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Research Report

Effect of combination of nano brown anchovy (*Stolephorus insularis*) nanoparticles and calcium hydroxide on inhibition *Streptococcus sanguinis* biofilm formation

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ABSTRACT

Background: *Lactobacillus acidophilus and Aggregatibacter actinomycetemcomitans are gram bacteria that can cause various problems in the oral cavity so that materials that have antibacterial potency are needed. Calcium hydroxide, chlorhexidine, and sodium hypochlorite as antibacterial ingredients have disadvantages such as forming tunnel defects, causing dysgeusia, and damaging periapical tissue, so it is hoped that there will be natural materials as alternatives. The nano brown anchovy has fluor as the active compound which has the potential as an antibacterial agent.* ***Purpose:*** *To analyze the effect of antibacterial potency of nano brown anchovy (Stolephorus insularis) on Lactobacillus acidophilus and Aggregatibacter actinomycetemcomitans.* ***Methods:*** *This research is a laboratory experimental in vitro with the post-test only control group design. Brown anchovy is made into nano and diluted by dilution method into several concentrations. The direct contact method was used between some concentrations and the two bacteria. The values of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Kill Concentration) were obtained by counting the number of bacterial colonies on Mueller Hinton Agar media. Bacterial colony growth was calculated manually in Colony Forming Units (CFU).* ***Results:*** *Antibacterial potency of nano brown anchovy on Lactobacillus acidophilus showed MIC results at 1.56% concentration and MBC at 3.125% concentration. Antibacterial potency of nano brown anchovy on Aggregatibacter actinomycetemcomitans showed MIC results at 3.125% concentration and MBC at 6.25% concentration.* ***Conclusion:*** *Nano brown anchovy has antibacterial activity on Lactobacillus acidophilus and Aggregatibacter actinomycetemcomitans.*

Keywords: *Nano brown anchovy; antibacterial; Lactobacillus acidophilus; Aggregatibacter actinomycetemcomitans; good health and well being*

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INTRODUCTION

The majority of Indonesia's population has experienced caries. Dental caries is an infection in the form of damage to the tooth structure due to the destruction of the chemical structure on the tooth surface caused by biofilm metabolic activity within a certain period of time.[1] In 2018, RISKESDAS data provides the fact that as many as 57.6% of Indonesian people have dental and oral health problems. The prevalence of dental caries in Indonesia reaches 45.3% and is one of the common diseases in the world (RIKESDAS, 2018). Tooth decay is the destruction of tooth tissue starting with the process of decalcification of the enamel layer and lysis of organic structures to form cavities. Cavities in the enamel and dentin if not handled properly can widen through the roof of the pulp to the pulp chamber.[2] The caries process is caused by multifactorial factors, namely the teeth themselves, microorganisms, substrate, and time.[3] caries in the pulp chamber is not treated, it can cause necrosis of blood vessels in the pulp and progress to pulp necrosis.[2]

EndodonticTreatment is carried out in conditions where the tooth has irreversible pulpitis, and necrosis accompanied by a periapical abscess is caused by bacteria that enter the root canal and extend to the periapical tissue. In root canals that experience necrosis there are many bacteria that have the potential to spread infection to the surrounding tissue.[4]

The goal of endodontic treatment is to remove microorganisms in the root canals.[5] Improper cleaning of root canal walls during biomechanical preparation can become a hiding place for bacteria, increase the apical gap, and reduce adhesion of root canal filling materials. Debris left in the root canal can reduce the adaptation of the filling material to the root canal walls. Poor adaptation of fillers can increase the possibility of treatment failure.[6]

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One of the bacteria present in the root canal is Streptococcus *sanguinis*, this was also confirmed by Bergenholtz et al., (2010) that Streptococcus *sanguinis* is often found in root canal cultures contaminated with saliva or invasion through temporary leakage of fillings. Streptococcus *sanguinis* or can also be called Streptococcus sanguinis is a member of the *Streptococcus viridans* group, and belongs to the genus *Streptococcus*. *Streptococcus sanguinis* belongs to gram-positive, facultative anaerobic, and cocci-shaped bacteria.[7]

Intracanal medicaments are one of the steps to eradicate bacteria in the root canal and to prevent reinfection, if bacteria are still left behind after obturation. The role of intracanal medicaments becomes more relevant and complex in root canal treatment in cases of pulpal necrosis and apical periodontitis. There is ample evidence in the literature that most root canals contain viable microorganisms after the chemomechanical preparation at the first visit is complete. Calcium hydroxide is an effective intracanal medicament in inhibiting microbial growth in the root canals.[8] The most commonly used intra-canal medicament today is calcium hydroxide (Ca(OH)2). This material has good antibacterial properties due to the decomposition of Ca2-+ and OH- ions.[9]

Pathogenic microorganisms found in the oral cavity produce acids from the fermentation of carbohydrates and cause demineralization. In addition, pathogenic microorganisms can cause various problems in the oral cavity such as dental caries, gingivitis, and periodontitis. Antibacterial materials are needed to inhibit the activity of pathogenic bacteria in the oral cavity. Several materials that have antibacterial properties are available and can be used in dentistry, including calcium hydroxide, chlorhexidine, and sodium hypochlorite. Calcium hydroxide is a material that has anti-bacterial potential, but has disadvantages such as dissolving over time, poor sealing, and forming tunnel defects.[10]

Biofilm is an association of microorganisms in which microbial cells attach to each other on living and non-living surfaces in a self-produced matrix of extracellular polymeric material.[11] Biofilms consist of microbial cells and extracellular polymeric substance (EPS). EPS can account for 50% to 90% of the total organic carbon of a biofilm and can be considered the primary matrix material of a biofilm. EPS (extracellular polymeric substance) can differ in chemical and physical properties, but mainly consists of polysaccharides.[12]

There are five stages of biofilm formation, namely initial attachment in which microbes attach to the surface of an object and can be mediated by fili or fine cell hairs. Permanent attachment, in which microbes attach with the help of exopolysaccharide maturation I, which is an early stage of maturation of biofilms. Maturation II is the last stage of maturation of biofilms, the microbes are ready to spread. Dispersion, that is, some bacteria will spread and colonize elsewhere.[13]

Biofilms can form on body surfaces and persist after treatment with various antibiotics. Biofilm cells can also withstand host immune responses, namely innate and adaptive, highly resistant to phagocytosis, and 10-1000 times more resistant to treatment with antibiotics compared to their planktonic forms.[14] Previous studies have shown that biofilm thickening can be due to the number of bacterial components.[12]

Indonesia is known as a maritime country in the form of islands and nearly two-thirds of Indonesia's territory is sea. Indonesia has abundant marine wealth, which is a source of food for people in Indonesia, one of which is fish. One of the fish habitats is in the tropics, therefore fish are easy to find in almost all waters in Indonesia. This fish lives in waters with a depth of between 0-50 m. Anchovies are distributed in the Indo-Pacific region in the northern part of the Indian Ocean (Gulf of Aden, eastward to Burma) and the West Pacific (Gulf of Thailand, Java Sea, Hong Kong, Fujian and the island of Taiwan). There are various types of anchovies, one of which is *Stolephorus insularis*.[15]

In this study *Stolephorus insularis* was used in the form of nanoparticles. Nanoparticles are particles less than 100 nanometers in size that have been used as biomaterials in medicine and dentistry. The main considerations in using nanoparticles are physical and chemical properties, including surface charge and degree of hydrophobicity, surface area, and the ability of nanoparticles to be absorbed by the surface of the biofilm. Nanoparticles have a smaller size and a large surface area so that their application does not require a large concentration. In addition, nanoparticles have good biocompatibility and low toxicity. The effectiveness of nanoparticles in preventing caries is due to the ability of nanoparticles to inhibit biofilms and increase remineralization.[15]

Calcium fluoride (CaF2) provides calcium and fluoride ions to form hydroxyapatite, fluorapatite, or fluorhydroxyapatite. This study aims to assess the activity of CaF2 obtained from *Stolephorus insularis* in a concentration of 1:1 to determine the effect on the thickness of the extracellular polymeric substances (EPS) of *S. sanguinis*.

This study aims to determine the effect of adding *Stolephorus insularis* (anchovy) to intracanal Ca(OH)2 material on *S. sanguinis* biofilm which will be seen from the decrease in *S. sanguinis* biofilm formation. This research was conducted by testing the concentration of the addition of Ca(OH)2 with a ratio of 1:1 to *Stolephorus insularis* + distilled water.

MATERIALS AND METHODS

The type of research used was an experimental in vitro laboratory study with The Post Test Only Control Group Design. The sample used was the stock of bacteria *S. sanguinis* obtained from the Research Center of the Faculty of Dentistry, Airlangga University.

In this study, researchers used brown anchovies obtained from Surabaya, East Java, Indonesia. 1 kg of brown anchovy cut into pieces then boiled for 1 hour then washed and dried in the sun for about 1 day. 100 g of dried anchovy was taken

and then macerated using 1 M HCL for 2 hours and cleaned. After that, the brown anchovies were dried using an oven at a temperature of 105°C for 2 hours. The dried anchovy was pounded using a mortar and then filtered through a 60 mesh filter. Brown anchovy powder was ground using HEM for 1 hour at a speed of 3000 rpm and a milling ball diameter of 0.5 mm. 20 grams of nano brown anchovy diluted using a saline solution into concentration level 12%.

The cultures of the *Streptococcus sanguinis* group were taken from the stock using a sterile osse. Bacteria were grown in tubes containing Brain Heart Infusion Broth (BHIB) and incubated for 24 hours at 37°C. The concentration of bacteria was adjusted to the standard of 0.5 McFarland (1.5 x 108 CFU/ml).

Ca(OH)2 paste was prepared by mixing pure Ca(OH)2 powder with sterile distilled water at a ratio of 1:1 (0.9 g Ca(OH)2 powder and 0.9 mL sterile distilled water). Pure Ca(OH)2 powder and sterile distilled water are stirred using a cement spatula on a glass slab until the consistency becomes like a paste. Anchovy extract paste was prepared by mixing anchovy extract powder with sterile distilled water at a ratio of 1:1 (0.9 g powdered anchovy extract and 0.9 mL sterile distilled water). The anchovy extract powder and sterile distilled water were stirred using a cement spatula on a glass slab until the consistency became like a paste. The treatment group was formed by mixing Ca(OH)2 and anchovy extract with a ratio of 1:1 (0.6 g of Ca(OH)2 powder and 0.6 mL of 12% anchovy extract).

After anaerobic incubation, rinsing and staining were carried out using 0.1% CV. Biofilms that have been stained with CV are measured through their absorbance by a 590 nm light wave spectrophotometer, which is then defined as the number of biofilms.

RESULTS

In this study, the aim was to examine the effect of giving a combination of anchovy nano extract and calcium hydroxide on the formation of *Streptoccocus sanguinis* bacterial biofilm, where the concentration of anchovy extract used in this study was 12%. This concentration is used to determine the anti-biofilm ability of *Streptoccocus sanguinis* bacteria. Anti-biofilm activity of a combination of jenki nano anchovy extract and calcium hydroxide against *Streptoccocus* bacteria. The effectiveness of the combination of nano anchovy (*S. insularis*) 12% and Ca(OH)2 to reduce the growth of *S. sanguinis* biofilms was tested and read using a spectrophotometer with a wavelength of 540 nm expressed in OD units. The results of the research OD reading can be seen in (Table 1):

Based on the results of reading the OD of S. sanguinis biofilm using a spectrophotometer. It was found that treatment group 1 (a combination of nano S. insularis 12% and Ca(OH)2) and positive control (Ca(OH)2) had a biofilm OD value lower than the OD value negative control.

Figure 1 show BHIB media without test bacteria stained with 200 µl of 0.1% CV dye for 15 min at room temperature. Figure 2 The test bacteria were soaked in Ca(OH)2 + distilled water paste for 24 hours stained with 200 µl of

Table 1. *S. sanguinis* bacterial biofilm formation

Sample	Control (+) (S. Sanguinis)	Control (-) (S. Sanguinis + $CaOH_2$)	Nano Brown Anchovy 12% and Calcium Hydroxide (1:1)
1	0.905	0.437	0.351
2	0.918	0.422	0.322
3	0.907	0.443	0.314
4	0.902	0.419	0.359
5	0.936	0.428	0.366
6	0.921	0.444	0.362
7	0.914	0.427	0.344
8	0.921	0.443	0.358
9	0.906	0.451	0.378
10	0.912	0.431	0.391

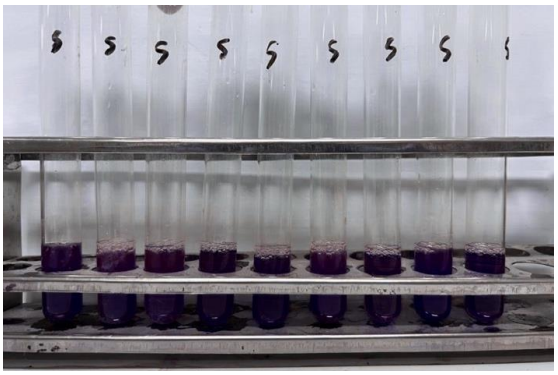

Figure 1. Negative Control Group (K-).

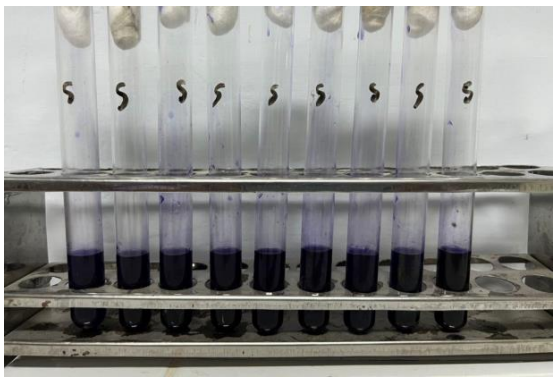

Figure 2. Positive Control Group (K+).

0.1% CV dye for 15 min at room temperature . Figure 3 The test bacteria were soaked in Ca(OH)2 with a ratio of 1:1 to anchovy extract (Stolephorus insularis) + distilled water for 24 hours stained with 200 µl of 0.1% CV dye for 15 min at room temperature. All Biofilms were washed gently with distilled water, bound dye was removed from cells with 100 µl of 98% ethanol. For full release of dye, the plate was kept on a shaker for 5 minutes.

From the table of biofilm OD readings, it can be calculated. Also, the average S. sanguinis biofilm OD value from each group can be calculated (Table 2).

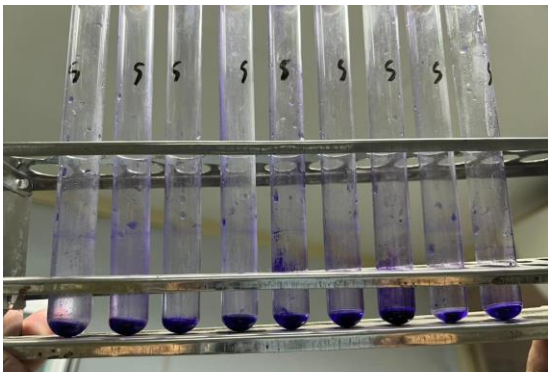

Figure 3. Treatment Groups (P).

Figure 4 shows a diagram between the positive, negative and treatment control groups. From the research results, the mean value and standard deviation of the biofilm formed were as follows, the value of the biofilm formed in the negative control group was 0.914 ± 0.010, then the biofilm value decreased in the positive control group was 0.435 ± 0.011, the value of biofilm formation in the treatment group also decreased from the positive control group of 0.435 ± 0.011. The decrease in concentration in the negative control group to the positive group was 52%, while the decrease in the positive group to the 12% jenki nano anchovy and calcium hydroxide (1:1) treatment group was 18%.

The data was then analyzed using statistical tests. Before carrying out statistical tests in each study group, normality and homogeneity tests were first carried out. The normality test for this data uses the Shapiro-Wilk Test to see if the data is normally distributed, then to test for homogeneity using the Levene Test, to see if the data is homogeneous.

The statistical test begins with a normality test for each group using the Shapiro-Wilk Test (n <50) to see if the data is normally distributed. The hypotheses used are 1) H0 = data normally distributed and 2) H1 = data not normally distributed. The results of the Shapiro-Wilk Test obtained a significance value (p-value) of 0.001 for the OD value of the biofilm in the positive control, negative control, and treatment group 1. The p-value or significance in all groups

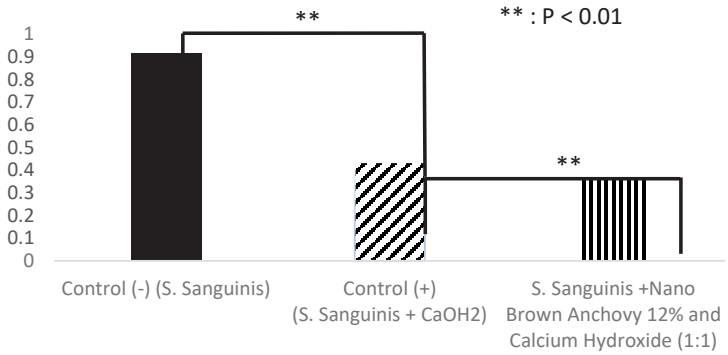

Figure 4. *S. sanguinis* bacterial biofilm formation.

Table 2. The mean concentration value and standard deviation of *S. sanguinis* bacterial biofilm formation

Group	N	Mean Concentration ± Standard Deviation
Control (+)	10	0.914 ± 0.010
Control (-)	10	0.435 ± 0.011
S. sanguinis + Nano Brown Anchovy 12% and Calcium Hydroxide (1:1)	10	0.355 ± 0.023

Table 3. *Shapiro-Wilk* Test Results for Normality

Normality Test Results (Shapiro-Wilk Test)	
p-value	0.000
α	0.05

Table 4. Levene Test Homogeneity Test Results

Homogeneity Test Results (Levene Test)	
p-value	0.000
α	0.05

Table 5. One Way Anova Difference Test Results

Difference Test Results (One Way Anova)	
p-value	0.001
α	0.05

Table 6. *Tukey HSD* Advanced Test Results

Group	K(-)	K(+)	P
K(-)	-	0.001*	0.001*
K(+)	-	-	0.001*
P	-	-	-

* significant difference (p-value < 0.05)

was valued at α (0.05) indicating that H0 is accepted, so it can be interpreted that the research data is normally distributed.

Table 3 shows the results of the Shapiro-Wilk Test obtained a significance value (p-value) of 0.001 for the OD value of the biofilm in the positive control, negative control, and treatment group 1. The p-value or significance in all groups was valued at α (0.05) indicating that H0 is accepted, so it can be interpreted that the research data is normally distributed.

After carrying out the normality test, a homogeneity test was carried out for each group using the Levene Test to see whether the data was homogeneous. The hypothesis used is 1) H0 = homogeneous data and 2) H1 = non-homogeneous data.

Table 4 shows the Levene Test results obtained a significance value (p-value) of 0.000 for the trimmed mean OD value of the biofilm in the positive control, negative control, and nano anchovy (S. insularis) 12% and Ca(OH)2 treatment groups. The p-value or significance in all categories is greater than the value of α (0.05) which indicates that H0 is accepted, so it can be interpreted that the research data is homogeneous.

From these results, the data above will be tested using a parametric test, namely the One Way Anova test (Table 5). Then it was obtained from the test results that P = 0.001 (P <0.05), it can be said that there was a significant effect on the combination of Stolephorus insularis nano extract and calcium hydroxide on the formation of biofilms of Streptococcus sanguinis bacteria, so this further test was used to see which group whichever is different. 1) H0 = there is no difference between groups and 2) H1 = there is a difference between groups. Further tests were carried out to see if there were significant (significant) differences between the treatment groups. In this study, the Post Hoc Tukey HSD test was used.

Table 6 obtained a significance value (p-value) of 0.001 in all comparisons between the negative control group, the positive control group, and the treatment group 1. The p-value or significance in the comparison between the negative control group and the positive control group and the treatment group 1 is smaller than the value α (0.05). This shows that H0 is rejected, so it can be interpreted that the negative control group has a significant difference with the positive control group and treatment group 1.

In the comparison between the positive control group and the negative control group and the treatment group 1, the p-value is smaller than the α value (0.05) so that it can be interpreted that the positive control group has a significant difference from the negative control group and the treatment group 1. Finally, the p-value in the comparison between treatment group 1 and the negative control group and the positive control group is also smaller than the α value (0.05) so that it can be interpreted that treatment group 1 has a significant difference from the negative control group and the positive control group. From the Post Hoc Test table above, it can be seen that all groups showed significant differences in inhibition of biofilm formation when compared to other groups (marked with an asterisk "*").

DISCUSSION

In this study, the results showed that there was an obstacle to the formation of biofilms by nano anchovies against *Streptococcus sanguinis* bacteria. There is an obstacle to the formation of biofilms in the anchovy nano because the anchovy contains an active compound in the form of fluoride which is able to inhibit bacterial growth. Results of phytochemical tests conducted at BPPKI Ketintang. showed that the fluoride value in nano anchovy was 4.03 mg/100g or in 100 grams of nano anchovy there was 4.03 mg of fluoride. The selected anchovy is jengki anchovy because jengki anchovy is the most common type of anchovy found in Indonesia. In addition, Brown anchovy contains fluorine which has an antibacterial effect. Brown anchovy was studied in nano form because nanoparticles have a higher activity of killing bacteria compared to particles that have a larger size.

Streptococcus sanguinis is often present in root canal cultures due to saliva contamination or also through leakage of temporary fillings. *Streptococcus sanguinis* has also been found in unsuccessful root canal treatments. The ratio of the combination of calcium hydroxide and nano anchovy used in this study is 1:1 because this ratio is the gold standard issued by the manufacturer.[18]

The ratio of the combination of calcium hydroxide and nano anchovy used in this study is 1:1 because this ratio is the gold standard issued by the manufacturer.[18] In this study, the concentration of nano anchovies used was 12%. In a previous study, nano anchovy with a concentration of 12% was used as an anti-biofilm material for *Streptococcus mutans* bacteria and was proven to be a minimal killing concentration as an initial concentration.[19] In preparing bacterial biofilms for this study, *S. sanguinis* (1×108CFU/ml) was grown in tubes containing BHIB and 5% sucrose and then incubated for 24 hours at 37°C.

The content of the active compound that acts as an antibacterial is fluorine. The phytochemical test on the anchovy was carried out at 21.05 mg/100g or in 100 grams of nano anchovy contained 21.05 mg CaF2. Besides CaF2, there is also fluorine as much as 4.03 mg/100g or in 100 grams of nano anchovy there is 4.03 mg F. Fluorine acts as an antibacterial agent because F− ions can inhibit the performance of two enzymes in bacteria, namely Enolase Enzyme and Enzyme F-ATPase. Enolase enzymes produce water to form phosphoenolpyruvate (PEP).[20] Enolase enzymes are inhibited by F− ions so that PEP production decreases which results in disrupted bacterial metabolism.[20] In addition, F− ions inhibit the F-ATPase enzyme in exporting protons so that the bacterial cytoplasm is more acidic (lower pH) than the outside environment of the cell. This lower cytoplasmic pH condition results in inhibition of acid excretion in cells. Disturbed bacterial metabolism and acidic conditions in the intracellular cause disruption of

bacterial cell growth and even bacterial death. In addition, F− ions work to disrupt the lipopolysaccharide (LPS) layer in gram-negative bacteria which functions to maintain the stability of the bacterial outer membrane structure and trigger a bacterial immune response. Disruption of F− ions causes the protection of bacteria in the face of antibacterial agents to decrease which in turn causes inhibition of bacterial growth and even death. [22,23]

After anaerobic incubation, rinsing and staining were carried out using 0.1% CV. Biofilms that have been stained with CV are measured by absorbance with a 590 nm light wave spectrophotometer, which is then defined as the number of biofilms. [23] In the untreated group (negative control), the *S. sanguinis* biofilm that formed had an average OD of 0.914 ± 0.010. The group treated with Ca(OH)2 (positive control) had an average OD of 0.435 ± 0.011 and the group treated with a combination of nano anchovies (*S. insularis*) 12% and Ca(OH)2 (group I) had an average OD of 0.435 ± 0.011.

In the group given Ca(OH)2, the average percentage reduction in *S. sanguinis* biofilm formation was 52%, while in the combination treatment group of nano anchovy (*S. insularis*) and Ca(OH)2, the average percentage reduction in S. sanguinis biofilm formation was obtained. sanguinis by 18%. The smaller the biofilm OD number that is read, the greater the percentage of reduction in biofilm formation. It can be seen that the combination of nano anchovy (*S. insularis*) and Ca(OH)2 has anti-biofilm properties against *S. sanguinis* bacteria.

From the results of the average decrease in biofilm growth, it can also be concluded that the anti-biofilm activity of the combination group of nano anchovy (*S. insularis*) and Ca(OH)2 against *S. sangunis* biofilm is more potent than the group that was only tested with Ca(OH)2. This is because, In an aqueous environment, Ca(OH)2 releases hydroxyl ions. The hydroxyl ion is a free radical that exhibits extreme reactivity towards several biomolecules. The lethal effect of hydroxyl ions on bacterial cells may cause damage to the bacterial cytoplasmic membrane, protein denaturation, or DNA damage. It is speculated that calcium hydroxide has the ability to damage the biofilm EPS matrix which is related to the bactericidal effect of the material.[15,16] In the presence of calcium fluoride nanoparticles, there is a decrease in adhesion or interference with the attachment of bacteria to the tooth surface. The addition of CaF2 nanoparticles with Ca(OH)2 is expected to result in nearly a 90% decrease in the concentration of polysaccharides which causes an increase in biofilm EPS permeability. Then, there was a disturbance in the EPS biofilm balance which resulted in the dissolving of EPS.[17] F- ions cause the EPS layer to be disrupted so that the stability of the bacterial outer membrane structure and the bacterial immune response also decrease. If this happens, it will inhibit the growth of bacteria. In addition, the administration of nanoparticles is thought to be associated with disruption of bacterial cell interactions. Nanoparticles can suppress c-di-G/AMP intracellular signals so that they can induce biofilm dispersion.[15]

REFERENCES

1. Hidaya, N., & Sinta, M. T. (2018) Gambaran Kejadian Karies Gigi Pada Anak Sekolah Dasar. Babul Ilmi Jurnal Ilmiah Multi Science Kesehatan, 9(1).
2. Mardiyantoro, F. (2017). Penyebaran Infeksi Odontogen dan Tatalaksana. Malang : UB Press.
3. Torabinejad, M. (2009). Walton, R.E. Endodontics Principles and Practice. China: Elsevier.
4. White, C., Stuart, Pharoah,& J. (2008). Michael.Oral Radiology Principles and Interpretation (6 th ed.). United Kingdom: Mosby Elsevier.
5. Cohen, S., dan Hargreaves, K. (2016). Cohen's Pathways of the Pulp, Eleventh Edition. Missouri: Elsevier
6. Dunavant, T. R., Regan, J. D., Glickman, G. N., Solomon, E. S., & Honeyman, A. L. (2006) Comparative Evaluation of Endodontic Irrigants against Enterococcus faecalis Biofilms. Journal of Endodontics, 32(6), 527–531.
7. Kakoli, P., Nandakumar, R., Romberg, E., Arola, D., & Fouad, A. F. (2009)The Effect of Age on Bacterial Penetration of Radicular Dentin. Journal of Endodontics, 35(1), 78–81
8. Permatasari, R., & Irbahani, M. (2021). Pemilihan Medikamen Intrakanal Pada Perawatan Saluran Akar . MDERJ, 1(3), 157–170
9. Novita, A., Dan, S., Endro, T. (2014). Root Canal Retreatment menggunakan Kombinasi Kalsium Hidroksida dan Chlorhexidine sebagai Medikamen Intra Kanal Insisivus Sentral Kiri Maksila.
10. Arandi, N. Z., & Thabet, (2021). M. Minimal Intervention in Dentistry: A Literature Review on Biodentine as a Bioactive Pulp Capping Material. BioMed Research International, 1–13.
11. Purbowati, R. (2016). Hubungan Biofilm dengan Infeksi: Implikaso pada Kesehatan Masyarakat dan Strategi Mengontrolnya. In Ilmiah Kedokteran (Vol. 5).
12. Homenta, H. (2016). Infeksi biofilm bakterial. Jurnal E-Biomedik (EBm), 4(1).
13. Madigan, M. T. Brock biology of microorganisms (J. M. Martinko, K. S. Bender, D. H. (Daniel H. Buckley, & D. A. Stahl, Eds.; Fourteenth edition.) [Book]. Pearson. 2015
14. de la Fuente-Núñez, C., Reffuveille, F., Fernández, L., & Hancock, R. E. (2013) Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Current Opinion in Microbiology, 16(5), 580–589.
15. Lei, L., Shao, M., Yang, Y., Mao, M., Yang, Y., & Hu, T. (2016). Exopolysaccharide dispelled by calcium hydroxide with volatile vehicles related to bactericidal effect for root canal medication. Journal of Applied Oral Science, 24(5), 487.
16. Mohammadi, Z., Shalavi, S., & Yazdizadeh, M. (2021) Antimicrobial Activity of Calcium Hydroxide in Endodontics: A Review. Chonnam Medical Journal, 48(3), 133.
17. Łukomska-Szymańska, M., Kleczewska, J., Nowak, J., Pryliński, M., Szczesio, A., Podlewska, M., Sokołowski, J., & Łapińska, B. (2016) Mechanical Properties of Calcium Fluoride-Based Composite Materials. BioMed Research International,
18. Pribadi, N. (2020). 'The solubility and water sorption properties of a combination of Ca(OH)2and propolis when used as pulp capping material', Indian Journal of Dental Research, 31(4), p. 557.
19. Fa'Izah, A., Wardani, I., Soesilo, D. (2016) . 'View of The

Effectiveness Of Anchovy Concentration (Stolephorus insularis) as Antimicrobial to Streptococcus mutans (In Vitro)', DENTAi, 10(1), pp. 71–78.
20. Putri BO, Isnawati I. (2018) Profil Miskonsepsi Buku Teks pada Konsep Enzim dan Metabolisme Sel Kelas XII SMA di Kabupaten Sidoarjo. BioEdu. Sep 30;7(3):468-77.
21. Pradiptama Y, Purwanta M, Notopuro H. Antibacterial Effects of Fluoride in Streptococcus mutans Growth in Vitro. Biomolecular and Health Science Journal. 2019 Jun 30;2(1):1-3.
22. Sunaryanto R, Tarwadi T. (2015) Isolasi dan Karakterisasi Bakteriosin yang Dihasilkan Oleh Lactobacillus lactis dari Sedimen Laut. Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan. Apr 21;10(1):11-8.
23. Roza, D. (2018). Peningkatan Imunitas Benih Ikan Kerapu Hibrid Cantik Dengan Lipopolisakarida (LPS). Jurnal Ilmu dan Teknologi Kelautan Tropis. 9(1):161-172.
24. Kamimura, R. (2022). 'Quantitative Analyses of Biofilm by Using Crystal Violet Staining and Optical Reflection', Materials, 15(19), pp. 1-9.