The Effect of Papain Towards mRNA Expression OF gtfB, gtfC, gtfD, gbpB and Streptococcus Mutans Biofilm Mass Formation

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Abstract

Papain is a proteolytic enzyme extracted from papaya fruit that can break down proteins into cysteine active side. *Streptococcus mutans* as the main pathogenic bacteria of dental caries is due to the virulence of glucosyltransferases (GtfB, GtfC, GtfD) and glucan-binding protein (GbpB) which together form the dental biofilm. The objective of this study is to obtain the appropriate papain concentration as an inhibitory agent of *S. mutans* biofilm formation. The effect of papain administration for 1 and 30 minutes towards *S. mutans* ATCC 25175 biofilm aged 24 hours was performed through examination of the gtfB, gtfD, gtfC, and gbpB mRNA expression, using the real-time PCR. The biofilm mass formation was examined using Safranin assay. Statistical analysis was performed by ANOVA, t-test, Pearson correlation, and regression analysis. The significance was determined based on the p-value<0.05. The effect of 15% papain administration on the mRNA expression of gtfB, gtfD, and gbpB, and Streptococcus mutans biofilm mass was equal to 0.2% chlorhexidine administration, except for the gtfC mRNA expression (p = 0.032). There was a significant decrease in the mRNA expression of gtfB (p = 0.030), gtfC (p = 0.024), gbpB (p = 0.010), and biofilm mass (p = 0.014), between pre- and post- papain administration for 30 minutes on *S. mutans* biofilm. Meanwhile the gtfD mRNA expression was increase although insignificant (p = 0.142). There was a very strong relationship between the time of papain administration and the mRNA expression of gtfB, gtfC, gtfD, and gbpB. In conclucion, papain with 15% concentration affected the decrease of mRNA expression of gtfB, gtfC, and gbpB, and also *S. mutans* biofilm mass.

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Introduction

Dental and oral health is important because it is part of the entire health of the community. Based on a survey conducted towards Indonesian children, as much as 90% of them were suffering from oral diseases.[1] According to Riset Kesehatan Dasar (Riskesdas), the caries incidence rate in Indonesia was still relatively high and more severe than other developing countries. The caries index for the 12-year-old age group in Year 2000 was 2.5. This value was much higher than the national DMF (decay, missing, filling) target for developing countries, which was 1.2 and the WHO target, which was 1.0.[2]

The multifactorial cause of dental caries is a complex interaction between specific oral bacteria and its products, saliva, and carbohydrates.[3] Prior researchers believe that pathogenic bacteria in dental plaque that become the major etiologists in forming tooth decay is *Streptococcus mutans*, often identified consistently as most prominent bacteria.[4] *Streptococcus mutans* is considered to be the most prominent dental caries bacteria due to their ability to form biofilms known as plaque on the tooth surfaces. *Streptococcus mutans* is the most cariogenic microorganism in the dental biofilm. The cariogenic properties possessed by these bacteria produce an enzyme called glucosyltransferase (Gtf). This enzyme is a virulence factor in the pathogenesis of caries

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since it converts disaccharides, especially sucrose, to glucan. This extracellular glucan is a component of the dental plaque matrix structure and serves as an early attachment medium for other bacteria on the surface of the tooth, facilitating bacterial accumulation, and as a source of extracellular polysaccharide reserves.[5] *Streptococcus mutans* produces at least three glucosyltransferase enzymes, GtfB which forms insoluble glucan polymer, GtfC which forms a mixture of insoluble and soluble glucans, and GtfD which forms soluble glucan.[6]

Several studies on the gene encoding of Gtf and Gbp were conducted by examining the mRNA that will be translated into a protein. Klein[3] examined the dynamics of rRNA expression of *Streptococcus mutans* genes in the development of single biofilms as well as mixed species. The results showed that *Streptococcus mutans* in the mixed biofilm community increased the expression of specific genes, in which gtfB, gtfC, dexA, and gbpB were associated with glucan synthesis and remodeling. A study conducted by Koo[7] on the effect of apigenin towards gtf expression of *Streptococcus mutans* showed that apigenin induction could decrease the gtfB and gtfC mRNA expression by more than 50% but increase the gtfD by 45%.

Also, the attachment of glucan towards the bacterial surface is caused by the presence of another protein known as Gbp.[8] Streptococcus mutans having at least four Gbp, they are GbpA, GbpB, GbpC, and Gbp, in which they are immunologically and biochemically different.[9] Beside functioning as the accelerator of the glucan synthesis, this Gbp also acts as a substrate for bacterial growth in the presence of aggregation capability and maintaining the structure of the biofilm.[10]

Currently, caries prevention aimed at preventing the formation of dental plaque or reducing the amount of *Streptococcus mutans* in dental plaque. Some chemical compounds often used in dental products to suppress the growth of the bacteria.[11] Chlorhexidine (bisbiguanide cation) is categorized as the golden standard of anti-plaque mouthwash due to its wide spectrum of antimicrobial and long-time effect, which makes it a strong dental plaque inhibitor.[11, 12] However, chlorhexidine has several side effects such as yellow-brownish discolouration on the one-third cervical crown, cemento-enamel junction, radicular surface, pit and fissure, composite restoration, and tongue. Other side effects including self-limiting dysgeusia; burning, soreness, and dryness on the oral tissue; desquamation lesions; and gingival mucosal ulcerations.[11-13] According to Gold,[13] chlorhexidine was not recommended for caries prevention, as some studies did not show high effectiveness in caries prevention, or clinical data was inconclusive.

There were many natural compounds used in research for caries prevention. Papain is an enzyme derived from papaya plants (*Carica papaya L*), which included to the *Caricaceae* family.[14] Papain is bactericidal, bacteriostatic, anti-inflammatory, and debridement materials.[15] The cysteine protease aspect for its catalytic activity is the high nucleophilic on the thiol group active side. Therefore, the active form of papain and its protease cysteine are comprised of the thiolate-imidazolium ion. Papain is able to break the bonds of amino peptides.[14] Papain breaks peptide bonds by involving amino acids, especially arginine, lysine, and the residues of phenylalanine. The mechanism of the function of papain in breaking the protein is performed through Cysteine-25 from the three active sides by attacking the carbonyl carbon on the peptide chain backbone thus releasing the amino-terminal part. The peptide bonding mechanism involves deprotonation of Cysteine-25 by Histidine-159. Asparagine-175 assists the orientation of imidazole ring of Histidine-159, resulting in deprotonation.[15]

Methods

Material

Streptococcus mutans ATCC 25175 was used in this research. The object of this study was a 15% papain powder (76220-25G) extracted from Carica papaya (Sigma Co.) stored at 2-8°C; and 0.2% chlorhexidine (Ultradent®).

Biofilm Manufacture

Streptococcus mutans biofilm was manufactured in the 2 ml microtube. The bacteria were grown In Mueller-Hinton culture medium added with artificial saliva with the ratio of saliva and bacterial culture was 60 µl:240 µl to form the initial biofilm.[15] The 24 hours-aged biofilm was later given 300 µl of papain and chlorhexidine (as positive control) for 1 and 30 minutes respectively, then incubated at the pH of 7.4. The next stages were the isolation of biofilm RNA, total isolation of RNA from cultured *S. mutans* ATCC 25175 cell, and cDNA synthesis.

Real Time PCR Procedure

The mRNA expression of gtfB, gtfC, gtfD, and gbpB was determined using absolute quantitative real-time PCR. The PCR reaction was performed using a real-time PCR detection system (Roche Light Cycler Nano) with the following cycles: Preincubation at 94 °C for 10 minutes was performed, followed by 40 cycles at the denaturation temperature of 95 °C for 20 seconds, annealing at 59 °C for 20 seconds, and extension at 72 °C for 20 seconds. The sampling was tested with Duplo analysis, whilst for fulfilling the gapd standard, the multilevel concentration of DNA samples was made starting from the concentration of 500 ng, 50 ng, 5 ng, 0.5 ng, 0.05 ng, and 0.005 ng. *S. mutans* ATCC 25175 was used for positive control. The Water Nuclease-Free was used for negative control. The primer used in this research (Table 1) were consisted of 5 pairs which were gtfB, gtfC, gtfD, gbpB, and gapd (control).

Primer	Forward Sequence	Reversed Sequence
gtfB	AGCAATGCAGCACTTACAAAT	ACGAACTTTGCCGTTATTGTCA
gtfC	CTCAACCAACCGCCACTGTT	GGTTTAACGTCAAAATTAGCTGTATTAGC
gtfD	CACAGGCAAAAGCTGAATTAACA	GAATGGCCGCTAAGTCAACAG
gbpB	ATGGCGGTTATGGACACGTT	TTTGGCCACCTTGAACACCT
Gapd	CTCTGCTCCTCCTGTTCGAC	GCCCAATACGACCAAATCC

Table 1. Primers used in this research[30].

Biofilm mass measurement using safranin assay

The remaining saliva and bacterial cultures of biofilms made on polystyrene microwell were discarded, then washed three times with the PBS. Afterwards, as much as 250 µL 0.1% (w/v) Safranin was added. Excessive safranin was later washed with aquadest. The microwell was dried overnight in an inverted position at 37 °C. Then resolved in 250 µL of 30% (v/v) glacial acetic acid. The biofilm mass was quantified in a microplate well using the ELISA plate reader at the OD of 490 nm.

Results

Real time PCR

The difference of gtfB, gtfC, gtfD and gbpB mRNA expression in the *Streptococcus mutans* biofilm before and after the administration of papain (Figure 1.A) and chlorhexidine (Figure 1.B) was shown in Figure 1. There was the increase of gtfB expression after papain and chlorhexidine administration for 1 minute, then the expression was decreased after 30 minutes. The decrease of mRNA expression of gtfB after papain administration for 1 and 30 minutes, was observed to be higher than after chlorhexidine administration. The gtfC mRNA expression was increased after papain administration for 1 minute, then decreased after 30 min. On the contrary, after the administration of chlorhexidine, there was the decrease of gtfC expression after administration for 1 minute, but then it was increasing after 30 minutes of administration. The gtfD mRNA expression was decreased after papain administration for 1 minute, then a significant increase observed after 30 minutes of administration. On the contrary, after the administration of chlorhexidine, there was a decreasing expression both after 1 minute and 30 minutes administration. Similarly, the gbpB expression also decreased after papain administration for 1 minute, then increased after 30 minutes. After chlorhexidine administration, there was a decrease of the gbpB expression after 1 and 30 minutes administration. The decrease of mRNA expression of gbpB after papain administration for 1 minute was observed more than chlorhexidine administration.

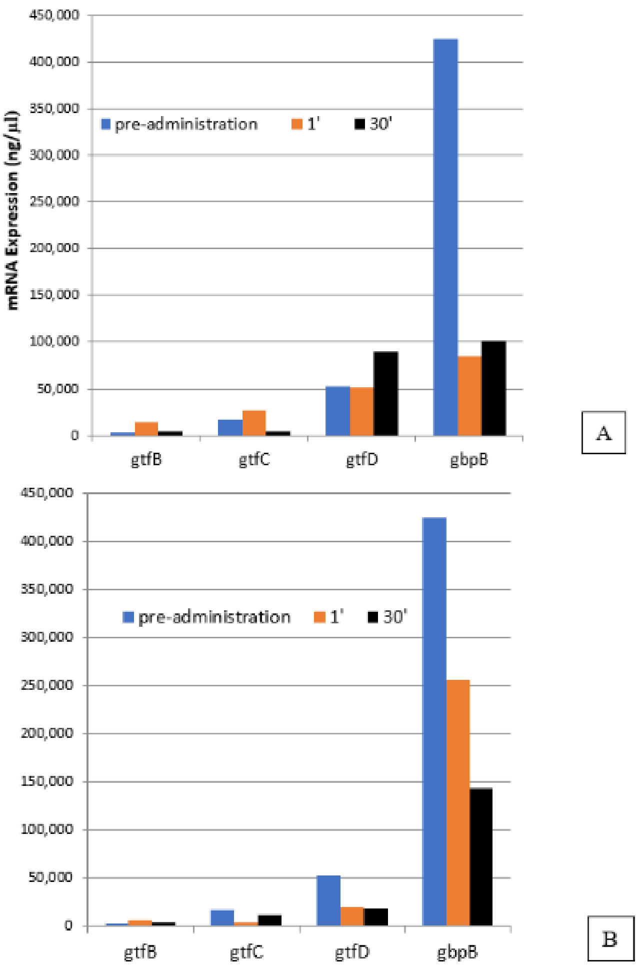

Figure 1. Effect of papain (A) and chlorhexidine (B) toward gtfB, gtfC, gtfD and gbpB mRNA expression of Streptococcus mutans biofilm.

Safranin assay

The results of Safranin examination by measuring the absorbance values of *Streptococcus mutans* biofilm using the ELISA/Microplate reader after papain and chlorhexidine administration for 1 and 30 minutes were presented in Figure 2.

Figure 2 showed the absorbance value average of *S. mutans* biofilms after papain and chlorhexidine administration for 1 and 30 minutes. The higher absorbance value, the bigger biofilm mass. The absorbance value in papain administration is less than chlorhexidine, it meant the biofilm mass formation after papain administration is less than chlorhexidine.

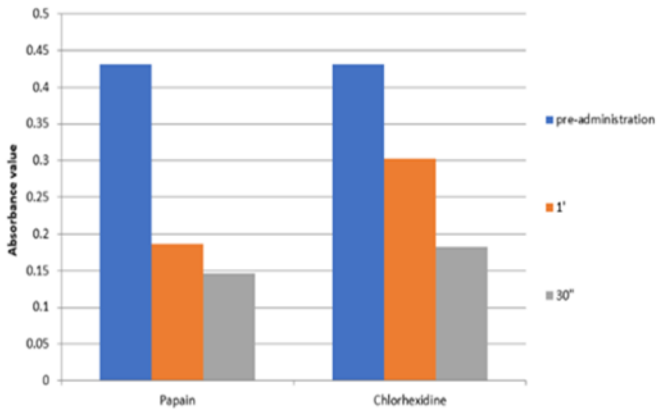

Figure 2. Effect of papain and chlorhexidine toward Streptococcus mutans biofilm mass.

The t-test analysis (Table 2) showed that there was a significant difference of mRNA expression of gtfB, gtfC, and gbpB before and after papain and chlorhexidine administration for 30 minutes. There was a significant decrease of gtfC and gbpB mRNA expression, and also biofilm mass after papain administration for 30 minutes, while the gtfB mRNA expression was increased. The gtfD mRNA expression also increased, although it was insignificant.

The t-test analysis after the administration of papain and chlorhexidine for 30 minutes showed insignificant difference in almost all genes and biofilm mass, which concludes that papain has the same inhibitory effect with chlorhexidine. The significant difference only occurred in the gtfC expression due to the decreasing expression in papain administration and increasing expression after the chlorhexidine administration. The decrease in the biofilm mass after papain administration was higher than chlorhexidine. Based on the t-test analysis, changes in the biofilm mass after administration of papain or chlorhexidine for 1 minute was shown to be very significant (p = 0.001).

Variable	Time (minutes)	Average and Deviation Standard	Papain	Chlorhexidine	p-Value
gtfB	1	$\bar{x}$	2,351	0,304	0,009*)
		Σ	0,386	0,081	
	30	$\bar{x}$	11,121	11,425	0,425
		Σ	1,461	1,380	
gtfC	1	$\bar{x}$	10,170	12,048	0,422
		Σ	10,274	6,048	
	30	$\bar{x}$	13,054	4,748	0,032*)
		Σ	2,682	1,630	
gtfD	1	$\bar{x}$	5,176	32,382	0,105
		Σ	0,801	21,288	
	30	$\bar{x}$	37,605	33,898	0,413
		Σ	7,284	19,594	
gbpB	1	$\bar{x}$	340,475	168,178	0,05
		Σ	38,730	74,861	
	30	$\bar{x}$	322,918	281,863	0,168
		Σ	22,488	40.335	
Masa Biofilm	1	$\bar{x}$	0,243	0,128	0,001*)
		Σ	0,009	0	
	30	$\bar{x}$	0,283	0,247	0,220
		Σ	0,05	0,018	

Notes : *) = significant

Table 2. Comparison between Papain and Chlorhexidine Administration to gtfB, gtfC, gtfD, gbpB mRNA Expression and Biofilm Mass.

Pearson correlation test, the coefficient of determination and linear regression (Table 3) showed that the duration of papain administration has a very strong relationship with mRNA expression of gtfB, gtfC, gtfD, and gbpB. The relationship between the duration of papain administration with gtfB, gtfC, and biofilm mass was negative, which means, the longer the duration of papain administration, mRNA expression of gtfB and gtfC, and biofilm mass would decrease. In contrast, the relationship with gtfD and gbpB was positive, which means that the longer the duration of papain administration, mRNA expression of gtfD and gbpB would increase. The regression equation for the biofilm mass was not made because the correlation was insignificant (p = 0.185).

Varible (Y)	Time (X)	r	r^2	p-Value	Regression
gtfB	1' 30'	-,984	0,968	0,0081752 *)	Y = 14,110 - 0,335 X
gtfC	1' 30'	-,953	0,908	0,0235638 *)	Y = 27,523 – 0,801 X
gtfD	1' 30'	,916	0,838	0,0422247 *)	Y = 50,498 + 1,316 X
gbpB	1' 30'	,661	0,437	0,0422247 *)	Y = 83,588 + 0,605 X
Biofilm mass	1' 30'	-,629	0,396	0,1853416	-

Notes : *) = significant
r = correlation coefficient
r^2 = determination coefficient

Table 3. Pearson Correlation, Determination Coefficient, and Linear Regression of The Length of Papain Administration toward gtfB, gtfC, gtfD, gbpB mRNA Expression and Biofilm Mass.

Discussion

The results of this study showed a decreasing expression of gtfB (p = 0.03), gtfC (p = 0.024), and gbpB (p = 0.010), before and after papain administration for 30 minutes, and insignificant increasing gtfD expression (Table 4). A study conducted by Koo et al.[7] on apigenin induction towards biofilms showed a decrease of gtfB and gtfC mRNA expression and increase of gtfD expression. A study conducted by Hasan[16] showed a decreasing gtfC expression by 74.6% due to the induction of the rough extract of *Zingiber officinale* towards *S. mutans* biofilms, whilst induction of the methanol fraction of the material caused a 59% reduction in the gtfC expression. According to Yousefi,[17] decenoic hydroxyl acids from royal jelly had caused downregulation of the gtfB and gtfC expression. Increasing gtfD expression after papain administration for 30 minutes was consistent with the gene regulation mechanism which worked in contradiction with the gtfB and gtfC.[18]

Figure 1 showed the differences of gtfB, gtfC, gtfD and gbpB mRNA expression between papain and chlorhexidine administration. This difference was presumably caused by the different working mechanism of both types of materials. According to Tezt[19], protease enzymes such as papain can cause extracellular protein and lipid degradation, but did not affect the bacteria[20-22], or unable to concentrate on the bacterial membranes.[23,24] The working mechanism of chlorhexidine was interfering the cell membrane transport and bacterial metabolism, causing the cell wall lysis.[12,13] Based on the t-test result showed that there was no difference of the mRNA expression of gtfB, gtfD, and gbpB, between papain and chlorhexidine administration for 30 minutes. The research of da Silva[25] showed an increasing of gtfC and gtfD expression of *Streptococcus mutans* UA 159 in the planktonic, and decreasing expression of gtfB, gtfC, and gtfD in the biofilm.

Variable	Average		p-Value
	Before	After	
gtfB	2,351	4,072	0,030 *)
gtfC	18,563	3,498	0,024 *)
gtfD	52,381	89,897	0,142
gbpB	424,668	101,750	0,010 *)
Biofilm Mass	0,431	0,147	0,014 *)

Notes : *) = significant

Table 4. Unpaired t-Test of gtfB, gtfC, gtfD, gbpB mRNA Expression and Biofilm Mass Before and After Papain Administration For 30 Minutes.

The decrease of gbpB mRNA expression after papain and chlorhexidine administration for 30 minutes did not show significant differences, which means papain had the same effect as chlorhexidine towards the gbpB gene. The results of the study conducted by Wen[26] showed a decreasing expression of spaP, gtfB, and gbpB of *Streptococcus mutans* when grown in multiple species biofilm with *Streptococcus sanguis* more than in the mono-species biofilm, although not statistically significant. Wen also studied the multiple species biofilm of *S. mutans* and *L. casei*, which showed that *S. mutans* was accumulated more than 2-fold in the biofilm, whilst the gtfB and gbpB expression were decreasing. This condition was presumably occurred due to the down-regulation of the gtfB and gbpB (and possibly some other members of gtfB and gbpB) when grown together with *L. casei*, altering the balance of the glucan ratio towards the glucan fastening proteins, or altering the glucan structure thus altering the biofilm architecture. A similar observation was also reported in several other groups.[27-29] It was concluded that papain had the same effect towards the mRNA expression of gtfB, gtfD, and gbpB with chlorhexidine, as the golden standard of mouthwash.

Based on the regression analysis, duration of the papain administration was related to mRNA expression of all genes examined. There was a very strong relationship with gtfB and gtfC in the negative ways, means that the longer the administration, the more decreasing mRNA expression of gtfB and the gtfC. Similarly, there was a very strong relationship with gtfD and gbpB in the positive ways, means that the longer the papain administration, the more increasing mRNA expression of gtfD and gbpB. According to Shemesh[30], all 30 genes examined in the biofilm were upregulated compared to the cells that grown planktonically. The highest increase of gene expression in the biofilm phase was gtfB. The biofilm formation was accompanied by an increase of 22 folds of mRNA of the gtfB coding gene, and 14.8 folds of mRNA of the gtfC coding gene. These enzymes synthesized the glucan polymer from sucrose that plays an important role in the formation of the tooth biofilms.[31, 32]

The gene expression depends on several factors, such as bacterial strains, phases and growth mode, and other environmental parameters such as growth media and carbohydrates. Further research on the mechanisms involved in biofilm regulation associated with gene expression by strains of *Streptococcus mutans* is needed for prevention of the biofilm formation.[30]

This study using the strains of *Streptococcus mutans* ATCC 25175.[33] Mattos-Graner[34] had used different strains of *Streptococcus mutans*, which was the UA130 and UA159 strains to compare the gbpB expression levels. The results showed that in the UA130 strain was found no significant increase in the gbpB expression towards the high salt response and low pH, whilst at the UA159 strain showed the upregulation of gbpB in response towards the osmotic pressure. The variability of gbpB transcription between the strains was consistent with the previous observation suggested that the protein production was also depending on the strain. Both osmotic and acidic stress induce the groEL and dnaK expression in the SJ32 strain but not on the UA159 strains, depends on the condition. Therefore, it can be concluded that bacterial strains were experienced different genetic modifications during the growth in the laboratory.

In this study, *Streptococcus mutans* biofilm mass after administration of papain and chlorhexidine for 1 and 30 minutes was decreasing (Figure 2). The papain administration effect was found more reliable due to the capability of the proteolytic enzyme contained in the papain is degrading large proteins into smaller peptides or amino acids.[14, 15] The working mechanism of chlorhexidine was by binding the cell membrane with the cation molecules thus affecting the permeability and going through bacterial metabolism resulting in the cell lysis.[12, 13] In the study conducted by Ccahuana-Vasquez[35], the biofilm weight was increasing, but the viability of bacteria was decreased thus the reduction in the exopolysaccharide amount was associated with the cell death, and was not the specific inhibitory effect of chlorhexidine towards the extracellular polysaccharide synthesis.

In previous research of Tetz[36] it was found that the extracellular DNA damaged by deoxyribonuclease could alter the characteristics of extracellular matrix thus the bacterial viability was reducing. It was suspected that the effect of the enzymes such as DNase I, papain, pancreatin, and chymotrypsin in the extracellular matrix were not penetrated into the bacterial membrane.[37] Tetz[19] also found that the nuclease, protease, and the combination of proteases and lipases were altering the characteristics of biofilm, including the genetic material transfer between bacteria. These data were consistent with the study results showed that some enzymes were affecting the biofilm characteristics and the extracellular matrix but not directly towards the bacteria.[20-22] Proteolytic and lipolytic enzymes did not affect the extracellular DNA but able to alter the extracellular matrix component. Genetic changes between bacteria in the biofilm after enzyme application were caused by the protein and extracellular lipids degradation.[23, 24]

According to an in-vitro study conducted by Koo,[38] the gtfB and gtfC genes were required for sucrose-dependent colonization on the hard surfaces by *S. mutans* and the pathogenesis of dental caries in-vivo.[39] Anggraini[40] dan Silvia[41] stated that a decrease in the value of denture base's surface roughness is followed by the amount of microorganism lessening. The reaction of glucosyltransferases in the dental pellicle and biofilms are different and complex thus further explanation was needed regarding how the exopolymer synthesised by these enzymes affecting the biophysical and diffusion of the matrix materials, that will enhance the understanding of the microcolonial formation and metabolic activity, and potentially have the ability to identify the therapeutic targets which can be used to interfere the pathogen biofilm effectively.

Conclusion

It was concluded that 15% papain had the same effect towards the mRNA expression of gtfB, gtfD, and gbpB as 0,2% chlorhexidine, which is known as the golden standard of mouthwash.

Conflict of Interest

Authors report that there is no conflict of interest and this article is not funded by any research grant.

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