ISSN No:-2456-2165

In-Vitro Antimicrobial Activity of Nano-Chitosan Hydroxyapatite against *Streptococcus Mutans* on Biofilm Formation

Trimurni Abidin[1], Fitri Yunita Batubara[1]
[1] Department of Conservative Dentistry,
Faculty of Dentistry, Universitas Sumatera Utara,
Medan, Sumatera Utara,
Indonesia

Harry Agusnar[2],
[2]Department of Chemistry,
Faculty of Natural and Mathematical Sciences,
Universitas Sumatera Utara, Medan,
Sumatera Utara, Indonesia

Basri A. Gani[3]
[3] Department of Oral Biology,
Faculty of Dentistry, Universitas Syiah Kuala,
Darussalam, Banda Aceh,
Aceh, Indonesia

Abstract:- ***Streptococcus mutans*** **are oral bacteria that have the most role in developing dental caries.** ***S. mutans*****can form colonies and biofilms through adhesion mechanisms on the tooth surface. The use of chitosan as an antimicrobial material has been widely developed in dentistry, especially on the nanoscale, because nanomaterials can spread quickly throughout the biofilm structure. The use of hydroxyapatite (HAP) in conservative dentistry has been developed to protect teeth from** ***S. mutans*****. This research aims to see the ability of nano chitosan hydroxyapatite (KNHP), if combined in gel form, does it prevent** ***S. mutans*****from forming biofilms on the enamel surface. The research material was** ***S. mutans*****serotype c and saliva collected from a volunteer. The biofilm mass profile was determined on the surfaces of teeth implanted on acrylic resin and incubated for 24, 48, and 72 hours, then observed under a counter colony lens. Determination of** ***S. mutans*** **CFU after interacting with nano chitosan hydroxyapatite for 24, 48, and 72 was then examined with spectrophotometry at a wavelength of 610 nm OD value of 0.08-0.1, which is equivalent to 0.5 McFarlan (1.5x 108). Then the determination of the interaction power of KNHP and HAP with** ***S. mutans*****was done by UV / VIS with a wavelength of 250-600 nm. The results showed that the mass profile of biofilms at 24 hours still showed colony growth after being given KNHP and HAP, whereas, at incubation times at 48 and 72 hours, there was no** ***S. mutans*** **growth. The interaction between KNHP and** ***S. mutans*** **with UV-VIS at 24, 48, and 72 hours incubation time has higher interaction power than HAP.**

Keywords:- *Biofilm, Nano chitosan hydroxyapatite, Streptococcus mutans.*

I. INTRODUCTION

The occurrence of caries is always associated with biofilms, the majority of which are *Streptococcus mutans*is a key in developing the caries process. The pathogenesis of caries begins when there is a process of attachment of *S. mutans*in salivary protein on a tooth's surface and forms a biofilm. *S. mutans* colonization in the formed biofilm layer is an initial process that induces caries(Lemos et al., 2019). Molecular pathogenesis of *S. mutans* so that it can cause dental caries consists of several stages namely the first stage, bacteria, requires a hard surface of the teeth to colonize and accumulate. In the second stage, adhesion is on the tooth's surface, preceded by the protein's interaction with lectin in the dental pellicle, which covers the tooth surface(Kozmos et al., 2021). The mineral hydroxyapatite in enamel is the third stage, soluble in lactic acid produced by bacterial metabolism. The concentration of damaging acids requires the accumulation of *S. mutans*, which are acidogenic. The accumulation process begins with the activity of glucosyltransferase (Gtf) and the help of glucan binding protein (Gbp)(Pribadi et al., 2017).

*S. mutans*serotype c bacterial strains play a significant role in the pathogenesis of teeth in humans because these bacteria are found in populations with high, low, and very low caries prevalence. This microorganism is a normal flora that triggers tooth demineralization, which results in dental caries(Krzyściak et al., 2014). Like other Gram-positive bacteria, *S. mutans* cells consist of cell walls, protoplasmic membranes, and cell wall matrices consisting of peptidoglycan. The most essential cell wall surface antigens are proteins, including glucosyltransferase enzymes that convert sucrose into dextran /glucans (glucose polymers) and interact with receptors in the pellicle(Krzyściak et al., 2014). The fucosyltransferase enzyme converts fructose into fructans, antigens I, II, I / II, and antigen II. Lipoteikoat acid (LTA) originates from the protoplasmic membrane but penetrates the cell wall and interacts with the pellicles (Zafar et al., 2021).

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Caries prevention can be done by intervening in S. mutans and tooth surface interaction. Several antimicrobial materials have been produced to prevent the colonization and growth of certain organisms in the biofilm community, including fluorine and chlorhexidine(Chen et al., 2020). Research conducted on groups of low caries sufferers, both fluorine and chlorhexidine, tended to show no significant difference in the caries index between groups who received therapy and those who did not receive treatment. This phenomenon explains that the caries process is multifactorial, which not only involves *S. mutans* but is also influenced by other factors such as plaque accumulation, carbohydrate and dietary acid patterns, and saliva(Schaeken et al., 1991).

The use of natural substances in Dentistry is widely used today. Chitosan, which is a polymer, is one of the materials that has been developed in the field of dentistry. Chitosan is a poly- (2-amino-2-deoxy- (1-4) -D-glucopyranose with molecular formula (C6H11NO4)n, which can be obtained by deacetylation of chitin(Fakhri et al., 2020). Chitosan has biocompatible, biodegradable, non-toxic and chelator properties. It can bind to bacterial cell walls and membranes because of its positive ions to provide bacteriostatic and bactericidal effects(Ahmad et al., 2019). Chitosan exposure to bacteria will affect the inhibition of bacterial growth over a long period. The activity of mouthwash containing chitosan 0.1% and 0.5% could significantly reduce plaque due to electrostatic interactions between the surface of bacterial cells in saliva and the surface of the pellicle on the teeth, where positive ion bonds are formed from chitosan and negative ions from bacteria that will form bridge bonds so that bacteria cannot form colonies on the surface of the tooth(Aranaz et al., 2018).

Caries process that begins with the demineralization of the enamel surface, it is necessary to consider anticaries material not only antibacterial but also needed materials that can condition the surface of the enamel resistant to acids from bacteria that are in the plaque so that it is not easy to dissolve the hydroxyapatite crystals due to the presence of these acids(Dai et al., 2019). Several studies have been carried out to determine the effect of toothpaste containing nano-hydroxyapatite on initial lesions in enamel under dynamic pH-cycling conditions. The results of these studies show that nano-hydroxyapatite can remineralize early enamel lesions(Juntavee et al., 2021). Nano-HA concentration of 10% is the optimal concentration for remineralization of enamel caries. The addition of nano-HA to Sports drinks can increase demineralization resistance and cause remineralization in early enamel lesions (Min et al., 2015).

This study aimed to see if hydroxyapatite was added to nano chitosan in the form of nano-chitosan hydroxyapatite gel can prevent the attachment of *S. mutans* to the tooth surface. Biomaterials produced are expected to provide the best solution for the community in preventing dental caries, which is currently a dental health problem in Indonesia and throughout the world. Besides that, it can increase dental protection efforts against the occurrence of caries through non-invasive care in support of care with a minimal intervention approach.

II. MATERIAL AND METHODS

The research obtained the Ethical Clearance No. 644 / TGL / KEPK FK USU-RSUP HAM/2019, Medical Faculty, Universitas Sumatera Utara, Medan Indonesia. As much 20 premolar teeth were obtained from the Oral Surgery clinic at USU Hospital. Teeth were free from caries, and there was no restoration and cleaned of plaque, calculus, and then separated corona and roots at the cementoenamel junction boundary. Dental crowns were planted in acrylic with the buccal surface facing up. Then the teeth were polished with 400-1000 grit sandpaper disks to get a flat surface. After that, the teeth were put in an ultrasonic bath for 30 minutes to get a clean sample, and the peripheral surface was given a nail varnish, then sterilized into the autoclave for 2 hours, and the sample was ready for use.

A. Chitosan Preparation

High molecular chitosan powder was obtained from the Center of Excellence Chitosan and Advance Material USU, mashed with a ball mill, then filtered. Nano-chitosan gel is made by dissolving 1 gram of chitosan in 50 ml of a weak acid solution (1% acetic acid), then distilled with a Jar test at 200 rpm for ± 30 minutes to obtain the gel. Then the chitosan solution was dropped with 20 drops of tripolyphosphate (TPP) solution while stirring. Chitosan solution mixture with TPP has stirred again with Jar Test for 30 minutes. The addition of TPP was done to smooth the solution's surface. The solution which had formed the gel became nano with a size of 180 nm. The resulting residue in the form of nano-chitosan gel was added to hydroxyapatite.

Hydroxyapatite was obtained from pearl shells (Nacre) sold commercially. Two grams of hydroxyapatite were added with 100 mL of aquadest and then stirred until homogeneous. Preparation of nano-chitosan hydroxyapatite was prepared by adding 25 mL of nano-chitosan solution with 25 mL of 2% hydroxyapatite solution slowly while stirring until homogeneous. To achieve a neutral solution, 0.05 M NaOH was added until the pH reached 7, seen with Universal pH(Szatkowski et al., 2015).

B. Saliva Preparation

The saliva of 50 mL was collected from a healthy volunteer using the spit-out method after chewing 1 g of paraffin wax for 5 min. Saliva collection was done before breakfast between 6.00 and 8.00 o'clock. An Adsorption Buffer Solution was prepared as much as 50 mMKCl; 1 mM potassium phosphate (0.35 mM K2HPO4 plus 0.65 mM KH2PO4); 1mM CaCl2; 0.1 mM MgCl2. The pH is equal to 6.5. The solution was stored at room temperature. Making saliva (clarified saliva) was done by mixing saliva with an adsorption buffer solution (ratio 1: 1, v / v) and added with 50 µl 0.1 M phenylmethyl-sulfonyl fluoride (PMSF). The solution mixture was centrifuged at 5,500g; 4º for 10 mins. Supranatan was taken and filtered with a 0.22 µm PES low protein-binding filter.

C. Culture of Streptococcus mutans

Samples of *S. mutans* ATCC 25175n were obtained from the Faculty of Dentistry, University of Syiah Kuala Banda Aceh, Indonesia. The *S. mutans* were cultured on the selective media of TYS20B for 72 hours at 37 ºC in an anaerobic jar furthermore incubated in media of TSB (Trypticase Soy Broth). Culture on this liquid media is kept in an anaerobic jar in which the gas pack was performed, then incubated for 72 h at 37ºC.

D. Biofilm Assay

Teeth with acrylic were soaked in saliva for 24 h and then stirred for 15 min at 1000x. Furthermore, a suspension of *S. mutans* (1.5x108) 1: 5 was added to the test material (chitosan nanoparticles and hydroxyapatite). Moreover, it was incubated for 24 h, 48 h, and 72 h. Each sample was then prepared to isolate the biofilm from the teeth with acrylic resin. Every 5 mL of 70% ethanol was added to the sample container, then stirred for 15 minutes at 2000x. The biofilm suspension is then put into a test tube and centrifuge for 20 min at 3000x. Then the supernatant is separated from the residue. Each residue was added with 100 µl methionine iodine, centrifuged again at 300 rpm for 50 mins, then incubated at room temperature for five h. In the last stage, 1% of each violet was given (1:10), and centrifuged again for 5 minutes at 300x. Subsequently, the supernatant taken 150 µl was put into 96 well plates (triple) to read the quantity of *S. mutans* biofilm due to the influence of chitosan nanoparticles and hydroxyapatite at a wavelength of 590 nm.

Biofilm mass profile determination was carried out on teeth embedded in acrylic resin. Samples incubated for 24 h, 48 h, and 73 h were then soaked in saline for 15 minutes. Then the tooth sample was given 1% violet crystal and soaked again for 24 hours. Then each sample is added with 1% glycerol every 2 mL in a sample container. Furthermore, it was incubated for 24 h. Then proceed with biofilm mass image capture on the surface of the teeth and acrylic resin. Biofilm mass profile after interacting with nanoparticle chitosan and hydroxyapatite was observed under a counter colony lens, where each sample was given emersil oil to clarify the biofilm mass(Gani et al., 2017).

E. Growth Assessment of S. mutans

Determination of *S. mutans* growth used by spectrometry is evaluated in 24 hours, 48 hours, and 72 hours then mixed for 30 min at 2000 rpm. Furthermore, each solution from the results of the immersion sample was centrifuged at 3000 rpm for 15 minutes. The residue from the solution was then separated and resuspended to reduce turbidity as an indicator of the growth of *S. mutans* colonies. Each of the 150 µL suspensions was put into 96 well plates. Then it was examined by spectrophotometry at a wavelength of 610 nm. An OD value of 0.08-0.1 was equivalent to 0.5 McFarlan ($1.5x10^8$)(Soraya et al., 2020).

F. UV-Vis Visual of S. mutans Inhibition

In this study, interaction power determination was done using the UV / VIS Spectrophotometry technique [13]. Determination of the interaction power of chitosan nanoparticle and hydroxyapatite test material was a continuation of CFU *S. mutans* assessment based on the turbidity of the solution. Before the sample suspension was examined, the cuvette was filled with the sample baseline solution encoded in the system as a control activity interaction between the test material and *S. mutans*. Then the sample spectrum was determined. Spectrum determination functions for scanning samples at a specific wavelength range (600-250 nm). Used to find out at what wavelength absorbance or% T is maximum. A cuvette containing a blank solution is inserted into the reference and sample sides in the first stage. Then click Baselinecorrection will take place according to a predetermined wavelength range. Then, click Start and enter the standard solution or sample to be measured to the side of the sample.

If there are any concerns, the scanning process will begin.If the curve does not appear scaled-down, click OK.Then, replace the filename with the name of the sample you want to print.To view the obtained spectrum's peak-to-peak report, click Operation.Simply click Peak Pick.Typically, the wavelength that produces the most excellent absorption value is assumed to be the maximum wavelength.It is frequently used as the wavelength for the quantitative measurement that follows.During this period, click Operation Peak Pick to determine the absorbance at a specific wavelength.The wavelength can be entered directly into the table and then converted to an absorbance value, or it can be selected by moving the cursor over the graph.To save the spectrum data, click File, choose Save As, type the File's name, and click Save.Finally, print the image by clicking on the peak or point pick table and then clicking print.

G. Statistical Analyses

A one-way ANOVA analysis was used to determine the formation of the *S. mutans* biofilm based on incubation time, followed by an Independent T-test on the test material. The Kruskal Wallis test was used to determine the growth rate of S. mutans as a function of incubation time.

III. RESULTS AND DISCUSSION

The study results are related to modeling dental caries by evaluating based on the pathogenesis of caries infection, including the formation of biofilms, growth of *S. mutans* colonies. Fig 1 shows the activity of *S. mutans* biofilm formation increased according to the incubation time ($p < 0.05$). Its means that time affects the intensity of biofilm formation. These results are used as a reference to measure the anti-biofilm power of chitosan nanoparticles and hydroxyapatite test materials. Figure 2 shows chitosan nanoparticles had a better ability to inhibit the formation of biofilms at incubation times of 24 hours and 48 hours. At 72 hours, hydroxyapatite had a better effect in inhibiting the formation of *S. mutans* biofilms even though both activities have a strong intensity in inhibiting the formation of *S.*

ISSN No:-2456-2165

mutans biofilms. At incubation t 24 hours and 48 hours, KNHP(Chitosan Nanoparticles Hydroxyapatite) had better anti-biofilm of *S. mutans*than HAP (Hydroxyapatite). Still, at the incubation time of 72 hours, HAP had a better anti-biofilm of *S. mutans.*The anti-biofilm of *S. mutans*after being treated by KNHP and HAP. They are a significant difference in the incubation time (Table 1), but the material assay did not show a substantial difference with the anti-biofilm of *S. mutans*(Fig2).

The biofilm mass of the tooth embedded in acrylic after being applied with KNHP and HAP showed that biofilm wasonly found in the periphery surface area of the tooth and also expanded to the acrylic, where the area that was coated with nail varnish did not contact with the test material (Fig 3). Fig 4 shows that the KNHP groups had a better effect on suppressing *S. mutants* growth thanat other incubation times. (0.08 nm = <50 CFU ($0.5x10^8$).

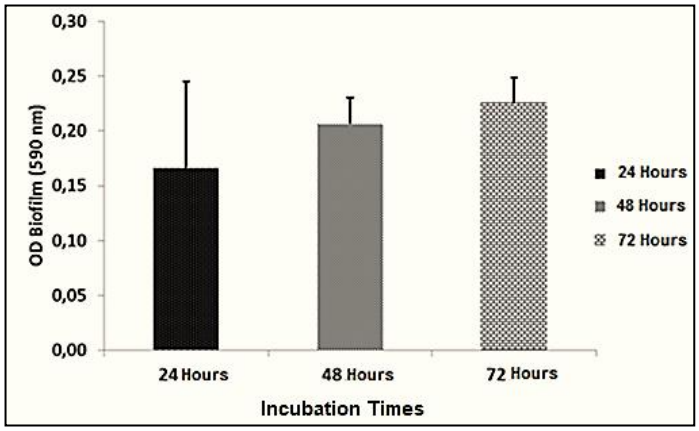


Fig 1:- Biofilm formation of *S. mutans* Biofilm. There was an increase in the formation of *S. mutans* biofilms based on incubation times of 24 hours, 48 hours, and 72 hours. Bar (OD Biofilm) and Bar error (Standard Deviation)

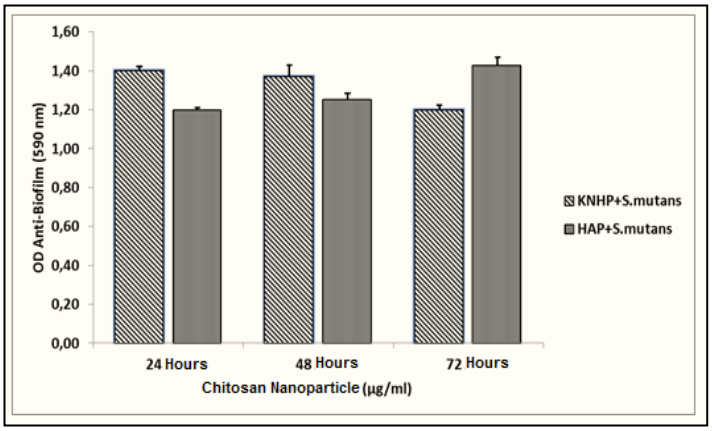


Fig 2:- Anti-biofilm formation. At 24 hours and 48 hours. KNHP (Chitosan Nano Particles) had better anti-forming properties of *S. mutans* biofilm than HAP (Hydroxyapatite). At the incubation time of 72 hours, HAP was higher in the biofilm's anti-formation properties than KNHP.

Analysis variables	Description of statistics					One Way Anova	T-test Independent
	N	Min	Max	Mean	S.Dev	AntiBiofilm Vs. Incubation Time	AntiBiofilm Vs. Material Test
Anti-Biofilm	18	0,18	0,30	0,21	0,03	p>0,05 (0,177) r=0,412	p>0,05 (0,55) r= -0,149
Incubation Times	18	1,00	3,00	2,00	0,84		
Test Material	18	1,00	2,00	1,50	0,51		

Table 1: Statistical analysis of the biofilms formation of *S. mutans*

Analysis variables	Description of statistics					Kruskal Wallis	T test Independent
	N	Min	Max	Mean	S.Dev	OD-CFU Vs Incubation Time	OD-CFU Vs Material Test
*OD-CFU	18	0,01	0,68	0,26	0,18	p>0,05 (0,623); r=0,218	p<0,05 (0,008); r= -0,613
Incubation Time	18	1,00	3,00	2,00	0,84		
Test Material	18	1,00	2,00	1,50	0,51		

Table 2: Statistical analysis of *S. mutans* colony growth by spectrophotometry

* OD (Optical Density); CFU (Colony-forming unit)

In this research, nano-Chitosan Hydroxyapatite biomaterial is used, which will be developed as an anticaries material applied to the enamel's surface by preventing the formation of biofilms that contain bacteria and attach to the tooth surface. The bacterium that has the most role in developing dental caries is *Streptococcus mutans* (*S. mutans*). Theasignificant relationship between the occurrence of caries and the number of *S. mutans* that form a biofilm mass on the tooth surface. It means that control of biofilm formation is the most appropriate method for caries prevention(Krzyściak et al., 2017).

The formation of *S. mutans* biofilm seems to increase with increasing incubation time (Fig 3). *S. mutans* is the most cariogenic type of bacteria among all types of Streptococcus in the oral cavity. *S. mutans* is a normal flora of the oral cavity, but if the environment is favorable and there is an increase in population can turn into pathogens(Ranganathan and Akhila, 2019). If the percentage of *S. mutans* in dental plaque reaches 2-10%, then caries risk is high. Conversely, if the percentage of *S. mutans* in dental plaque can be reduced to 0.1%, then the chance of caries is low. In addition, the most acid-

ISSN No:-2456-2165

producing bacteria are *S. mutans* serotype c, which is found in dental plaque(Banas and Drake, 2018)

The ability of *S. mutans* to attach to the tooth surface and form plaque is one of its virulence factors. In teeth that have been cleaned, saliva protein will attach to tooth enamel within a few minutes, called the acquired enamel pellicle (AEP)(Laputková et al., 2018). The formation of dental plaque by *S. mutans* begins with attaching bacterial adhesion molecules to glycoproteins in AEP, such as lectin proteins that can cover the tooth surface. *S. mutans* attachment to tooth enamel is then followed by the process of colonization(Kreth et al., 2020). The mechanism of bacterial development and adhesion will increase over time, which at first adheres to reversible bacterial adhesion, changing into irreversible adhesion(Dunne, 2002). It appears that the process of biofilm formation begins with (1) reversible adhesion of planktonic cells, occurring in seconds; (2) followed by the beginning of the colony attached irreversibly (in seconds to minutes); (3) cell growth and development (hours to days); (4) Cells produce LPS and form water channels (hours to days) and (5) Formation of a second colony and release of microbes to new places (days to months).

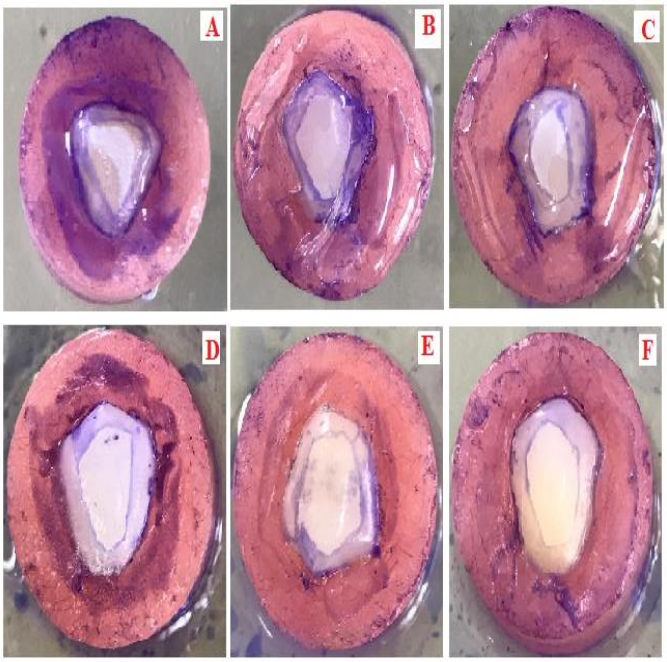
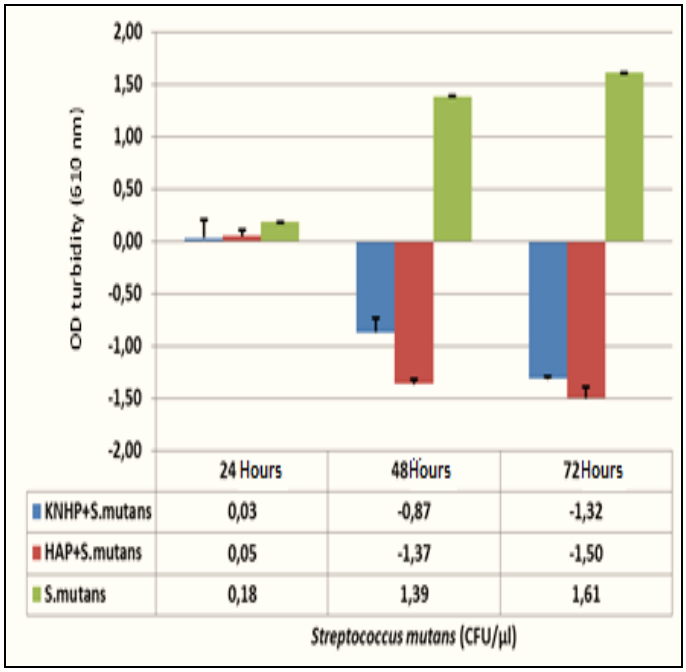


Fig. 3: Biofilm mass in the teeth surface.Inhibitory activity of *S. mutans* biofilm formation after preparation with nano chitosan (Aand D) 24 hours; (B and E) 48 hours; (C and F).



	24 Hours	48 Hours	72 Hours
KNHP+S.mutans	0,03	-0,87	-1,32
HAP+S.mutans	0,05	-1,37	-1,50
S.mutans	0,18	1,39	1,61

Fig. 4: Profile of *S. mutans* colonies based on Optical Density. At 24 hours, the growth of *S. mutans* colonies showed after preparation with Chitosan Nano-Particles and Hydroxyapatite. Whereas at the incubation time of 48 hours and 72 hours, there was no growth of *S. mutans*. Bar (growth of *S. mutans*) and Bar error (Standard deviation)

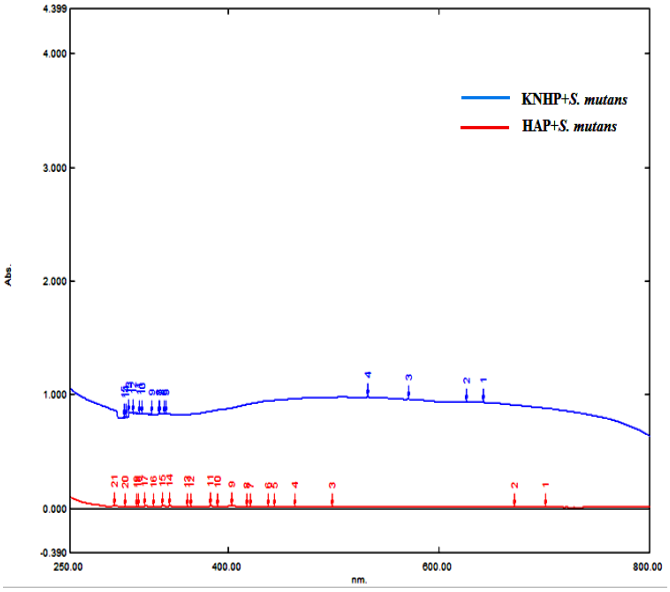


Fig. 5: The interaction power of test material against *S. mutans* with UV / Vis at 24 hours incubation time. KNHP (Chitosan nanoparticle Hydroxyapatite) had a higher interaction power than HAP (hydroxyapatite).

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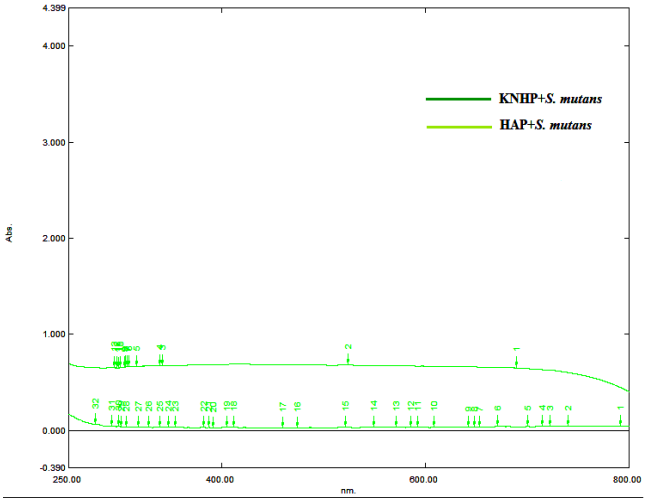

Fig. 6: Interaction power of test material against *S. mutans* with UV / Vis at 48 hours incubation time. KNHP (Chitosan nanoparticle Hydroxyapatite) had a higher interaction power than HAP (hydroxyapatite).

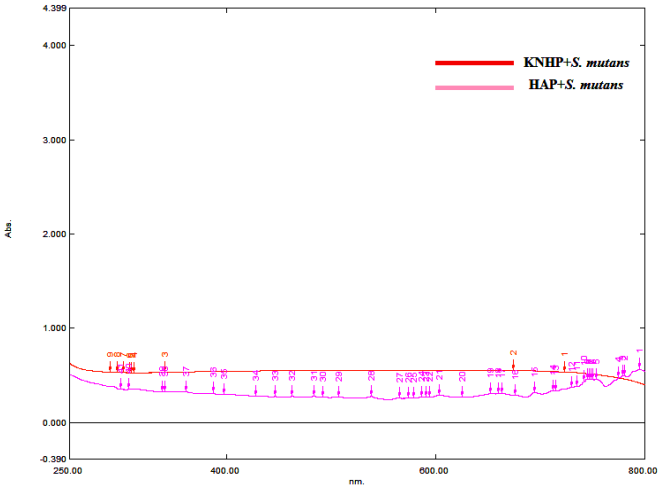

Fig. 7: Interaction power of test material against *S. mutans* with UV / Vis at 72 hours incubation time. KNHP (Chitosan nanoparticle Hydroxyapatite) had a higher interaction power than HAP (hydroxyapatite).

Provision of KNHP test material appears to have a better ability to inhibit the formation of biofilms at incubation times at 24 and 48 hours compared to HAP material (Fig 2 and 3). It is due to the ability of nano chitosan to bind electrostatically to bacterial cell walls, resulting in cell membrane damage. The alteration of the membrane potential, depolarization of the membrane, and loss of membrane integrity result in an imbalance in transportation, impaired respiration, disruption of energy transduction, cell lysis, and subsequent cell death(Choi and Lee, 2015). Chitosan also functions as a chelation material. It can bind micronutrients needed by bacteria for DNA replication so that DNA damage occurs that causes the death of bacterial cells. Aside from the protonation effect, the number of chitosan linking (C) amino groups is significant in electrostatic interactions.Ample amounts of amino acids can increase antibacterial activity so that chitosan with a high degree of deacetylation (DD) because the chitosan used as a test material is the high molecule, showing a more substantial inhibitory effect than chitosan with lower DD(Ikono et al., 2019, Saberpour et al., 2020).

Nano chitosan particles contain elements C, N, O, so the administration of nano chitosan on the surface of the enamel causes the exchange of ions, both cation and anion, resulting in the coordination of covalent bonds that can form a dense consistency. The presence of amino and hydroxyl groups that are bound to each other causes chitosan to have high chemical reactivity and contributes to the electrolyte properties of cation so that it acts as a substituted amino(Saberpour et al., 2020). It means that the adhesion ability of chitosan nanoparticles causes good sealing ability in forming layers or barriers on the enamel surface.

The addition of hydroxyapatite in nano chitosan can prevent the demineralization process caused by *S. mutans* from developing because hydroxyapatite causes an increased density of apatite in enamel(Chu et al., 2021). As a result, biofilm adhesion can be controlled in addition to *S. mutans* that exist on biofilms that the ability of nano chitosan has damaged. But at 72 hours, it was seen that HAP without the addition of nano chitosan had a better effect than KNHP in inhibiting the formation of biofilms on the enamel surface. However,statistical analysis did not show significant differences between antibiofilm, incubation time, and test material (Table 1) due to hydroxyapatite's influence, which causes dense email structure, which can cause email to be more robust against the demineralization process by *S. mutans*. The surface of the email will be difficult to be infiltrated by *S. mutans*(Abou Neel et al., 2016). HAP is very different from KNHP in preventing biofilm formation or suppressing the growth of *S. mutans* in carious pathogenesis, so it can be explained that HAP does not have antibacterial properties but is an organic element of teeth.

In contrast, chitosan does have antibacterial compounds (Nehra et al., 2018). As a positive control in this study, HAP is used as a material for modeling dental pellicles because it can have a dual role. Besides helping to maintain teeth, it can also withstand bacterial development by compacting dental hydroxyapatite to strengthen enamel surfaces.

In this research, a test was conducted to see the ability of Chitosan hydroxyapatite (KNHP) and Hydroxyapatite (HAP) test materials as anti-growth of *S. mutans* based on Optical Density. It was seen that KNHP at 24 hours of incubation had a better effect on suppressing the growth of *S. mutants* thanat 48 and 72 Hours (0.03 nm = <50 CFU (0.5x108). HAP had a lower impact than KNHP in inhibiting growth *S. mutans* (0.05 = <100 CFU (1x108) (Table 2) This can be explained by the presence of nano chitosan which plays a role in the damage of *S. mutans* cells by: (1) Interaction of positive ions or positive charges, where positive ions chitosan and negative ions in the cell membrane of *S. mutans* cause changes in the structure of the cell membrane and permeability, the occurrence of leakage /damage from proteins and other intercellular contents; and the outer membrane of negatively charged

bacteria, causing loss of replicative ability and ultimately leading to cell death, (3) Chitosan acts as a chelating agent that selectively removes metal catalysts which inhibit the production of toxins and the growth of *S. mutans*; (4) Chitosan on the cell surface can form an impermeable polymer layer that changes cell permeability and prevents nutrients from entering the cell; (5) Chitosan can absorb electronegative substances in cells which cause microbial cell contents to flocculate, damage the physiological activity of microorganisms and cause cell death (Afrasiabi et al., 2021).

At the incubation times of 48 and 72 hours, there was no longer any growth of *S. mutans*, meaning that time had an influence on the workability of the test material in inhibiting the growth of *S. mutans* (Fig 4). Statistical analysis showed no significant difference between bacterial growth (OD-CFU) and incubation time ($p > 0.05$), but there were significant differences between bacterial growth and test material ($p < 0.05$) (Table 2). From these results, it can be seen that KNHP can indeed cause the death of *S. mutans*during the incubation period of 24 hours, 48 hours, and 72 hours.

The interaction power of KNHP and HAP with *S mutans* was also evidenced by UV/VIS at 24, 48, and 72 hours incubation time. It is seen that the interaction power of the test material, KNHP has a higher interaction power than HAP (Fig 5 and 7). The two test materials interact with the relatively small *S. mutans* protein molecule, characterized by the number of peak absorbances detected at wavelengths between 250-300 kDa. By its nature, the two test materials can interact with endoproteinase to prevent the expression of *S. mutans* biofilm protein. Thus based on the data in figure 2, figure 4, and the picture of tooth surface given by KNHP and HAP (Fig 3), there is a close relationship with the study results analyzed by UV / VIS.

IV. CONCLUSION

This study can be concluded that the nano-chitosan hydroxyapatite gel could influence the mass profile of biofilms on the enamel surface at incubation times 24. 48 and 78 hours. At 24 hours of incubation, there was still growth of *S. mutans*, but at 48 and 72 hours, there was no growth of *S. mutans*. The interaction power of nanochitosan hydroxyapatite against *S. mutans* with UV / VIS at incubation t 24, 48, and 72 hours showed higher interaction power than hydroxyapatite. The results showed a close relationship with the results analyzed using UV/VIS.

ACKNOWLEDGMENT

The authors would like to acknowledge Grant 31/UN5.2.3.1/PPM/KP-TALENTA USU/ 2019, LembagaPenelitian, Universitas Sumatera Utara, Medan, Sumatera Utara,Indonesia

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