Giant Prawn Shell Nano-chitosan Degrades Streptococcus Mutans ATCC 25175 Biofilm in Vitro

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Abstract

The objective of this research was to determine the effect of giant prawn shell nano-chitosan (GPSN) on the degradation of S. mutans ATCC 25175 biofilm.

GPSN was extracted from giant prawn shells (Macrobrachium rosenbergii) through deproteination, demineralization, depigmentation, deacetylation, and ionic gelation method using sodium tripolyphosphate. Biofilm degradation assay was conducted by incubating varying concentrations of GPSN with 24 and 48-h S. mutans biofilms for 24-h. The remaining biofilm was then stained using crystal violet and the absorbance was read by spectrophotometer (λ=540 nm). Biofilms were also analyzed through biofilm viability staining and confocal laser scanning microscopy.

ANOVA showed significant differences among groups indicating that GPSN affected the degradation of 24 and 48-h S. mutans biofilms ($p < 0.05$). Post-hoc LSD tests showed a concentration-dependent effect of nano-chitosan in S. mutans biofilm degradation ($p<0.05$). The number of bacteria in the 24-h and 48-h S. mutans biofilms were significantly less after treatment with varying concentrations of GPSN ($p<0.05$).

In conclusion, GPSN has a biofilm degradation effect on 24-h and 48-h S. mutans biofilms.

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Introduction

Approximately up to 700 species of bacteria are present in the oral biofilm[1–3], a multibacterial community attached to a tooth surface and embedded in an extracellular polysaccharide matrix (EPS) [4,5]. Those various microorganisms interact with each other to make up complex oral biofilm communities[6]. When the biofilm community is already formed, the microbial metabolism and the host immune response may alter the local environment that supports or suppress the growth of certain organisms, triggering a dysbiotic state[7]. Factors such as food intake, consumption of certain drugs, and poor oral hygiene conditions can also alter the microbial balance in the biofilm[8]. Disruption of the biofilm balance leads to the pathological conditions in the oral cavity such as caries and periodontal disease [9,10].

Dental caries is one of the main oral health problems that occur due to dysbiosis of the biofilm-forming microorganisms[11]. Pathogenesis of dental caries is initiated by biofilm formation. The biofilm formation begins with the attachment of pioneer bacteria to the tooth surface and then followed by co-adhesion between the pioneer bacteria and other microorganisms. The biofilm then undergoes maturation after 24 to 48-hours[12]. At this stage, there is an increase in bacterial cell multiplication and EPS secretion[9,13]. The EPS functions to maintain biofilm stability, support bacterial attachment, facilitate nutrient transfer, and protect biofilms from external influences, thus causing biofilm resistance to antimicrobial substances[4,5,13].

A high sucrose diet will increase the growth of cariogenic bacteria in the biofilms [7,11]. Those bacteria can metabolize sucrose into acid, causing acidic atmosphere in the oral cavity, thus

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triggering the demineralization of the teeth leading to caries[4,11]. One of the bacteria that play an important role in the pathogenesis of dental caries is Streptococcus mutans [14]. Streptococcus mutans can adhere to the tooth surface and initiate the process of biofilm formation, able to ferment carbohydrate as a nutrient source, and forming quorum sensing[15]. In addition, the bacteria also metabolize sucrose, and survive in an acidic environment[4,12,15–17].

Various caries prevention strategies are performed, one of which is by using antimicrobial mouthwash to prevent biofilm formation. The most widely used antimicrobial mouthwash ingredients are chlorhexidine gluconate (Chx), povidone iodine, essential oils (EO), and cetylpyridinium chloride (CPC)[18]. Previous study proved that they were effective in preventing the attachment of bacteria to the tooth surface thereby reducing dental plaque formation[19–23]. However, some studies have shown that those antibacterial agents are not effective in disrupting the existing biofilms. A study by Takenaka et al. revealed that there was no biofilm removal after treated with ethanol, Chx, and other commercial mouthwash[23,24]. Other studies showed that CPC and EO were not effective in degrading biofilm due to their low penetration ability into the biofilm matrix [23,25,26]. In addition, several side effects such as tooth staining, mucosal irritation, burning sensation, and taste alteration have been reported due to the use of those mouthwashes for a long time[18,27]. Therefore, further studies are needed to explore alternative antimicrobial substance that are more effective in degrading biofilm with minimal side effects.

One of the materials developed that may have potency as an anti-biofilm is chitosan. Chitosan, a natural polysaccharide produced from chitin, consisting of 2-amino-2-deoxi-D-glycopyranose and 2-acetamide-2-desoxi-D-glycopyranose units connected by -1,4 glycosidic bonds2[8]. One of major chitosan source is giant prawn (Macrobrachium rosenbergii) shells2[9]. Giant prawn is widely cultured in tropical countries such as Indonesia, Vietnam, Thailand, China, and India. It belongs to superior commodity with high amount production due to the high market demand[30].

Chitosan is a potential anti-biofilm substance due to its positive charged amine and hydroxyl groups that can bind to the negative charged bacterial cell walls[28,31,32]. Some previous studies revealed that chitosan was able to prevent biofilm formation[31,33]. Other study by Chavez de Paz et al. demonstrated the potential biofilm degradation action by low molecular weight chitosan[34]. Smaller chitosan particle size was expected to increase its penetration ability into biofilm matrix and degrade the biofilm structure[34]. The purpose of this study was to determine the effect of GPSN on the S. mutans biofilm degradation.

Materials and methods

Extraction of Chitosan

Chitosan were extracted from giant prawn (Macrobrachium rosenbergii) shells through deproteination, demineralization, depigmentation, and deacetylation processes[35]. In short, giant prawn shells were washed and dried in an oven at 45°C for 3 days, then pulverized. Deproteination process was conducted by immersing the powder in 4% NaOH (Merck, Germany) solution with a ratio 1:10 (w/v), heated at 80°C and stirred for 60 minutes. The powder was washed, dried for 24-hours, then soaked in 1M HCl (Merck, Germany) with ratio 1:15 (w/v) for 3 hours for demineralization. For depigmentation process, the demineralized powder was immersed in 4% NaOCl solution. The deacetylation process was done by mixing the powder in 50% NaOH with ratio 1:15 (w/v) at 100°C and stirred for 3 hours. The deacetylation process was repeated three times and then dried for 24-hours to get chitosan isolates.

Synthesis of Giant Prawn Shell Nano-chitosan

Giant prawn shell nano-chitosan synthesis was carried out by the ionic gelation method[35]. In brief, chitosan was dissolved in 1% acetic, and then dropwisely added with Na-TPP (Xilong AR, China) until it reached a ratio of 2:1 and stirred with a magnetic stirrer at 1000 rpm. The solution was sonicated for 40 minutes, dried using a spray dryer, and redissolved in 1 M acetic acid (Merck, Germany) to obtain 2% GPSN suspension.

Bacteria Preparation

Streptococcus mutans ATCC 25175 was obtained from the Integrated Research Laboratory of the Faculty of Dentistry, Universitas Gadjah Mada. An overnight culture of S. mutans was suspended in Phosphate Buffered Saline (PBS) and adjusted to a turbidity of 0.5 Mc

Farland (1.5×10^8 CFU/mL).

Biofilm Degradation Assay

To induce biofilm formation, 10 µl S. mutans was incubated in 90 µl Brain Heart Infusion (BHI) broth containing 2% sucrose media for 24 and 48-h in 96-well microplates. After the biofilm had been formed, 100 µl nano-chitosan with concentrations of 0.5%, 0.25%, and 0.125% were added to the wells (to get final concentration of 0.25%, 0.125%, and 0.0625%) as well as chlorhexidine (positive control) and PBS (negative control). The plates were then incubated for 24-h at 37°C anaerobically. After the incubation period, the plates were washed using PBS, then the remaining biofilms were stained with 0.1% crystal violet. The optical density was read with a spectrophotometer (λ=540 nm) and the degradation of the biofilm (%) was calculated using formula:

$$\%\ degradation = \left[1 - \left(\frac{OD\ sample - OD\ blank\ sample}{OD\ control - OD\ blank\ control}\right)\right] x\ 100\%$$

Biofilm Viability Staining

Streptococcus mutans and BHI containing 2% sucrose media were filled in standard 24-microwell plates with sterilized glass coverslips, and then incubated anaerobically for 24-h and 48-h at 37°C. After the incubation period, varying concentrations of GPSN (the final concentrations of GPSN were 0.25%, 0.125%, and 0.0625%), as well as PBS and Chx (controls) were added. The plates were incubated anaerobically for 24-h at 37°C. The coverslips were then washed with 1.5 M PBS, stained with LIVE/DEAD BacLight Bacterial Viability Kit (Thermofisher, USA) according to the manufacturer instruction, and then observed with CLSM (Zeiss-Leica, Germany). When incubated with the SYTO 9 and propidium iodide nucleic acid stains included in the kit, live bacteria with intact cell membranes will appear green and dead bacteria with damaged membranes will appear red. To obtain the number of live and dead cells, the CLSM results were further quantified using Image-J software.

Statistical Analysis

All assays were repeated five times. All the quantitative data were statistically analyzed with One Way ANOVA and continued with the Post Hoc LSD test using SPSS software.

Results

Biofilm Degradation

The results of the biofilm degradation assays showed that GPSN degraded 24-h and 48-h *S. mutans* biofilm. The ability of GPSN to degrade biofilm was increased with an increase of the GPSN concentration; however, the degradation effect of GPSN towards 48-h biofilm was less than 24-h biofilm (Figure 1). Experimental data of biofilm degradation percentages were 45.10% ± 2.5, 71.55% ± 4.66, and 82.36% ± 4.23 when the 24-h biofilm were exposed to 0.0625%, 0.125%, and 0.25% GPSN, subsequently; while biofilm degradation percentage of Chx (positive control) was 64.88% ± 5.30.

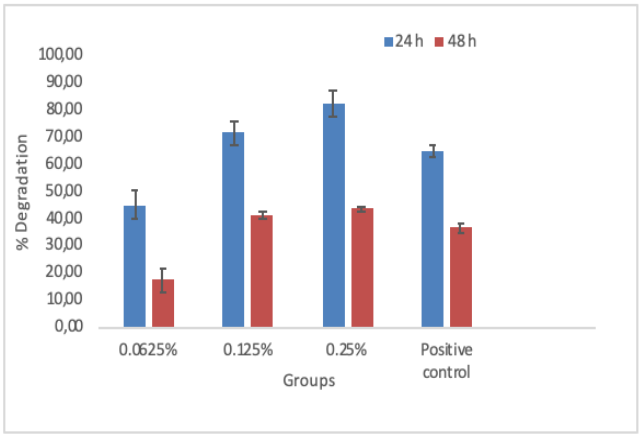

Figure 1. Biofilm degradation effects of GPSN. The higher concentration of GPSN showed a greater degradation effect ($p<0.05$). The degradation effect of GPSN towards 48-h biofilm was less than 24-h biofilm.

Experimental data of biofilm degradation percentages were 17.68% ± 1.74, 41.26% ± 1.03, and 43.76% ± 1.27 when the 48-h biofilm were exposed to 0.0625%, 0.125%, and 0.25% GPSN, subsequently; while biofilm degradation percentage of Chx (positive control) was 36.61% ± 4.36.

One-Way ANOVA statistical tests showed that there were significant differences among groups, indicating that GPSN affected 24-h and 48-h *S. mutans* biofilm degradation ($p<0.05$). Post-hoc LSD test showed that there were significantly differences between 0.25% GPSN and the other test groups on 24 and 48-h biofilms ($p<0.05$), indicating that 0.25% GPSN was the most effective concentration in degrading 24-h as

well as 48-h biofilm compared to the other test groups, including Chx.

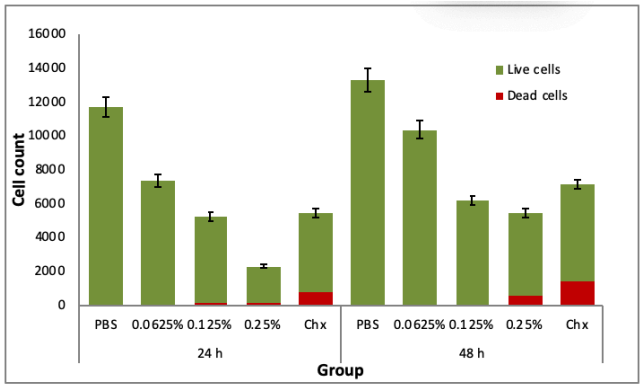

Figure 2. The number of 24-h and 48-h *S. mutans* cell biofilm after treatment with GPSN. The number of live cells is higher in 48-h biofilm than 24-h biofilm. The number of live cells becomes less as the concentration of GPSN increases ($p<0.05$). The number of dead cells increases as the concentration of GPSN increases ($p<0.05$).

Biofilm Viability Staining

Figure 2 shows that the number of the bacteria in the 48-h biofilm was more than 24-h biofilm. The result of the study demonstrated that the number of the bacteria in the 24-h and 48-h *S. mutans* biofilms became significantly less after treatment with varying concentration of GPSN ($p<0.05$), indicating that GPSN degraded 24-h and 48-h *S. mutans* biofilms (Figure 3). The ability of GPSN to degrade biofilm was increased significantly as the concentration increased ($p<0.05$). The degradation effect of GPSN towards 48-h biofilm was less than 24-h biofilm.

One-Way ANOVA statistical tests showed that there were significantly differences of the number of remaining cells among groups, indicating that GPSN affected the cell number of 24-h and 48-h *S. mutans* biofilms ($p<0.05$). Post-hoc LSD test showed that there were significant differences in the 24-h biofilm cell number between 0.25% GPSN and the other GPSN concentration groups ($p<0.05$) indicating that 0.25% GPSN was the most effective concentration in reducing the 24-h biofilm cells ($p<0.05$). There was significant difference in the 24-h biofilm cell number between 0.25% GPSN and Chx (positive control) ($p<0.05$), although no significant difference was found between 0.25% GPSN and Chx on 48-h biofilms ($p>0.05$).

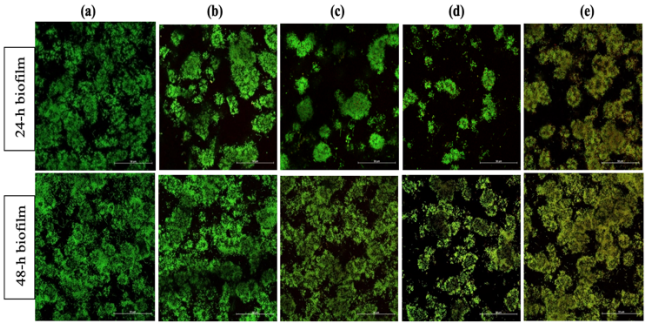

Figure 3. Representative CLSM images of Live/Dead (SYTO-9 and PI) stained of 24-h and 48-h *S. mutans* biofilms after treatment with varying concentration of GPSN (40X magnification): (a) negative control; (b) 0.0625% GPSN; (c) 0.125% GPSN; (d) 0.25% GPSN; (e) positive control. The 48-h biofilm is denser than 24-h biofilm. The biofilm masses decrease as the GPSN concentration increase. The degradation effect of GPSN towards 48-h biofilm is less than 24-h biofilm.

In addition, One-Way ANOVA statistical tests showed that there were significantly differences of the number of dead cells among groups, indicating that GPSN affected the cell number of dead cells in 24-h and 48-h *S. mutans* biofilms ($p<0.05$). The number of dead cells was increased as the concentration of GPSN increased. Post-hoc LSD test showed not significantly difference of the number of dead cells between 0.25% GPSN and Chx on 24-h and 48-h biofilms ($p>0.05$). These results indicated that 0.25% GPSN exhibited greater degradation effect toward 24-h and 48-h biofilms compared to the other GPSN concentrations and worked effectively as Chx to eliminate cells from the formed biofilms.

Discussion

Streptococcus mutans has been accepted as the primary causative agent of dental caries, an infectious and transmissible bacterial disease. As a result of various growing conditions, including carbohydrate source, pH, biofilm presence, and starvation, this bacteria's cariogenicity differs[36]. It is commonly acknowledged that S. mutans has high capacity to withstand low pH (aciduricity), which is crucial for its cariogenicity[37]. Biofilm has important effects on the aciduricity of the bacteria due to the complex nature of the oral environment.

Compared to planktonic bacteria, bacteria that are developing in oral biofilms are more resistant to various antimicrobial treatments[38]. Several studies have revealed that S. mutans in biofilms exhibited higher tolerance for antimicrobial treatments and increased adaptability to challenging environments. Therefore, biofilm is strongly related to the pathogenesis of dental caries. Biofilm formation begins with the attachment of the pioneer bacteria, continued by coaggregation, maturation, and detachment stages[12]. The maturation stage occurs at 24-48-hours and is characterized by an increase in bacterial colony number and the formation of an extracellular matrix that protects the biofilm structure[9,13]

Our findings proved that GPSN had ability in degrading 24 and 48-h S. mutans biofilms. The result of this study was in accordance with the previous studies that chitosan nanoparticles can degrades the biofilm structure[20,34,39,40]. In addition, the finding support our previous report that GPSN inhibited dual species (S. mutans and S. sanguinis) biofilm formation[35].

Chitosan is a natural polysaccharide that can be obtained from the exoskeleton of crustaceans, mollusks, and fungi through demineralization, deproteination, decoloration, and deacetylation processes[41]. Nano-chitosan is a chitosan derivative product with a size of 10-1,000 nm produced by the ionic gelation method[42]. The ability of nano-chitosan to degrade biofilms is influenced by its particle size, molecular weight, and physicochemical properties[20,34,43]. Degradation of biofilm structure by GPSN presumably occurred through the mechanism of destruction of the EPS matrix and inhibition of the quorum sensing system. GPSN that obtained in this study has an average molecular size of 432.9 nm, deacetylation degree (DD) of 77.61%, and classified as a low molecular weight nano-chitosan. It also has polydispersity index (PdI) of 0.288 and zeta potential value +51.2 mV (unpublished data). The EPS matrix has a negative charge so they interact more easily with positively charged GPSN[44]. This interaction results in changes in the permeability of the extracellular matrix so that GPSN molecules more easily diffuse through the biofilm matrix. Small and homogenous particle size, high zeta potential value, and regular particle shape can increase the diffusion ability of GPSN particles into the biofilm matrix through biofilm pores and water channels[20,40,45]. GPSN that has entered the biofilm structure will then interact with the bacterial cells that make up the biofilm and cause bacterial cell death[43]. GPSN is also thought to be able to inhibit the synthesis of molecules that play a role in the quorum-sensing system[46]. As a result, the integrity and structure of the biofilm were damaged.

In this study, we evaluated the antibiofilm potency of GPSN at mature phase of the biofilm. The result of the study showed that compared to a 24-hour biofilm, a 48-h biofilm was denser with a significantly larger number of viable bacteria. The ability of GPSN to degrade 48-h biofilm was less compared to the 24-h biofilm. It was suggested that mature biofilms might be more likely to resist biofilm disintegration and provide stronger cell protection under stressful environments, which was consistent with earlier studies' findings that compared to their younger counterparts, biofilm cells were more resistant to environmental stress and antimicrobial treatments. A study by Shen et al. revealed that compared to immature biofilms, bacteria in mature and nutrient-limited biofilms are more resistant to Chx[47]. One of the possible explanations for this finding that mature biofilm is harder for GPSN to penetrate than immature biofilm is because it is denser, more compact, and more complicated.

Moreover, as the biofilm mature, the inhabitants of the biofilm start to develop EPS, an adhesive matrix that allowing the cells to adhere to one another and form a layered biofilm. EPS is considered the main component of a biofilm, which constitute between 50% and 90% of the total organic matter in biofilms[48]. The main components of the matrix are exopolysaccharide, protein, and DNA. Theoretically, the increment of the biofilm matrix (e.g., EPS), which serves as a physical or chemical diffusion barrier, may prevent antibacterial agents from penetrating the biofilm during biofilm formation. Previous study by Bowen et al.[49] revealed that the volume of EPS matrix in mature biofilms was higher than that in younger biofilms, indicating that EPS production continued as biofilms develop. As the biofilm matures, EPS increased an impact on providing protection of the biofilm by limiting the antibacterial drugs penetration and triggering antimicrobial tolerance. In addition, the mature biofilms had higher cohesive forces at the cell-cell interface than did the younger biofilms[49].

Thus, the presence of EPS in mature biofilms is thought to reduce the effectiveness of GPSN in degrading biofilms.

Taken together, this study results showed potential activity of GPSN as an anti-biofilm agent due to its ability to degrade S. mutans biofilm in vitro. These findings suggested the further potential development of GPSN as an anti-caries ingredient in toothpaste, mouthwashes, or restorative materials.

Conclusions

It is concluded that GPSN degrades *S. mutans* biofilm. The most effective concentration of GPSN in degrading the biofilm is 0.25% GPSN.

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This study obtained ethical approval from the Ethics Committee of the Faculty of Dentistry, Universitas Gadjah Mada. (protocol number: 00797/KKEP/FKG-UGM/EC/2021).

Declaration of Interest

The authors report no conflict of interest.

References

1. Marsh PD. Dental plaque: Biological significance of a biofilm and community life-style. In: Journal of Clinical Periodontology. 2005;32 Suppl 6:7-15.
2. Hojo K, Nagaoka S, Ohshima T, Maeda N. Critical Review in Oral Biology & Medicine: Bacterial Interactions in Dental Biofilm Development. J Dent Res. 2009;88(11):982-990.
3. Dewhirst FE, Chen T, Izard J, et al. The human oral microbiome. J Bacteriol. 2010;192(19):5002-5017.
4. Xin X, Yuan Z, Wenyuan S, Yaling L, Xuedong Z. Biofilm and Dental Caries. In: Dental Caries: Principles and Management.; Berlin: Springer Verlag; 2016:27-58.
5. Saini R, Saini S, Sharma S. Biofilm: A dental microbial infection. J Nat Sci Biol Med. 2011;2(1):71-75.
6. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence. 2011;2(5):435-444.
7. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. Nat Rev Microbiol. 2018;16(12):745-759.
8. Larsen T, Fiehn NE. Dental biofilm infections – an update. APMIS. 2017;125(4):376-384.
9. Marsh PD. Dental Biofilms in Health and Disease. In: Understanding Dental Caries: From Pathogenesis to Prevention and Therapy.; Switzerland: Spinger; 2016: 41-52.
10. Fejerskov O, Nyvad B, Kidd EAM. Dental caries: what is it? In: Dental Caries : The Disease and Its Clinical Management. ; Oxford: Blackwell Munksgaard; 2015:282-382.
11. Oh DH, Chen X, Daliri EBM, Kim N, Kim JR, Yoo D. Microbial etiology and prevention of dental caries: Exploiting natural products to inhibit cariogenic biofilms. Pathogens. 2020;9(7):1-15.
12. Marsh PD. Dental plaque as a microbial biofilm. In: Caries Research. 2004;38(3):204-211.
13. Krzysciak W, Jurczak A, Piątkowski J. The Role of Human Oral Microbiome in Dental Biofilm Formation. In: Microbial Biofilms - Importance and Applications. ; InTech; 2016:329-382.
14. Jose AL, Hui W, Robert GQ, Hyun K. Pathogenic Mechanisms in Dental Caries. In: Oral Microbiology and Immunology. Vol 9.; Washington DC: ASM Press; 2020:267-285.
15. Soraya C, Syafriza D, Gani BA. Antibacterial Effect of Moringa Oleifera Gel to Prevent the Growth, Biofilm Formation, and Cytotoxicity of Streptococcus Mutans. J Int Dent Med Res. 2022;15(3):1053-1061.
16. Krzyściak W, Jurczak A, Kościelniak D, Bystrowska B, Skalniak A. The virulence of Streptococcus mutans and the ability to form biofilms. Eur J Clin Microbiol Infect Dis. 2014;33(4):499-515.
17. Gill J. Dental Caries: The Disease and its Clinical Management, Third Edition. Br Dent J. 2016;221(8):443-443.
18. Takenaka S, Sotozono M, Ohkura N, Noiri Y. Evidence on the Use of Mouthwash for the Control of Supragingival Biofilm and Its Potential Adverse Effects. Antibiotics. 2022;11(6):727.
19. Van Strydonck DAC, Slot DE, Van Der Velden U, Van Der Weijden F. Effect of a chlorhexidine mouthrinse on plaque, gingival inflammation and staining in gingivitis patients: A systematic review. J Clin Periodontol. 2012;39(11):1042-1055.
20. Ikono R, Vibriani A, Wibowo I, et al. Nanochitosan antimicrobial activity against Streptococcus mutans and Candida albicans dual-species biofilms. BMC Res Notes. 2019;12(1):383.
21. Langa GPJ, Muniz FWMG, Costa R dos SA, da Silveira TM, Rösing CK. The effect of cetylpyridinium chloride mouthrinse as adjunct to toothbrushing compared to placebo on interproximal plaque and gingival inflammation—a systematic review with meta-analyses. Clin Oral Investig. 2021;25(2):745-757.
22. Escribano M, Figuero E, Martín C, et al. Efficacy of adjunctive anti-plaque chemical agents: a systematic review and network meta-analyses of the Turesky modification of the Quigley and Hein plaque index. J Clin Periodontol. 2016;43(12):1059-1073.
23. Takenaka S, Trivedi HM, Corbin A, Pitts B, Stewart PS. Direct visualization of spatial and temporal patterns of antimicrobial action within model oral biofilms. Appl Environ Microbiol. 2008;74(6):1869-1875.
24. Takenaka S, Ohshima H, Ohsumi T, Okiji T. Current and future strategies for the control of mature oral biofilms-Shift from a bacteria-targeting to a matrix-targeting approach. J Oral Biosci. 2012;54(4):173-179.
25. Corbin A, Pitts B, Parker A, Stewart PS. Antimicrobial penetration and efficacy in an in vitro oral biofilm model. Antimicrob Agents Chemother. 2011;55(7):3338-3344.
26. Wakamatsu R, Takenaka S, Ohsumi T, Terao Y, Ohshima H, Okiji T. Penetration kinetics of four mouthrinses into Streptococcus mutans biofilms analyzed by direct time-lapse visualization. Clin Oral Investig. 2014;18(2):625-634.
27. Tartaglia GM, Tadakamadla SK, Connelly ST, Sforza C, Martín C. Adverse events associated with home use of mouthrinses: a systematic review. Ther Adv Drug Saf. 2019;10:1-16.
28. Montenegro Stamford TC, Montenegro T, Medeiros Cavalcante HM de, Oliveira R, de Campos-Takaki GM. Microbiological Chitosan: Potential Application as Anticariogenic Agent. In: Practical Applications in Biomedical Engineering. ; InTech Open; 2013:229-244.
29. Younes I, Rinaudo M. Chitin and chitosan preparation from marine sources. Structure, properties and applications. Mar Drugs. 2015;13(3):1133-1174.
30. Pillai BR, Ponzoni RW, Das Mahapatra K, Panda D. Genetic improvement of giant freshwater prawn Macrobrachium rosenbergii: A review of global status. Rev Aquac. 2022;14(3):1285-1299.

31. Costa EM, Silva S, Tavaria FK, Pintado MM. Study of the effects of chitosan upon Streptococcus mutans adherence and biofilm formation. Anaerobe. 2013;20:27-31.
32. Husain S, Al-Samadani KH, Najeeb S, et al. Chitosan biomaterials for current and potential dental applications. Materials (Basel). 2017;10(6):602.
33. Aliasghari A, Khorasgani MR, Vaezifar S, Rahimi F, Younesi H, Khoroushi M. Evaluation of antibacterial efficiency of chitosan and chitosan nanoparticles on cariogenic streptococci: An in vitro study. Iran J Microbiol. 2016;8(2):93-100.
34. de Paz LEC, Resin A, Howard KA, Sutherland DS, Wejse PL. Antimicrobial effect of chitosan nanoparticles on Streptococcus mutans biofilms. Appl Environ Microbiol. 2011;77(11):3892-3895.
35. Haniastuti T, Puspasari TA, Hakim ER, Tandelilin RTC. Potential Effect of Giant Freshwater Prawn Shell Nano Chitosan in Inhibiting the Development of Streptococcus mutans and Streptococcus sanguinis Biofilm in Vitro. Int J Dent. 2023;2023(2):1-9.
36. Jiang S, Chen S, Zhang C, Zhao X, Huang X, Cai Z. Effect of the biofilm age and starvation on acid tolerance of biofilm formed by Streptococcus mutans isolated from caries-active and caries-free adults. Int J Mol Sci. 2017;18(4):713.
37. Liu S, Tao Y, Yu L, et al. Analysis of small RNAs in streptococcus mutans under acid stress—A new insight for caries research. Int J Mol Sci. 2016;17(9):1529.
38. Jiao Y, Tay FR, Niu L na, Chen J hua. Advancing antimicrobial strategies for managing oral biofilm infections. Int J Oral Sci. 2019;11(3):28.
39. Neilands J, Sutherland D, Resin A, Wejse PL, Chávez De Paz LE. Chitosan nanoparticles affect the acid tolerance response in adhered cells of Streptococcus mutans. Caries Res. 2011;45(6):501-505.
40. Tan Y, Ma S, Leonhard M, et al. Enhancing antibiofilm activity with functional chitosan nanoparticles targeting biofilm cells and biofilm matrix. Carbohydr Polym. 2018;200:35-42.
41. Costa EM, Silva S, Vicente S, et al. Chitosan nanoparticles as alternative anti-staphylococci agents: Bactericidal, antibiofilm and antiadhesive effects. Mater Sci Eng C. 2017;79:221-226.
42. Divya K, Vijayan S, George TK, Jisha MS. Antimicrobial properties of chitosan nanoparticles: Mode of action and factors affecting activity. Fibers Polym. 2017;18(2):221-230.
43. Kawakita ERH, Ré ACS, Peixoto MPG, et al. Effect of chitosan dispersion and microparticles on older streptococcus mutans biofilms. Molecules. 2019;24(9):1808.
44. Fulaz S, Vitale S, Quinn L, Casey E. Nanoparticle–Biofilm Interactions: The Role of the EPS Matrix. Trends Microbiol. 2019;27(11):915-926.
45. Khan F, Pham DTN, Oloketuyi SF, Manivasagan P, Oh J, Kim YM. Chitosan and their derivatives: Antibiofilm drugs against pathogenic bacteria. Colloids Surfaces B Biointerfaces. 2020;185:110627.
46. Badawy MSEM, Riad OKM, Taher FA, Zaki SA. Chitosan and chitosan-zinc oxide nanocomposite inhibit expression of LasI and RhlI genes and quorum sensing dependent virulence factors of Pseudomonas aeruginosa. Int J Biol Macromol. 2020;149:1109-1117.
47. Shen Y, Stojicic S, Haapasalo M. Antimicrobial efficacy of chlorhexidine against bacteria in biofilms at different stages of development. J Endod. 2011;37(5):657-661.
48. Jiang Q, Jing Q, Ren B, et al. Culture Supernatant of Enterococcus faecalis Promotes the Hyphal Morphogenesis and Biofilm Formation of Candida albicans. Pathogens. 2022;11(10):1177.
49. Bowen WH, Burne RA, Wu H, Koo H. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. Trends Microbiol. 2018;26(3):229-242.