Oxygen tension during biofilm growth influences the efficacy antimicrobial agents

Eficácia dos antimicrobianos em biofilme formado por diferentes tensões de oxigênio

Raquel Pippi ANTONIAZZI[a], Gabriela Ocampo TROJAHN[a], Maísa CASARIN[b]*,
Camilla Filippi dos Santos ALVES[b], Roberto Christ Vianna SANTOS[b], Fabricio Batistin ZANATTA[b]

[a]Faculdade de Odontologia, UNIFRA – Centro Universitário Franciscano, Santa Maria, RS, Brasil
[b]UFSM – Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

Resumo

Objetivo: Comparar a eficácia antimicrobiana de clorexidina 0,12% (CHX) e solução de chá verde (CV) (*Camellia sinensis*) em biofilmes formados em diferentes tensões de oxigênio em um modelo *in situ*. **Método:** Vinte e cinco estudantes de odontologia foram elegíveis. Um dispositivo com espécimes de esmalte (EE) padronizados, voltado para o palato e para vestibular foram inseridos nos voluntários por 7 dias. Durante os primeiros quatro dias nenhum agente foi aplicado. A partir do quinto dia, ambos os agentes foram aplicados nos EEs no grupo teste e nenhum agente foi aplicado no grupo EE controle. Após 7 dias, os fragmentos EEs foram removidos dos dispositivos, sonicados, plaqueados em ágar, e incubado durante 24 h a 37 °C para determinar unidades formadoras de colonias (UFCs). **Resultado:** CHX teve significativamente melhor eficácia em comparação com CV nos EEs para vestibular (1.330 vs. 2.170 UFC/mL) e EEs voltados para o palatal (2.250 vs. 2.520 UFC/mL). Comparações intragrupos mostraram maior eficácia em EEs vestibulares do que as EEs voltadas para o palato (1.330 vs. 2.250 UFC/mL para CHX e 2170 vs, 2520 UFC/mL para CV) em ambas as soluções. Análise dos controles, mostrou a formação de biofilme significativamente maior em comparação com EEs palatinas e bucais. **Conclusão:** CHX tem uma eficácia maior do que o chá verde em biofilmes formados por 4 dias. A eficácia dos agentes foi reduzida para os biofilmes em ambiente com baixa tensão de oxigênio. A tensão de oxigênio parece influenciar na eficácia dos agentes testados.

Descritores: *Camellia sinensis*; clorexidina; placa dentária.

Abstract

Objective: To compare the antimicrobial efficacy of a 0.12% chlorhexidine (CHX) and herbal green tea (*Camellia sinensis*) solution on established biofilms formed at different oxygen tensions in an *in situ* model. **Method:** Twenty-five dental students were eligible for the study. *In situ* devices with standardized enamel specimens (ES) facing the palatal and buccal sides were inserted in the mouths of volunteers for a 7 day period. No agent was applied during the first four days. From the fifth day onward, both agents were applied to the test ES group and no agent was applied to the control ES group. After 7 days the ES fragments were removed from the devices, sonicated, plated on agar, and incubated for 24 h at 37 °C to determine and quantify the colony forming units (CFUs). **Result:** CHX had significantly higher efficacy compared to green tea on the buccal (1330 vs. 2170 CFU/μL) and palatal (2250 vs. 2520 CFU/μL) ES. In addition, intragroup comparisons showed significantly higher efficacy in buccal ES over palatal ES (1330 vs. 2250 CFU/μL for CHX and 2170 vs, 2520 CFU/μL for CV) for both solutions. Analysis of the ES controls showed significantly higher biofilm formation in palatal ES compared to buccal ES. **Conclusion:** CHX has higher efficacy than green tea on 4-day biofilms. The efficacy of both agents was reduced for biofilms grown in a low oxygen tension environment. Therefore, the oxygen tension environment seems to influence the efficacy of the tested agents.

Descriptors: *Camellia sinensis*; chlorhexidine; dental plaque.

INTRODUCTION

Dental biofilm is composed of extracellular exopolymers from bacteria permeated by bacterial products, saliva, and gingival exudate[1]. On the tooth surface, the initial bacterial colonizers allow other bacteria that do not have direct adhesion to the substrate to colonize the tooth surface through specific interactions[2]. This allows bacteria with contrasting characteristics, such as aerobic and anaerobic bacteria, to survive together in a microaerophilic environment created by the oxygen consumed by the aerobic bacteria in the inner layers

of the biofilm[3]. It has been hypothesized that biofilms that grow under conditions of oxygen deprivation, such as the subgingival biofilms that are etiologic agents of destructive periodontal diseases, can show qualitative and quantitative differences that also result in differences in their pathogenicity potential[2,3]. Furthermore, influences in the oral cavity, such as, decreased saliva flow in the dental substrate can increase bacterial activity in dental biofilm[3].

Antimicrobial resistance is, perhaps, one of the most striking features of bacteria structured as a biofilm[4]. Mechanical disruption of supragingival biofilm with fluoridated toothpaste is known to be the most effective form of oral hygiene for maintaining a balance between dental health and gingivitis. However, a large percentage of the population fails in performing routine oral hygiene self-care at home[2,3]. Chemical agents have been studied to minimize failures in oral hygiene. Among those agents, chlorhexidine (CHX) is considered the gold standard agent for reducing biofilm formation[5]. However, its effectiveness appears to be reduced with biofilm already established on the tooth surface. Zaura-Arite et al.[6] exposed biofilms, that had been established for 24 h and 48 h, to 0.2% CHX and found that the compound was ineffective on deeper biofilm layers. Another study, using *in situ* model devices, has corroborated these findings with established biofilms[7].

The natural products trade has increased in recent years, and green tea (*C. sinensis*) has been shown to have therapeutic effects and biological activity in the treatment of systemic diseases[8]. Recently, green tea has been investigated for its antimicrobial efficacy due to the action of components such as polyphenols and flavonoids[9]. Some authors have demonstrated that subgingival application of green tea, together with scaling and root planning, has shown better results than just scaling and root planning[10-12]. A preliminary, randomized clinical trial showed that green tea mouthwash was effective in reducing plaque and gingivitis scores[13]. Some evidence suggests that green tea extract has antimicrobial activity against anaerobic bacteria that are typically associated with periodontal diseases[10] and against cariogenic bacteria[14]. In addition, green tea inhibits the growth and adherence of *Porphyromonas gingivalis*[15], *Prevottela intermedia*[12] and several other bacteria[16]. This evidence suggests that the natural product may inhibit the adhesion phase of biofilm formation. However, there is currently a lack of information regarding the effect of green tea on an established oral biofilm.

Since the biofilm formation process has different characteristics, based on the available oxygen environment, the aim of this study was to compare the antimicrobial effect of CHX 0.12% and a green tea solution on established biofilms formed under different oxygen tensions.

METHOD

This study used an *in situ* model with *in vitro* microbial analysis and was conducted between June and December, 2012. The research ethics committee of the Franciscan University Center (CAAE: 06782812.7.0000.5306) approved the study protocol. The volunteers were selected from graduate dental students at the UNIFRA dental college. Eligibility criteria included good general health, no use of any antimicrobial / anti-inflammatory drugs for at least 3 months prior to the start of the study, not being mouth breathers, no periodontitis (defined as a loss of attachment > 3 mm in at least 2 proximal sites), non-smokers, and having no allergies to the substances used in the study. Any individuals that exhibited an allergic reaction to the compounds after the study was initiated were subsequently withdrawn and excluded from the analysis.

In situ Devices

Intraoral devices were fabricated on plaster models of the upper arch, which were obtained by molding with alginate (Avagel®, Dentsply, Petrópolis, RJ, Brazil) and special plaster casting (Asfer®, São José, SP, Brazil). Eighty bovine incisors were obtained for the preparation of enamel specimens (ES). Teeth with cracks or grooves were discarded. Square ES were prepared (5 mm × 5 mm × 1 mm) using discs, drills, and a digital caliper (Jomarca®, Guarulhos, SP, Brazil). The ES were then autoclaved and packaged in a suitable container until use. Eight ES were inserted into each *in situ* device. Four fragments were placed in the palatal area (to allow limited access to oxygen) and four fragments were placed in contact with the free oral environment.

Tested Solutions

Two antiseptic solutions were tested: 0.12% Chlorhexidine (Periogard®, São Paulo, SP, Brazil); and, a manipulated solution of 10% herbal green tea extract from the *Camellia sinensis* leaf. Dehydrated *Camellia sinensis* leaves (Yamamotoyamar®, São Miguel Arcanjo, Brazil) were obtained commercially. The green tea extract solution contained the following: 10% dry *Camellia sinensis* (Yamamotoyamar®, São Miguel Arcanjo, Brazil) leaf extract, 0.05% sodium fluoride, 0.15% mint aroma, 0.01% green dye, 0.01% sucralose, 0.1% EDTA, 0.5% nipagim solution/Nipasol, and water. The two solutions were delivered, in two distinct identified glasses with screw caps and application droppers, in order to apply them. In addition, a specially formulated toothpaste and a bottle containing saline solution were given to each participant. Both the green tea extract solution and the toothpaste were produced by a compounding pharmacy (Dermapelle®, Santa Maria, RS, Brazil).

Experimental Procedures

Two days befor the baseline assessment, volunteers began to use a toothpaste without fluoride or active compounds. At baseline, *in situ* devices were inserted into volunteers and maintained in them for 7 days. Participants were told to remove the devices only for meals, oral hygiene, and application of solutions (requiring immersion of the devices in the saline solution) during the study period. The participants were also instructed not to apply any solution to the devices during the first four days of the study (period of biofilm formation and maturation). The 21 participants were instructed to add one drop of each solution to the respective ES (on the palatal and bucal sides), every 12 hours (twice a day), starting on the fifth day. ES #1 received the 0.12% CHX solution, ES #4 received the green tea extract solution, and ES #2 and #3 were controls that did not receive either solution (biofilm only) (Figure 1).
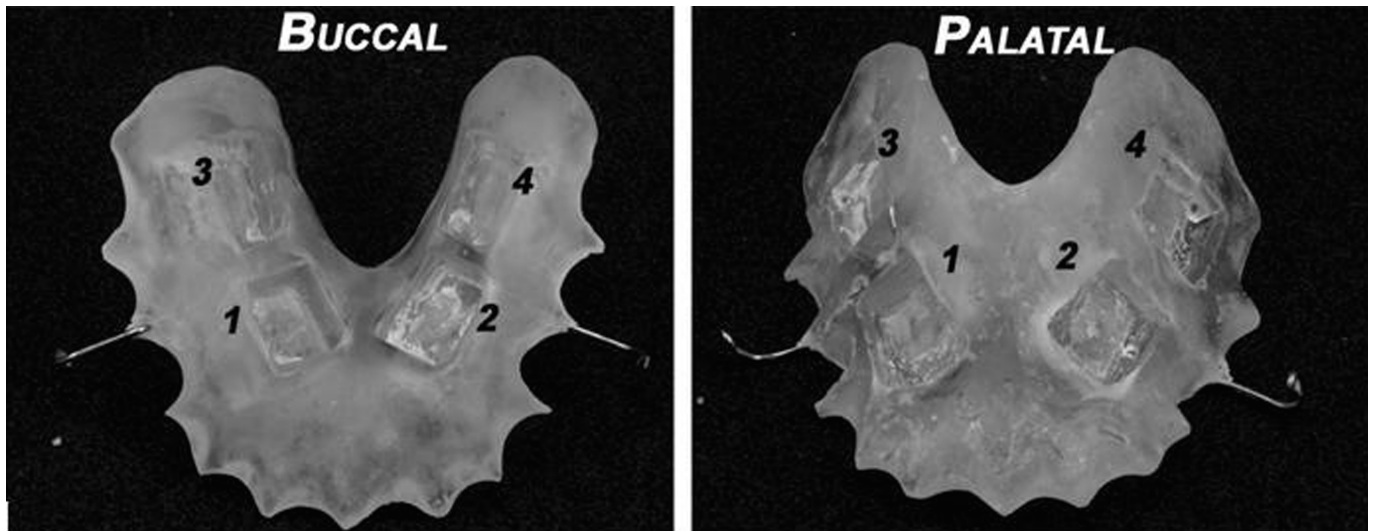
Figure 1. Views of both sides of the same *in situ* device. The left figure shows the buccal side of the device and right shows the palatal side of the device.

At the time of installation of the *in situ* devices, all participants received a detailed explanation of every step of the experiment. A written protocol containing detailed explanations of all steps and solutions was also provided. The experimental design is summarized in Figure 2. After the 7 day study period, the devices were removed from the oral cavity and each ES was analyzed in the laboratory.

For the bacterial vitality analyses, ES were removed from the *in situ* devices using tweezers and a spatula, and immediately were placed in test tubes with screw caps, each containing 5 ml of saline solution. Tubes were then sonicated (Ultrasonic Washer USC3380A, 37KHz, Unique, Brazil) for 15 min to detach bacteria that were adhered to the enamel surface. The sonication time of 15 min was previously determined in a pilot study that investigated the optimal time for detaching bacteria from the ES without resulting in significant cell death. After the samples were sonicated they were diluted in 0.9% NaCl and then a serial dilution was performed for each suspension. The diluted suspensions were plated on nutrient agar and incubated in aerobiosis for 24-48 h at 37 °C to determine and quantify the number of colony forming units (CFUs). The examiner analyzed the CFUs on plates in blinded fashion.

Intra-examiner Reproducibility

Prior to the beginning of the study, a training session was conducted for determining the viable cell count. During this process, the examiner analyzed petri dishes containing CFUs and compared the results with those of an expert examiner until there was agreement between the results. The trained examiner then calibrated the process by counting 5 plates in duplicate in a blinded fashion at an interval of 1 hour for each plate. This calibration process resulted in an intraclass correlation coefficient of 0.92.

The number of ES used for the application of each agent was determined in a pilot study where a mean difference of 2500 CFUs with a standard deviation of 3500 CFUs was found between the buccal and palatal side for each agent. Based on this mean difference, 80% beta, 5% alpha, and a paired design, it was estimated that 17 ES were needed on both the buccal and palatal sides, for each agent.
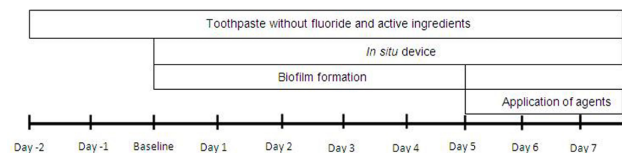

Figure 2. Experimental flow diagram.

When we included a dropout estimate, it was determined that 25 ES were needed on both the palatal and buccal sides. Therefore, we recruited 25 volunteers to participate in this study.

Data Analysis

The microbiological data analysis was presented using absolute and relative frequencies obtained from the palatal and lingual sides treated with the different agents. Different experimental groups were compared using analysis of variance (ANOVA) for repeated measures, and then ranked for analysis using a *post-hoc* Tukey test. For comparisons between the buccal and palatal sides receiving the same agent, a Student's paired t-test was used. The statistical significance level was set at 5%.

RESULT

25 volunteers were eligible for the study. However, 4 were excluded due to failure to utilize the *in situ* devices for the entire study period. The mean age of the participants who completed the study was 21.3 ± 2.3 years. There were 12 women and 9 men. Table 1 shows that the 0.12% CHX was significantly more effective for reducing CFUs than green tea on the buccal (1330 vs. 2170 CFUs/μl) and palatine sides (2250 vs. 2520 CFUs/μl), respectively. Both agents were significantly more effective than the controls that did not receive any solution. In addition, intragroup comparisons showed that the efficacy of both agents was significantly different between the buccal and palatal sides.

Table 1. Mean colony forming unit (CFU) counts from the different conditions

	Count (CFUs/µl) Mean ± standard deviation			
	Total	**Buccal**	**Palatal**	**p#**
0.12% CHX	2910 ± 2550[A]	1330 ± 1000[A]	2250 ± 2230[A]	0.004
Green Tea	3610 ± 3200[B]	2170 ± 2340[B]	2520 ± 2370[B]	0.015
Control	8120 ± 9390[C]	3360 ± 2850[C]	6440 ± 8790[C]	0.001
p*	< 0.001	< 0.001	< 0.001	

p* - ANOVA for repeated measures. p# - Paired t-test (comparisons within the group and between the buccal and palatal side). Uppercase letters, comparison in the column: A-B; B-C; A-C: p < 0.05. *Post-hoc* Tukey test.

DISCUSSION

The present study compared the efficacy of different agents on oral biofilms formed under different oxygen tensions. Our results showed that 0.12% CHX had significantly greater efficacy at reducing the CFUs than the green tea extract, the untreated, and the control. Moreover, our analysis found that both agents were less efficacious on the palatal side than on the buccal side.

Biofilm growth provides advantages for bacterial survival, including protection against competitive microorganisms, environmental factors (host defense mechanisms), and toxic substances in the environment (antimicrobial agents)[2,3]. Moreover, the habitat has a strong effect on the composition, metabolic activity, and virulence factors of the microorganisms present. Subgingival biofilms present qualitative and quantitative differences compared to supragingival biofilms because they develop in periodontal pockets where oxygen levels are reduced[2,3]. In order to simulate biofilm formation under different oxygen tensions, ES were inserted toward the palate and the oral cavity in the *in situ* device; thus, the ES facing the palate would have greater biofilm growth. This was confirmed by the greater number of CFUs that formed on the ES controls placed on the palatal side than on the buccal side. Furthermore, the fact that specimens facing the palate were less exposed to saliva may have influenced the results because of the important antimicrobial function[3] of the saliva. Our results showed that both agents had greater efficacy on the biofilms on the buccal side. It is possible that the lower efficacy observed with the palatal specimens was due to the higher number of bacterial cells present and the more dense exopolysaccharide matrix. Therefore, biofilms formed in environments with low oxygen tension seem to have greater resistance to antimicrobial agents. Ximénez-Fyvie et al.[17] showed that subgingival plaque is qualitatively and quantitatively different than supragingival plaque.

CHX is currently the gold standard agent for dentistry use. It is a bisdiguanide bi-positive salt with 12 hour substantivity, which means that it is maintained in certain environments and released gradually over 12 hours. In addition, CHX is a broad spectrum antimicrobial agent that acts on the bacterial cell wall and is effective against Gram positive, Gram negative, aerobic, and anaerobic bacteria as well as fungi and some viruses[18]. For this reason, CHX was used as the positive control in this study. Our results also confirmed the unique characteristics of CHX substantivity, since all ES received drops of either agent every 12 hours, which could have been too long for the optimal effects of the green tea extract. This was also supported by the greater efficacy of CHX compared to the green tea extract on both the buccal and palatal ES. However, 0.12% CHX has limited efficacy in the deeper layers of an established biofilmed compared to planktonic cultures[6], and this reduced action against an established biofilm seems to be clinically important regarding gingivitis[19].

Despite the lower efficacy of the green tea extract compared to CHX, our results demonstrated that the extract had a therapeutic effect on established biofilms compared to the control group (that did not receive any solution). The activity observed was most likely due to the polyphenols such as catechin found in the green tea leaf, which seem to affect the double layer of the cell membrane that is critical for bacterial resistance to antimicrobials as well as other cell functions[9]. Our results corroborate evidence demonstrating *in vitro* antimicrobial activity of green tea on various bacteria[15,16]. Furthermore, the consumption of green tea can provide clinical benefits in the treatment of gingivitis[13,20] and periodontitis with greater reductions in probing depth and gains in clinical attachment[9]. It is possible that this systemic effect is due to the powerful antioxidant effects of green tea, as it is known that oxidative stress plays an important role in the pathogenesis of periodontal diseases. Thus, it is thought that antioxidants may be beneficial in treating inflammatory diseases. Moreover, the systemic action of catechins seems to contribute to a decrease in the activity of proteinases[20] and a decrease in osteoclast formation[21], thereby interfering with the normal course of periodontal diseases.

The microbiological techniques used in this study are widely evident and referenced in the literature[22]. However, one important limitation of this type of study is the high sensitivity for detecting aerobic microorganisms, but low sensitivity for detecting anaerobic microorganisms. Our results showed a higher number of bacteria on the palatal ES, which was probably due to the formation of biofilms containing a greater number of anaerobic bacteria as a result of the local microaerophilic conditions. Our results were most likely not affected by the technique used for counting bacteria. Furthermore, it is known that the gold standard for assessing the antimicrobial effect in biofilms is confocal laser scanning microscopy because it allows for identification of the location and three-dimensional structure of molecules labeled with fluorochromes, can differentiate viable and non-viable microorganisms, and allows for visualization of the spatial distribution of species within biofilms[3].

The use of *in situ* models to evaluate the effect of topical antimicrobial agents in oral biofilms is a reliable experimental model[23]. These models can simulate biofilms that form on the surface of teeth and are exposed to different host protective factors present in the oral cavity. In this study, the effect of different solutions, on biofilms that had been growing for four days, was evaluated. This biofilm growth period was chosen due to the little change in quantitative and qualitative biofilm composition that occurs

after four days of growth[24]. Additionally, after 4 days of biofilm growth, a visible dental biofilm layer forms. High percentages of visible supragingival biofilms have been observed clinically within this time frame[25]. Therefore, the four day growth period was chosen to simulate a real clinical situation widely confirmed in previous studies. However, because we evaluated a surrogate outcome, our results cannot be extrapolated to clinical situations with real outcomes, such as the reduction of caries activity or gingivitis. The laboratory test, based on *in situ* biofilm models, of antiseptic substances can only simulate intra-oral situation.

This is an important step in selecting the agent, which should be used in clinical studies[7].

In conclusion, despite the study limitations we found that 0.12% CHX had better antimicrobial efficacy than green tea extract, and both agents were significantly better than the controls that did not receive either solution. However, oxygen tension appears to influence the effectiveness of the agents, which exhibited lower antimicrobial effect on biofilms grown under reduced oxygen conditions. Therefore, controlled clinical studies are needed to assess the clinical utility and efficacy of green tea extract.

REFERENCES

1. Park AW, Yaacob HB. A synopsis of the origins and function of dental plaque and pellicle. J Nihon Univ Sch Dent. 1994 Sep;36(3):157-74. PMid:7989958. http://dx.doi.org/10.2334/josnusd1959.36.157.

2. Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. Clin Oral Investig. 2003 Dec;7(4):181-8. PMid:14598129. http://dx.doi.org/10.1007/s00784-003-0236-1.

3. Marsh PD. Dental plaque as a biofilm and a microbial community: implications for health and disease. BMC Oral Health. 2006;6(Suppl 1):S14. PMid:16934115. http://dx.doi.org/10.1186/1472-6831-6-S1-S14.

4. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999 May;284(5418):1318-22. PMid:10334980. http://dx.doi.org/10.1126/science.284.5418.1318.

5. Swartz MN. Impact of antimicrobial agents and chemotherapy from 1972 to 1998. Antimicrob Agents Chemother. 2000 Aug;44(8):2009-16. PMid:10898668. http://dx.doi.org/10.1128/AAC.44.8.2009-2016.2000.

6. Zaura-Arite E, van Marle J, ten Cate JM. Conofocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. J Dent Res. 2001 May;80(5):1436-40. PMid:11437215. http://dx.doi.org/10.1177/00220345010800051001.

7. Auschill TM, Hein N, Hellwig E, Follo M, Sculean A, Arweiler NB. Effect of two antimicrobial agents on early in situ biofilm formation. J Clin Periodontol. 2005 Feb;32(2):147-52. PMid:15691343. http://dx.doi.org/10.1111/j.1600-051X.2005.00650.x.

8. Cho KN, Sukhthankar M, Lee SH, Yoon JH, Baek SJ. Green tea catechin (-)-epicatechin gallate induces tumour suppressor protein ATF3 via EGR-1 activation. Eur J Cancer. 2007 Nov;43(16):2404-12. http://dx.doi.org/10.1016/j.ejca.2007.07.020. PMid:17764926.

9. Kushiyama M, Shimazaki Y, Murakami M, Yamashita Y. Relationship between intake of green tea and periodontal disease. J Periodontol. 2009 Mar;80(3):372-7. PMid:19254120. http://dx.doi.org/10.1902/jop.2009.080510.

10. Kudva P, Tabasum ST, Shekhawat NK. Effect of green tea catechin, a local drug delivery system as an adjunct to scaling and root planing in chronic periodontitis patients: a clinicomicrobiological study. J Indian Soc Periodontol. 2011 Jan;15(1):39-45. PMid:21772720. http://dx.doi.org/10.4103/0972-124X.82269.

11. Chava VK, Vedula BD. Thermo-reversible green tea catechin gel for local application in chronic periodontitis: a 4-week clinical trial. J Periodontol. 2013 Sep;84(9):1290-6. PMid:23121459. http://dx.doi.org/10.1902/jop.2012.120425.

12. Hirasawa M, Takada K, Makimura M, Otake S. Improvement of periodontal status by green tea catechin using a local delivery system: a clinical pilot study. J Periodontal Res. 2002 Dec;37(6):433-8. PMid:12472837. http://dx.doi.org/10.1034/j.1600-0765.2002.01640.x.

13. Sarin S, Marya C, Nagpal R, Oberoi SS, Rekhi A. Preliminary clinical evidence of the antiplaque antigingivitis efficacy of a mouthwash containing 2% green tea: a randomised clinical trial. Oral Health Prev Dent. 2015;13(3):197-203. http://dx.doi.org/10.3290/j.ohpd.a33447. PMid:25610918.

14. Awadalla HI, Ragab MH, Bassuoni MW, Fayed MT, Abbas MO. A pilot study of the role of green tea use on oral health. Int J Dent Hyg. 2011 May;9(2):110-6. PMid:21356006. http://dx.doi.org/10.1111/j.1601-5037.2009.00440.x.

15. Sakanaka S, Aizawa M, Kim M, Yamamoto T. Inhibitory effects of green tea polyphenols on growth and cellular adherence of an oral bacterium, Porphyromonas gingivalis. Biosci Biotechnol Biochem. 1996 May;60(5):745-9. PMid:8704303. http://dx.doi.org/10.1271/bbb.60.745.

16. Cho Y-S, Oh J-J, Oh K-H. Antimicrobial activity and biofilm formation inhibition of green tea polyphenols on human teeth. Biotechnol Bioproc E. 2010 Apr;15(2):359-64. http://dx.doi.org/10.1007/s12257-009-0195-8.

17. Ximénez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol. 2000 Sep;27(9):648-57. PMid:10983598. http://dx.doi.org/10.1034/j.1600-051x.2000.027009648.x.

18. Addy M, Moran J. Comparison of plaque accumulation after topical application and mouth rinsing with chlorhexidine gluconate. J Clin Periodontol. 1983 Jan;10(1):69-71. PMid:6572636. http://dx.doi.org/10.1111/j.1600-051X.1983.tb01268.x.

19. Zanatta FB, Antoniazzi RP, Rösing CK. The effect of 0.12% chlorhexidine gluconate rinsing on previously plaque-free and plaque-covered surfaces: a randomized, controlled clinical trial. J Periodontol. 2007 Nov;78(11):2127-34. PMid:17970679. http://dx.doi.org/10.1902/jop.2007.070090.

20. Jenabian N, Moghadamnia AA, Karami E, Mir APB. The effect of Camellia Sinensis (green tea) mouthwash on plaque-induced gingivitis: a single-blinded randomized controlled clinical trial. Daru. 2012 Sep;20(1):39. PMid:23351842. http://dx.doi.org/10.1186/2008-2231-20-39.

21. Okamoto M, Sugimoto A, Leung KP, Nakayama K, Kamaguchi A, Maeda N. Inhibitory effect of green tea catechins on cysteine proteinases in Porphyromonas gingivalis. Oral Microbiol Immunol. 2004 Apr;19(2):118-20. PMid:14871352. http://dx.doi.org/10.1046/j.0902-0055.2003.00112.x.

22. Yun JH, Pang EK, Kim CS, Yoo YJ, Cho KS, Chai JK, et al. Inhibitory effects of green tea polyphenol (-)-epigallocatechin gallate on the expression of matrix metalloproteinase-9 and on the formation of osteoclasts. J Periodontal Res. 2004 Oct;39(5):300-7. PMid:15324350. http://dx.doi.org/10.1111/j.1600-0765.2004.00743.x.

23. Pantanella F, Berlutti F, Passeri D, Sordi D, Frioni A, Natalizi T, et al. Quantitative evaluation of bacteria adherent and in biofilm on single-wall carbon nanotube-coated surfaces. Interdiscip Perspect Infect Dis. 2011;2011: 291513. http://dx.doi.org/10.1155/2011/291513.

24. Wefel JS. Working Group Report 2: In situ caries models, saliva, microbiology, and statistical considerations. Adv Dent Res. 1995 Nov;9(3):335-7. PMid:8615953.

25. Ramberg P, Sekino S, Uzel NG, Socransky S, Lindhe J. Bacterial colonization during de novo plaque formation. J Clin Periodontol. 2003 Nov;30(11):990-5. PMid:14761122. http://dx.doi.org/10.1034/j.1600-051X.2003.00419.x.

CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

*CORRESPONDING AUTHOR

Maísa Casarin, Departamento de Estomatologia, UFSM – Universidade Federal de Santa Maria, Rua Dr. Bozano, 587, apto 503, Centro, 97015-001 Santa Maria - RS, Brasil, e-mail: maisa.66@hotmail.com