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Inhibition of *Streptococcus mutans* (ATCC 25175) biofilm formation on eugenol-impregnated surgical sutures

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The use of bioactive compounds as anti-infective coating on biomaterial surfaces has been studied as a tool against microbial adhesion and the establishment of biofilms. The objective of this work was to evaluate the antibacterial activity of eugenol, specifically the ability to interact with cotton suture threads for preventing adhesion and biofilm formation of *Streptococcus mutans* (ATCC 25175). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of eugenol were determined according to Clinical and Laboratory Standards Institute (CLSI), which showed respective concentration values of 250 and 1000 µg/ml. In addition, eugenol displayed marked activity against to biofilm formation in 96-well polystyrene plates against several strains from the *Streptococcus* genus, even at lower than bacteriostatic concentrations between 15 to 250 µg/ml. Moreover, eugenol formed an effective covering on cotton-suture surfaces that inhibited cell adhesion, which decreased the *S. mutans* (ATCC 25175) biofilm development, according to biomass and metabolic rate, quantified by crystal violet staining and XTT reduction, respectively. This research may help to explore the eugenol molecule as an antifouling coating on surfaces, bringing a new perspective to the prevention of infections associated with biomaterials.

Key words: Eugenol, biofilm formation, *Streptococcus mutans,* anti-infective surfaces.

INTRODUCTION

Biofilms are microbial communities physiologically distinct from their free-living counterparts, primarily because of the ability to produce an adhesive matrix rich in proteins and polymeric substances (Billings et al., 2015). The presence of this matrix provides the biofilm with structural stability and offers a protection barrier against foreign agents, besides facilitating adhesion to biological and synthetic surfaces. These cellular clusters can establish

relationships of synergism and mutualism, generating cooperative benefits that favor the microbial community (Okuda et al., 2012).

Microbial colonization on the inserted or implanted foreign body material surface is known for biomaterials-associated infections (BAI) (Busscher et al., 2012). Suture threads used in surgical repairs represent a convenient target for attachment of cells and biofilm formation (Costa Neto et al., 2015). Microbial colonization on these surfaces may trigger severe tissue damage and compromise the success of the surgery (Chu et al., 1984).

Many types of bacteria have the ability to colonize the surfaces of the suture threads, triggering an infectious process in the tissues (Selvi et al., 2016). Once the biofilm has been established; as a consequence of the higher microbial resistance, the local decontamination mechanisms become ineffective, since these structured communities of microorganisms may harbor cells that are 10 to 1000 times more resistant to antimicrobial agents compared to their planktonic forms (Stewart, 2015). Due to high risk of infections, research in this area has focused primarily on how to avoid microbial adhesion and consequently biofilm formation in biomaterials (Chen et al., 2015; Meghil et al., 2015). One of the most promising approaches to combat microbial adhesion is through surface coating with bioactive substances (Bazaka et al., 2011; Trentin et al., 2015; Aranya et al., 2017).

Since 2002, Vicryl Plus was the first commercial antimicrobial suture approved by the US Food and Drug Administration (US FDA), and since then, this suture was clinically used as a useful alternative to decrease the risk factors for Surgical Site Infection (SSI), risk, even in oral surgery (Venema et al., 2011; Hoshino et al., 2013; Sewlikar et al., 2015). However, the efficacy of this suture is still unclear, and its clinical efficacy has been questioned in randomized controlled trials (Wu et al., 2017).

Conversely, antimicrobial molecules extracted from plants also present a promising strategy due to their relatively low toxicity, biocompatibility and low cost (Savoia, 2012; Al-Jumaili et al., 2018). Among these is eugenol (4-allyl-2-methoxyphenol), an aromatic substance found as the majority constituent in clove, cinnamon and myrrh (Khalil et al., 2017), which has been extensively employed in various dental procedures as an analgesic, local anesthetic, antiseptic, and even used in combination with zinc oxide to prepare dental cement (OZE).

Since the prevention or treatment of infections by coating surfaces with antimicrobial substances appears promising, the aim of this work was to evaluate the antibiofilm activity of eugenol against several commercial strains of the genus *Streptococcus* and to test it as an anti-infective coating on cotton sutures by studying the effects on initial adhesion and biofilm development of *Streptococcus mutans* (ATCC 25175).

MATERIALS AND METHODS

Test compound - eugenol solution

Eugenol pure was commercially acquired from Sigma-Aldrich (ReagentPlus®, and used to prepare the stock solution at final concentration of 8000 µg/ml. Frist, eugenol was mixed onto 80 µl of DMSO (dimethyl sulfoxide), and then added fresh Brain Heart Infusion broth (BHI broth, Difco, Detroit, MI) up to 1 ml. Test solutions were made by serial-2-fold dilutions with concentrations ranging from 15 to 2000 µg/ml. To perform the antibiofilm assays on surgical sutures, a new eugenol solution was similarly prepared using PBS/DMSO buffer (sodium phosphate 100 mM with sodium chloride 150 mM, pH 12 and 8% DMSO), and diluted to different concentrations of 2000, 5000 and 8000 µg/ml. These concentrations were established to guarantee the eugenol covering on the suture samples. It was used as untreated group PBS/DMSO buffer, only. The amount of DMSO used to prepare the eugenol solution, as well as phosphate buffer has no effect on bacterial growth of the tested microbes.

Bacterial strains and culture conditions

Streptococcus mutans (ATCC 25175), *S. oralis* (ATCC 10517), *S. parasanguinis* (ATCC 503), *S. pyogenes* (ATCC 19615), *S. salivarius* (ATCC 7073) and *S. sp.* (ATCC 15300) were generously donated by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil). All bacteria were initially streaked from −80°C glycerol stocks onto BHI plates, and a fresh single colony was inoculated into BHI and cultured at 37°C under 5% of CO_2. Bacterial strains were collected by centrifugation (FS-15000CFN II, Vision Scientific, Daejeon, Korea) after 18 h growing at exponential phase, and the cell suspensions were adjusted against to 0.5 McFarland and recorded the optical densities at 620 nm by spectrophotometer (Biotrak II Reader - Amersham Biosciences, Cambridge, England), and then diluted until 10^6 CFU/ml using fresh BHI.

Planktonic susceptibility testing

The antibacterial activity of eugenol was verified according to the microdilution test in 96-well polystyrene plates according to the Clinical and Laboratory Standards Institute (CLSI, 2012). Different eugenol concentrations were prepared as described above and added to the plates at a volume of 100 µl. The same volume of bacterial suspension was then added in each well to a final volume of 200 µl with a density of 5 x 10^5 cells per well, and the plates were incubated for 24 h at 37°C under 5% of CO_2 (Shel Lab - TC2123-2E, Cornelius, USA). The MIC was defined as the lowest concentration that inhibits microbial growth detected by the unaided eye. To establish the MBC, 10 µl of broth from wells showing no visible microbial growth were plated onto BHI agar medium. After 24 h incubation under suitable conditions, the lowest concentration able to completely inhibit microbial growth was considered the

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MBC. BHI broth without eugenol was used as controls. All MIC/MBC tests were performed in triplicate.

Antibiofilm activity *in vitro*

This assay investigated the ability of the compound to interfere in the initial steps of biofilm formation by using a 96-well, flat-bottom microtiter plate (Kasvi K12-096, Parana, Brazil). The prevention of biofilm formation (bacterial inoculation and drug exposure occur simultaneously) was defined as the lowest concentration of antimicrobial agent that resulted in an optical density (OD) 640 nm difference of the mean of positive control well readings, afterwards shown as percentage of biofilm biomass (Macià et al., 2014). Briefly, 100 µl of the serial dilutions of eugenol was added to each well, similar to planktonic susceptibility test, using BHI with 2% sucrose to perform all dilutions. About 100 µl of each bacterial suspension containing 10^6 cells/ml was then transferred into each well to allow biofilm formation for 24 h at 37°C, anaerobically. The extent of residual biofilm formation was measured using the crystal violet (CV) assay, as described by O'Toole (2011). Each strain was tested in triplicate at three independent assays.

Eugenol-impregnated (E) sutures procedure

Cotton-sutures (Techno 3-0/30 mm, São Paulo, Brazil) in a standard-cut size of 1 cm and sterilized in glass Petri dishes by autoclaving were placed individually into polystyrene 24-well plates. One milliliter of eugenol dissolved in PBS/DMSO buffer at concentrations of 2000, 5000 and 8000 µg/ml was added to the plates. PBS/DMSO buffer was used as a control to avoid any interference from DMSO and/or the pH buffer. This group was called the control (C) suture. Afterwards, the threads were incubated with orbital agitation (Marconi-MA 410, Piracicaba, SP, Brazil) overnight at 37°C, 100 rpm, and subsequently dried for approximately 1 - 2 h at room temperature before being used for subsequent assays.

Effect of E-suture on initial adhesion of bacteria

After drying, E-sutures were placed in contact by immersion into 1000 µl of microbial suspension containing 10^6CFU/ml, and incubated for 6 h at 37°C with agitation of 100 rpm to allow the first step of initial adhesion of the biofilm formation process. During adherence analysis, sutures were washed twice using PBS buffer (*sodium phosphate* 100 mM, pH 7.0) and transferred to a new polystyrene 24-well plate with 1000 µl of same buffer to provide the detachment of the adhered cells through ultrasonic bath with 42 kHz and 0.17 kW. After 360 s of sonication, the bacterial suspension obtained was used to CFU count. Serial ten-fold dilution was prepared in fresh broth and plated onto BHI agar and incubated for 24 h at suitable conditions. The C-suture samples were used as a control group. The experiments were repeated at least three independent assays and were performed with six suture-samples per group.

Effect of E-suture on biofilm development

Sutures were incubated for 6 h to enable initial colonization, which was prepared as mentioned previously and afterward submitted to the biofilm formation tests by culturing for 48 h in 1000 µL of fresh BHI on 24-well polystyrene plate. Between intervals of 12 h, test (E) and control (C) sutures were analyzed for biomass production (12/12 h), as well as cellular viability (24/24 h) by crystal violet quantification and the XTT assay (sodium-3-(1-((phenylamino)-carbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate), respectively. For biomass quantification by crystal violet staining, the suture threads under biofilm development were washed twice with PBS-buffer (pH 7.0) and then sequentially transferred to new wells with fresh broth culture. This procedure was repeated at 12, 24, 36 and 48 h after 6 h of initial adhesion. For each time, the E- and C-sutures samples were transferred to new 24-well plate and washed twice to remove loosely attached cells, before to biomass quantification through CV staining.

The XTT reduction assay was used to quantify the bacterial viability in cells firmly attached on the surface of E- and C-threads. Briefly, the solutions of XTT (1 mg/ml) (Sigma, St. Louis, MO, USA) and menadione (0.4 mM) (Fluka, Newport News, VA, USA) were prepared in PBS buffer and DMSO, respectively, before use. After 24 h of biofilm culturing, the suture samples were rinsed twice with PBS buffer to remove loosely attached cells, and allowed to air dry using a newly opened polystyrene 24-well plate. A PBS/XTT/Menadione solution (volume ratio of 79/20/1), was added (1000 µl) in every individual well containing pre-washed suture, and left for 5 h at 37°C in the dark. The reduction of XTT (oxidative activity) was then measured at 492 nm (Biotrak II Reader, Amersham Biosciences). This procedure was performed at the 24 and 48 h intervals after 6 h of initial adhesion. For the measurement of background XTT-colorimetric levels, suture threads exposed to the same solutions but only using sterile culture media was used. Each strain was tested in triplicate.

Statistical analysis

The results of the respective tests were categorized in Microsoft Excel (Version 2012 for Windows) and were analyzed in GraphPad Prism software (Version 5.0 for Windows, San Diego, California, USA). Significant differences between the groups were verified using the one-way ANOVA test with Tukey's multiple comparison post-test. The results were considered to be statistically significant when $p < 0.01$.

RESULTS

Antibacterial activity *in vitro*

Eugenol inhibited significantly the planktonic growth of all species of *Streptococcus* tested at concentrations between 250 and 1000 µg/ml, especially *S. mutans* (ATCC 25175), which showed greater sensitivity (Table 1).

Preliminary results for antibiofilm activity in 96-well microplates indicate that eugenol interferes with the biofilm formation process, resulting in considerable inhibition of all the bacteria tested (Figure 1). For the most *Streptococcus* strains, the residual biomass formed was reduced approximately 75 to 90% at minimal tested eugenol concentrations, 15 µg/ml, unless to *S. oralis* (ATCC 10517), which showed dose-response relationship (Figure 1B). However, these results may be related to the specific antimicrobial activity from eugenol, since the concentrations found were lower to MIC values found for the most of microorganisms tested.

A promising result was manifested by *S. mutans*

Table 1. Minimal bacteriostatic and bactericidal concentration against planktonic cell culture of Eugenol against to oral *Streptococcus* strains.

Microorganism	Eugenol (µg/mL)	
	MIC	MBC
Streptococcus sp. ATCC 15300	500	1000
Streptococcus salivarius ATCC 7073	1000	1000
Streptococcus oralis ATCC 10517	500	1000
Streptococcus mutans ATCC 25175	250	1000
Streptococcus parasanguinis ATCC 503	500	500
Streptococcus pyogenes ATCC 19615	1000	Nd*

*Nd- Not Determined.

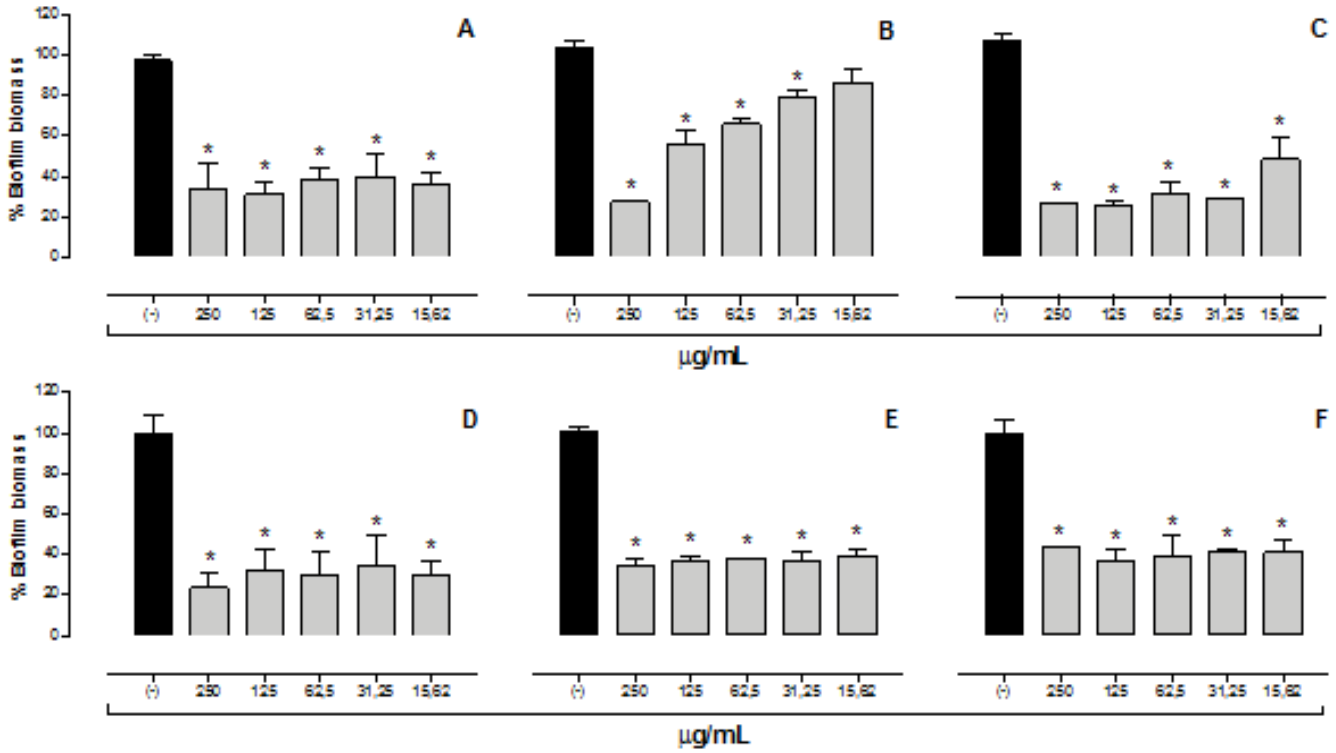

Figure 1. Inhibition of biofilm formation of eugenol at different subMIC concentrations against six species of *Streptococcus* reference strains represented as percentage of residual biofilm biomass. *Significantly different ($p<0.01$). *Streptococcus mutans* ATCC 25175 (A); *S. oralis* ATCC 10517 (B); *S. parasanguinis* ATCC 503 (C); *S. pyogenes* ATCC 19615 (D); *S. salivarius* ATCC 7073 (E); *S. sp.* ATCC 15300 (F). (black bar) Group control; (Gray bar) decreasing concentration of eugenol (15 to 250 µg/ml).

(ATCC 25175) indicated at Figure 1A. Beyond being bacterium with clinical relevance in the caries development, it exhibited one of the best responses regarding preliminary antibiofilm activity, performed on polystyrene surface (96-well plate). Even at lower concentrations, 15 µg/ml, it decreased around 60% of biofilm biomass compared to untreated group. So, this strain was selected to carry out the experimental biofilm formation on the suture-cotton threads.

Effect of eugenol (E)-suture on initial adhesion of bacteria

In previous antiadhesive tests using E-suture produced in neutral pH, the eugenol groups did not show any differences with the C-suture, control group, after 6 h of initial adhesion. This result is probably due to ionization, since the eugenol in alkaline solution (pH 12) showed better interaction with the cotton-suture (electrically

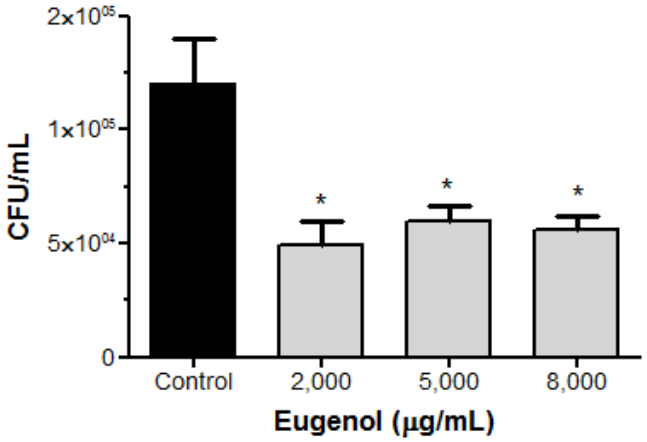

Figure 2. Antiadhesive effect of eugenol on *S. mutans* ATCC 25175 adhered on the surgical suture. (control) bacteria adhered on C-suture; (2000, 5000 and 8000 µg/ml) bacteria adhered on E-suture at different concentrations ($p < 0.01$).

neutral). After a period of initial adhesion, the E-suture treatment significantly inhibited the adhesion of viable *S. mutans* cells and compared to untreated group (Figure 2). A reduction of more than 50% of colony forming units (CFU) was observed compared to the C-sutures.

Effect of E-suture on biofilm development

The quantification of the *S. mutans* (ATCC 25175) biomass formed on E-suture (2000, 5000 and 8000 µg/ml) was performed by the crystal violet staining method at 0, 12, 24, 36 and 48 h after 6 h of initial adhesion. Significant differences between treatments and the negative control were observed only after 12 h. These differences continued until 48 h, which demonstrates that an early inhibition of the initial adhesion may be prolonged for a time, decreasing the biofilm development and biomass accumulation (Figure 3A). Metabolically active cells of *S. mutans* were monitored by XTT reduction at 24 and 48 h after initial adhesion (6 h) on E-suture at concentrations of 2000, 5000 and 8000 µg/ml. We observed a significant decrease in cellular metabolism in all E-suture groups, while the negative control presented a progressive rise of metabolic activity, proportional to the maturation period (Figure 3B).

At the end of the biofilm development (48 h), all of the E-sutures did not show any visible biofilm layer. In contrast, the control group formed a high amount of biomass with healthy metabolic activity, which was clearly observed through the high optical density values. These results demonstrate that eugenol acts to prevent the initial adhesion of *S. mutans* and, consequently, affects negatively biofilm development.

DISCUSSION

Several researchers have already demonstrated the antimicrobial activity of many essential oils rich in the eugenol are active against a wide range of fungi and Gram-negative and -positive bacteria (Yadav et al., 2013; Marchese et al., 2017). In this case, the analysis was performed against bacteria from the genus *Streptococcus*, which are intrinsically related to biofilm formation and development of diseases in the oral cavity (Tomás et al., 2013). The eugenol activity on bacterial cells is well-studied and the antimicrobial effect is attributed to lipophilic nature, as well as the presence of a free hydroxyl group in the molecule (Nazzaro et al., 2013). Among different hypothesized mechanisms of the eugenol action, the primary approach involves the plasma membrane disruption that increases nonspecific permeability and affects the transport of ions and ATP (Devi et al., 2010; Marchese et al., 2017). Additionally, eugenol is active against certain bacterial enzymes, such as proteases, histidine carboxylases, amylases, and ATPases (Devi et al., 2013). It is highly probable that this versatility of eugenol's mechanism of action reduces the potential of bacteria to develop resistance, which is a serious problem that evolves common antimicrobial agents, such as triclosan (Ciusa et al., 2012).

The effect eugenol may be related to the ability to prevent biofilm organization and to disaggregate micro colonies, as demonstrated by Yadav et al. (2015) against *Streptococcus pneumoniae*. Recently, comparing antibiofilm activity of eugenol and three other eugenol derivatives, researchers suggested that the C-1 hydroxyl unit, the C-2 methoxy unit, and C-4 alkyl or alkane chain on the benzene ring of eugenol play important roles against to Enterohemorrhagic *Escherichia coli* (EHEC) biofilm formation without affecting planktonic cell growth (Kim et al., 2016). Thus, the presence of this antimicrobial compound made the bacteria unable to form biofilms due to antifouling activity of sub-MIC eugenol concentrations (Yadav et al., 2013; Kim et al., 2016).

Sutures impregnated or coated with several antibacterial agents, including plant-derived metabolites, have been extensively explored by many researchers (Reinbold et al., 2017; Sudha et al., 2017). Therefore, the aim of this study was to suggest a safe and inexpensive eugenol-coating for surgical sutures. This molecule may represent a novel alternative for reducing bacterial accumulation and subsequent *S. mutans* biofilm formation on suture threads. Our results showed that, among the bacterial strains, *S. mutans* was one of the most sensitive to eugenol in planktonic and biofilm inhibition assays (Table 1). Thus, this strain was used for subsequent assays involving biofilm formation on cotton-suture threads.

In dental practice, sutures are frequently used in procedures within periodontics, endodontics, implant dentistry, as well as oral and maxillofacial surgery (Kathju

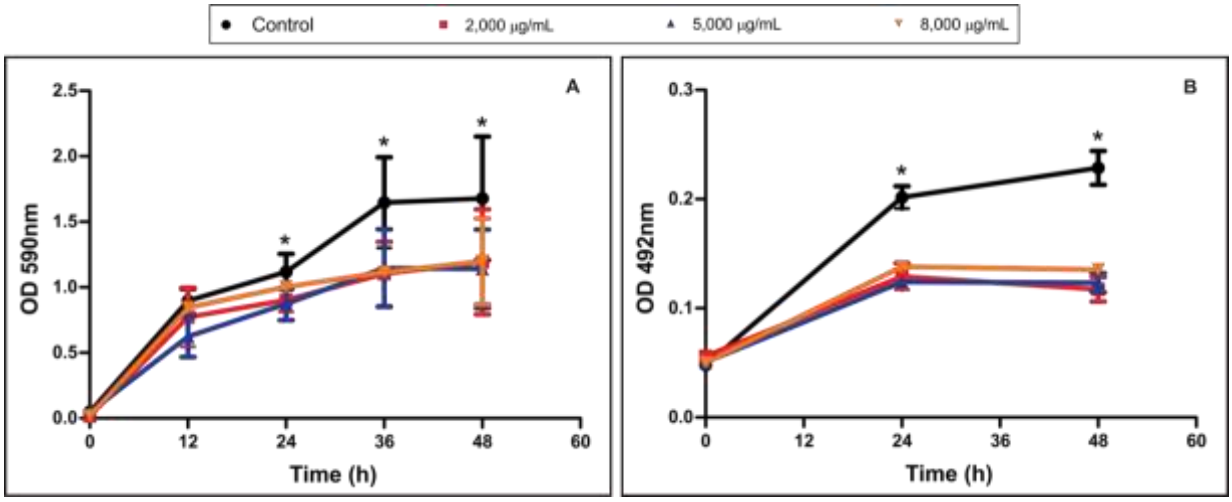

Figure 3. Antibiofilm effect of eugenol-impregnated sutures at different concentrations (2000, 5000 and 8000 µg/mL) on *S. mutans* (ATCC 25175) biofilm development. Cells adhered after 6 hours of initial adhesion were quantified during 48 h of development, with intervals of 12 h to biomass quantification, and 24 h to metabolic rate. Positive control was C-suture. (A) Biomass quantification measured using the dye crystal violet (CV), and (B) metabolic activity by XTT reduction assay. All of the experiments were done in three independent days ($p < 0.01$).

et al., 2009). However, these abiotic structures favor the adhesion and accumulation of pathogenic micro-organisms that can initiate an infectious process at the surgical site (Surgical site infection - SSI) and thereby compromise the healing process (Venema et al., 2011). Absorbable and non-absorbable sutures in patients after dental surgery have shown different bacteria adhered on surface. Among these, aerobic and anaerobic bacteria comprised >40 and >25%, respectively, and included pathogenic microorganisms that did not belong in the oral cavity (Otten et al., 2005). Thus, antimicrobial suture coatings have been developed to avoid bacterial adherence and colonization on suture surfaces (Serrano et al., 2015; Gallo et al., 2016; Reinbold et al., 2017).

The effect *in vitro* of eugenol on the cariogenic properties of *S. mutans* was demonstrated previously by Xu et al. (2013). Eugenol inhibited, significantly, the acid production from carbohydrate fermentation, and, as well as, the synthesis of water-insoluble glucans through glucosyltransferases (Gtf) of *S. mutans* inhibition (Li et al., 2012). Similar results were obtained using artificial mouth models with oral bacteria of the genus *Streptococcus*, wherein eugenol was able to eradicate *S. mutans* and *S. sobrinus* biofilms by inhibiting the synthesis of insoluble and soluble glucans (Xiao and Koo, 2010).

The biosynthesis of water-insoluble glucans through glucosyltransferase (Gtf) is an essential Streptococcal mechanism for the adhesion, co-adhesion and bacterial accumulation on the tooth surface, in addition to providing structural integrity for the biofilm matrix (Li et al., 2012). Similarly, there are specific water-insoluble glucans that promote the adhesive interactions of bacteria with solid surfaces, and inhibition of Gtf activity (particularly GtfC and GtfB) and polysaccharide synthesis is an approach to prevent biofilm-related diseases (Abachi et al., 2016).

Many Gtf inhibitors have been identified in plant-derived compounds, including eugenol, which was recognized for its anti-biofilm capacity using multiple mechanisms, including the direct inhibition of Gtf activity (Xu et al., 2013; Ren et al., 2016). Our results show that eugenol adsorbed to the E-suture surface may work as a Gtf inhibitor, resulting in a decrease of water-insoluble glucans synthesis which, in turn, reduced the number of bonded cells on the E-suture surface (Figure 2), as well as biofilm development (Figure 3). The results of crystal violet and XTT methods showed a minor level of attached cells in the E-suture treatment were able to develop into a biofilm; however, the amount was considerably statistically less than the untreated C-suture group.

Currently, cotton sutures are the most commonly used materials for skin closure, and such material is not commercially available with antimicrobial properties, such as Vicryl® Plus, which is coated by triclosan and considered the “gold standard”.

Bioactive compounds, such as eugenol, are a promising alternative to triclosan, which has demonstrated doubtful effectiveness for some surgical interventions (Sewlikar et al., 2015). Furthermore, triclosan has been classified as an "emerging environmental contaminant", that has been directly related to the spreading of antibiotic resistance in clinically important strains (Carey and McNamara, 2015).

Conclusion

Eugenol successfully exhibited modest antiadhesive and antibiofilm properties when used to cover the cotton-suture surface. Although there was no dose-dependent effect, it is noteworthy that the eugenol film adsorbed to the E-suture and promoted a high level of inhibition of cell adhesion and biofilm maturation of *S. mutans* (ATCC 25175). Thus, the present data show that eugenol is an inexpensive antifouling alternative to protect surgical sutures. However, new studies are strongly recommended to increase the knowledge regarding molecules that can control the initial adhesion and formation of the first cell aggregates of *S. mutans*. The continuation of this research may help to identify new compounds for the prevention of infections associated with biomaterials.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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