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AVALIAÇÃO DA AÇÃO ANTIMICROBIANA DA PRÓPOLIS VERMELHA BRASILEIRA EM MODELO DE BIOFILME *IN VITRO* COM MÚLTIPLAS ESPÉCIES BACTERIANAS

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razão de ter minhas forças renovadas a cada manhã, a toda a minha família
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“ Mas aqueles que esperam no Senhor

renovam suas forças. Voam alto como águias,

correm e não ficam exaustos, andam e não se cansam”.

Isaías 40:31

RESUMO

O biofilme periodontopatogênico é o principal agente etiológico das periodontites. Assim, a busca por novos agentes antimicrobianos naturais é importante, destacando-se a própolis vermelha brasileira devido a sua composição química distinta e atividade antimicrobiana. No entanto, não há relatos na literatura sobre a atividade da própolis vermelha sobre o biofilme subgengival multiespécie. Assim, o objetivo do presente trabalho foi avaliar o efeito do extrato bruto da própolis vermelha (EBPV) sobre modelo de biofilme subgengival multiespécie. O biofilme subgengival contendo 31 espécies relacionadas com a periodontite foi formado por 7 dias, utilizando o dispositivo de Calgary. Os tratamentos com EBPV à 1600; 800 400 e 200 µg/mL foram realizados 2x/dia, por 1 minuto cada, a partir do 3º dia, totalizando 8 tratamentos. O grupo controle positivo foi tratado com clorexidina 0,12% (Periogard, Colgate), e o grupo controle negativo os tratamentos foram realizados com veículo. Após 7 dias de formação do biofilme, foram avaliadas a atividade metabólica do biofilme por meio de reação colorimétrica e a composição microbiana por meio de checkerboard DNA-DNA hybridization. Os resultados foram submetidos à análise estatística por meio de ANOVA com dados transformados via BOX-COX seguido do post-hoc de Dunnett. EBPV a 1600 e 800 µg/mL reduziram a atividade metabólica do biofilme em 60 e 50 %, respectivamente e ambas concentrações apresentaram atividade antimicrobiana contra 21 espécies diferentes. EBPV demonstrou ser um promissor agente antimicrobiano que poderá ser útil no controle da doença periodontal. No entanto, novos estudos utilizando modelos *in vivo* de doença periodontal experimental devem ser realizados para comprovar tal efeito.

Palavras-chaves: Própolis, Biofilme, Biofilme subgengival, Doença periodontal

ABSTRACT

Perio-pathogenic biofilm is the main etiological agent of periodontitis. Thus, the search for new natural antimicrobial agents is noteworthy, standing out the Brazilian red propolis (BRP) due to its distinct chemical composition and antimicrobial activity. However, there are no reports in the literature regarding BRP properties on subgingival multispecies biofilms. Thus, the objective of the present work was to evaluate the effect of crude red propolis extract (EBPV) on a sub-gingival multi-species biofilm model. The subgingival biofilm containing 31 species related to periodontitis was formed during 7 days using the Calgary device. The treatments with EBPV at 1600; 800;400 and 200μg / mL were performed 2x / day, for 1 minute, from day 3, totaling 8 treatments, in the positive control group the treatments were performed with 0.12% chlorhexidine (Periogard, Colgate), and in the negative control group the treatments were performed with vehicle. After 7 days of biofilm formation, the metabolic activity of the biofilm was evaluated by means of a colorimetric reaction and the microbial composition by means of checkerboard DNA-DNA hybridization. The results were submitted to statistical analysis using ANOVA with data transformed via BOX-COX followed by Dunnett post-hoc. EBPV at 1600 and 800 μg / mL reduced the metabolic activity of the biofilm by 60 and 50%, respectively, and both concentrations presented antimicrobial activity against 21 distinct species. EBPV has been shown to be a promising antimicrobial agent that may be useful in the control of periodontal disease. However, novel studies using *in vivo* models of experimental periodontal disease should be performed to prove such an effect.

Keywords: Propolis, Biofilm, Subgingival biofilm, Periodontal disease

SUMÁRIO

1. INTRODUÇÃO

A ciência confirma que biofilmes virulentos são agentes de infecções, incluindo aquelas que ocorrem na boca. O biofilme dental bacteriano que se forma sobre a superfície do dente apresenta uma composição bacteriana e bioquímica variável dependendo de fatores intrínsecos e extrínsecos, podendo mudar de modo a tornar este biofilme patogênico (MARSH, 2005).

A microbiota subgengival, altamente complexa, desempenha um papel importante no estabelecimento da saúde periodontal, bem como no desenvolvimento de doenças periodontais e é composta principalmente por espécies orais comensais residentes as quais têm co-evoluído para colonizar a cavidade oral humana (JORN et al.,2005; SOCRANSKI et al., 2005; DEWHIRST et al., 2010).

No entanto, a existência de uma grande variedade de determinantes ecológicos no ecossistema oral pode proporcionar condições ideais para o estabelecimento de microrganismos que normalmente não são considerados residentes da microbiota oral normal. Embora ainda haja controvérsia sobre se essas espécies são meramente contaminantes ou membros transitórios, fortes evidências têm mostrado que elas podem de fato colonizar a microbiota oral (EKE et al., 2012; MEYLE et al., 2015).

Com o acúmulo do biofilme sobre a superfície dos dentes, bactérias anaeróbias irão se juntar a esse biofilme já formado e provocar um processo inflamatório nos tecidos moles adjacentes à estrutura dental levando ao desenvolvimento da doença periodontal, com consequente produção de citocinas, as quais exercerão efeito quimiotáxico sobre os leucócitos (JONES *et al.,* 1991). As doenças periodontais estão entre as doenças infecciosas orais mais comuns correlacionadas com o estabelecimento de um biofilme altamente patogênico que desencadeia uma resposta imune / inflamatória do hospedeiro, levando à destruição de tecidos periodontais de suporte e eventual perda dentária (EKE et al., 2012; MAYLE et al., 2015)

Esta doença global é um exemplo notável das conseqüências decorrentes de interações entre microrganismos e seus produtos, saliva do anfitrião e componentes imunológicos que conduzem ao desenvolvimento de biofilmes em superfícies dentárias suscetíveis (MARSH, 2003).

Desde a introdução da penicilina houve aumento considerável no número de classes de antibióticos disponíveis. Infelizmente ocorreu o desenvolvimento da resistência bacteriana

contra as diversas classes de antibióticos (GINZBURG et al., 2000),o que justifica a busca por outros agentes antimicrobianos.

Entre todas as novas drogas aprovadas pelo Food and Drug Administration (FDA) ou outras entidades equivalentes de outros países, 30% delas são totalmente de origem direta de produtos naturais e 44% são de derivados destes e 26% de sintéticas. Desta forma 74% de todas as novas drogas aprovadas atualmente são de fontes naturais ou derivadas de fontes naturais. (NEWMAN et al., 2016).

Entre os produtos naturais, a própolis tem destacando-se, tanto pelas suas diversas propriedades biológicas (KOO et al., 2002;CHEN et al., 2003; NAGAOKA et al., 2003; DUARTE et al., 2003; NAGAI et al., 2003; ASO et al., 2004; KUMAZAWA; HAMASAKA; NAKAYAMA, 2004; HAYACIBARA et al.,2005; CASTRO et al., 2007; ALENCAR et al., 2007; OLDONI, 2007), quanto pela sua aplicação nas indústrias de cosméticos e alimentos, utilizada como ingrediente na formulação de vários produtos (MATSUDA, 1994).

A própolis é definida como uma substância resinosa coletada pelas abelhas de diferentes partes da planta, como brotos, botões florais e exsudados resinosos. Possui coloração e consistência variada e é utilizada para fechar pequenas frestas, embalsamar insetos mortos bem como proteger a colméia contra a invasão de microrganismos (GHISALBERTI, 1979).

Diversas outras atividades da própolis têm sido descritas, tais como propriedades hepatoprotetoras (LIN et al., 1997; BANKSOTA et al., 2000), analgésica (PAULINO et al., 2003), atividade estrogênica (SONG et al., 2002a), atividade antiangiogênica (SONG et al., 2002b) e regenerativa de cartilagem e ossos, por meio do estímulo na proliferação de condrócitos (CARDILE et al., 2003).

Devido à grande complexidade química, a própolis é considerada uma das misturas mais heterogêneas já encontradas em fontes naturais. Os principais constituintes são os compostos fenólicos, representados pelos flavonóides, ácidos fenólicos e seus ésteres, e que possuem papel importante no organismo, pois podem agir como antioxidantes, antinflamatórios, antimicrobianos entre outras atividades biológicas. Um novo tipo de própolis, classificada como o 13º tipo de própolis brasileira (SILVA et al., 2008), denominada de própolis vermelha, demonstrou alta atividade biológica, principalmente atividade antimicrobiana contra microrganismos patogênicos em ensaios *in vitro* (ALENCAR et al., 2007), antioxidante (ALENCAR et al., 2007, SILVA et al., 2008). Além disso, os isoflavonóides neovestitol e vestitol foram isolados a partir da própolis vermelha e suas

propriedades biológicas contra biofilmes de *S. mutans* foram caracterizados (BUENO-SILVA et al., 2013).

No entanto não há relatos na literatura sobre a atividade da própolis vermelha sobre o biofilme subgengival complexo. Assim formulou-se a hipótese de que a própolis vermelha apresenta atividade antimicrobiana em modelo de biofilme complexo.

2 OBJETIVO

O objetivo deste estudo foi avaliar o efeito do extrato bruto da própolis vermelha (EBPV) sobre modelo de biofilme subgengival multiespécie.

2.1. Objetivos específicos

- Avaliar o efeito antimicrobiano das diferentes concentrações (1600; 800; 400 e 200 µg/ml) do EBPV;

- Comparar a atividade metabólica dos biofilmes tratados com o EBPV (1600; 800; 400 e 200 µg/ml) com o grupo tratado com a clorexidina 0,12% e com o grupo veículo controle;

- Comparar a composição microbiana dos biofilmes tratados com o EBPV com o biofilme tratado com o veículo controle.

3. ARTIGO CIENTÍFICO

Brazilian red propolis effects on *in vitro* dental multispecies biofilm

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Abstract

The sub-gingival biofilm is considered the periodontitis main etiological agent. Thus, the search for new natural antimicrobial agents is notable over the literature, standing out the Brazilian red propolis (BRP) due to its distinct chemical composition, anti-inflammatory and antimicrobial activity. However, there are no reports in the literature regarding BRP properties on sub-gingival multi-species biofilms. Thus, the objective of the present work was to evaluate the effect of BRP ethanolic extract (BRPEE) on a subgingival multi-species biofilm model. The subgingival biofilm containing 31 species related to periodontitis was formed for 7 days using the Calgary device. The BRPEE treatments at 1600; 800; 400 and 200 μg / mL were performed 2x / day for 1 minute each, starting from 3 days, totaling 8 treatments. In the positive control group the treatments were performed with 0.12% chlorhexidine (Periogard, Colgate), and in the negative control group the treatments were performed with vehicle.On the day 7, the biofilm metabolic activity was evaluated by means of a colorimetric reaction and the microbial composition by DNA-DNA hybridization. Results were submitted to statistical analysis using ANOVA followed by Tukey-test for metabolic activity and with data transformed via BOX-COX followed by Dunnett post-hoc. BRPEE at 1600 and 800 μg/mL reduced biofilm metabolic activity by 60 and 50%, respectively, when compared to vehicle control and were statically similarto chlorhexidine inhibition. Checkerboard DNA-DNA hybridization revealed that BRPEE at 1600 and 800 both μg/mL significantly reduced levels of 21 distinct species when compared to vehicle control. BRP has been shown to be a promising antimicrobial agent that may be useful in the control of periodontal disease. However, novel studies using *in vivo* models of experimental periodontal disease should be performed to prove such effect.

Keywords: Multispecies Biofilm; Brazilian Red Propolis; Antimicrobial.

Introduction

The multispecies dental biofilm is considered the primary etiological factor of periodontal disease. Different bacteria species grow at subgingival sites in the mouth and produce destructive factors and enzymes that degrade the extracellular matrix and human cell membranes in order to obtain nutrients for their evolution and tissue invasion. Due to subgingival biofilm development, an exacerbated inflammatory response is produced, what in turn lead to destruction of teeth supporting tissues, with progressive loss of insertion and eventual dental loss(TELES et al., 2013).

The microbiota composing the subgengival biofilm was classified into different complexes, denominated by distinct colors, according to the role of each microorganism in the development or not of the disease. The yellow complex, composed of several species of the genus *Streptococcus*, and purple complex, mainly *Actinomyces* and *Veillonella parvula*, initiate the formation of the subgingival dental biofilm, allowing the colonization by species of *Capnocytophaga*, *Eikenella* and *Aggregatibacter actinomycetemcomitans* serotype a, members of green complex. So far, this biofilm is considered health-friendly and will not cause a disease unless a specific shift in microbial composition starts to occur. If not controlled, this health biofilm creates conditions to colonization of orange complex, composed of *Fusobacterium*, *Campylobacter*, *Prevotella*, *Parvimonas* and certain *Streptococci* (*Streptococcus constellatus* group).The orange complex is associated with health-disease transition since they produce suitable environmental conditions for colonization by *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, microorganisms of the red complex that are considered as the main pathogens of periodontal disease (Socransky et al., 1998)

Periodontal disease affects 25 % of the world population (Pathirana et al., 2010) and, despite all the research conducted in this matter, still may be considered as a public health

problem (Batchelor, 2014; Dumitrescu, 2016). Therefore, there is an intense search over the literature to find novel antimicrobials that disrupts the complex subgingival multispecies biofilm and one of the mainly sources of new compounds are the natural products (Arbia et al., 2017; Lee et al., 2017; Lazar; Saviuc; Chifiriuc 2016; Slobodníková et al., 2016; Freires et al., 2015).

Among the natural products, Brazilian red propolis (BRP) stands out in drug discovery since it pursue several pharmacological properties such as antimicrobial (Bueno-Silva et al 2017a; Bueno-Silva et al., 2013a,b; Dantas Silva et al., 2017; Silva et al., 2008), anti-inflammatory (Bueno-Silva et al., 2017b, 2016, 2015) anti-tumor (Frozza et al., 2017), healing properties (Correa et al., 2017), antioxidant and antiparasitic (Dantas Silva et al., 2017). The BRP chemical composition is quite complex and the flavoinoids are considered as the main bioactives compounds (Barbosa Bezerra et al., 2017; Bueno-Silva et al., 2017c; Franchin et al., 2016; Lima Cavendish et al., 2015) Although the strong effort to research BRP pharmacological properties, no studies regarding its action on periodontal biofilm can be found over the literature. Therefore, the objective of this manuscript is to evaluate the anti-biofilm property of BRP on a subgingival multispecies biofilm *in vitro* model.

Material and Methods

BRP solution preparation

BRP was collected in a private land, with permission of the owner, by scraping the *Apis mellifera* bee's boxes sides, located in Maceio, Alagoas, Northeast, Brazil. The Brazilian red propolis ethanolic extract of (BRPEE) was obtained by adding 25g of propolis to 200ml of 80% ethanol (v/v) with continuous mixing during 45 min and then, the suspension was filtered with paper filter. Then, the solvent was evaporated and the ethanolic extract of BRP was obtained. Since we used same BRP samples of our previous manuscripts, the BRP chemical profile was confirmed in accordance with Bueno-Silva et al., (2016, 2017b). The

BRP ethanolic extract was diluted in ethanol 80% and phosphate buffer 10 % (v/v), at 1600, 800, 400 and 200µg/mL. The final ethanol concentration was 15%.

Multispecies biofilm *in vitro* model

The speciesused to form multispecies biofilm are listed on Table 1.

Table 1: List of microorganisms used to form multispecies biofilm.

Multispecies biofilm microorganisms	
A. gerencseriae ATCC 23840	*E. saburreum* ATCC 33271
A. israelii ATCC 12102	*F. nucleatum polymorphum* ATCC 10953
A.naeslundii ATCC 12104	*F. nucleatum vincentii* ATCC 49256
A.naeslundii II ATCC 43146	*F. periodonticum* ATCC 33693
A. odontolyticus ATCC 17929	*P. intermedia* ATCC 25611
V. parvula ATCC 10790	*E. nodatum* ATCC 33099
S.gordonii ATCC 10558	*A. actinomycetemcomitans* ATCC 29523
S. intermedius ATCC 27335	*T. forsythia* ATCC 43037
S.mitis ATCC 49456	*E. corrodens* ATCC 23834
S. oralis ATCC 35037	*C. ochracea* ATCC 33596
S.sanguinis ATCC 10556	*S. noxia* ATCC 43541
S.anginosus ATCC 33397	*G. morbillorum* ATCC 27824
C. gingivalis ATCC 33624	*P. micra* ATCC 33270
C. gracilis ATCC 33236	*S. constellatus* ATCC 27823
C. showae ATCC 51146	*P.gingivalis* ATCC 33277
C. sputigena ATCC 33612	

Most species, including *Actinomyces* subsp., *Streptococcus* subsp., And *Fusobacterium* subsp., were grown on tryptone soy agar with 5% sheep blood under anaerobic conditions (85% nitrogen, 10% carbon dioxide, and 5% hydrogen), while *Eubacterium* subsp. and *N. mucosa* were cultured on fastidious anaerobic agar with 5% ram blood. *P. gingivalis* and *P.melaninogenica* were grown on tryptone soy agar with yeast extract enriched with 1% hemin, 5% menadione and 5% sheep blood as *T. Forsythia* were grown on tryptone soy agar

with yeast extract enriched with 1% hemin, 5% menadione, 5% sheep blood, and 1% N-acetylmuramic acid. After 24 h of growth, all species were transferred to glass tubes with BHI culture medium (Becton Dickinson, Sparks, MD) supplemented with 1% hemin.

After 24 hours growth in BHI broth with 1% hemin, the optical density (OD) at 600 nm was adjusted to 0.1, corresponding to about 10^8 cells/ml of each species. The individual cell suspensions were diluted and 100 µl aliquots containing 10^6 cells from each species were mixed with 33 µL of BHI broth with 1% hemin and 5% sheep blood to give a final 45 ml biofilm inoculum.

The multiple biofilm species model was developed using the Calgary biofilm device (CBD) consisting of a 96-well plate (Nunc; Thermo Scientific, Roskilde, Denmark) which was used for the addition of 150 µL of inoculum per well containing 10^4 cells of each species and also a plate cover containing polystyrene pins (Nunc TSP system; Thermo Scientific, Roskilde, Denmark). The coated plates were incubated at 37°C under anaerobic conditions. After 72 h of incubation, plate covers were transferred to fresh 96-well plates with fresh broth (BHI broth with 1% hemin and 5% sheep blood) and kept at 37°C under anaerobic conditions until 7 days of biofilm formation.

BRPEE treatments

BRPEE treatments initiated after 72 h of biofilm formation and were performed twice a day during the next 4 days. Pins coated with the biofilms were transferred to 96-well plates containing different concentrations of ethanolic extract of BRPEE at 1600, 800, 400 and 200 µg/ml, vehicle negative control and chlorhexidine (Periogard, Colgate), used as positive control, for 1 minute each treatment. Then the plate returned to the original culture medium. Three experiments in triplicate for each of the test groups and respective controls were performed (Bueno-Silva et al., 2013b; Soares et al., 2015).

Biofilm metabolic activity

The percentage reduction of biofilm metabolic activity was determined using 2,3,5-triphenyltetrazolium chloride (TTC) (catalog No. 17779; Fluka analytical) and spectrophotometry. TTC is used for differentiation between metabolically active and inactive cells. The white substrate is enzymatically reduced to red formazan 1,3,5-triphenyl (TPHP) by bacterial live cells, due to the activity of several dehydrogenases. The change in substrate color is read by spectrophotometry to determine the rate of reduction, which is used as an indirect measure of bacterial metabolic activity. To measure the metabolic activity of biofilms, the pins were transferred to plates with 200 μL per well of fresh BHI medium containing 1% hemin with 10% of a 1% TTC solution. The plates were incubated under anaerobic conditions for 24 h at 37°C. The TTC conversion was read at 485 nm using a spectrophotometer (Soares et al., 2015)

Checkerboard DNA-DNA hybridization

Three 7-day biofilm coated pins of each of the groups was transferred to Eppendorf tubes containing 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7, 6]), and then 100 μL of 0.5 M NaOH were added. The tubes containing the pins and the final solution were boiled for 10 min and the solution neutralized with the addition of 0.8 ml of ammonium 5 M. The samples were analyzed individually for the presence and quantity of the 35 bacterial species, using DNA-DNA hybridization technique. Briefly, upon lysis of the samples, the DNA was plated onto a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After DNA attachment to the membrane, it was placed in a Miniblotter 45 (Immunetics). Digoxigenin labeled with DNA probes of the entire genome for the subgengival species used were hybridized to individual lanes of Miniblotter 45. After hybridization, the membranes were washed, and DNA probes were detected using a specific antibody to digoxigenin conjugated to phosphatase alkaline. The signals were detected using

AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL), and the results were obtained using Typhoon Trio Plus (Molecular Dynamics, Sunnyvale, CA). Two lanes in each race contained the standards with $10^5$and 10^6cells of each species. Signals obtained with the Typhoon Trio were converted to absolute counts, by comparison with the patterns on the same membrane. Failure to detect a signal was recorded as zero. The values obtained after the treatment with BRPethanolic extract were compared with the values of negative and positive controls (Soares et al., 2015).

Statistical analysis

Biofilm metabolic results were submitted to statistical analysis using ANOVA followed by Tukey post-hoc while for checkerboard results ANOVA, with data transformation via BOX-COX followed by Dunnett post-hoc were used.

Results

Biofilm metabolic activity

Figure 1 shows results of metabolic activity of biofilms treated with vehicle, different concentrations of BRPEE and chlorhexidine. Biofilms treated with BRPEE at 1600 and 800 µg/mL had no statistical difference from group treated with chlorhexidine 0.12% and these three groups were statistical different from vehicle controlresults, reducing respectively, 45, 40 and 55% of biofilm metabolic activity, when compared to vehicle results. Results of biofilms treated with BRPEE at 400 µg/mL had no statistical difference to any other group while BRPEE at 200 µg/mL biofilm metabolic activity were statistically higher than chlorhexidine group and did not present statistical significance to the other groups.

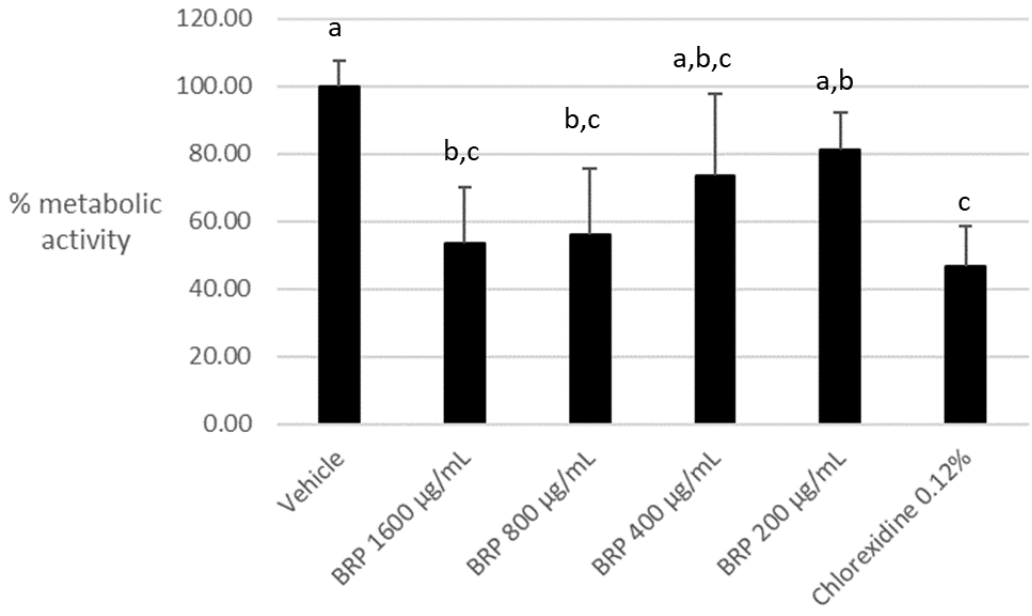

Figure 1: Metabolic activity of biofilms treated with vehicle (negative control), ethanolic extract of BRP at 1600, 800, 400, 200 µg/mL and chlorhexidine 0.12%. Vehicle results were considered as 100% of metabolic activity and different letters means statistical significance by ANOVA followed by Tukey test ($p \leq 0.05$).

Since BRPEE 400 and 200 µg/mL did not diminish the biofilm metabolic activity, we performed checkboard analysis only with the higher BRPEE concentrations (1600 and 800 µg/mL). In this way, BRPEE (1600 and 800 µg/mL) reduced levels of 21 distinct microorganisms according to DNA-DNA hybridization results (Figure 2). Microorganisms affected by BRPEE 1600 µg/mL includes *A.actinomycetemcomitans* 29523, *P.gingivalis* 33277, *T. forsythia* 43037, *S.gordonii* 10558.

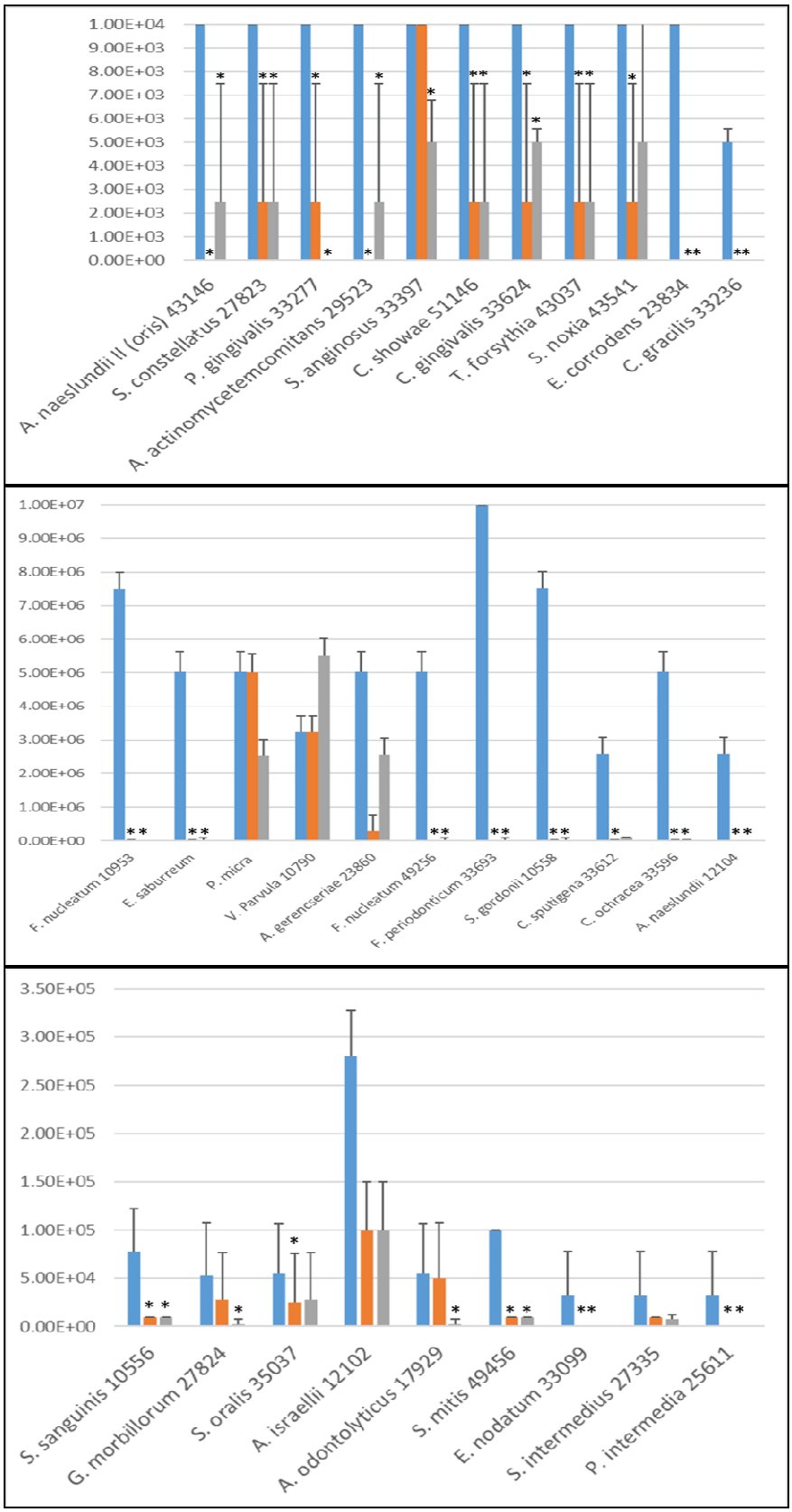

Figure 2: DNA-DNA hybridization results of biofilms treated with vehicle control and BRPEE (1600 and 800 µg/mL). *means statistical difference from vehicle control group.

Discussion

Biofilms are defined as an organized microbial community, embedded into an extracellular matrix of polysaccharide and attached to a rigid surface. They are considered as the etiological factor of many infectious diseases (Flemming et al., 2016).

One of these diseases is the periodontitis with a complex etiology including microbial communities capable of evasion fromhost immune system and provoking an exacerbated inflammatory reaction, which produces tissue destruction and even, loss of function and/or dental element. Nowadays, periodontitis is initiated by a dysbiotic microbial community and not by specific periodontopathogens (Hajishengallis & Lamont, 2012). Thus, *P. gingivalis* together with, mainly *Tannerella forsythia* and *Streptococcos gordonii* (and possibly other microorganisms), has recently been suggested as responsible for the dysbiosis in the buccal microbiota, promoting the transition from the original "healthy" biofilm to the now periodontopathogenic biofilm, and tissue destruction because of the exacerbated immunoinflammatory response (Hajishengalis, 2015).

Conventional Periodontal treatment includes scaling and root planning (SRP). However, just SRP every so often is not always sufficient to eradicate such oral pathogens, and association with the use of antimicrobials is frequently necessary through the combination of amoxicillin and metronidazol for 14 days. The effect of antibiotic therapy for the treatment of periodontitis on the subgingival microbiota was well studied, showing improvement of clinical parameters of periodontitis (Feres et al., 2015), but their effects on the whole human organism are not described. In this way, BRP appears as an adjuvant option to conventional periodontal treatment since natural products have been considered safe and healthy to human organism.

Soares et al (2015) demonstrated that a 24-hour treatment with amoxicillin and metronidazol, in levels like what is found in gingival crevicular fluid, reduce 16 % of metabolic activity of multispecies biofilms through a very similar model used here. In addition, this 16 % of reducing would be worse to the approximately 55 % reduction found for BRP 1600 and 800 µg/mL treatment, even without a statistical analysis to compare both results. However, the Soares's therapeutic scheme include a 24 hours treatment on 7-days biofilm while here, a twice daily 1-min treatment were chosen because to mimics a mouth rinse use as adjuvant to SRP.

Interestingly, according to DNA-DNA hybridization, BRP 1600 µg/mL reduced levels of *P.gingivalis*, *T. forsythia* and *S. gordonii* when compared to vehicle control treatment group. Moreover, BRP has a statistical similar effect of chlorhexidine on them. These microorganism's orchestra dysbiosis and then, all the mechanisms to bacteria evasion from the immune system (Hajishengallis 2015). Therefore, propolis effects on these three microorganisms are noteworthy.

Recently, BRP demonstrated immune-modulatory properties what may account to help to control periodontal disease since this disease has an important immune-inflammatory component. In fact, an effort has been done to find an immune-inflammatory agent to control periodontal disease (Preshaw 2018; Van Dyke 2017; Balta; Loos; Nicu; 2017; Sculley 2014). Therefore, the results found here are outstanding because this is the first work to demonstrate the effect of BRP on a multispecies biofilm model related to periodontal disease. Taken all together, the anti-inflammatory and the anti-periodontal biofilm properties of BRP turns this natural product as a promising agent to contribute to periodontal disease treatment.

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4. CONCLUSÃO

O extrato bruto da própolis vermelha demonstrou ser um promissor agente antimicrobiano no modelo de biofilme subgengival, que poderá ser útil no controle da doença periodontal. As concentrações de 1600 e 800 µg/ml inibiram o crescimento de 21 espécies diferentes do modelo de biofilme subgengival e estas concentrações reduziram a atividade metabólica do biofilme em valores estaticamente semelhantes a clorexidina 0,12%. Estudos futuros utilizando modelos *in vivo* de doença periodontal experimental devem ser realizados para comprovar tal efeito e determinar qual o melhor método de aplicação da própolis no biofilme subgengival.

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