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Research Article

# ANTIBACTERIAL EFFECT OF 0.2% CHLORHEXIDINE AND 1% CHITOSAN MOUTHWASH ON BACTERIA DURING ORTHODONTIC MINISCREW USE

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**ABSTRACT**

**Objective:** Inflammation is one of the most common complications observed when using orthodontic miniscrews. Chlorhexidine mouthwash can be used to prevent and reduce the degree of inflammation, but long-term use of this solution may lead to some side effects. This study sought to evaluate the peri-miniscrew antibacterial effect of 1% chitosan, a biomaterial with antibacterial properties, relative to 0.2% chlorhexidine mouthwash.

**Methods:** A randomized, double-blind clinical trial was conducted at the Dental Teaching Hospital and Oral Biology Research Laboratory at the University of Indonesia from February to June 2019. Thirty subjects (25 females and five males) were randomly assigned to rinse with 1% chitosan (n=10), 0.2% chlorhexidine digluconate (n=10), and Aquadest (n=10) in addition to their usual oral hygiene procedure for 4 days. Peri-miniscrew clinical inflammation signs were recorded and peri-miniscrew plaque collected before and after 4 days of rinsing. The total bacterial and red-complex bacteria count in plaque samples were evaluated by a real-time polymerase chain reaction.

**Results:** Chitosan and chlorhexidine showed antibacterial activity, reducing total bacterial count around orthodontic miniscrews ($p<0.05$). The antibacterial activity of chitosan on total bacteria was not significantly different from that of chlorhexidine ($p\geq0.05$). Regarding the antibacterial activity of chitosan on red-complex bacteria, the best result seen was a 58% bacteria count reduction in *Tannerella denticola*.

**Conclusion:** Chitosan has potential antibacterial activity and could be used in mouthwash to maintain peri-miniscrew hygiene.

**Keywords:** Orthodontic miniscrew, Chitosan, Chlorhexidine, Red-complex bacteria, Mouthwash.

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## INTRODUCTION

Nowadays, miniscrews have been widely used in orthodontic treatment due to their superiority relative to other anchorage devices. Miniscrews are versatile, easy to install and remove, function independently of the patient's cooperation, and are relatively affordable [1,2]. However, they also bear risks and impart complications that may occur during the installment procedure, orthodontic treatment, and the removal procedure [3]. Inflammation is a common complication that may appear around orthodontic miniscrews. The environment condition around the miniscrew neck – which is dark, anaerobic, and full of nutrition (amino acids and peptides) – promotes anaerobic bacterial growth that may lead to peri-miniscrew inflammation [4].

The previous studies have revealed that the bacteria in peri-implantitis are similar to those that caused the periodontitis [5]. The primary pathogenic bacteria in periodontitis cases in adults mostly include red-complex bacteria such as *Porphyromonas gingivalis*, *Tanerella forsythia, and Treponema denticola.* One of the ways to prevent bacterial growth is by rinsing with an antibacterial mouthwash. Chlorhexidine is the gold standard mouthwash that can be used to prevent and reduce bacterial growth and inflammation [6]. However, the use of chlorhexidine mouthwash on a daily basis may exhibit some side effects, such as oral mucosa irritation, taste perception alteration, burning sensation, and tooth staining [7].

On the other hand, recent studies have found that chitosan is a biomaterial that possesses antibacterial properties. Chitosan is produced from the deacytelation of chitin, a biopolymer that can be obtained from crustaceans [8,9]. However, studies on the antibacterial activity of chitosan as a mouthwash in clinical application remain minimal due to the material's water-insoluble properties. Ibrahim *et al.* [10] created a chitosan mouthwash solution from microcrystalline chitosan (chitosan that has been modified by minimizing its particle size); their results suggested that 1% microcrystalline chitosan solution can reduce the bacteria total plate count up to 99.05%. Therefore, this study sought to evaluate the antibacterial effect of 1% chitosan solution relative to 0.2% chlorhexidine mouthwash on a bacterial level around orthodontic miniscrews *in vivo*.

## METHODS

### Ethical clearance

This randomized double-blind clinical trial was conducted at the Dental Teaching Hospital and Oral Biology Research Laboratory at the University of Indonesia from February 2019 to June 2019. The trial was approved by the Ethics Committee of Research at the Faculty of Dentistry, University of Indonesia (ref. no. 4/ethical approval/FKG UI/I/2019). All participants signed the written informed consent before participating in the study.

### Subject criteria

Thirty subjects were randomly assigned to rinse with 1% chitosan solution (n = 10), 0.2% chlorhexidine digluconate mouthwash (n=10), and Aquadest (n=10) in addition to their usual oral hygiene procedure for 4 days. The inclusion criteria were orthodontic patients aged 10–65 years who have not consumed antibiotics in the past month, with miniscrews (Dual-Top Anchor System; JEIL Med. Corp., Korea) inserted at least 2 weeks before the sampling procedure, who were willing to participate in this study and signed the informed consent form. The exclusion criteria were patients with an allergic history to chitosan and chlorhexidine, those who were systemically compromised, and smokers.

**Clinical procedure and materials**

Every subject was instructed to rinse twice a day (after breakfast in the morning and before sleep in the evening) for 4 days with 10 mL of assigned solution for 30 s. The rinsing procedure was conducted after the usual oral hygiene procedure, which included tooth- and miniscrew-brushing. Subjects were not allowed to rinse/eat/drink for 30 min after the rinsing procedure. The chlorhexidine mouthwash used in this study is commercially available at a 0.2% chlorhexidine gluconate mouthwash (MINOSEP® PT. Minorock Mandiri, Depok, Indonesia). Separately, the chitosan solution used in this study is also commercially available with a composition of 1% chitosan with 0.25% acetic acid (KITOBE™; Berkah Inovasi Kreatif Indonesia, Bogor, Indonesia). The deacetylation degree (DD) of the chitosan is 85% with a high molecular weight (MW) (CV. Biochitosan Indonesia, Cirebon, Indonesia). Sterile Aquadest was used as rinsing solution in the control group. All rinsing solution was placed in 30 identical 100-mL bottles and randomly labeled as one to 30 by the first author's supervisor.

Peri-miniscrew clinical inflammation signs (i.e., redness, swelling, pain, and mobility) were recorded, and peri-miniscrew plaque was collected by a single operator both before and after 4 days of rinsing. Peri-miniscrew redness and swelling were detected visually. Pain was quantified based on the patient's opinion. Miniscrew mobility was evaluated visually with mouth mirror handles [11]. Any orthodontic auxiliaries were removed from the miniscrew, and the surface was air-dried. The selection of samples to be evaluated with real-time quantitative polymerase chain reaction (qPCR) was done by prioritizing miniscrews located in the right buccal of the maxilla from each patient. This location was chosen because the majority of miniscrews used in orthodontic treatment are inserted in the maxillary buccal region for retraction of the anterior teeth. The right side was chosen because a previous study showed that the right side is more at risk of experiencing miniscrew failure due to infection and inflammation [12]. Peri-miniscrew plaque was collected from the miniscrew neck surface using a sterile absorbent paper point (Dentplus #35). The paper points were swabbed clockwise around the miniscrew's neck, as shown in Fig. 1 and then stored in 1000-µL of phosphate-buffered saline in an Eppendorf tube. Subsequently, all the samples were refrigerated at −20°C until laboratory processing.

**Laboratory procedure and materials**

DNA was extracted and purified from each sample using GENEzol™ reagent by following the instructions of the manufacturer. The DNA concentration of each sample was measured using Qubit fluorometry. The Qubit® dsDNA HS (high-sensitivity) assay kit was used as Qubit reagent. The concentration of DNA was standardized to 100 ng/µL using nuclease-free water.

The bacterial counts in plaque samples were evaluated by real-time PCR qPCR. qPCR was used in this study because it is effective, efficient, reproducible, and sensitive for detecting periodontopathogenic bacteria [13]. All reactions were performed using the Step One Plus qPCR system (Applied Biosystems, Foster City, CA, USA) and SensiFAST™ SYBR® Hi-Rox kit. The primers used for qPCR are displayed in Table 1.

qPCR was performed in a final volume of 10 µL, consisting of 5 µL of SensiFAST™ SYBR® Hi-Rox, 0.5 µL of forward primers, 0.5 µL of reverse primers, and 4 µL of target template. The thermal profile consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min [14,15]. Each cycle-threshold (CT) of every sample was obtained at the end of qPCR. The CT mean value was inserted into a standard curve equation determined previously and $2^{-\Delta\Delta Ct}$ for relative quantification. The standard curve of total bacteria was generated from the known colony-forming unit (CFU/mL) of *Escherichia coli* serial dilution. The CT was measured and plotted against the $\log_{10}$ of the copy number. The equation of total bacteria was obtained from the standard curve resulting from qPCR, as shown in Fig. 2.

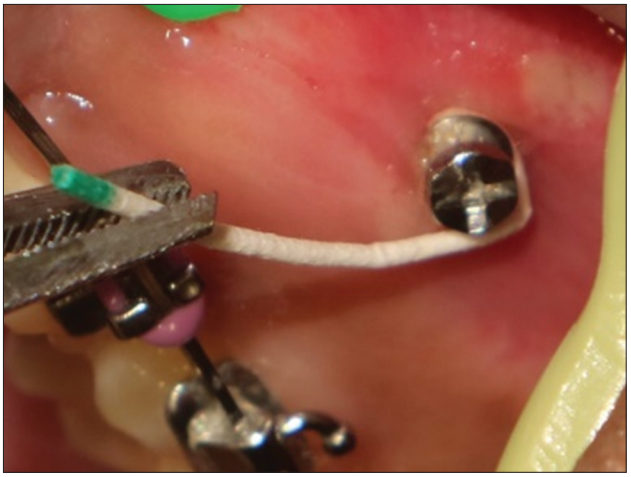

**Fig. 1: Conducting bacteria sample collection from the miniscrew neck**

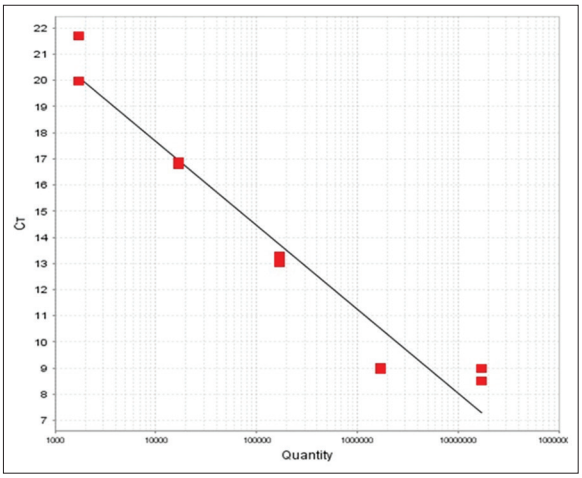


**Fig. 2: Standard curve for total bacteria; cycle-threshold = −3.02 (log quantity) + 30,461; $R^2$ = 0.994**

**Table 1: Primers used in this study**

| Bacteria | Sequence of primers (5'-3') | Target gene |
|---|---|---|
| Universal [14] (total bacteria) | Forward, TTA AAC TCA AAG GAA TTG ACG G<br>Reverse, CTC ACG ACA CGA GCT GAC GAC | 16S rRNA |
| *Porphyromonas Gingivalis* [15] | Forward, TAC CCA TCG TCG CCT TGG T<br>Reverse, CGG ACT AAA ACC GCA TAC ACT TG | 16S rRNA |
| *Tannerella forsythia* [15] | Forward, ATC CTG GCT CAG GAT<br>Reverse, TAC GCA TAC CCA TCC GCA | 16S rRNA |
| *Treponema Denticola* [15] | Forward, AGA GCA AGC TCT CCC TTA CCG<br>Reverse, TAA GGG CGG CTT GAA ATA ATG | 16S rRNA |

**Statistical analysis**

All statistical analyses were performed using the Statistical Package for the Social Sciences version 22 software program (IBM Corp., Armonk, NY, USA). Univariate analysis was performed to obtain the mean, standard deviation, minimum, and maximum values of all parameters. Quantitative differences of total bacteria before and after rinsing

treatment were assessed using the Wilcoxon test because the data varied significantly from a normal distribution ($p<0.05$). The differences in total bacteria and red-complex bacteria quantity alteration between the chlorhexidine, chitosan, and Aquadest groups were assessed using the Kruskal–Wallis test because the data varied significantly from a normal distribution ($p<0.05$).

## RESULTS AND DISCUSSION

### Peri-miniscrews clinical condition

Thirty patients (25 women and five men) according to the inclusion criteria underwent the complete procedure in this study with no complications. Subjects' ages ranged from 15 to 33 years (23.3±4.7). From 30 patients, 53 miniscrews inserted in various locations were evaluated for clinical signs of peri-miniscrew inflammation. Fig. 3 shows the distribution of clinical signs found from peri-miniscrews involved in this study.

Among all miniscrews implanted, 34 miniscrews' implantation sites (64.15%) were healthy; two miniscrews' implantation sites (3.77%) showed signs of peri-implantitis (e.g., mobility, pain, swelling, and redness); three miniscrews' implantation sites (5.66%) showed swelling, redness, and pain only; eight miniscrews' implantation sites (15.09%) showed swelling and redness; and six miniscrews' implantation sites (11.33%) showed redness of peri-miniscrew tissue.

Infection and inflammation in peri-miniscrew tissue need to be controlled to prevent the functional failure of the miniscrew implant as an anchorage device in orthodontic treatment. Although microbial infection is not the only causative factor of miniscrew failure, several studies have stated that infection and inflammation in relation to peri-miniscrew implantation are related to the miniscrew failure rate [16-19]. Osório *et al*. [20] confirmed that microbial colonization occurred in the first 24 h after exposure of miniscrew implants to the oral cavity, and the nature of microbial colonization did not change significantly if miniscrew hygiene was controlled.

One of the 30 plaque samples evaluated with qPCR showed clinical symptoms of peri-implantitis, including redness, swelling, pain, and mobility of the miniscrew. Peri-miniscrew infection is a main factor leading to miniscrew failure [16,19,21]. The results of qPCR demonstrated the detection of three red-complex bacteria in a peri-miniscrew plaque sample included in the chlorhexidine group. After using the chlorhexidine mouthwash twice a day for 4 days, peri-miniscrew swelling was observed to have subsided but the mobility, pain, and redness were seen to remain. The reduced clinical symptoms and bacterial counts in this peri-implantitis case indicate that chlorhexidine can be used to treat peri-implantitis even though miniscrew mobility cannot always be overcome immediately.

### Total bacteria quantification

One sample from each subject was evaluated with qPCR to obtain the quantity of peri-miniscrew bacteria. The results indicated a reduction in total bacteria count occurred in all groups after 4 days rinsing, as shown in Fig. 3. Normality testing using the Shapiro–Wilk test revealed that the data varied significantly from normal distribution ($p<0.05$). The Wilcoxon test showed that the number of total bacteria colonies was reduced significantly in both the chlorhexidine and chitosan groups but not in the control (Aquadest) group. This result supports that chlorhexidine and chitosan are effective as antibacterial agents to reduce the total bacteria around the miniscrew. The comparison of total bacteria reduction before and after 4 days of rinsing is shown in Fig. 4. The quantity of total bacteria reduction and the p-values from the Wilcoxon test are shown in Table 2.

The outcomes of Kruskal–Wallis testing among rinsing groups showed $p<0.05$ ($p=0.038$), which meant that the difference was statistically significant. *Post hoc* analysis using the Mann–Whitney U-test supported that the total bacteria count after rinsing differed significantly between the chlorhexidine and control groups ($p=0.041$). Moreover, the

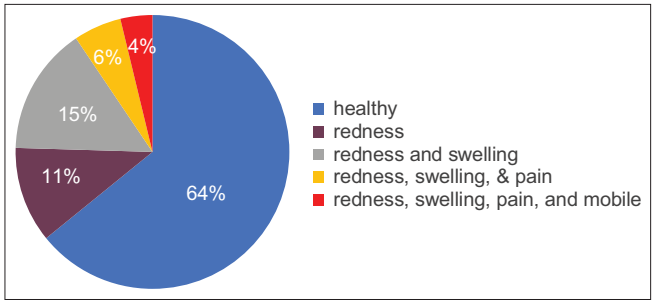


**Fig. 3: Peri-miniscrew clinical condition**

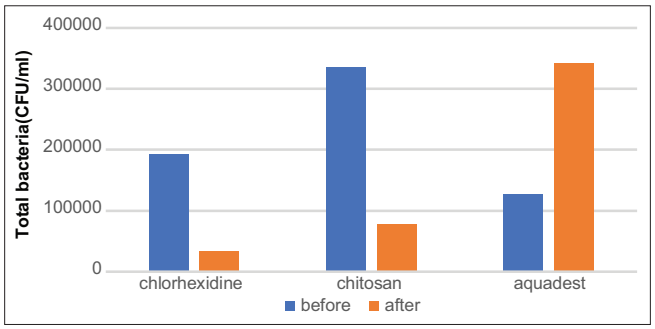


**Fig. 4: The total bacteria before and after 4 days of rinsing**

**Table 2: Total bacteria count reduction**

| Mouthwash | Total bacteria count before rinsing | Total bacteria count after rinsing | Total bacteria count changes | p-value (Wilcoxon test) |
|---|---|---|---|---|
| Chlorhexidine | 192.409 | 33.830 | −167.551 | 0.037* |
| Chitosan | 336.109 | 77.578 | −202.407 | 0.028* |
| Aquadest | 127.630 | 341.405 | 137.686 | 0.285 |

Negative values indicate a reduction after treatment. *$p<0.05$=significant

mean differed significantly between the chitosan and control groups ($p=0.019$). However, the total bacteria count after rinsing was not significantly different between the chlorhexidine and chitosan groups ($p=0.821$). These results suggest that chitosan was as effective as chlorhexidine in reducing the total bacteria count.

### Red-complex bacteria quantification

The effects of chlorhexidine, chitosan, and Aquadest rinsing on red-complex bacteria were evaluated using relative quantification with the $2^{-\Delta\Delta Ct}$ method [22]. Delta Ct (ΔCt) values in this study were obtained from differences between Ct values of target genes and total bacteria from the same sample. ΔΔCt values were obtained from differences between ΔCt before and after 4 days of rinsing treatment. The results showed that the chlorhexidine group presented reductions of 55.8% in *P. gingivalis* count, 25.3% in *T. forsythia* count, and 42.6% in *T. denticola* count. In the chitosan group, the reductions were 26% in *P. gingivalis* count, 17.1% in *T. forsythia* count, and 58.11% in *T. denticola* count. In this study, the control group also exhibited a reduction in red-complex bacteria, although the degree of such was quite low. Further, there was a 19.5% reduction in *P. gingivalis*, 18.8% reduction in *T. forsythia*, and 2.7% reduction of *T. denticola* in the control group. The comparison among bacteria count reduction between rinsing groups for each red-complex bacteria is shown in Figs. 5-7.

The results of qPCR showed a decrease in the total number of bacteria as well as specifically *P. gingivalis*, *T. forsythia,* and *T. denticola* after the use of chlorhexidine mouthwash. Apel *et al.* [23] investigated the microflora associated with failed and successful miniscrew cases, finding that

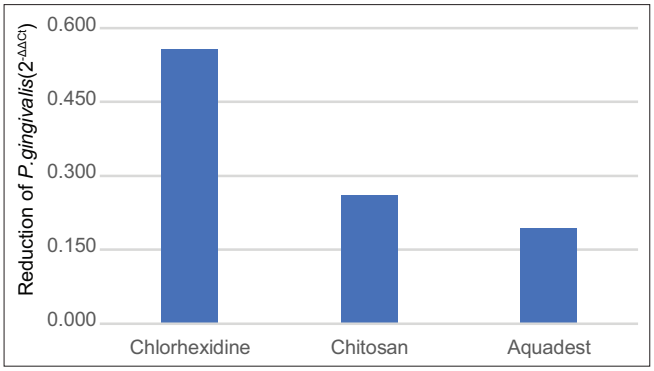


**Fig. 5: The effect of chlorhexidine, chitosan, and Aquadest on *Porphyromonas gingivalis* count reduction**

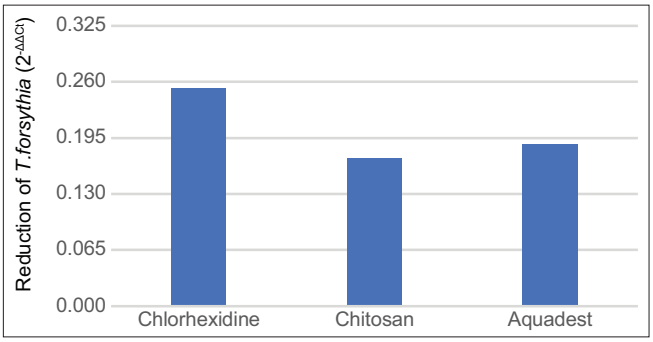


**Fig. 6: The effect of chlorhexidine, chitosan, and Aquadest on *Tannerella forsythia* count reduction**

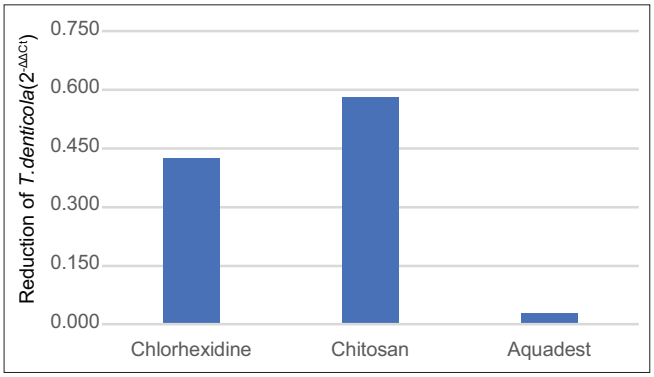


**Fig. 7: The effect of chlorhexidine, chitosan, and Aquadest on *Treponema denticola* count reduction**

*P. gingivalis* was not found in either healthy or failed miniscrews, but *T. forsythia* was found in 25% of failed miniscrews but no healthy miniscrews. In the present study, one out of 30 miniscrews exhibited clinical signs of peri-miniscrew implantitis (e.g., mobility, pain, swelling, and redness) as explained by Monga *et al.* [11]. Different from in the study by Apel *et al.* [23], in this investigation, all red-complex bacteria including *P. gingivalis, T. forsythia,* and *T. denticola* were detected peri-miniscrew with accompanying peri-implantitis clinical signs.

The present study also shows that chitosan has a comparable level of antibacterial activity to that of chlorhexidine in reducing the total bacteria count, including specifically against *T. denticola*. However, the antibacterial activity of chitosan against both *P. gingivalis* and *T. forsythia* was not significantly different from that of the control group (Aquadest) in this study. The Aquadest group in this study represented placebo rinsing, which has no antibacterial effect. This result showed that the chitosan solution in this study was not effective in reducing *P. gingivalis* or *T. forsythia*. In contrast, recent studies have revealed that chitosan possesses antibacterial activity against Gram-positive bacteria, Gram-negative bacteria, and fungi [8,9,24].

**Chitosan antibacterial modes of action**

The antibacterial activity of chitosan is influenced by its DD, MW, and acidity (pH) [8,25]. The characteristics of chitosan used in this study are DD: 85%, high MW, and pH: 6. There are several chitosan modes of action against bacteria cells. One of the modes of action is to disrupt bacterial cell membranes by provoking electrostatic interaction between the positive charges of chitosan and negative charges of the cell membrane [8]. The high-DD chitosan used in this study means that this chitosan has a high number of positive charges with which to interact with anionic parts of the lipopolysaccharides on bacterial cell membranes. This electrostatic interaction between chitosan and lipopolysaccharide leads to disruption of the cell membrane. Another chitosan mode of action involves penetrating the bacterial cell membrane [8]. The high-molecular-weight chitosan used in this study probably inhibited the ability of chitosan molecules to get inside the bacterial cells, especially those of Gram-negative bacteria, which have double membranes. This explains the inadequacy of chitosan to reduce the counts of *P. gingivalis* and *T. forsythia* in this study. The better antibacterial activity of chitosan on *T. denticola* relative to *P. gingivalis* and *T. forsythia* in the present study is allegedly due to the long helical-shaped morphology of *T. denticola*. The long helical shape of *T. denticola* means there is a wider membrane surface area that can interact with positive charges of chitosan as compared short rod-shaped *P. gingivalis* and *T. forsythia*. In the future study, it is recommended to use high DD chitosan with low MW or nanoparticle to evaluate its potential antibacterial value, especially on Gram-negative bacteria.

However, some limitations in this study should be noted. First, this study involved a limited amount of sample with a high variation of clinical condition of peri-miniscrew before treatment that may influence the result. Second, the oral hygiene standard of subjects was not controlled, which would bias the results. Nonetheless, the results of this study showed that chitosan is a potential antibacterial agent to be used as an active ingredient in mouthwash and further studies is required.

## CONCLUSION

This study reveals that chitosan has adequate antibacterial activity to reduce the total bacteria count peri-miniscrew and its effectiveness does not significantly differ from that of chlorhexidine. The antibacterial activity of chitosan on red-complex bacteria still needs to be evaluated. Nonetheless, chitosan has a potential antibacterial activity to be incorporated into mouthwash to maintain peri-miniscrew hygiene.

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## CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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