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Virulence traits-based behavior of *Streptococcus mutans* bacteria from dental plaque and dental caries conditions

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ABSTRACT

Streptococcus mutans has significant virulence factors associated with the etiology and pathophysiology of dental plaque and caries through adherence to the tooth surface and biofilm formation. These microbes can tolerate and survive the acidic environment. The study aimed to compare the behavior of the *S. mutans* isolates from dental plaque and caries conditions, in addition to the evaluation of the biofilm formation by *S. mutans* with oral microbiota (*Candida albicans*). A total of 106 *S. mutans* isolates were recovered including 56 isolates from soft caries lesions, 30 isolates from the plaque of caries active and 20 isolates from the plaque of caries-free samples. The isolates' virulence efficacy was assessed phenotypically by acid production, acid tolerance, and biofilm formation assays. PCR detected genes encoding these virulence traits. There was a significant difference ($p < 0.05$) between the three groups of isolates in biofilm formation, biofilm formation when co-cultivated with *C. albicans*, pH reduction by acid production, but there was no significant difference in withstanding acidic pH. *S. mutans* isolates harbored the genes glucosyltransferase B (*gtfB*), glucan binding protein B (*gbpB*), F1F0 membrane-bound proton-translocating ATPase C subunit (*atpH*) and lactate dehydrogenase (*ldh*) which are responsible for biofilm formation, sucrose-dependent adhesion, acid tolerance and acid production, respectively. In conclusion, *S. mutans* isolates from caries-free and caries active dental plaque and dental caries conditions showed different virulence efficacy-based behaviour. *C. albicans* enhances the biofilm formation by *S. mutans*. Further investigation of factors influencing variable behavior of *S. mutans* bacteria from different lesions is warranted.

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Introduction

Oral microbiota, one of the most complex microbial communities in the human body, contains about 700 kinds of microorganisms that inhabit the human mouth (Lu et al. 2019). Some microorganisms that colonize the oral cavity can form a dental plaque (Takahashi 2005). Plaque formation can lead to two of the most common oral

diseases; dental caries and periodontal diseases. The absence of oral hygiene, one of the essential reasons, causes the plaque to accumulate (Marsh 2003). The viridans group streptococci (VGS) is a highly diverse range of organisms within the genus *Streptococcus*, an essential member of oral microbiota. Oral streptococci are primarily members of the VGS, a group of 20 species that are commensal occupants of the oropharyngeal cavity, as well as the

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gastrointestinal and genital tracts of mammals, and are thought to be the principal cause of dental caries in mammals (Gomez et al. 2017; Hujoel et al. 2018). *S. mutans* bacteria are facultatively anaerobic gram-positive cocci that belong to the lactic acid-producing bacteria (Chavan et al. 2015). Break down of sugars and production of lactic acids is one of the important virulence traits of *S. mutans*, thus causing tooth decay (Lamont and Eglund 2015). In addition, *S. mutans* bacteria have an acid tolerance response (ATR) to combat the harmful acidic environment when exposed to sublethal pH values (Guo et al. 2015). Moreover, *S. mutans* secretes multiple exoenzymes, mainly glycosyltransferases Gtfs which, can cleave sucrose to produce extracellular glucans (Ranganathan and Akhila 2019; Alhobeira et al. 2021). The glucans are the primary keys that comprise the matrix in cariogenic biofilms (Oho et al. 2000; Swedan et al. 2018). Non-enzymatic glucan-binding proteins (GBPs) can bind to glucan and are assumed to take part in the sucrose-dependent adhesion and the cohesive nature of the dental plaque biofilm. The *gbpB* gene expresses an adhesion protein named glucan-binding protein B which facilitates the binding of glucan to the physical and biological surfaces (Wang et al. 2020; Rouabhia and Semlali 2021). The acid tolerance of *S. mutans* (acidouricity) is regulated by the *atpH* gene which encodes subunit C of a multisubunit enzyme F1F0-ATPase which, is involved in intracellular pH regulation and acid tolerance (Gong et al. 2009; Li et al. 2018). *S. mutans* bacteria can break down sugars and produce lactic acid (acidogenicity). The lactate dehydrogenase gene *Idh* is responsible for lactate production from the breaking down of sugars and has a vital role in plaque formation (Zhang et al. 2016; Park et al. 2018). Bacterium-fungus interactions are widespread in people and can impact the switch from a healthy to a diseased state within a specific host niche.

There are almost 85 species of fungi in the human mouth. The most prevalent fungus organism found on human mucosal surfaces, frequently contributes to producing polymicrobial biofilms on soft tissue and acrylic surfaces is *Candida* species (Lu et al. 2019). The presence of *C. albicans* increased the production of exopolysaccharides (EPS), such that cospecies biofilms form more biomass and contain more viable *S. mutans* cells than single-species biofilms. The biofilm architecture showed that *S. mutans* microcolonies surround fungal cells (Khoury et al. 2020).

The study aimed to compare the virulence-based efficacy of *S. mutans* bacteria isolated from dental plaque of both caries-free and caries active conditions and dental caries conditions, such as acid production, acid tolerance and biofilm formation, and detect some virulence encoding genes. Furthermore, to investigate the interaction of *S.*

mutans with the other oral microbiota (*Candida albicans*), which contributes to the production of dental biofilms formation.

Materials and Methods

Study population and clinical samples

The present study's ethical approval (No. ETH38) was issued by the Faculty of Dentistry, October University for Modern Sciences and Arts (MSA). A total of 140 clinical samples were collected from the patients of the faculty dental clinics. The samples comprised 70 plaque samples collected by swabbing the surface of the intact enamel of the teeth from caries-free (n = 35) and caries active (n = 35) cases, and 70 soft caries lesion samples from soft caries lesions obtained from the outermost layer of carious dentin and removed with a sharp, sterile excavator (Aas et al. 2008; Villhauer et al. 2017).

Sample processing and identification of *S. mutans* bacteria swabs and soft caries samples were placed in Eppendorf tubes containing one ml brain heart infusion broth (LAB M, England), then transferred to the microbiology laboratory within 10 - 15 min. Next, they were added to 10 ml brain heart infusion broth in the laboratory and incubated at 37°C for 24 h. In the next day, the growth in the brain heart infusion broth was inoculated onto the surface of the growth medium tryptone-yeast-cysteine-sucrose-bacitracin (TYCSB) agar by streaking [tryptone 15 gm/L (Techno Pharmchem, India), yeast extract (Pronadisa, Spain) 5 gm/L, disodium hydrogenphosphate.7 H₂O (Sigma, Germany) 1 gm/L, sodium bicarbonate (Oxford, India) 2 gm/L, sodium acetate trihydrate (Oxford, India) 20 gm/L, sucrose 200 gm/L, L-cysteine (Sigma, Germany) 0.2 gm/L, sodium sulfite (Oxford, India) 0.1 gm/L, sodium chloride (Adwic, Egypt) 0.1 gm/L, Agar (Oxoid, England) 15 gm/L, bacitracin (Sigma, Germany) 0.002 gm/L, distilled water 1000 ml], which is the most sensitive and selective media for the culture of *S. mutans* bacteria (Wan et al. 2002).

S. mutans bacteria were identified using the traditional microbiological methods, including culture identification based on colony morphology on the selective media (tiny white colonies within a clear outer zone) (Wan et al. 2002). Initial characterization for identification depends on (gram staining, microscopic and biochemical tests). *S. mutans* isolates were catalase-negative, and fermented the sugars mannitol, sorbitol, inulin and sucrose (Al-Jumaily et al. 2014).

The identification was confirmed by PCR assay using specific pairs of PCR primers targeting the *S. mutans* *gtfB* gene according to Oho et al. (2000). *S. mutans* isolates were sub-cultured routinely on brain heart infusion agar

and stored as glycerol stock at -20°C for long-term preservation.

Biofilm formation assay

Biofilm formation by *S. mutans* isolates was estimated using the microtiter plate crystal violet method according to Stepanović et al. (2000) and Zhou et al. (2018). *S. mutans* isolates from the glycerol stock were cultured on brain heart agar media (Oxoid, England) and incubated at 37°C in a candle jar for 48 h. A sterile plastic loop transferred a loopful of bacterial colonies into a tube containing 5 ml of isotonic saline and adjusted to match the 0.5 McFarland turbidity standard. Then, 100 μl from the standardized saline was transferred into 10 ml brain-heart broth (LAB M, England) and 200 μl of each of the diluted solutions was transferred to a sterile flat-bottom 96-well plate containing 100 μl of fresh media per well (Brain heart infusion broth + 5% sucrose) in triplicates and incubated at 37°C in a candle jar for 24 h. The negative control wells contained all components except the bacteria. Following incubation, the broth was removed, and the wells were gently washed three times with saline. The plates were left to dry, then followed by biofilm quantified using 200 μl of 0.1% crystal violet (Adwic, Egypt) for 15 min. The excess stain was washed off by saline and inverted on tissues and left to dry, after that resolubilized by 100 μl of 98% ethanol (Adwic, Egypt) for 15 min. The optical density (OD) was measured at 570 nm by Stat Fax-2600 microplate reader (Awareness Technology, USA). The results obtained by the microtiter plate were classified according to (Stepanović et al. 2000). We defined the cut-off OD (OD_c) of the negative control: $\text{OD} \leq \text{OD}_c$: Non-adherent, $\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c$: Weakly adherent, $2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c$: Moderately adherent, $4 \times \text{OD}_c < \text{OD}$: Strongly adherent.

The effect of *Candida albicans* on biofilm formation of *S. mutans* isolates

The effect of *C. albicans* species on *S. mutans* biofilm's formation was tested according to Tomé et al. (2017) dual-species biofilm assay. The bacterial isolates were cultured on brain heart agar media and incubated at 37°C in the candle jar for 48 h. *C. albicans* ATCC 1023 was cultured on sabouraud dextrose agar (Oxoid, England) (obtained from School of Medicine (Kasr Al-Ainiy, Cairo) and incubated at 37°C for 24 h. A loopful of *S. mutans* colonies were transferred into a tube containing 5 ml of isotonic saline and adjusted to match the 0.5 McFarland turbidity standard, then 100 μl from the standardized saline was transferred into 10 ml brain-heart broth. A 125 μl of each of the diluted solutions of *S. mutans* isolates was added to sterile flat-bottom 96-well plates in triplicates and 125 μl of the *C. albicans* ATCC 1023 suspension (equivalent to

0.5 McFarland turbidity standard) was added on each of the wells containing *S. mutans* isolates solution and incubated in a candle jar 37°C for 24 h. The negative control wells contained brain-heart broth only.

Quantitation of biofilms was performed using crystal violet-based microtitre plate assay according to Stepanović et al. (2000), after 24 h of incubation, the broth was removed, and the wells were gently washed three times with saline and all non-adherent bacteria were removed. The wells were stained with 200 μl of 0.1% crystal violet and left for 15 min. Excess stain was washed off by saline and plates were inverted on tissues and left to dry. The dye was resolubilized by 200 μl of 33% glacial acetic acid (Adwic, Egypt) per well for 15 min. The optical density (OD) was measured at 550 nm by Stat Fax-2600 microplate reader. We defined the cut-off OD (OD_c) as a negative control: $\text{OD} \leq \text{OD}_c$: Non-adherent, $\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c$: Weakly adherent, $2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c$: Moderately adherent, $4 \times \text{OD}_c < \text{OD}$: Strongly adherent.

Acid susceptibility assay

The ability of *S. mutans* to withstand acid stress was evaluated using the method described by Lembo et al. (2007). The isolates from the glycerol stock were grown in brain heart infusion broth for 24 h at 37°C . The cultures were measured by spectrophotometer (Shimadzu, Japan) and grown from OD 0.1 to OD 0.3 at 600 nm by incubating bacterial cultures in a shaking incubator (HYSC, Korea) for 1 h at 200 rpm then harvested by centrifugation (Sigma, Germany) at 10,000 rpm for 10 min and discarding the supernatant of all bacterial cultures. The pellets were washed once with 1 ml 0.1 M glycine buffer (Starchemie, China) (pH 7.0) and then undergo centrifugation again for 10 min at 10,000 rpm and resuspending the cell pellets in 1 ml of 0.1 M glycine buffer (pH 2.8) followed by incubation for 5 min. Cells resuspended in 0.1 M glycine buffer (pH 7.0) acted as positive controls. Following the incubation, surviving cells were estimated by inoculating diluted aliquots from each tube at pH 7.0 and pH 2.8 on the surface of BHI plates. The plates were incubated in the candle jar at 37°C for 48 h. The number of colony-forming units (CFU) was determined for each pH condition. The cell is considered acid susceptible when no cell is viable at pH 2.8 in relation to the total number of viable cells at pH 7.0

Glycolysis (Acidogenicity assay)

To evaluate the ability of *S. mutans* to lower the suspension pH (i.e. acid production), the glycolysis assay was performed as described by Arthur et al. (2011). Isolates from the glycerol stock were grown on brain heart agar plates and incubated at 37°C for 48 h in the candle jar. A loopful of bacterial colonies were collected and inoculated into brain heart infusion broth at 37°C for 18 h. Aliquots

of 5 ml broth cultures (approximately 1×10^8 CFU/ml) were put in falcon tubes, then centrifuged at 10,000 rpm for 10 min, the supernatant was discarded and the pellets were resuspended in 1 ml of 50 mM KCl (Adwic, Egypt) supplemented with 1 ml of 1 mM MgCl₂ (Adwic, Egypt). The pH of the solution was adjusted (by adding HCL for decreasing pH in case of alkaline pH of the solution or by adding NaOH in case of acidic pH) to 7.2 by pH meter previously calibrated with pH standards (pH 4.0 and pH 7); then 1 ml of 55.6 mM glucose (Loba Chemie, India) was added. The decrease in pH was then measured over 180 min using a pH meter.

PCR-based detection of virulence encoding genes

DNA extraction and PCR oligonucleotide primers

The bacterial isolates were cultivated on TYCSB and incubated anaerobically at 37°C for 48 h in a candle jar, then loopful colonies were transferred by a sterile plastic loop into 5 ml of brain heart broth, then incubated at 37°C for 24 h. Genomic DNA was extracted from *S. mutans* isolates by i-genomic BYF DNA Extraction Mini Kit (Intron, KOREA) following the manufacturer's instructions. PCR oligonucleotide primers used in this study to detect virulence genes, synthesized by Invitrogen (UK), are listed in Table 1. The primers used were previously published or designed in this study using the PCR primers designing tool available at NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) using *Streptococcus mutans* strain NG8 (NZ_CP013237.1) as a DNA template.

For PCR-based molecular identification of *S. mutans*, the following PCR primers were designed in this study: 5'- TTCGGGTGGCTTGGTTAAAGC -3' (forward primer) and 5'-TGCTTAGATGTCACTTCGGTTGT -3' (reverse primer). The expected PCR amplicon size is 123 bp. PCR primers were designed to be specific for *S. mutans* and include a conserved region among all *S. mutans* *gtfB* available sequences in the GeneBank database. The Primer-Blast analysis indicated the specificity at that time. The lyophilized primer powder was reconstituted using nuclease-free water to achieve a concentration of 100 pmol/μl and then was adjusted to the working solution of 10 pmol/μl.

PCR reactions

The primers sequences were selected to amplify genes responsible for *S. mutans* virulence traits are glucan production *GtfB*, glucan binding protein B *GbpB*, acid tolerance *ApH* and acid production *LdH* in (Table 1). Amplification reactions were carried out using 96 well PCR thermocycler (Applied Biosystems, USA), containing 1 μl of template DNA with 10 pmol of each forward and

reverse primer, 10 μl of 2× DreamTaq Green PCR Master Mix (DreamTaq DNA Polymerase, optimized DreamTaq Green buffer, MgCl₂, and dNTPs) and the volume was completed to 20 μl by adding nuclease-free water. The PCR run for 40 cycles on a thermal cycler under the following conditions: denaturation at 94°C for 5 min, primer annealing for 30 s, and extension at 72°C, followed by a final extension at 72°C for 7 min.

Ten μl of mixture reaction were analyzed by 1% TAE agarose (Electrophoresis-grade agarose powder, GIBCO Bethesda Research Lab, U.S.A) gel electrophoresis. The PCR products were stained with ethidium bromide (5 mg/ml) (Alliance Bio, USA) in a 1× TAE buffer at 85 Volt for 45 min and visualized sing by placing on a UV transilluminator (UVP, USA) and photographed directly. The size of the PCR products was estimated from the electrophoretic migration of products relative to 6 μl of GeneRuler (Thermo Scientific, USA) 50 bp and 100 bp DNA molecular weight markers.

Statistical analysis

The results are expressed in terms of relative frequency and frequencies or percentages. Statistical analysis was performed to examine if there is a significant difference in the results of the isolates recovered from the three groups of samples using the Chi-square test and considered significant at a $p < 0.05$. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

Results and discussion

A total of 106 *S. mutans* isolates were recovered from 140 clinical samples collected in the current study. *S. mutans* isolates were 56 isolates from soft caries lesion samples (n = 70) and 50 isolates from dental plaque samples (n = 70). The 50 plaque isolates comprised 30 isolates from the plaque of caries active samples (n = 35), and 20 isolates from the plaque of caries-free samples (n = 35). Many studies from the literature revealed that *S. mutans* bacteria could produce biofilm both *in vivo* and *in vitro* conditions (Kulshrestha et al. 2016; Ahn et al. 2018; Senpuku et al. 2019). Collectively, 24.5% (26/106) of all isolates was weak biofilm producer and 75.5% (80/106) was moderate biofilm producer (Table 2). There was a statistically significant difference in the biofilm formation ability among the three groups of isolates based ($p = 0.01$). This finding agreed with the results of Jiang et al. (2015) which showed that caries active and caries-free isolates exhibited significant differences in their abilities for biofilm formation. Notably, in this study, the ability to produce biofilm was higher among the soft caries lesion isolates than the plaque of caries active or plaque of caries-free isolates as the isolates from soft caries lesions showed that

Table 1 Virulence genes and sequences of PCR oligonucleotide primers used in this study

Virulence gene	Primers sequence (5' - 3')	Expected amplicon size (bp)	Ta / extension time	Source
<i>gtfB</i>	F: ACTACACTTTCGGGTGGCTTGG R: CAGTATAAGCGCCAGTTTCATC F: AGCAACAGAAGCACAACCATCAG	517	55°C / 45 s	Oho et al. (2000)
<i>gbpB</i>	R: CCACCATTACCCAGTAGTTTCC	150	55°C / 30 s	Sadeghinejad et al. (2016)
<i>atpH</i>	F: GCGTTAGCCTTGGTGAAGGA F: TGTTGAAGCAAGAAGCACGA	146	53°C / 30 s	This study
<i>ldh</i>	F: GGGCGAACAATACCATGTGC F: ATTCTCAGGTTTCCCTGCCG	456	53°C / 40 s	This study

- *gtfB* encodes for GtfB (Glucosyltransferases GTF-I); function: biofilm formation.

- *gbpB* encodes for GbpB (Glucan binding protein B); function: facilitates the binding of glucan to the physical and biological surfaces.

- *atpH* encodes for AtpH (F1F0 membrane-bound proton-translocating ATPase C subunit); function: aciduricity.

- *ldh* encodes for LdH (Lactate dehydrogenase); function: increase of lactic acid, which may cause the increase of acid production).

Table 2A Biofilm formation assay of *S. mutans* isolates from each sample type

Biofilm formation grade	<i>S. mutans</i> isolates		
	Soft caries lesion isolates (n = 56) ¹	Plaque of caries active isolates (n = 30)	Dental plaque isolates (n = 50) Plaque of caries-free isolates (n = 20)
Non-biofilm producer	0% ² (n = 0)	0% (n = 0)	0% (n = 0)
Weak biofilm producer	14.5% (n = 8)	30% (n = 9)	45% (n = 9)
Moderate biofilm producer	85.5% (n = 48)	70% (n = 21)	55% (n = 11)
Strong biofilm producer	0% (n = 0)	0% (n = 0)	0% (n = 0)

¹Number of *S. mutans* isolates recovered from each sample type.

²Percentage correlated to the total number of isolates from each sample type.

Table 2B Biofilm formation assays of *S. mutans* isolates using dual-species assay with *C. albicans*

Biofilm formation grade	<i>S. mutans</i> isolates		
	Co-species biofilm formation of soft caries lesion isolates (n = 56) ¹	Co-species biofilm formation of plaque of caries active isolates (n = 30)	Dental plaque isolates (n = 50) Co-species biofilm formation of plaque of caries-free isolates (n = 20)
Non-biofilm producer	0% ² (n = 0)	0% (n = 0)	0% (n = 0)
Weak biofilm producer	9% (n = 5)	100% (n = 30)	0% (n = 0)
Moderate biofilm producer	35% (n = 20)	0% (n = 0)	10% (n = 2)
Strong biofilm producer	56% (n = 31)	0% (n = 0)	90% (n = 18)

¹Number of *S. mutans* isolates recovered from each sample type.

²Percentage correlated to the total number of isolates from each sample type.

only 14.5% of the isolates was weak biofilm producer and 85.5% of the isolates was moderate biofilm producers. In addition, the isolates from the plaque of caries active samples showed a higher frequency of biofilm formation ability than isolates from the plaque of caries-free samples as 30% of the plaque of active caries isolates were weak biofilm producers and 70% of isolates were moderate

biofilm producers whereas 45% of the plaque of caries-free isolates were weak biofilm producer and 55% were moderate biofilm producer. This finding was consistent with the results of Valdez et al. (2017). *S. mutans* secretes glycosyltransferases Gtfs which is responsible for glucan production and biofilm formation (Ranganathan and Akhila 2019; Alhobeira et al. 2021; Oliveira et al. 2021). *S.*

mutans isolates were examined by PCR for the presence of *S. mutans* gene *gtfB*. All isolates harbored this gene and produced a detectable DNA band at the expected 517 bp PCR product (Oho et al. 2000) (Figure 1). The *gbpB* gene which facilitates the binding of glucan to the physical and biological surfaces (Wang et al. 2020; Rouabhia and

Semlali 2021) was also detected in all *S. mutans* isolates and produced the expected PCR product of 150 bp consistent with the study of Sadeghinejad et al. (2016) (Figure 1).

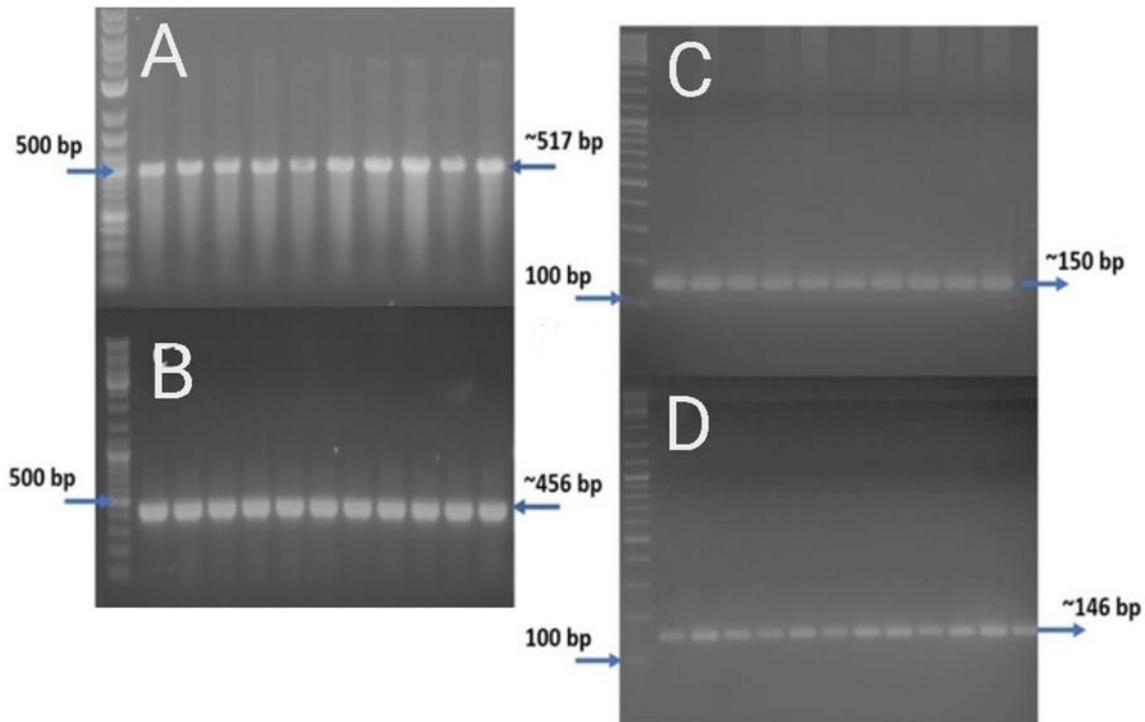


Fig 1. Agarose gel (1 %) of PCR-based detection of *S. mutans* virulence genes. A, *gtfB* DNA fragment of 517 bp; B, *ldh* gene of 456 bp; C, *gbpB* gene of 150 bp; D, *atpH* gene of 146 bp.

Biofilm formation ability in the presence of *C. albicans* was studied (Table 2). The interaction between *S. mutans* and *C. albicans* led to an increase in the biofilm formation ability of *S. mutans*. Relative to biofilm formation of *S. mutans* alone, 33% of isolates became weak biofilm producers, 21% moderate biofilm producers and 44% strong biofilm producers in the presence of *C. albicans*. There was a statistically significant difference in biofilm formation in the presence of *C. albicans* among the three test groups of isolates ($p < 0.0001$). These results indicated that *C. albicans* enhanced the biofilm formation capability of *S. mutans* isolated from soft caries lesion samples and plaque of caries-free samples, which agreed with many previous studies (Ellepola et al. 2017; Kim et al. 2017; Ikono et al. 2019; Sampaio et al. 2019). However, *C. albicans* reduced the biofilm formation capability of plaque of caries active *S. mutans* isolates. These results were consistent with the results of Eidt et al. (2019) study which revealed that *C. albicans* can reduce both the cariogenic

activity due to competition for nutrients and acidogenic potentials of *S. mutans* biofilms. Although *S. mutans* bacteria are considered the most cariogenic bacteria, studies showed that *C. albicans* can enhance the cariogenic potential of the *S. mutans* by altering the surrounding biofilm microenvironment to modulate the cariogenic potential of biofilms. and enhancing sugar metabolism and production of acids thus increasing the dentine demineralization (He et al. 2017; Sampaio et al. 2019). Other factors that may affect the interaction between both organisms with each other need further investigation.

S. mutans develop ATR to combat the destructive nature of the acidic environment it produces (i.e. aciduricity) (Guo et al. 2015). The acid tolerance of *S. mutans* is regulated by the *atpH* gene, which encodes subunit C of a multisubunit enzyme F1F0-ATPase which is involved in intracellular pH regulation and acid tolerance (Gong et al. 2009; Li et al. 2018). *atpH* gene was detected in all *S. mutans* isolates produced a detectable DNA band

of the expected size of 146 bp (Figure 1). Thus, in the current study, *S. mutans* isolates showed a high ability to withstand acidic pH which agreed with the results of many previous studies (Lembo et al. 2007; Liu et al. 2016; Qiu et al. 2017; Sun et al. 2018; Valdez et al. 2017). The ability of *S. mutans* isolates to withstand acid challenge is illustrated in Table 3. There was no significant difference in the acid

susceptibility among the three test groups of isolates ($p = 0.2323$) (Table 4). This finding is inconsistent with Valdez et al. (2017) results, which stated that *S. mutans* isolates from caries active cases were more acid-tolerant than those isolated from caries-free samples.

Table 3 Acid susceptibility assay of *S. mutans* isolates

Acid susceptibility (% cells killed by acids)	<i>S. mutans</i> isolates
0 - < 10%	0% (n = 0)
10 - < 20%	1.8% (n = 2)
20 - < 30%	0% (n = 0)
30 - < 40%	0.94 % (n = 1)
40 - < 50%	2.8% (n = 3)
50 - < 60%	1.8% (n = 2)
60 - < 70%	0.94% (n = 1)
70 - < 80%	5.6% (n = 6)
80 - < 90%	10.3% (n = 11)
90 - < 100%	75.4% (n = 80)

Table 4 Acid susceptibility assay of *S. mutans* isolates recovered from each sample type.

Acid susceptibility (% cells killed by acids)	Source of samples		
	Soft caries lesion (n=56)	Plaque of caries active patients (n = 30)	Dental plaque isolates (n = 50) Plaque of caries- free individuals (n = 20)
0 - < 10%	0% (n = 0)	0% (n = 0)	0% (n = 0)
10 - < 20%	3.57% (n = 2)	0% (n = 0)	0% (n = 0)
20 - < 30%	0% (n = 0)	0% (n = 0)	0% (n = 0)
30 - < 40%	1.78% (n = 1)	0% (n = 0)	0% (n = 0)
40 - < 50%	5.35% (n = 3)	0% (n = 0)	0% (n = 0)
50 - < 60%	3.57% (n = 2)	0% (n = 0)	0% (n = 0)
60 - < 70%	0% (n = 0)	0% (n = 0)	5% (n = 1)
70 - < 80%	5.35% (n = 3)	6.70% (n = 2)	5% (n = 1)
80 - < 90%	7.14% (n = 4)	23.30% (n = 7)	0% (n = 0)
90 - < 100%	73.1% (n = 41)	69.9% (n = 21)	90% (n = 18)

An important virulence trait of *S. mutans* is its ability to metabolize a variety of dietary carbohydrates such as sucrose and produce a large amount of acid by fermentation; thus, the isolates were able to drop the suspension pH from 7 to 3 to become acidic during the test three hours (Figure 1). There was a statistically significant difference in the ability of *S. mutans* to lower the suspension pH among the three test groups of isolates (Figures 2 - 5) ($p < 0.0001$), which was consistent with the results of other studies (Napimoga et al. 2004; Baker et al. 2017; Valdez et al. 2017; Shafiei et al. 2020). However,

comparing the acidogenicity among *S. mutans* isolates from the three groups revealed significant differences in their ability to reduce the pH ($p < 0.0001$) which did not agree with the results of Napimoga et al. (2004) and this difference may be because the samples were taken from the saliva, not from plaque or caries lesion conditions as in this study. The *Idh* gene responsible for lactate production has an important role in plaque formation, was detected in all *S. mutans* isolates and produced a detectable DNA band of the expected size of 456 bp (Zhang et al. 2016; Park et al. 2018) (Figure 1).

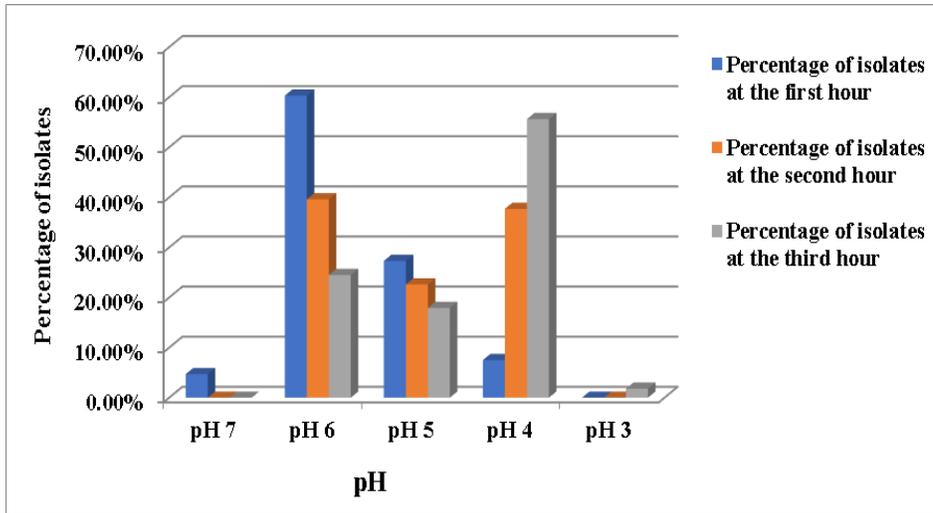


Fig 2. Frequencies of *S. mutans* isolates reducing pH values over the test three hours.

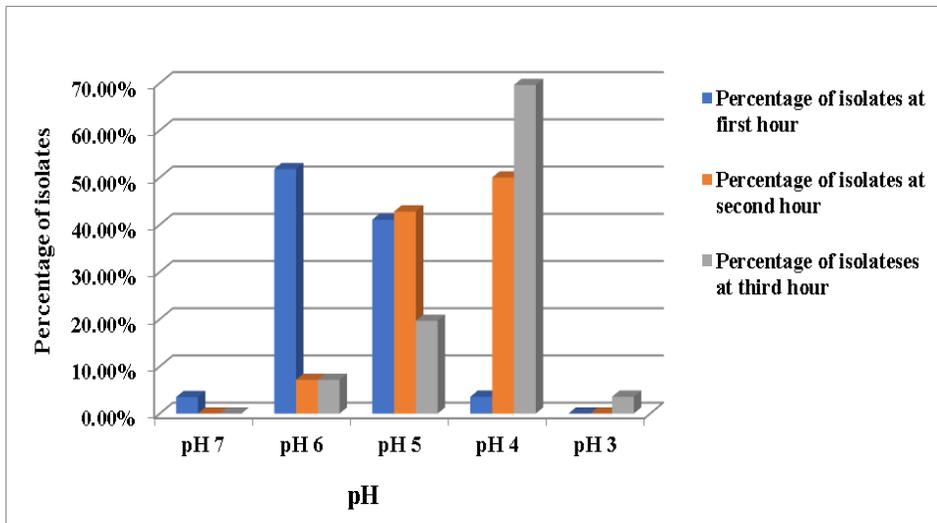


Fig 3. Frequencies of *S. mutans* isolates from soft caries lesion samples reducing pH values over the test three hours.

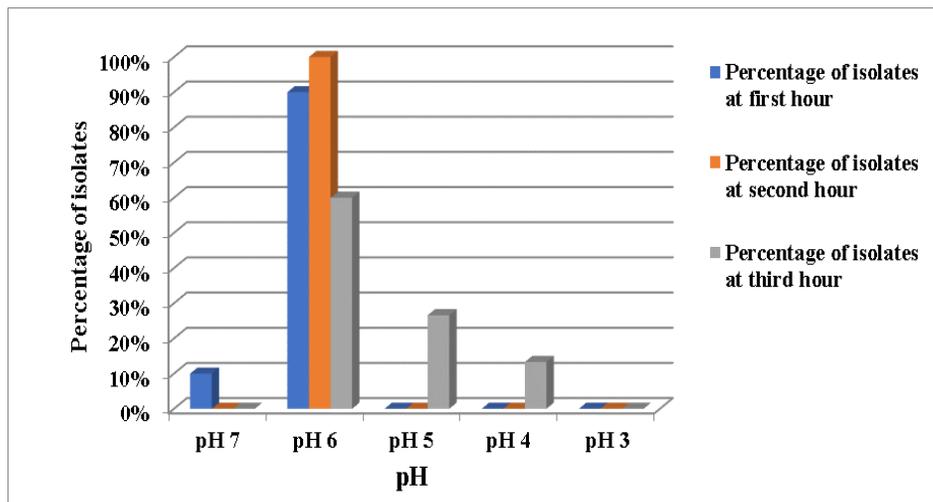


Fig 4. Frequencies of *S. mutans* isolates from the plaque of caries active samples reducing pH value over the test three hours.

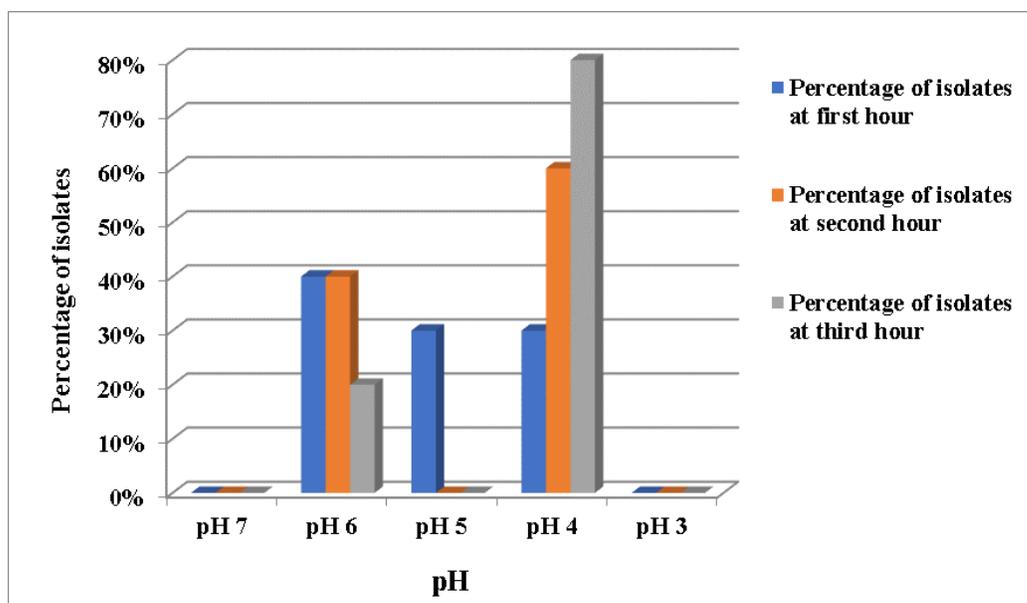


Fig 5. Frequencies of *S. mutans* isolates from the the plaque of caries-free samples reducing pH value over the test three hours.

Conclusion

There was a difference in the virulence-based behavior of *S. mutans* bacterial isolates from each soft caries lesion, the plaque of caries active and plaque of caries-free conditions concerning biofilm formation ability, biofilm formation when co-cultivated with *C. albicans*, ability to produce acid and thus reducing. However, there was not a significant difference in the results of the three groups in the ability to withstand acidic pH. *S. mutans* isolates harbored *gtfB*, *gbpB*, *atpH* and *ldh* genes responsible for the virulence traits including glucan production and biofilm formation, sucrose-dependent adhesion, acid tolerance and acid production, respectively. *C. albicans* may enhance the biofilm formation activity of *S. mutans* isolates. The variable behaviour of *S. mutans* bacterium from different disease stages or conditions may suggest different protocols for therapy. Although further investigation of factors influencing variable behavior and virulence efficacy of *S. mutans* bacteria from different disease lesions is warranted.

Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval

The research was granted confirmation of conductance from the responsible research ethics committee of Faculty of Dentistry, October University for Modern Science and Arts (MSA) with number: ETH38

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