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Chewable Lozenges using White Shrimp Waste (*Litopenaeus vannamei*) in Reduce Colonization of Bacteria *Streptococcus mutans* in the Case of Early Childhood Caries

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ABSTRACT

Background: Early childhood caries (ECC) is one of the most common caries in children. One reason is the bacterium *Streptococcus mutans*. Choosing the right diet can suppress the growth of *Streptococcus mutans*. Chitosan is a natural biopolymer from white shrimp waste as an antibacterial and can be combined with food or drinks including chewable lozenges.

Objective: This study aims to determine the effectiveness of chewable lozenges from white shrimp waste (*Litopenaeus vannamei*) in inhibiting *Streptococcus mutans* bacteria in ECC cases.

Research Methods: This type of research uses field and laboratory experimental research designs with pretest-posttest control group design. A sample of 30 children consisted of 3 groups, namely 10 children chewing xylitol candy, 10 children chewing chewable lozenges chitosan 2.5%, and 10 children chewing chewable lozenges chitosan 5%. Then, bacterial samples isolated from caries teeth before and after chewing chewable lozenges. Then, counted the number of *Streptococcus mutans* colonies using the colony counter method with the CFU unit. Data processing and analysis using SPSS version 25.0 for windows.

Results: The paired t-test results showed a significant decrease in the number of *Streptococcus mutans* colonies before and after chewable lozenges chitosan 2.5%. The average difference between groups before and after treatment of chewable lozenges was -17.9 CFU with a standard deviation of 20.306. The paired t-test results showed a significant decrease in the number of *Streptococcus mutans* colonies before and after chewable lozenges chitosan 5%. The average difference between groups before and after treatment of chewable lozenges was -15.4 CFU with a standard deviation of 9.24. Data test results obtained p = 0.021 (p <0.05). This shows a significant decrease in the number of *Streptococcus mutans* colonies in cases of early childhood caries.

Conclusion: Chewable lozenges chitosan 2.5% effective in reducing the number of *Streptococcus mutans* bacterial colonies in cases of early childhood caries in children.

Keywords: Chitosan, Chewable lozenges, *Streptococcus mutans*, *Litopenaeus vannamei*

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INTRODUCTION

Oral health problems that are often found in children throughout the world, especially in developing countries including in Indonesia are dental caries.[1] Caries process that begins with dissolving enamel with the formation of acid and microbial substrates, resulting in destruction of organic components tooth. The prevalence of caries in Indonesia reaches 90% of the population under five.[2] According to the Basic Health Research (Riskedas) in 2007, it shows that the increase in dental caries, especially in children under five and pre-school children, is from 24% to 28% while in children age 2-5 years increased by 70% including caries in early childhood.[3]

Early childhood caries (ECC) is the most important dental health problem occurring in infants and toddlers, which can affect the health and development of children's teeth. Early childhood caries is a term used to describe dental caries that appears in children. The American Academy of Pediatric Dentistry (AADP) defines early childhood caries as the presence of one or more carious teeth, extracted due to caries or the surface of a deciduous tooth that is patched in children aged <71 months.[4] Prevalence and severity of dental caries in children under the age of 5 years in some countries is quite high. In Indonesia, in 2001, the prevalence of caries in children aged 3-5 years in DKI Jakarta was 81.2%.[5] The prevalence of caries in children under five in Indonesia was around 90.05%.[6]

The caries process is influenced by host factors (tooth surface), microorganisms (bacteria that cause caries), substrates (fermentable carbohydrates), and time. Caries can occur if all of these factors are involved.[7] Dental plaque is a biofilm that forms naturally on the tooth surface. If the plaque is formed it will look gray.[8,9] Several studies have shown that dental plaque plays a role in oral disease and is associated with cariogenicity (the ability to potentially form caries) plaque bacteria, such as acid production, which results from the production of extra polysaccharides cellular.[10]

Streptococcus mutans is a bacterium that is closely related to acidogenicity as a trigger for caries in children. Some types of carbohydrates, such as sucrose, can be fermented by *Streptococcus mutans* to form acids, which can reduce plaque pH and cause enamel demineralization.[11] Prevention of caries can be done by the right diet. Diet is food and drinks consumed daily by individuals. Diet is one of the main factors for the beginning of caries development, so the choice of diet is important to note.[12]

The selection of the right diet can suppress the growth of *Streptococcus mutans*. Antibacterial product commonly used in chewing gum are phenols and flavonoids. That's why chitosan is an attractive alternative material as an antibacterial.[13] According to Kim[13], which states that the amino-polysaccharide chitosan is a natural material that has the molecular formula $C_8H_{12}NO_5$. The main source of chitosan is obtained from exoskeleton from marine invertebrate crustaceans (*crustaceans*), such as shrimp, crabs and lobsters.

Shrimp is one of the raw materials for aquatic products from the crustacean phyla. Range of shrimp waste in Indonesia reaches 298.642.255 tons per year. This shrimp is exported in a frozen state which produces a large amount of waste in the form of shrimp shells that are less than optimal utilization.[14] According to research conducted by Akbar[11], chitosan as an antibacterial against degradation in bacterial cell walls, resulting in damage to the cytoplasmic membrane, cytoplasmic nucleus out of the cell wall bacteria.

According to Visveswaraiah and Prasad[15] in their research proved that chitosan has organic ions which inhibit hydroxapatitic acid, which is very reactive with cariogenic foods. In addition, anticariogenic chitosan has been proven to be able to act as a mechanical protector against enamel.[16]

Utilization of waste in Indonesia into useful products is still very small, one of which is shrimp shell waste. *Litopenaeus vannamei* is a type of shrimp that is widely cultivated for export purposes. [17]

According to Achmad H and Feby Y *Litopenaeus vannamei* shrimp shells can be processed into chitosan which is proven to inhibit the growth of *Streptococcus mutans* bacteria that can cause caries.[18] Chewable lozenges is one of the preparations that can be used as an alternative for the treatment of antibacterial locally in the mouth because chewable lozenges can directly dissolve the caries active substance in the mouth.

The form and taste of chewable lozenges are expected to be preferable to other dosage forms such as tablets, syrups or solutions because they are easier to use and more attractive.[19] The basis of this research is the antibacterial properties of chitosan in chewable lozenges preparations having remineralisation ability, so that they have the potential to be agents of use treatment in cases of early childhood caries. The results of this study are chitosan from white shrimp shells (*Litopenaeus vannamei*) waste combinated with chewable lozenges in cases early childhood caries.

However, until now there has been no research on the effects of chitosan karyostatic from white shrimp skin waste (*Litopenaeus vannamei*) in chewable lozenges preparations. Therefore, interested researchers are interested in conducting research on chewable lozenges made from chitosan (*Litopenaeus vannamei*) in reducing *Streptococcus mutans* bacteria in cases of early childhood caries.

MATERIAL AND RESEARCH METHODS

This type of research is a true experimental research. The design of this study uses the method of pre and post test with control group design. This study has obtained ethical qualifications with number: 0131/PL.09/KEPK FKG-RSGM UNHAS/2019 and UH17120136 register number on April 1st 2019. This research was conducted at the Dharma Utama Kindergarten Hasanuddin University Makassar for sampling *Streptococcus mutans* bacteria in the teeth of children with early childhood caries. Hasanuddin University for making chewable lozenges, and Microbiology Laboratory, Faculty of Medicine, Hasanuddin University for the calculation of the number of *Streptococcus mutans* bacterial colonies in children with early childhood caries. The study was conducted on April 1st through August 6th, 2019. The population was kindergarten students of the Dharma Utama University of Hasanuddin Makassar, who experienced early childhood caries aged 5 years. Samples totaled 30 people. The sampling method used was simple random sampling because the research subjects were homogeneous. This research data is done by blinding. The inclusion criteria in this study were children aged at least 36 months and a maximum of 71 months, children with early childhood caries with caries of at least 2 teeth, willing to be the subject of research by filling out informed consent, brushing teeth twice a day, not having periodontal disease, and not taking drugs. The exclusion criteria in this study is that in the process of sampling the child suddenly refuses to be the subject and does not comply with the treatment process.

This research was begun by making chitosan from the skin of white shrimp (*Litopenaeus vannamei*). The first stage in making chitosan is the process of preparing samples, namely white shrimp shell waste washed shrimp waste with water. Furthermore, the shrimp shells are dried in an oven at 80°C, then mashed using a dry blender. The second stage is demineralization, done by soaking the shrimp shells in 3% hydrochloric acid solution. The third stage is deproteinization, done by soaking the shrimp shells with 4% sodium hydroxide solution. The fourth step is the process of forming and refining chitosan. Then the deacetylation process by immersing shrimp shells in 60% sodium hydroxide solution. After that, the chitosan that has been obtained is dried at 65°C.

Then a Nihydrin test was carried out, amounting to 0.1 g chitosan, sprayed with a nihydrin solution and then allowed to stand for 5 minutes. Chitosan which has been processed in the Laboratory of Biochemistry of the Faculty of Mathematics and Natural Sciences, Hasanuddin University, continued with making chewable lozenges chitosan 2.5% and 5%. After the chitosan powder of white shrimp skin (*Litopenaeus vannamei*) is produced, mix it with as much as 0.5 gram as an anticariogenic chewable lozenges. The chewable lozenges formula are 25 basis gelatin glycerin as much as 70 ml, aquadest 12 ml, gelatin 18 grams, methylparaben 0.4 grams, xylitol 0.5 grams, flavoring oil 3-4 drops. All the ingredients for making chewable lozenges are weighed then the aquadest is heated to boiling. Gelatin is poured into a container and soaked with boiling water as much as desired.

The mixture of gelatin and aquadest is allowed to stand for 15 minutes until fluffy. Glycerin is added little by little and stirred while heated on a water bath until all the gelatin is mixed with glycerin, then add the remaining glycerin slowly

while stirring until evenly mixed and free of lumps. This base is reheated for 45 minutes. Aspartame and metylparaben are added and stirred. The active ingredient as this idea is chitosan made from white shrimp skin (*Litopenaeus vannamei*) and citric monohydric acid is added, stirring until evenly mixed. The mixture is poured into molds and allowed to cool. If the mixture freezes when it is poured, it can be heated again and poured back. Quality control is a test that will be conducted to determine the physical properties of chewable lozenges from the experimental results. In this chewable preparation the quality control is almost the same as the quality control in the lozenges preparation because chewable is one type of lozenges. Quality control includes: product color and brightness, texture, appearance, preparation consistency, weight uniformity test, melting time test, elasticity test, and physical stability test. If it passes the test, it is put into the warehouse of the finished product, so that chewable lozenges are ready for consumption for children who have early childhood caries as a preventive caries strategy in children.

The research at Dharma Utama Kindergarten Hasanuddin University Makassar began by asking the parents of children to fill out a questionnaire asking about matters related to this study. The research group was divided into 3 groups, 10 children chewable lozenges xylitol, 10 children chewable lozenges chitosan 2.5%, and 10 children chewable lozenges chitosan 5%. The subjects did not see the toothpaste group to be tested.

The first sample is taken when the child has not chewed lozenges. Bacterial sampling using patient saliva stored in vial bottles. Then, the bacterial sample is stored in a physiological solution, ie 0.9% NaCl is stored in a vial of 60 bottles. After chewing chewable lozenges, the child is instructed not to eat or drink for 5 minutes. Then, the samples were taken after chewing chewable lozenges xylitol, chewable lozenges chitosan 2.5%, and chewable lozenges chitosan 5%, and the samples were stored in 0.9% NaCl solution.

To calculate the number of *Streptococcus mutans* bacterial colonies begins with the sterilization of tools and materials used in oral biology laboratories. Then, a 10^{-3} dilution was carried out using 0.9% NaCl solution. Then, selective medium was made, namely TYSB20 (*Tryptone Yeast Extract Cystein Sucrose Bacitracin 20*) which aims to select and identify *Streptococcus mutans* bacteria, so that only this type of bacteria can live as a research variable. Then, the pour plate method is carried out to isolate bacteria into the petri dish. As much as 1 ml of diluted bacteria was put into a petri dish, then pour 10 ml sterile TYSB20 (*Tryptone Yeast Extract Cystein Sucrose Bacitracin 20*) selective medium. Then the suspense is homogenized and put in an incubator at 37°C for 1x24 hours. After the bacteria are incubated, observation and calculation of the number of colonies can be done using the colony forming units (CFU/ml) method using a colony counter. Analysis of the data in this study using a computer program that is SPSS 25.0 for windows. To test the significance of differences in the number of *Streptococcus mutans* bacterial colonies before and after each group's treatment, a paired T-test was conducted.

RESULT

The results of the study are displayed in the following table:

Table 1: Descriptive of Each Control and Treatment

Treatment	Mean	N	Standard Deviation	P-Value
Pre Chitosan 2.5%	87.8000	10	104.99291	0.368
Post Chitosan 2.5%	69.9000	10	97.31444	
Pre Chitosan 5%	57.6000	10	37.34881	
Post Chitosan 5%	42.2000	10	33.69405	
Pre Xylitol	22.9000	10	19.48475	
Post Xylitol	50.7000	10	29.46203	

(Source: Primary Data, 2019)

Based on Table 1. shows the results of the univariate test in each treatment group before and after chewable chewable lozenges chitosan levels of 2.5%, 5% and market xylitol candy. The number of samples studied in each treatment

and control group were 10 samples. The results show that on the average treatment before 2.5% chitosan has an average bacterial colony of 87.8 while in the group after 69.9. There was a decrease in the number of bacterial colonies after chewable lozenges chitosan chewable treatment with a concentration of 2.5%. In the chewable lozenges chitosan chewable treatment with a concentration of 5% showed that the number of bacterial colonies before treatment was 57.6 while 42.2. In addition, in the market chewing xylitol brand candy obtained by the number of bacterial colonies before treatment was 22.9 while at the time after treatment was 50.7. In the treatment of market brand xylitol candy there is an increase in the number of bacterial colonies between before and after. Based on the normality test, it was found that the normal data for the control group and chewable lozenges chewing content of 2.5% and 5% because the p-value was greater than 0.05. The effect of the treatment given, testing is done using the paired t test as follows:

Table 2: T-Paired Test of Chewable Lozenges of Chitosan 2,5%

	Difference in a couple			
	Mean	Standard Deviation	T	P-value
Pair 1 Pre Chitosan 2.5% Post Chitosan 2.5%	-17.9	20.306	2.788	0.021

(Source: Primary Data, 2019)

Based on Table 2. shows the test results using t paired analysis on chewable lozenges chitosan chewing treatment with a concentration of 2.5%. The results show that the average difference between the groups before and after treatment was -17.9 with a standard deviation of 20.306. The negative average difference of -17.9 indicates that the number of *S. mutans* bacterial colonies decreased after chewable lozenges chitosan chewing with a concentration of 2.5%. So that the chewable lozenges chitosan chewable treatment with a concentration of 2.5% can reduce the number of *S. mutans* bacterial colonies. T paired test results obtained a p-value of 0.021. This shows that the p-value obtained is smaller than 0.05. So it can be concluded that the chewable lozenges chitosan chewing treatment with a concentration of 2.5% has a significant effect in reducing the number of bacterial colonies in the mouths of children with ECC.

Table 3: T Paired Test of Chewable Lozenges of Chitosan 5%

Paired Samples Test					
		Difference in a couple			
		Mean	Standard Deviation	T	P-value
Pair 1	Pre Chitosan 5% Post Chtosan 5%	-15.4	9.240	5.270	0.001

(Source: Primary Data, 2019)

In Table 3. the results obtained using the t paired analysis of the treatment group in the condition before and chewable chewable lozenges chitosan concentration of 5%. The difference between the average group before and after is -15.4 with a standard deviation of 9.240. The negative average difference of -15.4 showed that there was a decrease in the number of *S. mutans* bacterial colonies after being treated with chewable lozenges chitosan concentration of 5%. From the paired t test results obtained p-value of 0.001. This shows that the p-value obtained is smaller than 0.05 so it can be concluded that the chewable lozenges chitosan treatment concentration of 5% has a significant effect in reducing the number of bacteria in the mouths of children with ECC.

Table 4: T Paired Test of Chewable Lozenges of Xylitol

		Difference in a couple		T	P-value
		Mean	Standard Deviation		
Pair 1	Pre Xylitol - Post Xylitol	27.80000	10.993	7.997	0.000

(Source: Primary Data, 2019)

Based on table 4, the results of the control group test were obtained before and after chewing on the market-brand xylitol candy. The results show that the average difference between the control group before and after chewing the market brand xylitol candy was 27.8 with a standard deviation of 7.997. A positive average difference of 27.8 indicates that an increase in the number of bacterial colonies after chewing chewable lozenges pure xylitol. Based on t paired sales, the p-value is 0,000. This shows that the p-value obtained is smaller than 0.05 so that the control group chewing market xylitol brand candy is very significant in increasing the number of bacterial colonies in a child's mouth.

To see the most significant treatment group in reducing the number of bacteria, a comparison is performed before and after using the one-way ANOVA test, with the average difference data as follows:

Table 5: Anova Test of Control Group and Treatment Group

Treatment	Average Difference	F	Sig.
Pre Chitosan 2.5% - Post Chitosan 2.5%	-17.900		
Pre Chitosan 5% - Post Chitosan 5%	-15.400		
Pre Xylitol - Post Xylitol	27.800	32.019	0.000

(Source: Primary Data, 2019)

Based on Table 5. the results of the comparison of the average difference in each control group and the treatment before and after. It was shown that the largest negative average difference occurred in the chewable lozenges chitosan chewable treatment group concentration of 5%. While the treatment group chewable lozenges chitosan concentration of 2.5% had a difference before and after - 17.9. Whereas in the control group, the difference between the average before and after was 27.8. So that the overall test using the one way ANOVA test results obtained that the p-value of 0.05. A p-value less than 0.05 indicates that the treatment group was significantly different from the treatment group. To see the most influential treatment, further testing of LSD is carried out as follows:

Table 6: Average Difference of Treatment Control

		Mean Difference (I-J)	Std. Error	Sig.
Control	Chitosan 2,5%	45.70*	6.4215 5	0.000
	Chitosan 5%	43.20*	6.4215 5	0.000

(Source: Primary Data, 2019)

Based on the LSD follow-up test results, it was found that the difference in the average of the control group by chewable lozenges chitosan concentration of 2.5% had a difference of 45.7 while the difference between the control group and the chewable lozenges of chitosan treatment group concentration of 5% has a difference of 43.2. Each p-value obtained for testing the difference in average of 0.000. This shows that the p-value obtained is smaller than 0.05 so that the two treatment groups influence the decrease of bacteria, but the most influential is the chewable lozenges chitosan concentration of 2.5% because it has the difference in difference with the largest control group.

DISCUSSION

Sampling of bacteria by taking saliva of children who have caries. This is done because the saliva of the teeth that have caries has the number of *Streptococcus mutans* bacteria as the initiator of the occurrence of dental caries in the teeth. Bacterial samples (saliva) were stored in vial bottles followed by a 10^{-3} dilution method. 10^{-3} dilution is done because it is more effective in reducing the number of colonies and bacteria will be seen more clearly when observing according to research journals conducted previously. This study uses a selective medium, namely TYSB20 (*Tryptone Yeast Extract Cystein Sucrose Bacitracin 20*) to breed *Streprococcus mutans*.[20]

Dental caries or cavities are often associated with the role of *Streptococcus mutans*. The process of caries infection begins with the attachment of *Streptococcus mutans* to the tooth surface. This is because *Streptococcus mutans* has the enzyme glucosiltransferase which can break down sucrose into glucans in large quantities. Predominantly, *Streptococcus mutans* forms dextran chains that are insoluble in water and have a sticky power to colonize the tooth surface. Furthermore, these bacteria form organic acids from sucrose. Sucrose metabolism by *Streptococcus mutans* produces lactic acid which is an acid that can cause decalcification of teeth. [21,22,23]

This study uses chitosan with concentrations of 2.5% and 5% based on research conducted by Visveswaraiah[15], saying that chitosan is effective and safe for the body with concentrations of 2.5% and 5%. According to research conducted by Achmad[18], chitosan toothpaste with a concentration of 2.5% and 5% has been shown to reduce the number of *Streptococcus mutans* bacterial colonies after use by children with early childhood caries. Table 1. shows that chewable lozenges chitosan has a significant effect in reducing the number of *Streptococcus mutans* bacterial colonies, it appears that chewable lozenges chitosan 2.5% is a type of group that gives a very significant change compared to market brand xylitol candy and chewable lozenges chitosan 5%. Chewable lozenges chitosan works as an antibacterial agent by combining the ability of salivary flow when chewing lozenges in inhibiting bacterial cell division and chitosan in disrupting bacterial metabolism through inhibition of bacterial glycolysis.

Chitosan has also been widely used in the pharmaceutical industry in drug delivery systems in different forms, such as tablets, microspheres, and conjugates. Chitosan and its derivatives can be used in various forms of drug delivery systems through the mouth, nose and through the eyes. In addition, chitosan facilitates transmukosal absorption which is important in the delivery of some polar drugs such as peptides along with proteins to the nose and mouth. It is commonly used as an excipient in tablet formulations for oral drugs. High molecular weight chitosan will delay the release of active ingredients, extend the duration of drug activity, increase therapeutic efficiency and reduce the side effects of oral tablets.[22,24,25]

Chitosan has biocompatible properties, biodegradability, and low immunogenicity. The high density of positive charges makes chitosan have mucoadhesive properties. This property allows the delivery of drugs to mucosal tissue. Chitosan also has very low toxicity. Chitosan is a form of chitin which is partially deacetylated and has received attention as a potential new functional or excipient material in the pharmaceutical industry. Chitosan exhibits good superiority and chemical and physical stability.[23,24,25,26,27,28] Therefore, chewable lozenges chitosan has an excellent antibacterial in reducing the number of *Streptococcus mutans* colonies in cases of early childhood caries (ECC).

CONCLUSION

Based on research that has been done, it can be concluded as follows:

1. Chewable lozenges chitosan 2.5% and 5% have a significant effect on the growth of *Streptococcus mutans* bacteria, which decreases the number of Streptococcus mutans colonies after chewing for 5 minutes.
2. Chewable lozenges chitosan 2.5% more effective than chewable lozenges chitosan 5% and xylitol candy brand market, so that in this study, chitosan toothpaste 2.5% had a more significant effect in reducing the number of *Streptococcus mutans* bacteria.

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