The Efficacy of Chitosan Toothpaste Based White Shrimp (Litopenaeus Vannamei) to Decrease Streptococcus Mutans Colonies in Children with Early Childhood Caries

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Abstract. Caries are common in early childhood. The prevalence of early childhood caries according to the American Academy of Pediatric Dentistry is 57.7%. One of the causes is Streptococcus mutans bacteria. The most effective method to prevent plaque accumulation is to brush the teeth with toothpaste. Chitosan is a natural bi-opolymer that is safe to use as an antibacterial and can be combined with toothpaste for children with early childhood caries. The objective of this study is to determine the effectiveness of chitosan from white shrimp (Litopenaeus vannamei) as toothpaste to reduce S. mutans in early childhood caries. The results of a paired t-test using SPSS ver. 22.0 for Windows showed a significant decrease in the number of colonies of S. mutans from before to after brushing with 5% chitosan toothpaste. The number of bacterial colonies pretest was 120.4 CFU and that post-test was 11.4 CFU ($p < 0.05$). Chitosan toothpaste 5% was effective in decreasing the number of colonies of S. mutans in cases of early childhood caries.

Keywords: chitosan, white shrimp, toothpaste, *Streptococcus mutans*

1   Introduction

The most common plaque-mediated disease in children is dental caries, which is also one of the most common childhood diseases [1]. The first experience with dental caries in infants and children < 5 years old is defined as early childhood caries (ECC). ECC can interfere with growth and development of the child, disturb phonetics and articulation, and eventually cause low self-esteem. ECC is a significant problem in developing countries because it is worsened by low social economic status and malnutrition [2]. According to American Academy of Pediatric Dentistry, the global prevalence of ECC in children aged 12–36 months is 57.7%. The prevalence in Indonesia, is 90% of the childhood population [3, 4].

ECC is multi-factorial disease resulting from the interaction among the host, diet, and microflora on the tooth surface over a period of time, which leads to de-mineralization of hard tissues [5]. The most implicated bacteria in dental caries etiology is Streptococcus mutans for initiation and Lactobacillus acidophilus for progression of caries [6]. S. mutans is an acidogenic bacteria that plays a role as trigger for ECC.

Sucrose and carbohydrates that contain milk are fermented by S. mutans to form an acidic environment, causing demineralization of the enamel [7-9].

Daily use of an efficient anti-plaque compound, especially a form formulated into toothpaste, can be very beneficial for plaque control, as it suppresses the growth of bacteria, such as S. mutans. [7,10]. Some groups of toothpaste antibacterial compounds, such as phenol, hexetidine, fluoride, and chlorhexidine, have been studied thus far [11]. However, these antibacterial agents have limited use in children with hypersensitivity or allergies. Chitosan has received special attention as a natural biopolymer material because it is non-chemical and natural and has long been used in traditional medicine. Thus, chitosan is a promising antibacterial agent to add to toothpaste [12].

According to Kim, the natural amino-polysaccharides in chitosan have the molecular formula $C_8H_{12}NO_5$ with high levels of C, H, N, and O, respectively of 47%, 6%, 7%, and 40% [12]. The main source of chitosan is from the exoskeletons of marine crustaceans, such as shrimp, crabs, and lobsters. The shrimp is a fisheries sector commodity, with high economic value. One type of shrimp that is widely cultivated for export is white shrimp (Litopenaeus vannamei). Indonesian shrimp waste reaches 298,642.25 tons every year [13].

According to research conducted by Akbar, chitosan is an antibacterial agent that degrades bacterial cell walls [10]. Visveswaraiah demonstrated that chitosan inhibits hydroxyapatite acid, which is highly reactive with cariogenic foods [14]. Arnaud showed that the anticariogenic properties of chitosan can act as a mechanical barrier to the enamel [15].

The basis of this study was to test the efficacy of the antibacterial properties of chitosan added to a toothpaste that has the capability of remineralization. Thus, it could be a potential antibacterial agent for children with ECC. We prepared a toothpaste combined with chitosan-based white shrimp (L. vannamei) waste. However, until now, there is no research on the cariostatic effects of toothpaste combined with chitosan. Therefore, we were interested in conducting a study about the efficacy of chitosan toothpaste based white shrimp (L. vannamei) to decrease the number of S. mutans colonies in children with ECC.

2   Materials and Methods

Healthy kindergarten students who volunteered in the age range of 36–60 months, and who experienced ECC with at least four caries were recruited as the sample to obtain S. mutans. Other inclusion criteria were: brushed teeth regularly, no periodontal disease, and not taking drugs. In total, 30 subjects participated in the quasi experimental study with pretest, post-test, and control groups. The samples were divided into three toothpaste (TP) groups (n = 10): (G1) placebo-TP, 2.5% chitosan-TP, and 5% chitosan-TP. All volunteers provided informed consent to donate a bacterial sample, with approval of the Medical Ethics Committee at Hasanuddin University, Makassar (no: 8/ H04.8.4.5.31/PP36-KOMETIK/2017 and registration no. UH17010015), Indonesia. This research was conducted at TK Cipta Mandiri Makassar, Biochemistry Laboratory Faculty of Mathematics Sciences and Nature, Oral Laboratory Biology Faculty of Dentistry, Hasanuddin University during January 2–20, 2017.

This research begins with the preparation of the chitosan from white shrimp (L. vannamei) waste. White shrimp skin was washed with water. Next, shrimp shells were dried in an oven at 80°C and macerated using a dry blender. Next, the chitin was isolated in several stages. The first stage was demineralization by immersing the shrimp skin in 3% HCl solution. The second stage was deproteinization by immersing the shrimp skin in 4% NaOH solution. Then, the shrimp skin was soaked in 60% NaOH solution for deacetylation. After that, the chitosan was dried at 65°C. A 0.1 g portion of chitosan was sprayed with nihydrin solution and allowed to stand for 5 min. The chitosan was processed in the laboratory of Biochemistry Faculty of Mathematics and Natural Sciences of Hasanuddin University.

The 2.5% and 5% chitosan TPs made from white shrimp (Litopenaeus vannamei) were prepared by making the first mixture: 1.2 ml anise oil, 0.1 g menthol crystals, 1.0 ml perme oil, and leather chitosan powder were mixed with a mortar and pestle until homogeneous. Then, 13 g of magnesium carbonate and 15 g of calcium carbonate were stirred with a mortar and pestle until the paste was homogeneous. Then, 3 ml glycerin, 14.2 ml distilled water, 4 g polyethylene glycol, 1.5 ml triethanolamine, and 1.0 ml oleic acid was stirred with a mortar and pestle until the paste was homogeneous. The three mixtures were mixed together and stirred evenly into a fine paste that was ivory white. The paste was put in a tube.

All subjects brushed their teeth in the same way before sampling to minimize confounding variables. The S. mutans was sampled using a swab. Then, the bacterial samples were placed in 0.9% NaCl deposited in 60 vials. After brushing their teeth, the child was instructed not to eat or drink for 5 min. Then, the sample was taken after brushing and stored in 0.9% NaCl solution.

Then, a 10^{-3} dilution was performed using the 0.9% NaCl solution. Subsequently, a selective medium (TYSB20 (tryptone yeast extract cysteine sucrose Bacitracin 20)) was made to select and identify S. mutans. One ml of diluted bacteria was poured into a Petri dish, containing 10 ml sterile TYSB20. Once the suspension was homogenized, it was incubated at 37°C for 24 hours. After the incubation, a colony count was performed using the colony forming unit (CFU/ml) method and a colony counter. The data analysis was conducted with SPSS 22.0 for Windows software (SPSS Inc. Chicago, IL, USA). A paired t-test was performed to test the significance of the number of colonies of S. mutans before and after the treatment in each group. A p-value < 0.05 was considered significant.

3   Results

This research achieved a waiver of ethics with number: 8/ H04.8.4.5.31/PP36-KOMETIK/2017 and registration number UH17010015 on January 6, 2017. There was a decrease in the number of S. mutans colonies between the pre- and post-tests in both sexes (Table 1). Females had the highest number of S. mutans colonies, with a pretest colony count of 121.84 CFU and a 28.0 CFU post-test.

Table 1. Comparison of males and females

Gender	Pre	Post
	Mean ± SD	Mean ± SD
Male	120.8824 ± 6.07127	26.5882 ± 14.30498
Female	121.8462 ± 6.14880	28.0000 ± 14.08309
Total	121.3000 ± 6.01808	27.2000 ± 13.98127

A decrease in the number of S. mutans colonies was observed from pretest to post-test in both sexes. The highest number of S. mutans colonies was found in female's pretest, with 122.5 CFU and 44.75 CFU on the post-test. The pretest of the 2.5% chitosan toothpaste group yielded 122.2 CFU and 27.0 CFU post-test. The 5% chitosan toothpaste group was 120.75 CFU pretest and 12.5 CFU post-test.

The mean number of S. mutans bacterial colonies in the pretest placebo toothpaste group (Fig. 1) was 121.20 CFU and it was 42.9 CFU post-test. The mean difference was 145.399 CFU. The mean number of S. mutans colonies pretest in the 2.5% chitosan toothpaste group was 122.30 CFU and it was 27.3 CFU post-test. The difference in the mean value was 120.208 CFU. The mean number of S. mutans bacterial colonies pretest in the 5% chitosan toothpaste group was 120.40 CFU and it was 11.4 CFU post-test. The difference in the mean values was 318.64 CFU ($p < 0.05$).
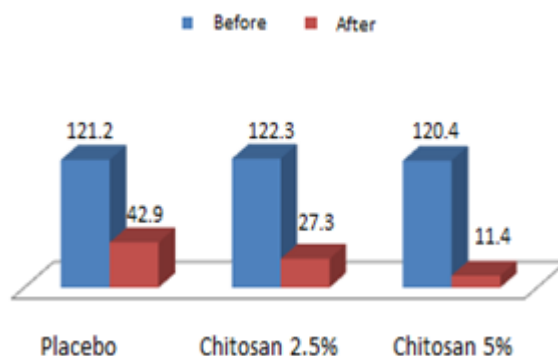

Fig. 1 Diagram of the mean number of Streptococcus mutans colonies before and after brushing with placebo toothpaste, 2.5% chitosan toothpaste, and 5% chitosan toothpaste

All toothpaste groups had significant changes. However, the 5% chitosan toothpaste group has the greatest difference among the placebo toothpaste and 2.5% chitosan toothpaste groups (Fig. 2).
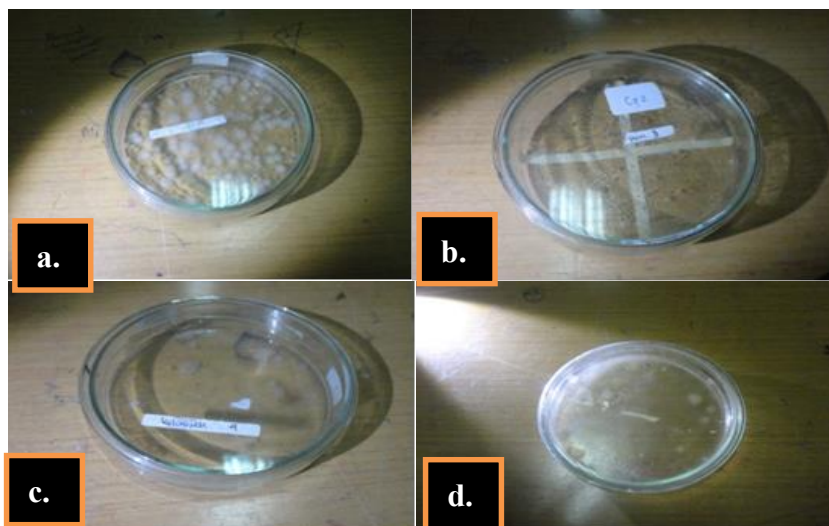
Fig. 2 A Number of colonies of Streptococcus mutans bacteria before brushing teeth; B. placebo toothpaste; C. chitosan toothpaste, 2.5%; D. chitosan toothpaste, 5%.

3 Discussion

Antibacterial effects were seen based on the ability of the toothpastes to kill bacterial colonies measured by counting the decrease in the number of bacterial colonies. The bacterial sample was deposited into a 0.9% NaCl as a physiological solution, followed by a 10^{-3} dilution because it was more effective for reducing the number of bacterial colonies. Previous research streaked S. mutans on blood agar, while in this study S. mutans were cultured on selective TYSB20 medium [16].

This study used 2.5% and 5% chitosan concentrations based on research conducted by Visveswaraiah (2016) who reported that 2.5% and 5% chitosan is effective and safe for the body [14]. Table 3 shows that the mean number of S. mutans colonies bacteria pretest in the 5% chitosan toothpaste group was 120.40 CFU, whereas it was 11 [4]. CFU post-test ($P < 0.05$). Chitosan works as an antibacterial agent by combining the ability of toothpaste to inhibit cell division of bacteria. Chitosan interferes with bacterial metabolism by inhibiting bacterial glycolysis. Akbar (2015) reported that chitosan degrades the bacterial cell wall, then destroys the cytoplasmic membrane which causes the nucleus to escape through the bacterial cell wall [10].

Table 2. Differences in Mean of Colony Bacteria of *Streptococcus mutans* by Group of Placebo Toothpaste, 2.5% Chitosan Toothpaste Before and After Brushing Teeth

Toothpaste Group	Pre Mean ±SD	Post Mean ±SD	t	P	P		
Placebo	121.2000±6.86052	42.9000±6.17252	145.399	.000	$\frac{1}{3}$.000±5.35516	27.3000±4.98999	
5% Chitosan	120.4000±6.23966	11.4000±3.94968	120.208	.000			
			318.640	.000			

Paired sample t-test p < 0.05; significant

The results of this study were supported by Ibrahim (2012), who suggested that chitosan interacts with bacteria through the NH3 glucosamine groups and the surface of negatively charged cells [17]. The structural attraction between the bacterial cell wall and chitosan is caused by the bacterial cell wall containing peptidoglycans whose basic main chain structure consists of N- acetylglucosamine and β-glycans. The initial interaction between polycationic chitosan and negatively charged cell wall polymers is influenced by electrostatic interactions and citric acid. Consequently, binding of chitosan to the cell wall polymer leads to destabilization and destruction of bacterial membrane function as a protector. Membrane permeability is impaired, leading to inhibited movement of bacterial substances across the membrane.

Cherissa (2016) demonstrated that chitosan has antibacterial properties because it inhibits glucosyltransferase (Gtf) in bacteria [18]. Gtf converts sucrose into two types of glucans, such as water-soluble glucan and insoluble glucan. Water-soluble glucans are also called mutants (1,3 α-glucans), whereas insoluble glucans include dextran (1,6 α-glucans). Glucans plays a role initiating adhesion of bacteria into a dental plaque. Chitosan may also inhibit the expression of mRNA that induces the formation of cyclooxygenase-2 (prostaglandin-endoperoxidase synthase 2) that plays a role in the mechanisms of inflammation and pain receptors [15].

The results of this study are supported by those reported by Andres (2007) who documented the interaction between chitosan and the outermost bacterial cell membrane, where the concetration effect on antibacterial power is high. Based on the results of other studies, chitosan antibacterial activity decreases with increasing chitosan concentration [19]. In other words, higher inhibition at lower chitosan concentrations, presumably due to the lower viscosity of the solution.

Two antibacterial mechanisms of chitosan against bacteria have been proposed. The first is based on the presence of amine functional groups on chitosan that form bonds with the cell wall of bacteria, resulting in leakage of intracellular constituents, which lyses the bacteria. The second hypothesis states that chitosan begins by destroying bacterial cell walls, engages in intracellular binding, blocks mRNA, and inhibits protein synthesis [19].

According to Setiyani (2015), the antibacterial effect of chitosan is derived from the free amine groups (-NH2) of chitosan, which are protonated into cationic amine groups (-NH3) and can interact with the surface of bacterial cells, which are negatively charged [20]. In addition, the chitosan component eliminates the integrity and function of the membrane so that the cell loses intracellular material. Furthermore,

cells cannot maintain their homeostatic balance and cell respiration is inhibited [10]. This is also supported by a study by Li, Poon, Li W, Zhu HY, Yeap SH, and Cao Y, et al (2011) which stated that chitosan has antibacterial properties due to inter-actions between positively charged chitosan molecules and the negatively charged cell membrane [21]. Microbes interfere with the microbial membrane and the break-down of microbial cell walls and alterations in the permeability of bacterial cells.

Therefore, chitosan toothpaste has a very good antibacterial effect of lowering the number of S. mutans colonies in cases of ECC.

4   Conclusion

Chitosan 5% toothpaste was more effective than chitosan 2.5% toothpaste and pla-cebo toothpaste. The chitosan 5% toothpaste had a greater effect on growth of S. mutans in cases of ECC.

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