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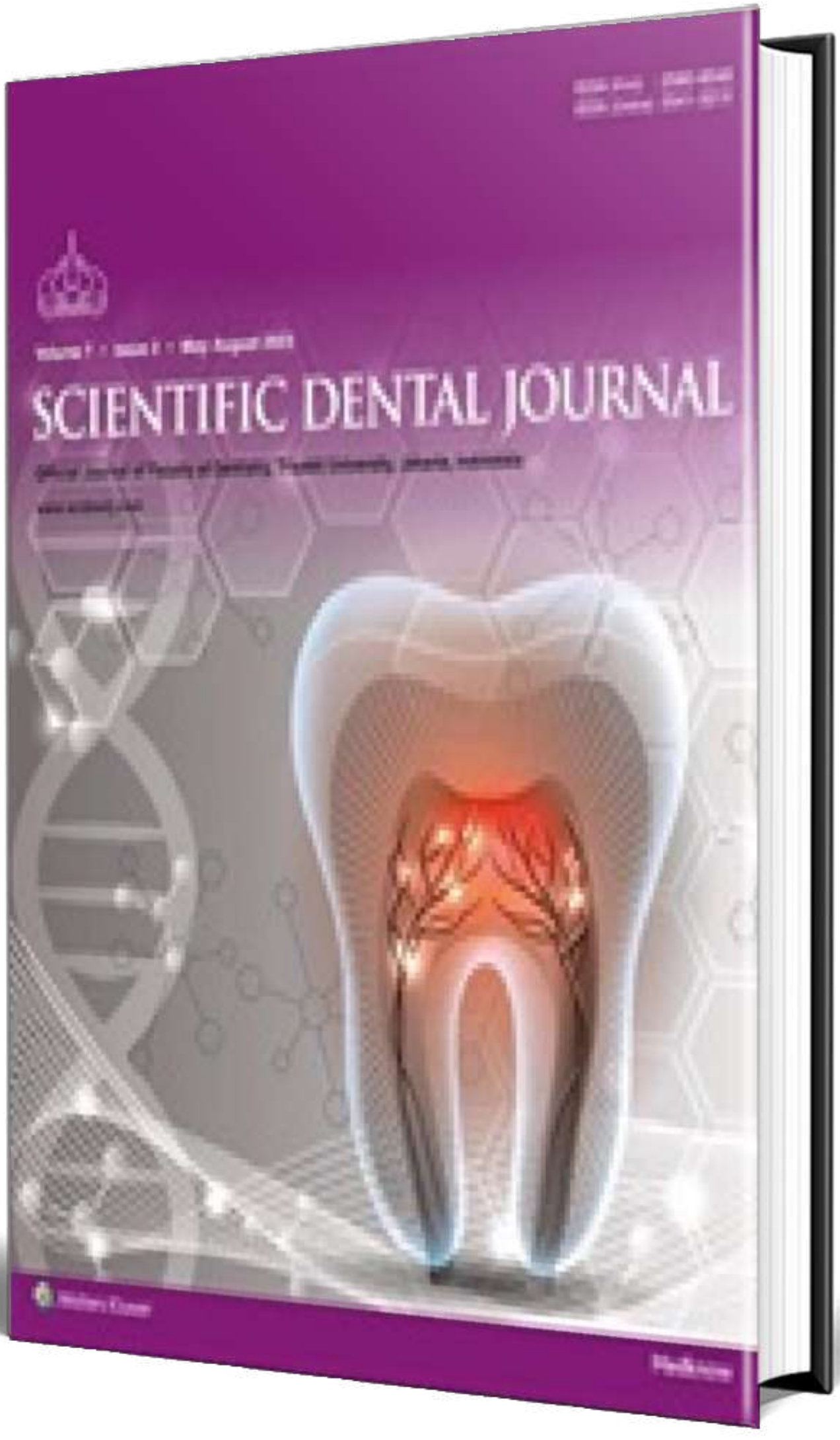
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## Comparing the Effectivities of Chitosan Citrate and Chitosan Acetate in Eradicating Enterococcus faecalis Biofilm (https://e-journal.trisakti.ac.id/index.php/dental/article/view/2290)

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Scientific Dental Journal

Original Articles

# Comparing the Effectivities of Chitosan Citrate and Chitosan Acetate in Eradicating *Enterococcus faecalis* Biofilm

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## ABSTRACT

**Background:** Adequate biomechanical preparations, antibacterial irrigants, and intracanal medications to promote the elimination of bacteria and their products are required to succeed root canal treatment. *Enterococcus faecalis* with its biofilm is known as an important etiological agent in endodontic treatment failures. Chitosan, as a natural product, has an antibacterial activity and is considered less toxic to the periapical tissue than other irrigants. However, the use of this natural product needs to be examined to determine its effectiveness as a root canal irrigant in endodontic treatment; this can be done by comparing it with the most common endodontic irrigant (NaOCl 5.25%) as a positive control. **Objective:** The objective of the study was to compare the effectiveness between 1–3% chitosan acetate (CA) and 1–3% chitosan citrate (CC) against *E. faecalis* biofilm formation after treatment for 15, 30, and 60 minutes. **Methods:** The study was conducted using 12 groups, including 1–3% CA, 1–3% CC, and control groups. *E. faecalis* biofilms in 96-well plates were exposed to each sample for 15, 30, or 60 minutes. Subsequently, the biofilms were stained with crystal violet solution, and the optical density value was measured using a microtiter plate reader at a wavelength of 600 nm. **Results:** CA and CC were effective in eradicating *E. faecalis* biofilm. However, the levels of effectiveness of CC and CA depended on the concentration and application time. Three-way analysis of variance (ANOVA) showed a significant difference between the irrigants ($p$ <0.05) and three application times ($p$ <0.05). The CA was effective in eradicating biofilm after 15 minutes of application, whereas the CC was more effective after 30 and 60 minutes of application. **Conclusion:** CC and CA are both effective in eradicating *E. faecalis* biofilm.

Keywords : biofilm, chitosan acetate, chitosan citrate, *Enterococcus faecalis*, irrigation

## Background

Endodontic treatment focuses on reducing the microbial load in the root canal to enable healing and prevent bacteria from re-entering the root canal in the future.[1] Previous *ex vivo* studies and clinical evidence have suggested that mechanical instrumentation alone will leave an unaffected portion of the infected root canal

wall, which indicates that the cleaning process of the root canal cannot be achieved via preparation alone.[2,3] To achieve a successful endodontic treatment, in addition to preparation, effective antibacterial irrigants are required to eliminate bacteria in the root canal.[4,5]

Recent studies have suggested that the presence of *Enterococcus faecalis* in the root canal can increase the risk of endodontic infections during or after the root canal treatment.[6,7,8] Studies on NaOCl and chlorhexidine as antimicrobial irrigants have shown that both irrigants are effective to be used as antibacterial agents.[9,10] However, NaOCl is potentially toxic to human osteoblast cells; such toxicity can affect tissue regeneration, thereby causing a delay in the healing process.[11,12] Therefore, the search for ideal root canal irrigants continues with the development of new materials and methods. One material that has been investigated in many studies is chitosan, which has been considered for many dental applications. As the second most abundant polysaccharide after cellulose, it is widely distributed in nature, especially in Indonesia.[13] Chitosan can be easily generated from prawn and crab shells.[14,15]

In a previous study on chitosan dissolved in citric acid, it was found that 0.1–0.6% chitosan citrate (CC) solution showed antibacterial activity, significantly better cleansing of the smear layer, and a lack of dentin erosion compared with 10% citric acid. A previous study using electronic micrographs showed that chitosan weakens and even damages Gram-positive bacterial cell walls and disrupts the cytoplasm membrane in Gram-negative bacteria.[16]

Previous research on the effect of chitosan on the attachment and formation of *Streptococcus mutans* biofilm has suggested that chitosan can inhibit the attachment and formation of biofilm, and it is able to remove existing biofilm.[17] Based on these findings in the literature, the aim of the present study was to evaluate the effectiveness of chitosan acetate (CA) and CC as antibacterial agents and their inhibition of *E. faecalis* biofilm formation *in vitro*.

# Material and Methods

## Bacterial Culture

The bacterial cultures were obtained by following the standard protocols at MiCORE Laboratory, Faculty of Dentistry, Trisakti University, Indonesia. Bacterial strains of *E. faecalis* ATCC 29212 were maintained in stock cultures frozen at –80°C in brain heart infusion (BHI) broth containing glycerol (20% v/v). For biofilm assays, *E. faecalis* colonies were inoculated in BHI broth and incubated at 37°C under anaerobic conditions using a gas pack jar system for 24 hours. Bacterial cells were then collected by centrifugation and suspended in fresh sterile BHI broth.

## Sample Solution Preparation

Chitosan acetate (CA) solution was prepared with different concentrations ranging from 1 to 3% by mixing 1–3 g of chitosan powder into 100 mL of 1% acetic acid. The pH level of the CA solution was adjusted to 3.5 with NaOH. This solution was stirred for 24 hours with a magnetic stirrer, using materials from Biotech Surindo.[18] The CC solution was prepared with different concentrations ranging from 1 to 3% by mixing 1–3 g of chitosan powder into 100 mL of 10% citric acid.[19] The pH level of the CC solution was adjusted to 3.2 with NaOH. This solution was stirred for 24 hours with a magnetic stirrer, using materials from Biotech Surindo.[18]

## Minimum Inhibitory Concentration (MIC)

A broth dilution test was performed to determine the minimum inhibitory concentration (MIC) of the CC and CA solutions. Three milliliters of BHI broth were inserted into 12 glass tubes, and then 100 µL of bacterial cell suspension ($1 \times 10^8$ CFU/mL) was added to each tube, followed by 100 µL solution of CC and CA at various concentrations (1%, 1.5%, 2%, 2.5%, and 3%) and 5.25% NaOCl as the positive control. A solution containing only BHI and chitosan was also made for a comparison of the optical density values. The plate was incubated at 37°C for 24 hours. The MIC was determined by observing the optical density value obtained using a microtiter plate reader.[12]

## Biofilm Assay

Two hundred microliters of bacterial suspension were inoculated in a 96-well microtiter plate, and the plate was incubated at 37°C under anaerobic conditions for 24 hours

to form biofilm. Subsequently, 200-µL samples of each irrigation solution (1%, 1.5%, 2%, 2.5%, 3% CA; 1%, 1.5%, 2%, 2.5%, 3% CC; and 5.25% NaOCl) were added to the *E. faecalis* biofilm-containing well plate. The plate was incubated for 15, 30, or 60 minutes. Afterward, the biofilms in each well were stained with 0.1% violet crystalline solution for 15 minutes and rinsed with phosphate-buffered saline (PBS); following this, 90% ethanol was added. The effectiveness of each irrigation solution in terms of disrupting the *E. faecalis* biofilm was analyzed by observing the optical density values of the crystal violet dyes absorbed by the biofilms.[20] The absorbencies of the remaining biofilms after being treated by CA, CC, and NaOCl were assessed at 600 nm using a microtiter plate reader. Five wells for each group were used in this study.

## Statistical Analysis

Differences between experimental groups were analyzed using three-way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant. Shapiro Wilk test was used to test for normality previously. Statistical calculations were performed with SPSS Statistics for Windows software version 20 (IBM, USA).

## Results

The MIC test results showed that the minimum concentration of CA and CC to inhibit *E. faecalis* when grown in the planktonic state was 1%. The data normality test, which was performed using the Shapiro–Wilk test for each group, showed a normal data on the optical density values of the CC (1%, 1.5%, 2%, 2.5%, 3%), CA (1%, 1.5%, 2%, 2.5%, 3%), and 5.25% NaOCl groups after 15, 30, and 60 minutes of application time ($p > 0.05$).

The biofilm assay result showed that, for the 15-minute application, the highest optical density values were evident in the group of negative controls, followed by 1% CC, 3% CC, 2.5% CC, 1.5% CC, 2% CC, 3% CA, 1% CA, 1.5% CA, 2% CA, and 2.5% CA; 5.25% NaOCl had the lowest value (Fig. 1). For the 30-minute application, the optical density values, ranging from highest to lowest, were obtained from the group of negative controls, 1% CA, 1.5% CA, 2% CA, 3% CA, 3% CC, 2.5% CC, 1% CC, 1.5% CC, 2.5% CA, 2% CA, and 5.25% NaOCl (Fig. 2). For the 60-minute application, the optical density values, ranging from highest to lowest, were obtained from the group of negative controls, 1% CA, 1.5% CA, 2% CA, 3% CA, 2.5% CA, 3% CC, 2.5% CC, 1% CC, 1.5% CC, 2% CC, and 5.25% NaOCl (Fig. 3).

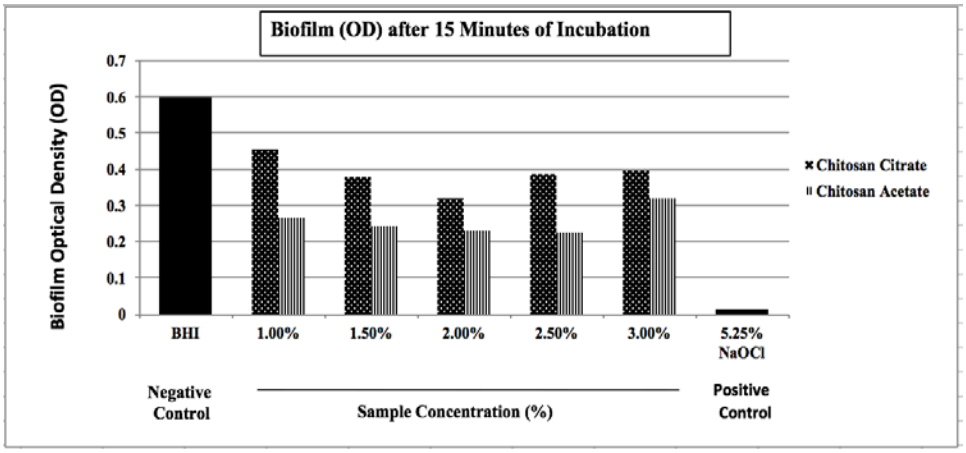


**Figure 1.** Optical density measurements of *Enterococcus faecalis* biofilm eradication using a microtiter plate reader after 15 minutes of incubation.

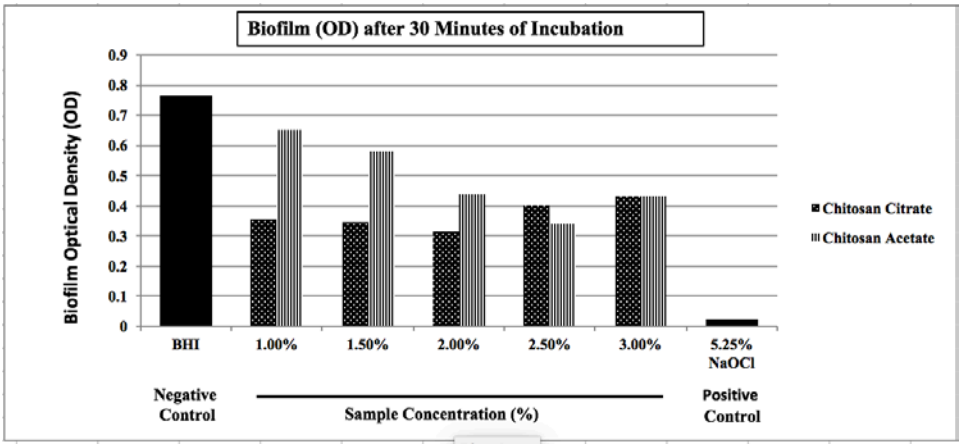


**Figure 2.** Optical density measurement of *Enterococcus faecalis* biofilm eradication using a microtiter plate reader after 30 minutes of incubation.

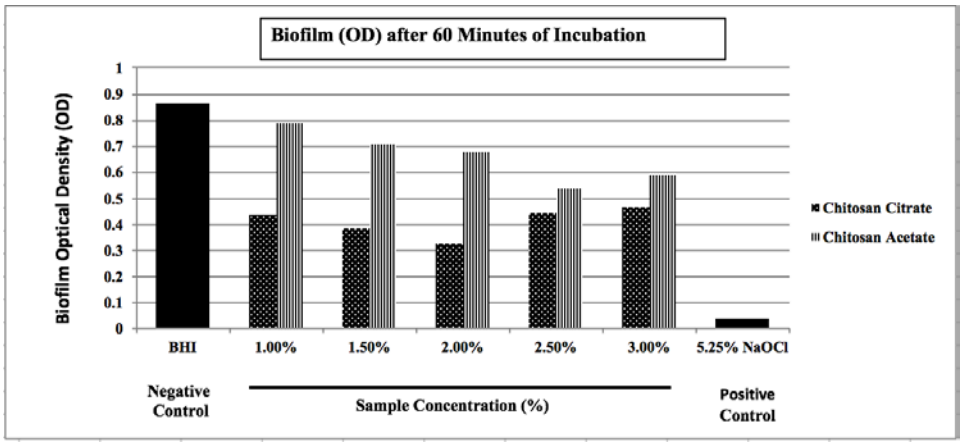


**Figure 3.** Optical density measurement of *Enterococcus faecalis* biofilm eradication using a microtiter plate reader after 60 minutes of incubation.

# Discussion

The results of three-way ANOVA on the crystal violet absorption assay of *E. faecalis* biofilms, after treatment with CC, CA, and 5.25% NaOCl, showed that both the CC and CA solutions at all given concentrations for application time of 15, 30, and 60 minutes generated significant differences between irrigation time ($F = 6.50$; $p < 0.05$) and each type of irrigant ($F = 2.92$; $p < 0.05$). The effectiveness of 1.5–3% CC was lower than that of 1–3% CA for 15 minutes; 1.5–2% CC and 1–3% CA for 30 minutes; 1–2% CC for 60 minutes; and 5.25% NaOCl for 15, 30, and 60 minutes. There was also a difference between the effectiveness levels of CC and CA in eradicating the *E. faecalis* biofilm, depending on the concentration and application time. There were no difference among 1.5–3% CC for 15 minutes, 1.5–2% CC, 1–3% CC for 30 minutes, 1–2% CC for 60 minutes of application, and 5.25% NaOCl in eradicating the *E. faecalis* biofilm.

Multiple comparison tests were performed using Tukey's HSD to investigate significant differences among each type of irrigant and irrigation time. The posterior multiple comparison test showed that there were some differences in the effectiveness of each irrigant, depending on the concentration and irrigation time. The following irrigants, with the indicated concentrations and irrigation times, suggested the same level of antimicrobial activity as 5.25% NaOCl for eradicating the *E. faecalis* biofilm: 1.5–3% CA for 15 minutes, 1–2% CC for 30 minutes, 1.5–2% CC for 60 minutes, 1–3% CA for 15 minutes, and 2.5% CA for 30 minutes.

A previous study concluded that there was no significant difference between CA and NaOCl solution in eradicating *E. faecalis* biofilm.[21] However, in the current study, each irrigant had different effective concentrations against bacterial biofilms, in contrast to the previous study. This may have been caused by the different sources and molecular weights of the chitosan between the two studies. In this study, the chitosan powder was extracted from crab shells, and it had a higher molecular weight of 900 kDa (>150 kDa). In this study, acetic acid and citric acid were used to prepare the chitosan solutions, as they have been commonly used as chitosan solvents. In addition, 10% citric acid is often used as a root canal irrigant, as it is ideal for cleansing the smear layer and exhibits antimicrobial activity.[22,23] The common concentration of citric acid is 10%, with pH 3.2. However, in this study, we used citric acid with a higher concentration and lower pH than 3.2 to dissolve the chitosan; still, the chitosan powder was not completely dissolved. The chitosan powder was successfully dissolved in 1% acetic acid at pH 3.5. Therefore, we suggest that acetic acid is more effective than citric acid as a solvent for chitosan powder.

The optical density values obtained at an application of 15 minutes were significantly better than those at 30 and 60 minutes. The least effective irrigant was in the group of 1% CA for an application time of 60 minutes, whereas the most effective one was found in the NaOCl group as the positive control. However, no significant differences were found in the effectiveness levels of NaOCl, 1–2% CC for 60 minutes, 2.5% CA for 30 minutes, 2% CC for 15 and 30 minutes, and 1–3% CA for 15 minutes. The most effective application time of 5.25% NaOCl was observed to be 15 minutes, whereas applications for 30 and 60 minutes showed less effective antimicrobial activity due to the instability of the NaOCl solutions and the loss of active chlorine compounds during the incubation period.[24] The current study also suggested that CC was more stable than CA, as observed in its effectiveness in eradicating the *E. faecalis* biofilms.

## Conclusions

Chitosan acetate (CA) and chitosan citrate (CC) are effective in eradicating *E. faecalis* biofilms. The levels of effectiveness of CC and CA differ depending on the concentration and application time. CA is more effective in eradicating biofilm after 15 minutes of application, whereas CC is more effective after 30 and 60 minutes of application. Both solutions may be used as alternative irrigants in root canal treatment. However, further research is needed to evaluate the interaction between the chitosan solutions to evaluate their cleaning efficacy *in vivo*.

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## Conflict of Interest

The authors declare that there are no conflicts of interest.

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# Comparing the Effectivities of Chitosan Citrate and Chitosan Acetate in Eradicating Enterococcus faecalis Biofilm

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# Comparing the Effectivities of Chitosan Citrate and Chitosan Acetate in Eradicating *Enterococcus faecalis* Biofilm

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## ABSTRACT

**Background:** Adequate biomechanical preparations, antibacterial irrigants, and intracanal medications to promote the elimination of bacteria and their products are required to succeed root canal treatment. *Enterococcus faecalis* with its biofilm is known as an important etiological agent in endodontic treatment failures. Chitosan, as a natural product, has an antibacterial activity and is considered less toxic to the periapical tissue than other irrigants. However, the use of this natural product needs to be examined to determine its effectiveness as a root canal irrigant in endodontic treatment; this can be done by comparing it with the most common endodontic irrigant (NaOCl 5.25%) as a positive control. **Objective:** The objective of the study was to compare the effectiveness between 1–3% chitosan acetate (CA) and 1–3% chitosan citrate (CC) against *E. faecalis* biofilm formation after treatment for 15, 30, and 60 minutes. **Methods:** The study was conducted using 12 groups, including 1–3% CA, 1–3% CC, and control groups. *E. faecalis* biofilms in 96-well plates were exposed to each sample for 15, 30, or 60 minutes. Subsequently, the biofilms were stained with crystal violet solution, and the optical density value was measured using a microtiter plate reader at a wavelength of 600 nm. **Results:** CA and CC were effective in eradicating *E. faecalis* biofilm. However, the levels of effectiveness of CC and CA depended on the concentration and application time. Three-way analysis of variance (ANOVA) showed a significant difference between the irrigants ($p$ <0.05) and three application times ($p$ <0.05). The CA was effective in eradicating biofilm after 15 minutes of application, whereas the CC was more effective after 30 and 60 minutes of application. **Conclusion:** CC and CA are both effective in eradicating *E. faecalis* biofilm.

Keywords : biofilm, chitosan acetate, chitosan citrate, *Enterococcus faecalis,* irrigation

## Background

Endodontic treatment focuses on reducing the microbial load in the root canal to enable healing and prevent bacteria from re-entering the root canal in the future.[1] Previous *ex vivo* studies and clinical evidence have suggested that mechanical instrumentation alone will leave an unaffected portion of the infected root canal

wall, which indicates that the cleaning process of the root canal cannot be achieved via preparation alone.[2,3] To achieve a successful endodontic treatment, in addition to preparation, effective antibacterial irrigants are required to eliminate bacteria in the root canal.[4,5]

Recent studies have suggested that the presence of *Enterococcus faecalis* in the root canal can increase the risk of endodontic infections during or after the root canal treatment.[6,7,8] Studies on NaOCl and chlorhexidine as antimicrobial irrigants have shown that both irrigants are effective to be used as antibacterial agents.[9,10] However, NaOCl is potentially toxic to human osteoblast cells; such toxicity can affect tissue regeneration, thereby causing a delay in the healing process.[11,12] Therefore, the search for ideal root canal irrigants continues with the development of new materials and methods. One material that has been investigated in many studies is chitosan, which has been considered for many dental applications. As the second most abundant polysaccharide after cellulose, it is widely distributed in nature, especially in Indonesia.[13] Chitosan can be easily generated from prawn and crab shells.[14,15]

In a previous study on chitosan dissolved in citric acid, it was found that 0.1–0.6% chitosan citrate (CC) solution showed antibacterial activity, significantly better cleansing of the smear layer, and a lack of dentin erosion compared with 10% citric acid. A previous study using electronic micrographs showed that chitosan weakens and even damages Gram-positive bacterial cell walls and disrupts the cytoplasm membrane in Gram-negative bacteria.[16]

Previous research on the effect of chitosan on the attachment and formation of *Streptococcus mutans* biofilm has suggested that chitosan can inhibit the attachment and formation of biofilm, and it is able to remove existing biofilm.[17] Based on these findings in the literature, the aim of the present study was to evaluate the effectiveness of chitosan acetate (CA) and CC as antibacterial agents and their inhibition of *E. faecalis* biofilm formation *in vitro*.

# Material and Methods

## Bacterial Culture

The bacterial cultures were obtained by following the standard protocols at MiCORE Laboratory, Faculty of Dentistry, Trisakti University, Indonesia. Bacterial strains of *E. faecalis* ATCC 29212 were maintained in stock cultures frozen at –80°C in brain heart infusion (BHI) broth containing glycerol (20% v/v). For biofilm assays, *E. faecalis* colonies were inoculated in BHI broth and incubated at 37°C under anaerobic conditions using a gas pack jar system for 24 hours. Bacterial cells were then collected by centrifugation and suspended in fresh sterile BHI broth.

## Sample Solution Preparation

Chitosan acetate (CA) solution was prepared with different concentrations ranging from 1 to 3% by mixing 1–3 g of chitosan powder into 100 mL of 1% acetic acid. The pH level of the CA solution was adjusted to 3.5 with NaOH. This solution was stirred for 24 hours with a magnetic stirrer, using materials from Biotech Surindo.[18] The CC solution was prepared with different concentrations ranging from 1 to 3% by mixing 1–3 g of chitosan powder into 100 mL of 10% citric acid.[19] The pH level of the CC solution was adjusted to 3.2 with NaOH. This solution was stirred for 24 hours with a magnetic stirrer, using materials from Biotech Surindo.[18]

## Minimum Inhibitory Concentration (MIC)

A broth dilution test was performed to determine the minimum inhibitory concentration (MIC) of the CC and CA solutions. Three milliliters of BHI broth were inserted into 12 glass tubes, and then 100 µL of bacterial cell suspension ($1 \times 10^8$ CFU/mL) was added to each tube, followed by 100 µL solution of CC and CA at various concentrations (1%, 1.5%, 2%, 2.5%, and 3%) and 5.25% NaOCl as the positive control. A solution containing only BHI and chitosan was also made for a comparison of the optical density values. The plate was incubated at 37°C for 24 hours. The MIC was determined by observing the optical density value obtained using a microtiter plate reader.[12]

## Biofilm Assay

Two hundred microliters of bacterial suspension were inoculated in a 96-well microtiter plate, and the plate was incubated at 37°C under anaerobic conditions for 24 hours

to form biofilm. Subsequently, 200-µL samples of each irrigation solution (1%, 1.5%, 2%, 2.5%, 3% CA; 1%, 1.5%, 2%, 2.5%, 3% CC; and 5.25% NaOCl) were added to the *E. faecalis* biofilm-containing well plate. The plate was incubated for 15, 30, or 60 minutes. Afterward, the biofilms in each well were stained with 0.1% violet crystalline solution for 15 minutes and rinsed with phosphate-buffered saline (PBS); following this, 90% ethanol was added. The effectiveness of each irrigation solution in terms of disrupting the *E. faecalis* biofilm was analyzed by observing the optical density values of the crystal violet dyes absorbed by the biofilms.[20] The absorbencies of the remaining biofilms after being treated by CA, CC, and NaOCl were assessed at 600 nm using a microtiter plate reader. Five wells for each group were used in this study.

## Statistical Analysis

Differences between experimental groups were analyzed using three-way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant. Shapiro Wilk test was used to test for normality previously. Statistical calculations were performed with SPSS Statistics for Windows software version 20 (IBM, USA).

## Results

The MIC test results showed that the minimum concentration of CA and CC to inhibit *E. faecalis* when grown in the planktonic state was 1%. The data normality test, which was performed using the Shapiro–Wilk test for each group, showed a normal data on the optical density values of the CC (1%, 1.5%, 2%, 2.5%, 3%), CA (1%, 1.5%, 2%, 2.5%, 3%), and 5.25% NaOCl groups after 15, 30, and 60 minutes of application time ($p > 0.05$).

The biofilm assay result showed that, for the 15-minute application, the highest optical density values were evident in the group of negative controls, followed by 1% CC, 3% CC, 2.5% CC, 1.5% CC, 2% CC, 3% CA, 1% CA, 1.5% CA, 2% CA, and 2.5% CA; 5.25% NaOCl had the lowest value (Fig. 1). For the 30-minute application, the optical density values, ranging from highest to lowest, were obtained from the group of negative controls, 1% CA, 1.5% CA, 2% CA, 3% CA, 3% CC, 2.5% CC, 1% CC, 1.5% CC, 2.5% CA, 2% CA, and 5.25% NaOCl (Fig. 2). For the 60-minute application, the optical density values, ranging from highest to lowest, were obtained from the group of negative controls, 1% CA, 1.5% CA, 2% CA, 3% CA, 2.5% CA, 3% CC, 2.5% CC, 1% CC, 1.5% CC, 2% CC, and 5.25% NaOCl (Fig. 3).

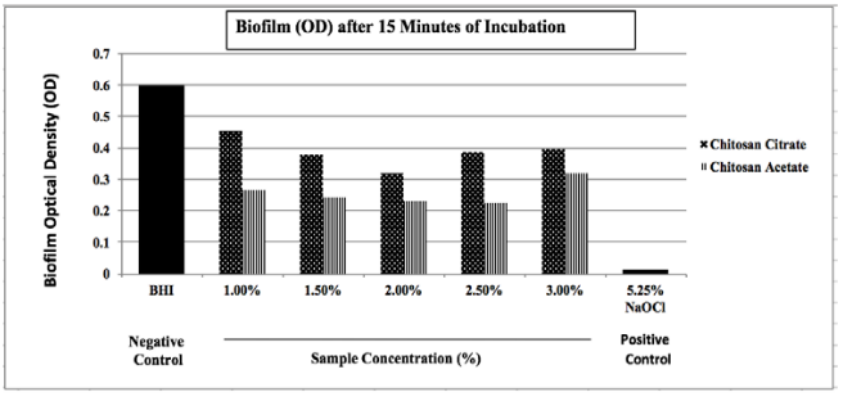


**Figure 1.** Optical density measurements of *Enterococcus faecalis* biofilm eradication using a microtiter plate reader after 15 minutes of incubation.

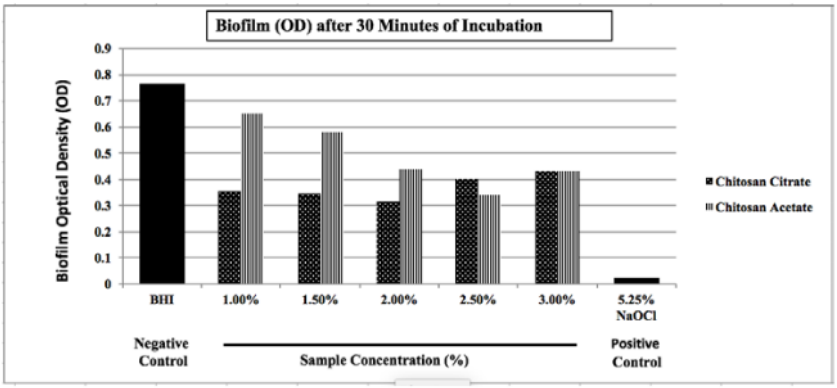


**Figure 2.** Optical density measurement of *Enterococcus faecalis* biofilm eradication using a microtiter plate reader after 30 minutes of incubation.

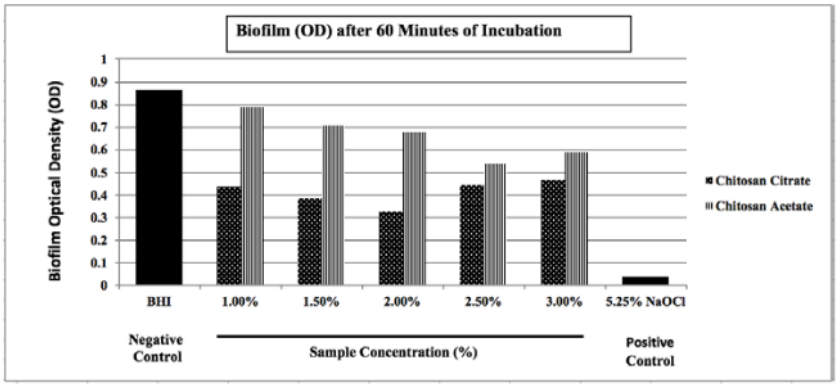


**Figure 3.** Optical density measurement of *Enterococcus faecalis* biofilm eradication using a microtiter plate reader after 60 minutes of incubation.

## Discussion

The results of three-way ANOVA on the crystal violet absorption assay of *E. faecalis* biofilms, after treatment with CC, CA, and 5.25% NaOCl, showed that both the CC and CA solutions at all given concentrations for application time of 15, 30, and 60 minutes generated significant differences between irrigation time ($F = 6.50$; $p < 0.05$) and each type of irrigant ($F = 2.92$; $p < 0.05$). The effectiveness of 1.5–3% CC was lower than that of 1–3% CA for 15 minutes; 1.5–2% CC and 1–3% CA for 30 minutes; 1–2% CC for 60 minutes; and 5.25% NaOCl for 15, 30, and 60 minutes. There was also a difference between the effectiveness levels of CC and CA in eradicating the *E. faecalis* biofilm, depending on the concentration and application time. There were no difference among 1.5–3% CC for 15 minutes, 1.5–2% CC, 1–3% CC for 30 minutes, 1–2% CC for 60 minutes of application, and 5.25% NaOCl in eradicating the *E. faecalis* biofilm.

Multiple comparison tests were performed using Tukey's HSD to investigate significant differences among each type of irrigant and irrigation time. The posterior multiple comparison test showed that there were some differences in the effectiveness of each irrigant, depending on the concentration and irrigation time. The following irrigants, with the indicated concentrations and irrigation times, suggested the same level of antimicrobial activity as 5.25% NaOCl for eradicating the *E. faecalis* biofilm: 1.5–3% CA for 15 minutes, 1–2% CC for 30 minutes, 1.5–2% CC for 60 minutes, 1–3% CA for 15 minutes, and 2.5% CA for 30 minutes.

A previous study concluded that there was no significant difference between CA and NaOCl solution in eradicating *E. faecalis* biofilm.[21] However, in the current study, each irrigant had different effective concentrations against bacterial biofilms, in contrast to the previous study. This may have been caused by the different sources and molecular weights of the chitosan between the two studies. In this study, the chitosan powder was extracted from crab shells, and it had a higher molecular weight of 900 kDa (>150 kDa). In this study, acetic acid and citric acid were used to prepare the chitosan solutions, as they have been commonly used as chitosan solvents. In addition, 10% citric acid is often used as a root canal irrigant, as it is ideal for cleansing the smear layer and exhibits antimicrobial activity.[22,23] The common concentration of citric acid is 10%, with pH 3.2. However, in this study, we used citric acid with a higher concentration and lower pH than 3.2 to dissolve the chitosan; still, the chitosan powder was not completely dissolved. The chitosan powder was successfully dissolved in 1% acetic acid at pH 3.5. Therefore, we suggest that acetic acid is more effective than citric acid as a solvent for chitosan powder.

The optical density values obtained at an application of 15 minutes were significantly better than those at 30 and 60 minutes. The least effective irrigant was in the group of 1% CA for an application time of 60 minutes, whereas the most effective one was found in the NaOCl group as the positive control. However, no significant differences were found in the effectiveness levels of NaOCl, 1–2% CC for 60 minutes, 2.5% CA for 30 minutes, 2% CC for 15 and 30 minutes, and 1–3% CA for 15 minutes. The most effective application time of 5.25% NaOCl was observed to be 15 minutes, whereas applications for 30 and 60 minutes showed less effective antimicrobial activity due to the instability of the NaOCl solutions and the loss of active chlorine compounds during the incubation period.[24] The current study also suggested that CC was more stable than CA, as observed in its effectiveness in eradicating the *E. faecalis* biofilms.

## Conclusions

Chitosan acetate (CA) and chitosan citrate (CC) are effective in eradicating *E. faecalis* biofilms. The levels of effectiveness of CC and CA differ depending on the concentration and application time. CA is more effective in eradicating biofilm after 15 minutes of application, whereas CC is more effective after 30 and 60 minutes of application. Both solutions may be used as alternative irrigants in root canal treatment. However, further research is needed to evaluate the interaction between the chitosan solutions to evaluate their cleaning efficacy *in vivo*.

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## Conflict of Interest

The authors declare that there are no conflicts of interest.

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