Tufts UNIVERSITY | School of Dental Medicine

# Comparison of The Effect of the Preoperative Use of Chlorhexidine, Essential Oil, and Cetylpyridinium Chloride Mouthwashes on Bacterial Contamination During Dental Implant Placement: A Randomized Controlled Clinical Trial

A Thesis

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by

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# ABSTRACT

**Introduction:** There is a linkage between the presence of bacteria and peri-implantitis as well as the long-term success rate of implant and a favorable response to antimicrobial agents. Failure to achieve and maintain a successful osseointegration around dental implants is caused by several factors, one of them is microbial infection. There is an agreement on the role of chlorhexidine and other mouthwashes after periodontal treatment, but scarce information can be found about the use of it as a preoperative mouthrinse routinely before surgeries, especially in periodontal procedures, including placement of dental implants.
**Aim**: The aim of this study was to evaluate and compare the efficacy of preoperative rinse with Chlorhexidine, Essential Oil-based, and Cetylpyridinium Chloride mouthwashes on bacterial contamination at the time of implant placement.
**Materials and Methods**: Patients who were qualified for dental implants placement and met the inclusion criteria were included in the study. They were randomly divided into four groups according the mouthwash used: (1) 15 ml of 0.12% Chlorhexidine for 60 seconds, (2) 15 ml of Essential oil-based for 60 seconds, (3) 15 ml of Cetylpyridinium Chloride for 60 seconds, and (4) 15 ml of saline solution for 60 seconds as the control group. On the day of the implant placement, three saliva samples were collected: before the procedure and before the rinse with any of the mouthwashes (pre), immediately after the use of the mouthwash but before the procedure (post), and at the end of the procedure and after suturing the flap (end). Real-time PCR (qPCR) was used to analyze the samples and quantify the targeted periodontal pathogens with the utilization of Propidium monoazide (PMA™) dye.
**Results**: Forty patients were included in the study. Real-time PCR analysis showed that all mouthwashes resulted in a significant reduction in the number of the pathogens in the saliva samples collected after the use of the mouthwashes compared to the saline group. There was a statistically significant difference between the groups for pre-post and pre-end samples ($P<0.001$, $P< 0.001$, respectively) but not for post-end ($P= 0.203$). There was a statistically significant difference between the chlorhexidine and the saline, essential oil and saline, and CPC and saline groups ($P< 0.001$, $P< 0.001$, $P< 0.001$, respectively). The use of PMA $^{TM}$ dye showed a significant difference in the bacterial counts compared to samples without the dye.
**Conclusions**: Within the limitations of this study, pre-operative use of examined mouthwashes could be advantageous in terms of reducing the bacterial load at the time of implant placement which may reduce the possibility of implant-related complications. PMA™ dye should be used with the qPCR to overcome the limitation of inability to distinguish dead and live bacteria. Additional randomized clinical trials with a larger sample size are needed to confirm the effect of the pre-operative use of mouthwashes.

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# TABLE OF CONTENTS

# LIST OF TABLES

# Comparison of The Effect of the Preoperative Use of Chlorhexidine, Essential Oil, and Cetylpyridinium Chloride Mouthwashes on Bacterial Contamination During Dental Implant Placement: A Randomized Controlled Clinical Trial

# INTRODUCTION

The oral cavity is normally populated by a large number of bacterial species which make it practically impossible to obtain aseptic environment.[1,2] As a result, there is an increased risk for infections during intra-oral surgical procedures. Infections are usually polymicrobial and are mainly due to wound contamination by intra-oral bacterial flora. Decreasing the number of bacterial flora can lessen the risk of postoperative infection and infective endocarditis, in the case of *Staphylococcus aureus*.[2,3,4] Preoperative use of antiseptic mouthwashes against postoperative infections has shown several benefits. The goals are to decrease the bacterial number in the oral cavity and to prevent microorganisms from entering a surgical wound, as several studies showed a positive relationship between the infection and the level of microorganisms.[1]

One of the most commonly used mouthrinses in dentistry is chlorhexidine. Its solution is bacteriostatic in low concentration where it induces changes in the cellular membrane and leakage of intracellular constituents of the bacteria. On the other hand, in high concentration, it is bactericidal where it causes irreversible precipitation of the cellular content. Specifically, for dental use, it inhibits the function of glycosyltransferase enzyme which is responsible for the accumulation of bacteria on dental surfaces. Also, it affects the sugar transportation and acid production in oral bacteria.[5,6] Chlorhexidine has a prolonged substantivity which allows it to bind to proteins, bacteria, the extracellular polysaccharides of the oral mucosa as well as the gradual release from the retention sites.[2,3,6] Additionally, it has a broad-spectrum antibacterial effect against gram-positive and gram-negative bacteria, yeasts, dermaphytes, and some lipophilic viruses. Therefore, it is considered as the "gold standard" for chemical plaque control.[6,7,8] Its use and effect postoperatively are well documented but there is lack of

information about its effect as a preoperative measure. Some studies showed that salivary bacterial count can be lowered by 10% to 20% of baseline after a single rinse and remains at this level for $\geq$ 7 hours, while others showed that effect lasts for probably > 12 hours.[8,9] As it is delivered orally and it binds strongly to tissues, chlorhexidine is poorly absorbed from the gastrointestinal tract and therefore it does not cause systemic toxicity, microbial resistance or supra-infections. Moreover, long-term oral use of chlorhexidine does not provide any detectable blood levels or teratogenic alterations. However, reported adverse effects include extrinsic brown staining of teeth and tongue, mucositis, reversible epithelial desquamation, taste alteration, and increased supragingival calculus.[9,10] Also, some of its disadvantages are the need for prescription and the increased cost. Chlorhexidine mouthrinse is available in different concentrations. Several studies showed that the concentration of 0.12% is as effective as the concentration of 0.2% if the volume is increased from 10 to 15ml, yielding an 18mg dose on each occasion.[6]

Another example of a commonly used mouthrinse as antiseptic is essential oil based, Listerine®. It is formed by a mixture of essential oils that include thymol, eucalyptol, menthol and methyl salicylate.[10,11]. It was promoted as a breath freshener until the mid-1970-80`s when it was positioned as a mouthrinse for prevention of plaque and gingivitis. Several studies showed that Listerine® has comparable effects to chlorhexidine in terms of antisepsis with a broad spectrum activity, substantivity, and significant effects on plaque inhibition.[11] Both the American Dental Association and the Food and Drug Administration granted Listerine® with the seal of acceptance in the mouthrinse category. It is believed that Listerine® affects gram-negative bacteria by removing lioppolysacchride, reducing co-aggregation, and working at a suboptimal dose. In a cross-over study investigating

bacteremia, subjects rinsed with Listerine® for 30 seconds followed by subgingival irrigation and ultrasonic scaling. The same subjects rinsed and irrigated with a placebo. Rinsing and irrigation with Listerine® resulted in reduction in blood borne bacteria by 88-90% compared to the placebo treatment.[11] Possible side effects of Listerine are burning sensation, bitter taste, and staining of teeth.[10]

To overcome taste-related problems associated with chlorhexidine and Listerine® and the alcohol content, an alcohol-free oral rinse was developed to prevent plaque accumulation and gingivitis. Its active ingredient is cetylpyridinium chloride which acts by penetrating bacterial cell membrane with subsequent of leakage of cell components, disruption of bacterial metabolism, inhibition of cell growth, and cell death.[12] Gunsolley made a comprehensive review about different agents in mouthrinses. He found that the evidence for cetylpyridimium chloride-based mouthrinse was inconsistent and dependent on the formulation of the mouthrinse. It was hard to make a conclusion about the efficacy of cetylpyridinium chloride from this review due to the rarity of available studies about the agent and the difference in the results of each formulation.[13] Another review was conducted by Hap et al., in which they reviewed six months and shorter interval studies. [13] They concluded that cetylpyridinium chloride mouthrinse has a significant effect in the control of plaque and gingivitis but nothing was mentioned about the preoperative use of the agent before periodontal surgical procedures. Several reviews did not have an idea about the clinical value of the agent.[13]

Mouthrinses can be used with various periodontal surgical procedures, such as dental implant placement. It is accepted that the use of a strict approach to minimize the floral contamination should be applied during the insertion of dental implants to achieve a successful osseointegration. Also, it is accepted that infections have destructive effects on the

success rate of dental implants and guided bone regeneration.[14] Microbial contamination of bone particles collected intra-orally is a normal event due to the presence of a very large number of microorganisms which may reach up to $10^9$ colony-forming units/ml saliva. As a consequence, implantation of these bone particles may cause iatrogenic contamination of the implant and the barrier membrane, and failure of the augmentation procedure.[14,15,16] Peri-implant diseases could be presented as a microbial plaque-induced inflammation and, if left untreated, may lead to progressive bone loss, implant loss, and the need for regenerative treatment. There is a linkage between the presence of bacteria and peri-implantitis as well as the long- term success rate of implant and a favorable response to antimicrobial agents. Several research studies showed that peri-implantitis may affect 56% of subjects and 12-46% of implant sites. Colonization of the peri-implant crevice by bacteria occurs soon after implant placement to build polymicrobial communities and a biofilm that is different from the one associated with the healthy implants.[17,18] A biofilm could be defined as "a functional consortium of microorganisms organized within an extensive exopolymer matrix".[13] Glycocalyx and its adhesive property plays a fundamental role in the development of the biofilm, and its production is known to be species-dependent and strain-dependent. Although biofilms can co-exist with their hosts safely, it is not well-understood why they cause infection around dental implants which is considered as a cornerstone in late failure of dental implants.[14] Dental implants are made from titanium due to its excellent surface characteristics and biocompatibility. During the healing stage of dental implants, adsorption of salivary pellicle, bacterial accumulation, and biofilm formation initiate the inflammatory process. Several factors can influence the formation of the biofilms which may include the composition of the biofilm, the speed of biofilm formation, the surface energy, the surface

roughness, and the chemical characteristics of the implant. Numerous studies showed that the quantity and the quality of plaque adhesion on the implant surface are important in the long-term success of dental implants.[19] There are different stages for biofilm formation in the oral environment which are association, adhesion, proliferation, micro-colonies, biofilm formation, and growth maturation.[19] The sequence of these stages is similar for dental implants and teeth, and species that colonize dental implants are the same species that are found in healthy gingiva and in gingivitis sites. Exposure of different implant materials to the oral environment results in an early colonization with streptococci which were predominant after 4 hours.[19] Streptococci prepare a favorable environment for late colonizing microbes which need more demanding growth conditions. Putative periodontal pathogens such as *Porphyromonas, Prevotella, Capnocytophaga*, and *Fusibacterium* species are responsible for periodontal infections and peri-implantitis.[19] The mechanism of action of these pathogens to cause peri-implantitis is believed to be through producing endotoxins such as collagenase, hyaluronidase, and chondroitin sulfates, which initiate the inflammatory response with subsequent loss of supporting bone and periodontal tissues around implants.[19]

Similarly, failure to achieve and maintain a successful osseointegration around dental implants is caused by several factors; one of them is microbial infection.[20,21] Several studies mentioned peri-implantitis microflora which may include *Fusobacterium species, Campylobacter rectus, Prevotella intermedia, Candida albicans, Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans, and Treponema denticola*. They showed that rapidly progressing peri-implantitis may resemble aggressive periodontitis, and slowly progressive peri-implantitis may approximate chronic periodontitis.[20-24] Longitudinal studies showed the possibility of transmission of periodontal pathogens from

periodontally affected sites to implant sites in the oral cavity. The same periodontal pathogens were identified in periodontal pockets and colonizing implants after 3 and 6 months. This clarifies the need for eradication of probable reservoirs of those pathogens before placing an implant.[14,18]

A few studies evaluated the effect of different mouthwashes over time. Kosutic et al. found a statistically significant reduction in oral bacterial counts in saliva samples taken 5 minutes after rinsing and those obtained at the end of the surgical procedure. Chlorhexidine had a stronger effect and longer duration of action compared to povidon-iodine and saline solutions. Also, they found that rinsing with saline gave a small, short, but statistically significant reduction of oral bacterial counts 5 minutes after rinsing. However, there was a fast increase in the bacterial counts toward the preoperative counts.[2] In another study, authors evaluated the effect of different solutions on the decontamination of the oral cavity during oral surgical procedures. Immediately after the treatment of saline, the bacterial load was reduced but it was not statistically significant compared to the preoperative counts. Although they did not provide the duration of the surgical procedures, they found that the reduction in the bacterial counts in the saline group postoperatively was also not statistically significant. As a result, saline cannot be considered as the standard preoperative mouthwash during surgical procedures.[4] Another study evaluated the long-term effect of cetylpyridinium chloride and essential oils mouthwashes on the bacterial counts. At the 3- and 6-month evaluation, there was not a statistically significant difference between two groups regarding the bacterial counts. For instance, at baseline, 49% of sites were positive for *porphyromonas gingivalis* whereas 43.9% of the sites were positive for the same bacteria at the 6-month evaluation.[12]

Identification of bacterial species from saliva can be achieved through different techniques, such as culture, polymerase chain reaction (PCR), and the more advanced technique, the real-time quantitative polymerase chain reaction (qPCR). PCR technique has been used since the early 1990s which enabled scientists to analyze the total microbial community structure and diversity within the environment. The concept of the PCR technique includes "multiple amplification cycles in which DNA template is initially denatured, followed by annealing of oligonucleotide primers targeting specific sequences, followed by subsequent extension of a complementary strand from each annealed primer by a thermo-stable DNA polymerase, resulting in an exponential increase in amplicon numbers during the PCR".[25] The main difference between qPCR and the conventional PCR is that qPCR records the number of amplicons in real-time during PCR through the detection of a fluorescent reporter in each cycle, so it will give both qualitative and quantitative results. The combination of the 5' nuclease assay with the fluorescence activity after the cleavage of an internal DNA probe, enables the accumulation of amplicons to be monitored after each cycle in real-time. The ability of PCR to specifically target particular taxonomic markers from different levels makes it a valuable method in the molecular microbiology. Thus, it helps in quantitative determination of the initial template gene numbers.[25] The target specificity of any qPCR assay is determined by the design of the primers allowing the quantification of functional gene markers to exist within a mixed community from the domain level. qPCR is a highly reproducible and sensitive way to identify and quantify different gene markers.[25] Nowadays, qPCR is used to determine the gene and/or transcript numbers present in different samples. The detection of the targeted DNA in a sample is achieved via recording the amplification of qPCR products through a corresponding increase in the fluorescence

associated with the product formation during each cycle in the reaction.[25] Quantification of gene numbers is done during the exponential phase of the qPCR reaction when the numbers of the amplicons detected are directly proportional to the initial numbers of the targeted DNA in the sample.

The number of the amplicons is recorded via detection of a fluorescent dye that denotes the accumulation of them during each cycle in the reaction. Two fluorescent reporter systems are commonly used, the intercalating SYBR green assay and the *Taq*Man probe system.[25] The mechanism of action of the SYBR green includes binding of the dye to all double-stranded DNA through intercalation between adjacent base pairs. When it binds to DNA, a fluorescent signal is released following light excitation. As DNA number is amplified after each cycle during the reaction, there is a corresponding increase in the fluorescence.[25] As the SYBR green dye binds to all double-stranded DNA nonspecifically, it is fundamental to use primer pairs that are highly specific to their target sequence to prevent the generation of nonspecific products which may affect the fluorescence signal and resulting in an overestimation of the targeted DNA. That is why qPCR needs a comprehensive optimization of the primer concentration that is going to be used in SYBR green qPCR assays to guarantee that only the targeted product is formed. Regardless of the fluorescent dye used in qPCR reaction, the quantification of the targeted template is done similarly. Fluorescent data from each amplification cycle during the reaction is collected and the increase in the fluorescent activity is plotted against the cycle number, resulting in the typical amplification curve. The amplification curve can be divided into four phases: background noise phase where the background fluorescence still exceeds that derived from initial exponential template accumulation, exponential amplification phase, linear amplification phase, and a plateau

phase. In the exponential phase, the amount of the target template amplified is proportional to the starting template and it is during these cycles that gene numbers are quantified using the cycle threshold ($C_t$) method. The cycle threshold is defined as the number of cycles required for the fluorescent signal to cross the threshold, i.e. exceeds the background level.[25]

Nevertheless, the qPCR method has some limitations, such as the inability to distinguish between live and dead bacteria in a sample. The bacterial DNA can remain in the samples for a long time after the use of antimicrobial agents and subsequent cell death. As a result, bacterial DNA still could be detected with the qPCR which may produce an overestimated result. In order to overcome this limitation, a method that is based on the use of special discriminating dyes, such as propidium monoazide (PMA) has been developed. It enables the differentiation between live and dead bacteria in samples, thus giving more reliable and valid results about the efficacy of antimicrobial agents. It is a DNA-intercalating dye with an azide group and it binds covalently with DNA bases with little or no sequence preference when exposed to bright visible light. It does not have the ability to penetrate the intact cell membranes and it can only bind to DNA from dead bacteria in which the cell membrane is disrupted. As a result, when it is used with qPCR, the PMA binding to DNA bases will detect live bacteria only [26].

There is an agreement on the role of chlorhexidine and other mouthwashes after the periodontal treatment, but scarce information can be found about the use of it as a preoperative mouthrinse routinely before surgeries, especially in periodontal procedures, including placement of dental implants.[1] At Tufts University School of Dental Medicine, periodontists use preoperative mouthrinses electively. It depends on the individual's experience and preference without a clear protocol. To our knowledge, this is the first study

aiming to compare the effect of the preoperative use of chlorhexidine, essential oil-based, and cetylpyridinium chloride mouthwashes on bacterial contamination with dental implant placement procedures. We believe this study will help in filling the gap in this aspect by examining the reduction of bacterial counts at the end of the procedure with each one of the tested mouthwashes.

# AIMS AND HYPOTHESES

The primary aim of this study was to evaluate and compare the efficacy of 60 seconds rinse with 0.12% chlorhexidine, essential oil-based mouthwash, cetylpyridinium chloride mouthwash, and saline solution on bacterial contamination when used preoperatively using the real-time Polymerase Chain Reaction (qPCR). We hypothesized that preoperative rinse with 0.12% chlorhexidine mouthwash would result in greater reduction in bacterial counts than with essential oil-based, cetylpyridinium chloride, or saline mouthwashes.

The secondary aim of this study was to evaluate the effect of the tested mouthwashes in reducing the bacterial counts over time. We hypothesized that the effect of the preoperative rinse with 0.12% chlorhexidine mouthwash would be greater at the end of the implant placement procedure than with essential oil-based, cetylpyridinium chloride, or saline mouthwashes.

# MATERIALS AND METHODS

**Study design:**

This was a single-blinded randomized controlled clinical trial. Patients who attended the postgraduate periodontology clinics at Tufts University School of Dental Medicine to have dental implant treatment were included. The study protocol was approved by the Tufts University Health Sciences Campus Institutional Review Board (IRB: 10951).

**Group allocation and randomization**:

The participants were randomly divided into four groups according the mouthwash used: (1) 15 ml of 0.12% chlorhexidine for 60 seconds (Paroex® Chlorhexidine Gluconate Oral Rinse USP, 0.12%. Schaumburg, IL, USA), (2) 15 ml of Essential oil-based for 60 seconds (Listerine Zero ®, Johnson & Johnson Healthcare products, Division of McNeil-PPC, Inc. Skillman, NJ, USA), (3) 15 ml of cetylpyridinium chloride for 60 seconds (Crest Pro-Health ®, Procter & Gamble, Cincinnati, OH, USA), (4) 15 ml of saline solution for 60 seconds as the control group.

Randomization of study subjects was done before the beginning of the study using the statistical software package R (Version 2.11.1)**.**

**Sample size calculation:**

A sample size calculation was conducted using nQuery Advisor (Version 7.0). Assuming means of 1.0, 1.0, 2.8, and 5.0 for the total bacterial reductions of Chlorhexidine, saline, Cetylpyridinuim chloride, and Listerine respectively, as well as a common standard deviation of 4.0[15,27,28], a sample size of n=30 per group is adequate to obtain a Type I error rate of 5%

and a power of 97%. However, at the end of the study, the total sample size was 40 with 10 subjects in each mouthwash group.

**Inclusion criteria:**

- Patients qualified for dental implant placement according to the standards of care in the Department of Periodontology at Tufts University School of Dental Medicine.
- Male or female patients aged 18 years and above.
- Partially edentulous patients.
- Absence of any active infection in the oral cavity.

**Exclusion criteria:**

- Antibiotic therapy within 2 weeks of the study.
- Allergy to any of the agents used in the study.
- History of or current systemic disease that could impair immune response such as poorly-controlled diabetes mellitus, immunological disorders, hepatitis or HIV.
- Pregnant female patients.
- Fully edentulous patients.
- Regular use of mouthwashes.

**Study visits**:

Recruiting the patients was done through flyers that were posted in different common areas in Tufts University School of Dental Medicine. Patients who were qualified for dental implants placement and willing to participate in the study were given an appointment for a screening visit. During that visit, an interview with the patient was done to give information about the study as well as to obtain demographic data, medical history, and a complete periodontal examination to evaluate the inclusion and exclusion criteria. If the patient agreed

to participate and he/she met the inclusion criteria, an informed consent was given to the patient to be signed.

The second visit was done at the time of the dental implant placement. At that visit and before starting the dental implant procedure, the participant was interviewed briefly again to evaluate any changes in the inclusion/exclusion criteria. If the participant was still eligible for the study, three saliva samples were collected according to a modified previously mentioned method[1]. Briefly, the participants were asked to keep their head facing downward and to let the saliva drop passively into a 50-millilitre-centrifuge tube (Karter Scientific Labware Manufacturing, LA, USA) avoiding skin contact with the tube. The first sample (**pre**) of 5 ml of whole saliva was collected before the rinse of the mouthwash and before the start of the dental implant procedure. Then, the participant was given the 15 ml of the allocated mouthwash based on the randomization results and was asked to rinse for 60 seconds. The second saliva sample (**post**) was collected similar to the first one immediately after rinsing with the mouthwash. Then, the dental implant surgical procedure was performed and at the end of it, a third sample (**end**) was collected in the same manner as the first two samples. The duration of the procedure was recorded for each participant. After collection of the third sample, all participants were given a $20 Visa gift card.

All of the saliva samples were stored in -20ºC until they were transferred to the Forsyth Institute for the microbiological analysis.

**Laboratory procedure:**

***Saliva preparation:***

All saliva samples within the centrifuge tubes were centrifuged for 4 minutes at 4°C using 1,500 RPM and the obtained supernatant diluted (1:1) with phosphate-buffered saline (PBS).

***DNA extraction:***

A commercially available DNA extraction kit was used according to the manufacturer's instructions (QIAamp DNA Mini Kit, QIAGEN, Inc. Valencia, CA, USA). All centrifugation steps were carried out at room temperature (15–25°C). The final yields were 3–12 µg of DNA. The details of the procedure were as follows: pipet 20 µl QIAGEN Protease (or proteinase K) (provided with the kit) into the bottom of a 1.5 ml microcentrifuge tube, add 200 µl from the saliva sample to the microcentrifuge tube, add 200 µl Buffer AL (provided with the kit) to the sample, mix by pulse-vortexing for 15 seconds, incubate at 56°C for 10 minutes, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid, add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 seconds, after mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid, carefully apply the mixture from the last step to the QIAamp Mini spin column in a 2 ml collection tube (provided with the kit) without wetting the rim, close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 minute, place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate, carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 (provided with the kit) without wetting the rim, close the cap and centrifuge at 6000 x g (8000 rpm) for 1 minute, place the QIAamp Mini spin column in a clean 2 ml collection tube (provided with the kit), and discard the collection tube containing the filtrate, carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 (provided with the kit) without wetting the rim, close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 minutes, add an extra wash with 80% ethanol (500ul), place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate, centrifuge at full speed for

1 minute, place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate, carefully open the QIAamp Mini spin column and add 200 µl Buffer AE (provided with the kit) or distilled water, incubate at room temperature (15–25°C) for 1 minute, and then centrifuge at 6000 x g (8000 rpm) for 1 minute. The DNA concentration was measured on a Nanodrop® 2000 UV-Vis Spectrophotometer (Thermo Scientific, NanoDrop products, Wilmington, DE, USA). All the pure extracted DNA from the samples were stored at -20°C for further quantification with qPCR analysis.

***Propidium monoazide (PMA) treatment:***

Some of the samples were mixed with PMA™ (Biotium Inc., Hayword, CA, USA) to differentiate between dead and live bacteria in those samples. For those subjects, the three samples were collected with the same manner as described before with the only difference of collecting two different samples at each time (i.e. pre, post, end). Each sample contained 2.5 ml whole saliva in two different centrifuge tubes. One of them was treated similarly to the previous samples while the other sample was mixed with the PMA dye. Following the manufacturer's recommendations; 1.25 ul of 20uM PMA were added to 2.5 ml of the saliva samples in the microcentrifuge tubes. Then, the samples were placed over ice to avoid excessive heating as they were subjected to light exposure for 30 min, using a 600 W halogen light source, placed 20 cm above the samples. After PMA photo-induced DNA cross-linking, 100 ul of 40ug/ul Deoxyribonucleic acid sodium salt from salmon testes (Sigma-Aldrich Co. LLC. St. Louis, MO, USA) were added to the samples in the centrifuge tubes. Then, all the samples were stored at -20°C until further analysis using qPCR.

***Quantification of the targeted bacteria using real-time Polymerase Chain Reaction (qPCR) analysis*:**

The microbiological analysis of the samples was done using the real-time PCR (qPCR) at the immunology department at the Forsyth Institute. In 1.5 ml-microcentrifuge tubes, the monoplex reaction included mixing of 1ul from each of saliva samples after the DNA extraction, 2ul of 10uM of the bacterium-specific primers (**Table 1**) (Integrated DNA Technologies, Inc. Skokie, IL, USA), 10ul of 2X Sybre Green qPCR reagent (Bio-Rad Laboratories, Inc. Hercules, CA, USA), and 7ul of distilled water to have a mixture of 20ul. The reaction mix, standards, and negative controls were loaded in 96-well qPCR clear plates (MicroAmp® Optical 96-Well Reaction Plate with Barcode, Applied Biosystems, Waltham, MA, USA). Then the microplates were placed in the LightCycler ® 480 Instrument II (Roche diagnostic corporation, Indianapolis, IN, USA). The thermal cycler was programmed as follows: 1 cycle of 95ºC for 4 minutes, 35 cycles of 95ºC for 20 seconds, 55ºC for 20 seconds, 72ºC for 33 seconds, 1 cycle of 95ºC for 5 seconds, 60ºC for 60 seconds, 1 cycle of 60-97ºC (in 5ºC increments) for 30 seconds, and finished by 1 cycle of 40ºC for 30 seconds. The qPCR data analysis was done using the absolute quantification by the cycle threshold ($C_T$). The $C_T$ values of saliva samples were compared to those of standards of known quantity using a standard curve. This analysis gave the number of bacterial copies in any given sample.

**<u>Statistical analysis</u>:**

To compare the bacterial counts among subjects in each group in terms of overall bacterial counts and each bacterium separately at different samples (i.e. pre, post, end),

Friedman's test was used. If it was significant, the Wilcoxon signed-rank test with Bonferroni correction was used.

To compare the different groups with each other in terms of the change as well as the percentage reduction of the overall bacterial count and each bacterium separately at different samples (i.e. pre, post, end), the Kruskal–Wallis test was used. If it was significant, the Mann–Whitney U test with Bonferroni correction was used.

To compare the bacterial counts in samples with and without the PMA$^{TM}$ dye, the Wilcoxon signed-rank test was used.

Additionally, descriptive statistics were used for all of the different groups with the median and inter-quartile range.

Results were determined to be statistically significant at $P < 0.05$, except when the Bonferroni correction was used. SPSS (Version 24) was used in all analyses.

# RESULTS

**Study subjects:**

A total of 40 subjects with a total of 150 samples were included in the study. Thirty out of the 40 subjects' samples (i.e. 90 samples) were analyzed with the qPCR without the PMA™ dye, whereas the other 10 subjects' samples (i.e., 60 samples) were examined by the qPCR with and without the PMA™ dye. All of the mouthwashes were safe for the patients and no adverse reactions were observed during the study.

There were 10 subjects in each group. The age range was from 25 – 80 years with the mean ± SD age of 56.5 ± 14.4 years. There were 21 (52.5%) males and 19 (47.5%) females. In terms of race, there were 36 (90%) whites, 2 (5%) Asians, and 2 (5%) identified as others (**Table 2**).

**Subjects' samples without PMA™ dye.**

**Comparing the overall bacterial counts and each bacterium separately in each group:**

**(Tables 3 and 4)**

***Chlorhexidine group:***

**Overall**. Friedman's test was used to compare the overall bacterial counts and it showed that there was a statistically significant difference between the subjects' samples ($P<0.001$). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples for pre-post and pre-end ($P=0.005$, 0.005, respectively) but not for the post-end samples ($P= 0.169$).

**Each bacterium separately**.

*P. intermedia*: Friedman's test was used to compare the count of *P. intermedia* separately and it showed that there was a statistically significant difference between the subjects'

samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

*P. gingivalis*: Friedman's test was used to compare the count of *P. gingivalis* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.011 for pre-post, pre-end, post-end, respectively).

*A. Actinomycetemcomitans*: Friedman's test was used to compare the count of *A. Actinomycetemcomitans* separately and it showed that there was not a statistically significant difference between the subjects' samples (P=0.773).

*T. Denticola*: Friedman's test was used to compare the count of *T. Denticola* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

*T. forsythia*: Friedman's test was used to compare the count of *T. forsythia* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni

correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

*F. nucleatum*: Friedman's test was used to compare the count of *F. nucleatum* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.007, 0.005 for pre-post, pre-end, post-end, respectively).

***Essential-oil group:***

**Overall.** Friedman's test was used to compare the overall bacterial counts as well as each bacterium separately among the essential oil group. For the overall bacterial load, Friedman's test showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

**Each bacterium separately**.

*P. intermedia*: Friedman's test was used to compare the count of *P. intermedia* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples for pre-post and post-end (P= 0.005, 0.005, respectively) but not for the pre-end (P= 0.022).

*P. gingivalis*: Friedman's test was used to compare the count of *P. gingivalis* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

*A. Actinomycetemcomitans*: Friedman's test was used to compare the count of *A. Actinomycetemcomitans* separately and it showed that there was not a statistically significant difference between the subjects' samples (P=0.148).

*T. Denticola*: Friedman's test was used to compare the count of *T. Denticola* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (P=0.005, 0.005, 0.005, pre-post, pre-end, post-end respectively).

*T. forsythia*: Friedman's test was used to compare the count of *T. forsythia* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

*F. nucleatum*: Friedman's test was used to compare the count of *F. nucleatum* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects'

samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

***CPC group:***

**Overall.** Friedman's test was used to compare the overall bacterial counts as well as each bacterium separately among CPC group. For the overall bacterial load, Friedman's test showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

**Each bacterium separately**.

*P. intermedia*: Friedman's test was used to compare the count of *P. intermedia* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

*P. gingivalis*: Friedman's test was used to compare the count of *P. gingivalis* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (P= 0.005, 0.005, 0.013 for pre-post, pre-end, post-end, respectively).

*A. Actinomycetemcomitans*: Friedman's test was used to compare the count of *A. Actinomycetemcomitans* separately and it showed that there was not a statistically significant difference between the subjects' samples (P=0.539).

*T. Denticola*: Friedman's test was used to compare the count of *T. Denticola* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples pre-post and pre-end (P=0.005, 0.005, respectively) but not for the post-end samples (P=0.037).

*T. forsythia*: Friedman's test was used to compare the count of *T. forsythia* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (i.e. pre, post, end) (P=0.005, 0.005, 0.005 for pre-post, pre-end, post-end, respectively).

*F. nucleatum*: Friedman's test was used to compare the count of *F. nucleatum* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples for post-end (P=0.005) but not for the pre-post or pre-end (P=0.047, 0.059, respectively).

***Saline group:***

**Overall.** Friedman's test was used to compare the overall bacterial counts as well as each bacterium separately among the saline group. For the overall bacterial load, Friedman's test

showed that there was a statistically significant difference between the subjects' samples (P<0.001). However, the pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction did not show a statistically significant difference between the subjects' samples (P=0.114, 0.047, 0.285, for pre-post, pre-end, post-end respectively).

**Each bacterium separately**.

*P. intermedia*: Friedman's test was used to compare the count of *P. intermedia* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples for pre-post and pre-end (P=0.005, 0.005, respectively), but not for post-end (P=0.202).

*P. gingivalis*: Friedman's test was used to compare the count of *P. gingivalis* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (P=0.005, 0.009 for pre-post, pre-end, respectively), but not for post-end (P=0.203).

*A. Actinomycetemcomitans*: Friedman's test was used to compare the count of *A. Actinomycetemcomitans* separately and it showed that there was not a statistically significant difference between the subjects' samples (P=0.165).

*T. Denticola*: Friedman's test was used to compare the count of *T. Denticola* separately and it showed that there was a statistically significant difference between the subjects' samples (P=0.002). The pairwise comparisons with Wilcoxon signed-rank test with

Bonferroni correction did not show a statistically significant difference between the subjects' samples (P=0.114, 0.059, 0.241, pre-post, pre-end, post-end respectively).

*T. forsythia*: Friedman's test was used to compare the count of *T. forsythia* separately and it showed that there was a statistically significant difference between the subjects' samples (P<0.001). The pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction showed a statistically significant difference between the subjects' samples (P=0.005 for the pre-post), but not for pre-end or post-end (P=0.386, 0.028 respectively).

*F. nucleatum*: Friedman's test was used to compare the count of *F. nucleatum* separately and it showed that there was not a statistically significant difference between the subjects' samples (P=0.002). However, the pairwise comparisons with Wilcoxon signed-rank test with Bonferroni correction did not show a statistically significant difference between the subjects' samples (P=0.386, 0.879, 0.047 for pre-post, pre-end, post-end respectively).

**Comparing the bacterial reduction among different mouthwash groups over time, in terms of median difference: (Tables 5 and 6)**

**Overall**. The Kruskal-Wallis test was used to compare the overall bacterial counts and it showed that there was not a statistically significant difference between the groups for pre-end, pre-post or post-end (P= 0.282, 0.129, 0.466 respectively).

**Each bacterium separately**.

The Kruskal-Wallis test was used to compare the change in the bacterial counts for each bacterium separately and it showed that there was a statistically significant difference between the groups for pre-post samples among *F. Nucleatum* (P=0.004) and pre-end samples among *F. nucleatum and T. Forsythia* (P= 0.021, 0.016, respectively) but not for all other comparisons among the other bacteria. The analysis with the Mann-Whitney U test

with Bonferroni correction for the pre-post samples among *F. Nucleatum* showed a statistically significant difference between the chlorhexidine and saline groups (P= 0.005), the essential oil and the saline groups (P= 0.005), and the CPC and saline groups (P= 0.003) but not for all the other comparisons. For the pre-end samples among *T. Forsythia*, the Mann-Whitney U test with Bonferroni correction showed a statistically significant difference between the CPC and saline groups (P=0.007). The same statistically significant difference for the pre-end samples among *F. nucleatum* was shown between chlorhexidine and saline groups (P=0.007) and between the CPC and saline groups (P=0.006).

**Comparing the bacterial reduction among different mouthwash groups over time, in terms of proportion difference: (Tables 7 and 8)**

**Overall**. The Kruskal-Wallis test was used to compare the overall bacterial percentage change and it showed that there was a statistically significant difference between the groups for pre-post and pre-end samples (P<0.001, P< 0.001, respectively) but not for post-end (P= 0.203). The analysis with Mann-Whitney U test with Bonferroni correction for the pre-post samples showed a statistically significant difference between the chlorhexidine and the saline, essential oil and saline, and CPC and saline groups but not for the other comparisons (P< 0.001, P< 0.001, P< 0.001, respectively). Regarding the pre-end samples using the same test, there was a statistically significant difference between the chlorhexidine and the saline, essential oil and saline, and CPC and saline groups but not for the other comparisons (P< 0.001, P< 0.001, P< 0.001, respectively).

**Each bacterium separately**.

The Kruskal-Wallis test was used to compare the percentage change for each bacterium separately and it showed that there was a statistically significant difference between the

groups for pre-post samples among *P. gingivalis, T. denticola, T. forsythia, and F. Nucleatum* (P<0.001, P<0.001, P=0.003, P=0.044, respectively), pre-end samples among *T. Denticola and T. Forsythia* (P< 0.001, P= 0.015, respectively), and for post-end samples among *P. intermedia and P. gingivalis* (P= 0.007, 0.003, respectively) but not for all other comparisons among the other bacteria. The analysis with Mann-Whitney U test with Bonferroni correction for the pre-post samples among *P. gingivalis* showed a statistically significant difference between chlorhexidine and saline groups (P= 0.001), essential oil and saline (P=0.001), and CPC and saline groups (P=0.003). Regarding the pre-post samples among *T. denticola,* there was a statistically significant difference between chlorhexidine and saline groups (P<0.001), essential oil and saline (P<0.001), and CPC and saline groups (P<0.001). For the pre-post samples among *T. forsythia,* there was a statistically significant difference between essential oil and saline groups (P=0.002), and CPC and saline groups (P=0.002). The pre-post samples of *F. Nucleatum* showed a statistically significant difference between essential oil and saline groups (P=0.007). Also, the analysis with the Mann-Whitney U test with Bonferroni correction for the pre-end samples among *T. denticola* showed a statistically significant difference between chlorhexidine and saline groups (P<0.001), essential oil and saline groups (P< 0.001), and CPC and saline groups (P<0.001). Additionally, the pre-end samples among *T. forsythia* showed a statistically significant difference between the essential oil and saline groups (P= 0.003). Moreover, the Mann-Whitney U test with Bonferroni correction showed a statistically significant difference in terms of the post-end samples among *P. gingivalis* between essential oil and saline groups (P= 0.001). The same statistically significant difference was found among *P. intermedia* between essential oil and saline groups (P= 0.003).

**Subjects' samples with PMA™ dye.**

Comparing the bacterial counts in samples with and without the PMA dye was done in this category as there were not enough samples in each mouthwash group. Paired comparisons were done for the overall bacteria as well as each bacterium separately in terms of bacterial counts. The results showed a statistically significant difference in the bacterial counts at different samples with and without the PMA™ dye with lower bacterial counts in the PMA™ samples. The only exception was *P. intermedia* at the end samples which did not show a statistically significant difference. **(Table 9)**.

# DISCUSSION

The objective of this randomized controlled clinical trial was to compare the efficacy of chlorhexidine, essential oil, and Cetylpyridinium chloride mouthwashes in reducing the bacterial count when used immediately preoperatively with dental implant procedures. A direct comparison with previous studies was difficult because we believe that this study was the first one to evaluate such aims. Also, other studies were varied in several ways, such as the duration of the study and the microbiological analyses that were used to evaluate the outcome of the bacterial count. Additionally, most of the studies that evaluated the essential oil and CPC mouthwashes tested their effects on plaque and gingivitis, rather than their effects on the bacterial counts. Moreover, there is a lack of a standardized or generally accepted protocol regarding the use of preoperative mouthwashes for oral cavity decontamination, especially before dental implant placement procedures. Some clinicians may use systemic or local antibiotics as a prophylaxis to prevent postoperative infections. However, their use is associated with possible allergic reactions, increased bacterial resistance, and the cost. Additionally, systemic antibiotics which are used preoperatively do not reach effective concentration in saliva, which makes them effective only against bacteria that enter the bloodstream during oral surgeries.[2] These undesirable side effects could be prevented by using mouthwash solutions which give similar effects in reducing local postoperative infections after oral surgical procedures.[2,4]

In this study, when each group was evaluated individually, there was a statistically significant reduction in the overall bacterial count which was observed for all the mouthwashes except for saline which was the control group. Similar observations were found for chlorhexidine and its effect in the reduction of the bacterial count in a study by de

Albuquerque et al.[1]. However, in that study the evaluation of the bacterial count was done with culture to obtain the count of viable bacteria and they were looking for salivary *staphylococcus aureus* and mutans group *streptococci* instead of the periodontal pathogens that were evaluated in this study.

When the evaluation was made for each bacterium specifically, both the chlorhexidine and essential oil groups showed a statistically significant difference between pre and end samples in all of the targeted six periodontal pathogens except *A. actinomycetemcomtians*. This is in agreement with previous studies which showed that 0.12% chlorhexidine was effective against anaerobic bacteria for a prolonged duration. It resulted in a significant reduction in the bacterial count during oral surgical procedures.[2,10] Moreover, several studies showed that essential oil mouthwash has comparable results to chlorhexidine in terms of its broad spectrum activity, substantivity, and antimicrobial activity. For instance, a study showed that essential oil mouthwashes resulted in 88-90% reduction in blood borne bacteria.[11] In another study by Young et al., they collected bone particles at the time of implant placement and they found a statistical significant difference in the colony forming units (CFU) of viable bacteria between the samples collected after the preoperative rinse with chlorhexidine and the samples collected after the rinse with sterile water. The authors recommended the preoperative use of chlorhexidine to reduce the number of bacteria that may cause infections especially if the collected autogenous bone is to be used for augmentation procedures around dental implants.[14] Similarly for the CPC group, there was a statistically significant reduction for all of the bacteria as well as among the different sample times except for *A. actinomycetemcomtians*. This could be due to the fact that there is evidence from the literature about the invasive nature of *A. actinomycetemcomtians*. and its

ability to penetrate deeper into the tissues, persistence of it after mechanical treatment, and the need for systemic antibiotics to eradicate the bacteria for a successful treatment. The statistically significant reduction of the targeted periodontal pathogens in this study could be an indication of a successful dental implant treatment. Several studies showed that the most common bacteria in the peri-implantitis sites were *P. gingivalis, P. intermedia*, *T. forsythia,* and *A. actinomycetemcomtians*. They concluded that there is a necessity to remove or significantly reduce the number of those periodontal pathogens from peri-implant sites.[19-20] Reduction in the bacterial load may have a significant effect in decreasing the chance of postoperative infection.[1,4] Some authors think that bacterial reduction while the flap is still opened is more important than reducing the bacterial load before making the incision or after it has been sutured.[2]

The saline group showed a statistically significant reduction only in *P. intermedia, P. gingivalis,* and *T. Forsythia* in addition to the different sample times. A possible explanation for the variation in the results between the different mouthwashes could be the ability of the saline mouthwashes to maintain their effect in reducing the bacterial count over time. There was a statistically significant difference between different sample times which means the number of the bacteria increased to a higher level at the time of the second sample as a result of the weak effect of both CPC and saline. Although previous studies mentioned that saline or distilled water could result in a reduction in the bacterial count, that effect was minimal and less significant than other mouthwashes that have a therapeutic effect.[1] Similarly, another study by Kosutic et al. found that the bacterial reduction by saline lasted for a short period of time and then increased to the pre-operative levels which was similar to the results in this study.[2]

When inter-group comparisons were done, there was a statistically significant difference between the groups in terms of the overall bacterial counts between pre and end samples. When further evaluating the difference between the groups, the only significant difference was found between the chlorhexidine and saline groups. Moreover, there was a statistically significant difference in the *T. denticola* count between pre and end samples. The only difference was found between the chlorhexidine and CPC groups when compared to the saline group. Also, a significant difference was found in the *F. nucleatum* count between pre and post samples with the only difference between the essential oil and saline groups. There was a statistically significant difference in the *T. forsythia* count between pre and end samples with the significant difference between chlorhexidine and essential oil when compared to saline group. These results are in agreement with evidence from previous studies which found clinically comparable results for chlorhexidine, essential oil, and CPC mouthwashes. In a 6-month study to compare the effect of rinsing with essential oil and CPC mouthwashes, it was found that rinsing with both mouthwashes resulted in reduction of bacterial loads which did not reach statistical significance. However, that study is different from our study in terms of the duration of rinsing and the microbiological analyses as they evaluated the bacterial count using the DNA checkerboard method.[12] On the other hand, several studies showed that the presence of these periodontal pathogens individually does not necessarily indicate a diseased status. However, if they are present together at the same time, a diseased status may be produced. Also, it was mentioned that peri-implant diseases are multifactorial and a combination of genetic, inflammatory process, and occlusal-related factors may be involved.[19-22]

This study had some limitations. First, it had a small sample size which may have led to greater imprecision in the results. Second, the utilization of qPCR as a microbiological test to evaluate the bacterial count would detect the whole bacterial DNA in the samples without differentiation between live and dead bacteria. That makes it difficult to evaluate the actual effect of the mouthwashes in killing the bacteria. Instead, a better way to detect live bacteria only could be the use of cultures, which nevertheless has its own limitations, such as low sensitivity especially for gram negative bacteria, more specifically for *T. denticola* and *T. forsythia*.[21] However, the use of PMA™ dye would be a good addition to the qPCR to overcome the limitation of the inability to detect live bacteria only. This was demonstrated in this study with the limited number of the samples which were evaluated with that dye. This is in an agreement with a previous study which confirmed the ability of the PMA™ dye in detecting viable DNA only. In that study they could detect viable *P. gingivalis*, *A. actinomycetemcomitans,* and *F. nucleatum* successfully without affecting the efficiency of qPCR. The authors recommended the use of PMA-qPCR method to evaluate the number of live and dead bacteria after an antimicrobial treatment.[26]

Future research should evaluate the incidence of any implant-related complications, such as postoperative infections or implant failures, and correlate that with the preoperative use of mouthwashes. Additionally, future similar studies should utilize the PMA™ dye to take advantages of both the qPCR in terms of high sensitivity and faster reaction time, and the PMA™ dye in terms of detecting live bacteria only.

# CONCLUSION

Within the limitations of this study, pre-operative use of examined mouthwashes could be advantageous in terms of reducing the bacterial load at the time of implant placement which may reduce the possibility of implant-related complications. PMA™ dye should be used with qPCR to overcome the limitation of inability to distinguish dead and live bacteria. Additional randomized clinical trials with a larger sample size are needed to confirm the effect of the pre-operative use of mouthwashes.

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# APPENDIX A: Tables

**Table 1**: Primers used for quantification of genomic DNA from the target bacteria.

| bacteria | 3'primer | 5'primer | Length (bp) |
|---|---|---|---|
| *Fn* | ATGACGGTACCAACAGAAGA AGTGACGGCTAA | CCAATAAATCCGGATAAC GCTCGTGACATA | 32 |
| *Td* | GGTATCCGGCCTGAGAGGGT GAACGGACA | TTCTTAGCTGCTGCCTCCC GTAGGAGTTT | 29 |
| *Pg* | TTGAATGTACCGTAAGAATA AGCATCGGCTAAC | CTCGCATCCTCCGTATTAC C | 33 |
| *Tf* | AATGCATAGAGATCACGCAG AACTCCGATT | TTGATACCCACGCTTTCGT GCTTCAGTGT | 30 |
| *Pi* | CCACATATGGCATCTGACGT GGACCAAAGA | GGGCCGTTACCCGCACCA ACAAGCTAATC | 30 |
| *Aa* | GCACAAATCGTTGGCATTCTC GGCGAA | AAAGTGCGGGAAACTTCT TGTTTAGCT | 27 |

*Fn: Fusobacterium nucleatum. Td: Treponema denticola. Pg: Porphyromonas gingivalis. Tf: Tannerella forsythia. Pi: Prevotella intermedia. Aa: Aggregatibacter actinomycetemcomitans.*

**Table 2**: Demographics of the subjects

| | Chlorhexidine | Essential oil | CPC | Saline | Total |
|---|---|---|---|---|---|
| Gender | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) |
| Female | 6 (60) | 5 (50) | 4 (40) | 4 (40) | 19 (47.5) |
| Male | 4 (40) | 5 (50) | 6 (60) | 6 (60) | 21 (52.5) |
| Total | 10 (100) | 10 (100) | 10 (100) | 10 (100) | 40 (100) |
| Race | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) |
| White | 10 (100) | 9 (90) | 9 (90) | 8 (80) | 36 (90) |
| Asian | 0 | 0 | 1 (10) | 1 (10) | 2 (5) |
| Other | 0 | 1 (10) | 0 | 1 (10) | 2 (5) |
| Total | 10 (100) | 10 (100) | 10 (100) | 10 (100) | 40 (100) |
| Age (years) | Mean $\pm$ SD (Min – Max) | Mean $\pm$ SD (Min – Max) | Mean $\pm$ SD (Min – Max) | Mean $\pm$ SD (Min – Max) | Mean $\pm$ SD (Min – Max) |
| | 55.6 $\pm$ 11.6 (42 - 80) | 54.8 $\pm$ 20.8 (25 - 76) | 60.2 $\pm$ 11.9 (35 - 78) | 55.4 $\pm$ 12.9 (33 - 70) | 56.5 $\pm$ 14.4 (25 – 80) |

**Table 3**: Descriptive statistics of the different mouthwash groups

| | Chlorhexidine | Essential oil | CPC | Saline |
|---|---|---|---|---|
| | Median (IQR) (Min – Max) | Median (IQR) (Min – Max) | Median (IQR) (Min – Max) | Median (IQR) (Min – Max) |
| Overall | | | | |
| Pre | 1.02E12 (2.01E12) (6.07E9 - 5.50E12) | 4.54E11 (2.16E12) (6.287E9 - 9.14E12) | 4.66E11 (8.24E11) (7.35E9 - 4.12E12) | 4.16E11 (5.59E11) (9.74E6 - 4.12E12) |
| Post | 2.79E10 (4.7E10) (2.5E7 - 1.46E11) | 7.68E10 (1.70E10) (2.48E7 - 1.05E10) | 1.5E10 (4.37E10) (4.95E7 - 3.67E10) | 2.62E11 (3.65E11) (1.29E8 - 1.08E12) |
| End | 5.96E10 (1.49E10) (2.74E8 - 3.39E10) | 8.75E10 (1.13E10) (8.82E8 - 1.32E10) | 1.01E10 (3.92E10) (7.00E8 - 8.27E10) | 3.49E11 (5.40E11) (4.67E9 - 1.33E12) |
| *Pi* | | | | |
| Pre | 9.84E6 (6.99E6) (2.34E5 – 1.21E9) | 2.64E6 (2.52E8) (373 – 2.06E9) | 1.12E7 (1.28E8) (5.27E5 – 1.35E10) | 3.28E7 (6.53E7) (3.21E5 – 3.15E8) |
| Post | 708 (2420) (116 – 2.78E6) | 456.5 (434) (154 – 32400) | 1395 (14239) (154 – 68000) | 1.19E6 (3.99E6) (588 – 9.64E6) |
| End | 3.58E5 (2.68E6) (2020 – 2.69E7) | 2.28E5 (6.26E5) (766 – 3.16E7) | 3.02E5 (1.17E6) (2200 – 9.11E6) | 3.23E6 (4.13E6) (2450 – 1.75E7) |
| *Pg* | | | | |
| Pre | 2.39E8 (6.73E9) (1.21E6 – 6.07E10) | 4.77E7 (3.17E8) (1.02E6 – 4.44E10) | 2.42E7 (3.29E7) (2.82E6 – 7.41E9) | 5.61E7 (3.00E7) (1.85E6 – 2.01E8) |
| Post | 2.41E5 (2.09E5) (2680 – 5.53E5) | 5000 (1.32E5) (1010 – 56000) | 35300 (48000) (1670 – 55300) | 2.26E6 (4.48E6) (2.56E5 - 5.78E7) |
| End | 7.27E5 (5.93E6) (2.03E5 – 9.15E6) | 3.37E6 (3.43E6) (1.36E5 – 8.31E7) | 1.08E6 (1.66E6) (2.45E5 – 4.75E6) | 3.67E6 (6.70E6) (5.40E5 – 7.52E7) |
| *Aa* | | | | |
| Pre | 2.26E9 (2.61E10) (2.26E9 - 9.08E11) | 5.15E9 (5.07E9) (2.26E9 – 6.90E10) | 3.93E9 (4.97E9) (2.26E9 – 2.75E10) | 4.58E9 (1.92E10) (2.26E9 - 3.20E11) |
| Post | 2.83E9 (3.37E9) (2.26E7 - 1.44E11) | 2.26E9 (3.47E9) (2.26E7 – 6.63E10) | 2.93E9 (2.03E9) (2.26E7 – 4.35E10) | 2.26E9 (2.06E9) (2.26E7 - 3.24E11) |
| End | 2.26E9 (8.60E8) (2.26E8 – 9.64E9) | 2.26E9 (5.4E8) (8.10E8 – 8.10E10) | 2.26E9 (4.22E9) (6.48E8 – 2.04E10) | 3.59E9 (3.03E10) (2.26E8 - 2.86E10) |

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

**Table 3 (Continued)**: Descriptive statistics of the different groups

| | Chlorhexidine | Essential oil | CPC | Saline |
|---|---|---|---|---|
| | Median (IQR) (Min – Max) | Median (IQR) (Min – Max) | Median (IQR) (Min – Max) | Median (IQR) (Min – Max) |
| *Td* | | | | |
| Pre | 7.94E11 (2.35E12) (6.04E8 - 5.49E12) | 4.38E11 (2.11E12) (1.97E9 - 9.06E12) | 4.42E11 (7.99E11) (3.36E8 - 4.05E12) | 4.09E11 (5.62E11) (2.65E9 - 3.79E12) |
| Post | 1.74E9 (4.88E9) (1.45E5 – 1.48E7) | 7.63E8 (2.61E9) (1.04E6 – 1.19E10) | 2.42E9 (1.45E10) (5.69E5 – 3.96E10) | 1.54E11 (2.99E11) (3.35E7 - 1.07E12) |
| End | 8.26E9 (7.76E10) (1.53E6 – 9.35E10) | 1.48E10 (6.48E10) (1.31E7 - 1.18E10) | 1.58E (2.85E10) (2.31E6 - 7.63E10) | 3.30E11 (5.37E11) (3.60E8 - 1.32E12) |
| *Tf* | | | | |
| Pre | 6.53E10 (1.14E10) (1.27E8 - 4.79E10) | 4.98E10 (1.31E10) (3.01E8 - 6.80E10) | 1.05E (3.40E10) (3.27E9 - 9.74E10) | 7.66E9 (4.83E10) (2.72E8 – 9.9E10) |
| Post | 1.17E9 (4.58E9) (1.81E - 1.55E10) | 4.47E7 (2.68E9) (1.27E5 – 2.21E10) | 6.98E7 (4.86E8) (6.29E - 2.21E10) | 2.72E9 (1.84E10) (4.05E6 – 6.76E10) |
| End | 3.36E9 (3.83E10) (5.04E6 - 1.73E10) | 7.41E9 (2.12E10) (4.83E6 – 6.61E10) | 2.61E9 (4.88E10) (3.24E6 - 3.74E10) | 4.03E9 (4.34E10) (2.10E7 - 2.33E10) |
| *Fn* | | | | |
| Pre | 5.72E10 (3.21E10) (2.13E9 - 1.39E10) | 7.73E10 (4.41E10) (1.21E9 - 2.98E10) | 5.18E10 (2.85E10) (2.28E9 - 3.89E10) | 2.75E10 (2.35E10) (1.65E9 – 8.68E10) |
| Post | 1.01E10 (1.95E10) (1.22E5 – 3.04E10) | 1.22E9 (1.34E10) (3.025 – 3.29E10) | 351E9 (1.44E10) (4.45E6 - 1.04E10) | 1.23E10 (30.0E10) (1.06E7 – 6.45E10) |
| End | 2.50E10 (5.54E10) (2.35E6 – 8.13E10) | 2.79E10 (3.83E10) (3.25E6 – 5.23E10) | 4.05E10 (3.30E10) (1.56E7 - 1.53E10) | 2.89E10 (3.26E10) (3.45E8 – 9.48E10) |

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

**Table 4**: Comparison of the overall bacterial count and each bacterium in each group (P–values)

| | Chlorhexidine | Essential oil | CPC | Saline |
|---|---|---|---|---|
| Overall | <0.001* | <0.001* | <0.001* | <0.001* |
| Pre-Post | 0.005^ | 0.005^ | 0.005^ | 0.114 |
| Pre-end | 0.005^ | 0.005^ | 0.005^ | 0.047 |
| Post-end | 0.169 | 0.005^ | 0.005^ | 0.285 |
| *Pi* | <0.001* | <0.001* | <0.001* | <0.001* |
| Pre-Post | 0.005^ | 0.005^ | 0.005^ | 0.005^ |
| Pre-end | 0.005^ | 0.022 | 0.005^ | 0.005^ |
| Post-end | 0.005^ | 0.005^ | 0.005^ | 0.202 |
| *Pg* | <0.001* | <0.001* | <0.001* | <0.001* |
| Pre-Post | 0.005^ | 0.005^ | 0.005^ | 0.005^ |
| Pre-end | 0.005^ | 0.005^ | 0.005^ | 0.009^ |
| Post-end | 0.011^ | 0.005^ | 0.013^ | 0.203 |
| *Aa* | 0.003* | 0.004* | 0.003* | 0.005* |
| Pre-Post | 0.412 | 0.025 | 0.610 | 0.151 |
| Pre-end | 0.161 | 0.051 | 0.259 | 0.083 |
| Post-end | 0.508 | 0.795 | 0.837 | 0.608 |
| *Td* | <0.001* | <0.001* | <0.001* | 0.002* |
| Pre-Post | 0.005^ | 0.005^ | 0.005^ | 0.114 |
| Pre-end | 0.005^ | 0.005^ | 0.005^ | 0.059 |
| Post-end | 0.005^ | 0.005^ | 0.037 | 0.241 |
| *Tf* | <0.001* | <0.001* | <0.001* | 0.001* |
| Pre-Post | 0.005^ | 0.005^ | 0.005^ | 0.005^ |
| Pre-end | 0.005^ | 0.005^ | 0.005^ | 0.386 |
| Post-end | 0.005^ | 0.005^ | 0.005^ | 0.028 |
| *Fn* | <0.001* | <0.001* | 0.001* | 0.002* |
| Pre-Post | 0.005^ | 0.005^ | 0.047 | 0.386 |
| Pre-end | 0.007^ | 0.005^ | 0.059 | 0.879 |
| Post-end | 0.005^ | 0.005^ | 0.005^ | 0.047 |

* Significant with Friedman's test ($P < 0.05$).

^ Significant with Wilcoxon signed-rank test with Bonferroni correction ($P < 0.017$).

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

**Table 5**: Comparison of the bacterial reduction among different mouthwash groups over time, in terms of median difference.

| | Chlorhexidine | Essential oil | CPC | Saline | |
|---|---|---|---|---|---|
| | Median (IQR) | Median (IQR) | Median (IQR) | Median (IQR) | P-value |
| Overall | | | | | |
| Pre-Post | 1.02E12 (2.01E12) | 4.49E11 (2.16E12) | 4.62E11 (8.20E+11) | 1.59E11 (5.83E11) | 0.282 |
| Pre-end | 1.02E12 (1.99E12) | 4.43E11 (2.15E12) | 4.44E11 (7.49E11) | 2.12E10 (3.83E10) | 0.129 |
| Post-end | -3.08E09 (1.20E10) | -3.11E09 (9.45E7) | -9.27E09 (3.36E10) | -9.41E10 (5.91E11) | 0.466 |
| *Pi* | | | | | |
| Pre-Post | 9.40E6 (6.99E7) | 2.63E6 (2.52E8) | 1.11E7 (1.28E8) | 3.15E7 (5.57E7) | 0.669 |
| Pre-end | 4.01E6 (6.98E) | 2.47E6 (2.52E8) | 1.11E7 (1.26E8) | 3.05E7 (6.20E7) | 0.583 |
| Post-end | -2.94E5 (5.73E5) | -2.19E5 (6.30E5) | -2.72E5 (1.24E6) | -2.84E5(1.91E6) | 0.988 |
| *Pg* | | | | | |
| Pre-Post | 2.39E8 (6.73E9) | 4.77E7 (3.16E8) | 2.38E7 (3.24E7) | 5.28E7 (3.82E7) | 0.447 |
| Pre-end | 2.39E8 (6.72E9) | 4.73E7 (3.15E8) | 2.23E7 (3.17E7) | 4.56E7 (4.60E7) | 0.435 |
| Post-end | -6.82E5 (5.95E6) | -3.34E6 (3.37E6) | -7.73E5 (1.38E6) | -1.08E6 (6.32E6) | 0.577 |
| *Aa* | | | | | |
| Pre-Post | 0 (3.45E9) | 2.69E9 (5.07E9) | 2.45E8 (3.66E9) | 1.16E9 (8.18E9) | 0.693 |
| Pre-end | 8.68E8 (1.88E10) | 2.01E9 (6.52E9) | 6.20E8 (3.15E9) | 1.16E9 (3.19E9) | 0.913 |
| Post-end | 4.70E8 (2.13E9) | 0 (9.67E8) | 0 (3.44E9) | -1.02E8 (2.25E10) | 0.822 |
| *Td* | | | | | |
| Pre-Post | 7.94E11 (2.35E12) | 4.37E11 (2.11E12) | 4.41E11 (7.99E11) | 1.51E11 (5.86E11) | 0.510 |
| Pre-end | 7.89E11 (2.35E12) | 4.31E11 (2.11E12) | 4.28E11 (7.23E11) | 2.50E10 (4.45E10) | 0.263 |
| Post-end | -3.43E9 (6.86E10) | -1.11E09 (6.43E10) | -1.42E09 (2.65E10) | -9.07E10 (5.87E11) | 0.863 |
| *Tf* | | | | | |
| Pre-Post | 6.13E10 (1.15E10) | 4.62E10 (1.31E10) | 1.05E10 (3.18E10) | 5.42E9 (2.31E10) | 0.264 |
| Pre-end | 4.40E10 (9.14E10) | 4.21E10 (1.23E10) | 1.05E10 (2.64E10) | 1.37E9 (5.35E9) | 0.016* |
| Post-end | -1.19E9 (1.79E10) | -1.43E9 (8.00E9) | -2.32E9 (4.88E10) | -1.67E9 (4.73E9) | 0.977 |
| *Fn* | | | | | |
| Pre-Post | 4.86E10 (4.04E10) | 7.54E10 (4.38E10) | 4.18E10 (3.01E10) | 1.46E10 (2.03E10) | 0.004* |
| Pre-end | 9.40E9 (5.40E10) | 4.12E10 (6.48E10) | 2.05E10 (4.04E10) | 1.55E9 (3.16E10) | 0.021* |
| Post-end | -1.17E09 (3.70E10) | -6.94E9 (2.74E10) | -2.98E09 (3.30E10) | -1.19E09 (1.85E10) | 0.673 |

* Significant with Kruskal-Wallis test ($P < 0.05$).

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*

**Table 6**: Inter-group Comparison of the bacterial reduction over time (P-values)

| | Chlorhexidine | Essential oil | CPC | Saline |
|---|---|---|---|---|
| *Tf* (Pre-end) | | | | |
| Chlorhexidine | ------ | 0.821 | 0.880 | 0.008 |
| Essential oil | ------ | ------ | 0.940 | 0.016 |
| CPC | ------ | ------ | ------ | 0.007* |
| Saline | ------ | ------ | ------ | ------ |
| *Fn* (Pre-post) | | | | |
| Chlorhexidine | ------ | 0.226 | 1.000 | 0.005* |
| Essential oil | ------ | ------ | 0.364 | 0.005* |
| CPC | ------ | ------ | ------ | 0.003* |
| Saline | ------ | ------ | ------ | ------ |
| *Fn* (Pre-end) | | | | |
| Chlorhexidine | ------ | 1.000 | 0.496 | 0.007* |
| Essential oil | ------ | ------ | 0.880 | 0.326 |
| CPC | ------ | ------ | ------ | 0.006* |
| Saline | ------ | ------ | ------ | ------ |

* Significant with Mann-Whitney U test with Bonferroni correction ($P < 0.008$).

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

**Table 7**: Comparison of the bacterial reduction among different mouthwash groups over time, in terms of proportion difference.

| | Chlorhexidine | Essential oil | CPC | Saline | P-value |
|---|---|---|---|---|---|
| Overall | Median (IQR) | Median (IQR) | Median (IQR) | Median (IQR) | |
| Pre-Post | 0.995 ± 0.011 | 0.997 ± 0.012 | 0.996 ± 0.007 | 0.636 ± 1.988 | <0.001* |
| Pre-end | 0.992± 0.01 | 0.993 ± 0.161 | 0.98 ± 0.061 | 0.108 ± 0.154 | <0.001* |
| Post-end | 0.672 ± 1.383 | 0.874 ± 0.703 | 0.837 ± 0.407 | 0.55 ± 2.085 | 0.203 |
| *Pi* | | | | | |
| Pre-Post | 0.991 ± 0.085 | 0.99 ± 0.412 | 0.999 ± 0.005 | 0.95 ± 0.07 | 0.107 |
| Pre-end | 0.897 ± 0.563 | 0.9 ± 1.783 | 0.986 ± 0.29 | 0.918 ± 0.071 | 0.628 |
| Post-end | 0.908 ± 0.046 | 0.944 ± 0.098 | 0.915 ± 0.284 | 0.512 ± 1.32 | 0.007* |
| *Pg* | | | | | |
| Pre-Post | 0.999 ± 0.02 | 0.998 ± 0.004 | 0.986 ± 0.013 | 0.873 ± 0.186 | 0.001* |
| Pre-end | 0.977 ± 0.341 | 0.867 ± 0.244 | 0.943 ± 0.072 | 0.876 ± 0.214 | 0.125 |
| Post-end | 0.941 ± 0.128 | 0.986 ± 0.103 | 0.716 ± 0.659 | 0.102 ± 1.266 | 0.003* |
| *Aa* | | | | | |
| Pre-Post | -0.053 ± 0.535 | 0.039 ± 0.994 | -0.052 ± 0.57 | 0.17 ± 0.503 | 0.742 |
| Pre-end | 0 ± 1.041 | 0.324 ± 0.683 | 0.052 ± 0.483 | 0.329 ± 0.628 | 0.868 |
| Post-end | 0 ± 1.041 | 0.324 ± 0.683 | 0.052 ± 0.483 | 0.329 ± 0.628 | 0.916 |
| *Td* | | | | | |
| Pre-Post | 1 ± 0.001 | 1 ± 0.001 | 0.999 ± 0.002 | 0.637 ± 2.05 | <0.001* |
| Pre-end | 0.998 ± 0.007 | 0.994 ± 0.083 | 0.994 ± 0.031 | 0.138 ± 0.152 | <0.001* |
| Post-end | 0.913 ± 0.415 | 0.952 ± 0.343 | 0.938 ± 0.492 | 0.586 ± 2.373 | 0.143 |
| *Tf* | | | | | |
| Pre-Post | 0.924 ± 0.251 | 0.98 ± 0.064 | 0.991 ± 0.079 | 0.643 ± 0.399 | 0.003* |
| Pre-end | 0.645 ± 0.433 | 0.896 ± 0.091 | 0.846 ± 0.276 | 0.163 ± 1.144 | 0.015* |
| Post-end | 0.283 ± 0.72 | 0.874 ± 0.798 | 0.904 ± 0.424 | 0.419 ± 0.647 | 0.158 |
| *Fn* | | | | | |
| Pre-Post | 0.764 ± 0.286 | 0.948 ± 0.132 | 0.894 ± 0.35 | 0.498 ± 1.633 | 0.044* |
| Pre-end | 0.264 ± 0.353 | 0.537 ± 0.42 | 0.392 ± 0.41 | 0.106 ± 2.698 | 0.147 |
| Post-end | 0.571 ± 0.737 | 0.784 ± 0.855 | 0.879 ± 0.538 | 0.467 ± 0.383 | 0.398 |

* Significant with Kruskal-Wallis test ($P < 0.05$).

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*

**Table 8**: Inter-group comparisons in terms of percentage change between the groups at different sample times (P-values).

| | Chlorhexidine | Essential oil | CPC | Saline |
|---|---|---|---|---|
| Overall (Pre-post) | | | | |
| Chlorhexidine | ------ | 0.597 | 0.650 | <0.001* |
| Essential oil | ------ | ------ | 0.326 | <0.001* |
| CPC | ------ | ------ | ------ | <0.001* |
| Saline | ------ | ------ | ------ | ------ |
| Overall (Pre-end) | | | | |
| Chlorhexidine | ------ | 0.227 | 0.013 | <0.001* |
| Essential oil | ------ | ------ | 0.326 | <0.001* |
| CPC | ------ | ------ | ------ | <0.001* |
| Saline | ------ | ------ | ------ | ------ |
| *Pi* (Post-end) | | | | |
| Chlorhexidine | ------ | 0.406 | 0.880 | 0.008 |
| Essential oil | ------ | ------ | 0.345 | 0.003* |
| CPC | ------ | ------ | ------ | 0.016 |
| Saline | ------ | ------ | ------ | ------ |
| *Pg* (Pre-post) | | | | |
| Chlorhexidine | ------ | 0.940 | 0.112 | 0.001* |
| Essential oil | ------ | ------ | 0.049 | 0.001* |
| CPC | ------ | ------ | ------ | 0.003* |
| Saline | ------ | ------ | ------ | ------ |
| *Pg* (Post-end) | | | | |
| Chlorhexidine | ------ | 0.034 | 0.325 | 0.023 |
| Essential oil | ------ | ------ | 0.041 | 0.001* |
| CPC | ------ | ------ | ------ | 0.070 |
| Saline | ------ | ------ | ------ | ------ |

* Significant with Mann-Whitney U test with Bonferroni correction (P < 0.008).

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

**Table 8 (Continued)**: Inter-group comparisons in terms of percentage change between the groups at different sample times (P-values).

| | Chlorhexidine | Essential oil | CPC | Saline |
|---|---|---|---|---|
| *Td* (Pre-post) | | | | |
| Chlorhexidine | ------ | 0.450 | 0.174 | <0.001* |
| Essential oil | ------ | ------ | 0.545 | <0.001* |
| CPC | ------ | ------ | ------ | <0.001* |
| Saline | ------ | ------ | ------ | ------ |
| *Td* (Pre-end) | | | | |
| Chlorhexidine | ------ | 0.174 | 0.096 | <0.001* |
| Essential oil | ------ | ------ | 0.821 | <0.001* |
| CPC | ------ | ------ | ------ | <0.001* |
| Saline | ------ | ------ | ------ | ------ |
| *Tf* (Pre-post) | | | | |
| Chlorhexidine | ------ | 0.290 | 0.450 | 0.013 |
| Essential oil | ------ | ------ | 0.880 | 0.002* |
| CPC | ------ | ------ | ------ | 0.002* |
| Saline | ------ | ------ | ------ | ------ |
| *Tf* (Pre-end) | | | | |
| Chlorhexidine | ------ | 0.650 | 0.940 | 0.023 |
| Essential oil | ------ | ------ | 0.650 | 0.003* |
| CPC | ------ | ------ | ------ | 0.016 |
| Saline | ------ | ------ | ------ | ------ |
| *Fn* (Pre-post) | | | | |
| Chlorhexidine | ------ | 0.226 | 0.706 | 0.082 |
| Essential oil | ------ | ------ | 0.406 | 0.007* |
| CPC | ------ | ------ | ------ | 0.059 |
| Saline | ------ | ------ | ------ | ------ |

* Significant with Mann-Whitney U test with Bonferroni correction (P < 0.008).

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

**Table 9**: Comparison of the bacterial counts in samples with and without the PMA dye (P-values).

| | All mouthwash groups with vs. without PMA dye |
|---|---|
| Overall | |
| Pre | 0.0002* |
| Post | 0.007* |
| End | 0.003* |
| *Pi* | |
| Pre | 0.01* |
| Post | 0.006* |
| End | 0.33 |
| *Pg* | |
| Pre | 0.002* |
| Post | 0.02* |
| End | 0.04* |
| *Aa* | |
| Pre | 0.0001* |
| Post | 0.0001* |
| End | 0.0002* |
| *Td* | |
| Pre | 0.0007* |
| Post | 0.02* |
| End | 0.02* |
| *Tf* | |
| Pre | 0.0002* |
| Post | 0.03* |
| End | 0.0004* |
| *Fn* | |
| Pre | 0.0002* |
| Post | 0.007* |
| End | 0.004* |

* Significant with Wilcoxon-signed rank test ($P < 0.05$).

**Pre**: Samples before the rinse, **Post**: Samples immediately after the rinse, **End**: Samples at the end of the procedure.

*Pi: Prevotella intermedia. Pg: Porphyromonas gingivalis. Aa: Aggregatibacter actinomycetemcomitans. Td: Treponema denticola. Tf: Tannerella forsythia. Fn: Fusobacterium nucleatum.*