

Screening and evaluation of multifunctional excipients: a novel approach for the local delivery of chlorhexidine against streptococcus mutans biofilms

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**SCREENING AND EVALUATION OF MULTIFUNCTIONAL
EXCIPIENTS: A NOVEL APPROACH FOR THE LOCAL
DELIVERY OF CHLORHEXIDINE AGAINST
STREPTOCOCCUS MUTANS BIOFILMS**

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**A thesis submitted in partial fulfilment of the requirements of the
University of Wolverhampton for the degree of Doctor of Philosophy**

October 2021

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ABSTRACT

Dental caries is a major infectious condition involving biofilms that affects billions of people worldwide. *Streptococcus mutans* (NTCC 10449) is the known primary etiologic agent in the development of dental caries, which initiates biofilm formation on the surfaces of teeth. In this study, the antimicrobial activity of chlorhexidine diacetate (CHX) was tested as the drug of choice. This bioactive compound disrupts *S. mutans* biofilms and is a potential treatment for dental caries. It has no systemic side effects and microorganisms do not develop resistance against it. This study aimed to evaluate the effect of sugars (sucrose, fructose, lactose, maltodextrin) and polyols (xylitol, mannitol, and sorbitol) at a concentration range of 2 - 40% w/v, alone and in combination with the antimicrobial agent CHX on planktonic and biofilm forms of *S. mutans*.

Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of CHX were found to be 0.625 µg/ml and 1.25 µg/ml respectively. The sugars were included to simulate the oral environment upon consumption of simple carbohydrates and to study their effect on the formation of dental plaque. Studies with sugars and polyols under both aerobic and anaerobic conditions in planktonic state stimulated growth of *S. mutans*. Overall, polyols supported significantly less biofilm formation when compared to sugars in combination with CHX. Chlorhexidine alone was successful in inhibiting biofilm formation at a higher concentration of 4 µg/ml. According to the results, only fructose at 40%w/v concentration in combination with a lower concentration of chlorhexidine (0.1µg/ml) resulted in lower biofilm density when compared to using lower concentrations of the sugar or high concentration of CHX alone. Xylitol and sorbitol at 2- 40%w/v concentration resulted in significantly less *S. mutans* biofilm formation when combined with chlorhexidine (0.5µg/ml). This study indicates that both sorbitol and xylitol have synergistic inhibitory effects with lower concentration of chlorhexidine on biofilm formation by *S. mutans*.

Cell vitality and EPS production are two crucial factors in the establishment and survival of biofilms. CLSM analysis of biofilm formation by *S. mutans* grown for 72 hours in combination with 5% xylitol and 0.1 µg/ml of CHX showed 13% cell vitality compared to 63% in control biofilms and 53% reduction in EPS production at 5% xylitol with 1.0 µg/ml of CHX. *S. mutans* has the potential for colonization of hard tissues in the human oral cavity and mediation of cariogenic biofilms through dietary sugar metabolism. Gene expression study was performed to discriminate 11 chosen genes known to be predominantly engaged in *S. mutans* planktonic and biofilm formation by

assessing expression in biofilm conditions when exposed to CHX and xylitol. According to quantitative real-time PCR studies, all the genes evaluated were differentially expressed in the biofilm relative to cells cultured in planktonic conditions. The genes encoding extracellular polysaccharide-producing enzymes, *gtfB* and *gtfC*, showed the greatest induction in the biofilm phase. *smu_104* and *smu_105* encoding for carbohydrates uptakes and *wapA* encoding for cell wall associated with protein in intercellular competition were expressed less when compared to control as these genes were inhibited by CHX and xylitol. Lozenge tablets for controlled release of CHX were developed, designed, and optimised using multi-functional excipients by heat congealing and direct compression methods. The pre-formulation studies were carried out by assessing flowability, moisture content, particle size distribution, powder X-ray diffraction (PXRD), morphology and SEM. Physical properties of the lozenges tablets, such as hardness ($11.5 \pm 0.13 \text{Kp}$), friability (<1%), tablet erosion, stability, and drug release (100% under 20 minutes) were also evaluated. During the stability analysis, there were no adverse changes in the appearance and performance of the tablet over a period of six months. In addition to studying the impact of sugars and polyols on biofilm formation, this study has allowed for the selection of polyols as excipients instead of sugars in the formulation of chlorhexidine lozenges to treat dental plaque.

One of most significant findings of this study was that polyols in the presence of chlorhexidine decreased biofilm formation in *S. mutans* when compared to sugars which promoted biofilm formation. However, the use of sugar in conventional chlorhexidine lozenges was defeating the action of CHX on biofilm. Therefore, replacing sugars with polyols not only overcomes the biofilm promoting effects of sugars but also provide a sugar free alternative to be used in diabetic patients and children. Another interesting finding of this study using Confocal microscopy showed morphological changes in EPS (53%) decreased in EPS production compared to the control. There were also 50% decrease in cell vitality. Chlorhexidine in the presence of polyols gave synergistic activity that could work as a mouthwash. However, formulation of lozenges tablet will increase the contact time of the API in the oral cavity when compared to a mouthwash. Hence, the lozenges would increase the effectiveness compared to mouthwash. Another significant findings of this study was the decrease in the amount of antimicrobial agent needed to inhibit biofilm which could lead to addressing the problem antimicrobial resistance and side effect of chlorhexidine which cause teeth staining. This systemic study emphasized the needs to adopt a holistic approach from starting from characterizing the API and excipients followed by evaluation of microbiological effects and mechanism of action of new formulations.

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DEDICATION

I dedicate this work to my family members especially my mother, Habibah Othman and my beloved husband Nidzamuddin Ahmad, without whom, I wouldn't be able to achieve this. It has been a tough journey and I couldn't have imagined myself getting to this stage without your encouragement and support. You have strengthened me, stood by my side, prayed for me, and backed me with all resources. No matter how much I try, words fail to express my love and gratitude to you. Finally, I would like to say thank you from the heart to my children and family to their care, love, encouragement, and support throughout my study.

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List of Abbreviations

ANOVA	Analysis of variance
API	Active Pharmaceutical ingredient
BHI	Brain Heart Infusion
BP	British Pharmacopoeia
C	Celsius
cDNA	complimentary Deoxyribose Nucleic Acid
CLSM	Confocal laser scanning microscopy
CHX	Chlorhexidine
CI Carr's	Compressibility Index Carr's
Con A	Concanavalin A
CV	crystal violet
D10%	Particle diameter at 10% volume distribution
D50%	Particle diameter at 50% volume distribution (median diameter)
D90%	Particle diameter at 90% volume distribution
EMA	European Medicines Agency
EP	European Pharmacopeia
EPS	Extracellular polysaccharides
Eq	Equation
FDA	Food and Drug Administration
FLC	Fractional Inhibited Concentration
G	gram
GCP	Good Clinical Practice
<i>GTF</i>	<i>glucosyltransferase</i>
<i>gtfB</i>	<i>glucosyltransferase B</i>
<i>gtfC</i>	<i>glucosyltransferase C</i>
<i>glk</i>	<i>glucosyl kinase</i>
GI	gastrointestinal

HR	Hausner Ratio
ICH	International Counsel of Harmonisation
kp	kilopones
LOD	limit of detection
LOQ	limit of quantification
mg	milligram
mg/mL	milligram per millilitre
MgSt	magnesium stearate
Min	minute
MIC	Minimum Inhibitory Concentration
MLC	Minimum Lethal Concentration
mm	millimetre
nm	nanometre
mw	molecular weight
%	Percentage
ODTs	Orally Disintegrating Tablets
PBS	Phosphate buffer saline
PI	Propidium Iodide
PSD	Particle Size Distribution
rpm	round per minute
RNA	Ribosomes Nucleic Acid
RT- PCR	Real Time - Polymerase Chain Reaction
SD	Standard Deviation
SEM	Scanning electron microscopy
SMCC	Silicified microcrystalline cellulose
<i>S. mutans</i>	<i>Streptococcus mutans</i>
S9	Syto 9
TGA	Thermogravimetric analysis

USP	United States Pharmacopoeia
VMD	Volume median diameter
wapA	wall associated polysaccharides A
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight
wgs	whole genome sequencing
XRD	X-ray diffractometry

CHAPTER ONE

Literature Review

1.1 Introduction

The oral cavity has more than 700 different bacterial species and is a complex, dynamic system. These bacterial communities live in symbiosis under normal conditions, without harming their host. However, changes in environmental or stress signals could convert these commensals into pathogenic bacteria leading to oral diseases such as dental caries, gingivitis, and periodontitis, (Avila, 2009 and Kilian, 2016). In 2000, oral diseases were classified as "silent epidemic" by the US Surgeon General. Almost 20 years later, every year there are still 3.5 billion people affected by tooth decay. This problem needs to be urgently addressed as oral health plays an important role in maintaining global health.

The global economic burden of dentistry cost \$544.41 billion in 2015. Seventy-nine percent of dental diseases are caused due to severe tooth loss and untreated dental caries (Righolt *et al.*, 2018). The prevalence of dental caries has significantly increased, and caries is now reported to be the most common chronic disease in many countries. According to the 2016 Global Burden of Diseases, Injuries and Risk Factor Study, (GBD 2016), the most prevalent disease world-wide was permanent tooth decay (Vos *et al.*, 2017). This prevalence of dental cavities have been reported to be high in developed countries. In the United Kingdom, for instance, in 2003, 55% - 72% dental diseases were prevalent in children aged 5 to 15. The prevalence of dental caries was 5-78% for children aged 5 to 17 years in the United States. Thus in 2009 and in recent years in 2011-2012, almost three

out of five teens from 12 to 19 years of age had dental caries, five times more than asthma (Bagramian *et al.*, 2009).

1.2 Aetiology of Dental caries and Gingivitis

Dental caries is one of the most prevalent infectious disease in human oral cavity. This compromises people not only in childhood or adolescence, but throughout their lifetimes. The pathological state of caries can be either at the end of the dental crown enamel or at the root-coating cement or dentine that causes the dental tissue to decalcify and break down. Dental plaque is an example of oral biofilm, a requisite factor of dental caries aetiology. This oral biofilm has bacterial aggregation embedded in exopolysaccharides and other extracellular polymers that bind to the tooth surface (Marsh *et al.*, 2010; Koo *et al.*, 2013). In the absence of oral hygiene, dental plaque naturally accumulates at specific sites on tooth surfaces and can achieve a point of "maturation" after one to two days. The maintenance of oral health & hygiene is required for controlling bacterial build as biofilm on teeth. Other cause like the presence of fermentable sugars, host factors, the presence of cariogenic/ acid producing microbial flora and other associated environmental factors also contributed to dental caries (Rathee and Sapra, 2021).

1.2.1 *Streptococcus mutans*

Evidence from studies on dental caries such as oral streptococci and especially from *S. mutans*, *Lactobacillus* spp., members of mites, *S. anginosus* and *Streptococcus salivarius* group, as well as *Propionibacterium*,

Enterococcus faecalis and *Scardovia* showed that different bacterial species could also be associated with the development of dental caries (Tanzer *et al.*, 2001; Wade, 2013).

Streptococci were the most dominant genus that was confirmed not only from culture based methods but also from 16S rRNA next generation sequencing (NGS). Streptococci were abundantly represented in hard palate (44%), oral mucosa (65%), and keratinized gingiva (66%). However, they were found to a smaller extent (12–23%) of the total microbiome in the tongue (12%), supragingival (13%) and subgingival plaque (15%). According to WGS analysis, *Streptococcus mitis* showed to be the most common (9.5% of the total detected species), followed by *S. oralis*, *salivarius*, and *sanguinis* (in order of abundance). The cariogenic *S. mutans* was found in small amounts, accounting for 0.003% of the total species (Caselli, *et al.*, 2020).

These infections caused by oral microflora have showed a linkage with the current Covid-19 pandemic. Metagenomic analyses reported that patients with severe acute respiratory syndrome have high number of cariogenic pathogens. The connection between oral microbiome and Covid-19 indicated the bacteria involved in the pathogenesis of respiratory diseases were also linked with comorbidities including type 2 diabetes, hypertension, and cardiovascular disease (Chakraborty S, 2020). Oral bacteria play a role in the pathogenesis of respiratory infections through aspiration of oral pathogens into respiratory organs, variation of the respiratory epithelium by periodontal-associated cytokines, and oral mucosal surfaces provided to

promote respiratory pathogen adhesion. Research showed that dental hygiene treatments significantly improve clinical results and decrease mortality in patients with pneumonia. One in every ten pneumonia-related deaths in nursing home residents over the age of 65 is thought to be avoidable by improving oral hygiene. Optimal oral care has been shown to lower the occurrence of ventilator-associated pneumonia in intensive care unit patients (Azarpazhooh & Leake, 2006). More metagenomic research and clinical trials are needed to characterise co-infections COVID-19 and the correlation between the role of the oral microbiome and virus complications. These findings are critical in determining whether poor oral hygiene is a modifiable risk factor for COVID-19 complications and whether there is a contribution for good oral hygiene promotion as a preventive public health strategy during the pandemic (Manger *et al.*, 2017).

Streptococcus mutans (*S. mutans*) is a major pathogen commonly implicated in dental caries. *S. mutans* can attach to the tooth surface and produce lactic acid from dietary carbohydrates, primarily sucrose and glucose (Van Houte *et al.*, 1991; Boisen *et al.*, 2021). *S. mutans* is capable of demineralizing the hard teeth by the creation of such acidic environments. They are constantly active in the mixed species environment, and impact on colonization and acid production. These bacteria are the most cariogenic dental pathogen found in the oral cavity. The natural habitat of *S. mutans* is in the human mouth, particularly associated with development of dental plaque. Furthermore, when the plaque on the tooth surface has forming a layer which contains bacteria reside, they are presenting a biofilms. They

contribute to dental biofilm formation by producing large amounts of organic acids and allow non-cariogenic species to flourish at low pH levels. In the oral cavity there is dynamic environments, where pH, nutrient sources, oxygen tension, temperature and osmolality fluctuate at rapid paces (Lemos *et al.*, 2005). *S. mutans* is forming biofilms on the hard tissues particularly in gum and teeth area. This microorganism adherence to surfaces is the initial step in the formation of biofilms by sugar-dependent and sugar-independent mechanisms (Lemos *et al.*, 2019). It also can bind to salivary pellicle and penetrate through the teeth. Sucrose-dependent adherence is mediated by glucan binding proteins and water-insoluble glucans produced from sucrose by glucosyltransferase (*gtf*) enzymes (Durso *et al.*, (2014). Moreover, human saliva has a high-molecular-weight of glycoprotein that mediates the adhesion and aggregation of *S. mutans* via the cell wall. When *S. mutans* biofilms mature, then the diseases of oral cavities are produced. *S. mutans* is a facultatively anaerobic, Gram-positive cocci commonly found in the human oral cavity. [Figure 1.1](#) shows a microscopic image of *S. mutans* using Gram staining technique.

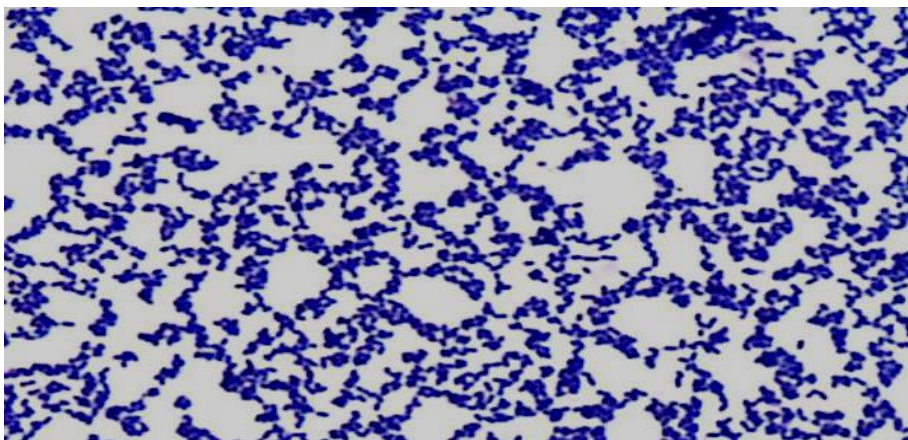


Figure 1.1: Gram- stained of *S. mutans* found in the oral cavity. The colonies were stained with Crystal Violet, iodine, and safranin on the glass slide (Magnification x100).

One common disease that can cause dental caries among humans is gingivitis. Gingivitis is a disease that causes inflammation, swelling, redness, and bleeding from tissues around the teeth due to infection caused by *S. mutans*. These bacteria are considered to play the main role during gingivitis as it is dependent on adherence of bacteria to the tooth surface and formation of biofilms (Forssten *et al.*, 2009). This ability of oral bacteria to survive and persist in biofilms depends on their capacity to respond to environmental changes at genetic, physiological, and biochemical levels (Kolenbrander, 2000).

Gingivitis is also caused by the action of acids on the enamel surface of tooth which are produced when sugars from food and drink is utilised by bacteria present in the biofilm (plaque) on the tooth surface. The acid dissolves minerals in the hard enamel which leads to gap formation between teeth. It induces mineral loss due to production of acid from fermentable carbohydrates which is also responsible for the low pH in the oral cavity. The acid produced causes loss of calcium and phosphate from the enamel and this process is called demineralisation. At early stages, caries can be found as a small patch of demineralized enamel on tooth surface. With poor supervision, the destruction could be spread into softer and sensitive part of tooth beneath the enamel causing gingivitis (Burne, 1998). Importantly, gingivitis is not just a disease seen in adults, but majority of children and adolescents reported having signs of mild to moderate gingival inflammation, making management and prevention of gingivitis an important public health

concern. [Figure 1.2](#) show a difference between gums of a healthy person and one having gingivitis.

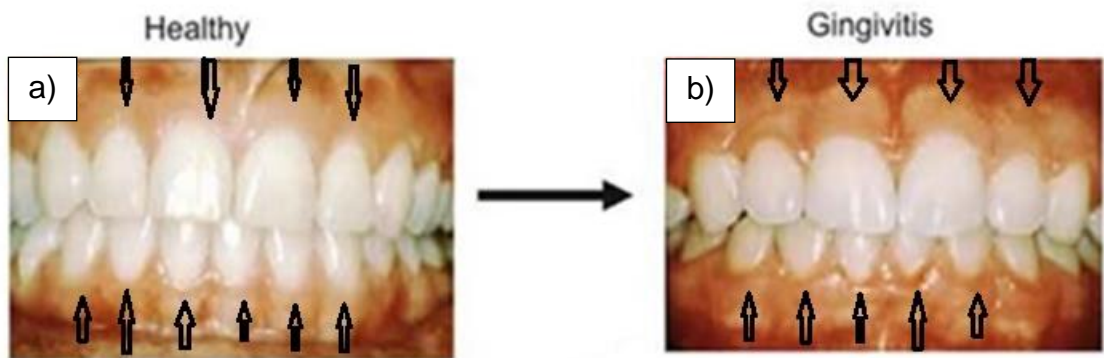


Figure 1.2: Inflammation of gums around teeth in a healthy individual (a) compared to a Gingivitis patient (b). Adapted from (Rutter, P. 2013)

The inflamed gum tissue around the teeth as shown in [Figure 1.2 \(b\)](#) on the gingivitis patient can easily bleed during brushing. The mechanical removal of plaque results in the disappearance of the clinical signs of gingival inflammation. Tooth decay refers to the damage of tooth structure caused by the acids created by plaque bacteria as they break down sugar in the mouth. A cavity or hole in the tooth enamel can form if the demineralization is not treated. The stages of tooth decay are show as in [Figure 1.3](#).

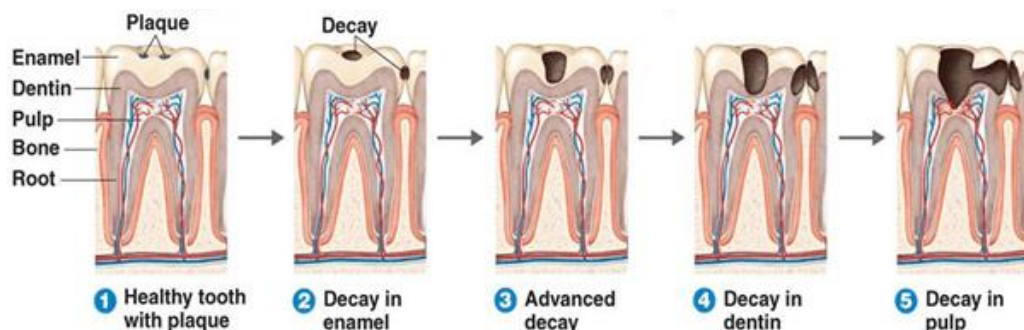


Figure 1.3: The stages of tooth decay. Adapted from (Hertel et al., 2017)

Therefore, regular mechanical removal of all bacterial plaque from oral surfaces is considered as primary treatment. It means to prevent and stop the progression of gingivitis disease. They could reduce the overall rate of accumulation of new plaque and also reduce or remove existing plaque. Although considerable progress has been made, the search for a safe and effective chemical agent for the control of plaque is still under consideration (James *et al.*, 2017).

1.2.2 Biofilm

Biofilms are communities of microorganisms, encapsulated in an extracellular matrix of polymers, that live at interfaces. While biofilms may grow at liquid interfaces or liquid/gas interfaces, they are typically attached to a biotic or abiotic surface that interfaces with liquid or gas. Most bacteria can form biofilms but biofilms do not solely comprise of bacteria and may include algae, archaea, fungi, and protozoa. The extracellular matrix is made up of varying combinations of polysaccharides, proteins, and nucleic acids. Bacteria when living as biofilms are many times more resistant to antibiotics, biocides, and hydrodynamic shear forces than when the same bacteria employ a planktonic stage. Species richness and the extracellular matrix act as buffers against changing conditions, although when conditions do change the composition of the community and matrix changes over time (Liu *et al.*, (2011).

1.2.2.1 Biofilm Formation

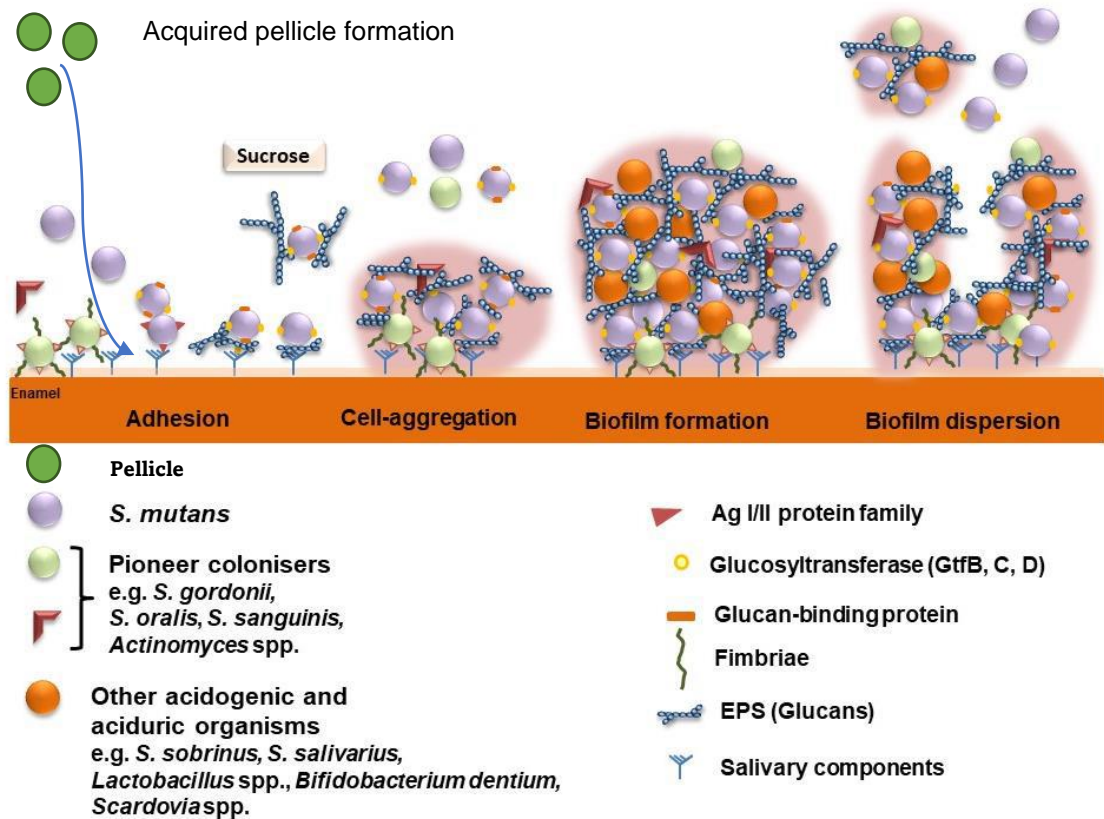


Figure 1.4: Oral biofilm formation. Adapted from (Koo, 2013)

Four key stages generate oral biofilms on the tooth surface which known as adhesion, cell aggregation, biofilm formation and biofilm dispersion as shows in Figure 1.4. Starting with oral bacteria, particularly pioneer colonizers adhere to salivary components on tooth surfaces. Early colonizers aggregate to adherent bacteria on the tooth surface once irreversible adhesion occurs. This cell-to-cell aggregation increases the density of cells adhering to the tooth surface as well as the concentration of cell signaling molecules. Cell signaling molecules, also known as quorum sensing molecules, cause cells to form a biofilm. To form a biofilm, a portion of the biofilm and some cells

within the biofilm structure can be detached, and some detached cells can reattach to a new surface (Koo, 2013).

Oral bacteria adhere to salivary components such as lysozyme, amylase, proline-rich proteins, and mucin-coated tooth surfaces. Pioneer colonizers like *S. gordonii*, *S. sanguinis*, *S. mitis*, *S. oralen* and *Actinomyces spp.* in absence of sucrose can bind on the tooth surface to salivary components. In this process, *S. mutans* can bind directly with their adhesions to salivary components (Ahn *et al.*, 2008).

Then, early colonizers such *S. mutans*, assemble around on previously bonded pioneer colonizers (Kolenbrander *et al.*, 2010; Peterson *et al.*, 2011 and Koo *et al.*, 2013). According to Koo *et al.*, (2013) *S. mutans* produces exoenzymes called glucosyltransferases (Gtfs) in the pellicle and attach to microbial surface in the presence of sucrose, and then produce glucans from. Because Gtfs can adhere to salivary components and insoluble glucan binds to guanylate-binding proteins (Gbps), both Gtfs and insoluble glucan facilitated in the adhesion of *S. mutans* and the co-aggregation of oral bacteria (Nobbs *et al.*, 2009). After the primary colonizers and early colonizers adhere irreversibly on the tooth surface, other oral bacteria can adhere to the tooth surface such as *Capnocytophaga spp.*, *Actinomyces spp.*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis*.

Cell-to-cell communication plays an important role in biofilm formation using quorum-sensing system. For example, the adherent cells differentiate to become sessile cells and start forming biofilm (Leung *et al.*, 2015). Competence stimulating peptide (CSP), a short signaling peptide, and the ComDE two-component signal transduction system make up the quorum-sensing system in *S. mutans*. The CSP-ComDE system in *S. mutans* is activated under stress conditions such as acidic pH, amino acid deficiency, and oxidative stress. This system governs a variety of phenotypes after activation, including biofilm formation, acid tolerance, competence, and bacteriocin production (Perry *et al.*, 2009; Leung *et al.*, 2015). Bacteria on the surfaces of teeth create a three-dimensional, (3-D) structures by producing extracellular polymeric substances (EPS) such as polysaccharides, proteins, and eDNA. These 3-D structures are important for biofilm formation and cell- to cell communication.

Biofilm formations are formed from a complex aggregation of microorganisms growing on a solid substrate. They can occur on organic or inorganic surfaces such as dental plaque. Biofilms could be characterized by structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances. The matrix protects the cells and increases their resistance to antimicrobials. Many antimicrobial agents have a difficult time eliminating organisms in a biofilm.

1.2.2.2 Biofilm Dispersal

Biofilm dispersal can be defined in three steps between detachment of biofilm cells, translocation of the detached biofilm cells to a new location, and attachment of the biofilm cells to the new location (Kaplan, 2010). There are two types of dispersal such as passive and active dispersal (Kaplan, 2010; Fleming & Rumbaugh, 2017). The passive dispersal biofilm refers to external force such as mechanical intervention (tooth brushing, tongue movement, fluid, and solid shear). Whereas active dispersal refers to a process supported by biofilm contained microbes (inside the biofilm structure). The active dispersal is mediated by many factors such as nutrient starvation, toxic by-products, and antimicrobial stress. This is an important phase in the cycle of biofilms, which contributes to bacterial survival and disease transmission (Kaplan, 2010; Fleming & Rumbaugh, 2017). Kaplan reported that biofilm dispersal has three modes such as erosion, sloughing, and seeding. The erosion of a biofilm refers to the release of small clusters or single cells. Sloughing is the term used to describe the sudden release of huge amounts of biofilm. Whereas the release of a high number of single cells from hollow spaces in a biofilm structure is referred to as seeding dispersal (Kaplan, 2010). However, in the case of *S. mutans*, biofilm dispersion may serve as a mechanism for the bacteria to propagate to other locations (Lee *et al.*, 1996). Biofilm dispersion in the oral cavity is mediated by salivary flow, mastication, and tongue movement (Das *et al.*, 2011). An endogenous surface protein releasing enzyme (SPRE) activity acquired from *S. mutans* has been reported to be the cause of active *S. mutans* biofilm

dispersal (Vats & Lee, 2000). Biofilm detached cells exhibit different physiological features than planktonic cells and sessile cells, according to *Liu et al.*, (2013). These detachment cells might adhere to other surfaces and form a new biofilm.

1.2.2.3 Gene expression involved in biofilm formation

The identification and characterization of genes that are differentially expressed during biofilm formation could allow scientists to better understand how *S. mutans* responds to environmental signals in the oral cavity. Sucrose and glucosyltransferases (*gtf*) have been implicated in the production of *S. mutans* biofilms in previous research. Several genes related with genetic competence, including the *ccpA* and *brpA* (*lytR*) genes, as well as a potential response regulator, *wapA*, have been implicated in biofilm development. Signal transduction cascades also determine the environment and control a variety of transport, protease development and biofilm formation.

1.2.2.3.1 Gene expression during EPS production

In the community of microbial species in natural biofilms, microorganisms coexist and interact with each other. The competition in the community drives microorganisms to evolve survival mechanisms (Xavier & Foster, 2007, Xavier *et al.*, 2009). Some bacteria might produce substances to combat other bacteria, especially during adhesion on the same area. For instance, biosurfactants produced by a strain of *Lactobacillus casei* reduces gene expression of *gtfB*, *gtfC*, and *fff* in *S. mutans* and this reduced expression

might reduce the adhesion of *S. mutans* subsequently due to lower amounts of glucans synthesized (Savabi *et al.*, 2014).

As mentioned previously, the accumulation of EPS matrix in biofilms provides a protective barrier against antimicrobial agents. Targeting the EPS of *S. mutans* biofilms has been investigated in many studies. Since glucans are major components in EPS, inhibiting *gtfs* expression and *Gtfs* activity reduces EPS accumulation (Falsetta *et al.*, 2012, Bueno-Silva *et al.*, 2013, Wang *et al.*, 2016). Many compounds have been reported to inhibit *gtfs* expression such as a novel combination therapy (2 mM myricetin, 4 mM tt-farnesol, 250 ppm of fluoride) reduced the expression of *gtfB* (Falsetta *et al.*, 2012) and glass-ionomer cements (GIC) containing a quaternary ammonium monomer (dimethylaminododecyl methacrylate, DMADDM) reduced the expression of *gtfB*, *gtfC* and *gtfD* (Wang *et al.*, 2016).

Targeting to EPS synthetic components has been reported in number of studies. 7-epiclusianone, a novel compound isolated from *Rheedia brasiliensis*, inhibits the activity of *gtfB* and *gtfC* resulting in the reduction of insoluble glucan synthesis (Murata *et al.*, 2010). Neovestitol-vestitol (NV), isolated from Brazilian red propolis, inhibits *gtfD* activity resulting in decrease amount of soluble glucan (Bueno-Silva *et al.*, 2013). Glucan, particularly insoluble glucan formation, is another important virulence factor that contributes to *S. mutans* cariogenicity. A dental biofilm contains 10–20% EPS (Bowen *et al.*, 2011), the majority of which is glucans synthesized by microbial glucosyltransferases (*Gtfs*) from the substrate sucrose (Paes Leme *et al.*, 2006). *S. mutans* makes a major contribution to the production of

glucans in dental biofilm (Koo *et al.*, 2010). Three GTF enzymes catalyze the synthesis of extracellular glucans from sucrose in *S. mutans*. *gtfB* primarily synthesizes water-insoluble glucans; *gtfC* catalyzes the synthesis of both water-insoluble and soluble glucans; and *gtfD* only synthesizes water-soluble glucans (Hanada & Kuramitsu, 1988 and Hanada & Kuramitsu, 1989). Surface-adsorbed *gtfB* and *gtfC* synthesize insoluble glucans, which represent as binding sites for *S. mutans* on the solid surface and facilitate co-adherence with other bacterial cells. It is important in mediating the transition from initial cell attachment and clustering to the formation of microcolonies and multi-microcolonies aggregates. In a rat model, deletion of both *gtfB* and *gtfC* resulted in the greatest reduction in *S. mutans* extracellular polysaccharide matrix production and biofilm formation, as well as a lower incidence of smooth surface caries. Therefore, the inhibition of *gtfD* reduces the EPS accumulation (Venkitaraman *et al.*, 1995).

There is a method to detect the gene expression. One of the common method is Reverse Transcription – Polymerase Chain Reaction (RT-PCR) which is a highly sensitive and precise approach that is useful for detection of mRNA transcripts available in small quantities. There are many advantages of using RT-PCR over other methods to quantify gene expression. While reproducibility is often a prerequisite, extreme precision is not possible: most studies do not concentrate on calculating small changes or the specific number of molecules but rather on increasing or decreasing expression levels by at least 1-2 fold. Thereby, despite the higher precision of recently developed techniques, semi-quantitative procedures are still commonly used

and suitable for many purposes (Shemesh *et al.*, 2007). RT-PCR can also differentiate between messenger RNAs (mRNAs) with almost identical sequences, requires much less RNA template than other gene expression analysis methods, and provided the correct equipment, can be relatively high throughput. The two techniques use the same process except that RT-PCR has an added step of reverse transcription of RNA to DNA, to allow for amplification. The major disadvantage to real-time PCR is due to costly equipment and reagents. In addition, sound experimental design and an in-depth knowledge of normalization techniques are crucial for precise conclusions due to its extremely high sensitivity (Higuchi *et al.*, 1993).

1.2.2.3.2 Possible strategies to target *S. mutans* biofilm

Several lines of evidence suggest possible strategies to combat biofilms, including those produced by *S. mutans*, by targeting the stages of biofilm formation, such as the inhibition on the surfaces bacterial adhesion, the inhibition of biofilm development and differentiation, the inhibition of biofilm cells, and the induction of biofilm dispersion (Yang *et al.*, 2012; Scharnow *et al.*, 2019). The adhesion of *S. mutans* cells is one of the important process to form biofilms on the tooth surfaces. Many studies have focused on inhibiting *S. mutans* biofilm formation at the adhesion step. *S. mutans* planktonic cells adhere on the tooth surfaces through a sucrose-dependent and sucrose-independent mechanism which are targeted differently.

In the presence of sucrose, *S. mutans* glucosyltransferases (*gtfB*, *gtfC*, *gtfD*) and glucan binding protein (Gbps) are essential for attachment and biofilm

formation. Molecules obstructing adhesion of *S. mutans* planktonic cells have been widely studied. Piceatannol and resveratrol have been discovered to be anti-adhesion molecules (Newbrun *et al.*, 1983; Kwon *et al.*, 2010) inhibit *S. mutans* biofilm formation by targeting *gtfB* and *gtfC*. Moreover, a quinoxaline derivative has been investigated to inhibit *gtfC* activity and reduce the adhesion of *S. mutans*' biofilm (Ren *et al.*, 2015). A small molecule, derived from a structure-based discovery study of the *gtfC* catalytic domain, inhibits *gtfC* activity and biofilm formation in *S. mutans*. The small molecule has no effect on *S. mutans* and intracellularly oral growth (Zhang *et al.*, 2017). Scharnow *et al.*, (2019) have revealed two *gtfs* inhibitors which are (i) hydroxychalcones, that have an aromatic ketone and enone forming central core, and (ii) molecules with high heteroatom density, an atom other than carbon or hydrogen, especially nitrogen. These findings might help to guide the development of the new compounds that are potent GTF inhibitors for use against *S. mutans* biofilms.

In the absence of sucrose, sortase A facilitates the attachment of Agl/II, *GbpC*, *FruA*, *wapA* and *wapE* on *S. mutans*' cell wall and these surface proteins have been confirmed to play important roles in adhesion, colonization, and biofilm formation (Ajdic *et al.*, 2002; Lévesque *et al.*, 2005). These reasons lead sortase A to be an attractive target for *S. mutans* anti-biofilm activity. Hu *et al.*, (2013) found that curcumin could inhibit the sortase A of *S. mutans* without affecting *S. mutans* viability. They was hypothesised that enone functionalities in curcumin are specific sites for irreversible modification by Cys184 in sortase A of *S. mutans*. This inhibition resulted in

the release of Pac protein (antigen I/II, P1, and Spa P1) on *S. mutans* cell surfaces and reduce the ability to form biofilms by *S. mutans*. A trans-chalcone, a chalcone flavonoid, inhibits sortase A and also inhibits biofilm formation by *S. mutans* (Wallock-Richards *et al.*, 2015). Mass spectrometry revealed that the trans-chalcone forms an adduct with the cysteine at the active site of sortase A (Wallock-Richards *et al.*, 2015).

Eugenol, a major component of essential oil from clove buds, inhibited biofilm formation from *S. mutans* (Adil *et al.*, 2014). Eugenol reduces the *S. mutans* cells adhesion by reducing the expression of Agl/II. Furthermore, an active compound from *Trachyspermum ammi* seeds was found to inhibit biofilm formation from *S. mutans* through a variety of mechanisms such as reducing adhesion and glucan synthesis and inhibiting the reduction in pH (Khan *et al.*, 2010). Therefore, the most dominant mechanism seems to be the inhibition of adhesion and destroy the integrity of cell wall *S. mutans*'s due to the reduction of hydrophobicity (Scharnow *et al.*, 2019).

1.2.2.3.3 Targeting EPS

The disruption of EPS structure is an attractive means to break *S. mutans* biofilms, which allows access of antimicrobial agents to kill microbes within biofilm. A previous study by Gao *et al.*, (2016) generated catalytic nanoparticles (CAT-NP) with peroxidase-like activity, containing Fe₃O₄. CAT-NP produced free-radicals, which could degrade the EPS matrix, particularly the water-insoluble glucan, and kill the embedded bacterial cells by causing DNA damage. The CAT-NP also reduced the acid dissolution of

hydroxyapatite. The clinical plaque disclosing/visualising agent erythrosine, when used as a photosensitiser in a photodynamic system, was found to kill *S. mutans* cells in a 200 µm-thick biofilm in a constant-depth film fermenter. The excited state of erythrosine could react with biomolecules in *S. mutans* cells to produce free radicals or could react with an oxygen molecule to produce singlet oxygen. The free radicals or singlet oxygen can cause oxidation of cellular constituents such as cell membrane, and DNA, causing cell death (Wood *et al.*, 2006).

Quorum sensing systems modulate virulence factors of *S. mutans* including biofilm formation, genetic competence, bacteriocin production, and acid tolerance response (Perry *et al.*, 2009; Leung *et al.*, 2015). Therefore, targeting quorum sensing systems has been widely studied. For example, specifically (or selectively) targeted antimicrobial peptides (STAMPs) and other selective antimicrobial peptides (i.e., C16G2, M8G2, C16-33, and M8-33) were constructed based on the CSP-derived peptides. Beneficially, a low concentration of these molecules is required to target the quorum sensing system but did not affect other oral streptococci (*S. gordonii* and *S. sanguinis*) (Eckert *et al.*, 2006).

Within the biofilm structure lactic acid produced by *S. mutans* is a major cause of demineralisation of the enamel surface. Several previous studies have focused on targeting either acidogenicity or aciduricity to inhibit biofilm formation. Acidogenicity mean bacteria producing acid while aciduricity means capability of growth in an acid environment. Pandit *et al.*, (2015) found that a fraction of hexane-dissolved extract from *Withania somniferous*

composed of palmitic, linoleic, and oleic acids, exhibits the strong anti-acidogenic, activity against *S. mutans*. The reduction in acid production and secretion might be due to an inhibitory activity of linoleic and oleic acids on F1F0-ATPase (Pandit *et al.*, 2015). There are significance of selected genes in development of *S. mutans* biofilm however other oral microflora would encodes to simplify their activity within development.

1.3 Management of the disease

1.3.1 Treatment

The primary goals of dental caries prevention are to reduce the risk of disease incidence by improving oral health. Other strategies may delay or stop the progression of caries lesions by implementing protective factors or reducing the impact of pathological factors (Featherstone, 2004). Preventing dental caries in children and adolescents is both preferable and more cost-effective than treating dental caries, so it is a main priority for dental services. When making preventive treatment plans, it is important to understand each patient's specific needs, and preventive care is an important aspect of treatment planning for all paediatric patients. **Table 1.1** shows different antibiotics are using to treat gingivitis and periodontitis on the current market.

Table 1.1: Gingivitis and periodontitis treatment using different antibiotics

Drug	Microorganism	Reference
Amoxicillin	<i>Aggregatibacter actinomycetemcomitans</i>	(Jorgensen, & Slots, 2000)
Amoxicillin clavulanate Potassium	<i>Porphyromonas spp</i>	(Tancawan <i>et al.</i> , 2015)
Azithromycin	<i>Porphyromonas gingivalis</i>	(Lai & Walters, 2016)
Ciprofloxacin	<i>Actinobacillus actinomycetemcomitans</i>	(Tezel <i>et al.</i> , 2005)
Metronidazole	<i>Porphyromonas gingivalis</i> , <i>Streptococci spp.</i>	(Razzaq <i>et al.</i> , 2018)
Nitroimidazoles	<i>Aggregatibacter actinomycetemcomitans</i> , <i>Porphyromonas gingivalis</i> and <i>Fusobacterium nucleatum</i>	(Al-Zubaydi <i>et al.</i> , 2017)
Macrolides	<i>Streptococci spp.</i>	(Seiple <i>et al.</i> , 2016)
Erythromycin	<i>Streptococcus mutans</i>	(Shahabooei <i>et al.</i> , 2015)

1.3.2 Prevention of dental caries using antimicrobial agents

Preventive techniques should not be limited to standard dental care approaches and models. It is important to use a strategy that enlists the help of dental, medical, and other health-care professionals. The assessment of public health should include steps of caries prevention in addition to an individualised approach. Children's caregivers play a vital role in avoiding dental caries (Pahel *et al.*, 2011). Multiple strategies have been proposed to treat dental caries. Dental practitioners are responsible for selecting the appropriate preventive measures for each clinical condition based on information from the patient's dental profile, and several measurements may be needed for caries prevention (Frencken *et al.*, 2012). These measurements could include diet therapy, sugar replacements, fluoridated agent, and antimicrobial agents.

Diet therapy and sugar replacements are an important consideration that may include controlling the formation of dental caries in relation to sugar and other fermentable carbohydrates intake. These dietary control measures are particularly necessary for people who are at high risk of caries and for whom the use of various fluoride agents would not be an alternative (Frencken *et al.*, 2012). There are associations between cariogenic food intake, oral hygiene, saliva availability, and fluoride could be considered to reduce enamel dentine demineralization caused by cariogenic food consumption. As a result, sugar replacements like xylitol and sorbitol can be used to help minimize cariogenic consumption. Study from Chou *et al.*, (2013) shown that chewing sugar-free gum with a sugar substitute immediately after meals has slowed the development of existing cariogenic lesions (Kuribayashi *et al.*, 2012).

Fluoride is a natural chemical that can be found in water and plants (Petersen and Lennon, 2004). When used systemically by mixing it with water, milk, and salt, or topically by using it as a toothpaste, gel, varnish, or mouthwash by a professional or self-applying, it helps to prevent dental caries (Frencken *et al.*, 2012). Fluoride can be used in the treatment of caries to prevent enamel corrosion and speed up remineralization, as well as having antimicrobial properties at low pH levels (Lynch *et al.*, 2011).

Fluoridated water can be used as treatment in water system among populations with systematic fluoride application (Iheozor-Ejiofor *et al.*, 2015). Moreover, study from Yeung *et al.*, (2005) has reported that fluoridated milk to promote schoolchildren's permanent dentition, and the effect of fluoridated

salt in the prevention of caries in permanent dentition has also been demonstrated (Yeung *et al.*, 2005; Stangvaltaite-Mouhat *et al.*, 2021). Fluoride also added through toothpaste, mouth rinses, gels, and fluoridated varnishes are effective in preventing caries, regardless of whether another fluoride route has been used at the same time (Marinho *et al.*, 2003; Walsh *et al.*, 2009). In addition, fluoride types such as stannous fluoride and amine fluoride have demonstrated antimicrobial activity. When targeting for dental plaque, a mixture of fluoride with insoluble calcium compound, arginine in toothpaste has the potential to minimize biofilm pathological factors and thus avoid the onset of the caries phase (Gaffar *et al.*, 1997). Study from Cummins, (2013) revealed that tooth brushing does not eliminate all the biofilms that has formed on surfaces.

Another way of treating dental caries is to use antimicrobial agents in different forms. Antibiotics are the most common agents used to treat and prevent infections caused by bacteria. While investigating the nature of tooth decay, Ruiz-Linares *et al.*, evaluated that antimicrobials such as alexidine (ALX), chlorhexidine (CHX) and cetrimide (CTR) reduce plaque which aimed to control dental caries (Ruiz-Linares *et al.*, 2014). ALX and CHX are bisbiguanide disinfectants previously used as a mouth wash and contact lens solution. Therefore, they were evaluated for endodontic treatment and it was found that 1% and 2% concentration of ALX lead to eradication of *Enterococcus faecalis* biofilm (Kim *et al.*, 2013). However, Borges *et al.*, (2012) reported that 2% of CHX effectively reduced total bacterial count of *S. mutans* and *lactobacilli* (Borges *et al.*, 2012). Besides that, CTR is cationic

surfactant demonstrated its effectiveness against Gram-positive and Gram-negative bacteria. Moreover, Baca *et al.*, (2011) emphasised that only 0.2% of concentration of CTR are used to give effectiveness in endodontics.

1.3.2.1 Chlorhexidine (CHX)

Chlorhexidine (CHX) is an antiseptic agent widely used in mouth rinses and ophthalmic solutions. CHX can be characterized as a strong cationic base. It is available as a white or yellowish material in both free-base and stable salt forms. There are several salt forms which include CHX diacetate (CHA), CHX dihydrochloride (CHCl), CHX digluconate (CHDG), CHX gluconate (CHG) and CHX phosphanilate (CHP). These solutions are colourless, odourless and have an extremely bitter taste. In healthcare or commercial use, CHG is one of the most common used forms of the CHX salts due to its ability to dissolve in water and deliver the molecule in an effective way (Zeng *et al.*, 2009). [Figure 1.5](#) show a chemical structure of CHX.

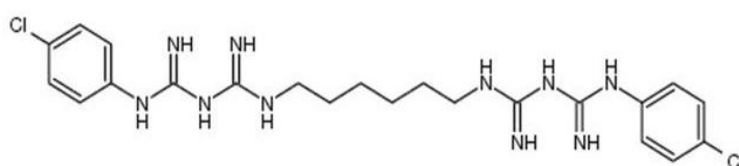


Figure 1.5: Chemical structure of CHX. Adapted from Fisherscie.se

1.3.2.2 Mechanism of CHX

CHX is a broad-spectrum biocide for bacteria that works against both Gram positive and Gram-negative bacteria and fungi. Because of the higher killing rate, CHX inactivates a wider-spectrum of microorganisms in comparison

with other antimicrobials, like antibiotics. While killing bacteria, in terms of mechanism of action, based on the concentration used, it is both bacteriostatic and bactericidal. By breaking up the cell membrane through disrupting cell membrane and subsequent leakage of cytoplasmic components, CHX exerts its microbicidal function by killing almost 100% of Gram positive and Gram negative bacteria within 30 seconds during *in vitro* application (Sköld-Larsson *et al.*, 2009). Since CHX formulations can kill most categories of microbes, the risk of developing infections is limited (Ribeiro *et al.*, 2007; Cieplik *et al.*, 2019).

1.3.2.3 Topical Application

The CHX is shown to a unique ability when it applied to the topical application. CHX binds readily to proteins in the skin and mucous membranes, for example, and has a low absorption rate. CHX that has been attached to proteins is slowly released, resulting in an extended action. This is referred to as substantivity, and it enables for extended antibacterial action against a wide range of bacteria and fungi. Sköld-Larsson *et al.*, (2009) studied that CHX's antimicrobial activity has been documented to last at least 48 hours on the skin. Unlike povidone-iodine, CHX is not affected by the presence of body fluids such as blood. When it comes to the topical application, the CHX has a special ability.

1.3.2.4 Oral Application

S. mutans is one of the most common causes of gingivitis in the mouth area. Infection persistence is influenced by the attachment of bacteria to medical

implants and host tissue, as well as the formation of a mature biofilm. The creation of a biofilm, as well as the encasement of cells in a polymer-based matrix, reduces antimicrobial susceptibility and immune defence, giving these infections difficult to eliminate. During infection, cell disperse from the biofilm can result in infection transmission to other sites and development of the infection.

Apel *et al.*, (2013) reported that biofilm might adhere to and on surfaces and interfaces, including the microbial aggregates and floccules. They are adherents in porous media in the biofilm areas. In the treatment of *S. mutans*' biofilm, CHX exposure will detach sensitive planktonic cells and metabolically active cells near the biofilm surface. However, extended cells and restful physiological cells in the biofilm will survive and remain protected from the immune system by the biofilm matrix. Treatment with this CHX, will improve the efficiency of antibiotic penetration and facilitates clearance. After matrix decomposition the specific antibiotic tolerant will survive the cells, then responsive antibiotic cells in biofilm are attributed and destructed (Wang & Ren, 2017).

In addition, CHX, stannous fluoride, a mix of essential oils and triclosan are among the control agents for gingivitis and supra-gingivitis. They are found to be beneficial under the recommended conditions of use. However, the most potent chemotherapeutic agent currently available is CHX. Although CHX has demonstrated short-term usefulness in situations such as postoperative plaque control, where mechanical cleaning is impaired or impossible long-term maintenance of gingival health is limited by side effects, primarily

staining and taste. The other agents listed above are clearly less effective than CHX in the prevention and treatment of gingivitis.

Improvement of oral health can influence by the life of quality people, so new prevention and treatment methods has been developed and produced which are safe, effective, and economical. One of the method is well established is mouthwash that could inhibit dental plaque and are widely used to maintain oral hygiene. However, Hasturk (2004), mentioned that mouthwashes are non-sterile aqueous solutions. They are used for reducing oral bacteria, cleaning food remnants and for decreasing oral malodour Since many people cannot remove dental plaque properly and mechanical plaque control alone is not enough, chemical plaque control measures such as mouthwash can be suggested. However, for this project a solid dosage form such as a lozenge tablet is proposed for antimicrobial drug release.

1.4 Formulation of lozenges for oral delivery

Lozenges are solid preparations that contain one or more medicaments, usually in a sweetened base, that are intended to be sucked and held in the mouth. They are easy to handle, the dose is apportioned, and the excipients have a demulcent effect on the throat since they are released slowly and spread uniformly over the affected mucosal membrane (Bader *et al.*, 2013). Lozenges are generally formulated to relieve oropharyngeal conditions caused by local infection. Historically, they have been used for the relief of minor throat pain and irritation and as topical anaesthetics, and as antibacterial agents (Codds *et al.*, 1998). Fungilin lozenges are formulated to

treat infection in the mouth, throat or tongue caused by yeast. Similarly, Flurbiprofen lozenges are used to relieve pain and inflammation in sore throat. Lozenges are used for patients who have difficulty swallowing of solid oral dosage forms (Pattanayak & Das, 2012). They are also intended to achieve local effect, releasing medicaments slowly to yield a constant level of drug in the oral cavity. Lozenges can also be employed for systemic delivery provided the drug is well absorbed through the mucosal and buccal linings in the oral cavity (Tietz *et al.*, 2018). For example, nicotine lozenges are used for helping smokers to quit smoking. It is a smoking deterrent and works by providing low systemic levels of nicotine, which may help quit smoking by lessening the physical symptoms of withdrawal. Furthermore, lozenges provide numerous benefits to formulation pharmacists. These are administered easily to the geriatric and child-care patients due to pleasant taste. Lozenges tablet also have extends time for drug to produce the in the oral cavity at specific effect. They could be readily prepared, with minimum equipment and time which do not require an intake of water for administration. Besides that, lozenges have some disadvantages. Lozenges tablets could be a mistaken by children due to taste and appearance. So that, lozenges should keep out of reach from them (Mulholland & Chang, 2009).

There is an approved guidance on how to establish Bioequivalent (BE) in solid dosage forms administered for local action in the oral cavity. In general, very limited information is available *in-vitro* dissolution studies for lozenges tablet products. Most international pharmacopoeias do not contain

information about how drug releases of lozenges can be determined. The USP lists 5 individual lozenge monographs, but the method of dissolution is only described in 3 of the monographs (2 lozenge with local action and one lozenge with systemic action). Therefore, database of three additional dissolution testing methods for systemically lozenges is listed in the FDA recommended dissolution methods ([Table 1.2](#)) adapted from Tietz *et al.*, (2018).

Table 1.2: USP- listed monographs for lozenges. Table adapter from (Tietz *et al.*, 2018)

Drug name	Dosage form	Mode of action	USP Apparatus	Speed (rpm)	Medium	Volume (ml)	Recommended sampling times (mins)
Calcium carbonate	Lozenges	Systemic	-	-	-	-	-
Menthol	Lozenges	Local	-	-	-	-	-
Zinc and Vitamin C	Lozenges	Systemic	II (paddle)	75	0.1 N hydrochloric acid	900	60
Nystatin	Lozenges	Local	Disintegration	29 – 32	Water	Not specified	90
Clotrimazole	Lozenges	Local	II (basket)	50	0.1 N hydrochloric acid, deaerated	500	45

1.4.1 Type of lozenges

Lozenges can be produced in various shapes with varying hardness. These solid dosage forms contain a medicinal agent and a flavouring substance, intended to be dissolved slowly in the oral cavity for localized or systemic effect. They are also called troches or pastilles. Lozenges are designed to dissolve slowly in the mouth without disintegration. Their ingredients should be stable with heat. There are several types of lozenges such as hard, soft, and chewable lozenges (Pothu & Yamsani, 2014).

1.4.1.1 Hard Lozenges

Hard lozenges were prepared by heating and congealing technique are made from mixtures of sugars (sucrose, dextrose, maltose, and lactose) and other carbohydrates. These lozenges require high temperature (145°C) to melt the sugar base. Many lozenges have hard bases of sugar and syrup and often incorporate adhesive such acacia gum. Additionally, citric acid, tartaric acid, malic acid or fumaric acid are added in the formulation to strengthen the sugar base (Pothu & Yamsani, 2014). The melted mixed mass is poured into pre-lubricated mould to get required shape and uniform sizes. Hard lozenges offer slow, uniform drug dissolution or erosion over 5 to 10 minutes, do not disintegrate, have a smooth surface texture and pleasant flavour for masking the drug taste. A primary disadvantage of hard candy lozenges is the high temperature required for their preparation. Hard lozenges eventually become grainy but the speed at which this occurs is dependent upon the excipients that are used. The incorporation of corn syrup solids at a concentration greater than

50% decreases the grainy texture but can increase moisture absorption (hygroscopicity) which increases product stickiness and excipient incompatibility during storage.

If the active ingredient or excipients for hard lozenges are thermo labile, direct compression method could be the alternative to prepare lozenges (Pundir & Verma, 2014). In addition, direct compression and wet granulation can be used to produce compressed tablet lozenges. The excipients are thoroughly mixed and then condensed when compressed directly. However, for wet granulation, the sugar mixtures are grinded to a fine powder then use wet granulation method by mechanical concentrations (40-80 mesh size) (Ficzere *et al.*, 2021). The drug is added and carefully mixed. The blended excipients are then screened through a 2-8 mesh screen. After that, the product is dried and milled to a size of lozenges desired. Prior to direct compression, flavour and lubricant will be added after all excipients are blended. The direct compressed lozenges are made by using different compression forces. Compressed lozenges tablet have content vehicle with sugar such as dextrose, sucrose. In additional of sugar free vehicle for e.g., xylitol, mannitol, sorbitol, polyethylene glycol (PEG) 6000 and 8000 can be used. Besides that, other filler such as calcium phosphate, calcium sulphate, calcium carbonate, lactose, microcrystalline cellulose can also be incorporated (Rapaille *et al.*, 2003).

These lozenge tablets are generally formulated in different sizes (between 5mm to 12.5 mm, flat-faced, weight between 100 to 700 mg). The tablets have high hardness between 2 to 15 kp. These physical attributes lead to ease of use in the mouth and contribute to the desired slow dissolution. The formulation

factors primarily responsible for controlling dissolution, hardness, and mouth feel are the presence of a high strength, dissolution retarding binder and the absence of a disintegrating agent (Macek *et al.*, 2012).

1.4.1.2 Soft lozenges

Soft lozenges provide a slow release to the mouth during chewing. The soft lozenges are prepared by melt and mould techniques. The fillers are made from PEG 1000 or 1450 and contain acacia and silica gel in some soft candy products. Acacia is used to ensure texture and smoothness while silica gel is used as a suspending agent to prevent materials being moved into the bottom of the mould during cooling (Yadav *et al.*, 2015). The formulation involves a 50°C heating process, making it only suitable for heat-resistant ingredients. The moisture content and weight of soft lozenge should be in between, 0.5 - 1.5% and 1.5-4.5 g respectively. These should undergo a slow and uniform dissolution or erosion over 5- 10 minutes and they should not disintegrate (Batheja *et al.*, 2006).

1.4.1.3 Chewable Lozenges

The chewable lozenges are made of gelatine that has been glycerinated. The gelatine base for these chewable lozenges is identical to glycerine suppositories, which are made up of 70% glycerine, 20% gelatine, and 10% filtered water. These are chewable tablets that deliver the substance to the gastrointestinal tract for systemic absorption. They also have a strong flavour with slightly acidic. The melted excipients were poured into moulds or onto a consistent thickness sheet to make these chewable. These lozenges tablets

have several advantages, including drug administration in the oral cavity, which makes it easier for patients who have trouble swallowing medications. Apart from that, lozenges are simple to provide to both children and the elderly (Ramadhany *et al.*, 2020). Furthermore, chewable lozenges are a great technique of giving medication products due to the taste of the drug may frequently be well masked, *e.g.*, with fruit-flavoured. They have a nice flavour and will keep a medication in the mouth cavity for longer, eliciting local activity (Modyala *et al.*, 2014).

1.4.2 Excipients used in the formulation of lozenges

Excipients are the building blocks used to formulate drug delivery systems and provide certain functionalities for the drug to maintain its efficacy, stability, and safety (Pifferi *et al.*, 1999). The recent developments in direct compression of tablets involved the introduction of excipients especially modified by physical or chemical methods to achieve desired target product profiles such as fast disintegration in the mouth, good mechanical strength, and acceptable friability levels (Pothu & Yamsani, 2014). However, a wide range of excipients have been used in formulating lozenges tablets. The emphasis of this work is on preparation of lozenges tablet is in [Table 1.3](#). summarizes a list of excipients widely employed in the production of lozenges using compressed and melted techniques, along with their roles and advantages and disadvantages. All excipients are compatible with pre-compaction techniques such as direct compression, granulation, and moulding.

Table 1.3: Selection of excipients that used in production of lozenges tablets.

Excipient	Role and other information	Advantages	Disadvantages
Aspartame	Sweetening agent, poor solubility in water	For masking taste	Incompatible with magnesium stearate and alcohol
Calcium carbonate	Binder, Tasteless, water soluble	Stable and non- toxic	Affect the absorption of other medications in the gastrointestinal tract
Calcium phosphate	Diluent, insoluble in ethanol and water	Good flowability and compaction properties,	Capping at high compaction forces, affect the other medication (API and excipients)
Citric acid	Effervescent, flavour enhancer, acidic taste	Safe to use	None
HPMC	Binder, solubilising agent, hygroscopic, soluble in cold water	Stable in any temperature	Incompatible with oxidising agents
Lactose	Binder, diluent, odourless	Good flowability	Not suitable for lactose intolerant
Liquid glucose	Sweetener, taste masking	Preventing from crystallization in sucrose and dextrose	Not suitable for diabetic, obesity
Magnesium stearate	Lubricant	Stable	Incompatible with strong acids or alkalis
PVP	Binder, soluble in water, adhesive agent, hygroscopic	Good flowability	None
Starch	Binder, disintegrant, diluent, expand immediately when contact with water	Act as anti-lubricant	Not suitable for celiac
Silicon dioxide	Disintegrant, absorbent, insoluble in organic solvents, water, and acid	Good flowability	None
Sucrose	Sweetener, diluent, hygroscopic	Very sweet, good flowability	Incompatible with acids or alkalis
Tartaric acid	Effervescent, acidic taste, odourless	Improve intestinal function	Cause paralysis if exceed dose
Xylitol	Sweetener, masking agent,	Good stability, cooling sensation	Hygroscopic and incorporate with oxidising agent

1.5 Aim and objectives

Aim: To formulate a targeted dosage form of an antimicrobial agent (CHX) with minimal excipient load for the effective treatment of *S. mutans* infections in the oral cavity

The objectives of this study:

- 1- To screen, evaluate and optimize the concentration of excipient for antimicrobial and anti-biofilm activity.
- 2- To evaluate the effect of sugars and polyols on the growth of *S. mutans* in planktonic state under aerobic and anaerobic conditions.
- 3- To investigate and optimise biofilm formation in *S. mutans* NTCC 10499 under aerobic and anaerobic conditions and to evaluate the effect of sugars and polyols on biofilm inhibition and dispersal.
- 5- To characterise biofilms and EPS using imaging methods such as Confocal Laser Scanning Microscopy (CLSM), and to study differential gene expression during biofilm formation and upon exposure to selected sugars and polyols.
- 6- Screening and characterisation of selected sugars and polyols by physicochemical techniques and methods for formulation of lozenges.
- 7- To develop, optimise and characterise directly compressed lozenges using multi-functional excipients.

CHAPTER TWO

**Evaluation of the antimicrobial activity of chlorhexidine in
controlling *Streptococcus mutans* in planktonic state**

2.1 Introduction

CHX is an antimicrobial agent used in dental practice to control plaque and biofilm formation. The clinicians often recommend the application of CHX to inhibit the development of plaque, which is a common treatment for human dental caries (Van Strydonck *et al.*, 2012). CHX influences the metabolic action of bacteria by acting as a bacteriostatic agent at low concentrations, causing both, changes in cell membrane function and leakage of intracellular contents and serving as a bactericidal agent at high concentrations by triggering irreversible cellular precipitation (James *et al.*, 2017).

S. mutans is the main cause of human dental caries, mainly due to its ability to adhere to dental surfaces and to survive in acidic environment (Zeng and Burne, 2013). These bacteria produce three enzymes which are known as glucosyltransferases (*gtfB*, *gtfC*, and *gtfD*) which synthesise soluble and insoluble glucan polymers from sugar i.e., sucrose (Lemos *et al.*, 2019; Huang *et al.*, 2021). The action of glycosyltransferases which helps in accumulation of bacteria on the dental surface was found to be inhibited by CHX and it also influences transport of sugar and production of acid in oral bacteria. Moreover, sugar enhances the colonization of cariogenic bacteria and promotes the formation of dental plaque on tooth surfaces. Therefore, when CHX disrupts the cell wall of *S. mutans*, it changes the membrane's extracellular composition and inhibits plaque formation (Lemos *et al.*, 2019). Although studies have already proved that CHX has been effective in plaque control, few studies have focused on structural or molecular changes that

may occur with minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) concentration in cells and the biofilm matrix (Gunsolley, 2006; Uzer Celik *et al.*, 2016; Haghgoo *et al.*, 2017) particularly in the presence of sugars and polyols. MIC and MLC were determined against planktonic forms so that the concentration required for eradication of biofilm can be estimated.

The aim of this chapter was to investigate the antimicrobial effects of CHX on *S. mutans* planktonic cells. In addition, the effects of sugars and polyols on the activity of CHX were investigated to check the feasibility of their incorporation into CHX formulations. This work was carried out to mainly find out if sugars and polyols interfered with CHX or if they act synergistically.

2.2 Materials

2.2.1 Microbial strains and blood

S. mutans control strain (NTCC 10449) was obtained from University of Wolverhampton microbiology library laboratory strain collection.

Defibrinated horse blood was purchased from TCS Biosciences Ltd (UK) and stored at 4°C.

2.2.2 Microbiological and cell culture media

BHI agar and BHI broth were obtained from Lab M (UK). Muller Hinton broth (MHB), Tryptone Soy broth (TSB) were also purchased from Lab M (UK).

Table 2.1 show composition of media that used in the experiment.

Table 2.1:Composition of media used in the experiments.

Type of Media/ Broth	Chemical/Composition	Quantity (g/l)	Company
BHI broth	Brain heart infusion mixture	17.5	Lab M, UK
	Tryptose	10.0	Lab M, UK
	Glucose	2.0	Lab M, UK
	Sodium chloride	5.0	Lab M, UK
	Disodium hydrogen phosphate	2.5	Lab M, UK
TSB	Soy Peptone	5	Sigma-Aldrich, UK
	Casein peptone	15	Sigma-Aldrich, UK
	NaCl	5	Sigma-Aldrich, UK
MHB	Beef Extract	2	Sigma-Aldrich, UK
	Acid Hydrolysate of Casein	17.5	Sigma-Aldrich, UK
	Starch	1.5	Sigma-Aldrich, UK

All media were sterilised prior to experimental use.

2.2.3 Materials and chemicals

Chlorhexidine and xylitol were purchased from Sigma Aldrich (UK). Lactose, mannitol, sorbitol were free gift from Azelis (UK). Whereas sucrose, fructose maltodextrin were free sample from Sheffield Bioscience (UK).

Whereas disposable items such as sterile plastic petri dishes plates and 96 well micro-titre plate were purchased from Sarstedt (UK). 2-micron Millipore membrane filters were bought from Merck Millipore Ltd. (Ireland). Honeycombs well plates were purchased from Thermo Fisher Scientific (UK) for growth experiments using Bioscreen C spectrophotometric plate reader, Thermo LabSystems (Finland). Bioscreen C is an automated turbidity plate reader linked to an integrated PC, manufactured by LabSystems, Helsinki, Finland. The system can measure changes in the turbidity of up to 200 microtitre wells (2 × 100 multiwell plates) and can provide growth curves from each well either directly to the monitor or in the form of a data file suitable for further examination (Lambert *et al.*, 1998).

2.3 Methods

2.3.1 Preparation of microbial growth media

BHI agar and BHI broth were prepared according to manufacturer's recommendations (see in Appendix A2.1). MHB and TSB media were also prepared according to manufacturer's recommendations. These media were sterilized by autoclaving at 121°C for 15 minutes at 15 psi pressure. BHI agar was cooled to 45°C prior by adding 5% of defibrinated horse blood per 100ml of broth then pouring into sterile plastic petri dishes.

2.3.2 Preparation of microbial cultures

The stock plate was prepared prior to experimental use. Briefly, a vial with *S. mutans* stored at -80°C was thawed and grown on petri dish containing BHI agar plates supplemented with 5% defibrinated horse blood. The plate was then incubated for 72 hours anaerobically under atmosphere of 5% carbon dioxide and 95% oxygen at 37°C. A single bacterial colony was inoculated into 5 ml of BHI broth and cultured in a universal bottle at 37°C for 24 hours in aerobic orbital shakers operating system at 50 rpm (Series 25, New Brunswick Scientific Co. Inc. Edison, New Jersey, U.S.A.).

2.3.3 Preparation of antimicrobial agents

Different concentrations of the antibacterial agent, CHX were prepared from stock solutions at a concentration of 10µg/ml using double sterile distilled water and volumetric flask. This concentrated solution known as stock solution was filtered with sterile disposable filter with a pore size 0.2 µm.

Xylitol solution at 20% concentration was prepared by adding 2g of xylitol into 10ml of BHI media and also filtered with sterile disposable filter with a pore size 0.2 µm.

2.3.4 Preparation of sugars and polyols

Different sugar concentration was prepared for the growth study. Selection of sugars used were sucrose, lactose, fructose, and maltodextrin. In addition, polyols such as mannitol, sorbitol, and xylitol were also used to evaluate their effect on growth. Briefly, 2g of sucrose was dissolved in 10ml

of BHI media and was filtered with sterile disposable filter with a pore size 0.2 μm . The various preparation for these sugars and polyols were prepared by varying the mass and are summarised in the [Table 2.2](#) below.

Table 2.2: Preparation of different concentrations (%w/v) of sugars (fructose, lactose, sucrose, maltodextrin) and polyols solution (mannitol, sorbitol, xylitol).

Percentages of sugars/polyols (%w/v)	Mass (g)	Volume of BHI media (ml)
2	0.2	10
5	0.5	10
10	1.0	10
20	2.0	10
40	4.0	10

2.3.5 Optimization of culture media for the growth of *S. mutans*

To determine the optimum media which would support the growth of *S. mutans*, several media had been explored. These media, identified from literature search, were Muller Hinton broth (MHB), (Deshpande *et al.*, (2014), Tryptone Soy broth (TSB), (Murray, 1979) and BHI broth (BHI) (Woljtes *et al.*, (1981). The isolated bacteria of *S. mutans* were cultured in TSB, MHB and BHI broth at 37°C for 24 hours. The culture then tested for microbial growth through Miles and Misra technique (Miles *et al.*, 1938). Viable counts were also determined on each sample by plating onto MHB, TSA and BHI agars.

2.3.6 Minimum Inhibitory concentration (MIC) and minimum lethal Concentration (MLC)

The MIC is defined as the lowest concentration of an antimicrobial agent that completely inhibits bacterial growth and is determined by the highest dilution at which no visible growth is observed (Uzer Celik *et al.*, 2016).

Whereas MLC is defined as the lowest concentration of an antimicrobial agent that kills 99.9% of the initial inoculum in 24 hours using a plate count of viable cells at 37°C (Liu, *et al.*, 2012). The antimicrobial agent CHX was diluted in a series of half-fold dilutions from its stock solution (from 10µg/ml to 0.078 µg/ml) to obtain the working concentrations.

An overnight culture *S. mutans* strain (NTCC 10449) was grown at 37°C in BHI broth and initial optical density at 600nm (OD₆₀₀) was immediately measured using a spectrophotometer (Cecil 1000 Series, Cecil Instruments, Cambridge, United Kingdom). The overnight cultures corresponding approximately to 1x 10⁹ colony forming units/ml (CFU/mL) were diluted 100-fold to get 1x10⁷ CFU/mL. Briefly, 0.5ml of bacterial culture were added into 4.5ml sterile clean test tube with contain BHI broth and mix with vortex which was labelled as 1(i). Then aseptically 0.5ml from 1(i) was taken into a new sterile clean test tube which contains 4.5ml BHI broth and this test tube was labelled as 2(i). Test tube 2(i) is designated as the working inoculum which give 1 x 10⁷ CFU/mL.

Ten test tubes were prepared with 5ml of BHI broth then labelled as 1 to 10. After that, 5 ml of drug solution of CHX at 10µg/ml concentration was added into first test tube and diluted in series in test tubes 1 to 8. Then 0.1ml of working inoculum from test tube 2(i) is added into test tube until test tube 9. Each dilution had final volume of 10 ml. The diluted inoculum was at a final concentration of between 1x10⁵ to 1x10⁶ (CFU)/mL per tube. Tubes were incubated for 24 hours at 37°C and inspected for visible growth (turbidity).

The MIC was taken to be the lowest concentration along the series with no visible growth. Samples from concentrations with no visible growth were plated on BHI agar media and the MLC was determined as the lowest concentration showing no visible colony formation after incubation for 24 hours at 37°C. All tubes in the test series were plated to determine the MLC through Miles and Misra technique. All these determinations represent the mean of three independent experiments i.e., biological replicates, (n=3). MIC and MLC values determined this way were used as guideline concentrations for the subsequent time kill studies and determination of synergism between the combined agents.

2.3.7 Checkerboard assay, micro dilution and time kill study

BHI broth and BHI agar were prepared according to the manufacturer's instructions. The antimicrobial agents, CHX and xylitol were prepared at concentration 10 µg/ml and 20% Xylitol respectively as described in section 2.3.3. *S. mutans* overnight cultures were prepared and diluted to 1×10^7 cells/ml. The MIC and MLC were determined by use of the broth dilution method section 2.3.6, whilst the checkerboard assay using 96 microtiter plates. The stepwise protocol for the checkerboard assay is summarized in the [Table 2.3](#) below. However, [Table 2.4](#) was shown to determine synergistic action of the antimicrobial agents.

Table 2.3: Method of checkerboard assay using 96 microtiter plate.



<p>Filled columns 2 up to column 9, except row A, with sterile BHI (150µL) by using an eight-channel pipette.</p>
<p>Filled row A, from column 2 up to column 8 with 300µL of the prepared CHX solution and consequently added 150µL of the prepared solution to 150µL of sterile BHI and serially diluted from B to H using the eight-channel pipette. After serially dilution, disposed/discarded the final 150µL of each well in row H.</p>
<p>Filled column 1, up to row H with 300µL of the prepared second agent (Xylitol) and serially diluted up to column 8 (follow to step 3) and disposed the final 150µL of each well in column 8.</p>
<p>Inoculated all the wells with 10µL of the working stock inoculum of <i>S. mutans</i>.</p>
<p>To prepare a positive control, in the same plate, wells were filled with sterile BHI alone on column 10,11 and 12. Added 10µl of antimicrobial agent on column 10 and 11. Both column 10 (CHX) and 11 (XYL) are drug controls. Added 10 µl the working stock inoculum of <i>S. mutans</i> on column 12. Column 9 would be negative control, because it has filled wells with sterile BHI alone. Both controls demonstrated microbial growth and media sterility, respectively.</p>
<p>Plated all clear wells (an aliquot of 20µl) on sterile BHI Petri dishes to demonstrate the bactericidal effect of the antimicrobial agents combined</p>

Table 2.4: Checkerboard assay synergistic action of the antimicrobial agent.

	1	2	3	4	5	6	7	8	9	10	11	12
A									BHI	BHI CHX	BHI XYL	BHI S.M
B		X	X	X	X	X	X	X	DISCARD			X
C		X	X	X	X	X	X	X	DISCARD			X
D		X	X	X	X	X	X	X	DISCARD			X
E		X	X	X	X	X	X	X	DISCARD			X
F		X	X	X	X	X	X	X	DISCARD			X
G		X	X	X	X	X	X	X	DISCARD			X
H		X	X	X	X	X	X	X	DISCARD			X

Legend:

DISCARD

	Sterile BHI 150µl
	10 µg/ml of CHX 300µl
	20% Xylitol 300µl
	1 st serial dilution of CHX across the entire row (except column 1)
	2 nd serial dilution for 40% Xylitol across the entire column
X	Inoculate with <i>S. mutans</i> 20µl

The two-dimensional microdilution analyses with 96 micro-titre wells plate was to evaluate the synergy if any, between CHX and Xylitol. This aim to assess the effect of the combination to be synergistic, indifferent, or antagonistic. Fractional inhibitory concentration index (FICI) was calculated. The following formulas, Eq1, Eq2 and were used to calculate the FICI of a combination (Karpanen *et al.*, 2008; Low *et al.*, 2011).

$$FIC \text{ of compound A} = \frac{(MIC \text{ of compound A in combination})}{(MIC \text{ of compound A alone})} \quad \text{Eq 1}$$

$$FIC \text{ of compound B} = \frac{(MIC \text{ of compound B in combination})}{(MIC \text{ of compound B alone})} \quad \text{Eq 2}$$

$$FICI = \sum FIC \text{ of compound A} + FIC \text{ of compound B} \quad \text{Eq 3}$$

Synergy was defined as FLCI index value of ≤ 0.5 . **Indifference or absence** of interaction was defined as FICI value >0.5 but <4 . **Antagonism** was defined as an FICI of >4 (Hasan *et al.*, 2014).

Time Kill study

The time kill study involved conical flasks set up containing 200 ml of sterile BHI and 5 ml of *S. mutans* inoculum of 1×10^7 CFU/mL colony forming units (CFU)/mL culture as control. The concentration of Xylitol of 40% and CHX at 10 $\mu\text{g/ml}$ were chosen as stocks concentration. The flasks were then incubated at 37°C in orbital shakers, and samples were collected at time point 12, 14, 16, 18, 20, 36 and 38 hours. At each sample time a 50 μl aliquot was withdrawn from each conical flask then replaced with fresh broth. A 10-fold dilutions were prepared in BHI. A 20 μl of the diluted sample was then plated onto BHI agar plates. These plates were incubated at 37°C for 24

hours, and colony counts were determined. Synergy also defined as a ≥ 100 -fold or 2-log_{10} reduction in colony count at 24 hours of incubation by the combination compared to single agent/compound showing maximum activity and as a ≥ 100 -fold decrease in colony count compared with the starting inoculum. Contrastingly, indifference (or additivity) was defined as a < 10 -fold change in colony count at 24 hours of incubation by the combination compared with the most active agent/compound alone. Antagonism was defined as a ≥ 100 -fold increment in colony count at 24 hours of incubation by the combination compared with that by the most active agent/compound (Hasan *et al.*, 2014).

2.3.8 Growth study of *S. mutans* in anaerobic and aerobic state at planktonic stage

This study was designed to assess the phenotypic impact of the presence and absence of oxygen on *S. mutans* growth. This will improve understanding of how growth is affected under aerobic and anaerobic conditions and its impact on the formation of pathogenic biofilms. The overnight cultures of *S. mutans* were prepared and diluted to 1×10^7 cells/ml. For aerobic growth, 200 μl of the diluted overnight culture and 100 μl BHI broth were prepared aseptically into 100 well honeycomb well plates (Thermo Fisher Scientific, UK). However, for anaerobic growth, the sample of honeycomb wells needed to be overlaid with 30 μl mineral oil to prevent any oxygen going into the well. After that, the plate was incubated at 37°C in a Bioscreen C machine (Labsystems, Thermo Fisher Scientific, Finland) for 72 hours. Readings were taken in every 5 hours up to 72 hours. The

samples were prepared in triplicates (n=3) for optimisation. The growth study was carried out at different concentrations of sugars and polyols, (2, 5, 10, 20 and 40%w/v) which were added to BHI broth. The samples were prepared in triplicates (n=3) for optimisation and reproducibility.

2.4 Results and discussion

2.4.1 Optimization of culture media and growth condition for *S. mutans*

From the observation, both MHB and TSB media did not support the growth of *S. mutans* both on liquid (broth) and solid media. This was evident from the lack of turbidity in broth cultures after 48 hours as well as no viable cells from the agar plates after 24 hours. Furthermore, MHB and TSB did not provide the correct medium or growth conditions for the strain. Therefore, this bacterium required specific nutrients, proper pH and salt concentration and the right temperature to grow. At lower pH, *S. mutans*' cell is response to acid tolerance during survival specially maturation in biofilm formation. In biofilm formation, the cells are more resistant compared to planktonic in acid stress condition (Welin-Neilands and Svensäter, 2007). The strain of *S. mutans* used in these studies is NCTC 10449, which is classified as a serotype c strain (Kondo *et al.*, 2019).

S. mutans can be categorised into four different serological groups based on the rhamnose-glucose cell surface polysaccharide composition. The strains

are isolated from 75% dental plaque isolate known as strain c, 20% serotypes e, and remaining 5% serotypes f and k (Lemos *et al.*, 2019). Most of the published studies used UA159 strain which is among the common strains for *S. mutans* because this strain is naturally competent and include all of the genes required for competence and quorum sensing (Adjic *et al.*, 2002; Dong *et al.*, 2012; Salvadori *et al.*, 2019; Ricomini Filho, 2019). Dong *et al.*, suggested that the bacteria could produce more bioactive molecules effective against medically and agriculturally important bacteria depending on factors such as culture media, nutrients, oxygen, and temperature. These factors are really important for growing *S. mutans* as these bacteria can be identified as fastidious organisms. Other than *S. mutans*, *Helicobacter pylori* and *Lactobacillus spp* are also examples of fastidious organisms. Therefore, for fastidious organism the media suitable for growth was observed to be BHI media. Whereas MHB and TSB are used for non-fastidious organisms such as *Escherichia coli* and *Staphylococcus spp* (Lagier *et al.*, 2015). Furthermore, these factors enhanced the accuracy and ease of enumeration of *S. mutans* when compared to other media, which significantly supports their growth.

2.4.2 Effect of mineral oil on the growth of *S. mutans* using Spectrophotometry

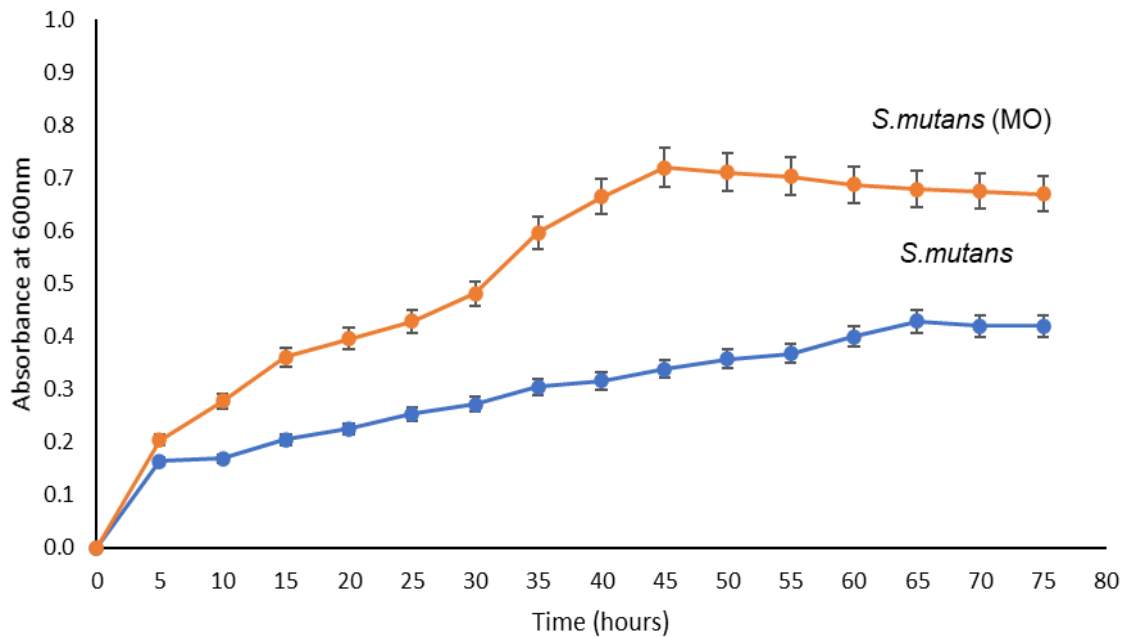


Figure 2.1: Growth Curve for *S. mutans* in the presence (anaerobic) and absence (aerobic) of mineral oil. Culture growth was monitored using the Bioscreen system at 37°C for 72 hours. Absorbance (optical density) at 600 nm was measured every 5h for a duration of 72 hours. Values represent means; n=3. \pm SD, $p < 0.05$

According to the growth curves of Figure 2.1, *S. mutans* growth is shown under aerobic (with oxygen) and anaerobic (without oxygen) conditions. This investigation was carried out by determining the *S. mutans* growth in BHI medium in the presence and absence of mineral oil. To stimulate anaerobic conditions, 30 μ l sterile mineral oil was added on top of the broth culture to trap any oxygen from entering the well. The result revealed growth rates of cells as measured by increases in optical density at 600nm within 72 hours. Meanwhile, anaerobic growth (without oxygen) conditions that has mineral oil overlaid prevents oxygen from entering into the cells and exit of carbon dioxide released by cells from the wells. The anaerobic growth curve shows doubling OD when compared to aerobic condition between 35-50 hours. The growth shows that a long exponential phase and the number of *S. mutans*

cells were increased with increase in time as determined by the optical density. In this experiment, oxygen is a major factor influencing *S. mutans* activity during aerobic and anaerobic conditions (Ahn *et al.*, 2007). As shown in [Figure 2.1](#), the results supported that anaerobic condition is higher when compared to aerobic growth in *S. mutans*. The anaerobic growth promotes the development oral biofilm on clean enamel surface which results in formation of plaques (Yu *et al.*, 2017). Under anaerobic conditions, the biofilm formation is considerably enhanced, and some other factors such as pH, nutrients contribute to the physiological development of cells (Ahn *et al.*, 2007; Ahn *et al.*, 2009). Moreover, study reported by Ahn *et al.*, 2009 revealed that the glycolytic rate was reduced by aeration (aerobic), while the production of intracellular storage polysaccharides was increased. Despite the fact the aeration decreased *S. mutans*' acid tolerance, aerobically grown cells had greater F-ATPase activity (Kühlbrandt, 2019). Enzyme F-ATPase enhances acid production in the growing cells of *S. mutans*. However, aeration altered the architecture of biofilms but did not alter *S. mutans*' ability to interact with salivary agglutinin (Ahn *et al.*, 2009). Beside from that, aerobic growth was slower than anaerobic growth, which could be due to the significant amount of energy required to maintain proper NADH/NAD⁺ balances in the presence of oxygen (Ahn *et al.*, 2020).

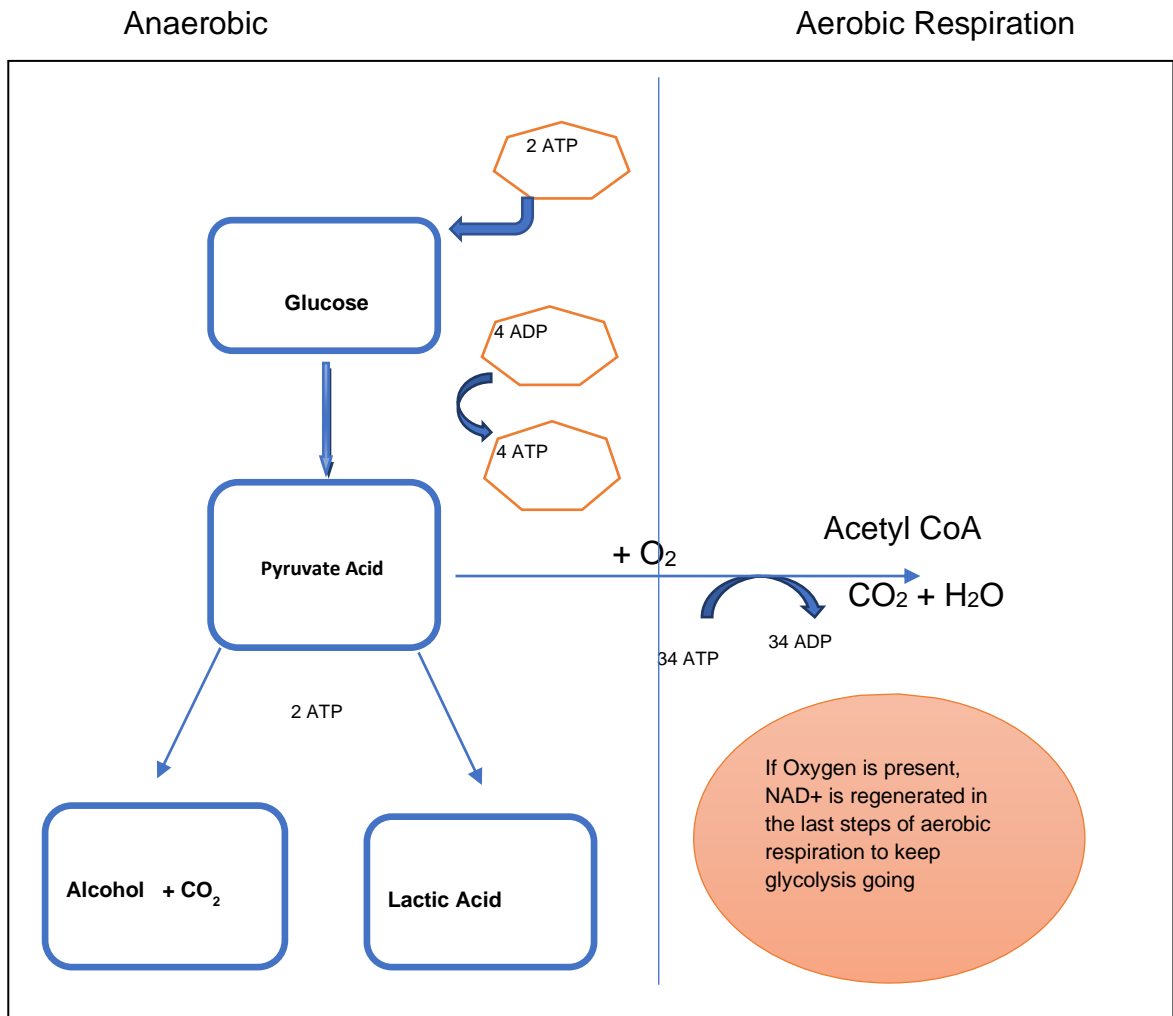


Figure 2.2: Cellular respiration is the process that converts the energy from chemical bonds in food to a form of energy that the cell can use, ATP. Adapted from (Shan *et al.*, 2021).

A study undertaken by Higuchi *et al.*, looked into the removal of oxygen during anaerobic growth in *S. mutans* which mainly involves enzymes NADH oxidases and NADH peroxidases, that oxidizes NADH to NADP⁺ and which transform oxygen and some of its metabolites into water or hydrogen peroxide (Higuchi *et al.*, 1999). The partial tricarboxyl acid (TCA) cycle, identified by genome sequence, has become an important factor for *S. mutans* that grow in aerated conditions to regenerate NADH. Under aerobic conditions, pyruvate is activated by the addition of coenzyme A,

decarboxylated and dehydrogenated, to form acetyl-CoA by a complex of three closely linked enzymes known as the pyruvate dehydrogenase complex (PDH), [Figure 2.2](#). Because end product accumulation highly inhibits the PDH complex, the vast majority of pyruvate produced is simply converted to lactate and eliminated during more with higher organism (excess unoxidized NADH) or aerobic metabolism with normal fatty acid levels (sufficient acetyl CoA levels). This reproduces NAD⁺ and enables glycolysis to take place. For that, kinase-mediated phosphorylation and inactivation of the complex can be reversed by phosphatase-mediated cleavage of pyruvate. Thus, these activation components (the acetyl-CoA/CoASH, NADH/NAD⁺, and ATP/ADP) enable carbohydrate use for inhibiting pyruvate and access to final oxidation in the TCA cycle (Ahn *et al.*, 2009).

S. mutans is also known as a facultative anaerobe, which means it can live in the presence or absence of oxygen. It may survive in the presence of oxygen or ferment in the absence of oxygen with anaerobic respiration. Glycolysis and lactic acid fermentation are the process by which the plaque forms whereby glucose is transformed to pyruvate and lactic acid. It has been shown that the ability of the bacteria to form biofilms on the teeth decreases by 80% in the presence of oxygen under aerobic conditions (Krzysciak *et al.*, 2014).

Similarly, if carbohydrate was diverted away from ATP production, that would result in a decrease in yield for aerobically cultured cells. Therefore,

these results show that *S. mutans* can alter their pathogenic potential dramatically in response to oxygen exposure, suggesting that the organism's phenotype can be highly variable.

2.4.3 Effect of mineral oil on the growth of *S. mutans* with supplementary nutrients sugars and polyols using UV Spectrophotometry

Oxygen has a significant effect on the function of physiological and biochemical pathways in bacteria such as *S. mutans*. From observation, oxygen greatly altered the virulence-related phenotypic properties of *S. mutans*, as a primary etiological agent of human dental caries. One of the examples of phenotypic properties of *S. mutans* is the ability of *S. mutans* to metabolize fermentable sugars (sucrose) and, as a result, the production of various organic acids, which lower the pH of the oral medium. The low pH of the medium causes demineralization of the dental element over time, resulting in the development of dental caries which involves *S. mutans*' growth and processes such as glycolysis (Burne, 1998; Abou Neel *et al.*, 2016). *S. mutans* virulence and pathogenesis in dental caries is due to its capability to withstand low pH. Therefore, *S. mutans* has evolved a number of stress response mechanisms to combat acid stress in the oral cavity. As a result, it can thrive in low pH conditions, giving it an advantage over other bacteria commonly found in the oral environment (Zhu *et al.*, 2017).

The quantitative growth study of *S. mutans* in planktonic state with supplementary nutrients (Figure 2.3 to Figure 2.9) showed high microbial metabolism of sugars and polyols. The growth curve with increase of xylitol concentration from 2% to 40% in aerobic and anaerobic conditions is shown in Figure 2.9.

These figures were similar in observation to the main growth curve (Figure 2.1) as reference. These findings, in addition to the metabolic activity profiles, suggest that *S. mutans* cells at aerobic and anaerobic conditions at similar metabolic stage shows increased growth with increases in percentage of nutrients which is shown by increases of OD. Figure 2.3 until Figure 2.9 shows the OD₆₀₀ of a planktonic *S. mutans* culture supplemented with variable percentage of 2%, 5%, 10%, 20% and 40% of sugars and polyols. These results show that increase in percentage of sugars and polyols would result in an increase in OD values which is in accordance with the literature studies reported by Hulbah *et al.*, and Mäkinen (Hulbah *et al.*, 2021 and Mäkinen, 2011). However, 5% fructose at anaerobic conditions showed decrease in OD at 35 hours compared to aerobic condition. Hence this concentration inhibited the growth. In addition, Hulbah *et al.*, (2021) reported that 2 and 4% of polyols (mannitol and xylitol) inhibited growth. Furthermore, Mäkinen evaluated that polyols are a significant dietary resource in the prevention of dental caries (Mäkinen, 2011).

2.4.3.1 Sugar

Figure 2.3 until Figure 2.6 shows the growth profile of *S. mutans* with different concentrations of sugars (2-40%) under aerobic and anaerobic conditions. The growth enhancement effect was greatest at high concentration (40%) and intermediate at the sugar concentration of 10% compared to other sugar concentrations in the presence of aerobic and anaerobic conditions. Anaerobic conditions were stimulated by the addition of 30 μ l of mineral oil to prevent oxygen from diffusing into the media. However, aerobic cell cultivation has shown the duration of lag phase to be between 5-10 hours in control *S. mutans* (black dotted line) and a substantial decreased in growth rate when compared to that in anaerobic conditions. Overall growth of *S. mutans* was monitored for a total period of 72 hours. Between 40-45 hours most of the *S. mutans* growth plateaus i.e., entered stationary phase. Ahn *et al.*, (2009) reported that doubling periods and the growth rates of *S. mutans* bacteria cells under anaerobic state increased with increase in resulting OD. However, in the presence of different sugars, (fructose, sucrose, lactose, and maltodextrin) there were observed changes in the pattern of growth curve as shown by changes in OD. Therefore, the sugars in general promoted the growth of *S. mutans* in both aerobic and anaerobic development processes compared to control.

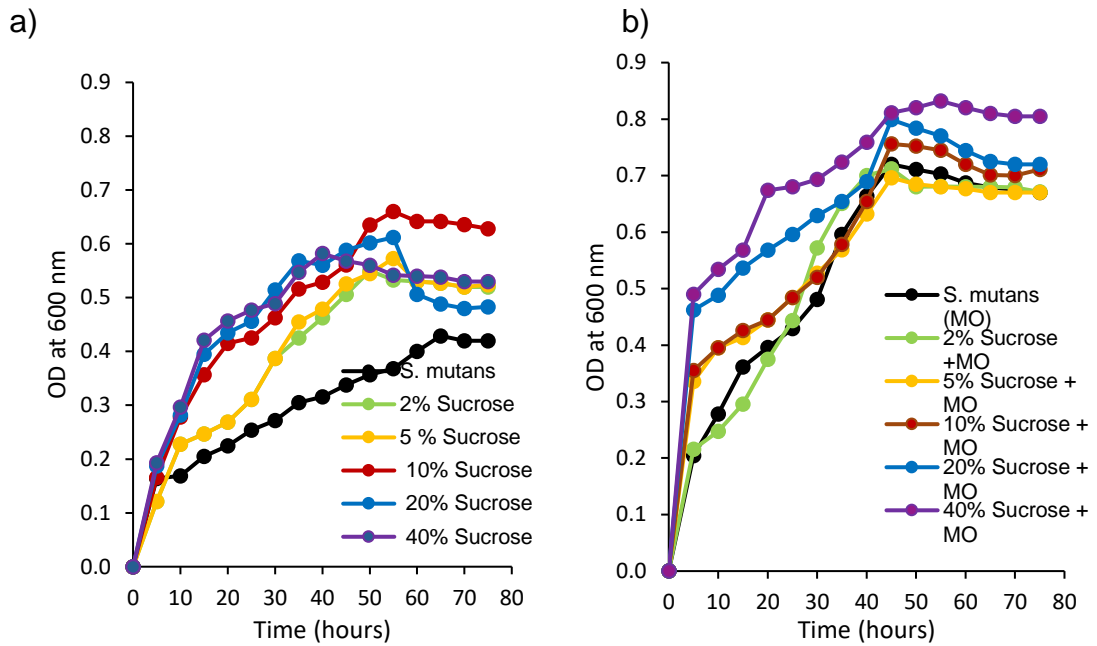


Figure 2.3: Growth curve of *S. mutans* with different concentrations of sucrose (2-40%) under a) aerobic and b) anaerobic conditions measured by monitoring optical density at 600nm for 75h, n=3

Figure 2.3 shows a growth curve for sucrose at aerobic and anaerobic conditions. From 0-5 hours, the growth increases at 2% to 40% concentration of sucrose. This is because the sucrose metabolite the carbohydrate which enhances the growth of bacterial cells of *S.mutans*. 2% Sucrose (green dotted line) has 78% increase in OD when compared to control at aerobic conditions at 5 hours. However, when 2% sucrose at anaerobic (with mineral oil) was compared to *S. mutans* control (black dotted line), at 5 hours the graph shows no difference in OD as both lines were overlapped. At 40% sucrose (purple dotted line) from 0 to 30 hours, there were a big increase in growth when compared to control at both aerobic and anaerobic conditions. The percentage increased in OD to be significant (range 50-60%) for 40% sucrose under anaerobic conditions (0.534 of OD) (with mineral oil) compared to *S. mutans* control (0.278 of OD) (see in

Appendix A2.4). Sucrose is one of the carbohydrate sources from sugar that is found in dietary food. This sugar can easily stick or attach to the tooth surface in the oral cavity. Ahn and Burn, (2007) study revealed that sugar easily binds to salivary pellicles by colonized *S. mutans* bacteria cell on the teeth (Ahn and Burn, 2007). Therefore, with increasing amounts of sucrose in the growth media from 2% to 40% between 5-30 hours under anaerobic conditions, the curve shows doubling growth as shown by increase in the OD.

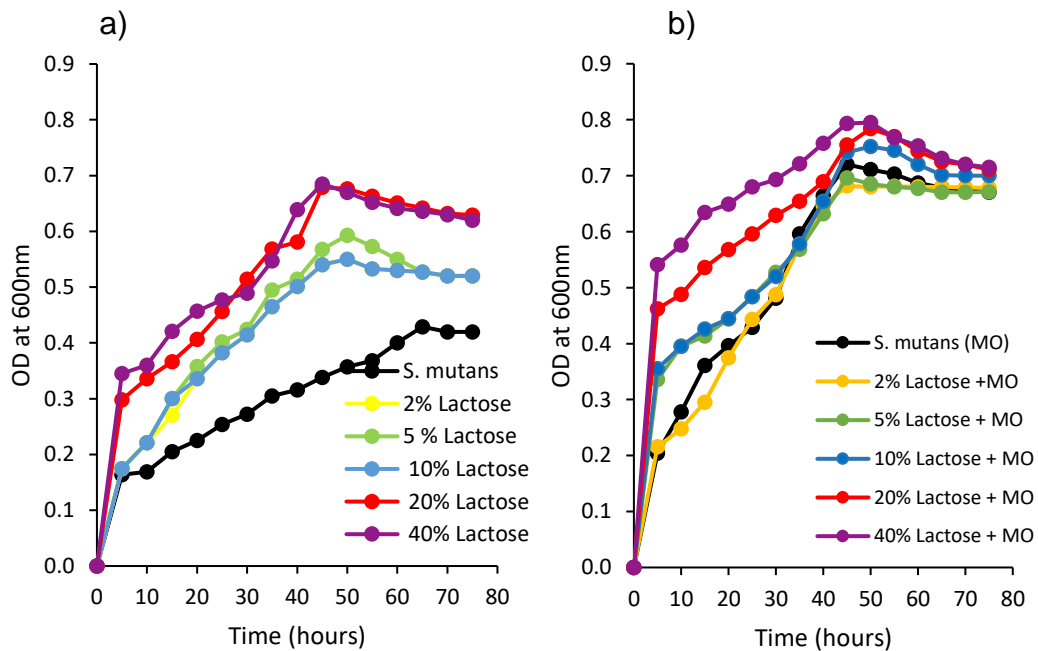


Figure 2.4: Growth curve of *S. mutans* with different concentrations of Lactose (2-40%) under a) aerobic and b) anaerobic conditions measured by monitoring optical density at 600nm for 75h, n=3

Growth curve for lactose in Figure 2.4 indicates that an increase in concentration of lactose from 2% to 40%, enhances growth of *S. mutans* in both aerobic and anaerobic conditions. The growth in 20% lactose source (red dotted line) has showed that the highest growth in aerobic condition compared to *S. mutans* control (black dotted line). However, under

anaerobic conditions, 40% lactose (purple dotted line) showed the highest growth of *S. mutans*.

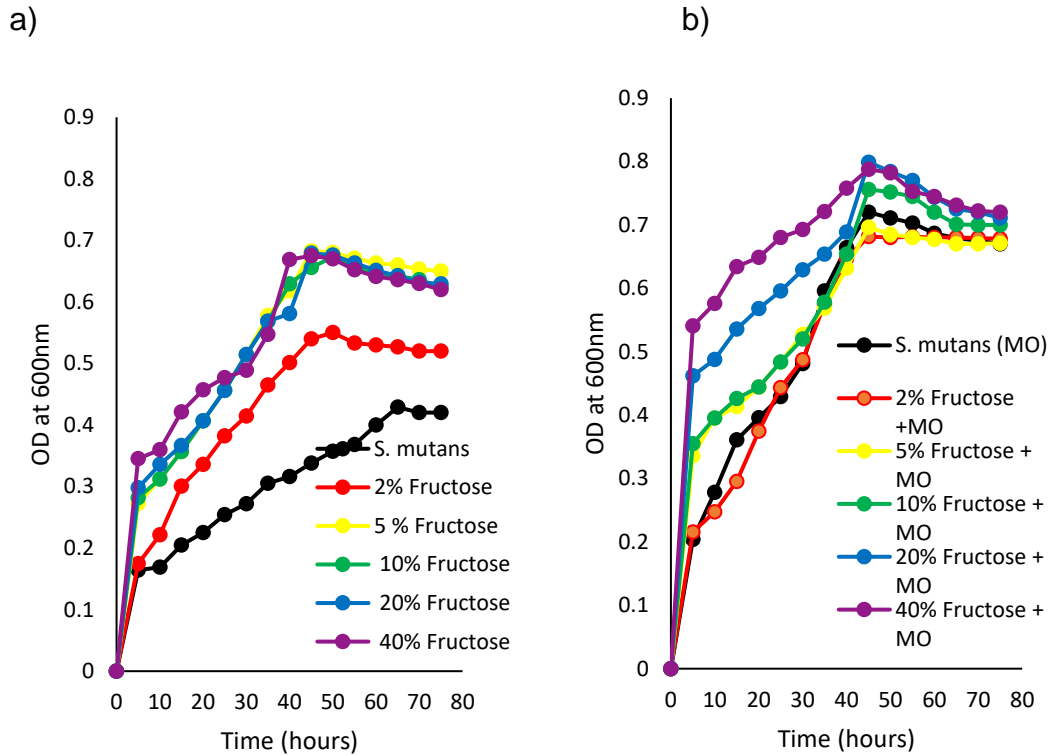


Figure 2.5: Growth curve of *S. mutans* with different concentrations of Fructose (2-40%) under a) aerobic and b) anaerobic conditions measured by monitoring optical density at 600nm for 75h, n=3

Figure 2.5 shows growth curve for fructose in both conditions. In aerobic condition 5% fructose (yellow dotted line) showed the highest concentration that gave high OD values compared to *S. mutans* control. However, in anaerobic conditions, 40% fructose (purple dotted line) showed the highest concentration compared to 40% fructose in aerobic and anaerobic conditions which gave highest OD compared to *S. mutans* control. There was increase in OD for each concentration of fructose with increase in time. However, at 35 hours, there was 2% reduction in growth for 5% fructose (yellow dotted line) between aerobic and anaerobic conditions (as shows in Appendix A2.6). This could be due to entry into stationary phase when

growth rate of *S. mutans* cell become equal or death rate. In addition, sugars at all concentrations promoted growth of *S. mutans* when compared to control.

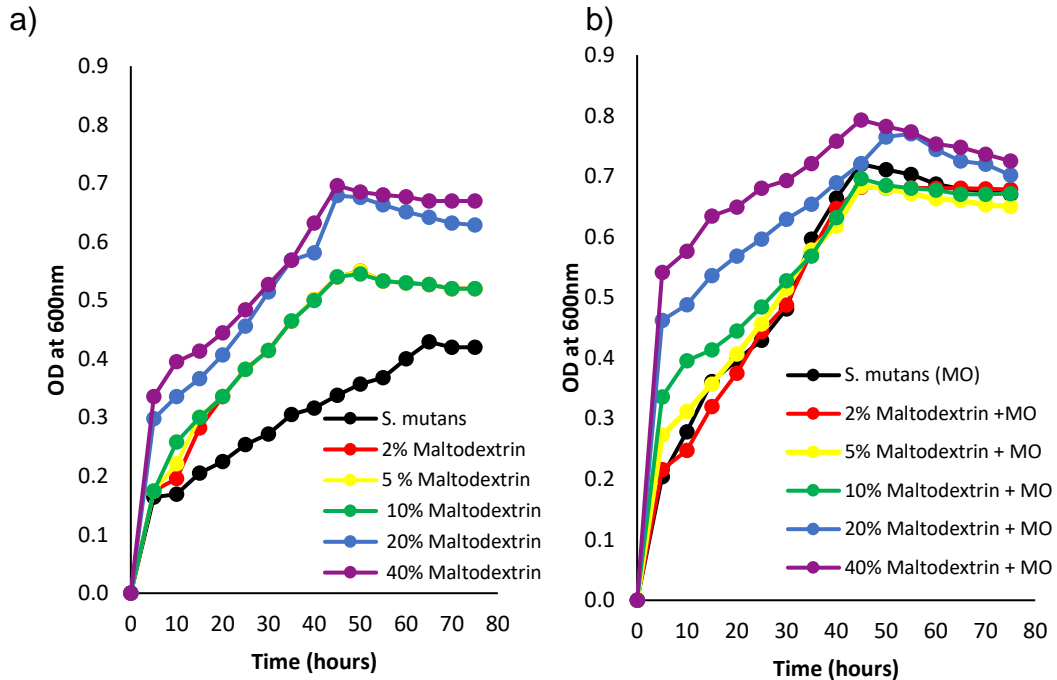


Figure 2.6: Growth curve of *S. mutans* with Percentage of Maltodextrin in presence of under a) aerobic and b) anaerobic conditions measured by monitoring optical density at 600nm for 75h, n=3

Figure 2.6 shows the growth of *S. mutans* with time in the presence of increasing concentrations of maltodextrin in aerobic and anaerobic conditions. The growth curve at both conditions showed that 40% maltodextrin (purple dotted line) has the highest rate when compared to *S. mutans* control (black dotted line). At 5 hours, 10% maltodextrin (green dotted line) has showed 92% increase in OD values between aerobic and anaerobic condition. Besides, 2%, 5% and 10% of maltodextrin in anaerobic condition showed overlap and were lower than *S. mutans* control between 20 to 72 hours. This was due to stationary and death phase (Renyne *et al.*, 2004; Gefen *et al.*, 2014). According to study Renyne *et al.*, the number of

culturable bacteria decreased as the bacteria were starved for longer periods of time. Moreover, Gefen *et al.*, studies revealed that after the initial phase of exponential growth, the trapped bacteria could starve at the OD observed at stationary phase. This is caused by entry into dormancy or by equal single cell probabilities to die or grow. Hence, the rate of division of *S. mutans* cell decreased and the nutrients supplied in the form of maltodextrin did not accumulate in bacteria.

2.4.3.2 Polyol

The growth of *S. mutans* during consumption of three types of polyols (sorbitol, mannitol, and xylitol) at difference percentages between 2 and 40% with absence and presence of mineral oil has been presented in [Figure 2.7](#) to [Figure 2.9](#). These growth curves show that with an increase in percentage of polyols in the presence of mineral oil, the ODs were doubled when compared to the control.

According to Abbe *et al.*, *S. mutans* cells upon exposure to oxygen have increased NADH and pyruvate levels, which suggests that the pyruvate catabolism rate has been reduced. *S. mutans* catabolize sugar under anaerobic conditions by glycolysis and convert it into lactic dehydrogenase-mediated lactate. Metabolism of the major products occurred when sugar is in excess. *S. mutans* produced sugar-limited formate, acetate, and ethanol. Therefore *S. mutans* can create a low pH environment and can withstand low pH stress (Abbe *et al.*, 1991; Zhu *et al.*, 2017).

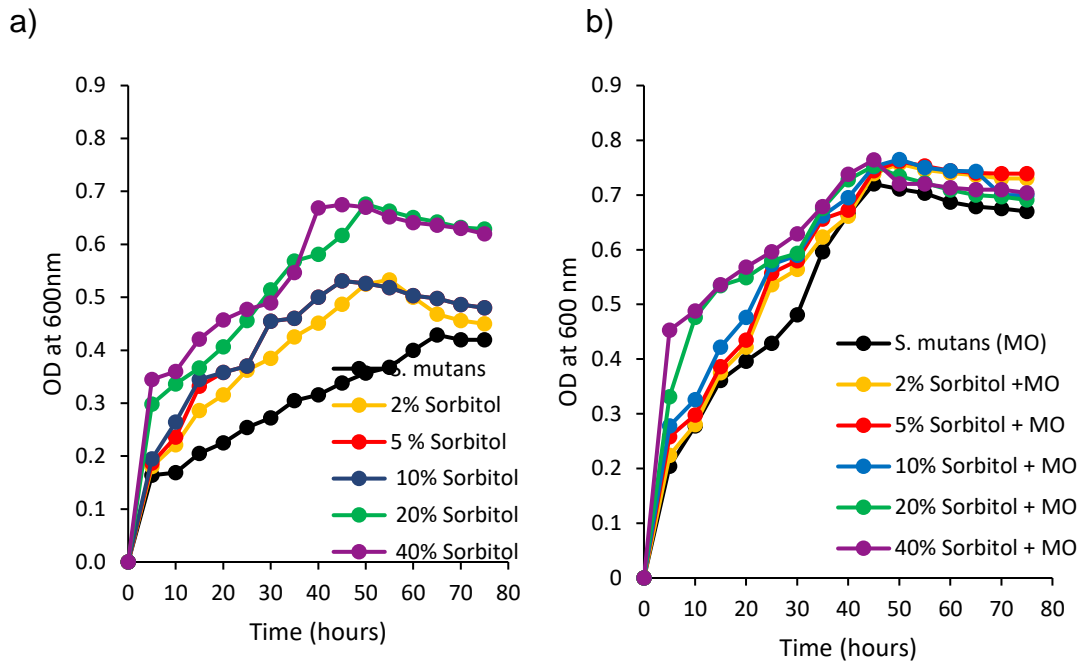


Figure 2.7: Growth curve of *S. mutans* with Percentage of Sorbitol in presence of a) aerobic and b) anaerobic conditions measured by monitoring optical density at 600nm for 75h, n=3

The growth curve with an increase of sorbitol from 2% to 40% in aerobic and anaerobic conditions is presented in Figure 2.7. In aerobic and anaerobic condition, 40% (purple line) shows the highest growth with an increase in time. Moreover, in anaerobic condition, as percentage of sorbitol increased, the growth of *S. mutans* also increased as compared to *S. mutans* control. Therefore, the sorbitol promotes the growth in the planktonic form. It was contradiction to Takahashi-Abbe *et al.*, studies (2001). They reported polyols such as sorbitol inhibit *S. mutans in vitro* in anaerobic condition and produced acid during *in vivo* by recording changes in pH. However, they found at different oxygen concentrations, the rate of acid production from sorbitol was not changed.

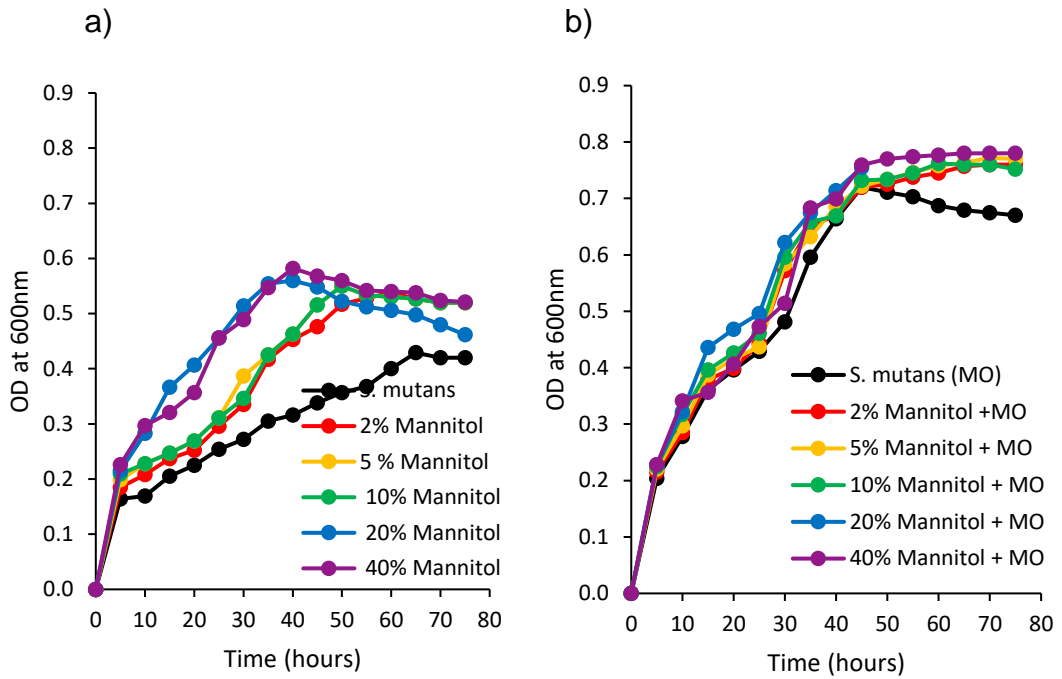


Figure 2.8: Growth curve of *S. mutans* with Percentage of Mannitol in presence of a) aerobic and b) anaerobic conditions measured by monitoring optical density at 600nm for 75h, n=3

Figure 2.8 shows a growth curve for mannitol for both conditions. At aerobic and anaerobic conditions, 40% of mannitol (purple dotted line) showed the highest concentration which has the highest OD value compared to *S. mutans* control (black dotted line) with time. At 5 hours, 2% of mannitol (red dotted line) showed 16% increase between aerobic and anaerobic condition. However, at the similar time point (5 hours) 40% of mannitol only showed 1% increased between both conditions. 40% of mannitol showed slow reaction as lower concentrations of mannitol initially were sufficient to promote growth of *S. mutans*. However, when the time is increased mannitol at 40% showed increased changes in OD (see in Appendix A2.9). The effects of oxygen on *S. mutans* growth with mannitol were studied under aerobic and strictly anaerobic conditions by (Higuchi, 1984). Higuchi reported that oxygen significantly aided the growth of two of the oxygen-

tolerant strains. In his study, all eight strains, the oxygen changed mannitol metabolism from heterolactic to homolactic fermentation and that no enzyme pyruvate formate-lyase activity was detected in cells grown under aerobic conditions. Role of this enzyme is to convert pyruvate and CoA into acetyl CoA and formate under anaerobic conditions in bacteria. (Abbe *et al.*, 1982). This enzyme is very sensitive to oxygen and plays a crucial role in pyruvate metabolism as part of the ATP production and/or NADH cycle. The *S. mutans* can withstand oxygen depending on the ability of strains to induce enzyme NADH oxidase and superoxide dismutase. Therefore, the aerobic condition rate showed correlation between these enzymes activities from the bacteria. Moreover, the activities of NADH oxidase and superoxide dismutase increased during aeration of anaerobically grown cells of oxygen-tolerant strains, but not in strains which were inhibited by oxygen. Hence, these activities of enzymes in aerobically conditions in mannitol have shown sensitivity to oxygen in strains were similar to those in anaerobic conditions (Higuchi, 1984).

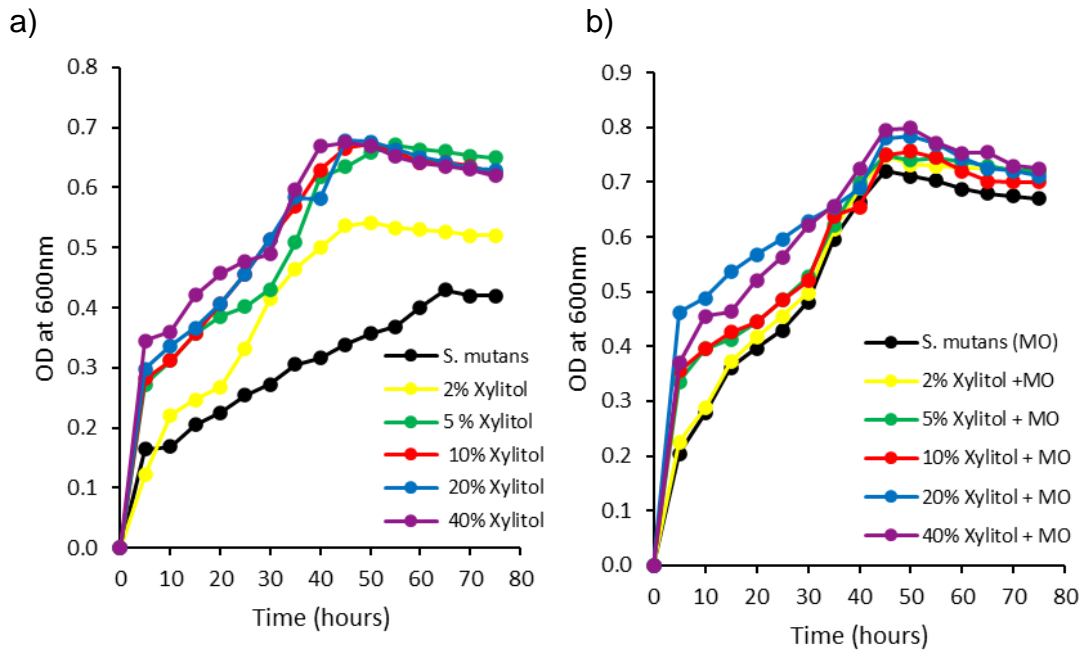


Figure 2.9: Growth curve of *S. mutans* with Percentage of Xylitol in presence of a) aerobic and b) anaerobic conditions measured by monitoring optical density at 600nm for 75h, n=3

The growth curve with increase of xylitol concentration from 2% to 40% in aerobic and anaerobic conditions is shown in Figure 2.9. Under both conditions, 40% xylitol (purple dotted line) shows the highest growth as increase the time. With increase in percentage of xylitol concentration, in both conditions, the growth of *S. mutans* in the also increased. At the 5 hours, there was 86% increase in growth at 2% xylitol (yellow dotted line) which shows there is a rapid growth under aerobic condition when compared to anaerobic condition. Whereas, at the same time (5 hours), 40% of xylitol (purple dotted line) demonstrated only 7% increase in growth under anaerobic condition when compared to aerobic condition (see in Appendix A2.10). This implied that the highest percentage (of 40%) showed xylitol already saturated when interacting with bacteria *S. mutans* cells. Hence, the percentage showed very low difference between the two conditions.

2.4.4 Determination of Minimum Inhibitory concentration (MIC) and minimum lethal concentration (MLC) of Chlorhexidine (CHX) against *S. mutans* NCTC 10449

MIC and MLC are typically obtained with planktonic cells (Souza *et al.*, 2019). Most oral microorganisms including *S. mutans* exist in complex oral biofilm communities also referred to as dental plaque. Test tube 9 was a positive control which has turbidity and shows visible growth in both BHI and BHI agar media. However, test tube 10 showed clear in the media with no visible growth in the BHI agar. In this studied the MIC and MLC values of CHX against *S. mutans* were found to be 0.625 and 1.25µg/ml respectively as shown in [Table 2.5](#). This is supported by research by Liu *et al.*, 2012, who found the MIC was 0.63µg/ml and the MLC was 2µg/ml. According to their studies, they used a different strain of *S. mutans*, UA159. Dong *et. al.* also worked on UA159 strain of *S. mutans* to determine the MIC/MLC of CHX, which resulted both MIC/MLC have 2.5µg/ml with addition of 1% sucrose into the BHI. However, their MIC and MLC have slightly higher result even though they used the same strain of the microorganism (Dong *et al.*, 2012). [Table 2.5](#) shows the MIC and MLC results of CHX in the planktonic stage in (BHI) media and BHI agar with 5% defibrinated horse blood. Moreover, the observation MIC (10%) and MLC (20%) of Xylitol in the planktonic stage from using media BHI and BHI agar with 5% defibrinated horse blood is also presented in [Table 2.6](#).

Table 2.5: Determination of Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of CHX against *S. mutans* in the planktonic stage in BHI media. The sample was placed at stationary incubation at 37 °C 24 hours. MIC is highlighted in yellow and MLC in green.

Test tube	Dilution factor	Observation in test tube	Inference	Growth observed on agar plate	Concentration (µg/ml)	Concentration (% w/v)
1	½	Clear	No Growth	No growth	10	1.0 x 10 ⁻³
2	¼	Clear	No Growth	No growth	5.0	5.0 x 10 ⁻⁴
3	1/8	Clear	No Growth	No growth	2.5	2.5 x 10 ⁻⁴
4	1/16	Clear	No Growth	No growth	1.25	1.25 x 10 ⁻⁴
5	1/32	Clear	No Growth	Growth	0.625	6.25 x 10 ⁻⁵
6	1/64	Turbid	Growth	Growth	0.313	3.13 x 10 ⁻⁵
7	1/128	Turbid	Growth	Growth	0.156	1.56 x 10 ⁻⁵
8	1/256	Turbid	Growth	Growth	0.078	7.80 x 10 ⁻⁶
9	Positive control	Turbid	Growth	Growth	-	
10	Negative control	Clear	No Growth	No Growth	-	

Table 2.6: Determination of Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of Xylitol against *S. mutans* in the planktonic stage in BHI media. The sample was placed at stationary incubation at 37 °C 24 hours. MIC is highlighted in yellow and MLC in green.

Test tube	Dilution factor	Observation in test tube	Inference	Growth observed on agar plate	Concentration (% w/v)
1	½	Clear	No Growth	No growth	40
2	¼	Clear	No Growth	No growth	20
3	1/8	Clear	No Growth	Growth	10
4	1/16	Turbid	Growth	Growth	5
5	1/32	Turbid	Growth	Growth	2.5
6	1/64	Turbid	Growth	Growth	1.25
7	1/128	Turbid	Growth	Growth	0.625
8	1/256	Turbid	Growth	Growth	0.313
9	Positive control	Turbid	Growth	Growth	-
10	Negative control	Clear	No Growth	No Growth	-

2.4.5 Checkerboard Dilution Assay

Checkerboard dilution assay was adapted from Low *et al.*, (2011), method. The checkerboard assay method was used to define the lowest concentration of two antimicrobial agents combined resulting in a potential synergistic effect. [Table 2.7](#) shows (10×10^{-4} of CHX and 20 of Xylitol in (%w/v) were included to demonstrate the outcome of the first part of this experiment i.e., determination of antimicrobial activity of two agents combined at different concentrations. CHX, is long considered for gingivitis treatment showed killing capacity only at concentration greater than 4x MIC. However, for this checkerboard assay concentration for CHX is chosen 10 times greater than MIC ($0.625 \mu\text{g/ml}$) and 20% of xylitol due to its solubility to determine series of dilution in the checkerboard assay. These results found synergy to be present in two wells of the 96 microtiters plates which are highlighted in blue in [Table 2.7](#). The xylitol and CHX combination concentration correspondent to 1.25×10^{-4} of CHX at 0.16% Xylitol and 6.25×10^{-4} of CHX and 10% Xylitol in (%w/v).

Table 2.7: Checkerboard assay for the combination of 10×10^{-4} of CHX and 20 of Xylitol in (%w/v) against *S. mutans* for 24 hours.

	1	2	3	4	5	6	7	8	9	10	11	12
A		1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	BHI	BHI+ 10×10^{-4} of CHX	BHI+ 20 of XYL	BHI <i>S. mutans</i>
	20	10	5	2.5	1.25	0.63	0.31	0.16				
		-	-	-	-	-	-	-	-	-	-	-
B		5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	BHI	BHI+ 10×10^{-4} of CHX	BHI+ 20 of XYL	
	20	10	5	2.5	1.25	0.63	0.31	0.16				
		-	-	-	-	-	-	-	-	-	-	-
C		2.5×10^{-4}	2.5×10^{-4}	2.5×10^{-4}	2.5×10^{-4}	2.5×10^{-4}	2.5×10^{-4}	2.5×10^{-4}	BHI	BHI+ 10×10^{-4} of CHX	BHI+ 20 of XYL	
	20	10	5	2.5	1.25	0.63	0.31	0.16				
		-	-	-	-	-	-	-	-	-	-	-
D		1.25×10^{-4}	1.25×10^{-4}	1.25×10^{-4}	1.25×10^{-4}	1.25×10^{-4}	1.25×10^{-4}	1.25×10^{-4}	BHI	BHI+ 10×10^{-4} of CHX	BHI+ 20 of XYL	
	20	10	5	2.5	1.25	0.63	0.31	0.16				
		-	-	-	-	-	-	-	-	-	-	-
E		6.25×10^{-5}	6.25×10^{-5}	6.25×10^{-5}	6.25×10^{-5}	6.25×10^{-5}	6.25×10^{-5}	6.25×10^{-5}	BHI	BHI+ 10×10^{-4} of CHX	BHI+ 20 of XYL	
	20	10	5	2.5	1.25	0.63	0.31	0.16				
		-	+	+	+	+	+	+	-	-	-	-
F		3.13×10^{-5}	3.13×10^{-5}	3.13×10^{-5}	3.13×10^{-5}	3.13×10^{-5}	3.13×10^{-5}	3.13×10^{-5}	BHI	BHI+ 10×10^{-4} of CHX	BHI+ 20 of XYL	
	20	10	5	2.5	1.25	0.63	0.31	0.16				
		+	+	+	+	+	+	+	-	-	-	-
G		1.56×10^{-5}	1.56×10^{-5}	1.56×10^{-5}	1.56×10^{-5}	1.56×10^{-5}	1.56×10^{-5}	1.56×10^{-5}	BHI	BHI+ 10×10^{-4} of CHX	BHI+ 20 of XYL	
	20	10	5	2.5	1.25	0.63	0.31	0.16				
		+	+	+	+	+	+	+	-	-	-	-
H		7.8×10^{-6}	7.8×10^{-6}	7.8×10^{-6}	7.8×10^{-6}	7.8×10^{-6}	7.8×10^{-6}	7.8×10^{-6}	BHI	BHI+ 10×10^{-4} of CHX	BHI+ 20 of XYL	
	20	10	5	2.5	1.25	0.63	0.31	0.16				
		+	+	+	+	+	+	+	-	-	-	-

Legend:

 CHX	 Xylitol 20%	 Growth on agar	 Synergism	+	Bacterial on growth/ Turbid	-	No bacterial growth
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2.4.6 Fractional Lethal Concentration Index (FLCI)

Table 2.7 shows the active *in vitro* combination of agents used against *S. mutans* to be indifferent. It also illustrated FLCI indices according to Karpanen *et al.*, studies (Karpanen *et al.*, 2008). However, the values for the combination Xylitol and CHX are used against *S. mutans* indicated indifferent interactions with FLCL of 1.0 (Table 2.8) and 1.008 (Table 2.9). Although the values for the combination used against *S. mutans* indicated indifferences, both values were considerably lower than antagonist value of > 4.0.

Table 2.8: Estimation of Fractional Lethal Concentration (FLC) and Fractional Lethal Concentration Index (FLCI) of Xylitol and CHX against *S. mutans* E2

Microorganism	MLC of CHX (agent A in combination/ alone % w/v)	FLC of CHX	MLC of Xylitol (agent B in combination/ alone % w/v)	FLC of Xylitol	FLCI	Inference
<i>S. mutans</i>	$\frac{6.25 \times 10^{-5}}{1.25 \times 10^{-4}}$	0.5	$\frac{10}{20}$	0.5	1.0	Indifference

Table 2.9: Estimation of Fractional Lethal Concentration (FLC) and Fractional Lethal Concentration Index (FLCI) of Combination Xylitol and CHX against *S. mutans* D8

Microorganism	MLC of CHX in combination/ alone (%w/v)	FLC of CHX	MLC of Xylitol (0.16% in combination/ alone % w/v)	FLC of Xylitol	FLCI	Inference
<i>S. mutans</i>	$\frac{1.25 \times 10^{-4}}{1.25 \times 10^{-4}}$	1	$\frac{0.16}{20}$	0.008	1.008	Indifference

2.4.7 Time Kill Studies

Bactericidal activity is defined as greater than 3 log₁₀ -fold decrease in colony forming units (surviving bacteria), which is equivalent to 99.9% killing of the inoculum according to guideline M26 Method (Clinical Laboratory Standard Institute, 1999). Synergism between xylitol and CHX was further determined by a time-kill test, xylitol alone was found to decrease bacterial viability when the incubation time was increased as compared to control with an initial inoculum of 10⁷ CFU/mL. The effect of CHX on *S. mutans* viable cell count with time was studied as shown as in (Figure 2.10).

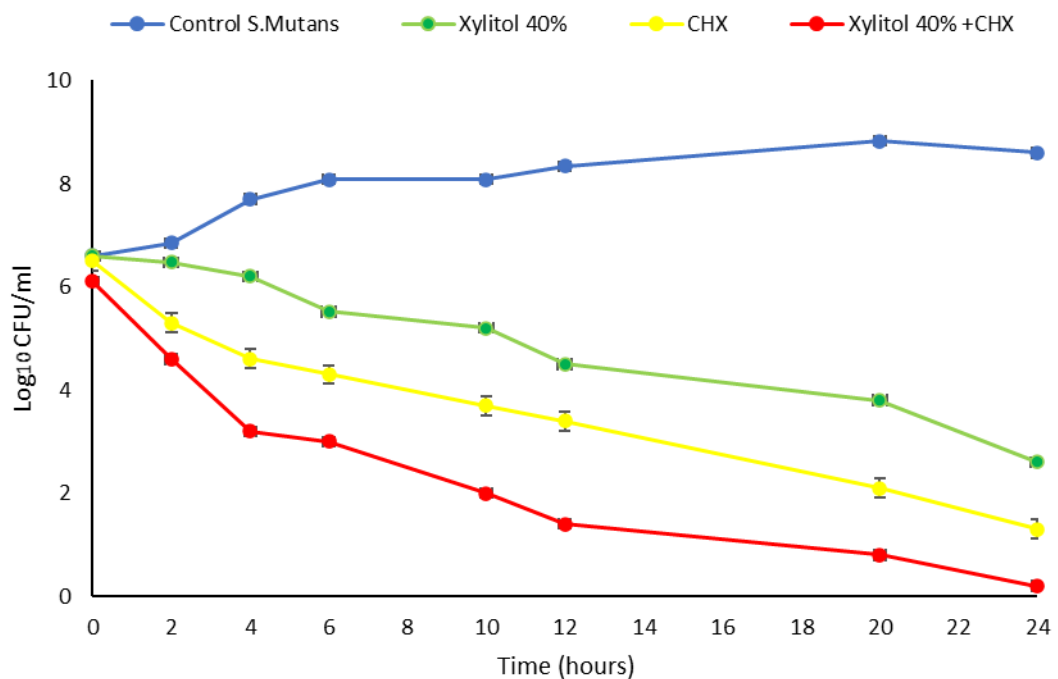


Figure 2.10: Time kill- curve of *S. mutans* exposed to CHX, Xylitol, and Xylitol-CHX. Log CFU indicated for absorbance reading for duration time for 24 hours. Values represent, Mean (n=3), \pm SD and ($p < 0.05$)

The pattern of the time–kill curves presented here for *S. mutans* are similar to those reported by Hasan *et al.*, (2014) for clinical isolates treated with anticariogenic agents, Quercitrin and Deoxynojirimycin (Hasan *et al.*, 2014). However, the results show that at the MLC, cell viability was decreased within the first 60 minutes and eventually by 2 log, for both the MLC CHX and xylitol, and the positive control showed no change in cell viability over the tested period. However, 40% Xylitol-CHX combination showed a further decrease from initial incubation times and a further decrease in CFU/mL compared with Xylitol after 24 hours of incubation. Incubation time is chosen for 24 hours due to *S. mutans* growth in the planktonic form. Log reduction in CFU/mL of the combination 40% Xylitol-CHX show decreased by more than 3 log₁₀ when compared to xylitol alone. Furthermore, log reduction in CFU/mL of the combination 40% Xylitol-CHX show decreased by 2- log₁₀ when compared to CHX alone.

When CHX are bound to extra cellular soluble protein and cell walls of *S. mutans*, it causes disruption and affects the integrity of bacterial cell walls at high concentrations thereby disrupting cells (McDonnell and Russell, 1999; Zhu *et al.*, 2019). Hence, CHX is classified as bacteriostatic at low concentrations. However, Xylitol has been reported to have a potential antimicrobial activity (Nayak *et al.*, 2014). Xylitol is known as a sugar alcohol that is not fermentable in the most plaque bacteria. It also does not transform into end-products of cariogenic acid. Xylitol works by inhibiting the growth of plaque and oral microorganisms when it intracellularly accumulates in the microorganism (Bader *et al.*, 2013). In Nayak *et al.*

studies, they found *S. mutans* transport the xylitol into the cell resulted in growth inhibition (Nayak *et al.*, 2014). According to the findings of Nayak *et al.*, *S. mutans* transport xylitol into the cell is an energy-consuming cycle. *S. mutans* converts xylitol to xylitol-5-phosphate via phosphoenolpyruvate: fructose phosphotransferase reaction. This metabolic reaction of xylitol resulted in the development of intracellular vacuoles and degradation of *S. mutans* cell membrane. This eventually results in microorganism starvation and growth inhibition (Nayak *et al.*, 2014). Therefore, the mechanism of action of xylitol in inhibiting *S. mutans* demonstrated that xylitol- inhibited the growth. Moreover, combination of CHX with Xylitol would increase the inhibiting of *S. mutans* activity. Hence, the disruption of cell in *S. mutans* was increased.

2.5 Conclusion

These findings show that *S. mutans'* changes dramatically in response to oxygen and changes in sugar and polyol concentrations. This suggests that survival of this pathogenic organism in the human oral cavity is highly variable depending on diet, which contribute significantly to the maturation of the dental biofilm *i.e.*, plaque. The effect of mineral oil was used in anaerobic respiration of *S. mutans* at different percentage of sugars and polyols (2, 5, 10, 20, 40) were found to increase OD in the growth study. The results showed changes in growth rate between aerobic and anaerobic conditions with increasing time. However, based on these analyses, there were changed in pattern of growth in 5% fructose and 40% xylitol (from 35 to 75

hours). Both of these concentrations shown significant percentage changes in growth rate at both aerobic and anaerobic condition when these agents inhibited growth of *S. mutans*. Study by Linke *et al.*, (2014) emphasized the growth rate of *S. mutans* at higher levels of xylitol has always been reduced during development of *S. mutans*. The result showed contradicted to Linke *et al* studies, the highest concentration of xylitol demonstrated very low level in growth under anaerobic conditions when compared to aerobic conditions. However, this study was supported by Söderling *et al.*, who reported that growth inhibition of *S. mutans* with low concentration of xylitol at 0.01% inhibited the *S. mutans* cells (Söderling *et al.*, 2008).

Moreover, this study showed MIC planktonic cells were extremely receptive to xylitol in combination with CHX. Xylitol and CHX have synergistic effects which inhibited antimicrobial efficacy against *S. mutans*. Although the values for the combination used against *S. mutans* indicated indifferent interactions using chequerboard assay, both values were considerably lower than the antagonistic value of >4.0.

Furthermore, time –kill data, *S. mutans* responded well to the combinations with xylitol and CHX at an early stage of exposure, with CFU/mL reducing to the lowest detectable level between 1 and 10 hours of treatment. There was 3 log reduction in CFU/mL with the combination of CHX-xylitol confirming synergy. This could be confirmed throughout colony count which were obtained using the Miles and Misra technique. According to results, combination of these antimicrobial agents are vital to obtain an effective reduction of this pathogenic microorganism causing gingivitis. Therefore,

xylitol is the best replacement of sugar for formulation of lozenges tablet to treat gingivitis.

CHAPTER THREE

**Evaluation of antimicrobial activity of chlorhexidine in
controlling *S. mutans* biofilms in combination with sugars
and polyols**

3.1 Introduction

A biofilm is formed when bacteria attach to a biotic or abiotic surface. Bacteria are enclosed in an extracellular polymeric material which consists of components such as extracellular polysaccharides (EPS), proteins, extracellular DNA (eDNA) and metabolites (Ahn *et al.*, 2018). There is 97% of water built up in the biofilm (Welch *et al.*, 2012). In the case of *S. mutans*, an EPS comprises of glucans which helps in adherence of *S. mutans* to the surface of teeth as well as cell to cell interactions (Koo *et al.*, 2010 ; Kim *et al.*, 2018 and Zhu *et al.*, 2018). Moreover, *S. mutans* can form EPS in the presence of sucrose, fructose, and glucose (Costa Oliveira *et al.*, 2021; Zero, 2014). Costa Oliveira *et al.*, discovered that *S. mutans* produces both glucans and fructans from sucrose. The presence of dextranases and fructanase in *S. mutans* is required for the degradation of these EPS compounds. Study by Costa Oliveira *et al.*, found that because sucrose is metabolized by *S. mutans*' EPS synthetic machinery, it can have a significant impact on intracellular polysaccharide (IPS) metabolism and extending acid production during starvation. EPS has long been considered to be an important factor in *S. mutans* virulence, and this study reveals a relatively novel mechanism for virulence enhancement.

The aim of this study is to investigate the effect of antimicrobial activity of CHX and growth of *S. mutans* biofilm in the presence of sugars and polyols. Biofilm development of *S. mutans* was monitored over 72 hours to evaluate

the activity of sugars and polyols through estimation of biofilm density during biofilm inhibition and dispersal assays.

3.2 Materials

3.2.1 Microbial strains

See Chapter two in section 2.2.1

3.2.2 Microbiological and cell culture media

See Chapter two in section 2.2.2

3.2.3 Materials and chemicals

See Chapter two in section 2.2.3. The additional chemicals are listed below:

Phosphate buffered saline (PBS) was purchased from Sigma Aldrich, (U.K). 0.1% of Crystal Violet and 95% of Ethanol were obtained from Sigma Aldrich, (UK). 0.9% saline was purchased from Fisher Scientific, (U.K).

SYTO 9, (S9), was purchased from Life Technologies, (U.K) and Propidium Iodide, (PI), was purchased from Thermo Fisher Scientific, (U.K).

6, 24 and 96-well flat-bottom and polystyrene plates were purchased from Sarstedt, (U.K.).

3.3 Methods

3.3.1 Preparation of biofilm assay

Initially, an overnight culture of *S. mutans* was prepared in BHI. 100µl diluted 1×10^7 cells/ml colony forming units (CFU)/mL of *S. mutans* and 100 µl BHI

were added into 96-well flat-bottom polystyrene plate under aseptic conditions and incubated at 37°C for 72 hours in static incubator which contain atmosphere of 5% carbon dioxide and oxygen 95%.

PBS was prepared according to manufacturer at pH 7.4. After incubation for 72 hours, the plates were gently washed with 100µl of PBS using a multichannel pipette. The biofilm formation was examined using the crystal violet-based microtiter plate assay originally described by O'Toole (O'Toole *et al.*, 2000). The washing step was repeated three to four times with PBS to ensure all the planktonic bacteria are removed. After washing, 100 µl of 0.1 % of crystal violet was added to the plates and left for 1 hour at room temperature. Excess crystal violet was removed by rinsing the plate with sterile distilled water using technique of submerging the plate in a tub of water, shaking out and blotting vigorously on a stack of paper towels to get rid of excess dye (O'Toole *et al.*, 2000). The plates were turned upside down and left to dry overnight. 125µl of 95% ethanol were added into each well of 96-well plate to solubilize the biofilm. The plate was then incubated at room temperature for 30 minutes. After that, 125µl of solubilized biofilm stained with crystal violet were transferred into a new 96- well plate. The plate was then analysed to get the absorbance at 600nm (Promega Glomax Multi Detection System, U.K.), using 95% ethanol as the blank.

3.3.2 Preparation of biofilm inhibition assay for different inoculum sizes

The method in section 3.3.1 was repeated using different inoculum volumes (200, 400, 600 and 800µl) of diluted 1×10^7 cells/ml colony forming units (CFU)/mL of *S. mutans* and different concentration from (0.1, 0.5, 1.0, 2.0 and 4.0µg/ml) of the antimicrobial agent CHX were added into 24-well flat-bottom polystyrene plate. This range was developed to study the effect of differing cultural size on the formation of biofilms. 200µl of BHI were added to each well then the plate were incubated overnight at 37°C for 72 hours in static incubator which contain atmosphere of 5% carbon dioxide and oxygen 95%. After 72 hours the biofilms plates were washed, stained, solubilized, and quantified as described in section 3.3.1.

3.3.3 Preparation of biofilm dispersal assay

Biofilms inhibition of *S. mutans* were grown as described in section 3.3.1. After biofilm maturation (after development within 72 hours), different concentrations of sugar (2, 5, 10, 20 and 40%), polyols (2, 5, 10, 20 and 40%), and antimicrobial agent CHX (0.1, 0.5, 1.0, 2.0 4.0µg/ml) were added into biofilm plates and incubated for further 24 hours. After 24 hours of exposure to CHX/polyol/sugar, the biofilms were washed, stained, solubilised, and quantified as described in section 3.3.1.

3.3.4 Microbial vitality by Confocal Laser Scanning Microscopy (CLSM)

The biofilm inhibition method in 3.3.1 was prepared by adding 5ml of diluted 1×10^7 cells/ml colony forming units (CFU)/mL of *S. mutans* into each 6 well flat-bottom polystyrene plate incubated at 37°C for 72 hours in static incubator. The incubation plates were washed with 0.9% saline according to manufacturer guidelines instead of phosphate buffer as phosphate buffer will interfere with staining, Sigma Aldrich (UK).

Working solutions were prepared by using 3 µl of SYTO 9, (S9), stain 5mM and 3 µl of Propidium Iodide, (PI), 20 mM in 1ml of filtered -sterilized water. These dyes are part of the Live/Dead BacLight Bacterial Viability Kit (Invitrogen, Darmstadt, Germany), which helps in assessing the membrane integrity status and allows the differentiation between living (intact membranes, green fluorescence) and non-vital/dead cells (compromised membranes, red fluorescence) (Decker, 2014).

One coupon or well only requires 200µl of stain diluted in water. 200µl of mixture staining solution was gently transferred onto the biofilm sample to avoid disturbance on the biofilm. The plates were covered with lid and the sample incubated for 20 minutes at room temperature protected from light. After incubation, the samples were gently rinse with filtered- sterilized water to remove excess stains. The plates were inverted to let them dry for analyse through CLSM. The dyes S9 and PI were excited at 488nm and 543nm respectively and images were obtained using a CLSM (LSM700, Carl Zeiss,

Munches- Hallbergmoss, Germany). The scanning images were analysed three- dimensionally using imaging software (Imaris ®, Bitplane AG, Zurich, Switzerland) and convert to image J for further quantification. The samples were prepared in duplicates for optimisation and percentage of vital *S. mutans* biofilm was calculated using following equation (Decker *et al.*, 2014):

$$\% \text{ Vitality} = \frac{[\text{Vital (S9, green) bacteria}]}{[\text{Vital bacteria(S9,green) + dead (PI, red) bacteria}]} \times 100\%$$

3.3.5 Statistical methods

All data was expressed as mean with \pm standard deviation (SD). The statistical significance of the result was determined by analysis of variance (ANOVA) using a one way and a two- way ANOVA in Excel. P-values less than 0.05 were considered as statistically significant.

3.4 Results and discussions

3.4.1 Effect of varying inoculum size and CHX concentration on *S. mutans* biofilm formation

Inoculum size plays a very important role in the development of biofilm by *S. mutans*. Different volumes from (100-800 μ l) of the inoculum were tested to assess the amount of biofilm formed by *S. mutans*. As shown in [Figure 3.1](#) biofilm density increased as the size of the inoculum was increased from 100-800 μ l.

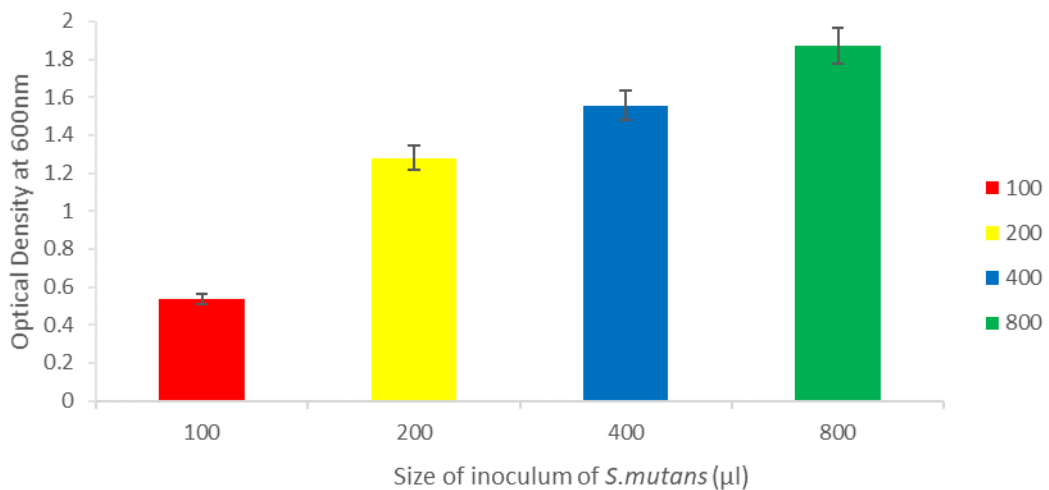


Figure 3.1: Effect of inoculum size (100-800 μ l) on the growth of *S. mutans* biofilms formation at 72 hours incubated at 37°C with volume of the inoculum in microlitres (μ l) on the x-axis and biofilm density measured as optical density at 600nm on the y-axis. Values represent mean of 3 replicates with \pm SD, $p < 0.05$.

There is a significant difference between size of inoculum and the amount of biofilm produced by *S. mutans* measured as OD values (ANOVA, $p < 0.05$ & F value $> F$ critical). The results demonstrated that the biofilm density of *S. mutans* when the size of the inoculum was 200 μ l was optimum due to the

optical density reading of 1AU at 600nm. This also allowed the measurement of OD₆₀₀ after biofilm inhibition and dispersal by CHX.

The present study found that inoculum size of 200µl was optimum and was thus selected to study biofilm formation and effect of different concentrations of CHX on *S. mutans* (Figure 3.2).

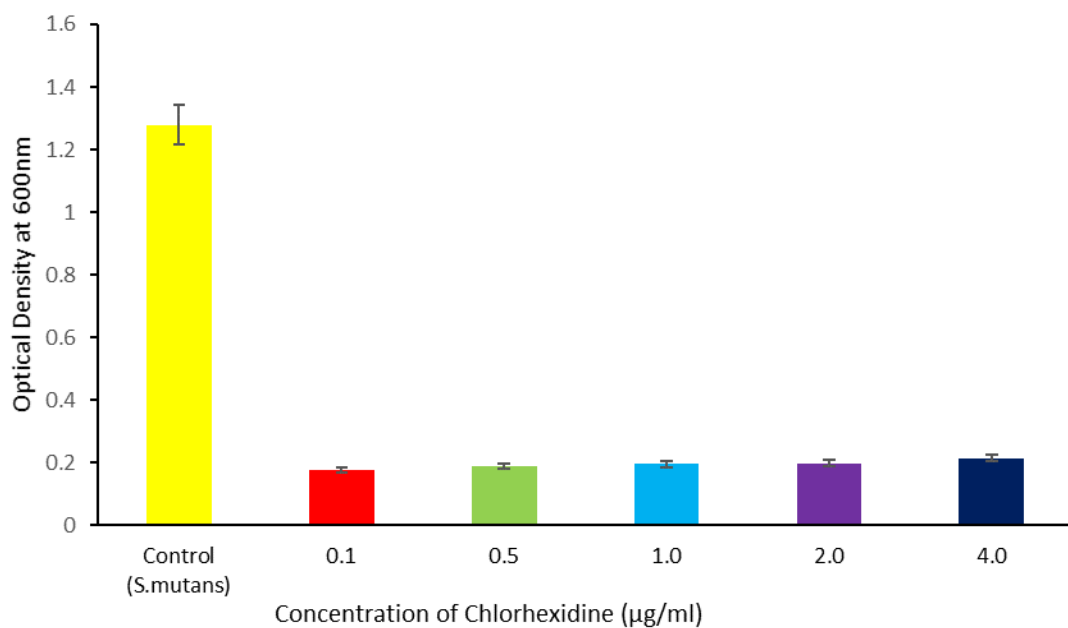


Figure 3.2: Size of inoculum of *S. mutans* with different concentrations of CHX on the x-axis and biofilm density measured as optical density at 600nm on the y-axis. 200µl of size volume inoculum was used to obtain the OD (OD 600nm). Values represent mean of 3 replicates with +/- SD, p<0.05.

The graph showed that optical density of *S. mutans* control biofilm was decreased when exposed to CHX concentration from 0.1 to 4.0 µg/ml. At 200µl size of inoculum, most of the biofilm formations were susceptible to different concentrations of antiseptic CHX. Therefore, the bactericidal activities of CHX against *S. mutans* suppressed biofilm formation and affected bacterial growth. The changes observed in the biofilm density and matrix development are inoculum dependent as reported by Veloz *et al.*, who

investigated changes in biofilm density with varying inoculum size (Veloz *et al.*, 2019). The biofilm density decreased significantly in comparison to control when exposed to CHX at concentration from 0.1 to 4.0µg/ml (ANOVA, $p < 0.05$). Hence, 200µl size of inoculum can be used to determine biofilm activity either inhibition or dispersal of *S. mutans* biofilm as well as interaction with sugars and polyols. Moreover, when the effect of different CHX concentrations on biofilm density was evaluated using ANOVA analysis, no statistically significant difference was found because the p value was less than 0.05, ($p < 0.05$).

3.4.2 Evaluation of the effect of different sugars and polyols on *S. mutans* biofilms

Study by Decker *et al.*, (2011) investigated the availability of dietary carbohydrates in *S. mutans* biofilms employing diverse research techniques. *S. mutans* biofilms grown was measured by culture growth, vitality, respiratory activity, EPS production and glucose consumption. In this findings, *S. mutans* biofilms were found to have increased vitality across all biofilm layers, as well as primary metabolic consumption of glucose with subsequent sucrose utilization. Furthermore, sucrose-induced changes in biofilm morphology were noticed such as the formation of microcolonies. Moreover, biofilm bacteria are known to be 10-to 1000-fold more resistant to antibiotics and antimicrobial peptides than to planktonic bacteria. Biofilms are also a public health issue due to increased resistance to antibiotics that restrict treatment options. Many studies have illustrated the significance of

sugars as a factor in the development of caries (Boonyanit *et al.*, 2011; Liu *et al.*, 2011).

The purpose of this study was to evaluate the effect of sugars and polyols at different concentrations and in combination with the antimicrobial agent CHX on the formation of biofilms by *S. mutans*. The sugars were to stimulate the oral environment upon consumption of simple carbohydrates. The sugars studied in these experiments are fructose, sucrose, lactose, and maltodextrin. However, maltodextrin is a polysaccharide that is used as a food additive which consists of chains of glucose. It could also be one of the common carbohydrates that can be found in the diet due to being inexpensive and easy to produce (Hofman *et al.*, 2016). Besides sugars, polyols that were used are mannitol, sorbitol, and xylitol. The concentration of sugars and polyols, (2, 5, 10, 20 and 40%) and their interaction with CHX at concentrations 0.1, 0.5, 1, 2, 4 µg/ml was studied during biofilm formation and dispersal in *S. mutans*.

3.4.2.1 Effect of sugars on biofilm formation by *S. mutans*

Results suggested an increase in formation of biofilm with fructose, [Figure 3.3\(a\)](#). The addition of CHX concentration of 0.1µg/ml was ineffective with increase in concentration of fructose between 2 to 10%. However, as the fructose concentration was further increased from 20 to 40%, the biofilm formation density was decreased by 40%. Overall, it was determined that by increasing the concentration of the fructose, the biofilm density gradually

decreased, and the inhibition is statistically significant ($p < 0.05$) which is in accordance with the findings of Costa Oliveira *et al.*, (2021).

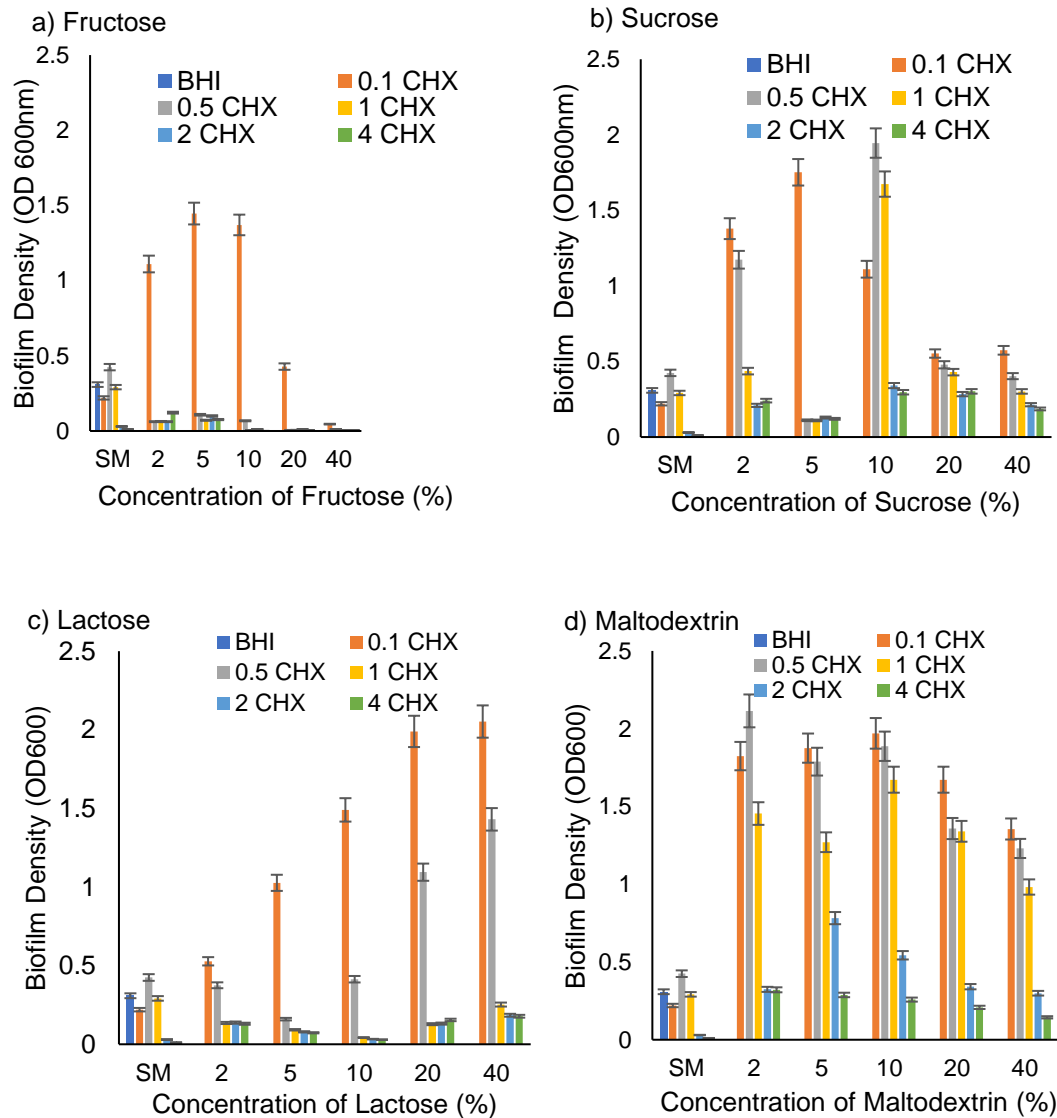


Figure 3.3: Biofilm formation of *S. mutans* at 72 hours and interaction with various sugars concentrations; (a) fructose, (b) sucrose and (c) lactose and (d) maltodextrin from concentration of 2, 5, 10, 20 and 40%, and concentration of CHX from (0.1, 0.5, 1, 2 and 4 µg/ml) OD 600nm, mean \pm SD, $n=3$, $p < 0.05$

This Figure 3.3(a) also clearly shows that at 0.5 µg/ml CHX is more effective than 0.1 µg/ml in the inhibition of *S. mutans* biofilms. When biofilm formation was compared between 0.1 and 0.5µg/ml of CHX at 2% fructose, percentage reduction was 83.40%. However, 1 µg/ml of CHX showed an even greater

reduction in biofilm formation at 2% fructose (percentage reduction = 95.29%) compared to 2% fructose with 0.1µg/ml CHX. Meanwhile at 0.1µg/ml of CHX, the biofilm formation decreased with an increase in fructose concentration to 40%. Therefore, the biofilm formation with fructose did show a significant change ($p < 0.05$).

The results clearly show that the growth of *S. mutans* biofilm formation is stimulated by sucrose, [Figure 3.3\(b\)](#). At concentration of 2% and 5% sucrose with 0.1µg/ml CHX, biofilm formation of *S. mutans* shows an increase. However, with a further increase in sucrose concentration from 10% to 40% in combination with 0.1µg/ml CHX, the density of the biofilm decreased. Overall, by increasing the concentration of CHX in the presence of sucrose there was a decrease in the density of the biofilm. Besides that, at lower concentration of CHX (0.1µg/ml) both percentage of 20% and 40% of sucrose were effective in reducing the biofilm density when compared to lower concentration of sucrose.

At 1µg/ml CHX, biofilm formation was not disrupted in the presence of 10% sucrose, however when the concentration of CHX was further increased (up to 4 µg/ml), biofilm formation was significantly decreased ($p < 0.05$). Whereas 2 and 4 µg/ml CHX were both effective concentrations of the antimicrobial agent as CHX inhibits the growth of *S. mutans*, although the concentration of sucrose has risen to 40%. However, there was a significant difference between these two concentrations in relation to inhibition of biofilm formation ($p < 0.05$). It was previously reported that density of biofilm did not change when different sucrose levels have interacted with oral bacteria (Leme *et al.*,

2006 and Durso *et al.*, 2014). Sucrose is a sugar that is most cariogenic as it is fermented by *S. mutans*. Furthermore, sucrose provides a substrate for the synthesis of extracellular (EPS) and intracellular (IPS) polysaccharides in addition to being fermented by oral bacteria as it resulted in low pH which promotes biofilm formation. Furthermore, when the amount of carbohydrate exposure is increased, the plaque is exposed to a longer period below the essential pH for enamel demineralization (Atkinson *et al.*, 2021). However, when the sucrose concentration is increased, the pH decreases more rapidly. These latter conditions would develop *S. mutans* leading to the transformation of a healthy biofilm into a disease and a resulting increased demineralization. Along these finding, the current study supported that at 5% sucrose with 0.5 and 1µg/ml of CHX concentration has shown reduction in biofilm formation when compared to the control (*S. mutans* exposed of CHX alone).

At concentration of 0.1µg/ml, CHX, had no effect on *S. mutans* biofilm formation in the presence of lactose from (2 to 40%). This can be seen from [Figure 3.3\(c\)](#) as biofilm formation of *S. mutans* increased gradually with an increase in concentration lactose (2 to 40%) in the culture media. Meanwhile, when the concentration of CHX was increased to 0.5µg/ml, the biofilm formation was reduced at both 2% and 5% lactose concentrations. Moreover, at the same concentration (0.5µg/ml) of CHX with 10 to 40% of lactose concentrations, the density of biofilms increased when compared to control. When the biofilm inhibition was compared at these two lowest concentrations of CHX (0.1 and 0.5µg/ml), the results shown were statistically significant, ($p < 0.05$). However, the biofilm formation of *S. mutans* in presence of lactose

concentration from 2 to 5% shows inhibition at all concentration of CHX except 0.1µg/ml. Furthermore, there was a significant difference between biofilm formation of *S. mutans* in presence of lactose 2 to 40% when exposed to 2 µg/ml and 4µg/ml of CHX ($p>0.05$). Besides that, the graphs showed the biofilm formation of *S. mutans* increases as concentration of lactose was increases from 10 to 40%, in the presence of 0.1 to 0.5 µg/ml of CHX. Therefore, these concentrations led to significantly different values of biofilm density ($p>0.05$)

Figure 3.3(d) demonstrates the CHX effect on biofilm inhibition with maltodextrin at a concentration of 2 to 40%. The biofilm formation shows it decreased when there was an increase of presence of maltodextrin concentrations (2 to 40%) at higher concentration of CHX (2 and 4ug/ml). Moreover, at 2% maltodextrin with the lowest CHX (0.1µg/ml) concentration, compared to the effect of the highest concentration (4µg/ml) of the antimicrobial agent was shown to be significantly different ($P<0.005$). Furthermore, the biofilm inhibition in the presence of maltodextrin was effective when the concentration of CHX increased to 4µg/ml. From overall result of maltodextrin biofilm formations when exposed to the antimicrobial agent, CHX in combination were shown to unsuccessfully inhibit the development of biofilm when compared to *S. mutans* control biofilm. Studies by Sultana *et al.*, 2016 on maltodextrin showed to be more effective in decreasing viable *S. aureus* biofilms cell density. According to their findings, biofilm formation using electrochemically generated H_2O_2 and maltodextrin were more successful in reducing viable biofilm cell density. In comparison to untreated biofilm, this combination resulted in the greatest reduction in

biofilm density. The reduction in viable biofilm cell density by e-scaffold increased with increasing osmolarity in the case of *S. aureus* biofilms, was reaching a maximum reduction at 30 mM maltodextrin. After the maximum reduction in viable biofilm, cell density was established in both biofilms, increasing the maltodextrin concentration inhibited treatment efficacy, most likely by preventing H₂O₂ diffusion.

3.4.2.2 Effect of polyols on biofilm formation by *S. mutans*

Moreover, fermentation of sugars by oral bacteria such as *S. mutans* produces acidic products in a biofilm forming a thin plaque. When the supply of sugar is high in the diet, the formation of biofilm by *S. mutans* increased (Leme *et al.*, 2016). However, sugar alcohol can be introduced as it can aid in diet control, i.e., reducing calorie intake. These sugar alcohols, also known as polyols, can be found in fruits, vegetables, and sugar free- sweeteners (Chan *et al.*, 2020).

In literature by Chan *et al.*, (2020), xylitol and sorbitol have been described as non-cariogenic sweeteners, which have properties that inhibit the formation of cariogenic biofilms (Marttinen *et al.*, 2012). These polyols also have advantages in promoting dental health by reducing cariogenic sugars' consumption, which is beneficial to develop new strategies for preventing this disease. The sugar alcohol i.e., polyols have been reported to decrease biofilm formation of *S. mutans* (Marttinen *et al.*, 2012; Lim *et al.*, 2018; Staszczyk *et al.*, 2020).

This study was carried out to evaluate the effect of polyols on biofilm formation and to determine synergy present, if any, between polyols and CHX. The selection of polyols tested were, mannitol, sorbitol, xylitol, in the concentration ranges of 2 to 40%, in the presence of CHX at a concentration ranges of 0.1 to 4.0µg/ml.

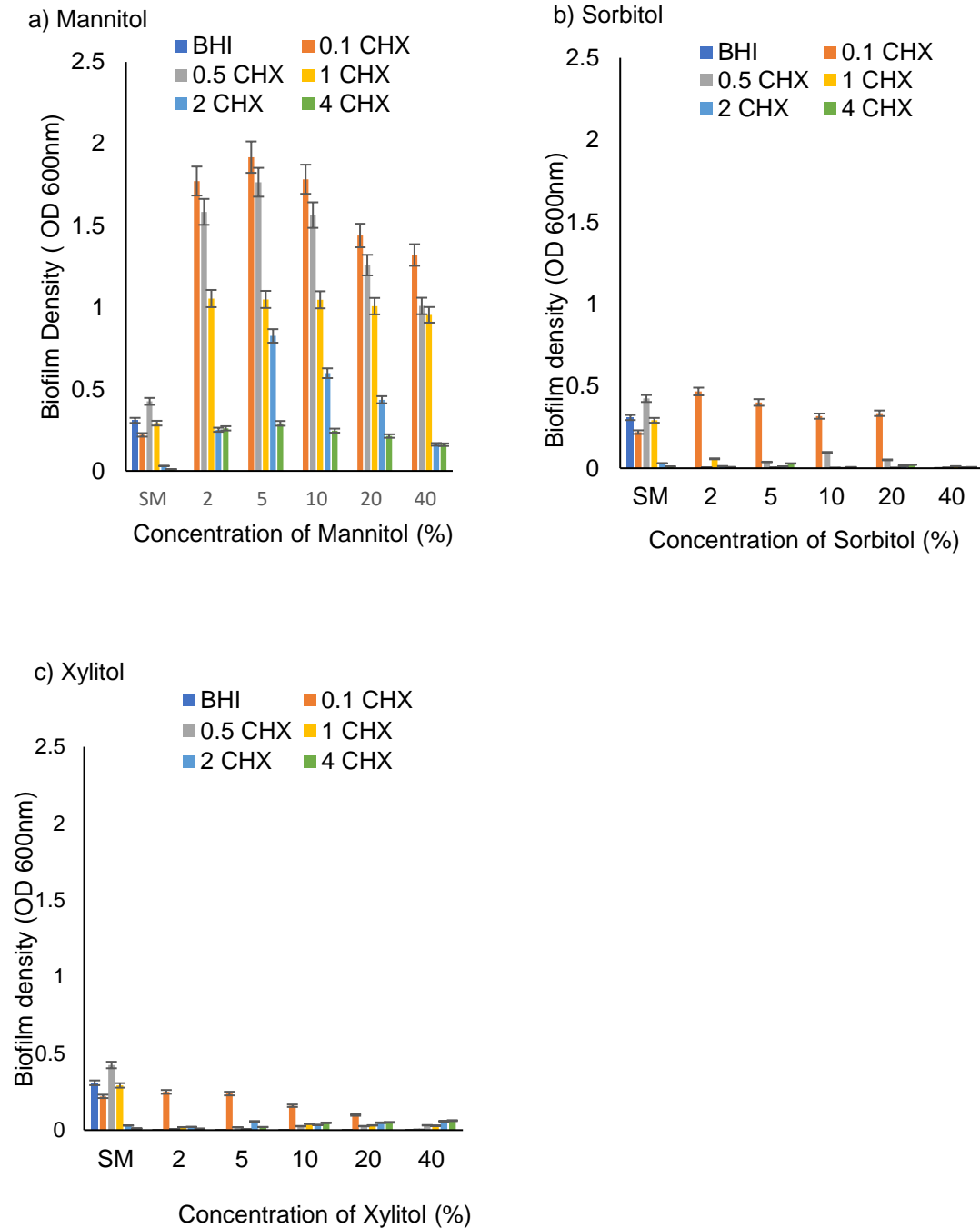


Figure 3.4: Biofilm formation of *S. mutans* at 72 hours and interaction with various polyols concentrations; (a) mannitol, (b) sorbitol and (c) xylitol from concentration of 2, 5, 10, 20 and 40%, and CHX concentration of CHX from (0.1, 0.5, 1, 2 and 4 $\mu\text{g/ml}$) OD 600nm, mean \pm SD, n=3, p<0.05.

Figure 3.4(a) below shows that mannitol enhances the growth of *S. mutans* at the concentrations tested. The results indicate that CHX at the concentration of 0.1 and 0.5µg/ml was not effective for the complete inhibition of biofilm formation. However, the difference between these two groups was statistically significant ($p < 0.005$). At concentration 1µg/ml of CHX, biofilm formation was slightly reduced compared to both concentrations (0.1 and 0.5µg/ml) of CHX. However, CHX at 1µg/ml does not have sufficient strength to completely inhibit the growth of *S. mutans* in the presence of mannitol. As the concentration of CHX increased to 2 and 4µg/ml, both strengths were effectively reducing the biofilm formation in the presence of 5% to 40% mannitol. However, at 40% mannitol, biofilm formation showed no reduction with 2 and 4µg/ml CHX.

When the study was extended using sorbitol, it was clearly evident, Figure 3.4(b) that the presence of sorbitol in addition to CHX decreased the density of the biofilm at a low concentration of 0.5µg/ml of CHX compared to *S. mutans* control. Sorbitol at concentration (2 to 40%) combined with 2µg/ml CHX had a complete inhibitory effect on biofilm formation. In addition, inhibition with 40% sorbitol and CHX at 2µg/ml was similar to inhibition of biofilm with 4 µg/ml of CHX. There showed a significance different between both concentrations, ($p < 0.05$).

At low concentrations (0.1µg/ml) of the antimicrobial agent CHX, xylitol has an inhibitory impact on biofilm formation, Figure 3.4(c). It was clearly visible in the, Figure 3.4(c) that increasing concentration of xylitol from 2 to 40%,

resulted in gradually decreased biofilm density at 0.1µg/ml of CHX. Moreover, between 2% and 40% of xylitol there was 85% decreased in biofilm density at 0.1µg/ml of CHX. However, when comparing 0.5µg/ml concentration of CHX with presence of 10% xylitol, the biofilm inhibition showed a significant difference ($p < 0.014$). There was no substantial improvement in the inhibitory effect on biofilm between in all concentrations of the antimicrobial agent (CHX) as well as in increasing concentration of xylitol compared to control. The explanation for this was that the combination of xylitol and at low concentration of CHX has synergy and resulted in effective inhibition of *S. mutans* biofilm (Söderling *et al.*, 2008). According to Söderling's research, 0.01% xylitol has a very low inhibitory effect on the *S. mutans* biofilm. Furthermore, the lowest level of inhibition was significantly weaker than compared to 0.1 and 1% xylitol. The findings indicate that low xylitol concentrations of 0.1% could inhibit *S. mutans* in vivo.

Besides that, research by Kõljalg *et al.* (2020) revealed the impact of xylitol and erythritol on the growth and biofilm formation of cariogenic bacteria, including a set of clinical *mutans streptococci* and *Scardovia wiggisiae* (*S. wiggisiae*) (Kressirer *et al.*, 2017). This research also evaluated the potential synergistic influence of these two polyols. Both, xylitol and erythritol were discovered to inhibit the growth of cariogenic bacteria. The combination of 10% polyol and an excess of erythritol was found to be synergistic in inhibiting the growth of *S. mutans*. Furthermore, the combination with excess xylitol was more effective in inhibiting the growth of *S. sobrinus* and *S. wiggisiae*. In biofilm inhibition, solutions containing 10% polyols in various

combinations and 15% single polyols were found to be effective against mutans streptococci. Simultaneously, higher biofilm formation of *S. wiggisiae* was detected in different polyol concentrations for up to 34% compared to experiments without polyols. Finally, erythritol and xylitol, as well as their combinations, show inhibited the growth of various cariogenic bacteria.

3.4.2.3 Effect of sugars on dispersal of biofilms formed by *S. mutans*

The mechanism of dispersion of biofilms was described by Kaplan (2010) in three levels: detachment of biofilm cells, translocation of the detached biofilm cells to a new location, and attachment to the current position of the biofilm cells (Kaplan, 2010). In the dispersal study, biofilms of *S. mutans* were initially grown without any added sugar/polyol or antimicrobial agents. The biofilms of *S. mutans* were incubated in 24 well plates for 72 hours and afterwards the sugar/polyol or antimicrobial agent was added and incubated for further 24 hours before the plates were examined. A variable concentration of CHX (0.1, 0.5, 1, 2, and 4µg/ml), sugars and polyols at (2, 5, 10, 20 and 40%) were evaluated on developed biofilm with an objective of assessing the dispersal effect of CHX in the presence of sugars and polyols on biofilms.

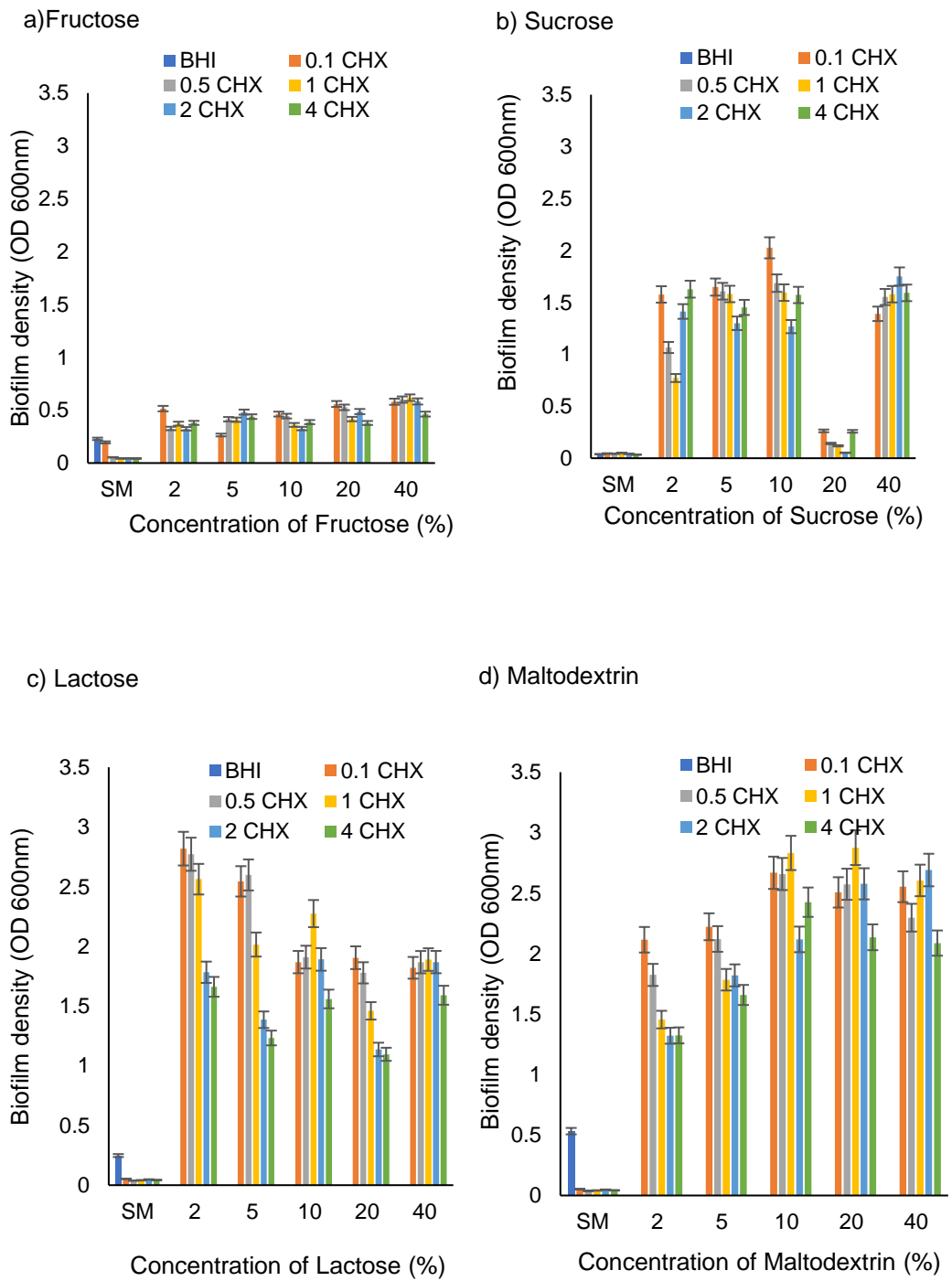


Figure 3.5: Biofilm dispersal of *S. mutans* after 24 hours of exposure to various sugar concentrations; (a) fructose, (b) sucrose, (c) lactose (d) maltodextrin from concentration of 2, 5, 10, 20 and 40%, and CHX concentration of CHX from (0.1, 0.5, 1, 2 and 4µg/ml) OD 600nm, mean \pm SD, n=3, p<0.05.

There was no major difference in the antimicrobial effect in 0.1 to 1µg/ml CHX with the presence of fructose ($p>0.05$). However, the biofilm density was similar with 5 to 40% fructose, and the increased sugar content did not contribute to a decreased biofilm as seen in the [Figure 3.5\(a\)](#). However, there was no significant difference in dispersal effect between 0.1 and 0.5µg/ml CHX ($p>0.05$) with fructose 2% compared to *S. mutans* control. Meanwhile as fructose percentage was increased from 5 to 40% with increased CHX concentration, the biofilm dispersal of *S. mutans* showed similar trend.

[Figure 3.5\(b\)](#) represents sucrose percentage from 2 to 40% on biofilm dispersal. At 20% sucrose with an increase of CHX concentrations from 0.1 to 2µg/ml, there was a decrease in biofilm density of *S. mutans*. However, at this percentage of sucrose only at 2µg/ml showed a decrease in biofilm density compared to control. Therefore, there was a significant difference between the biofilm dispersal of *S. mutans* within all concentration of CHX ($p<0.05$).

The biofilm dispersal of *S. mutans* in the presence of lactose, [Figure 3.5\(c\)](#), showed decreasing trend in biofilm density between concentration 2 and 4µg/ml of CHX. There was a significant difference in the biofilm density of *S. mutans* in the presence of 2 and 5% of lactose when exposed to 2 µg/ml CHX ($p<0.05$). However, there is significant increase in biofilm density at all concentrations of lactose compared to the control.

The [Figure 3.5\(d\)](#), showed the effect of maltodextrin in the biofilm dispersal formation of *S. mutans*. Concentration of CHX at 0.1 and 0.5 µg/ml showed

no significant changes in the presence of all concentration maltodextrin with respect to biofilm dispersal of *S. mutans* ($p>0.05$). Furthermore, when the lowest concentration (0.1 μ g/ml) and the highest concentration (4 μ g/ml) of CHX were compared in the presence of 2% maltodextrin, it was shown that the antimicrobial effect also was not significant ($p>0.05$). Meanwhile, when maltodextrin concentrations, increased from 2% to 40% with the presence of all antimicrobial agent concentrations there was a significant increase in biofilm density when compared to control ($p<0.005$). According to Guilhen *et al.*, (2017), biofilm dispersal includes numerous complicated ways for dissemination of bacteria with distinct features which could be obtained through EPS degradation or protein processing in the biofilm structure.

3.4.2.4 Effect of Polyols on Biofilm dispersal by *S. mutans*

Figure 3.6 shows that polyols inhibit biofilm dispersal of *S. mutans* by CHX.

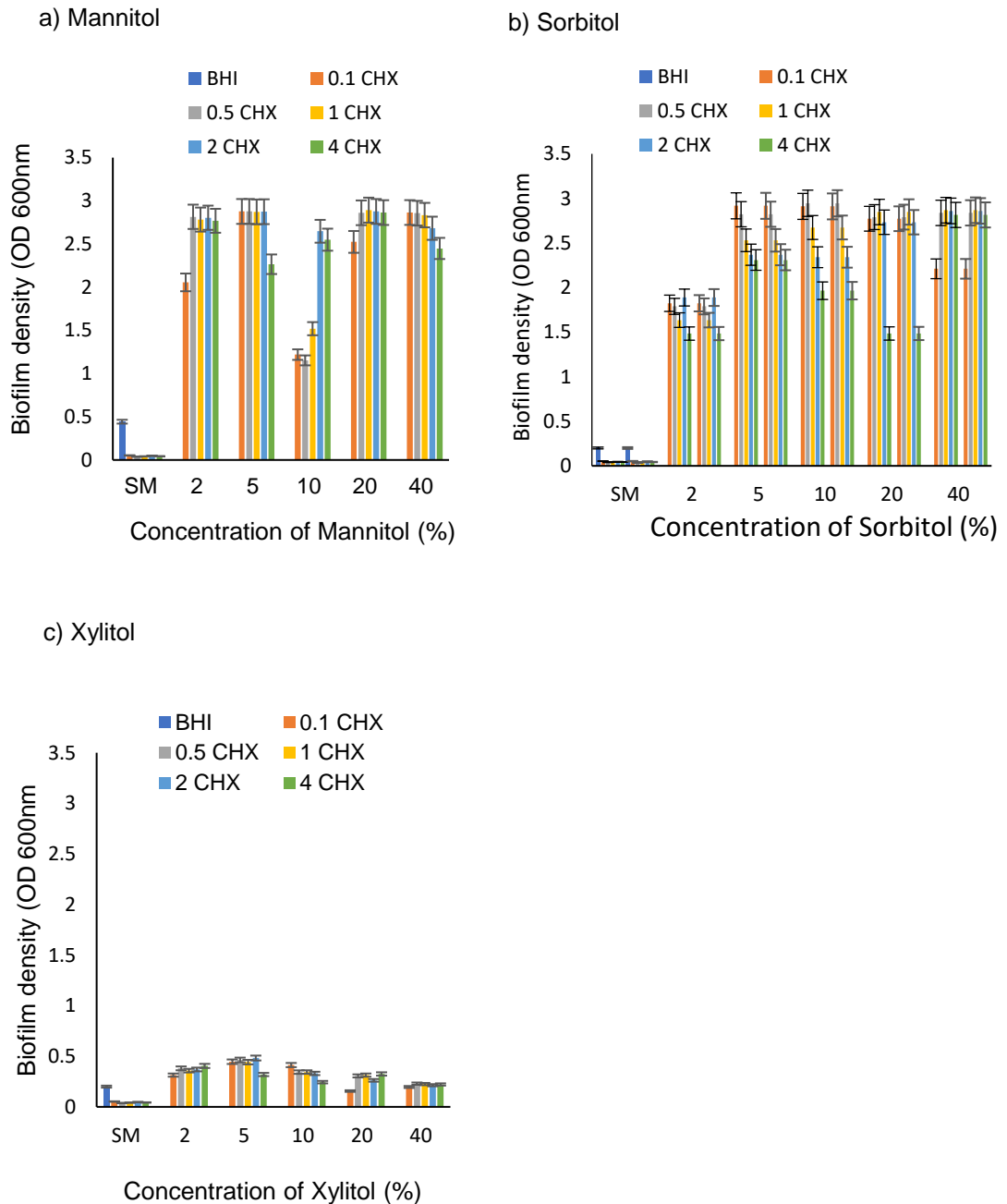


Figure 3.6: Biofilm dispersal of *S. mutans* after 24 hours of exposure to various polyols concentrations; (a) mannitol, (b) sorbitol and (c) xylitol from concentration of 2, 5, 10, 20 and 40%, and CHX concentration from (0.1, 0.5, 1, 2 and 4 µg/ml), OD 600nm, mean ±SD, n=3, p<0.05.

Figure 3.6(a) shows a concentration of mannitol increases from 2% to 40%, biofilm density increases as well as with increase in the concentration (0.1-4µg/ml) of CHX. Only at 10% of mannitol shows a decreased in biofilm density between 0.1 and 0.5µg/ml of CHX concentration. The difference between these two groups was statistically significant ($p < 0.05$). Moreover, at 10% of mannitol with concentration of CHX 2 and 4 µg/ml, both concentrations were reduced by 10% of the biofilm density of *S. mutans*. Hence, CHX treatment at the presence of 10%mannitol, did result in significant changes in biofilm dispersal of *S. mutans* ($p < 0.05$). There were changes in the overall results compared to the control as the concentration of mannitol and CHX increased.

As percentage concentration of sorbitol, was increased Figure 3.6(b), from 5% to 20%, only at high concentration of CHX (4µg/ml) showed a decrease in the biofilm density of *S. mutans*. The difference between these three concentrations (5, 10 and 20%) of sorbitol showed a statistically significant difference ($p < 0.05$). At 5% of sorbitol, the graph showed biofilm dispersal decreased as increases of concentration of CHX from 0.1 to 4µg/ml. This concentration of sorbitol within all concentrations of CHX shows a significance difference ($p < 0.05$). The overall effect shows that as sorbitol concentration increases, biofilm density increases even in the presence of increasing concentration of CHX except when CHX is at 4µg/ml where a decrease in biofilm density is observed at 20% sorbitol concentration.

Figure 3.6(c) demonstrated the effect of xylitol on biofilm dispersal of *S. mutans* in combination with CHX at different concentrations. At 10% of

xylitol, the biofilm density decreased with increase in concentration of CHX from 0.1 to 4µg/ml. The difference between these CHX concentrations were show statistically significant ($p<0.05$). However, with increase in concentration of xylitol from 5 to 40%, the graph showed there were decreased in biofilm density at 1µg/ml of CHX and changes were statistically significant ($p<0.05$). Meanwhile, biofilm dispersal with 2 µg/ml of CHX has decreased biofilm density from 5 to 40% of xylitol.

Through microarray research, Liu *et al.*, (2017) identified a nuclease (DeoC) as a *S. mutans* biofilm dispersal modifying component by neutrophil extracellular traps (NETs) . The DeoC deletion mutants showed a dispersal deficiency in *in vitro* experiments, while functional analyses with pure protein indicated that DeoC has biofilm dispersal potential. Neutrophils are an important host response factor that prevents bacteria from spreading by forming neutrophil extracellular traps (NETs), which are made up of a nuclear DNA backbone and antimicrobial peptides. Dispersed *S. mutans* would use DeoC to degrade NETs and prevent immune system detection. *S. mutans* caused NET formation when it interacted with neutrophils, and the presence of NETs increased *S. mutans'* DeoC expression. Study by Berkowitz and Jones, (1985) reported *S. mutans* can be transmitted to an infant in the oral cavity when it breaks free from dental biofilms in a mother's mouth (Berkowitz and Jones, 1985). Salivary flow can also transport detached *S. mutans* cells to surrounding teeth (Liu *et al.*, 2017). Through gingival incisions after dental surgery, dispersed *S. mutans* from biofilm can easily enter the bloodstream and cause infective endocarditis (Jung *et al.*, 2015). Different host defence mechanisms exert endogenous inhibition of *S. mutans*

development, with neutrophils being a significant host component in the response to bacterial infection. For pathogen removal, activated neutrophils produce NETs, which include antimicrobial proteins attached to a DNA scaffold.

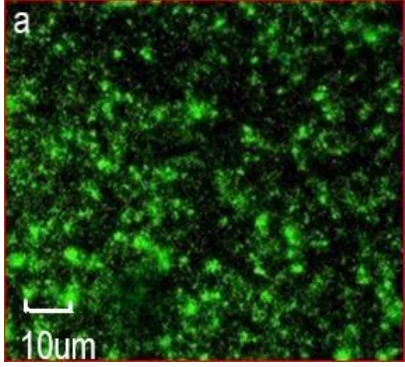
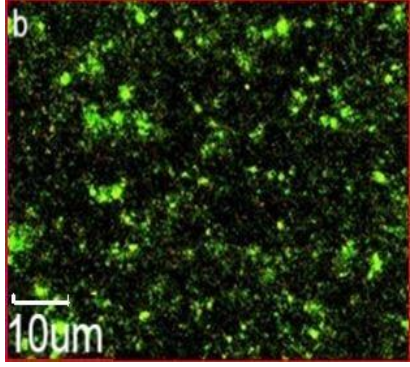
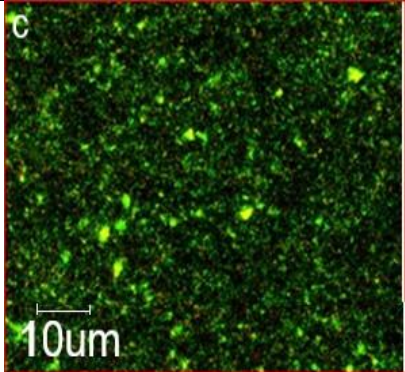
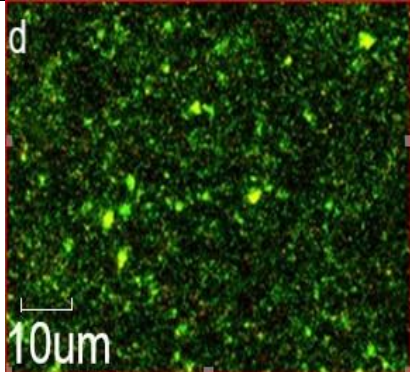
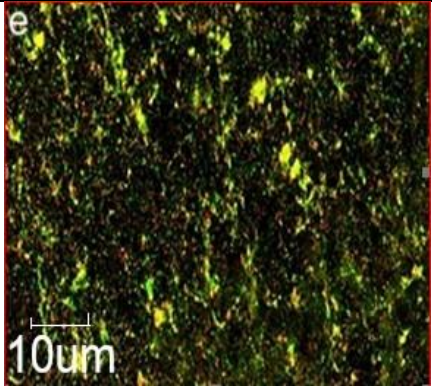
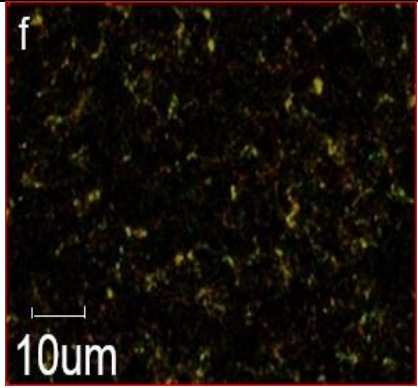
The production of various exo-enzymes to degrade the EPS, such as proteases and nucleases, is the primary strategy used by various bacterial species to actively disperse biofilm (Lister and Horswill, 2014). According to their finding, a deoxyribonuclease encoded by DeoC as a potential active component in *S. mutans* biofilm. The importance of DeoC in biofilm dispersal was first demonstrated by a *S. mutans* DeoC mutant's reduced capacity to disperse cells, however this ability was not fully lost. Because *S. mutans* contains a number of other putative ribonucleases and endonucleases (Ajdic *et al.*, 2002), they hypothesized that the partial biofilm dispersal ability of the DeoC mutant was due in part to the presence of these nucleases. Our recent study has shown that polyols exhibit no dispersal efficacy with increase in polyol concentrations.

3.4.3 Morphological Characterization and Vitality Analysis of *S. mutans* biofilms formed by Confocal Laser Scanning Microscopy (CLSM) upon exposure to sugars, polyols and CHX

The CLSM-based analyses of the microbial vitality of aerobic *S. mutans* was performed after 72 hours biofilm inhibition formation. In addition to the microbiological sample testing, sugars, polyols, and CHX concentrations, were added into the biofilm of *S. mutans* to quantify and investigate their biofilm structures. This technique also determined the architecture and distribution of vital cells within the oral biofilm. It has been refined by treating viable and nonviable bacteria as distinct populations and comparing the spatial distribution trends between these two components to produce vitality profile. This method was adopted from Mizuho *et al.*, and Decker *et al.*, (Mizuho *et al.*, 2006; Decker *et al.*, 2014).

Biofilm of *S. mutans* structures that were obtained from CLSM indicated that biofilm formation has mixtures of compounds such as extracellular polysaccharides (EPS), proteins and other cells. *S. mutans* produce microcolonies separated by water channels, which allow soluble nutrients to reach cells deep within the biofilm and metabolic waste products to disperse (Motegi *et al.*, 2006). *S. mutans* are the predominant acidogenic component of dental plaque, metabolizing food energy such as sugar or polyols and producing lactic acid as a by-product (Lemos *et al.*, 2019). Eventually, lactic acid accumulation in the plaque biofilm triggers a localized pH decrease followed by demineralization of tooth enamel, indicating the beginning of dental caries.

For morphological characterisation and viability analysis by CLSM, *S. mutans* biofilm was incubated for 72 hours in 6 well plates. The results showed very thick biofilm formation and clusters of live cells as in [Figure 3.7\(a\)](#) which is indicated as the control biofilm of *S. mutans*. CLSM images found *S. mutans* biofilm control group demonstrated viable bacteria as all colonies remained green stained with (S9) fluorescence indicating that *S. mutans* were viable and intact during the period evaluated. Non-viable/ dead colonies, red colonies stained with (PI) were seen after treated with variable concentration of CHX, polyols and sugars. There was a decrease in the number of *S. mutans* viable cells observed at different concentrations of CHX (0.1 and 0.5µg/ml), xylitol at (2, 5, 40 %w/v of concentration) and fructose at 20 and 40% of concentrations. The colour of *S. mutans* colonies changed to a yellowish or orange (red) indicated the death of *S. mutans* after exposure to concentrations of CHX with 5%w/v xylitol ([Figure 3.7\(f\)](#)) and 40% xylitol ([Figure 3.7\(h\)](#)); fructose 20% ([Figure 3.7\(j\)](#)) and 40% ([Figure 3.7\(l\)](#)). Biofilm formation of *S. mutans* in media containing fructose was thicker than that in media containing polyols ($p < 0.05$). The biofilms formed in media containing polyols appeared to consist of homogeneous clumps and larger microcolonies, whereas biofilms formed in media containing fructose showed a more uniform, reticulated structure (Decker *et al.*, 2014). However, these biofilm formations showed a gradual decrease of viable bacterial cells of *S. mutans* with increase in amount of CHX (Loimaranta *et al.*, 2020).

	Control	+ treated with CHX 0.1 µg/ml
<i>S. mutans</i> only	<p>a</p>  <p>10µm</p>	<p>b</p>  <p>10µm</p>
	Control	+ treated with CHX 0.5 µg/ml
<i>S. mutans</i> with 2% Xylitol	<p>c</p>  <p>10µm</p>	<p>d</p>  <p>10µm</p>
	Control	+ treated with CHX 0.1 µg/ml
<i>S. mutans</i> with 5% Xylitol	<p>e</p>  <p>10µm</p>	<p>f</p>  <p>10µm</p>
	Control	+ treated with CHX 0.1 µg/ml

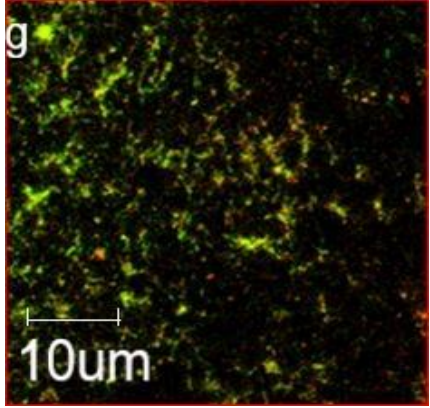
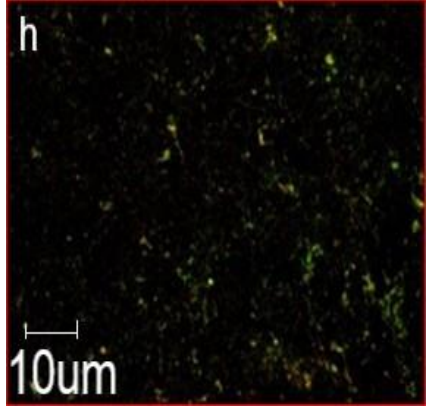
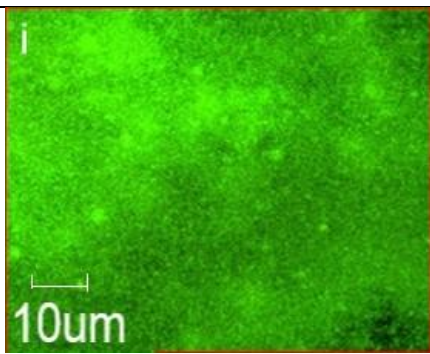
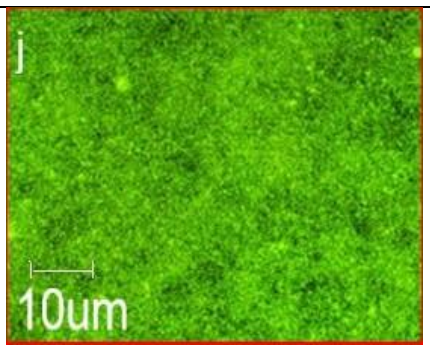
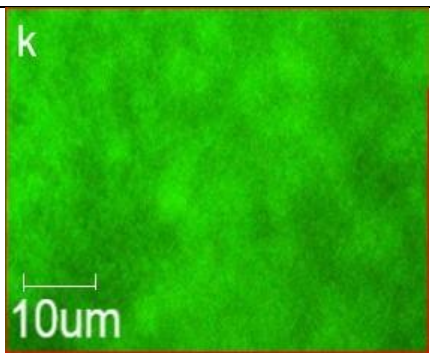
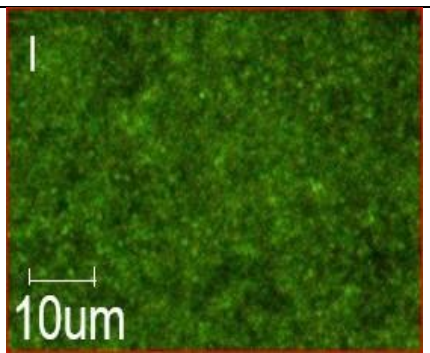
<p><i>S. mutans</i> with 40% Xylitol</p>	<p>g</p>  <p>10µm</p>	<p>h</p>  <p>10µm</p>
	<p>Control</p>	<p>+ treated with CHX 1.0 µg/ml</p>
<p><i>S. mutans</i> with 20% Fructose</p>	<p>i</p>  <p>10µm</p>	<p>j</p>  <p>10µm</p>
	<p>Control</p>	<p>+ treated with CHX 0.1 µg/ml</p>
<p><i>S. mutans</i> with 40% Fructose</p>	<p>k</p>  <p>10µm</p>	<p>l</p>  <p>10µm</p>

Figure 3.7: CSLM images of biofilm formation of *S. mutans* biofilm grown in BHI medium (a) control (b) treated with 0.1µg/ml CHX, (c) treated with 2% xylitol (d) treated 2% xylitol and 0.5µg/ml CHX, (e) treated with 5% xylitol (f) treated with 5% xylitol and 0.1µg/ml CHX, (g) treated with 40% xylitol (h) treated with 40% xylitol and 0.1µg/ml CHX, (i) treated with 20% fructose (j) treated with 20% fructose and 1.0µg/ml CHX, (k) treated with 40% fructose (l) treated with 40% fructose with 0.1µg/ml CHX. Stained with S9 (live cell) and PI (dead cell). Scale bar: 10µm with magnification 60X. Mean=3, p<0.05.

When the *S. mutans* biofilm was treated with 1.0µg/ml of CHX, [Figure 3.7\(b\)](#), the patches of live cell (green) were getting less with increase in concentration of CHX to 2.0µg/ml. However, when biofilm of *S. mutans* was exposed to xylitol, it was inhibited the viability of *S. mutans*. This result was supported by Decker *et al.*, 2014. They evaluated the vitality of biofilms formation at 1% of xylitol and reported a decrease in the biofilm formation (Decker *et al.*, 2014).

Fructose was a chosen to be a dietary cariogenic sugar compared to sucrose, lactose and maltodextrin as concluded in Chapter section 3.4.2.1. This was due to *S. mutans* biofilm formation showed low optical density with fructose when compared to the sugars (Bray *et al.*, 2004). [Figure 3.7\(i\)](#) showed at 20% fructose increases cell vitality in all biofilm layers when compared to 40%, [Figure 3.7\(k\)](#). However, when both concentrations of fructose (20% and 40%) treated with 0.1ug/ml of CHX, the viability cell in biofilm formation were decreased. Viability of dead cell showed in [Figure 3.7\(j\)](#) and [Figure 3.7\(l\)](#) revealed that fructose would support adherence of the cells to the surface in a biofilm (Ma *et al.*, 2013). According to the study undertaken by Ma *et al.*, (2013) the cariogenicity of *S. mutans*, in the presence of fructose, may vary considerably from its cariogenicity in the presence of sucrose. Sucrose is known as the most potent cariogenic sugar because of approach it promotes *S. mutans* colonization and accumulation. The authors reported that *S. mutans* biofilms grown in presence of fructose had a higher acidogenicity than those grown in the presence of sucrose. Therefore, *S. mutans* biofilm formation increased acidogenic characteristics in the presence of fructose however sucrose is more cariogenic as the

biofilm formed in presence of sucrose was thicker when compared to fructose. Result from [Figure 3.7](#) was quantified using Image J software and [Table 3.1](#) shows a summary of percentage vitality of *S. mutans* biofilm.

3.4.4 Evaluation of biofilm formation of *S. mutans* using CLSM

This study helped estimate cell vitality during biofilm formation. [Table 3.1](#) shows the calculation of vitality of samples of biofilm using equation, (Decker *et al.*, 2014):

$$\text{Percentage of vitality} = \frac{[\text{vital, (S9, green) bacterial}]}{[\text{vital, (S9, green) + dead (PI, red) bacterial}]} \times 100\%$$

Table 3.1: Shows vitality of *S. mutans* biofilms as a percentage at various combinations of selected sugars and polyols with CHX. The samples were taken after biofilm formation by *S. mutans* in the presence of CHX at a concentration of 0.1, 0.5, 1.0, 2.0 and 4.0 µg/ml and 2, and 5 % xylitol, 40% sorbitol and 20% and 40% fructose. Mean, (n=3), ±SD.

Samples of Biofilm Formation	Green Cell count	Red Cell count	Vitality %
<i>S. mutans</i>	70124	41633	63
<i>S. mutans</i> with 0.1µg/ml CHX	5752	5056	53
<i>S. mutans</i> with 0.5µg/ml CHX	6347	10246	38
<i>S. mutans</i> with 1.0µg/ml CHX	10037	18410	35
<i>S. mutans</i> with 2.0µg/ml CHX	2983	6427	32
<i>S. mutans</i> with 4.0µg/ml CHX	10783	29128	27
<i>S. mutans</i> +2% Xylitol	9863	10109	49
<i>S. mutans</i> + 2% Xylitol + 0.5 µg/ml CHX	10711	12594	46
<i>S. mutans</i> + 5% Xylitol	6798	36743	16
<i>S. mutans</i> + 5% Xylitol 0.1 µg/ml CHX	7598	52347	13
<i>S. mutans</i> +40% Sorbitol	19629	59027	25
<i>S. mutans</i> +40% Sorbitol 1.0 µg/ml CHX	11737	43985	21
<i>S. mutans</i> +20% Fructose	10974	12696	46
<i>S. mutans</i> +20% Fructose +1.0 µg/ml CHX	10943	14972	42
<i>S. mutans</i> +40% Fructose	9394	15535	38
<i>S. mutans</i> +40% Fructose +0.1 µg/ml CHX	6630	14640	31

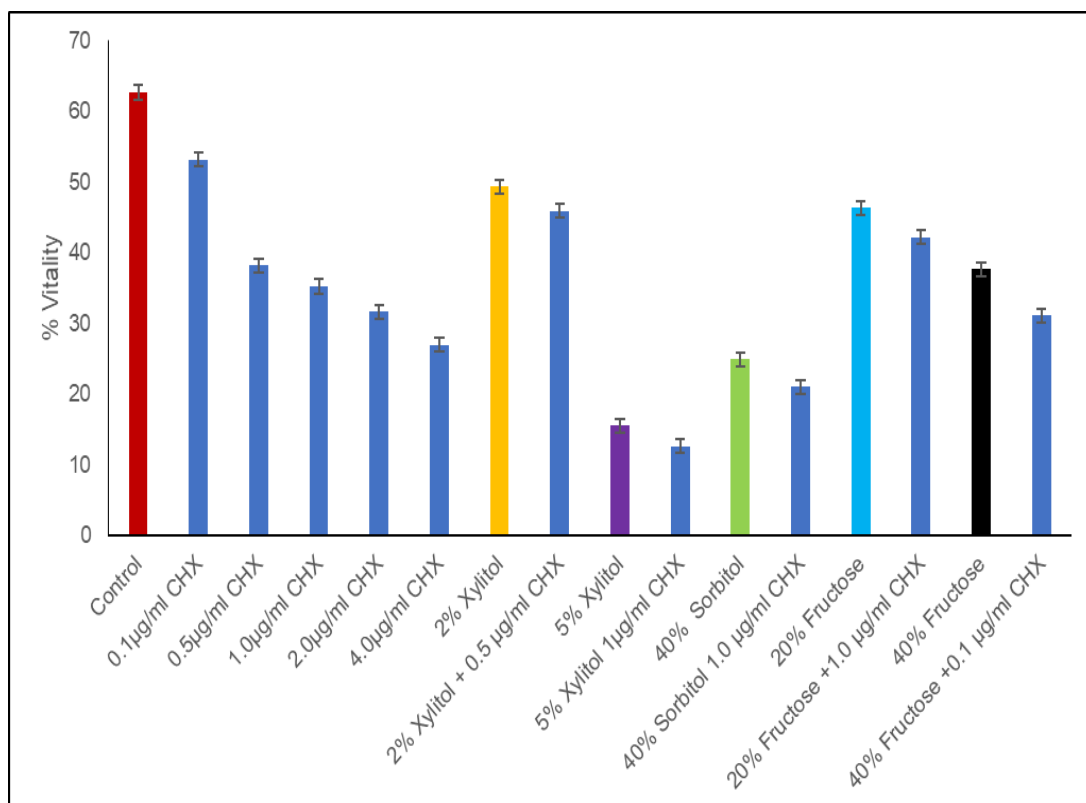


Figure 3.8: Evaluation of percentage vitality biofilm formed by *S. mutans* using CLSM. The control of *S. mutans* was compared to samples exposed to concentration of CHX (0.1, 0.5, 1, 2 and 4 µg/ml) where 2% and 5% of Xylitol, 40% Sorbitol and 20% and 40% Fructose. Mean, n=3, ±SD, p<0.05.

In the present study, cell vitality of *S. mutans* biofilm was estimated using Image J software and expressed as a percentage as indicated in Figure 3.8. The findings of the present study indicated that *S. mutans* control biofilm formation showed the highest percentages of vital cells, 63%. However, with increase in concentration of CHX, the vitality of cells in biofilm decreases. When 2% xylitol was added to *S. mutans* control, there is also 14% decrease of vitality. Moreover, 2% xylitol was added with 0.5 µg/ml of CHX, the vitality was further decreased by 3% compared to control. Following increases of percentage of xylitol 5% in biofilm of *S. mutans*, there was a big decrease in vitality of from 63% to 16% between both control and treated. However, when 5% xylitol biofilm was combined with CHX concentration at 1.0 µg/ml,

the vitality showed a decrease to 13%. This showed that a huge decrease in percentages of vitality of *S. mutans* biofilm with increased of CHX concentration. Similar findings are published by Decker *et al.*, (2014), where the authors reported the vitality of biofilms grown in xylitol and was statistically decreased with increase in concentration of CHX. This is due to the availability of carbohydrate substrates decreased and a declining nutrient concentration. In additional, the authors revealed that the EPS production in biofilm formation was exposed to xylitol. The ability of *S. mutans* to produce EPS is influenced by growth stages, nutritional availability, and environmental factors (Pal and Paul, 2013). The EPS production was decreased when *S. mutans* was exposed to xylitol.

Meanwhile, sorbitol with concentration of 40% showed 25% vitality during biofilm formation by *S. mutans*. This sample was treated with 1.0µg/ml of CHX also showed 4% of vitality decreased from untreated sample. This is because sorbitol inhibited *S. mutans* production of acid (Takahashi-Abbe *et al.*, 2001). These findings show that an elevated NADH/NAD ratio during sorbitol metabolism caused by oxygen inactivation of pyruvate formate-lyase hindered glyceraldehyde-phosphate dehydrogenase, which in turn prevented acid generation in *S. mutans* and dental plaque. This *S. mutans* biofilm formation treated with sorbitol *in vivo* is anticipated to proceed in a similar way as *in vitro*. There is also the possibility that sorbitol inhibits the sugar metabolism of other plaque bacteria.

Selection of fructose with 20% and 40% concentration also showed decrease in vitality as the percentages of sugars increased. Both sugars treated with 1.0 µg/ml showed the vitality decreased 4% and 7% after *S. mutans* biofilm formation, respectively. Additionally, as an increases concentration of fructose with combination of CHX showed a significantly decreased in biofilm. Ma *et al.*, (2013) undertook studies to characterise adherence and biofilm formation by *S. mutans* grown *in vitro* in media containing fructose. These results are in agreement with show decreased in percentage of adherence and decreased biofilm formation of *S. mutans* in comparison to the presence of sucrose.

In this study, synergism was observed when CHX combined with different concentrations of xylitol was tested on the biofilm formation of *S. mutans*. The inhibition was greater when the antimicrobial agent, CHX and xylitol were used in combination rather than CHX alone. This characteristic of xylitol is supported by Bader (Bader *et al.*, 2013), which concluded that xylitol did not only show inhibitory effects on the formation of biofilm, but it prevents the formation of multi-species biofilm formation. However, the effect of combination between CHX concentrations and xylitol exposures on the *S. mutans* formation of biofilms was also determined from the Paula *et al.*, study. This study was confirmed that combination 1% of CHX-xylitol in chewing gum exposure to biofilm, resulted an efficient treatment in controlling biofilm and suppressing *S. mutans* (Paula *et al.*, 2010). Furthermore, study from Marya *et al.*, (2017) reported combination CHX and xylitol have significant efficacies in a mouthwash against *S. mutans* showed a decreases in plaque index and gingival index (Marya *et al.*, 2017).

3.5 Conclusion

Results from inhibition and dispersal biofilm assays of *S. mutans* with sugars and polyols showed that inhibition of biofilm formation with polyols was affected more clearly. According to the results on biofilm formation with sugars, only fructose at 40% combination with a lower concentration of CHX, (0.1µg/ml) resulted in lower biofilm density when compared to lower concentrations of the sugar or high concentration of CHX alone. Furthermore, biofilm formation by *S. mutans* with polyols (mannitol, sorbitol, and xylitol) supported significantly less biofilm formation when compared to sugars (fructose, sucrose, lactose, and maltodextrin) in combination with CHX concentration. At 0.1µg/ml CHX was successful in inhibiting biofilm formation at a concentration of with both 20% sorbitol and xylitol. Regardless of concentration, polyols (sorbitol and xylitol) were found more effective than sugars in inhibiting the biofilm growth of *S. mutans*. The findings revealed that polyols exhibited greater inhibitory effect on biofilm formation than dispersal effect because polyols are present from the initiation of biofilm formation and therefore are in contact for longer duration of time during inhibitory assay (72 hours) as opposed to dispersal assay where they are in contact with *S. mutans* for 24 hours after biofilm formation has taken place.

In addition, xylitol, and sorbitol at 2-40% concentration resulted in significantly less in *S. mutans* biofilm formation when combined with CHX at concentration 0.5µg/ml. These biofilms have a variety of attributes that contribute synergistically to the process of antibiotic resistance (Dincer *et al.*, 2020; Wilton *et al.*, 2015). These attributes include, but are not limited to an

exopolysaccharide matrix, pH, and nutrients (Decker *et al.*, 2014). In this study indicates that both sorbitol and xylitol have synergistic inhibitory effects with CHX at low concentrations on biofilm formation. Therefore, xylitol and sorbitol are a good choice of excipients in the formulation of CHX oral dosage forms.

Furthermore, the presence of CHX disrupted the biofilm of *S. mutans*, resulting in a reduction in biofilm density. This is because when polyol is combined with CHX, a synergistic mechanism is demonstrated. Because polyols have anti-microbial activity, they were used to combine with CHX, resulting in a synergistic effect (Nayak *et al.*, 2014).

In summary, the study revealed that the biofilm formation of pathogenic bacteria, *S. mutans* could be efficiently killed by corresponding treatment with low concentration of xylitol and CHX. This strategy for the control of pathogenic biofilms has proved that synergistic effect between xylitol and CHX through vitality observation of CLSM images. The interaction between xylitol and CHX showed a significant effect in the inhibition of biofilms, as they disrupted the biofilm as well as having bacteriocidal effect on cells by effecting their vitality.

CHAPTER FOUR

**Extra Polymeric Substance (EPS) Matrix Evaluation and
Semiquantitative RT-PCR analysis to assess the expression
levels of multiple genes during biofilm formation by**

S. mutans

4.1 EPS Matrix

S. mutans virulence enables accumulation of cariogenic microorganisms in the oral cavity that result in dental demineralization. The most widely studied virulence factor from this disease is the accumulation by *S. mutans* in an extra polymeric substance (EPS) matrix molecules. The EPS matrix interacts by attaching the biofilm onto the surface and allowing any dissolved substances and nutrients from environment to diffuse into the biofilm and aid biofilm formation (Watnick and Kolter, 2000). Besides that, EPS formation is genetically controlled as differential gene expression regulates its composition and development in biofilm state.

4.1.1 EPS Role and significance

There are several virulence factors and biological and physiological processes such as EPS synthesis, adherence to tooth surface and biofilm formation by *S. mutans* responsible for its pathogenesis. Cells that are present in this biofilm potentially accompany major changes in gene expression patterns, which are unique compared to planktonic environment. The established cariogenic characteristics of *S. mutans* biofilm are regulated by various genes involved in five important metabolic pathways: microbial adhesion (Shemesh *et al.*, 2007) biofilm formation (Banu *et al.*, 2010), EPS production (Senadheera *et al.*, 2005), carbohydrate uptake (Sztajer *et al.*, 2008) and acid tolerance (Gong *et al.*, 2009).

S. mutans can be attached to substrates by sucrose-independent and sucrose-dependent mechanisms (Banas, 2004). Many sucrose-independent

adhesions factors are expressed by *S. mutans* and facilitates the attachment to the pellicle (Wan *et al.*, 2003; Lévesque *et al.*, 2005). The pellicle is a film that coats tooth surface and is made from salivary proteins and bacterial exoenzymes such as glucosyltransferases (*gtfs*). *Gtfs* are enzymes which are multifunctional as they are not only part of the pellicle but also adsorb to surfaces where they synthesise glucans from sucrose. The formation of extracellular glucans by exoenzymes such as glucosyltransferases (*gtfs*) in *S. mutans* involves sucrose-dependent adhesion. The extra cellular glucans are part of the biofilm matrix and offer adhesion sites for oral bacteria (Koo *et al.*, 2013). In addition cariogenicity of *S. mutans* is enhanced by binding with the aid of glucan binding protein (*GbpA*, *GbpB* and *GbpC*) (Lynch *et al.*, 2007; Lynch *et al.*, 2013).

4.1.2 Semiquantitative RT-PCR analysis

Changes in the expression levels of *gtfB*, *gtfC*, *glt*, *glk*, *comD*, *sacB*, *wapA*, *smu_104*, *smu_105*, *smu_307c*, and *vicR* genes involved in the development of *S. mutans* biofilm when exposed to different experimental conditions were investigated using semi- quantitative RT-PCR analysis and agarose gel electrophoresis and were compared to their expression levels in the biofilm formation state. RT-PCR is a highly sensitive and specific method to detect rare transcripts or analysing samples in limited quantities (Carding *et al.*, 1992).

The aim of this study was to determine levels of expression of target genes (*comD*, *gtfB*, *gtfC*, *gltA*, *glk*, *sacB*, *vicR*, *wapA*, *smu_1037c*, *smu_104* and *smu_105*) under different biofilm formation conditions when exposed to

xylitol and CHX compared to *S. mutans* biofilm control sample using semi-quantitative RT-PCR analysis.

4.2 Materials and chemicals

The reagents used in the investigation of EPS formation and semi-quantitative RT-PCR analysis are listed below:

- SYTO 9, (S9), and Alexa Fluor 633 were purchased from Life Technologies, (U.K)
- Qiagen RNA extraction kits GmbH, Hilden, (Germany). HotStart Taq master mix bought from Qiagen, (UK)
- Transcriptor High Fidelity cDNA Synthesis kits were purchased from Roche, (Germany).
- The oligo primers were purchased from Sigma, (U.K).
- Agarose gels, , Nancy500 and TAE buffer was purchased from Sigma Aldrich, (U.K)
- 1.5 kb DNA ladder molecular weight marker was purchased from (New England Biolabs, (U.K)
- 6-well flat-bottom and polystyrene plates were purchased from Sarstedt, (U.K).

4.3 Methods

4.3.1 EPS Evaluation during biofilm formation using Confocal Laser Scanning Microscopy (CLSM)

Biofilm inhibition assay as described in method section 3.3.2 was prepared using 5 ml of diluted 1×10^7 colony forming units (CFU)/mL of *S. mutans* added into each 6 well flat-bottom polystyrene plate (Sarstedt, Leicester, U.K.) followed by incubation overnight at 37°C for 72 hours in a static incubator. The incubated plates are washed with 0.9% saline according to the manufacture instructions (Fisher Scientific, U.K) instead of phosphate buffer as phosphate buffer will interfere with staining.

Working solution were prepared by using 5 µl of SYTO 9 stain at 5µmol/L concentration (Life Technologies, U.K) and 3 µl of Alexa Fluor 633, labelled as lectin Concanavalin A, (Con A) at concentration of 0.96µmol/l (Thermo Fisher Scientific, U.K.) and combined into 5ml of filtered-sterilized water (see in Appendix A4.1). One coupon or well only requires 200 µl of stain diluted in water. Transfer 200 µl of staining solution gently onto the biofilm sample to avoid disturbance on the biofilm. The plates were covered with lid and the sample incubated for 20 minutes at room temperature protected from light. After incubation, the samples were gently rinsed with filtered- sterilized water to remove excess of the stains. The plates were inverted to allow them to dry before CLSM analyses. The fluorescent dyes were excited by the argon laser (488 nm, for S9) and the Helium Neon (HeNe) laser (633 nm, EPS-Alexa Fluor 633)(Con A). Con A is a marker for glycoconjugates, which are essential components of EPS, and binds selectively to -glucopyranosyl

molecules such as glucans. The samples were prepared in duplicate for optimisation. The images were analyzed to calculate percentage EPS production using the formula as shown in Eq 1, (Decker *et al.*, 2014):

$$\% \text{ EPS Production} = \frac{[\text{EPS} - \text{related fluorescent (Con A red)}]}{[\text{bacteria (S9,green)} + \text{EPS} - \text{related fluorescent (Con A,red)}]} \times 100$$

(Eq 1)

4.3.2 Extraction and quantification of total RNA

S. mutans overnight cultures and biofilm were prepared as described in method sections 2.2.1 and 3.3.1., Isolation, extraction, and quantification of total RNA from each culture was performed according to the manufacturer's instructions (Qiagen RNA extraction kits GmbH, Hilden, Germany), see further procedures in Appendix A4.2. The results from RNA extraction for each sample using the NanoDrop spectrophotometer as shown in Table 4.1.

Table 4.1: RNA samples extracted from planktonic and biofilm of *S. mutans* cells with xylitol and CHX from Nanodrop

	Samples	Nucleic Acid (concentration (ng/μl))	A260	A280	Yield: A260 /A280
1	<i>S. mutans</i> Planktonic	35.1	1.804	0.855	2.109
2	<i>S. mutans</i> Biofilm	17.2	1.32	0.644	2.038
3	<i>S. mutans</i> Biofilm + 1% Xylitol	22.2	2.492	1.312	1.899
4	<i>S. mutans</i> Biofilm + 5% Xylitol	16.4	2.228	1.022	2.180
5	<i>S. mutans</i> Biofilm + 1% Xylitol + 0.25μg/ml CHX	18.2	2.063	0.955	2.160
6	<i>S. mutans</i> Biofilm + 1% Xylitol + 0.50μg/ml CHX	19.4	1.686	0.796	2.118
7	<i>S. mutans</i> Biofilm + 5% Xylitol + 0.25μg/ml CHX	28.6	2.442	1.326	1.842
8	<i>S. mutans</i> Biofilm + 5% Xylitol + 0.5 μg/ml CHX	35.0	2.523	1.205	2.093
9	<i>S. mutans</i> Biofilm + 0.25 μg/ml CHX	27.0	1.521	0.785	1.938
10	<i>S. mutans</i> Biofilm + 0.50 μg/ml CHX	30.2	2.142	1.026	2.087

4.3.3 Complementary DNA (cDNA) synthesis

Transcriptor High Fidelity cDNA Synthesis kit was purchased from Roche, (Germany). Total RNA (3µg of from each sample) was prepared as described in the Methods section 4.3.2. To each RNA sample, 1µl of Anchored- oligo(dT)18 Primer at a concentration of 50 pmol/µl and 11.4µl of water, PCR Grade were added. All the reagents were mixed well by pipetting and centrifuged briefly. The template-primer mixture was denatured by heating the tube for 10 min at +65°C in a block cycler PTC-200 Peltier Thermal Cycler (MJ research laboratories) with a heated lid (to minimize evaporation). This step ensures denaturation of RNA secondary structures. Following incubation, samples were immediately cooled on the ice. In each sample mixture, 4µl of 5X concentrate transcriptor High Fidelity Reverse Transcriptase Reaction Buffer 0.5 µl Protector RNase Inhibitor (40 U/l), 2 µl of deoxynucleotide mix (10 mM dNTP), 1 µl DTT and 1.1 µl Transcriptor High Fidelity Reverse Transcriptase were added to a final volume of 20µl. Each sample mixture was then transferred in a thermal block cycler, PTC-200 Peltier Thermal Cycler (MJ research laboratories), with a heated lid to minimize evaporation and samples were heated at +85°C for 5 min to inactivate transcriptor high fidelity reverse transcriptase and then at +45° C 30 min. Following incubation, the samples were placed on ice prior to RT-PCR or stored at -20°C until further use.

4.3.4 RT-PCR primers design

RT-PCR primers for each target gene were designed using Primer 3 software: (<http://primer3.ut.ee>). A full report with the FASTA sequence is generated by the NCBI software and the sequence for the strain *S. mutans* UA159 was selected. The sequence was copied and pasted in the box of Sequence Entry in the open online resource tool called Primer Quest. Primers were designed to ensure they spanned no greater than 700 bp. The oligo primers were ordered via Sigma (Sigma, UK), which were designed with a melting temperature (T_m) set between 55-60 °C and were received at a concentration of a 100 μ M. The list of primer sequences is show in [Table 4.2](#).

Table 4.2: Selection of genes involved in biofilm formation and primers designed to study the expression profile of the selected genes of *S. mutans*

Gene	Enzyme/ gene product	Description/ function	Primer sequence ('5-3')		Annealing Temperature (C°)	Amplicon Size Bp
			Forward	Reverse		
<i>comD</i>	Sulfoxyruvate decarboxylase subunit alpha	Competence stimulation peptide	CTGAGATGGAGTTGCTTGAT	TTGGAGCCTTTAGTGAATAG	55	540
<i>GtfB</i>	Glucosyltransferase -I	Water soluble and insoluble glucan production	CGGAGCAGGCTATGTCTTAAA	CACCATCTTCACCGAGATACAG	60	608
<i>GtfC</i>	Glucosyltransferase -I	Water soluble and insoluble glucan production	CGTCTGTCCGCTATGGTAAAG	GTGGCGAAAGCTTGGAATTAG	60	478
<i>GitA</i>	Glutamate synthases	Regulation of the glutamate synthase operon	CTCAGCCTTTCCGCTTCTTA	AGGGATTGATCTTGGTGGAAAC (Sense) Hairpin Blast	62	351
<i>Glk</i>	Glucose kinase synthases	Glucose uptake	CCAGGAGATCCCATTCCAATAC (AntiSense)	CCAGGAGATCCCATTCCAATAC (AntiSense)	62	604
<i>sacB</i>	Glycosyl hydrolase	Sucrose microbial adhesion	GAACGCTATGCTATCCCTTATT	GTAGCTGAACCTGACCATTC	58	204
<i>vicR</i>	Transcriptional regulatory protein	Two- component regulatory system	CTGCGTCGTACCGAAACTAT	GACGACTTGGTGTGTCTTCA	56	256
<i>wapA</i>	Cell wall-associated protein precursor	Intercellular competition	CTTTCCAGATGAAGTCAGTAT	ACCAGCAGATTGAGAAGAAG	55	138
<i>smu_1037c</i>	Histidine Kinase	Acid tolerance	GCTACCATTTGGCAGTGA	GTCGGATAGTTCCTTCTGTAAG	56	315
<i>smu_104</i>	Putative alpha-glucosidase glycosyl hydrolase	Biofilm formation hypothetical protein	GATCCAATTTGGCGTGTTAG	GCCACAGTTCCTTGACTATT	56	402
<i>smu_105</i>	Putative transcriptional regulator repressor of sugar transport operon	Biofilm formation hypothetical protein	TCAGTCGAGGAACTGTATCA	CACTGGTAATAAGAGGGACATC	58	263

4.3.5 Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis

Previously, RT-PCR was used to analyse genes that are differentially expressed in the *S. mutans* biofilm when compared to cells grown in planktonic conditions (Whiteley *et al.*, 2001). In this study, RT-PCR analysis for each sample was performed by adding 10 µl of HotStart Taq master mix, 1 µl of each of the primers (10 pmoles/µl), 6 µl of RNase free water and 2 µl cDNA of each sample into a 0.5ml centrifuge tube to a final volume of 20ul. The samples were mixed well with pipetting before the RT-PCR reaction. RT-PCR was performed using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, UK). The cycling conditions were as follows 15 minutes at 95°C for HotStart heat activation, followed by 35 cycles each one consisting of 30 seconds at 95°C for primer denaturation, 30 seconds at 58°C for primer annealing, 30 seconds at 72°C for primer extension. A final extension step for 10 minutes at 72°C was done.

4.3.5.1 Agarose Gel Electrophoresis

For agarose gel electrophoresis, a 2% agarose gel was prepared by dissolving 2 g of agarose in 100 ml of 1 X TAE buffer. For the preparation of 1 X TAE buffer, 20 mls of 10 X TAE buffer were added to 980 mls of distilled water. A 100 ml of 1 X TAE buffer solution with agarose was microwaved for 1-2 minutes until completely dissolving the agarose and was left to cool at room temperature after which 10 µl of Nancy 520 was added into the gel solution and mixed. In each PCR product 2 µl of 6 X loading dye were added and mixed into before loading them into the gel. The first well contained 10 µl

of a 1.5 bp DNA ladder and the rest of the wells contained 20 µl of PCR product. Gel was allowed to run at 100 volts for 45 minutes.

4.3.5.2 Quantification of gel analysis

The semi-quantitative measurement of PCR amplicons was achieved by digital analysis of PCR-electrophoresis gels using the Gel Doc™ EZ imager and Syngene Gel software. Images of the agarose gels were acquired under UV fluorescence light and uploaded onto the imager. The band intensity from each PCR product was measured and expressed in arbitrary units. The arbitrary units were normalized using *S. mutans* biofilm control to determine the relative gene expression values. Data was analysed through MS Excel and represent as relative gene expression for each target gene sample compared to control conditions. Mean and standard deviation of all experiments performed were calculated after normalization. The formula for this gene expression is follow, Eq 2:

$$\text{Relative gene expression} = \frac{\text{Expression of the target gene in each sample}}{\text{Expression of the target gene in the biofilm control}} \quad \text{Eq (2)}$$

4.4 Results and discussion

4.4.1 EPS Production in Biofilm Formation

The objective of this study was to report a methodology for quantification of *S. mutans* biofilm formation by determining the structural distributions of extracellular components of matrix. The method consists of distinct but interconnected steps involving biofilm growth, staining, CLSM imaging of biofilms, biofilm structural analysis and visualization, and statistical analysis of structural parameters. To quantify the EPS production in biofilm matrix formation, there is a novel 3-D parameter analysis using CLMS. The biofilm of *S. mutans* was stained using S9 and Alexa Fluoro (Con A) to visualize live bacteria and EPS component (Flemming *et al.*, 2002).

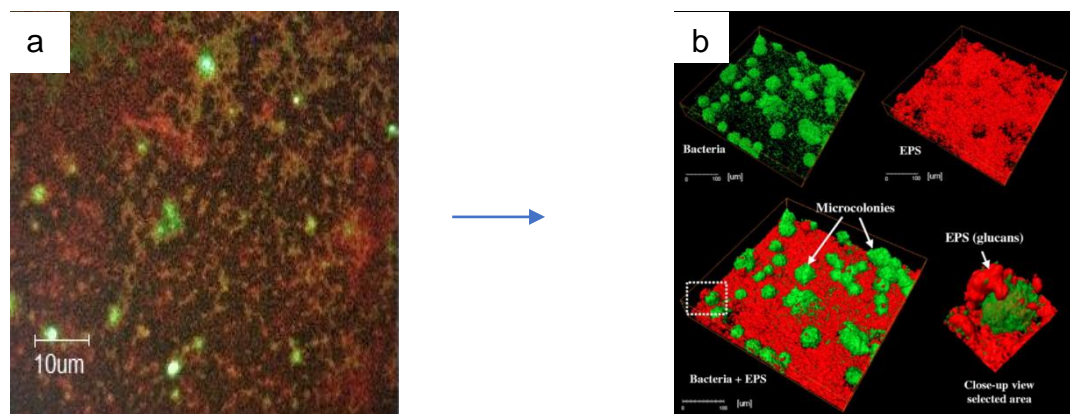


Figure 4.1: a) CLSM image of EPS production during biofilm formation by *S. mutans* grown in BHI medium for 72 hours as a control stained with Con A and S9 dyes. *S. mutans* biofilm characterized by clustered microcolonies, red-oranges patches showed EPS production which were clearly visible. Resolution at 10 μ m. Figure b) The image of EPS production of *S. mutans* was adapted from Koo *et al.*, (2010).

Biofilm formation by *S. mutans* in BHI medium for 72 hours formed a distinctive three – dimensional (3-D) biofilm structure on the 6 well plates. The [Figure 4.1](#) show their EPS production during biofilm formation by *S. mutans* which was taken as a control. The average EPS production of *S.*

mutans biofilms detected by Con A in red-orange colour can be estimated using this method (Decker *et al.*, 2014). When both dyes (S9 and Con A) are used Con A represents a marker for glycoconjugates, which are essential components of EPS, and binds selectively to -glucopyranosyl (carbohydrates) molecules such as glucans and S9 binds to DNA and RNA of both live and dead cells and emits green fluorescence (Rosenberg *et al.*, (2019).

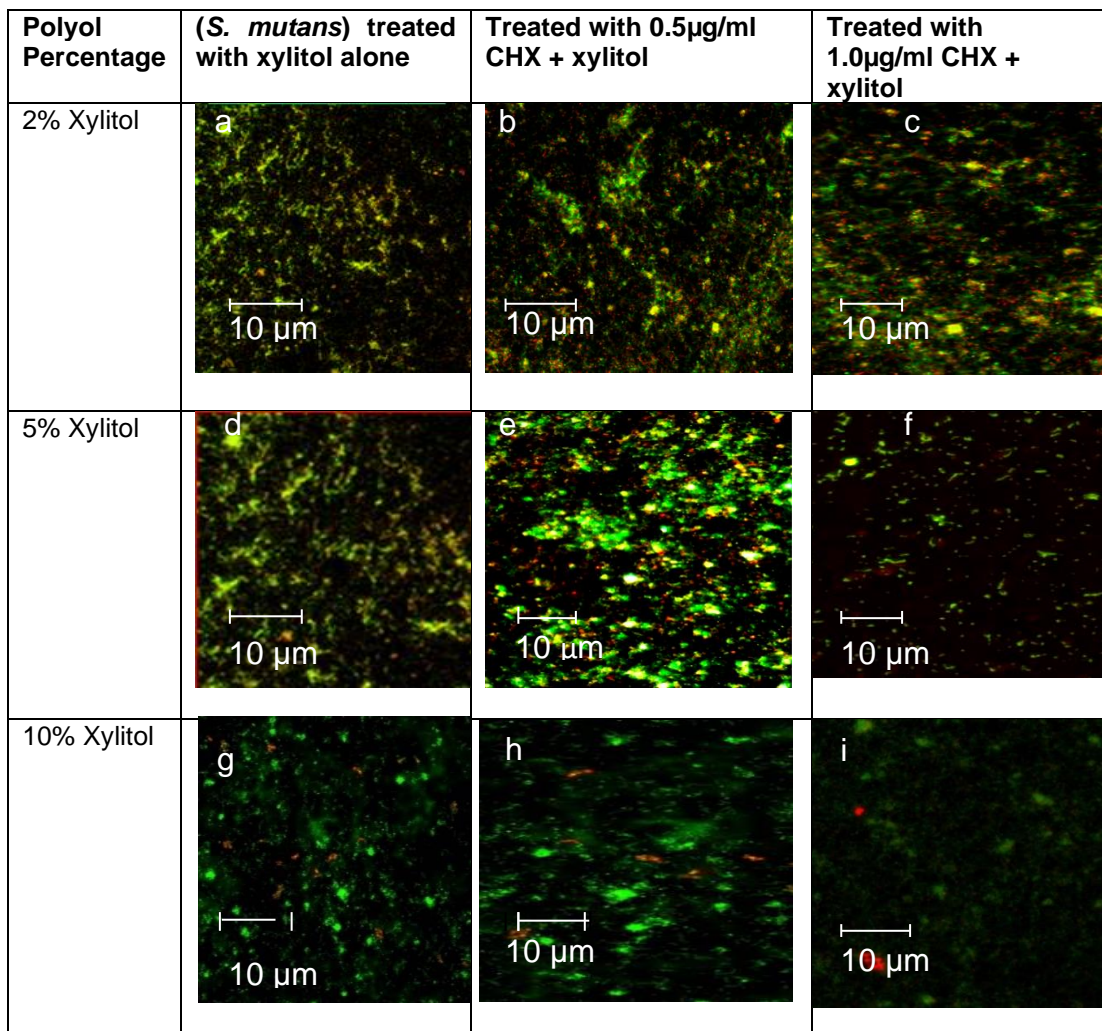


Figure 4.2: CLSM images of EPS production in *S. mutans* biofilm formation during inhibition assay for 72 hours treated with 2% of Xylitol (a-c) 5% of Xylitol (d-f) and 10% of Xylitol (g-i) ; treated with 0.5 and 1.0 µg/ml of CHX concentration stained with Syto 9 and Alexa Fluro Con A. EPS production shows the orange- red micro-colonized clusters when stained with Alexa Fluro Con A.

The EPS is produced during biofilm formation in the inner, middle, and outer biofilm layers regardless of the carbohydrates present (Decker *et al.*, 2014). The EPS patches (orange- red) are less visible with increasing concentrations of xylitol (2, 5, 10%) and CHX (0.5-1.0 $\mu\text{g/ml}$), as show in [Figure 4.2\(a-i\)](#).

EPS production depends on productivity of *S. mutans*, availability of nutrients and environmental conditions. However, when *S. mutans* biofilm treated with xylitol and CHX, both treatments inhibited the biofilm as well as lead to decreased EPS production. The finding of Loimaranta *et al.*, (2020) discovered that xylitol and erythritol inhibited real-time biofilm formation by all of the nine *S. mutans*' strains. They found the addition of 1% xylitol and erythritol during biofilm formation by *S. mutans* between 12- 14 hours showed a reduction in the production of biofilm matrix. This suggests that the matrix composition and the surface can be altered by polyols besides reduced proliferation of bacterial cells (Loimaranta *et al.*, 2020). Similar finding was found from Söderling *et al.*, (2008) and Ghezelbash *et al.*, (2012) which reported low concentration, 0.01% of xylitol inhibited *S. mutans* biofilm growth.

Morphology of *S. mutans* cell and EPS in the biofilm formation images showed in [Figure 4.1](#) and [Figure 4.2](#) could be compared with Decker *et al.*, (2014) studies. The biofilms *S. mutans* show clusters of cells which are enclosed in the matrix. However, it is hard to distinguished individual cells due to the small size of *S. mutans*, less than size 10 μm when compared to CSLM images of organisms such as *Staphylococcus aureus* where the cells

are clearly seen (Warraich *et al.*, 2020). Similar CSLM images were obtained on 24 hours *S. mutans* biofilm studied by Decker *et al.*, 2014. They clarified that cells were clustered at 50 μm resolution during observation for vitality and EPS production in the biofilm of *S. mutans*. For our studies, biofilm formation of *S. mutans* were performed for 72 hours, therefore the cells appear clustered and suppressed due to the lack of nutrients which would reduce their sizes and attenuate biofilm formation.

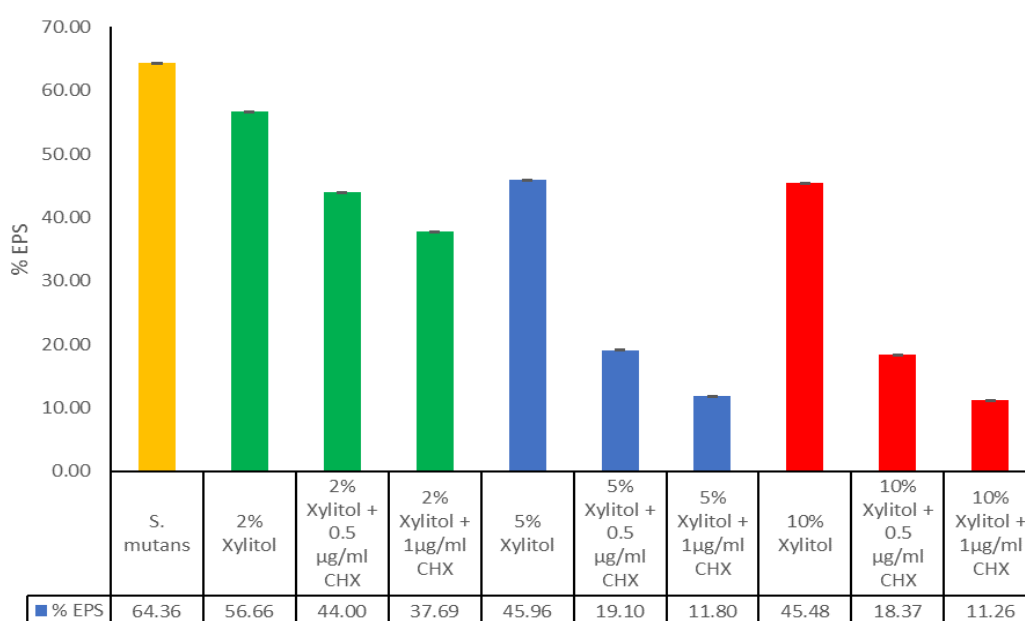


Figure 4.3 Percentages EPS production in *S. mutans* biofilms during inhibition assay at 72 hours with (2%, 5% and 10%) of xylitol treated with 0.5 and 1.0 $\mu\text{g/ml}$ CHX concentration, $n=3 \pm\text{SD}$, $p<0.05$

The percentage of EPS produced in the presence of xylitol 2%, 5% and 10% and concentration of CHX at 0.5 $\mu\text{g/ml}$ and 1.0 $\mu\text{g/ml}$ was calculated by the formula, Eq 3, described in section 4.3.1. and is shown in Figure 4.3. Control *S. mutans* show the highest production of EPS (64.36%) compared to other samples. This dye Con A detected the production of EPS in the inner, middle, and outer biofilm layer. This is due to no other additives or antimicrobial agents that were added into the BHI medium during the

inhibition assay. However, when 2% of xylitol was added into the biofilm medium, EPS production decreases compared to the control by 7.7%. Moreover, when 0.5 and 1.0 µg/ml of CHX added into each well during development of biofilm of *S. mutans*, the production of EPS showed a decrease to 44% for 2% xylitol combined with CHX 0.5 µg/ml and 37.69% for 2% xylitol combined with CHX 1.0 µg/ml. However, the biofilm formation of *S. mutans* with 5% xylitol showed 45.96% EPS production. When this biofilm combined with 0.5 µg/ml of CHX, the development of EPS decreases to 19.10% which was almost 3 times reduced when compared to control *S. mutans* biofilm formation. While this biofilm formation of *S. mutans* exposure to 5% xylitol combined with 1.0 µg/ml of CHX, the production of EPS was reduced to 11.80%. Both samples showed slightly reduced EPS production with increase in concentration of CHX from 0.5 to 1.0 µg/ml.

The exposure of *S. mutans* biofilm formation to 10% xylitol, the production of EPS was 45.48%. There was a reduction of EPS production compared to *S. mutans* control. However, when 10% xylitol combined with 0.5 µg/ml, the EPS production was show reduce 18.37% meanwhile with 1.0 µg/ml it was 11.26%. Both concentrations indicated reduction of EPS with increase in concentration of CHX.

Overall EPS production of *S. mutans* during biofilm formation grown in BHI medium containing increasing concentrations of xylitol and CHX showed tremendous decrease. In this study, exposure to xylitol conditions were used to stimulate oral cariogenic condition for *S. mutans* biofilm formation (Decker

et al., 2014). The range of 1% to 5% of xylitol concentration is used in this study based on *in vitro* studies conducted by So"derling *et al.*, (2008) and (Badet *et al.*, (2008). However, in their studies, only concentration of 1% xylitol was chosen to use prior to clinical studies as this concentration simulates the oral environment with saliva in 10 minutes after the use of xylitol containing products such as toothpaste (Marttinen *et al.*, 2012).

4.4.2 Quantification of gene expression during biofilm formation by *S. mutans* by semi-quantitative reverse transcription polymerase chain reaction (RT- PCR) analysis

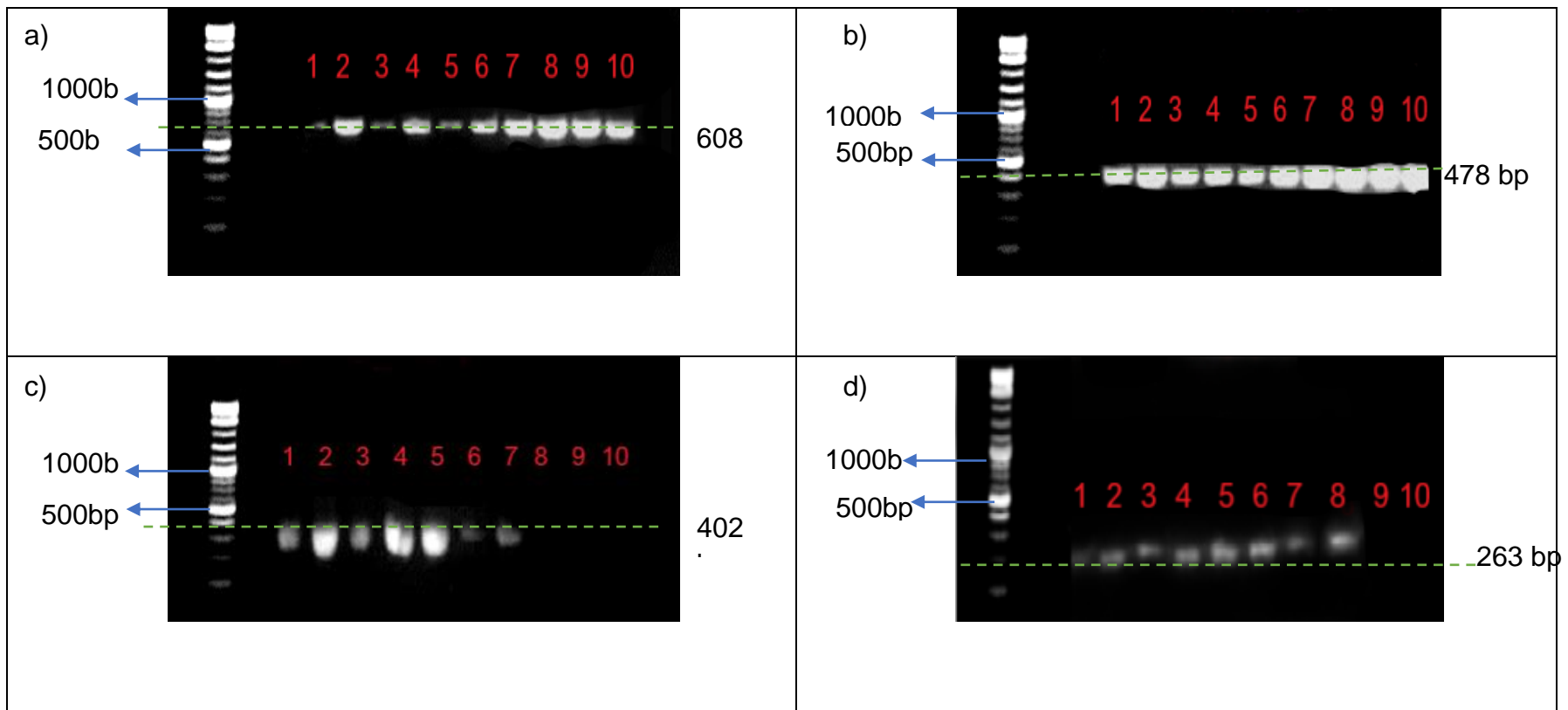
Genes associated with EPS formation were only selected including *gtfB*, *gtfC*, *sacB* and *vicR* was investigated. During the biofilm formation phase, genes *sacB*, *smu_104* and *smu_105* involved in the carbohydrate uptake, were investigated. However, during microbial adhesion, genes *sacB*, *vicR* and *wapA* were selected to study their expression. Finally, expression levels of genes involved in acid tolerance including *comD* and *smu_1037c* were measured by semi-quantitative RT-PCR.

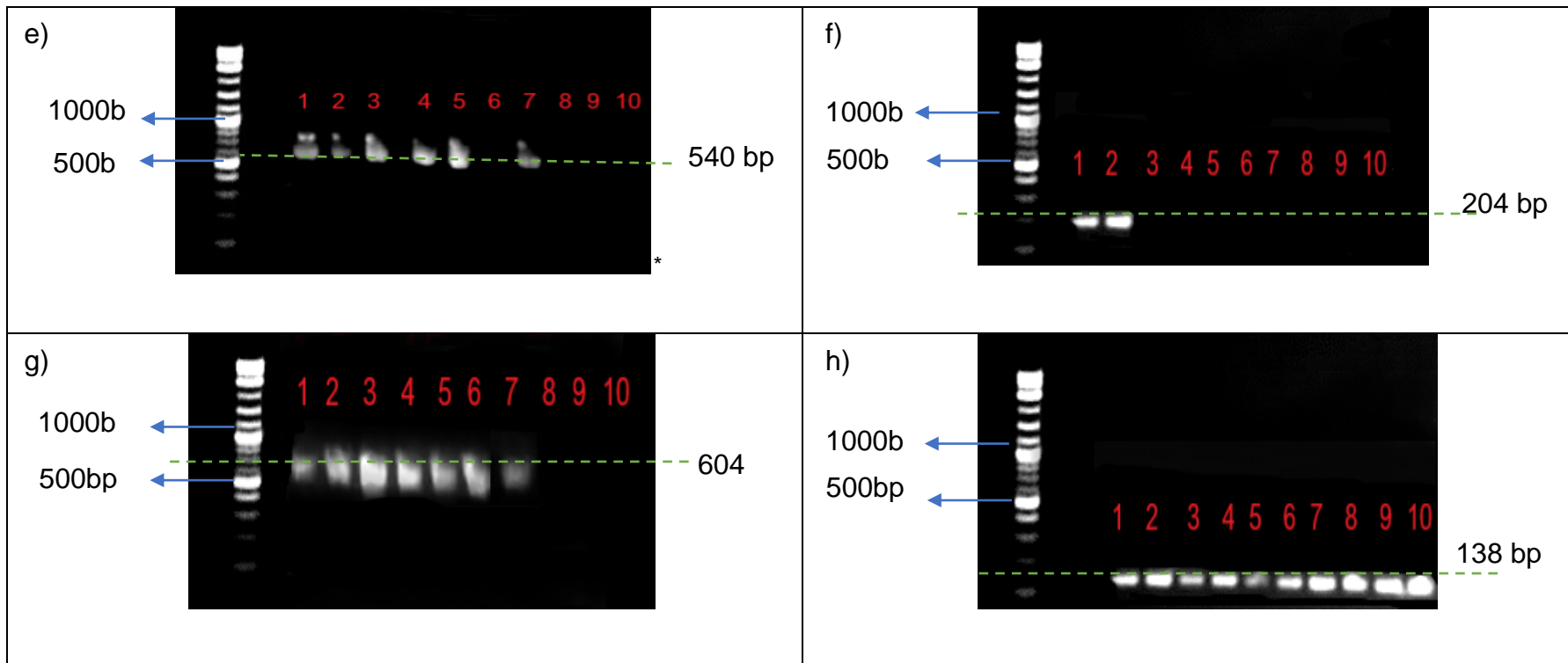
An *in vitro* comparative expression analysis was performed to differentiate 11 target genes known to be primarily involved in EPS of *S. mutans* biofilm formation by comparing their expression levels under different conditions of xylitol and CHX. RT- PCR analysis revealed expression patterns of 7 target genes *gtfB*, *gtfC*, *glk*, *comD*, *wapA*, *smu_104* and *smu_105* during biofilm formation by *S. mutans* at two concentrations of xylitol; 1% and 5% when

combined with CHX at 0.25 and 0.5 µg/ml. The intensity band on the gel electrophoresis was summarized in [Table 4.3](#).

Table 4.3: Qualitative evaluation of the band intensity using gel electrophoresis and biofilm *S. mutans* control as housekeeping gene

Gene name	Band intensity on the gel
<i>smu_105, glk, sacB, comD</i>	Less intensity
<i>gtfB, gtfC, smu_104, wapA</i>	Higher intensity
<i>smu_307c, gltA and vicR</i>	No band





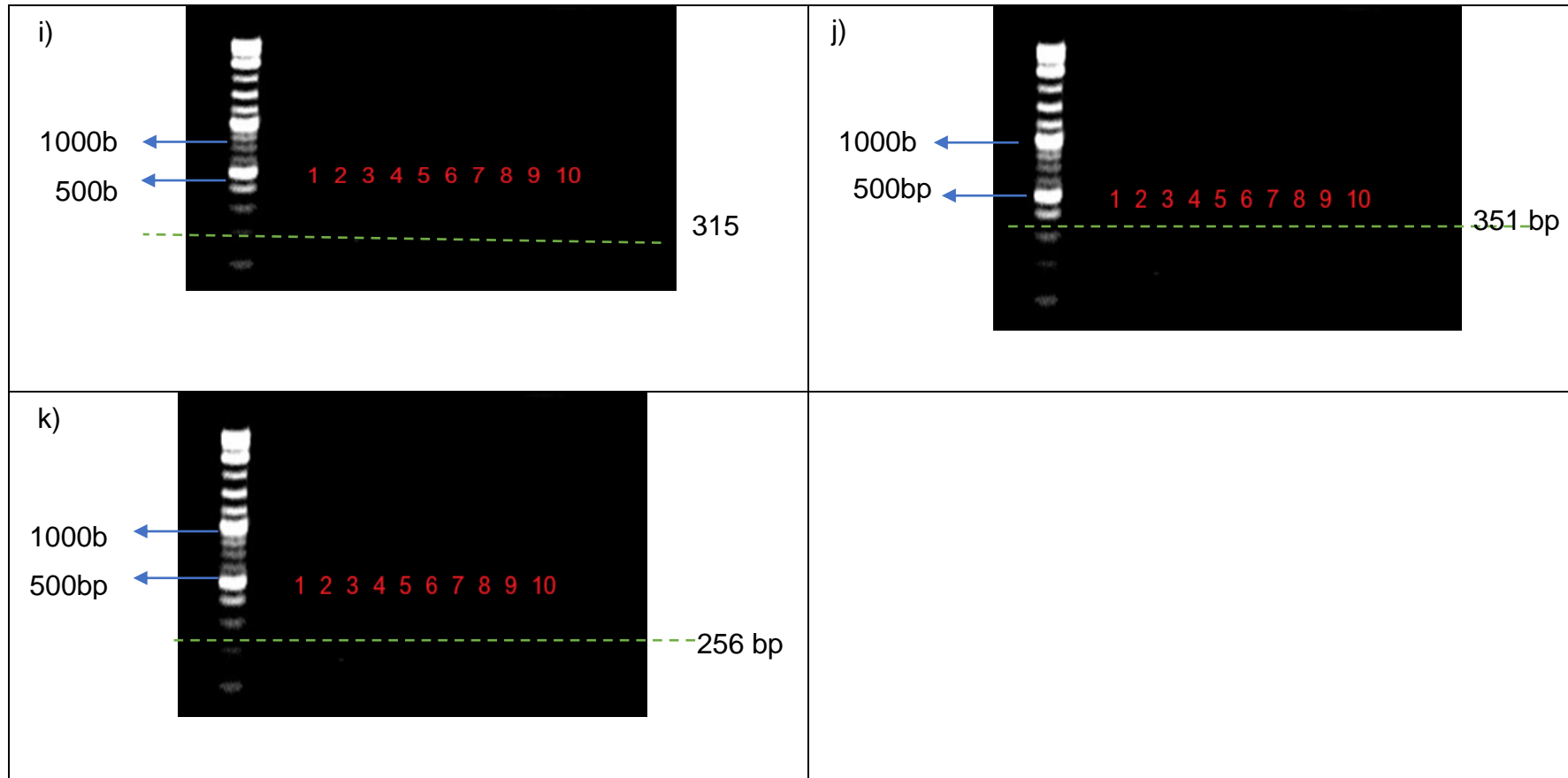


Figure 4.4: Gel Electrophoresis results from semi-quantitative RT-PCR analysis. Lanes 1-10 denote samples as follows. (1: Planktonic *S. mutans*, 2: Biofilm *S. mutans*, 3: Biofilm + Xylitol 1%, 4: Biofilm +5% Xylitol, 5: Biofilm +1% Xylitol + 0.25 $\mu\text{g/ml}$ CHX, 6: Biofilm +1% Xylitol + 0.5 $\mu\text{g/ml}$ CHX, 7: Biofilm +5% Xylitol+ 0.25 $\mu\text{g/ml}$ CHX, 8: Biofilm +5% Xylitol+ 0.5 $\mu\text{g/ml}$ CHX, 9: Biofilm + 0.25 $\mu\text{g/ml}$ CHX, 10: Biofilm + 0.5 $\mu\text{g/ml}$ CHX): Gene expressions; (a) *gtfB*, (b) *gtfC*, (c) *smu_104*, (d) *smu_105*, (e) *comD*, (f) *sacB*, (g) *glk*, (h) *wapA*, (i) *smu_370c*, (j) *gltA* and (k) *vicR*.

Figure 4.4 shows result of size and intensity of bands that quantified from gel electrophoresis on 1.5% agarose gel and visualised under UV light after staining with ethidium bromide. The size of band was identified as gene expression. In [Figure 4.4\(a\)](#) and [Figure 4.4 \(b\)](#) *gtfB*, and *gtfC* genes that are involved in biofilm formation showed the highest expression under xylitol and CHX concentration conditions due to their role in EPS production. Indeed, studies revealed that these two genes (*gtfB* and *gtfC*) encode glucosyltransferases which are responsible for EPS synthesis (Duque *et al.*, 2011 and (Zeng & Burne, 2013). RT-PCR analysis showed expression of *gtfB*, and *gtfC* genes that are that are also associated with carbohydrate-dependent adhesion, which is significantly increased in response to presence of xylitol and CHX. These two genes also responded to biofilm *S. mutans* treated with xylitol and CHX at different conditions. According to Koo *et al.*, (2010) study, *S. mutans* develops at least two genetically different *gtfs* (*gtfB* and *gtfC*), that are required for the EPS matrix to be formed. *gtfB* appears to be responsible for formation of microcolonies. *gtfB* synthesises mainly insoluble glucan rich in-1,3-linkages, while *gtfC* produces a mixture of soluble (mostly-1,6-linkages) and insoluble glucans. In addition to this, adsorbed *gtfB* and *gtfC* surface glucan synthesis is required to create a matrix which improves bacterial cells' stability and adhesion to indifferent surfaces, which enables the production of highly organised and dense cell clusters (Koo *et al.*, 2010). Tahmourespour *et al.*, (2011) studies indicated that *gtfB* and *gtfC* are essential for the sucrose-dependent attachment of *S. mutans* cells to hard surfaces. Therefore, these genes have become a potential target for protection against dental caries. Hence, *S. mutans* have a

much greater impact on the formation and composition of the plaque than its population would appear in the biofilm (Monchois *et al.*, 1999 and Van Hijum *et al.*, 2006).

RT-PCR analysis also revealed expression of both *smu_104*, and *smu_105* in at least 7 out of 10 conditions assessed in this study. These genes were found to play a role in carbohydrate uptake and biofilm formation of *S. mutans* (Decker *et al.*, 2014). *smu_104* was expressed under seven conditions when treated with xylitol and CHX at different concentrations although no expression of this gene was detected when samples were treated with high concentrations of CHX (lane 8, 9 and 10). *smu_105* expressed in eight samples when treated with xylitol and CHX at different concentrations. However, there were two samples with no expression of this gene when biofilm *S. mutans* treated with 0.25 (lane 9) and 0.5µg/ml (lane10) of CHX only.

Expression of *comD* was only expressed in six samples when treated with xylitol + 0.5 µg/ml of CHX. There were four samples with no expression of this gene when treated higher concentration CHX. *comD* is a competence stimulation peptide which showed expression in 7 out of 10 conditions as shown in [Figure 4.4 \(e\)](#). Study from Yoshida and Kuramitsu, (2002), revealed that *comD* gene mutants were defective in biofilm formation on abiotic surfaces. They also found that the biofilm formation was reduced when other regulatory genes involved such as *comC* and *comE* were deleted.

Expression of *sacB* was detected in sample 1 which was planktonic state and sample 2 the control biofilm of *S. mutans*. This gene is related to

microbial adhesion and encoding levansucrase enzyme (fructosyltransferase) during biofilm formation by *S. mutans* (Klein *et al.*, 2010). The adherence represents the start of the colonisation process where the surface has physical and molecular interaction with microbes during biofilm growth.

Figure 4.4 (f) clearly shows that *sacB* was not expressed (lane 3 to 10), in biofilms exposed to different concentration of xylitol and CHX when compared to both planktonic forms and biofilm control sample. Hence, both xylitol and CHX inhibit expression of this gene and may prevent adhesion of *S. mutans* to surfaces.

glk encodes glucose uptake and expression was detected in 7 out of 10 conditions. There were no expression of this gene in lane 8, 9 and 10 when the samples were treated with high concentration of CHX (0.25 and 0.5µg/ml of CHX) and 5% xylitol. In

Figure 4.4(h) *wapA* which is a cell wall associated protein involved in intercellular competition was found to be expressed in *S. mutans* under different concentrations of xylitol and CHX. In addition, *wapA* is involved in maintaining the cell surface structure, cell aggregation, cell chain length and in the development of biofilm (Zhu *et al.*, 2006). Findings from Zhu *et al.*, (2006) upon exposure to 0.5% sucrose *wapA* plays an important structural role on the cell surface, which affects sucrose-independent cell–cell aggregation and biofilm architecture. *wapA* may also be involved in protecting the bacteria from some forms of environmental stress such as oxidative stress.

Three genes, *vicR*, *smu_1037c*, and *glt*, did not show any expression in any of the conditions tested in this study (Figure 4.4 (i), (j) and (k)). This could be due to genes not expressed under these experimental conditions. Furthermore, the primer for these genes may not have bound to the target sequence due to degradation.

4.4.2.1 Gene expression levels involved in different metabolic pathways in *S. mutans* biofilms

Several entities' biofilm growth was shown to be regulated by the medium's nutritional concentration (Carlson, 2000; Gilmore *et al.*, 2003). As a result, the effects of various nutritional components on gene expression in *S. mutans* biofilm under different conditions has been extensively studied. The gene expression profiles of biofilm on polystyrene surfaces (6-well plates) were evaluated using a variety of conditions. The expression of target genes that are involved in different metabolic pathways in biofilm formation of *S. mutans* was measured after 72 hours. Each metabolic pathway has different compounds associated in the development of *S. mutans* biofilm. Since the role of xylitol in *S. mutans* biofilm formation has been well documented by Loiramanta *et al.*, (2020), our research concentrated on the combined effect of xylitol and CHX affects gene expression in this organism.

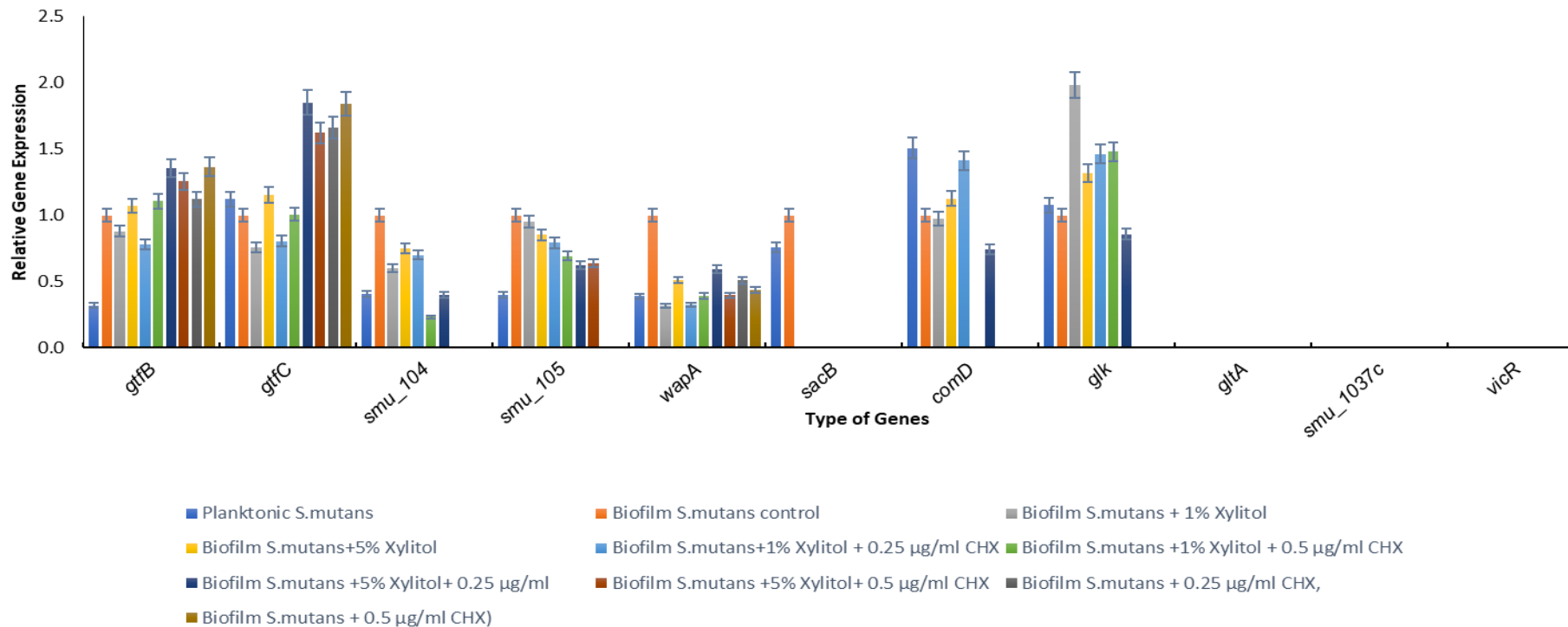


Figure 4.5: Expression of genes involved in different metabolic pathways in a 72 hours *S. mutans* biofilm treated with xylitol 1%, 5% only, and combined with 0.25 µg/ml and 0.50 µg/ml of CHX. These genes showed differential regulation based on analysis of RT-PCR products by gel electrophoresis. (Mean= 2, ±SD, p<0.05)

To produce [Figure 4.5](#), the gene expression was normalised with biofilm *S. mutans* control for each gene using formula as show in [Eq 2](#). Expression of *gtfB* was highly upregulated in all conditions including xylitol combined with CHX of biofilm *S. mutans*. This gene was associated with sucrose-dependent adhesion and was upregulated in response to xylitol and CHX compared to growth without treatment (lane 1 to 10). *gtfB* was also shown to be critical for adherence to a smooth tooth surface and essential for virulence (Stipp *et al.*, 2013). Moreover, *gtfC* was also upregulated in the presence of xylitol and CHX conditions. Relatively *gtfC* expression levels were higher in the biofilm *S. mutans* treated with 5% xylitol and 0.25 µg/ml CHX. Studies by Loimaranta *et al.*, (2020) revealed that increasing expression of the *gtfs* genes found in 10-hour xylitol biofilms also reflects the higher accumulation of polysaccharides in xylitol biofilms than biofilm without supplemented of xylitol. Decker *et al.* (2014) was studied when *S. mutans* biofilms exposed to 1% xylitol of glucose-containing media, the results showed upregulation in the genes of *gtfB*, *gtfC* and *gtfD* at 24-hours biofilm formation. They suggested a metabolic imbalance for xylitol because of the mitigation of these levels of expression.

The genes involved in carbohydrates uptakes (*smu_104* and *smu_105*) showed decreased expression in all biofilm treated samples with xylitol and CHX. *smu_104* only showed expression in 7 out of 8 condition tested. However, three other lane treated with 5% xylitol, with both lower (0.25µg/ml) and higher CHX concentration (0.5µg/ml) of CHX showed no expression on the gel. Furthermore *smu_105* showed expression in 6 out of 8 conditions.

However, this gene did not express under two conditions when *S. mutans* biofilm was treated with both concentrations of CHX alone as this gene is only involved in carbohydrate uptake (Sims *et al.*, 2011).

Decreased expression of *wapA* was detected in all conditions suggesting its association with cell wall and intercellular competition during biofilm formation by *S. mutans*. On the contrary, *sacB* was only expressed in conditions with planktonic and biofilm *S. mutans*. *sacB* is a gene that encodes for glycosyl hydrolase involved in sucrose dependent microbial adhesion associated with carbohydrates- adhesion in the biofilm at different growth conditions such as pH and nutrients. Moreover, adhesion of carbohydrates to biofilm enhanced the stability of the biofilm structure (Hasan *et al.*, 2012). Furthermore, *sacB* was found to be promoting *S. mutans* colonization in the biofilm (Tsumori *et al.*, 1997).

ComD gene associated with competence stimulation peptide showed increased expression during biofilm formation when treated with xylitol and combined with CHX at all conditions. However, there was no expression of *comD* in the sample treated with highest percentage of xylitol and highest concentration of CHX. This gene would facilitate the internal communication mechanism by *S. mutans* to change their gene expression (Bachtiar *et al.*, 2016). Hence, when this mechanism has changed their cell population density, it can contribute to a quorum-sensing signalling system which essential for genetic competence in *S. mutans* biofilm formation (Liu *et al.*, 2011).

Glk showed increased expression in seven lanes exposed to xylitol and CHX, except 3 samples treated with highest xylitol percentage and biofilm with CHX only. *glk* is associated with glucose uptake in biofilm, hence the absence of the gene expression in these samples. Furthermore, no expression of *smu_307c*, *gltA* and *vicR* was detected in any of the samples. This might be explained due to incorrect primer binding in RT-PCR analysis.

4.5 Conclusion

In conclusion, *S. mutans* biofilms development in the presence of xylitol combined with CHX after 72 hours resulted in a reduction in the EPS production. *S. mutans* biofilm have an effectively disrupted when treated at different concentration of xylitol and CHX. Combination of xylitol and CHX induced changes in biofilm morphology and development of microcolonies. Moreover, development of biofilm formation of *S. mutans* with xylitol combined with CHX was also effective in influencing the significant of gene expression using semi quantitative RT-PCR. The result showed upregulation of glycosyltransferases in the combined presence of xylitol and CHX. The gene expression studies provided new information of xylitol and CHX effects on *S. mutans* biofilm formation. The selected virulence genes such as *gtfB* and *comD* involved in EPS production showed increased expression when exposed to xylitol and CHX at different concentrations. Genes *smu_104*, *smu_105* and *wapA* were expressed less than control as these genes were successfully inhibited by xylitol and CHX at various concentrations.

CHAPTER FIVE

Development, Optimisation and Validation of UV Spectroscopic Method for Quantification of Chlorhexidine

5.1 Introduction

Ultraviolet-visible (UV–vis) spectroscopy is a quantitative analysis method that has been widely used in modern laboratories. This is due its simplicity, accuracy, efficiency, and cost-effectiveness. This is also an important analytical method to determine the concentration of drug in a given formulation. The development of an analytical method helps to understand and mitigate the effect of important process parameters on accuracy and precision (Dipali and Hrishikesh, 2018). Besides that, analytical methods have a vital role to play in the production of medicines in bioequivalence, analytical risk assessment and management (Shah *et al.*, 1991). It helps to define product-specific acceptance requirements and stability of results. Therefore, the analytical methods need to be performed in accordance with GMP and GLP guidelines and established in conjunction with protocols and requirements of acceptance in the ICH Guidelines Q2 (R1) (ICH, 2005).

Validation method is a procedure of assessment to verify numerous parameters or method for suitable analytical system providing reproducible analytical data. These methods follow a standardized set of experimental procedures to produce data at the required level of accuracy and precision. This process is noted as a standard operating procedure (SOP). After validation or verification of methods, the responsible person should be formally authorized for routine use in the laboratory. The development and validation of successful analytical methods can lead to significant

improvements in accuracy and reduction in bias errors. Hence, they can also help to prevent time and expensive experimentation (Bouabidi *et al.*, 2010).

One factor in the validation of the analytical method is the determination of its reproducibility from laboratory to laboratory. This is an important element to refer within assessment in the laboratory depending on analytical records, procedure, and chemical data. Reproducibility of analytical method can be defined as precision between the measurements obtained at different laboratories. Sometimes an error occurs, and a term reproducibility is used for internal laboratory studies at the level of intermediate precision. In addition, reproducibility of the analytical method could require a reassessment, including laboratory transfers, instrument changes or changes in critical reagents (Liao *et al.*, 2006).

The analytical method used for analysis of chlorhexidine (CHX) was UV-vis spectroscopic. The UV-vis spectroscopic method was developed for determination of chlorhexidine (CHX) in pharmaceutical lozenge formulation. The maximum wavelength of UV absorption of CHX was found at 254 nm. The main objective was to develop and validate the UV spectroscopic method for CHX so that drug concentration in lozenge formulation can be studied as per International Conference on Harmonisation (ICH) guidelines. Method validation including linearity, detection limit, quantification limit, accuracy and precision was carried out to ensure consistency and reproducibility of results.

5.2 Materials

Chlorhexidine diacetate was purchased from Sigma Aldrich, UK. Double distilled filtered water was used for all the experiments.

5.3 Methods

5.3.1 UV/Visible Spectrophotometer (UV) Assay

UV spectroscopy was employed for quantitative analysis of CHX and was adapted from Sirorat (Sirorat *et al.*, 2016). The maximum wavelength of absorption of CHX was quantified using a UV/Visible spectrophotometer (Novaspec II, Pharmacia Biotech, U.K) between 190 to 400 nm. Method validation was done based on the International conference on Harmonization (ICH) guidelines for validation of analytical procedures (ICH, 2005). Calibration curve was prepared from dilution of stock solution of CHX (50 µg/ml) using water as solvent. The absorbance of the dilutions of stock solutions were determined at a wavelength of 254 nm. Six-point calibration curves were obtained in a concentration range from 0-25 µg/ml for CHX. Calibration curve was validated against specificity, linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). Three independent stock solutions were prepared followed by serial dilutions to obtain mean absorbance. All values reported include mean +/- standard deviation.

5.3.2 UV Assay Validation

The UV method was validated according to ICH guidelines in terms of specificity, accuracy, precision, linearity, limits of detection/ quantification and stability of CHX solutions (Shah *et al*, 1991).

5.3.2.1 Specificity (Identification and Assay)

50mg of Chlorhexidine diacetate was dissolved in 1000 ml of distilled water in volumetric flask and assayed using 10 mm path length quartz cuvette between wavelength ranging from 190 to 400 nm. The maximum wavelength was detected at absorbance of λ_{254} nm. CHX percentage recovery (the calculated concentration of CHX relative to the actual concentration used) was calculated using the regression equation obtained from linearity experiments below.

5.3.2.2 Linearity

A Beer-Lamberts calibration curve was developed by plotting mean absorbance against CHX concentration. Linearity was described by a linear regression plot of known concentration versus response using a minimum of 6 different concentrations. This linearity was produced by computing the regression line of the calibration curve for CHX concentration from 0 to 25 $\mu\text{g/ml}$ and the correlation coefficient (R^2) was calculated.

5.3.2.3 Accuracy

Accuracy is the percent of analyte recovered by assay from a known concentration. 50 $\mu\text{g/mL}$ of sample from standard solutions were analysed

and the protocol described for linearity above followed. The absorbance for CHX were recorded and the percentage recovery was calculated using the regression equation.

5.3.2.4 Precision

Operator precision was determined by developing the CHX calibration curve in triplicates. The precision of the method was determined by intra-assay and the inter-assay and the repeatability was expressed as the relative standard deviation (RSD). RSD was verified by assaying samples of CHX in aqueous solution, at the same concentration, within one day and under the same experimental conditions. The intermediate precision was evaluated by comparing the assay results from three different days. The absorbance and relative standard deviation (RSD) were calculated using the equation below:

$$RSD = \frac{\textit{standard deviation}}{\textit{mean}} \quad \text{Eq 4}$$

Calibration curves were repeated (n=3) on different days using freshly prepared solution and the RSD calculated.

5.3.2.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined according to the guidance for bioanalytical method validation described by ICH guidelines. The measurements were performed at room temperature of 25°C. The experiments were run with three repeats in series using aqueous media dilution from 25 to 10 µg/ml. The standard deviation of the recorded peak areas was multiplied by 3.3 and 10 to calculate the LOD and LOQ

respectively. This was repeated three times for two different samples of the blank, in order to validate the obtained results (Thomas *et al.*, 2008). LOD and LOQ values were calculated using below equations:

$$\text{LOD}=3.3 \delta /S$$

$$\text{LOQ}=10 \delta /S$$

Where, δ = standard deviation of residuals from the curve; S=slope of the curve

5.3.2.6 Stability of CHX Solutions

50 $\mu\text{g/ml}$ CHX solution was freshly prepared in triplicates and immediately analysed on the UV Spectrometer system. The solutions were stored in the fridge (2-8°C) and quantified after three days (72 hours). The peak areas and RSD were recorded.

5.3.2.7 Statistical Analysis

Statistical analysis was done using Regression Statistics, one-way analysis of variance (ANOVA) and pair-wise multiple comparisons were used to compare data groups by using mean values and standard deviation (SD). The significant difference was determined using the probability value of 95% ($P < 0.005$).

5.4. Results and discussion

5.4.1 UV Method Development for Quantification of CHX

UV spectroscopy method development and validation are routinely used for the determination of active ingredients in medicines and is a very important analytical tool for quality control and product safety (Rosenthal *et al.*, 2004). These methods are simple, rapid, sensitive, and accurate for the routine quantitative analysis of samples to reduce unnecessary preparations, cost of materials and labour. In this chapter, we developed a UV spectroscopy method to quantify the amount of CHX which was also validated as per ICH guidelines.

5.4.2 Linearity

Calibration curve data was constructed from the stock solution with CHX concentration of 50 µg/ml. From this stock solution, six various concentrations (0.781, 1.563, 3.125, 6.25, 12.5 and 25.0 µg/ml) of each solution were transferred to 100 ml volumetric flasks and diluted with distilled water. The UV absorbance readings of these solutions were measured at 254 nm using UV/Visible spectrophotometer. Distilled water was used as a reference. Then, the absorbance versus concentration of solutions was plotted to obtain the calibration curve which resulted in a very good R-square value. The response of the drug was linear in the concentration range investigated and the equation was found to be $y = 0.0367x + 0.0775$. The correlation coefficient (R^2) of the standard curve was found to be **0.9994** and presented in [Figure 5.1](#). The calibration curve was made by dissolving 50

$\mu\text{g/ml}$ CHX in volumetric flask contained distilled water and serial dilutions made to achieve concentrations in the range of 0- 25 $\mu\text{g/ml}$. The absorbance, OD was measured at 254nm. Value denotes, mean \pm SD, from at least three experiments [Figure 5.1](#). However, parameter of the regression coefficient is presented in [Table 5.1](#).

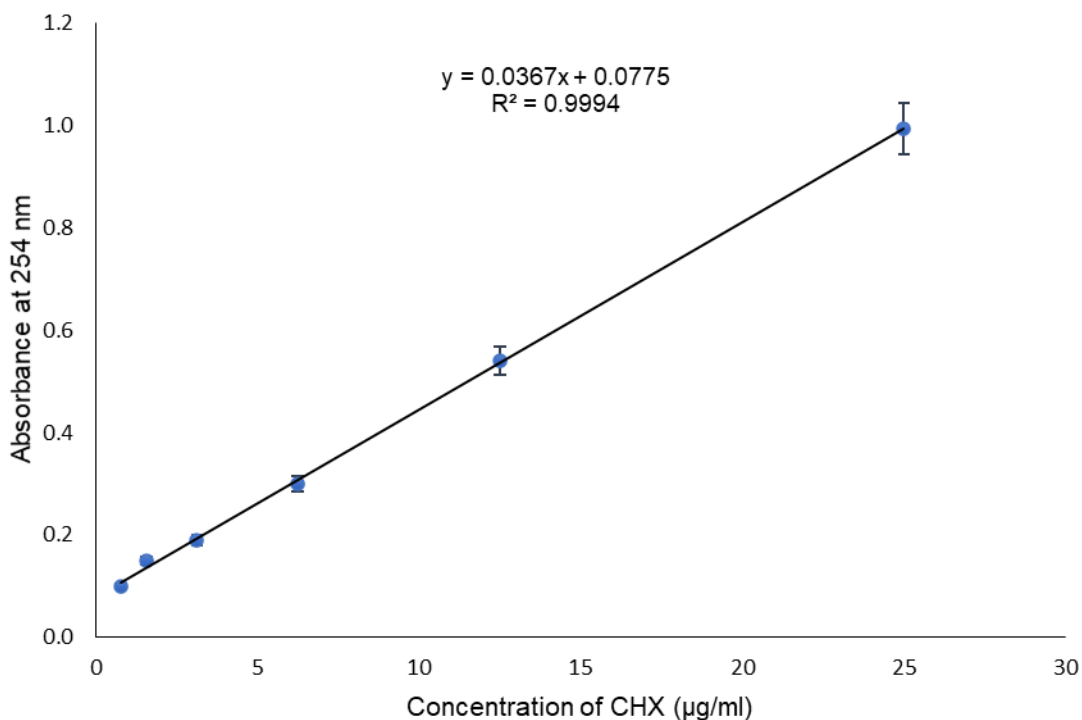


Figure 5.1: The calibration curve was made by dissolving 50 $\mu\text{g/ml}$ CHX in volumetric flask contained distilled water and serial dilutions made to achieve concentrations in the range of 0- 25 $\mu\text{g/ml}$. The absorbance OD was measured at 254nm. Value denotes, mean \pm SD, from at least three experiments.

Table 5.1: Factors of the regression equation CHX concentration to the analytical response (area under the peak) from concentration range 0-25 $\mu\text{g/ml}$. The calculation of LOD and LOQ was adaptation from method 5.3.2.5 into the graph.

Parameter	Value
Linearity range, $\mu\text{g/ml}$	0-25
LOD, $\mu\text{g /ml}$	1.2000
LOQ, $\mu\text{g /ml}$	3.6354
Slope of regression equation	0.0367
Intercept	0.0775
Correlation coefficient	0.9994

5.4.3 Repeatability (instrument and operator precision)

Precision of the analytical method was carried out according to the guidance for bioanalytical method validation described by ICH guidelines. Table 5.2 shows result of intra-day repeatability and %RSD of the samples. The developed method was found to be precise as the % RSD values for the repeatability and precision (was done in triplicates) were within the range of 0.5 to 9.9 % as shown in Table 5.2. This ties in with studies from Shah *et al.*, (2000), who stated that the acceptable % RSD for evaluating precision in analysis of pharmaceuticals, was between the range of 0-20% (Shah *et al.*, 2000).

Table 5.2: Intra-day UV spectroscopy validation parameters for CHX method carried out on the same day, from concentration range 0-25 µg/ml to assess method reproducibility (n=3)

Concentration of CHX (µg/ml)	Absorbance, mean ±SD	% RSD
0.781	0.090 ± 0.009	0.595
1.563	0.157 ± 0.010	0.422
3.125	0.295 ± 0.008	2.648
6.25	0.428 ± 0.002	6.650
12.5	0.794 ± 0.005	9.962
25.0	0.995 ± 0.014	6.304

Table 5.3: Factors of the regression equation CHX concentration to the intra-day UV spectroscopy validation parameter analytical response (area under the peak) from concentration range 0-25 µg/ml. The calculation of LOD and LOQ was adaptation from method 5.3.2.5 into the graph

Parameter	Value
LOD, µg /ml	0.5390
LOQ, µg /ml	1.6333
Slope of regression equation	0.0346
Intercept	0.0804
Correlation coefficient	0.9954

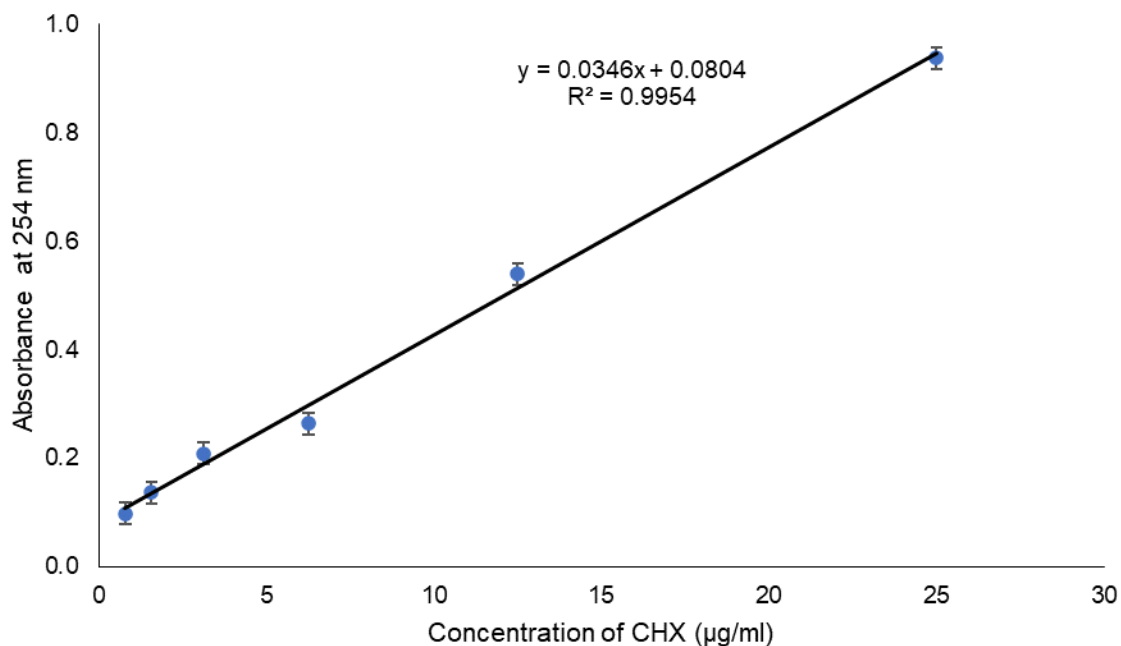


Figure 5.2: Intra- day standard curve was made by dissolving 50 µg/ml CHX in volumetric flask contained distilled water and serial dilutions made to achieve concentrations in the range of 0- 25 µg/ml. The absorbance OD was measured at 254nm. Value denotes, mean ±SD, from at least three experiments.

Inter-day precision was determined by establishing calibration curves on different days – results are shown in. [Figure 5.3](#) Inter-day standard curve was made by dissolving 50 µg/ml CHX in volumetric flask contained distilled water and serial dilutions made to achieve concentrations in the range of 0- 25 µg/ml and [Table 5.4](#). The overall %RSD values ranged from 0.595 – 10.08% as stated in [Table 5.5](#). These values for %RSD are within the limits for precision and accuracy.

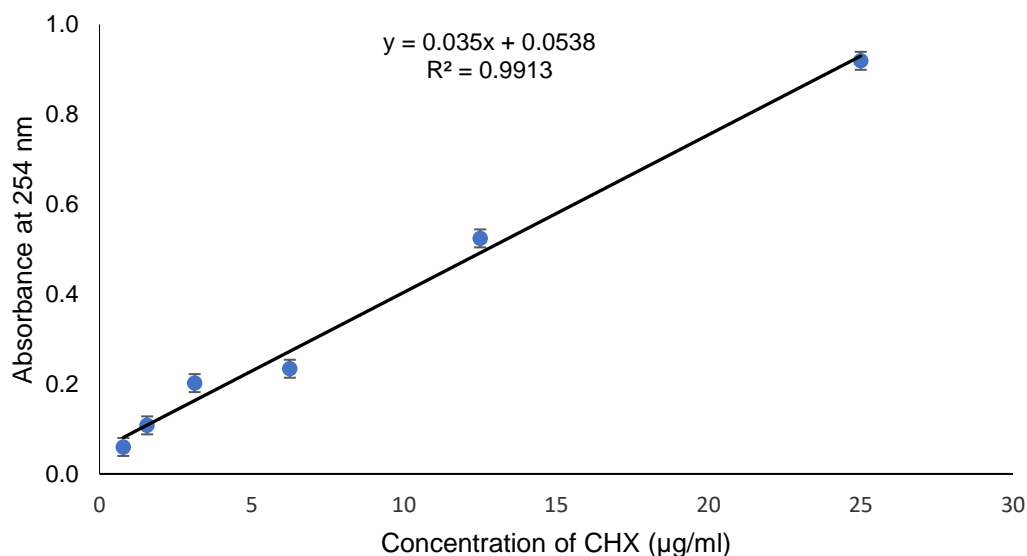


Figure 5.3: Inter-day standard curve was made by dissolving 50 µg/ml CHX in volumetric flask contained distilled water and serial dilutions made to achieve concentrations in the range of 0- 25 µg/ml. The absorbance was measured at 254nm. Value denotes, mean ±SD, from at least three experiments.

Table 5.4: Factors of the regression equation CHX concentration to the inter-day UV spectroscopy validation parameter analytical response (area under the peak) from concentration range 0-25 µg/ml. The calculation of LOD and LOQ was adaptation from method 5.3.2.5 into the graph.

Parameter	Value
Linearity range, µg/mL	0-25
LOD, µg /mL	0.7428
LOQ, µg /mL	2.2510
Slope of regression equation	0.0035
Intercept	0.0538
Correlation coefficient	0.9913

Table 5.5: Inter-day UV spectroscopy validation parameters for CHX method carried out on the different day, from concentration range 0-25 µg/ml to assess method reproducibility (n=3+SD)

Concentration of CHX (ug/ml)	Absorbance on day 1	RSD	Absorbance on day 2	RSD	Overall RSD
0.781	0.075± 0.002	0.59	0.077 ± 0.072	3.25	1.92
1.563	0.152± 0.026	0.42	0.144 ± 0.011	16.13	8.27
3.125	0.311 ± 0.04	2.65	0.231 ± 0.021	15.10	8.87
6.250	0.422 ± 0.03	6.65	0.499 ± 0.024	8.37	7.51
12.50	0.784 ± 0.07	9.96	0.655 ± 0.030	10.19	10.08
25.00	0.998 ± 0.03	0.20	0.894 ± 0.020	15.02	8.30

5.4.4 Limit of Detection (LOD) & Limit of Quantification (LOQ)

The LOD and LOQ were calculated from the standard curve (Figure 5.2), as well as from the standard deviation of the response and slope were found to be 1.2 and 3.6 µg/mL respectively in Table 5.1. Thus, the lowest amount of CHX that can be detected and quantified respectively, within the limits of accuracy and precision, using this analytical method were 1.2 and 3.6 µg/mL (Holbrook, 1958).

5.4.5 Stability of CHX Aqueous Solutions

Results of stability of 50 µg/mL CHX solutions stored in the fridge for 72 hours are shown in Table 5.6. The percent recovery was calculated using the regression equation of the calibration curve. The overall RSD of 1.92 – 10.10% showed that CHX solutions can be stored in the fridge for up to three days without deterioration. This is in line with results obtained by Huizinga *et al.*, who reported that CHX solutions remained stable in the fridge for up to 5 days (Huizinga *et al.*, 1991).

Table 5.6: Stability of 25 µg/mL CHX solutions after storage at 2-8 °C for 72 hours (n=3). The solution was made by dissolving CHX in water and serial dilution made to achieve concentration range of 0-25 µg/mL. The absorbance was measured at 254nm. The results showed that CHX solutions were stable for 3 days when stored in the fridge.

Concentration of CHX (ug/ml)	Absorbance on day 0	Absorbance on day 3
0.781	0.073± 0.001	0.073 ± 0.020
1.563	0.150± 0.02	0.144 ± 0.011
3.125	0.318 ± 0.04	0.310 ± 0.021
6.25	0.402 ± 0.03	0.399 ± 0.024
12.5	0.764 ± 0.06	0.655 ± 0.030
25.0	0.995 ± 0.02	0.872 ± 0.024

5.5 Conclusion

The aim of this work was to optimise and validate a simple, rapid and efficient method for CHX quantification by UV, which can be reproduced in any laboratory at room temperature. The method was validated on different days and complied with the ICH guidelines for UV method validation. Thus, the developed method is rapid with short runtime, at room temperature with simple UV detector, which can be employed for quantification of CHX from any sample. The proposed methods can be successfully applied for assay in lozenges tablet dosage form without any interference.

CHAPTER SIX

Formulation and Characterisation of Chlorhexidine lozenge formulation

6.1 Introduction

The most popular route to deliver active pharmaceutical ingredients (APIs) to the human body is the oral route. This is due to ease of self-administration, accuracy of dosing, and the most importantly, patient compliance (Spireas, 2002). Furthermore, because oral formulations do not require sterile conditions for manufacture, they are less expensive to produce and store (Liew *et al.*, 2014). For these reasons, the majority of therapeutic agents considered for systemic drug delivery are generally taken orally (Srikanth *et al.*, 2013).

The most common types of solid dosage forms used in the oral cavity to achieve either a systemic or local action are lozenges. The lozenges are defined as solid, single-dose preparations and flavoured medicated dosage formulations containing sweetened base intended to be sucked and held in the mouth (Peters, 2005). According to United States Pharmacopoeia (USP) and the European Pharmacopoeia (Ph. Eur.), lozenges are intended to alleviate oropharyngeal symptoms, which are usually caused by local infections, and can also be used to deliver drugs systemically through absorption by the buccal lining. They are usually placed in the oral cavity between cheek and gum, for local and or systemic delivery (Batheja *et al.*, 2006).

Although the time of lozenge dissolution is about 20-30 minutes, this depends on the patient as the patient controls the rate of dissolution and absorption by sucking the lozenge until it dissolves.

The aim of this investigation was to develop, optimise and characterise a stable lozenge formulation to deliver CHX. As per the work described in the previous chapter, there was a clear synergistic effect of polyols in the presence of CHX on the integrity of the biofilm and survival of the bacteria. Therefore, we carried out a systematic investigation in two key areas: process and method of manufacture of lozenges and material optimisation. Work presented in this chapter describes the application of heat and congealing process to formulate lozenges and subsequent switch to direct compression. This strategy enabled us to identify the challenges of heat and congealing method and to develop and simplify the manufacturing process by using traditional methods employed in direct compression.

6.2 Materials

Chlorhexidine and citric acid were obtained from Sigma Aldrich (UK). Menthol and orange flavour were free samples from Sheffield Bioscience (Sheffield, U.K). Sucrose, fructose, sorbitol, and liquid glucose were gifted by Roquette (Lancaster, U.K). HPMC, PVP, silicified microcrystalline cellulose (Avicel PH-102 NF) and magnesium stearate were gifted by JRS Pharma (Belgium). Xylitol was purchased from Bulk Powder (Hampshire, U.K).

6.3 Methods

6.3.1 Preparation, characterization, and process optimisation of pre-blend excipients

6.3.1.1 Powder flow

Individual excipient sample was weighed accurately and gently poured into a 10 mL measuring cylinder using a glass funnel. The bulk volume of each powder was recorded, and then the cylinder was tapped 200 times using a tapping machine (JV 1000, Copley Scientific, UK) under laboratory conditions and the tapped volume after each tapping was recorded. Initial experiments showed that the 200 taps were adequate to reach the maximum reduction in the volume of the powder beds. Bulk and tap density were calculated according to [Eq \(1\)](#) and [Eq \(2\)](#).

$$\text{Bulk Density, } D_b = \frac{\text{Mass of the powder (M)}}{\text{Volume of the powder (V}_0\text{)}} \quad \text{Eq (1)}$$

$$\text{Tapped density} = \frac{\text{Mass of powder blend (M)}}{\text{Tapped volume of powder blend}} \quad \text{Eq (2)}$$

Where, D_b is the bulk density (g/ml), M : mass of the powder blend (g) and V_0 is the bulk volume.

Compressibility is one factor contributing to flow. The Carr's index, (CI), Eq(3) and Hausner's ratio (HR) were calculated as the percentage change in the volume of constant mass of powder because of tapping. These values were derived using the following formula (Bowker *et al.*, 2008):

$$CI = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100 \quad \text{Eq (3)}$$

and Hausner's ratio (HR), Eq (4), were calculated using this formula (Beddow, 1995):

$$HR = \frac{\text{Tapped density}}{\text{Bulk density}} \quad \text{Eq (4)}$$

Where V_0 is bulk volume and V_t is the tapped volume.

6.3.1.2 Particle size distribution analysis

Volume-weighted particle size analysis of excipient formulation powders were conducted using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Germany). Laser diffraction particle size analyzer was equipped with a dry sampling system (Aero S, Malvern Instruments, UK) to measure sizes in the range of 0.1 μm to 3500 μm . Before measurement, a background reading was taken. The air pressure was adjusted to 2.0 bar and a feed rate of 30%

was applied. The measurement time was 5 s. The particle sizes at 10% (d10%), 50% (d50%), and 90% (d90) of the volume distribution, and the volume mean diameter (VMD, the average diameter based on the unit volume of a particle) was calculated automatically using the Malvern Software (Version 2.20). The span of the volume distribution, Eq (5), was used as a measure of the width of the distribution of size relative to the median diameter as shown formula as follow:

$$\text{Span} = \frac{d_{90\%} - d_{10\%}}{d_{50\%}} \quad \text{Eq (5)}$$

Three samples were measured for each excipient and results were averaged.

6.3.1.3 Powder crystallinity

This is an analytical technique used in the identification of crystalline phase by studying the diffraction patterns to determine the crystallinity of the material. Powder PXRD patterns of excipient formulations were collected on Empyrean PAN analytical powder diffractometer (Philips: PW1770 UK). The tube voltage and amperage were set at 40 kV and 40 mA, respectively. The monochromator slit was set at 20 mm sample size. Each sample was scanned between 5° and 45° in 2θ with a step size of 0.01° at 1 step/s. The sample stage was spun at 30 rpm. The instrument was calibrated before use using a silicon standard.

6.3.1.4 Moisture content

A thermogravimetric analyser, Pyris 1 TGA from Perkin Elmer (Massachusetts, USA) was used to measure the moisture content of all powders. 2-5 mg of each sample was loaded onto the TGA pan and heated between 30-300°C at a scanning rate of 30°C/min and held for 5 minutes at 100°C under a nitrogen stream. Pyris Manager Software (version 5.00.02) was used for analysing the obtained thermograms. Moisture content was obtained by calculating Δy for each run between 70°C and 130°C. All samples were analysed in triplicate (n=3).

6.3.1.5 Scanning Electron Microscopy (SEM)

All samples during this research were analyzed using a Zeiss EV050–EP scanning electron microscope with EM scope of (Sc 500) (Polaron equipment, Watford, UK), operated using beam current of 10 μ A at an accelerated voltage of 10 KV; the samples preparation was done by sticking few milligrams of each formulation on a separate aluminium stub using an adhesive carbon tab then sputter coating them with gold. The SEM images were obtained at different magnitudes with the aim to ascertain their morphological characteristics.

6.3.2 Preparation of tablet lozenges

6.3.2.1 Preparation using heat and congealing

Lozenge tablets were made by heat and congealing method. These lozenge tablets were prepared as shown in [Table 6.1](#) and [Table 6.2](#). All excipients such as sucrose, liquid glucose and polymer were mixed at 150°C for 30 minutes on the magnetic stirrer until the syrup was thick (Mendes *et al.*, 2006). After that, the temperature was brought down to 40- 45°C and the flavours, chlorhexidine (CHX) and citric acid were added to the molten sugar. The sugar mixtures were stirred for another 10 minutes to get uniform distribution of medicament before pouring into pre-lubricated ice-mould. Once the formulated lozenges were cooled for 30 minutes, then they were packed in an aluminium foil for physico-chemical characterisation tests.

Table 6.1: List of formulation code

Polymer Used	Concentration (%w/v)	Formulation code
Melting lozenges without polymer	-	F1, F2, F3,
PVP	1	F4
	2	F5
	3	F6
HPMC	1	F7
	2	F8
	3	F9

Table 6.2: Formulation of Chlorhexidine by heating and congealing

Excipient (mg)	F1	F2	F3	F4	F5	F6	F7	F8	F9
Sucrose	1520	1500	1470	1490	1460	1430	1490	1460	1430
Liquid glucose	1200	1220	1250	1200	1200	1200	1200	1200	1200
PVP	-	-	-	30	60	90	-	-	-
HPMC	-	-	-	-	-	-	30	60	90
Orange flavour	100	100	100	100	100	100	100	100	100
Menthol flavour	150	150	150	150	150	150	150	150	150
Citric acid	10	10	10	10	10	10	10	10	10
Chlorhexidine	20	20	20	20	20	20	20	20	20
Total weigh (each/ mg)	3000	3000	3000	3000	3000	3000	3000	3000	3000

6.3.2.2 Preparation of lozenges using direct compression

The aim of this investigations was to develop and evaluate lozenge tablets based on polyols i.e., xylitol. Excipient composition is listed in [Table 6.3](#). All the excipients were blended for 5 minutes in a V-shaped powder blender (CapsulCN®) except silicified microcrystalline cellulose and magnesium stearate. Then silicified microcrystalline cellulose was added into the mixtures and blended for another 3 minutes. Finally, magnesium stearate was added to the mixture and blended for another 1 minute. The steps for manufacturing of lozenges tablet by the direct compression method were as follows:

Weighing → Blending → Lubrication → Compression

Table 6.3: Formulation of compressed lozenges tablets

Excipients	% w/w	mg (1pc)
Xylitol	65.5	327.5
Chlorhexidine	4	20
Menthol	4	20
Orange flavour	1	5
Silicified Microcrystalline cellulose	25	125
Magnesium Stearate	0.5	2.5
Total	100	500

The samples of each excipient were accurately weighed as in [Table 6.3](#) and then compressed on 10 mm punch and die using a single- punch manual tableting machine (Globpharma, USA) at a fixed pressure of 2000 psi.

6.3.3 Method of physico-chemical analysis of lozenge tablets

6.3.3.1 Thickness and diameter

The thickness and diameter of lozenges were determined using electronic digital Vernier callipers (MSC- Limited, U.K). 10 lozenges from each batch were used and average values were calculated.

6.3.3.2 Hardness

The hardness of the lozenges was determined by using hardness tester (Varian, VK 200, Benchsaver Series, U.K) where the force required to break the lozenges were noted. 10 lozenges tablets were selected, for every batch of formulation to analyse and data were recorded.

6.3.3.3 Weight variation

The weight variation was conducted by weighting by 10 hard lozenges tablets individually and calculating the average weight and comparing the average lozenges weight to the average value.

6.3.3.4 Friability

Twenty hard lozenges tablets were weighed and placed in the friabilator (Charles Ischi AG, AE-1, U.K.), and rotated 100 times at 25 rpm for 4 minutes. After revolutions the tablets were dedusted and weighed again. The percentage friability, Eq (6), was calculated for analysis using below equation:

$$\text{Friability} = \frac{W_i - W_f}{W_i} \times 100 \quad \text{Eq (6)}$$

where W_i is the initial weight and W_f is the final weight

6.3.3.5 Lozenge tablets compression at different forces

Excipients were blended as outlined in section 6.3.2.2. The resultant blend was directly compressed using a single-punch tablet machine with size 10mm flat faced die at different compression forces of 4, 6 and 8 tonnes.

6.3.3.6 Lozenge tablet erosion/swelling

To evaluate the effect of compression force on each formulation, lozenges tablet diameter and thickness was recorded. The tablets were then placed in a small container with a lid which contained 18 ml of distilled water at room

temperature. At various time intervals (0, 5, 15 and 40 min), diameter and thickness of tablets were measured by using electronic digital Vernier callipers. The percentage increase in erosion/swelling of each tablet due to water uptake was calculated using the following equation, Eq (7).

$$\text{Erosion} = \frac{M_t - M_0}{M_0} \times 100 \quad \text{Eq (7)}$$

where M_t is the diameter of tablet at time 't' and M_0 is the diameter of tablet at time 0. The experiments were performed in triplicate for each time point. Fresh samples were used for each time point.

6.3.3.7 Dissolution study

Dissolution testing was performed to study in-vitro dissolution and the rate at which the drug is released from lozenge. A USP dissolution apparatus II (paddle method) was used with a rotational speed of 100 rpm. The dissolution testing system comprised of a VK7010 dissolution apparatus (Varian, USA) and an automated sampling manifold (Varian, UK). The dissolution test was performed according to the USP Pharmacopoeia. The dissolution media consisted of 900 ml of distilled water equilibrated to $37^\circ\text{C} \pm 0.5^\circ\text{C}$. 5ml of samples were withdrawn from the dissolution medium and replaced with the same volume of fresh media using a syringe at intervals of 2 for 20 minutes. The absorbances of CHX were recorded using a UV spectrophotometer at λ_{254} nm by NovaSpec II, Pharmacia Biotech, U.K. Three tablets were tested for each formulation.

6.3.3.8 Stability Study

Long term stability studies were conducted by placing the lozenges in individual foil and placed in airtight dark container in the desiccator according to ICH guidelines at $25\pm 2^{\circ}\text{C}$ and relative humidity at $60\pm 5\%$ for 6 months. The stability of lozenges tablets was evaluated by studying the physicochemical parameters such as weight uniformity, diameter, thickness, hardness, friability, and dissolution at different time intervals of 0, 1, 2, 3, and 6 months.

6.3.3.9 Statistical methods

The statistical significance of the result was determined by analysis of variance (ANOVA) using a one way and a two- way ANOVA in Excel. P-values less than 0.05 were considered as statistically significant ($p < 0.05$).

6.4 Result and discussion

6.4.1 Powder characterisation studies

Pre- formulation investigation is an integral part of drug and formulation development. Key focus during this phase includes assessment of physicochemical properties of the drug compound to inform product development.

To begin, the properties of excipients or drug compound must be characterized. This includes understanding physicochemical characterization such as solubility in various solvents, the dissociation constant (pKa), partition or distribution coefficient (logP or logD), pH, permeability, solid state stability, and solution state stability. Selection of appropriate salt, polymorph,

solvent form, and amorphous form are also included in this development. Furthermore, the morphology, particle size, bulk density needs to be evaluated. The final activity in pre-formulation development is excipient compatibility studies, which are required to test the stability of the drug molecule in the presence of excipients. In our study, these different characterisation studies are essential to produce lozenge tablets with desirable properties. It also ensures that lozenges tablets maintain their mechanical integrity (Kumar, 2016).

6.4.1.1 Particle size, crystallinity, and morphological studies

In powder characterisation studies, the key elements determining rheological behaviour and packing efficiency of the powder are particle properties. Various investigations have been conducted to identify relationships between powder properties and rheological behaviour. A wide particle size distribution (PSD) enhances packing density but reduces powder flowability. The coarse particles have greater flowability than their fine counterparts (Muller *et al.*, 2015). Moreover, spherical particles promote both powder packing efficiency and powder flowability.

PSD of CHX and the excipients are shown in [Table 6.4](#). The results show that polyols including xylitol and sorbitol had the highest mean volume diameter. Similarly, sucrose, fructose, HPMC and PVP had significantly larger mean volume diameter when compared to other excipients such as menthol and chlorhexidine. Furthermore, magnesium stearate showed a wider PSD (high polydispersity) in comparison to other excipients.

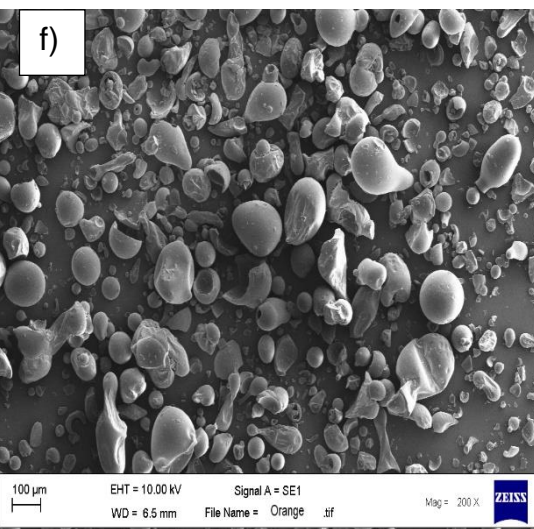
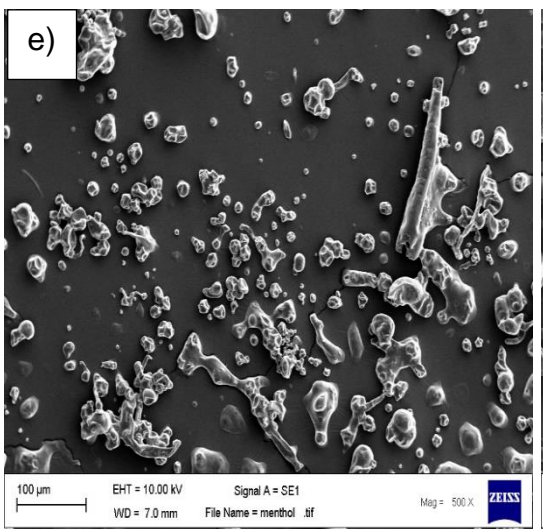
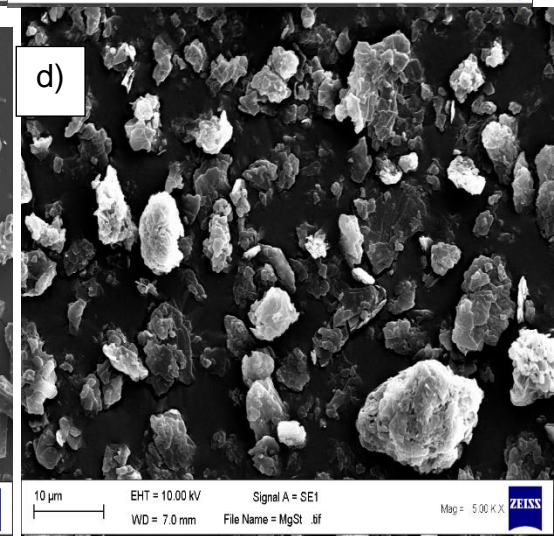
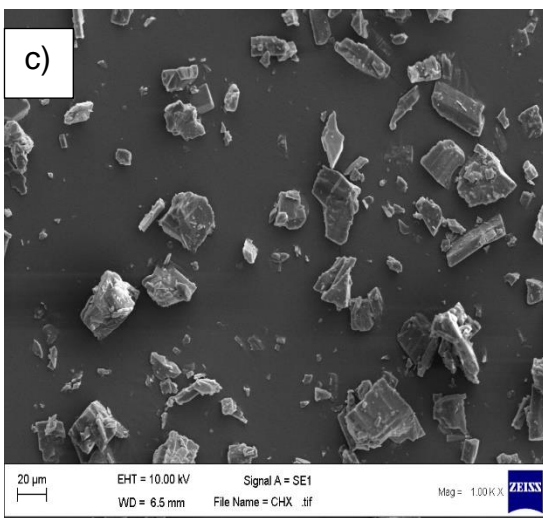
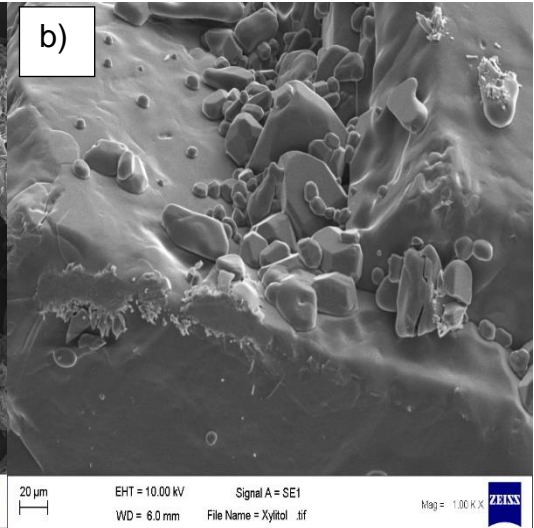
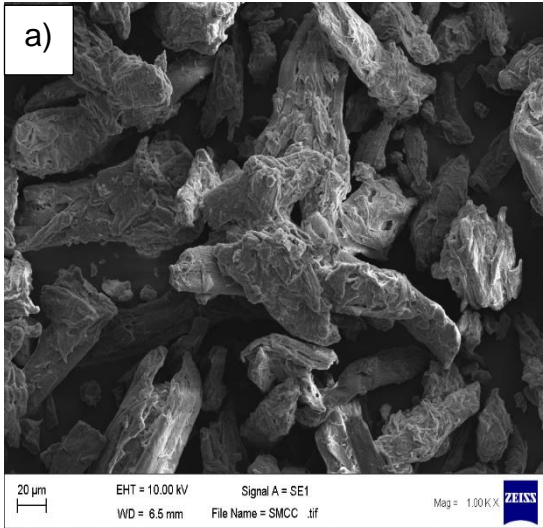
Table 6.4 : Flow properties of excipients showing the particles size analysis parameters [particle size at 10%, (d_{10%}), 50% (d_{50%}), 90% (d_{90%}), volume mean diameter, (VMD) and span: mean ±, SD, n =3]

Excipients	d10 (µm)	d50 (µm)	d90 (µm)	VMD	Span (µm)
Xylitol	19.6±0.14	370±0.07	700±0.03	544 ± 0.09	1.84 ± 0.03
Sorbitol	119.0±0.06	254±0.04	394± 0.12	255± 0.10	1.08 ± 0.01
Sucrose	43.4±0.04	160±0.13	286± 0.10	163± 0.06	1.52 ± 0.02
Fructose	19.7±0.12	74.6±0.08	178± 0.02	88.1± 0.17	2.12 ± 0.04
HPMC	26.5±0.05	70.8±0.05	202± 0.04	94.2± 0.05	2.48 ± 0.07
PVP	11.8±0.10	38.4±0.04	74.5± 0.06	432.± 0.03	1.63 ± 0.05
Maltodextrin	17.8±0.03	68.0±0.02	123± 0.05	69.8± 0.10	1.55 ± 0.15
Magnesium Stearate	2.26±0.08	10.9±0.09	58.3± 0.01	21.1± 0.07	5.14 ± 0.11
Orange flavour	9.06±0.07	39.1±0.03	95.9± 0.18	46.6± 0.12	2.22 ± 0.04
Menthol	8.06±0.16	31.8±0.10	84.2± 0.02	51.2± 0.04	2.39 ± 0.03
SMCC	21.8±0.05	62.2±0.02	120 ±0.09	67± 0.06	1.58 ± 0.01
CHX	4.95±0.03	15.7±0.05	45.6±0.06	21.3± 0.12	2.59 ± 0.03

CHX (21.3±0.12 µm) and Magnesium stearate (21.1±0.07 µm) had the lowest average particle size (VMD) with span of distribution value of 2.59±0.03 µm and 5.14±0.11 µm respectively, suggesting the presence of agglomerates due to the presence of fine particles. The cohesivity of powders affects flowability and sometimes leads to problems with powder flow. However, static charges during the filling process and therefore moderate cohesiveness is sometimes beneficial, particularly in some cases where an active pharmaceutical ingredient has a high proportion of fine particles (Shah *et al.*, 2008).

SEM images of excipients showed different particle morphologies. There were variations in morphological features between all excipients except sorbitol which had needle shape and the smallest span values of 1.08 µm, [Figure 6.1\(I\)](#). Xylitol had spherical particles with nearly uniform (regular)

shape. The SEM images revealed that the surface structure of xylitol was smooth, whereas the surface structure of sorbitol was rough. When comparing particle shapes at the same magnification, sorbitol particles appeared to be more symmetrical and spherical than xylitol particles, [Figure 6.2\(b\)](#). The xylitol particles were coarser than the sorbitol particles.



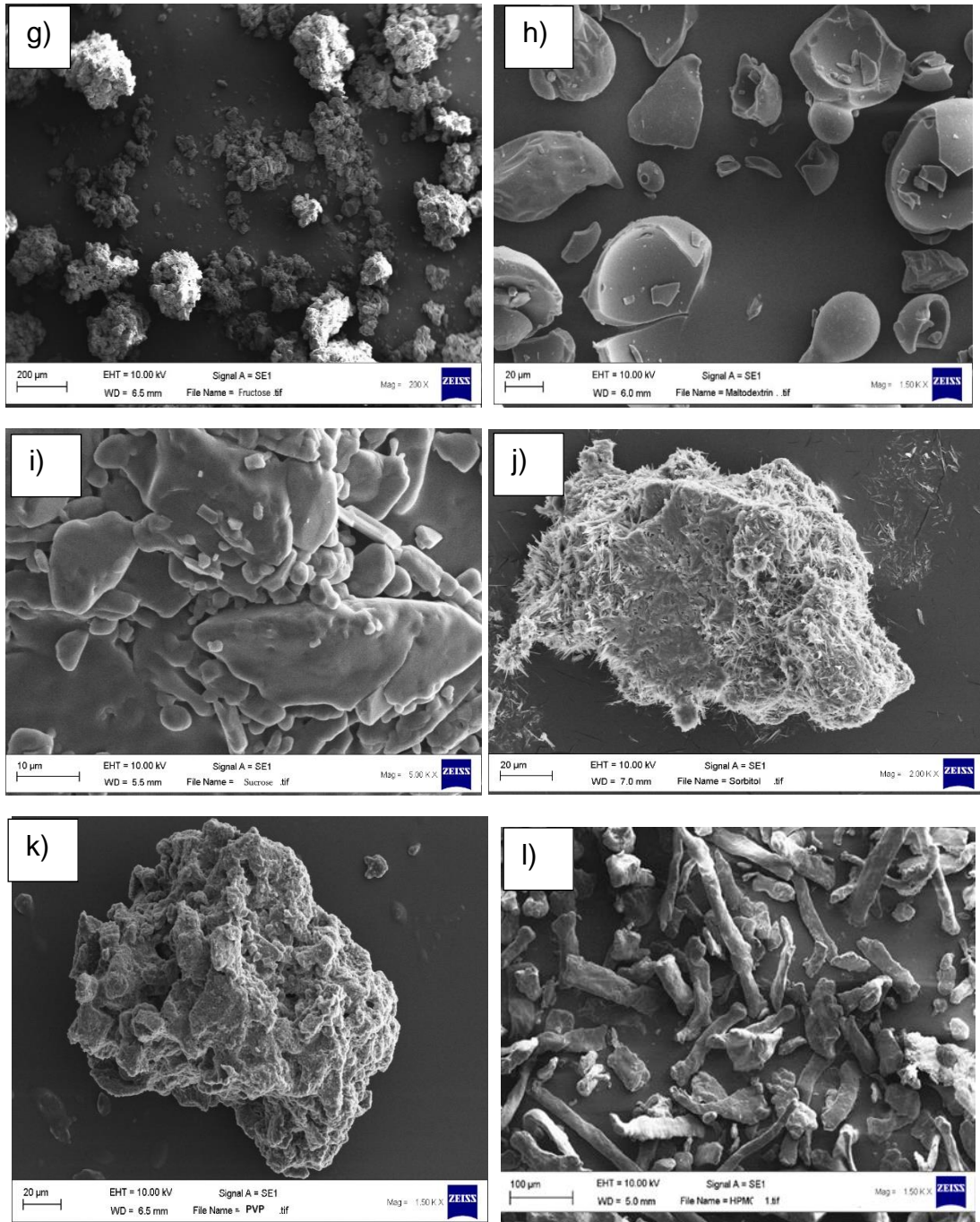


Figure 6.1: SEM images of excipient lozenges tablet pre-formulation (a: CHX, b: Xylitol, c: SMCC, d: MgSt, e: menthol, f: orange, g: fructose, h: maltodextrin, i: sucrose, j: sorbitol, k: PVP and l: HPMC)

Powder X-ray Diffraction (PXRD) analysis were performed to check the crystallinity of the excipient. The PXRD patterns of excipients are shown either in crystalline or amorphous stage as in [Figure 6.2](#). XRD results of excipients were show in appendices A6.1. The diffraction pattern of sorbitol, xylitol, sucrose, and fructose demonstrates that they have a very crystalline solid state in nature with sharp peaks throughout the scan. The PXRD pattern of PVP, appears to be amorphous which agrees with studies reported by Abdelrazek *et al.*, (2018). The PXRD pattern of CHX displayed intense and sharp peaks at 2θ values of 22.73° , 20.50° and 17.08° affirming its crystalline nature.

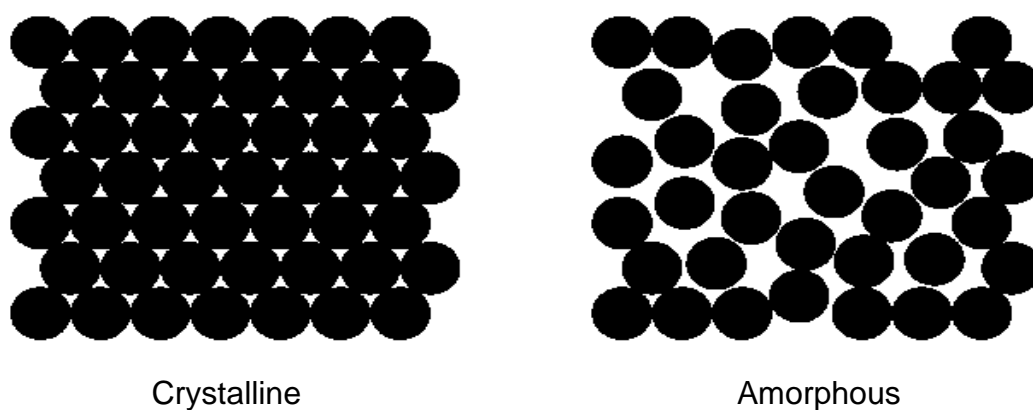


Figure 6.2: Different structure of crystalline and amorphous stage of excipients

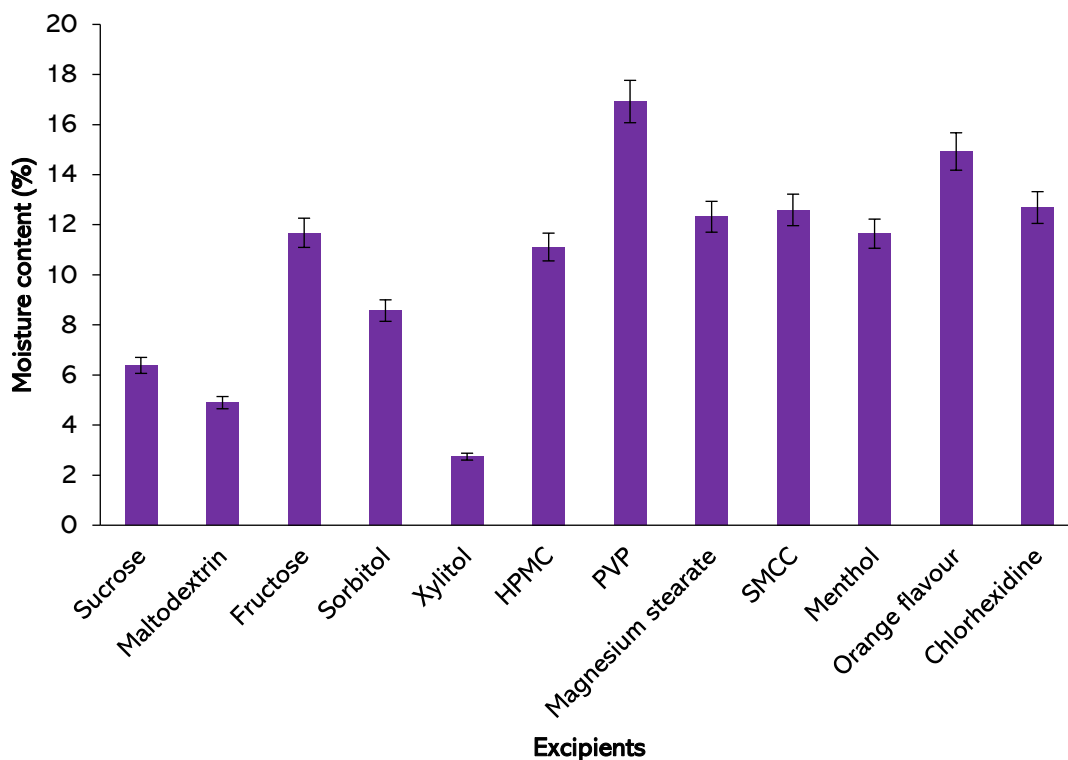


Figure 6.3: Percentage moisture content of excipients (n=3±SD) p<0.05.

Figure 6.3 shows percentage of moisture content. PVP showed the highest moisture content compared to other excipients. Study by Kurakula and Rao, (2020), found PVP is highly hygroscopic, polar, and soluble in water. They reported PVP is the most common polymer found in controlled release dosage forms due to its water imbibing property. Xylitol showed the lowest percentage of moisture content which indicates that it can be used as a good filler-binder to formulate tablet lozenges (Bolhuis *et al.*, 2009). The moisture content plays an important role in the manufacture of the final product. It may have an impact on the physical and chemical as well as microbiological properties of the final product. The hardness of a tablet can be affected by high and extra-low moisture content in the direct compression process. Room temperature and humidity must be retained within certain limits in

order to achieve satisfactory tablet hardness. According to studies reported by Ahlneck and Zografi (1990), moisture may be present as crystallization water and/or as adsorbed water. When moisture levels exceed a certain threshold, it can have an impact on product stability and increase the probability of bacterial contamination. The moisture content has an impact on the manufacture of the solid dosage formulation. It can cause poor powder flow, which can lead to inconsistent tablet performance. It may also cause problems with sticking on the tablet's surface. When moisture is present in moderation, it improves flow and also enhances the binding of the API and excipients (Ahlneck and Zografi, 1990).

6.4.1.2 Evaluation of flowability and compressibility

Powder flowability is an important factor that affects content uniformity and integrity of the solid dosage form. [Table 6.5](#) shows flowability guidelines from studies by Carr, (1976), on flowability and compressibility of the powder and granules. Furthermore, data from our work showed that the flowability of the various powder blends ranged between good, passable and very poor ([Table 6.6](#)).

Table 6.5: Guideline of CI and HR in flowability

Compressibility index (%) (CI)	Flow character	Hausner ratio (HR)
1-10	Excellent	1.00-1.11
11-15	Good	1.12-1.18
16-20	Fair	1.19-1.25
21-25	Passable	1.26-1.34
26-31	Poor	1.35-1.45
32-37	Very poor	1.46-1.59
> 38	Very, very poor	> 1.60

Table 6.6: Evaluation CI and HR of excipients, (n=3 ±SD)

Excipients	Bulk density	Tap density	CI	HR	Flow character
Sucrose	0.76	0.96	21.21	1.27	Passable
Maltodextrin	0.39	0.50	22.22	1.29	Passable
Fructose	0.48	0.62	22.62	1.29	Passable
Sorbitol	0.56	0.74	25.00	1.33	Passable
Xylitol	0.60	0.70	14.00	1.16	Good
HPMC	0.56	0.73	24.07	1.32	Passable
PVP	0.38	0.48	20.51	1.26	Fair
Magnesium stearate	0.23	0.33	30.23	1.43	Poor
SMCC	0.25	0.33	25.00	1.33	Passable
Menthol	0.33	0.57	41.67	1.71	Very poor
Orange flavour	0.40	0.71	44.00	1.79	Very poor

Poor flowability can be attributed to greater inter particle interaction and greater difference between bulk and tapped density. Xylitol has spherical particles with nearly uniform (regular) shape and passable flow properties. Sorbitol powder showed lower CI and HR than xylitol. Both excipients show good and passable flowability. Furthermore, HPMC showed passable flowability as it had high CI and HR. Amongst the binders, HPMC had higher HR values compared to PVP.

This was consistent with the findings of Patel *et al*, (2011) who discovered that using PVP as a binder instead of HPMC improved granule flow when combined with lactose as a filler (Patel *et al.*, 2011).

6.4.2 Evaluation of heat and congealing as a method to prepare lozenges

6.4.2.1 Evaluation of physico-chemical properties of lozenges

Following evaluation of material properties, the next stage was to prepare lozenge tablets and assess properties of finished dosage form. Heat and congealing which involves molten excipients followed by addition of drug and other excipients at lower temperature was employed to formulate lozenges.

All nine formulations were tested for physical parameters such as weight variation, hardness, thickness, drug content and stability. Morphological appearance of the lozenge tablets is presented in [Figure 6.4](#).

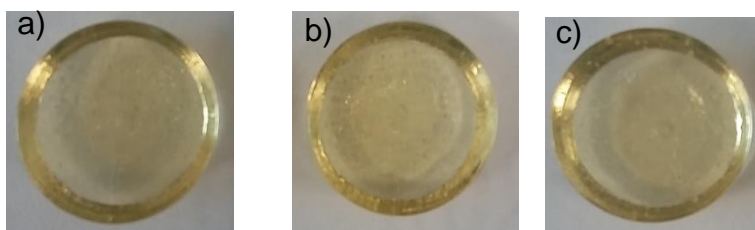


Figure 6.4: Appearance of lozenges produced by heat and congealing method. These lozenges were selected from different categories of formulations a) F1, b) F4 and F7

Table 6.7: Physical test of heat congealed lozenge tablets

Formulation	Weight variation (mg)	Thickness (mm)	Hardness (kp)	Friability (%)	Drug Content (%)
F1	3000±1.14	7.20±0.02	8.00±0.02	0.12±0.08	90.50±0.33
F2	3000±1.34	7.22±0.03	8.01±0.10	0.15±0.14	91.00±0.21
F3	3000±1.12	7.21±0.02	8.06±0.25	0.11±0.02	93.50±0.15
F4	3000±1.17	7.23±0.04	8.20±0.02	0.12±0.10	95.35±0.18
F5	3000±1.20	7.20±0.02	8.21±0.22	0.12±0.17	90.40±0.02
F6	3000±1.02	7.25±0.03	8.40±0.15	0.10±0.17	93.10±0.43
F7	3000±1.14	7.22±0.06	8.11±0.02	0.14±0.02	95.20±0.02
F8	3000±1.14	7.20±0.05	8.09±0.02	0.16±0.01	92.00±0.16
F9	3000±1.14	7.20±0.08	8.02±0.02	0.15±0.05	90.24±0.08

[Table 6.7](#) showed weight variation was found to be in the range of 3000± 1.02 to 3000± 1.34 (mg). Thickness of the formulations was in the range of

7.20±0.08 to 7.25±0.03 mm which indicated all formulations met the requirement for uniformity. Hardness of the formulations were in the range of 8.00±0.02 to 8.40±0.15 kp. Friability was between 0.10±0.13 and 0.16±0.07%. The results of hardness and friability indicated that the CHX lozenges formulations were mechanically stable. Drug content was found to be in the range of 90.24±0.08 to 95.35±0.18%.

The hardness of the tablets depends on the degree of binding that depends on the amount of the binder and the force of compression. The higher hardness of the tablet with the binder may be related to its ability to form film and its cohesive strength to form solid bonds between the particles. As a result, it is possible that the binder was forced into inter-particle spaces resulting in a more solid bond between excipients. Formulation, F6 containing PVP 3% was found to have the highest hardness values in comparison to those matrices containing other polymer ($p < 0.05$), in [Table 6.2](#). According to a study by Bajelan *et al.*, (2014), PVP is used as a common binder in the pharmaceutical industry for preparing lozenges tablet. This may be due to the formation of stronger hydrogen bridge linkages between the primary particles of liquid glucose and PVP. It can be hypothesised that both glucose and polymer had stronger binding characteristics, resulting in greater accessible hydrogen bonding space outside of the physically joined polymer chains. This was affirmed in studies reported by Tongalairoum *et al.*, (2015) who employed PVP as a mucoadhesive inner fiber between liquid glucose to improve the mechanical properties of the formulation.

HPMC was chosen in formulation, F6-F9, due to its gelling and swellable property. However, when HPMC was included as a binder in the formulation, the hardness values were lower compared to the other formulation without polymer (F1-F3). It is possible that the presence of other excipients (glucose) and the method of preparation involving high temperature together with high moisture content resulted in weakening of the inter particle bonds as reported by Nokhodchi *et al.*, (1997).

6.4.2.2 Evaluation of drug release studies

Lozenge tablet dissolution was performed using 900 mL of ultrapure water, using USP dissolution apparatus 2 (paddle method) with a rotational speed of 100 rpm. The results showed that all formulations allowed the release of the drug irrespective of the type and the particle size of the polymer, [Table 6.2](#). The highest drug release at 95.35% was formulation F4 which contained 1% PVP. Whereas the lowest drug release at 90.24% was presented in formulation F9 which was made of 3% HPMC as a binder. It was interesting to note that the difference in drug release was not noticeable when the concentration of the binder polymer was varied. For instance, when no polymer was incorporated, there was a lag phase with no release in the first 4 minutes. However, inclusion of polymer showed nearly 10% drug release in the first 2 minutes. It is possible that the polymeric chains in the formulation create a network of channels which ensures that the formulation is robust and the same time allows release of the drug upon contact with the dissolution media.

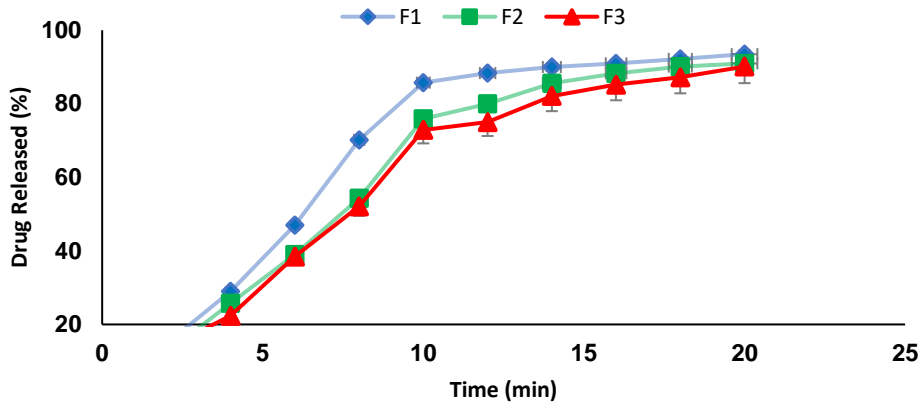


Figure 6.5: Drug release profile of CHX from melting lozenges without polymer in water at $37\pm 0.5^\circ\text{C}$ at 100 rpm. Each point represents mean \pm SD (n=3) $p < 0.05$

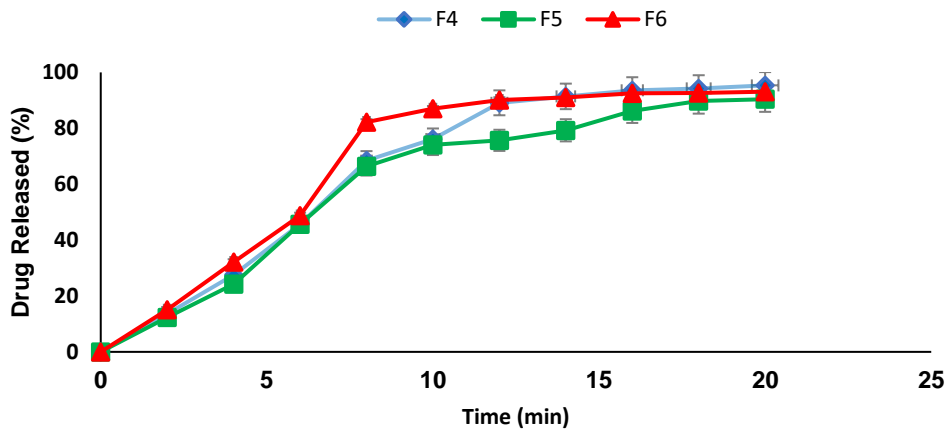


Figure 6.6 : Drug release profile of CHX melting lozenges with PVP in water at $37\pm 0.5^\circ\text{C}$ at 100 rpm. Each point represents mean \pm SD (n=3) $p < 0.05$

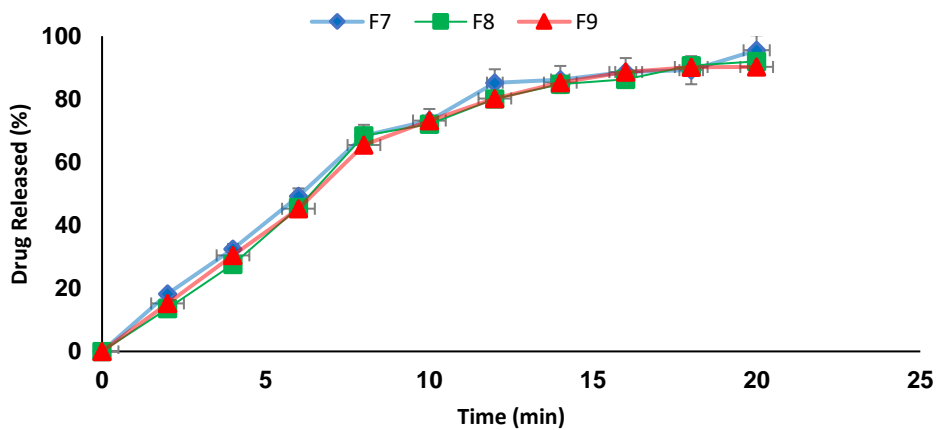


Figure 6.7: Drug release profile of CHX melting lozenges with HPMC in water at $37\pm 0.5^\circ\text{C}$ at 100 rpm. Each point represents mean \pm SD (n=3)

6.4.2.3 Evaluation of stability studies

The purpose of the stability study is to establish physical and chemical stability of the product and the shelf life of the finished dosage form. All samples of lozenge tablets were placed in stability chamber at long term ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}/ 60\% \text{RH} \pm 5\% \text{RH}$) conditions recommended by ICH Q1A(R2) (ICH, 2003). The samples were monitored at day 0, 30, 60, 90, 120, 150 and 180 by analysing tablets hardness, friability, and drug release. All the formulated lozenges showed good physical shape at the beginning when tested for hardness test. However, the shapes of lozenges tablets changed at day 30. This is possibly due to the melting of glucose (common across all the formulations) upon storage thereby compromising the integrity of the formulation. The liquid glucose was utilized as a binder and blended with various excipients to improve the mechanical and release qualities of these lozenge tablets. According to Sriram *et al.*, (2020), the proportion of liquid glucose to sucrose should be 60 to 40% to achieve transparent and smooth surface. However, with the use of 13, 18, and 20% sucrose and liquid glucose in the current study, sufficient transparency was achieved but it negatively impacted the stability of the formulation. Furthermore, when the HPMC in the formulation was heated, it became translucent. To overcome the poor stability of the finished dosage form, we decided to move away from heat and congealing method and evaluate the feasibility of formulating lozenges using direct compression.

6.4.3 Evaluation of Direct Compression Lozenges Tablets

6.4.3.1 Physical properties of the tablets

Direct compression as a tableting method does not require heat and solvents and therefore any potential issues surrounding heat and solvent drug/excipient can be easily overcome.

The first step was to evaluate the impact of different compression forces on lozenge properties to ensure sufficient mechanical integrity and low friability.

The physical properties of the lozenge tablet formulations are presented in [Table 6.8](#). All the lozenge tablet formulations showed higher hardness and strengths with the increase in compression force. This could be due to the higher force, from 4 to 8 tonnes, that was used to compress the lozenge formulations. Blending time and lubricating of the tablets also contributed to the high strength. All formulated lozenges at four different compression forces showed acceptable levels of friability of less than 1%. Moreover, the resultant tablets showed acceptable weight variation in the range 499.97-500.0mg. Therefore, all the lozenges tablet formulations exhibited acceptable physical characteristics for further study.

Table 6.8: Physical properties of lozenges tablets at different compression forces. Each point represents mean \pm SD (n=3)

	Compression force (tonnes)			
	2	4	6	8
Weight (mg)	499.97 \pm 0.04	499.99 \pm 0.02	500.00 \pm 0.01	500.00 \pm 0.09
Hardness (kp)	9.97 \pm 0.04	11.92 \pm 0.01	12.25 \pm 0.07	13.93 \pm 0.03
Friability (%)	1.00 \pm 0.02	0.60 \pm 0.01	0.30 \pm 0.09	0.20 \pm 0.04
Diameter (mm)	10.00 \pm 0.09	10.00 \pm 0.03	10.00 \pm 0.01	10.00 \pm 0.02
Thickness (mm)	4.55 \pm 0.02	4.32 \pm 0.07	4.30 \pm 0.01	4.30 \pm 0.08

6.4.3.1 Effect of Compression Force on Hardness

Hardness is a measurement of tablet strength. It provides insight into the force required to break a tablet and its ability to withstand breakage, crumbling or chipping under the conditions of storage, transportation, and handling. Generally, hardness is dependent on type and concentration of binder, tablet diameter and compression force. Figure 6.8 shows there was a significant effect on the hardness of the tablet with four different compression forces. ANOVA analysis revealed a significant difference in tablet hardness with increase in compression pressure ($p < 0.005$). This could be due to gas displacement from the powder bed in the die as compression pressure increases when bringing particles in close contact. This therefore causes increase in the number of particles in contact within the material thereby increasing particle-particle interaction leading to formation of a strong bond which increases the mechanical strength of the tablet at high compression pressure.

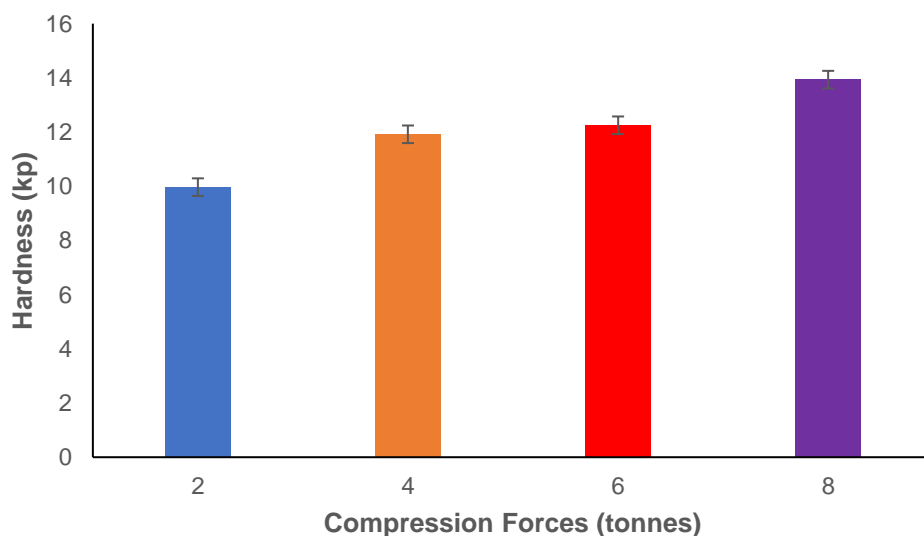


Figure 6.8: Hardness of tablet lozenges in different compression forces. Each point represents mean \pm SD (n=3) $p < 0.05$

6.4.3.2 Effect of Compression Force on Friability

The friability test for tablets is used to assess the ability of the tablets to withstand shock and abrasion encountered during packaging, transportation, and handling. The friability values decreased as compression pressure increased for all the formulations as presented in Figure 6.9. The graph shows decrease in friability with the increase in compression force. This could have been due to the formation of more solid bonds which led to the formation of tablets with increasing hardness and more resistance to fracture and abrasion.

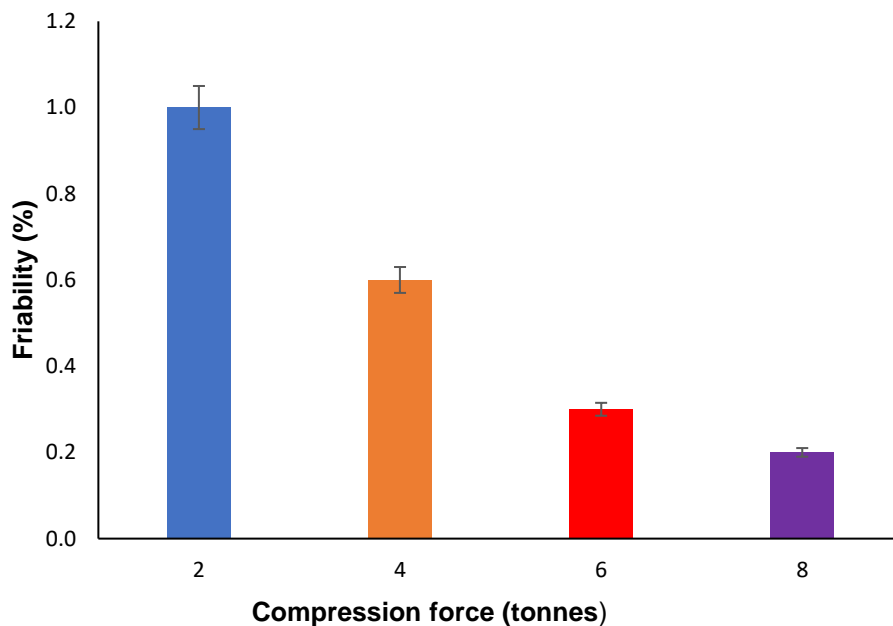


Figure 6.9: Friability of tablet lozenges under different compression forces. Each point represents mean \pm SD (n=3) p<0.05

6.4.3.3 Characterization of Tablet Erosion

The lozenge tablets were placed in a 6-well plate with water as a media for evaluating erosion/swelling over 45 minutes. The observation was timed from 0 minutes until there were no change in thickness and diameter of lozenges tablets.

Figure 6.10 shows that the thickness of the lozenges increased with time. Furthermore, Figure 6.11 shows that the diameter of the lozenges tablets also increased with time. SMCC is widely used excipient in producing tablets by direct compression. It is hygroscopic thereby affects disintegration and drug dissolution due to diffusion of the media into a tablet matrix (Raijada *et al.*, 2015).

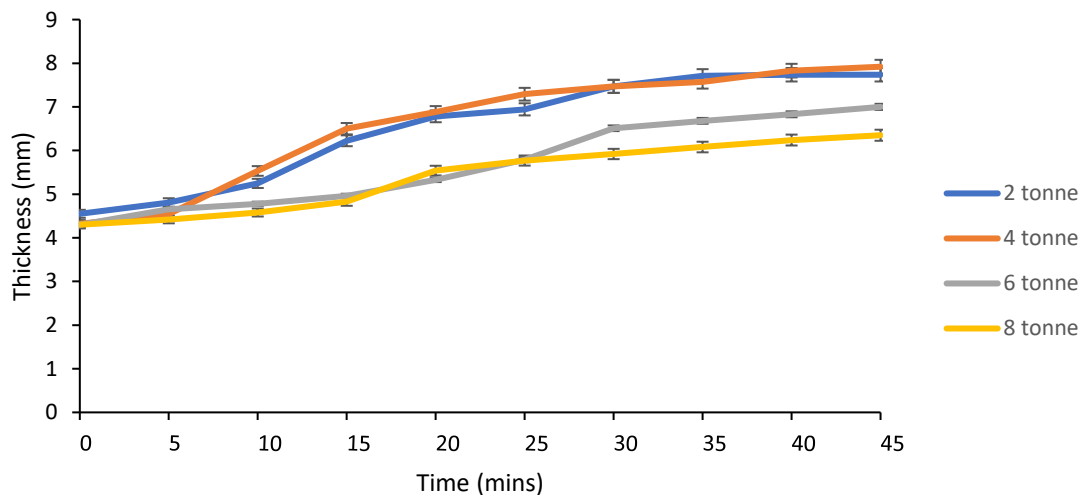


Figure 6.10: Evaluation of thickness of tablet lozenges formulation at different compression forces. Each point represents mean \pm SD (n=3) $p < 0.05$

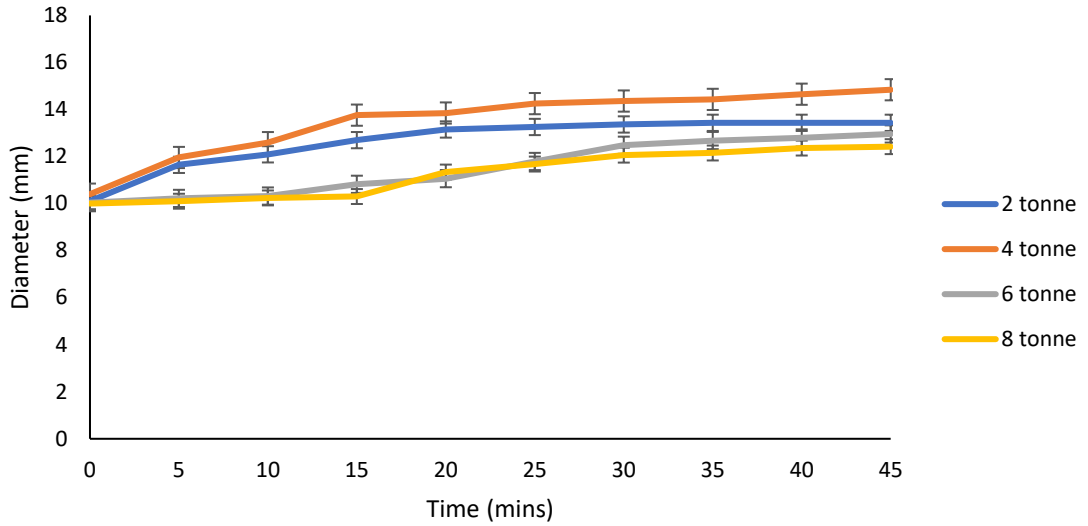


Figure 6.11: Evaluation of diameter of tablet lozenges formulation at different compression forces. Each point represents mean \pm SD (n=3) $p < 0.05$

The tablet erosion data, at different compression forces, was used to streamline the next stage of investigations.

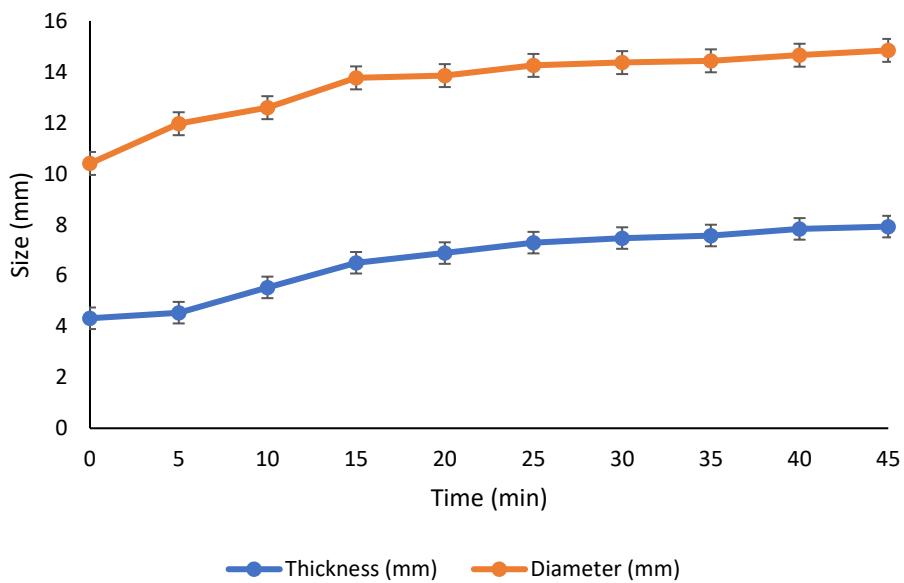


Figure 6.12: Comparison of tablet lozenges between thickness and diameter profiles at 4 tonne compression force. Each point represents, mean \pm SD (n=3) $p < 0.05$

6.4.4.4 Drug Release Study by Dissolution

Drug absorption from oral solid dosage form depends on drug release and dissolution under physiological conditions and permeability through gastrointestinal tract. Because of the critical nature of these conditions, *in vitro* dissolution can be relevant to predict *in vivo* performance.

Figure 6.13 shows the drug release of lozenges made at four different compression forces. As the compression force increases, the powder becomes densely packed together with no inter-particulate void spaces for any relative particle movement. At this stage, stress starts to build-up at the particle contact point in the die and the material begins to deform. The particles would have deformed above the elastic limit of the material. But if the force is not strong enough for it to exceed the elastic limit of the particle, the tablet would be unstable and crumble. Once compression is below the limit, materials will not form a coherent compact while compression above this limit, materials form a coherent tablet with increasing strength as the compression force increases (Garr and Rubinstein, 1990).

In this study, we observed that increases in compression force at four different compression forces showed a significant difference in the percentage of drug release rate.

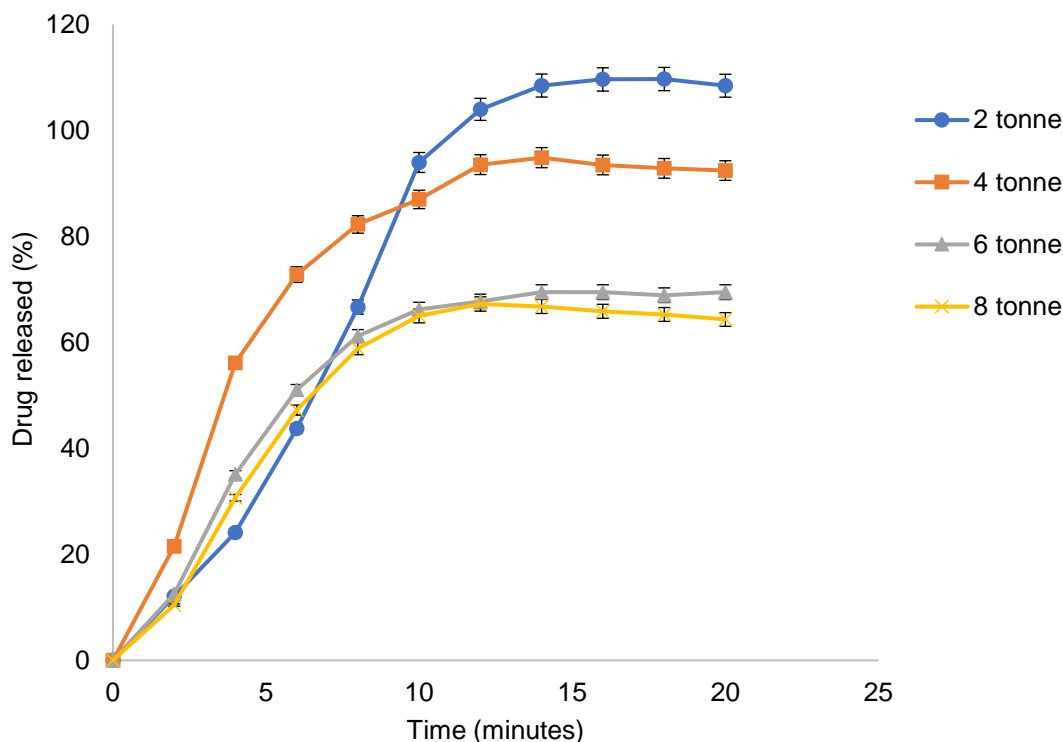


Figure 6.13: Evaluation of drug release of CHX lozenge tablet at different compression forces. Each point represents mean \pm SD (n=3) $p < 0.05$

Figure 6.13 shows percentage of drug release for tablets made at four different compression forces. At two tonne, drug release was 108%, four tonne was 92%, six tonne was 69% and eight tonne was 64% in the first 20 minutes. Drug released from matrix tablets takes place because of hydration with the dissolution medium. The hydrated portion leads to the formation of channels for drug to diffuse out. It causes the rate determining step in matrix tablet dissolution. While disintegration is not desirable in a matrix tablet, dissolution occurs from the dosage form. The design of such delivery system requires inhibitory action on biofilm formation as well as synergism with CHX tablet to remain intact and gradually disintegrate to release its content, without drug dumping.

There was a significant effect of compression pressure on the mechanical properties of the matrix tablets. However, the release properties of CHX lozenges tablet formulations were significantly influenced with increase in compression pressure. It can therefore be concluded that the matrix forming polymer and the material properties of the drug together with the compression pressure influenced drug release profiles (Gonnissen *et al.*, 2007).

6.4.4.5 Stability Studies

The aim of stability studies is to provide evidence on how the performance of the formulation containing API changes over time as a result of a variety of environmental factors. Moreover, this study was used to evaluate drug release from the matrix lozenges tablets *in vitro*. Stability studies were carried out on lozenges compressed at 4 tonnes. Due to the amount of work involved in preparing the scaled-up batches and the following analytical characterization, 4 tonnes was chosen as an optimal compression for stability evaluation. During this stability study, lozenges tablets were characterized for physicochemical analysis such as hardness, friability, weight uniformity, and drug release.

Table 6.9: Evaluation of Stability Studies of Chlorhexidine Lozenge Tablets

Time (day)	Hardness (Kp)	Friability (%)	Weigh uniformity (mg)	Drug content (%)
1	11.5±0.13	0.07±0.06	500.00±0.34	98.41 ±0.04
30	11.4±0.15	0.08±0.12	500.00±0.40	96.62 ±0.10
60	10.9±0.04	0.09±0.24	500.00±0.46	95.62 ±0.07
90	10.6±0.08	0.11±0.03	500.00±0.57	92.43 ±0.06
180	10.6±0.25	0.12±0.17	500.00±0.58	90.13 ±0.05

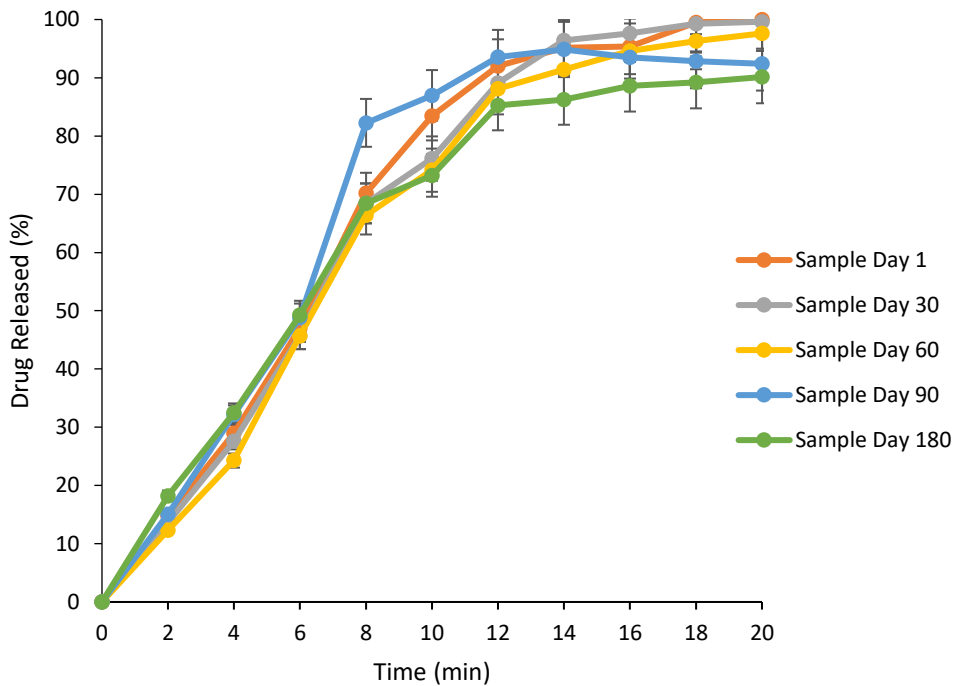


Figure 6.14: Evaluation of drug release of CHX lozenge tablet according to stability study for day (1, 30, 60, 90 and 180) days. Each point represents mean \pm SD (n=3) $p < 0.05$

The results on [Table 6.9](#) indicated no significant changes in the physical properties of the formulations during 6 months (180 days) of stability studies. Similarly, drug release data remain unchanged throughout the stability period ([Figure 6.14](#)). Each sample of lozenges tablets from long term stability periods (from 1 to 180 days) were analysed using *in vitro* dissolution study to measure drug released within 20 minutes. Results from this investigation has shown that chlorhexidine-based lozenges can be prepared using direct compression method and that the impact of storage conditions is minimal on the integrity and stability of the dosage form.

6.5 Conclusion

The systematic approach to dosage form development included assessment of material properties such as flowability, moisture content, morphology and crystallinity. Data from these studies helped inform the understanding of the formulation process and the assessment of finished dosage form properties. Heat congealing was used to study the impact of type and concentration of polymer on the finished lozenges. Formulation with 1% PVP, formulation F4 showed the best drug released within 20 minutes. Despite the ability to alter the drug release properties by incorporating different polymer-based binders, these units failed to remain stable beyond 30 days. The relationships between the excipient and stability are the most important for formulating of lozenges tablets. These are the most important criteria for developing drug release formulations, so that the formulation lozenges can withstand environmental conditions and have an impact on the quality of the product's shelf life.

On the other hand, evaluation of heat free method of tableting, direct compression enabled the formulation of lozenges with different release profile. It was interesting to note impact of compression force on drug release profiles; for instance, compression force can be used to alter profile without changing the composition of the formulation. This is a significant advantage for the formulator whereby alteration of manufacturing conditions without changing the composition allows control of physical properties and drug release profiles.

CHAPTER SEVEN

General Discussion

7.0 General Discussion

7.1.1 Discussion

S. mutans is one of the most common bacteria that causes dental caries because it promotes demineralization when it forms a biofilm on the tooth surface. This research aimed to investigate the effect of excipients alone and in combination with chlorhexidine for the effective treatment of gingivitis in the oral cavity. This study was carried out by screening commonly studied excipients for anti-microbial and anti-biofilm activity against *S. mutans*. Different concentrations of excipients such as sugars and polyols were evaluated on the growth of *S. mutans* in planktonic state under aerobic and anaerobic conditions. Results indicate that the pathogenic organism's survival in the human oral cavity is highly dependent on nutrition, which plays an important role in the formation of the dental biofilm, or plaque. Mineral oil was used to simulate anaerobic respiration of *S. mutans* and the impact of various sugars and polyols was studied in the concentration range between 2 - 40% w/v. With increasing time, the results revealed changes in growth rate between aerobic and anaerobic conditions. The growth rate doubled under anaerobic respiration when compared to aerobic respiration. However, these findings confirmed that the growth rate patterns in the presence of 5 percent fructose and 40 percent xylitol had changed (from 35 to 75 hours). When these agents inhibited *S. mutans* growth, both of these concentrations showed significant changes in growth rate, (-2% with 5 percent fructose and +8% with 40 percent xylitol) in both aerobic and anaerobic conditions.

According to a study by Linke *et al.*, (2014) the growth rate of *S. mutans* in the presence of higher concentration of xylitol inhibited biofilm formation in *S. mutans*. On the other hand, Söderling *et al.*, (2008), found that a low concentration of xylitol at 0.01% reduced the proliferation of *S. mutans* cells.

In addition, planktonic cells were found to be particularly responsive to xylitol when combined with CHX in this investigation. Synergistic effects of xylitol and CHX exhibited antibacterial effect against *S. mutans*. Using the checkerboard assay, the data for the combination utilized against *S. mutans* demonstrated indifferent interactions, but both values (FLCI of 1.0 and 1.008) were significantly lower than the antagonistic value. According to time-kill data *S. mutans* responded well to the combinations of xylitol and CHX at an early stage of exposure, with CFU/mL decreasing to the lowest detectable level between 1 and 10 hours of treatment. The combination of CHX and xylitol resulted in reduction in CFU/mL, indicating synergy. This was confirmed by colony counts acquired using the Miles and Misra method. These results also supported by Hasan *et al.*, (2014) for their clinical isolates treated with anticariogenic agents, Quercitrin and Deoxynojirimcin (Hasan *et al.*, 2014). According to the findings, a combination of antimicrobial agents is required to effectively reduce the pathogenic bacterium that causes gingivitis. As a result, xylitol is the ideal sugar substitute for developing lozenges tablets to treat gingivitis (Bader *et al.*, 2013).

According to the data on biofilm formation with sugars, only fructose at 40% combined with a lower concentration of CHX (0.1µg/ml) resulted in decreased biofilm density when compared to lower sugar concentrations or a high concentration of CHX alone. Additionally, when compared to sugars (fructose, sucrose, lactose, and maltodextrin) in combination with CHX at different concentrations, biofilm formation by *S. mutans* with polyols (mannitol, sorbitol, and xylitol) supported much less biofilm formation. At a concentration of 20% sorbitol and xylitol, CHX was successful in inhibiting biofilm development at 0.1g/ml. Polyols (sorbitol and xylitol) were found to be more efficient than sugars in suppressing *S. mutans* biofilm development regardless of concentration. Moreover, polyols reduced biofilm development and interacted with CHX at various concentrations. Furthermore, when combined with CHX at a concentration of 0.5µg/ml, xylitol and sorbitol at 2-40 percent concentration resulted in 85% reduction in biofilm density. These biofilms have a number of characteristics that work together to promote antibiotic resistance (Dincer *et al.*, 2020; Wilton *et al.*, 2015). Some of these characteristics that contribute to development of biofilm are exopolysaccharide matrix, pH, and nutrition. According to this study, at low concentrations, both sorbitol and xylitol had synergistic inhibitory effects on biofilm formation by *S. mutans*. Decker *et al.*, (2014) also found that at low concentrations of CHX, both sorbitol and xylitol inhibit biofilm formation. As a result, xylitol and sorbitol were chosen as appropriate excipients in the formulation of CHX oral dosage forms.

Furthermore, the presence of CHX disrupted the *S. mutans* biofilm, resulting in a decreased biofilm density. This is because a synergistic mechanism is

established when polyol and CHX are combined. Polyols were combined with CHX because they provide antimicrobial activity, resulting in a synergistic effect (Nayak *et al.*, 2014). In conclusion, the study found that pathogenic bacteria *S. mutans* biofilm development may be effectively eliminated by appropriate treatment with low concentrations of xylitol and 0.1µg/ml of CHX. Through vitality observation by CLSM has proven that xylitol and CHX have a synergistic impact. Xylitol at 5% showed 50% decrease in vitality compared to control in the present of 0.1µg/ml of CHX. *S. mutans* biofilms formation showed a characteristic 3-D clusters in CLMS using dyes, S9 and PI to visual the live/dead cell. These dyes help in assessing the membrane integrity status and allows the differentiation between living (intact membranes) and non-vital/dead (compromised membranes) (Decker, 2001). Besides that, many typical biofilm architectural and behavioural features are present in the mature plaque biofilm, including an abundant matrix with extensive channelling, intercellular communication and signalling, pH and O₂ gradients, and cell cluster detachment. The interaction of xylitol and CHX had a significant effect on biofilms as they disrupted the biofilm while also having a bactericidal effect on cells by affecting their vitality.

S. mutans cariogenicity is aided by glucan production, which promotes the sticky and cohesive qualities of *S. mutans* biofilms. Glucan-binding productions have also been discovered to contribute to the sticky and cohesive qualities of *S. mutans* biofilms, as well as to influence their architecture in vitro. The *S. mutans* biofilm formation can be detected by EPS production by visualizing CLSM using dyes S9 and Alexa Fluor 633, labelled

as lectin Con A. Con A is a marker for carbohydrates glycoconjugates which are essential components to detect EPS and bind selectively to glucopyranosyl molecules such as glucans. The EPS synthesis of *S. mutans* biofilms in the presence of xylitol and CHX was shown to be reduced after 72 hours. With increase in their concentrations, xylitol and CHX have efficiently affected *S. mutans* cells. Changes in biofilm morphology and microbial cells vitality were caused by combining xylitol with CHX. Xylitol and CHX have effectively disrupted *S. mutans* cells with increases in their concentrations. Combination of xylitol and CHX were induced changes in biofilm morphology and development of microcolonies. This synergistic effect can be explained due to a combination of effects such as decrease in cell viability as confirmed by time kill studies and decrease in EPS as confirmed by CLSM. This finding is further substantiated by studies on *S. mutans* biofilm inhibition using erythritol and xylitol, which lead to reduction in cell viability and quantity of polysaccharide in the EPS matrix (Loimaranta *et al.*, 2020).

Furthermore, xylitol coupled with CHX was effective in regulating gene expression during *S. mutans* biofilm formation as these gene are commonly expressed in other dominant oral microbes such as *lactobacilli*, *staphylococci* and *corynebacterial* (Krzysciak *et al.*, 2014). In the presence of xylitol and CHX, the findings demonstrated overexpression of glycosyltransferases. The gene expression experiments revealed additional details about the effects of xylitol and CHX on the production of *S. mutans* biofilms. Glucan – binding proteins were found to contribute to the adhesive and cohesive properties of *S. mutans* biofilms and affected the architecture of these biofilm in vitro.

Therefore, the results showed upregulation of glycosyltransferases in the presence of xylitol and CHX. This is due to xylitol, a sugar alcohol, commonly present in dietary carbohydrates which is metabolized to glucose. Hence, the increased expression of glycosyltransferases could be in response to increasing concentration of xylitol. Gene expression studies provided new information of xylitol and CHX effects on *S. mutans* biofilm formation. The selected virulence genes such as *gtfB* and *comD* involved in EPS production showed increased expression when exposed to xylitol and CHX at different concentrations (Tahmourespour *et al.*, 2011). Genes *smu_104*, *smu_105* and *wapA* involved in biofilm formation with intercellular competition were expressed less than control as these genes were successfully inhibited by xylitol and CHX at various concentrations (Decker *et al.*, 2014).

UV–vis spectroscopy is an extensively used quantitative analysis method in modern laboratories. This is owing to its ease of use, accuracy, efficiency, and low cost. This is also a useful analytical approach for determining the drug concentration in a formulation. The creation of an analytical approach aids in the understanding and mitigation of the impact of critical process factors on precision and accuracy. The method was validated on several occasions and followed ICH criteria for UV method validation. As a result, the established approach is efficient, with a short runtime, and can be used to quantify CHX from any material at room temperature (Rosenthal *et al.*, 2004).

The most commonly used delivery route is oral delivery, which is popular due to its non-invasiveness and general ease of administration. A variety of oral

drug delivery systems have been developed, with different set of advantages and disadvantages, but the directly compressed lozenges tablet remains the most popular. Direct compressed lozenges tablet have good storage stability, transportability, and simplicity for the patient, while they are appealing to manufacturers due to the low costs and complexity involved in production (Pundir & Verma, 2014).

The lozenge tablet is the most common type of solid dosage form used in the oral cavity to achieve systemic or local action (Srikanth *et al.*, 2013). They are solid, single-dose preparations and flavoured medicated dosage formulations with a sweetened base that are intended to be sucked and held in the mouth (Peters, 2005). These lozenges are intended to relieve oropharyngeal symptoms, which are typically caused by local infections, and can also be used to deliver drugs systemically via buccal lining absorption (Batheja *et al.*, 2006). They are typically placed in the oral cavity between the cheek and gum for local and/or systemic administration. Lozenge tablets were formulated by direct compression using xylitol as a binder based on drug and excipient optimisation studies in microbiology. The results demonstrated that excipient consolidation in the powder bed increases with increase in compression force. The lozenges tablets showed excellent properties based on physico-chemical studies such as friability, hardness and erosion. Following optimisation of the compression force, 4 tonnes was selected for drug release and stability studies. Furthermore, the development of formulations using both heat congealing and direct compression methods had no difference when drug release was compared. However, tablets

produced by heat congealing failed to retain their physical integrity thereby negatively impacting on the overall stability of the formulation.

The results demonstrate the feasibility and assessment of directly compressed lozenges but further work is required to develop drug dissolution method to simulate the limited availability of saliva in the oral cavity and the impact of pressure/forces exerted by the tongue on the formulation. Based on the current findings, CHX lozenges tablet preparation was successfully formulated. They demonstrated good physical properties, drug release, and stability. As a result, the lozenges tablets were developed which could be used as a potential treatment for gingivitis patients. The replacement of sugars with polyols in lozenges tablet offers a good alternative formulation to diabetic patients and children with gingivitis.

Xylitol and magnolia bark extracts were investigated in high risk adults in the clinical trial to prevent caries. Three groups were categorised as follows (1) polyols (pols), (2) xylitol (xyl) and (3) xylitol plus magnolia (xyl + mag) which were added into the chewing gum. The clinical examination of the enrolled sample was performed at baseline (t_0) and repeated at the end of the chewing-gum administration period (12 months, t_2) and at the end of the experimental period (24 months, t_3). The outcomes such as caries lesions, gingival bleeding, *mutans streptococci* (MS), and plaque pH were re-evaluated after 2 years in 64 Pols, 66 Xyl and 64 Xyl + Mag subjects. This clinical study found that the xyl + Mag and Xyl ($p = 0.01$ and <0.01 , respectively) groups had significantly lower gingival scores. Chewing-gum

with Xylitol and Magnolia has a greater preventive effect than Xylitol alone (Campus *et al.*, 2011).

7.1.2 Main Conclusions

The results presented here confirm that the synergistic effect of polyols in the presence of CHX. Excipient xylitol play as multifunctional for local delivery of CHX against *S. mutans* biofilms.

Xylitol is a sugar alcohol used in the development of lozenges. Pre-formulation studies indicate that xylitol is a good choice which is supported by microbiological data generated. The present study demonstrates that that lozenges tablet with xylitol and CHX exhibited good drug release and stability. Moreover, the method of production, physico-chemical characterisation, in vitro drug release study and stability study of CHX lozenges tablets were carried out to optimise the concentrations of active constituents and excipients for potential treatment of oral diseases.

7.1.3 Future Work

Clinical research is required to determine drug release in the oral cavity, patient perception and acceptance, and the in- vivo effect of the developed lozenge tablets on *S. mutans* biofilm in the oral cavity. Moreover, one of the CHX side effects is teeth staining, which should be tested in vitro and in vivo, despite the fact that the concentration of CHX used in this formulation is much lower than the dose available in marketed products. There is a possibility that the lower concentration of CHX combined with xylitol used in

the developed oral dosage form will lead to minimal teeth staining. Furthermore, there are commercially available dosage forms in solutions for treating gingivitis. Lozenge based dosage form offers convenience and acceptability, especially when travelling and in the community. As a result, the research conducted and methods developed will provide a solid foundation for the adoption of dosage forms containing novel combinations of API and functional excipients in the future.

In addition, a clinical study will be needed to assess the efficacy of the newly formulated lozenges tablet in clinical cases of gingivitis. The efficacy of the CHX- xylitol formulation could be tested on multispecies biofilm. A robust analysis could be used for HPLC development to optimize drug release experiments.

Furthermore, research on other oral microbes should be conducted in order to study this infectious disease using technique such as whole genome sequencing (WGS). Finally, a clinical study evaluating the effect of different polyols formulations in combination with antimicrobial agent should be conducted in different target population (children, adults, elderly and patients with other comorbidities such as diabetes, asthma etc.).

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APPENDICES

A2.1 Preparation of broth and agar

Broth/ media	Quantity (g/l)	Agar	Quantity (g/l)
BHI	37	BHI	47
MHB	23	MHA	38
TSB	30	TSA	45

A2.2 The Bioscreen

The machine Bioscreen plate reader is an automated turbidity reader linked to an integrated PC, manufactured by LabSystems, Helsinki, Finland. The system can measure changes in the turbidity of cell up to 200 microtitre wells (2 × 100 multiwell plates) and can provide growth curves from each well either directly to the monitor or in the form of a data file suitable for further examination.

A2.3 Time kill study result

Time	Log ₁₀ CFU/mL			
	Control <i>S.mutans</i>	Xylitol 40%	CHX	Xylitol 40%+CHX
0	6.50	6.43	6.31	6.12
2	6.80	6.20	5.45	4.40
4	7.60	6.10	4.62	3.20
6	8.20	5.72	4.50	3.00
10	8.65	6.21	4.21	2.00
12	9.10	5.60	3.80	1.60
20	9.20	4.80	2.40	1.15
24	9.00	3.50	1.76	0.32

A2.4 Percentage of increased in sucrose

Time (hours)	2% Sucrose	2% Sucrose +MO	% Increased	5 % Sucrose	5% Sucrose + MO	% Increased	10% Sucrose	10% Sucrose + MO	% Increased	20% Sucrose	20% Sucrose + MO	Increased	40% Sucrose	40% Sucrose + MO	% Increased'
0	0	0		0	0		0	0		0	0		0	0	
5	0.121	0.216	78%	0.121	0.308	155%	0.187	0.355	90%	0.174	0.462	166%	0.196	0.490	150%
10	0.228	0.247	9%	0.228	0.395	73%	0.250	0.395	58%	0.274	0.488	78%	0.278	0.534	92%
15	0.247	0.295	20%	0.247	0.413	67%	0.362	0.426	18%	0.344	0.536	56%	0.385	0.568	48%
20	0.269	0.375	39%	0.269	0.445	65%	0.425	0.445	5%	0.415	0.568	37%	0.444	0.674	52%
25	0.311	0.443	43%	0.311	0.484	56%	0.456	0.484	6%	0.477	0.596	25%	0.425	0.680	60%
30	0.387	0.572	48%	0.387	0.527	36%	0.463	0.520	12%	0.489	0.629	29%	0.463	0.693	50%
35	0.425	0.651	53%	0.455	0.568	25%	0.510	0.578	13%	0.5	0.654	31%	0.546	0.724	33%
40	0.463	0.700	51%	0.479	0.632	32%	0.546	0.654	20%	0.582	0.689	18%	0.529	0.759	43%
45	0.506	0.712	41%	0.526	0.696	32%	0.588	0.756	29%	0.568	0.799	41%	0.561	0.811	45%
50	0.550	0.680	24%	0.545	0.685	26%	0.602	0.752	25%	0.56	0.784	40%	0.635	0.820	29%
55	0.533	0.681	28%	0.573	0.680	19%	0.612	0.745	22%	0.542	0.770	42%	0.660	0.832	26%
60	0.530	0.680	28%	0.530	0.677	28%	0.506	0.720	42%	0.54	0.744	38%	0.642	0.820	28%
65	0.527	0.680	29%	0.527	0.670	27%	0.488	0.701	44%	0.538	0.725	35%	0.642	0.810	26%
70	0.520	0.679	31%	0.520	0.670	29%	0.480	0.700	46%	0.53	0.720	36%	0.636	0.805	27%
75	0.520	0.671	29%	0.524	0.670	28%	0.483	0.711	47%	0.53	0.720	36%	0.628	0.805	28%

A2.5 Percentage of increased in lactose

Time (hours)	2% Lactose	2% Lactose +MO	%Increased	5 % Lactose	5% Lactose + MO	% Increased	10% Lactose	10% Lactose + MO	% Increased	20% Lactose	20% Lactose + MO	% Increased	40% Lactose	40% Lactose + MO	% Increased
0	0	0		0	0		0	0		0	0		0	0	
5	0.175	0.216	24%	0.175	0.336	92%	0.175	0.369	111%	0.298	0.462	55%	0.345	0.541	57%
10	0.221	0.247	12%	0.221	0.395	79%	0.221	0.395	79%	0.336	0.488	45%	0.360	0.576	60%
15	0.271	0.295	9%	0.301	0.413	38%	0.301	0.426	42%	0.367	0.536	46%	0.421	0.634	51%
20	0.336	0.375	12%	0.358	0.445	24%	0.336	0.445	32%	0.407	0.568	40%	0.457	0.649	42%
25	0.382	0.443	16%	0.402	0.484	20%	0.382	0.484	27%	0.456	0.596	31%	0.477	0.68	43%
30	0.414	0.487	18%	0.425	0.527	24%	0.414	0.520	25%	0.514	0.629	22%	0.489	0.693	42%
35	0.465	0.575	24%	0.495	0.568	15%	0.465	0.578	24%	0.568	0.654	15%	0.547	0.721	32%
40	0.501	0.647	29%	0.515	0.632	23%	0.501	0.654	30%	0.581	0.689	19%	0.639	0.758	19%
45	0.540	0.682	26%	0.568	0.696	23%	0.540	0.742	37%	0.679	0.755	11%	0.685	0.793	16%
50	0.550	0.680	24%	0.593	0.685	16%	0.550	0.752	37%	0.676	0.784	16%	0.67	0.795	19%
55	0.533	0.681	28%	0.573	0.680	19%	0.533	0.745	40%	0.663	0.770	16%	0.652	0.768	18%
60	0.530	0.680	28%	0.550	0.677	23%	0.530	0.720	36%	0.651	0.744	14%	0.641	0.753	17%
65	0.527	0.680	29%	0.527	0.670	27%	0.527	0.701	33%	0.642	0.725	13%	0.636	0.731	15%
70	0.520	0.679	31%	0.520	0.670	29%	0.520	0.700	35%	0.632	0.720	14%	0.63	0.72	14%
75	0.520	0.678	30%	0.520	0.671	29%	0.520	0.700	35%	0.629	0.711	13%	0.62	0.715	15%

A2.6 Percentage of increased in fructose

Time (hours)	2% Fructose	2% Fructose +MO	% Increased	5 % Fructose	5% Fructose + MO	% Increased	10% Fructose	10% Fructose + MO	% Increased	20% Fructose	20% Fructose + MO	% Increased	40% Fructose	40% Fructose + MO	% Increased
0	0	0		0	0		0	0		0	0		0	0	
5	0.175	0.216	24%	0.273	0.336	23%	0.282	0.355	26%	0.298	0.462	55%	0.345	0.541	57%
10	0.221	0.247	12%	0.312	0.395	27%	0.312	0.395	27%	0.336	0.488	45%	0.360	0.576	60%
15	0.276	0.344	25%	0.357	0.413	16%	0.357	0.426	19%	0.367	0.536	46%	0.421	0.634	51%
20	0.336	0.375	12%	0.407	0.445	9%	0.407	0.445	9%	0.407	0.568	40%	0.457	0.649	42%
25	0.382	0.443	16%	0.456	0.484	6%	0.456	0.484	6%	0.456	0.596	31%	0.477	0.68	43%
30	0.414	0.487	18%	0.514	0.527	3%	0.514	0.552	7%	0.514	0.629	22%	0.489	0.693	42%
35	0.465	0.575	24%	0.578	0.568	-2%	0.568	0.578	2%	0.568	0.654	15%	0.547	0.721	32%
40	0.501	0.647	29%	0.618	0.632	2%	0.629	0.654	4%	0.581	0.689	19%	0.669	0.758	13%
45	0.540	0.682	26%	0.683	0.696	2%	0.656	0.756	15%	0.679	0.799	18%	0.675	0.788	17%
50	0.550	0.680	24%	0.680	0.685	1%	0.673	0.752	12%	0.676	0.784	16%	0.67	0.782	17%
55	0.533	0.681	28%	0.671	0.680	1%	0.660	0.745	13%	0.663	0.770	16%	0.652	0.753	15%
60	0.530	0.680	28%	0.663	0.677	2%	0.642	0.720	12%	0.651	0.744	14%	0.641	0.745	16%
65	0.527	0.680	29%	0.660	0.670	2%	0.642	0.701	9%	0.642	0.725	13%	0.636	0.731	15%
70	0.520	0.679	31%	0.653	0.670	3%	0.636	0.700	10%	0.632	0.720	14%	0.63	0.722	15%
75	0.520	0.678	30%	0.650	0.671	3%	0.624	0.700	12%	0.629	0.711	13%	0.62	0.72	16%

A2.7 Percentage of increased in maltodextrin

Time (hours)	2% Malto dextrin	2% Malto dextrin +MO	%Increased	5 % Malto dextrin	5% Malto dextrin + MO	% Increased	10% Malto dextrin	10% Malto dextrin + MO	% Increased	20% Malto dextrin	20% Malto dextrin + MO	% Increased	40% Malto dextrin	40% Malto dextrin + MO	% Increased
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0.175	0.216	24%	0.175	0.273	56%	0.175	0.336	92%	0.298	0.462	55%	0.336	0.541	61%
10	0.196	0.247	26%	0.221	0.312	41%	0.258	0.395	53%	0.336	0.488	45%	0.395	0.576	46%
15	0.282	0.320	13%	0.301	0.357	19%	0.300	0.413	38%	0.367	0.536	46%	0.413	0.634	53%
20	0.336	0.375	12%	0.336	0.407	21%	0.336	0.445	32%	0.407	0.568	40%	0.445	0.649	46%
25	0.382	0.443	16%	0.382	0.456	19%	0.382	0.484	27%	0.456	0.596	31%	0.484	0.68	41%
30	0.414	0.487	18%	0.414	0.514	24%	0.414	0.527	27%	0.514	0.629	22%	0.527	0.693	31%
35	0.465	0.575	24%	0.465	0.578	24%	0.465	0.568	22%	0.568	0.654	15%	0.568	0.721	27%
40	0.501	0.647	29%	0.501	0.618	23%	0.500	0.632	26%	0.581	0.689	19%	0.632	0.758	20%
45	0.540	0.682	26%	0.540	0.683	26%	0.540	0.696	29%	0.679	0.721	6%	0.696	0.793	14%
50	0.550	0.680	24%	0.550	0.680	24%	0.545	0.685	26%	0.676	0.765	13%	0.685	0.782	14%
55	0.533	0.681	28%	0.533	0.671	26%	0.533	0.680	28%	0.663	0.770	16%	0.680	0.773	14%
60	0.530	0.680	28%	0.530	0.663	25%	0.530	0.677	28%	0.651	0.744	14%	0.677	0.753	11%
65	0.527	0.680	29%	0.527	0.660	25%	0.527	0.670	27%	0.642	0.725	13%	0.670	0.748	12%
70	0.520	0.679	31%	0.520	0.653	26%	0.520	0.670	29%	0.632	0.720	14%	0.670	0.736	10%
75	0.520	0.678	30%	0.520	0.650	25%	0.520	0.671	29%	0.629	0.702	12%	0.670	0.725	8%

A2.8 Percentage of increased in sorbitol

Time (hours)	2% Sorbitol	2% Sorbitol +MO	% Increased	5 % Sorbitol	5% Sorbitol + MO	% Increased	10% Sorbitol	10% Sorbitol + MO	% Increased	20% Sorbitol	20% Sorbitol + MO	% Increased	40% Sorbitol	40% Sorbitol + MO	% Increased
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0.180	0.224	24%	0.187	0.258	38%	0.195	0.278	43%	0.298	0.331	11%	0.345	0.453	31%
10	0.221	0.280	27%	0.236	0.298	26%	0.264	0.326	23%	0.336	0.476	42%	0.360	0.488	36%
15	0.286	0.375	31%	0.332	0.386	16%	0.345	0.422	22%	0.367	0.534	46%	0.421	0.536	27%
20	0.316	0.422	34%	0.358	0.435	22%	0.358	0.476	33%	0.407	0.549	35%	0.457	0.568	24%
25	0.362	0.536	48%	0.370	0.557	51%	0.370	0.573	55%	0.456	0.580	27%	0.477	0.596	25%
30	0.384	0.564	47%	0.455	0.580	27%	0.455	0.590	30%	0.514	0.593	15%	0.489	0.629	29%
35	0.425	0.623	47%	0.460	0.656	43%	0.460	0.662	44%	0.568	0.675	19%	0.547	0.679	24%
40	0.451	0.661	47%	0.500	0.672	34%	0.553	0.695	26%	0.581	0.728	25%	0.669	0.738	10%
45	0.487	0.739	52%	0.531	0.744	40%	0.540	0.752	39%	0.617	0.753	22%	0.675	0.764	13%
50	0.525	0.757	44%	0.526	0.720	37%	0.536	0.765	43%	0.630	0.734	17%	0.676	0.761	13%
55	0.533	0.746	40%	0.518	0.720	39%	0.528	0.750	42%	0.652	0.722	11%	0.663	0.753	14%
60	0.500	0.740	48%	0.503	0.713	42%	0.513	0.744	45%	0.641	0.709	11%	0.651	0.744	14%
65	0.468	0.736	57%	0.498	0.710	43%	0.508	0.743	46%	0.636	0.700	10%	0.642	0.740	15%
70	0.456	0.730	60%	0.486	0.710	46%	0.506	0.700	38%	0.630	0.697	11%	0.632	0.739	17%
75	0.450	0.730	62%	0.480	0.704	47%	0.500	0.700	40%	0.620	0.691	11%	0.629	0.739	17%

A2.9 Percentage of increased in mannitol

Time (hours)	2% Mannitol	2% Mannitol +MO	% Increased	5 % Mannitol	5% Mannitol + MO	% Increased	10% Mannitol	10% Mannitol + MO	% Increased	20% Mannitol	20% Mannitol + MO	% Increased	40% Mannitol	40% Mannitol + MO	% Increased
0	0	0	0	0	0		0	0	0	0	0	0	0	0	0
5	0.185	0.215	16%	0.199	0.220	11%	0.209	0.225	8%	0.215	0.228	6%	0.226	0.228	1%
10	0.208	0.285	37%	0.228	0.297	30%	0.228	0.317	39%	0.283	0.322	14%	0.297	0.341	15%
15	0.237	0.379	60%	0.247	0.387	57%	0.247	0.396	60%	0.367	0.436	19%	0.321	0.357	11%
20	0.252	0.399	58%	0.269	0.416	55%	0.269	0.426	58%	0.407	0.468	15%	0.357	0.407	14%
25	0.296	0.443	50%	0.311	0.437	41%	0.311	0.461	48%	0.456	0.496	9%	0.456	0.473	4%
30	0.335	0.572	71%	0.387	0.584	54%	0.346	0.596	69%	0.514	0.622	21%	0.489	0.514	5%
35	0.417	0.651	56%	0.425	0.632	49%	0.425	0.658	49%	0.554	0.675	22%	0.547	0.683	25%
40	0.453	0.670	48%	0.463	0.686	42%	0.463	0.669	48%	0.560	0.714	28%	0.582	0.699	20%
45	0.476	0.720	51%	0.516	0.722	30%	0.516	0.732	40%	0.548	0.755	38%	0.568	0.759	34%
50	0.517	0.725	40%	0.550	0.734	33%	0.550	0.734	33%	0.522	0.762	46%	0.56	0.770	38%
55	0.528	0.738	40%	0.533	0.746	38%	0.533	0.745	40%	0.512	0.771	51%	0.542	0.774	43%
60	0.538	0.745	38%	0.530	0.758	41%	0.530	0.762	43%	0.506	0.777	54%	0.54	0.777	44%
65	0.527	0.757	44%	0.527	0.763	45%	0.527	0.760	45%	0.498	0.786	58%	0.538	0.780	45%
70	0.520	0.760	46%	0.520	0.772	46%	0.520	0.760	48%	0.480	0.780	63%	0.524	0.780	49%
75	0.520	0.760	46%	0.520	0.770	46%	0.520	0.752	48%	0.462	0.780	69%	0.521	0.780	50%

A2.10 Percentage of increased in xylitol

Time (hours)	2% Xylitol	2% Xylitol +MO	% Increased	5 % Xylitol	5% Xylitol + MO	% Increased	10% Xylitol	10% Xylitol + MO	% Increased	20% Xylitol	20% Xylitol + MO	% Increased	40% Xylitol	40% Xylitol + MO	% Increased
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0.121	0.225	86%	0.273	0.336	23%	0.282	0.355	26%	0.298	0.462	55%	0.345	0.369	7%
10	0.221	0.288	30%	0.312	0.395	27%	0.312	0.395	27%	0.336	0.488	45%	0.360	0.455	26%
15	0.247	0.372	51%	0.357	0.413	16%	0.357	0.426	19%	0.367	0.536	46%	0.421	0.463	10%
20	0.268	0.416	55%	0.386	0.445	15%	0.407	0.445	9%	0.407	0.568	40%	0.457	0.521	14%
25	0.332	0.455	37%	0.402	0.484	20%	0.456	0.484	6%	0.456	0.596	31%	0.477	0.563	18%
30	0.414	0.497	20%	0.430	0.527	23%	0.514	0.520	1%	0.514	0.629	22%	0.489	0.62	27%
35	0.465	0.614	32%	0.508	0.622	22%	0.568	0.638	12%	0.584	0.654	12%	0.597	0.657	10%
40	0.500	0.688	38%	0.618	0.699	13%	0.629	0.654	4%	0.581	0.689	19%	0.669	0.725	8%
45	0.536	0.748	40%	0.635	0.750	18%	0.666	0.750	13%	0.679	0.780	15%	0.675	0.795	18%
50	0.542	0.732	35%	0.658	0.740	12%	0.673	0.756	12%	0.676	0.784	16%	0.67	0.799	19%
55	0.533	0.730	37%	0.671	0.745	11%	0.660	0.745	13%	0.663	0.770	16%	0.652	0.772	18%
60	0.530	0.725	37%	0.663	0.737	11%	0.642	0.720	12%	0.651	0.744	14%	0.641	0.753	17%
65	0.527	0.725	38%	0.660	0.730	11%	0.642	0.701	9%	0.642	0.725	13%	0.636	0.754	19%
70	0.520	0.722	39%	0.653	0.720	10%	0.636	0.700	10%	0.632	0.720	14%	0.63	0.73	16%
75	0.520	0.720	38%	0.650	0.720	11%	0.624	0.700	12%	0.629	0.711	13%	0.62	0.725	17%

A3.1 Size of inoculum

Statistical ANOVA Analysis

One- way ANOVA (Appendix)

SUMMARY

<i>Size of inoculum (μl)</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
100	3	1.604	0.53466	2.0333E-05
200	3	3.733	1.24433	0.00143033
400	3	4.42	1.482	0.015132
800	3	5.587	1.86233	7.2333E-05

ANOVA

<i>Source of Variation</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	3	0.93670	224.967	4.61E-08	4.066181
Within Groups	8	0.03331	0.00416		
Total	11	2.84343			

Anova: Two-Factor Without Replication

<i>SUMMARY</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
100ul	6	1.541	0.256833	0.019191
200ul	6	2.253	0.3755	0.196076
400ul	6	2.61	0.435	0.301665
800ul	6	2.878	0.479667	0.464859
Control (S.mutans)	4	5.245	1.31125	0.323544
0.1	4	0.755	0.18875	0.000101
0.5	4	0.78	0.195	9E-05
1	4	0.797	0.19925	5.29E-05
2	4	0.816	0.204	2.47E-05
4	4	0.889	0.22225	5.69E-05

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	0.167799	3	0.055933	1.043772	0.401817	3.287382
Columns	4.105146	5	0.821029	15.32132	1.97E-05	2.901295
Error	0.80381	15	0.053587			
Total	5.076755	23				

A3.2 Size of inoculum only 200µl

Anova: Two-Factor Without Replication

<i>SUMMARY</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
200	6	1.474	0.245667	0.015686
0	6	1.054	0.175667	0.007477
0	6	1.007	0.167833	0.007025
0	3	0.5	0.166667	0.083333
0.1	3	0.563	0.187667	0.000144
0.5	3	0.584	0.194667	0.000134
1	3	0.603	0.201	6.1E-05
2	3	0.613	0.204333	3.63E-05
4	3	0.672	0.224	6.7E-05

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	0.022039	2	0.011019	0.757274	0.494044	4.102821
Columns	0.005428	5	0.001086	0.074599	0.994814	3.325835
Error	0.145514	10	0.014551			
Total	0.17298	17				

A3.3 Size of inoculum with CHX Concentration

Two-way ANOVA

SUMMARY	CHX concentration ($\mu\text{g/ml}$)					Total
	0.1	0.5	1.0	2.0	4.0	
<i>Size inoculum 100</i>						
Count	3	3	3	3	3	15
Sum	0.55	0.57	0.58	0.60	0.647	2.962
Average	0.18	0.19	0.19	0.20	0.215	0.1974
Variance	3.6E-05	1.7E-05	9.3E-06	3E-06	2.6E-05	0.00013
<i>Size inoculum 200</i>						
Count	3	3	3	3	3	15
Sum	0.52	0.55	0.58	0.57	0.632	2.863
Average	0.17	0.18	0.19	0.19	0.210	0.1908
Variance	3.6E-05	3.9E-05	1E-05	3.6E-05	1.6E-05	0.00018
<i>Size inoculum 400</i>						
Count	3	3	3	3	3	15
Sum	0.59	0.62	0.63	0.61	0.674	3.14
Average	0.19	0.20	0.21	0.20	0.224	0.2093
Variance	7.3E-05	7E-05	3E-05	4.6E-05	6.7E-05	0.0003

Variance	1.4E-05	4.3E-05	9E-06	2.5E-05	3.7E-05	0.00011
Count	<i>Size inoculum 800</i>					15
Sum	0.539	0.549	0.569	0.6	0.674	2.922
Average	0.17967	0.18058	0.18742	0.2	0.22467	0.1948
Variance	5.6E-05	4.9E-05	5.6E-05	2.5E-05	4.2E-05	0.00033
Count	<i>Total</i>					12
Sum	2.21	2.287	2.369	2.394	2.627	
Average	0.18417	0.19058	0.19742	0.1995	0.21892	
Variance	0.00011	0.00014	0.00011	3.8E-05	6.2E-05	
ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	0.00285	3	0.00095	33.752	4.8E-11	2.83875
Columns	0.00824	4	0.00206	73.2374	7.3E-18	2.60597

	0.00		8.6E	3.04	0.004	2.0034
Interaction	103	12	-05	614	01	6
	0.00		2.8E			
Within	112	40	-05			
	0.01					
Total	324	59				

A3.4 Effect of fructose with CHX on *S. mutans* biofilms inhibition

Fructose

<i>SUMMARY</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
0.02	3	1.567667	0.783834	0.627573
0.05	3	1.554	0.777	0.895121
0.1	3	1.104999	0.5525	0.46948
0.2	3	0.435	0.2175	0.088901
0.4	3	0.057778	0.028889	0.000563
0.1 µg/ml	5	4.301	0.8602	0.364614
0.5 µg/ml	5	0.418445	0.83689	0.007872

<u>ANOVA</u>					
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>
	<i>F crit</i>				
Rows	0.915726	4	0.228932	1.594742	0.331103
			6.388233		
Columns	1.507423	1	1.507423	10.50074	0.031659
			7.708647		
Error	0.574216	4	0.143554		
Total	2.997366	9			

A3.5 Effect of sucrose with CHX on *S. mutans* biofilms inhibition

Sucrose

<i>SUMMARY</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
0.02	3	1.619957	0.809979	0.647621
0.05	3	1.8725	0.93625	1.330896
0.1	3	1.407039	0.703519	0.331314
0.2	3	0.854998	0.427499	0.031326
0.4		0.76	0.38	0.075272
0.1 µg/ml	5	5.368201	1.07364	0.268965
4 µg/ml	5	1.146292	0.229258	0.005925

ANOVA

<i>Source of</i>						
<i>Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F</i>
Rows	0.465586	4	0.116396	0.734389	0.61396	
Columns	1.782452	1	1.782452	11.24617	0.028475	
Error	0.633977	4	0.158494			
Total	2.882014	9				

A3.6 Effect of lactose with CHX on *S. mutans* biofilms inhibition

<i>SUMMARY</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
2	3	0.977222	0.488611	0.256408
5	3	1.099667	0.549833	0.45474
10	3	1.52	0.76	1.067748
20	3	2.144444	1.072222	1.683816
40	3	2.230667	1.115333	1.756771
0.1	5	7.406333	1.481267	0.298605
4	5	0.565667	0.113133	0.003721

ANOVA

<i>Source of</i>						
<i>Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	0.66929	4	0.167324	1.23941	6.38823	
Columns	4.67947	1	4.679472	34.66208	7.70864	
Error	0.54001	4	0.135003			
Total	5.888777	9				

A3.7 Effect of maltodextrin with CHX on *S. mutans* biofilms inhibition

<i>SUMMARY</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>	
0.02	3	0.6448828	0.322441	4.4E-06	
0.05	3	1.07	0.535	0.122018	
0.1	3	0.801	0.4005	0.040613	
0.2	3	0.549	0.2745	0.008845	
0.4	3	0.443	0.2215	0.012013	
2 µg/ml	5		0.457192	0.042487	
4 µg/ml		5	1.2219243	0.244385	0.004954

ANOVA

<i>Source of</i>						
<i>Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	0.1194	4	0.02987	1.70029	0.3098555	6.388233
Columns	0.1132	1	0.11321	6.44421	0.0640797	7.708647
Error	0.0702	4	0.01756			
Total	0.30298	9				

A3.8 Effect of mannitol with CHX on *S. mutans* biofilms inhibition

<i>SUMMAR</i>	<i>Cour.</i>	<i>Sum</i>	<i>Average</i>	<i>Varianc</i>
0.02	3	2.82590	1.41295	0.25840
0.05	3	2.96626	1.48313	0.37835
0.1	3	2.829	1.414	0.27158
0.2	3	2.446	1.223	0.09331
0.4	3	2.274	1.137	0.06697

0.1 µg/ml 5 8.23247 1.64649 0.06447

1 µg/ml 5 5.108693 1.021739 0.001776

ANOVA

Source of

<i>Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	0.17216	4	0.043042	1.854662	0.282165	
Columns	0.97580	1	0.975803	42.04694	0.002916	
Error	0.0928	4	0.023207			
Total	1.2408	9				

A3.9 Effect of sorbitol with CHX on *S. mutans* biofilms inhibition

<i>SUMMARY</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
2	3	0.422	0.0844	0.024819
5	3	0.338667	0.067733	0.009462
10	3	0.306833	0.061367	0.003045
20	3	0.253667	0.050733	0.000822
40	3	0.183167	0.036633	0.000582

0.1µg/ml	3	0.864667	0.172933	0.019047
0.5µg/ml	3	0.108167	0.021633	9.39E-05
1µg/ml	3	0.124333	0.024867	0.000185
2µg/ml	3	0.217667	0.043533	0.000254
4µg/ml	3	0.1895	0.0379	0.000486

ANOVA

<i>Source of</i>						
<i>Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	0.006444	4	0.001611	0.349157	0.840774	3.006917
Columns	0.081099	4	0.020275	4.394341	0.01379	3.006917
Error	0.073821	16	0.004614			
Total	0.161363	24				

A3.10 Effect of xylitol with CHX on *S. mutans* biofilms inhibition

<i>SUMMARY</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>	
	2	3	0.422	0.0844	0.024819
	5	3	0.338667	0.067733	0.009462
0.1µg/ml			0.604333	0.302167	0.008149
0.5µg/ml			0.025	0.0125	7.61E-05
1µg/ml	3	0.024	0.012	9.8E-05	
2µg/ml	3	0.077667	0.038833	0.000636	
4µg/ml		3	0.029667	0.014833	5.34E-05

ANOVA

Source of

<i>Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	0.00069	1	0.000694	0.59432	7.70864	
Columns	0.12880	4	0.032201	0.01059	6.38823	
Error	0.00831	4	0.00208			
Total	0.137818	9				

A4.1 Preparation of stock solution of Alexa Fluor 633

Stock solution can be made at 1-5mg/ml in 0.1M sodium bicarbonate (approximately pH 8.3).

Alexa Fluor was diluted 20 times in 2.29ml of solvent (0.1 M sodium bicarbonate and store at -20°C. (20 x (19.2 $\mu\text{mol/L}$) of stock solution.)

A4.2 Extraction of total RNA

Briefly, 5 mls of overnight cultures were transferred into 1.5 ml centrifuge tubes. Microbial cultures were centrifuged at 5000 x g for 3-5 minutes at 4°C after which the supernatant was discarded. The pellet was loosened by flicking the bottom of the tube and 350 μl of Buffer RLT was added to each sample and vortexed vigorously.

For RNA extraction from biofilms, the supernatant was discarded and biofilm cell development from 6- well plate was scrapped with sterile loop and pipetting. The cells were rinsed twice with 100 μl PBS buffer and were then transferred into a centrifuge tube where 350 μl RLT buffer was added and samples were sonicated for 1 minute at room temperature. Following lysis of cells, 250 μl of ethanol (96-100%) were added into each tube. Each sample was then transferred into a RNeasy mini spin column and centrifuged for 15 sec at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Buffer RW1 (700 μl) was then added onto each RNeasy column and centrifuged for 15 sec at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Each RNeasy column was then transferred into a new 2-ml collection tube. Buffer RPE (500 μl) was pipetted onto each RNeasy column and

centrifuged for 15 sec at $\geq 8000 \times g$ ($\geq 10,000$ rpm). After centrifugation, each RNeasy column was transferred into a new 1.5ml collection tube and 30–50 μ l of RNase-free water were pipetted directly onto the RNeasy membrane and samples were centrifuged for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute the RNA. RNA extraction was prepared in triplicates per sample. RNA samples were analysed with a Nanodrop UV-Vis 2000 (Thermo Scientific, UK) spectrophotometer to assess their quality and quantity.

A4.3 Quantification of RNA

Concentration and purity of each RNA sample were quantified by NanoDrop ND-2000 spectrophotometer according to the manufacturer's instructions (ThermoFisher Scientific, UK). Briefly, 2 μ l of RNase free-water was loaded into the sample reader and the RNA setting was used to establish a blank standard. The sample reader was cleaned with distilled water and 2 μ l of each RNA sample was loaded into the spectrophotometer for absorbance measurement at 260 nm (A_{260}) and 280 nm (A_{280}). If the A_{260}/A_{280} ratio achieved a value of 1.9-2.1, this was indicative of high purity RNA, RNeasy mini handbook, Qiagen, (U.K). This was repeated for all the RNA samples before storage at -80°C for degradation prevention.

A4.4 RT-PCR Method

10 μ l of HotStart Taq master mix bought from Qiagen was added into 0.1ml Eppendorf tube. 1 μ l of each of the primers (10 pmoles/ μ l) were purchased from Sigma were added into the tube. Meanwhile 2 μ l cDNA of sample synthesised as describe in section 4.2.3 were added into the tube. 6 μ l of RNase free water was added as final aliquot. Total volume of sample would

be 20ul. These aliquoted sample was mixed well with vortexed before the PCR reaction.

The RT-PCR was performed using a thermal cycling condition block. The cycling conditions were as follows: initial heat 15 minutes at 95°C for activation. However, 35 cycles were adapted at 30 seconds for denaturation at 95°C, 30 seconds for annealing time (T_m) (according to manufacturer below 5°C has been suggested) at 58°C of primers, 30 seconds for extension time at 72°C. For final extension, 10 minutes at 72°C and a final cooling at 4°C for 10 minutes.

A4.5 Calculation for normalization of gene expression

	<i>gftB</i>				S1	S2	Average	Normalisation (Gene of interest/S.mutans)	
Lane 1	Planktonic S.mutans				13573	13597	13585	42456	0.319978331
Lane 2	Biofilm S.mutans control				42368	42544	42456	42456	1
Lane 3	Biofilm S.mutans + 1% Xylitol				38904	35872	37388	42456	0.880629357
Lane 4	Biofilm S.mutans+5% Xylitol				45236	45648	45442	42456	1.070331637
Lane 5	Biofilm S.mutans+1% Xylitol + 0.25 µg/ml CHX				33689	32542	33115.5	42456	0.77999576
Lane 6	Biofilm S.mutans +1% Xylitol + 0.5 µg/ml CHX				48283	45660	46971.5	42456	1.10635717
Lane 7	Biofilm S.mutans +5% Xylitol+ 0.25 µg/ml				59825	55471	57648	42456	1.357829282
Lane 8	Biofilm S.mutans +5% Xylitol+ 0.5 µg/ml CHX				53343	53459	53401	42456	1.257796307
Lane 9	Biofilm S.mutans + 0.25 µg/ml CHX,				41923	53459	47691	42456	1.123304127
Lane 10	Biofilm S.mutans + 0.5 µg/ml CHX)				58765	57230	57997.5	42456	1.366061334

	<i>gftC</i>				S1	S2	Average	Normalisation (Gene of interest/S.mutans)	
Lane 1	Planktonic S.mutans				44967	54872	49920	44431	1.123541261
Lane 2	Biofilm S.mutans control				44338	44523	44431	44431	1
Lane 3	Biofilm S.mutans + 1% Xylitol				33638	33425	33532	44431	0.754695536
Lane 4	Biofilm S.mutans+5% Xylitol				52412	50128	51270	44431	1.153937048
Lane 5	Biofilm S.mutans+1% Xylitol + 0.25 µg/ml CHX				35240	36213	35727	44431	0.804098536
Lane 6	Biofilm S.mutans +1% Xylitol + 0.5 µg/ml CHX				44546	44859	44703	44431	1.006121921
Lane 7	Biofilm S.mutans +5% Xylitol+ 0.25 µg/ml				82968	81524	82246	44431	1.851115788
Lane 8	Biofilm S.mutans +5% Xylitol+ 0.5 µg/ml CHX				71550	72572	72061	44431	1.621881365
Lane 9	Biofilm S.mutans + 0.25 µg/ml CHX,				72423	75498	73961	44431	1.664633529
Lane 10	Biofilm S.mutans + 0.5 µg/ml CHX)				82673	81024	81849	44431	1.842169231

	<i>glk</i>				S1	S2	Average	Normalisation (Gene of interest/S.mutans)	
Lane 1	Planktonic S.mutans				337987	453948	395968	368161.5	1.075526637
Lane 2	Biofilm S.mutans control				382043	354280	368162	368161.5	1
Lane 3	Biofilm S.mutans + 1% Xylitol				739435	721647	730541	368161.5	1.984294936
Lane 4	Biofilm S.mutans+5% Xylitol				484609	487591	486100	368161.5	1.320344468
Lane 5	Biofilm S.mutans+1% Xylitol + 0.25 µg/ml CHX				523452	554208	538830	368161.5	1.463569656
Lane 6	Biofilm S.mutans +1% Xylitol + 0.5 µg/ml CHX				532417	557078	544748	368161.5	1.479642765
Lane 7	Biofilm S.mutans +5% Xylitol+ 0.25 µg/ml				330732	301245	315989	368161.5	0.858287735

	smu_104				S1	S2	Average	Normalisation (Gene of interest/S.mutans)	
Lane 1	Planktonic S.mutans				262824	264540	263682	650849.5	0.405135135
Lane 2	Biofilm S.mutans control				659177	642522	650850	650849.5	1
Lane 3	Biofilm S.mutans + 1% Xylitol				404082	382150	393116	650849.5	0.604004459
Lane 4	Biofilm S.mutans+5% Xylitol				491048	487574	489311	650849.5	0.751803604
Lane 5	Biofilm S.mutans+1% Xylitol + 0.25 µg/ml CHX				433297	478652	455975	650849.5	0.700583622
Lane 6	Biofilm S.mutans +1% Xylitol + 0.5 µg/ml CHX				144565	156422	150494	650849.5	0.231226267
Lane 7	Biofilm S.mutans +5% Xylitol+ 0.25 µg/ml				270430	250014	260222	650849.5	0.399819006

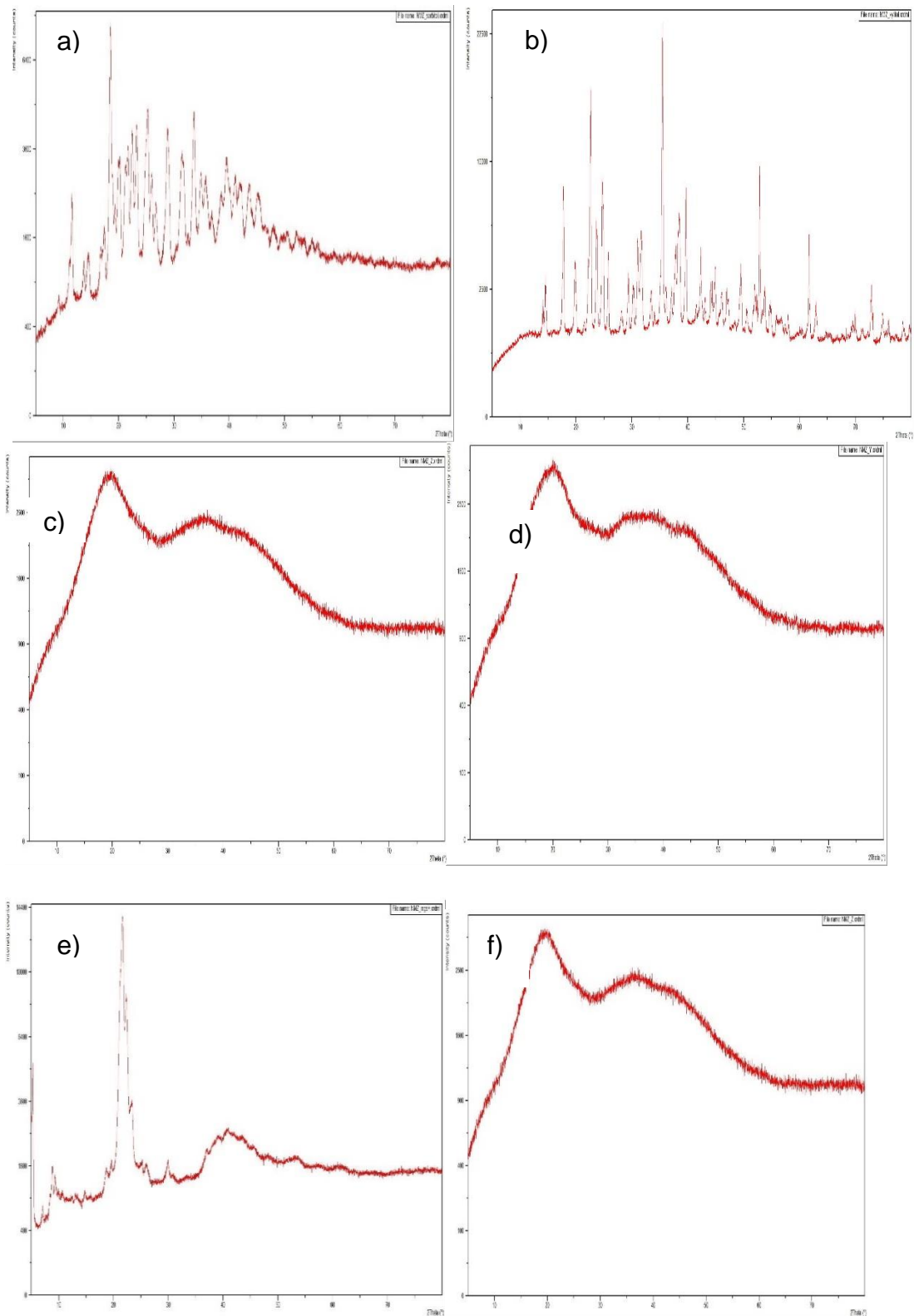
	smu_105				S1	S2	Average	Normalisation (Gene of interest/S.mutans)	
Lane 1	Planktonic S.mutans				183042	152452	167747	421768	0.397723393
Lane 2	Biofilm S.mutans control				433208	410328	421768	421768	1
Lane 3	Biofilm S.mutans + 1% Xylitol				402362	401112	401737	421768	0.952507065
Lane 4	Biofilm S.mutans+5% Xylitol				364593	354872	359733	421768	0.852915584
Lane 5	Biofilm S.mutans+1% Xylitol + 0.25 µg/ml CHX				335991	333120	334556	421768	0.793221629
Lane 6	Biofilm S.mutans +1% Xylitol + 0.5 µg/ml CHX				296881	287890	292386	421768	0.693237752
Lane 7	Biofilm S.mutans +5% Xylitol+ 0.25 µg/ml				269327	254582	261955	421768	0.621086711
Lane 8	Biofilm S.mutans +5% Xylitol+ 0.5 µg/ml CHX				277295	261155	269225	421768	0.638324861

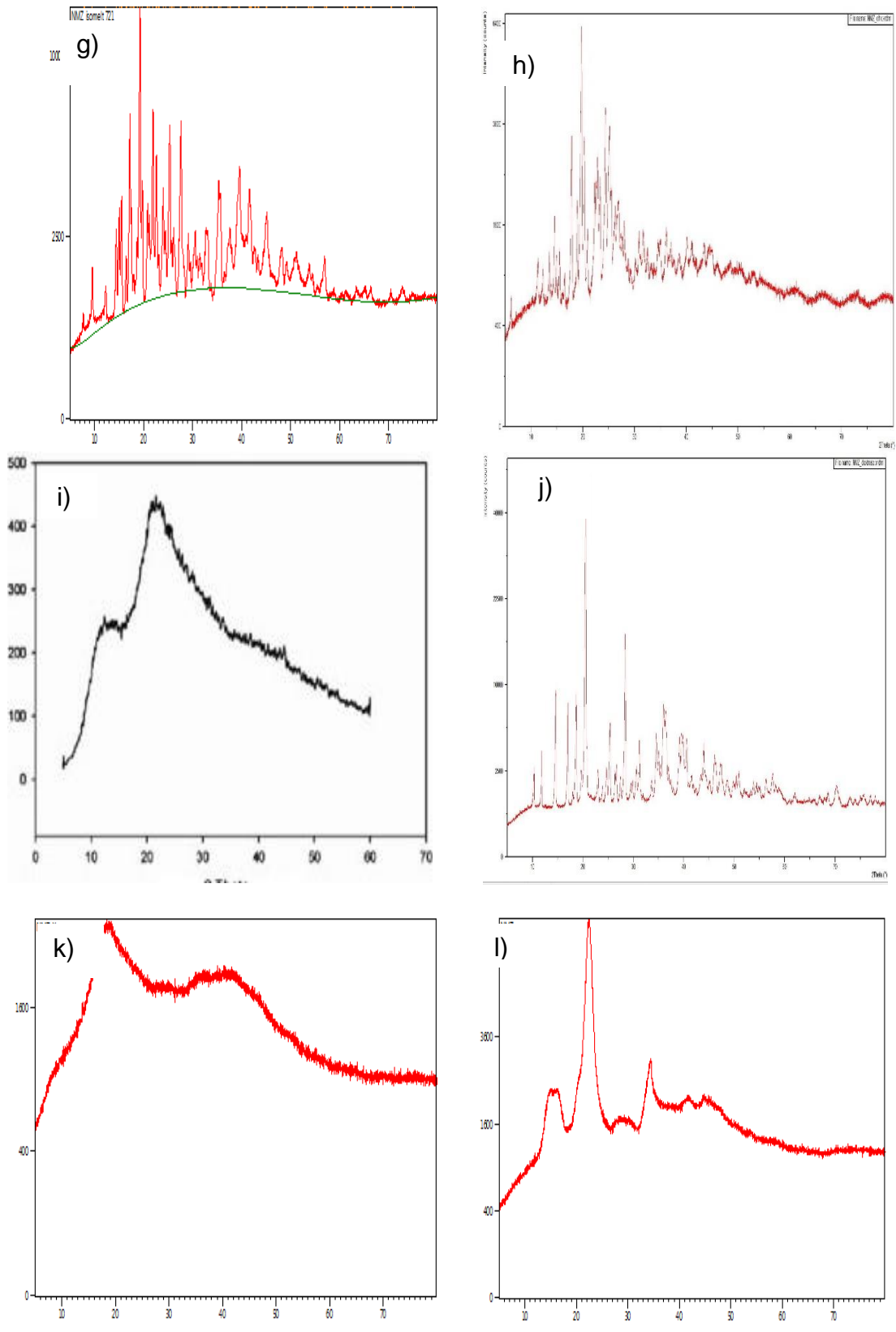
	<i>wapA</i>			S1	S2	Average	Normalisation (Gene of interest/S.mutans)	
Lane 1	Planktonic S.mutans			517068	503360	510214	1309654	0.389579232
Lane 2	Biofilm S.mutans control			1313435	1305873	1309654	1309654	1
Lane 3	Biofilm S.mutans + 1% Xylitol			425293	402150	413722	1309654	0.315901375
Lane 4	Biofilm S.mutans+5% Xylitol			688790	650012	669401	1309654	0.51112813
Lane 5	Biofilm S.mutans+1% Xylitol + 0.25 µg/ml CHX			421350	422236	421793	1309654	0.322064454
Lane 6	Biofilm S.mutans +1% Xylitol + 0.5 µg/ml CHX			519663	505001	512332	1309654	0.391196453
Lane 7	Biofilm S.mutans +5% Xylitol+ 0.25 µg/ml			795935	756422	776179	1309654	0.592659206
Lane 8	Biofilm S.mutans +5% Xylitol+ 0.5 µg/ml CHX			539284	500014	519649	1309654	0.396783425
Lane 9	Biofilm S.mutans + 0.25 µg/ml CHX,			677198	653002	665100	1309654	0.507844057
Lane 10	Biofilm S.mutans + 0.5 µg/ml CHX)			591151	554652	572902	1309654	0.437444928

	<i>comD</i>			S1	S2	Average	Normalisation (Gene of interest/S.mutans)	
Lane 1	Planktonic S.mutans			198242	152450	175346	116208	1.508897838
Lane 2	Biofilm S.mutans control			132364	100052	116208	116208	1
Lane 3	Biofilm S.mutans + 1% Xylitol			117994	108457	113226	116208	0.974334813
Lane 4	Biofilm S.mutans+5% Xylitol			140939	120369	130654	116208	1.124311579
Lane 5	Biofilm S.mutans+1% Xylitol + 0.25 µg/ml CHX			174565	154210	164388	116208	1.414597102
Lane 6	Biofilm S.mutans +1% Xylitol + 0.5 µg/ml CHX			0	0	0	116208	0
Lane 7	Biofilm S.mutans +5% Xylitol+ 0.25 µg/ml			97010	75465	86238	116208	0.742096069

	<i>sacB</i>			S1	S2	Average	Normalisation (Gene of interest/S.mutans)	
Lane 1	Planktonic S.mutans			693332	652550	672941	887540	0.758209207
Lane 2	Biofilm S.mutans control			892517	882563	887540	887540	1

A6.1 XRD data





PXRD of pattern excipients (a: sorbitol, b: maltodextrin , c: magnesium stearate, d: xylitol, e: orange, f: menthol, g: CHX, h: PVP, i: sucrose, j: fructose, k: HPMC, l: SMCC)