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Original Research Article

Antibacterial activity of Gel Nanohydroxyapatite-abalone as Remineralization Agent against *Lactobacillus acidophilus, Streptococcus sanguinis,* and *Streptococcus mutans*

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ABSTRACT

Dental health issues in Indonesia require urgent attention, particularly periodontal disease and tooth decay, which can lead to cavities. Dental caries are multifactorial diseases influenced by bacteria, including *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus.* The application of nano hydroxyapatite (n-HA) can be used as an alternative to help remineralize tooth enamel. Mouthwash, toothpaste, gum, and gel are generally used to reduce the risk of dental caries. This study chooses gel formulation because it is easy to apply and can increase the contact time between the active ingredient and tooth enamel. This work aims to investigate the antibacterial properties of gel nano hydroxyapatite abalone (gel n-HA Abalone) with concentration variations of 20,30, and 40 wt%. The antibacterial analysis used the Kirby-Bauer disk diffusion method. This study analyzes the calcium/phosphate (Ca/P) molar ratio and the ability of the gel n-HA Abalone to inhibit the growth of key cariogenic bacteria. An increase in gel concentration was associated with an upward shift in the particle size distribution of the gel n-HA Abalone. Energy dispersive X-ray spectroscopy (EDS) showed that the Ca/P molar ratio of gel n-HA Abalone 40 wt% was 1.65, *providing optimal ion availability for* remineralization. Antibacterial testing revealed that the gel n-HA Abalone 40 wt% exhibited the most effective inhibitory activity, with inhibition zone diameters of 15.6 ± 0.2 mm for *Streptococcus mutans*, 15.63 ± 0.1 mm for *Streptococcus sanguinis*, and 15.27 ± 0.3 mm for *Lactobacillus acidophilus*. The gel n-HA Abalone 40 wt % can be a natural antibacterial agent for dental caries prevention.

Keywords: Gel Nano Hydroxyapatite Abalone, Dental Caries, Antibacterial Agent, Diffusion Method

Introduction

Dental and oral health plays an essential role in body health. Some aspects of quality of life that are influenced by dental and oral health include influencing speech function, chewing, and facial structure. Dental health issues in Indonesia require urgent attention, particularly periodontal disease and tooth decay, which can lead to cavities. [1,2] Based on the Basic Health Research (Riskesdas) results in 2018, the prevalence of dental and oral health problems in Indonesia was 57.6%. [3] In particular, 88.8% of the Indonesian population experienced caries, with the prevalence in the 15-24 age group being relatively high at 75.3%. [2] Dental caries is a multifactorial disease influenced by various factors, including host-related elements (teeth and saliva), microorganisms (plaque), dietary components (carbohydrates), and the duration of exposure. [4] Bacterial infections can also cause dental caries. Bacteria that cause dental caries include *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus.* [5]

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Additionally, several predisposing factors contribute to the severity and progression of dental caries, such as previous caries experience, socioeconomic status,age, gender, geographic location, and oral hygiene practices and behaviors toward dental health. [6,7]The high prevalence of caries in society is caused by low awareness of maintaining dental and oral health and consuming instant foods and foods containing high carbohydrates. [8,9] In addition, public awareness of the need to check dental health and hygiene with the dentist routinely every year is also low. [10] The most common caries prevention effort is remineralization. Remineralizing dental hard tissue is the process by which calcium and phosphate ions are supplied from external sources to the tooth to promote ion deposition into cavities in demineralized enamel. [11,12] The demineralization and remineralization processes are regulated by the degree of saturation of the oral cavity with mineral apatite. The development of caries lesions begins when the pH of saliva/plaque on the enamel surface reaches a critical value of 5.5, and organic acids from bacteria diffuse into the enamel. At low pH, phosphate groups release calcium phosphate from the enamel surface and decrease microhardness. [11,13]. Remineralizing tooth enamel can be done in several ways, including adding fluoride to toothpaste, educating on dietary rules, and maintaining oral hygiene. [12] The remineralization process occurs when calcium (Ca) and phosphate (P) ions fill the cavities of demineralized tooth enamel. Although remineralization can occur naturally with the help of saliva, it can be accelerated by increasing calcium and phosphate. [14,15] The application of biomaterial science can be used as an alternative to help remineralize tooth enamel. Biomaterials are natural or synthetic functional materials that, in their application, can be used to interact with biological systems. These materials are metals, ceramics, polymers, and composites. [11]Biomaterials are widely used in the medical field, such as in dentistry.

For this material to be safe to use when interacting with biological systems, the biomaterial must interact with living tissue (biocompatible), dissolve in tissue without side effects (biodegradable), and be non-toxic. Biomaterials that can be applied to dentals for remineralizing tooth enamel are hydroxyapatite (HA). [12,13]
(HA; ($(Ca_{10}[PO_4]_6[OH]_2$) represents the calcium phosphate (CaP) family, which is the main component of human bones and teeth, commonly used for medical procedures. [12,14] HA has a molar ratio of calcium (Ca) and phosphate (P) of 1.67, [11,12,15,16] according to the natural conditions of bones and teeth. [17] The similarity of the chemical composition content found in HA and teeth makes HA widely applied in the biomedical field. HA crystals are the main component that makes up tooth enamel, causing teeth to be less brittle, so the hardness of the enamel depends on the amount of HA minerals in the enamel. [18] In the microscopic structure of tooth enamel, HA fills the delicate pores of the tooth surface in almost all tooth enamel so that teeth are not brittle. HA is considered promising because of its similarity to the structure of tooth minerals, which are biocompatible and bioactive. [19,20] Adding HA to teeth is expected to increase remineralizations. [21] The remineralization effect can be more optimal if the HA particle size can be reduced to nano size. [20,22] Nanohydroxyapatite (n-HA) acts as a filler by repairing small holes and depressions in the enamel surface — a function enhanced by the small size of the particles that make it up. [12] This research used n-HA based on biogenic materials from Indonesian abalone mussel shells (*Haliotis asinina*) as a matrix composition with the carbomer to form gel n-HA Abalone, as used in the previous study. [11] It contains high concentrations of calcium carbonate ($CaCO_3$) as a natural precursor for the fabrication of n-HA, which is 90–95% $CaCO_3$. [11] Mouthwash, toothpaste, gum, and gel are generally used to reduce the risk of dental caries. [12] This study chooses gel formulation because it is easy to apply and can increase the contact time between the active ingredient and tooth enamel. [12] In addition, the gel formulation from natural ingredients was carried out because rat tooth gel has been developed using porcine trypsin enzymes. [23] The preparation and concentration of a material influences the absorption process of a substance in the body. High ion concentrations can increase the potential for remineralization compared to saliva. [24] This research used carbomer-based gel preparations because they can easily flow and enter the tooth enamel surface, as used in the previous study. [12] In fabricating gel n-HA, a homogenization method is needed to disperse small particles of n-HA into liquid. This method uses mechanical force to break particles and disperse them homogeneously in a liquid. [25] The advantages of homogenization in making gel n-HA are that it can produce gel n-HA with uniform particle size, high viscosity, and properties that follow the desired application. In previous studies, [12] the fabrication of gel n-HA Abalone was only focused on physicochemical characterization and cell viability analysis without analysis of antibacterial properties. This property is essential for preventing dental caries against post-implantation infection and inflammation. In this study, the Ca/P molar ratio of gel n-HA Abalone will be examined using EDS and antibacterial properties will be analyzed to evaluate the potential antibacterial power of gel n-HA abalone against bacteria that cause dental caries, including *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus*.

Materials and Methods

The experimental procedure consisted of two main stages: the fabrication procedure of gel n-HA Abalone and its antibacterial power against bacteria that cause dental caries, including *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus*. The schematic procedure for this study can be referred to in Figure 1. The materials for this work, including n-HA and gel n-HA Abalone with concentration variations of 20 wt%, 30 wt%, and 40 wt%, were adapted from previous study. [12] Bacteria, including *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus*, were obtained from the Faculty of Dentistry (FKG), Universitas Airlangga, Surabaya, Indonesia, and were included in the antibacterial analysis.

Fabrication of gel n-HA Abalone
According to previous research methods, the fabrication of gel n-HA Abalone with concentration variations of 20 wt%, 30 wt%, and 40 wt% were fabricated. [12] The homogenization method was used to fabricate gel n-HA. In this method, n-HA powder was dissolved in 100 mL of distilled water and heated to 50 ℃. Carbomer was added to the n-HA solution and stirred until it was homogeneous. Then, 10 mL of glycerin and 5 mL of propylene glycol were added to the n-HA and carbomer solutions mixture and stirred until the solution became a gel. After the gel was formed, the gel was stored at room temperature (around at 30℃) for 24 h. [12]

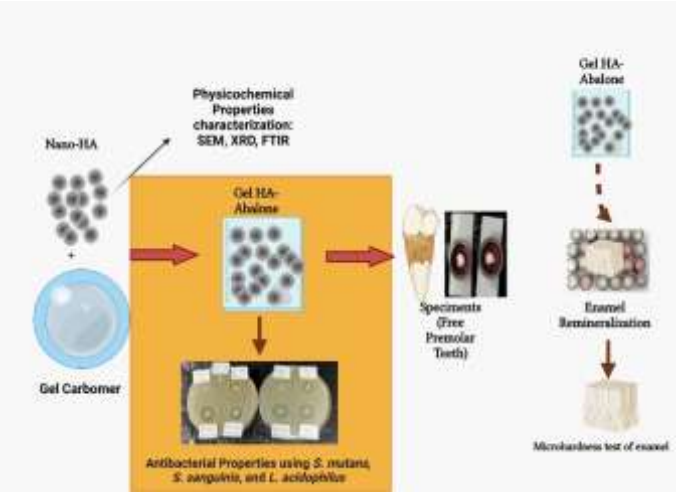

Figure 1: The Schematic design used for fabrication of gel n-HA abalone and antibacterial properties analysis (Created with BioRender.com)

Analysis of the percentage of mineral elements in the gel n-HA Abalone
As shown in previous research, [12] SEM (Joel JSM-6510LA-1400, Japan) was used to observe the morphology of the gel n-HA Abalone with concentration variations of 20 wt%, 30 wt%, and 40 wt%. The particle grain size distribution of the gel n-HA Abalone was calculated according to the measurements of 100 randomly selected particles using ImageJ software version 2006 (National Institutes of Health (NIH), Bethesda, MD, USA). The analysis of the percentage of mineral elements in the gel n-HA Abalone with concentration variations of 20 wt%, 30 wt%, and 40 wt% was specified by EDS. These products were applied to analyze the molar ratio of Ca/P in the gel n-HA Abalone samples. The Ca/P molar ratio in these products was calculated using the following equations: [26]

$$ratio_{Ca/P} = \frac{\frac{Ca\ mass}{relative\ atomic\ mass\ of\ Ca}}{\frac{P\ mass}{relative\ atomic\ mass\ of\ P}} = 0.775\,\frac{wt.\%\ Ca}{wt.\%\ P} \quad (1)$$

Antibacterial properties of gel nano hydroxyapatite abalone
Antibacterial properties are the ability of a material to inhibit the growth and development of bacteria. Materials that have these properties are usually called antibacterial agents. These antibacterial agents are usually obtained naturally, available from nature, or fabricated using artificial processes. [27,28] The antibacterial test was conducted to determine the inhibition of the gel against bacteria that cause dental caries. The bacterial inhibition test was performed by measuring the diameter of the clear zone of the sample. [12] The method used is the Kirby-Bauer disk diffusion method. [29] Samples that have antibacterial properties will form a clear zone around the sample. This clear zone indicates that no bacteria live because of the influence of the sample given. The clear zone is also called the zone of inhibition (ZOI). The formed ZOI is then measured in diameter with a caliper vertically or horizontally. Observing the zone of inhibition is carried out for 24 h to ensure that the sample effectively inhibits bacterial growth. [30,31]

Statistical analysis
All average diameter of inhibition rate data were presented as the mean

± standard deviation (SD) and one-way ANOVA was used to analyze the obtained results, followed by Tukey's test. Tukey's test was multiple comparisons with the family-wised confidence level; however, the gradual density of factor components separated each group, and the maximum one decreased. [32] *p*-values < 0.05 were considered statistically significant.

Results and Discussion

The morphology and particle grain size distribution of gel n-HA Abalone 20 wt%, gel n-HA Abalone 30 wt%, and gel n-HA Abalone 40 wt% was determined using scanning electron microscopy (SEM), as shown in Figure 2 and Table 1. The results of this analysis have been studied in previous research, [12] where the particle grain size distribution for all gel n-HA Abalone at 500 nm is also shown in Figure 2.

Table 1: Particle grain size distribution analysis of gel n-HA Abalone [12]

No	Gel with concentration variation	Particle Grain Size (nm)
1	Gel n-HA Abalone 20 wt%	487.60 ± 13.35
2	Gel n-HA Abalone 30 wt%	498.68 ± 12.65
3	Gel n-HA Abalone 40 wt%	574.00 ± 27.62

Based on SEM results, gel n-HA Abalone with concentrations of 20, 30, and 40 wt% shows small clumps and solid structures. The clumpy and evenly distributed structure at the bottom and in the middle is shown in gel n-HA Abalone 20 wt% (Figure (2a)) and 30 wt% (Figure (2b)). As shown in Figure 2c, the gel n-HA Abalone 40 wt% has grains with uniform grain morphology. Moreover, some finger-like crystals are irregularly distributed on this gel. In addition, the addition of higher gel concentration tends to produce gel n-HA Abalone with higher particle size distribution. The molar ratio of Ca/P tended to increase when the concentration of gel n-HA Abalone increased, as shown in Table 2. Gel n-HA Abalone 40 wt% exhibited a Ca/P molar ratio of 1.65, which approaches the stoichiometric ratio of natural HA. A gel n-HA with a Ca/P molar ratio close to 1.67 can mimic the natural enamel more effectively, providing optimal ion availability for remineralization and supporting apatite crystal regrowth. An increased concentration of gel n-HA Abalone contains a higher local supply of Ca^{2+} and PO_4^{3-} ions at the enamel surface. [33] Therefore, a higher concentration gel n-HA Abalone with a balanced Ca/P molar ratio close to 1.67 is ideal because it provides a larger reservoir of bioavailable ions. Moreover, it can enhance the remineralized layer's crystal growth and mechanical integrity. [34]

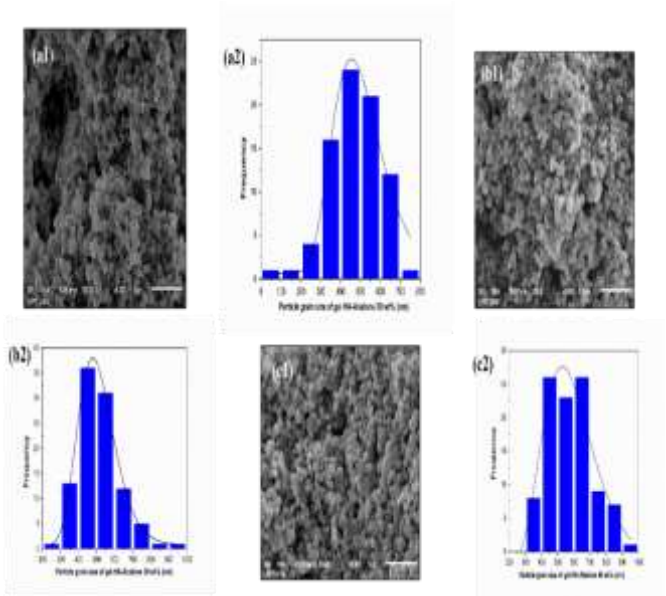

Figure 2: Morphology and particle grain size distribution of (a) gel n-HA Abalone 20 wt%, (b) gel n-HA Abalone 30 wt%, and (c) gel n-HA Abalone 40 wt%. [12]

Table 2: Ca/P Molar ratio of gel n-HA Abalone

No	Gel with concentration variation	Ca and P (%)		Ca/P Molar Ratio
		Ca	P	
1	Gel n-HA Abalone 20 wt%	25.68	12.64	1.57
2	Gel n-HA Abalone 30 wt%	22.75	11.78	1.50
3	Gel n-HA Abalone 40 wt%	34.12	16.03	1.65

Antibacterial activity was evaluated for three gel n-HA

Abalone samples with concentration variations of 20, 30, and 40 wt%. The assessment aimed to determine the inhibitory effect of the gel n-HA Abalone formulations against the growth and colonization of cariogenic bacteria associated with dental caries, as presented in Figure 3. Although the oral cavity harbors a diverse microbiota, only a limited number of species—primarily *Lactobacillus acidophilus, Streptococcus sanguinis, and Streptococcus mutans*—are recognized as the principal etiological agents of dental caries. [12] The bacterial growth zone, Lactobacillus acidophilus bacterial (Figure 3(a)) testing in gel n-HA Abalone with concentrations of 20 wt%, 30 wt%, and 40 wt% showed a diameter value of 8.87 ± 0.2 mm, 12.4 ± 0.2 mm, and 15.27 ± 0.3 mm, respectively. Streptococcus sanguinis bacterial (Figure 3(b)) testing in gel n-HA Abalone with concentrations of 20 wt%, 30 wt%, and 40 wt% showed a diameter value of 9.35 ± 0.4 mm, 12.72 ± 0,1 mm, and 15.63 ± 0.1 mm, respectively. In the bacterial testing of *Streptococcus mutans (*Figure 3(c)), gel n-HA Abalone with concentrations of 20 wt%, 30 wt%, and 40 wt% showed a diameter value of 9.67 ± 0.5 mm, 12.68 ± 0.4 mm, and 15.6 ± 0.2 mm, respectively. Gel n-HA 40 wt% has the highest diameter inhibition rate value and thus has the strongest *Lactobacillus acidophilus, Streptococcus sanguinis, and Streptococcus mutans* inhibition compared to the gel n-HA with concentrations of 20 wt% and 30 wt%. Therefore, the concentration of gel n-HA abalone significantly affects its antibacterial activity, with higher concentrations generally leading to greater inhibition of bacterial growth. [35]

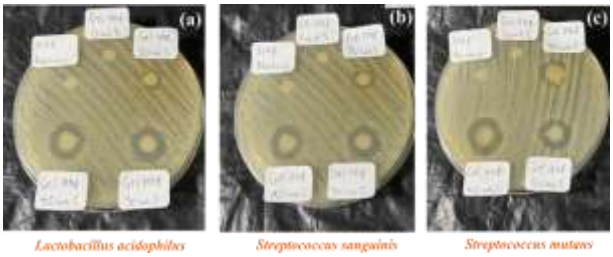

Figure 3: The test results of antibacterial activity of gel n-HA Abalone samples with concentration variations of 20, 30, and 40 wt% against (a) *Lactobacillus acidophilus*, (b) *Streptococcus sanguinis*, and (c) *Streptococcus mutans* using disk diffusion method

As shown in Figure 4 and according to the one-way ANOVA to determine the effect of concentration of gel n-HA Abalone on the diameter of inhibition rate value, the *p*-values < 0.05 reflected a significant difference in the average diameter of inhibition rate in the three groups. Tukey test revealed that *Streptococcus sanguinis and Streptococcus mutans exhibited a larger inhibition zone diameter than Lactobacillus acidophilus, suggesting that Streptococcus sanguinis and Streptococcus mutans were more affected by the presence of gel n-HA Abalone.*

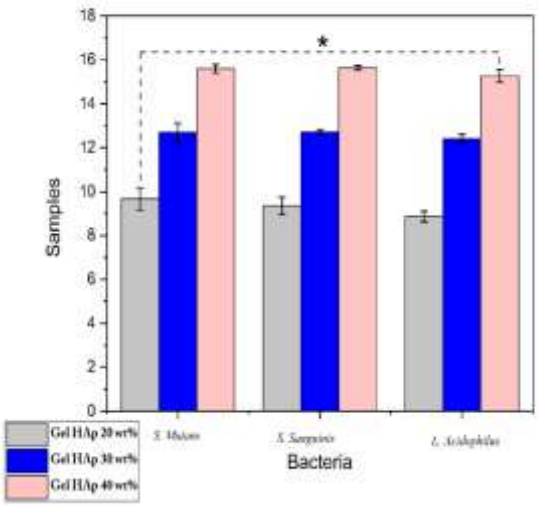

Figure 4: Average diameter of inhibition rate using *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus*

The concentration of gel n-HA significantly influences its antibacterial activity against *Lactobacillus acidophilus*, *Streptococcus sanguinis*, and *Streptococcus mutans*. As the concentration of n-HA increases, the amount of active surface areas available for interaction with bacterial cells increases. Higher concentrations of gel n-HA abalone 40 wt%, provide a greater density of nanoparticles, which can enhance ion release (particularly Ca dan P ions) into the surrounding environment. These released ions promote remineralization of demineralized enamel, which disrupts bacterial cell membranes, interferes with metabolic activities, and inhibits bacterial adhesion and biofilm formation. The n-HA has demonstrated the ability to inhibit bacterial and fungal growth. [13] In this study, the n-HA were synthesized via a precipitation method, which is straightforward and capable of producing nanoparticles with tiny dimensions. The size of these particles affects their antibacterial properties due to the faster release of ions. [36] Since it has a greater surface area, leading to greater contact with the environment with smaller particles, it becomes more solubility. Consequently, amorphous HA nanoparticles at the nanoscale may exhibit superior antibacterial activity due to their accelerated ion release compared to particles with larger grain sizes. [37] Moreover, additional mechanisms, such as generating reactive oxygen species (ROS) induced by HA and electrostatic interactions between n-HA and bacterial cell walls, further contribute to their antibacterial effects. These interactions can hinder the penetration of n-HA into bacterial cells and interfere with cell wall formation and bacterial growth. Therefore, the antibacterial potency of biomaterials can vary depending on the structural characteristics of different bacterial species. [38]

Conclusion

This study presents the successful antibacterial analysis of gel n-HA Abalone with concentration variations of 20 wt%, 30 wt%, and 40 wt% against bacteria that cause dental caries, including *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus* using the Kirby-Bauer disk diffusion method. The morphology and particle grain size distribution of gel n-HA Abalone 20 wt%, gel n-HA Abalone 30 wt%, and gel n-HA Abalone 40 wt% have been studied in previous research. [12] As shown in the antibacterial analysis, the larger the zone of inhibit bacteria, the greater the inhibition of the gel to inhibit caries-causing bacteria. The gel n-HA Abalone 40 wt% exhibited the greatest inhibition zone diameter, indicating the most potent antibacterial activity against *Lactobacillus acidophilus*, *Streptococcus sanguinis*, and *Streptococcus mutans* when compared to gels formulated with concentrations of 20 wt% and 30 wt%. Therefore, gel n-HA Abalone 40 wt% can help treat teeth problems, such as demineralization and cavities, so it can be an antibacterial agent against bacteria that cause dental caries.

Conflict of Interest

The author`s declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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