Probiotic-based anti-biofilm therapy for bacterial-fungal mixed species biofilms

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DECLARATION

I hereby declare that this thesis is my original work and has been written by me in its entirety. All the sources have been duly acknowledged wherever used in this thesis.

This thesis has not been submitted for any degree in any University previously.

Neha Srivastava
A0161105X
August 18, 2017
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I would like to thank all the staff and lab assistants in the Oral Sciences Faculty of Dentistry lab for their consistent support. I would also like to thank all my colleagues and friends, especially Kassapa Ellepola, who helped me directly or indirectly for the successful completion of this research.

Last but not the least; I would like to express my regards to my husband Shubham and family who provided a firm mental support and encouragement during my research.
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Summary

Dental caries is a major oral health problem worldwide, particularly among industrialized countries. Early childhood caries (ECC) refers to an aggressive form of dental caries that affects children under six years of age. If left untreated, ECC may lead to cavitation and subsequent pulpal infection causing severe pain which will require expensive treatment. *Streptococcus mutans*, an acidogenic bacteria is considered a key pathogen associated with ECC forming a virulent plaque biofilm on the enamel which is considered the main contributory factor for the development of ECC. Previous studies have demonstrated that *Streptococcus mutans* and human fungal pathogen *Candida albicans* develop a symbiotic relationship that enhances the highly pathogenic mixed-species biofilm formation in dental plaque. Thus, this complex microenvironment such as the dental plaque displays resistance to most antimicrobials. Use of probiotics as a strategy for controlling dental plaque biofilms has recently gained significant interest. Studies have demonstrated the promising anti-biofilm activity of probiotic-based strategies against *S. mutans* and *C. albicans* single-species biofilms. However, its effect on mixed-species biofilms remains unexplored. Taking this knowledge gap into consideration, the present study aimed to evaluate the efficacy of secretory components of *Lactobacillus plantarum* against *S. mutans* and *C. albicans* mixed-species biofilms.

For this study, supernatant of the *lactobacillus plantarum* strain was obtained using standard methodology. In the presence of probiotic supernatant, *S. mutans* and *C. albicans* single and mixed-species biofilms were formed according to previously established protocol. In order to examine the anti-biofilm activity of cell-free *L. plantarum* supernatant in single and mixed-species biofilms, two assays were conducted. Firstly, *L. plantarum* supernatant was introduced into the wells at the beginning (0 h) along with bacterial or fungal cell suspensions to evaluate the
preventive ability of the supernatant against biofilms. For the therapeutic assay, respective *S. mutans* and *C. albicans* biofilms were formed for 12 h and subsequently the probiotic supernatant was introduced to these pre-formed biofilms. The efficacy of probiotic supernatant against foregoing microorganisms in single and mixed species biofilms was evaluated using biofilm quantification assays such as XTT reduction assay, crystal violet assay, colony forming unit (CFU) counting. In the presence and absence of *L. plantarum* supernatant, *S. mutans* and *C. albicans* single and mixed-species biofilms were visualized by confocal laser scanning microscopy for both preventive and therapeutic groups. *L. plantarum* supernatant mediated inhibition of *S. mutans* and *C. albicans* single and mixed-species biofilms were further validated at genetic level. Quantitative real time polymerase chain reaction (qPCR) was performed to evaluate the expression of genes associated with the glucosyltransferase activity of *S. mutans* i.e. *gtfB, gtfC* and *gtfD* and hyphal growth associated *Candida* genes *HWP1, ALS1* and *ALS3* after probiotic treatment. These genes play a crucial role in biofilm development and subsequently in the pathogenesis of caries.

The major findings of this study are summarized as below:

1. Biofilm quantification assays demonstrated that the active component/s of *L. plantarum* supernatant were not only able to significantly inhibit the early colonization of *S. mutans* and *C. albicans* but also successfully inhibited the preformed biofilms in both single and mixed-species biofilms.

2. Confocal laser scanning microscopy showed that probiotic supernatant significantly inhibited the biofilm formation of *S. mutans* and *C. albicans* single and mixed-species biofilms. However, preformed biofilms treated with probiotics had comparatively more residual cells than the biofilms of the inhibitory experiment assay.
3. qPCR analysis demonstrated that *L. plantarum* supernatant was able to down-regulate the expression of *S. mutans* genes associated with glucosyltransferase activity (*gtfB, gtfC* and *gtfD*) and *C. albicans* hyphal specific genes (*HWP1, ALS1* and *ALS3*).

Thus, this study provides a substantial evidence for the anti-biofilm activity of *L. plantarum* supernatant on *S. mutans* and *C. albicans* mixed-species biofilms. However, further studies are required to decipher the exact antimicrobial compound/s of this probiotic supernatant prior to clinical applications. If proven feasible, probiotic-based anti-biofilm strategy will be highly useful to treat bacterial-fungal mixed-species biofilm infection, including ECC.
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**Abbreviation**

<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo nucleic acid</td>
</tr>
<tr>
<td>ECC</td>
<td>Early Childhood Caries</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polysaccharide</td>
</tr>
<tr>
<td>GMM</td>
<td>Glucose minimal medium</td>
</tr>
<tr>
<td>GTF</td>
<td>Glucosyltransferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>H</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydro chloric acid</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td><em>Lactobacillus plantarum</em></td>
</tr>
<tr>
<td>MRS</td>
<td>de Man, Rogosa and Sharpe</td>
</tr>
<tr>
<td>Min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>ml</td>
<td>Millimeter(s)</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propodium idodide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribo nucleic acid</td>
</tr>
<tr>
<td>Sap</td>
<td>Secreted aspartyl prteinase</td>
</tr>
<tr>
<td>S. mutans</td>
<td><em>Streptococcus mutans</em></td>
</tr>
<tr>
<td>UFTYE</td>
<td>Ultra filtrate tryptone yeast extract</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>WL</td>
<td>Wave length</td>
</tr>
<tr>
<td>XTT</td>
<td>Tetrazolium salt 2, 3 - bis (2 - methoxy - 4 - nitro - 5- sulfophenyl) -5- [(phenylamino) carbonyl]- 2H- tetrazolium hydroxide</td>
</tr>
</tbody>
</table>
Preface

The investigations mentioned in this thesis were performed in the department of Oral Sciences, Faculty of Dentistry, National University of Singapore, Singapore. Most of the findings described in this thesis have been submitted for publication and have also been reported at scientific meetings as below.

Publications


Presentations at scientific meetings

Chapter 1: Introduction

1.1 Introduction

Dental caries is a major oral health problem worldwide. Early childhood caries (ECC) refers to an aggressive form of dental caries that affects children under six years of age. According to World Health Organization, the prevalence of ECC is reported to be 17% in two-year old children, 48% in four-year old children and 70% in six-year old children (Rodriguez et al., 2016). If left untreated, ECC may lead to cavitation and subsequent pulpal infection causing severe pain which will require expensive treatment (Berkowitz et al., 2009).

*Streptococcus mutans*, an acidogenic bacteria is considered a key pathogen associated with ECC (Takahashi and Nyvad, 2011) forming a virulent plaque biofilm on the enamel which is considered the main contributory factor for the development of ECC (Socransky and Haffajee, 2000). *S. mutans* is able to rapidly utilize fermentable dietary carbohydrates such as sucrose and subsequently synthesize extracellular glucans through several exoenzymes, such as glucosyltransferases (Gtfs). This extracellular glucan enhances the bacterial adhesion to the tooth surface, as well as aids in bacterial coaggregation leading to the development of highly virulent mixed-species biofilms in the oral cavity (Bowen and Koo, 2011). These complex biofilms encourage the development of an acidogenic microenvironment, and later cause the initiation of dental caries (Koo et al., 2013).

Previous studies have demonstrated that *S. mutans* forms mixed-species biofilms with the human fungal pathogen *Candida albicans* in dental plaque (Carvalho et al., 2006; Raja et al., 2010). *S. mutans* derived Gtfs binds firmly to the cell surface of *C. albicans* (Gregoire et al., 2011). Adhesion between the foregoing
microorganisms has been shown to be significantly enhanced in the presence of sucrose (Falsetta et al., 2014; Metwalli et al., 2013; Pereira-Cenci et al., 2008). Gtfs bound to the C. albicans cell surface produce large amounts of glucans in the presence of sucrose. These glucans in turn increases the binding sites for S. mutans (Branting et al., 1989; Gregoire et al., 2011) resulting in a highly pathogenic mixed-species biofilm (Falsetta et al., 2014). We recently demonstrated that S. mutans gtfB is able to augment the aggregation of C. albicans in mixed-species biofilms (Ellepola et al., 2017). Moreover, S. mutans was able to up-regulate the expression of C. albicans hypha associated genes such as HWP1, ALS1 and ALS3 which can be attributed to an increase in virulence of the organism in mixed-species biofilms. On the other hand, C. albicans also enhanced the development of S. mutans microcolonies in mixed-species biofilms (Kim et al., 2017). Taken together, S. mutans and C. albicans demonstrate a symbiotic relationship in the mixed-species biofilm with complex inter-species interactions. Thus, a complex organized microbial community such as the dental plaque displays resistance to most antimicrobials (Seneviratne et al., 2008).

Use of probiotics as a strategy for controlling dental plaque biofilms has recently gained significant interest (Saha et al., 2014; Soderling et al., 2011). According to the 2001 definition by the World Health Organization, probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Ohshima et al., 2016). However, administration of live microorganisms may not be an ideal therapeutic option in certain conditions (Bandara et al., 2017). Therefore, focus has shifted towards testing the antimicrobial activity of secretory components of probiotic bacteria. Probiotic bacteria are known to produce various antimicrobial compounds that can efficiently inhibit bacterial adhesion and disrupt biofilm formation (Ohshima et al., 2017).
Studies have demonstrated the promising anti-biofilm activity of probiotic-based strategies against *S. mutans* and *C. albicans* single-species biofilms (James et al., 2016; Lee and Kim, 2014; Matsubara et al., 2016a; Saha et al., 2014). However, there are no studies in literature investigating the probiotic activity against mixed-species biofilms. Taking this knowledge gap into consideration, the present study aimed to evaluate the efficacy of secretory components of *Lactobacillus plantarum* against *S. mutans* and *C. albicans* mixed-species biofilms.

### 1.2 Objectives

i. To investigate the efficacy of secretory component/s of *Lactobacillus plantarum* on single and mixed species biofilms formed by *Candida albicans* and *Streptococcus mutans*.

ii. To examine the effect of secretory component/s of *Lactobacillus plantarum* on the virulence attributes of *Candida albicans* and *Streptococcus mutans* biofilms.
Chapter 2: Literature review

2.1 Dental caries

2.1.1 Prevalence of dental caries

Dental caries is a major oral health issue in most of the countries worldwide that leads to tooth decay mostly in toddlers (60 - 90%) and a vast majority of adults (Petersen et al., 2005). Even though dental caries is not a very severe condition affecting human life, it significantly impacts the quality of human health because of the severe pain associated with pulpal infections and subsequent tooth loss (Petersen, 2003). In recent years, the prevalence of dental caries has increased significantly in children of 2 - 5 years age group worldwide. It is considered as one of the most common chronic childhood disease and thus this age group needs to be given a higher priority for treatment of dental caries (Dye et al., 2010).

Figure 1: Prevalence of dental caries worldwide

Adapted from: (Petersen et al., 2005)
General health of an individual is closely related to oral health and negligence of oral health may increase the risk towards systemic diseases like cardiovascular disease and diabetes (Petersen, 2003). Oral health problems are a considerable economic burden and most industrialized countries allocate around 5 - 10% of their total public health budgets to oral diseases (Media, 2012). WHO's report on the Global Problem of Oral Diseases, emphasizes that dental caries, periodontitis (gum disease) and oral and pharyngeal cancers are global health problem in both industrialized and the developing nations (Petersen, 2003).

2.1.2 Early childhood caries (ECC)

Early childhood caries (ECC) refers to an aggressive form of dental caries that affects children under six years of age. Even though the number of cases of dental caries have noticeably reduced worldwide, it has been highlighted by WHO that ECC is still a major health concern. This is because of its rapid growth and accompanied challenges in prevention (Petersen, 2003; Wendy et al., 1999).

According to WHO, the global prevalence of ECC is reported to be 17% in two-year old children, 48% in four-year old children and 70% in six-year old children (Rodriguez et al., 2016). In a recent review, National Health and Nutrition Examination Survey (NHANES) reported 23% of children in 2 - 5 years age group in United States were diagnosed with caries in primary dentition (Pitts et al., 2017). Aforementioned survey also reported that 10% of these children did not receive any treatment for dental caries. If left untreated, ECC may lead to cavitation and subsequent pulpal infection causing severe pain which will require expensive treatment (Berkowitz et al., 2009).
2.1.3 Hypothesis regarding dental caries

In order to explain dental caries, the first hypothesis, known as "Chemico-parasitic theory" was proposed by Miller in 1890. Miller proposed that oral microbes have the tendency to decompose carbohydrates to acids which subsequently dissolves hydroxyapatite and releases calcium and phosphates (Miller, 1890). Subsequently, others proposed several hypotheses namely specific plaque hypothesis, non-specific plaque hypothesis and ecological hypothesis with advanced scientific evidence on the disease.

Walter J. Loesche in 1979 proposed a specific plaque hypothesis which states that only a few of the many species found in dental plaque biofilm were actively involved in etiology of caries (Loesche, 1979). Experimental results revealed that bacterial species belong to mutans streptococci (MS) are associated with dental caries. On the other hand, the nonspecific plaque hypothesis argued that dental caries is caused because of the overall activity of the total microorganisms in the dental plaque.

The extended caries ecological hypothesis was proposed by Takahashi et al in 2011 (Takahashi and Nyvad, 2011). According to this hypothesis, the caries development comprises of three stages that are reversible. In the primary stage, non-\textit{mutans streptococci} and \textit{Actinomyces} reside on the tooth surface. This stage is referred to as dynamic stability stage wherein the amount acid production is less and hence equilibrium of the demineralization/ remineralization is feasible. In the second stage, due to increased availability of sugar, frequent acid production takes place which results into demineralization. This subsequently initiates the development of dental caries, and thus this stage is known as acidogenic stage. In case the acidic conditions prevail, the more acidic bacteria becomes hyperactive and hence this last
stage is called as aciduric stage wherein several acidogenic and aciduric bacteria contribute to the development of dental caries.

### 2.1.4 Pathogenesis of dental caries

Dental caries is the mostly localized, continuous and irreversible decay of tooth. Dental caries is caused by demineralization of inorganic substances and depreciation of organic substances in the tooth (enamel, dentine and cementum), which consequently leads to cavity formation (Fejerskov et al., 2015).

There are several factors which are responsible for the initiation of dental caries such as microorganisms, type and frequency of food intake, host factors, and time (Figure 2). Oral cavity has several ecological niches that provide an ideal habitat for the microorganisms owing to its suitable humidity and temperature conditions. Oral microbes stick to different surfaces of the oral cavity such as, teeth, tongue, epithelial cells lining roof of the mouth and the cheeks (Loesche, 1986).

Additionally, the frequent food intake provides a continuous nutrient supply for the microbial growth. Bacteria feed on fermentable carbohydrates and produce various organic acids as a byproduct of metabolism, such as lactic acid, formic acid, acetic acid, and propionic acids (Hicks et al., 2003).
The critical pH for demineralization of tooth substance varies between 5.2 to 5.5 among individuals (Dawes, 2003). The acids produced by the bacteria, lower the plaque pH below the critical value which results in diffusion of calcium and phosphate ions of the tooth. Demineralization rate depends on the absolute pH decrease and time duration for which pH remains below the critical pH.

2.1.5 Dental caries therapeutic treatment modalities

Etiology of dental caries suggests the contribution of several detrimental factors in the development of caries. These factors are related to consumption of high sucrose containing food, regular intake of carbohydrates, improper oral hygiene, bacterial accumulation spots in oral cavity, insufficient fluoride level in drinking water and dehydration (Yadav and Prakash, 2016). Invasion of pathogenic microorganisms into the dental tissues leads to severe tooth pain, tooth loss, facial cellulitis, osteomyelitis and may also lead to spread of infection to systemic circulation.
The existing treatment modalities aim at inhibiting the caries growth and thereby restoring the structure of affected tooth are summarized in Table 1.

**Table 1: Preventive and therapeutic treatment modalities for dental caries**

<table>
<thead>
<tr>
<th>Prevention</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit the substrate</td>
<td>Restoration of carious tooth</td>
</tr>
<tr>
<td>Reduce the sucrose intake</td>
<td>Restorative material</td>
</tr>
<tr>
<td>Modify oral microflora</td>
<td>Endodontic procedures</td>
</tr>
<tr>
<td>Bactericidal mouth wash</td>
<td>Pulpotomy</td>
</tr>
<tr>
<td>Topical fluoride treatment</td>
<td>Pulpectomy</td>
</tr>
<tr>
<td>Plaque disruption</td>
<td>Root filling</td>
</tr>
<tr>
<td>Brushing</td>
<td></td>
</tr>
<tr>
<td>Flossing</td>
<td></td>
</tr>
<tr>
<td>Modify tooth</td>
<td></td>
</tr>
<tr>
<td>Topical fluoride</td>
<td></td>
</tr>
<tr>
<td>Systemic fluoride</td>
<td></td>
</tr>
<tr>
<td>Stimulate salivary flow</td>
<td></td>
</tr>
<tr>
<td>Eat non cariogenic fibrous food</td>
<td></td>
</tr>
<tr>
<td>Use sugarless chewing gums</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2 Biofilm formation

Microbial biofilms are referred to as structures comprising of a matrix of extracellular polymeric substances (EPS) and an aggregate of microorganisms that form clusters by adhering to each other and/or to a surface (Kolenbrander, 2010). In their natural environment, most of the microbes are attached to the surfaces and form a structured biofilm (Seneviratne et al., 2008). Studies have shown that around 65% of all microbial infections are associated with biofilm formation (Potera, 1999). Microorganisms in biofilms demonstrate a better survival characteristic in hostile
environment as compared to their planktonic mode. Hence, biofilms are about 10 to 1000 times more resistant to antimicrobial agents (Mah and O’Toole, 2001).

The biofilm when formed on the tooth surface is called dental plaque. Dental plaque is associated with dental caries and periodontal diseases. Formation of dental plaque is a multi-step process which results in functionally and structurally organized microbial biofilm (Marsh et al., 2000). There are several stages involved in the formation of biofilm.

2.2.1 Formation of pellicle

Pellicle formation is the first step of biofilm formation which occurs immediately after tooth eruption or brushing. Salivary proteins, non-salivary-derived proteins, carbohydrates, and lipids are the major constituents of pellicle (Siqueira et al., 2012). Saliva contains amylase and carbonic anhydrase like proteins which aid pellicle formation through electrostatic bond with tooth surface (Hay, 1973). Following this, these proteins enhance further saliva accumulation on tooth surface by protein-protein interactions, thereby forming a thick pellicle within the first hour after tooth cleaning (Hannig, 1999). Pellicle helps in regulating microbial colonization, mineral homeostasis and host defense (Hanniga and Joinerb, 2006).

2.2.2 Bacterial attachment to tooth surface

*Streptococcus mutans, Streptococcus sanguinis, Streptococcus oralis* and *Streptococcus mitis* along with *Actinomyces* are known to be the primary colonizers that aid dental plaque formation (Li et al., 2004). Studies have shown that there are two stages of bacterial attachment to the tooth surface (Cowan et al., 1986).
**Figure 3**: Stages of biofilm formation.

First, microbial adhesion to a surface. Second, microcolony formation; third, EPS production and maturation of biofilm three dimensionally; and finally, detachment and dispersion of progeny cells.

Adapted from: (Jahid and Sang-Do, 2014)

The first stage is a reversible process where bacterial cells get attached to the acquired pellicle on tooth surface through van der Waal’s forces, which is basically a weak adherence that acts when the separation gap is more than 50nm. Most of the bacteria and tooth surface possess negative electrostatic charge which causes mutual repulsion within the organisms. Electrostatic repulsion occurs when the separation gap is 10nm – 20nm (Cowan et al., 1986).

The second stage is irreversible attachment. Bacterial adhesins consist of hydrophobic or lectin properties and are found on the filamentous surfaces of bacteria such as pili or fimbriae. In this stage, these adhesins act like a bridging gap between bacterial cells and pellicle (Gibbons, 1984). The hydrophobic surface components (adhesin) eliminate the water films between bacterial surfaces and pellicle thereby overcoming the energy barrier that eventually leads to short range (less than 1.5nm) irreversible interaction (Huizhen, 2014).
2.2.3 **Adhesion of secondary colonizers**

This stage witnesses enhanced diversity of the plaque which is caused by specific adhesin-receptor interactions among bacteria. Secondary colonizers increase the microbial density on the tooth surface which eventually cause either adhesive interaction or inhibitory effect of bacteria upon each other (Houte, 1982).

2.2.4 **Development of mature biofilm**

Bacterial cell division results in proliferation and production of extracellular polysaccharide (EPS). EPS comprises of proteins, and extracellular deoxyribonucleic acid (DNA) which eventually help in formation of a well-structured biofilm (Flemming et al., 2007). The nutrient supply and communication through signaling molecules is facilitated by the well-established water channels in the mature biofilms (Dufour et al., 2010).

2.2.5 **Detachment and dispersion of biofilm**

Owing to environmental changes, bacteria get detached from the surfaces. This is driven by few enzymes that have the potential to hydrolyze the fimbriae associated adhesions (Cavedon, 1993). Subsequently, dispersed cells adhere to other suitable surfaces (Huižhen, 2014; Sokunrotanak et al., 2013).
2.2.6 Biofilm quantification

Several biofilm quantification assays have been used to evaluate the biofilm formation. In the present study, *L. plantarum* mediated single and mixed-species biofilms inhibition was quantified by three different assays *i.e.* XTT reduction assay, crystal violet assay, and colony forming unit (CFU) counting method. Although aforementioned biofilm quantification assays have been extensively used, they have associated advantages and disadvantage that basically make them complimentary to each other. XTT assay is a sensitive method which quantifies the biofilm biomass on the metabolic activity of the living cells. Owing to inter-species and inter-strain variation in metabolic activity, consideration should be put in while comparing growth kinetics of various species on the basis of XTT readings (Kuhn et al., 2003). Crystal violet assay has a definite advantage of being a simple and effective assay which measures the total biomass of biofilm cells and extracellular matrix. But, this method stains both living and dead cells (Monteiro et al., 2015) and hence it is not appropriate to analyze the dead cells in biofilm. CFU assay is also a simple and inexpensive method, but it has a disadvantage that some viable cells are not cultivable in agar medium.

i. **XTT reduction assay**

XTT assay is a Colorimetric assay for assessing cell metabolic activity. Viability of cells is based on the capacity of mitochondrial dehydrogenase enzymes present in living cells. This enzyme reduces the XTT into water soluble formazan (orange color) as an end product. Total amount of formazan is calculated by using plate reader or spectrophotometer at a specific wave length of 490 nm. The measured absorbance of the cell supernatant is directly related to the number of metabolic activity of cells. This value represents the relative cell viability (Kuhn et al., 2003).
XTT assay has been widely used for quantification of viable bacterial and yeast cells in biofilm as well as in planktonic cultures.

**ii. Crystal violet assay**

Crystal violet was synthesized by Alfred Kern in 1883. Later, in 1884, this stain became more popular and was used to stain bacteria by Hans Christian Gram. Crystal violet is basically a triarylmethane dye. Crystal violet staining method was first explained by Christensen et al. in 1985 and subsequently it was revised to enhance its accuracy for biofilm biomass quantification (Stepanovic et al., 2000).

Crystal violet adheres to negatively charged molecules of bacterial cell wall components including nucleic acids and acidic polysaccharides. When dissolved in 95% ethanol, it has an absorbance at 590 nm with blue violet color. Crystal violet assay is used for quantification of the total biofilm biomass because the amount of bacteria in the sample is directly proportional to the crystal violet binding.

**iii. Colony forming unit counting**

The quantitative analysis of determining bacterial viability is done by counting the number of colony forming units (CFU) after plating of serial dilutions of culture samples. The purpose of serial dilution is to estimate the number of microbial colonies in the test sample by counting the number of colonies formed after serial dilution of the sample. This count is then compared with the number of colonies formed in untreated group.
2.3 *Streptococcus mutans*

*Streptococcus mutans* is a Gram-positive, facultative anaerobic bacteria. It is known as a key pathogen associated with dental caries. Human oral cavity is a common habitat for *S. mutans*. It is an early colonizer on the tooth surface.

**Figure 4: Streptococcus mutans (x10,000)**

![Streptococcus mutans](image)

Adapted from: (Streptococcus mutans, 2017)

2.3.1 **Prevalence of *Streptococcus mutans***

The name *Streptococcus mutans* was given by Clarke in 1924. This microorganism was isolated from caries lesions. Since these organisms were oval in shape and appeared as a mutant form of *Streptococcus*, these were named as *Streptococcus mutans* (Clarke, 1924).

This finding was later confirmed by Maclean in 1927 and later by Onisi and Nucolls in 1958 who investigated the deeper part of lesions and found microorganisms very similar to *S. mutans* (Edwardsson, 1968). *S. mutans* were found in the dental plaque and saliva of toddlers. It was found to be associated with caries risk enhancement (Thenisch et al., 2006). Later it was verified by Parisotto et al in 2010
that *S. mutans* is a major risk factor for dental caries in toddlers (Parisotto et al., 2010).

### 2.3.2 Virulence factor of *Streptococcus mutans*

*S. mutans* is most commonly found pathogen in oral cavity that plays an significant role in dental caries formation (Hudson and III, 1990). The important virulence factors of *S. mutans* associated with the pathogenesis of dental caries include microbial adhesion, acidogenicity, acid tolerance, biofilm formation and capability of maintaining the intracellular pH.

#### 2.3.2.1 Adhesion

*S. mutans* adherence in dental plaque is a two-stage process, namely; sucrose-independent and sucrose-dependent adhesion. Previous studies have shown that the interaction between *S. mutans* and the salivary pellicle commences through sucrose-independent process. Subsequently, structured colonies are formed through sucrose-dependent adhesion (Banas, 2004).

i) **Sucrose independent adhesion**

Antigen I/II and surface protein are known to be responsible for sucrose-independent adhesion. Other similar protein found on oral *streptococci* are P1, SpaP, Sr, PAc, and antigen B (Ma et al., 1991). Previous studies revealed that antigen I/II family proteins are structurally similar to amino acids but they bind to salivary agglutinins, salivary pellicle components, and other plaque bacteria in a different manner (Petersen, 2002; Whittaker et al., 1996). Two different forms of amino acids;
alanine-rich and proline-rich are the crucial for binding between antigen I/II and salivary components (Banas, 2004; Crowley et al., 1993; G Hajishengallis et al., 1994). The role of antigen I/II in adhesion of *S. mutans* to saliva-coated hydroxyapatite has been verified by several researchers (Douglas and Russell, 1982; Song et al., 1989).

Bowen et al studied this phenomenon further and found that isogenic mutant lacking P1 (antigen I/II) did not bind well to saliva-coated hydroxyapatite contrary to the wild-type strain. They also showed that both mutant and wild type strains were bounded similarly to hydroxyapatite surface coated with saliva and in situ formed glucan (Bowen et al., 1991).

### ii) Sucrose dependent adhesion

The mechanism of sucrose-dependent adhesion is comparatively more established than sucrose-independent adhesion. *S. mutans* consumes sucrose and synthesizes glucan in the presence of glucosyltransferase enzyme (Fig. 5). This glucan is the most critical virulence factor contributing to dental caries formation which enables bacteria to strongly adhere to the tooth surface and leads to biofilm formation. Hydrogen bonding of glucan polymer is believed to be the root cause for interaction between bacteria and pellicle (Banas, 2004).

Glucosyltransferases comprises of three distinct gtf enzymes; gtfB, gtfC and gtfD, which helps in synthesis of glucan. Properties of these three gtf enzymes are described below.
Table 2: Gtf enzymes and associated glucan synthesis (Bowen and Koo, 2011)

<table>
<thead>
<tr>
<th>Gtf types</th>
<th>Type of glucan</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>gtfB</td>
<td>Water insoluble glucans</td>
<td>alpha 1, 3</td>
</tr>
<tr>
<td>gtfC</td>
<td>Mixture of soluble and insoluble</td>
<td>alpha-1,6</td>
</tr>
<tr>
<td>gtfD</td>
<td>Water-soluble glucans</td>
<td>alpha-1,6-glycosidic</td>
</tr>
</tbody>
</table>

All the Gtfs bind to tooth surface, although the strength of the adhesion may differ (Vacca-Smith and Bowen, 1997). Studies demonstrated that Gtf adheres better to the saliva coated hydroxyappetite (HA) disks as compared to uncoated HA (Steinberg, 1996; Venkitaraman et al., 1995). Hamada et al (1978) demonstrated that gtfB has the strongest affinity towards HA whereas gtfD is weakest in this regard (Hamada et al., 1978). Due to the formation of soluble, quickly metabolizable polysaccharides, gtfD acts as a primer for gtfB. Gtf has the potential to interact with other oral bacteria including those which do not synthesize gtfs, like Actinomyces spp. (Hamada et al., 1978; McCabe and Donkersloot, 1977). The glucan molecules facilitate the adhesion of S. mutans to tooth surfaces as well as to other microorganisms. This results in structured microbial community i.e. biofilms.
Microbial adhesion to acquired enamel pellicle in the presence of sucrose and subsequent cariogenic biofilm formation.

Adapted from: (Bowen and Koo, 2011)

2.3.2.2 Acidogenicity

*S. mutans* consume fermentable carbohydrates and produce lactic acid, formate, ethanol, and acetate as a byproduct of carbohydrate metabolism (Ajdic et al., 2002). Yield of these fermentation products is dependent on the availability and type of carbohydrate consumption. For example, when glucose is available in sufficient amount, the production of lactic acid is more (Dashper and Reynolds, 2000). Studies have also demonstrated that production of fermentation products is significantly reduced when strains are deficient in lactate dehydrogenase, lowering the risk of caries development (Fitzgerald et al., 1989; Hillman and Socransky, 1987).
It is believed that ecological changes in the dental plaque is due to acid production by *S. mutans*, which results in the further colonization of acidogenic and acid-tolerant bacteria. Hence, due to the presence of such bacteria, plaque pH reduces to lower levels and subsequently the recovery to neutral pH is delayed (Banas, 2004). Demineralization of enamel and progression of dental caries is taken place when plaque pH is maintained below the critical pH value of 5.5 (Barron et al., 2003).

2.3.2.3 Acid tolerance

*S. mutans* are known to have noticeable acid tolerance capability. The acid tolerance response (ATR) of *S. mutans* is dictated by ATPase proton pump and is also related to change in gene and protein expression (Banas, 2004).

Studies suggest that acid-tolerance of *S. mutans* is attribute to the synthesis of water-insoluble glucan and biofilm formation. *S. mutans* cells within a biofilm have shown better resistance against acid attack as compared to the planktonically grown bacteria (Banas, 2004). ATR may also be related to the quorum sensing systems and physical characteristics of biofilm (Banas, 2004). This was verified by a study by McNeill and Hamilton in 2003, demonstrating that *S. mutans* biofilm is more resistant to acid attack (pH lower than 4.4) than planktonic cells (K. McNeill and Hamilton, 2003). Furthermore, acid production was found to be faster when *S. mutans* are grown in sucrose as compared to that in glucose (Houte et al., 1989).
2.3.2.4 Maintaining intracellular pH

*S. mutans* consume fermentable carbohydrates and produce various organic acids as a by-product of metabolism, as a result the external environment becomes more acidic. This subsequently causes increased permeability of *S. mutans* membranes and acidifying the bacterial cytoplasm.

Due to low intra-cellular pH, cells become active and in order to maintain the cytoplasmic pH, the membrane bound protons ATPase or F-ATPase start translocating. This fact was confirmed by Hamilton and Buckley, wherein it was found that *S. mutans* FATPase activity increased at low pH to maintain a pH gradient (Belli and Marquis, 1991; Hamilton and Buckley, 1991).

2.3.2.5 Biofilm formation

Dental plaque basically comprises of a community of microorganisms which are established in an extracellular matrix of polymers of host and bacterial origin (Marsh et al., 2000; Socransky and Haffajee, 2002).

Microbial growth in biofilm is different from that in planktonic environment. This is attributed to the several factors such as adhesion, nutrient flow, and coaggregation that might affect the growth rate, gene expression and quorum sensing in biofilm. Thus, in recent studies a significant emphasis has been put on examining the expression of virulence genes within biofilms. Variability of gtfB and gtfC gene expression in response to environment is one such relevant example.

Studies have shown that in comparison to planktonic cells, biofilm mode of bacterial growth is resistant to anti-bacterial agents that results in enhanced pathogenicity of dental caries.
2.4 Candida albicans

2.4.1 Prevalence of Candida albicans

*Candida albicans* is a commensal fungus that harmlessly resides in various parts of the human body, such as oral cavity, gastrointestinal tract, vagina, and skin (Mayer et al., 2013). In a few cases where the individual is immuno-compromised, *Candida* causes infections or candidiasis that might lead to superficial infections and even to life-threatening systemic diseases (Mayer et al., 2013). This is also known to be the most commonly found pathogen in lethal blood stream infections (Seneviratne et al., 2011).

Total 154 species of *Candida* have been discovered till date. Among this large plethora of species, six of the species are most commonly found in human body infections. These are, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krasie*, and *Candida lusitaniae*. Among these, *C. albicans* is the most commonly occurring pathogenic fungus of all forms of candidiasis. Majority of *Candida* infections (50 - 80%) are caused by *C. albicans* (Pfaller and Diekema, 2004). *C. albicans* is the most prevalent fungi in oral cavity (Carvalho et al., 2007; Wendy et al., 1999). Candidiasis has several oral manifestations, of which oral thrush and denture stomatitis are the most common (Signoretto et al., 2009).

*C. albicans* is commonly associated with early childhood caries (ECC). Clinical research has established a precise quantification of *C. albicans* presence in the carious lesions to be 62.3% in pre-school children and 71.4% in schoolchildren (Rozkiewicz et al., 2006). It has been demonstrated that oral biofilm in toddlers suffering from dental caries have significantly high prevalence of *C. albicans* as
compared to children without caries (Carvalho et al., 2007; Moalica et al., 2001; Thaweboon et al., 2008).

**Figure 6: Candida albicans**

![Image of Candida albicans biofilm](image)

Adapted from: (Kim, 2015)

*C. albicans* is a dimorphic fungi that assumes several morphological forms under varied environmental conditions such as budding yeast cells (blastospores), pseudohyphae (elongated cells appear as filamentous cell chains), true hyphae, and chlamydomspores. *C. albicans* forms biofilms which are difficult to eliminate due to significantly higher antifungal resistance (Seneviratne et al., 2008).

Only a few classes of antifungal drugs like polyenes, azoles, echinocandins, allyl amines, and flucytosine are known to be effective for treatment of fungal infections (Sanglard et al., 2009). Still, none of the presently available antifungal agents are known to have ideal properties for biofilm infections (Wong et al., 2014).

### 2.4.2 Virulence factor of Candida albicans

Pathogenicity of a *C. albicans* is determined by its ability to adapt to the surrounding environment and to tolerate the lytic activity of defense system of the
host. Virulence factors play an important role in the growth of pathogen. This aids tissue adhesion and penetration which results in resistance against defense system of host. A few prominent virulence factors of \textit{C. albicans} are phospholipase production, production of secreted aspartyl proteinase (SAP), expression of drug resistance genes, adhesion, hyphal formation and biofilm formation.

\textbf{2.4.2.1 Phospholipase production}

\textit{C. albicans} secrets enzymes that aids invasion of host tissues which subsequently results in pathogenesis of diseases. Since the cell membranes consist of lipids and proteins, these are prone to enzyme attack. These enzymes are categorized into two groups; proteinases (Hube, 1998) and phospholipases (Ibrahim et al., 1995). Proteinases hydrolyzes the peptide bonds whereas phospholipases hydrolyzes phospholipids. In this way, the hydrolytic enzymes produced by \textit{C. albicans} helps to destroy and de-functionalize the host tissue membrane (Ghannoum, 2000). The study done by Silverman et al. (1992) demonstrated the role of phospholipases in the lysis of host cell membranes and subsequent adhesion at the time of Rickettsia infection (Silverman et al., 1992).

\textbf{2.4.2.2 Expression of drug resistance genes}

\textit{C. albicans} has been identified as the most common yeast found in humans (Navarro-Garcia et al., 2001). \textit{Candida} infections are prominent in immunocompromised patients i.e AIDS, diabetes, various cancers and organ transplant patients worldwide (Wisplinghoff et al., 2004). Fungal infections are treated with antifungals such as polyenes, azoles, fluoro pyrimidines and echinocandins (Ruhnke et al., 2008). The extensive use of antifungal drugs has resulted in emergence of drug
resistant *Candida* strains. The expression of drug resistance genes is known to promote drug resistance capability of *Candida*. A study done by Prasad and Kapoor demonstrated the phenomenon wherein the over expression of genes regulating drug efflux pumps helps to ouster toxic molecules and drugs (Prasad and Kapoor, 2005). They showed that ATP binding cassette transporters encoded by CDR1 and CDR2 (drug resistance genes) and major facilitator transporters encoded by MDR1 (multidrug-resistance gene) play an important role in drug resistance of *C. albicans* (Prasad and Kapoor, 2005).

### 2.4.2.3 Production of Secreted aspartyl proteinase (SAP)

Secreted aspartyl proteinases (SAP) is also known to be a virulence factor in *C. albicans*. This proteinases enzyme disrupts the host defense through breakdown of membrane protein. SAP are responsible for hydrolyzing several proteins such as albumin, hemoglobin, keratin, collagen, laminin, fibronectin, mucin, salivary lactoferin, cystatin A, and Immunoglobulin A (Hube, 1998). Subsequently it provides a pathway for the invasion of *C. albicans* and spread of the infection. SAP acts as a catalyst in this process by hydrolysing the peptide bonds (CO-NH) in proteins (Bernardis et al., 1995).

SAP family comprises of ten proteins (SAPs 1-10), which aid tissue invasion. Previous studies have revealed that SAPs 1, 2 and 3 are expressed in yeast form and SAPs 4, 5 and 6 are expressed in hyphal formation (Naglik et al., 2004). SAPs 9 and 10 are expressed in both yeast and hyphal forms (Albrecht et al., 2006). Several researchers have verified the role of SAPs in microbial adhesion, colonization and tissue penetration (Hube and Naglik, 2001; Naglik et al., 2004). It has also been demonstrated that SAP 5, 6 and 9 genes are over expressed in biofilm mode of growth as compared to that in planktonic mode.
2.4.2.4 Adhesion

Adhesion characteristics of *Candida* to various host tissues are known to be a crucial virulence factor in *Candida* infections. *Candida* adhesion to host tissue is a complex process that acts through different types of adhesins. These proteins are involved in maintaining the cell wall integrity and hyphal growth and are responsible for pathogenesis due to adhesion and invasion on the host tissues (Finkel and Mitchell, 2011). The adhesin proteins are generally expressed on cell surface during transition of yeast to hyphae. A few prominent adhesins are ALS (agglutinin like sequences) and Hwp1 (hyphal wall protein). The important proteins on the hyphal cell surface are Als1p, Als3p and Als5p, which aids fungal adhesion to the epithelial surfaces (Hoyer, 2001). Hwp1 is another important adhesin protein on the hyphal wall which helps in covalent adhesion between fungal cells and human epithelial cells. Studies done by Chaffin et al in 1998 have shown that *HWP1* deficient mutant have reduced adherences in murine models (Chaffin et al., 1998).

2.4.2.5 Hyphal formation

*C. albicans* normally exists in yeast form but its ability to form hyphae under specific environment conditions is the most significant virulence factor that leads to infection (Nantel et al., 2002). Dissemination in tissues and other hosts is mostly associated with yeast form of *C. albicans*. On the other hand, hyphal form causes tissue invasion and damage. Studies have shown that hyphal cell walls contain more chitin and have better adhesion capability towards human endothelial and epithelial cells in comparison to yeast cells. Hypha produces adhesion proteins that aids the fungal cells to stick to the host cells, also releases proteases that causes host tissue invasion (Martin and Konopka, 2004). Morphological transition of *C. albicans* from
yeast to hyphae is dependent on several factors like temperature, pH, carbon source, nitrogen source, cell concentration and serum (C. Gale et al., 2001).

### 2.4.2.6 Biofilm formation

*C. albicans* is able to adapt to different environments and form microbial aggregation on host surfaces. The biofilm formation ability is a major virulence factor of *C. albicans*. Studies have verified the association between biofilm and the diseases caused by *Candida* species (Chandra et al., 2001).

Biofilm formation capability of *C. albicans* is strongly dependent on several genes that aid in adhesion and penetration. Adhesins encoded by *ALS1, ALS2, ALS3, ALS4, ALS5, HWP1*, and *EAP1* are responsible for adhesion to the endothelial and epithelial cells (Finkel and Mitchell, 2011). Several studies have demonstrated the up-regulation of *ALS* family genes in biofilm forming cells as compared to planktonic cells.*C. albicans* biofilms are known to be resistant to antifungal agents and are thus difficult to eradicate (Seneviratne et al., 2008).

### 2.5 Probiotics

#### 2.5.1 History and Definition

The term probiotic was first introduced in 1965 by in Lilley and Stillwell. The term "probiotic" has been derived from the Greek word that means "fit for life. Probiotics are used to describe molecules released by one microorganism that stimulates the growth of another microorganism. This definition is basically the opposite of antibiotic (Lilly and Stillwell, 1965).
In 1989, probiotics were defined by Fuller as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance". Therefore, at that time, probiotics were considered only for ‘intestinal microbiota’. The nutritional benefits of probiotics were studied by Metchnikoff in 1907. In this study, life span of Bulgarian peasants was analyzed and it was found that their long life span was because of the large quantity of lactic acid rich yoghurt that they consume (Twetman et al., 2009). This observation triggered a noticeable interest in the study of benefits of probiotics worldwide.

General health benefits of probiotics were demonstrated in subsequent studies wherein its effect on the improvement of immunity, prevention of urinary and respiratory tract infections and allergic conditions in infants, were emphasized (Gourbeyre et al., 2011). Owing to these benefits of probiotics, Salminen et al. (1998) redefined it as "a viable microbial food supplement which beneficially influences the health of the host" (Salminen et al., 1998). In 2001, probiotics were identified by World Health Organization (Media) as the suitable agents for immune defense system in cases where the common antibiotics fail to provide the desired successful treatment due to antibiotic resistance. As per a formal definition by Food and Agriculture Organization and World Health Organization, probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Reid et al., 2003). Various probiotic microorganisms are listed below.

**Lactobacillus species:** L. acidophilus, L. casei, L. fermentum, L. bulgaricus, L. cremoris, L. lactis, L. plantarum, L. salivarius.

**Bifidobacterium species:** B. bifidum, B. breve, B. lactis, B. longum,

**Streptococcus species:** S. thermophilus.

**Enterococcus species:** E. faecium.
2.5.2 Probiotics and general health

The benefits of probiotics on human health have been established since a long time. Tisser made a significant observation more than hundred years back that bifidobacteria was dominantly existent in the healthy breast fed infants. Whereas, the same was absent in infants suffering from diarrhoea. A series of studies have been conducted since then to evaluate the health benefits of probiotics.

Probiotic bacteria are known to be advantageous in the treatment of gastrointestinal infections (Hatakka and Saxelin, 2008) and respiratory tract infections (Lehtoranta et al., 2014). Effectiveness of probiotics in the treatment of antibiotics associated diarrhoea (Parvez et al., 2006) have been evident in several clinical studies. Previous studies in this regard have reported that probiotics are also effective in treatment of cardiovascular disease, urogenital infections, oropharyngeal infections and cancers (Gueimonde and Salminen, 2006; Vrese and Schrezenmeir, 2008). However, a recent control trial study in old patients demonstrated that lactobacilli and bifidobacteria were not effective against antibiotics associated diarrhea. The benefits of probiotics on human health are strain specific and act by the different mechanisms.

2.5.3 Probiotics and oral health

Study of probiotics and its effect on general health has already established to a significant level. However, the study of effect of probiotics on oral health has recently gained interest worldwide. A series of clinical trials have demonstrated that use of probiotic strains has the potential to prevent dental caries. Various carriers and delivery modes (milk, straw, tablet, chewing gum, yogurt, ice cream, powder, lozenges and drops) have been used for administering lactobacilli or Bifidobacterium,
for different treatment durations. Most of such studies have demonstrated a strong potential of probiotic bacteria to inhibit the *S. mutans* growth and thus prevent dental caries. Caglar et al. (2006) and Toiviainen et al. (2015) conducted double-blinded, randomized clinical trials using probiotic tablet and lozenges on adult population with oral health problems. They noticed the trend that probiotics decrease the *S. mutans* growth. Ashwin et al. (2015) also found the decrease in *S. mutans* count after probiotic treatment in young children. In vitro studies also reported a similar trend wherein inhibition of *S*.mutans biofilm was observed after co-culturing with different probiotic strains (Kang et al., 2011; Kojima et al., 2016; Saha et al., 2014). Few significant clinical trials and in-vitro studies related to beneficial effect of probiotics on dental caries are summarized in Table 3.

Table 3: Summary of studies that investigated beneficial effect of probiotics against *streptococcus mutans*.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study design</th>
<th>Carriage of test strains</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Nase et al., 2001)</td>
<td>Study: RCT</td>
<td>Milk containing <em>Lactobacillus rhamnosus</em></td>
<td>After 7 month treatment reduction in <em>S. mutans</em></td>
</tr>
<tr>
<td></td>
<td>Participant: 1-6 year old children</td>
<td>Sample size: 594</td>
<td></td>
</tr>
<tr>
<td>(Caglar et al., 2006)</td>
<td>Study: RCT</td>
<td><em>L. reutai</em> tablet</td>
<td>After 3 week oral intake salivary <em>S. mutans</em> decreased</td>
</tr>
<tr>
<td></td>
<td>Participant: 21-24 year old adults</td>
<td>Sample size: 120</td>
<td></td>
</tr>
<tr>
<td>(Toiviainen et al., 2015)</td>
<td>Study: RCT</td>
<td>Lozenges containing <em>L. rhamnosus GG</em> and <em>bifidobacterium</em></td>
<td>Reduction in <em>S. mutans</em> was observed after 4 weeks</td>
</tr>
<tr>
<td></td>
<td>Participant: healthy adults</td>
<td>Sample size: 29</td>
<td></td>
</tr>
<tr>
<td>(Ashwin et al., 2015)</td>
<td>Study: RCT</td>
<td>Ice cream containing <em>Bifidobacterium lactis Bb-12, L. acidophilus La-5</em></td>
<td>Reduction in <em>S. mutans</em> was observed after 7 days</td>
</tr>
<tr>
<td></td>
<td>Participant: 6–12 years old</td>
<td>Sample size: 60</td>
<td></td>
</tr>
</tbody>
</table>
In vitro studies

<table>
<thead>
<tr>
<th>(Kang et al., 2011)</th>
<th>Efficacy of probiotic strain against \textit{S. mutans} biofilm</th>
<th>\textit{L. reuteri} strains (KCTC 3594 and KCTC 3678), \textit{L. reuteri} Strains (KCTC 3594 and KCTC 3678)</th>
<th>Probiotic inhibited \textit{S. mutans} biofilm through release of hydrogen peroxide and bacteriocin like compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Saha et al., 2014)</td>
<td>Efficacy of probiotic strains against \textit{S. mutans} biofilm</td>
<td>\textit{L. fermentum}, \textit{L. reuteri} and \textit{L. acidophilus}</td>
<td>\textit{L. reuteri} inhibited \textit{S. mutans} biofilm below the detection limit. \textit{L. fermentum} coaggregated with \textit{S. mutans}</td>
</tr>
<tr>
<td>(Kojima et al., 2016)</td>
<td>Inhibition assays of insoluble glucan production by \textit{S. mutans} with probiotic supernatant</td>
<td>\textit{L. plantarum}, \textit{L. fermentum}, \textit{L. paracasei}, \textit{L. casei}</td>
<td>Probiotic strains inhibited \textit{S. mutans} biofilm</td>
</tr>
</tbody>
</table>

Although the efficacy of probiotics on caries and periodontitis has been considerably studied, the data of probiotics effect on oral candidiasis are scarce. A double-blinded, randomized clinical trial in elderly groups conducted by Hatakka et al. (2007) found decrease in \textit{Candida} count after consumption of probiotic cheese. However, there was no change in mucosal symptoms. On the contrary, a series of studies reported improvement in the patients suffering from oral candidiasis after probiotic treatment (Kraft-Bodi et al., 2015; Mendonca et al., 2012; Sutula et al., 2013). Several in vitro studies have been performed which have examined the effect of probiotics on oral \textit{Candida} infections. The reports suggested a beneficial effect of probiotics on candidiasis (Hasslof et al., 2010; James et al., 2016; Kheradmand et al., 2014; Matsubara et al., 2016b). Few clinical trials and in-vitro studies related to beneficial effect of probiotics on \textit{C. albicans} are summarized in Table 4.
Table 4: Summary of studies that investigated beneficial effect of probiotics against *Candida albicans*.

<table>
<thead>
<tr>
<th>Reference (1st author, year)</th>
<th>Study design</th>
<th>Carriage of test strains</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Hatakka et al., 2007)</td>
<td>Study: RCT</td>
<td>Cheese containing <em>L. lactis</em>, <em>L. helveticus</em>, <em>L. rhamnosus GG</em>, <em>P. freudenreichii</em></td>
<td>After 16 weeks treatment 10% reduction in <em>Candida</em> count</td>
</tr>
<tr>
<td></td>
<td>Participant: elderly group size: 276</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Mendonca et al., 2012)</td>
<td>Study: RCT</td>
<td>Commercial probiotic drinks containing <em>L. casei</em></td>
<td><em>Candida</em> prevalence decreased after 30 days, increment of sIgA level</td>
</tr>
<tr>
<td></td>
<td>Participant: individuals older than 65 years of age Sample size: 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Sutula et al., 2013)</td>
<td>Study: RCT</td>
<td>Commercial probiotic drinks containing <em>L. casei</em></td>
<td>After 4 weeks treatment no reduction in CFU count of <em>Candida</em></td>
</tr>
<tr>
<td></td>
<td>Participant: healthy adults 32 year old Sample size: 22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Kraft-Bodi et al., 2015)</td>
<td>Study: RCT</td>
<td><em>L. reuteri</em> (lozenges)</td>
<td>Improved candida score</td>
</tr>
<tr>
<td></td>
<td>Participant: elderly patients staying in nursing home Sample size: 174</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vitro study</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Hasslof P, 2010)</td>
<td>Agar overlay interference tests</td>
<td><em>L. plantarum</em>, <em>L. paracasei</em>, <em>L.rhamnosus GG</em>, <em>L. acidophilus</em>, <em>L. reuteri</em>.</td>
<td>Probiotic inhibited <em>Candida</em> growth. However less reduction in <em>S. mutans</em> biofilm</td>
</tr>
</tbody>
</table>
| Shokryazdan, P. et al., 2014  | Pathogenic microorganism was grown in the presence of probiotics | *L. fermentum*  
*L. casei*,  
*L. acidophilus*,  
*L. buchneri* | Antagonistic activity against pathogens due to release of organic acids. |
| (Kheradmand et al., 2014)     | *C. albicans* co-culture with selenium NPs-enriched *Lactobacillus* species | *L. johnsonii*,  
*L. plantarum* | Exometabolites of probiotics are novel antifungal agent |
| (James et al., 2016)          | Inhibitory effect with probiotic supernatants evaluated in vitro biofilm formation | *L. plantarum*,  
*L. helveticus CBS*,  
*S. salivarius* | Reduction in *C. albicans* biofilm formation and expression of hyphal growth associated genes |
(Matsubara et al., 2016b) Biofilm quantification and structural analysis (CLSM and SEM) of *Candida* in the presence of probiotics supernatant  | *L. rhamnosus*, *L. casei*, *L. acidophilus*  | Exometabolites in supernatant reduced *Candida albicans* colonization and hyphal differentiation |

### 2.5.4 Mechanism of action

Lactic acid bacteria and bifidobacteria are the most frequently used probiotic strains. Although positive therapeutic effect of probiotic has been shown, the mechanism by which probiotics exert anti-microbial activity has not been fully elucidated yet. The possible mechanism of action includes competitive exclusion of pathogenic microorganisms, adhesion to intestinal mucosa and epithelium, enhancement of the epithelial barrier, secretion of anti-microbial agents and modulation of the immune system.
**2.5.4.1 Release of antimicrobial agents**

Release of low molecular weight antimicrobial substances (organic acids, hydrogen peroxide and bacteriocins) has been proposed as one of the mechanism of action of probiotics (Reid et al., 2003).
i) Organic acids

Many *Lactobacilli* produce lactic acid and other organic acids which possess strong inhibitory effect against the pathogens (Taniguchi et al., 1998). Organic acid permeabilizes the outer membrane of pathogens, thereby letting the lactobacilli molecules to penetrate into the bacteria and eventually, lead to cell death of pathogen due to lowering of the intracellular pH (Alakomi et al., 2000). Sookkhee et al (2001) studied the antimicrobial activity of lactobacilli at acidic and alkaline pH. They suggested that the antimicrobial activity of *lactobacilli* could only partly be attributed to organic acids, because the antimicrobial effect was more active at acidic pH than that at alkaline pH. They further adjusted the pH of the supernatant fluid to 7.0 and found 10-20% reduction in bacterial growth inhibition. Therefore, they concluded that organic acids were not the only antimicrobial agents (Sookkhee et al., 2001). In contrast, a study conducted by Rossland et al in 2005 to investigate the growth inhibition of *Bacillus cereus* after treatment with lactic acid producing bacteria. They showed that the initial rate of lactic acid production by *Lactobacilli* has a strong effect on inhibition of growth of *Bacillus cereus*. They also demonstrated that *B. cereus* inhibition was maximum when treated with fast lactic acid producing strains. They concluded that lactic acid was the primary agent for pathogen inhibition and the effect from other antimicrobial metabolites like bacteriocins and hydrogen peroxide was minor or negligible (Rossland et al., 2005).

However, the recent study conducted by Hasslof et al. (2010) depicted a different mechanism of action. This study found that a few *Lactobacillus* strains that have lesser acid production, like *L. reuteri* ATCC 55730, showed inhibition for both *mutans streptococci* and *Candida*. This study revealed that there are antimicrobial substances produced by probiotic bacteria other than lactic acid (Hasslof et al., 2010).
ii) **Bacteriocin**

Bacteriocin mediated inhibition was first discovered by A. Gratia in 1925. These are proteinaceous toxins ribosomally synthesized single polypeptides produced by *lactobacillus*. Bacteriocins inhibit the growth of closely related bacterial strains by pore formation in the membrane surrounding the bacteria (Hassan et al., 2012). Based on primary structure, molecular composition and properties, bacteriocins were classified into three groups (Cotter et al., 2005).

Class I - lanthionine-containing (Lantibiotics)

Class II - Non-lanthionine-containing bacteriocins

Class III - Heat-labile murein hydrolases (Bacteriolysins)

Awaisheh and Ibrahim (2009) studied the antibacterial activity of neutral supernatant of lactic acid bacteria (LAB) and found significant inhibition on different pathogenic strains (Awaisheh and Ibrahim, 2009). Bacteriocin was sensitive towards proteolytic enzymes. A series of studies have demonstrated the bacteriocin mediated inhibition of different pathogenic strains. Plantaricin secreted by *L. plantarum* inhibits yeast form of *Candida* (Sharma and Srivastava, 2014) whereas Nisin (*Lactococcus lactis*) was effective against the hyphal growth of *C. albicans* (Lay et al., 2008).

### 2.5.4.2 Hydrogen peroxide

Hydrogen peroxide is known to be another available mechanism by which probiotics act against the pathogens. This hypothesis was studied by Sookkhee S et al (2001) wherein they found that after treatment with catalase such as trypsin and pepsin, various *lactobacillus* strain showed a noticeable reduction in antimicrobial
activity. It was also noticed that there was a growth of blue pigment around the developing bacterial colonies when the medium was supplemented with tetramethyl benzidine and horseradish peroxidase. This study therefore concluded that hydrogen peroxide is another antimicrobial agent produced by probiotic bacteria which inhibits the growth of pathogens (Sookkhee et al., 2001).

Other studies have also supported the wide spread effect of hydrogen peroxide on planktonic bacteria, but have also highlighted that the effect decreases significantly on biofilm (Perumal, 2014).

2.5.4.3 Competition for adhesion sites

Probiotic bacteria battle out with the invading pathogens for adhering to epithelial cells and the mucus layer. Competition between probiotic bacteria and invading pathogen may occur for the available nutrients and mucosal binding sites. In order to gain a competitive advantage over the pathogens, probiotic bacteria modulates the environment so as to make it less suitable for pathogens. The environment modulation is done by secreting antimicrobial compounds. Reid and co-workers (1985) explained the adhesive property of *Lactobacillus* of urovagina and concluded that it could inhibit the attachment of uropathogens with respective epithelial cells (Raphael et al., 1985; Reid et al., 1985).

The most effective action of probiotics is obtained only if these microorganisms attach to the mucosal cells. However, it is still not clear whether or not the exogenously administered probiotics are equally effective in human body. Few studies have been done to understand the adherence and colonization of probiotic strains in the gut (Juntunen et al., 2001).
2.5.4.4 Stimulation of immunity

Probiotic bacteria have been known to affect the gut lumina, epithelial and mucosal barrier function, and the mucosal immune system. The cells which are involved in the immune responses like epithelial cells, dendritic cells, monocytes/macrophages, B cells, and T cells, are affected by the probiotic microorganisms and subsequently aids the modulation of immune response (Hart et al., 2004; Takeda et al., 2006).

Previous studies have demonstrated that probiotics have the ability to catalyze the regulatory cytokine IL-10 and attenuate the proinflammatory cytokines (TNF) in the mucosa of patients suffering from ulcerative colitis and pouchitis (N Borruel et al., 2002; Pathmakanthan et al., 2004).

2.5.4.5 Interference with quorum sensing signaling

Quorum sensing is a phenomenon by which bacteria communicate with each other and the surrounding environment through signaling molecules referred to as auto-inducers (Miller and Bassler, 2001). Effective cell to cell signaling enhances the colonization of bacteria leading to infection in the host tissues (Kendall and Sperandio, 2007). Previous study demonstrated that Lactobacillus acidophilus releases an active compound which either inhibits the quorum sensing signal or disrupts the colonization of pathogen by directly interacting with bacterial transcription of E. coli O157 gene (Medellin-Peña et al., 2007).
Chapter 3: Materials and methods

List of materials and apparatus used in various experimental stages are available in the appendix.

3.1 Methodology

Outline of experimental design for studying the efficacy of *Lactobacillus plantarum* against *Streptococcus mutans* and *Candida albicans* single and mixed species biofilms is described in Figure 8.

**Figure 8: Experiment design**
3.1.1 Strains and culture conditions

Two bacterial strains i.e. *Streptococcus mutans UA159* and *Lactobacillus plantarum* 108 and one fungal strain *i.e. Candida albicans SC5314*, were used in this study. The three microbial strains were taken from the archival collection of the Oral Sciences laboratory of the Faculty of Dentistry, National University of Singapore. All microbial strains were maintained at a temperature of -80°C in a sterile 50% (v/v) glycerol solution. For routine use, *S. mutans* and *L. plantarum* were sub-cultured on Brain-heart infusion (BHI) agar (Sigma-Aldrich, Singapore) and de Man, Rogosa and Sharpe (MRS) agar (Sigma-Aldrich, Singapore) plates, respectively. *C. albicans* was sub-cultured on Glucose minimal medium (GMM; 1.2% w/v agar, 20% w/v glucose, and 6.7% w/v Yeast nitrogen base, Sigma-Aldrich Singapore) agar plates. BHI agar plates and MRS agar plates were incubated at 37°C for 24 h under 5% CO₂ conditions. GMM agar plates were incubated at 37°C for 24 h under aerobic conditions. After bacterial and fungal colony formation, these agar plates were stored at 4°C during the experimental period with sub culturing into new agar plates every second week. Purity of cultures was checked frequently by Gram-staining.

3.1.2 Preparation of standard cell suspensions

Prior to the experiment, ultra filtered tryptone yeast extract medium (UFTYE) was prepared by adding 2.5% w/v Yeast extract (Sigma-Aldrich, Singapore) and 1.5% w/v Tryptone (Sigma-Aldrich, Singapore). Two different UFTYE solutions of pH 7 and pH 5.5 were prepared by adding NaOH and HCl, respectively. The pH was confirmed using pH meter (Fisher Scientific accumet AB150, Singapore). These solutions were stored at 4°C during the experimental period.
A loopful culture of *S. mutans* and *C. albicans* were taken from agar plates and inoculated in UFTYE supplemented with 1% (w/v) glucose (Sigma-Aldrich, Singapore) at pH 7 and pH 5.5 for *S. mutans* and *C. albicans*, respectively. Then it was subjected to incubation for 18 h at 37°C in orbital shaker incubator (ES-20 Biosan, Singapore) at 80 rpm. The standard cell suspensions of *S. mutans* and *C. albicans* were prepared according to the previously established protocol (Ellepola et al., 2017). After 18h incubation, the cells were centrifuged (6000xg) for 10min at 4°C. Following centrifugation, the cells were washed twice in phosphate-buffered saline (PBS). Subsequently, optical density of bacterial and fungal cell suspensions in PBS was adjusted using Mcfarland standards. Optical density (OD) of the *S. mutans* cell suspension was adjusted to 0.300 at a wavelength (WL) of 520nm, which is equivalent to 10^7 cells/mL in the suspension. Similarly, the optical density of *C. albicans* was adjusted to 0.375 at a wavelength of 520nm, equivalent to 10^7 cells/mL. Freshly prepared standard cell suspensions were immediately used for biofilm formation.

### 3.1.3 Preparation of *Lactobacillus plantarums* supernatant

*L. plantarum* loopful culture was taken from agar plate, inoculated in MRS broth and cells were grown to mid exponential phase for 18 h at 37°C. This overnight culture was subjected to centrifugation (4000xg, 10 min, 4°C) following which, it was washed twice with PBS. The optical density of the standard cell suspension was adjusted to 0.9 at a WL of 600 nm by using a spectrophotometer(UV-1700 Shimadzu, Tokyo, Japan). For supernatant preparation, this standard cell suspension was further incubated for 24 h at 37°C. Following 24 h of incubation, the bacterial culture was subjected to centrifugation (4,000xg for 10 min, 4°C) and subsequently filtered through sterilized 0.22 mm pore size membrane (Surfactant-free cellulose acetate,
Ministart syringe filter, Sartorius, Singapore) and supernatant was collected. This freshly prepared cell free supernatant was used to check the inhibitory activity.

3.1.4 Antimicrobial activity against planktonic Streptococcus mutans and Candida albicans

Planktonic cells of S. mutans (2 × 10⁶ CFU/mL) and C. albicans (1 × 10⁶ CFU/mL) were incubated with UFTYE broth containing 2% (w/v) sucrose in pre-sterilized, polystyrene, flat-bottomed, 96-well microtiter plate (Greiner Bio-One, Singapore) in the presence and absence of L. plantarum supernatant. Subsequently this plate was incubated for 24 h at 37°C and the optical density of the microbial cultures was measured at a wavelength of 600 nm. The growth was measured at 30-min intervals for 24 h by using plate reader (Multiskan™ GO, Thermo Scientific, Singapore). The data obtained from growth kinetics was directly used for analyzing the effect of supernatant on planktonic cells of S. mutans and C. albicans.

3.1.5 In vitro biofilm formation

Biofilm formation was carried out in commercially available pre-sterilized, polystyrene, flat-bottomed, 96-well microtiter plate (Greiner bio-one, Singapore) according to the previously established protocol with slight modification (Ellepola et al., 2017). For S. mutans and C. albicans single-species biofilm formation, a cell suspension containing a cell concentration of 1 × 10⁶ CFU/mL yeast cells or 2 × 10⁶ CFU/mL bacterial cells was used. A volume of 75 μl of UFTYE broth containing 2% (w/v) sucrose were pipetted into each well of the microtiter plate. For mixed species biofilm formation, equal volumes of standardized cell suspension of S. mutans and C. albicans were used. Similar to the single species biofilms, 75μl of UFTYE containing 2% (w/v) sucrose was added into each well of the microtiter plate.
A volume of 100 μl *L. plantarum* supernatant was added into each well of treatment groups whereas equal amount of MRS broth (100 μl) was used as the negative control.

To demonstrate the ability of *L. plantarum* supernatant against biofilm formation and preformed biofilms, supernatant was added at two different time points. In order to evaluate the efficacy of probiotic supernatant in preventing biofilm formation, *L. plantarum* supernatant was introduced into the wells at the beginning (0 h) along with bacterial or fungal cell suspensions. For the therapeutic assay, the biofilms were formed for 12 h and subsequently the probiotic supernatant was introduced to these pre-formed biofilms. Further, microtitre plates were incubated at 30°C for 24h in orbital shaker incubator (ES-20 Biosan, Singapore) at 80 rpm. Three replicates of each samples were examined.

### 3.1.6 Quantification of biofilms

To assess the efficacy of *L. plantarum* supernatant on bacterial and fungal cells in single and mixed species biofilms, it is important to determine the relative reduction in viable cells due to the treatment. Therefore, biofilms were quantified by three different techniques, namely XTT reduction assay, crystal violet (CV) assay and colony forming units (CFU) counting. XTT assay quantifies the metabolic activity of the biofilms, CV assay estimates the total biomass of biofilms including extracellular matrix, whereas CFU assay indicates the number of biofilm cells.

#### i XTT reduction assay

XTT assay was performed as previously described with minor modifications (Seneviratne et al., 2008). The stock solution of XTT tetrazolium salt were prepared
by adding 0.5 gm/L in PBS and filtered using filter of 0.22 μm pore size (Surfactant-free cellulose acetate, Ministart syringe filter, Sartorious, Singapore). This stock was stored at 4°C for future experiments. Prior to the experiments, 0.4 mM Menadione solution (Sigma Aldrich, Singapore) was prepared and filtered through sterilized 0.22 μm pore size filter. Prior to the test, thawed XTT solution was mixed with menadione solution. After appropriate incubation, suspension was removed from the wells and the biofilms were washed once with PBS (200 μl) to remove non-adherent cells and then 200 μl of XTT-menadione solution was pipetted into each wells. Further, plates were incubated in dark at 37°C for 20min. Following incubation, 200 μl of solution was transferred to new wells and the colorimetric changes were measured by using a plate reader at 490 nm (Multiskan™ GO, Thermo Scientific, Singapore). The absorbance values for the treatment groups were compared with the absorbance values of respective control groups to analyze the efficacy of probiotic supernatant in treatment groups.

**ii Crystal violet assay**

Crystal violet (CV) assay was performed according to a previously published protocol with slight modifications (O'Toole, 2011). After appropriate incubation period, the cell suspensions were aspirated from each well and biofilms were washed gently with PBS to remove the non-adherent cells. Following the washing, plates were air dried for 20 min. Further, 200 μl of 2% formalin was pipetted into each washed wells and incubated at room temperature for 15 min in order to fix the cells in the biofilm. Subsequently, 200 μl of 1% (w/v) crystal violet stain (Sigma-Aldrich, Singapore) was added into each well for 5 min and then washed three times with PBS to remove the excess stain. After staining, 200 μl of 95% ethanol was added into each well.
well and incubated for 15 min at 80 rpm. Absorbance of 95% ethanol was measured at 570 nm using a plate reader (Multiskan™ GO, Thermo Scientific, Singapore).

**iii Colony Forming Units counting**

Colony-forming units (CFUs) counting is used as a quantification method to count the number of viable cells in biofilm community. CFU counting was performed using a previously established protocol (Thein et al., 2007). After the biofilm development in the presence and absence of probiotic supernatant, the cell suspensions were aspirated. Biofilms were washed gently with PBS and then 100 μl of PBS was pipetted into each well. Subsequently the biofilm was cautiously scraped off from the bottom of each well, the suspension was collected and transferred into PBS (900 μl). A dilution series was prepared in PBS for the cell suspensions obtained from both single and mixed species biofilms. A volume of 100 μl from each dilutions was plated out on BHI and GMM agar to enumerate the *S. mutans* and *C. albicans*, respectively.

For quantification of viable cells in mixed species biofilms, BHI and GMM agar plates were supplemented with 8 μg/ml of amphotericin B (to prevent fungal growth) and 8 μg/ml gentamicin sulfate salt-Sigma-Aldrich (to prevent bacterial growth), respectively. Three replicates were prepared for both control and treatment groups of single and mixed species biofilms. Further, BHI plates were incubated at 37°C for 24 h and the GMM plates were incubated at 30°C for 48 h. The bacterial and fungal colonies were counted and the corresponding log CFU values were calculated.

**3.1.7 Confocal laser scanning microscopy (CLSM)**

For CLSM, *S. mutans* and *C. albicans* single and mixed-species biofilms were formed on pre-sterilized, 8-well chamber slide (Nunc, Thermo Scientific, Lab-
Briefly, 8-well chamber slide were placed on the bottom of a sterile plastic petri dish with caution and bacterial and fungal standard cell suspensions with UFTYE broth containing 2% (w/v) sucrose were pipetted into each well. The test samples were treated with probiotic supernatant at two different time points (0 h and 12 h) and then incubated at 30°C in a orbital shaker incubator (80 rpm) to allow the biofilm formation for 24 h. After biofilm formation, the cell suspensions were removed and each well was washed gently with PBS. Following this, biofilms were stained for CLSM imaging according to a previously described protocol (Ellepola et al., 2017).

Biofilms were fixed with 4% (v/v) paraformaldehyde and stained by adding 200 μl of propidium iodide (Live/Dead BacLight Bacterial Viability kit; Invitrogen, Singapore) and 0.001% (w/v) calcofluor white (Sigma-Aldrich, Singapore) into each wells and subjected to further incubation for 20 min in the dark. Propidium iodide (excitation/emission 535/617 nm) stained S. mutans cells while and calcofluor white (excitation/emission 365/435 nm) stained the C. albicans cells.

Prior to the visualization using CLSM, stains were removed by washing once with PBS. Stained single and mixed species biofilms were visualized using an Olympus-FluoviewFV1000 TIRF confocal microscope with the 60X water lens. Z sections were collected from (top to bottom of biofilms) several microscopic fields in three biological replicates. The single and mixed-species biofilms architecture was analyzed using Imaris software.

3.1.8 Gene expression analysis by qPCR

To gain further insight of L. plantarum supernatant mediated inhibition of S. mutans in single and mixed species biofilms, we analyzed the expression of genes associated with the glucosyltransferase activity i-e gtfB, gtfC and gtfD of S. mutans
which play a crucial role in the biofilm formation (Bowen and Koo, 2011). Similarly, the expression of hyphal growth associated Candida genes *HWP1*, *ALS1* and *ALS3* were also examined in the presence of probiotic supernatant. These *Candida* genes *HWP1* (hyphal wall protein 1), *ALS1* (agglutinin-like sequence 1) and *ALS3* (agglutinin-like sequence 3) contribute in maintaining the integrity of cell wall and hyphal growth in biofilm and are responsible for pathogenesis due to adhesion and invasion on the host tissues (Finkel and Mitchell, 2011).

Analysis of gene expression was performed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Expression of gene in the sample is a multi-step procedure; RNA extraction, cDNA synthesis, PCR reaction, and finally the data analysis by using $2^{-\Delta\Delta C_t}$ relative expression method to calculate the fold changes.

### RNA extraction

Fresh cell suspension of *S. mutans* and *C. albicans* was added in each well of 24-well microtiter plate (Greiner Bio-One, Singapore) with UFTYE broth containing 2% (w/v) sucrose. Probiotic supernatant was diluted in MRS (1:10 ratio). The test samples were treated with this diluted supernatant and then incubated at 37°C to allow biofilm formation for 24 h. After incubation, biofilms were washed once with PBS and biofilms were carefully scraped off from each well and collected in 1.5 ml microtubes (Eppendorf). Microtubes were centrifuged (10,000 g for 10 min) and the cell pellets were collected.

Trizol reagent (Invitrogen life Tech, Ambion, Singapore) was used to extract the total RNA from cell pellets of treated and untreated samples according to the previously published protocol (Rio et al., 2010). In brief, 1ml of Trizol reagent per sample was added and cell pellets were quickly dissolved in the Trizol by passing
through a pipette several times. Samples were incubated at room temperature for 5 min and subsequently 200μl of chloroform (aids phase separation) was added in each samples and mixed vigorously by hands for 2 to 3 min. This mixture was kept at room temperature for 5 min and then subjected to centrifugation at 10,000g for 20 min. Following centrifugation, the mixture got separated into two phases: lower red phase containing protein and upper phase colorless aqueous phase containing RNA. The upper aqueous phase was transferred carefully to a fresh tube without disturbing the inter phase which contains DNA.

RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropanol per 1 ml of Trizol. Following this, the sample was incubated at room temperature for 20 min. Subsequently, all samples were subjected to centrifugation at 10,000 g for 10 min at 4°C. Thereafter, supernatant was removed, RNA pellets were washed twice with ice-cold 75% ethanol (1 ml) diluted with DEPC-treated water and finally, precipitated RNA was collected. Left over ethanol was removed by brief centrifugation and RNA pellets were air dried for 10 min at room temperature. After air drying, RNA pellets were dissolved in nuclease free deionized distilled water (13 μl ) by passing through a pipette several times.

NanoDrop ND1000 spectrophotometer (Thermo Scientific, Singapore) was used to determine the concentration, purity and quality of the isolated RNA by measuring the absorbance ratio at 260/280 nm and 260/230 nm. Purified RNA was stored at -80°C for future experiments.

ii Reverse Transcription (cDNA synthesis)

For cDNA synthesis, 500 ng RNA, 1 μl dNTP (10 mM), 1 μl of oligodT (50mM) and nuclease free deionized distilled water were added in each reaction mixture. Subsequently, this mixture was incubated at 65°C for 5 min followed by 4°C
for 5 min. Followed by this, M-MLV 5x reaction buffer (4 μl), RNase inhibitor (0.5 μl) and M-MLV reverse transcriptase (1 μl) were added to this mixture. Finally, this was incubated at 50°C for 1 h followed by 85°C for 5 min and subsequently put on ice to cool down. The obtained cDNA samples were diluted three times in Nuclease-Free Water (Ambion) and stored at -20°C for real time PCR experiments.

iii Real-time PCR

Reaction mixture (20 μl) was prepared by adding diluted cDNA (1 μl), gene specific forward and reverse primers (0.8 μl),(10.8 μl) SYBR Green (KAPA SYBR FAST qPCR Kit,Kapa Biosystems, Singapore) and Nuclease-Free Water (7.8 μl). Subsequently this mixture was pipetted into each well of a MicroAmp optical 96-well plates (Applied Biosystems, Singapore). Plate was sealed properly by using optical adhesive film (Thermo fisher scientific, Singapore) and briefly centrifuged the plate to spin down the contents and to remove the air bubbles. Finally, qPCR was performed using the Step One Plus™ Real-Time PCR system (Thermo Fisher Scientific, Singapore) as described in previous protocol (Ellepola et al., 2017). This was done under adequate thermo cycling conditions (Holding stage 95°C for 3min, Cycling stage 95°C for 3s, 60°C for 1min , and Melting curve stage 95 °C for 1s, 60°C for 1 min, and 95°C for15s) for 40 cycles. Specificity of all the primers were evaluated using melting curve scanning (Ellepola et al., 2017).

iv Data Analysis

All the reactions were performed in triplicates on the same plate and the C_T values were obtained from the system. The resultant C_T values of the target genes of interest were normalized to the C_T values of the respective housekeeping gene (16sRNA for S. mutans and PMA1 for C. albicans). Results were analyzed using the
$2^{-\Delta \Delta CT}$ relative expression method to calculate the fold changes (Schmittgen and Livak, 2008).

A list of *S. mutans* gene specific forward and reverse primers (*gtfB, gtfC, and gtfD*) for target and housekeeping gene (16s RNA) used in q-PCR are mentioned in Table 5. *C. albicans* gene specific primers (*HWP1, ALS1, and ALS3*) and housekeeping gene (*PMA1*) and their sequences are summarized in Table 6.

**Table 5:** Primer sequence of *Streptococcus mutans* genes used for qPCR (Lee and Kim, 2014)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
</table>
| Sm-16sRNA | F: CCT ACG GGA GGC AGC AGT AG  
         | R: CAA CAG AGC TTT ACG ATC CGA AA   |
| Sm-GtfB  | F: AGC AAT GCA GCC AAT CTA CAA AT  
         | R: ACG AAC TTT GCC GTT ATT GTC A   |
| Sm-GtfC  | F: GGT TTA ACG TCA AAA TTA GCT GTA TTA GC  
         | R: CTC AAC CAA CCG CCA CTG TT   |
| Sm-GtfD  | F: ACA GCA GAC AGC AGC CAA GA  
         | R: ACT GGG TTT GCT GCG TTT G   |

**Table 6:** Primer sequence of *Candida albicans* genes used for qPCR (Theberge S, 2013)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
</table>
| PMA1 | F: TTGAAGATGACCACCCAAATCC  
         | R: GAAACCTCTGGAAGCAAATCG   |
| HWP1 | F: GCTCAACTTTGCTATCGCTATTACA  
         | R: GACCCTCTACCTGTGGGGAGAG   |
| ALS1 | F: GAC TAG TGA ACC AAC AAA TAC CAG A  
         | R: CCA GAA GAA ACA GCA GGT GA |
| ALS3 | F: AATGGTCTTATGAATCCCATCTCTA  
         | R: GAGTTTTTATCATCTTTGATTTCAT |
3.1.9 **Statistical analysis**

All experiments were carried out in triplicate and on three different occasions. The obtained data were expressed as mean values with the corresponding standard deviations (SD). The error bar in each graph indicates mean + standard deviation (SD). Pair wise comparison was performed between treated groups and control groups for all sets of data. Student's *t*-test or Mann-Whitney *U* test was used for these comparisons. P-value <0.05 was considered statistically significant(*).
Chapter 4: Results

4.1 Effect of *Lactobacillus plantarum* supernatant on planktonic *Streptococcus mutans* and *Candida albicans* cells

Planktonic cells of *S. mutans* and *C. albicans* were grown in the presence and absence of probiotic supernatant as described in chapter 3 (section 3.1.4). Growth kinetics assay showed that *L. plantarum* supernatant has a significant inhibition on both *S. mutans* and *C. albicans* planktonic mode of growth compared to control group without supernatant (Figure 9A-B). In control groups, *S. mutans* and *C. albicans* cells were grown exponentially whereas there was no significant difference in the growth kinetics of the group treated with *L. plantarum* supernatant (Figure 9A-B).

Therefore, the differences in *S. mutans* and *C. albicans* planktonic mode of growth in both control and treatment groups was due to secretory component of *L. plantarum*. 
Figure 9: Growth kinetics of *S. mutans* (A) and *C. albicans* (B) planktonic cells in the presence of *L. plantarum* supernatant (Sup).

4.2 Effect of *Lactobacillus plantarum* supernatant on *Streptococcus mutans* single-species biofilms

Standard cell suspension of *S. mutans* was co-cultured in the presence and absence of probiotic supernatant and biofilm formation was quantified using XTT reduction assay, crystal violet assay and CFU counting as mentioned in chapter 3 (section 3.1.6). In the presence of *L. plantarum* supernatant, the XTT assay showed a
significant reduction (89%) in *S. mutans* biofilm (Figure 10A, *P* < 0.05). Similar to the XTT assay, the CV assay and CFU counting method also showed a similar pattern in biofilm inhibition when treated with probiotic supernatant. CV assay demonstrated that total biofilm biomass was reduced by 75% (Figure 10B, *P* < 0.05). CFU counting demonstrated 99% reduction in cell counts in the test group compared to the untreated control (Figure 10C, *P* < 0.05).

Activity of probiotic supernatant against *S. mutans* preformed biofilms was also quantified using aforementioned techniques. *S. mutans* preformed biofilms also showed a considerable inhibition. In XTT reduction assay, metabolic activity of *S. mutans* in preformed biofilms was reduced by 33% (Figure 10A, *P* < 0.05). CV assay showed a 36% reduction in total biofilm biomass after treatment with probiotic supernatant (Figure 10B, *P* < 0.05). Similarly, CFU counting method showed 99% reduction in *S. mutans* cultivable cells following treatment with probiotic supernatant (Figure 10C, *P* < 0.05).

These results suggest that *L. plantarum* supernatant was not only able to inhibit the initial colonization of *S. mutans* biofilm, but also inhibits preformed biofilms.
Figure 10: Effect of *L. plantarum* supernatant on *S. mutans* single-species biofilms.

Biofilms were analyzed by various quantification methods: XTT reduction assay (A), Crystal Violet assay (B) and colony forming unit (CFU) counting (C).
4.3 Effect of *Lactobacillus plantarum* supernatant on *Candida albicans* single-species biofilms

*C. albicans* biofilms were developed in the presence and absence of probiotic supernatant as described in Chapter 3 (section 3.1.5). Biofilms were quantified by XTT reduction assay, crystal violet assay and colony forming unit (CFU) counting as described in Chapter 3 (section 3.1.6). There was a significant inhibition of *C. albicans* biofilms when treated with probiotic supernatant. XTT assay showed a 90% reduction in the metabolic activity of *C. albicans* cells treated with supernatant (Figure 11A, p < 0.05). Similarly, CV assay demonstrated that total biofilm biomass was reduced by 80% (Figure 11B, p < 0.05). CFU counting method demonstrated 91% reduction in *C. albicans* cultivable cells in the test groups with respect to their control groups (Figure 11C, p < 0.05).
Probiotic supernatant was also successfully eradicated the preformed C. albicans biofilms. The XTT assay showed a 34 % reduction in C. albicans preformed biofilm upon treatment with probiotic supernatant (Figure 11A, p < 0.05). Similar to the XTT assay, CV assay and CFU counting method were also demonstrated a considerable reduction in preformed biofilms by 42% and 46 %, respectively ( Figure 11B-C, p < 0.05).

These results indicated that L. plantarum supernatant was able to inhibit the initial colonization biofilms as well as the C. albicans preformed biofilms.

**Figure 11:** Effect of L. plantarum supernatant on C. albicans single-species biofilms. Biofilms were analyzed by various techniques; XTT reduction assay (A), Crystal Violet assay (B) and colony forming unit (CFU) counting (C).
4.4 Effect of *Lactobacillus plantarum* supernatant on *Streptococcus mutans* and *Candida albicans* mixed-species biofilms

We further evaluated the efficacy of *L. plantarum* supernatant against *S. mutans* and *C. albicans* mixed-species biofilm formation and preformed biofilms (12h mature). The XTT assay, CV assay and Colony forming unit counting methods were used to quantify the mixed species biofilms of treated and untreated groups as described in chapter 3 (section 3.1.6).

Interestingly, a significant reduction in mixed species biofilm formation was observed when treated with *L. plantarum* supernatant. The XTT reduction assay showed 85% reduction in the metabolic activity of the treated cells in mixed-species biofilms compared to the control groups without supernatant (Figure 12A, p < 0.05). CV assay demonstrated 86% reduction in total biofilm biomass (Figure 12B, p < 0.05). The CFU counting method showed a significant reduction in both *S. mutans* and *C. albicans* cells in the probiotic-treated biofilm samples compared to the control without supernatant. The probiotic supernatant was able to reduce the *S. mutans* and *C. albicans* cells by 99.99% and 99.34%, respectively, in the mixed-species biofilms (Figure 12C-D, p < 0.05).

Further, *L. plantarum* supernatant was also found to inhibit the preformed *S. mutans* and *C. albicans* mixed-species biofilms. The XTT assay, and CV assay showed a reduction in the preformed mixed-species biofilm by 33% and 50%, respectively (Figure 12A-B, p < 0.05). In the presence of probiotic supernatant, cultivable *S. mutans* and *C. albicans* cells were reduced by 99.96% (Figure 12C, p <0.05) and 43.68% (Figure 12D), respectively.

Foregoing data demonstrated that *L. plantarum* supernatant was effective against *S. mutans* and *C. albicans* mixed-species biofilms.
Figure 12: Effect of *L. plantarum* supernatant (Sup) on *S. mutans* (Sm) and *C. albicans* (Ca) mixed-species biofilms.

Biofilms analyzed by various techniques; XTT reduction assay (A), Crystal Violet assay (B) and colony forming unit (CFU) counting for *S. mutans* (C), *C. albicans* (D).
4.5 Structural analysis of single and mixed species biofilms by confocal laser scanning microscopy

CLSM was used to further validate the efficacy of *L. plantarum* supernatant against *S. mutans* and *C. albicans* single and mixed-species biofilms. *S. mutans* and *C. albicans* single and mixed-species biofilms were developed on 8-well chamber slide and stained with propidium iodide and calcofluor white as described in chapter 3 (Section 3.1.7). CLSM images of the control groups showed a dense accumulation of *S. mutans* (Figure 13A) and *C. albicans* (Figure 13D) cells in the single-species biofilm depicting a typical biofilm architecture. Closely aggregated *S. mutans* and *C. albicans* yeast cells clustering together with intermittent hyphal distribution was observed in the *S. mutans*-*C. albicans* control mixed-species biofilm (Figure 13G). On the contrary, probiotic supernatant significantly inhibited the biofilm formation of *S. mutans* and *C. albicans* single and mixed-species biofilms (Figure 13B, E, H).

However, mixed-species preformed biofilms treated with probiotic supernatant comparatively had more residual cells than the biofilms of the inhibitory experiment assay. (Figure 13C, F, I).

Conclusively, confocal microscopic images showed the reduction in biofilm biomass and showed poorly developed biofilm architecture after probiotic treatment. These observations were consistent with the data obtained from the biofilm quantification assays.
**Figure 13:** Confocal laser scanning microscopic examination (Scale bar 30μm)

CLSM examination of *S. mutans* (Sm) and *C. albicans* (Ca) single and mixed-species biofilm formation in the presence and absence of *L. plantarum* supernatant (Sup); *S. mutans* single-species biofilms (A) control group, (B) treated with supernatant at 0 h, (C) preformed biofilm treated with the supernatant at 12 h. *C. albicans* single-species biofilms (D) control group, (E) treated with supernatant at 0 h, (F) preformed biofilm treated with the supernatant at 12 h. *S. mutans* and *C. albicans* mixed-species biofilms (G) Control group, (H) treated with supernatant at 0 h, (I) preformed biofilm treated with the supernatant at 12 h.
4.6 Gene expression analysis

4.6.1 L. plantarum supernatant down-regulated the gtf gene expression in Streptococcus mutans single-species biofilms

To evaluate the L. plantarum supernatant mediated inhibition of S. mutans gtf genes, quantitative reverse transcription polymerase chain reaction (qPCR) was performed as described in chapter 3 (section 3.1.8). 16sRNA was used as housekeeping gene. In single-species biofilm, qPCR analysis demonstrated that the probiotic supernatant significantly down-regulated the expression of the genes associated with glucosyltransferase activity of S. mutans. Hence, the probiotic supernatant down-regulated the gtfB, gtfC, and gtfD gene expression by 48.8%, 44.7%, and 65.7% respectively, compared to the control groups without treatment (Figure 14, p < 0.05). The histogram highlights the relative expression fold change of treated group with respect to the control group. Relative fold change compared to control and p values are summarized in Table 7.

Figure 14: Effect of L. plantarum supernatant on the expression of gtf genes in S. mutans single-species biofilms.
4.6.2  *L. plantarum* supernatant down-regulated the *gtf* gene expression of *S. mutans* in mixed-species biofilms

The efficacy of supernatant against the expression of *S. mutans* *gtf* genes in *S. mutans* and *C. albicans* mixed-species biofilms was further investigated. Interestingly, the expression of *gtf* genes in mixed-species biofilms were also significantly down regulated when treated with probiotic supernatant. Expression of *gtfB*, *gtfC*, and *gtfD* were down-regulated by 21.8%, 29.3%, and 35.6% , respectively as compared to the untreated control groups (Figure 15, p < 0.05). Melt curves showed the absence of non-specific products in all amplification reactions. Relative expression fold change of treated group with respect to the control group was shown in the histogram. Relative fold change compared to control and p values are summarized in supplementary Table 7.

Gene expression results were found to be consistent with the inhibitory activity of probiotic supernatant as shown by biofilm quantification assays and confocal imaging.
Figure 15: Effect of *L. plantarum* supernatant on the expression of *gtf* genes in *S. mutans* and *C. albicans* mixed-species biofilms.

![Graph showing expression of gtf genes](image)

Table 7: *S. mutans* *gtf* gene expression after incubation with *L. plantarum* supernatant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Single species</th>
<th>Mixed species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>Treated with supernatant</td>
</tr>
<tr>
<td></td>
<td>Fold change</td>
<td>Fold Change</td>
</tr>
<tr>
<td><em>gtfB</em></td>
<td>1.00</td>
<td>0.512</td>
</tr>
<tr>
<td><em>gtfC</em></td>
<td>1.00</td>
<td>0.553</td>
</tr>
<tr>
<td><em>gtfD</em></td>
<td>1.00</td>
<td>0.343</td>
</tr>
</tbody>
</table>
4.6.3 *L. plantarum* supernatant down-regulated the expression of *HWP1*, *ALS1* and *ALS3* genes in *C. albicans* single-species biofilm

For further validation of antifungal efficacy of *L. plantarum* supernatant against *C. albicans* biofilm, expression of *HWP1*, *ALS1* and *ALS3* genes in the presence of supernatant were analyzed. qPCR was conducted for treated and untreated samples where *PMA1* was used as an internal control as described in chapter 3 (section 3.1.8). The expressions of *HWP1*, *ALS1* and *ALS3* genes in the *C. albicans* single-species biofilms treated with *L. plantarum* supernatant were strongly down-regulated with respect to the untreated control groups. *HWP1*, *ALS1* and *ALS3* genes were down-regulated by 84.3%, 84.4%, and 72.9%, respectively (Figure 16, p < 0.05). The histogram highlights the relative expression fold change of treated group with respect to the control groups. Relative fold change compared to control and p values are summarized in Table 8.

**Figure 16:** Effect of *L. plantarum* supernatant on the expression of *C. albicans* hyphal growth associated genes in single-species biofilm.
4.6.4 *L. plantarum* supernatant down-regulated the expression of *C. albicans* *HWP1, ALS1* and *ALS3* genes in mixed-species biofilms

Efficacy of *L. plantarum* supernatant was further explored against *C. albicans* *HWP1, ALS1* and *ALS3* genes expression in *S. mutans* and *C. albicans* mixed-species biofilms. qPCR was performed for the analysis of *HWP1, ALS1* and *ALS3* gene expression in the presence of supernatant and *PMA1* was used as an internal control gene as described in chapter 3. Interestingly, gene expression of these hyphal growth associated genes were down-regulated when treated with supernatant. Down-regulation of *HWP1* (58.3%), *ALS1* (33.9%) and *ALS3* (39.1%) genes were observed when treated with probiotic supernatant (Figure 17, p < 0.05). Fold change relative to the control and p value are summarized in supplementary

**Table 8**

Gene expression results were found to be consistent with the inhibitory activity of probiotic supernatant as shown by biofilm quantification assays and confocal imaging.
Figure 17: Effect of *L. plantarum* supernatant on the expression of *C. albicans* hyphal growth associated genes in mixed-species biofilm.

Table 8: *C. albicans* *gtf* gene expression after incubation with *L. plantarum* supernatant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Single species</th>
<th>Mixed species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>Treated with supernatant</td>
</tr>
<tr>
<td></td>
<td>Fold change</td>
<td>Fold Change</td>
</tr>
<tr>
<td><em>HWPI</em></td>
<td>1.00</td>
<td>0.157</td>
</tr>
<tr>
<td><em>ALS1</em></td>
<td>1.00</td>
<td>0.156</td>
</tr>
<tr>
<td><em>ALS3</em></td>
<td>1.00</td>
<td>0.271</td>
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</table>
Chapter 5: Discussion and Conclusion

5.1 Discussion

Probiotic based strategies have shown promising results in the prevention of *S. mutans* and *C. albicans* single-species biofilms. The presented study observed that *L. plantarum* supernatant inhibits not only the initial colonization but also the preformed *S. mutans* and *C. albicans* single as well as mixed-species biofilms. *L. plantarum* supernatant was most effective during the early phase of biofilm formation and had comparatively reduced activity against the pre-formed biofilms. Numerous *in-vitro* studies have shown that different probiotic strains inhibit *S. mutans* (Lee and Kim, 2014; Saha et al., 2014) and *C. albicans* (James et al., 2016; Matsubara et al., 2016b) single-species biofilm formation. Clinical studies have also shown the inhibitory capacity of various probiotics against *S.mutans* (Ashwin et al., 2015; Toiviainen et al., 2015) and *C. albicans* (Kraft-Bodi et al., 2015; Sutula et al., 2013) in patients suffering from oral health problems.

The antimicrobial effect of the probiotic supernatant could be attributed to the presence of a mixture of antimicrobial peptides and other antimicrobial compounds in the secretome of *L. plantarum*. Previous studies have attempted to purify secretory component of *L. plantarum* LR/14 (Tiwari and Srivastava, 2008). The secreted low molecular weight compound was designated as plantaricin and was found to have strong bactericidal characteristics, heat stability and tolerance to acids (Tiwari and Srivastava, 2008). Subsequently, plantaricin was found to be proteinaceous in nature and exerted its antifungal activity by causing leakage of the intra-cellular contents leading to cell death (Sharma and Srivastava, 2013). Hence, it is likely that the *L. plantarum* supernatant used in the present study may also possess molecules with similar properties; however other mechanisms of action cannot be ruled out. For example, the nature of the interaction of *L. plantarum* supernatant with biofilms
might be physiochemical. It can be assumed that secretory component of *L. plantarum* supernatant might have altered the surface energies of the microorganisms and sabotaged the development of complex biofilms by preventing the microbial coaggregation (Ceresa et al., 2015).

Furthermore, as shown by CLSM images, *L. plantarum* supernatant may also have molecules that inhibit the adhesion of *S. mutans* and *C. albicans* to solid surfaces. This can be attributed to the biosurfactants and exometabolites of supernatant that account for reduction in the hydrophobicity of surface substratum by interfering with microbial adhesion and desorption process (Rodrigues et al., 2006). Similar findings have been reported in other studies wherein biosurfactants reduce the microbial adhesion to solid surfaces (Ceresa et al., 2015).

Eventhough the inhibitory effect of biosurfactants on bacterial adhesion and biofilm formation has been studied by several researchers, the specific mechanism of action of this behaviour has not yet been reported. The mechanism of action is believed to depend on property of target bacteria and the type of biosurfactant. Studies done by Walencka et al (2008) demonstrated that biosurfactant affects the bacterial adhesion by influencing the surface tension and charge of bacterial cell-wall (Walencka et al., 2008). These factors enhance the electrostaic repulsion between the bacterial cell surface and its substrate. So, the results indicate that *L. plantarum* derived biosurfactant may affect both cell to cell and cell to substrate intercation.

Production of glucans from *S. mutans* is regarded as a crucial virulence factor in the pathogenesis of dental caries. Interestingly, our findings revealed that *L. plantarum* supernatant significantly down-regulated all three *gtf* genes i.e. *gtfB*, *gtfC*, and *gtfD* in *S. mutans* single and mixed-species biofilms. Regulatory mechanisms of genes encoding Gtf enzymes in *S. mutans* were complex and have not been fully elucidated. However, it can be assumed that the active components in the probiotic
interfere with the gtf enzymes production at the gene expression level. As a result, it reduces the S. mutans attachment and biofilm formation. There are also other studies that demonstrate the ability of probiotic strains to down regulate gtf genes in S. mutans (Lee and Kim, 2014).

ALS3 and ALS1 genes belong to the ALS family of adhesins which are generally over expressed during in vitro adhesion of C. albicans to the epithelial cells (Sudbery, 2011). The HWP1 is known to encode the C. albicans protein which is responsible for maintenance of cell wall integrity, hyphal development and intracellular signaling (Sudbery, 2011). HWP1 and ALS3 mutants of C. albicans are defective in biofilm formation (Nobile et al., 2006a; Nobile et al., 2006b). Recently, we demonstrated that S. mutans derived gtfB is able to up regulate the expression of these genes of C. albicans in mixed-species biofilms (Ellepola et al., 2017). Interestingly, in this study, we found that probiotic supernatant significantly down regulated the expression of HWP1, ALS1 and ALS3 of C. albicans in single and mixed-species biofilms. Our results corroborated previous studies that reported down regulation of these genes after treatment of C. albicans with probiotic strains (Bandara et al., 2013; James et al., 2016). Foregoing findings explain the inhibitory effect of L. plantarum supernatant on C. albicans single and mixed-species biofilms.

5.2 Conclusion

In this study, efficacy of L. plantarum supernatant was investigated against S. mutans and C. albicans in single and mixed species biofilms. Biofilm quantification assays and confocal microscopy demonstrated that supernatant was not only able to inhibit the initial colonization of foregoing microorganisms, but also inhibited the preformed biofilms. Furthermore, glucan synthesis by S. mutans and yeast - hypha transition in C. albicans in the presence of L. plantarum supernatant were examined
at genetic level. The results showed a noticeable down regulation of the concerned genes. Altogether, the data demonstrated that L. plantarum supernatant was able to inhibit the S. mutans and C. albicans mixed-species biofilms. Therefore, this study provides a new insight on potential of probiotic-based strategies for the bacterial-fungal mixed-species biofilms.

5.3 Limitations

The scope of this study is limited to demonstrating the inhibitory effect of L. plantarum supernatant on the S. mutans and C. albicans single and mixed species biofilms. However, dental plaque is known to comprise of several other microorganisms that interact with host surface leading to biofilm formation. The explorations done in this thesis focus on the interaction of S. mutans and C. albicans and does not cover possible interactions between other oral microorganism like Streptococcus sobrinus and C. albicans. S. sorbinus bacteria is known to be associated with childhood caries in the oral cavity (Conrads et al., 2014).

5.4 Future work

The investigation of antimicrobial mechanism of active component/s in the L. plantarum supernatant at the molecular level remains to be explored and would be an interesting future study.

The results in this study establish the inhibitory activity of L. plantarum supernatant alone. The study of synergistic effect of L. plantarum supernatant with antifungal/ anti-bacterial drugs would be one area of exploration in future.
Another recommended future study would be evaluate the long term clinical trials of *L. plantarum* supernatant in the early childhood caries affected population.
# APPENDIX

1. List of culture media and materials used for various experimental stages

**Table 9: Materials used for various experiments**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Culture media and chemical used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbial cultivation</td>
<td>Brain heart infusion (BHI) agar- <em>Streptococcus mutans</em></td>
</tr>
<tr>
<td></td>
<td>Glucose minimal medium (GMM) agar- <em>Candida albicans</em></td>
</tr>
<tr>
<td></td>
<td>de Man, Rogosa and Sharpe (MRS) agar- <em>Lactobacillus plantarum</em></td>
</tr>
<tr>
<td>Biofilm formation</td>
<td>UFTYE: Yeast extract (2.5% w/v) + Tryptone (1.5% w/v)</td>
</tr>
<tr>
<td></td>
<td>UFTYE pH 7+1%glucose- for <em>S. mutans</em> inoculation</td>
</tr>
<tr>
<td></td>
<td>UFTYE pH 5.5+1%glucose- for <em>C. albicans</em> inoculation</td>
</tr>
<tr>
<td></td>
<td>MRS - for <em>L. plantarum</em> inoculation</td>
</tr>
<tr>
<td></td>
<td>UFTYE pH 7+2%sucrose- growth media</td>
</tr>
<tr>
<td></td>
<td>Phosphate buffered saline (PBS)</td>
</tr>
<tr>
<td>pH adjustment</td>
<td>NaOH</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
</tr>
<tr>
<td>Crystal violet assay</td>
<td>Crystal violet (CV)</td>
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<tr>
<td></td>
<td>2% Formalin</td>
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<tr>
<td></td>
<td>95% Ethanol</td>
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<td>XTT assay</td>
<td>XTT sodium salt</td>
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<td></td>
<td>Menadione</td>
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<td>Colony forming unit (CFU/ml) counting</td>
<td>Gentamicin sulfate salt -to prevent bacterial growth</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B - to prevent fungal growth</td>
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<tr>
<td>Confocal laser scanning microscopy</td>
<td>4% (v/v) Paraformaldehyde</td>
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<tr>
<td></td>
<td>Calcofluor white (Sigma - Aldrich) (excitation/emission 365/435 nm)</td>
</tr>
<tr>
<td></td>
<td>Propidium iodide (Live/Dead BacLight Bacterial Viability kit) (excitation/emission 535/617 nm)</td>
</tr>
<tr>
<td>Experiment</td>
<td>Material used</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>---------------------------------------------------</td>
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<tr>
<td>Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) Analysis</td>
<td>Trizol (Invitrogen life Tech)</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
</tr>
<tr>
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<td>Isopropanol</td>
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<td>1- RNA Extraction</td>
<td>75% Ethanol</td>
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<td>Nuclease free water (Hypure TM Molecular Biology)</td>
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<td>2- Gene expression analysis</td>
<td><strong>cDNA synthesis</strong></td>
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<tr>
<td></td>
<td>dNTP (10mM)</td>
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<td>OligodT(50mM)</td>
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<tr>
<td></td>
<td>M-MLV 5X Reaction buffer</td>
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<td>RNase inhibitor</td>
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<tr>
<td></td>
<td>M-MLV Reverse transcriptase</td>
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<tr>
<td><strong>PCR</strong></td>
<td>Sybr green + Rox high (Kappa biosystems)</td>
</tr>
<tr>
<td></td>
<td><em>C. albicans</em> and <em>S. mutans</em>primers</td>
</tr>
</tbody>
</table>

2. **Equipment used**

A list of laboratory equipment used in this study are mentioned below.

- Centrifuge
- Orbital shaker incubator
- Spectrophotometer- (Division)
- Plate reader- (Multiskan™ GO, Thermo Scientific)
- Olympus-Fluoview FV1000 TIRF confocal microscope
- NanoDrop ND 1000 spectrophotometer (Thermo Scientific)
- One -step real-time PCR detection system (Applied biosystems)
- pH meter
REFERENCES


Taniguchi, M., Nakazawa, H., Takeda, O., Kaneko, T., Hoshino, K., and Tanaka, T. (1998). Production of a mixture of antimicrobial organic acids from lactose by co-culture of *Bifidobacterium longum* and *Propionibacterium*


