# CHAPTER V

# ANALYSIS AND DISCUSSION

## 5.1 The Effect of Different Solvents on the Final Yield of *A. Paniculata* Extract

In this study of plant extraction, alcohol-based solvent was used instead of an aqueous (water-based) solvent as the polar solvent. This is because the objective is to focus on alcoholic solvents. Furthermore, the findings of several studies show that alcoholic extract is the best polar solvent for plant extracts (Nirmala & Kanchana, 2018; Mizhir, 2022).

According to the findings of this study, different solvents have a significant effect on the final yield of crude extract for *A. paniculata* (Figure 4.1). The extract with the most extractable solids is methanol, followed by acetone, dichloromethane, and *n*-hexane. This includes both the flower (weighing 200g, 180g, 150g, and 100g, respectively) and the leaves (weighing 217g, 195g, 170g and 158g, respectively). This indicates that as the polarity of the solvent used increases, so will the crude extract. Most studies concluded that polar solvents have the greatest weight impact because, due to the presence of polar chemicals in plant material, they can dissolve and separate polar molecules in polar solvents such as water, methanol, and ethanol (Truong et al., 2019). Polar molecules tend to have a positive or negative charge and are attracted to the polar solvent molecules. When the polar solvent comes into contact with the material being extracted, it dissolves and separates the polar molecules, resulting in a larger extract. Furthermore, polar solvents can often dissolve and separate a broader range of compounds than non-polar solvents, which can only dissolve and separate non-polar

compounds. Previous research on apple pomace (Kobus et al., 2017) and bean seeds (Nawaz et al., 2020) also concluded that the extraction solvents could have a significant impact on the yields of crude extract obtained.

On the other hand, n-hexane has a low yield of extract compared to alcoholic solvents because it is a nonpolar solvent and does not dissolve polar compounds such as carbohydrates, proteins, and other polar organic compounds (Abubakar & Haque, 2020). Many plant extracts, for example, contain a mixture of both polar and nonpolar compounds. As mentioned earlier, the polar compounds are typically more soluble in alcoholic solvents such as ethanol or methanol, while the nonpolar compounds are more soluble in nonpolar solvents such as n-hexane. Therefore, when using n-hexane as a solvent for plant extraction, it will only extract non-polar compounds and leave behind the polar compounds. This results in a lower yield of extract compared to using alcoholic solvents, which can extract both polar and non-polar compounds (Putra et al., 2018).

## 5.2 Antibacterial Screening of APL and APF using Disc Diffusion Assay

The four solvents were chosen based on their degree of polarity. Non-polar compounds were extracted using *n*-hexane (APHE) and DCM (APDE), while semi-polar and polar compounds were extracted using acetone (APAE) and methanol (APME), respectively.

According to the results, only APLHE, APLME, APFHE and APFDE exhibit antibacterial properties by expanding the zone of inhibition against *S. mutans* on agar. These findings revealed that the inhibitory zone for all extracts significantly increased in a concentration-dependent manner ($p<0.05$). Through various studies, most agreed

upon bioactive compounds with antibacterial and antiseptic characteristics, such as the phenol group, have been isolated using polar solvents (Altemimi et al., 2017; Truong et al., 2019). However, in this study, *n*-hexane and methanol leaves extract were found to be the most effective at inhibiting the growth of *S. mutans* with significantly different at $p<0.05$. In the case of flower extract, *n*-hexane and DCM solvents were found to have greater antibacterial activity ($p<0.05$) when compared with acetone and methanol.

In general, polar solvents, such as water, alcohols, and aqueous solutions, have been shown to have some antibacterial activity, largely due to their ability to dissolve polar substances and disrupt the membrane structure of bacteria. However, non-polar solvents, have also been shown to have antibacterial activity, as they can penetrate and disrupt the non-polar regions of bacterial membranes (Saluja & Khan, 2018). There are also many reports have found that antibacterial activity is dependent not just on the solvent used but also on the chemical structure of the extracts and the strain being studied. Simorangkir et al. (2019) show in their study on *C. fragrans* leaves extract against two different types of bacteria that semi-polar extract had the greatest inhibition value on Gram-positive *S. aureus*, while non-polar extracts had a greater impact on Gram-negative *E. coli*. Thus, it can be indicated that the results may vary depending on not only the solvent polarity but also the type of bacteria used. In addition, Truong et al. (2019) also mentioned that different organic solvent extracts included different phytochemical components in varying concentrations, which resulted in variances in the inhibitory zone's effect. Therefore, in this research, APLHE, APLME, APFHE and APFDE were selected for further study.

## 5.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC values are considered the "benchmark" for determining an organism's susceptibility to antimicrobials, and thus they are used to evaluate the efficacy of all other susceptibility testing procedures. MIC is defined as the lowest concentration of a drug at which an organism's observable growth is inhibited during incubation. The value is determined by a microdilution assay with MTT as an indicator. The formazan colour in the MTT assay indicated that the bacteria were alive, whereas the yellow colour indicated that the bacteria were inhibited when exposed to the extract. In this study, the MIC values for APLHE and APLME were both at 25 mg/mL, while those for APFHE and APFDE were at 12.5 mg/mL.

Following the completion of the MIC, the MBC test was performed in stages. This test identifies the lowest concentration of an antimicrobial agent required to kill a certain bacterium, and it is useful for comparing the bacteria-killing activity. As for APLHE and APLME, the MBC values were 50 mg/mL and 100 mg/mL, respectively. Meanwhile, APLHE and APLME have the same value of 50 mg/mL. The antimicrobial agent is considered to be bacteriostatic when the MBC/MIC ratio for bacteria is greater than or equal to 16, and the agent is considered to be bactericidal when this ratio is less than or equal to 4 (Neethu et al., 2018; Platania et al., 2022). This means that the concentration of the agent required to kill bacteria is relatively close to the concentration required to inhibit their growth.

As can be seen here, the results for both leaves and flowers of *A. paniculata* extracts (APL and APF, respectively) reveal that the MBC values are not more than four times the MIC values. Therefore, it can be said that the *A. paniculata* extracts are bactericidal agents towards *S. mutans*, as they have effectively proved that the crude

extracts of this plant exhibit high antibacterial action against pathogens (Mostafa et al., 2018; Chassagne et al., 2021). However, in addition, when comparing APL and APF extracts, we may conclude that the APF extract is much more effective, as it requires a lower concentration to provide antibacterial activity. Hence, *A. paniculata* can be further studied for the creation of novel antibiotics.

## 5.4 Antibiofilm Assay

Biofilm formation is a natural process for bacteria and does not require any specific treatment or conditioning. Bacteria can form biofilms on various surfaces, including natural and artificial materials such as rocks, soils, medical implants, and pipes, which can provide a suitable environment for growth and survival. *Streptococcus mutans* is a bacterial population that adheres to the tooth surface. One of the virulence factors found in this bacterium is its ability to form a biological layer on the tooth surface that leads to plaque formation. In this experiment, non-treated *S. mutans* acts as a negative control because, in the absence of treatment, bacteria can continue to multiply and produce extracellular polymeric substances (EPS), which helps to form the biofilm structure and maintain its stability. Therefore, it does not help in reducing biofilm formation. Whereas for the positive control, which was treated with penicillin at a concentration of 0.04 mg/mL, almost 84% reduction was observed ($p<0.05$). As we all know, penicillin is an effective antimicrobial agent that is commonly used to treat bacterial infections. Besides, penicillin has a broad spectrum of activity, making it effective against a wide range of bacteria that can form biofilms by targeting the bacterial cell wall, which is essential for biofilm formation, and interfering with the biosynthesis of the EPS matrix that holds the biofilm together (Fymat, 2017; Yip &

Gerriets, 2022). On this factor, a low concentration is sufficient to help inhibit the growth of biofilm bacteria.

However, as reported earlier, research has revealed that bacteria are already susceptible to antibiotics, including penicillin (González-Bello, 2017). Bacteria can evolve and become resistant to antibiotics via a variety of mechanisms. One of the primary mechanisms is the formation of mutations in their DNA (Sommer et al., 2017; Mancuso et al., 2021). In some cases, these mutations can alter the structure of the bacteria by modifying antibiotic-binding proteins (PBPs) or producing enzymes that break down antibiotics (Egorov et al., 2018). Another factor, the overuse and misuse of antibiotics, can hasten the development of resistance. When bacteria are continuously exposed to antibiotics, they can evolve and develop resistance to those antibiotics (Yin et al., 2020).

Therefore, this study on developing *A. paniculata* as a new medicine to fight pathogens in teeth has shown positive results when there has been a reduction in the biofilm formation of *S. mutans*. These in vitro biofilms were treated with APLHE, APLME, APFHE and APFDE. Based on the results in Figure 4.3, there is a significant difference of biofilm formation between all extracts with the control ($p<0.05$) and the reduction pattern was dependent on the concentration, where the higher the concentration of the extract, the lower the biological activity of the bacteria. With an average difference of nearly 20%, the *A. paniculata* flower extracts (APFHE and APFDE) were stronger than both *A. paniculata* leaves extracts (APLHE and APLME). However, when comparing both flower extracts, APFDE is the most efficient at preventing the development of *S. mutans* biofilm.

In conclusion, the effects of all samples on the biofilm formation activities of *S. mutans* were concentration-dependent. This indicated that samples with a higher

concentration contained more bioactive compounds than those with a lower concentration. As many previous researchers have agreed, the concentration of the extract is also a key factor in determining the antibacterial activity of certain plants (Altemimi et al., 2017; Manilal et al., 2020; Hanafiah et al., 2020; Salehuddin et al., 2020).

## 5.5 Time Kill Assay

To further test the compound's bioactive effect on *S. mutans*, the time-kill assay was used to investigate the rate of kill and the effectiveness of the sample's bactericidal activity. From the results in Figures 4.4 (APLHE), 4.4 (APLME), 4.6 (APFHE) and 4.7 (APFDE), we can see that both bacteriostatic and bactericidal effects on *S. mutans* did occur, but at different times and concentrations. Hanafiah et al. (2015) stated that bactericidal activity is achieved when the extracts reduce the initial inoculum by more than 3 $\log_{10}$ colony-forming units (CFU). Meanwhile, bacteriostatic activity is obtained when antibacterial agents reduce the initial inoculum by 3 $\log_{10}$ CFU. This study demonstrated that after 8 hours of incubation, APFD and APFH extracts (both at the MIC value) significantly decreased the number of *S. mutans* by more than 3 $\log_{10}$ CFU of the original amount ($p<0.05$), which then led to the killing of the bacteria. In comparison to flower extracts, it was discovered that leaves extracts (APLH and APLM) had a lower level of antibacterial activity. Flowers of the *A. paniculata* plant have more commonly been used traditionally to alleviate toothaches than *A. paniculata* leaves (Krishna et al., 2014). They were pounded into a powder, which was then applied to the surface of the tooth to alleviate the pain.

As studies have reported, bacteriostatic drugs prevent the development and multiplication of bacterial cells. Loree and Lappin (2021) stated that this was accomplished by interfering with the bacterial cell's metabolic pathways, most commonly protein production. Even though this does not result in complete cell death, it does effectively prevent bacterial cells from growing and replicating their DNA. When bacteriostatic agents are utilized, they will control the number of bacteria in the cells; the bacteria will not be eliminated, but they will not multiply as they are kept in the stationary phase of growth (McCall et al., 2019). However, increasing bacteriostatic agent treatment levels (for example, by increasing the extract concentration) may eventually result in bactericidal properties, such as irreversible action and/or cell death (Loree & Lappin, 2021). At this stage, the drugs targeted microorganisms by altering their cell wall, lipids, enzymes, or protein synthesis (Khameneh et al., 2019; Baquero & Levin, 2021).

According to the time-kill data obtained in this study, the antibacterial efficacy of *A. paniculata* extracts against *S. mutans* biofilm formation was time- and concentration-dependent. This is supported by the findings of Minami et al., (2019), who also found that Cyanidin 3-O-glucoside (C3G), one of the components of LCE, inhibited the formation of biofilm by *P. gingivalis* in a concentration-dependent manner. When APL and APF extracts were compared, APF extracts appeared to be the best because they require less concentration to have effects on *S. mutans*. As for that, APF extracts demonstrated significantly higher levels of antibacterial activity and potency than APL extracts, this investigation was continued to identify the phytochemicals contained in both APFHE and APFDE.

## 5.6 Morphology of *S. mutans* Biofilm when Treated with APFHE and APFDE under Scanning Electron Microscope (SEM)

As a continuation of this study, the morphological changes of *S. mutans* were observed under SEM and TEM after being treated with APFHE and APFDE. This was done to see how the treatment affected the bacteria and if any physical changes had occurred. As mentioned earlier, *S. mutans* biofilm is coccus-shaped and structured in chains (Zhou et al., 2018). From Figure 4.9 (A), we have proven that the cell shape of untreated *S. mutans* was normal, smooth, with a round surface, and linked in clusters.

According to studies, antibacterial agents can cause changes in the morphology of bacteria. Depending on the type and mechanism of action of the antibacterial agent, it can cause structural changes in the bacterial cell, such as damage to the cell wall, cell membrane, or cytoplasmic contents (Lei et al., 2019; Grabowski et al., 2021). Therefore, as seen in the SEM micrograph for treated *S. mutans*, the morphology was altered after 4 hours of incubation with both APFHE and APFDE (Figure 4.9 B–C). The cells began to shrink and rupture. However, when comparing APFHE and APFDE, the APFHE treatment shows that the cells can still form clumps and chains. In contrast to the condition of APFDE treatment, where it is difficult to see the cells in chains because most of the cells expand and the holes that form on the cells are larger and more numerous. Furthermore, many cells shrank and lysed when compared to the APFHE treatment.

However, the difference is less visible at different concentrations, most likely due to the short incubation time, but both conditions are sufficient to demonstrate that there is a change in cell morphology following drug administration after 4 hours of exposure. This indicated that there is an effect on cellular membrane disruption, cell clumping, and intracellular component leakage (Naeem et al., 2018). As a result, this

study discovered that changes in bacterial cell morphology have a direct correlation to the antibacterial analysis test (Azizan et al., 2020). When APFHE and APFDE are compared, there is a clear difference, with the cells exposed to APFDE being more scattered and nearly all the cells expanding from their original shape. When contrasted to those exposed to APFHE, it is still possible to see chained cells, and the shapes do not expand as much as those tested with APFDE.

## 5.7 Transmission Electron Microscope (TEM) Analysis on *S. mutans* Biofilm when Treated with APFHE and APFDE

Transmission Electron Microscopy (TEM) is another type of microscopy that can be used to study bacteria and their response to treatment with antibacterial agents. Unlike scanning electron microscopy (SEM), which provides high-resolution images of the surface structure of the bacteria, TEM allows researchers to examine the internal structure of the bacteria at a very high magnification. TEM works by passing a beam of electrons through a thin section of the bacteria, which creates an image based on the interaction of the electrons with the sample (Akhtar et al., 2018). Therefore, when bacteria are treated, researchers can see what happens to the cell wall, cell membrane, cytoplasm, and organelles in the bacterial cell.

Similar in the SEM micrograph, Figure 4.10 (A) shows the non-treated *S. mutans*'s cytoplasmic membrane and cell wall were intact under normal conditions, with no obvious cell lysis or generation of cell debris. But, when exposed to both APFHE and APFDE (Figure 4.10 B-C), the overall shape of the *S. mutans* or even the cell membrane was dramatically changed after 4 hours of treatment.

Both findings under SEM and TEM have to some extent, explained why there was a decrease in the percentage of biofilms after 4 hours of exposure to *A. paniculata* flower extracts. Most of the antibacterial drugs penetrate through the cell membrane and then attack the targeted area (Bechinger & Gorr, 2017; Vasconcelos et al., 2018; Jackson et al., 2018). Bioactive compounds from plant extracts were found to affect lipid hydrophobic bonding in cell membranes (Godlewska-Żyłkiewicz et al., 2020; Nishimura & Matsumori, 2020). This hydrophobic contact is a crucial aspect of cell membrane integrity to observe. Hanafiah et al. (2020) did mentioned in their study that changes in the shape of *S. mutans* were caused by the hydrophobic interaction between plant chemical groups and lipids from the cell membrane. They found that plant extraction contained acid hexadecenoic, and they suggested the compound was interacting with phospolipid in the membrane cell to cause lysis of the bacteria.

## 5.8 Gas Chromatography-Mass Spectrometry

As stated earlier, this study focuses on alcoholic extraction. Therefore, the selection of LCMS is not appropriate. Water extraction will incorporate with nanoparticles for further study. GC-MS is well-suited for the analysis of volatile and semi-volatile compounds in plant extracts, such as terpenes, flavonoids, and other small molecular weight compounds that have a high vaporization temperature (Wang et al., 2018; Alonso et al., 2022). GC-MS allows for the separation of these compounds based on their boiling points, and can provide high resolution and sensitivity for the detection of individual compounds in complex mixtures (Moldoveanu & David, 2018; Pramod et al., 2020).

HS-SPME on the other hand is also a popular method particularly useful for the analysis of volatile and semi-volatile compounds, which may not be easily analyzed by other techniques (Lancioni et al., 2022). Compounds retained on the fibre can be desorption directly in a GC injector. Although this desorption method carries the risk of introducing air into the system and resulting in the loss of most volatile compounds, it is the most used method due to its simplicity and does not require the use of solvents or complicated apparatus (Zhakupbekova et al., 2019). A relatively small sample is required for SPME isolation, and its preparation is quick. The main advantages of this method where it can provide enhanced sensitivity for the analysis of volatile compounds, allowing for the detection of compounds that may not be detectable by other techniques (Monedeiro et al., 2019). Besides that, HS-SPME is a selective technique that can reduce interference from the sample matrix, leading to improved selectivity for the analysis.

The major compounds of APFHE and APFDE in phytochemical analysis were fatty acids such as palmitic, stearic, oleic and linolenic acids, which are also major and common constituents in most plants (Fatiha, 2019; Peng et al., 2020). As shown in Tables 4.4 and 4.5, APFHE and APFDE contain all the major fatty acid components, with hexadecenoic and oleic acid being the most abundant. An earlier study discovered that hexadecenoic acid, oleic acid and linoleic acids had antibacterial properties against pathogenic microorganisms. Hexadecanoic acid (palmitic acid) is a fatty acid with the chemical formula $C_{16}H_{32}O_2$ (Carta et al., 2017). According to Lalthanpuii and Lalchhandama (2019), hexadecanoic acid has antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. In addition, Shaaban et al. (2021) discovered that hexadecanoic acid was the main compound in clove alcoholic extract and possessed antibacterial activities against

pathogen bacteria isolated from diabetic patients' swabs. On the other hand, oleic acid compounds are monosaturated fatty acids with the chemical formula $C_{18}H_{34}O_2$ (Bahadi et al., 2021). According to Ghavam et al. (2021) in their study, the high concentration of oleic acid in the rose essential oil has contributed to its potential antifungal activity against *A. brasiliensis* and *C. albicans* and they believe that oleic acid could be a promising natural compound for the prevention and treatment of fungal diseases in humans and food spoilage. Furthermore, this compound possessed antibacterial properties. Previous research discovered that oleic acid inhibited the growth of *Staphylococcus aureus* by blocking the bacteria's carrier protein enzyme. (enoyl-acyl carrier protein reductase, FabI) (Alsenani et al., 2020; Ustadi et al., 2022). Like many other fatty acids, linoleic acid ($C_{18}H_{32}O_2$) can disrupt or damage cell membranes or the cell wall under certain conditions. Linoleic acid has previously been shown to alter peptidoglycan synthesis in *S. aureus* (Casillas-Vargas et al., 2021), where peptidoglycan is a necessary component of bacterial cell walls. In some cases, linoleic acid can cause cell membrane damage by promoting oxidative stress, which can lead to the production of free radicals that can damage cell membranes (Singh et al., 2019). Also, linoleic acid can interact with other molecules in the cell membrane, which can change the structure of the membrane and make it leaky or less good at keeping the cell intact (Yoon. et al., 2018). Fatty acids will stimulate their antibacterial activities depending on their structure, shapes, number of carbons, positions and orientation of double bonds (Toshkova-Yotova et al., 2022). Thus, they disrupted bacterial membranes and inhibited fatty acid synthesis in microorganisms.

Besides that, as we can see, those fatty acids contain more than 10 carbon atoms. Panjaitan et al. (2022) reported that fatty acids with more than 10 carbon atoms can lyse the bacteria as they can become incorporated into the cell membrane, changing its

physical properties. The cell membrane is composed of a lipid bilayer, which is made up of two layers of phospholipids, and the fatty acid composition of these phospholipids can influence the structure and function of the membrane (Nagy & Tiuca, 2017). Fatty acids with longer carbon chains, such as those with 12 or more carbons, tend to be more hydrophobic (water-repelling) than shorter chain fatty acids (Florindo, 2018). This can lead to a more rigid and less fluid membrane, which can affect the ability of the cell to move or change shape. Additionally, longer chain fatty acids can also affect the permeability of the membrane, altering the ability of molecules to pass through it (Fecchio et al., 2018). This can have implications for various cellular processes, such as nutrient uptake and waste removal.

So, the mechanism is that the presence of long-chain fatty acids (LCFAs) in the cell membrane can modify its structure and function in different ways. These modifications include changing the arrangement of phospholipids by inserting between their tails, disrupting the organisation of lipid rafts, which are important for cell signalling, and interacting with proteins that are responsible for shaping and remodelling the membrane. These alterations can affect the curvature and fluidity of the membrane, which in turn may impact cellular processes that depend on membrane shape and flexibility.

## 5.9 Gene Expression

### 5.9.1 Downregulated Genes

Transcriptomic profiling of *S. mutans* treated with *A. paniculata* extracts was performed to investigate gene regulation, particularly in peptidoglycan synthesis. RNA sequencing is a new transcriptome analysis and profiling technology that can provide

quantitative analysis of all transcripts with high accuracy and sensitivity (Kafantaris et al., 2021). Furthermore, RNA sequencing can reveal specific biological functions that are affected after treatment with natural products or drugs (Li et al., 2019).

The results of the molecular analysis confirmed the findings from the SEM, TEM, and phytochemical analyses, which showed that fatty acids disrupted the membrane cells of *S. mutans*. In this study, exposure of *S. mutans* to APFDE leads to a downregulation of peptidoglycan biosynthesis genes (Table 4.9 A). This is consistent with a previous report using *P. aeruginosa*, in which transcriptional changes occurred as a result of drugs that inhibited the *murC* and *murD* gene regulations, and when examined under SEM, the cells were lysed (Moon et al., 2014). Downregulated genes in APFDE-treated *S. mutans* were associated with cell envelope biosynthesis as a result of electron transport disruption and low ATP production. Many metabolic processes in bacteria, including cell wall biosynthesis and protein synthesis, rely on ATP (Walker & White, 2017). This has been proven by the results shown in Tables 4.9 B and D, where the citrate cycle, glycolysis and pyruvate metabolism were also downregulated, resulting in no production of energy molecules. *MurC* and *MurD* genes are involved in the accumulation of L- and D-amino acids to form UDP-MurNAc-L-Ala-c-D-Glu-meso-A2pm-D-Ala-D-Ala of peptidoglycan (Das et al., 2011; Furchtgott et al., 2011; Egan et al., 2020). Therefore, inhibition of both genes will disrupt the process of creating the peptidoglycan which is sufficient to determine the cell shape (Figure 5.1).

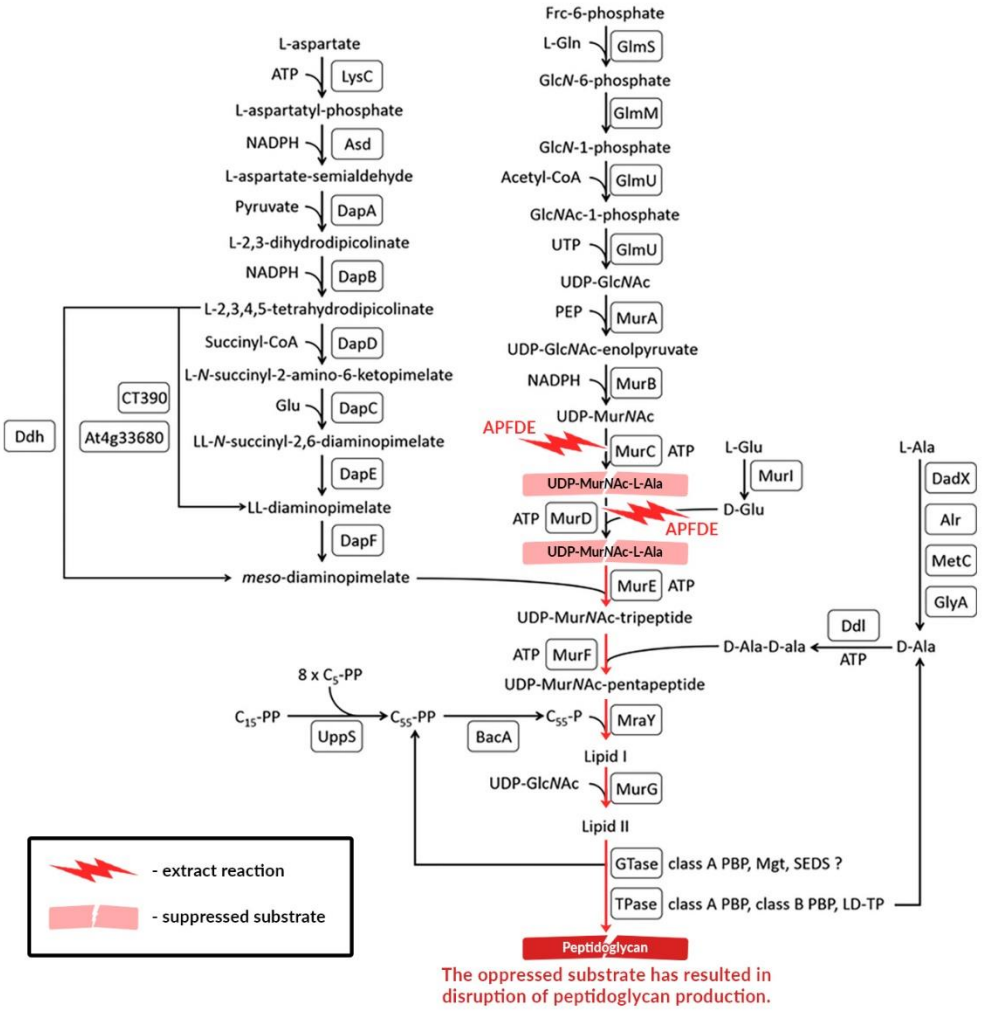


**Figure 5.1:** Disruption of Peptidoglycan Pathway by APFDE (MIC) at *MurC* and *MurD* Genes.

The transcriptomic profile in Table 4.9 (A) also revealed that DNA replication genes in *S. mutans* were downregulated after APFDE treatment. The *dnaG* (primase) and *ssb* genes encode hexameric helicase enzymes that act as replicative DNA polymerase during DNA synthesis (Bogutzki et al., 2019). DNA primase is an important molecule that produces RNA primers that are used to form Okazaki fragments on DNA strands (Gao et al., 2019). It is expected that inhibiting the primase genes (*dnaG* and *ssb*) will stop DNA replication and inhibit cell proliferation (Ilic et al., 2018). It has

been demonstrated that inhibiting DNA replication has antibacterial activity against bacterial pathogens. As a result, DNA primase could be a clinical target for new antibiotics. Wheeler et al. (2013) stated that the inhibition of DNA replication will have an impact on the growth of several bacteria. Aragaw et al. (2022) also discovered that a griselimycin peptide targeted DNA replication gene and killed *Mycobacterium tuberculosis*. In their study, Ilic et al. (2018) reinforces that protein and DNA replication genes such as *ssb, dnaG, rnh3*, and *hol b* have the potential to be drug discovery targets. Therefore, the results of this study show that APFDE inhibits the reproduction of the *S. mutant* by targeting its DNA replication (Figure 5.2).

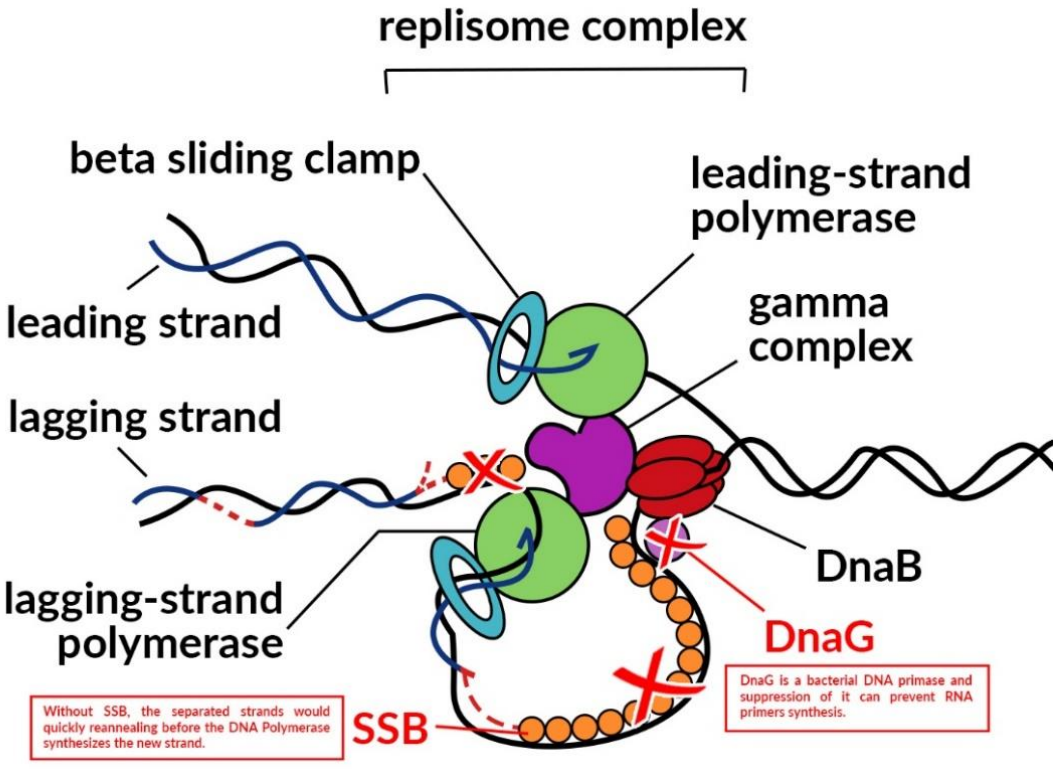


**Figure 5.2:** Disruption of DNA Replication Pathway by APFDE (MIC) at *ssb* and *dnaG* Genes.

Dental plaque is a complex bacterial biofilm community whose composition is influenced by bacterial adhesion, co-aggregation, and environmental growth and survival (Bacali et al., 2022). Caries pathogenesis is thought to be aided by the formation of stable biofilms (Huang et al., 2011; Chen et al., 2020). As mentioned earlier, *S. mutans* is a well-known member of the dental plaque population and uses *gtf* to generate extracellular adherent glucans by synthesising extracellular polymeric substances (EPSs) with dietary sucrose, allowing oral bacteria to accumulate on tooth surfaces (Bowen & Koo, 2011; Ren et al., 2016). EPSs have a key role in the development, adherence and structural integrity of dental biofilms (Koo et al., 2017). They clump together with bacteria cells on the tooth surface and through an EPS-mediated mechanism, produce microcolonies, which contribute to biofilm formation (Koo et al., 2017). However, the results of the current study show that APFDE suppresses biofilm formation by inhibiting growth and *gtf* activity, as it was found that *gtfB* and *gtfC* were downregulated after treatment (Table 4.9 D). Veloz et al. (2016) revealed in their findings that Chilean propolis inhibits genes related to *S. mutans* virulence and adherence via glucosyltransferase suppression, indicating that polyphenols from propolis have anticariogenic potential beyond *S. mutans* growth inhibition. The transcriptomic data also indicated that the relative gene expression of the *gbp* gene decreased when cells were treated with APFDE. The interaction of the *gbp* and *gtf* functions is important because sucrose cannot generate glucan polymers without *gtf*, and glucan polymers cannot bind for energy production without *gbp*. As for that, a deficiency of these two genes will affect the formation of the biofilm. Figure 5.3 shows the genes involved in the formation of the extracellular matrix biofilm of *S. mutans* and the effects of disrupting those genes.

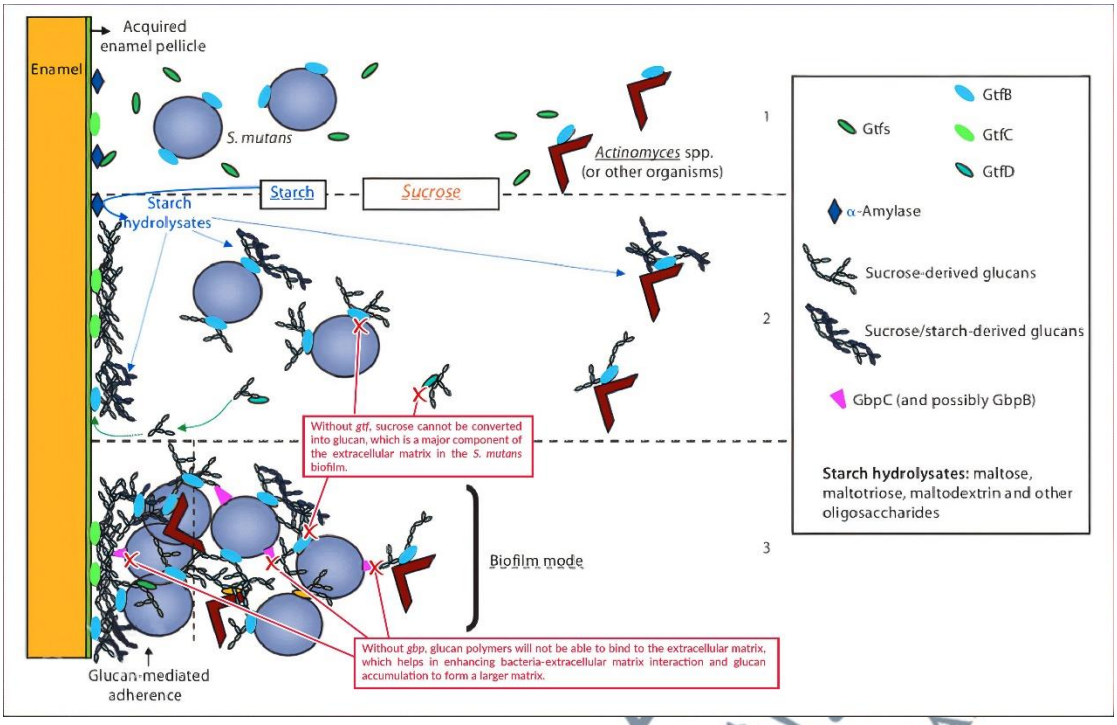


**Figure 5.3:** The *gtf*-glucan-mediated Bacterial Adhesion and Cariogenic Biofilm Development Model.

In addition, relative gene expression showed that the *brpA* gene is downregulated. These genes encode cell surface proteins to help *S. mutans* attach to tooth surfaces and it was the most adhesion-force-sensitive gene as well as a strongly expressed gene in adhering streptococci (Wang et al., 2019). Inhibition of this gene has caused the cells to be unable to congregate with each other. This study has demonstrated the action of APFDE on cells through SEM analysis, where the micrographs show that cells have begun to rupture and separate within the bacterial chains.

The interaction between bacteria and the environment causes biofilm formation. This interaction is aided through a system known as quorum sensing. In Gram-positive bacteria, quorum sensing is composed of three components: the peptide signal, the histidine kinase sensor and the regulatory response in the cell. Among the genes involved in this quorum sensing are *ComC, ComD* and *ComE*, where they encode the proteins histidine kinase and proteins for regulatory responses in cells that play an

important role in forming *S. mutans* biofilms (Wasfi et al., 2018). However, Table 4.9 (D) showed APFDE did suppress the *ComD* and *ComE* genes in *S. mutans.* This was proven by the results in Figure 4.3, where there is a reduction in biofilm percentage after daily exposure to APFDE.

### 5.9.2 Upregulated Genes

In molecular biology, "gene upregulation" refers to the process by which a gene is expressed at a higher level than its normal baseline expression in a particular cell or tissue under certain conditions. This means that the cell's transcriptional machinery, which reads DNA and creates RNA molecules, is triggered to produce more mRNA from a specific gene. This increased mRNA production often results in an increase in protein synthesis, which can affect the behaviour or function of the cell. There are several factors that can lead to gene upregulation, including environmental cues, hormonal signals, cellular stress, and developmental signals (Zhang & Sonnewald, 2017). In response to these signals, certain transcription factors and other regulatory molecules can bind to the DNA upstream of a gene and increase its transcription, leading to upregulation.

As we can see from Table 4.10, genes that were strongly upregulated when treated with APFDE were those encoding proteins involved in microbial metabolism. As a result, it is clear that the bacterial environment has changed, causing the genes to be upregulated. This will aid *S. mutans* in adapting to their surroundings and optimising their use of available resources, which is especially important for survival in harsh environments.

### 5.9.3 Gene Ontology

Gene ontology (GO) is a system used to describe and categorise the functions of genes and their products. GO provides a standardised language and structure for annotating the molecular, biological, and cellular functions of each gene in various species (Consortium, 2019; Thomas et al., 2019). Thomas (2017), in his book, described that GO consists of three main aspects: 1.) Cellular component: categories that describe the location where active protein is found within a cell, such as membrane, nucleus, or cytoplasm, 2.) Molecular function: categories that describe the activity or task performed by a protein, such as a specific enzyme or DNA-binding protein, 3.) Biological process: categories that describe the biological pathway involved in the function of a gene or protein, such as a metabolic pathway or a cell signalling pathway. Based on the Figures 4.18 – 4.20 we can see that almost all of the genes in this study were downregulated, indicating that cell function and metabolism were altered as a result of a treatment that disrupted cell function.

### 5.9.4 KEGG Pathway

KEGG analysis is a curated database dealing with biological pathways, genomes, diseases, drugs and chemical substances (Kanehisa et al., 2021). As shown in Figure 4.21, the extract induced differential expression of genes that were downregulated, such as those in the ribosome, DNA replication, peptidoglycan biosynthesis and the bacterial secretion systems of *S. mutans*, which are important factors for the survival of the bacteria (Shields et al., 2018). Aside from that, several pathways were upregulated, including microbial metabolism in a diverse environment and the biosynthesis of secondary metabolites in *S. mutans* bacteria cells. Like other

living things, in stressful situations, bacteria will create a competitive environment to protect themselves (Riglar & Silver, 2018). As a result, there is a disruption in *S. mutans'* viability after exposure to *A. paniculata* extract.

Dental caries is typically initiated by *S. mutans*. The findings in this study have proven that APFDE has antibacterial activity that disrupts cell function, especially by preventing DNA replication in *S. mutans* and lysing the cell wall of the bacteria. This discovery will lead to a worldwide decrease in cases of dental caries.

### 5.9.5 Novel Gene

The discovery of novel genes (unknown genes) that have not been characterised before can provide several benefits, such as an advance in our understanding of biological processes. Novel genes can provide important insights into the fundamental biological processes that govern life. By identifying new genes and their functions, researchers can gain a more comprehensive understanding of how cells and organisms work at the molecular level (Klasberg et al., 2018). Besides that, it may help in disease diagnosis and treatment. Novel genes can also provide important clues for the diagnosis and treatment of diseases (Wangler et al., 2017). For example, a previously unknown gene that is found to be associated with a certain disease may become a target for developing new therapies. The discovery of novel genes can also shed light on the evolution of species. In terms of technological advancements, novel genes can be used to develop new tools and techniques. For example, a novel gene that produces a valuable protein may be used to develop a new drug or improve the production of an existing one (Sonehara & Okada, 2021). Overall, the discovery of novel genes can have broad

implications for many areas of research and technology and can significantly advance our understanding of the natural world.