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ORIGINAL ARTICLE

A FEASIBLE *IN VITRO* METHOD TO EVALUATE BACTERIAL INFILTRATION IN THREE IMPLANT-ABUTMENT CONNECTION SYSTEMS

Método *in vitro* factible para evaluar la infiltración bacteriana en tres sistemas de conexión implante-pilar

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ABSTRACT

Introduction: Microorganism infiltration through the implant-abutment interface causes oral health problems such as periimplantitis, leading to implant loss.

Materials and Methods: A feasible new method to quantify the *Streptococcus mutans (S. mutans)* infiltration through the implant-abutment interface gap is introduced in the present work. Internal hexagon (IH; n = 10), external hexagon (EH; n = 10), Morse taper (MT; n = 10), and a control for each group (n = 1) were tested. Bacteria suspension was prepared at $1.5x10^8$ CFU/mL (CFU: colony forming units), and the implants were individually submerged up to the connection level, allowing the bacteria to contact it. The abutment was removed, and bacteria count was performed.

Results: The implant sets were tested under normal bacterial growth and early and late biofilm growth conditions. Colony-forming units per mL were obtained, and the results were compared among groups. Differences in bacterial count between the MT and EH (p<0.001) and the MT and IH (p<0.001) groups were significantly higher in the MT-type implant. There was a significant increment of bacterial infiltration in the MTs submitted to late biofilm growth conditions. EH and IH connections are more effective in preventing bacterial infiltration independent of the growth condition.

Conclusions: The proposed methodology is feasible to evaluate the infiltration of microorganisms through the implant-abutment interface.

Keywords: Biofilm; Bacteria; infiltration; *Streptococcus mutans*; Dental implants; *In vitro* techniques.

RESUMEN

Introducción: La infiltración de microorganismos a través de la interfaz implante-pilar provoca problemas de salud bucal como la periimplantitis, que conduce a la pérdida del implante.

Materiales y Métodos: En el presente trabajo se presenta un nuevo método factible para cuantificar la infiltración de *Streptococcus mutans* (*S. mutans*) a través de la brecha de la interfaz implante-pilar. Se probaron el hexágono interno (IH; n = 10), el hexágono externo (EH; n = 10), el cono Morse (MT; n = 10) y un control para cada grupo (n = 1). Se preparó una suspensión de bacterias a $1,5x10^8$ UFC/mL y los implantes se sumergieron individualmente hasta el nivel de conexión, permitiendo que las bacterias entraran en contacto con él.

Resultados: Se retiró el pilar y se realizó recuento de bacterias. Los conjuntos de implantes se probaron en condiciones de crecimiento bacteriano normal y de crecimiento temprano y tardío de biopelículas. Se obtuvieron unidades formadoras de colonias por ml y los resultados se compararon entre grupos. Las diferencias en el recuento bacteriano entre los grupos MT y EH (p<0,001) y MT e IH (p<0,001) fueron significativamente mayores en el implante tipo MT. Hubo un incremento significativo de la infiltración bacteriana en los MT sometidos a condiciones tardías de crecimiento de biopelículas. Las conexiones EH e IH son más efectivas para prevenir la infiltración bacteriana independientemente de las condiciones de crecimiento

Conclusión: La metodología propuesta es factible para evaluar la infiltración de microorganismos a través de la interfaz implante-pilar.

Palabras Clave: Biopelícula; Bacterias; *Streptococcus mutans;* Implantes dentales; Técnicas *in vitro*.

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INTRODUCTION

The implant fixation success depends on the adjacent alveolar bone crest, where the junction between the implant and the abutment is located. Previous studies showed that microbial colonization related to periodontitis at the implant-abutment interfaces was evident in patients whose implants were surrounded by infection compared to those covered by healthy tissues.[1] It is clinically evident that the internal portion of implants with peri-implantitis shows a prevalence of anaerobic bacteria such as Prevotella intermedia, *Peptostreptococcus* micros, and Fusobacterium nucleatum in the peri-implant sulcus.[2] Both anaerobic microorganisms and *Staphylococcus aureus* were already found on the external and internal abutment surfaces as opportunistic microbe, particularly in the early stages of peri-implantitis.[3] The peri-implant infection may be transmitted into bone sites or internal implant locations by the implant-abutment interface during the fixation surgery.[4,5]

External connection systems have been the most commonly used as they were one of the first materials adopted in implantology. Nonetheless, their design shows drawbacks, such as a poor contact length between the abutment and the hexagonal portion of the implant head, which allows some degree of rotation between the external hexagon (EH) of the platform and the internal hexagon (IH) of the abutment, as well as generates pressure over the fastening screw, transferring tension to the screw, which tends to loosen and/or fracture it easily.[6,7]

A comparison of the implant-abutment system efficacy in avoiding bacterial infiltration or leakage is found based on different experimental designs.

A previous study[8] reported microbial leakage evaluation in four dental implant-connection systems and found that leakage occurred in almost all analyzed systems. IH and conical implant-abutment sets were also analyzed according to microbial leakage. They reported a high leakage of microorganisms,[9] as was the evaluation of microgap size and microbial leakage, where the microgap size is directly related to the number of colony-forming bacteria/mL.[10] Nonetheless, reports on *in vitro* techniques evaluating bacterial infiltration through the implant-abutment connection are scarce and needed.

Two Morse taper connections were assessed,[11] where one was made of gold and the other made of PEEK (polyetheretherketone) copings, and results showed that both sets allowed minimal bacterial infiltration. In another study,[12] the percentage of bacterial infiltration in three implant-abutment systems (IH, EH, and MT) was evaluated after inoculating them with two bacteria, *Escherichia coli* and *Streptococcus sanguinis*, and no significant differences among the tested systems were found. Three internal conical connections of implant-abutment systems were tested by immersing them in *E. coli* suspension for 48h, and after that period, all systems showed bacteria infiltration.[13]

The present work reports a new, practical, accessible, and inexpensive method to evaluate the capacity of micro-infiltration of *Streptococcus mutans* in three types of internal oral implant-abutment systems.

MATERIALS AND METHODS

Studied materials - Implant-abutment sets

In the present study, three types of implant-abutment sets were used to evaluate a new technique proposed to quantify bacterial infiltration in the implant-abutment connection gap.

Three types of implant platforms were evaluated in the present study: Morse taper (MT), external

hexagon (EH), and internal hexagon (IH) (Implacil De Bortoli® brand, São Paulo, SP, Brazil). EH and IH implants consist of a cylindrical body with a seating platform of 4 mm diameter.

While the EH has a hexagonal external connection, the IH has an internal hexagonal connection. MT consists of a conical-shaped body in the longitudinal axis of the implant's upper part. A central line enables fixation of the abutment by a screw with a diameter of 4.00 mm.

The study design consisted of three groups with ten implants each (n=10, Ntotal=30), plus a negative control implant for each group (n=1, Ntotal=3). The abutment was fixed to the implant using a precalibrated 30 Newtons per centimeter (Ncm) wrench, as recommended by the manufacturer (Implacil De Bortoli®, São Paulo, SP, Brazil). Im-plant components were sterilized before the experiments. All procedures were carried out in a laminar flow hood using sterile techniques.

Bacterial growth conditions

Streptococcus mutans ATCC™ 25175® (*S. mutans*) were used as the experimental model. The microorganism culture was obtained from loops containing the lyophilized microorganisms (CultiLoops®. Oxoid®). An initial bacterial culture (mother-plaque) was kept under refrigeration for up to one month to keep bacteria in the same 3rd passage during the experiments. Fresh colonies were harvested weekly from the mother plaque at each procedure. *S. mutans* was grown on Brain Heart Infusion Agar (BHIA, Oxoid®) medium and incubated for 48h at 36.0°C, under aerobic conditions. For the experiments, the respective broth media were used (BHIB, Oxoid®).[14]

Bacteria were collected from the culture plates to prepare 400 mL of bacterial suspension in the BHIB medium, the final concentration of which was 2.6 x 10^8 CFU/mL. So, 9.5 mL of the bacterial suspension was transferred to the MT and EH test tubes, and 9.7 mL of the same suspension was transferred to the IH test tubes.

A test tube containing only BHIB medium without inoculum was used as a negative control. The test tubes were identified, capped with gauze, and then arranged on a test tube stand.

Bacterial biofilm growth conditions

Streptococcus mutans ATCC™ 25175® was also tested in biofilm growing conditions, so the bacteria were cultivated in a BHIB medium prepared with the addition of 5% sucrose and a final bacterial suspension concentration of 1.05x10^8 CFU/mL. To establish the bacteria concentration, the bacterial-cell unit viability counts in agar plates after a ten-fold serial dilution, expressed in colony-forming units / mL.

Streptococcus mutans ATCC™ 25175® (S. mutans) early biofilm was established after a 16-hour growth, while late biofilm was established after a 48-hour growth under aerobic conditions. In the late biofilm, a 5% sucrose-enriched medium change was made after 24h to keep an adequate biofilm growth condition during the experiment.

The implant-abutment infiltration technique

Preparation of the syringe platforms and coupling implant-abutment to syringe platforms. Each syringe is prepared as the platform to sup-port one implant-abutment set. So, the syringe bodies were serrated at the five mL mark (Figure 1A). The diameter at the needle adapter area was adjusted to 2.35 mm (Figure 1A). Two holes measuring 3.95 mm diameter were made in the upper part of the syringe (Figure 1B), by the side of the needle adapter. The syringe-platform height that supports the implant-abutment set is approximately 45 mm (Figure 1C), and the implant-abutment size is 20 mm to 25 mm, depending on the type of implant (Figure 1C).

Each implant-abutment set was mounted and torqued at 30 Ncm according to the manufac-

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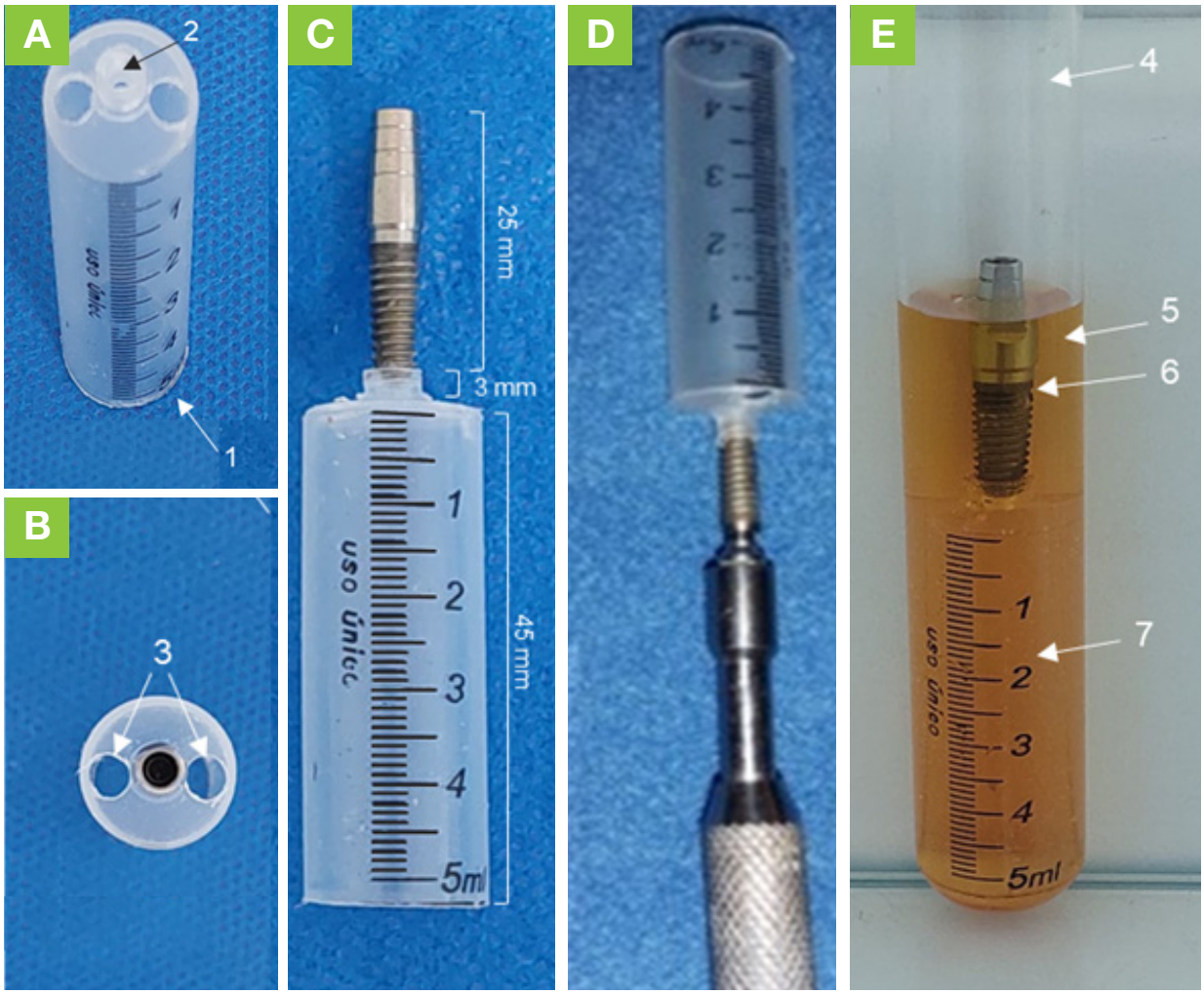
Figure 1. Assembly of the syringe platform and implant-abutment set coupling.



A. Syringe platform, (**1.** Needle adapter area. **2.** Five mL mark cut line).
B. Two holes at the upper part of the syringe platform. (**3.**Two holes measuring 3.95 mm diameter).
C. Measurement of the syringe platform implant-abutment assembly.
D. Implant-abutment set coupled to the syringe platform.
E. Syringe platform-implant-abutment set inserted into test tubes containing broth medium inoculated with bacteria. (**4.** Test tube. **5.** Broth medium, **6.** Implant-abutment interface gap. **7.** Syringe platform).

turer's specifications in sterile laminar flow. The implant-abutment set was coupled to the syringe platform at the needle adapter (Figures 1B and Figure 1C), one set to one platform, as seen in Figure 1D. The platform-implant-abutment sets are inserted into test tubes measuring approximately 16 cm long, 1.42cm internal diameter, and 1.46 mm external diameter (Figure 1E).

Testing bacteria infiltration

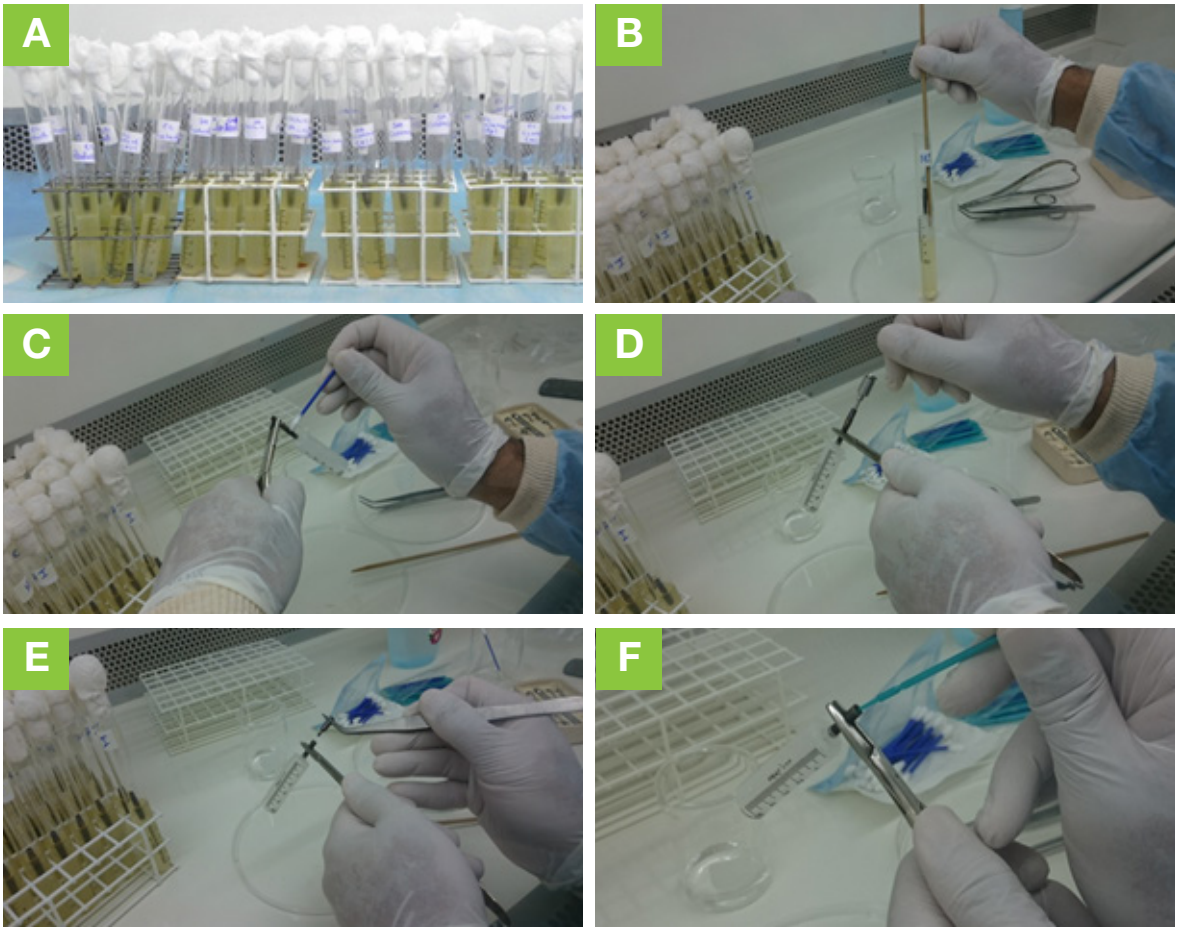
The syringe platform-implant-abutment set (Figure 1E) was immediately inserted into the corresponding test tube (Figure 1E) containing the inoculated medium (Figure 1E) and conveniently arranged into the tube stand (Figure 2A).

The tube stand was kept under aerobic incubation at 36°C, for the time established for the normal growth conditions, for the early, and late biofilm growth conditions. The inoculated medium level must cover the implant-abutment interface gap (Figure 1E).

Bacterial count

Removing the syringe platform-implant-abutment set from the test tube. After the incubation period,

Figure 2. Bacterial count techniques.



A. Test tubes arranged in test tube stand.
B. Technique of removing syringe platform-implant-abutment set from test tube.
C. Cleaning the outside of the implant-abutment set with a sterile swab containing chlorhexidine gluconate 0.12%.
D. Detorquing abutment from the implant.
E. Removing abutment from the implant with a sterile clampwith bacteria.
F. Removing internal bacterial content with a sterile microbrush.

each syringe-platform-implant-abutment set was removed from the tube with a sterilized thin rod inserted in one of the upper holes previously made in the syringe platform (Figure 2B). After removing the implant-abutment set from the tube and the syringe platform with sterilized gauze, it was cleaned with 0.12% chlorhexidine gluconate with a sterile swab (Figure 2C), and detorqued (Figure 2D and Figure 2E).

Removing total bacteria from the inner part of the implant for counting CFUs. With the aid of a sterilized 100 µL microbrush (Cavibrush Dentscare, Joinville, SC, Brazil; Figure 2F), the bacteria that was released from the biofilm localized at the external area of the implant set that could penetrate and remain in the internal area of the implant were removed and immediately placed in 1.5 mL vials containing one mL of sterile saline solution.

The vials were homogenized in a vortex for 30 seconds, and a 10-fold serial dilution was made so that 100 µL of the first vial was transferred to the second vial containing 900 µL of sterile saline solution. Subcultures of each bacterial dilution were placed on Petri dishes containing BHIA, so each plate received 100 µL of the respective

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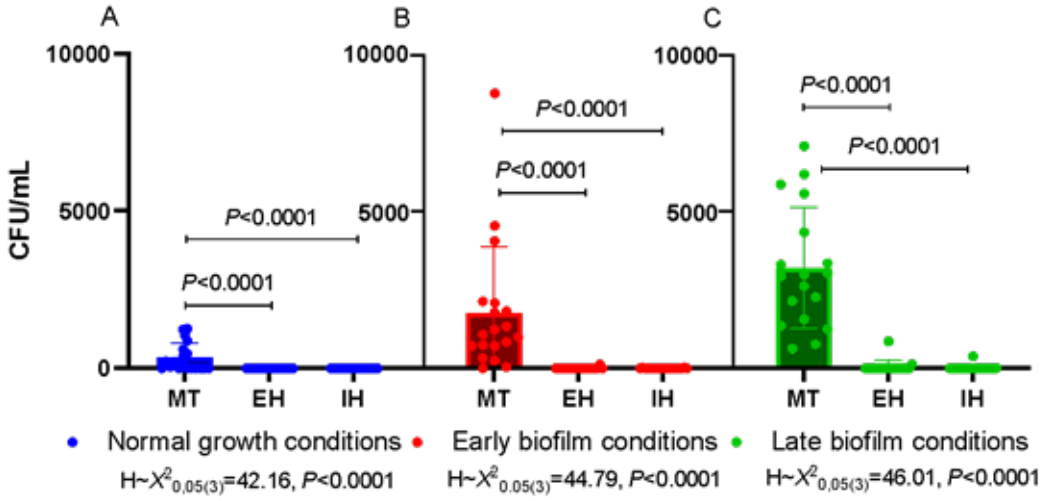
Figure 3. Results of the bacterial infiltration in Morse taper (MT), external hexagon (EH), and internal hexagon (IH) implants in the technique assay performed with *Streptococcus mutans* ATCC25175. Statistics were calculated based on Kruskal-Wallis followed by Dunn's post-test ($\alpha<0.05$).



A. Bacterial count under normal growth conditions.
B. Bacterial count under early biofilm growth conditions.
C. Bacterial count under late biofilm growth conditions.

dilution in du-plicate. The subcultures were incubated at 36°C for 24 h, and then the colony-forming units were counted.

Statistical analyses

Two independent experiments were done with the three groups of implant-abutment sets. The results given in CFU/mL obtained from both experiments were grouped and used in the calculations. The premises of t-tests and variance tests were considered, respecting independence, normality, and homoscedasticity. Data were submitted to the Shapiro-Wilk or the Kolgomorov-Smirnoff normality.

The null hypothesis was considered if there were no differences between or among medians in all the experiments. As data did not corroborate normality, non-parametric Kruskal-Wallis, followed by Dunn's post-test, and the two-tailed Mann-Whitney test were used in the biofilm test analysis. The significance level was considered $\alpha<0.05$ to reject the null hypothesis, and differences among medians were considered if $p<0.05$ (statistical package GraphPad Prism 9.5.1). The limitations of the present work remain the following:

(a) only three implant-sets were analyzed;

(b) a one-microorganism biofilm condition was adopted in the present analysis so that a more controlled assay could be used to standardize the technique.

RESULTS

Streptococcus mutans infiltration

Figure 3A shows the results of the experiment performed with *S. mutans* under usual growth conditions. According to the Kolgomorov-Smirnoff test, results obtained from groups MT ($p=0.0233$), EH ($p<0.0001$), and IH ($p<0.0001$) did not show normality, so the non-parametric

Kruskal-Wallis test followed by the Dunn's post-test was applied (H~X20,05(3)=42.16, p<0.0001, number of treatments=3, number of values=59). Differences between medians were found, and results report that the MT group significantly showed more bacterial infiltration than that observed for the EH (p<0.0001) or IH (p<0.0001) groups.

Streptococcus mutans in early biofilm growth condition

Figure 3B shows the results of the experiment performed with *Streptococcus mutans* in early biofilm conditions. According to the Kolgomorov-Smirnoff test, groups MT, EH, and IH did not show normality (p<0.001). For that reason, the non-parametric test Kruskal-Wallis followed by Dunn´s post-test was applied (H~X20.05(3)=44.79, p<0.0001, number of treatments=3, number of values=60). Differences between medians were found, and results report that the MT group significantly showed a higher bacterial count than the EH (p<0.0001) and IH (p<0.0001) groups.

Streptococcus mutans in late biofilm growth condition

Figure 3C shows the results obtained from the technique experiment with *Streptococcus mutans* in late biofilm conditions. According to the Kolgomorov-Smirnoff test, group MT passed normality (p>0.1000), while groups EH (p<0.0001) and IH (p<0.0001) did not give normality. For that reason, the non-parametric Kruskal-Wallis test followed by Dunn´s post-test was applied (H~X20,05(3)=46.01, p<0.0001, number of treatments=3, number of values=59).

Differences between medians were found, and results report that the MT group significantly showed a higher bacterial count in comparison to both EH (p<0.0001) and IH (p<0.0001) groups.

DISCUSSION

Bacterial contamination at the interface between the dental implant-abutment connection has long been studied for its influence on the inflammation of adjacent tissues as the leading cause of peri-implantitis and consequent dental implant loss.[15-18] The present work aimed at quantifying the number of bacteria infiltrating into the gap of three different implant-abutment systems, using a benchtop model of bacterial analysis where the bacteria is submitted to usual conditions and early and late biofilm conditions.
S. mutans is a Gram-positive bacterium that can form a biofilm in solid surfaces such as dental tissues or implants.[19]
Biofilms are complex structures that allow bacteria to grow as colonies that become more resistant to antibiotics and reinforce the colony's defensive power protecting the colony.[20]

Bacteria are essential to developing oral diseases such as caries, and to developing the initial steps of biofilm formation over implants resulting in periodontitis or peri-implantitis.[21] *Streptococcus mutans* play an indispensable role, particularly in the early phases of biofilm formation.
Evaluating *Streptococcus mutans* invasive capacity in implants using *in vitro* biofilm models seeks to reproduce the clinical reality of implant loss in a static model.

The biofilm formation in implants respects the same sequence observed in biofilms formed on healthy teeth surfaces, respecting a step-by-step development initiated with the association of pioneering microorganisms, predominantly of the genus *Streptococcus*. Then, anaerobe microorganisms such as *Prevotella spp.*, *Prophyromonas spp.*, *Fusobacterium spp.*, and *Caphnocytophaga spp.*[22] bind to the pioneer *Streptococci* and produce toxins that directly attack the protein structures surrounding the healthy tooth, such as periodontal

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tissue. These microorganisms and their toxins cause a localized inflammatory process, leading to bone loss and, eventually, tooth loss.[23] The same process happens to implants, except for ligament loss. Due to its significance in the biofilm formation, *S. mutans* was used in the present assays.

Our findings showed that the bacteria found in the implant's inner side was higher in MT implants than in IH or EH. Theoretically, MT implants should seal the gap between the implant and the abutment once designed to favor a reduction of the gap. The EH and IH implants were less susceptible to bacterial infiltration in both normal and biofilm conditions, as we described. Under biofilm conditions, there was a significant increase in the amount of bacteria in MT implants. According to the characteristics of *S. mutans*, their size, and shape did not influence the infiltration process in the MT.

It was also observed that the MT implant-abutment set showed that bacterial count in the inner side of the implant was lower in the group where bacteria was grown in normal conditions compared to the groups in which MT sets were submitted to both early and late biofilm growth conditions. In contrast, the MT implant-abutment set tested in late biofilm growth conditions presented a higher degree of bacterial infiltration when compared to the other two groups, EH and IH. Two different morse implants, one tapped-in (Bicon®) and the other screwed-in (Ankylos®), showed bacterial leakage in the implant-abutment gap.[24]

Another study also reported bacterial internal colonization in Cone Morse implants,[25] Although those results are in accordance with our findings, a recent review reports the results of twelve *in vitro* assays comparing the efficacy in preventing bacterial leakage among MT, EH, and IH implants, and they concluded that MT is more efficient than the other ones, despite MT present some degree of allowing for bacterial infiltration.[26] The definition of biofilm was accessed in a previous work,[27] described as *"a sessile community derived from microorganisms characterized by cells that are irreversibly linked to, or interacting with, a substrate embedded in a matrix of extracellular polymer substances produced by microbes."*

The implants were not submitted to analysis in a classic biofilm in the present study. Bacteria were cultured in a medium prepared to support biofilm growth, *i.e.*, supplied with 5% sucrose, an essential component for extracellular glycans. In the present work, only one microorganism was cultured in this medium.

However, a previous study[14] showed that a single microorganism such as *S. mutans* could be cultivated in a sucrose-enriched medium and have its growth behavior changed, particularly in the presence of a substrate. Under these conditions, bacteria get further resistant to antimicrobials and allow their colonies to grow stronger. Our present findings corroborate this information. MT, IH, and EH implant systems that underwent analyses under biofilm growth conditions showed more susceptibility to bacterial infiltration over time, particularly the MT set.

CONCLUSION

The development of a new feasible assay aiming to evaluate the microorganism infiltration in implant-abutment sets was conducted in three different implant systems using *S. mutans.* The assay showed reproducibility, ease of execution, and low cost, making it an accessible and inclusive method to be adopted by all laboratories.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

ETHICS APPROVAL

The authors declare that the current work does not need ethical approval.

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AUTHORS' CONTRIBUTIONS

Alexandre Cavalcante de Queiroz: conceived, designed, and supervised the experiment; performed experiments; review and/or revision of the manuscript

Ivana Barbosa Suffredini: conceived, designed and supervised the experiment, performed experiments, analysis and interpretation of data; review and/or revision of the manuscript

José Dimas de Oliveira: Conceived the experiments; review and/or revision of the manuscript

Jefferson de Souza Silva: Performed experiments; review and/or revision of the manuscript

Eduardo Fernandes Bondan: conceived, designed and supervised the experiment, analysis, and interpretation of data, review and/or revision of the manuscript.

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