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High Glucosyltransferase B gene Expression and Biofilm Formation of *Streptococcus mutans* after Exposure to Carbohydrates

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Abstract: *Streptococcus mutans* is the leading cause of dental caries. This bacterium causes the production of insoluble glucans from sucrose to cause the accumulation of this bacterium on the tooth surface and create a biofilm. The GlucosyltransferaseB (*GtfB*) gene of *S. mutans* seems to have responsibility for the formation and maturation of biofilm. Starch and sucrose can Increase the count of *S. mutans*. In this study, we assessed the influence of starch, sucrose, and starch plus sucrose on biofilm formation and *gtfB* gene Expression of *S. mutans* ATCC 35668 strain. The biofilm formation was detected by a 96-well microtiter plate crystal violet method containing Luria-Bertani (LB) broth, starch and sucrose. Gene expression of biofilm-forming (*GtfB*) was quantified through Real-Time PCR. The biofilm formation of *S. mutans* was increased to 1.54 times in 1% starch concentration, 3.30 times in 1% sucrose, and 2.26 times in the 0.5% starch plus 0.5% sucrose compared to biofilm formation in the media without sugars. The starch, sucrose, and starch plus sucrose carbohydrate also increased its expression by affecting the *gtfB* gene effective in colonization. These results indicate how carbohydrates directly affect *gtfB* gene and biofilm formation by *S. mutans*. Glucosyltransferase B showed great potential for diagnosis and treatment proposes in *S. mutans*.

Keywords: *Streptococcus mutans*; biofilm; GlucosyltransferaseB; carbohydrates; decay.

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1. Introduction

Streptococcus mutans is humans' main cause of tooth decay and periodontitis [1,2]. Tooth decay is one of the most common chronic infectious diseases affecting everyone globally [3]. Oral diseases are a silent epidemic in the world that threatens children and adults [4-6]. One of its important pathogenic properties is the ability to form a biofilm known as dental plaque on tooth surfaces [7].

A bacterial biofilm is an accumulation of microbes surrounded by exopolysaccharide or extracellular matrix, nucleic acids, and proteins [8,9]. Biofilm can cause resistance of

bacteria against host attack, antibiotics, and environmental stress [9]. *S. mutans* possesses several glucosyltransferases (*GTFB*, GTFC, and GTFD) enzymes which play a role in the pathogenesis, formation of biofilm, and adhesion-dependent on sucrose [10]. In addition, these enzymes are coded by *GtfB*, *GtfB*, and *GtfD* [11]. *GtfB* forms primarily water-insoluble α-1,3-linked glucosidic polymers, while *GtfD* forms water-soluble α-1,6-linked glucosidic polymers. In addition, *GtfC* produces both water-soluble and water-insoluble glucans [9]. Water-insoluble glucans synthesized by *gtfB/C*-encoded enzymes are important for colonizing *S. mutans* [12]. These genes could coordinate to produce dental biofilm leading to tooth caries induction [12].

Carbohydrates can cause various changes in the structure and performance of this microorganism which, in the case of bacterial cultivation in the presence of these carbohydrates, changes in the expression of some genes are observed [13]. Sucrose is fermentable for all kinds of bacteria, and *S. mutans* can use it as a substrate for synthesizing polysaccharides in dental biofilms [14,15]. Also, starch hydrolysates can serve as acceptors for exopolysaccharides (EPS) production by *S. mutans* exoenzymes [16]. The combination of starch and sucrose also affects not only the composition and structure of the polysaccharide matrix but also the expression of *S. mutans* genes in biofilms, which may increase the caries potential of dental biofilms [16,17].

The objective of this study is to detect the effect of sucrose, starch, and sucrose plus starch carbohydrates on the formation of biofilm of *S. mutans* and also to detect the effect of sucrose, starch, and sucrose plus starch carbohydrates on *gtfB* genes expression that is responsible for the formation of biofilm. Starch and sucrose carbohydrates are selected for a detailed study of their effect on gene expression because they are usually used as important carbohydrates in food.

2. Materials and Methods

2.1. Bacterial culture and growth conditions.

S. mutans strain ATCC 35668 was selected to elected the effect of 1% starch (Himedia, India), 1% sucrose (Himedia, India), 0.5% sucrose plus 0.5% starch on biofilm formation and gene expression. For Biofilm assays, we cultured the strain in a carbohydrate-free Luria Bertani (LB, Merck, Germany) broth and in LB medium containing sucrose and starch, in combination and alone, and compared the test results. All microorganisms were incubated at 37°C for 24 to 48 hours [18].

2.2. The effect of carbohydrates on biofilm formation.

To evaluate the effect of starch and sucrose on the biofilm formation of *S. mutans*, cultures were grown in their respective media, as mentioned above. Later, the suspension was adjusted to 0.5 McFarland turbidity standards. The formation of *S. mutans* biofilm was evaluated in the presence or lack of starch and sucrose carbohydrates. Plates were incubated at 37^{o}C for 24 and 48 hrs. After 48 hours, Biofilms were fixed with methanol and stained with crystal violet solution (0.1%). Biofilms stained with crystal violet were dissolved in 33% acetic acid and incubated at room temperature. Quantitation biofilm was investigated by 96 wells of assay microtiter plates [19]. The suspensions' optical density (OD) was wavelength at 570 nm using a microtiter plate reader. Each sample was tested three times, and the mean ODs were

recorded. The positive control for biofilm formation was *Pseudomonas aeruginosa* ATCC22924 [18].

2.3. RNA extraction.

RNA extraction was performed using Invitrogen Purelink RNA/DNA Mini Kit (Invitrogen) according to manufacturer protocol. Then we used biofilm-forming bacteria to continue the process. First, we dissolved the biofilm-forming strain in the physiological serum. Then, we prepared the lysate solution according to the kit instructions. In the next step, we will purify the genome, which we will do according to the following steps:1- Added the prepared lysate to the spin column in the collection tube 2- It was centrifuged for 1 minute at 6800xg, and the material was transferred to a new microtube 3- It was washed with 500 µl of Wash buffer and centrifuged at 6800xg for 1 minute Then the sediment was transferred to a new microtube 4-50 microliters of RNase-free water was added to the microtube and centrifuged at 10,000xg for 1 minute 5- DNA removal by adding DNase kit RNase-free Thermo scientific DNase I To remain content RNA 6- Quality of RNA was evaluated on agarose gel and quantified using Biophotometer plus. cDNA was synthesized from extracted RNA using PrimeScript reagent kit (Takara Bio Inc, Japan) according to manufacturer protocol [20].

2.4. Real-time PCR.

The effect of starch and sucrose on *S. mutans gtfB* gene expression was evaluated by Real-time PCR (Applied Biosystems StepOnePlusTM). *gtfB* gene expression was evaluated with Real-time PCR. *S. mutans 23S rRNA* was used as a housekeeping gene for the target genes. *S. mutans* specific *23S rRNA* and *gtfB* for Real-time PCR are used in Table 1 [12]. The reactions were performed in a Real-Time PCR detection system. Each reaction mixture Included7.5µl SYBR Green PCR Mix, Taq DNA polymerase, MgCL2, dNTP, 0.5µl Forward primer, 0.5µl Reverse primer of sample cDNA, and 4.5µl sterile deionized water. Amplification was performed at denaturation at 94^{o}C for 5mins followed by 40 cycles of 94^{o}C for 10secs, 49^{o}C for 10 secs, and 72^{o}C for 10 secs. All samples were performed in triplicate and relative quantification was performed using the $2^{-\Delta\Delta C}$t method.

Table 1. The sequence of primers of *gtfB* gene and *23S rRNA* for Real-time PCR.

Gene	Primer name	Primer sequences
gtfB	*gtfB* Forward	ACACTTTCGGGTGGCTTG
	gtfB Reverse	GCTTAGATGTCACTTCGGTTG
House keeping (*23S rRNA*)	*23S rRNA* Forward	CCTATCGGCCTCGGCTTAG
	23S rRNA Reverse	AGCGAAAGACAGGTGAGAATCC

2.5. Statistical analysis.

The ΔΔCT method was used to analyze the relative expression, and a P-value below 0.05 was considered significant. The Pfaffle formula was used to compare groups for significance using GraphPad Prism version 9.

3. Results and Discussion

3.1. Bacterial culture and growth conditions.

S. mutans was maintained in Tryptic Soy Broth (TSB) broth containing 20% glycerol. *S. mutans* ATCC 35668 was used to assay the gene expression and biofilm formation affected

by starch and sucrose. These microorganisms were incubated at 37ºC in an anaerobic jar for 24 to 48hrs.

3.2. Biofilm formation.

The results obtained from measuring the speed of biofilm formation showed that *S. mutans* ATCC 35668 forms a severe biofilm in the presence of sucrose and starch carbohydrates. The biofilm formation of *S. mutans* ATCC 35668 was increased to 1.54 times in 1% starch concentration, 3.30 times in 1% sucrose, and 2.26 times in the 0.5% starch plus 0.5% sucrose in comparison to biofilm formation in the media without carbohydrate. The optical density (OD) of violet crystals is shown in Figure 1 and Table 2.

Table 2. Influence of 1% starch, 1% sucrose, and 0.5% starch plus 0.5% sucrose on the biofilm formation of *S. mutans* ATCC 35668.

Experimental groups:	Average of ODs*	Standard deviation (SD)	Control
No carbohydrate	0.6290	0.09	0.1
1% starch	0.9683	0.28	0.140
1% starch	2.073	0.16	0.137
0.5% starch+ 0.5% sucrose	1.420	0.19	0.126

*OD: Optical density at 570 nm

3.3. Effect of carbohydrates on gtfB expression.

The expression analysis of *gtfB* gene in *S. mutans* ATCC 35668 isolate indicated starch, sucrose, and starch plus sucrose increased the expression of the *gtfB* gene. *gtfB* was a 95% increase in the Face starch, sucrose, and starch plus sucrose in comparison to the no carbohydrates (control) group. (Respectively $p< 0.034$, $p< 0.001$and $p< 0.001$) (Figure 2).

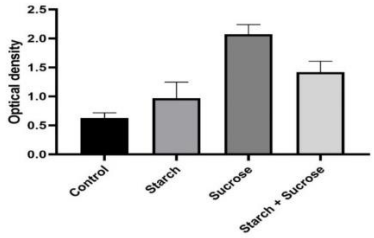

Figure 1. Influence of 1% starch, 1% sucrose, and 0.5% starch plus 0.5% sucrose on the biofilm formation of *S. mutans* ATCC 35668.

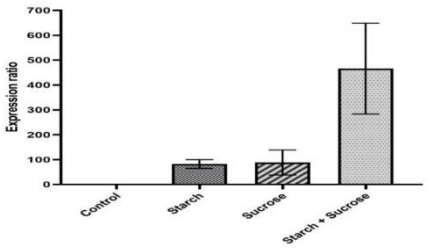

Figure 2. Effect of starch, sucrose, and starch plus sucrose carbohydrate on *gtfB* gene expression responsible for biofilm formation in *S. mutans* ATCC 35568.

3.4. Discussion.

In this study, the effect of starch and sucrose carbohydrates on biofilm formation and gene expression was evaluated in *S. mutans* ATCC 35668. The results indicate that this microorganism and biofilm formation increased gene expression after exposure to starch,

sucrose, and sucrose. *S. mutans* is one of the main causes of dental infections, including tooth decay and periodontitis [21,22]. The pathogenic factors of this bacterium are acid production, acid tolerance, saliva, and carbohydrates [23,24]. In the presence of sucrose, it synthesizes water-insoluble glucans, which are deposited to form dental plaque and provide a substrate for establishing a multispecies microbial community [25]. Any strategy directed at reducing the number of *S. mutans* is beneficial for maintaining oral health [26]. Newbrun *et al*. [27] have identified sucrose as the main culprit of tooth decay. This initial hypothesis based on the results obtained from animal experiments has shown that Tooth decay was higher in animals that received sucrose-containing foods than those that received other carbohydrates [27,28]. Walker *et al*. [28] expressed doubts about the main role of sucrose and other sugars in causing tooth decay, While other people, such as Ismail and Burt [29], confirm the relationship between the consumption of high amounts of sucrose and caries as part of a multifactorial process. Xylitol mints, such as xylitol alone, inhibit the growth and adhesion of *S. mutans*, while sucrose products promote their development [14]. A study conducted by Hasibul *et al*. [30] showed that D-tagatose prevents the growth and formation of *S. mutans* biofilm by interfering with GTF activity. In our study, the biofilm produced by *S. mutans* in the presence of sucrose is more than the combination of sucrose and starch, and it is more than starch.

This study evaluated the effect of starch, sucrose, and starch plus sucrose on gtfB gene expression of S. mutans ATCC 35668. *gtfB* is responsible for synthesizing insoluble glucans, which in turn form biofilm formation and provide food for another bacterium that can cause damage to the oral cavity [26,31]. The expression of *gtfB* level in saliva has a direct relationship with the presence of caries [32]. Moshe Shemesh *et al*. [33] showed that glucose and fructose strongly increase the expression of *gtfB* gene. Duart *et al*. [34] showed that *gtfB* gene expression in the biofilm formed by sucrose increased significantly. *gtfB* is considered to be the virulence factor, which needs to be reduced to prevent dental caries [26]. The results of our study showed that starch, sucrose, and starch plus sucrose carbohydrates increased *gtfB* gene expression together and separately. An increase in *gtfB* is directly related to the accumulation of more insoluble glucans, increasing the likelihood of tooth decay.

4. Conclusions

In conclusion, starch, sucrose, and starch plus sucrose increased the biofilm formation of *S. mutans* ATCC 35668 and *gtfB* gene expression responsible for biofilm formation and maturation. To investigate whether these effects are unique to starch and sucrose, it is suggested that further studies be conducted using other dietary carbohydrates. Still, additional research is needed to target other genes responsible for biofilm syntheses like *gpbA*, *gpbB*, *gpbC*, *gtfC*, *gtfD*, *ftf*, *Spa P*, and *BrpA* to find out the perfect mechanism involved in the reduction of *S. mutans* biofilm formation.

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Conflicts of Interest

The authors declare no conflict of interest.

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