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*Original Article*

# Effect of oral moisturizers containing chitosan and poloxamer 407 on biofilm formation of *Candida* species and *Streptococcus mutans: in vitro*

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**Abstract**

Oral moisturizers are topical agents which can directly relieve xerostomia and hyposalivation. This study evaluated the effects of three newly developed oral moisturizers (1) a formulation containing 0.5% chitosan (CHI) with 15% poloxamer 407 (P407), (2) a formulation containing only 0.5% CHI, and (3) a formulation containing only 15% P407, on adhesion and biofilm formation of three *Candida* strains, i.e., *Candida albicans* DMST 5815, *Candida krusei* and *Candida tropicalis* from clinical isolates and *Streptococcous mutans* DMST 18777, compared with a commercially available oral moisturizer by using crystal violet assay. The results showed, in contrast to the commercial oral moisturizer, a significant reduction of *Candida* species and *S. mutans* adhesion and biofilm formation after exposure to the three formulations ($P < 0.05$). The formulation containing CHI with P407 exhibited the strongest anti-adhesion and anti-biofilm properties against *Candida* species. At the same time, these combinations of CHI with P407 formulation may not be good for against *S. mutans* adhesion and biofilm. In contrast, the formulation containing only CHI displayed strikingly stronger anti-adhesion and anti-biofilm effects against *S. mutans* than the CHI with P407 formulation. This evidence confirms the great potential of anti-adhesion and anti-biofilm properties of CHI against *Candida* species and *S. mutans*.

**Keywords**: oral moisturizer, chitosan, poloxamer 407, *Candida* species, *Streptococcus mutans*

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## 1. Introduction

Xerostomia and salivary gland dysfunction are conditions that disturb oral homeostasis and significantly affect function in the oral cavity. Consequently, negative effects on quality of life can culminate in oral diseases, including dental caries, oral candidiasis, taste disturbances, burning sensation and difficulty chewing, swallowing, and speaking (Villa *et al*., 2015). A compromised oral cavity allows for increased fungal infections (*Candida* species), which remain the leading cause of oral candidiasis and multidrug-resistant fungal infections. In addition, bacterial infections, such as *Streptococcus mutans*, are the primary cause of dental caries (Ligtenberg & Almståhl, 2015; Sardi, Scorzoni, Bernardi, Fusco-Almeida, & Mendes Giannini, 2013). Treatment of xerostomia can be divided into two categories: systemic sialagogues and topical agents. Topical agents, such as artificial saliva or oral moisturizer, are first-line treatments recommended for xerostomia. Most readily available oral moisturizers mainly use mucoadhesive polymers such as sodium carboxymethylcellulose, xanthan gum, hydroxyethylcellulose and polyethene glycol to lubricate and hydrate the oral tissues. The polymers generally increase the viscosity of the products and prolong adhesion at the surface of the oral cavity (Hu, Andablo-Reyes, Mighell, Pavitt, & Sarkar, 2021). However, to date, the combinations of the mucoadhesive polymer of chitosan (CHI) with poloxamer 407 (P407) have not been formulated and investigated as oral moisturizers.

CHI is a polysaccharide prepared from the deacetylation of chitin, which is found in the shell of shrimp and crab. CHI has several biological properties such as biocompatibility, biodegradability, lack of allergenicity, non-toxicity, film-forming ability and serving to moisturize (Wang *et al*., 2020). CHI has potent antimicrobial activity against various microorganisms, including viruses, fungi, bacteria, and algae (Goy, Britto, & Assis, 2009). In addition, oral care products containing CHI have exhibited pharmacological actions such as prevention of caries, control of biofilm formation, and alleviation of oral mucositis symptoms (Carlson, Taffs, Davison, & Stewart, 2008; Mahattanadul *et al*., 2018; Mahima, Patil, Kulkarni, Tayal, & Keshari, 2015; Pasquantonio *et al*., 2008). P407 is a thermo-responsive hydrogel that is widely used in mucosal drug delivery systems, with several beneficial properties such as non-irritation, good solubilizing capacity, low toxicity, good drug-release characteristics, and compatibility with numerous biomolecules and chemical excipients (Giuliano, Paolino, Fresta, & Cosco, 2018). However, P407 rapidly dissolves in aqueous media resulting in a short residence time on the oral mucosa. The addition of mucoadhesive polymers has improved the mechanical and mucoadhesive properties of P407 (Gratieri *et al*., 2010). At present, a variety of oral moisturizers are available to the public with various claims for efficacy. However, there has been very little research on the effects of such products on adhesion and biofilm formation of oral microorganisms. The biofilms are surface-attached communities of microorganisms embedded in an extracellular matrix of biopolymeric substances, and may be the leading cause for the failure of antibiotic treatments and can cause many chronic infections (O'Toole, Kaplan, & Kolter, 2000). An increasing biofilm formation may contribute to the development of consequent oral diseases. Therefore, newly developed oral moisturizers require an understanding of the influence of their components on the biofilm formation of main oral pathogenic microorganisms. However, no reported study on the effects of an oral moisturizer containing CHI with P407 on biofilm formation of *Candida* species and *S. mutans* seems to be available. Thus, this study was designed to evaluate the effects of a newly developed oral moisturizer containing CHI with P407, a formulation containing only CHI, and a formulation containing only P407, on the biofilm formation of *Candida* species and *S. mutans*, compared to a commercial oral moisturizer.

## 2. Materials and Methods

### 2.1 Research design

This study was a laboratory-based comparison of three newly developed oral moisturizers and a commercial oral moisturizer on the adhesion and biofilm formation of *Candida* species and *S. mutans* developed on glass coverslips (Figure 1).

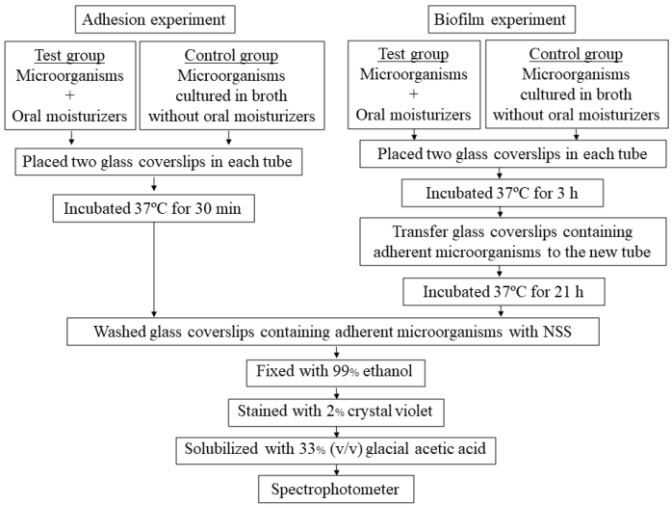


Figure 1. The experimental design of the study

### 2.2 Preparation of oral moisturizers

The three formulations of oral moisturizer were manufactured by the Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The three different formulations were (1) a formulation containing 0.5% CHI (average viscosity molecular weight of 414 kDa and 91% degree of deacetylation; Sigma-Aldrich, Iceland) with 15% P407 (Chanjao Longevity Company Limited, Thailand), (2) a formulation containing only 0.5% CHI, (3) a formulation containing only 15% P407 (Table 1). Additives including magnesium sulfate, potassium chloride, calcium chloride, sodium fluoride, methylparaben, propylparaben, xylitol, and flavors, were used in all formulations. The viscosity of the developed oral moisturizer was determined using a viscometer (Model DV-III Ultra, Brookfield, USA), spindle no.31, speed 50 rpm (or spindle no. T-F, speed 0.5 rpm). Mucoadhesive properties of the formulations were determined by using a Texture analyzer (TA.XT Plus, Stable Micro Systems, United Kingdom). The pH was measured using a digital pH meter (Mettler, Toledo, Switzerland). The commercial oral moisturizer consisted of purified water, glycerin, xanthan gum, xylitol, PEG-60, hydrogenated castor oil, VP/VA copolymer, flavor, sodium benzoate, methylparaben, propylparaben, sodium saccharin, and cetylpyridinium chloride.

### 2.3 Microorganisms and growth conditions

*C. albicans* DMST 5815 and *S. mutans* DMST 18777 were obtained from the culture collection of the Department of Medical Sciences, Ministry of Public Health, Thailand. *C. krusei* and *C. tropicalis* were isolated from patients who sought dental treatment at the Faculty of Dentistry, Khon Kaen University, Khon Kaen, Thailand. *Candida* species and *S. mutans* were grown on Sabouraud's dextrose agar (SDA) (Difco, Detroit, USA) and brain heart infusion (BHI) agar (HiMedia Pvt. Ltd. Mumbai, India), respectively. A preculture was obtained by inoculating 10 ml of Sabouraud's dextrose broth (SDB) and BHI broth with a single colony of *Candida* species and *S. mutans* initially grown on agar, respectively. The cultures were incubated at 37˚C for 24 h and used as inoculum in all experiments.

### 2.4 Determination of adhesion capacity of *Candida* species and *S. mutans*

The adhesion of each isolate on glass coverslips after 30 min incubation in each oral moisturizer and a commercial oral moisturizer was determined using the method described previously (Jin, Samaranayake, Samaranayake, & Yip, 2004; Lachica *et al*., 2019) with modifications. Briefly, 2% inocula (v/v) were inoculated into fresh media and incubated at 37ºC for 24 h. After incubation, the microorganisms were adjusted to an optical density (OD) at 540 nm of 0.8-0.9 in fresh media equivalent to $1.38 \times 10^7$ colony-forming units (CFU)/mL of *Candida* species and $9.80 \times 10^7$ CFU/mL of *S. mutans*. An equal volume of each microorganism suspension was added and mixed with each oral moisturizer in a 50 mL conical tube. Then two glass coverslips (22 x 22 mm) were placed in each tube. Tubes containing each microorganism cultured in the broth without oral moisturizer served as a control. The tubes were incubated at 37ºC for 30 min to allow the microorganism adhesion to the glass coverslips. Then the glass coverslips containing adherent microorganisms were washed with 15 mL of normal saline solution (NSS). The attached microorganisms, representing a 30-min adhesion culture, were fixed with 15 mL of 99% ethanol for 15 min, and the glass coverslips were dried at room temperature. The glass coverslips were stained for 5 min with 2% crystal violet and washed 3 times to remove excess stain. The dye bound to the adherent cells was solubilized with 33% (v/v) glacial acetic acid. The OD of the dye solubilized from each glass coverslip was measured at 630 nm using a spectrophotometer (Genesys20, Thermo Scientific, USA). Each assay was performed on four separate occasions, with duplicate determinations each time. The results reported are the mean±SEM from these 4 independent experiments.

### 2.5 Determination of biofilm-forming capacity of *Candida* species and *S. mutans*

The quantitative estimation of the biofilm produced was determined according to a method described previously with some modification (Taweechaisupapong *et al*., 2005). Briefly, 2% inocula (v/v) were inoculated into fresh media and incubated at 37ºC for 24 h. After incubation, the microorganisms were adjusted to an optical density (OD) at 540 nm of 0.8-0.9 in fresh media equivalent to $1.38 \times 10^7$ colony-forming units (CFU)/mL of *Candida* species and $9.80 \times 10^7$ CFU/mL of *S. mutans*. An equal volume of each microorganism suspension was added and mixed with each oral moisturizer and a commercial oral moisturizer in a 50 mL conical tube. Then two glass coverslips (22 x 22 mm) were placed in each tube. Tubes containing each microorganism cultured in the broth without oral moisturizer served as a control. The tubes were incubated at 37ºC for 3 h to allow the microorganism adhesion to the glass coverslips. Thereafter, the glass coverslips were transferred to the new tubes

Table 1. Composition and physicochemical characterization of oral moisturizer formulations

| Formulation components (%) | CHI with P407 | CHI | P407 |
|---|---|---|---|
| CHI | 0.5 | 0.5 | 0 |
| P407 | 15 | 0 | 15 |
| Additives | qs | qs | qs |
| pH | 6.34±0.01 | 6.30±0.01 | 6.42±0.01 |
| Viscosity (cp) | 271.70±1.20 | 17.60±1.60 | 1,416,667±28,868 |
| Gelation temperature (ºC) | N/A | N/A | 32 |
| Mucoadhesive force (N) | 0.21±0.11 | 0.39±0.02 | 0.12±0.03 |

qs quantity sufficient; cp centipoise; ºC degree Celsius; N newton

containing an equal volume of fresh medium and oral moisturizer and incubated at 37ºC for an additional 21 h. Then the glass coverslips containing adherent microorganisms were washed with 15 mL of NSS. The attached microorganisms, representing a 1-day biofilm culture, were fixed with 15 mL of 99% ethanol for 15 min, and the glass coverslips were dried at room temperature. The glass coverslips were stained for 5 min with 2% crystal violet and washed 3 times to remove excess stain. The dye bound to the adherent cells was solubilized with 33% (v/v) glacial acetic acid. The OD of the dye solubilized from each glass coverslip was measured at 630 nm using a spectrophotometer. The biofilm formation of each isolate was determined in 4 independent experiments, and the results reported are the mean±SEM from these 4 independent experiments. The percentage inhibiting effects of adhesion and biofilm formation were calculated using the formula [1- (OD sample /OD control)] x 100.

### 2.6 Statistical analysis

Comparing the mean±SEM of either adhesion or biofilm formation of *Candida* species and *S. mutans* between the three formulations of oral moisturizers, control and a commercial oral moisturizer were done using one-way analysis of variance (ANOVA) and Bonferroni *post hoc* test. Differences between groups were significant at a *P*-value of <0.05. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

## 3. Results

### 3.1 Effect of oral moisturizers on adhesion and biofilm formation of *Candida* species

The three newly developed oral moisturizers significantly decreased the adhesion and biofilm formation of *Candida* species compared to the commercial oral moisturizer (*P<0.05*) (Figures 2-4). The formulation containing CHI with P407 significantly decreased *C. albicans* adhesion (by 79%) over the control, 33% more than that of the formulation containing only CHI, 20% more than that of the formulation containing only P407, and 954% over the commercial oral moisturizer. The formulation containing CHI with P407 significantly decreased *C. krusei* adhesion (by 73%) over the control, 0.5% less than that of the formulation containing only CHI, 20% more than that of the formulation containing only P407, and 830% over the commercial oral moisturizer. The formulation containing CHI with P407 significantly decreased *C. tropicalis* adhesion (by 79%) over the control, 1% less than that of the formulation containing only CHI, 26% more than that of the formulation containing only P407, and 1,405% over the commercial oral moisturizer. The formulation containing CHI with P407 decreased *C. albicans* biofilm formation by 88% over the control, 39% more than that of the formulation containing only CHI, 14% more than the formulation containing only P407, and 1,002% over the commercial oral moisturizer. The formulation containing CHI with P407 significantly decreased *C. krusei* biofilm formation (by 96%) over the control, 1% less than the formulation containing only CHI, 4% more than that of the formulation containing only P407, and 186% over the commercial oral moisturizer. The formulation containing CHI with P407

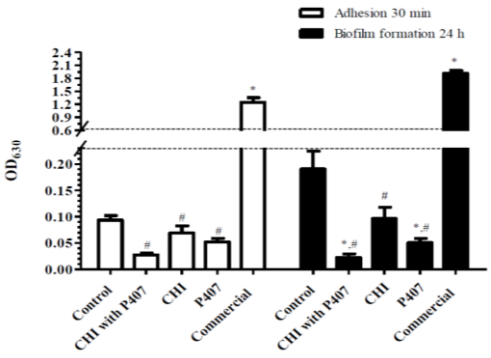


Figure 2. Adhesion and biofilm formation of *C. albicans* after exposure to 3 formulations of oral moisturizers, control and commercial oral moisturizer for 30 min and 24 h, respectively. Values are mean±SEM from 4 independent experiments (n=8).
$^{*}P < 0.05$ compared with control.
$^{\#}P < 0.05$ compared with commercial oral moisturizer.

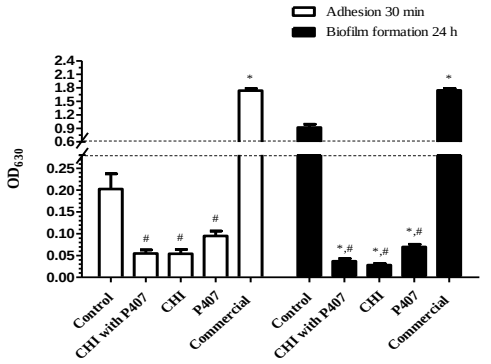


Figure 3. Adhesion and biofilm formation of *C. krusei* after exposure to 3 formulations of oral moisturizers, control and commercial oral moisturizer for 30 min and 24 h, respectively. Values are mean±SEM from 4 independent experiments (n=8).
$^{*}P < 0.05$ compared with control.
$^{\#}P < 0.05$ compared with commercial oral moisturizer.

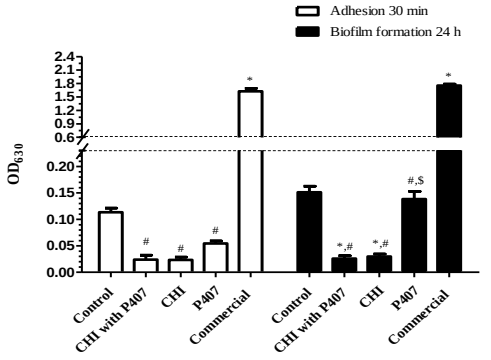


Figure 4. Adhesion and biofilm formation of *C. tropicalis* after exposure to 3 formulations of oral moisturizers, control and commercial oral moisturizer for 30 min and 24 h, respectively. Values are mean±SEM from 4 independent experiments (n=8).
$^{*}P < 0.05$ compared with control.
$^{\#}P < 0.05$ compared with commercial oral moisturizer.
$^{\$}P < 0.05$ compared with formulation containing CHI with P407.

significantly decreased *C. tropicalis* biofilm formation (by 83%) over the control, 3% more than that of the formulation containing only CHI, 75% more than that of the formulation containing only P407, and 1,144% over the commercial oral moisturizer.

### 3.2 Effects of oral moisturizers on adhesion and biofilm formation of *S. mutans*

*S. mutans* adhesion and biofilm formation after exposure to three newly developed oral moisturizers were statistically significantly less than that of the commercial oral moisturizer ($P<0.05$). Among the treatments, the formulation containing only CHI possessed the strongest anti-adhesion and anti-biofilm properties toward *S. mutans* (Figure 5). The formulation containing only CHI decreased *S. mutans* adhesion by 72% over the control, 1,082% over that of the formulation containing CHI with P407, 25% over that of the formulation containing only P407 and 2,062% over that of commercial oral moisturizer. Furthermore, the formulation containing only CHI decreased *S. mutans* biofilm formation by 92% over the control, 508% over the formulation containing CHI with P407, 21% over the formulation containing only P407 and 743% over the commercial oral moisturizer. The percentage inhibiting effects of *Candida* species and *S. mutans* adhesion and biofilm formation are summarized in Table 2.

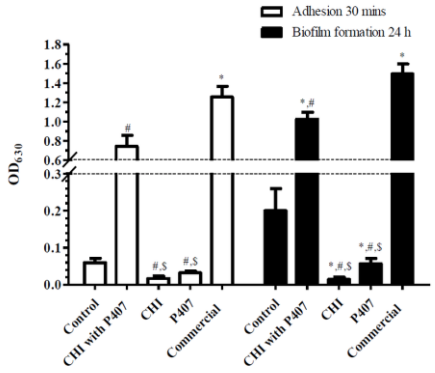


Figure 5. Adhesion and biofilm formation of *S. mutans* after exposure to 3 formulations of oral moisturizers, control and commercial oral moisturizer for 30 min and 24 h, respectively. Values are mean±SEM from 4 independent experiments (n=8).
*$P < 0.05$ compared with control.
#$P < 0.05$ compared with commercial oral moisturizer.
$$P < 0.05$ compared with formulation containing CHI with P407.

## 4. Discussion

It is well-known that CHI is a bioadhesive polymer with a well-established antimicrobial activity and inhibits biofilm formation, especially for *C. albicans* and *S. mutans* (Carlson *et al*., 2008; Pasquantonio *et al*., 2008). Therefore, CHI was selected to be one of the main constituents in the newly developed oral moisturizers for this study. A previous case-control study by Mahima *et al*. (2015) reported that 0.5% CHI mouthwash was effective and safe for treating oral mucositis following radio-chemotherapy. In addition, Mahattanadul *et al*., (2018) reported that 0.5% CHI mouthwash completely inhibited the biofilm of *C. albicans in vitro*, and *C. albicans* were not found in denture stomatitis patients after using the mouthwash for two weeks. Thus, 0.5% concentration of CHI was used in the oral moisturizer formulations for this study. The results in this study indicate that exposure of three *Candida* species to the formulation containing only CHI and the formulation containing CHI with P407 reduced adhesion and biofilm formation compared with the unexposed controls. These results are in line with previous studies (Carlson *et al*. 2008; Costa, Silva, Tavaria, & Pintado, 2014a; Tan, Leonhard, Moser, & Schneider-Stickler, 2016), and with Carlson *et al*. (2008) which showed that CHI could reduce *C. albicans* adhesion up to 99%. In addition, anti-adhesion and anti-biofilm effects against *S. mutans* of the formulation containing only CHI found in the present study, are in accordance with previous reports indicating antibacterial activity and anti-biofilm effect of CHI against *S. mutans* (Pasquantonio *et al*., 2008). It was found that the antimicrobial activity of CHI has a strong dependence on molecular weight and degree of acetylation characteristics and also varied according to microorganism strain (Goy *et al*., 2009). Several reports showed that minimum inhibitory concentrations (MIC) of CHI with different molecular weights towards *C. albicans* range from 0.8 to 3 mg/mL (Costa *et al*., 2014a; Qin *et al*., 2006), while former studies on CHI with *S. mutans* showed MIC values of 0.5-2 mg/mL (Pasquantonio *et al*., 2008; Costa *et al*., 2014b). The final concentration of CHI in the formulations used in this study was 2.5 mg/mL. Therefore, it may be possible that the reduction of biofilm formation of *Candida* species and *S. mutans* found in this study might be due to the antimicrobial activity of CHI. However, it is generally accepted that the first phase in the biofilm formation of *C. albicans* is the initial adherence of the round yeast cells to a surface to form a basal layer, follow by the growth and proliferation of hyphal cells (Gulati & Nobile, 2016). Thus, the transformation of *C. albicans* from yeast to hyphae as well as the ability of these hyphae to adhere to one another is required for proper biofilm formation. A previous study demonstrated that CHI could inhibit several Ada2-

Table 2. Percentage inhibition of *Candida* species and *S. mutans* adhesion and biofilm formation

| Formulations | Inhibition of adhesion (%) | | | | Inhibition of Biofilm formation (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | *C.albicans* | *C.krusei* | *C.tropicalis* | *S.mutans* | *C.albicans* | *C.krusei* | *C.tropicalis* | *S.mutans* |
| CHI with P407 | 79 | 73 | 79 | -1010 | 88 | 96 | 83 | -415 |
| CHI | 46 | 73 | 80 | 72 | 50 | 97 | 80 | 92 |
| P407 | 59 | 53 | 53 | 47 | 74 | 92 | 8 | 71 |
| Commercial | -875 | -757 | -1326 | -1990 | -913 | -90 | -1061 | -651 |

mediated cell wall-related genes, i.e. *ALS2*, *PGA45*, and *ACE2* (Shih, Liao, Tseng, Deng, & Lin, 2019). *ACE2* is one of the transcription factors that stimulate the pseudohyphal formation and the morphological switch in *C. albicans*. Therefore, the inhibitory effect of CHI on *ACE2* may be one of the reasons that prevent biofilm development of *C. albicans*.

There is some evidence that poloxamer-based surfactants have an important role in biofilm management (Percival *et al*., 2019). In this study, the formulation containing only P407 also reduced the adhesion and biofilm formation of *Candida* species and *S. mutans*. It decreased *C. albicans* and *S. mutans* adhesion by 59% and 47% less than control, respectively. For the biofilm formation of *C. albicans* and *S. mutans*, the formulation containing only P407 exhibited 74% and 71% anti-biofilm activity compared with control, respectively. This finding is similar to the previous report showing the capability of P407 to reduce *S. epidermidis* attachment and biofilm formation (Treter *et al*., 2014). It is well known that the first crucial step of biofilm formation is adhesion and Van der Waals' electrostatic and hydrophobic interactions are involved during initial attachment of microorganisms to the surface. Therefore, P407 might modify these interactions and then interrupt the ability of *C. albicans* and *S. mutans* to adhere. Further, species- and poloxamer-specific effects have been demonstrated in several studies (Jeon, Lee, Andrade, & De Gennes, 1991; Nejadnik *et al*., 2008). The different anti-adhesion and anti-biofilm properties of the formulation containing only P407 against *Candida* species and *S. mutans* found in the present study were similar to these reports.

Interestingly, the adhesion and biofilm formation of *S. mutan*s affected by the formulation containing CHI with P407 differed from those of *Candida* species. These might be due to the different mechanisms of biofilm formation between *Candida* species and *S. mutans*. Adherence of *S. mutans* to surfaces is mediated by sucrose-dependent and sucrose-independent mechanisms (Cvitkovitch, Li, & Ellen, 2003). The bacterium produces a variety of glucans with distinct functions and structures and also produces multiple glucan-binding proteins, which are able to promote adhesion. It was found that the combination of CHI and P407 in oral moisturizer significantly promoted the adhesion and biofilm formation of *S. mutan*s, as shown in Figure 5. Since the formulation containing CHI with P407 showed higher viscosity than the CHI only formulation (Table 1), it is possible that available CHI protonated amino groups interact with negative charges on bacterial cell walls. This could interfere in combination with the high viscosity of the oral moisturizer and decrease the antimicrobial activity of the formulation. Further, the newly developed oral moisturizers have various additives, including xylitol. Previous research has demonstrated that xylitol can induce the expression of several carbohydrate-associated genes involved in adhesion, biofilm formation and extracellular polysaccharides synthesis in *S. mutans* biofilms (Decker, Klein, Schwindt, & von Ohle, 2014). In addition, this study also showed that xylitol exposure did not inhibit the viability of *S. mutans* biofilms (Decker *et al*., 2014). When taken together, the less antimicrobial activity of the formulation containing CHI with P407 compared to the formulation containing only CHI and the availability of xylitol in the formulation may lead to a greater adherence of live bacteria and a greater amount of biofilm formation. However, the interaction between CHI and P407 in the formulation remains to be clarified. This study used crystal violet staining that does not discriminate between living and nonliving microorganisms in the biofilm. These assays are used for screening microorganisms for biofilm-forming capacity (Lee *et al*., 2005). To address this limitation, further research to elucidate the mechanism of action of the formulation by analyzing its effect on survival of microorganisms in the biofilm as well as ultrastructural analysis of biofilm architecture should be done. In addition, the use of mucoadhesive polymer to increase oral moisturizer retention time should be studied to prevent the adhesion and biofilm formation of pathogenic microorganisms. Indeed, the study of oral pathogens' adhesion and biofilm should be the starting point for developing oral moisturizers.

## 5. Conclusions

The combination of CHI with P407 in these formulations possessed potent activity against *Candida* species adhesion and biofilm. This formulation should be designated for patients particularly susceptible to the development of oral candidiasis. At the same time, these combinations of CHI with P407 formulation may not be good for inhibiting *S. mutans* adhesion and biofilm. In contrast, the formulation containing only CHI exhibited significant anti-adhesion and anti-biofilm properties against *S. mutans*. Accordingly, the formulation containing only CHI should be developed for prescribing in high caries risk patients. This evidence confirms the great potential of the anti-adhesion and anti-biofilm properties of CHI against *Candida* species and *S. mutans*.

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