

In vitro* antimicrobial activity of chitosan mouthwash against *Streptococcus mutans* and *Lactobacillus casei**Kultida Thongaroon^{1,*}, Araya Phonghanyudh¹, Rudee Surarit², Petcharat Kraivaphan³ and Nuttawee Niamsiri⁴**¹*Department of Pediatric Dentistry, Faculty of Dentistry, Mahidol University, Bangkok 10400, Thailand*²*Department of Oral Biology Faculty of Dentistry, Mahidol University, Bangkok 10400, Thailand*³*Department of Pharmacology Faculty of Dentistry, Mahidol University, Bangkok 10400, Thailand*⁴*Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

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Abstract

Objective: The aim of this study was to investigate the antimicrobial effect of chitosan mouthwash against *Streptococcus mutans* and *Lactobacillus casei* in the laboratory.

Materials and Methods: The chitosan mouthwashes were prepared from 3 different molecular weights (30 kDa, 890 kDa and 1,700 kDa). The solutions of the chitosan mouthwash were prepared for 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml by dissolving chitosan powder with 3 molecular weights in each concentration. Chitosan mouthwash was formulated from chitosan powder, acetic acid, saccharin, blueberry flavor, sodium hydroxide and glycerol. *Streptococcus mutans* and *Lactobacillus casei* were cultured on BHI agar and MRS agar respectively and were transferred to 5 ml of BHI broth and MRS broth respectively and incubated for 24 hours. Measure the optical density (OD) of both bacteria and adjusted the initial load of bacteria to 2×10^6 CFU/ml. Determined the antimicrobial effect of chitosan mouthwash against both bacteria using time-dependent killing assay by viable count technique with various concentrations of 5mg/ml, 2.5 mg/ml and 1.25 mg/ml. Samples were collected at 0 hour, 30 mins, 2 hours, 6 hours, and 12 hours. The inhibition of bacterial growth was determined by standard plate count technique. The tube with 0.12% chlorhexidine mouthwash was used as positive control and mouthwash solution without chitosan and Phosphate buffer saline (PBS) were used as negative controls.

Result: The results showed 5mg/ml, 2.5mg/ml and 1.25mg/ml of the chitosan mouthwash have bactericidal effect against *Streptococcus mutans* and *Lactobacillus casei*. All concentrations of the chitosan mouthwash showed an ability to inhibit the bacterial growth more than 3 log CFU/ml against *Streptococcus mutans* within 12 hours. For *Lactobacillus casei*, the lowest concentration that could inhibit growth of bacteria more than 3 log CFU/ml within 12 hours is 2.5 mg/ml. The lower molecular weight (30 kDa and 890kDa) of chitosan mouthwash could inhibit the growth of *Streptococcus mutans* more than the higher molecular weights (1700 kDa). Interestingly, the higher molecular weight (890 kDa and 1,700 kDa) of chitosan mouthwash could inhibit the growth of *Lactobacillus casei* more than the lower molecular weight (30 kDa).

Keywords: Chitosan mouthwash, antimicrobial effect, *Streptococcus mutans*, *Lactobacillus casei*

Introduction

FDI World Dental Federation reported that one of the most prevalent oral disease is dental caries (Hobdell, 2003). Dental caries is a multifactorial disease that is developed from the interaction among host, microorganism and environment (Daboor, 2015). *Streptococcus mutans* and *Lactobacillus casei* are the main etiologies of dental caries in term of microorganism factors (Selwitz, 2007; Karpinski, 2013). Antimicrobial mouthwash was considered as an alternative method for home care use for the reduction of oral bacteria (Oyanagi, 2012). Chlorhexidine mouthwash was considered as the gold standard of alternative home care use, but still having the unfavorable outcomes and that new agents are needed to be studied (Cunha, 2017; Flotra, 1971; Flotra, 1973). Chitosan is a polymer obtained from chitin with antimicrobial property and is focused from the researcher to be an alternative agent (Atai, 2017; Tanikonda, 2014; Costa, 2014; Costa, 2014). Chitosan mouthwash was considered as alternative method due to its broad antimicrobial spectrum, antifungal activity, biocompatible, biodegradable and low cytotoxicity (Costa, 2014). Moreover, it provide clinical benefits for plaque control and extended retention time on the oral mucosa (Ahu, 2012). The aim of this study was to investigate the antimicrobial effect of chitosan mouthwash against *Streptococcus mutans* and *Lactobacillus casei* in the laboratory.

Materials and Methods

Chitosan mouthwash preparation: Chitosan mouthwash were prepared from 3 molecular weights (30 kDa, 890 kDa and 1,700 kDa) of commercially available food-grade chitosan samples obtained from Marine Bio Resources Co., LTD (Thailand) with degree of deacetylation more than 90%.

A solutions of chitosan mouthwash were prepared for 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml by dissolving chitosan powder with 3 molecular weights in each concentration, in 1 % v/v acetic acid solution, 35 ml, stirring with magnetic stirrer at room temperature until chitosan was completely dissolved. Add 0.004 g of saccharin and 175 μ l of blueberry flavor into the solution. Gradually added 1M NaOH solution into pH 6.10. After that, add 5 ml of glycerin and distilled water until the solution reached 50 ml.

Agar medium preparation: Prepared Brain Heart Infusion agar (BHI agar) from 52 g of dehydrated BHI medium and a liter of distilled water. Boiled and stirred with magnetic stirrer and sterilized by autoclaving for 20 minutes. Poured the agar into sterile petri dish to a uniform depth of 4 mm and allowed to solidify. Stored in the refrigerator inside airtight plastic bags at 2-8°C for up to 4 weeks. De Man, Rogosa and Sharpe agar (MRS agar) were prepared with the same method by using 55 g of dehydrated MRS medium instead.

Bacterial suspension preparation: *Streptococcus mutans* was cultured on BHI agar and inoculate in anaerobe jar at 37°C. The single colony was transferred to culture in 5 ml BHI broth and incubated for 24 hours. *Lactobacillus casei* was cultured on MRS agar and inoculated in anaerobe jar at 37°C then transferred the single colony to culture in 5 ml MRS broth and incubated for 24 hours. Measure the optical density (OD) of both bacteria and adjusted the initial load of bacteria to 2×10^6 CFU/ml.

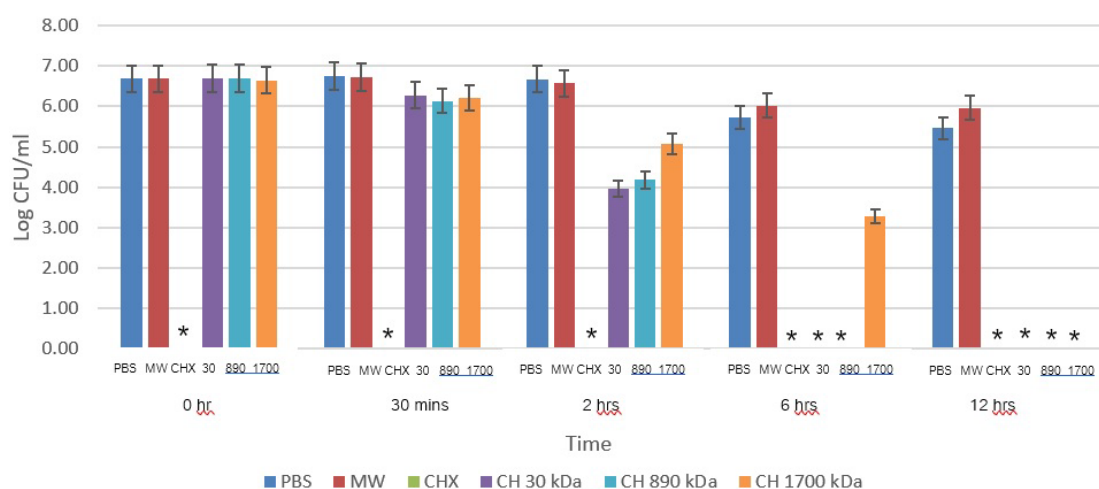
Time-dependent killing effect by viable count technique: Determined the antimicrobial effect of chitosan mouthwash against *Streptococcus mutans* and *Lactobacillus casei* using Time-dependent killing effect by viable count technique with various concentrations of 5mg/ml, 2.5 mg/ml and 1.25 mg/ml. Briefly, *Streptococcus mutans* and *Lactobacillus casei* were inoculated and adjusted an overnight culture to 2×10^6 CFU/ml in BHI broth. Mixed 1 ml of inoculum with 1 ml of chitosan mouthwash at 10 mg/ml, 5 mg/ml and 2.5 mg/ml in the tubes, after mixing the concentration would decrease to be 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml then incubated in anaerobe jar at 37°C for 12 hrs. Samples were collected at 0 hour, 30 mins, 2 hours, 6 hours, and 12 hours. The reduction of bacterial was determined by standard plate

count technique. The tube with 0.12% chlorhexidine mouthwash was used as positive and mouthwash solution without chitosan and PBS were used as negative controls.

Data analysis: The data were statistically analyzed by one-way ANOVA analysis of variances with Duncan's multiple range test at significance level of 0.05 using SPSS program version 22.0. All microbial experiments were performed with triplicate samples.

Results and Discussion

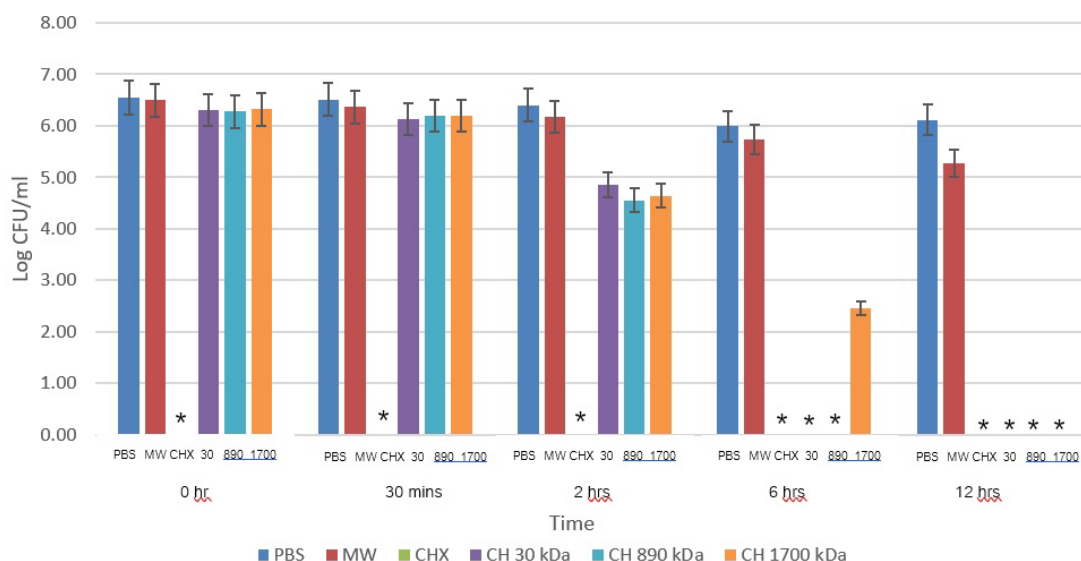
1. Time-dependent killing assay against *Streptococcus mutans* by 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml chitosan mouthwash with different molecular weights (30 kDa, 890 kDa, 1,700kDa)



* indicated for the amount of bacteria that were not shown in the figure were less than 100 CFU/ml

Figure 1 Amount of *Streptococcus mutans* by 5 mg/ml chitosan mouthwash with different molecular weights (30 kDa, 890 kDa, 1,700kDa) (PBS=Phosphate Buffer Saline, MW=Mouthwash Solution without chitosan, CHX=0.12%Chlorhexidine mouthwash, CH=Chitosan mouthwash).

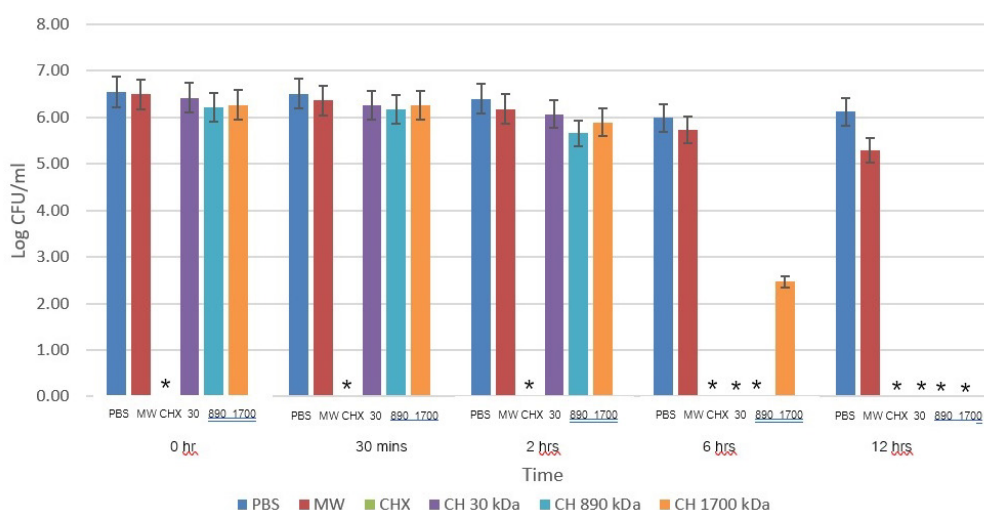
The 5 mg/ml Chitosan mouthwash with 30 kDa, 890 kDa and 1,700 kDa could reduce 2.64 log CFU/ml, 2.51 log CFU/ml and 1.58 log CFU/ml from the initial load respectively at 2 hours. At 6 hours, chitosan mouthwash with 30 kDa and 890 kDa could reduce *Streptococcus mutans* more than 3 log CFU/ml while 1,700 kDa reduced lesser. The bacterial reduction of chitosan mouthwash against *Streptococcus mutans* showed more than 3 log CFU/ml within 12 hours in all the different molecular weights (Figure 1)



* indicated for the amount of bacteria that were not shown in the figure were less than 100 CFU/ml

Figure 2 Amount of *Streptococcus mutans* by 2.5 mg/ml chitosan mouthwash with different molecular weights (30 kDa, 890 kDa, 1,700kDa) (PBS=Phosphate Buffer Saline, MW=Mouthwash Solution without chitosan, CHX=0.12%Chlorhexidine mouthwash, CH=Chitosan mouthwash).

In 2.5 mg/ml chitosan mouthwash, the pattern of bacterial reduction was similar to the 5 mg/ml chitosan mouthwash. Chitosan mouthwash with 30 kDa, 890 kDa and 1,700 kDa could reduce 1.51 log CFU/ml, 1.73 log CFU/ml and 1.68 log CFU/ml respectively from the initial load at 2 hours, then at 6 hours, chitosan mouthwash with 30 kDa, 890 kDa and 1,700 kDa can reduce *Streptococcus mutans* more than 3 log CFU/ml. The bacterial reduction of chitosan mouthwash showed more than 3 log CFU/ml within 12 hours in all the different molecular weights. (Figure2).

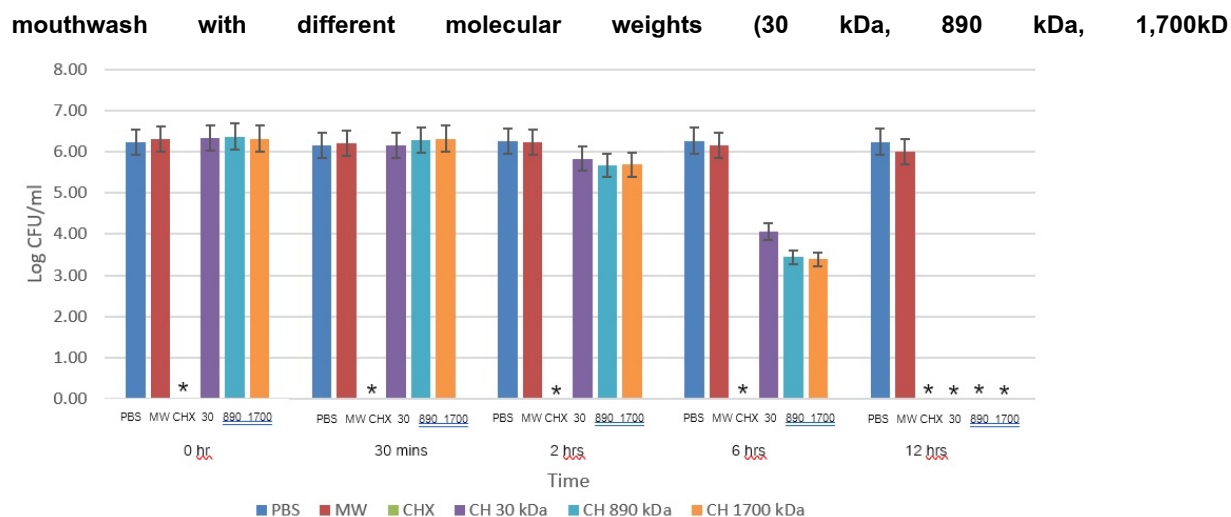


* indicated for the amount of bacteria that were not shown in the figure were less than 100 CFU/ml

Figure 3 Amount of *Streptococcus mutans* by 1.25 mg/ml chitosan mouthwash with different molecular weights (30 kDa, 890 kDa, 1,700kDa) (PBS=Phosphate Buffer Saline, MW=Mouthwash Solution without chitosan, CHX=0.12%Chlorhexidine mouthwash, CH=Chitosan mouthwash).

For 1.25 mg/ml concentration, chitosan mouthwash with 30 kDa and 890 kDa could reduce *Streptococcus mutans* more than 3 log CFU/ml at 6 hours while at 12 hours, chitosan mouthwash with all molecular weights could reduce more than 3 log CFU/ml. (Figure3).

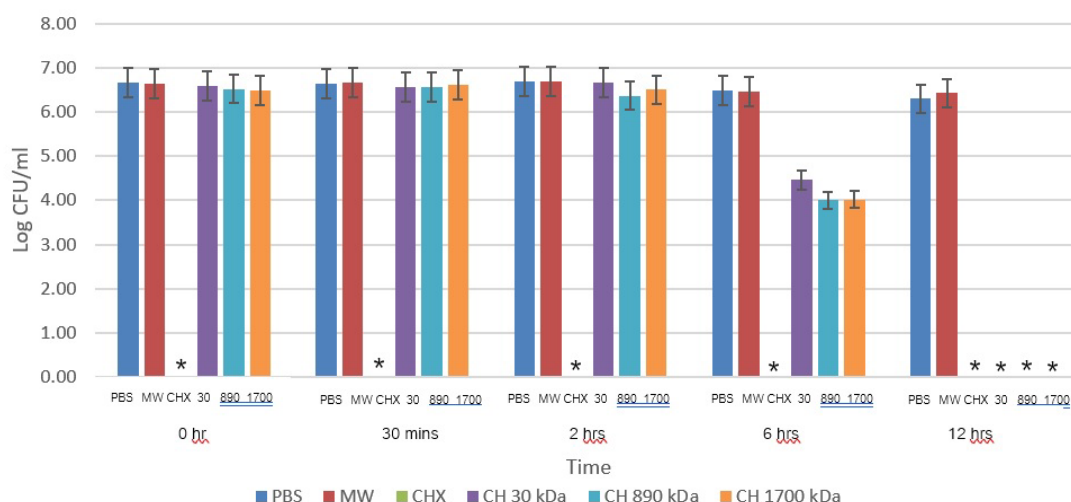
1. Time-dependent killing assay against *Lactobacillus casei* by 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml chitosan



* indicated for the amount of bacteria that were not shown in the figure were less than 100 CFU/ml

Figure 4 Amount of *Lactobacillus casei* by 5 mg/ml chitosan mouthwash with different molecular weights (30 kDa, 890 kDa, 1,700kDa) (PBS=Phosphate Buffer Saline, MW=Mouthwash Solution without chitosan, CHX=0.12%Chlorhexidine mouthwash, CH=Chitosan mouthwash).

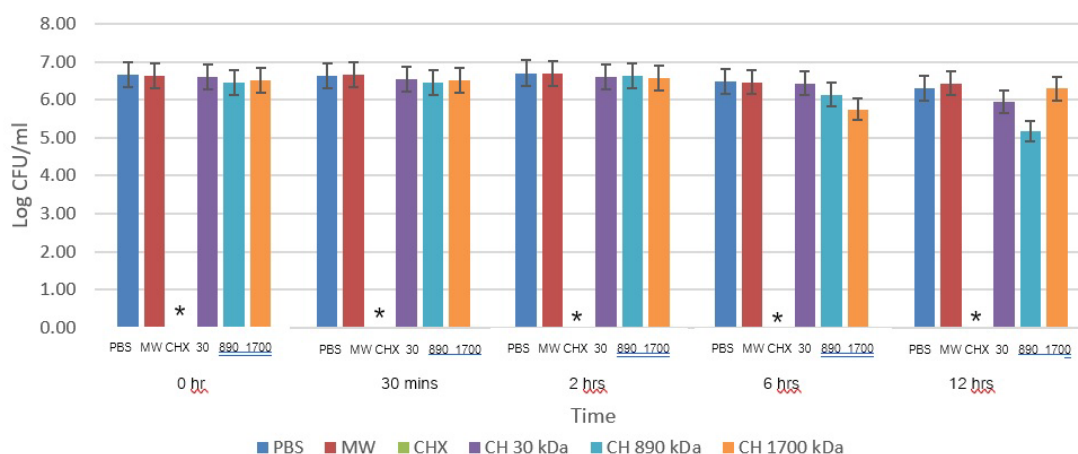
The 5 mg/ml Chitosan mouthwash with 30 kDa, 890 kDa and 1,700 kDa could reduce 2.27 log CFU/ml, 2.94 log CFU/ml and 1.93 log CFU/ml from the initial load respectively at 6 hours. The bacterial reduction of chitosan mouthwash against *Lactobacillus casei* showed more than 3 log CFU/ml within 12 hours in all the different molecular weights. (Figure4).



* indicated for the amount of bacteria that were not shown in the figure were less than 100 CFU/ml

Figure 5 Amount of *Lactobacillus casei* by 2.5 mg/ml chitosan mouthwash with different molecular weights (30 kDa, 890 kDa, 1,700kDa) (PBS=Phosphate Buffer Saline, MW=Mouthwash Solution without chitosan, CHX=0.12%Chlorhexidine mouthwash, CH=Chitosan mouthwash).

In 2.5 mg/ml chitosan mouthwash, the pattern of bacterial reduction was similar to the 5 mg/ml chitosan mouthwash. Chitosan mouthwash with 30 kDa, 890 kDa and 1,700 kDa could reduce 2.14 log CFU/ml, 2.56 log CFU/ml and 2.48 log CFU/ml respectively at 6 hours, the bacterial reduction of chitosan mouthwash showed more than 3 log CFU/ml within 12 hours in all the different molecular weights. (Figure 5).



* indicated for the amount of bacteria that were not shown in the figure were less than 100 CFU/ml

Figure 6 Amount of *Lactobacillus casei* by 1.25 mg/ml chitosan mouthwash with different molecular weights (30 kDa, 890 kDa, 1,700kDa) (PBS=Phosphate Buffer Saline, MW=Mouthwash Solution without chitosan, CHX=0.12%Chlorhexidine mouthwash, CH=Chitosan mouthwash).

In 1.25 mg/ml chitosan mouthwash with 30 kDa and 890 kDa could reduce only 0.49 log CFU/ml, 0.97 log CFU/ml respectively at 12 hours while chitosan mouthwash with 1700kDa increase the amount of bacteria 0.54 log CFU/ml. (Figure 6)

This study showed the lowest concentration that have bactericidal effect against *Lactobacillus casei* within 12 hours is 2.5 mg/ml.

From the results, they were shown that 5 mg/ml and 2.5 mg/ml chitosan mouthwashes inhibited growth of *Streptococcus mutans* and *Lactobacillus casei* within 12 hours. The amount of bacteria less than 100 CFU/ml were not shown from the study. Only 1.25 mg/ml chitosan mouthwash was less likely to inhibit growth of *Lactobacillus casei*. The killing effect of bacteria from chitosan was based on the higher degree of deacetylation (Goy, 2009). The chitosan used in this study was more than 90% degree of deacetylation. The degree of deacetylation is the determinant in the solubility and the charge development (Goy, 2009). Moreover, *Streptococcus mutans* and *Lactobacillus casei* are gram-positive bacteria. The teichoic acid which are essential polyanionic polymers in cell wall of gram-positive bacteria contribute to the negative charge of cell wall. The polycationic of chitosan interacts cell surface of bacteria and causing extensive cell surface alterations, leakage of intracellular substances leading to cell death (Raafat, 2009). The study of Costa et al. also reported that when chitosan interacted with cell wall, it could generate pore formation and completely membrane disruption which leads to impairment of vital bacterial activities (Costa, 2012)

Our study showed that the lower molecular weight (30 kDa and 890kDa) of chitosan mouthwash could inhibit the growth of *Streptococcus mutans* more than the higher molecular weights (1700 kDa) within 2 hours. This might be explained that the mobility attraction and ionic interaction of small chains of low molecular weight chitosan are easier than the large chain facilitating the adoption of an extended conformation and an effective binding to the membrane surface of *Streptococcus mutans* (Goy, 2009). Interestingly, the higher molecular weight (890 kDa and 1,700 kDa) of chitosan mouthwash could

inhibit the growth of *Lactobacillus casei* more than the lower molecular weight (30 kDa). This result agrees with the study of Sette-de-Souza et al. that reported that the higher molecular weight of chitosan has greater the antimicrobial activity against gram-positive bacteria (Sette-de-Souza, 2015).

Conclusions

The chitosan mouthwash in this study could inhibit the growth of *Streptococcus mutans* and *Lactobacillus casei*. The chitosan mouthwash can reduce the amount of *Streptococcus mutans* faster than *Lactobacillus casei*. The high molecular weight (1700kDa) can inhibit the growth of *Lactobacillus casei* more than the lower molecular weight while the low molecular weight (30 kDa) can inhibit *Streptococcus mutans* more than the higher ones. The higher concentration of the chitosan mouthwash can inhibit more amount of *Streptococcus mutans* and *Lactobacillus casei* than the lower concentration at the same interval time. The lowest concentration that effective in inhibiting *Streptococcus mutans* is 1.25 mg/ml while the lowest concentration for *Lactobacillus casei* is 2.5 mg/ml.

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