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ORIGINAL ARTICLE

Spectrophotometric Analysis of *Streptococcus mutans* Growth and Biofilm Formation in Saliva and Histatin-5 Relate to pH and Viscosity

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ABSTRACT

Objective: To analyze the ability of saliva in controlling the growth and the biofilm formation of *Streptococcus mutans* (*S. mutans*) as well as the effect of histatin-5 anti-biofilm relate to pH and saliva viscosity. **Material and Methods:** The *S. mutans* biofilm assayed by crystal violet 1% and its growth measured by spectrophotometer. The saliva viscosity was analyzed by viscometer, and pH of saliva was measured by pH meter. **Results:** Based on the optical density values, growth of *S. mutans* in saliva ranged <300 CFU/mL (0.1 nm) at concentrations of 25%, 12.5% and 6.25% for 24 hours. Whereas at the 48 h and 72 h period of incubation shown an increase in growth of *S. mutans* ranged 300-600 CFU/mL (0.2-0.36 nm). The inhibitory biofilm formation of *S. mutans* in saliva was significantly higher at concentrations of 12.5% and 6.25% at 24 h incubation times on a moderate scale, whereas the histatin-5 was effective to inhibit *S. mutans* biofilm on the 50 and 25 ppm. The saliva possessed a higher inhibitory of biofilm *S. mutans* than histatin-5 and good level viscosity (0.91-0.92 cP). **Conclusion:** The saliva was able to control the growth of *S. mutans*, and histatin-5 can inhibit the biofilm formation *S. mutans*. Furthermore, the saliva was also able to respond to the pH change with good viscosity of saliva.

Keywords: Saliva; Histatins; Viridans Streptococci; Biofilms; Hydrogen-Ion Concentration.

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Introduction

The saliva contains 99% of water, inorganic and organic compounds. It has produced in glandular of the parotid, sub-mandibular, and mandibular. The cholinergic receptor controls the secretion of them through the stimulation of mechanical [1]. The Saliva harbour sIgA, histatin, lactoferrin, polypeptide, and oligopeptide. These protein roles in maintaining the oral mucosa and dental pellicle [2]. The saliva has effect antifungal and antibacterial because it has lysozyme, lactoperoxidase, lactoferrin, and histidine riched polypeptide, which plays a role in controlling the oral pathogen and salivary pH change [3]. At the lower salivary pH, it could support the colonization of pathogen to encourage interaction with mucosal epithelial cells [4] regarding the increase of salivary glycosylated haemoglobin. These changes contribute to the growth in the colonization of *S. mutans* as one of the oral pathogens that mainly involved in the pathogenesis of dental caries [5]. It was reported that positive dental caries is associated with *S. mutans* saliva scores [6]. The activity is influenced by some *S. mutans* virulent factors. In addition to growth factors, the ability to form biofilms in dental pellicles is one of the destructive factors of *S. mutans* to be aware of [7], because it has related the biofilms formation and oral bacteria quorum sensing that involved in caries pathogenesis.

The saliva serves to maintain the biological balance of the oral cavity and generally controls the development of oral pathogens and prevents the interaction of S. mutans with dental pellicles and caries [8,9]. Dental caries caused by multiple cariogenic agents like mutans streptococci, lactobacilli, Scardovia wiggsiae, and Actinomyces species [10]. Human salivary protein or histatin-5 to be bacteriocidal against some oral bacteria such as *E. faecium*, *E. cloacae*, *A. baumannii*, and *C. albicans* [11]. Besides, the response to changes in pH and viscosity of saliva is a determinant of the development of *S. mutans* [12]. Animireddy et al. reported that caries could reduce the salivary flow rate, salivary pH, salivary buffer capacity, and can significantly increase salivary viscosity. Physiochemically shows that it has a close relationship with the prevalence of caries [13]. This study evaluated the function of saliva as a control for *S. mutans* growth, and together with histatin-5 assessed the sensitivity of *S. mutans* biofilm formation, which was confirmed by an adaptation response to changes in pH and viscosity of saliva.

Material and Methods

Laboratory Procedures

The critical saliva was obtained from children unstimulated and stored in phenylmethylsulfonyl fluoride (1%) 1:10 to avoid damage to salivary protein, then diluted to a concentration of 50%, 25%, 12.5%, and 6.25%. Salivary histatin-5 (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used as an in-vitro model of one salivary protein with a concentration (ppm). The critical saliva and histatin-5 were employed to evaluate their effectiveness in controlling the growth and biofilms formation of *S. mutans* and the effect of interactions between *S. mutans* and saliva to the adaptation response to pH changes and viscosity of saliva. Bacteria of *S. mutans* ATCC 25175 was obtained from glycerol 50% stock, refreshed by re-culture on Tryptic soy broth (TSB) media (Merck KGaA, Darmstadt, Germany. Then it was synchronized with McFarlan 0.5 ($1.5x10^8$).

Streptococcus mutans Growth Assessment

The spectrophotometric assessment of *S. mutans* growth initiated with the preparation of critical saliva at concentrations of 50%, 25%, 12.5% and 6.25% with Chlorhexidine (CHX) 0.2% as a positive control. In the 96-well plate, 50 µL of TSB medium was added to each well and incubated for 15 min and then washed twice

with PBS (pH 7.0). Subsequently, *S. mutant* was prepared into a 25 µL well in the medium and incubated at room temperature (27°C) for 15 min. Critical saliva was added with a predetermined concentration into each well of 100 µL (1: 4) and then was incubated in the anaerobic atmosphere for 24 h, 48 h, and 72 h. The growing quantity of *S. mutans* was read based on its vigour by spectrophotometry-Elisa Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with Optical density (OD) 620 nm. OD 0.08-0.1 similar to Mc. Farland 0.5 ($1.5x10^8$) or equivalent to <300 CFU [14].

Biofilm Assay

According to the method conducted previously, the formation of the S. mutans biofilm was carried out by the 1% violet crystal method [15]. The saliva test material was prepared at various concentrations of 50%, 25%, 12.5%, and 6.25%, while the histatin-5 was prepared in 50 ppm, 25 ppm, 12.5 ppm, and 6.25 ppm consecutively. 96-well plate was coated by 100 µL TSB medium, incubated for 15 min, rinsed by using PBS (pH 7.0) and to each well, 25 µl *S. mutant* was added followed by adaptation at ambient temperature for 15 min. Subsequently, both saliva and histatin-5 were added to each well with a different test (triple serial), then homogenized on the shaker at 1000 x g for 5 min, incubated anaerobically for 24 h, 48 h, and 72 h. The assessment of *S. mutans* biofilms formation was initiated by removing all the solutions in wells and then washing them with PBS and dishwasher at 1000 g for 5 m (repeated twice). Subsequently, into each well plate, 150 µL of 1% violet crystal was injected, and then the crystalline violet dye and biofilm protein were homogenized by using a shaker at 100 x g for 10 min. Each well was then washed with 150 µL PBS for 5 min, then discarded and resumed with 150 µL of 70% ethanol for 1 min. 96-well plates containing biofilms were then marked based on the absorption of violet crystalline dyes and incubated at room temperature for 15 min. The biofilm mass of *S. mutans* measured by spectrophotometer at 560 nm. The Anti-biofilm assessment according to OD spectrophotometry, OD≥0.4 (strong); OD=0.2-3.9 (moderate); OD=0.05-0.1 (low); OD<0.05 (no biofilm formation).

Saliva pH Measurement

The measurement of salivary pH adaptation response to *S. mutans* begins by preparing saliva concentrations of 50%, 25%, 12.5%, and 6.25%. 500 µL *S. mutans* was adapted in 10 mL of saliva on the shaker at 1000 x g at the room temperature then incubated anaerobically for 24 h, 48 h and 72 h. Then, the salivary pH change was checked (3 repetitions) using a pH meter (Eutech Instruments Pte Ltd, Singapore).

Saliva Viscosity Measurement

Saliva viscosity was examined using Ostwald viscometer [13]. The saliva density was measured using a pycnometer, an empty pycnometer, and the lid were weighed using an analytical balance. Then, 5 ml of saliva was added to the pycnometer re-weighed with three replications to obtain a constant weight.

Statistical Analysis

Data on differences in growth of *S. mutans* with the biofilms formation in both saliva and histatin-5 were analyzed by paired T-test. The correlation with the incubating time variable was analyzed by One-Way ANOVA and Kruskal-Wallis, with $p<0.05$ as a significant reference.

Ethical Aspects

The research was approved by the Ethics Committee of Medical Faculty, Universitas Sumatera Utara, Medan, Indonesia (No.41/TGL/KEPK FK USU-RSUP HAM/2018).

Results

Figure 1 illustrates that the incubation time of hours showing the intensity of *S. mutans* growth with an average OD of 0.1 nm (<300 CFU). One-Way ANOVA analysis showed no significant difference in the growth of *S. mutans* among the incubation times of 24 h, 48 h, and 72 h ($p>0.05$). Meanwhile, the varied concentration contributed to the difference in *S. mutans* growth for each saliva ($p<0.05$). Optical Density 0.08-0.1 nm (Mc Farland 0,5; <300 CFU), OD 0.11-0.29 nm (Mc Farland 1; 300-600 CFU); OD 0.3-0.49 nm (Mc Farland 2; 600-1200 CFU).

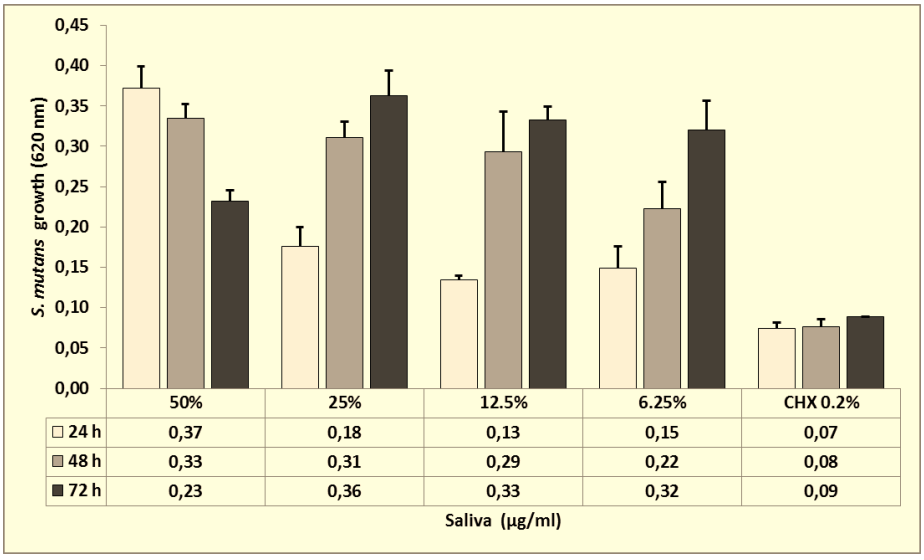

Figure 1. The growth of *S. mutans* in critical saliva at various concentrations. The graph depicted that the concentrations of saliva determined the population of *S. mutans* (CFU/ml). CHX 0.2% was able to inhibit the growth of *S. mutans* greater compared to the saliva at various concentrations. Bar (*S. mutans* growth (CFU/ml) and bar error (standard deviation).

Figure 2 shows the salivary concentration provides a varied response to the *S. mutans* biofilm formation. On the 24 and 48 h have a hight effect to adherence the biofilm formation of *S. mutans* at 12.5% and 6.25% salivary concentrations compared 50% and 25%. ANOVA analysis did not show significant differences ($p>0.05$), but the intensity of biofilm formation changed between incubation times of 24 h, 48 h, and 72 h ($p<0.05$). Figure 3 depicted that histatin-5 at concentrations of 50 ppm and 25 ppm has a moderate effect of anti-biofilm formation on *S. mutans*. The distribution and frequency of histatin-5 anti-biofilm formation of *S. mutans* in Table 1, both at the incubation times of 24 h, 48 h, and 72 h ($p>0.05$; One Way ANOVA), but increase as respect to the concentration ($p>0.05$; Kruskal-Wallis).

Figure 4 shows that saliva in each concentration has good viscosity (0.91-0.92 cP) based on the growth and biofilm inhibition of *S. mutans* in all of the concentrations of saliva. This viscosity value illustrates that protein components in saliva can respond to S. mutans activities that indicate the minimum damage to salivary proteins that correlate with lower the saliva's viscosity. Table 2 shows that all salivary concentrations can respond to pH changes after 24 h, 48 h, and 72 h of incubation; on the concentration of 50% salivary shown the best interaction with S. mutans and lower pH change of saliva. Kruskal-Wallis analysis showed no

significant difference (p>0.05) with a moderate correlation (r = 0.5). While the incubating time variable also shows insignificant changes in the pH of saliva (p>0.05) with a weak correlation (r=0.2).

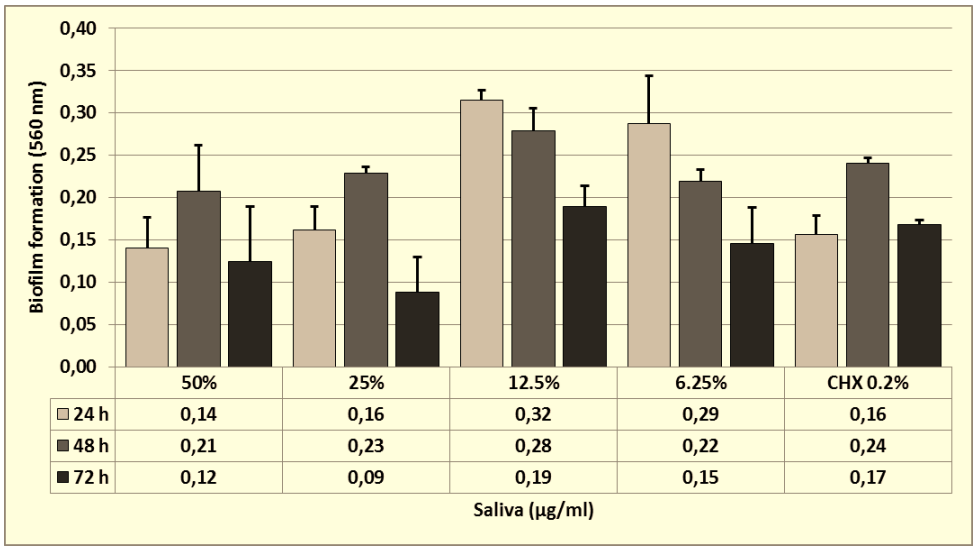

Figure 2. The biofilm formation of *S. mutans*. Generally, the saliva at the concentration of 50%, 25%, and CHX 0.2% show the more significant effect in decreasing *S. mutans* biofilm compared to the concentrations of 12.5% and 6.25%. Bar (Biofilm formation and bar error (standard deviation).

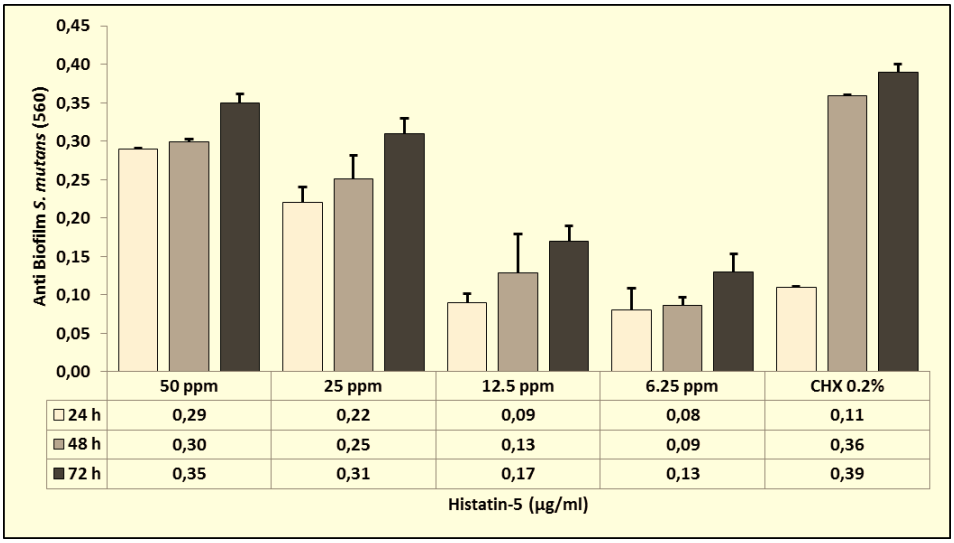

Figure 3. Histatin-5 anti-biofilm formation of *S. mutans*. The varying concentrations have shown the anti-biofilm effect against *S. mutans*, 50 ppm, and 25 ppm showed better results according to a positive reference (CHX 0.2%). Bar (histatin-5 anti-biofilm) and bar error (standard deviation).

Table 1. Distribution and frequency of histatin-5 anti-biofilm formation of *S. mutans*.

Histatin-5 (ppm)	24 h				48 h				72 h			
	OD Anti-Biofilm	SDV	%	Scala	OD Anti-Biofilm	SDV	%	Scala	OD Anti-Biofilm	SDV	%	Scala
50	0.29	0.00	43%	Moderate	0.30	0.00	39%	Moderate	0.35	0.01	36%	Moderate
25	0.22	0.09	32%	Moderate	0.25	0.07	33%	Moderate	0.31	0.02	32%	Moderate
12.5	0.09	0.01	13%	Low	0.13	0.15	17%	Low	0.17	0.12	18%	Low
6.25	0.08	0.03	12%	Low	0.09	0.09	11%	Low	0.13	0.02	14%	Low

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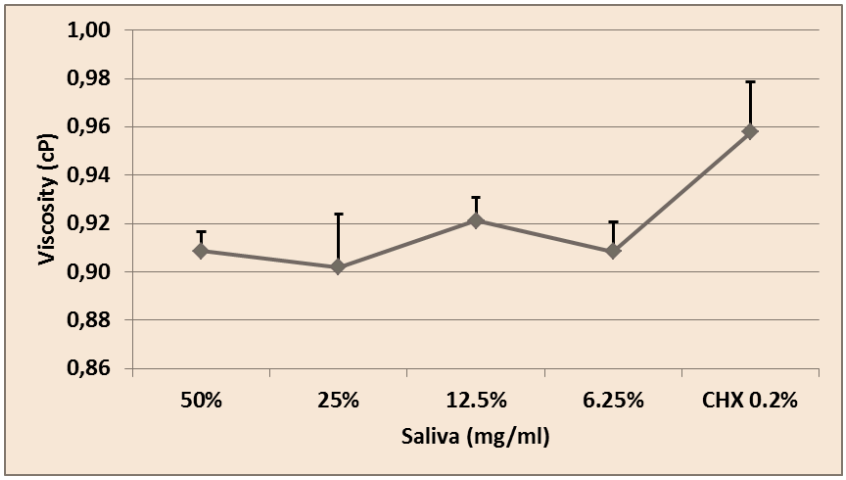

Figure 4. The viscosity of saliva at various concentrations after interacted with *S. mutans*. Saliva 25% has the best viscosity compared others concentrations: line (saliva viscosity) and bar error (standard deviation).

Table 2. The pH saliva adaptation after interacted with *S. mutans*.

Sample Analyses	Salivary Response of pH Changes After Interacted with *S. mutans*					
	pH	SD	Quantity pH Changes	%	Saliva pH Response %	Categories
24 hours						
Saliva 50%	6.41	±0.15	0.97	19.1%	80.9%	Good
Saliva 25%	6.62	±0.05	1.00	19.8%	80.2%	Good
Saliva 12.5%	6.80	±0.06	1.03	20.3%	79.7%	Moderate
Saliva 6.25%	6.83	±0.04	1.03	20.4%	79.6%	Moderate
CHX 0.2%	6.81	±0.11	1.03	20.4%	79.6%	Moderate
48 hours						
Saliva 50%	6.26	±0.32	1.05	19.6%	80.4%	Good
Saliva 25%	6.80	±0.10	1.14	21.2%	78.8%	Moderate
Saliva 12.5%	5.40	±0.26	0.90	16.9%	83.1%	Moderate
Saliva 6.25%	6.88	±0.03	1.15	21.5%	78.5%	Moderate
CHX 0.2%	6.66	±0.21	1.11	20.8%	79.2%	Moderate
72 hours						
Saliva 50%	5.96	±0.32	0.89	17.7%	82.3%	Good
Saliva 25%	6.83	±0.12	1.02	20.3%	79.7%	Moderate
Saliva 12.5%	7.10	±0.10	1.06	21.1%	78.9%	Moderate
Saliva 6.25%	6.98	±0.07	1.04	20.7%	79.3%	Moderate
CHX 0.2%	6.80	±0.10	1.02	20.2%	79.8%	Moderate

Discussion

Theoretically, saliva has the role to preventing dental caries which acts as a mechanical cleaning agent that (A) reduces plaque accumulation, as well as (B) reduces enamel solubility through calcium, phosphate, and fluoride, and (C) neutralizes acids generated by carcinogenic organisms or as a result of carbohydrate glycolysis and has an antibacterial role [3]. *S. mutans* in saliva caused the aciduric and acidogenic conditions as a result of glycolysis of glucose that impacted acid production. Furthermore, the saliva viscosity to determine of *S. mutans* development in dental caries pathogenesis [16]. The carious lesion is caused by the oral ecosystem diversity in saliva, where the *S. mutans* slightly contribute to the quorum-sensing bacterium. As the commensal, *S. mutans* present in saliva does not mean that the patient will have dental caries [17].

As can be seen in Figure 1, the research finding shows that salivary concentration could affect the growth of S. mutans, even though there was no significant difference between the incubating time of 24 h, 48 h,

and 72 h ($p>0.05$). At 50% salivary concentration, the growth of *S. mutans* was in line with the incubation time, when the incubation time of 72 h, it exhibits a slow growth, while at other concentrations, the longer the incubating time (72 h), the more rapid the growth of *S. mutans* according to spectrophotometry measurement. It can be assumed that the high concentration of saliva directly proportional to the protein quantity of saliva till stable up to 72 hours. It phenomena line with the result, where the concentration of saliva influenced the inhibition of *S. mutans* growth ($p<0.05$). Generally, the concentration of 50% and 25% with the incubating time of 24 hours was able to take control on the growth of *S. mutans* with an average OD 0.1, which equal to McFarlan 0.5 (<300 CFU) based on Mc Farland Standard for in vitro use only [14]. As a comparison, it was reported that the utilization of xylitol could inhibit the growth of *S. mutans*, which is dictated by the concentration of the xylitol [18].

When confirmed with dental caries, the results of this study show that at the saliva concentration of 50% and 25% could control the growth of *S. mutans*. Theoretically, it has been reported that saliva plays an essential role in controlling the growth of oral bacteria, especially *S. mutans*, by inhibiting the synthesis of some glucans from sucrose by *S. mutans*. As a result, the colonization in oral pellicle is prevented. Moreover, it also avoids acidogenic and aciduric [19]. Previous authors reported that using 1% sucrose in-vivo, the colonization of *S. mutans* in modelled rats significantly increase when the rats were pretreated by critical saliva; meanwhile, sIgA saliva specifically capable decreasing the growth *S. mutans* [20]. According to research, the critical saliva utilized in this research was indirectly related to maintaining *S. mutans* growth, which probably due to sIgA saliva but not limited to other components such as salivary peroxidase and catalase. While, in the saliva, *S. mutans* exhibit several sucrose catabolism pathways that produce acids [21], with the support of glycosyltransferase (Gtfs) through the conversion of sucrose to polymeric glucans leading to the biofilm formation by interacting and communicating with other oral pathogens [22]. The *S. mutans* has been reported as active bacteria in producing biofilm protein on the dental surface, which relayed on the synthesis of sucrose to be conducted by interaction with pellicle saliva deposited on the dental surface [23].

Figure 2 illustrated that *S. mutant* was capable of forming the biofilm in saliva at a medium and low level. It was indicated that several concentrations of saliva in this work affected *S. mutans* capability of forming the biofilm. The concentration of 50% and 25% shows better degradation of biofilm formation of S. mutans as respect to the incubating times compared to the concentration of 12.5% and 6.25%. Therefore, it is assumed that both incubating times and concentrations are associated with the quality of biofilm proteins production by *S. mutans*. It can be furthered presumed that the proteins content of saliva has the ability to prevent the enzymatic activity of glycosyltransferase (Gtf) of *S. mutans*, thus inhibiting the synthesis of sucrose, which support the bacterial colonization [24]. It was reported that Gtf has a special role in the formation of *S. mutans* biofilm and the increase in biofilm formation associated with the decrease in pH at the area of colonization of *S. mutans* with other oral pathogens [25]. This scientific information indicates that individual with a neutral pH of saliva (6-7) dental caries is not likely to be found [26].

Saliva contained some specific proteins that play a crucial role in preventing bacterial growth or controlling the oral cavity's biological balance. Histatin-5 is a histidine cation-rich peptide produced from human saliva and primates. Histatin-5 has 85% saliva protein and has strong antibacterial effects *acquired enamel pellicle* component (AEP) [27]. The research findings have a good agreement with our work as depicted by Figure 3, where both saliva and histatin-5 at each concentration has anti-biofilm formation against *S. mutans* with varied frequency and distribution (Table 1). The concentration of 50 ppm and 25 ppm showed better anti-biofilm of *S. mutans*, as referred to the positive control (CHX 0.2%). These findings were coherent with the data

depicted in Figures 1 and 2, the concentration of 50% and 25% of saliva was the optimum concentration to inhibit the *S. mutans* biofilm formation. This phenomenon implies that both saliva and histatin-5 play an important role in the cycle of infection initiation of caries by *S. mutans*, even though at a moderate level.

Histatin-5 is very likely to have higher antifungal activity against *Candida albicans*. He stated that the antibiofilm activity occurs via non-lytic depending on the energy [11]. Furthermore, previous authors found that histatin-5 was able to inhibit the growth of *S. mutans* at the concentration of 27.2 µg/mL and 54.4 µg/mL, either individually or when mixed with lysozyme (in a total concentration of 54.4 µg /mL) [28]. Helmerhorst et al. stated that synthetic histatin was able to decrease the biofilm in several oral bacteria significantly [29]. As a comparison, histatin-5 was also able to prevent the transition of blastospore hyphae of *C.albicans* and also contributes to reducing biofilm thickness. Combined histatin-5 and lactoferrin saliva has shown in vitro cytotoxicity against *Candida albicans* biofilm [30].

The capability of histatin inhibiting *S. mutans* biofilm in this research indicated the tendency of histatin-5 to change the amino acids generated by bacteria in developing biofilm through the elimination of N-terminal of four frequently used amino acids by anti-bacteria due to bacteriocidal properties [31]. Besides, as a synthetic peptide, histatin-5 could interfere with the interaction with cell membranes of bacteria by inhibiting PtxA blocking system of Phosphotransferase *Streptococcus mutans*. As a result, the peptide translocation like L-ascorbate, which aimed to interfere with the biofilm formation of *S. mutans* can be bothered [32]. As cellular response strategy, histatin act to stabilize the bacterial cell membranes assimilated with the surface of bacterial cells, thus promoting cell damage through interaction with the bacterial cell membrane to generate a hydrophilic channel [33].

In this study, we also measured the saliva viscosity upon the interaction with *S. mutans* for 72 h (Figure 3). The viscoelastic property was essential for lubrication and humidity to promote mucosa integrity. However, the increase of the saliva's viscosity probably associates with the rise of dental caries risk and periodontal disease [34]. Moreover, the saliva's viscosity was important to predict the tendency of S. mutans to initiate dental caries [35]. The low viscosity, however, indicated that several saliva glycoproteins have a better ability to responding the *S. mutans*. Dental caries prevalence in 7-8-year-old children was in line with saliva viscosity [36].

Figure 4 depicted the lowest viscosity of saliva at a concentration of 25% compared to other concentrations, but according to viscosity value (cP) in each concentration and CHX 0.2% showed a good level after being interacted with *S. mutans*, it means that both saliva and CHX 0.2% were able to control the biological activity of *S. mutans* growth, biofilm formation and the response to the change in pH of saliva. The viscosity range of the saliva in this research was 0.90-0.96 cP. On the 6.25% of saliva presents a viscosity value more similar to saliva at 25%, not 12.5%, and It has related to the intensity of *S. mutans* when interacting with active components of saliva. It has assumed that the concentration does not determine the changes in salivary viscosity. However, all saliva concentrations have relatively good viscosity values. The value confirmed that some protein in saliva gave a positive response to the *S. mutans*, thus minimizing the damage of saliva protein, which correlated to the viscosity. The viscosity of saliva shows an insignificant correlation to dental caries ($p>0.05$) as well as an insignificant correlation, also showed between gingiva inflammation and the viscosity of saliva [36]. In a previous study, saliva's viscosity in both working men and women were 1.05 cP and 1.29 cP, respectively, while in the depressed condition was 1.3 cP-1.5 cP [37]. There was no significant effect of the addition of amyl α-amylase or saliva with the suspension of bacteria [38].

Saliva viscosity is always associated with changes in pH as an indicator of increasing or decreasing salivary pH. At low salivary pH indicates changes in the structure of salivary proteins after being influenced by *S. mutans* and vice versa if the viscosity of saliva is low, then some salivary protein components can control the cellular regulation of S. carbohydrate receptor *S. mutans*. The results in Table 2 show that 50% and 25% of salivary concentrations have a better pH change response to S. mutans activity than other concentrations, especially at 24-hour incubation times. There is a possibility that incubation time shows the limitation of the ability of salivary protein components to work, more than 24 hours, a higher protein decomposition response occurs by *S. mutans* even though in this study it still shows a reasonable level and is responding to changes in salivary pH after being influenced by *S. mutans*. Cell density and increased biofilm growth could modulate *S. mutans* to adapt to acid changes and improve communication between bacterial biofilm cells [39].

Other findings from this study are that the smaller the change in salivary pH, the better the salivary response of *S. mutans* (Table 2), so it is possible to interpret that the concentration of saliva affects the intensity of the response to the growth of *S. mutans*, the ability to form biofilms in saliva and histatin-5. Biologically, it can be stated that the saliva and histatin-5 can interfere with the acid-tolerant response produced by *S. mutans*. This phenomenon can be justified that not all *S. mutans* that grow in the oral cavity can undergo pathogenesis of infection; normally, bacterial colonization was always limited with other pathogen populations so that not all growing bacteria tend to form biofilms and affect salivary conductivity and response to changes in changes salivary pH. The findings from these results can be explained that the biofilms formation of *S. mutans* is always influenced by environmental factors such as pH and temperature; in general, saliva has an excellent pH change response after being interacted with *S. mutans* that influenced by salivary carbohydrate receptors. *S. mutans* adhesion on the surface of dental pellicles is an essential step in the development of the concept of acid tolerance in the quorum sensing-biofilm of *S. mutans* to prevent dental caries [40].

Conclusion

Saliva can control the growth of *S. mutans* and together with histatin-5 inhibited the biofilm formation of *S. mutans* while stabilizing saliva viscosity and response to changes in salivary pH after being interacted with *S. mutans*. Salivary and histatin-5 concentrations, which have a better effect on the biological activity of *S. mutans*, are 50 and 25 (% and ppm).

Authors' Contributions

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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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