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INFLUENCE OF SUGAR AND STEVIA TO THE CARIOGENICITY OF ORAL STREPTOBACILLI

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INFLUENCE OF SUGAR AND STEVIA TO THE CARIOGENICITY

OF ORAL STREPTOBACILLI

By

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ABSTRACT

INFLUENCE OF SUGAR AND STEVIA TO THE CARIOGENICITY
OF ORAL STREPTOBACILLI

Sai Mei Ying

Dental caries is one of the major health concerns caused by the acidogenic and cariogenic plaque bacteria which are frequently exposed to sugar. The colonization of rare oral streptobacilli could induce the occurrence of dental caries as they are biofilm-forming and acid producing oral bacteria. This project aims to study three aspects that involve in the cariogenicity which are the biofilm-forming strength, acidogenicity, and thickness of biofilm formation. Hence, three different growth conditions, including sugar free condition, BHI broth supplemented with 1% (w/v) sugar (sugar-dependent, SD), and BHI broth supplemented with 0.15% (v/v) stevia (stevia-dependent, StD) were implemented. A total of three target isolates were isolated from the dental plaque in which two were Gram-positive, catalase-positive and one was Gram-negative, catalase negative. In the MTP assay, biofilm-forming strength of the isolates were assessed quantitatively based on the intensity of solubilized 0.1% (w/v) crystal violet stained in the biofilm. One target isolate showed significant outcome ($p<0.05$) of moderate biofilm-forming strength in SD condition and weak biofilm-forming strength in StD condition whereas another two isolates were excluded as they were unable to grow in broth medium and lead to poor

outcome in the MTP assay, respectively. The Gram-positive target isolate was identified as *Bacillus* sp. using 16S rRNA sequencing Besides, the scanning electron microscopic examination showed significant increased ($p<0.05$) in the thickness of biofilm formation of the *Bacillus* sp. under SD and StD conditions. Furthermore, the acidogenicity of the target isolate was determined via the acid detection test. The target isolate demonstrated a significant decreased ($p<0.05$) of pH in SD condition as compared to the StD condition. Overall, the outcomes demonstrated that *Bacillus* sp. may cause cariogenic effects under the presence of sugar.

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DECLARATION

I hereby declare that this final year project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.
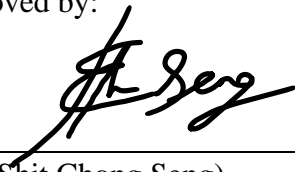
Emily
_____

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APPROVAL SHEET

This final year project report entitled **"<u>INFLUENCE OF SUGAR AND STEVIA TO THE CARIOGENICITY OF ORAL STREPTOBACILLI</u>"** was prepared by SAI MEI YING and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Honours) Microbiology at Universiti Tunku Abdul Rahman.

Approved by:

Date: 8 May 2023

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PERMISSION SHEET

It is hereby certified that **SAI MEI YING** (ID No: **18ADB03426**) has completed this final year project report entitled "**INFLUENCE OF SUGAR AND STEVIA TO THE CARIOGENICITY OF ORAL STREPTOBACILLI**" under the supervision of DR. SHIT CHONG SENG from the Department of Biological Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project dissertation in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

I hereby give permission to my supervisor to write and prepare manuscript of these research findings for publishing in any form, if I did not prepare it within six (6) months time from this date provided that my name is included as one of the authors for this article. Arrangement of the name depends on my supervisor.

Yours truly,

(SAI MEI YING)

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LIST OF ABBREVATIONS

BHI   Brain Heart Infusion

dH$_2$O   Distilled Water

EPS   Extracellular Polymeric Substances

MTP   Microtiter Plate

NTC   Non-template Control

PBS   Phosphate Buffered Saline

PCR   Polymerase Chain Reaction

RTD   Ready-to-drink

SBA   Sheep Blood Agar

SD   Sugar-dependent

SEM   Scanning Electron Microscopy

StD   Stevia-dependent

TSA   Tryptic Soy Agar

CHAPTER 1

INTRODUCTION

Biofilms are mainly composed of dense micro-communities that colonize and grow on a solid surface which is enclosed in the extracellular polymeric substances (EPS) (Berger, et al., 2018). The oral cavity is considered as an ideal environment for the formation of biofilms due to the moisture level, presence of salivary pellicle and nutrients that empowering the growth of the oral biofilms (Mosaddad, et al., 2019). In fact, oral biofilm occurs naturally in the oral cavity as majority of the oral microbiota are commensal microorganisms (Meyer, et al., 2021). Diverse microorganisms colonize the oral cavity in which most of them are commensal oral microbiota that are beneficial to the host's health by secreting inhibitory molecules against the colonization of pathogenic microbes originated from external sources, such as food. In contrast, the host diet that is rich in dietary sugars, especially sucrose, is the main factor that contributes to the formation of cariogenic biofilms that eventually lead to the changes in the microbial homeostasis, in which nutrients availability and pH condition in the oral cavity could undergo changes (Lamont, Koo and Hajishengallis, 2018). Biofilm composition could undergo changes due to above-mentioned conditions, leading to periodontal disease (Muhsin, et al., 2015).

Besides, the early stage of oral biofilm is well-known as the dental plaque in which the appearance of pale-yellow deposits on the surface of the teeth via the

colonization of a variety of microorganisms, including *Streptococcus* spp., *Bacillus* spp., *Actinomyces* spp. and *Neisseria* spp. These oral bacteria could be aerobes, facultative anaerobes, and anaerobes. The primary colonizers are usually the facultative anaerobes and they play an important role in assembling a complex microbiota in the oral cavity (Abranches, et al., 2018). The interaction of microbial communities that grow on the tooth surface could be cooperative or antagonistic by giving a significant impact on the formation of dental plaque (Lamont, Koo and Hajishengallis, 2018).

As previously mentioned, the host diet rich in dietary sugars could eventually trigger the acid production. As the consequences of the metabolism of dietary carbohydrates that being utilized by the cariogenic oral bacteria. The cariogenic oral bacteria that have the capability to produce acid and tolerate the acid are known as notorious acidogenic species, such as *Streptococcus mutans*, *Scardovia wiggsiae* and *Lactobacillus salivarius* (Abranches, et al., 2018). Saccharification process causes the changes in the pH condition in the oral cavity creating an acidic environment that is preferred by cariogenic biofilm for formation (Chen, et al., 2020). Acidity is the main driving force behind the biofilm-mediated disease because as the production of acid in damage the minerals of the dental apatite leading to enamel demineralization (Chen, et al., 2020).

Furthermore, cariogenicity of the oral bacteria trigger the occurrence of the dental caries in human. Dental caries is categorized as the biofilm-mediated

disease that seriously affects human health in which it causes painful and inflammation resulting in extraction of tooth (Chen, et al., 2020). According to World Health Organization (2022), dental caries are becoming increasingly more serious nowadays due to the uncontrollable host diet rich in dietary sugars. Meantime, repetitive exposure to sugars could initiate the colonization of the cariogenic oral bacteria causing carious lesions due to their acidogenicity and ability to produce extracellular polymeric substances (EPS) (Zeng, et al., 2020). According to previous study, non-mutans organisms could cause caries as well such as other acid producing bacterial species (Chen, et al., 2020). In this study, biofilm-forming and acid producing oral bacteria were being examined and compared on the aspects of cariogenicity. The aim of this study was to evaluate the influence of sugar and stevia sweetener which has been approved by Food and Drug Administration (FDA), on the acidogenicity and biofilm-forming strength of selected oral isolates (Razak, et al., 2017). This study provides the groundwork of potential cariogenic oral isolates is indispensable for further research on the selected cariogenic oral isolates.

Hence, the objectives of this study were:

i.   To isolate potential cariogenic oral bacteria from dental plaque.

ii. To estimate the biofilm-forming strength of selected isolates after exposure to sugar and sweetener via quantitative analysis.

iii. To determine the acidogenic property of the selected isolates after exposure to sugar and sweetener.

iv.   To evaluate the biofilm-forming strength of the selected isolates after exposure to sugar and sweetener via qualitative analysis.

v.   To perform molecular identification of the selected bacterial species.

CHAPTER 2

LITERATURE REVIEW

2.1 Oral Bacteria

2.1.1 Common Oral Bacteria

In 2012, the Human Microbiome Project (HMP) established the term "microbiome". The aim was to delineate major microbiome in human body comprising of 18 different sites including the oral cavity. Diversity of microorganisms in the oral cavity composes of more than 700 different physiological types of species identified. Normal oral flora typically includes *Actinomyces* spp., *Streptococcus* spp., *Veillonella* spp., *Neisseria* spp., *Bacillus* spp., and other microorganisms. These oral bacteria could be aerobes, facultative anaerobes, and anaerobes. In addition, these living organisms could be commensal, pathogenic, and symbiotic microorganisms, in which they serve as the determiners of human's health and disease state (Mosaddad, et al., 2019).

In fact, bacteria of the genus *Streptococcus* are the first inhabitants of the oral cavity, may be acquired shortly after birth and play a major role in building the oral microbiota followed by the colonization of *Actinomyces* spp. (Abranches, et al., 2018). Both *Streptococcus* spp. and *Actinomyces* spp. are the major contributors to the early stage of biofilms formation on the tooth surface (Jain, et al., 2013). They are well known as the plaque bacteria that dominant the oral

cavity with the ability of reversible adherence followed by the interaction of microbial communities that grow on the tooth surface could be cooperative or antagonistic by giving significant impact on the formation of dental plaque (Lamont, Koo and Hajishengallis, 2018).

Besides, the Gram-positive *Bacillus* spp. are considered as the rare oral bacteria that contributes to the dental plaque formation. *Bacillus* spp. have found to be the transient normal flora in the oral cavity and undergo the sugar metabolism process that leads to the production of acids as the by-products. This event could trigger the plaque-mediated diseases, due to the frequent exposure to the sugary and starchy food that seriously affects an individual oral health's status. The unique features of *Bacillus* spp., in which they are capable in tolerating the harsh acidic microenvironment in the oral cavity enable them to survive and initiate the formation of cariogenic biofilms as production of EPS enhances the plaque structure (Jain, et al., 2013).

2.1.2 Cariogenic Oral Bacteria

Cariogenic oral bacteria are defined as the oral microorganisms that pose several virulence factors to further enhance the cariogenic biofilms formation in the oral cavity. One of the most significant virulence factors is the ability to produce EPS which poses the pathogenicity effect on the host. The EPS production provides protection to the microbial community and enables quorum-sensing that involves the interaction and attachment of multiple species to further reinforce the cariogenic biofilm structure (Nishikawara, et al., 2007). The cariogenic oral

bacteria tend to demonstrate acidogenic property as they have the ability to produce, tolerate, and survive in the acidic microenvironment in the oral cavity. Acid is the main causes of the occurrence of the plaque-mediated diseases such as dental caries and periodontitis due to the enamel demineralization (Chen, et al., 2020).

Development of cariogenic oral bacteria is mainly due to the modern host diet that includes over consumption of dietary carbohydrates that causes the imbalance in the resident normal flora and enhances the maturation of the later colonizers refer to the cariogenic oral bacteria. The cariogenic oral bacteria encounter a significant shift of low plaque biomass to high plaque biomass resulting in formation of cariogenic biofilms that tend to cause carious lesions (Marsh, 2006). *Streptococcus mutans* is the well-known cariogenic oral bacteria that cause dental caries as they produce mutacins which is a protein that could actively against other species of oral bacteria. The mutacins are proteinaceous antibacterial compounds that are effective in killing and destroying the neighbouring peroxigenic bacteria such as *Streptococcus gordonii* and *Streptococcus sanguinis* (Huang, Li and Gregory, 2011). Eventually, they become dominant in the oral cavity under the frequent exposure to dietary carbohydrates especially sucrose that enable them to synthesize a virulence glue-like extracellular polymer glucan. Interestingly, they able to survive under very low acidic condition in which below the critical pH 5.5 (Abranches, et al., 2018).

2.2 Dental Plaque as Oral Biofilm

2.2.1 Microbial Homeostasis in Oral Biofilm

Oral biofilm is made up of diverse physiological types of microorganisms that inhabit in the oral cavity and enclosed in EPS secreted by themselves. The EPS plays the role in protecting the microorganisms and enhance nutrients uptake by the microorganisms within the oral biofilm. The formation of oral biofilm involves four different stages includes formation of acquired pellicle, initial adhesion, maturation, and dispersion. In addition, the formation of oral biofilm requires the host's salivary glycoproteins for better attachment to the tooth enamel (Huang, Li and Gregory, 2011).

According to Marsh (2006), oral biofilm is known as dental plaque in which it plays the role in causing polymicrobial biofilm disease. Microbial homeostasis in oral biofilm refers to the stable microenvironment condition achieved over certain period. The balance of microbial homeostasis within the oral biofilm is maintained by the growth of the majority normal microflora and minority growth of the pathogenic oral bacteria that usually has no significant harmful effect to the host. However, the host diet in which frequent intake of dietary sugars especially sucrose could disrupt the balance of the microbial homeostasis in oral biofilm and subsequently lead to the shift of the formation of cariogenic oral biofilm. This phenomenon occurs due to the thresholds of the ecological perturbations have been exceeded and triggers the overgrowth of the pathogenic and cariogenic oral bacteria (Marsh, 2006).

The changes of microbial homeostasis in the oral biofilm includes the availability of nutrients uptake by the pathogenic oral bacteria and the low pH condition that enhance the colonization of cariogenic plaque bacteria includes *Streptococcus* spp., *Lactobacillus* spp., and *Bacillus* spp. (Jain, et al., 2013; Lamont, Koo and Hajishengallis, 2018). They become dominated in the oral cavity by supressing the growth of other normal microflora in which they utilize the dietary sugars and produce the EPS and acids. The produced acids result in low acidic microenvironment that triggers the gene expression of the cariogenic oral bacteria to protect themselves under the stress condition. Thus, they develop the ability to adapt, tolerate, and survive under the acidic condition. The further decreased in the pH level of 5.5 and below leads to the occurrence of enamel demineralization in which the minerals in the tooth are being damaged and resulted in caries lesions (Marsh, 2006).

2.2.2 Cariogenic Biofilm Formation

Development of cariogenic biofilm is mainly due to the hosts diet of frequent consumption of dietary carbohydrates that are fermentable and being utilized by the cariogenic oral bacteria such as *Streptococcus* spp. (Meyer, et al., 2021). In addition, colonization of certain rare oral bacteria such as *Bacillus* spp. could occurs due to their ability to undergo the sugar metabolism process that further leads to the formation of cariogenic biofilm (Jain, et al., 2013). The cariogenic plaque bacteria tend to produce EPS which is a virulence factor by providing the surface adhesion that further enhance the binding of the cariogenic plaque bacteria to each other on the tooth surface. This interaction of microorganisms is irreversible and resulting in stronger binding that makes the cariogenic biofilm

difficult to be removed from the tooth surface. Hence, the formation of cariogenic biofilm is usually appearing to be thicker than the non-cariogenic biofilm due to its composition changed in which higher plaque biomass being accumulated under frequent exposure to sugar (Meyer, et al., 2021).

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Besides, the cariogenic plaque bacteria undergo the sugar metabolism process via the phosphotransferase (PTS) system to induce the formation of cariogenic biofilm with the optimization of sugar uptake. The PTS system enables the increased of sugar uptake under neutral condition. Persistent operation of PTS system due to frequent exposure to sugar would further lead to the acidic condition in which the cariogenic biofilm tends to form. The thick cariogenic biofilm formed would reduce the oxygen content that favours the colonization and growth of anaerobic bacteria. These anaerobic oral bacteria undergo glycolysis to metabolize the sugar to produce energy for survival as well as acids which are the by-products. The diffusion of acids in the cariogenic biofilm could leads to the enamel demineralization at the critical pH 5.5 and below that causes tooth decay (Meyer, et al., 2021).

2.3 Cariogenicity of Dental Plaque

2.3.1 Etiology of Dental Caries

The "Ecological Plaque Hypothesis" explained that the imbalance of microbial homeostasis in the oral biofilm composes of beneficial microflora and pathogenic oral bacteria triggers the occurrence of plaque-mediated diseases. Naturally, the ratio of cariogenic oral bacteria is low in the oral cavity due to

their less competitive under the neutral pH condition. However, the composition of cariogenic oral bacteria could change due to the modern host's diet rich in fermentable carbohydrates (Marsh, 2006). The colonization of plaque bacteria is elevated under frequent exposure to sugar in which they are capable to undergo sugar metabolism process and produce more acids that subsequently lead to the plaque-mediated diseases. The acids produced lower the pH condition in the oral cavity that could trigger the enamel demineralization to occurs when the critical pH 5.5 and below is reached (Meyer, et al., 2021).

Besides, the common plaque bacteria such as *Streptococcus mutans* and *Lactobacillus* sp. usually demonstrated acidogenic property under the acidic pH condition. The degree of caries lesions caused by these plaque bacteria would be more significant as they are able to produce more acids at faster rate (Marsh, 2006). Similarly, other plaque bacteria such as *Bacillus* sp. in which they are the transient microflora that are being introduce via food consumption could also cause the caries to occur, but at a slower rate. They require a longer period to undergo maturation stage in which it is sufficient to cause dental caries (Sánchez-Vargas, et al., 2013). Hence, the occurrence of dental caries is mainly due to the dominance colonization of acidogenic and cariogenic plaque bacteria in which they supress the growth of the beneficial oral microflora (Marsh, 2006).

2.3.2 Role of Sugar in Biofilm Formation

Sugar is the main culprit that causes dental caries as it is capable in disrupting the balance of microbial homeostasis in the oral biofilm and changing the

composition of oral biofilm by altering the biochemical process that further induces caries to occur. Sugar especially sucrose which is the common sugar used in our daily life that posses the highest cariogenic effect as compared to other sugars such as fructose, glucose, and lactose. Sucrose serves as the fermentable source of carbohydrates for the cariogenic and acidogenic oral bacteria to form dental biofilms. Caries lesions are formed due to the increase in the accumulation of the cariogenic oral bacteria at the dental biofilms that undergo sugar metabolism process which produce more acids as the by-products. These acids trapped in the plaque would further dissolve the minerals in the tooth and lead to caries lesions (Leme, et al., 2006).

Sucrose is categorized as disaccharide composes of glucose and fructose that favours the colonization of cariogenic plaque bacteria and decrease the growth of non-cariogenic oral bacteria. It provides more energy sources for the cariogenic plaque bacteria to produce EPS due to more energy is being released during the break down of α-1,2-glycosidic bond in the non-reducing sugar (Dwivedi, 1978). As the result, the period of enamel demineralization at the critical pH 5.5 and below is prolonged due to the frequent exposure to fermentable sugar. Thus, it could damage the host's health condition and lead to chronic diseases (Hasnor, et., 2006).

2.3.3 Effect of Sweetener in Biofilm Formation

Sweetener is a sugar substitute used to replace the common sugar in order to maintain the sweetness in some ready-to-drink (RTD) beverages such as tea,

coffee, carbonated drinks, and juice drinks. In addition, sweetener could help to reduce the risk of dental caries as the cariogenic oral bacteria are unable to utilize it and undergo the metabolism process to produce acid (Razak, et al., 2017). Stevia is classified as natural sweetener that extracted from *Stevia rebaudiana* Bertoni plant. It is approximately 200 to 400 times sweeter than sugar due to the steviol glycosides which are the nature components found in the leaves of the plant (de Slavutzky, 2010). Stevia sweetener resulted in a significant decreased of dental plaque formation as it is a non-fermentable product and posed anti-cariogenic effect. Meantime, the plaque bacteria are unable to utilize it efficiently to produce acids (Razak, et al., 2017).

According to Razak, et al. (2017), as an alternative sweetener stevia was effective in reducing the EPS production and enhanced the biofilm porosity that resulted in thin biofilm. The outcomes demonstrated that implementation of stevia sweetener would not trigger the occurrence of enamel demineralization since the acids produced would not be able to reach the critical pH. The microbial homeostasis in the oral biofilm could be maintained as the microflora are not dominated by the cariogenic plaque bacteria. Thus, stevia that formed the biofilm would not cause caries lesions in the tooth and did not have negative impact on dental caries.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Design

The overview of this study is summarized as shown in Figure 3.1 below included three main stages.
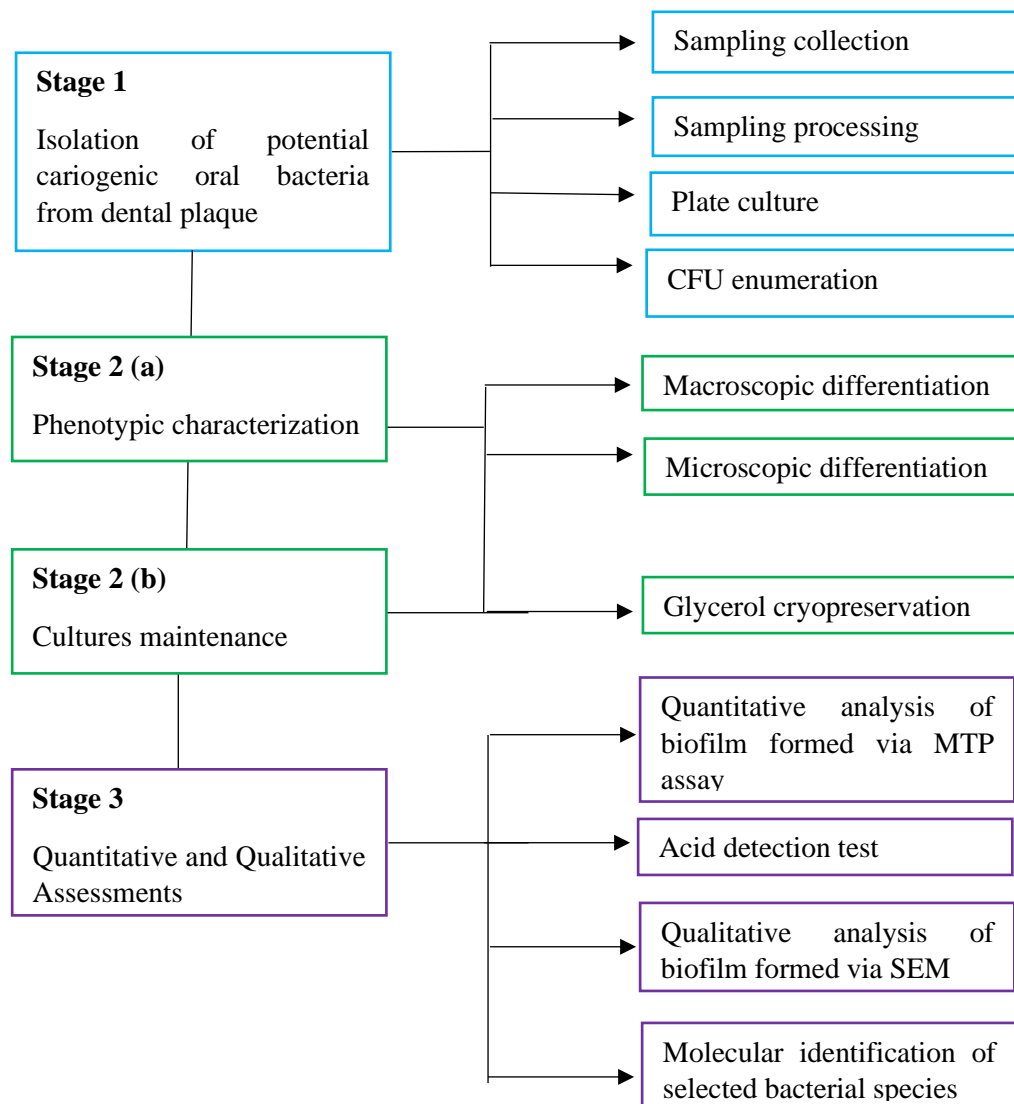
Figure 3.1: Experimental design of this study.

3.2 Experiment and Apparatus

The equipment and apparatus used in this study are listed in Appendix A.

3.3. Chemicals and Media

The chemicals and media used in this study are listed in Appendix A.

3.4 Media Preparation

The media that are required in this study were prepared at room temperature. The media were autoclaved at 121°C for 15 minutes to ensure the sterility and stored at room temperature prior to use unless specific conditions were stated otherwise.

3.4.1 Acetic acid, 30% (v/v)

A volume of 70 mL of sterile dH_2O was prepared in a beaker with addition of 30 mL of glacial acetic acid (System, Malaysia). Then, the mixture of solution with a final volume of 100 mL was stirred gently and poured into a 250 mL autoclaved Schott bottle. The sterilization process was not required for this solution.

3.4.2 Brain Heart Infusion (BHI) Broth

A final volume of 1 L of BHI broth was prepared by adding 37 g of BHI broth powder (HiMedia, India) into dH_2O. The mixture of solution was heated and

stirred gently using a hot plate for 1 minute to ensure the broth solution was completely dissolved. Then, the BHI broth was autoclaved.

3.4.3 Brain Heart Infusion (BHI) Broth Supplemented with 1% (w/v) Sugar

BHI broth was first prepared as stated in Section 3.4.2. A final volume of 1 L of BHI broth was prepared by adding 10 g of sugar (MSM Prai, Malaysia). The mixture of solution was heated and stirred gently using a hot plate for 1 minute to ensure the broth solution was completely dissolved. Then, the broth was autoclaved.

3.4.4 Brain Heart Infusion (BHI) Broth Supplemented with 0.15% (v/v) Stevia

BHI broth was first prepared as stated in Section 3.4.2. According to the manufacturer's protocol, it stated that 1 squeeze was equivalent to 0.3 ml per serving. A final volume of 1 L of autoclaved BHI broth was prepared by adding 0.15 ml which was equivalent to 150 µL of stevia (Equal, Malaysia). The mixture of solution was swirled gently.

3.4.5 Crystal Violet Solution, 0.1% (w/v)

A final volume of 50 mL of crystal violet solution was prepared by adding 0.05 g of crystal violet (HiMedia, India) in dH_2O. The sterilization process was not required for this solution.

3.4.6 Ethanol, 25%, 50%, 75%, 95% (v/v)

A final volume of 20 mL of ethanol (RCI Labscan, Thailand) with concentration of 25% (v/v) was prepared by mixing the 5 mL of absolute ethanol into dH_2O in a ratio of 1:3.

A final volume of 20 mL of ethanol (RCI Labscan, Thailand) with concentration of 50% (v/v) was prepared by mixing the 10 mL of absolute ethanol into dH_2O in a ratio of 1:1.

A final volume of 20 mL of ethanol (RCI Labscan, Thailand) with concentration of 75% (v/v) was prepared by mixing the 15 mL of absolute ethanol into dH_2O in a ratio of 3:1.

A final volume of 20 mL of ethanol (RCI Labscan, Thailand) with concentration of 95% (v/v) was prepared by mixing the 19 mL of absolute ethanol into dH_2O in a ratio of 19:1.

3.4.7 Glutaraldehyde in Phosphate Buffered Saline (PBS), 2.5% (v/v)

A final volume of 150 mL with final concentration of 2.5% (v/v) glutaraldehyde in PBS was prepared by adding 15 mL of concentrated glutaraldehyde in water with a stock concentration of 25% (v/v) (Acros Organics, Belgium) into 135 mL of autoclaved PBS. The sterilization process was not required in the preparation for this solution.

3.4.8 Glycerol Solution, 30% (v/v)

A final volume of 10 mL glycerol solution with concentration of 30% (v/v) was prepared by adding 3 mL of anhydrous glycerol stock solution (System, Malaysia) into 7 mL dH_2O.

3.4.9 Hydrogen peroxide (H_2O_2), 3% (v/v)

A final volume of 10 mL with a final concentration of 3% (v/v) of H_2O_2 (Merck Schuchardt OHG, Germany) was prepared by adding 1 mL of H_2O_2 with a stock concentration of 30% (v/v) into 9 mL of dH_2O. The mixture of solution was swirled gently. The sterilization process was not required for this solution.

3.4.10 Phosphate Buffered Saline (PBS), pH 7.3 ± 0.2

A final volume of 900 mL of PBS was prepared by adding 9 tablets of phosphate buffered salt tablets (ChemSoln, England) into dH_2O. The solution was autoclaved.

3.4.11 Saline Solution, 0.45% (w/v)

A final volume of 100 mL with concentration of 0.45% (w/v) was prepared by adding 0.45 g of sodium chloride (Merck, USA) in dH_2O. The mixture of solution was swirled gently to ensure the solution dissolve completely. The solution was autoclaved.

3.4.12 Tryptic Soy Agar (TSA)

A final volume of 1 L of TSA was prepared by adding 40 g of TSA powder (Merck, USA) in dH_2O. The mixture of solution was heated and stirred gently using a hot plate for 1 minute to ensure the broth solution was completely dissolved. Then, the BHI broth was autoclaved. After autoclaved, the medium was cooled to approximately 50 to 55°C in laminar flow and then poured into the respective Petri dishes. The agar was allowed to solidify at room temperature in the laminar flow. The fresh agar plates were then sealed and stored at 4°C and used within two months.

3.5 Sample Collection

3.5.1 Standard of Subject Selections

The selection of subjects that suitable for this study were screened via oral interview to further understand the oral history as well as the oral health condition of the participants. The subjects were chosen based on the age ranging from 20 to 25 years old. The consideration of selecting the age of the subjects was important as the group of people were believed to have good status of oral health (Mosha, et al., 1994). According to Heller et al. (2016), there were several exclusion criteria were required to take into consideration prior to oral sampling. The reason was to ensure that the personal factors would not cause impact on the nature structure of the oral bacteria and the collection of the dental plaque. The standard of subject selections included: (i) Full dentition as an adult have 32 teeth; (ii) Non-pregnancy; (iii) Non-smoker; (iv) No medication record of antibiotics consumption in the past 3 months before sampling collection; (v) No

long-term intake of anti-inflammatory medication; (vi) None detectable oral disease included caries lesions and gingivitis disease; (vii) None detectable systemic disease that might affect the salivary function. Thus, the oral consent was provided to the participants to protect their privacy.

3.5.2 Dental Plaque Sampling

Subjects that were fit for this study as stated in Section 3.5.1 were requested to abstain from any oral hygiene practices for a duration 18 to 24 hours for the purpose of sample collection. The sampling collection process was conducted in the morning before tooth brushing. The dental plaque on the tooth surface was scraped using sterile toothpicks and transferred into 1.0 mL of 0.45% (w/v) saline solution in a 1.5 mL of sterile microcentrifuge tube. The sample was then stored at 4°C for further used.

3.6 Isolation of Potential Cariogenic Oral Bacteria

3.6.1 Sample Processing

The collected dental plaque sample in the saline solution was processed after sample collection process. Vigorous vortexing (Stuart, UK) of the collected dental plaque sample was required under the conditions of 2, 500 rpm for three minutes to break the visible clumps and homogenize the sample. Then, the sample was subjected to vigorous pipetting (Eppendorf, Germany) for several times to suspend the remaining clumps. The suspension was further required for serial dilution and plate culture.

3.6.2 Serial Dilution and Plate Culture

Ten-fold serial dilution was performed with total volume of 300 µL of each suspension from Section 3.6.1 by adding 30 µL of suspension into 270 µL of 0.45% (w/v) saline solution as the diluent in a sterile 1.5 mL microcentrifuge tube. The dilution was performed up to 10^{-5} dilution. Then, the mixture of suspension was inverted gently to mix well.

Plate culture was performed by adding 100 µL of each dilution (10^{-1} to 10^{-5}) onto sterile TSA plate (Merck, USA) in two duplicates sets. The TSA plates were spread evenly using sterile spreader and allowed to dry in laminar flow (Esco, Singapore) to avoid any kind of contamination. The plates were sealed and incubated aerobically for Set 1 and microaerobically for Set 2 in a candle jar (Oxoid, UK) at 37°C for 48 hours.

3.6.3 Colony-forming Unit (CFU) Enumeration

After incubation, the TSA plate with cultures from Section 3.6.2 were proceeded with CFU enumeration. The CFU/mL enumeration was performed manually for each set by selecting the plates that contained CFU between 30 to 300 colonies using the Equation-1 as stated below (Sieuwerts et al., 2008).

$$CFU/mL = \frac{\text{(Number of colonies counted on plate (CFU))}}{\text{(Dilution} \times \text{Volume plate (mL))}} \quad \textbf{(1)}$$

3.7 Phenotypic Characterization

3.7.1 Macroscopic Differentiation

The different colonies morphologies of the selected plates obtained in Section 3.6.2 were observed and recorded. The macroscopic features included the shape, elevation, margin, size, colour, opacity, texture and appearance were recorded (Supriya, 2022).

3.7.2 Subculture of Oral Bacteria

The unique and distinct morphologies of colonies obtained in Section 3.7.1 were selected for subculture to obtain pure culture. The selected colony was inoculated with a sterile inoculating loop and streaked onto a sterile TSA in laminar flow. The inoculated TSA plates were incubated aerobically and microaerobically at 37°C for overnight (Zhou and Li, 2021).

3.7.3 Gram Stain

According to Smith and Hussey (2005), Gram stain was the basic need for phenotypic characterization of bacteria. Gram stain was performed to differentiate the bacteria based on the peptidoglycan structure in which Gram-positive has a thick peptidoglycan and Gram-negative has a thin peptidoglycan. A positive control (*Streptococcus mitis* ATCC 6249) and a negative control (*Actinomyces viscosus* ATCC 43146) were included. A thin smear of the pure culture of the bacteria colony obtained in Section 3.7.2 was made on a clean glass slide and allowed to dry in the laminar flow. Then, heat-fix was applied

onto the air-dried smear. The slide was stained with crystal violet for one minute. The stained slide was rinsed with running tap water. Next, the slide was covered with Gram's iodine for one minute and then rinsed with running tap water. Later, the decolourizing agent was added drop by drop slowly onto the slide for 15 seconds and then rinsed with running tap water. Lastly, the slide was counterstained with safranin for 30 seconds and then rinsed with running tap water. The slide was blotted dry and proceeded with microscopic examination with different magnifications included 4×, 10×, 40×, and 100× objective lens with immersion oil. The cells that stained purple colour indicated as Gram-positive. The cells that stained pink colour indicated as Gram-negative. The cells that stained with mixture of purple and pink colours indicated as Gram-variable.

3.7.4 Catalase Test

A positive control (*Actinomyces viscosus* ATCC 43146) and a negative control (*Streptococcus mitis* ATCC 6249) were included. According to Reiner (2010), catalase test was performed using the slide drop method in which a little amount of the pure culture of the bacterial colony obtained in Section 3.7.2 was inoculated on a clean glass slide using sterile inoculating loop. Then, a drop of 3% (v/v) hydrogen peroxide (H_2O_2) was placed onto the smear and the duration for the bubble formation was observed and recorded. Catalase-positive was indicated by the formation of bubble within the duration of 10 seconds. Catalase-negative was indicated by absence of bubble formation within the duration of 10 seconds (Engelkirk and Duben-Engelkirk, 2008).

3.7.5 Glycerol Cryopreservation

The pure culture obtained from Section 3.7.2 was stored via glycerol cryopreservation for a short period. Two loopfuls of colonies of each pure culture were inoculated into 15 mL of BHI broth (Himedia, India). The broth culture was incubated in shaking incubator (Cole-Parmer, USA) at 37°C, 200 rpm for overnight. The optical density at 600 nm (OD_{600}) of the broth culture was measured using UV-Vis spectrophotometer (Thermo Scientific, USA) within the range between 0.08 to 0.1 in which this range referred to 0.5 McFarland standard (Bai, Jiang and Jiang, 2014).

The glycerol stock culture of each pure culture with 15% (v/v) of final concentration was prepared by adding 500 µL of culture and 500 µL of 30% (v/v) glycerol solution (System, Malaysia). A volume of 1 mL of the mixture was then transferred into cryovials and stored at -20°C for further used.

3.7.6 Detection of Haemolytic Activity

An α-haemolytic positive control (*Streptococcus mitis* ATCC 6249), β-haemolytic positive control (*Staphylococcus pasteuri* ATCC 51128) and γ-haemolytic positive control (*Actinomyces viscosus* ATCC 43146) were included. A sterile cotton swab was dipped into the pure BHI broth culture and swab onto the sterile Columbia agar supplemented with 5% sheep blood (ISOLAB, Malaysia) (designated as "sheep blood agar (SBA)"). The respective plates were incubated aerobically and microaerobically in a candle jar at 37°C for overnight.

The results of haemolytic pattern of each of the pure culture was observed under a light source. According to Buxton (2005), it stated that α-haemolytic referred to partial haemolysis that appeared as greenish colour on SBA due to the oxidation of iron in the haemoglobin followed by β-haemolytic referred to complete haemolysis as the formation of clear zone surrounding the colonies due to the toxins produced by the bacteria that able to lyse the red blood cells and γ-haemolytic referred to no haemolysis occurs.

3.7.7 Culture Maintenance

The pure culture was maintained by performing cell viability test prior to glycerol cryopreservation by subculturing on sterile TSA once a week.

3.8 Assessment of the Biofilm-forming Strength via 96-well Microtiter Plate (MTP) Assay

The 96-well microtiter plate (MTP) assay was categorized as a static system to access the biofilm-forming strength by measuring the intensity of the stained biofilm cells. This assay was commonly used in examining the formation of biofilm in the early stages (Merritt, Kadouri and O'Toole, 2005). In this study, the biofilm-forming strength of each target isolates was accessed via polystyrene flat-bottom 96-well microtiter plates, tissue culture treated (Nest Biotech, China). The protocol of the MTP assay with slight modification applied in this study was as stated by Stepanović, et al. (2007). In addition, the washing step of the biofilm cells was one of the most important steps in the MTP assay to ensure all the unadherent bacterial cells have been removed (Merritt, Kadouri and O'Toole,

2005). There are four different stages in the MTP assay as stated in Section 3.8.1 to Section 3.8.4. Optical density at 570 nm (OD_{570}) referred to the quantification of the stained biofilm in the MTP after being solubilized using 30% (v/v) acetic acid. Then, the mean of the biofilm-forming strength of each target isolates referred as (OD_M) was determined by performing triplicates for each target isolates with the respective growth medium included (i) sugar free BHI broth, (ii) sugar-dependent (SD) BHI broth supplemented with 1% sugar (w/v) and (iii) stevia-dependent (StD) BHI broth supplemented with 0.15% stevia (v/v).

3.8.1 Inoculum Preparation

A positive control (*Streptococcus mitis* ATCC 6249) was included in this part. Two loopfuls of colonies of each target isolates and control were inoculated into 15 mL of BHI broth. The suspensions were incubated at 37°C for overnight. The optical density at 600 nm (OD_{600}) of the broth culture was measured using UV-Vis spectrophotometer (Thermo Scientific, USA) within the range between 0.08 to 0.1 in which this range referred to 0.5 McFarland standard (Bai, Jiang and Jiang, 2014). A volume of 1 mL of diluted broth culture was transferred into 15 mL Falcon tube and centrifuged at 5, 000 ×g, room temperature for 10 minutes. The supernatant was then discarded and the pellet was maintained and supplemented with respective growth medium. The pellet was subjected to vortex until dissolved completely. This procedure was to standardize the cell density of each isolate.

3.8.2 Cultivation of Biofilm

Biofilm cultivation was carried out by inoculating triplicates of 200 µL of the standardized culture and uninoculated sterile BHI broth served as the blank into each 96-well MTP and the plate was covered. The plate was incubated aerobically at 37°C for overnight. The blank played the important role in the calculation for the biofilm-forming strength in Section 3.8.4 and this referred as the unique cut-off OD_{570} (OD_C).

After the overnight of incubation, the content of each well in the MTP was removed slowly by pouring out the planktonic cells into a waste tray and shake it gently. The planktonic cells were removed by submerging the plate into a tray containing phosphate buffered saline (PBS) in a 45°angle to avoid the bubbles formation in the well. Then, the plate was flicked slowly to remove the excess PBS. The plate was blot dry with tissue paper and further allowed to air-dry in an inverted position at room temperature in the laminar flow for 45 minutes. Later, the plate was subjected to heat fixation at 60°C for an hour. This step was required to fix the biofilm by stopping the bacteria to continue to grow.

3.8.3 Quantification of Biofilm

Biofilm quantification was carried out by aliquoting 150 µL of 0.1% (w/v) crystal violet solution into each well to stain the fixed biofilm cells. The plate was incubated at room temperature for 10 minutes. After that, the excess crystal violet solution was poured out slowly into a waste tray and shake it gently. The crystal violet solution was further removed by submerging the plate into three

trays containing dH_2O, respectively. The plate was blot dry with tissue paper and covered to further allow to air-dry in an inverted position at room temperature for overnight.

On the next day, a volume of 150 µL of 30% (v/v) acetic acid was placed into each stained well. This step was used to solubilize the crystal violet stain in the biofilm. The plate was then covered to prevent acetic acid evaporation and incubated at room temperature for 30 minutes to allow the dye to solubilize. The solubilized crystal violet was transferred into a new 96-well plate accordingly. Lastly, the optical density at 570 nm (OD_{570}) was measured using microplate reader (BMG Labtech, Germany).

3.8.4 Assessment of Biofilm-forming Strength

In this study, the mean OD (OD_M) was calculated for the positive control and each of the target isolate as described in Section 3.8. The cut-off value (OD_C) was used to interpret the results obtained by defining between the biofilm producers and the non-biofilm producers by using the Equation-2 as shown below.

$$OD_C = \text{average } OD_{570} \text{ of negative control} + (3 \times \text{standard deviation of } OD_{570} \text{ of negative control}) \quad \textbf{(2)}$$

The calculated OD_M and OD_C of each target isolate were compared to indicate the biofilm-forming strength, respectively. A negative control was required to

include in each of the 96-well MTP to obtain the cut-off value (OD_C). Thus, there were four different categories of biofilm-forming strength including "non-biofilm producer", "weak biofilm producer", "moderate biofilm producer" and "strong biofilm producer" as shown in Table 3.1 below. In this study, the non-biofilm producer and weak biofilm producer were excluded while the moderate biofilm producer and strong biofilm producer were included for further analysis.

Table 3.1: Categorization of biofilm-forming strength of each isolate according to the calculated mean OD_{570}.

Biofilm-forming strength	Mean OD_{570} (OD_M)[a]
Non-biofilm producer	$\mathbf{OD_M} \leq OD_C$
Weak biofilm producer	$OD_C < \mathbf{OD_M} \leq 2 \times OD_C$
Moderate biofilm producer	$2 \times OD_C < \mathbf{OD_M} \leq 4 \times OD_C$
Strong biofilm producer	$4 \times OD_C < \mathbf{OD_M}$

[a] The calculated OD_M of each target isolate was compared with the calculated OD_C to indicate the biofilm-forming strength, respectively.

3.8.5 Statistical Analysis

Statistical analysis of MTP assay was conducted using Microsoft Excel based on the one-tailed *t*-test at 95% confidence interval. This step was used to further conclude the biofilm-forming strength after exposure to sugar and stevia, respectively based on the statistical significance. The null hypothesis, H_0 referred to there was no significant difference in the biofilm-forming strength of

the target isolate after exposure to sugar and stevia, respectively. When the p-value was more than or equal to 0.05 ($p \geq 0.05$), the H_0 was accepted in this test. However, the H_0 was rejected in this test when the p-value was less than 0.05 ($p < 0.05$) and this stated that there was a significant difference in the biofilm-forming strength of the target isolate.

3.9 Measurement of Acidogenicity

3.9.1 Acid Detection Test

The protocol of the acid detection test with slight modification applied in this study was as stated by Ma, et al. (2013). A positive control (*Streptococcus mitis* ATCC 6249) was included in this part. A triplicate of two loopfuls of colonies of each target isolates with strong biofilm-forming strength and control were inoculated into 15 mL of BHI broth. The suspensions were incubated at aerobically at 37°C for overnight. The optical density at 600 nm (OD_{600}) of the broth culture was standardized using UV-Vis spectrophotometer (Thermo Scientific, USA) within the range between 0.08 to 0.1 in which this range referred to 0.5 McFarland standard (Bai, Jiang and Jiang, 2014). A volume of 5 mL of diluted broth culture was transferred into 50 mL Falcon tube and centrifuged at 5, 000 ×g, room temperature for 10 minutes. The supernatant was then discarded and the pellet was maintained and supplemented with respective growth medium included (i) sugar free BHI broth with culture free, (ii) sugar-dependent (SD) BHI broth supplemented with 1% sugar (w/v) and (iii) stevia-dependent (StD) BHI broth supplemented with 0.15% stevia (v/v). The pellet was subjected to vortex until dissolved before overnight incubation. After

overnight incubation, the respective cultures were centrifuged at $5,000 \times g$, room temperature for 10 minutes. A volume of 10 mL of supernatant was collected and transferred into 15 mL Falcon tube. The pH of the supernatant was measured using pH meter (Mettler Toledo, USA).

3.9.2 Statistical Analysis

Statistical analysis of acid detection test was conducted using Microsoft Excel based on the one-tailed t-test at 95% confidence interval. This step was used to further conclude the acidogenic property of the target isolates after exposure to sugar and stevia, respectively based on the statistical significance. The null hypothesis, H_0 referred to there was no significant difference in the acidogenic property of the target isolates of the target isolate after exposure to sugar and stevia, respectively. When the p-value was more than or equal to 0.05 ($p \geq 0.05$), the H_0 was accepted in this test. However, the H_0 was rejected in this test when the p-value was less than 0.05 ($p < 0.05$) and this stated that there was a significant difference in the acidogenic property of the target isolate.

3.10 Qualitative Analysis of Biofilm Formation via Scanning Electron Microscope (SEM)

3.10.1 Cultivation of Biofilm on Glass Coverslip

A positive control (*Streptococcus mitis* ATCC 6249) was included in this part. The isolates that posed acidogenic property were selected in this part. Two loopfuls of colonies of each target isolates and control were inoculated into 10

mL of BHI broth. The suspensions were incubated at aerobically at 37°C for overnight. The optical density at 600 nm (OD_{600}) of the broth culture was standardized using UV-Vis spectrophotometer (Thermo Scientific, USA) within the range between 0.08 to 0.1 in which this range referred to 0.5 McFarland standard (Bai, Jiang and Jiang, 2014). A volume of 5 mL of cell suspensions was transferred into 50 mL Falcon tube and centrifuged at 5, 000 ×g, room temperature for 10 minutes. The supernatant was then discarded and the pellet was maintained and supplemented with respective growth medium included (i) sugar free BHI broth with culture free, (ii) sugar-dependent (SD) BHI broth supplemented with 1% sugar (w/v) and (iii) stevia-dependent (StD) BHI broth supplemented with 0.15% stevia (v/v). The pellet was subjected to vortex until dissolved completely. The coverslips that served as the base to cultivate the biofilm were cut into size of 0.5 cm^2 approximately. The glass coverslips were submerged into Listerine solution (McNeil Consumer Healthcare, USA) for disinfection and washed with sterile dH_2O. The glass coverslips were placed into the bacterial cultures with the respective growth medium as mentioned above and incubated at 37°C for overnight.

3.10.2 Preparation of Specimen

Preparation of specimen was conducted according to the protocol provided by the skilled lab officer from Department of Chemical Science, Faculty of Science, UTAR. The cultivation of biofilms on glass coverslips as stated in Section 3.10.1 referred as "specimen". The suspensions of each tube were poured out slowly onto the Petri dishes, respectively. A volume of 10 mL of 2.5% (v/v) glutaraldehyde in PBS was added into the Petri dish. The specimen was fixed at

room temperature for 10 minutes. After fixation, the glutaraldehyde solution was poured out gently. The specimen was then washed using 5 mL of 0.01M PBS. The washing step was repeated for three times. The specimen was placed at room temperature for 10 minutes. Next, serial dehydration of the specimen was performed using final volume of 10 mL of ethanol for each of the following orders: 25% ethanol for 5 minutes; 50% ethanol for 10 minutes; 75% ethanol for 10 minutes and 95% ethanol for 10 minutes to remove the moisture from the specimen. Later, the specimen was placed into absolute ethanol for 10 minutes to further remove the remaining moisture from the specimen. The dehydrated specimen was dried in the vacuum oven (Sheldon Manufacturing Inc., USA) according to the conditions of 150 mBar, 50°C for 3 hours.

3.10.3 Specimen Imaging

The specimens of each target isolates were sputter coated with platinum in JFC-1600 Auto Fine Coater (JEOL, USA). The specimens were examined at magnification of 1, 000× and 2, 000× in a JSM-6701F Scanning Electron Microscope (JEOL, USA) at 4 kV acceleration voltage, a range of 6.9 mm to 9.9 mm of working distance and 1 µm objective aperture. The biofilm thickness was measured in a scale of µm via the EOS 6701 integrated image software. Then, the mean of the biofilm thickness was obtained.

3.10.4 Statistical Analysis

Statistical analysis of qualitative analysis of biofilm formation via SEM was conducted using Microsoft Excel based on the one-tailed t-test at 95%

confidence interval. This step was used to further conclude the thickness of the biofilm of the target isolates after exposure to sugar and stevia, respectively based on the statistical significance. The null hypothesis, H_0 referred to there was no significant difference in the thickness of the biofilm of the target isolates after exposure to sugar and stevia, respectively. When the *p*-value was more than or equal to 0.05 ($p \geq 0.05$), the H_0 was accepted in this test. However, the H_0 was rejected in this test when the *p*-value was less than 0.05 ($p < 0.05$) and this stated that there was a significant difference in the thickness of the biofilm of the target isolate.

3.11 Genotypic Identification

3.11.1 Genomic DNA Extraction of Bacteria

Genomic DNA extraction of each target isolate was conducted according to the DNA extraction kit protocol provided (Macherey-Nagel NucleoSpin® Microbial DNA, Germany). The protocol was stated in Appendix B.

3.11.2 Universal 16S rDNA Polymerase Chain Reaction (PCR)

In this part, PCR was conducted to amplify the 16S rDNA region of the target isolates. According to Lu, et al. (2000) provided that a pair of universal 16S forward and reverse primers referred as F1 and R1, respectively were used with the expected size of the amplicon of 996 bp. The nucleotide sequence of the forward primer (F1) and reverse primer (R1) were designed as:

F1: 5'-CCAGCAGCCGCGGTAATACG-3'

R1: 5'-ATCGGYTACCTTGTTACGACTTC-3'

For a PCR mix of 50 µL reaction, it contained 1× FirstBase PCR master mix (FirstBase, Singapore), 0.8 µM of each forward and reverse primers and 150 ng of template DNA were prepared. A non-template control was prepared as well by replacing the template DNA with nuclease-free H_2O. A positive control *Streptococcus mitis* ATCC 6249 was included in this part. Amplification of the target gene was conducted in thermocycler (Eppendorf, Germany) according to the following PCR thermal profile as stated by Lu, et al. (2000). The conditions of the PCR thermal profile included initial denaturation, denaturation, primer annealing, primer extension and final extension as described in Table 3.2 below. The amplicons were held at 10°C at the end of the PCR cycle.

Table 3.2: The PCR thermal profile.

Cycling steps	Temperature / Time	Number of cycles
Initial denaturation	94°C / 10 mins	1
Denaturation	94°C / 1 min	
Primer annealing	55°C / 1 min	35
Primer extension	72°C / 1 min	
Final extension	72°C / 10 mins	1

3.11.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was conducted for the target amplicons obtained from Section 3.11.2 using 1.3% (w/v) agarose gel at 80 V for 40 minutes. A volume of 1 µL loading dye with DNA stain (GeneDireX, USA) with 5 µL of 0.1 µg/µL of 100 bp DNA Ladder (SMOBIO Technology Inc., China) were loaded into the first well. Then, a volume of 1 µL loading dye with DNA stain (GeneDireX, USA) with 5 µL of each of the target amplicons were loaded into the respective wells. The electrophoresed gel image was viewed under gel imaging system and TransUV illuminator (Bio-Rad, USA).

3.11.4 Purification of Amplicon and DNA Sequencing

Amplicons with the expected size of ~996 bp were used to proceed with the spin column-based nucleic acid purification process according to the spin column PCR purification kit protocol provided (Bio Basic Inc., Canada). The protocol was stated in Appendix C. After purification, the DNA concentration of the purified amplicons were measured using NanoDrop^{TM} (Thermo Scientific, USA) to ensure a minimum DNA concentration of 40 ng/µL as well as DNA purity of A_{260}/A_{280} ratio of 1.80-2.00 in order to meet the requirements stated by the sequencing company.

3.11.5 Sequence Analysis

The sequencing results obtained from Section 3.11.4 were proceeded to sequence analysis. The identity of the target isolates was determined using the

Basic Local Alignment Search Tool (BLAST) system which was the nucleotide BLAST (BLASTn) provided by the National Center for Biotechnology Information (NCBI). The results of the DNA sequences were analyzed using the Molecular Evolutionary Genetics Analysis software designated as "MEGA 11" to determine the high purity bases. Then, the identified high purity bases were set as the query sequence to compare with the subject sequence of the existing 16S rRNA gene of Bacteria and Archaea in GenBank. The criteria of sequence homology included high percentage identities of more than or equal to 97% and the low E-value which was closed to zero.

CHAPTER 4

RESULTS

4.1 Isolation of Potential Cariogenic Oral Bacteria

4.1.1 Isolation of Primary Isolates

The primary isolates were selected from the processed and serially diluted dental plaque sample onto the TSA plates under the respective incubation conditions which were aerobically for Set 1 and microaerobically for Set 2. For both aerobically (Set 1) and microaerobically (Set 2), serial dilution of 10^{-4} plates of each were selected as these plates fulfilled the criteria of colony-forming unit (CFU) enumeration within the range of 30-300 colonies (Sieuwerts, et al., 2008). The CFU enumeration of the respective plates were listed in Table 4.1. Besides, Figure 4.1 shows the appearance of primary isolates on TSA of the selected plates. Total of three isolates (ST, BETA and GAMMA) with distinct colony morphology were obtained prior to microscopic characterization.

Table 4.1: Colony-forming unit (CFU) enumeration of the primary isolates on 10^{-4} dilution TSA.

Primary plate	Number of colonies counted	CFU/mL
Set 1	54	0.054
Set 2	173	0.173
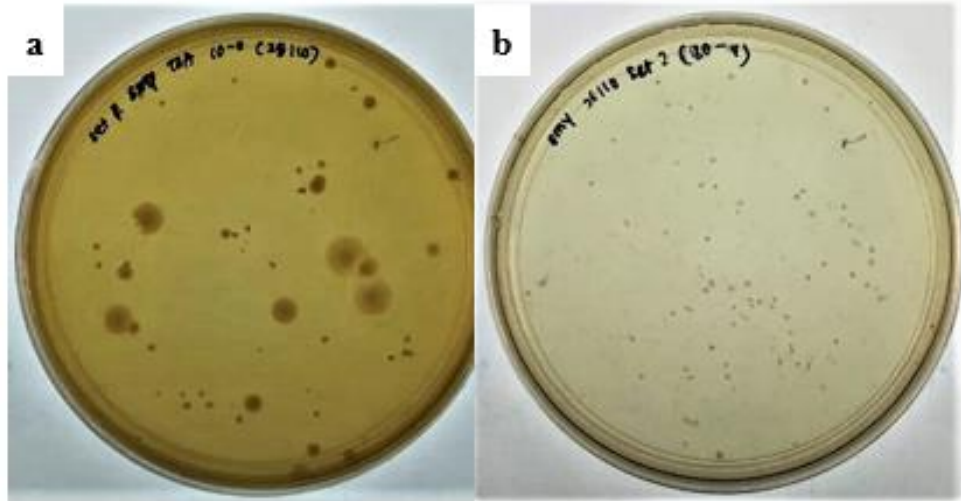
Figure 4.1: Appearance of primary isolates on TSA of serial dilution 10^{-4} incubated for 2 days (a) aerobically and (b) microaerobically.

4.2 Phenotypic Characterization

4.2.1 Macroscopic Differentiation

Pure cultures of ST, BETA and GAMMA were obtained on the TSA plates for further microscopic differentiation. These three target isolates tend to produce milky yellow or whitish colours colonies on TSA. Further descriptions of the colony morphologies of the target isolates were summarized in Table 4.2 in Section 4.2.4. The macroscopic morphologies of the pure cultures of the selected isolates appeared on TSA were illustrated in Figure 4.2.
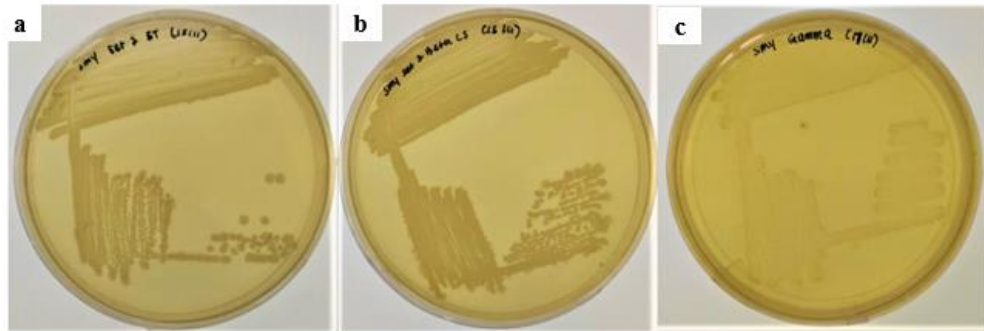
Figure 4.2: Macroscopic morphologies of the target cultures (a) ST, (b) BETA, and (c) GAMMA on tryptic soy agar (TSA) after incubation at 37°C for 24 hours.

4.2.2 Microscopic Differentiation

Gram stain was performed on the pure cultures of ST, BETA and GAMMA isolates obtained in Section 4.2.1 with a positive control (*Streptococcus mitis* ATCC 6249) and a negative control (*Actinomyces viscosus* ATCC 43146). Notably, ST and BETA isolates were Gram-positive, whereas GAMMA isolate was Gram-negative. The microscopic morphologies of the target isolates were short rod in shape with the arrangement of cells of either in pairs (diplobacilli) or in chains (streptobacilli). Figure 4.3 shows the representative results of the positive control and negative control of Gram stain. Further microscopic morphologies of the target isolates were summarized in Table 4.2 in Section 4.2.4.
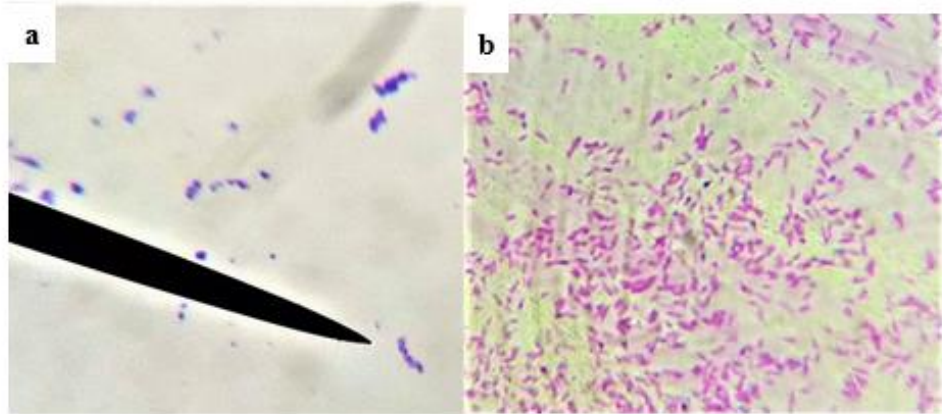
Figure 4.3: Representative results of Gram stain of (a) positive control (*S. mitis* ATCC 6249) and (b) negative control (*Actinomyces viscosus* ATCC 43146).

4.2.3 Catalase Test

Catalase test was performed on the pure cultures of ST, BETA and GAMMA isolates obtained in Section 4.2.1 with a positive control (*A. viscosus* ATCC 43146) and a negative control (*S. mitis* ATCC 6249). As the results, there were two isolates tested catalase-positive and one isolate tested catalase-negative. Figure 4.4 shows the representative results of catalase test of the positive control and negative control. The detail results of the catalase test of the target isolates were summarized in Table 4.2 in Section 4.2.4.
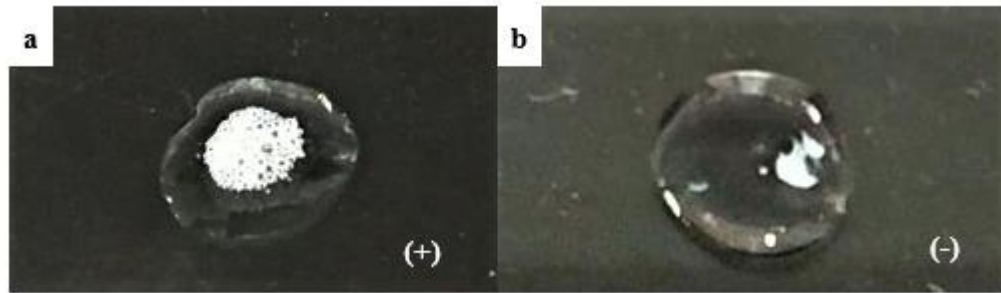
Figure 4.4: The representative results of catalase test.

Catalase-positive was represented as "(+)" and catalase-negative was represented as "(-)". **(a)** Catalase test reaction of positive control, (*A. viscosus* ATCC 43146) and **(b)** negative control (*S. mitis* ATCC 6249).

4.2.4 Detection of Haemolytic Activity

Haemolytic activity of the pure broth cultures of ST, BETA and GAMMA isolates were detected on SBA along with an α-haemolytic positive control (*S. mitis* ATCC 6249), β-haemolytic positive control (*Staphylococcus pasteuri* ATCC 51129) and γ-haemolytic positive control (*A. viscosus* ATCC 43146). Notably, the three target isolates resulted in different haemolytic patterns. As the results, α-haemolytic activity resulted in partial haemolysis that appeared as greenish colour surrounding the colonies followed by β-haemolytic activity resulted in complete haemolysis as the formation of clear zone surrounding the colonies and γ-haemolytic activity resulted in no haemolysis occurred. Similarly, the respective SBA plates were placed under a light source to further confirm the haemolytic patterns. Figure 4.5 shows the representative results of haemolytic activity of α-haemolytic, β-haemolytic, and γ-haemolytic activities, respectively. The detail results of the haemolytic activity of the target isolates were summarized in Table 4.2 in Section 4.2.4.
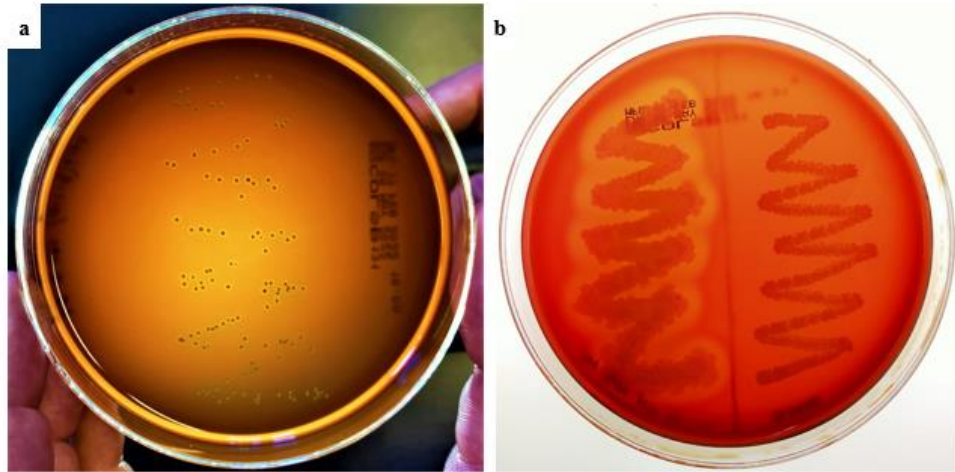
Figure 4.5: The representative results of haemolytic activity of α-haemolytic, β-haemolytic, and γ-haemolytic activities, respectively.

(a) Alpha haemolytic of positive control, (*S. mitis* ATCC 6249);

(b) Beta haemolytic of positive control (*S. pasteuri* ATCC 51129) (left) and Gamma haemolytic of positive control (*A. viscosus* ATCC 43146) (right).

Table 4.2: Summary of macroscopic differentiation, microscopic differentiation, results of catalase test and results of haemolytic activities of the target isolates.

Target isolates	Characteristics											
	Macroscopic										Microscopic	Catalase test[b]	Haemolytic activity
	Shape	Elevation	Margin	Size[a]	Colour	Opacity	Texture	Appearance	Gram type	Cells arrangements		
ST	Irregular	Flat	Undulate	Moderate	Milky yellow	Opaque	Dry, mucoid	Dull	Positive	Streptobacilli	+	α-haemolytic
BETA	Irregular	Flat	Undulate	Moderate	Pale yellow	Opaque	Dry, mucoid	Dull	Positive	Streptobacilli	+	β-haemolytic
GAMMA	Circular	Raised	Entire	Small	Whitish	Translucent	Shiny, viscous	Shiny	Negative	Diplobacilli	_	γ-haemolytic

[a] The size of colony was measured in millimetre (mm). Punctiform: < 1mm; Small:1 to 2 mm; Moderate: 2 to 8 mm; Large: > 8 mm (Caldwell, 2013).
[b] Catalase-positive (+): Formation of bubble within the duration of 10 seconds; Catalase-negative (-): Absence of bubble formation within the duration of 10 seconds (Engelkirk and Duben-Engelkirk, 2008).

4.3 Assessment of the Biofilm-forming Strength via 96-well Microtiter Plate (MTP)

MTP assay was performed under three conditions including sugar free condition, sugar-dependent (SD) condition and stevia-dependent (StD) condition. BHI broth was selected as the growth medium for all the conditions. In this part, ST and BETA isolates were subjected to the biofilm-forming strength assessment but, GAMMA isolate was excluded as it was unable to grow in broth. One-tailed t-test yielding the p-value of less than 0.05 ($p<0.05$) indicated as statistically significant whereas p-value of more than or equal to 0.05 ($p\geq0.05$) indicated as not significant. The mean OD_{570} (OD_M) was assigned as mean $\pm$ standard deviation.

The mean OD_{570} (OD_M) of the positive control (*S. mitis* ATCC 6249) and the target isolates (ST and BETA) under the three respective conditions followed by the biofilm-forming strengths for triplicates were recorded in Table 4.3, Table 4.4, and Table 4.5, respectively. The two target isolates (ST and BETA) demonstrated differences in the biofilm-forming strength after exposure to sugar and stevia, respectively. As shown in Table 4.3, the positive control (*S. mitis* ATCC 6249) and the two target isolates (ST and BETA) were classified as non-biofilm producer under the sugar free condition. In contrast, based on the results in Table 4.4, the biofilm-forming strength of the positive control (*S. mitis* ATCC 6249) and the two target isolates (ST and BETA) increased in the presence of sugar as compared to the sugar free condition. The positive control (*S. mitis* ATCC 6249) resulted in weak biofilm producer with supplementation of sugar. The ST isolate demonstrated as moderate biofilm producer with supplementation

of sugar. The BETA isolate categorized as weak biofilm producer with supplementation of sugar.

On the other hand, the biofilm-forming strength of the positive control (*S. mitis* ATCC 6249) and the two target isolates (ST and BETA) increased slightly in the presence of stevia as compared to the sugar free condition. As shown in Table 4.5, the positive control (*S. mitis* ATCC 6249) resulted in non-biofilm producer with supplementation of stevia. The ST isolate demonstrated as weak biofilm producer with supplementation of stevia. The BETA isolate categorized as non-biofilm producer with supplementation of stevia.

Based on Figure 4.6, it shows the graph of quantitative analysis of biofilm-forming strength of the two target isolates (ST and BETA) and positive control (*S. mitis* ATCC 6249), respectively. Sugar-enriched BHI medium induced BETA isolate to have an increased of at least 70% in the biofilm-forming strength whereas stevia-enriched BHI medium induced BETA isolate to have increased of at least 30% only in the biofilm-forming strength. Sugar-enriched BHI medium induced ST isolate to have at least 3.7-fold increased in biofilm-forming strength whereas stevia-enriched BHI medium induced ST isolate to have at least 1.3-fold increased in the biofilm-forming strength. Sugar-enriched BHI medium induced positive control (*S. mitis* ATCC 6249) to have an increased of at least 120% in the biofilm-forming strength whereas stevia-enriched BHI medium caused *S. mitis* to have an increased of approximately 21.9% only in the biofilm-forming strength.

Quantitative Analysis of Biofilm-forming Strength

- BHI only
- BHI+1% sugar
- BHI+0.15% stevia
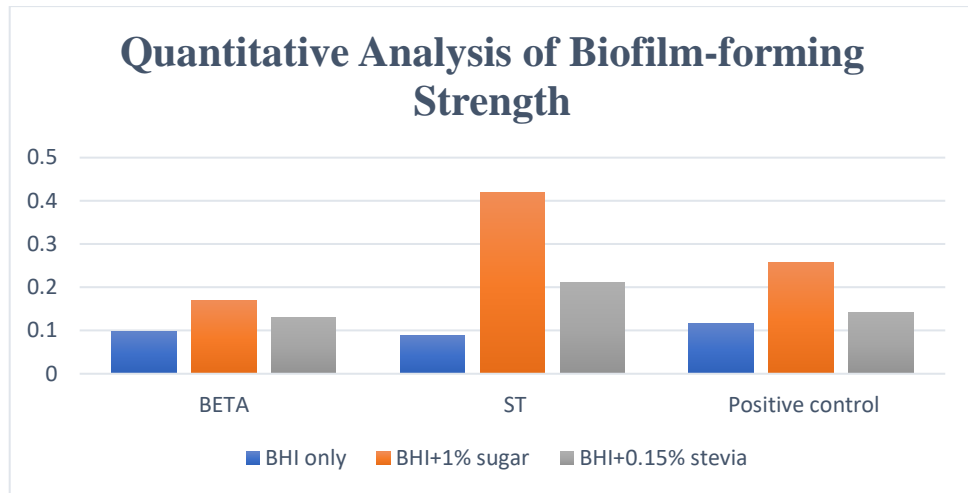
Figure 4.6: Graph of quantitative analysis of biofilm-forming strength of the two target isolates (ST and BETA) and positive control (*S. mitis* ATCC 6249) under sugar free, SD, and StD conditions, respectively.

According to the results of statistical analysis of the MTP assay, there was significant increased ($p<0.05$) of the biofilm-forming strength of the two target isolates (ST and BETA) as well as the positive control (*S. mitis* ATCC 6249) under the SD and StD conditions as compared to sugar free condition. The ST isolate posed the most significant *p*-value as compared to the BETA isolate and positive control (*S. mitis* ATCC 6249). This indicated that ST isolate posed the highest cariogenic effect as compared to BETA isolate and positive control (*S. mitis* ATCC 6249). On the other hand, the *p*-value of BETA isolate and positive control (*S. mitis* ATCC 6249) were quite similar as both isolates were slightly cariogenic.

Overall, sugar has the greatest impact in inducing the respective bacteria to produce more biofilms as compared to stevia. Thus, the influence of sugar and

stevia on the biofilm-forming strength in this assay were valid. The outcomes indicated that the influence of sugar and stevia on the biofilm-forming strength were significant. The *p*-value obtained for the target isolates (ST and BETA) and positive control (*S. mitis* ATCC 6249) were tabulated in Appendix E. In this MTP assay, moderate biofilm producer and weak biofilm producer were determined. Based on Figure 4.7, it shows the representative results of the intensity of solubilized crystal violet stained in the biofilm for moderate, weak, and non-biofilm producers. The raw data of the MTP assay under the sugar free, SD and StD conditions were tabulated in Appendix D.
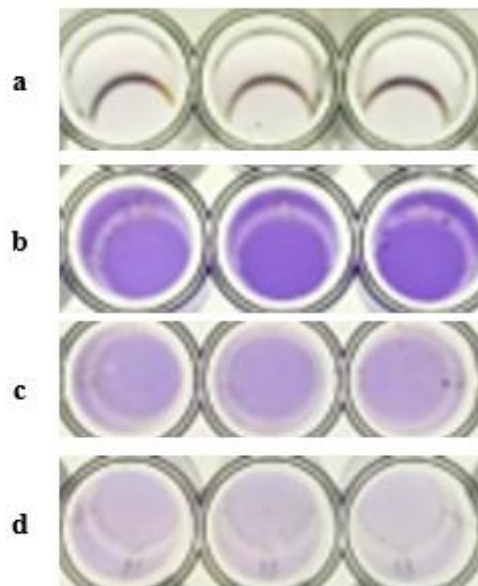
Figure 4.7: The representative results of the biofilm-forming strength based on the intensity of solubilized crystal violet stained in the biofilm.

The intensity of the solubilized crystal violet is directly proportional to the biofilm-forming strength. **(a)** Blank; **(b)** Moderate biofilm producer; **(c)** Weak biofilm producer; **(d)** Non-biofilm producer.

Table 4.3: Assessments of the biofilm-forming strength via MTP assay under sugar free condition.

Isolate	$OD_C{}^a$	$OD_M{}^b$	Biofilm-forming strength
ST	-0.054	0.088 ± 0.004	None
BETA	-0.044	0.098 ± 0.003	None
S. mitis ATCC 6249	-0.027	0.116 ± 0.007	None

[a] Cut off value of the biofilm-forming strength of negative control.
[b] Mean OD_{570} was determined by performing triplicates for each target isolates and positive control.

Table 4.4: Assessments of the biofilm-forming strength via MTP assay under sugar-dependent (SD) condition.

Isolate	$OD_C{}^a$	$OD_M{}^b$	Biofilm-forming strength
ST	2.000	0.420 ± 0.019	Moderate
BETA	0.027	0.169 ± 0.007	Weak
S. mitis ATCC 6249	0.810	0.257 ± 0.020	Weak

[a] Cut off value of the biofilm-forming strength of negative control.
[b] Mean OD_{570} was determined by performing triplicates for each target isolates and positive control.

Table 4.5: Assessments of the biofilm-forming strength via MTP assay under stevia-dependent (StD) condition.

Isolate	$OD_C{}^a$	$OD_M{}^b$	Biofilm-forming strength
ST	0.480	0.210 ± 0.011	Weak
BETA	-0.012	0.131 ± 0.014	None
S. mitis ATCC 6249	0	0.142 ± 0.013	None

[a] Cut off value of the biofilm-forming strength of negative control.
[b] Mean OD_{570} was determined by performing triplicates for each target isolates and positive control.

4.4 Measurement of Acidogenicity

Acid detection test was performed to determine the acidogenic property of the target isolates as the bacteria were capable to lower the pH condition after exposure to sugar and stevia, respectively. This study involved the three respective conditions included sugar free condition, sugar-dependent (SD) condition and stevia-dependent (StD) condition. The three respective conditions used BHI broth as the growth medium. In this part, ST isolate was subjected to proceed with the acid detection test as the isolate formed biofilms under SD and StD conditions. However, BETA isolate was excluded as the isolate had poor outcome in biofilm-forming strength as compared to ST. One-tailed *t*-test yielding the *p*-value of less than 0.05 ($p<0.05$) indicated as statistically significant in the acidogenic property of the target isolates after exposure to sugar and stevia, respectively whereas *p*-value of more than or equal to 0.05 ($p\geq0.05$) indicated as not significant. The calculated mean pH was assigned as mean ± standard deviation. The raw data of the acid detection test under the sugar free, SD, and StD conditions were tabulated in Appendix D.

The mean pH of the positive control (*S. mitis* ATCC 6249) and the target isolate (ST) under the three respective conditions for triplicates followed by the significance difference of the mean pH were recorded in Table 4.6. Based on the results in Table 4.6, the mean pH of ST isolate decreased significantly ($p<0.05$) after overnight of incubation under both SD and StD conditions as compared to the sugar free condition. In contrast, the mean pH of positive control (*S. mitis* ATCC 6249) decreased significantly ($p<0.05$) as well under SD condition as compared to the sugar free condition whereas the mean pH of positive control

(*S. mitis* ATCC 6249) shows slightly increased ($p \geq 0.05$) under StD condition as compared to the sugar free condition.

Overall, the ST isolate showed statistically significant in the acidogenic property under both SD and StD condition whereas the positive control (*S. mitis* ATCC 6249) showed statistically significant in the acidogenic property under SD condition only and showed non-statistically significant in the acidogenic property under StD condition. Based on Figure 4.8, it demonstrated that ST isolate has the ability to lower the pH condition referred as acidic pH under both SD and StD conditions. However, the positive control (*S. mitis* ATCC 6249) only has the ability to lower the pH condition under SD condition and the isolate was nearly neutral under StD condition. Thus, sugar has the greatest impact in inducing the respective bacteria to produce acids as compared to stevia.
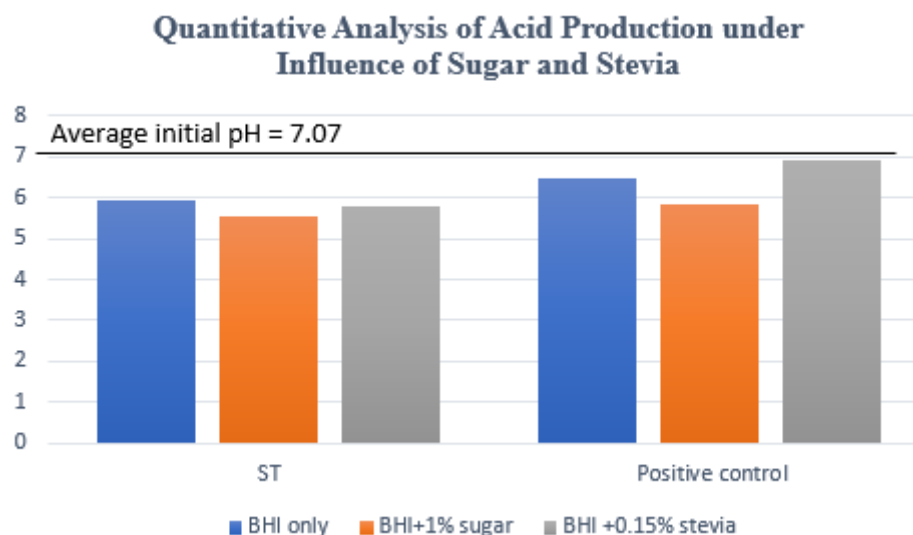
Figure 4.8: Graph of quantitative analysis of acid production of the target isolate (ST) and positive control (*S. mitis* ATCC 6249) under sugar free, SD, and STD conditions, respectively.

Table 4.6: Results of mean pH and significant difference of the mean pH of the target isolate and positive control (*S. mitis* ATCC 6249) under sugar free, sugar-dependent (SD), and stevia-dependent (StD) conditions, respectively.

Isolate	Sugar free[a]	SD[b]	StD[c]	p-value (Significance) under SD	p-value (Significance) under StD
ST	5.960 ± 0.026	5.557 ± 0.015	5.780 ± 0	<0.001 (Significant)	<0.001 (Significant)
S. mitis **ATCC 6249**	6.497 ± 0.373	5.863 ± 0.112	6.903 ± 0.051	0.024 (Significant)	0.067 (Non-significant)

[a] Initial pH of sugar free condition (BHI only): 6.970 ± 0.010.
[b] Initial pH of sugar-dependent (SD) condition (supplemented with 1% of sugar): 7.133 ± 0.058.
[c] Initial pH of stevia-dependent (StD) condition (supplemented with 0.15% of stevia): 7.113 ± 0.015.

4.5 Qualitative Analysis of Biofilm Formation via Scanning Electron Microscope (SEM)

SEM examination was conducted for the positive control (*S. mitis* ATCC 6249) and the target isolate (ST) to observe the thickness of the biofilm formation under sugar free, sugar-dependent, and stevia-dependent conditions, respectively as both isolates were capable to form biofilms in the MTP assay and posed acidogenic property in the acid detection test. The three conditions used BHI broth as the growth medium to cultivate the biofilm on glass coverslips. One-tailed *t*-test yielding the *p*-value of less than 0.05 ($p<0.05$) indicated as statistically significant in the thickness of biofilm formation of the target isolates after exposure to sugar and stevia, respectively whereas *p*-value of more than or equal to 0.05 ($p\geq0.05$) indicated as not significant. The calculated mean thickness of biofilm formation was assigned as mean ± standard deviation. The

raw data of the thickness of biofilm formation test under the sugar free, SD and StD conditions were tabulated in Appendix D.

The mean thickness of biofilm formation of the positive control (*S. mitis* ATCC 6249) and the target isolate (ST) under the three respective conditions for triplicates followed by the significance difference of the mean thickness of biofilm formation were recorded in Table 4.7. The scanning electron micrographs imaged at 1, 000$\times$ and 2, 000$\times$ magnifications were as shown in Figure 4.9. As the results shown in Figure 4.9 (a) and (b), the biofilms formed in SD condition were thicker as compared to sugar free and StD conditions. Based on the results in Table 4.7, the mean thickness of biofilm formation of ST isolate increased significantly ($p<0.05$) after overnight of incubation under both SD and StD conditions as compared to the sugar free condition. In contrast, the mean thickness of biofilm formation of positive control (*S. mitis* ATCC 6249) slightly increased ($p<0.05$) as well under both SD and StD conditions as compared to the sugar free condition. Meantime, the biofilm formation of ST isolate was thicker than the positive control (*S. mitis* ATCC 6249). Overall, there was significant increased in the thickness of biofilm of ST isolate and positive control (*S. mitis* ATCC 6249) after exposure to the addition of sugar and stevia, respectively compared to BHI only which was the sugar free condition.

Table 4.7: Results of mean thickness of biofilm formation and significant difference of the mean thickness of biofilm formation of the target isolate and positive control (*S. mitis* ATCC 6249) under sugar free, sugar-dependent (SD), and stevia-dependent (StD) conditions, respectively.

Isolate	Sugar free[a]	SD[b]	StD[c]	*p*-value (Significance) under SD	*p*-value (Significance) under StD
ST	1.127 ± 0.206	11.660 ± 1.346	9.690 ± 0.478	<0.001 (Significant)	<0.001 (Significant)
***S. mitis* ATCC 6249**	1.640 ± 0.840	8.127 ± 0.637	4.220 ± 0.248	<0.001 (Significant)	<0.001 (Significant)

[a] Sugar free condition (BHI only).
[b] Sugar-dependent (SD) condition (supplemented with 1% of sugar).
[c] Stevia-dependent (StD) condition (supplemented with 0.15% of stevia).
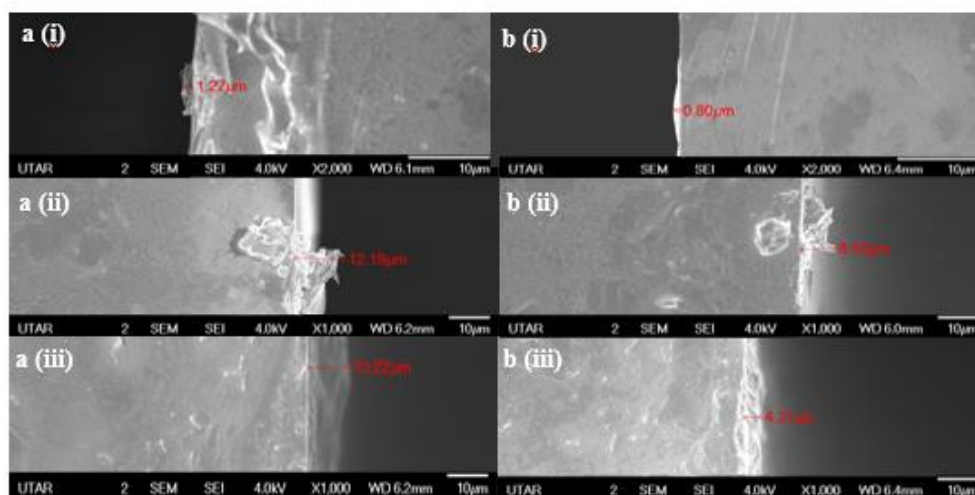
Figure 4.9: The thickness of biofilm formation shown via scanning electron micrographs under the three respective conditions.
(a) Target isolate (ST); (b) Positive control (*S. mitis* ATCC 6249); (i) Represent sugar free condition; (ii) Represent sugar-dependent condition; (iii) Represent stevia-dependent condition.

4.6 Genotypic Identification

4.6.1 Amplification of Bacterial Universal 16S rDNA

Bacterial genomic DNA extraction was performed for the positive control (*S. mitis* ATCC 6249) and the target isolate (ST) by using the DNA extraction kit (Macherey-Nagel NucleoSpin® Microbial DNA, Germany). The DNA template used with concentration of approximately 150 ng/µL was proceeded with PCR in order to detect and amplify the 16S rDNA region. The analysis of electrophoresed gel image via PCR was shown in Figure 4.10. Based on the results obtained, there were presence of expected bands with the size of (~996 bp) in both positive control (*S. mitis* ATCC 6249) and the target isolate (ST). Notably, there was great intensity of the expected band in the target isolate (ST) and absence of band with expected size in the non-template control (NTC).
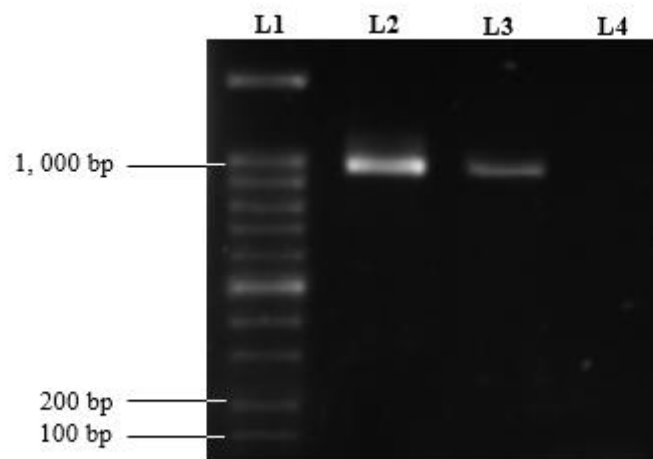
Figure 4.10: Analysis of electrophoresed gel image via gel imager.
The PCR products were analysed on a 1.3% (w/v) agarose gel. **Lane 1**: GeneDireX 100 bp DNA Ladder; **Lane 2**: Isolate ST; **Lane 3**: Positive control, *S. mitis* ATCC 6249; **Lane 4**: Non-template control (NTC).

4.6.2 Purification of Amplicon for Sequencing

The target amplicon with the expected size of ~996 bp obtained from the PCR reaction were subjected to the spin column PCR purification kit (Bio Basic Inc., Canada). The DNA concentration and DNA purity of A_{260}/A_{280} ratio were recorded in Table 4.8. Notably, the target ST isolate achieved the requirement of pure DNA concentration for sequencing. The spectrophotometry graph of ST isolate was provided in Appendix F.

Table 4.8: Results of DNA concentration and DNA purity of the target isolate.

Isolate	DNA concentration (ng/µL)[a]	DNA purity of A_{260}/A_{280} ratio[b]
ST	276.7	1.82

[a] Required a minimum of DNA concentration of 40 ng/µL as stated by the sequencing company (FirstBase, Singapore).
[b] Indicator of pure DNA sample within the range of 1.80-2.00.

4.6.3 Sequence Analysis

The forward and reverse DNA sequences of ST were analysed using the Molecular Evolutionary Genetics Analysis software designated as "MEGA 11" to determine the high purity bases. Expectedly, there was high purity bases of ST being identified. The identified high purity bases referred as the query sequence to compare with the subject sequence of the existing 16S rRNA gene of Bacteria and Archaea in GenBank via BLASTn. The summary of BLASTn for ST were shown in Table 4.9. The results generated form BLASTn system was provided in Appendix G.

According to the outcomes in Table 4.9, the generated identity of ST was referring to the *Bacillus* sp. with the identity score of 99% and 0.0 of E-value in which the criteria of sequence homology have been achieved. Although presence of *Bacillus* sp. in oral cavity is rare, still isolation of *Bacillus* sp. from the oral sample was possible as *Bacillus* sp. was able to form biofilms and tolerate as well as survive in harsh condition referred to the low pH condition in the oral cavity (Pauter, et al., 2022).

Table 4.9: Results of BLASTn analysis of the target isolate.

Isolate	Query cover	E-value	Identity score	Total score	Identity	Accession number
ST	99%	0.0	99%	1709	*Bacillus* sp. (in: firmicutes)	LC472522.1

CHAPTER 5

DISCUSSION

5.1 Dental Plaque Sampling

5.1.1 Selection of Sample

Optimal moisture level, presence of salivary pellicle, and nutrients availability making oral cavity a favourable environment for the colonization and growth of oral bacteria. Several surfaces in the oral cavity are available to be colonized by oral bacteria such as tooth, dorsum, gingival crevices, and tongue. Still, dental plaque on tooth surfaces were chosen as the tooth surfaces are rough and tight for the primary adhesion and colonization of the plaque bacteria (Mosaddad, et al., 2019). In this study, the oral biofilm producers were the focus as they are capable to metabolize the dietary carbohydrates and produce the extracellular polymeric substances (EPS) due to their cariogenicity (Zeng, et al., 2020). In order to get the desired cariogenic oral bacteria, the dental plaque formed on the tooth surfaces was selected as the sample rather than the saliva (Gotouda, et al., 2018). According to Gotouda, et al (2018), dental plaque composes of higher content of cariogenic oral bacteria as compared to the saliva. The cariogenic oral bacteria isolated from the dental plaque could be used in the assessment of dental caries activity (Gotouda, et al., 2018). On the other side, the saliva sample may not fit in this study as most oral bacteria in saliva are planktonic. Thus, the possibility to isolate cariogenic oral bacteria is higher in the dental plaque sample than the saliva sample (Gotouda, et al., 2018).

5.2 Isolation of Potential Cariogenic Oral Bacteria

Incubation conditions were important to obtain the primary isolates with the consideration to mimic the physiological environments that enable the growth of different physiological types of oral bacteria that further contribute to the primary biofilm formation (Peng, et al., 2020). Hence, the samples from dental plaque were incubated aerobically and microaerobically as these oral bacteria could be aerobes, facultative anaerobes, and anaerobes. The primary colonizers are usually the facultative anaerobes including *Streptococcus* spp., *Actinomyces* spp., and *Neisseria* spp. (Lamont, Koo and Hajishengallis, 2018). However, other different types of oral bacteria should not be neglected as the oral cavity involves the colonization of a variety of microorganisms. Therefore, regular incubation was applied for aerobic condition whereas candle jar was applied for microaerobic condition. The aerobes and facultative anaerobes tend to grow well in aerobic condition of 37°C in the regular incubator. In contrast, the anaerobes grow in the microaerobic condition refers to the candle jar (Peng, et al., 2020). According to Wilson (2005), it stated that the microaerophilic bacteria grow well at 2-10% of oxygen and the capnophilic bacteria grow well at 5-10% of carbon dioxide. The used of lighted candle in an enclosed candle jar reduced approximately 10% of the concentration of oxygen eventually and increased approximately 3-5% of the concentration of carbon dioxide. In addition, the duration of incubation applied in this study was 24-48 hours. The growth of aerobes and facultative anaerobes could be observed after overnight incubation as they grow in the oxygenated environment. In contrast, the anaerobes have slower growth rate as they grow in the absence of oxygen and undergo fermentation process. So, the growth of anaerobes may be observed after 48

hours of incubation. It is not surprise that the incubation duration of anaerobes is longer than the aerobes since there are certain rare oral bacteria includes oral spirochetes and oral bacilli require one week of incubation (Peng, et al., 2020; Pauter, et al., 2022). Thus, both aerobic and microaerobic conditions after 24 hours of incubation were applied to increase the possibility to isolate different physiological types of oral bacteria and prevent overgrowth of the oral bacteria.

5.2.1 Colony-forming Unit (CFU) Enumeration

The CFU enumeration is a basic method to estimate the number of viable colonies present in the original sample that undergo cell division and replication processes. It is a simple and accessible technique as it requires the culturing of microorganisms on the solid agar plate supplemented with nutrients (McBain, 2019). According to Abranches, et al. (2018), the diversity of microorganisms in the oral cavity composes of more than 700 different bacterial species that have been identified. Unfortunately, there are still approximately 50% of the bacterial species remained unculturable. According to Takeshita, et al. (2016), it stated that the total oral bacteria could be up to 10^9 CFU upon cultivation with synthetic hydroxyapatite disk as the mineral-rich substate. The total oral bacteria may include the unculturable oral bacteria as well. Meantime, this study only focused on the culturable oral bacteria as the outcome in CFU count. The calculated CFU/mL for the two primary plates were in the ten thousandth (10^4) which was for lesser than the above-mentioned quantity is because the sample that used in this study was just focused on dental plaque excluding the planktonic bacteria in saliva and other oral bacteria on tongue surface as well as other soft tissues in the oral cavity.

5.3 Phenotypic Characterization

5.3.1 Colony and Cellular Morphology of Selected Isolates

The selected isolates were cultured in TSA plates to obtain the pure culture. Out of the three target isolates, one produced milky yellow colonies with moderate size, one produced pale yellow colonies with moderate size, and one produced whitish colonies with small size. ST isolate and BETA isolate were quite similar to each other as both were Gram-positive and catalase-positive while the GAMMA isolate resulted in Gram-negative and catalase-negative. The application of Gram stain and catalase test contributed to further verification of the phenotypic characterization of the target isolates, respectively (Peng, et al., 2020).

According to Tankeshwar (2013), it stated that the appearance of *Bacillus* spp. colonies is milky yellow colour, moderate size, irregular shape and opaque that corresponded to the two isolates (ST and BETA) in this study. Besides, most of the *Bacillus* spp. are Gram-positive streptobacilli with chains arrangement under Gram stain that subsequently corresponded to the two isolates as well. The Gram-positive bacteria tend to have a thick peptidoglycan with nature of multi layers that trap the CV-I complexes in the cell and resulted in purple colour (Smith and Hussey, 2005).

5.3.2 Catalase Test

According to Reiner (2010), catalase test is one of the most common biochemical tests to differentiate between catalase-positive and catalase-negative bacteria. The implementation of catalase test is valuable in which it helps to clearly differentiate between the catalase-positive *Bacillus* from the catalase-negative aerotolerant *Clostridium*. Based on a previous study by Juven and Piersen (1996), it stated that most of the aerobes tend to produce catalase enzyme in which *Bacillus* spp. are included as well. The *Bacillus* spp. have the enzyme, catalase as their defence mechanism to neutralize the H_2O_2 and repair the oxidative damage of superoxide radicals. As the result, this further verified that the target ST isolate in this study was catalase-positive that eventually lead to the bubbles formation due to the reaction of H_2O_2 with catalase enzyme and produced water and oxygen as the by-products.

5.3.3 Haemolytic Activity

Blood agar is an enriched medium supplemented with the mammalian blood such as rabbit, sheep, or horse bloods (Buxton, 2005). This study implemented the used of 5% of SBA to detect the haemolysis patterns produced by the target isolates (ST, BETA, and GAMMA). The supplementation of X-factor in the SBA is to allow the pathogenic species to grow (Becton Dickenson, 2013). Haemolysis refers to the lysis of erythrocytes in which the microorganisms are able to produce hemolysin which is a toxin that aid in this process. The three isolates posed different haemolytic activities in which ST isolate resulted in α-haemolytic activity, BETA isolate resulted in β-haemolytic activity and

GAMMA isolate resulted in γ-haemolytic activity. In this study, the target ST isolate that resulted in α-haemolytic activity was the focus. The α-haemolytic activity resulted in partial or incomplete haemolysis that appeared as greenish colour surrounding the colonies on the SBA due to the oxidation of iron in the haemoglobin. This reaction subsequently lead to the production of methaemoglobin (Buxton, 2005). Thus, the α-haemolytic oral bacteria tend to cause the tissue invasion and prevent the host immune response mainly lead to the periodontitis (Wong, et al., 2016).

5.4 Role of Sugar in Biofilm Formation

In the MTP assay, the growth medium was supplemented with sugar to evaluate the influence of sugar on the biofilm-forming strength of the target isolates. Dietary carbohydrates especially sucrose is needed to examine and compare the biofilm-forming strength of the target isolates on the aspects to cariogenicity. The metabolism process of the dietary carbohydrates that are being utilized by the target isolates eventually lead to the acid production as the by-products (Hasnor, et al., 2006). The reason of selecting common sugar rather than other dietary carbohydrates as it is the most commonly used sugar in our daily life and poses highest cariogenic effect that causes plaque-mediated diseases such as dental caries. Sucrose serves as the fermentable source of carbohydrates for the cariogenic oral bacteria to form the dental biofilms that further increase the accumulation of the cariogenic oral bacteria (Leme, et al., 2006). According to Dwivedi (1978), it stated that sucrose is a disaccharide composes of glucose and fructose that provide more energy sources for the cariogenic oral bacteria to produce EPS due to more energy is being released during the break down of α-

1,2-glycosidic bond in this non-reducing sugar. However, other types of sugars include fructose, glucose and lactose demonstrated lesser impact on the thickness of biofilms formed (Yang, et al., 2006).

Besides, the BHI (HiMedia, India) growth medium used initially contained 2 g/L of glucose and such concentration of glucose insufficient to cause a decrease in pH level in the oral cavity as it is five times lower as compared to the concentration of the supplemented sugar into the medium. Meantime, the influence of sugar on the biofilm-forming strength of the target isolates were determined based on the significant increased in the biofilm-forming strength under SD condition as compared to sugar free condition. Supplementation with 1% sugar was to indicate the average amount of sugars left after meal (Kleinberg, 1961). In addition, the concentration of dietary carbohydrates that are found in most of the food may not overreach 10% even after being diluted by the saliva, except for certain types of food such as honey or sweets that consist of higher amount of sugar (Jenkins, 1966). The changes in pH level to a more acidic environment in the oral cavity was detected even in the presence of 1% (w/v) sugar (Kleinberg, 1961). Thus, 1% (w/v) sugar was implemented in this study to evaluate its influence on (i) biofilm-forming strength via MTP assay, (ii) acidogenicity and (iii) thickness of biofilm formation via SEM.

5.5 Effect of Sweetener in Biofilm Formation

In this study, the growth medium was implemented with stevia sweetener to evaluate the influence of this sweetener on the biofilm-forming strength of the

targets. Sweetener serves as the sugar substitute to reduce the risk of dental caries as the cariogenic oral bacteria unable to utilize it and undergo metabolism process to produce acids (Razak, et al., 2017). Stevia is categorized as natural sweetener that extracted from *Stevia rebaudiana* Bertoni plant. The sweetness produced is due to the stevia glycosides which are the nature components found in the leaves of the plant (de Slavutzky, 2010). The stevia extract sweetener is commercially available in the markets was used in this study to examine and compare the biofilm-forming strength of the target isolates on the aspects to cariogenicity. According to de Slavutzky (2010), it reported that there was a significant decreased of dental plaque formation when tested with stevia as it is a non-fermentable product and posed anti-cariogenic effect. Stevia as the alternative sweetener was effective in reducing the EPS production and enhanced the biofilm porosity that resulted in thin biofilm formation (Razak, et al., 2017).

Besides, the stevia sweetener used contained zero calories. As the results, there was no implementation of sucrose in BHI growth medium that contained only 0.2% (w/v) glucose concentration that was insufficient to cause a decrease in pH level in the oral cavity. Meantime, the influence of stevia on the biofilm-forming strength of the target isolates were determined based on the significant increased of the biofilm-forming strength under StD condition as compared to the sugar free condition. According to the product labelling, the used of 0.15% (v/v) stevia was implemented in this study to evaluate its influence on (i) biofilm-forming strength via MTP assay, (ii) acidogenicity and (iii) thickness of biofilm formation via SEM.

5.6 Assessment of the Biofilm-forming Strength via 96-well Microtiter Plate (MTP) Assay

5.6.1 Sugar free Condition

Formation of dental plaque depends on the ability of the oral bacteria to metabolize the dietary sugars into acids and creating acidic microenvironment to further enhance the biofilm-forming strength of the microbes (Chen, et al., 2021). The accumulation of plaque bacteria and the produced EPS referred as the plaque biomass (Costa Oliveira, Cury and Ricomini Filho, 2017). The oral diseases include periodontitis and dental caries usually resulted in an increased of plaque biomass that subsequently leads to the inflammation stage (Abusleme, et al., 2013). Hence, the estimation of biofilm-forming strength of the plaque is important as it serves as an indication to the oral health status of an individual.

Under sugar free condition, the biofilm-forming strength of the positive control (*S. mitis* ATCC 6249) and the two target isolates (ST and BETA) can be estimated. The biofilm-forming strength of the target isolates (ST and BETA) reflected the degree of the dental plaque formation. Absence of additional sugar making these three isolates demonstrated as the non-biofilm producers that show the insignificance in the biofilm-forming strength. The non-biofilm producers may not cause the impact on the dental plaque formation as they unable to form the EPS which serves as virulence factor that drives the development of cariogenic biofilms (Chen, et al., 2021).

5.6.2 Sugar-dependent (SD) Condition

Under sugar-dependent (SD) condition, the BHI growth medium supplemented with 1% (w/v) sugar was expected to have an increased in the intensity of the solubilized crystal violet which is directly proportional to the biofilm-forming strength (Stepanović, et al., 2007). The increase in the biofilm-forming strength indicated the increase of plaque biomass (Costa Oliveira, Cury and Ricomini Filho, 2017). The positive control (*S. mitis* ATCC 6249) and the target isolate (BETA) demonstrated as weak biofilm producers that show slightly increased ($p<0.05$) of the biofilm-forming strength under supplementation of 1% (w/v) sugar. The weak biofilm producer indicated lower plaque biomass that contributes to lower probability production of dental biofilms (Sanchez, et al., 2013). On the other hand, the target isolate (ST) demonstrated as the moderate biofilm producer that show a significant increased ($p<0.05$) of the biofilm-forming strength under supplementation of 1% (w/v) sugar. In addition, the moderate biofilm producers are more virulence than the non and weak biofilm producers as they have the ability to form dental plaque with higher plaque biomass that eventually lead to the cariogenic effects (Sánchez-Vargas, et al., 2013). Hence, the influence of sugar on the biofilm-forming strength on the aspect to cariogenicity was accessed on the ST isolate.

5.6.3 Stevia-dependent (StD) Condition

Under stevia-dependent (StD) condition, the BHI growth medium supplemented with 0.15% (v/v) stevia was implemented to study the effect of sweetener on the biofilm-forming strength on the solubilized crystal violet which is directly

proportional to the biofilm-forming strength (Stepanović, et al., 2007). The increase in the biofilm-forming strength indicated the increase of plaque biomass (Costa Oliveira, Cury and Ricomini Filho, 2017). The positive control (*S. mitis* ATCC 6249) and the target isolate (BETA) demonstrated as the non-biofilm producers that show the insignificance in the biofilm-forming strength under supplementation of 0.15% (v/v) stevia. The non-biofilm producers may not cause impacts on the dental plaque formation as they unable to utilize the dietary carbohydrates to form the EPS which is a virulence factor that drives the development of cariogenic biofilms (Chen, et al., 2021). On the other hand, the target isolate (ST) demonstrated as the weak biofilm producer that show slightly increased (p<0.05) of the biofilm-forming strength under supplementation of 0.15% (v/v) stevia. The weak biofilm producer indicates lower plaque biomass that contributes to lower probability production of dental biofilms (Sanchez, et al., 2013). Hence, the influence of stevia on the biofilm-forming strength on the aspect to cariogenicity was accessed on the ST isolate.

5.7 Significance of Target Isolate

Gram-positive ST isolate identified as *Bacillus* sp. was one of the contributors in the dental plaque formation. *Bacillus* sp. has found to be the transient normal flora in the oral cavity, and it has the ability to utilize the dietary carbohydrates especially sucrose that eventually lead to the dental biofilms formation. Frequent exposure to sugar would trigger the formation of cariogenic biofilms as production of EPS enhances the further uptake of nutrients (Jain, et al., 2013).

The target isolate (ST) was selected due to the significant outcomes of the biofilm-forming strength in both SD and StD conditions in the MTP assay that enabled to compare the degree of biofilm-forming strength under supplementations of sugar and stevia, respectively. ST isolate demonstrated a significant decreased in the biofilm-forming strength under StD condition as compared to SD condition. As the results, there was a significant decreased of plaque formation. These significant outcomes indicated the ability of the *Bacillus* sp. to tolerate and survive in harsh condition referred to the low pH condition in the oral cavity (Pauter, et al., 2022). Thus, the ST isolate was selected to determine its acidogenicity on the aspects to cariogenicity.

5.8 Measurement of Acidogenicity

Saccharification process causes the changes of pH condition in the oral cavity in which cariogenic biofilms prefer to form at low acidic pH condition. Acid is the main driving force that causes the biofilm-mediated disease to occur as production of acid results in damaging the minerals of the dental apatite refers to enamel demineralization (Chen, et al., 2020). According to Meyer, et al., (2021), it stated that the occurrence of enamel demineralization is usually at pH 5.5 and below which is the critical pH that favours the colonization of cariogenic oral bacteria. The tooth tissues are further being damaged due to the lost of phosphate, calcium, and carbonate ions upon demineralization. In other words, the formation of biofilms after exposure to sugar provides lesser calcium-binding sites for the minerals to bind.

Under sugar-dependent (SD) condition, the target isolate (ST) and the positive control (*S. mitis* ATCC 6249) demonstrated a significant decreased ($p<0.05$) in the pH level as compared to the sugar free condition. The decrease in the pH level indicates that the isolate is cariogenic oral bacteria that have the ability to produce acid and tolerate the acidic environment (Abranches, et al., 2018). The target isolate (ST) was considered as acid producing oral bacteria since it posed a decreased in pH at approximately 5.5. As mentioned, ST isolate reached the critical pH that triggers the occurrence of enamel demineralization that further leads to dental caries (Meyer, et al., 2021). Many different types of organic acids that are being produced include lactic acid, propionic acid, acetic acids and others. In fact, the lactic acid is the major cause of enamel demineralization because it has higher acid dissociation constant that indicates the strength of the acid (Walsh, 2006). However, the positive control (*S. mitis* ATCC 6249) posed a decreased in pH at approximately 5.8. As the results, *S. mitis* did not reach the critical pH and unable to cause the enamel demineralization. In context, the *S. mitis* still able to produce acid as the by-products, but the acid produced may not have direct impact in causing the enamel demineralization (Meyer, et al., 2021).

Under stevia-dependent (StD) condition, the target isolate (ST) demonstrated a significant decreased ($p<0.05$) in the pH level as compared to the sugar free condition. The ST isolate posed a decreased in pH at approximately 5.8. In contrast, the positive control (*S. mitis* ATCC 6249) demonstrated a slightly increased in the pH level as compared to the sugar free condition. *S. mitis* posed an increased in pH at approximately 6.9. As the results, ST isolate and *S. mitis* did not reach the critical pH and unable to cause the enamel demineralization.

Even though acids produced by S. mitis have higher acid dissociation constant, still unable to cause pH reduction since the initial pH of stevia is slightly alkaline (Giacaman et al., 2013). The drop of pH level in both SD and StD conditions further verified that ST isolate have the ability to produce acid and tolerate the acidic environment (Abranches, et al., 2018). Hence, the ST isolate posed the acidogenic property as the indicator of cariogenic oral bacteria.

5.9 Qualitative Analysis of Biofilm Formation via Scanning Electron Microscope (SEM)

SEM served as a further confirmatory analysis on the thickness of biofilm formation due to the limitation of MTP assay that was based on the intensity of the solubilized crystal violet. The side view of visualization of biofilm formation allows the measurement of the thickness of biofilm formed (Relucenti, et al., 2021). The thickness of biofilm formation reflected the cariogenicity of the oral bacteria after exposure to sugar and sweetener, respectively (Razak, et al., 2017). In other words, the visualization of the thickness of biofilm formation via SEM enables to perform comparative analysis such as evaluation of the cariogenicity of the oral bacteria after exposure to sugar and sweetener, respectively (Relucenti, et al., 2021).

Under sugar-dependent (SD) condition, it is expected that the biofilm formation is thicker than the sugar free condition and stevia-dependent (StD) condition (Razak, et al., 2017). The higher rate of sugar metabolism results in thicker biofilm formation by the cariogenic oral bacteria (Abranches, et al., 2018). The

target isolate (ST) which was a moderate biofilm producer demonstrated a significant increased ($p<0.05$) in the thickness of biofilm formation as compared to the positive control (*S. mitis* ATCC 6249) which was a weak biofilm producer. Thus, the moderate biofilm producers are more virulence than the non and weak biofilm producers as they have the ability to form dental plaque with higher plaque biomass that eventually lead to thicker biofilm formation (Sánchez-Vargas, et al., 2013).

Under stevia-dependent (StD) condition, it is expected that there is biofilm formation as the alternative sweeteners still have the ability to promote the biofilm formation, but with lower plaque biomass due to lower adherence of plaque bacteria (Razak, et al., 2017). The target isolate (ST) which was weak biofilm formation demonstrated a slightly increased in the thickness of biofilm formation as compared to the positive control (*S. mitis* ATCC 6249) which was a non-biofilm producer. The weak biofilm producers have lower probability production of dental biofilms formation with lower plaque biomass (Sánchez-Vargas, et al., 2013). According to Razak, et al., (2017), it stated that stevia as the alternative sweetener was effective in reducing the EPS production and enhanced the biofilm porosity that resulted in thin biofilm formation. Thus, the target isolate (ST) has higher cariogenic effects under SD condition than in StD condition.

.5.10 Significance of the Study

Sugars as the dietary carbohydrates have the highest cariogenic effect that cause the plaque-mediated diseases such as dental caries and periodontitis. This fermentable carbohydrate favours the colonization of the plaque bacteria to form the dental biofilms that further increase the accumulation of the cariogenic oral bacteria (Leme, et al., 2006). The identified target ST isolate (*Bacillus* sp.) has found to be the transient normal flora in the oral cavity. *Bacillus* sp. is categorized as the transient colonizer in the formation of dental biofilm (Jain, et al., 2013). Unlike *Streptococcus mutans* that is commonly being studied as the aetiology of the plaque-mediated diseases, the study of the isolation of *Bacillus* sp. also important in maintaining an individual's oral health status due to its cariogenicity (Bhattacharjee et al., 2018). The significant outcome of biofilm-forming strength after exposure to 1% (w/v) sugar indicated that ST isolate (*Bacillus* sp.) has the ability to utilize the dietary carbohydrates especially sucrose that eventually lead to the dental biofilms formation. Besides, ST isolate (*Bacillus* sp.) posed the acidogenic property as the indicator of cariogenic oral bacteria. Furthermore, ST isolate (*Bacillus* sp.) demonstrated as a moderate biofilm producer resulted in a significant in the thickness of biofilm formation after exposure to sugar. Hence, this study proposed that *Bacillus* sp. posed cariogenic effect after exposure to sugar. Studies that have been done previously have yet to demonstrate the significant relationship between *Bacillus* sp. and cariogenicity. Thus, the findings of this study would be indispensable for further research studies due to the significant of the target ST isolate (*Bacillus* sp.).

5.11 Recommendations and Future Prospects

As the *Bacillus* sp. able to produce EPS via sugar metabolism process, it is suggested to implement different final concentrations of sugar to evaluate the aspects of the influence of sugar to the cariogenicity (Jain, et al., 2013). In this study, the used of 1% (w/v) sugar indicate the average amount of sugars left in oral cavity after meal. On the other sides, the consequences of the consumption of other types of food that consist higher amount of sugar need to take into consideration as well. Meantime, the used of different final concentrations of sugar could help to strengthen the measurement of acidogenicity of the target oral bacteria as the pH level drops significantly when the concentration of sugar increases (Kleinberg, 1961).

Besides, the incubation period upon supplementation of sugar could be increased in order to access the biofilm-forming strength of the target isolate accurately. The development of moderate biofilm producers requires longer period to undergo the maturation stage that triggers the increase of plaque biomass (Sánchez-Vargas, et al., 2013). The maturation stage of biofilm formation initiates after 48 hours of incubation (Samaranayake and Matsubara, 2017). In this study, the incubation period implemented was 24 hours in which it may be inadequate to study the biofilm-forming strength to the aspects of cariogenicity.

Lastly, implementation of different types of alternative sweeteners that are available in the market could further enhance the evaluation of cariogenic oral bacteria based on their ability to utilize the sweeteners to produce EPS. In this

study, the used of 0.15% (v/v) stevia resulted in weak biofilm producer of the target ST isolate (*Bacillus* sp.) that have lower cariogenic effect as compared to sugar. The used of alternatives sweeteners as sugar substitutes is to reduce the risk to dental caries Thus, more alternative sweeteners could be tested as an indicator of antiplaque agents (Razak, et al., 2017).

CHAPTER 6

CONCLUSION

In this study, total of three target oral isolates were isolated from the dental plaque. A total of three different growth conditions, including sugar free condition, BHI broth supplemented with 1% (w/v) sugar, and BHI broth supplemented with 0.15% (v/v) stevia were implemented in three parts of the study included: (i) assessment of biofilm-forming strength via MTP assay; (ii) measurement of acidogenicity; and (iii) qualitative analysis of biofilm formation via SEM. The two growth conditions were important to evaluate the influence of sugar and stevia sweetener on the aspects of acidogenicity and cariogenicity of the target oral isolates. In the MTP assay, ST and BETA isolates were being assessed as GAMMA isolate was excluded as it was unable to grow in the broth medium. Both isolates were found to be the biofilm producers with different strengths. ST posed the highest cariogenic effect as it demonstrated as moderate biofilm producer under SD condition and weak biofilm producer under StD condition with significantly different ($p<0.05$) of biofilm-forming strength. In contrast, BETA was demonstrated as weak biofilm producer under SD condition and non-biofilm producer under StD condition. Hence ST was selected to proceed with the acid detection test to determine its acidogenic property whereas BETA was excluded due to its poor outcome in the MTP assay. In the acid detection test, ST (identified *Bacillus* sp.) posed a decreased of pH to approximately pH 5.5 which indicated the critical pH under SD condition and a

decreased of pH to approximately pH 5.8 under StD condition. The outcomes of acid detection test indicated *Bacillus* sp. is categorized as acid producing oral bacteria. In addition, the thickness of the biofilm formation of ST (identified *Bacillus* sp.) was viewed via SEM to further validate the outcomes in the MTP assay. It showed significant increased ($p<0.05$) of thickness of biofilm formation under SD condition as compared to the sugar free condition. Thus, the findings of this study showed that sugar has the greatest impact in inducing the *Bacillus* sp. to produce more biofilm and acids as compared to stevia. Similarly, sugar has the highest cariogenic effect that causes the plaque-mediated diseases such as dental caries to occur. Lastly, further study of transient normal flora in the oral cavity is required as they could be the contributors to dental caries as well.

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REFERENCES

Abranches, J., Zeng, L., Kajfasz, J.K., Palmer, S.R., Chakraborty, B., Wen, Z.T., Richards, V.P., Brady, L.J. and Lemos, J.A., 2018. Biology of oral streptococci. *Microbiology Spectrum*, *6*(5), pp.6-5.

Abusleme, L., Dupuy, A.K., Dutzan, N., Silva, N., Burleson, J.A., Strausbaugh, L.D., Gamonal, J. and Diaz, P.I., 2013. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *The ISME Journal*, *7*(5), pp.1016-1025.

Becton Dickenson, 2013. *BDTM columbia agar with 5% sheep blood*. [online] Available at: <http://www.bd.com/resource.aspx?IDX=8968> [Accessed 5 March 2023].

Berger, D., Rakhamimova, A., Pollack, A. and Loewy, Z., 2018. Oral biofilms: development, control, and analysis. *High-throughput*, *7*(3), p.24.

Bhattacharjee, S., Nath, S., Bhattacharjee, P., Chouhan, M. and Deb, B., 2018. Efficacy of toothpastes on bacteria isolated from oral cavity. *International Journal of Medicine and Public Health*, *8*(2).

Buxton, R., 2005. *Blood Agar Plates and Hemolysis Protocols*. [online] Available at: <https://asm.org/getattachment/7ec0de2b-bb16-4f6e-ba07-2aea25a43e76/protocol-2885.pdf.> [Accessed 23 October 2022].

Caldwell, F.E., 2013. *The students reference guide to bacteria*. North Carolina: Lulu.com.

Chen, X., Daliri, E.B.M., Chelliah, R. and Oh, D.H., 2020. Isolation and identification of potentially pathogenic microorganisms associated with dental caries in human teeth biofilms. *Microorganisms*, *8*(10), pp.1596.

Chen, X., Daliri, E.B.M., Tyagi, A. and Oh, D.H., 2021. Cariogenic biofilm: Pathology-related phenotypes and targeted therapy. *Microorganisms*, *9*(6), p.1311.

Costa Oliveira, B.E., Cury, J.A. and Ricomini Filho, A.P., 2017. Biofilm extracellular polysaccharides degradation during starvation and enamel demineralization. *PLoS One*, *12*(7), p.e0181168.

de Slavutzky, S.M.B., 2010. Stevia and sucrose effect on plaque formation. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, *5*, pp.213-216.

Dwivedi, B.K., 1978. *Low calorie and special dietary foods*. Florida: CRC Press.

Engelkirk, P.G. and Duben-Engelkirk, J., 2008. *Laboratory diagnosis of infectious diseases: essentials of diagnostic microbiology*. Philadelphia: Lippincott Williams & Wilkins.

Giacaman, R.A., Campos, P., Munoz-Sandoval, C. and Castro, R.J., 2013. Cariogenic potential of commercial sweeteners in an experimental biofilm caries model on enamel. *Archives of Oral Biology*, *58*(9), pp.1116-1122.

Gotouda, H., Shinozaki-Kuwahara, N., Taguchi, C., Endo, H., Ohta, M., Shimosaka, M., Tamamura, R., Matsune, K., Kono, Y., Okada, H., Kurita-Ochiai, T. and Ito, T., 2018. Comparison of evalutating the ratio of cariogenic bacteria in plaque and saliva samples. *Journal of Oral Health and Dental Management*, 17(2). [online] Available at: <https://www.omicsonline.org/open-

access/comparison-of-evaluating-the-ratio-of-cariogenic-bacteria-in-plaque-and-saliva-samples.pdf> [Accessed 10 January 2023].

Gupta, P., Gupta, N., Pawar, A.P., Birajdar, S.S., Natt, A.S. and Singh, H.P., 2013. Role of sugar and sugar substitutes in dental caries: a review. *International Scholarly Research Notices*, *2013*.

Huang, R., Li, M. and Gregory, R.L., 2011. Bacterial interactions in dental biofilm. *Virulence*, *2*(5), pp.435-444.

Jain, K., Parida, S., Mangwani, N., Dash, H.R. and Das, S., 2013. Isolation and characterization of biofilm-forming bacteria and associated extracellular polymeric substances from oral cavity. *Annals of Microbiology*, *63*, pp.1553-1562.

Jenkins, G.N., 1966. The refinement of food and caries. In: P.H. Staple, ed. *Advances in oral biology, volume 2*. London: Academic Press.

Kleinberg, I., 1961. Studies on dental plaque. I. The effect of different concentrations of glucose on the pH of dental plaque in vivo. *Journal of Dental Research*, 40(6), pp. 1087-1111.

Lamont, R.J., Koo, H. and Hajishengallis, G., 2018. The oral microbiota: dynamic communities and host interactions. *Nature Reviews Microbiology*, *16*(12), pp.745-759.

Leme, A.P., Koo, H., Bellato, C.M., Bedi, G. and Cury, J.A., 2006. The role of sucrose in cariogenic dental biofilm formation—new insight. *Journal of Dental Research*, *85*(10), pp.878-887.

Lu, J.J., Perng, C.L., Lee, S.Y. and Wan, C.C., 2000. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. *Journal of Clinical Microbiology*, *38*(6), pp.2076-2080.

Ma, R., Sun, M., Wang, S., Kang, Q., Huang, L., Li, T. and Xia, W.W., 2013. Effect of high-fructose corn syrup on the acidogenicity, adherence and biofilm formation of *Streptococcus mutans*. *Australian Dental Journal*, *58*(2), pp.213-218.

Marsh, P.D., 2006, June. Dental plaque as a biofilm and a microbial community–implications for health and disease. In *BMC Oral health* (Vol. 6, No. 1, pp. 1-7). BioMed Central.

McBain, A.J., 2009. In vitro biofilm models: an overview. *Advances in Applied Microbiology*, 69, pp. 100-126.

Merritt, J.H., Kadouri, D.E. and O'Toole, G.A., 2011. Growing and analyzing static biofilms. *Current Protocols in Microbiology*, *22*(1), pp.1B-1.

Meyer, F., Enax, J., Epple, M., Amaechi, B.T. and Simader, B., 2021. Cariogenic biofilms: Development, properties, and biomimetic preventive agents. *Dentistry Journal*, *9*(8), p.88.

Mosaddad, S.A., Tahmasebi, E., Yazdanian, A., Rezvani, M.B., Seifalian, A., Yazdanian, M. and Tebyanian, H., 2019. Oral microbial biofilms: an update. *European Journal of Clinical Microbiology & Infectious Diseases*, *38*(11), pp.2005-2019.

Nishikawara, F., Nomura, Y., Imai, S., Senda, A. and Hanada, N., 2007. Evaluation of cariogenic bacteria. *European Journal of Dentistry*, *1*(01), pp.031-039.

Pauter, K., Railean-Plugaru, V., Złoch, M., Pomastowski, P., Szultka-Młyńska, M. and Buszewski, B., 2022. Identification, structure and characterization of Bacillus tequilensis biofilm with the use of electrophoresis and complementary approaches. *Journal of Clinical Medicine*, *11*(3), p.722.

Peng, X., Ren, B., Li, Y., Zhou, X., Xie, J., Zhou, C., Zhang, D., Zheng, X. and Zhou, X., 2020. Techniques for oral microbiology. *Atlas of Oral Microbiology: From Healthy Microflora to Disease*, pp.25-80.

Razak, F.A., Baharuddin, B.A., Akbar, E.F.M., Norizan, A.H., Ibrahim, N.F. and Musa, M.Y., 2017. Alternative sweeteners influence the biomass of oral biofilm. *Archives of Oral Biology*, *80*, pp.180-184.

Reiner, K., 2010. Catalase Test Protocol. Available at: https://asm.org/getattachment/72a871fc-ba92-4128-a194-6f1bab5c3ab7/Catalase-Test-Protocol.pdf [Accessed 23 October 2022].

Relucenti, M., Familiari, G., Donfrancesco, O., Taurino, M., Li, X., Chen, R., Artini, M., Papa, R. and Selan, L., 2021. Microscopy methods for biofilm imaging: focus on SEM and VP-SEM pros and cons. *Biology*, *10*(1), p.51.

Samaranayake, L. and Matsubara, V.H., 2017. Normal oral flora and the oral ecosystem. In: A.B.R. Santosh and O.E. Ogle, eds. *Clinical Microbiology for the General Dentist*, 61(2). USA: Elsevier.

Sanchez, C.J., Mende, K., Beckius, M.L., Akers, K.S., Romano, D.R., Wenke, J.C. and Murray, C.K., 2013. Biofilm formation by clinical isolates and the implications in chronic infections. *BMC Infectious Diseases*, *13*(1), pp.1-12.

Sánchez-Vargas, L.O., Estrada-Barraza, D., Pozos-Guillen, A.J. and Rivas-Caceres, R., 2013. Biofilm formation by oral clinical isolates of Candida species. *Archives of Oral Biology*, *58*(10), pp.1318-1326.

Sieuwerts, S., De Bok, F.A., Mols, E., De Vos, W.M. and van Hylckama Vlieg, J.E.T., 2008. A simple and fast method for determining colony forming units. *Letters in Applied Microbiology*, *47*(4), pp.275-278.

Smith, A. and Hussey, M., 2005. *Gram Stain Protocols*. [online] Available at: <https://asm.org/getattachment/5c95a063-326b-4b2f-98ce-001de9a5ece3/gram-stain-protocol-2886.pdf#:~:text=The%20Gram%20stain%20was%20discussed%20in%20detail%20at> [Accessed 23 October 2022].

Stepanović, S., Vuković, D., Hola, V., Bonaventura, G.D., Djukić, S., Ćirković, I. and Ruzicka, F., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis*, *115*(8), pp.891-899.

Supriya, N., 2022. *What is the Colony Morphology of Bacteria?* [online] Available at: <https://biologyreader.com/colony-morphology-of-bacteria.html> [Accessed 23 October 2022].

Takeshita, T., Kageyama, S., Furuta, M., Tsuboi, H., Takeuchi, K., Shibata, Y., Shimazaki, Y., Akifusa, S., Ninomiya, T., Kiyohara, Y. and Yamashita, Y., 2016. Bacterial diversity in saliva and oral health-related conditions: the Hisayama Study. *Scientific reports*, *6*(1), pp.1-11.

Tankeshwar, A., 2013. *Characteristics Shape of Pathogenic Bacteria*. [online] Available at: <https://microbeonline.com/characteristics-shape-of-pathogenic-bacteria/> [Accessed 13 February 2023].

Walsh, L.J., 2006. Dental plaque fermentation and its role in caries risk assessment. International Dentistry, 8(5), pp. 34-40.

Wong, B.K., McGregor, N.R., Butt, H.L., Knight, R., Liu, L.Y. and Darby, I.B., 2016. Association of clinical parameters with periodontal bacterial haemolytic activity. *Journal of Clinical Periodontology*, *43*(6), pp.503-511.

World Health Organisation, 2017. *Sugars and dental caries* [online] Available at: <https://www.who.int/news-room/fact-sheets/detail/sugars-and-dental-caries> [Accessed 4 November 2022].

Yang, Y., Sreenivasan, P.K., Subramanyam, R. and Cummins, D., 2006. Multiparameter assessments to determine the effects of sugars and antimicrobials on a polymicrobial oral biofilm. *Applied and Environmental Microbiology*, *72*(10), pp.6734-6742.

Zeng, Y., Youssef, M., Wang, L., Alkhars, N., Thomas, M., Cacciato, R., Qing, S., Ly-Mapes, O. and Xiao, J., 2020. Identification of non-streptococcus mutans bacteria from predente infant saliva grown on mitis-salivarius-bacitracin agar. *Journal of Clinical Pediatric Dentistry*, *44*(1), pp.28-34.

Zhou, X. and Li, Y. eds., 2021. *Atlas of oral microbiology: From healthy microflora to disease*. Springer Nature.

Zhu, J., Liu, J., Li, Z., Xi, R., Li, Y., Peng, X., Xu, X., Zheng, X. and Zhou, X., 2021. The Effects of Nonnutritive Sweeteners on the Cariogenic Potential of Oral Microbiome. *BioMed Research International*, *2021*.

The complete list of equipment and apparatus which includes consumables used in this study is as follow:

Table A1: List of equipment and apparatus.

Equipment/Apparatus	Manufacturer
37°C incubator	Memmert, Germany
60°C drying incubator	Memmert, Germany
96-well plate (tissue culture treated)	Nest Biotech, China
Candle jar	Oxoid, UK
Compound Light Microscope	Meyer Instrument, USA
Coverslip	Marienfeld, Germany
Cryovials	Greiner Bio-One, Germany
Electrophoresis system	Major Science, USA
Falcon tube	Nest Biotech, China; TPP, Switzerland
Gel imaging system and TransUV illuminator	SynGene Bio Imaging, UK
High speed centrifuge machine	Hettich, Germany
Microcentrifuge machine	Sigma, Germany
Microcentrifuge tube, 2.0 mL	Sorenson, USA
Microplate reader	BMG Labtech, Germany
Microscopic glass slide	Sail Brand, China
Microvolume UV-Vis NanoDrop^{TM} spectrophotometer	Thermo Scientific, USA
Mini spin centrifuge	Tomy Kogyo, Japan
Orbital incubator shaker	Cole-Parmer, USA
PCR thermocycle	Eppendorf, Germany
Petri dish, 90mm × 15 mm	Nest Bitech, China

pH meter	Mettler Toledo, USA
Scanning electron microscope	JEOL, USA
Sputter coater	JEOL, USA
UV-Vis spectrophotometer	Thermo Scientific, USA
Vacuum oven	Sheldon Maunufacturing Inc., USA
Vortex mixer	Stuart, UK

The complete list of chemicals and media used in this study are as follow:

Table A2: List of chemicals and media.

Chemicals/Media	Manufacturer
2× FirstBase PCR master mix	FirstBase, Singapore
100 bp DNA ladder	GeneDireX, USA
Absolute ethanol	RCI Labscan, Thailand
Agarose	FirstBase, Singapore
Bacterial DNA extraction kit	Macherey-Nagel NucleoSpin® Microbial DNA, Germany
Brain heart infusion broth	HiMedia, India
Columbia agar with 5% sheep blood	ISOLAB, Malaysia
Crystal violet	HiMedia, India
Acetic acid, glacial	Systerm, Malaysia
Glutaraldehyde, 25% (v/v)	Acros Organics, Belgium
Glycerol, anhydrous	System, Malaysia
Phosphate buffered saline (Dulbecco A)	Chem Soln, Malaysia
Primers (F1 and R1)	FirstBase, Singapore
Sodium chloride	Merck, USA
Spin column PCR purification kit	Bio Basic Inc., Canada
Sugar	Prai, Malaysia
Tryptic soy agar	Merck, USA

APPENDIX B

The protocol of bacterial DNA extraction provided in the extraction kit (Macherey-Nagel NucleoSpin® Microbial DNA, Germany) is as follow. The reagents mentioned are as named by the manufacturer.

Sample preparation in which the cells from a culture was harvested by pipetting 1.5 mL of the bacterial culture and subjected to centrifugation at $6,000 \times g$ for 2 minutes. The supernatant was removed and ensured 40 mg of wet weight microbial cell culture pellet was obtained. A volume of 100 µL Elution Buffer BE was added and the cells were resuspended. The cell suspension was then transferred into the NucleoSpin® Bead Tube Type B. A volume of 40 µL Buffer MG was added followed by the addition of 10 µL Liquid Proteinase K and the tube was closed to lyse the sample. The NucleoSpin® Bead Tube was agitated on a swing mill. Then, the NucleoSpin® Bead Tube was subjected to centrifugation at $11,000 \times g$ for 30 seconds to clean the lid. A volume of 600 µL Buffer MG was added and mixed via vortexing for three seconds to adjust DNA binding conditions. The suspension was centrifuged at $11,000 \times g$ for 30 seconds. A volume of approximately 500 to 600 µL of supernatant was transferred onto the NucleoSpin® Microbial DNA column and placed in a 2 mL Collection Tube. It was then subjected to centrifugation at $11,000 \times g$ for 30 seconds. The collection tube was discarded with flow through. The column was put into a fresh 2 mL Collection Tube. A volume of 500 µL Buffer BW was added and subjected to centrifugation at $11,000 \times g$ for 30 seconds. The flow-through was discarded

and placed the column back into the Collection Tube. A volume of 500 µL Buffer B5 was added to the column and subjected to centrifugation at $11,000 \times g$ for 30 seconds. The flow-through was discarded and placed the column back into the Collection Tube. The column was centrifuged at $11,000 \times g$ for 30 seconds to dry the silica membrane. The NucleoSpin® Microbial DNA column was placed into a 1.5 mL nuclease-free tube. A volume of 100 µL Buffer BE was added onto the column followed by incubation at room temperature for one minute. Then, final centrifugation was conducted at $11,000 \times g$ for 30 seconds to eluate highly pure DNA. The eluate was stored at -20°C for further used.

APPENDIX C

The protocol of amplicon purification provided in the spin-column purification kit (Bio Basic Inc., Canada) is as follow. The reagents used as described below are as named by the manufacturer.

The amplicons with the expected size of approximately 996 bp were selected. A volume of 45 µL of the selected amplicon was aliquoted into a sterile 1.5 mL microcentrifuge tube. Then, a volume of 200 µL of Buffer B3 was added. The mixture of solution was loaded into a spin column followed by incubation at room temperature for two minutes. The column was subjected to centrifugation at $10, 000 \times g$ for two minutes. The flow-through was then removed. A volume of 750 µL of Wash Solution was added into the column and subjected to centrifugation at $10, 000 \times g$ for two minutes. The centrifugation was then repeated again at $10, 000 \times g$ for one minute to remove the excess Wash Solution. The column was transferred into a sterile 1.5 mL microcentrifuge tube and allowed it to dry for one minute for the ethanol evaporation. Next, a volume of 45 µL of Elution Buffer was added in the column membrane followed by incubation at room temperature for two minutes. It was then subjected to centrifugation at $10, 000 \times g$ for two minutes to remove the bound DNA. Lastly, the DNA concentration   of a minimum concentration of 40 ng/µL and DNA purity of A_{260}/A_{280} ratio of 1.80-2.00.

The raw data for MTP assay are as follow.

ST

Abs	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0870	0.0923	0.0908		0.0921	0.0920	0.0887		0.0863	0.0796	0.0838	
B												
C	0.4155	0.4460	0.4237		0.3661	0.4216	0.4394		0.3934	0.3991	0.3794	
D												
E	0.2158	0.1919	0.2017		0.2261	0.2157	0.2130		0.1595	0.1842	0.2058	
F												
G	0.1684	0.1475	0.1673		0.1617	0.1439	0.1274		0.1493	0.1476	0.1487	
H												

BETA

Abs	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0935	0.0941	0.0975		0.1017	0.0999	0.1003		0.0998	0.1430	0.1008	
B												
C	0.1428	0.1700	0.1735		0.1958	0.1500	0.1603		0.1647	0.1801	0.1639	
D												
E	0.1403	0.1417	0.1067		0.1310	0.1479	0.0873		0.1176	0.0943	0.1291	
F												
G	0.1046	0.1282	0.1137		0.1126	0.1363	0.1274		0.0948	0.1168	0.1121	
H												

Positive control (*S. mitis* ATCC 6249)

Abs	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1127	0.1146	0.1177		0.1160	0.1026	0.1163		0.1163	0.1261	0.1260	
B												
C	0.1726	0.2354	0.2547		0.2826	0.2871	0.2385		0.2568	0.2450	0.2090	
D												
E	0.1285	0.1205	0.1339		0.1377	0.1424	0.1575		0.1592	0.1431	0.1552	
F												
G	0.1629	0.2022	0.2011		0.2269	0.2153	0.2075		0.1972	0.2099	0.2042	
H												

The raw data for acid detection test are as follow.

Initial pH

pH BHI only	6.97	6.96	6.98
pH BHI + sugar	7.1	7.2	7.1
pH BHI +stevia	7.13	7.11	7.1

ST

pH BHI only	5.95	5.94	5.99
pH BHI + sugar	5.54	5.57	5.56
pH BHI + stevia	5.78	5.78	5.78

Positive control (*S. mitis* ATCC 6249)

pH BHI only	6.07	6.66	6.76
pH BHI + sugar	5.78	5.82	5.99
pH BHI +stevia	6.89	6.96	6.86

The raw data for SEM examination are as follow.

ST

BHI only	0.89	1.22	1.27
BHI + sugar	12.66	12.19	10.13
BHI + stevia	10.22	9.56	9.29

Positive control (*S. mitis* ATCC 6249)

BHI only	0.8	1.64	2.48
BHI + sugar	8.63	8.34	7.41
BHI + stevia	4.03	4.13	4.5

APPENDIX E

The p-value obtained from one-tailed t-test for MTP assay is as shown below.

Isolate	p-value
ST	< 0.001
BETA	< 0.001
Positive control (*S. mitis* ATCC 6249)	< 0.001

Result obtained from NanoDrop^{TM} measurement for ST isolate.

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1		Admin	13/1/2023 12:09:34 PM	276.7	ng/µl	5.533	3.041	1.82	1.39	DNA	50.00
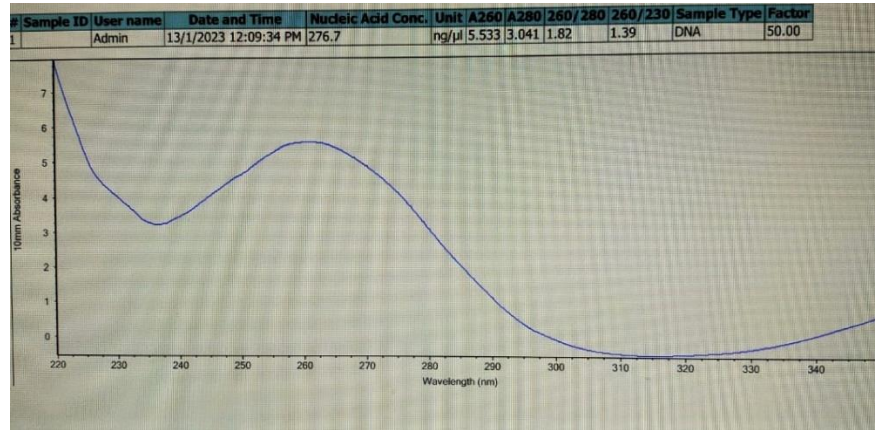
Figure F1: NanoDrop result of ST isolate.

Result obtained from BLASTn for ST isolate.

Figure G1: BLASTn result of ST isolate.