = positive control (490 µmol l⁻¹ chlorhexidine gluconate); tyrosol at 50 (c), 100 (d) and 200 mmol l⁻¹ (mM) (e). Magnification: x 5000. Bar: 5.0 µm..... **71**
- FIGURE 8 -** Scanning electron micrographs of pre-formed biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite and treated with tyrosol at 100 mmol l⁻¹ (mM) (c) and 200 mmol l⁻¹ (mM) (d). NC (a) = negative control; PC (b) = positive control (490 µmol l⁻¹ chlorhexidine gluconate). Magnification: x 5000. Bar: 5.0 µm..... **72**

Lista de Tabelas

LISTA DE TABELAS

Table 1 -	Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of tyrosol and chlorhexidine gluconate against the tested strain.....	62
Table 2 -	Mean (SD) pH values obtained with the acid production assay by single biofilms of <i>S. mutans</i> ATCC 25175 formed on hydroxyapatite in the presence of tyrosol at 11.25 mmol l ⁻¹ (11.25T) and 22.50 mmol l ⁻¹ (22.50T). NC: negative control (non-treated biofilm); PC: positive control (0.45 µmol l ⁻¹ chlorhexidine gluconate).....	63
Table 3 -	Mean (SD) protein and carbohydrate contents (from extracellular matrix) for <i>S. mutans</i> ATCC 25175 biofilms obtained after treatment with tyrosol at 200 mmol l ⁻¹ (200T). NC: negative control (non-treated biofilm); PC: positive control (490 µmol l ⁻¹ chlorhexidine gluconate).....	64

Lista de Abreviaturas e Símbolos

LISTA DE ABREVIATURAS E SÍMBOLOS

⁶⁰Co	Cobalto
a.m.	Ante Meridiem
Abs/Absorb	Absorbance
Abs cm⁻²	Absorbance per square centimeter
ANOVA	Análise de Variância/Analysis of Variance
AS	Artificial Saliva
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CaCl₂	Calcium chloride
cells ml⁻¹	Cells per milliliter
CFU(s)	Colony-forming unit (s)
CFU cm⁻²	Colony-forming unit (s) per square centimeter
CHG	Chlorhexidine gluconate
cm	Centimeter
CO₂	Gás carbônico
CV	Crytal Violet
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
Fig.	Figure
g	Gramme
g	Gravity
h	Hora/Hour
HA	Hidroxiapatita/Hydroxyapatite
KCl₂	Potassium chloride
KGy	Kilogray
l	Litro/Liter
Log₁₀	Logaritmo na base 10/ Logarithm to the base 10
MBC	Minimum Bactericidal Concentration
M	Molar
MEV	Microscopia Eletrônica de Varredura
mg	Miligramma/Milligram
mg g⁻¹	Miligramma por grama/Milligram per gramme
MIC	Minimum Inibitorium Concentration
min	Minuto/Minute
ml	Mililitro/Milliliter
mm	Milímetro/Millimeter

Lista de Abreviaturas e Símbolos

mM/ mmol l⁻¹	Milimolar/Millimolar
NaCl	Cloreto de Sódio/Sodium Chloride
nm	Nanômetro/Nanometer
°C	Graus Celsius/Degrees Celsius
p	Probabilidade/Probability
PBS	Phosphate-buffered saline
pH	Potencial Hidrogeniônico/Hydrogen potential
p.m.	Post Merediem
QS	Quorum Sensing
Ra	Rugosidade/Roughness
rev min⁻¹	Revolução por minuto/Revolution per minute
s	Segundo/Second
SD	Desvio Padrão/Standard Deviation
SEM	Scanning Eletron Microscopy
t	Tonelada/Tonne
T	Tirosol/Tyrosol
UFC(s)	Unidades Formadoras de Colônias
UNESP	Universidade Estadual Paulista
v	Volume/Volume
v v⁻¹	Volume por volume/Volume per volume
W	Watts
XTT	(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)
α	Alfa
θ	Teta
μl	Microlitro/Microliter
μm	Micrômetro/Micrometer
μmol l⁻¹	Micromolar/Micromolar

Sumário

SUMÁRIO

TITLE PAGE	30
ABSTRACT	31
INTRODUCTION	33
MATERIALS AND METHODS	35
RESULTS	43
DISCUSSION	46
ACKNOWLEDGEMENTS	51
REFERENCES	51
FIGURE CAPTIONS	59
ANEXOS	73

Effect of tyrosol on *Streptococcus mutans* biofilms

Running headline: Tyrosol against *Streptococcus mutans*

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*De acordo com as instruções aos autores do periódico Journal of Applied
Microbiology (Anexo A).

Abstract

Aim: the aim of this study was to evaluate the effect of tyrosol at different concentrations on the acid production and biofilm formation by *Streptococcus mutans*, as well as to quantify pre-formed biofilms of this species developed on hydroxyapatite (HA) surfaces and treated with tyrosol.

Methods and Results: *S. mutans* biofilms were formed on HA in the presence of different concentrations of tyrosol, during 48 h. Next, the acid production was assessed by pH determination, while the biofilm formation was determined through quantification of total biomass, metabolic activity and number of colony-forming units (CFUs). Moreover, pre-formed biofilms (24 h) were treated with tyrosol, twice a day for three days, and the antimicrobial activity was evaluated through quantification of total biomass, metabolic activity, number of CFUs and composition of biofilms' extracellular matrix. Data were analyzed by one-way ANOVA, followed by Tukey's and Holm-Sidak's tests ($\alpha = 0.05$). Scanning electron microscopy (SEM) observations were performed in order to analyze biofilms' structure. Tyrosol was not able to significantly reduce acid production by *S. mutans* biofilms. However, biofilms formed in the presence of tyrosol showed significant decreases in metabolic activity and number of CFUs. For pre-formed biofilms, in general, tyrosol did not promote significant reductions. SEM observations confirmed the results of CFU enumeration. **Conclusion:** Tyrosol showed better effects on biofilm formation than on pre-formed biofilm of *S. mutans*.

Significance and Impact of the Study: This is the first study showing the effect of tyrosol on *S. mutans* biofilms. These results may be useful in the development of topical therapies focused on controlling biofilm-associated oral diseases, such as dental caries.

Keywords: Antimicrobials, Biofilms, Hydroxyapatite, Quorum sensing, Streptococci, Tyrosol.

Introduction

Biofilms are defined as structured microbial communities attached to an inert or living surface and surrounded by an extracellular matrix into an aqueous medium (Costerton *et al.* 1999; Jakubovics and Kolenbrander 2010). The human oral cavity provides an environment with hard and soft tissues, which favors the formation of polymicrobial biofilms (Jakubovics and Kolenbrander 2010). The relationship among microorganisms within biofilms may enhance their resistance and chances of survival (De Sordi and Mühlshlegel 2009; Jakubovics and Kolenbrander 2010). In this sense, imbalances in these communities lead to the increase of pathogenic species (Spratt and Pratten 2003), contributing to the emergence of biofilm-related oral diseases such as dental caries. Yee and Sheiam (2002) reported that in third world countries, dental caries is the fourth most expensive disease to treat. Also, this oral disease affects approximately 80-90 % of developing countries (Petersen 2004).

Streptococcus mutans are Gram-positive bacteria regarded as the major causative agent of dental caries (Loesche 1986; Marsh 1999; Krzysciak *et al.* 2014), freely identified in saliva or located on teeth, mucosal surfaces and tongue dorsum (Liljemark and Gibbons, 1972). Their resistance to acidic environments and ability to produce an extracellular polysaccharide matrix are characteristics that confer a high degree of virulence to these bacteria (Loesche 1986; Krzysciak *et al.* 2014). In addition, some studies show that *S. mutans* is able to interact with other species in oral polymicrobial biofilms, such as *Candida albicans*, further increasing its virulence (Raja *et al.* 2010; Metwalli *et al.* 2013; Falsetta *et al.* 2014). Microbial biofilms, including those formed by *S. mutans*, have already developed resistance to some of the

conventional forms of treatment (Feverstein 2012; Taff *et al.* 2013; Jalal *et al.* 2015; Kouidhi *et al.* 2015), thereby contributing to disease chronicity.

To overcome the resistance problem, new strategies have been studied in order to control pathogenic biofilms. An alternative therapy involves the use of quorum sensing (QS) molecules (Weber *et al.* 2010). These molecules are secreted by the microbial cells in response to cell density within the biofilms, generating a cell-cell communication circuit that modifies the expression of target genes, resulting in the regulation of several intra- and inter-species physiological activities (biofilm formation, virulence, etc.) (Miller and Bassler 2001; Alem *et al.* 2006; Martins *et al.* 2007; De Sordi and Mühlischlegel 2009; Weber *et al.* 2010; Gori *et al.* 2011; Jeon *et al.* 2011).

Farnesol and tyrosol are the main QS molecules isolated from *C. albicans*. Farnesol is an aliphatic alcohol with antibiofilm activity against various species of fungi and bacteria (Ramage *et al.* 2002; Koo *et al.* 2003; Jeon *et al.* 2011). In *C. albicans*, this molecule inhibits the yeast-to-hyphae switch (Ramage *et al.* 2002; Weber *et al.* 2010). In contrast, tyrosol is an aromatic alcohol which induces the yeast-to-hyphae transformation (Chen *et al.* 2004; Chen and Fink 2006; Alem *et al.* 2006), but its effect on biofilms has been less studied. Shanmughapriya *et al.* (2014) found a synergistic antibiofilm effect of tyrosol in combination with amphotericin B against *C. krusei* and *C. tropicalis* isolated from intrauterine device users. Furthermore, it was recently demonstrated that high concentrations of tyrosol alone or combined with amphotericin B, itraconazole and fluconazole reduced biofilms formed by several strains of *C. albicans* and *C. tropicalis* (Cordeiro *et al.* 2015).

Although there are some reports regarding tyrosol activity on *Candida* biofilms, its effect on *S. mutans* biofilms remains unknown. Therefore, the objective of this study

was to evaluate the effect of tyrosol at different concentrations on the acid production and biofilm formation by *S. mutans*, as well as to quantify pre-formed biofilms of this species developed on hydroxyapatite surfaces and treated with tyrosol. The tested hypothesis was that tyrosol has an inhibitory effect on *S. mutans* biofilms.

Materials and methods

Tyrosol and artificial saliva

Tyrosol (2-[4-hydroxyphenyl] ethanol) was purchased from Sigma-Aldrich (St Louis, USA) and working solutions of this compound were directly prepared in artificial saliva (AS; pH 6.8). AS was based on the protocol of Lanfom *et al.* (2003), and its composition for 1 l of deionized water was as follows: 2 g yeast extract (Sigma-Aldrich), 5 g bacteriological peptone (Sigma-Aldrich), 2 g glucose (Sigma-Aldrich), 1 g mucin from porcine stomach (Sigma-Aldrich), 0.35 g NaCl (Sigma-Aldrich), 0.2 g CaCl₂ (Sigma-Aldrich) and 0.2 g KCl (Sigma-Aldrich).

Strain and growth conditions

A reference strain of American Type Culture Collection (ATCC) was used in this study: *Streptococcus mutans* ATCC 25175. *S. mutans* was cultivated on Brain Heart Infusion Agar (BHI; Difco, Le Pont de Claix, France) in 5 % CO₂ at 37 °C. Afterwards, *S. mutans* colonies were inoculated in 10 ml BHI broth (Difco) and incubated statically overnight (18 h) in 5 % CO₂ at 37 °C. After the incubation period, the bacterial cells

were harvested by centrifugation (6500 g, 5 min) and washed twice in phosphate buffered saline (PBS; pH 7, 0.1 mol l⁻¹). *S. mutans* cells were adjusted spectrophotometrically to 1 x 10⁸ cells in AS (Optical density_{640 nm} = 1.6) (Anexo B).

Cell surface hydrophobicity

An automatic goniometer (Kruss DSA, 100S, Kruss GMBH, Hamburg, Germany) was employed to determine the cell surface hydrophobicity through the sessile drop method, using the technique of Busscher *et al.* (1984b) (Anexo C). Briefly, cell suspensions of *S. mutans* 25175 were resuspended in PBS (pH 7, 0.1 mol l⁻¹) to a concentration of 1 x 10⁹ cells ml⁻¹ and filtered under vacuum using cellulose acetate membranes (0.22 µm). These membranes were cut in three pieces, deposited on glass slides and inserted into petri dishes containing 1 % agar and 10 % glycerol (Busscher *et al.* 1984b). After 3.5 h, the contact angles were measured by deposition of five drops (3 µl each drop) of deionized water on the cell layers deposited on each membrane piece. The experiments were carried out on three different occasions at 22 ± 2 °C.

Determination of tyrosol minimum inhibitory concentration (MIC) against planktonic cells

The broth microdilution method was applied to determine the MIC of tyrosol (Anexo D). In short, cell suspensions of *S. mutans* were standardized to a concentration of 0.5 of McFarland standard in saline solution, and subsequently diluted in BHI broth. Tyrosol was diluted in deionized water in geometric progression, and after, diluted in BHI broth

to yield concentrations varying from 0 to 300 mmol l⁻¹. Next, 100 µl of each cell suspension + 100 µl of each tyrosol concentration were pipetted into wells of a 96-well microplate (Costar, Tewksbury, USA). The plate was incubated at 37 °C for 48 h, and then the MIC was visually defined as the lowest concentration inhibiting the growth of microorganisms. Chlorhexidine gluconate (CHG) was used as positive control (PC). Also, the contents of the wells (from the MIC endpoint) were plated on BHI agar to determine the minimum bactericidal concentration (MBC). All assays were performed in triplicate on three independent occasions.

Confection and characterization of hydroxyapatite (HA) specimens

The HA specimens were obtained through the pressing of 0.650 g HA powder (Sigma-Aldrich) inside a stainless steel matrix (13 mm diameter), between two steel tablets with smooth surfaces. The matrix was taken to a hydraulic press (SKAY, São José do Rio Preto, Brazil) and submitted to a load of 2.5 t. Next, the matrix parts were separated and the HA specimen (a disc with 13 mm diameter x 3 mm thickness) was released from the matrix (Anexo E).

In order to select specimens with similar surface patterns for biofilm assays, HA specimens had their surface roughnesses (Ra - µm) measured through a profilometer (Mitutoyo SJ-401; Mitutoyo Corp., Tokyo, Japan) (Monteiro *et al.* 2015). Three measurements for each specimen were obtained using a cut-off of 0.25 mm and speed of 0.5 mm s⁻¹. Moreover, the surface hydrophobicity of these specimens was also characterized by using an automatic goniometer (Kruss DSA 100S, Kruss GMBH, Hamburg, Germany) (Busscher *et al.* 1984a). Three drops of deionized water were

deposited at distinct locations on each specimen surface and the contact angle (θ) values were obtained (Anexo F). The assays were carried out at 22 ± 2 °C. Lastly, all specimens were rinsed three times in deionized water, dried at 37 °C for 3 h and sterilized by irradiation (^{60}Co ; 25 KGy) before microbiological assays.

Effect of tyrosol on acid production by *S. mutans*

Specimens of HA were placed into 24-well microtiter plates (Costar, Tewksbury, USA) containing 1 ml of *S. mutans* inoculum (10^8 cells ml^{-1} in AS). After incubation in 5 % CO_2 at 37 °C for 2 h (adhesion phase), AS medium was removed and the wells were gently washed with 1 ml of PBS in order to remove non-adherent cells. Tyrosol was diluted in AS at concentrations of 11.25 and 22.5 mmol l^{-1} (corresponding to 1/8 and 1/4 of the MIC determined for planktonic cells). Each tyrosol solution was then added to the wells containing the adhered cells on the surface of the specimens. Plates were incubated at 37 °C in 5 % CO_2 to form biofilms during 48 h. Tyrosol-containing AS was renewed every 24 h. Acid production was assessed by the determination of the pH of the treatment solutions, both prior to treatment and after 48 h (Hasan *et al.* 2012) (Anexo G). Positive and negative controls were CHG at 0.45 $\mu\text{mol l}^{-1}$ (1/4 of MIC) and AS without tyrosol, respectively.

Effect of tyrosol on biofilm formation

Single biofilms of *S. mutans* ATCC 25175 were formed on the surfaces of HA specimens within 24-well plates (Costar). One milliliter of the standardized cell

suspension (1×10^8 cells ml^{-1} in AS) was added to the wells containing the HA specimens. The plates were incubated in 5 % CO_2 at 37 °C for 2 h to allow the cell adhesion to surfaces. Afterwards, the AS medium was removed and the specimens were gently washed with 1 ml of PBS (pH 7, 0.1 mol l^{-1}). Tyrosol was then diluted in AS at 50, 100 and 200 mmol l^{-1} , inoculated into the wells containing the specimens with adhered cells, and the plates incubated at 37 °C for 48 h to enable biofilm formation. After 24 h, 500 μl of AS medium with tyrosol was removed and an equal volume of fresh AS with tyrosol was added. CHG at 490 $\mu\text{mol l}^{-1}$ (272 x MIC) was used as PC. HA specimens inoculated with AS without tyrosol were used as negative controls (NC).

Quantification of total biofilm biomass. Total biofilm biomass was quantified by crystal violet (CV) staining assay (Monteiro *et al.* 2013) (Anexo H). In brief, after 48 h of biofilm formation in the presence of tyrosol, the resultant biofilms were fixed with 1 ml of 99 % (v v⁻¹) methanol (Sigma-Aldrich) during 15 min and stained with 1 % CV (v v⁻¹, Sigma-Aldrich) for 5 min. Next, 1 ml of acetic acid (33 % v v⁻¹, Sigma Aldrich) was added to release CV from the biofilms. The absorbance of the solutions was determined in a microplate reader (Eon Microplate Spectrophotometer; BioTek, Winooski, USA) at 570 nm wave length, and standardized according to the specimens' surface area (absorbance cm^{-2}).

Quantification of metabolic activity. Metabolic activity of biofilm cells was colorimetrically quantified by the XTT (2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Sigma-Aldrich) reduction assay (Silva *et al.* 2010) (Anexo I). Thus, 1 ml of a solution comprising 150 mg XTT l^{-1} and

10 mg of phenazine methosulphate l^{-1} (Sigma-Aldrich) was added to each well containing the HA specimens with biofilms (48 h). The plates were incubated (120 rev min^{-1}) in the dark at 37°C for 3 h and then aliquots of 200 μl of the supernatant were transferred to 96-well microplates and the absorbance was read at 490 nm. The absorbance values were standardized per unit area of specimens (Abs cm^{-2}), and the blanks were defined as wells containing specimens immersed in AS without microorganisms. All assays were repeated in triplicate on three different occasions.

Quantification of cultivable cells. The number of cultivable cells from biofilms formed on HA specimens, in the presence of tyrosol, was assessed by enumeration of colony-forming units (CFUs) (Anexo J). The resultant biofilms were washed once with 1 ml of PBS ($\text{pH } 7, 0.1 \text{ mol } l^{-1}$), inserted in falcon tubes containing PBS and then sonicated at 30 W for 30 s. Biofilm suspensions were vigorously vortexed for 90 s and serial decimal dilutions (in PBS; $\text{pH } 7, 0.1 \text{ mol } l^{-1}$) were plated on BHI agar. After 24-48 h of incubation at 37°C , the number of CFUs was counted, expressed in \log_{10} and standardized per unit area ($\text{Log}_{10} \text{ CFU cm}^{-2}$) of HA specimens. The assays were carried out in triplicate on three different occasions.

Effect of tyrosol on pre-formed biofilms

Single biofilms of *S. mutans* ATCC 25175 were allowed to grow on HA specimens into 24-well plates containing 1 ml of inoculum, as described above. Plates were incubated at 37°C in 5 % CO_2 , for 24 h. Following this, pre-formed biofilms were treated twice/day (9 a.m. and 3 p.m.), for 1 min (Koo *et al.* 2005) with tyrosol diluted in AS at

concentrations of 100 and 200 mmol l⁻¹, for 3 days. At the end of the experiment (96-h biofilms), AS was removed and the wells were washed with PBS to remove loosely attached cells. Total biofilms biomass, metabolic activity and number of CFUs were quantified as described above. PC and NC were CHG at 490 µmol l⁻¹ and AS without tyrosol, respectively.

Composition of extracellular matrix of pre-formed biofilms

For this analysis, *S. mutans* ATCC 25175 biofilms were grown on the bottom of the wells of a 24-well plate, instead of growing on the surface of HA specimens, in order to obtain larger amounts of biomass. Each well contained 1 ml of inoculum, and biofilms were treated with tyrosol diluted in AS at 200 mmol l⁻¹, besides 490 µmol l⁻¹ CHG and AS without tyrosol, as described above.

Extraction of extracellular matrix. After a 3-day treatment period, 96-h biofilms were scraped from the bottom of the wells and re-suspended in PBS. Biofilm suspensions were sonicated at 30 W for 30 s and subsequently vortexed for 120 s. Following this, suspensions were centrifuged at 3000 g for 10 min and the supernatants were filtered in nitrocellulose filters (0.22 µm) (Monteiro *et al.* 2014) (Anexo K).

Determination of biofilms' dry weight. A sample of each scraped biofilm was vacuum filtered on pre-weighed cellulose acetate membrane (0.22 µm) and dried at 60 °C until a constant weight was achieved (Monteiro *et al.* 2013) (Anexo K). Biofilm's dry weight was then determined by subtracting final weight of the membrane from the initial value.

Quantification of protein and carbohydrate in the extracellular matrix. The protein content of the extracellular matrix was quantified by the bicinchoninic acid method (BCA kit; Sigma-Aldrich), using BSA as the standard (Monteiro *et al.* 2013). Briefly, 25 μ l of the supernatants obtained after biofilm centrifugation were transferred to individual wells of a 96-well plate containing 200 μ l of the mixture of reagents A and B from BCA kit (Anexo K). Plates were incubated at 37 °C for 30 min and the absorbance was read at 562 nm.

The carbohydrate content of the extracellular matrix was determined according to Dubois *et al.* (1956), using glucose as the standard. Supernatants of the centrifuged biofilms (500 μ l) were mixed with 500 μ l of 9 % phenol (Sigma-Aldrich) and 2.5 ml of 95-98 % sulfuric acid (Sigma-Aldrich) in glass test tubes. Solutions were homogenized in vortex and allowed to rest for 15 min (Anexo K). The absorbance was read at 490 nm. BSA and glucose were used in the calibration curves for protein and carbohydrate analyses, respectively. The results were expressed as a function of the biofilms' dry weight (mg g^{-1} dry weight).

Structural analysis of the biofilms

In order to analyze the structure of *S. mutans* ATCC 25175 biofilms formed in the presence of tyrosol and pre-formed biofilms treated with this compound, scanning electron microscopy (SEM) was performed (Anexo L). After each experimental period, all biofilms were rinsed with PBS, as previously described. For imaging, biofilms were dehydrated with ethanol in three steps (70 % for 10 min, 95 % for 10 min and 100 % for 20 min) and air dried in a desiccator (Monteiro *et al.* 2015). Samples were mounted on

aluminum stubs, coated with gold and then observed in a scanning electron microscope (Electron Microscope FEG-VP Supra 35, Carl Zeiss, Jena, Thuringen, Germany).

Statistical analysis

The type of treatment (CHG and different concentrations of tyrosol) was considered as variation factor. Data passed the normality test (Shapiro-Wilk) and were submitted to one-way ANOVA, followed by Tukey's and Holm-Sidak's tests (SigmaPlot 12.0 software, Systat Software Inc., San Jose, USA). All tests were performed with a significance level of 5 %.

Results

Characterization of cell and specimen surfaces

S. mutans ATCC 25175 showed mean (SD) contact angle of 17.82° (1.02), characterizing a hydrophilic behavior ($\theta < 65^\circ$) (Vogler 1998). Concerning the surface roughness, HA specimens presented mean (SD) value of $0.06\ \mu\text{m}$ (0.01). Moreover, the low contact angles indicated hydrophilic HA surfaces ($9.55^\circ \pm 2.73$).

MIC and MBC

MIC and MBC values of tyrosol were the same ($90\ \text{mmol l}^{-1}$) and a thousand-fold higher than those observed for CHG (Table 1).

Determination of acid production by *S. mutans*

Treatments with tyrosol at 11.25 and 22.50 mmol l⁻¹ were not able to significantly reduce acid production by *S. mutans* ATCC 25175 biofilms developed on HA specimens (Table 2).

Effect of tyrosol on biofilm formation

S. mutans ATCC 25175 biofilm formed on HA in the presence of different concentrations of tyrosol showed no significant differences in biomass when compared to the controls (Fig. 1).

The results of XTT reduction for single biofilms of *S. mutans* ATCC 25175 (Fig. 2) demonstrated that the treatments with tyrosol and CHG promoted significant reductions (ranging from 23.4 % to 85.5 %; $p < 0.001$) in the metabolic activity of biofilm cells, compared with the NC. In short, tyrosol at 100 and 200 mmol l⁻¹ led to better results than at 50 mmol l⁻¹, and behaved similarly to CHG.

Regarding CFU enumeration (Fig. 3), the highest decreases in the number of CFUs were achieved in the presence of CHG and tyrosol at 200 mmol l⁻¹ (4.4-log₁₀ and 4.54-log₁₀, respectively; $p < 0.001$), when compared to the NC group. Tyrosol at 50 and 100 mmol l⁻¹ was also able to significantly reduce the number of CFUs ($p = 0.003$).

Effect of tyrosol on pre-formed biofilms

None of the treatments was able to promote significant reductions on total biomass of pre-formed biofilms (Fig. 4).

For XTT reduction assay (Fig. 5), the treatments with CHG and tyrosol at 200 mmol l⁻¹ promoted significant reductions in metabolic activity of 71.4 % (p < 0.001) and 40 % (p = 0.015) when compared with the negative control, respectively.

However, CFU enumeration assay (Fig. 6) showed that only CHG was able to promote significant reduction in the number of cultivable cells when compared with the negative control, with a reduction of 1.8-log₁₀ (p = 0.002).

Analysis of the composition of biofilms' extracellular matrix

Treatment with tyrosol at 200 mmol l⁻¹ promoted a significant increase in the protein content of *S. mutans* ATCC 25175 biofilm when compared with the negative control (Table 3). For carbohydrate content, no significant differences were observed among the treatments.

Structural analysis of the biofilms

S. mutans biofilm formed in the presence of tyrosol at 50 mmol l⁻¹ (Fig. 7c) showed a compact structure consisting of a network of bacterial cells covering the surface (Fig. 7a), similar to that observed for the untreated biofilm (Fig. 7a). Biofilms formed in the presence of CHG (Fig. 7b) and tyrosol at 100 (Fig. 7d) and 200 mmol l⁻¹ (Fig. 7e) revealed less compact structures and lower amounts of bacterial cells, when compared to the NC group. These resultant biofilms consisted of clusters of bacterial cells

partially covering the surfaces. The major disruption was visible for the biofilm treated with CHG (Fig. 7b).

Untreated pre-formed biofilm exhibited a dense network of cocci embedded on a thin extracellular matrix covering the surface of HA (Fig. 8a). None of the treatments seemed to affect the biofilm structure, except for the biofilm treated with CHG (Fig. 8b), which presented a slightly less compact structure and a less amount of cells, when compared to the NC.

Discussion

Biofilm resistance is a major clinical concern (Mah and O'Toole 2001; Taff *et al.* 2013; Jalal *et al.* 2015), which has motivated the scientific community to develop novel agents capable of combating important biofilm-associated oral diseases, such as dental caries (Koo *et al.* 2003; Feverstein 2012). In this regard, the use of QS molecules has generated good prospects when directed to the control of certain bacteria and fungi (Ramage *et al.* 2002, Koo *et al.* 2003; Weber *et al.* 2010; Jeon *et al.* 2011). Given that *S. mutans* consists in major oral pathogen responsible for the development of the above-mentioned disease, the present study investigated the effect of tyrosol on the acid production and biofilm formation by *S. mutans*, as well as on pre-formed biofilms of this species developed on HA surfaces. The study's hypothesis was partially accepted since tyrosol significantly influenced some of the parameters assessed.

Regarding the effect of QS molecules on planktonic cells, different MIC values of tyrosol and farnesol for several *Candida* and *Streptococcus* species have been reported, ranging from 40 $\mu\text{mol l}^{-1}$ to 5 mmol l^{-1} (Koo *et al.* 2002; Jabra-Rizk *et al.*

2006; Shanmughapriya *et al.* 2014; Cordeiro *et al.* 2015). Comparing with the above-mentioned studies, the MIC value observed for tyrosol in the present study can be considered high (Table 1). The discrepant MIC results found in these studies might be explained by different susceptibility profiles of the various strains and different protocols for determining the MIC. Further, in the current study, tyrosol was directly diluted in BHI broth instead of the initial dilution in ethanol/methanol performed in most of other studies (Koo *et al.* 2002; Jabra-Rizk *et al.* 2006). The dilution in ethanol/methanol may have favored the antimicrobial effect by the synergistic action of these alcohols with tyrosol/farnesol.

In order to mimic clinical conditions related to dental caries, HA specimens were employed for the *in vitro* biofilm formation. All specimens were standardized by roughness and hydrophobicity because these properties can interfere with the adhesion and biofilm formation processes. Usually, surfaces with higher roughness and hydrophobicity degree (Bulad *et al.* 2004; Das *et al.* 2011), as well as higher cell surface hydrophobicity values (Li *et al.* 2003) favor microbial adhesion. Following this rationale, it would be expected a poor biofilm formation due to low roughness and contact angle values found in this study. However, the quantification assays and SEM observations showed that *S. mutans* ATCC 25175 was able to form biofilms on HA surfaces. The cell adherence and the subsequent biofilm formation are also related to the connection of adhesins of the cell wall or membrane to specific cell receptors (Cotter and Kavanagh 2000).

Considering that the virulence factors have been used as a target for the development of antimicrobial therapies (Perfect 1996), the present study assessed the effect of tyrosol on acid production by *S. mutans* ATCC 25175 biofilms, but this QS

molecule at sub-inhibitory concentrations was shown to be ineffective in this regard (Table 2).

In contrast, the evaluation of XTT reduction demonstrated that the treatments with tyrosol and CHG were very effective in reducing the metabolic activity of *S. mutans* ATCC 25175 biofilms (Fig. 2). Tyrosol at 100 and 200 mmol l⁻¹ displayed higher reductions than at 50 mmol l⁻¹, and behaved similarly to CHG (Fig. 2). Interestingly, the treatment with tyrosol at 200 mmol l⁻¹ resulted in the highest decrease in the number of CFUs, with significant differences between this treatment and the others (Fig. 3). SEM observations are in line with these results and revealed less compact structures for biofilms formed in the presence of CHG and tyrosol at 100 and 200 mmol l⁻¹ (Fig. 7). On the other hand, the results of total biomass showed no reductions caused by the tyrosol treatments (Fig. 1). To explain this issue, it should be highlighted that the CV assay quantifies both living and dead cells plus extracellular matrix. Thus, this method must always be used as a complement to the XTT and CFU assays.

According to the literature, CHG's mechanism of action starts with damage to the outer cell layers of the bacterium or yeast (El-Moug *et al.* 1985), with subsequent attacks on the cytoplasmic membrane and leakage of intracellular components (Hiom *et al.* 1993). However, the mechanism of action of tyrosol is unknown. It has been hypothesized that tyrosol also might act on the integrity of the cell membrane (Monteiro *et al.* 2015), based on a study with *Staphylococcus aureus*, where a relationship between the K⁺ ion loss and the antimicrobial activity of terpene alcohols had been established (Inoue *et al.* 2004).

To evaluate the effect of tyrosol against pre-formed biofilms, the tyrosol concentrations with better results on *S. mutans* biofilm formation (100 mmol l⁻¹ and 200 mmol l⁻¹) were selected. The results of total biomass (Fig. 4) and CFU quantification (Fig. 6) are in line with SEM observations (Fig. 8), and demonstrate that tyrosol failed to promote significant reductions in pre-formed biofilms. Mature biofilms, as assessed in the present investigation (96 h), create an environment that enhances antimicrobial resistance. Among the resistance mechanisms, the presence of extracellular matrix protects the embedded cells and impairs drug penetration in deeper layers of the biofilm (Lewis 2001; Mah and O'Toole 2001; Sutherland 2001). Moreover, the expression of resistant genes, the high cellular density within the biofilm, as well as the presence of persistent cells are factors that might help to explain the lack of effect of tyrosol against the *S. mutans* pre-formed biofilms (Mah and O'Toole 2001; Jabra-Rizk *et al.* 2004; Kuhn and Ghannoum 2004).

On the other hand, the treatment with tyrosol at 200 mmol l⁻¹ caused significant reduction in the metabolic activity of *S. mutans* pre-formed biofilm. (Fig. 5). This result demonstrates that despite 200 mmol l⁻¹ tyrosol was not able to reduce the number of CFUs (Fig. 6), it did reduce the metabolic activity, indicating that the cells within the biofilm remained alive, but with reduced metabolism. It is possible that this reduction in cell metabolism could lead to a decreased virulence, besides reducing the potential of infection of other areas when these cells are released from the biofilm.

In the analysis of extracellular matrix composition, despite tyrosol was shown to have no effect on carbohydrate content, it significantly increased the protein content of extracellular matrix (Table 3). Such increase, however, is not coherent with the results of total biomass and metabolic activity for pre-formed biofilms (Figs. 4 and 5). The

reasons for these discrepant results are not apparent, and therefore should be further investigated. In addition, it must be pointed out that results of extracellular matrix composition cannot be directly compared with total biofilm biomass, given that biofilm used for this assay was formed on 24-well plates (instead of HA specimens); this modification was necessary in order to provide higher amounts of biofilm needed for protein and carbohydrate determination.

Overall, CHG promoted significant reductions on biofilm metabolic activity and CFU enumeration at a lower concentration when compared with tyrosol. Despite other antimicrobials possibly being more effective than tyrosol (a similar effect with a lower concentration), CHG presents some disadvantages and side-effects (Karpiński and Szkaradkiewicz 2015). Because of this, new compounds could potentially be used as substitutes to or in combination with traditional drugs (Jabra-Rizk *et al.* 2006, Shanmughapriya *et al.* 2014; Cordeiro *et al.* 2015). This possibility is supported by published data that evaluated the cytotoxicity of tyrosol in different cells (Babich and Visioli 2003; Loru *et al.* 2009; Anter *et al.* 2014) showing that apart from not being toxic against kidney (Loru *et al.* 2009) and salivary gland (Babich and Visioli 2003) cells, tyrosol has an antigenotoxic effect (Anter *et al.* 2014).

In conclusion, tyrosol showed better effects on biofilm formation than on pre-formed biofilm, and this QS molecule was not able to reduce the acid production by *S. mutans* ATCC 25175 biofilms. Finally, future studies addressing the cytotoxic effect of tyrosol at the concentrations used in the current work, as well as its action in combination with conventional drugs against a larger number of strains must be conducted. The completion of novel research with higher clinical reproducibility might

collaborate in the development of tyrosol-containing formulations that may prevent and/or control oral diseases such as dental caries.

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Conflict of interest

No conflict of interest declared.

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Figure captions

Fig. 1. Mean absorbance values per cm^2 obtained with crystal violet staining assay for single biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite specimens in the presence of different concentrations of tyrosol. NC: negative control (biofilm formed without tyrosol); PC: positive control (490 $\mu\text{mol l}^{-1}$ chlorhexidine gluconate); 50T, 100T and 200T: tyrosol at 50, 100 and 200 mmol l^{-1} , respectively. Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups ($p < 0.05$, using one-way ANOVA).

Fig. 2. Mean absorbance values per cm^2 obtained with XTT reduction assay for single biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite specimens in the presence of different concentrations of tyrosol. NC: negative control (biofilm formed without tyrosol); PC: positive control (490 $\mu\text{mol l}^{-1}$ chlorhexidine gluconate); 50T, 100T and 200T: tyrosol at 50, 100 and 200 mmol l^{-1} , respectively. Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups ($p < 0.05$, using one-way ANOVA with Holm-Sidak post-hoc test).

Fig. 3. Mean values of the logarithm of colony forming units per cm^2 (\log_{10} CFU cm^{-2}) obtained for single biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite specimens in the presence of different concentrations of tyrosol. NC: negative control (biofilm formed without tyrosol); PC: positive control (490 $\mu\text{mol l}^{-1}$ chlorhexidine gluconate); 50T, 100T and 200T: tyrosol at 50, 100 and 200 mmol l^{-1} , respectively. Error bars indicate standard deviations of the means. Different letters represent

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Fig. 4. Mean absorbance values per cm^2 (crystal violet) for pre-formed biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite, and treated with tyrosol at 100 mmol l^{-1} (100T) and 200 mmol l^{-1} (200T). NC: negative control (non-treated biofilm); PC: positive control ($490 \mu\text{mol l}^{-1}$ chlorhexidine gluconate). Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups ($p < 0.05$, using one-way ANOVA).

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Fig. 7. Scanning electron micrographs of *S. mutans* ATCC 25175 biofilms formed on hydroxyapatite in the presence of different concentrations of tyrosol. NC (a) = negative control; PC (b) = positive control (490 $\mu\text{mol l}^{-1}$ chlorhexidine gluconate); tyrosol at 50 (c), 100 (d) and 200 mmol l^{-1} (mM) (e). Magnification: x 5000. Bar: 5.0 μm .

Fig. 8. Scanning electron micrographs of pre-formed biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite and treated with tyrosol at 100 mmol l^{-1} (mM) (c) and 200 mmol l^{-1} (mM) (d). NC (a) = negative control; PC (b) = positive control (490 $\mu\text{mol l}^{-1}$ chlorhexidine gluconate). Magnification: x 5000. Bar: 5.0 μm .

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of tyrosol and chlorhexidine gluconate against the tested strain.

Species	Tyrosol		Chlorhexidine gluconate	
	MIC (mmol l ⁻¹)	MBC (mmol l ⁻¹)	MIC (μmol l ⁻¹)	MBC (μmol l ⁻¹)
<i>S. mutans</i> ATCC 25175	90	90	1.8	7.4

Table 2. Mean (SD) pH values obtained with the acid production assay by single biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite in the presence of tyrosol at 11.25 mmol l⁻¹ (11.25T) and 22.50 mmol l⁻¹ (22.50T). NC: negative control (non-treated biofilm); PC: positive control (0.45 μmol l⁻¹ chlorhexidine gluconate).

Treatments	Initial pH	Final pH
NC	7.0 (0.19)	4.7 (0.09) ^A
PC	7.0 (0.10)	4.7 (0.07) ^A
11.25T	6.8 (0.05)	4.6 (0.08) ^A
22.50T	6.9 (0.19)	4.7 (0.06) ^A

Different letters represent significant differences among the treatments (one-way ANOVA, $p < 0.05$)

Table 3. Mean (SD) protein and carbohydrate contents (from extracellular matrix) for *S. mutans* ATCC 25175 biofilms obtained after treatment with tyrosol at 200 mmol l⁻¹ (200T). NC: negative control (non-treated biofilm); PC: positive control (490 µmol l⁻¹ chlorhexidine gluconate).

Biofilm	Protein (mg g ⁻¹ of biofilm dry weight)			Carbohydrate (mg g ⁻¹ of biofilm dry weight)		
	Treatments			Treatments		
	NC	PC	200T	NC	PC	200T
<i>S. mutans</i>	5.58	3.02	13.03	39.22	53.84	30.48
ATCC 25175	(3.31) ^a	(2.62) ^a	(2.37) ^b	(10.44) ^A	(12.16) ^A	(3.18) ^A

Different lowercase and uppercase letters represent significant differences among the treatments, respectively for protein and carbohydrate contents (one-way ANOVA and Tukey's test, $p < 0.05$)

Fig. 1. Mean absorbance values per cm^2 obtained with crystal violet staining assay for single biofilms of *S. mutans* ATCC 25175 formed on hydroxyapatite specimens in the presence of different concentrations of tyrosol. NC: negative control (biofilm formed without tyrosol); PC: positive control (490 $\mu\text{mol l}^{-1}$ chlorhexidine gluconate); 50T, 100T and 200T: tyrosol at 50, 100 and 200 mmol l^{-1} , respectively. Error bars indicate standard deviations of the means. Different letters represent significant differences among the groups ($p < 0.05$, using one-way ANOVA).

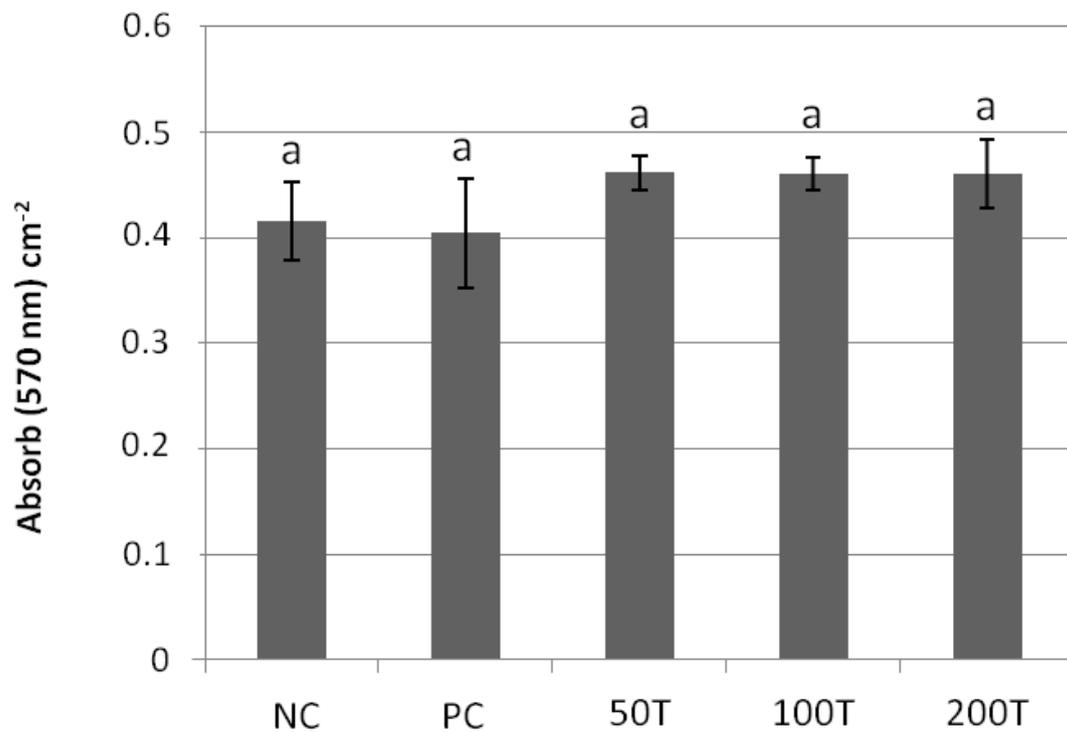


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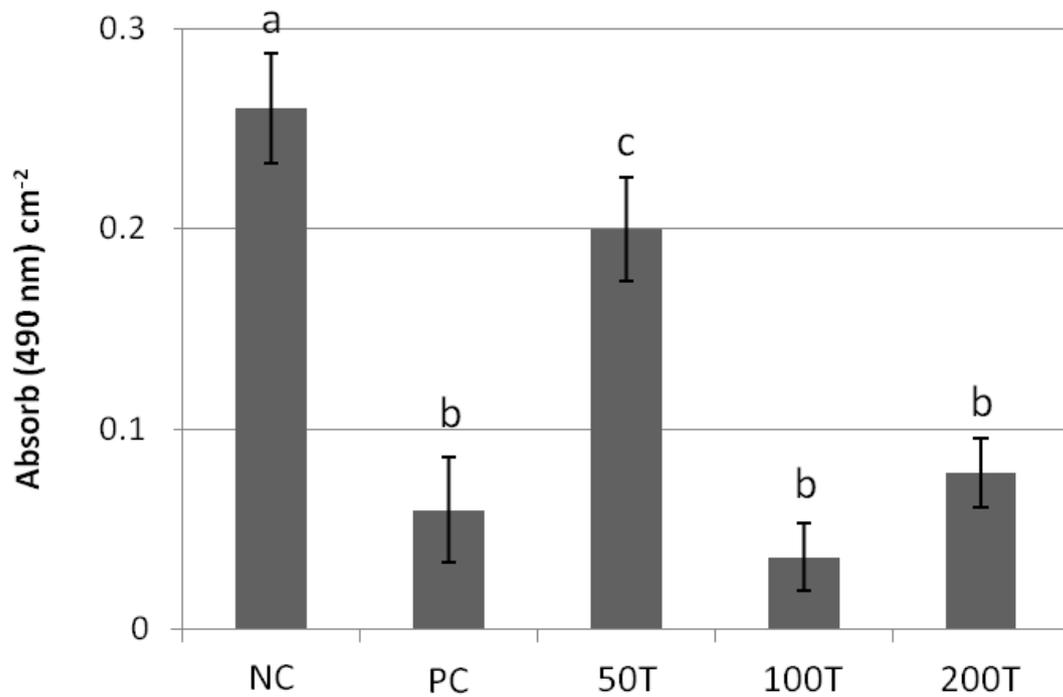


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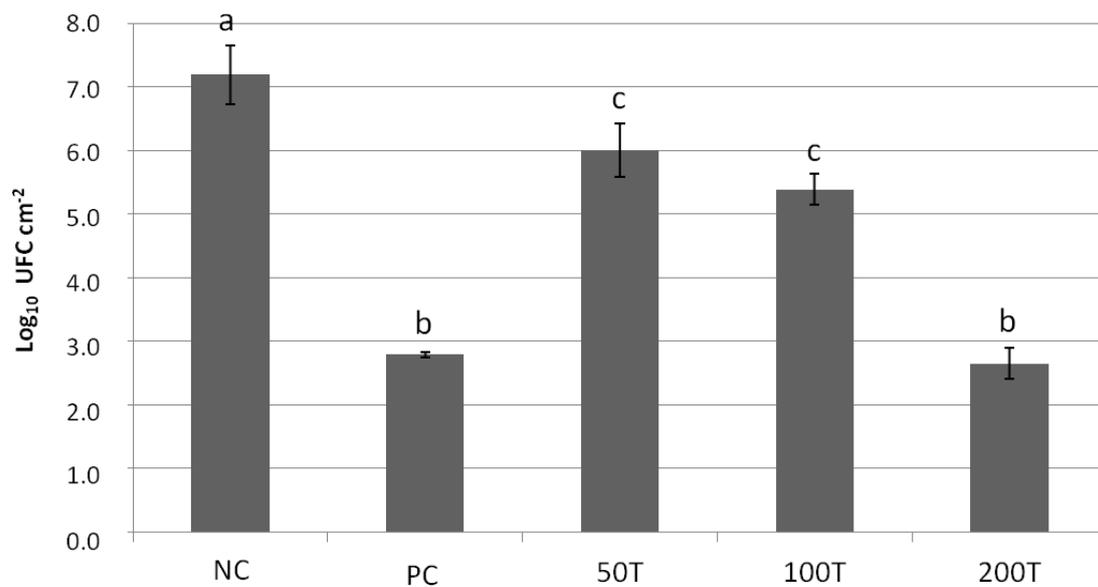


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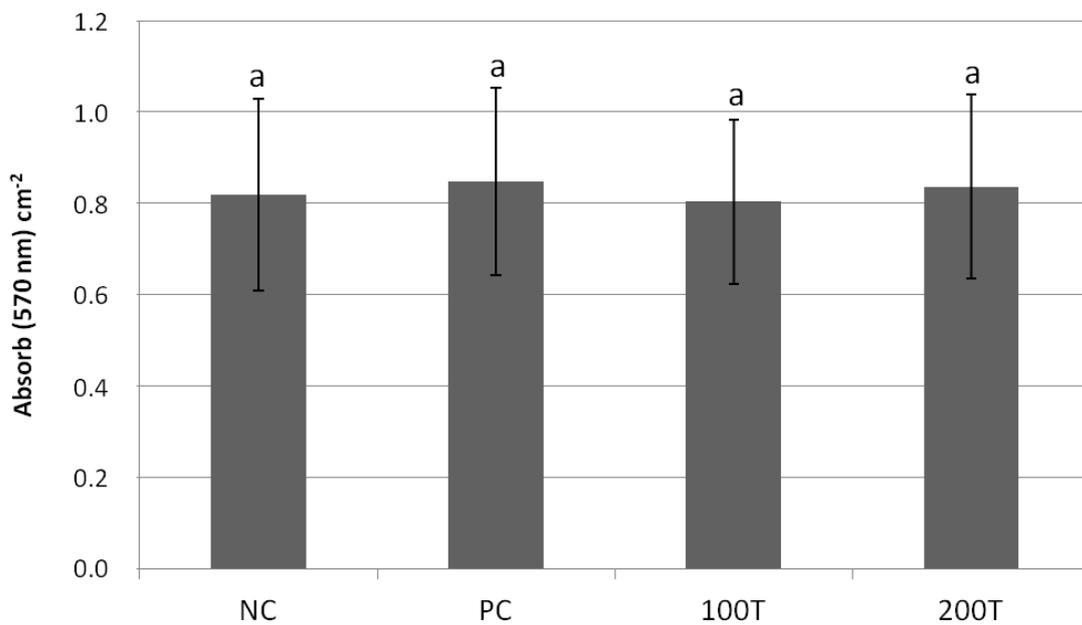


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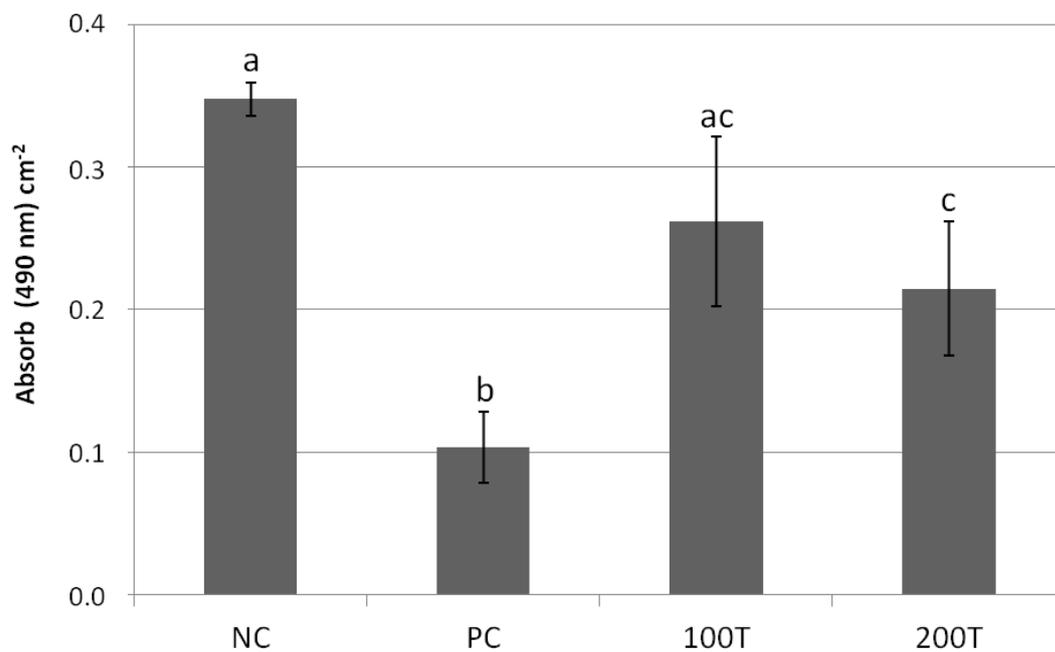


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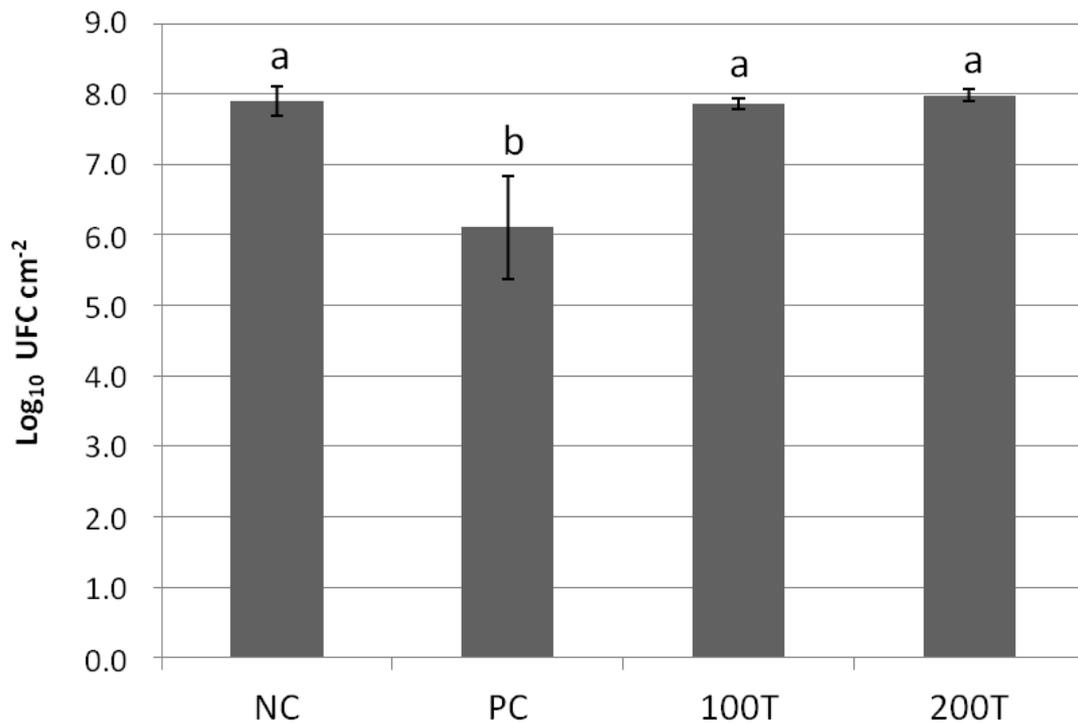


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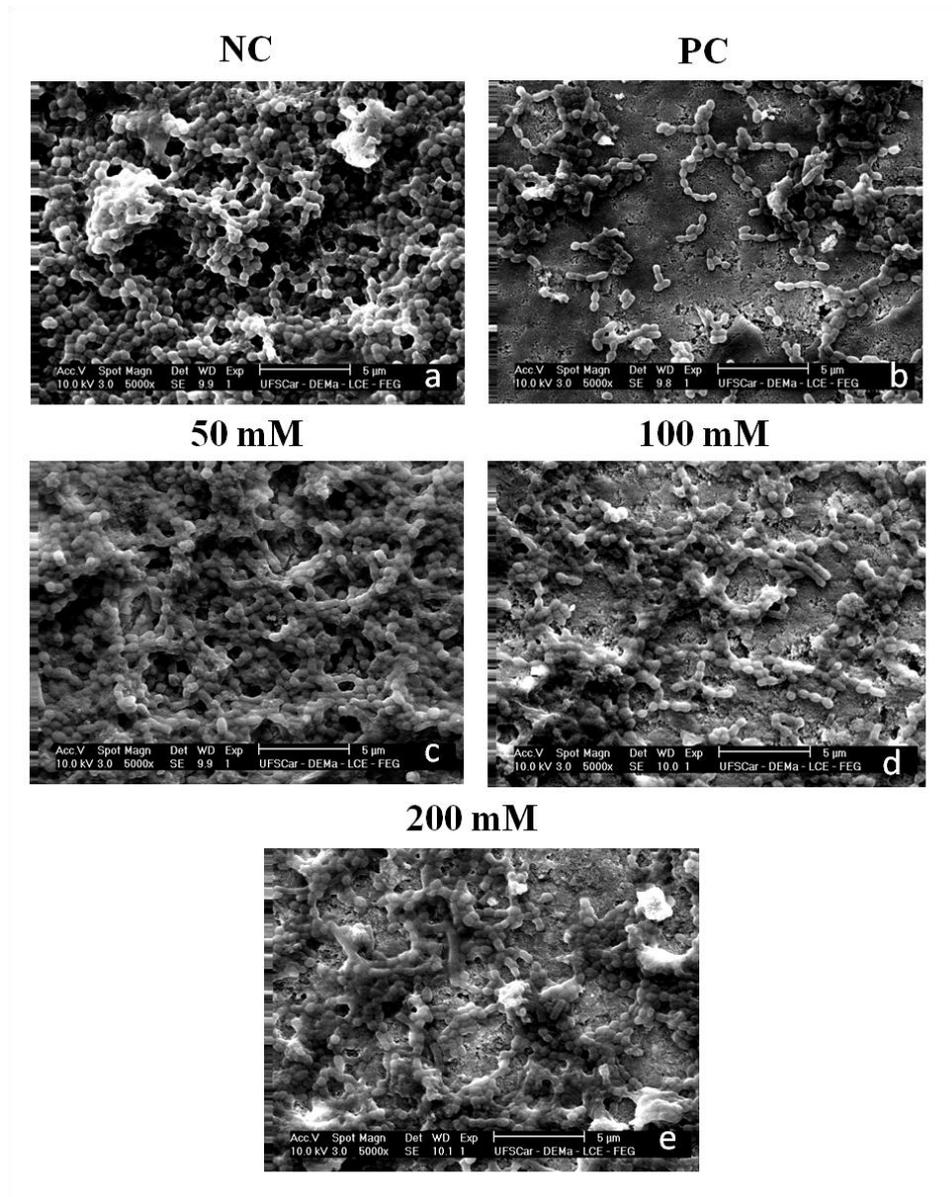
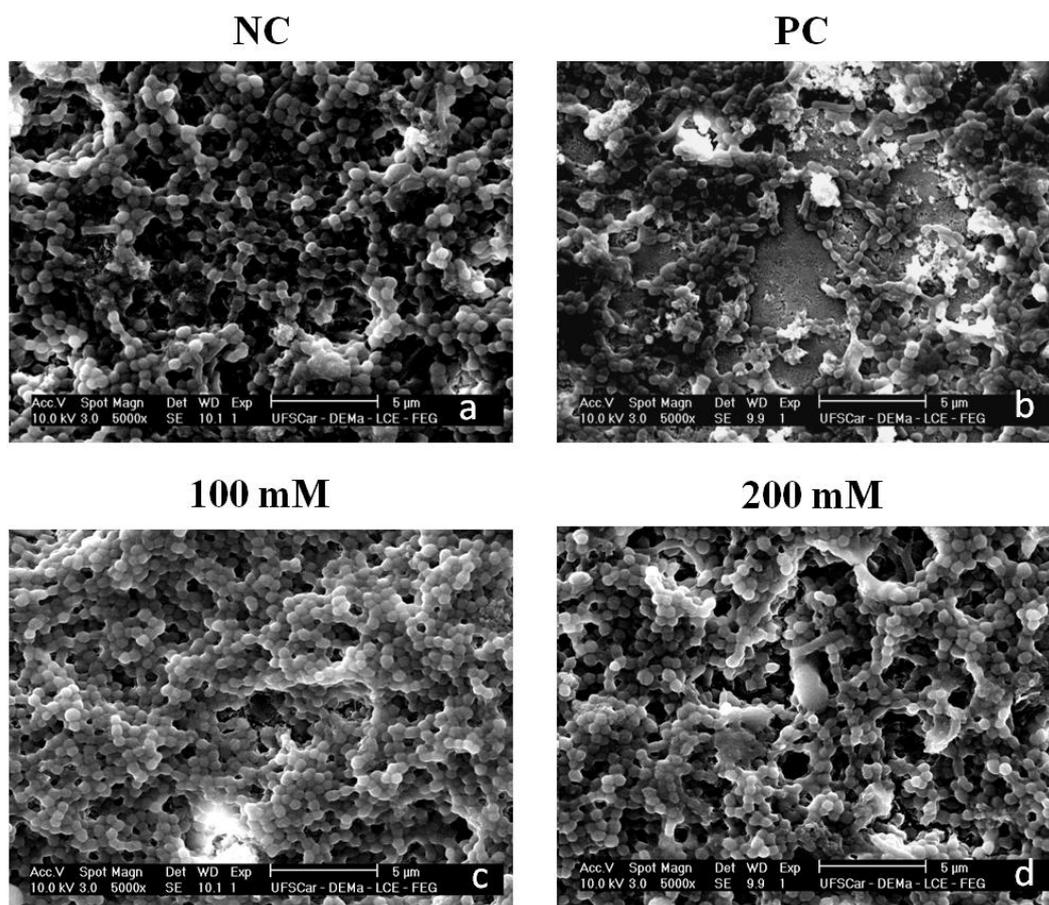


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Anexos

ANEXO A
INSTRUÇÕES AOS AUTORES

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Antibiotic antimicrobial testing and microbial resistance

A number of methods like disc diffusion, Etest, agar dilution, broth microdilution and broth macrodilution, are suitable for *in vitro* antimicrobial susceptibility testing. However, the test used must be performed in accordance with an internationally accepted procedure; for example tests published by the Clinical and Laboratory Standards Institute (CLSI), the British Society for Antimicrobial Chemotherapy (BSAC), the Deutsches Institut für Normung e.V. (DIN) and the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM). Further guidance and interpretation of MIC 50 and MIC 90 values as well as guidance for the interpretation of multiresistance can be found in Schwarz *et al.* J. Antimicrobial Chemother 2010; 65: 601-604.

Data availability

Data that is integral to the paper must be made available in such a way as to enable readers to replicate, verify and build upon the conclusions published in the paper. Any restriction on the availability of this data must be disclosed at the time of submission. Data may be included as part of the main article where practical. We recommend that data for which public repositories are widely used, and are accessible to all, should be deposited in such a repository prior to publication. The appropriate linking details and identifier(s) should then be included in the publication and where possible the repository, to facilitate linking between the journal article and the data. If such a repository does not exist, data should be included as supporting information to the published paper or authors should agree to make their data available upon reasonable request.

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been reported in another species unless a comparison with related sequences contributes important new information.

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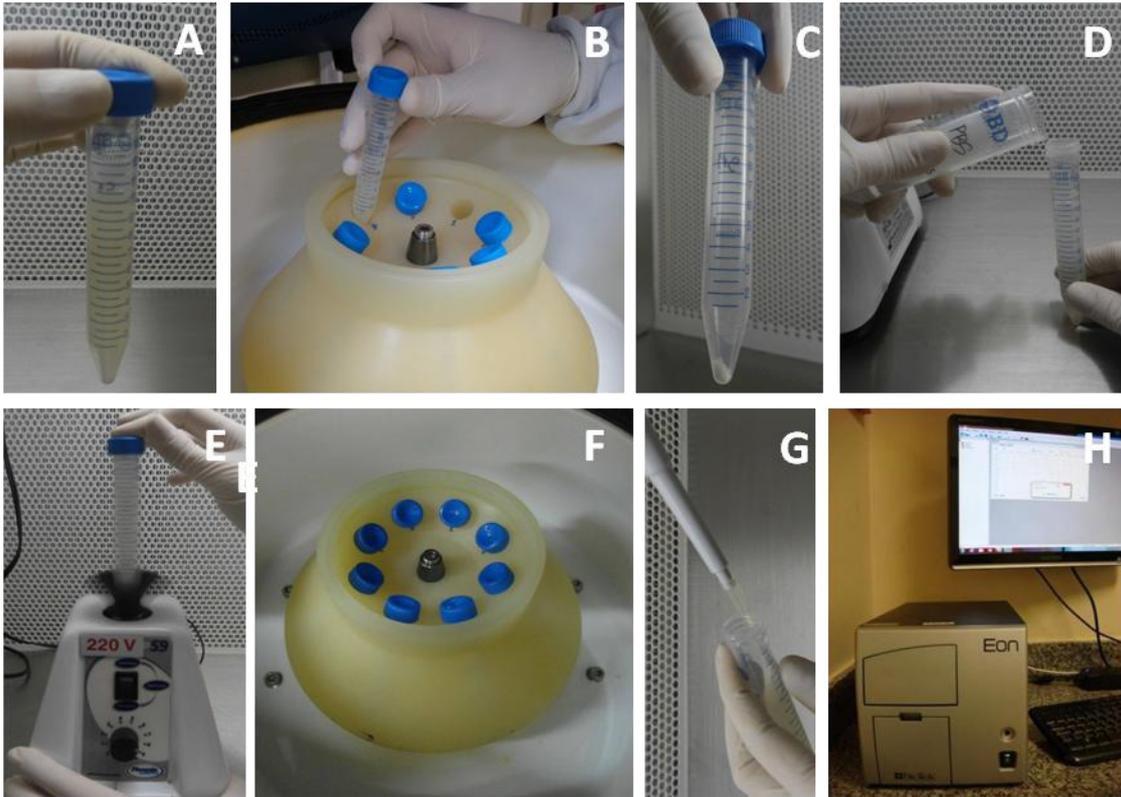
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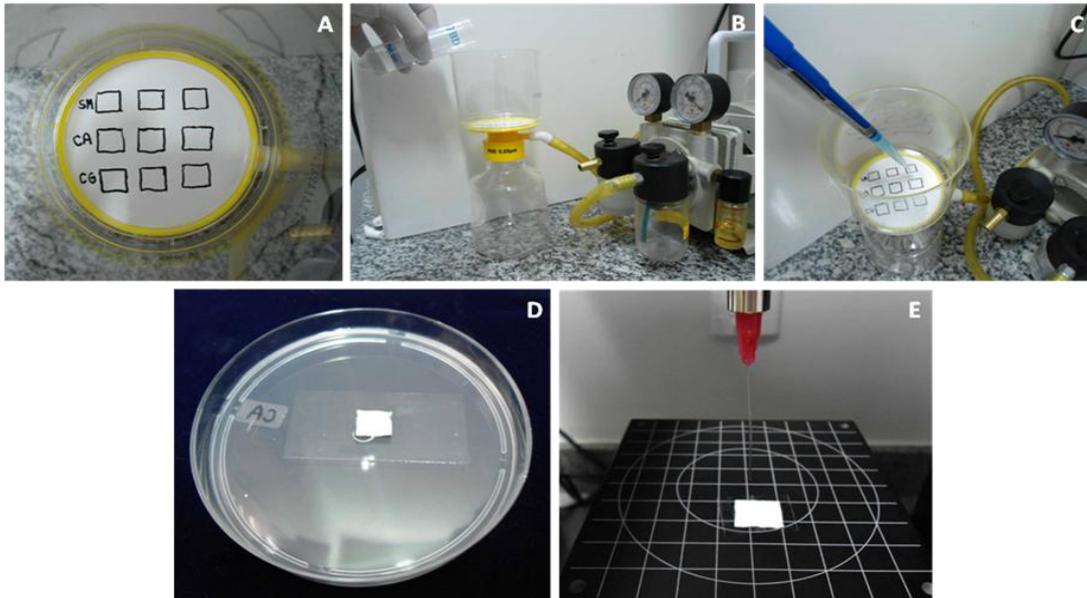
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ANEXO B

ILUSTRAÇÃO ESQUEMÁTICA DA PREPARAÇÃO DO INÓCULO DA CEPA DE *S. MUTANS* (10^8 CÉLULAS/ML EM SALIVA ARTIFICIAL)

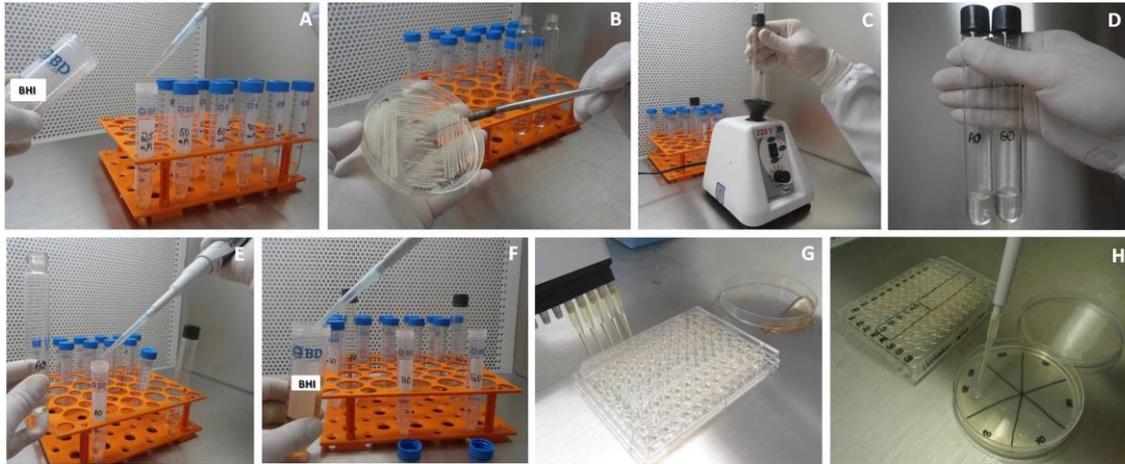
(A) Preparação do pré-inóculo de *S. mutans* ATCC 25175; (B) O inóculo foi centrifugado a 6500 *g* durante 5 min; (C) Obtenção do pellet de células e descarte do sobrenadante; (D) Na sequência, as células de *S. mutans* foram lavadas com PBS, (E) homogeneizadas em vórtex e (F) centrifugadas novamente (foram realizadas duas lavagens com PBS); (G) Alíquotas de 200 µl foram adicionadas aos poços de placas de 96 poços e levadas para leitura em (H) espectrofotômetro a 640 nm a fim de ajustar o número de células de *S. mutans* para 10^8 células/ml em saliva artificial.

ANEXO C

DETERMINAÇÃO DO GRAU DE HIDROFOBICIDADE DAS SUPERFÍCIES CELULARES

(A) Marcações (~1 x 1 cm) feitas na membrana do filtro a vácuo Stericup (0,22 μm) para receberem o inóculo da cepa de *S. mutans*; (B) Molhamento prévio da membrana do filtro (já acoplado a uma bomba a vácuo) com 10 ml de água destilada; (C) Filtragem de 2 mL do inóculo de *S. mutans* (10^9 células/ml) nas respectivas marcações da membrana; (D) Inserção do pedaço de membrana (contendo a camada de células filtradas) sobre lâminas de vidro no interior de placas de petri com 1 % de ágar e 10 % de glicerol; (E) Pedaço de membrana fixado na plataforma do Goniômetro para mensuração do ângulo de contato usando água deionizada como líquido teste.

ANEXO D

DETERMINAÇÃO DA CONCENTRAÇÃO INIBITÓRIA MÍNIMA DE TIROSOL

(A) Diluição (1:5) de cada concentração de tirosol no meio de cultura BHI caldo; (B) inoculação de colônias de *S. mutans* em solução salina 0,85%; (C) homogeneização em vórtex da suspensão da cepa; (D) ajuste da turbidez da suspensão da cepa ao padrão 0,5 da escala McFarland; (E) diluição (1:5) da suspensão celular em solução salina; (F) diluição (1:20) da suspensão celular no meio BHI caldo; (G) inserção, com auxílio de pipeta multicanal, de 100 μ l de cada concentração de tirosol diluído em BHI caldo + 100 μ l da suspensão de *S. mutans* em BHI caldo; (H) plaqueamento do conteúdo de cada poço após 48 horas de incubação para determinação da concentração bactericida mínima de tirosol.

ANEXO E

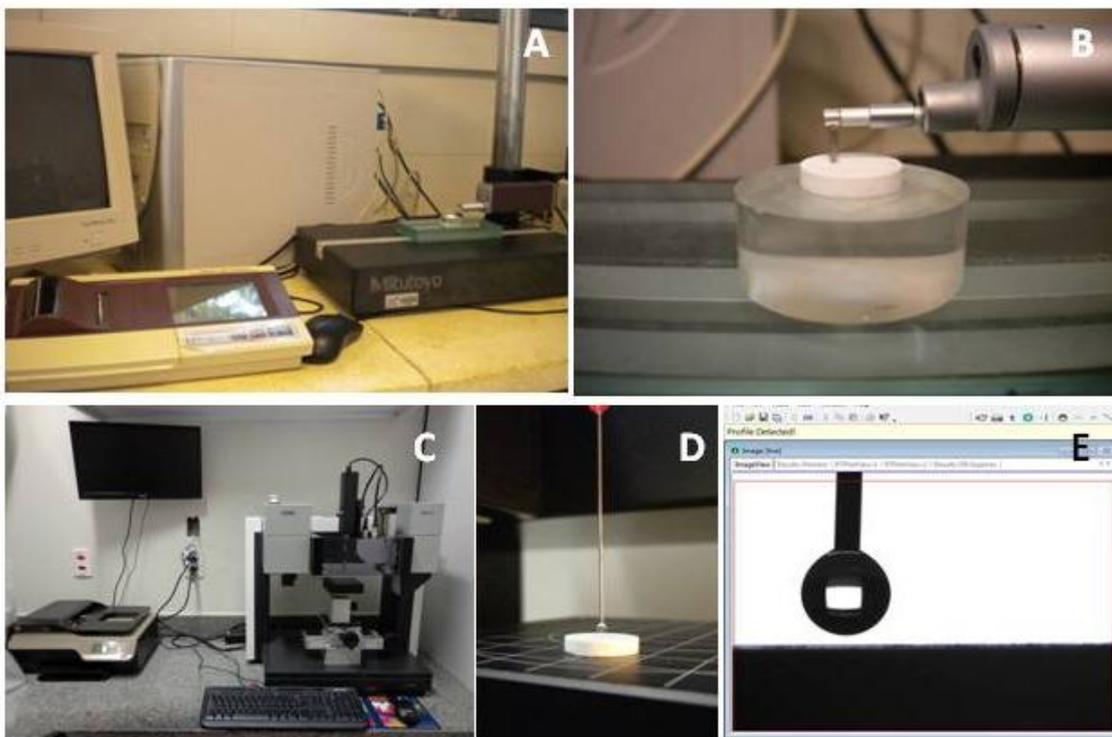
OBTENÇÃO DOS ESPÉCIMES DE HIDROXIAPATITA (HA)



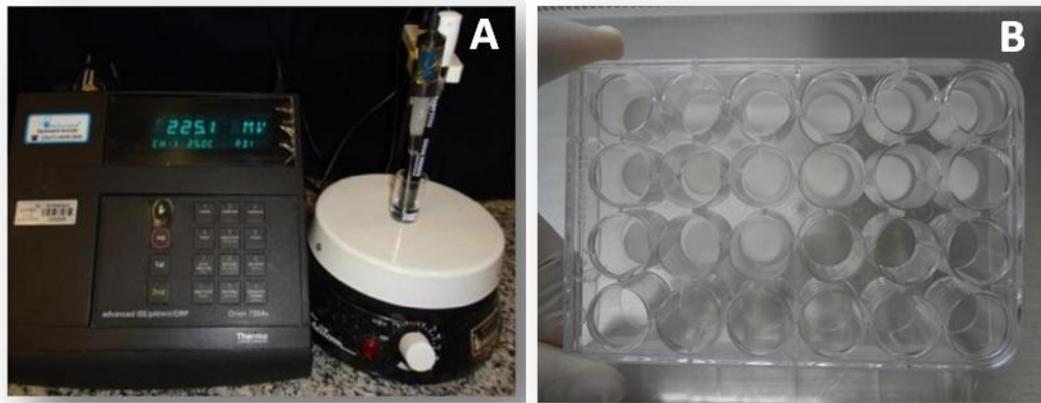
(A) Pesagem de 0,65 g do pó de hidroxiapatita (HA- Sigma Aldrich); (B) Inserção do pó de HA no interior da matriz de aço inoxidável (13 mm de diâmetro); (C) HA em pó no interior da matriz e pastilha de aço sendo inserida na matriz; (D) prensagem do pó de HA com carga de 2,5 toneladas; (E) partes da matriz de aço utilizada na confecção dos espécimes; (F) aspecto do espécime de HA finalizado.

ANEXO F

CARACTERIZAÇÃO DA SUPERFÍCIE DOS ESPÉCIMES DE HA



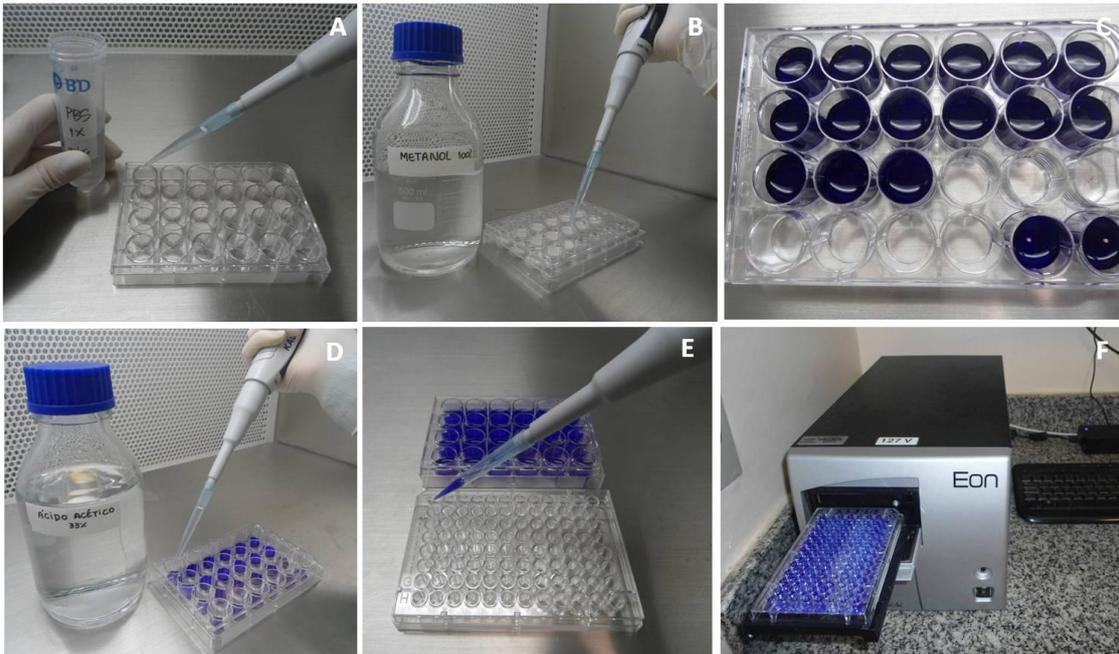
(A) Rugosímetro Surftest SJ-400, Tóquio, Japão; (B) ponta analisadora do rugosímetro deslizando sobre a superfície do espécime de HA; (C) Goniômetro automático Kruss, DAS 100 Standard, Alemanha; (D) espécimes de HA posicionados na plataforma do goniômetro para análise do grau de hidrofobicidade; (E) imagem do software evidenciando a gota de água deionizada (3 μ l) dispensada sobre a superfície dos espécimes.

ANEXO G***AValiação DA PRODUÇÃO DE ÁCIDO POR S. MUTANS***

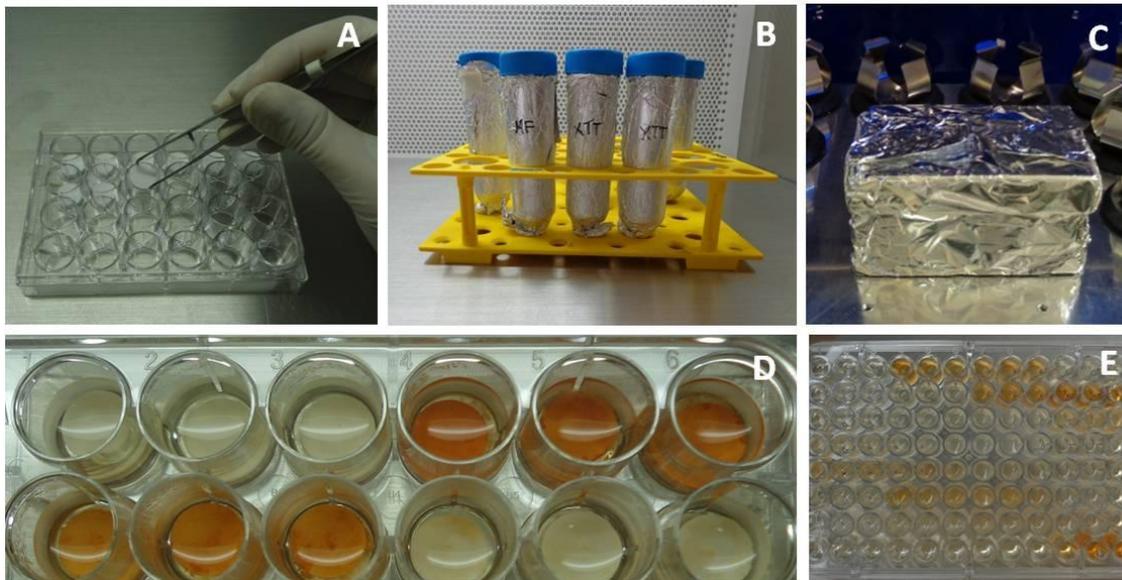
(A) Microeletrodo de referência (Analyser Comércio e Indústria Ltda, São Paulo, SP) acoplado ao analisador de íons Orion 720A (Orion Research, Inc.) utilizado na determinação dos valores de pH inicial e final (avaliação da produção de ácido por *S. mutans*); (B) espécimes de HA posicionados no fundo dos poços de placa de 24 poços contendo biofilmes expostos ao tirosol nas concentrações de $11,25 \text{ mol l}^{-1}$ e $22,5 \text{ mol l}^{-1}$, prontos para a avaliação da produção de ácido por meio da mensuração do pH do meio (saliva artificial).

ANEXO H

QUANTIFICAÇÃO DA BIOMASSA TOTAL DOS BIOFILMES



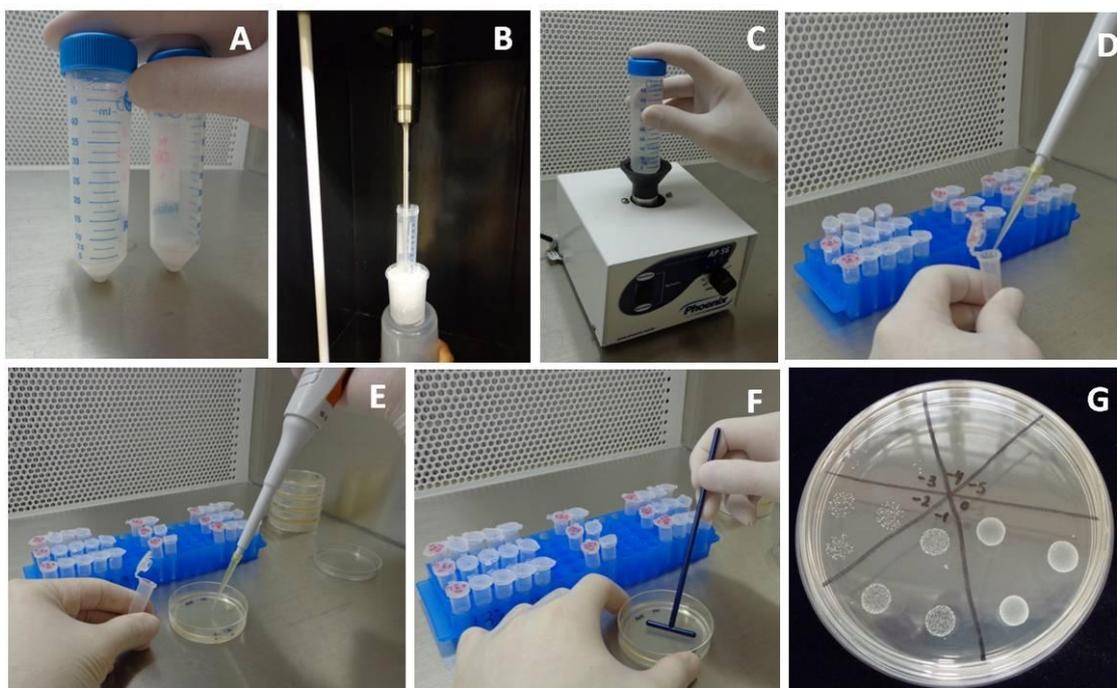
(A) Lavagem dos espécimes com PBS para remoção das células não aderidas; (B) fixação dos biofilmes com metanol 99 %; (C) adição de 1 ml de cristal violeta 1 % para corar os biofilmes; (D) remoção do cristal violeta e adição de 1 ml de ácido acético 33 %; (E) transferência de 200 µl da solução final obtida para poços de placas de microtitulação de 96 poços; (F) leitura de absorbância (570 nm) da solução obtida.

ANEXO I***AVALIAÇÃO DA ATIVIDADE METABÓLICA DAS CÉLULAS DOS BIOFILMES***

(A) Transferência dos espécimes contendo os biofilmes para novas placas de 24 poços; (B) soluções de XTT e metassulfato de fenazina (MF) protegidas da luminosidade; (C) incubação das placas com solução de XTT + MF (1ml/poço) (protegidas da luminosidade) por 3 horas a 37 °C; (D) diferentes tonalidades (dependendo da maior ou menor atividade metabólica das células) da solução de XTT após o período de incubação; (E) transferência de 200 µl da solução final para poços de placas de microtitulação de 96 poços para leitura de absorbância (490 nm).

ANEXO J

QUANTIFICAÇÃO DAS UNIDADES FORMADORAS DE COLÔNIAS (UFCs) DOS BIOFILMES



(A) Transferência dos espécimes para tubos falcon contendo 3 mL de PBS; (B) sonicação e (C) agitação em vórtex para desprendimento e desagregação dos biofilmes das superfícies dos espécimes; (D) diluições decimais seriadas das suspensões de biofilmes em PBS; (E) e (F) plaqueamento das diluições no meio de cultura BHI ágar; (G) crescimento das UFCs de *S. mutans* em BHI ágar.

ANEXO K

QUANTIFICAÇÃO DE PROTEÍNA E CARBOIDRATO DA MATRIZ EXTRACELULAR DOS BIOFILMES

(A) Secagem das amostras de biofilmes após tratamento com tirosol em membrana de acetato de celulose (previamente pesada), para determinação do peso seco dos biofilmes; (B) amostra sonicada a 30 w por 30 s e (C) homogeneizada em vórtex; (D) centrifugação das amostras durante 10 min a 3000 g; (E) filtragem do sobrenadante obtido anteriormente; (F) kit BCA utilizado para quantificação do conteúdo proteico da matriz extracelular; (G) pipetagem de 200 μ l da mistura dos reagentes do kit em poços de placa de 96 poços; (H) pipetagem de 25 μ l do sobrenadante da amostra de biofilme para quantificação de proteínas; (I) para determinação do conteúdo de carboidrato da matriz extracelular, 500 μ l do sobrenadante das amostras foram adicionados à mistura de 500 μ l de fenol a 9% e 2,5 ml de ácido sulfúrico em tubos de ensaio de vidro; (J) diferentes tonalidades de cores nos tubos demonstrando diferentes teores de carboidrato; (K) as quantidades de proteína e carboidrato da matriz dos biofilmes foram determinadas colorimetricamente por leitura em espectrofotômetro a 562 e 490 nm, respectivamente.

ANEXO L

MICROSCÓPIO ELETRÔNICO DE VARREDURA (MEV) UTILIZADO NA ANÁLISE DA ESTRUTURA DOS BIOFILMES



(A) Microscópio Eletrônico de Varredura (Electron Microscope FEG-VP Supra 35, Carl Zeiss, Jena, Thuringen, Germany) utilizado na análise da estrutura dos biofilmes; (B) espécimes já metalizados em ouro e posicionados no MEV para posterior obtenção das imagens.