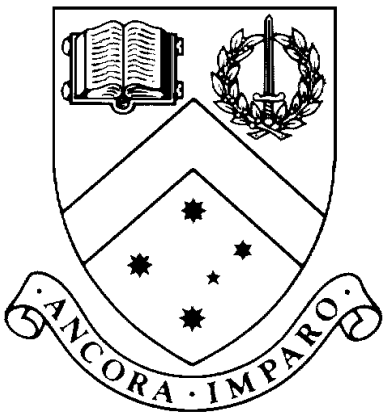

Monash University

Modulation of Attachment of Oral Bacteria to Surfaces by Tea Extracts and Mathematical Modeling of the Physicochemical Process of Bacterial Attachment

Yi Wang

A thesis submitted for the degree of Doctor of Philosophy

at Monash University in June 2013

Faculty of Science

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This thesis includes two original papers published in peer reviewed journals and five unpublished publications. The core theme of the thesis is Modulation of Attachment of Oral Bacteria to Surfaces by Tea Extracts and Mathematical Modeling for Bacterial Attachment. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Science under the supervision of Professor Gary Dykes.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 1, Chapter 2a, Chapter 2b, Chapter 2c, Chapter 3, Chapter 4, Chapter 5b my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
Chapter 1	The physicochemical process of bacterial attachment to abiotic surfaces: challenges for mechanistic studies, predictability and the development of control strategies.	Submitted	I was responsible for 90% of drafting and writing.
Chapter 2a	The inhibitory effect of tea on attachment of oral Streptococci to hydroxyapatite and other abiotic surfaces is associated with a reduction in bacterial surface hydrophobicity.	Submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 2b	Growth in the presence of sucrose may decrease attachment of some oral bacteria to abiotic surfaces.	Submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 2c	Inhibitory effects of saliva as a suspending fluid on attachment of oral bacteria to hydroxyapatite.	Submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 3	Inhibition of attachment of oral bacteria to immortalized human gingival fibroblasts (HGF-1) by tea extracts and tea components.	Published	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 4	Potential mechanisms for the effects of tea extracts on the attachment, biofilm formation and cell size of *Streptococcus mutans*.	Published	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 5b	A mathematical approach for modeling the physical process of bacterial attachment to abiotic surfaces.	Submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: …… ……

June 12, 2013

Date: ……

Additional Publications by the Author Relevant to the Thesis but not Forming Part of it

Tan, M.S.F., Wang, Y. and Dykes, G.A. (In press) Attachment of *Salmonella enterica* and *Listeria monocytogenes* to a bacterial cellulose derived plant cell wall model. *Foodborne Pathogens and Disease* DOI: 10.1089/fpd.2013.1536.

Teh, A.H.T., Wang, Y. and Dykes, G.A. (Submitted) The influence of antibiotic resistance gene carriage on biofilm formation by two *Escherichia coli* strains associated with urinary tract infections. *Canadian Journal of Microbiology*.

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Abstract

The effects of five tea extracts (green, oolong, black, pu-erh and chrysanthemum) on five oral bacterial strains (*Streptococcus mutans* ATCC 25175, *Streptococcus mutans* ATCC 35668, *Streptococcus salivarius* ATCC 13419, *Streptococcus mitis* ATCC 49456 and *Actinomyces naeslundii* ATCC 51655) were investigated with respect to cell surface properties (hydrophobicity, charge and auto-aggregation), attachment to, detachment from and biofilm formation on three hard surfaces (glass, stainless steel and hydroxyapatite). The effects of the tea extracts on the attachment of the oral strains to cultured human gingival fibroblasts were also determined. The influences of saliva and sucrose on the cell surface properties, attachment and biofilm formation of the bacterial strains were examined. The mechanism of the inhibition of attachment and biofilm formation by tea was studied. The extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theory was used to calculate the energy of interactions in the attachment systems and to predict attachment. A new mathematical approach consisting of an empirical model and a probability distribution model was developed to study the predictability and stochasticity of bacterial attachment.

The results indicated that the tea extracts and tea compounds could alter cell surface properties of the strains. Oolong tea extract was found to inhibit the attachment of all strains to the hard surface most effectively by 0.2 to 2.2 log CFU/cm^2 while pu-erh tea extract was found to inhibit the biofilm formation by the Streptococcal strains on all surfaces most effectively by 0.5 to 2.6 log CFU/cm^2. The mechanism of the inhibitory effects of oolong tea and pu-erh tea on the attachment and biofilm formation by *Streptococcus mutans* was found to be the coating of cell surfaces by tea components including flavonoids, tannins and indolic compounds. Pu-erh tea and chrysanthemum tea extracts were found to remarkably inhibit the attachment of the Streptococcal strains to cultured human gingival fibroblasts by up to 4 log CFU/well. Saliva as a suspending fluid was found to reduce the attachment of the Streptococcal strains to hydroxyapatite by 1.4 to 1.7 log CFU/cm^2. Sucrose was found to affect cell surface properties and the

attachment of the strains (either increase or decrease) and enhance biofilm formation by four strains on all surfaces by 0.4 to 1 log CFU/cm^2.

Positive correlations were found between cell surface hydrophobicity of the Streptococcal strains and their attachment to all surfaces with R^2s ranging from 0.37 to 0.91. The XDLVO theory indicated that hydrogen bonding energy dominated the overall interaction energy in all cases. This suggests that the coating by tea components blocked the hydrogen bonding sites on the cell surfaces, which reduced cell surface hydrophobicity and in turn inhibited attachment.

XDLVO failed to predict the experimental results of the attachment assays while the new modeling approach could however effectively predict in both deterministic and probabilistic ways. The empirical model (R^2=0.814) revealed that hydrophobic interaction is the most important parameter in a bacterial attachment system and established a range for each cell/substratum properties within which the resultant attachment is stochastic and unpredictable.

Keywords

Bacterial attachment, biofilm, oral bacteria, tea, cell surface hydrophobicity, XDLVO, mathematical modeling, probability distribution.

List of Abbreviations

AB	Lewis acid-base
AFM	Atomic force microscopy / Atomic force microscope
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ASB	Aluminum sec-butoxide
ATCC	American type culture collection
BATH	Bacterial attachment to hydrocarbon
BMA	Butyl methacrylate
CAM	Contact angle measurement
CFM	Chemical force microscopy
CFU	Colony forming unit
CSH	Cell surface hydrophobicity
EAcAc	Ethyl acetoacetate
EDMA	Ethyl dimethacrylate
EOS	Electro-osmotic stream
DLVO	Derjaguin-Landau-Verwey-Overbeek
DMEM	Dulbecco's modified Eagle's medium
EGCg	Epigallocatechin gallate
EL	Electrostatic
FBS	Fetal bovine serum
Fn	Fibronectin
GAE	Gallic acid equivalent
GTF	Glycosyltrasferase
HA	Hydroxyapatite
HGF-1	Human gingival fibroblast-1
HIC	Hydrophobic interaction chromatography
HPLC	High-performance liquid chromatography
LW	Lifshitz-van der Waals

MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MSA	Mitis salivarius agar
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate buffered saline
PVPP	Poly(vinylpolypyrrolidone)
QE	Quercetin equivalent
RMS	Root mean square
SAT	Salting-out aggregation test
SD	Standard deviation
SEM	Scanning electron microscopy
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
WapA	Wall-associated protein A
XDLVO	Extended Derjaguin-Landau-Verwey-Overbeek
ZP	Zeta potential

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Chapter 1

Literature Review

The review presented in this chapter has been partly submitted for peer review:

Wang, Y., Lee, S.M. and Dyke, G.A. (Submitted) The physicochemical process of bacterial attachment to abiotic surfaces: challenges for mechanistic studies, predictability and the development of control strategies. *Critical Reviews in Microbiology*.

Declaration for Thesis Chapter 1

Declaration by candidate

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was responsible for 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Sui Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidate's Signature	[redacted]	**Date** June 12, 2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University, Sunwasy Campus**

Signature 1	[redacted]	**Date** June 14, 2013
Signature 2	[redacted]	**Date** June 13, 2013

1.1 Introduction

The attachment of oral bacteria to dental surfaces is the first step in the formation of dental caries and other oral diseases (Kaufman and Kleinberg, 1973). Attachment initiates biofilm formation by oral bacteria on dental surfaces, dental plaque formation and eventually causes tooth decay and compromises oral health (Kaufman and Kleinberg, 1973; Hamilton-Miller, 2001).

Tea is a popular drink generally recognized to be of benefit to oral health with respect to its ability to kill oral bacteria (Yoshino et al., 1996; Rasheed and Haider, 1998; Hamilton-Miller, 2001). Tea has also been suggested to be a potential inhibitor of attachment to surfaces of oral bacteria (Hamilton-Miller, 2001) but research in this area is limited.

In addition to oral health, bacterial attachment to surfaces is of concern in many other spheres associated with human activities and health (Goulter et al., 2009). Studying the mechanisms of bacterial attachment to surfaces is therefore crucial for controlling it and improving health. The mechanisms of bacterial attachment to surfaces in general have been widely studied but are not well understood due to their complexity (Warning and Datta, 2013). As a result bacterial attachment is difficult to be effectively predicted (Bos et al., 1999). In addition, based on our current understanding of the mechanisms of attachment it is not clear what role stochasticicty plays in this process (Nguyen et al., 2011a).

1.2 Oral bacteria and dental caries

Dental caries are one of the most common chronic infectious oral diseases in the world (Aas et al., 2008). Epidemiological studies of virulence factors for dental caries have focused on the behavior of oral bacteria in the oral cavity (Kolenbrander and London,

1993). Three etiological hypothesis are usually used to explain dental caries: 1. The specific plaque hypothesis, which suggests that only a few specific species of bacteria (mainly S*treptococcus*) are actively involved in dental caries (Loesche, 1992); 2. The non-specific plaque hypothesis which claims that the entire plaque micro-flora are responsible for caries (Theilade, 1986); and 3. The ecological plaque hypothesis which proposes that caries are a result of a shift in the balance of the resident microbiota driven by changes in local environmental conditions (Marsh, 1994).

Although the etiology is arguable several α-hemolytic mutans and non-mutans groups of oral Streptococci (e.g. *Streptococcus mutans*, *Streptococcus salivarius* and *Streptococcus mitis*), as well as other primary colonizers of tooth surfaces (such as *Actinomyces naeslundii*), have been implicated as the primary and major agents that are responsible to dental caries (Hamilton-Miller, 2001). They produce glycosyltrasferase (GTF) that hydrolyzes sucrose to glucose and fructose and polymerize glucose into an adherent water-insoluble polymer glucan. Glucan builds up dental plaque that acts as a matrix housing over 300 types of microorganisms. These microorganisms are low-pH tolerant and can ferment carbohydrate generating acids that demineralize tooth enamel, eventually causing caries. Fructose can also be used by oral bacteria causing an accumulation of lactic acid around the enamel surface and eventually resulting in demineralization and tooth decay (Kaufman and Kleinberg, 1973).

The pathogenesis of dental caries may therefore involve three processes: 1. Attachment of oral bacteria to elements of the oral cavity, such as the enamel, tongue, saliva or gum; 2. Formation and accumulation of dental plaque due to the synthesis of glucan by GTF; and 3. Biofilm formation by oral bacteria that are able to metabolize carbohydrates at a low pH and demineralize enamel (Kaufman and Kleinberg, 1973; Kolenbrander and London, 1993; Hamilton-Miller, 2001).

1.3 Tea (Camellia sinensis)

Natural products are extensively used by humans in many biological systems due to their wide array of bioactivities. Tea is generally agreed to have a range of bioactivities in both microbiological and cell biology systems (Baer and Chen, 2005) and is suggested to benefit human health with respect to a variety of diseases such as heart disease, bone disease, obesity and oral diseases (Hara, 2001).

Tea is the second most popular drink in the world (after water) and is grown in approximately 30 countries but is consumed worldwide and particularly in China, Japan, India and the UK. It is produced from the processed leaves of *Camellia sinensis* by fermentation and into different products including green tea (non-fermented), oolong tea (partially fermented), black tea (fully fermented) and pu-erh tea (post-fermented) (Peterson et al., 2005). The so-called fermentation is in fact an enzymatic conversion of tea polyphenols (mainly epicatechins) from monomers to oligomers (theaflavins) or polymers (thearubigins) by leaf enzymes (e.g. polyphenoloxidase and peroxidase) (Engelhardt, 2010).

Tea contains a variety of chemical compounds including vitamins, minerals, caffeine, amino acids and phenolic compounds (Engelhardt, 2010). Epigallocatechin gallate (EGCg), for example, is generally recognized as the 'signature' compound of tea flavonoids as it constitutes approximately 10% of the dry weight of green tea (Peterson et al., 2005). It is established that EGCg has strong activities in a wide variety of *in vitro* and *in vivo* biological systems (Friedman, 2007). This compound has therefore been suggested to be a major source of bioactivities of tea (Friedman, 2007). Another phenolic compound, gallic acid, is an important precursor of many tea flavonoids, a blood metabolite of tea components and makes up approximately 0.5% dry weight of black tea (Cabrera et al., 2003). Gallic acid has also been reported to possess a range of bioactivities, such as its antimicrobial activity against *Campylobacter* and *Salmonella* strains (Friedman et al., 2003; Nohynek et al., 2006).

1.4 Tea and dental health

Many *in vitro* studies have indicated that tea exhibits strong beneficial effects on oral health. Its beneficial effects in the mouth encompass anti-cariogenic activities (Otake et al., 1991) including direct bactericidal effects against oral bacteria (Rasheed and Haider, 1998), inhibition of bacterial adhesion to tooth surfaces (Ferrazzano et al., 2011), inhibition of glycosyltransferase activity and the associated biosynthesis of dental plaque (Hada et al., 1989) and inhibition of human and bacterial amylase activity which limits available sugar (Zhang and Kashket, 2000). These beneficial effects have been investigated using animal studies (Ooshima et al., 2000) but have rarely been investigated in human clinical trials (Hamilton-Miller, 2001). A limited number of studies have been conducted on the bacterial attachment inhibitory effects of tea (Otake et al., 1991; Matsumoto et al., 1999) but the mechanism behind this inhibitory effect has yet to be investigated.

1.5 Bacterial attachment

Bacterial attachment to a surface is the initial step in microbial colonization and biofouling (Bos et al., 1999). It directly or indirectly results in the formation of biofilms and is of concern in many areas including medicine (Goulter et al., 2009), food (Rivas et al., 2007; Warning and Datta, 2013) and engineering (Li and Logan, 2004). Understanding the mechanisms of bacterial attachment, and controlling and minimizing it is therefore important. The mechanical process of attachment has been extensively studied but is still not well understood (Goulter et al., 2009; Warning and Datta, 2013).

Bacterial attachment can be affected by physicochemical factors and interactions within the systems as well as by biological factors associated with bacterial cells (Bos et al., 1999; Goulter et al., 2009). Research on bacterial attachment has focused on the

physicochemical aspect of the process, such as thermodynamic and electrostatic interactions (van Oss, 1989; Hermansson, 1999). Mechanistic models have been developed based on these interactions and have been used in an attempt to explain the physical process of bacterial attachment (Hermansson, 1999). Due to the complexity of bacterial attachment and the limitations of those models, however, they often do not give robust explanations to the processes involved (Warning and Datta, 2013).

Development of mechanistic models strongly relies on an understanding of the interactions involved in attachment and the methodologies used to study these (Bos et al., 1999). Existing methodologies are diverse and well developed but are not standardized and often fail to control for many factors that have a significant influence. These methodologies therefore fail to eliminate the effect of interference and noise (Dillon et al., 1986; Wilson et al., 2001; Goulter et al., 2009).

Control strategies for bacterial attachment have been developed based on studies of attachment interactions and on also on the mechanistic models derived from these (Hori and Matsumoto, 2010). A number of methods used to control bacterial attachment are successful in minimizing biofouling. The limitations of these methods include, for example, effects which are bacterial species/strain-dependent and easily compromised by environmental factors (Hori and Matsumoto, 2010).

In this review the specific physicochemical interactions and factors in bacterial attachment systems are outlined, the value of the generally recognized mechanistic models are discussed, frequently used methods to study bacterial attachment interactions are compared and attachment control strategies are summarized. This review describes, using examples, the issues in studying bacterial attachment using existing methods, the difficulties in developing mechanistic models to predict bacterial attachment based on these methods and the challenges in developing strategies to control bacterial attachment based on the mechanisms of bacterial attachment as

currently understood.

1.6 Specific interactions and factors affecting bacterial attachment

Interactions and factors involved in bacterial attachment systems are diverse and complex. In this section, the impacts of hydrophobic interaction, electrostatic interaction and substratum surface roughness on attachment of bacteria to abiotic surfaces are discussed and summarized (Figure 1.1).

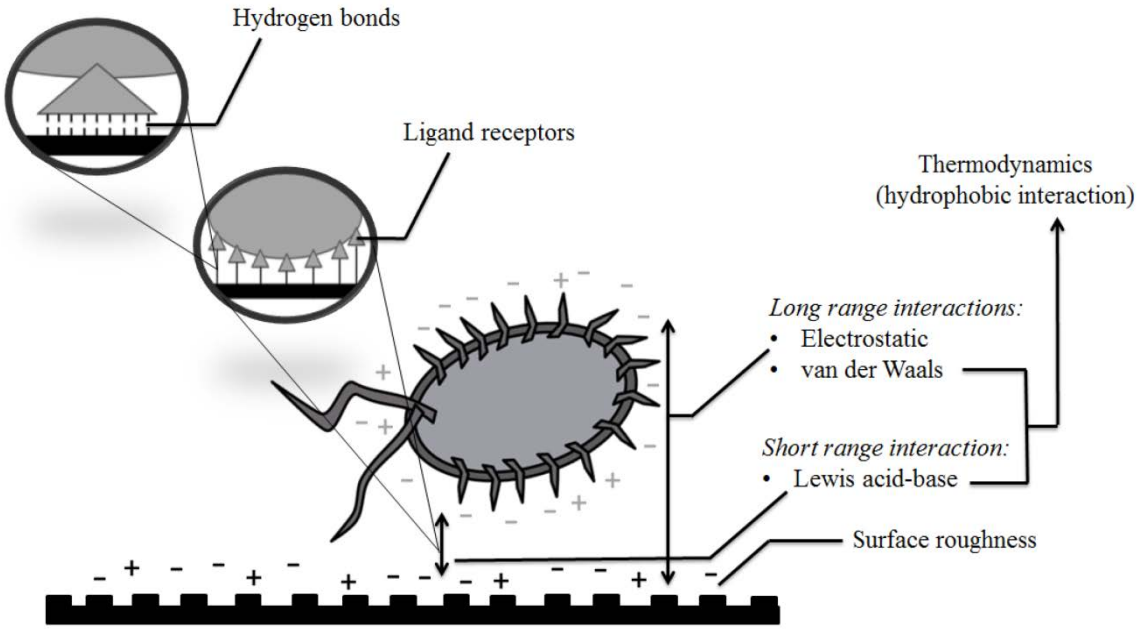


Figure 1.1 Physicochemical interactions/factors in systems of bacterial attachment to abiotic surfaces.

1.6.1 Hydrophobic interactions

Hydrophobic interactions that mediate thermodynamic forces occur between bacterial cells, substratum surfaces and the liquid suspension medium (van Oss, 1989). These interactions ($\Delta G^{Hydrophobic}$) consist of long range Lifshitz-van der Waals (LW) forces (ΔG^{LW}) and short range Lewis Acid-base forces (AB) the latter of which is specifically

dominated by hydrogen bonding (ΔG^{AB}) (Figure 1.1). These interactions and can be calculated according to Bos et al. (1999):

$$\Delta G^{Hydrophobic} = \Delta G^{LW} + \Delta G^{AB} = \gamma_{bs} - \gamma_{bl} - \gamma_{sl} = -2\left(\sqrt{\gamma_b^{LW}} - \sqrt{\gamma_l^{LW}}\right)\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_l^{LW}}\right) + 2\left[\left(\sqrt{\gamma_b^+} - \sqrt{\gamma_s^+}\right)\left(\sqrt{\gamma_b^-} - \sqrt{\gamma_s^-}\right) - \left(\sqrt{\gamma_b^+} - \sqrt{\gamma_l^+}\right)\left(\sqrt{\gamma_b^-} - \sqrt{\gamma_l^-}\right) - \left(\sqrt{\gamma_s^+} - \sqrt{\gamma_l^+}\right)\left(\sqrt{\gamma_s^-} - \sqrt{\gamma_l^-}\right)\right] \quad (1.1)$$

where γ^{LW} is the LW component of surface tension, $\gamma+$ is the electron acceptor parameter and $\gamma-$ is the electron donor parameter of AB component of the surface tension. The subscript b, s and l refer to bacteria, hard surface and liquid medium, respectively.

On the surface of a bacterial cell there are a number of ligand receptor sites (e.g. ~12 for *Escherichia coli* and ~60 for *Streptococcus*), on each of which multiple hydrogen bonds can form with the substratum surface (Chen et al., 2011) (Figure 1.1). In many cases the energy of a single short range bond is comparable or even higher than the total long range forces (Chen et al., 2011). The overall interaction in a bacterial attachment system is therefore often dominated by the short range hydrogen bonding energy.

Hydrophobic interactions can be affected by bacterial cell surface hydrophobicity [which is a result of the presence of residues and structures on the cell surfaces (van der Mei et al., 1991)], substratum surface hydrophobicity [which is a result of the chemical composition of the material and affected by the surface roughness (Quirynen et al., 1990)], and the surface tension of the liquid medium (van Oss et al., 1986). Some studies investigating the relationship between cell surface hydrophobicity and bacterial attachment to surfaces have found positive correlations between them (Zita and Hermansson, 1997; Rad et al., 1998; Liu et al., 2004; Norhana et al., 2009; Hui and Dykes, 2012). Investigations of the correlation between substratum surface hydrophobicity and bacterial attachment have also shown positive correlations (Li and Logan, 2004; Boks et al., 2008; Salerno et al., 2009). Many other authors, however,

have found a lack of correlation between bacterial/substratum hydrophobicity and attachment (Li and McLandsborough, 1999; Hassan and Frank, 2004; Li and Logan, 2004; Rivas et al., 2007). A possible reason for the apparent lack of correlation between cell/substratum surface hydrophobicity and attachment could be that the bacterial model used in many of these studies was *Eschericia coli* which is regarded as highly hydrophilic (Li and Mclandsborough, 1999; Rivas et al., 2005). The impact of hydrophobic interactions on attachment in the system may therefore have been too weak to be observed. An alternative reason could be that the difference in hydrophobicity between the bacteria or between the substratum surfaces used in these studies were small, and therefore did not result in significantly different numbers of bacteria attaching. We suggest that the bacteria or substratum models used to study the impact of hydrophobic interactions on bacterial attachment should use systems with larger differences in hydrophobicity. This should in turn result in significantly different and measurable numbers of bacteria attaching. In addition, the range of hydrophobicities within which attachment cannot be differentiated should be determined in future studies in order to gain a fundamental understanding of the impact of hydrophobic interactions in these systems.

1.6.2 Electrostatic interactions

Electrostatic (EL) interactions are a long range force between bacterial cells and the substratum surface (Chen et al., 2011) (Figure 1.1). These interactions can be affected by cell surface charge (ξ_b) [which is dependent on the presence of an excess of carboxyl and phosphate groups located on the cell surfaces (Goulter et al., 2009)], substratum surface charge (ξ_s) [which is dependent on the physicochemical nature of the material (Li and Logan, 2004)], and the ionic strength of the liquid medium (Bos et al., 1999). Electrostatic interaction energies (ΔG^{EL}) over the separation distance (l) between bacterial cells and the substratum surface can be calculated as described by Bos et al. (1999):

$$\Delta G^{EL} = \pi\varepsilon_0\varepsilon r\left(\xi_b^2 + \xi_s^2\right)\left\{\frac{2\xi_b\xi_s}{\xi_b^2 + \xi_s^2} ln\left[\frac{1 + exp(-\kappa l)}{1 - exp(-\kappa l)}\right] + ln\left[1 - exp(-2\kappa l)\right]\right\} \quad (1.2)$$

where ε_0 denotes the dielectric permittivity of vacuum and ε denotes the relative permittivity of the liquid medium, r is the radius of the cells and κ is the inverse Debye layer thickness (which is directly proportional to the ionic strength of the medium).

Studies on the relationship between electrostatic interactions and bacterial attachment have shown both positive correlation and an absence correlation. Ukuku and Fett (2002) and Norhana et al. (2009), for example, found a positive correlation between the surface charge of a variety of food-borne pathogens and the their ability to attach to food contact surfaces. In the majority of studies investigating the role of charge in bacterial attachment, however, no correlation was found. For example, Rivas et al. (2007) found no correlation between surface charge of *Escherichia coli* strains and their attachment to stainless steel, while Boks et al. (2008) found no correlation between surface charge of six different bacteria and their attachment to dimethyldichlorosliane coated glass. Li and Logan (2004) and Salerno et al. (2004) also found no correlation between cell substratum surface charge and bacterial attachment, but they found a positive correlation between the ionic strength of the liquid medium and attachment. This suggests that electrostatic mediated attachment is more strongly attributable to the length of the electrostatic double layer rather than the intensity of the charge.
The reason that the role of electrostatic interaction in bacterial attachment has often been found to not be significant may be that bacterial cells are generally considered to be hard spherical particles whose surface electric potential is often approximated by the zeta potential [the potential located at the electrokinetic plane of shear (de Kerchove and Elimelech, 2004)]. This approximation is, however, accurate as bacterial cells carry a charged ion-permeable polymer layer on the cell surfaces which interferes with the electrostatic and electrokinetic interactions of bacterial cells (Ohshima and Kondo, 1991). These polymer layers consist mainly of lipopolysaccharides, proteins and cell surface appendages (e.g. pili and fimbriae) (Madigan et al., 2003). Ohshima and Kondo

(1989) developed the Soft Particle Theory to calculate the surface electrostatics and electrokinetics of particles taking into account the presence of the ion-permeable layer. This theory has been used in an attempt to explain bacterial attachment but has failed in many cases. Poortinga et al. (2001) found that the attachment of *Streptococcus salivarius* and *Staphylococcus epidermidis* to glass was not dependent on the softness of bacterial cells, while de Kerchove and Elimelech (2005) found that the Soft Particle Theory could not explain the attachment of *Escherichia coli* strains to silica beads. The reason that the Soft Particle Theory fails to explain bacterial attachment in many cases may be that the cell surface softness parameters derived from the theory do not reflect the cell surface structure or morphology. In addition, the accuracy of the quantification of cell surface charge by the theory is highly dependent on the thickness of the ion-permeable layer as it is usually more accurate for cells with thick layers (Rodriguez et al., 2007).

Arguments with respect to the significance of electrostatic interactions in bacterial attachment have been raised (Kang and Choi, 2005; Goulter et al., 2009; Salerno et al., 2009; Warning and Datta, 2013) and some authors suggest its importance may be overrated (Poortinga et al., 2002). In addition, the influence of electrostatic interactions may only be significant when other interactions are relatively weak with, for example, electrostatic interactions having a greater contribution to attachment in systems containing hydrophilic but highly charged bacteria.

1.6.3 Substratum surface roughness

Surface roughness is a two-dimensional parameter of a substratum surface (Tang et al., 2007) and has been suggested to play a role in bacterial attachment (Quirynen et al., 1990; Medilanski et al., 2002; Tang et al., 2006; Wang et al., 2009; Gouter et al., 2011) (Figure 1.1). Czarnecki and Warszyński (1987) and Bhattacharjee et al. (1998) suggested that surface roughness significantly affects the surface interaction energy between a particle and a substratum surface, particularly at short separation distances.

The influence of surface roughness on bacterial attachment is still not clear (Tang et al., 2007). Some authors suggested that bacterial attachment is directly correlated with surface roughness. For example, Quirynen et al. (1993) found that oral bacteria tended to attach to rougher titanium surfaces. McAllister et al. (1993) reported that *Escherichia coli* attached better to rougher plastic surfaces. Bames et al. (1999) found that *Staphylococcus aureus* attached in lower numbers to smoother stainless steel. Faille et al. (2002) found a positive correlation between the roughness of inert surfaces and the attachment of *Escherichia coli* strains to those surfaces. Wang et al. (2002) reported a positive linear correlation between the roughness of fruit and metal surfaces, and the attachment of *Escherichia coli* O157:H7 strains. By contrast Goulter et al. (2011) reported that *Escherichia coli* strains attached better to smoother stainless steel surfaces. Still other authors suggest that roughness does not influence attachment. For example, An et al. (1995) found that surface roughness did not affect the attachment of Staphylococci to titanium surfaces. Oliveria et al. (2006) reported that the attachment of *Salmonella* Enteritidis was surface roughness-independent. Ortega et al. (2010) found no correlation between surface roughness and the attachment of *Escherichia coli* strains to stainless steel. Importantly, Medilanski et al. (2002) and Verran et al. (2010) reported an optimum roughness value for bacterial attachment exists below or above which the number of attached bacteria dropped proportionally. It is therefore necessary to include a larger range of roughness values in studies investigating the role of surface roughness in bacterial attachment. Medilanski et al. (2002) and Verran et al. (2010) also suggested that the optimum roughness value is dependent on the topographical feature of the hard surface and is determined by the shape and size of attaching bacterial cells. This suggests that the interactions between hard surface topography and cell morphology also play a role in attachment. In addition, roughness may also affect other substratum surface properties that influence bacterial attachment. For instance, roughness increases the effect of surface tension and affects surface hydrophobicity (Quirynen et al., 1990). The studies described above indicate that the contribution of roughness to attachment could be a complex process the role of which is not easily resolved.

1.7 Mechanistic models to explain the physicochemical process of bacterial attachment

1.7.1 Thermodynamic model

Mechanistic models based on physicochemical interactions in bacterial/substrate systems have been used in an attempt to explain the attachment process (Warning and Datta, 2013) (Table 1.1). The thermodynamic model, for example, has been used to predict the potential of bacterial attachment to a solid surface. It uses the free energy in the system as an indicator of the potential of attachment (i.e. a decreased thermodynamic energy indicates that the attachment process is favored in the system) (Absolom et al., 1983). The free energy consists of LW and AB interaction energies and therefore represents the hydrophobic interaction energy [see Equation (1.1) above] (van Oss et al., 1986). This model assumes that attachment processes are irreversible, which is usually not the case (Hori and Matsumoto, 2010). Absolom et al. (1983) reported that the thermodynamic model could predict the attachment of a range of bacteria to different abiotic polymeric surfaces. In many other cases, however, the model could not fully explain or failed to explain the process of attachment (Simões et al., 2007; Warning and Datta, 2013).

1.7.2 Derjaguin-Landau-Verwey-Overbeek model

Another mechanistic model that is often used to predict bacterial attachment is the Derjaguin-Landau-Verwey-Overbeek (DLVO) model, which calculates the overall interaction energy using LW and EL interaction energies as a function of the separation distance between a bacterial cell and the substratum surface. The total interaction energy is given by van Oss (1989) as:

$$\Delta G^{DLVO} = \Delta G^{LW} + \Delta G^{EL} \tag{1.3}$$

In this model, bacterial attachment is described as a two-step process including a reversible physical step and an irreversible cellular/molecular step depending on separation distance (An and Friedman, 1998). Based on the above parameters the DLVO energy profile is usually plotted over distance and displays two energy minima and an energy barrier (Hermansson, 1999). Marshall et al. (1971) were the first to use the DLVO model to describe bacterial attachment. They found that the DLVO theory could explain the differences in attachment of marine bacteria to glass surfaces resulting from different ionic strengths of the liquid medium. It has, however, been repeatedly demonstrated that the DLVO theory is not able to universally predict bacterial attachment (Hermansson, 1999). For example, it could not fully explain the attachment of 12 different bacterial species to glass and Teflon (Rijnaarts et al., 1995) or the attachment of *Pseudomonas* spp. to surfaces in a sand column (Simoni et al., 1998). In both these cases the authors assumed that hydrogen bonding between bacterial cell surfaces and the substratum played a role in the attachment process.

1.7.3 Extended Derjaguin-Landau-Verwey-Overbeek model

Van Oss developed the extended DLVO model (XDLVO) which takes hydrogen bonding energy (AB) and osmotic interaction (as a function of separation distance) into account (van Oss, 1989; van Oss, 1993). Since osmotic interaction energy is too low to be considered of consequence, the total XDLVO attachment energy is expressed as:

$$\Delta G^{XDLVO} = \Delta G^{LW} + \Delta G^{EL} + \Delta G^{AB} \qquad (1.4)$$

which is equivalent to Equation (1.1) + Equation (1.2).

Jucker et al. (1996) found that the attachment of *Stenotrophomonas maltophila* to glass, which was predicted by the classical DLVO model to attach in higher numbers as compared to *Pseudomonas putida*, exhibited the same ability to attach to this substrate as *Pseudomonas putida*. This difference was due to the contribution of AB interaction

and was explainable by the XDLVO model (Jucker et al., 1997). In many other cases, however, the XDLVO theory has failed to fully predict bacterial attachment. For example, the model could not predict the attachment of *Pseudomonas aeruginosa* strains to poly(ethylene oxide) brushes (Roosjen et al., 2006). Chia et al. (2011) investigated the predictability of the attachment of different *Salmonella enterica* serotypes to four abiotic surfaces by the XDLVO model. They reported that the model could only predict attachment in a limited number of combinations of bacteria and hard surfaces studied, but not all of them. Nguyen et al. (2011a) studied the attachment of *Campylobacter jejuni* strains and various *Salmonella* serotypes to stainless steel and used XDLVO model to predict their attachment. These authors found that the model could explain the attachment of *Campylobacter jejuni* but not *Salmonella*.

Although the XDLVO model cannot explain bacterial attachment in all cases, it has highlight the importance of hydrogen bonding in the process. The interaction energy of hydrogen bonding is usually higher than that of LW and EL interactions by a number of orders of magnitude (Roosjen et al., 2006; Chen et al., 2011; Nguyen et al., 2011a). This results in an extremely deep energy minimum without an energy barrier (Hermansson, 1999; Chia et al., 2011; Nguyen et al., 2011a).

A number of reasons can be forwarded as to why these mechanistic models cannot effectively explain bacterial attachment. In addition to the obvious fact that attachment processes are not purely physical, all these models assume perfectly smooth and uniform surfaces (Hermansson, 1999; Salerno et al., 2009) which in reality do not exist. In addition these models are strongly deterministic and do not take biological variability into account. Chen et al. (2011) attempted to use a Poisson distribution model to predict the probability of the formation of each hydrogen bond between a cell surface and a substratum, and the probability of a given number of hydrogen bonds forming between the same cell surface and a substratum. These authors demonstrated that the hydrogen bonding force distribution derived from a Poisson analysis can deterministically and

probabilistically interpret the AB interactions from the thermodynamic and the XDLVO models. This study indicated that using a probability distribution may be an effective way to take noise into consideration and to probabilistically explain bacterial attachment. Future development of mechanistic models for bacterial attachment should take surface roughness into account and consider the biological variability by using probability distribution models.

Table 1.1 Mechanistic models used to explain the physicochemical process of bacterial attachment.

Mechanistic models	Equations	References	Notes
Thermodynamic model	$\Delta G^{total} = \Delta G^{LW} + \Delta G^{AB}$	(Absolom et al., 1983)	The free energy comprises both LW and AB interaction energies and therefore represents the hydrophobic interaction energy of the system. The model does not take EL interaction into account.
DLVO model	$\Delta G^{DLVO} = \Delta G^{LW} + \Delta G^{EL}$	(An and Friedman, 1998)	Bacterial attachment is considered as a two-step process depending on separation distance. The energy profile displays two energy minima and an energy barrier. The model does not take AB interaction into account.
XDLVO model	$\Delta G^{XDLVO} = \Delta G^{LW} + \Delta G^{EL} + \Delta G^{AB}$	(van Oss, 1989)	This model takes AB interactions into account in addition to the other interactions in the DLVO model and highlights the importance of hydrogen bonding.

DLVO: Derjaguin-Landau-Verwey-Overbeek; XDLVO: extended Derjaguin-Landau-Verwey-Overbeek; LW: Lifshitz-van der Waals interactions; EL: electrostatic interactions; AB: Lewis acid-base interactions.

1.8 Methods to study bacterial attachment and interactions in a system

Various methods to measure bacterial attachment and cell/substratum surface properties have been developed and used. These methods measure the properties directly or indirectly and often measure bulk properties (population behavior) rather than the interactions of individual units within population. Common methods to study bacterial attachment are critically compared below and are summarized in Table 1.2.

1.8.1 Bacterial cell surface hydrophobicity

Bacterial cell surface hydrophobicity is an important parameter in bacterial attachment to surfaces. There is, however, no direct measurement for cell surface hydrophobicity available (van der Mei et al., 1987). The existing methods can only measure the interaction between cells and a hydrophobic/hydrophilic material as an index of hydrophobicity.

A common method for hydrophobicity measurement is the Bacterial Attachment to Hydrocarbon (BATH) method developed by Rosenberg et al. (1980). It determines the percentage of bacterial cells in an aqueous suspension migrating to a hydrophobic hydrocarbon phase spectrophotometrically. Using this method discrepancies can result if different types and volumes of hydrocarbon are used (Dillon et al., 1986), but the accuracy of the method can be improved by using three hydrocarbons each over a range of volumes (Rosenberg et al., 1983).

Contact angle measurement (CAM) is the technique most often used to determine the surface tension energy of bacterial cells for mechanistic models (Busscher et al., 1984; Chia et al., 2011; Nguyen et al., 2011b). It measures the contact angle of a sessile drop of water deposited on a lawn of bacterial cells. Hydrophobicity determinations using this method are highly dependent on the wetness of the bacterial lawn and are therefore

often highly variable (Nguyen et al., 2011a).

Hydrophobic interaction chromatography (HIC) is another technique to determine cell surface hydrophobicity by spectrophotometrically measuring the percentage of bacterial cells remaining in a hydrophobic column (Clark et al., 1985). This method is generally less reproducible than the other methods since a small hydrophobic fraction on the cell surface can result in a high affinity of the cells for the column (Dillon et al., 1986). This means that a small number of hydrophobic residues on the cell surface can result in a high hydrophobicity value.

Another approach used to determine hydrophobicity is the salting-out aggregation test (SAT) which determines the lowest salt concentration that can cause a visible aggregation of bacterial cells (Lindahl et al., 1981). Cellular aggregation can, however, be affected by cell surface charge (van der Mei et al., 1987) and cell concentration (Dillon et al., 1986).

The different methods described above define hydrophobicity in different ways and reliance on the results obtained from one method is inadequate (Dillon et al., 1986). Mozes and Rouxhet (1986) suggested that results obtained from a combination of different methods are more accurate.

1.8.2 Bacterial cell surface charge

The electrostatic charge density on a bacterial cell surface is usually expressed as zeta potential, which is defined as the difference between the electric potential of the cell surface double layer and the dispersion medium. Zeta potential of relatively small particles, such as bacterial cells, cannot be directly measured (Lytle et al., 1999) and it is therefore necessary to employ indirect means.

Microelectrophoresis is the most common method to determine bacterial cell surface

charge (Wilson et al., 2001). This method measures the velocity of the movement of bacterial cells in an electric field and this can be converted into a zeta potential value (van der Mei et al., 1997). The electric mobility of bacterial cells can be affected by temperature, pH and the ionic strength of the liquid medium. The drawback of this method is that it is usually time consuming (Pedersen, 1981).

Electrostatic interaction chromatography is another technique for cell surface charge measurement. It measures the retention time of bacterial cells in an ion-exchange resin column (Pedersen, 1981). It generates reproducible results but does not give direct or indirect measurements of zeta potential.

Aqueous two-phase partitioning is a less common method employed to determine bacterial cell surface charge. It uses a polyethylene glycol-dextran two phase system and allows cells to preferentially partition into either the more hydrophobic polyethylene glycol phase or the less hydrophobic dextran phase depending on their surface polarity, which is influenced by cell surface charge (Liang et al., 1993). The results yielded by his method are highly dependent on cell surface hydrophobicity and like electrostatic interaction chromatography cannot be converted into zeta potential.

Isoelectric equilibrium analysis was modified by Sherbet et al. (1972) for bacterial surface charge measurements. It determines the migration of bacterial cells over a pH gradient to their isoelectric positions. This method can generate zeta potential values but is extremely time consuming (Wilson et al., 2001).

Electrophoretic light scattering is a relatively new technique that utilizes the same basic principles as microelectrophoresis, but with a modified measurement method. It directly measures the movement of bacterial cells in an electric field by determining the frequency of change of the laser light they scatter and thereby gives the zeta potential values (Blake et al., 1994). This method has been shown to be an easy and rapid way to

measure cell surface charge (Blake et al., 1994; Morris et al., 1995; Li and McLandsborough, 1999).

The most commonly used technique nowadays is microelectrophoresis or its modified form of electrophoretic light scattering. Many laboratory instruments have been developed for this technique to reduce the time for experiments (Wilson et al., 2001).

1.8.3 Hard surface hydrophobicity

The most common method used to determine hard surface hydrophobicity is water contact angle measurements (Bos et al., 1999; Chia et al., 2011; Nguyen et al., 2011a). The results of this method can be used to calculate surface tension using Young's equation and it is the standard method for input into mechanistic models (Bos et al., 1999).

Ducker and Senden (1992) used atomic force microscopy (AFM) to measure the surface forces between colloidal particles and hard surfaces at a nanoscopic scale which were determined to represent hydrophobicity. In this method, an AFM cantilever is coated with standard colloidal particles before tapping on a sample surface. The force between the standard colloidal particles and the sample surface is measured as a function of the displacement of the cantilever. This method is, however, expensive and time consuming.

1.8.4 Hard surface charge

Hard surface charge is generally represented by zeta potential and is an important parameter to establish with respect to electrostatic interactions between bacteria and surfaces.

The electrostatic fieldmeter method is a traditional technique used to measure hard surface charge. This method uses an electrostatic probe to measure the voltage induced

by surface charge (Faircloth and Allen, 2003). Results obtained using this technique are dependent on the distance between the probe and the surface, and can be influenced by discharge between the probe and the surface (Seaver, 1995).

Another technique used to determine hard surface charge is the electrostatic voltmeter method. This method determines the voltage on a surface with the aid of a voltage amplifier which nullifies the electric field. This technique can overcome the influence of discharge and also the distance between the surface and the probe (Smith and Rungis, 1975).

Hard surface charge can also be measured using electrophoretic mobility (Ballona and Drewes, 2005). This method determines the mobility of reference colloidal particles in an electrolyte solution under an electric field generated by hard surface charge which is then converted into hard surface zeta potential.

Streaming potential measurement is also widely used to measure hard surface change. It determines the potential induced when an electrolyte solution flows across a stationary and charged surface (Childress and Elimelech, 1996).

In addition to the methods listed above, zeta potential can also be determined by other electrokinetic measurements such as sedimentation potential and electro-osmosis assays (Childress and Elimelech, 1996). Many laboratory instruments measuring hard surface charge have been developed based on these techniques.

1.8.5 Hard surface roughness

Surface roughness can be measured using a Perthometer or an AFM (Quirynen et al., 1990; Goulter et al., 2011). These instruments measure the average displacement of a cantilever mapping the surface (Goulter et al., 2011). Authors such as Medilanski et al. (2002) have also optically measured the average of the deviation in depth/height and

width of scratches on a surface from a digitized scanning electron micrograph obtained by an AFM to measure roughness.

1.8.6 Quantification of bacterial attachment

Quantification of bacterial attachment to a hard surface can be achieved in various ways. The measurements can be at a population level or at an individual cell level (Warning and Datta, 2013). It is still unclear whether the results of experiments conducted at the two different levels are similar. If they are it is reasonable to assume that bacterial attachment is not a random process as the population behavior can be reflected in the individual behavior. In this case bacterial attachment is non-stochastic and is therefore predictable by mechanistic models. Combining the two levels of measurement may give a more accurate measurement and a better understanding for bacterial attachment.

Quantification of bacterial attachment to slides of different materials is a frequently used method to determine attachment at a population level. In these methods, a slide of the substratum material is immersed in a bacterial suspension, with or without shaking, to allow attachment. Attached cells are enumerated by direct microscopic observation (Clark and Gibbons, 1977; Absolom et al., 1983; Hood and Zottola, 1997; Wang et al., 2013a) or by spread plating after detachment of the cells from the surface by stomaching or sonication (Morra et al., 1996; Faille et al., 2002; Cassat et al., 2007; Chia et al., 2011; Wang et al., 2013b).

Beads of substratum material, as opposed to slides, are also commonly used. This method is essentially the same as the slide methods but is distinguished by having a larger substratum surface area relative to the volume of the cell suspension. In the bead methods cells attached to the beads are quantified indirectly using radioactive labeling or spectrophotometry (Clark et al., 1978; Staat et al., 1980; Wassall et al., 1995; Groessner-Schreiber et al., 2004).

The flow chamber method and rotating disc method are also used to measure attachment in flow systems at a population level (Abbott et al., 1983; Christersson et al., 1987). In flow systems, attachment occurs at a low substratum area to suspension ratio which gives a constant cell density at maximum surface coverage (Bos et al., 1999). These methods allow bacterial attachment to take place under carefully controlled hydrodynamic and mass transport conditions (Busscher and van der Mei, 1995). An example of such systems is a parallel plate flow chamber system (Christersson et al., 1987; Sjollema et al., 1989), which is usually equipped with a microscope and allows *in situ* observation and image analysis (Busscher et al., 1995; van Hoogmoed et al., 1997; Mueller et al., 1992). By using a flow system, liquid-air interface passages are effectively avoided (Busscher and van der Mei, 1995), conditioning films can be applied to substratum surfaces (Busscher et al., 1992; Bradshaw et al., 1997) and antibiotics and detergents can be introduced in the system (Bos et al., 1999). Flow chamber techniques may therefore be considered as superior to static systems (Bos et al., 1999) and due to the well controlled hydrodynamic forces produced by these systems, non-quantitative statements such as "gentle rinsing to remove loosely attached cells" (Morra et al., 1996; Wang et al., 2013a) can be reduced by using them.

The use of AFM allows the measurement of bacterial attachment strength at a single cell level. This method determines the attachment strength by measuring the shear force at which an attached cell is detached (Fang et al., 2000). An AFM cantilever applies an opposing lateral force to the cell while the surface carrying the cell moves up and down (Warning and Datta, 2013). A force-distance curve of the variation of the interaction force of the cell approaching the cantilever can be generated (Ducker and Senden, 1992; Fang et al., 2000). Alternatively, attachment strength can be measured by determining the interaction forces between a cantilever coated with bacterial cells and the substratum surface (Ong et al., 1999; Lower et al., 2000). In addition to the ability of AFM to measure bacterial attachment at an individual cell level, a major advantage that this technique provides is that it can provide information on cell/substratum surface

properties and interaction forces simultaneously (Katsikogianni and Missirlis, 2004). The AFM approach is limited by the need to firmly fix the cells to a probe on a cantilever using physicochemical treatments which may alter cell surface properties and by the inability to take bacterial morphology into account (Katsikogianni and Missirlis, 2004).

Bacterial attachment and specific interactions in the above systems are usually under a delicate balance and easily influenced by experimental conditions. These conditions are often not under control in most of the experimental setups described above, resulting in non-reproducible, uninterpretable and non-comparable results from different laboratories.

Table 1.2 Methods to study specific interactions and factors affecting bacterial attachment.

Specific interactions (factors)	Affecting parameters	Methods to study the parameters	References	Notes
Hydrophobic interactions	Bacterial surface hydrophobicity	Bacterial attachment to hydrocarbon (BATH)	(Rosenberg et al., 1980)	Discrepancies can result from using different types and volumes of hydrocarbon.
		Contact angle measurement (CAM)	(Busscher et al., 1984)	Results are highly dependent on the wetness of the bacterial lawn.
		Hydrophobic interaction chromatography (HIC)	(Clark et al., 1985)	A small hydrophobic fraction on the cell surface can result in a high affinity.
		Salting-out aggregation test (SAT)	(Lindahl et al., 1981)	Results can be affected by cell surface charge.
	Substratum surface hydrophobicity	Contact angle measurement (CAM)	(Bos et al., 1999)	A standard method for the mechanistic models.
		Atomic force microscopy (AFM)	(Ducker and Senden, 1992)	This method is expensive and time consuming.

Table 1.2 (continued)

Electrostatic interactions	Bacterial surface charge	Microelectrophoresis	(Wilson et al., 2001)	This method is time consuming
		Electrostatic interaction chromatography	(Pedersen, 1981)	This method does not give direct or indirect measurements of zeta potential.
		Aqueous two-phase partitioning	(Liang et al., 1993)	Results can be influenced by cell surface hydrophobicity and cannot be converted to zeta potential.
		Isoelectric equilibrium analysis	(Sherbet et al., 1972)	This method is time consuming.
		Electrophoretic light scattering	(Blake et al., 1994)	This method was developed based on the principles as microelectrophoresis and has been shown to be an easy and rapid approach.
	Substratum surface charge	Electrostatic fieldmeter	(Faircloth and Allen, 2003)	Results can be affected by measuring distance and discharges.
		Electrostatic voltmeter	(Smith and Rungis, 1975)	This method overcomes the influence of measuring distance and discharges.
		Electrophoretic mobility	(Ballona and Drewes, 2005)	A commonly used method.
		Streaming potential measurement	(Childress and Elimelech, 1996)	A commonly used method.
Substratum surface roughness		Perthometer	(Quirynen et al., 1990)	A commonly used method.
		Atomic force microscopy (AFM)	(Goulter et al., 2011))	A commonly used method.

1.9 Strategies to control bacterial attachment

Bacterial cells attach to almost all hard surfaces by a wide range of possible means. They utilize any favorable interactions to approach surfaces in order to avoid a "homeless" state and protect themselves by forming biofilms. In addition, cells in a sessile condition express different phenotypes from planktonic cells and may resist hostile environments (Hori and Matsumoto, 2010). For this reason it is often difficult to prevent bacterial cells from attaching to a hard surface or to remove attached cells from a surface without using antimicrobial agents. Effective control of bacterial attachment has been reported by many authors and some of these studies are described below.

1.9.1 Treatment of substratum surfaces: chemical approaches

Bacterial attachment to a surface relies on specific interactions between cells and the substrate which are directly influenced by the corresponding cell and substrate surface properties. Chemical or physical treatment of substratum surfaces to modify the surface properties could significantly reduce the potential of attachment. For example, Park et al. (1998) found that modifying polyurethane surfaces with poly(ethylene glycol) significantly reduced surface hydrophobicity and consequently reduced the attachment of *Staphylococcus epidermidis* and *Escherichia coli* by up to 2 log CFU cm^{-2}. Roosjen et al. (2003) covalently coated glass surfaces with poly(ethylene oxide)-brushes and demonstrated a reduction in attachment of five bacterial and two yeast strains. Due to the high mobility and extremely large exclusion volumes of poly(ethylene oxide) it is difficult for particles to approach the surface (Harris, 1992). In addition Roosjen et al. (2003) noted a reduced LW interaction energy between cells and the surface after coating resulted in a more than 98% reduction in the attachment of four bacteria. The effect of the poly(ethylene oxide)-brush coatings on the attachment of *Pseudomonas aeruginosa* was weaker since theses bacteria can release surfactants that form hydrogen bonds with poly(ethylene oxide). Tiller et al. (2001) found that a covalent coating of

poly(4-vinyl-*N*-alkylpyridinium bromide) (a polycation material that has been reported to kill bacteria) on glass can reduce attachment of 1 to 2 log of airborne *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Escherichia coli* on the surface.

1.9.2 Treatment of substratum surfaces: physical approaches

Many authors apply physical treatments to modify the electrostatic properties of substratum surfaces to control bacterial attachment. For instance, van der Borden et al. (2004a, 2004b) used electric current to detach *Staphylococcus epidermidis* from surgical stainless steel. They established that an electric current of 60 to 100 μA, applied either as a direct current or as a block current, to a surgical stainless steel detached 75% of adhering *Staphylococcus epidermidis* and caused a 2 log reduction in the viability of the remaining cells (van der Borden et al., 2004a). They also subsequently established that a 100 μA direct current resulted in a 78% removal of *Staphylococcus epidermidis* biofilms (van der Borden et al., 2004b). The electric current did not only kill or inhibit cells but induced a strong electrostatic repulsion which removed the cells from the surface. The electro-assisted method has been widely applied to control bacterial attachment, biofilm formation and marine biofouling (Nakasono et al., 1993; Nakayama et al., 1998; Wake et al., 2006). Bacteria that have been detached by a direct electric current can re-accumulate on hard surfaces since some cells inactivated by a direct constant cathodic or anodic current tend to remain on the surface, and may serve as seeds for other bacteria to attach (Wagner et al., 2004). To overcome this limitation a block current method entailing periodic shifting between cathodic and anodic currents has been widely used to control biofouling. Application of block currents has been demonstrated to be an effective approach to increase bacterial detachment, inhibit bacterial attachment and to increases cell inactivation (Wake et al., 2006). In addition, the application of block currents has been suggested to be better than direct currents with respect to heat dissipation (Hong et al., 2008).

1.9.3 Treatment of bacterial cells: application of phytochemicals

The application of phytochemicals to bacterial cells is another approach used to control bacterial attachment. Nostro et al. (2004) studied the effect of the ethanol extracts of *Helichrysum italicum* on the attachment of *Streptococcus mutans* to glass. They found that *Helichrysum italicum* inhibited 90% of the attachment by reducing cell surface hydrophobicity. Hui and Dykes (2012) screened the water extracts of a range of Malaysian herbs for their effects on the attachment of five food related bacterial strains onto glass and stainless steel. Inhibitory effects were observed and suggested to be due to the effects of the extracts on cell surface hydrophobicity. Matsumoto et al. (1999) found that both polar and non-polar oolong tea fractions inhibited the attachment of *Streptococcus mutans* to hydroxyapatite by 40% to 70%. The potential mechanism of the attachment inhibition was assumed to be a reduction in cell surface hydrophobicity. In a study by Wang et al. (2013a), the mechanism of the inhibitory effect of oolong tea on *Streptococcus mutans* attachment to glass and hydroxyapatite was established. Oolong tea treated cells were found to be coated by flavonoids, indolic compounds and tannins. The coating activity was suggested to neutralize cell surface hydrophobicity, alter cell surface charge and block attachment sites (e.g. sites for hydrogen bond formation) on cell surfaces. From the literature in general, it is apparent that plants and plant components usually inhibit bacterial attachment by modifying bacterial cell surface properties and in particular hydrophobicity. Plants that can reduce bacterial surface hydrophobicity may therefore have potential as agents to control attachment. Razak et al. (2006), for example, found that the surface hydrophobicity of a range of early colonizers of dental plaque can be reduced by *Piper betle* and *Psidium guajava* extracts by approximately 10% and 50% (attachment to hexadecane), respectively. Dykes et al. (2002) reported that ethanol extracts of bearberry leaves reduced the hydrophobicity of *Staphylococcus aureus* by 43% (attachment to xylene). It should be noted that plant extracts may also be effective in reducing bacterial hydrophobicity and in some cases may increase it. In the same study by Dykes et al. (2002) reported above, ethanol extracts of bearberry leaves were also found to increase the hydrophobicity of a

large range of other bacteria by 2 to 67% attachment to xylene. Voravuthikunchai and Limsuwan (2006) reported that ethanol extracts of *Punica granatum* and *Quercus infectoria* increased the hydrophobicity of *Helicobacter pylori* strains. In addition, the ethanol extracts of a range of Thai medicinal plants were found to increase the hydrophobicity of *Escherichia coli* O157:H7 strains determined using SAT (Voravuthikunchai et al., 2006). Ethanol extracts of *Quercus infectoria* were subsequently established to increase the hydrophobicity of Shiga toxigenic *Escherichia coli* strains by 10 to 50% attachment to toluene and these effects were attributed the removal of pili from the cell surfaces by the plant extracts (Voravuthikunchai and Suwalak, 2009). The application of phytochemicals to modify bacterial cell surface hydrophobicity has practical potential but these effects tend to be bacterial strain specific and a compound effective against all bacteria is unlikely to exist. It is however possible to develop strategies to control attachment using phytochemicals based on the hydrophobicity nature of the target bacteria. Future research should entail identification of phytochemicals that work for either hydrophobic or hydrophilic bacteria.

In addition to the strategies listed above bacterial attachment can be controlled by many other means such as shear force and altering temperature (Nejadnik et al., 2008; Nguyen et al., 2010). Regardless of the methods used and due to the fact that different bacteria attach to surfaces by different physicochemical means the development of a universal method to control the attachment of all bacteria to all surfaces is unlikely.

1.10 Concluding remarks

A substantial amount of research has been conducted, and advances made, in understanding the mechanisms of bacterial attachment to hard surfaces. Sufficient experimental evidence is available to support the idea that bacterial attachment can be explained using physicochemical approaches, although the process can also be affected by many other parameters, such as biological properties of bacterial cells. Furthermore

the ability to explain bacterial attachment phenomena using physicochemical approaches often decreases with an increasing complexity of the interactions. This is especially apparent when the impact of a weak interaction is masked by that of stronger ones or when surface roughness is involved. It is therefore not sufficient to study bacterial attachment by considering only one or a very few interactions in a system. In addition, it is often difficult to study attachment if surface properties across a small range of values are considered as the resultant numbers of attaching bacteria are usually not significantly different and the value of these studies is therefore limited. This difficulty leads to a need to determine a range of values for each surface property within which bacterial attachment cannot be differentiated and which may assist in designing more useful studies.

A universally valid physicochemical model for predictions of bacterial attachment is still not available. Limitations of the existing models are apparent. In addition to the fact that biological factors play a role in attachment, these models assume ideal conditions (e.g. perfectly smooth, uniform and inert surfaces) which in reality do not exist. They also tend to predict attachment in a solely deterministic way resulting in poor outcome because of noise. Additional factors such as substratum surface roughness and cell to cell interactions need to be considered in future model developments. The application of probability distributions to mechanistic models in order to take biological variability into account may be one approach to solve some of these problems.

The development of a universal approach to control bacterial attachment to hard surface is unlikely based on our current understanding of the attachment process. Strategies that address specific needs with respect to the control of bacterial attachment have been developed. For example, chemical and physical treatments such as coating and electro-assisted methods have been used to protect particular surfaces against bacterial attachment. In addition phytochemical treatments that alter cell surface hydrophobicity have been employed to prevent particular bacteria from attaching to surfaces. As these

strategies are limited in scope a better understanding of the mechanisms of bacterial attachment to hard surfaces is required in order to develop new ones.

Considerable progress has been made in the development of methods used for the study of bacterial attachment, but the lack of process control and standardization means that there are still substantial challenges in model development based on these methodologies. In addition, experimental approaches which examine interactions at both the population and individual cell level may help overcome the challenges in establishing systems that allow accurate prediction of microbial colonization.

In conclusion, in order to develop a better understanding and predication of the process of bacterial attachment as well as to develop better control strategies, future research needs to focus on optimizing and standardizing methodologies for bacterial attachment studies, and this needs to be accomplished by multidisciplinary research teams consisting of microbiologists, surface chemists, engineers and statisticians.

1.11 Aims of this project

A review of the literature indicated that information on the inhibitory effects of tea on attachment of oral bacteria to surfaces is limited, and that the mechanisms, predictability and stochasticity of bacterial attachment to abiotic surfaces are not well understood. The following questions were therefore raised: 1. Which tea product(s) or tea component(s) inhibit attachment and biofilm formation by oral bacteria on surfaces? 2. How do these components inhibit attachment and biofilm formation? 3. Is bacterial attachment a stochastic process? 4. If it is not stochastic how and to what extent can it be predicted?

Based on the questions raised this project aimed:

1. To screen the extracts of a range of commercial tea products and tea components for their effects on a number of oral bacterial strains in terms of their cell surface properties and ability to attach and form biofilm on abiotic surfaces. In addition, to determine the most effective tea extract(s) that inhibit attachment and biofilm formation and to determine the bacterial cell surface properties that affect attachment most (Chapter 2a).

2. To investigate the effects of dietary sucrose on cell surface properties of oral bacteria and their colonization of hard surfaces (Chapter 2b).

3. To investigate the influence of the interaction of saliva on oral bacterial attachment to hydroxyapatite (Chapter 2c).

4. To investigate the effects of the tea extracts and tea components on oral bacterial attachment to cultured human gingival fibroblasts (Chapter 3).

5. To investigate the mechanisms of the inhibition of the attachment and biofilm of *Streptococcus mutans* on hard surfaces by the most effective tea extract(s) determined in Chapter 2a (Chapter 4).

6. To study the predictability of oral bacterial attachment to hard surfaces determined in Chapter 2a using the XDLVO theory (Chapter 5a).

7. To develop a mathematical approach to model bacterial attachment to hard surfaces in general, and to study the predictability and stochasticity of bacterial attachment using this model (Chapter 5b).

Chapter 2

Effect of Tea Extracts, Sucrose and Saliva on Attachment of Oral Bacteria to Hard Surfaces

The work presented in this chapter represents the following publications submitted for peer review:

Wang, Y., Lee, S.M. and Dykes G.A. (submitted) The inhibitory effect of tea on attachment of oral Streptococci to hydroxyapatite and other abiotic surfaces is associated with a reduction in bacterial surface hydrophobicity. *PLOS ONE*.

Wang, Y., Lee, S.M. and Dykes G.A. (submitted) Growth in the presence of sucrose may decrease attachment of some oral bacteria to abiotic surfaces. *Letters in Applied Microbiology*.

Wang, Y., Lee, S.M. and Dykes G.A. (submitted) Inhibitory effects of saliva as a suspending fluid on attachment of oral bacteria to hydroxyapatite. *Journal of Basic Microbiology*.

Declaration for Thesis Chapter 2a

Declaration by candidate

In the case of Chapter 2a, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Sui Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidate's Signature	[redacted]	**Date** June 12, 2013

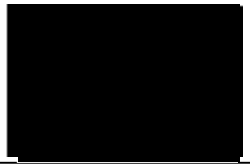
Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University, Sunwasy Campus**

Signature 1		**Date** June 14, 2013
Signature 2		**Date** June 13, 2013

Chapter 2a Effect of Tea Extracts on Surface Properties of Oral Bacteria and Their Colonization of Hard Surfaces

2a.1 Introduction

The colonization of tooth surfaces by oral pathogenic bacteria is of concern since it can lead to oral disease such as caries (Aas et al., 2008). This colonization is initiated by bacterial attachment and ultimately results in biofilm formation on teeth (Hamilton-Miller, 2001). Bacterial attachment is a complex process involving a large number of factors including bacterial cell surface properties (hydrophobicity and charge) and substratum surface properties (surface tension, charge and roughness) (Goulter et al., 2009). The process of biofilm formation is even more complex. In addition to initial attachment many other factors, such as cellular auto-aggregation, extracellular polymer synthesis and virulence genes, can influence formation of biofilms (Sorroche et al., 2012).

Tea (*Camellia sinensis*) is a popular drink worldwide and has proven benefits to oral health (Hamilton-Miller, 2001). Research on the beneficial effects of this drink in preventing oral disease has focused on its direct bactericidal capability (Hamilton-Miller, 2001; Otake et al., 1991; Friedman, 2007). Only a limited number of studies have been conducted with respect to its ability to prevent bacterial attachment and biofilm formation (Matsumoto et al., 1999).

In this study, extracts of a range of commercial tea products and tea components were tested for their ability to modify surface properties (hydrophobicity, charge and auto-aggregation) of five strains of oral bacteria and their colonization (attachment and biofilm formation) of hydroxyapatite (a tooth model surface) (Apella et al., 2008) and of glass and stainless steel (as reference surfaces with different surface properties). The

impact of cell surface properties on colonization were established in order to determine any factor(s) that may have a significant influence on attachment and biofilm formation.

2a.2 Materials and methods

2a.2.1 Bacterial culture and suspension preparation

Five strains of oral bacteria namely *Streptococcus mutans* (ATCC 25175 and ATCC 35668), *Streptococcus salivarius* (ATCC 13419), *Streptococcus mitis* (ATCC 49456) and *Actinomyces naeslundii* (ATCC 51655) were used in this study and obtained from the American Type Culture Collection (Manassas, USA). All strains were maintained on Mitis Salivarius Agar (MSA; Difco, US) at 4°C and grown for experiments in Tryptic Soy Broth (TSB; Merck, USA) at 37°C for 24 h (48 h for *A. naeslundii* ATCC 51655) with shaking at 150 rpm. Bacterial suspensions were prepared by centrifuging 20 mL of the TSB cultures at 7669 g for 15min. Cells were gently washed with 150 mM phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mMKH_2PO_4, 137 mM NaCl, pH 7.4; 1st BASE, Singapore) and the final pellet was resuspended in 20 mL PBS for all experiments unless otherwise stated.

2a.2.2 Tea extract preparation

Five commercial tea products, namely green tea, oolong tea, black tea, pu-erh tea and chrysanthemum tea (Ten Ren Tea Co. Ltd., Taiwan) were extracted using 90% (vol/vol) acetone (Sigma-Aldrich, USA) at a ratio of 1:20 (g:mL) for 2 h (Wang et al., 2013b). The resultant extracts were evaporated under vacuum at 40°C, freeze dried and stored at -20°C for further use.

Tea extract solutions for experiments were prepared by dissolving dried tea extracts in PBS or distilled water containing 1% (vol/vol) methanol (Systerm, UK) to a desired concentration and filter sterilizing though a 0.2 μm filter (Millipore, USA).

Epigallocatechin gallate [EGCg; 95% (vt/vt); Sigma-Aldrich] and gallic acid (Sigma-Aldrich) were also used in this study as they are major flavonoid components of most teas especially low degree fermented teas (Peterson et al., 2005), and were prepared in the same way as the tea extract solutions.

2a.2.3 Antimicrobial susceptibility tests

The minimal inhibitory concentrations (MICs) of the tea extracts and pure compounds against the five bacterial strains were determined using the micro-broth dilution method as previously described by James (1990). This was done in order to determine the concentrations of tea extracts and tea compounds to apply to the bacteria in subsequent assays without killing them or inhibiting their growth. Briefly, 100 µL of each tea extract solution or pure compound water solution (10 mg/mL) was subject to doubling dilution in a microtitre plate (Jet Biofil, China), mixed with 100 µL of TSB containing suspended bacterial cells (approximately 10^4 CFU/mL, determined by a turbidity standard) and incubated at 37°C for 48 h. Growth was determined by assessing the turbidity in the wells. In subsequent experiments, each bacterial strain was treated with tea extracts at the concentration below the lowest MIC value among all tea extracts to make the studies comparable. In the case of the pure tea compounds bacterial strains were treated at a concentration below each of their individual MICs.

2a.2.4 Effect of tea extracts on bacterial cell surface properties

2a.2.4.1 Cell surface hydrophobicity (CSH) determination

The CSH was determined using the Bacterial Attachment to Hydrocarbon (BATH) method as described by Gibbons and Etherden (1983) with slight modifications. Briefly, bacterial cells were suspended in PBS containing dissolved tea extracts / pure compounds and the suspensions were adjusted to an optical density (OD) of 1.0 ± 0.2 at 550 nm to eliminate cell concentration effects. PBS without tea extracts or pure

compounds was used as a control. The samples were incubated at 37°C for 1 h. A 3 mL aliquot of each sample was mixed with 1 mL of hexane (Sigma-Aldrich) and vortexed for 2min. The mixtures were then allowed to separate for 1 h. The OD_{550} of the aqueous phase was measured before (A_0) and after (A) addition of hexane. Blanks were prepared using the suspending liquids without bacterial cells. The index of hydrophobicity was calculated as *% Binding to hexane = $(1 - A / A_0) \times 100\%$*.

2a.2.4.2 Cell surface zeta potential (ZP) measurements

Bacterial cell surface ZP was measured as previously described by Bayoudh et al. (2009). Bacterial suspensions (with or without tea extracts / pure compounds) at a concentration of 10^7 CFU/mL (pH 7.4) were incubated at 37°C for 30 min. The ZP of a 1 mL aliquot of each sample was measured using a zetasizer (Nano ZS-ZEN3600; Malvern Instruments Ltd., UK).

2a.2.4.3 Auto-aggregation assay

Auto-aggregation assays were performed as described by Ellen and Balcerzak-Raczkowski (1977). A 1 mL aliquot of bacterial suspensions (with or without tea extracts / pure compounds) was adjusted to an OD of 0.25 ± 0.05 at 600 nm and incubated at 37°C. The OD_{600} was taken before (A_i) and after (A_f) 6 h incubation at 37°C. Aggregation percentage was expressed as *% Auto-aggregation = $(1 - A_f / A_i) \times 100\%$*.

2a.2.5 Effect of tea extracts on bacterial attachment, detachment and biofilm formation on hard surfaces

2a.2.5.1 Hard surface preparation

Hydroxyapatite surfaces were prepared by coating hydroxyapatite powder onto glass slides as described by Wang et al. (2013a). Briefly, glass slides were painted with 40%

(wt/vol) bonding adhesive (60% limestone, 30% kaolin, 8% ethylene glycol, 1% SiO_2 and 1% TiO_2, %wt; Over Sea Plaster Supply and Construction Sdn. Bhd., Malaysia) and partially dried in air for 3 min. The slides were then coated with a paste consisting of 10% cement (56% CaO, 40% SiO_2, 3% $Al_2O_3 \cdot Fe_2O_3$ and 1% MgO, %wt; Over Sea Plaster Supply and Construction Sdn. Bhd., Malaysia), 40% hydroxyapatite powder (Sigma-Aldrich, USA) and 50% distilled water (%wt) followed by air drying for 16 h.

Glass (75 × 25 mm; Thermo Fisher Scientific, USA), stainless steel (75 × 25 mm; type 302, # 4 finish, 1 mm thickness) and hydroxyapatite (75 × 25 mm) slides were degreased by soaking in acetone for 30 min, rinsed in distilled water and sterilized by autoclaving.

2a.2.5.2 Scanning electron microscopy (SEM) and atomic force microscopy (AFM)

The three hard surfaces were visualized under a scanning electron microscope (S-3400N; Hitachi, Japan) at 2,000 times magnification at a scanning voltage of 5 kV. Their roughness was determined using an atomic force microscope (10×10 mm; Veeco Instruments, Inc., Canada). Images for surface roughness (5 × 5 μm) were obtained using a silicon nitride cantilevers (Budget Sensors, Bulgaria) with a spring constant of 0.06 N/m at a scan rate of 2 Hz. The roughness of hydroxyapatite was not measurable using this method as material broke off from the surface and attached to the point of the cantilever during the mapping process. Surface roughness was analyzed using Nanoscope software (version 5, Digital Instruments, Canada) and reported as the root mean square (RMS; nm).

2a.2.5.3 Attachment assay

The attachment assays were carried out as described by Wang et al. (2013a). Bacterial cells were suspended in 20 mL PBS containing tea extracts / pure compounds and incubated with the hard surface slides at 37°C for 30 min without shaking to allow

attachment to take place. The slides were then removed from the suspensions, gently rinsed three times with PBS and stained by 0.1% (wt/vol) crystal violet (for glass; Sigma-Aldrich) or 0.1% (wt/vol) acridine orange (for hydroxyapatite and stainless steel; Sigma-Aldrich). The number of attached cells was counted using a microscope (BX51; Olympus, Japan) under light or epifluorescence. A total of 50 fields were counted for each slide and the number of attached cells was expressed as mean log CFU/cm^2. The *A. naeslundii* ATCC 51655 strain was non-enumerable under a microscope due to its morphology. After rinsing slides with *A. naeslundii* ATCC 51655 cells attached were placed in a stomacher bag (Gossenlin, France) containing 50 mL of PBS, and massaged in a stomacher (400P, BigMixer®, France) for 10 min to remove the attached cells. The massaged slides were stained and visualized under a microscope for 50 fields. No cells were observable and therefore stomaching was considered to be an effective means for the removal of attached cells. Aliquots of PBS were serially diluted, spread plated on Tryptic Soy Agar (TSA; Merck) and quantified after 48 h incubation at 37^{o}C (Chia et al., 2011). Chia et al. (2011) determined that attachment results obtained from the two methods were not significantly different.

2a.2.5.4 Detachment assay

Hard surface slides were incubated at 37^{o}C for 30 min with 20 mL aliquots of bacterial suspensions with a cell density at approximately 10^6 CFU/mL to allow for attachment. After incubation the slides were removed, rinsed three times with PBS and transferred into 20 mL PBS containing tea extracts / pure compounds (PBS was used as a control) prior to incubation for 16 h at 37^{o}C. The cells remaining on the slides were quantified using direct counting method with a microscope for Streptococcal strains as described above. The detachment assay was not carried out for *A. naeslundii* ATCC 51655 (spread plating method) as cells remaining on the slide did not grow on TSA after stomaching.

2a.2.5.5 Biofilm formation assay

A biofilm formation assay was performed as described by Wang et al. (2013). A 10 mL aliquot of water containing tea extracts / pure compounds was added to 10 mL pre-autoclaved TSB at a double concentration. The mixture was inoculated with a 0.1 mL of a 24 h TSB culture and incubated with a slide at 37°C for 72 h without shaking. Controls were prepared by using distilled water instead of tea extract solutions. After incubation the slide was washed three times with PBS and transferred into a Falcon tube (TPP®, Switzerland) containing 40 mL of PBS. The tube was sonicated for 10 min using a water bath sonicator (LC-130H; ELMA, Germany) at room temperature at a frequency of 35 kHz to detach the biofilm cells into the surrounding PBS. An aliquot of the PBS was serially diluted, spread plated on TSA and incubated at 37°C for 48 h before enumeration.

2a.2.6 Statistical analysis

All assays were carried out in triplicate with independently grown cultures and all values were expressed as mean ± standard deviation. A one way ANOVA (Tukey's comparison) or a Student's *t*-test was performed on all data sets. A nested ANOVA was performed to compare the hard surfaces in terms of bacterial attachment and biofilm forming ability with respect to tea extract / pure compound treatments, and also to determine the most effective tea extracts in inhibiting attachment and biofilm formation with consideration of all bacteria on all surfaces. The relationships between bacterial cell surface properties and their colonization abilities were determined using regression plots. All analysis was conducted using SPSS software (PASW Statistics 18; SPSS Inc.) at a 95% confidence level.

2a.3 Results

2a.3.1 Antimicrobial susceptibility tests

The MIC values of the tea extracts and pure compounds against the five bacterial strains used in this study are shown in Table 2a.1. Most of the tea extracts exhibited inhibitory effects against the bacterial strains. Green tea and oolong tea extracts had lower MICs (625 to 2500 μg/mL) than the other extracts (2500 to 5000 μg/mL). EGCg also generally had lower MICs (125 to 500 μg/mL) against the bacteria than gallic acid (250 to 4000 μg/mL).

Table 2a.1 Minimum inhibitory concentrations (MIC) of five tea extracts and two pure compounds against five oral bacteria.

	MIC (μg/mL)						
	Green tea	Oolong tea	Black tea	Pu-erh tea	Chrysanthemum tea	EGCg	Gallic acid
S. mutans ATCC 25175	2500	2500	5000	>5000	5000	500	4000
S. mutans ATCC 35668	2500	2500	5000	>5000	5000	250	2000
S. salivarius ATCC 13419	2500	2500	5000	5000	5000	250	2000
S. mitis ATCC 49456	1250	1250	5000	5000	5000	125	2000
A. naeslundii ATCC 51655	625	625	2500	2500	2500	250	250

2a.3.2 Effect of tea extracts on bacterial cell surface properties

Figure 2a.1 shows the effects of tea extracts and pure compounds on the cell surface properties (hydrophobicity, charge and auto-aggregation) of the five bacterial strains used in this study. All strains showed high surface hydrophobicity ranging from 60 to 90% (Gibbons and Etherden, 1983). The tea extracts and EGCg reduced the

hydrophobicity of the strains (by between 4 to 58%; $p<0.05$) in most of the cases. Chrysanthemum tea extract showed the strongest effect (58%; $p<0.05$). Gallic acid on the other hand had no effect ($p>0.05$) on cell surface hydrophobicity of the strains. All strains were negatively charged with ZP ranging from -4 to -12 mV. The net ZP of both *S. mutans* strains was increased by all tea extracts and EGCg by between 4 to 20 mV ($p<0.05$), while that of *S. mitis* ATCC 49456 was only increased by green tea and pu-erh tea extracts by 8 and 9 mV, respectively ($p<0.05$). The ZP of *S. salivarius* ATCC 13419 and *A. naeslundii* ATCC 51655 were not affected by any of the tea extracts or pure compounds ($p>0.05$). All strains exhibited moderate to relatively high auto-aggregation ranging from 22 to 45% (Ellen and Balcerzak-Raczkowski, 1977). The oolong tea extract increased the auto-aggregation of *S mutans* ATCC 35668 and *S. salivarius* ATCC 13419 by 9 and 7%, respectively ($p<0.05$). The black tea extract increased the auto-aggregation of *S. mutans* ATCC 35668 by 10% ($p<0.05$) but decreased that of *S. salivarius* ATCC 13419 by 11% ($p<0.05$). The pu-erh tea extract increased the auto-aggregation of *A. naeslundii* ATCC 51655 by 15% ($p<0.05$). The auto-aggregation of *S. mutans* ATCC 25175 and *S. mitis* ATCC 49456 were not affected by any of the tea extracts ($p>0.05$). Gallic acid showed increased the auto-aggregation of all strains by 8 to 30% ($p<0.05$) except for that of *S. mitis* ATCC 49456 ($p>0.05$), while EGCg increased the auto-aggregation of *S. mutans* ATCC 35668 only by 24% ($p<0.05$).

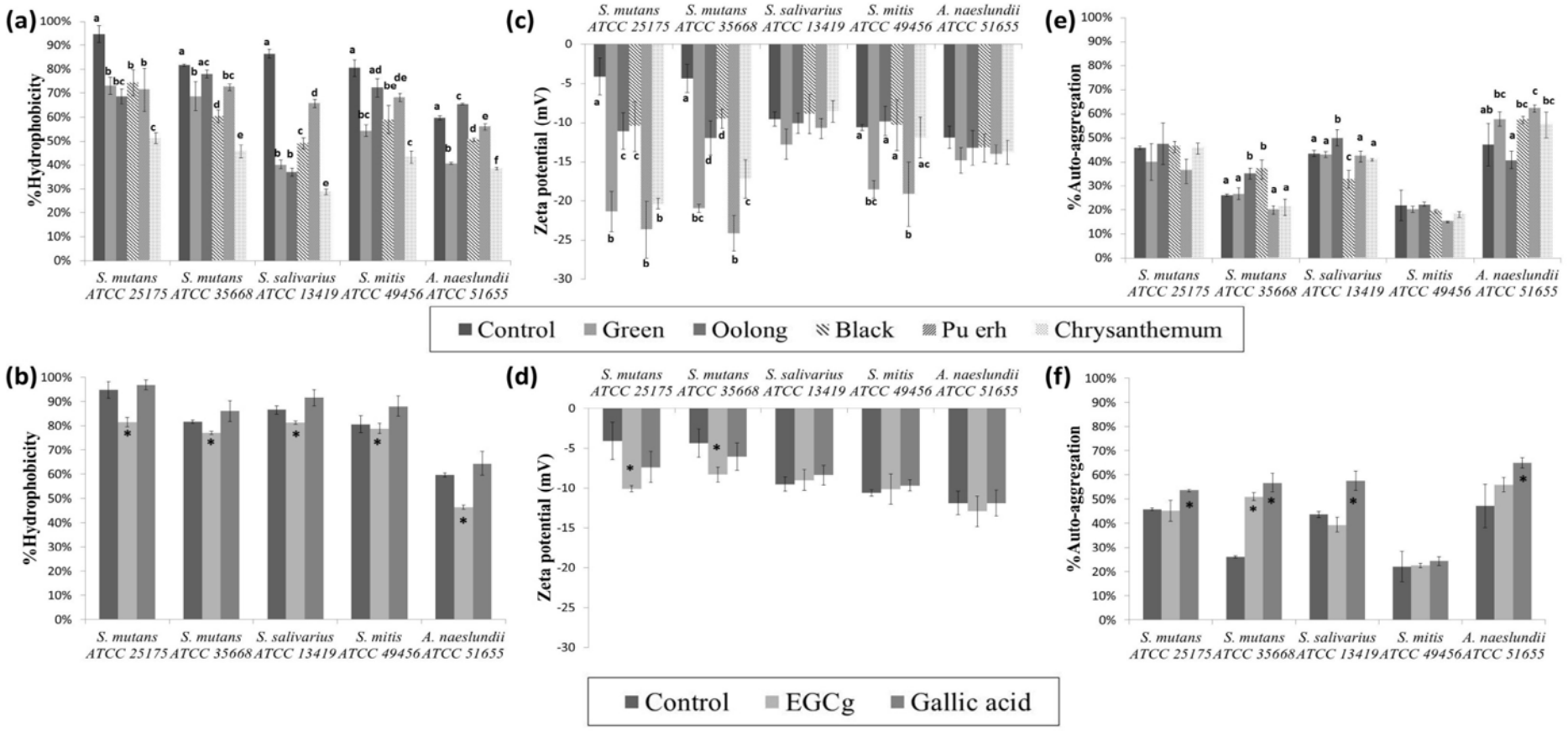


Figure 2a.1 Effect of tea extracts and pure compounds on bacterial cell surface properties. The effect of tea extracts (first letter) and pure compounds (second letter) on hydrophobicity (a, b), charge (c, d) and auto-aggregation (e, f). Values labeled with the same letter are not significantly different ($p>0.05$) among the treatments within a strain. Values labeled with the * symbol are significantly different from the control ($p<0.05$).

2a.3.3 Effect of tea extracts on bacterial attachment, detachment and biofilm formation on hard surfaces

The effects of the tea extracts and compounds on the attachment of the oral bacteria to the hard surfaces used in this study are presented in Figure 2a.2. All strains exhibited a similar ability to attach to the surfaces (~6.1 to 6.5 log CFU/cm^2) except for *A. naeslundii* ATCC 51655 which attached in significantly lower numbers (~5.5 log CFU/cm^2; $p<0.05$). The tea extracts exhibited inhibitory activities on attachment but not in all cases. Most notably the oolong tea extract was effective in reducing attachment of all strains to all surfaces by between 0.3 to 2.2 log CFU/cm^2 ($p<0.05$). A nested ANOVA indicated that this extract was the strongest inhibitor of attachment among all the tea extracts tested ($p<0.05$). In addition, the attachment of *A. naeslundii* ATCC 51655 was inhibited to a greater degree by all tea extracts as compared to the other bacterial strains tested ($p<0.05$). Exposure to EGCg reduced attachment by between 0.3 to 1.5 log CFU/cm^2 for all strains ($p<0.05$) except for *S. salivarius* ATCC 13419 and *S. mitis* ATCC 49456 to hydroxyapatite ($p>0.05$). Gallic acid reduced attachment of all strains to hydroxyapatite surface ($p<0.05$) with the exception of *S. salivarius* ATCC 13419 ($p>0.05$). It was established (Nested ANOVA) that hydroxyapatite was the most favorable surface for bacterial attachment among the three surfaces followed by stainless steel and then glass ($p<0.05$). No interaction ($p>0.05$) was found between the type of hard surface and tea extract / pure compound treatments, indicating that the type of hard surface did not affect the effects of tea extracts / pure compounds on bacterial attachment.

The results of detachment assays indicated that the tea extracts and pure compounds did not detach any of the bacterial strains from any of the surfaces ($p>0.05$) (Appendix I).

Figure 2a.3 shows the results of the biofilm formation assays. Results of this part of the study indicated that the *S. mutans* strains formed a significantly greater ($p<0.05$) amount of biofilm on hydroxyapatite (~6.6 to 7.1 log CFU/cm^2) than on the other two surfaces

tested (~5.2 to 5.8 log CFU/cm^2). Greater ($p<0.05$) amounts of biofilm were also formed by the two *S. mutans* on hydroxyapatite than that formed by the other stains on all surfaces (~4.7 to 5.6 log CFU/cm^2). The Pu-erh tea extract reduced biofilm formation by the four Streptococcal strains on all surfaces by 0.5 to 2.4 log CFU/cm^2 ($p<0.05$). The black tea extract reduced biofilm formation by *S. mutans* ATCC 25175 on hydroxyapatite by 1.2 log CFU/cm^2 ($p<0.05$). Chrysanthemum tea extract reduced biofilm formation by *S. mutans* ATCC 25175 and *S. mutans* ATCC 35668 on hydroxyapatite by 1.1 and 1.0 log CFU/cm^2, respectively ($p<0.05$). Biofilm formation by *A. naeslundii* ATCC 51655 was enhanced by the pu-erh tea extract by 0.5 log CFU/cm^2 on both glass and hydroxyapatite ($p<0.05$). EGCg had no effect on biofilm formation by any of the bacteria on any of the surfaces ($p>0.05$) while gallic acid enhanced the biofilm formation by *A. naeslundii* ATCC 51655 on all surfaces by 1.0 to 1.2 log CFU/cm^2 ($p<0.05$). A nested ANOVA indicated that the pu-erh tea extract was the most effective inhibitor of biofilm formation for the Streptococcal strains ($p<0.05$) and that for all strains hydroxyapatite was the most favorable surface for biofilm formation followed by glass and then stainless steel ($p<0.05$). No interaction ($p>0.05$) was found between the type of hard surface and tea extract / pure compound treatments, indicating that the type of hard surface did not influence the effect of tea extracts / pure compounds on biofilm formation.

Correlations between cell surface properties and attachment on different surfaces after tea extract / pure compound treatments are presented in Table 2a.2. Correlations between cell surface hydrophobicity and attachment were greater as compared to those between surface charge and auto-aggregation / attachment for the Streptococcal strains and especially for *S. salivarius* ATCC 13419 (R^2s of 68 to 91% for hydrophobicity). No significant correlation was found between biofilm formation and cell surface properties or attachment ($p>0.05$).

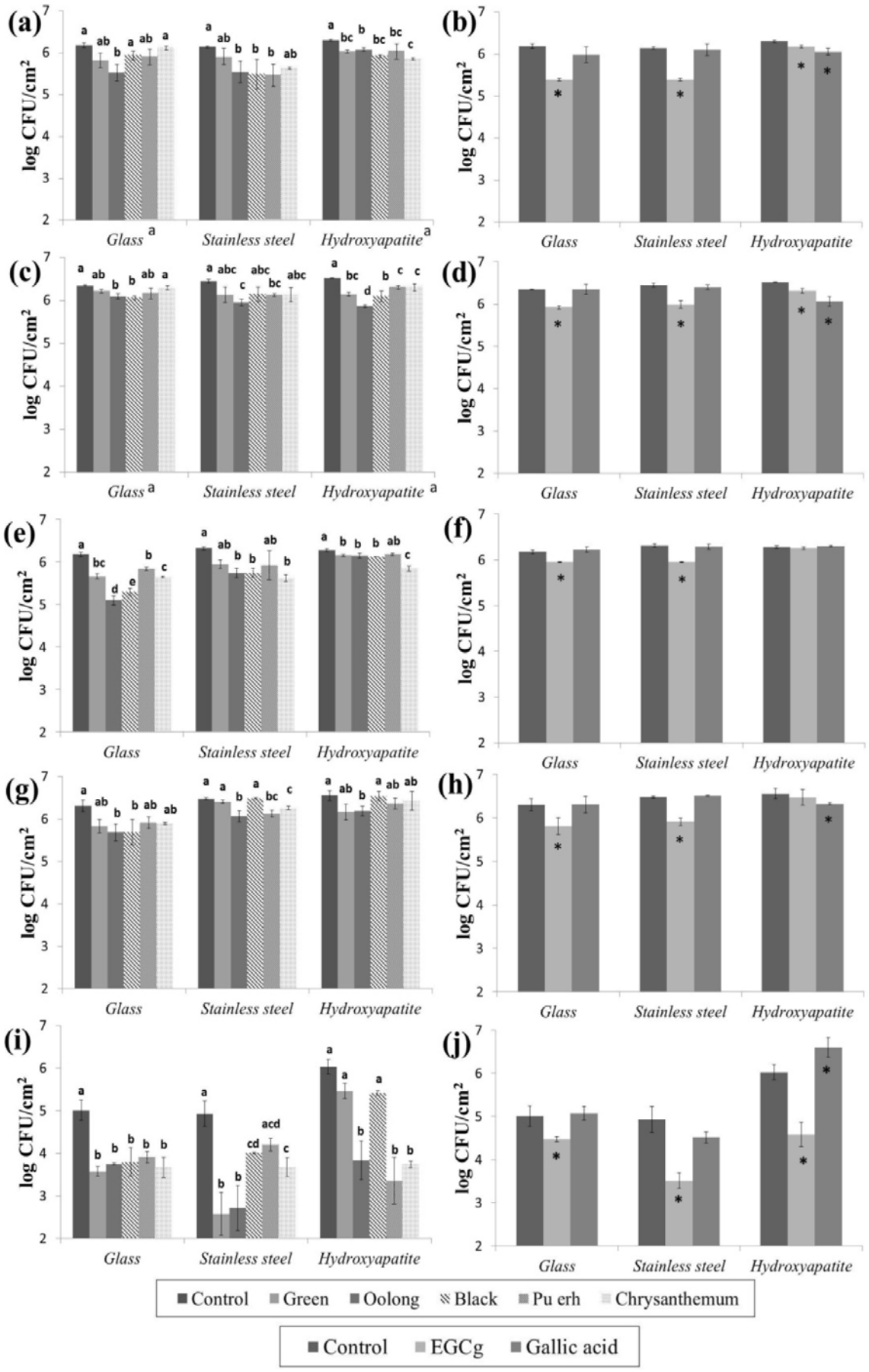

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
log CFU/cm²
Glass
Stainless steel
Hydroxyapatite
Control
Green
Oolong
Black
Pu erh
Chrysanthemum
Control
EGCg
Gallic acid

Figure 2a.2 Effect of tea extracts and pure compounds on bacterial attachment to hard surfaces. The effect of tea extracts (first letter) and pure compounds (second letter) on the attachment of *S. mutans* ATCC 25175 (a, b), *S. mutans* ATCC 35668 (c, d), *S. salivarius* ATCC 13419 (e, f), *S. mitis* ATCC 49456 (g, h) and *A. naeslundii* ATCC 51655 (i, j). Values labeled with the same letter are not significantly different ($p>0.05$) among the treatments on the same surface. Values labeled with the * symbol are significantly different from the control ($p<0.05$).

[a]The effect of tea extracts on the attachment of *S. mutans* ATCC 25175 and *S. mutans* ATCC 35668 to glass and hydroxyapatite were previously reported (Wang et al., 2013a).

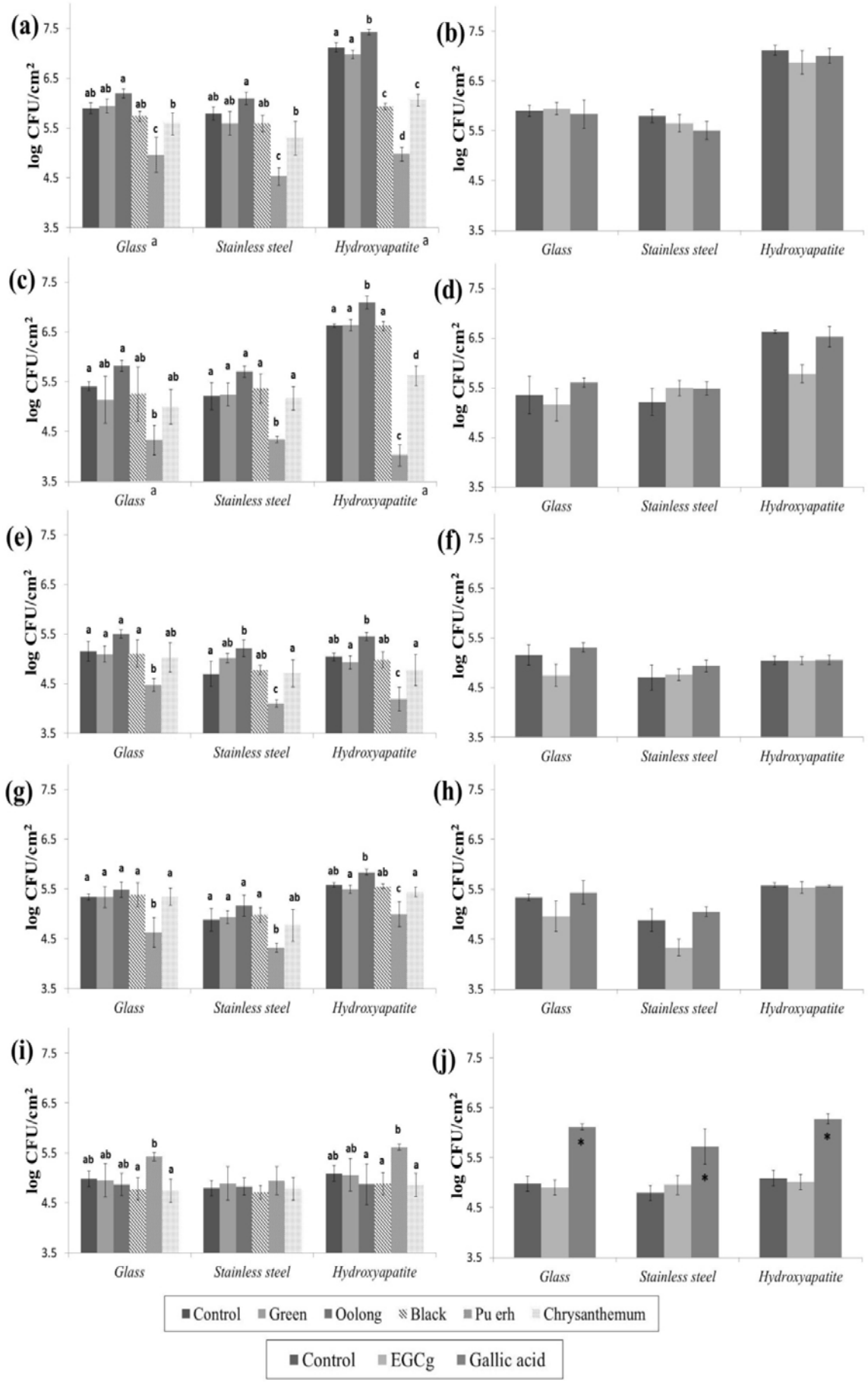

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
log CFU/cm²
7.5
6.5
5.5
4.5
3.5
Glass
Stainless steel
Hydroxyapatite
Control
Green
Oolong
Black
Pu erh
Chrysanthemum
Control
EGCg
Gallic acid

Figure 2a.3 Effect of tea extracts and pure compounds on biofilm formation on hard surfaces. The effect of tea extracts (first letter) and pure compounds (second letter) on the biofilm formation by *S. mutans* ATCC 25175 (a, b), *S. mutans* ATCC 35668 (c, d), *S. salivarius* ATCC 13419 (e, f), *S. mitis* ATCC 49456 (g, h) and *A. naeslundii* ATCC 51655 (i, j). Values labeled with the same letter are not significantly different ($p>0.05$) among the treatments on the same surface. Values labeled with the * symbol are significantly different from the control ($p<0.05$).

[a]The effect of tea extracts on biofilm formation by *S. mutans* ATCC 25175 and *S. mutans* ATCC 35668 on glass and hydroxyapatite were previously reported (Wang et al., 2013a).

Table 2a.2 Correlations between surface properties (cell surface hydrophobicity, charge and auto-aggregation) of five bacteria and their attachment to three abiotic surfaces.

		Cell surface hydrophobicity			Cell surface charge			Auto-aggregation		
		R^2	F	p	R^2	F	p	R^2	F	p
S. mutans ATCC 25175	Glass	**0.40**	**6.86**	**0.005**	0.01	0.19	0.671	0.09	1.04	0.372
	Stainless steel	**0.65**	**19.12**	**<0.001**	**0.20**	**5.49**	**0.029**	0.12	1.41	0.267
	Hydroxyapatite	**0.41**	**7.35**	**0.004**	**0.29**	**8.84**	**0.007**	0.06	0.66	0.530
S. mutans ATCC 35668	Glass	**0.44**	**8.32**	**0.002**	0.02	0.41	0.526	0.29	4.26	0.28
	Stainless steel	**0.37**	**6.28**	**0.007**	**0.22**	**6.09**	**0.022**	0.10	1.21	0.320
	Hydroxyapatite	0.01	0.14	0.873	0.03	0.61	0.443	0.16	2.03	0.157
S. salivarius ATCC 13419	Glass	**0.91**	**102.18**	**<0.001**	0.09	2.34	0.140	0.23	3.15	0.064
	Stainless steel	**0.68**	**22.55**	**<0.001**	0.02	0.35	0.560	0.25	3.47	0.050
	Hydroxyapatite	**0.79**	**39.31**	**<0.001**	0.01	0.03	0.870	0.20	2.56	0.101
S. mitis ATCC 49456	Glass	**0.52**	**11.38**	**<0.001**	0.05	1.22	0.280	**0.43**	**7.79**	**0.003**
	Stainless steel	0.17	2.20	0.135	0.02	0.42	0.523	0.05	0.57	0.573
	Hydroxyapatite	0.01	0.02	0.985	0.09	2.14	0.157	0.03	0.33	0.720
A. naeslundii ATCC 51655	Glass	**0.25**	**3.54**	**0.047**	**0.83**	**49.81**	**<0.001**	0.12	1.37	0.276
	Stainless steel	0.19	2.47	0.109	**0.52**	**11.51**	**<0.001**	<0.01	0.04	0.965
	Hydroxyapatite	0.24	3.36	0.054	**0.51**	**11.03**	**0.001**	**0.42**	**7.66**	**0.003**

Correlations were plotted based on arcsine transformed % hydrophobicity values, ZPs, arcsine transformed %auto-aggregation values and attachment values (CFU/cm^2) using quadratic regression (with an equation of $y = ax^2 + bx + c$). The surfaces property and attachment values were obtained after tea extract / pure compound treatments. The cases in bold face were found to be significantly correlated ($p<0.05$).

Figure 2a.4 shows the SEM and AFM images of the hard surfaces. It can be seen from the SEM images that the surface of hydroxyapatite was significantly rougher than the other two surfaces. The AFM images present the mapping of glass and stainless steel surfaces with RMS values. The roughness of stainless steel surface (RMS: 42 nm) was about 10 times higher than that of glass (RMS: 0.49 nm).

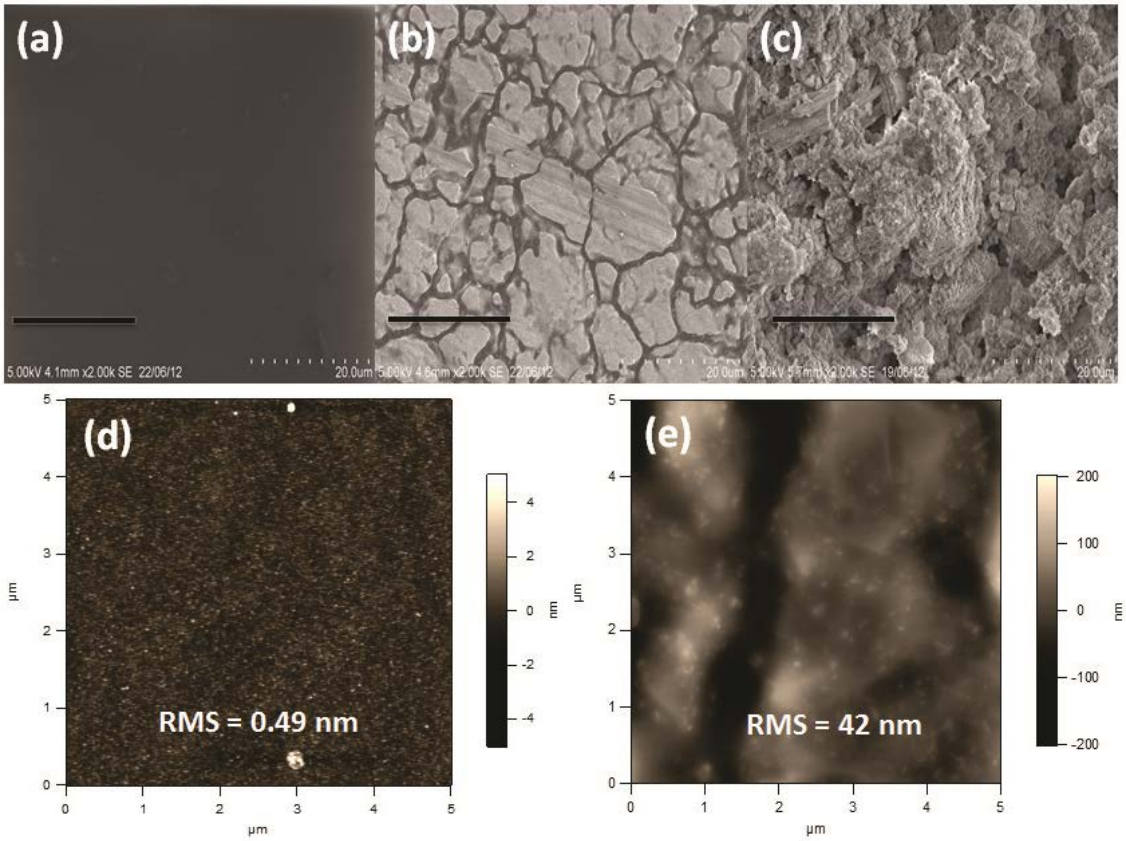


Figure 2a.4 Scanning electron microscopic image (scale bar: 20 µm) of (a) glass, (b) stainless steel and (c) hydroxyapatite, and atomic force microscopic images of (d) glass and (e) stainless steel.

2a.4 Discussion

Positive correlations were found between cell surface hydrophobicity and attachment to surfaces for the four Streptococcal strains used in this study but not for *A. naeslundii* ATCC 51655. This suggests that the tea extracts may have inhibited the attachment of

the Streptococci by reducing their surface hydrophobicity resulting in an increased surface tension between the cells and the substratum surface but a reduced surface tension between the cells and the liquid medium (aqueous) (van Oss et al., 1986). According to the colloidal thermodynamic theory hydrophobic interactions consist of Lifshitz van der Waals (LW) and Lewis acid-base (AB) (hydrogen bonding) interactions (van Oss et al., 1986). The extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theory was used to calculate the energies of the interactions [including LW, AB and electrostatic (EL) interactions] involved in the attachment systems in this study (Table 5a.2) and the results indicated that AB interaction energy dominated the overall energy in the case of attachment of all bacteria to all surfaces examined. Hydrogen bonding could therefore be the key mechanism for the attachment of the oral bacterial strains in the system used. Based on this assumption, the effects of the tea extracts on the attachment of the Streptococci could be due to interference with the hydrogen bonding of the bacteria with surfaces. A previous study found that tea components inhibited the attachment of *Streptococcus mutans* to surfaces by coating the cell surfaces with tannins, flavonoids and indolic compounds (Wang et al., 2013a). It is therefore suggested that a potential mechanism for the inhibitory effect of the tea extracts on Streptococci at the molecular levels is that the tea extracts block the hydrogen boding sites on the bacterial cell surfaces by coating them with tea components. This coating phenomenon had a less profound effect on cell surface charge and auto-aggregation than on hydrophobicity and could not detach cells from surfaces. The attachment of *A. naeslundii* ATCC 51655 to surfaces in the current study correlated more to cell surface charge than to hydrophobicity. This apparent correlation was not considered to be a valid one because the slopes of the regressions tended to be infinite [i.e. tea had no significant effect on the ZP of *A. naeslundii* ATCC 51655 ($p>0.05$; Figure 2a.1) but significantly inhibited their attachment ($p<0.05$; Figure 2a.2)]. Based on these observations there was deemed to be no significant correlation between the attachment of *A. naeslundii* ATCC 51655 and any of the cell surface properties. Similarly, no relationship was found between biofilm formation and any of the cell surface properties, suggesting that the mechanisms of

biofilm formation is far more complex than attachment and may involve many other factors. Biofilm formation by the bacteria tested was therefore not explainable by the cell surface properties used in this study.

Both attachment and biofilm formation were greater on hydroxyapatite than on the other two surfaces and this may be because hydroxyapatite surface was rougher than the other surfaces at a micron scale (Figure 2a.4) giving the cells a larger surface area to attach to and form biofilms on. Stainless steel was ten-fold rougher at a nano scale than glass. In general bacteria attached to stainless steel in higher numbers and formed less biofilm on it than on glass. This may indicate that the bacteria examined tend to attach more easily to a rougher surface but form biofilm more easily on a smoother one. This result contrasts with the findings of some other authors. For example Goulter et al. (2011) reported that *E. coli* attached in greater numbers to smoother stainless steel surfaces than to rougher ones, while Medilanski et al. (2002) found that four species of bacteria (*Desulfovibrio desulfuricans, Pseudomonas aeruginosa, Pseudomonas putida* and *Rhodococcus* sp. C125) attached in a greater number to both smoother and rougher stainless steel and in a minimal number to stainless steel surfaces with a medium roughness. There may therefore be other factors affecting attachment to hard surfaces in this study that were not measured. Medilanski et al. (2002), for example, suggested that the influence of hard surface roughness on bacterial attachment is associated with the size and shape of bacterial cells. It was also found that the type of hard surface did not influence the effects of tea extracts / pure compounds on bacterial attachment and biofilm formation. This suggests that in the attachment system used the tea extract / pure compound treatments only affected the bacterial cells and had no effect on the hard surfaces (e.g. the tea extracts / pure compounds did not coat the hard surfaces, a phenomenon which is usually not the case in the oral cavity).

Based on this *in vitro* study, tea extracts may have the potential to inhibit the colonization of oral pathogens on tooth surfaces and improve oral health. In addition,

the concentrations of tea extracts applied to the bacteria in this study (which were below the MIC levels) were lower than the concentration in a cup of fresh brewed tea. The concentration of compounds in fresh brewed tea, especially green tea and oolong tea, may reach levels inhibitory or lethal to many oral bacteria, such as *S. mutans* (Xu et al., 2012). It is possible that tea could kill or inhibit a portion of oral bacteria on tooth surfaces immediately after consumption and suppress colonization of the rest of the bacteria after being diluted by saliva to concentrations below the MIC levels. These suggestions need to be further confirmed by *in vivo* studies.

2a.5 Conclusions

In summary, this study found that tea extracts could inhibit attachment and biofilm formation by oral pathogenic bacteria on hydroxyapatite, glass and stainless steel surfaces. It suggested a potential mechanism of the inhibitory effect of tea on the attachment of Streptococcal strains, namely the ability of the tea extracts to reduce bacterial surface hydrophobicity. These findings were, however, not applicable to *A. naeslundii* ATCC 51655. This study also indicated that different types of hard surface result in different levels of bacterial attachment and biofilm formation but do not affect the inhibitory effects of the tea extracts.

Declaration for Thesis Chapter 2b

Declaration by candidate

In the case of Chapter 2b, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Sui Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidate's Signature	[redacted]	**Date** June 12, 2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University, Sunwasy Campus**

Signature 1		**Date** June 14, 2013
Signature 2		**Date** June 13, 2013

Chapter 2b Effect of Sucrose on Surface Properties of Oral Bacteria and Their Colonization of Hard Surfaces

2b.1 Introduction

Many oral bacteria can colonize tooth surfaces, resulting in the development of dental plaque and induction of dental caries (Weerkamp and Jacobs, 1982; Kolenbrander and London, 1993). Primary colonizers, such as oral Streptococci and *Actinomyces naeslundii*, synthesize water-insoluble, adhesive glucan from dietary sucrose by the enzymatic action of glucosyl transferase (Hamada et al., 1978; Sato et al., 1990; Ozek, 2011) and form early plaque which can be colonized by over 350 species of bacteria. Glucan synthesis can occur on the surfaces of bacteria cells and form a layer of glucan film coating the cells (Hamada et al., 1978). This cell-bound glucan can induce bacterial cellular aggregation (Vickerman and Jones, 1995) which enhances the ability of primary colonizers to adhere and grow on tooth surfaces (Kolenbrander and London, 1993). This may help to explain the ability of oral bacteria to attach to various abiotic surfaces including glass, steel wire, hydroxyapatite, and extracted and artificial teeth (Slade, 1976). Other cell surface physicochemical properties that influence bacterial colonization, such as hydrophobicity and charge (Goulter et al., 2009), may also be affected by cell-bound glucan.

In this study, five strains of primary colonizing bacteria were grown in culture medium with or without 2% sucrose. The effects of the addition of sucrose in the culture medium were investigated with respect to bacterial cell surface physicochemical properties (hydrophobicity, charge and auto-aggregation) and two colonization activities (attachment and biofilm formation) on hydroxyapatite (a tooth model; Apella et al., 2008) and two other abiotic surfaces (glass and stainless steel) with different surface properties.

2b.2 Materials and methods

2b.2.1 Bacterial cultures and growth conditions

Five bacteria of oral origin, namely *Streptococcus mutans* ATCC 25175, *Streptococcus mutans* ATCC 35668, *Streptococcus salivarius* ATCC 13419, *Streptococcus mitis* ATCC 49456 and *Actinomyces naeslundii* ATCC 51655, were obtained from the American Type Culture Collection (Manassas, USA) and used in this study. All bacteria were maintained on Mitis Salivarius Agar (MSA; Difco, USA) at 4°C and grown in Tryptic Soy Broth (TSB; Merck, USA) with or without 2% sucrose at 37°C for 24 h (48 h for *A. naeslundii* ATCC 51655) with shaking at 150 rpm. Bacterial suspensions were prepared by centrifuging 20 mL of TSB cultures at 7669 g for 15 min. The pellets were washed with 150 mM phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl, pH 7.4; 1st BASE, Singapore) and resuspended in 20 mL PBS for all experiments.

2b.2.2 Cell surface hydrophobicity measurements

The cell surface hydrophobicity was determined using Bacterial Attachment to Hydrocarbon (BATH) method as previously described by Rosenberg et al. (1980) with slight modification. Bacterial suspensions were adjusted to an optical density (OD) of 1.0 ± 0.2 at 550 nm. A 3 mL aliquot of each sample was mixed with 1 mL of hexane (Sigma-Aldrich, USA) and vortexed for 2 min. The mixture was allowed to separate for 1 h at 37°C. The OD_{550} of the aqueous layer was measured before (A_0) and after (A) addition of hexane. The cell surface hydrophobicity was expressed as % binding to hexane = $(1 – A/A_0) \times 100\%$.

2b.2.3 Cell surface charge measurements

Bacterial cell surface charge was measured as described by Bayoudh et al. (2009). A 1 ml aliquot of bacterial suspension at a cell density of 10^7 CFU/mL (pH 7.4) was measured for cell surface charge using a zetasizer (Nano ZS-ZEN3600; Malvern Instruments Ltd., UK). Cell surface charge was expressed as zeta potential (mV).

2b.2.4 Cellular auto-aggregation measurements

Auto-aggregation measurements were performed as described by Ellen and Balcerzak-Raczkowski (1977). A 1 mL aliquot of bacterial suspension was adjusted to an OD of 0.25 ± 0.05 at 600 nm prior to incubation at 37°C for 6 h. The OD_{600} was measured before (A_i) and after (A_f) incubation. Aggregation percentage was expressed as % Auto-aggregation = $(1 – A_f / A_i) \times 100\%$.

2b.2.5 Hard surface preparation

Hydroxyapatite surfaces were prepared by coating hydroxyapatite powder (Sigma-Aldrich) onto glass slides as previously described by Wang et al. (2013a). Glass slides (75 × 25 mm; Thermo Fisher Scientific, USA) were first coated with bonding adhesive (containing 60% limestone, 30% kaolin, 8% ethylene glycol, 1% SiO_2 and 1% TiO_2, %wt; Over Sea Plaster Supply and Construction Sdn. Bhd., Malaysia) mixed with distilled water at a ratio of 6:4 (wt:vol) and partially dried in air for 3 min. The slides were then coated with a paste consisting of 10% cement powder (containing 56% CaO, 40% SiO_2, 3% $Al_2O_3 \cdot Fe_2O_3$ and 1% MgO, %wt; Over Sea Plaster Supply and Construction Sdn. Bhd., Malaysia), 40% hydroxyapatite powder and 50% distilled water (%wt) followed by air drying for 16 h.

Glass (75 × 25 mm), stainless steel (75 × 25 mm; type 302, #4 finish) and hydroxyapatite slides were degreased by soaking in acetone for 30 min, rinsed in

distilled water and sterilized by autoclaving.

2b.2.6 Attachment assays

The attachment assays were carried out as previously described by Wang et al. (2013a). A 20 mL bacterial suspension containing 10^7 CFU/mL was incubated with a hard surface slide at 37°C for 30 min without shaking. After incubation the slide was removed from the suspension, gently washed three times with PBS to remove loosely attached cells and stained by 0.1% (wt/vol) crystal violet (for glass; Sigma-Aldrich) or 0.1% (wt/vol) acridine orange (for hydroxyapatite and stainless steel; Sigma-Aldrich). The attached cells were counted under a light or epifluorescence microscope (BX51; Olympus, Japan). A total of 50 fields were counted for each slide and the number of attached cells was calculated and expressed as log CFU/cm^2. The attached cells of *A. naeslundii* ATCC 51655 were morphologically not countable under a microscope and were therefore enumerated using a method adapted from Chia et al. (2011). After rinsing slides with *A. naeslundii* ATCC 51655 cells attached were placed in a stomacher bag (Gossenlin, France) containing 50 mL of PBS, and pummeled in a stomacher (400P, BigMixer®, France) for 10 min at a speed setting of 1 to remove the attached cells. The pummeled slides were stained and visualized under a microscope for 50 fields. No cells were observable and stomaching was therefore considered to be an effective means for the removal of attached cells. Aliquots PBS were serially diluted, spread plated on Tryptic Soy Agar (TSA; Merck) and quantified after 48 h incubation at 37°C. Chia et al. (2011) determined that attachment results obtained from the two methods were not significantly different.

2b.2.7 Biofilm formation assays

Biofilm formation assays were performed as described by Wang et al. (2013a). A 0.1 mL aliquot of 24 h TSB culture was inoculated into 20 mL of TSB and incubated statically with a hard surface slide at 37°C for 72 h. After incubation the slide was

washed three times with PBS and placed in a Falcon tube (TPP®, Switzerland) containing 50 mL of PBS. The tube was sonicated for 10 min using a water bath sonicator (LC-130H; ELMA, Germany) at room temperature at a frequency of 35 kHz to detach the biofilm cells into the surrounding PBS. An aliquot of the PBS was serially diluted, spread plated on TSA and incubated at 37°C for 48 h prior to enumeration.

2b.2.8 Statistical analysis

All assays were carried out in triplicate with independently grown cultures. A Student's *t*-test was performed on all data sets at a 95% confidence level using SPSS software (PASW Statistics 18; SPSS Inc.).

2b.3 Results and discussion

2b.3.1 Effect of sucrose on cell surface hydrophobicity, charge and auto-aggregation

The effects of sucrose on the cell surface properties of the five strains studied are shown in Figure 2b.1. It was found that all strains were hydrophobic (as defined by Gibbons and Etherden, 1983), ranging from 60 to 90% adhesion to the hydrocarbon. The addition of sucrose in the culture medium significantly reduced ($p<0.05$) the cell surface hydrophobicity (Figure 2b.1a) of the two *S. mutans* strains and the *S. salivarius* strain by 62, 52 and 44%, respectively. The addition of sucrose significantly increased ($p<0.05$) the hydrophobicity of the *A. naeslundii* strain by 31% but had no effect ($p>0.05$) on that of the *S. mitis* strain. All strains were negatively charged (Figure 2b.1b) with zeta potentials ranging from -4 to -12 mV. The net charges of the strains were not affected ($p>0.05$) by the addition of sucrose to the culture medium. All strains exhibited moderate to relatively high auto-aggregation (as defined by Ellen and Balcerzak-Raczkowski, 1977) (Figure 2b.1c), ranging from 22 to 45%. The addition of sucrose to the culture medium significantly reduced ($p<0.05$) the auto-aggregation of *S. mutans* ATCC 25175 by 13%, significantly increased ($p<0.05$) that of the *S. mitis* strain

by 21% and had no effect ($p>0.05$) on that of the other three strains.

The effect of the formation of cell-bound glucan on cell surface hydrophobicity might be due to the hydrophilic nature of glucan, which reduces the surface tension between cells and the aqueous medium resulting in a reduced hydrophobicity (Van Oss et al., 1986). This was, however, not the case for the *S. mitis* and *A. naeslundii* strains suggesting that cell surface hydrophobicity is not solely dependent on surface tension. Based on the assumption that glucan can affect surface tension the reduced auto-aggregation seen in this study was expected. Vickerman and Jones (1995) on the other hand indicated that synthesis of extracellular glucan enhances cellular aggregation of dental plaque associated bacteria. The changes in auto-aggregation observed in this study however did not have a uniform pattern for all five strains. It was also found that glucan did not affect cell surface charge of the strains tested. Based on the soft particle theory (Ohshima, 2009) this suggests that the glucan film on the cell surfaces was ion-penetrable and did not carry charge.

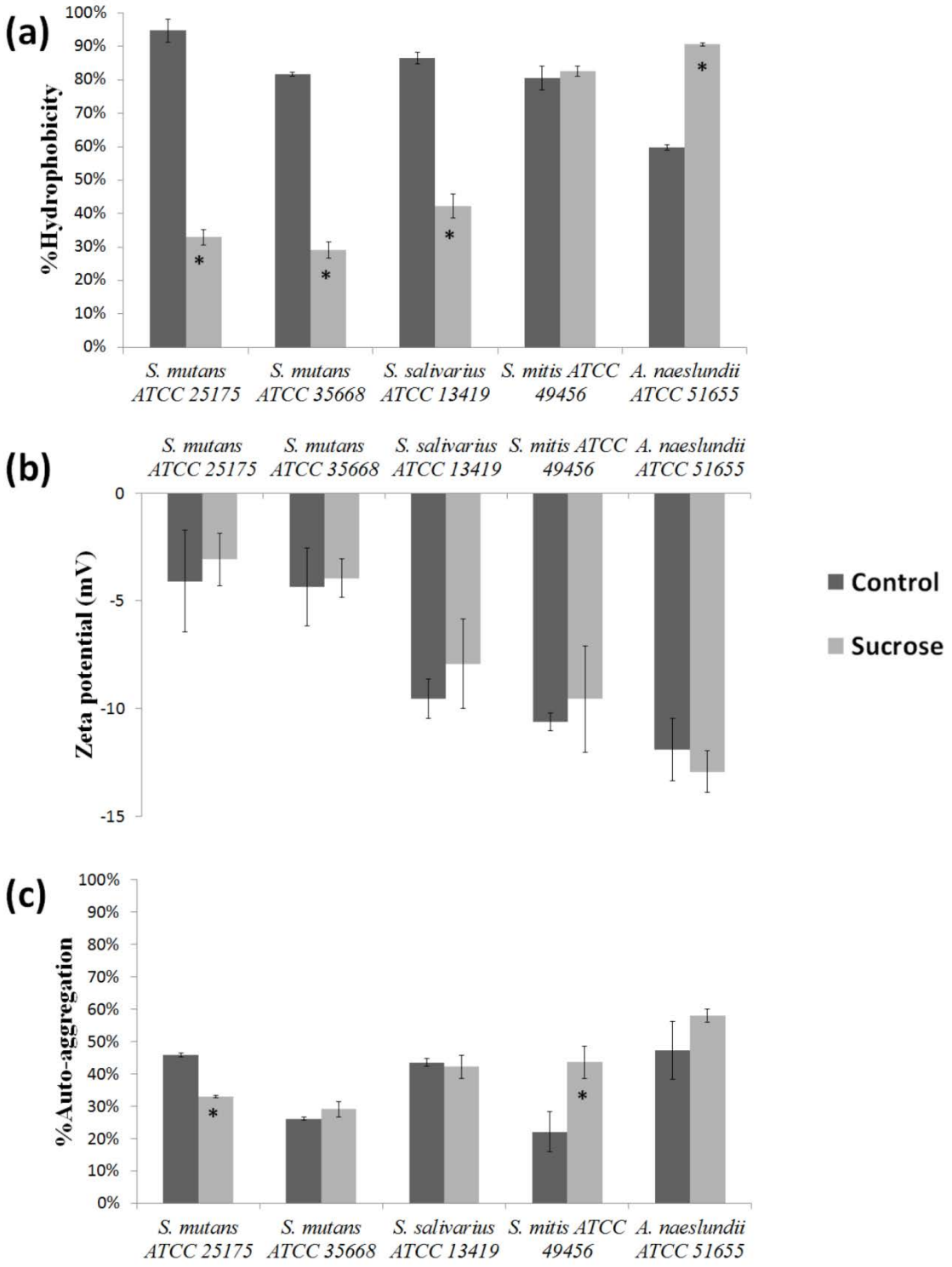


Figure 2b.1 The effects of sucrose on (a) surface hydrophobicity, (b) charge and (c) auto-aggregation of five bacterial strains of oral origin. All results are presented in mean ± SD with n = 3. Values labeled with the * symbol are significantly different from the control ($p<0.05$).

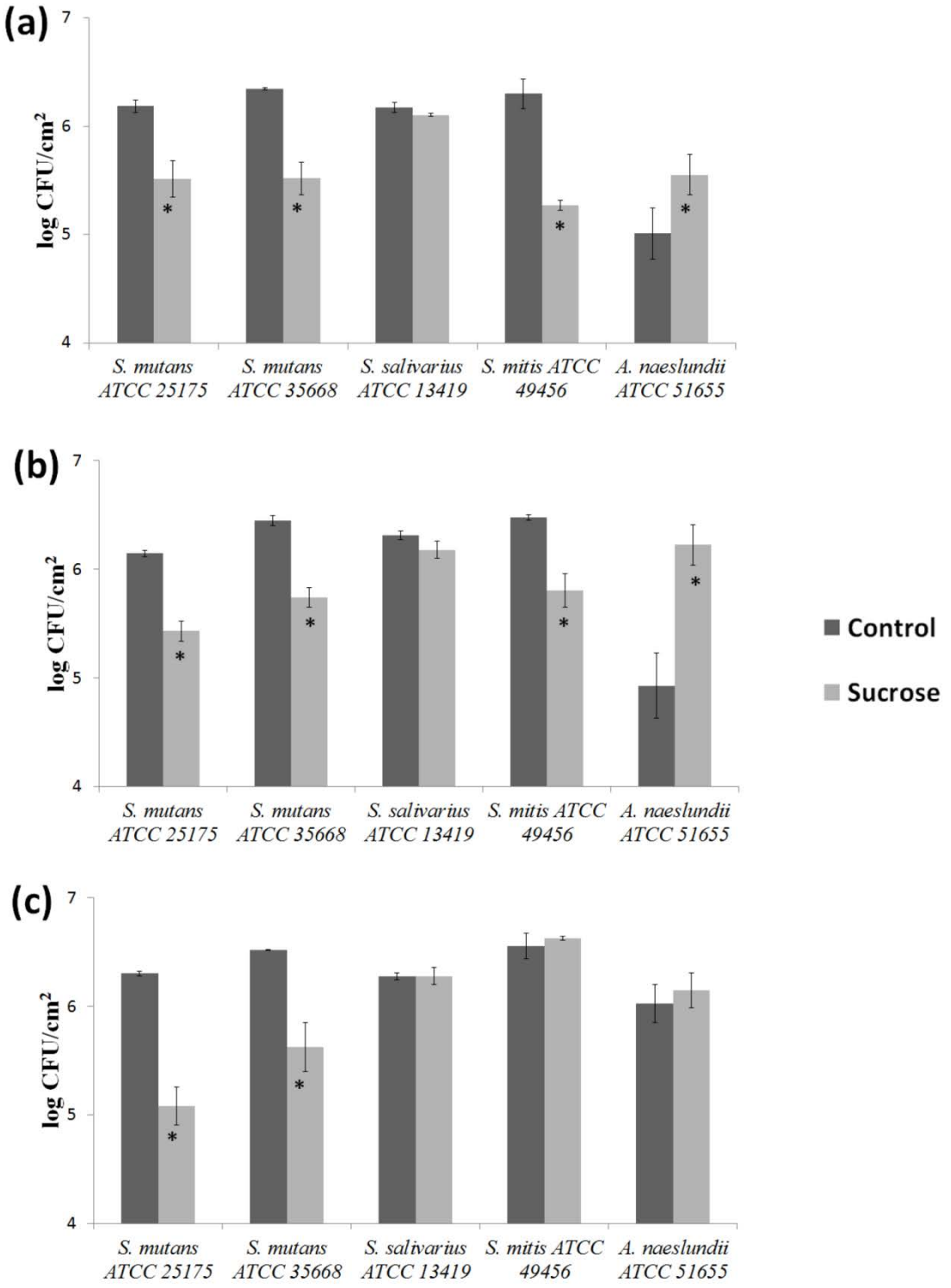


Figure 2b.2 The effects of sucrose on attachment to (a) glass, (b) stainless steel and (c) hydroxyapatite of five bacterial strains of oral origin. All results are presented in mean ± SD where n = 3. Values labeled with the * symbol are significantly different from the control ($p<0.05$).

2b.3.2 Effect of sucrose on bacterial attachment

The effects of sucrose on the attachment of the five bacterial strains to hydroxyapatite, glass and stainless steel are presented in Figure 2b.2. The four Streptococci attach to the hard surfaces in similar numbers to each other ranging from 6.1 to 6.5 log CFU/cm^2. The *A. naeslundii* strain attached in significantly lower ($p<0.05$) numbers (~5.5 log CFU/cm^2) than the Streptococci did. The addition of sucrose to the culture medium reduced ($p<0.05$) the attachment of the two *S. mutans* strains and *S. mitis* strain by 0.6 to 1.3 log CFU/cm^2 to all of the three hard surfaces, except for the attachment of the *S. mitis* strain to hydroxyapatite ($p>0.05$). The addition of sucrose to the culture medium also enhanced ($p<0.05$) the attachment of the *A. naeslundii* strain to glass and stainless steel by 0.5 and 1.3 log CFU/cm^2, respectively. It had no effect ($p>0.05$) on the attachment of *S. salivarius* strain to all surfaces.

It is generally agreed in the literature that cell-bound glucan enhances the level and strength of oral bacterial attachment (Marshall et al., 1971; Vickerman and Jones, 1995). The results in this study, however, showed the opposite for three strains. Mukasa and Slade (1973) indicated that the attachment of *S. mutans* to glass surfaces could only be increased by post-formed glucan but not pre-formed glucan. Clark and Gibbsons (1977) reported a reduction in the attachment of a *S. mutans* strain grown in sucrose condition to hydroxyapatite, a finding which is in agreement to this study. This could be due to the inability of free Streptococcal cells coated with glucan films to adopt the ideal steric arrangement and strongly interact with the substratum surface (Clark and Gibbsons, 1977) and that an 'active' form of glucan is required to enhance attachment (Mukasa and Slade, 1973). Cell-bound glucan may also block the attachment sites on the cell surfaces and result in reduced attachment (Vickerman and Jones, 1995). The *S. salivarius* may have behaved differently to the other strains due to its larger cell size as compared to other oral Streptococci (Sherman et al., 1943). The *A. naeslundii* strains may have behaved differently to the other strains due to its clumped and filamentous morphology (Coleman et al., 1969) which may affect attachment. In addition,

cell-bound glucan had a different effect on the attachment of the *S. mitis* and *A. naeslundii* strains to hydroxyapatite as compared to the other two abiotic surfaces. This may be due to the enlargement of cell size by the glucan film resulting in a different spatial arrangement of cells on hard surfaces displaying differences in roughness (Medilanski et al., 2002).

2b.3.3 Effect of sucrose on biofilm formation

The effects of sucrose on biofilm formation by the five strains on hydroxyapatite, glass and stainless steel are shown in Figure 2b.3. Both *S. mutans* strains formed a significantly greater ($p<0.05$) amount of biofilm on hydroxyapatite (6.6 to 7.1 log CFU/cm^2) than on the other two surfaces tested (5.2 to 5.8 log CFU/cm^2). The biofilm formed by the two *S. mutans* strains on hydroxyapatite were also significantly greater ($p<0.05$) than the biofilm formed by the other stains on all surfaces (4.7 to 5.6 log CFU/cm^2). The addition of sucrose to the culture medium significantly enhanced ($p<0.05$) biofilm formed by the two *S. mutans* strains, the *S. salivarius* strain and the *A. naeslundii* strain on all of the surfaces by 0.4 to 1 log CFU/cm^2. It had no effect ($p>0.05$) on biofilm formation by the *S. mitis* strain.

It was reported by many authors that cell-bound glucan enhanced biofilm formation by oral bacteria (Abbott et al., 1983; Lynch et al., 2007) which concurs the finding of this study. This was probably because the glucan was formed after attachment in the assays used and is due to the 'sticky' nature of glucan (Jordan and Keyes, 1966) that 'glued' the cells to the hard surfaces. Bofilm formation by the *S. mitis* strain was not enhanced by glucan and this could be due to the fact that the amount of glucan produced by *S. mitis* is approximately 10 fold lower as compared to other oral bacteria (Hamada et al., 1978).

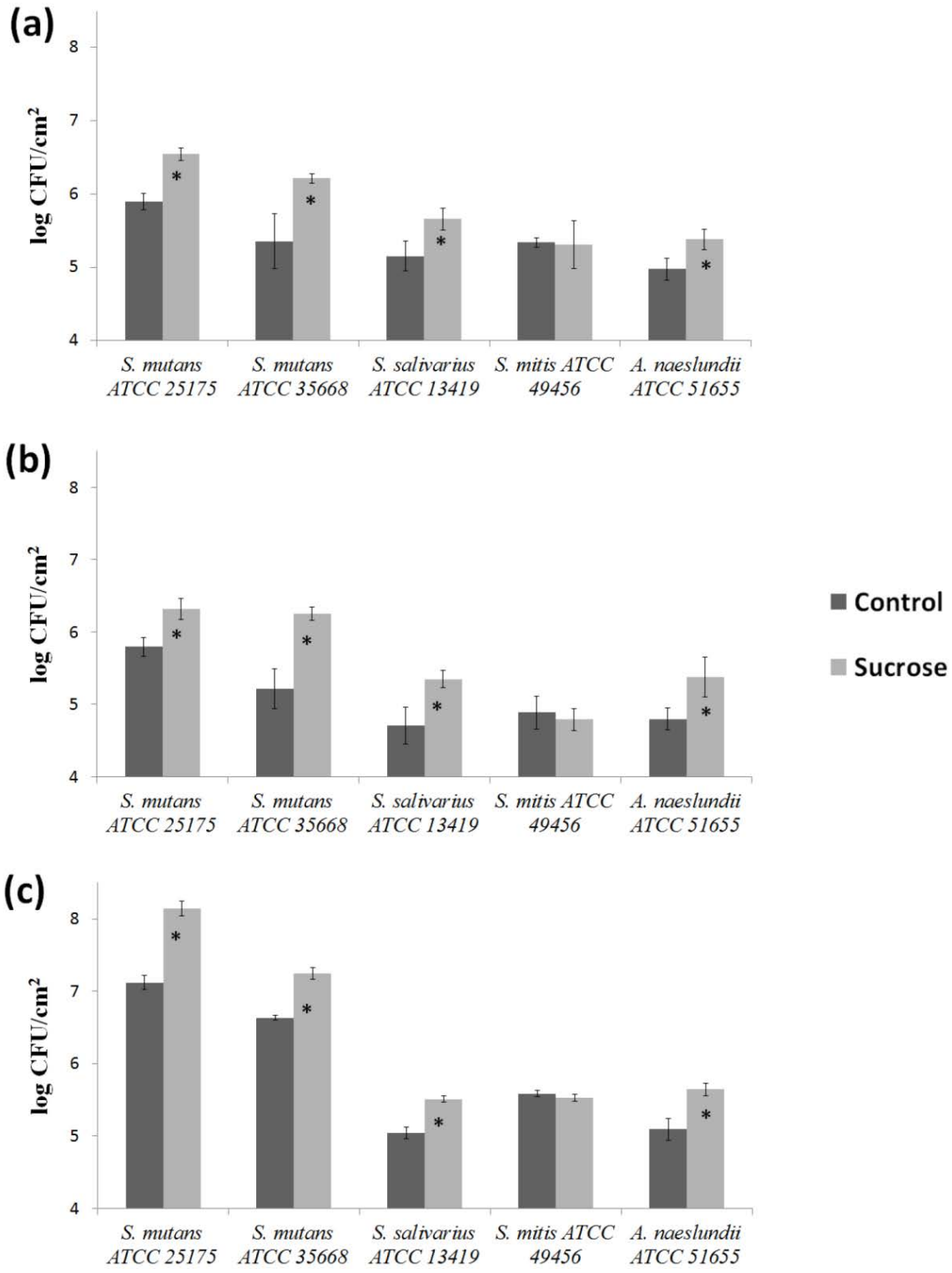


Figure 2b.3 The effects of sucrose on biofilm formation on (a) glass, (b) stainless steel and (c) hydroxyapatite of five bacterial strains of oral origin. All results are presented in mean ± SD where n = 3. Values labeled with the * symbol are significantly different from the control ($p<0.05$).

2b.4 Conclusions

This study found that the synthesis of cell-bound glucan can affect cell surface physical properties, colonization behavior on abiotic surfaces of some dental plaque forming-associated bacteria but not all of them. There was, however, no clear relation between the changes in cell surface properties induced by sucrose and the changes in the colonization behavior. This suggests that the effects of glucan on bacterial attachment and biofilm formation were strain-dependent and were not purely due to the physical influence of glucan on the cells. It also indicates that glucan on cell surfaces does not always enhance bacterial colonization. In conclusion, the role that sucrose plays in oral bacterial colonization may be dependent on the species, the strain of bacteria and the form of the glucan synthesized. Consumption of dietary sucrose may therefore not always be harmful to dental health but this finding needs to be further confirmed by *in vivo* studies.

Declaration for Thesis Chapter 2c

Declaration by candidate

In the case of Chapter 2c, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Sui Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidate's Signature	[redacted]	**Date** June 12, 2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University, Sunwasy Campus**

Signature 1	[redacted]	**Date** June 14, 2013
Signature 2	[redacted]	**Date** June 13, 2013

Chapter 2c Effect of Saliva as a Suspending Fluid on Attachment of Oral Bacteria to Hydroxyapatite

2c.1 Introduction

The attachment of oral bacteria to the surface of teeth initiates biofilm formation and a buildup of dental plaque which eventually causes dental caries (Marsh, 1994). It has been reported that conditions in the oral cavity can influence bacterial attachment (van Houte and Green, 1974; Whittaker et al., 1996). The presence saliva, for example, is a factor that can influence oral bacterial attachment to surfaces due to the interactions between salivary components and bacterial cell surfaces (Gibbons and Qureshi, 1978; Whittaker et al., 1996).

Studies using saliva coated hydroxyapatite as a substratum for oral bacterial attachment have found that the coating by saliva enhanced the attachment of oral bacteria to hydroxyapatite (Gillece-Castro et al., 1991; Ligtenberg et al., 1992). Studies using salivary components instead of whole saliva as a supplemental material to cell suspensions, however, have found that the salivary components inhibited the attachment (Williams and Gibbons, 1975; Murray et al., 1992).

Studies have often used saliva as a coating material on hydroxyapatite to model bacterial attachment in the oral cavity (Otake et al., 1991; Lee et al., 1992; Sharma et al., 1993; Vacca-Smith and Bowen, 1998; Matsumoto et al., 1999; Daglia et al., 2002) but seldom consider the influence of saliva as a suspending fluid for bacteria. In this study the influence of saliva as a suspending fluid on the attachment of five oral bacteria to hydroxyapatite (a tooth model surface) (Apella et al., 2008) was studied.

2c.2 Materials and methods

2c.2.1 Bacterial cultures and growth conditions

Five oral bacterial strains (*Streptococcus mutans* ATCC 25175, *Streptococcus mutans* ATCC 35668, *Streptococcus salivarius* ATCC 13419, *Streptococcus mitis* ATCC 49456 and *Actinomyces naeslundii* ATCC 51655) obtained from the American Type Culture Collection (Manassas, USA) were used in this study. All strains were maintained on Mitis Salivarius Agar (MSA; Difco, USA) at 4°C, and grown in Tryptic Soy Broth (TSB; Merk, USA) at 37°C for 24 h (48 h for *Actinomyces naeslundii*) with shaking at 150 rpm. Bacterial suspensions were prepared by centrifuging 20 mL of TSB cultures bacterial cultures at 7669 g for 15 min and resuspending the pellet in 20 mL of 150 mM phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 150 mM NaCl, pH 7.4; 1st BASE, Singarpore) or saliva (prepared as described below).

2c.2.2 Saliva collection and preparation

Saliva was collected from 10 adult donors (age from 20 to 25) and stored at 4°C before treatments. Saliva suspending fluid was prepared following the method described by Matsumoto et al. (1999) with modifications. Briefly, saliva was centrifuged at 12,000 g for 10 min to eliminate the suspended particles, heated at 60°C for 30 min to inactivate enzymes, and centrifuged again at 110,000 g for 10 min. The supernatant was filter sterilized (0.2 μm) and stored at 4°C for further use.

2c.2.3 Bacterial attachment to hydroxyapatite assays

Bacterial attachment to hydroxyapatite was measured as previously described by Wang et al. (2013a) with modifications. Briefly, hydroxyapatite slides (75×20mm; Clarkson Chromatography Products Inc., USA) were immersed and incubated at 37°C for 30 min in 20 mL of a saliva suspension of bacterial cells (containing ~10^7 CFU/mL) or in a

PBS suspension as a null control. The slides were then removed from the suspension, gently rinsed three times with PBS, placed in a stomacher bag (Gossenlin, France) containing 50 mL of PBS and pummeled in a stomacher (400P, BigMixer®, France) for 10 min at a speed setting of 1. An aliquot of the stomacher liquid was serially diluted, plated on Tryptic Soy Agar (TSA; Merk), and incubated for 48 h at 37°C before enumeration. The numbers of attached cells were expressed as log CFU/cm^2.

2c.2.4 Statistical analysis

All assays were performed in triplicate with independently grown cultures. A Student's *t*-test was performed on all data sets using SPSS software (PASW Statistics 18; SPSS Inc.) at a 95% confidence level.

2c.3 Results and discussion

The effects of saliva as a suspending fluid on the attachment of oral bacteria to hydroxyapatite are shown in Figure 2c.1. The numbers of bacteria suspended in saliva that attached to hydroxyapatite were significantly lower ($p<0.05$; by 1.4 to 1.7 log CFU/cm^2) than for bacteria suspended in PBS for the four Streptococcal strains. In the oral cavity a cell density of up to 10^4 cells/mL is required for bacteria suspended in saliva to initiate attachment to tooth surfaces (van Houte and Green, 1974). High molecular weight saliva components, such as salivary mucin, may inhibit the attachment of Streptococci to teeth by interrupting the interactions between bacterial cell surfaces and tooth surfaces (Williams and Gibbons, 1975; Gibbons and Qureshi, 1978; Murray et al., 1992). Those components can bind to bacterial surfaces and occupy the receptor sites for attachment (Murray et al., 1992). For example, cell surface receptors of many species of oral Streptococci can be saturated by a group of free salivary components known as blood-group-reactive glycoproteins (Williams and Gibbons, 1975; Koga et al., 1990). In addition, wall-associated protein A (WapA) on the surfaces of *Streptococcus*

mutans and *Streptococcus salivarius* cells has great binding ability to salivary components (Han et al., 2006).

The numbers of *Actinomyces naeslundii* cells suspended in saliva that attached to hydroxyapatite were not significantly different ($p>0.05$) from that of the cells suspended in PBS (Figure 2c.1). By contrast to the attachment of Streptococci, which is largely mediated by cell surface receptors, the attachment of *Antinomyces naeslundii* is usually mediated by fimbriae (Whittaker et al., 1996) and therefore is unlikely to be affected by salivary components.

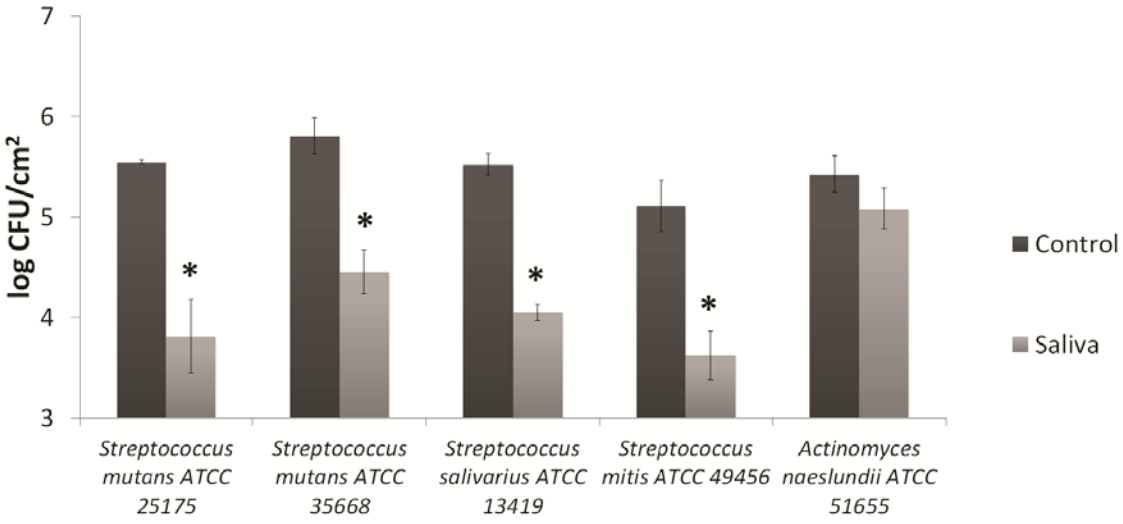


Figure 2c.1 Effect of saliva as a suspending fluid on attachment of five oral bacteria to hydroxyapatite. All results are presented in mean ± SD where n = 3. Values labeled with the * symbol are significantly different from the control ($p<0.05$).

As mentioned above, glycoproteins were found to inhibit oral bacterial attachment to surfaces. Some authors, however, have reported that immobilized glycoproteins (used as a coating material) promoted the attachment of many oral bacterial species, including *Streptococcus*, *Actinomyces* and *Fusobacterium*, to hydroxyapatite (Clark et al., 1989; Kishimoto et al., 1989; Gillece-Castro et al., 1991; Ligtenberg et al., 1992). This suggests that saliva components immobilized on hydroxyapatite may serve as receptors for dental surfaces to attract oral bacteria and therefore promote oral bacterial

attachment. This explains the results from previous studies that show that saliva coatings on hydroxyapatite promoted oral bacterial attachment to surfaces but salivary components as a supplemental material to cell suspensions inhibited the attachment (Williams and Gibbons, 1975; Gillece-Castro et al., 1991; Ligtenberg et al., 1992; Murray et al., 1992). As compared to coating saliva on hard surfaces only, using saliva as a suspending fluid as well may represent a more realistic model of the situation in the oral cavity for studying oral bacterial attachment.

2c.4 Conclusions

In summary, this *in vitro* study using saliva as a suspending fluid for oral bacteria indicates that the reported binding ability of salivary components to oral bacterial cell surfaces may not promote but inhibit oral bacterial attachment to hydroxyapatite. This suggests that saliva could be of benefit to dental health with respect to inhibiting attachment of oral bacteria. The finding also suggests that future studies investigating the role of saliva in oral bacterial attachment to surfaces should use saliva as the suspending fluid for bacterial cells as well as a coating material for hard surfaces. This method is, however, limited by the difficulty in collecting a large amount of saliva. These findings also need to be further confirmed by *in vivo* studies.

Chapter 3

Effect of Tea Extracts on Attachment of Oral Bacteria to Cultured Human Gingival Fibroblasts

The work presented in this chapter represents a peer reviewed publication:

Wang, Y., Chung, F.F.L., Lee, S.M. and Dykes, G.A. (2013) Inhibition of attachment of oral bacteria to immortalized human gingival fibroblasts (HGF-1) by tea extracts and tea components. *BMC Research Notes* **6**, 143-147.

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Felicia Chung	FC was responsible for 2% of writing and the review of the publication.	2%
Sui Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 6% of writing and the review of the publication.	6%

Candidate's Signature	[redacted]	**Date** June 12, 2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University, Sunwasy Campus**

Signature 1	[redacted]	**Date** June 12, 2013
Signature 2	[redacted]	**Date** June 14, 2013
Signature 3	[redacted]	**Date** June 13, 2013

3.1 Introduction

Oral Streptococci such as *Streptococcus mutans* are pathogens commonly associated with dental plaque and the formation of caries (Hamilton-Miller, 2001). In order to initiate disease these bacteria must attach to components of the oral cavity such as the enamel, tongue, saliva or gums (Kolenbrander and London, 1993).

Plant extracts and phytochemicals can inhibit bacterial attachment to abiotic and biotic surfaces by altering cell surface properties including hydrophobicity, surface charge and the presence of structures such as flagella (Sklodowska and Matlakowska, 1998; Nostro et al., 2004; Goulter et al., 2009). Tea is one such potential attachment inhibitor (Tagashira et al., 1997). Non-fermented teas or partially-fermented teas, such as green tea and oolong tea, have strong bactericidal activity and may inhibit bacterial attachment to some elements of the gastrointestinal tract (Otake et al., 1991; Yoshino et al., 1996; Rasheed et al., 1998; Xu et al., 2011; Lee et al., 2006). Fully fermented teas, such as black tea and pu-erh tea, have less effective bactericidal activity but may inhibit attachment of bacteria to dental plaque (Friedman, 2007).

Previous studies investigating bacterial attachment and inhibition by phytochemicals to components of the oral cavity have focused on attachment to hard surfaces such as enamel (Clark and Gibbons, 1977; Abbott et al., 1983; Xu et al., 2011). Attachment of bacteria to soft tissues in the mouth can also initiate disease and for this reason we investigated the effects of tea extracts and tea components on attachment of oral pathogenic bacteria to an immortalized line of connective gingival fibroblasts *in vitro*.

3.2 Materials and methods

3.2.1 Bacteria and growth conditions

Five strains of bacteria, namely *Streptococcus mutans* (ATCC 25175), *Streptococcus mutans* (ATCC 35668), *Streptococcus mitis* (ATCC 49456), *Streptococcus salivarius* (ATCC 13419) and *Actinomyces naeslundii* (ATCC 51655), were selected for this study and obtained from the American Type Culture Collection (Manassas, USA). All bacteria were maintained on Mitis Salivarius Agar (MSA; Difco, USA) at 4°C and grown in Tryptic Soy Broth (TSB; Merck, USA) at 37°C for 24 h with shaking at 150 rpm for all experiments. Bacterial suspensions were prepared by centrifuging 20 mL of TSB cultures at 7669 g and 4°C for 15 min, washing the resultant pellet gently with phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na2HPO4, 17 mM KH2PO4, 137 mM NaCl, pH 7.4; 1st BASE, Singapore) and resuspending it in 20 mL PBS, tea extract solutions or tea component solutions prepared as described below.

3.2.2 Preparation of tea extracts and tea components

Commercial green tea, oolong tea, black tea, pu-erh tea and chrysanthemum tea (Ten Ren Tea Co. Ltd., Taiwan) extracts were prepared using 90% acetone (Sigma Aldrich, USA) at the ratio of 1:20 (wt/vol) for 2 h. The resultant extracts were evaporated under vacuum at 40°C, freeze dried and stored at -20°C until further use. Using this method reportedly allows for extraction of more than 95% of the phenolic compounds in tea, including catechins, myricetin, quercetin and kaempherol (Perva-Uzunalić et al., 2006). Epigallocatechin gallate [EGCg; 95% (vt/vt); Sigma-Aldrich] and gallic acid (Sigma-Aldrich) were also used as they are major phenolic components of teas. Specifically, EGCg constitutes approximately 10% of the dry weight of green tea and its level decreases with increasing degree of fermentation (Peterson et al., 2005). Levels of gallic acid, on the other hand, increase with fermentation and constitute approximately 0.5% dry weight of black tea (Cabrera et al., 2003). The stock solutions for all

experiments were prepared by dissolving 100 mg of tea extracts or tea component in 10 mL PBS containing 1% (vol/vol) methanol (Systerm, UK) and the resultant solutions were filter sterilized though a 0.2 μm filter (Millipore, USA).

3.2.3 Determination of total phenolic, total tannin and total flavonoid content

Total phenolic and total tannin contents of the tea extracts were determined using the Folin-Ciocalteau colorimetric method (Megat et al., 2012). To determine the total phenolic content, a 15 μL tea extract solution (1 mg/mL) was added to 80 μL of 7.5% (wt/vol) sodium carbonate (R&M Chemicals, Malaysia) and 75 μL of 10% (vol/vol) Folin-Ciocalteau reagent (R&M Chemicals) in a well of a microtitre plate (Jet Biofil, China). The plate was incubated in the dark for 30 min before measuring the absorbance at 765 nm. To determine the total tannin content, 0.5 mL of the sample solution was mixed with 0.5 mL of distilled water and 50 mg of poly(vinylpolypyrrolidone) (PVPP; Sigma-Aldrich) which has a high affinity to tannins. The mixture was vortexed, incubated at 4°C for 15 min and vortexed again prior to centrifuging at 1409 g for 10 min in order to remove tannins. The supernatant containing non-tannin phenolics was then quantified using the Folin-Ciocalteau method described above. The difference between the total phenolic content and the non-tannin phenolic content is the measure of tannins. A standard curve was plotted using gallic acid, and the total phenolic and total tannin contents were expressed as μg gallic acid equivalent (GAE)/mg.

Total flavonoid content was measured using the aluminum chloride colorimetric method (Chang et al., 2002). A 50 μL aliquot of tea extract sample dissolved in methanol (1 mg/mL) was added to 10 μL of 10% (wt/vol) aluminum chloride (Bendosen, Malaysia), 10 μL of 1 M potassium acetate (R&M Chemicals) and 80 μL of distilled water in the wells of a microtitre plate. The plate was incubated at room temperature for 30 min before the absorbance was measured at 435 nm. The blank was prepared using distilled water in place of aluminum chloride. A standard curve was plotted using quercetin (Sigma-Aldrich) and the total flavonoid content was expressed as μg quercetin

equivalent (QE)/mg.

3.2.4 Cell culture

Immortalized human gingival fibroblast-1 HGF-1 (ATCC CRL-2014) were obtained from the American Type Culture Collection and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 4 mM L-glutamine (Sigma-Aldrich) and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich). No antibiotic supplement was used. Cells were incubated at 37°C in 5% CO_2 atmosphere, fed every 48 h and routinely sub-cultured every 5 days with a split ratio of 1:3 using 1 × trypsin-EDTA (0.05%; Sigma-Aldrich) for 3 min at 37°C.

3.2.5 Bacterial attachment assay

Bacterial attachment assays were as described by Mellor et al. (2009) with some modifications. Briefly, monolayers of HGF-1 cells were grown in 24-well tissue culture plates (Jet Biofil) to a density of 1.8 (±0.2) × 10^5 cells per well (approximately 100% coverage). Prior to the attachment assay the culture medium in each well was removed and the cell monolayer was washed with PBS. The monolayer was incubated at 37°C for 30 min with 2 mL aliquots of tea extracts or tea components (PBS as control) containing suspended bacteria (~1 × 10^7 CFU/mL). The concentrations of the tea extracts and compounds used to suspend bacteria were previously determined by antimicrobial susceptibility assays and cytotoxicity assays to not kill or inhibit the bacteria or the HGF-1 cells at the concentrations used in this study (Table 2a.1 and Appendix III). After incubation the supernatant in each well was removed and the wells were washed three times with 2 mL PBS. The monolayer with bacteria attached was then detached by incubating with 400 µL 0.3 × trypsin-EDTA (at which concentration trypsin does not kill or inhibit the bacteria) at 37°C for 5 min. The detached bacteria were then serial diluted, spread plated on Tryptic Soy Agar (TSA; Merck) and

quantified after 24 h incubation. The ability of bacteria to attach to wells without HGF-1 cells was also determined in order to ensure that the bacteria attached to HGF-1 cells but not to the plastic material of the plate. The numbers of bacteria attached to the cell line was expressed as log CFU/well.

3.2.6 Statistical analysis

A one way ANOVA (Tukey's comparison) was performed on all data sets using MINITAB software (MINITAB 15.1; Minitab Inc., USA) at a 95% confidence level. All assays were performed in triplicate with independently grown cultures.

3.3 Results and discussion

The results of total phenolic, total tannin and total flavonoid content assays are presented in Table 3.1. The total phenolic and total flavonoid content decreased and total tannins increased, with an increasing degree of fermentation from green tea to oolong tea to black tea to pu-erh tea. These differences between teas are probably due to the polymerization of flavonoids (especially flavon-3-ols) into large molecule polyphenols (tannins) which occur during the fermentation process (Peterson et al., 2005). Chrysanthemum tea, which is a blend of black tea and dried chrysanthemum, had similar levels of total phenolic and total tannin to pu-erh tea and similar levels of flavonoids to green tea. This suggests that dried chrysanthemum is rich in flavonoids.

Baseline data for attachment of the bacterial strains to the cell line and empty wells are shown in Table 3.2. Bacterial attachment to the cell line was ~2 log higher ($p<0.05$) than that to the plastic in the wells indicating that 90% to 99% of bacteria were attached to the cell line and validating the assay.

The results of assays investigating the effect of the tea extracts and tea components on bacterial attachment to the cell line are presented in Figure 3.1. All strains exhibited a similar ability to attach to the cell line except for *Streptococcus salivarius* ATCC 13419 which attached in significantly lower numbers as compared to *Streptococcus mitis* ATCC 49456 ($p<0.05$). Green tea extracts, oolong tea extracts and black tea extracts inhibited the attachment of *Streptococcus mitis* ATCC49456 by between ~1 and ~2 log CFU/well (90-99% attachment inhibition; $p<0.05$), but had no effect on the other strains ($p>0.05$). Pu-erh tea extracts and chrysanthemum tea extracts, on the other hand, reduced the attachment of all *Streptococcus* strains to cells by between ~2 and ~4 log CFU/well (99–99.99% attachment inhibition; $p<0.05$). The attachment of *Actinomyces naeslundii* ATCC 51665 to cells was not affected by any of the tea extracts tested ($p>0.05$). Of particular note is that the extract of chrysanthemum tea, which, as mentioned above, is a blend of black tea and dried chrysanthemum, had a greater ($p<0.05$) effect on inhibiting attachment than the black tea extract alone. This suggests that the active compounds in the chrysanthemum tea extract were contributed by the chrysanthemum components and not the black tea components of the mix. Pu-erh tea and chrysanthemum tea extracts, which were found to contain relatively higher levels of tannin, had a greater effect ($p<0.05$) than the non-fermented or partially-fermented tea extracts on *Streptococcus* strains, suggesting that Streptococci may be more sensitive to polymeric flavonoids or other large molecule polyphenols with respect to their attachment to HGF-1.

Table 3.1 Total phenolic, total tannin and total flavonoid contents of the tea extracts.

	Total phenolic content (μg GAE / mg)	Total tannin content (μg GAE / mg)	Total flavonoid content (μg QE / mg)
Green tea	527±34 (a)	149±26 (a)	7.30±0.68 (a)
Oolong tea	469±28 (a, b)	161±35 (a, b)	4.89±0.14 (b)
Black tea	411±20 (b, c)	241±19 (b, c)	2.97±0.59 (c)
Pu-erh tea	349±35 (c)	305±34 (c)	1.68±0.68 (c)
Chrysanthemum tea	376±13 (c)	280±8 (c)	7.61±0.42 (a)

All results are presented as the means followed by SDs. Values labeled with the same letter are not significantly different ($p>0.05$) among the tea extract samples. Tukey's comparisons were conducted separately for each assay.

Table 3.2 Baseline data for bacterial attachment to the HGF-1 cell line and empty wells.

	Mean±SD attachment (log CFU/well)				
	Streptococcus mutans ATCC25175	*Streptococcus mutans* ATCC35668	*Streptococcus salivarius* ATCC13419	*Streptococcus mitis* ATCC49456	*Actinomyces naeslundii* ATCC51655
Attachment to cell line	4.67±0.25	4.75±0.35	3.68±0.77	5.11±0.14	4.68±0.34
Attachment to empty wells	2.37±0.18	2.38±0.27	2.14±0.49	2.34±0.47	2.19±0.08

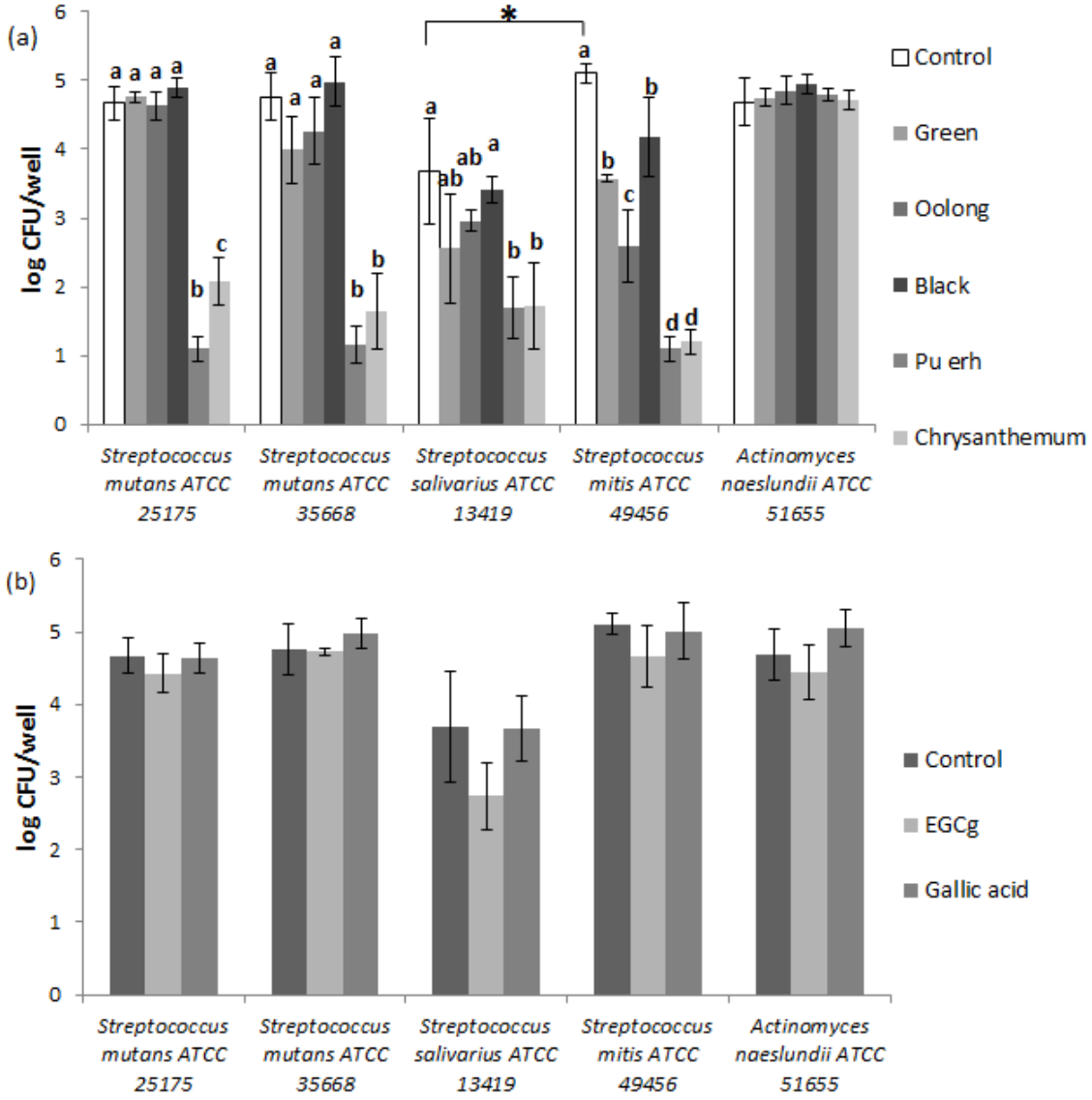


Figure 3.1 Effect of extracts and compounds on attachment of oral bacteria. Effect of tea extracts (a) and EGCg and gallic acid (b) on attachment of five oral bacteria to HGF-1 gingival cell line (log CFU/well, n=3). Values labeled with the same letter are not significantly different ($p>0.05$) among the treatments within a strain. Tukey's comparisons were conducted separately for each strain. The * symbol indicates that the attachment of *Streptococcus salivarius* was significantly different from that of *Streptococcus mitis* ($p<0.05$).

Non-fermented or partially-fermented teas, such as green tea and oolong tea, have been previously shown to inhibit the attachment of *Streptococcus mutans* to collagen and tooth surfaces (Friedman, 2007). As indicated, in our study extracts of these teas only slightly inhibited the attachment of one bacterial strain to the gingival cell line. In addition, EGCg and gallic acid were found to have no significant effect ($p>0.05$) on the ability of all strains to attach to the cell line. This finding suggests a possible reason for the relative ineffectiveness of the lower degree fermented tea extracts (green tea and oolong tea), which are rich in these compounds, in inhibiting adhesion. Fibronectin (Fn) is located on the outer surface of the HGF-1 plasma membrane and acts as a receptor protein for oral bacteria such as *Treponema denticola* (Ellen et al., 1994). *Streptococcus mutans* and *Streptococcus salivarius* have wall-associated protein A (wapA) in their outer membrane that allows them to bind collagen and a wide range of extracellular matrix molecules including type I collagen, laminin, keratin and Fn (Weerkamp and Jacobs, 1982; Han et al., 2006). Tea catechins, such as EGCg, have been reported to impair the adhesion promoting ability of Fn (Ogata et al., 1995), and inhibit the interactions between Fn and attaching cells by binding to the Fn receptor integrin β1 (Suzuki et al., 2006). These catechins should theoretically inhibit attachment but this was not the case in our study.

3.4 Conclusions

This study suggests that the mechanisms of inhibition of attachment of oral pathogens to gingival cells by tea or tea extracts may be different than that of inhibition to other components of the oral cavity. Based on this *in vitro* study extracts of pu-erh tea and chrysanthemum tea, in particular, may have the potential to reduce attachment of oral pathogens to gingival tissue and improve the health of oral soft tissues but this finding needs to be confirmed by *in vivo* studies. In order to further assess the situation in the oral cavity testing fresh brewed teas (hot water extracts) for adhesion inhibitory effect is required. The experimental setup used in this study could also be used to evaluate the

effect of tea on the adhesion of other oral pathogenic microorganisms, such as *Candida albicans*, which have been reported to adhere to human buccal epithelial cells and cause oral candidosis (Ellepola and Samaranayake, 1998).

Chapter 4

Potential Mechanism of the Inhibitory Effect of Tea Extracts on Attachment and Biofilm Formation by Oral Bacteria on Hard Surfaces

The work presented in this chapter represents the following peer reviewed publication:

Wang, Y., Lee, S.M. and Dykes, G.A. (2013) Potential mechanism of the effects of tea extracts on *Streptococcus mutans* attachment, biofilm formation and cell size. *Biofouling* **29**, 307-318.

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Sui Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidate's Signature	[redacted]	**Date** June 12, 2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University, Sunwasy Campus**

Signature 1	[redacted]	**Date** June 14, 2013
Signature 2	[redacted]	**Date** June 13, 2013

4.1 Introduction

Attachment and biofilm formation on tooth surfaces are regarded as virulence factors for oral bacteria. These processes initiate a buildup of dental plaque and eventually cause dental caries (Marsh, 1994). The virulence of *Streptococcus mutans*, which is the major aetiological agent responsible for dental caries (Aas et al., 2008), has been studied widely, including its ability to attach and form biofilms (Hamilton-Miller, 2001).

Tea (*Camellia sinensis*) is the second most popular drink worldwide after water. Commercial tea products are classified into four types based on their degree of fermentation. These types are green tea (non-fermented), oolong tea (partially fermented), black tea (fully fermented) and pu-erh tea (post-fermented) (Peterson et al., 2005). The fermentation process condenses tea components (such as epicatechins) into dimers (theaflavins) and polymers (thearubigins). The degree of fermentation therefore influences the polymerisation level of tea phenolics (Peterson et al., 2005). Tea has been demonstrated to benefit oral health through anti-cariogenic activities (Otake et al., 1991). These activities include direct bactericidal effects against oral bacteria (Rasheed & Haider, 1998), the inhibition of bacterial adhesion to tooth surfaces (Tagashira et al., 1997), the inhibition of glycosyltransferase activity and biosynthesis of glucan (Hada et al., 1989) and the inhibition of human and bacterial amylase with an associated limitation on sugar availability for bacterial metabolism (Zhang & Kashket, 2000).

Studies on *S. mutans* indicate that tea can reduce the attachment of the bacterium to hydroxyapatite (HA), which is a calcium mineral often used as a model of tooth surfaces. Otake et al. (1991) showed that green tea could inhibit the attachment of *S. mutans* to saliva-coated HA, and that this inhibition was due to the interactions between the tea components and the bacterial cells. Matsumoto et al. (1999) found that both small and large molecules from oolong tea could bind to the surface proteins of *S.*

mutans and via this mechanism could reduce the cell surface hydrophobicity, induce auto-aggregation and prevent bacterial attachment to HA. These studies, while indicating the potential of tea in inhibiting attachment of *S. mutans* to teeth, did not examine the outcomes for biofilm formation. Furthermore, these studies only examined the inhibition of attachment of *S. mutans* to HA and not other model tooth surfaces, and did not study the mechanisms responsible for the inhibition to any great extent.

In the current study, extracts of a range of commercial tea products were screened for the ability to inhibit attachment and biofilm formation by two strains of *S. mutans* on glass and HA substrata. Some inhibitory effects were established and the potential mechanisms behind these were investigated using scanning electron microscopy (SEM) and phytochemical screening.

4.2 Materials and methods

4.2.1 Bacterial cultures and growth conditions

Two strains of *S. mutans* (ATCC 25175 and ATCC 35668) were used in this study and were obtained from the American Type Culture Collection (Manassas, USA). Both of the strains were maintained on Mitis Salivarius Agar (Difco, USA) at 4°C. Experimental cultures were grown in Tryptic Soy Broth (TSB) (Merck, USA) at 37°C for 24 h with shaking at 150 rpm, unless otherwise stated.

4.2.2 Preparation of tea extracts

Extracts of five commercial tea products namely green tea, oolong tea, black tea, pu-erh tea and chrysanthemum tea (Ten Ren Tea Co. Ltd, Taiwan) were prepared by mixing 1 g of tea product with 20 mL of 90% (vol/vol) acetone (Sigma-Aldrich, USA) for 2 h (Perva-Uzunalić et al., 2006). The resulting extracts were evaporated under vacuum at 40°C, freeze dried and stored at -20°C for further use.

Stock solutions of the tea extracts were prepared by dissolving 100 mg of the extract in 10 mL of phosphate buffered saline (PBS) (2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl, pH 7.4, 1st BASE, Singapore) containing 1% (vol/vol) methanol (Systerm, UK) and filter sterilizing though a 0.2 μm filter (Millipore, USA).

4.2.3 Preparation of hard surfaces

Glass slides were painted with 40% (wt/vol) bonding adhesive [60% limestone, 30% kaolin, 8% ethylene glycol, 1% SiO_2 and 1% TiO_2 (all wt), Over Sea Plaster Supply and Construction Sdn Bhd, Malaysia] and partially air dried for 3 min. The slides were then coated with a paste consisting of 10% cement [56% CaO, 40% SiO_2, 3% $Al_2O_3 \cdot Fe_2O_3$ and 1% MgO (all wt), Over Sea Plaster Supply and Construction Sdn Bhd, Malaysia], 40% (wt) HA powder (Sigma-Aldrich, USA) and 50% (wt) distilled water. Slides were then air dried for 16 h and sterilized by autoclaving.

Glass slides (5 × 5 mm) were used to model the smoothness of the surface of teeth (Forssten et al., 2010), and HA slides (5 × 5 mm) were used as a model for the chemical composition of teeth (Apella et al., 2008). The model surfaces were degreased by soaking in acetone for 30 min, rinsed in distilled water and sterilized by autoclaving.

4.2.4 Attachment and biofilm formation assays

The attachment assays were carried out as described previously (Goulter et al., 2011) with modifications. Briefly, bacterial cells were pelleted by centrifuging 20 mL of TSB cultures at 7669 g for 15 min at 4°C. The cell pellets were washed gently with PBS and resuspended in the tea extract solutions at 1.25 mg/mL (containing ~10^7 CFU/mL). PBS without the addition of bacterial cells was used as a null control. Preliminary antimicrobial susceptibility testing had established that the concentration of tea extract used did not kill or inhibit the bacteria (Table 2a.1). Glass or HA slides were incubated

in the cell suspensions at 37°C for 30 min without shaking. The slides were then removed from the suspensions and gently rinsed three times with PBS to remove any loosely attached bacterial cells. The attached cells on the glass substratum were stained with 0.1% (wt/vol) crystal violet (Sigma-Aldrich), on HA substratum 0.1% (wt/vol) acridine orange (Sigma-Aldrich) was used. The number of cells on the respective substrata was determined using light or epifluorescence microscopy (BX51; Olympus, Japan). A total of 50 fields of view were counted for each slide and the number of attached cells was expressed as log CFU/cm^2.

To study the formation of biofilms, 10 mL of filter sterilized tea extract (with a final concentration of 1.25 mg/mL) and the attachment substratum (glass or HA) were added to 10 mL of autoclaved TSB (at double the normal concentration). These flasks were then inoculated with 0.1 mL of a 24 h old TSB culture and incubated at 37°C for 3 days without shaking. The same volume of TSB without tea extract was used as a null control. The biofilm formed was quantified using a method adapted from Cassat et al. (2007). Briefly, after incubation, slides were washed with PBS (×3) and transferred into Falcon tubes (TPP®, Switzerland) containing 50 mL of PBS. To detach the cells from the biofilm, the tubes were sonicated for 10 min using a water bath sonicator (LC-130H; ELMA, Germany) at room temperature with a frequency of 35 kHz. An aliquot of the PBS was serially diluted, spread plated on Tryptic Soy Agar (Merck, USA) and incubated at 37°C for 24 h before enumeration. The method was verified and results indicated that sonication detaches 95-99% of the biofilm cells without killing them (Appendix II).

4.2.5 SEM study

The experimental and control slides, with attached cells or biofilms, were washed in PBS, air dried and fixed with 4% (vol/vol) glutaraldehyde (Sigma-Aldrich, USA) in PBS. The fixed slides were washed a second time in PBS and air dried, followed by snap freezing with liquid nitrogen and freeze drying. The slides were then

platinum-sputtered using a sputter coater (Q150RS; Quorum, UK) prior to examination with a SEM (S-3400N; Hitachi, Japan).

4.2.6 Extraction of tea components associated with bacterial surfaces

A 1 L TSB culture of *S. mutans* (ATCC 25175 or ATCC 35668) was treated with tea extracts under the same conditions of exposure as those used for the attachment or biofilm assays. After incubation, cells were pelleted at 12,090 g for 15 min at 4°C using a large volume centrifuge (6930; Kubota, Japan). To ensure cells were clean, the pellet was resuspended in 500 mL of PBS before being centrifuged for a second time. The resulting pellets were resuspended in 1 L of acetone and incubated for 24 h at 40°C with magnetic stirring at 700 rpm. The suspension was then filtered through 0.2 μm bottle top filters (Corning, USA). The filtrate was evaporated under vacuum at 40°C and freeze dried. Controls containing untreated cells were prepared in the same way. The dried product was dissolved in 2mL of methanol (Systerm, UK) prior to phytochemical screening.

4.2.7 Phytochemical screening

Phytochemical screening tests were conducted qualitatively for major phytoconstituents. The methods are shown in Table 4.1. Total phenolic, total tannin and total flavonoid contents were determined quantitatively as indicated below.

Total phenolic and total tannin contents were measured using the Folin-Ciocalteau method before and after treatment with polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, USA), which has a high affinity for tannins. Briefly, to determine total phenolic content, 20 μL of the sample were added to 100 μL of 10% (vol/vol) Folin-Ciocalteau's reagent (R&M Chemicals, Malaysia) and 80 μL of 7.5% (wt/vol) Na_2CO_3 (R&M Chemicals, Malaysia) in a well of a microtitre plate (Jet Biofil, China). The plate was placed in the dark for 30 min before the absorbance was measured at 765

nm. Gallic acid (Sigma-Aldrich, USA) was used to produce a standard curve. To determine total tannin content, about 0.5 mL of the sample was first mixed with 50 mg of PVPP and 0.5 mL of distilled water. The mixture was vortexed, incubated at 4°C for 15 min and subsequently vortexed again prior to centrifuging at 1409 g for 10 min in order to remove tannins. The non-tannin phenolics in the supernatant were then quantified using the Folin-Ciocalteau method described above. The difference between the total phenolic content and the simple phenolic content is the measure of tannins. Both the total phenolic and the total tannin contents were expressed as µg of gallic acid equivalent (GAE)/10^9 cells (Kaur & Kapoor, 2001).

The total flavonoid content was determined using the aluminium chloride colorimetric method. A 50 µL aliquot of the sample was added to 10 µL of 10% (wt/vol) $AlCl_3$ (Bendosen, Malaysia), 10 µL of 1 M CH_3CO_2K (R&M Chemicals, Malaysia) and 130 µL of distilled water in a well of a microtitre plate. The plate was incubated at room temperature for 30 min before the absorbance was measured at 435 nm. The blank was prepared using distilled water in place of aluminium chloride and the standard curve was obtained using quercetin as the substrate (Sigma-Aldrich, USA). The total flavonoid content was expressed as µg quercetin equivalent (QE)/10^9 cells (Chang et al., 2002).

4.2.8 Statistical analysis

A one-way ANOVA with post hoc Tukey's comparison was performed to compare the attachment and biofilm formation between control and treatments with tea extract, and to compare the quantities of phytochemicals in the cell surface coatings between the two strains of *S. mutans*. A nested ANOVA was performed to determine which tea extracts were most effective at inhibiting attachment and biofilm formation. All analysis was conducted using SPSS software (PASW Statistics 18; SPSS Inc.) at a 95% confidence level. All experiments were performed in triplicate with independently grown cultures and all values were expressed as means ± SD.

Table 4.1 Methods for qualitative phytochemical screening.

Phytochemical	Method	Reference
Alkaloid	A 0.1 mL of sample was added to 0.5 mL of 2 M HCl (Merck, USA) and 0.5 mL of Dragendroff reagent (R&M Chemicals, Malaysia). The mixture was then centrifuged at 1409 g for 5 min. The presence of orange brown precipitate indicated a positive result.	(Kumar et al. 2009)
Saponin	A 0.1 mL of sample was boiled with 0.9 mL of distilled water for 15 min. The solution was then cooled, mixed vigorously and left to stand for 3 min. Formation of froth indicated a positive result.	(Kaur and Arora 2009)
Glycoside	A mixture of 0.1 mL of sample and 0.1 mL of 10% (wt/vol) ethanolic α-naphthol solution (Sigma-Aldrich, USA) was added to 0.5 mL of 98% H_2SO_4 (Merck, USA). A purple ring appearing at the interface indicated the presence of glycosides.	(Silva et al. 1998)
Tannin	A mixture of 0.1 mL of sample and 0.2 mL of 2% (wt/vol) NaCl solution (R&M Chemicals, Malaysia) was centrifuged at 1409 g for 5 min. The supernatant was mixed with 0.5 mL of 1% (wt/vol) gelatin solution (R&M Chemicals, Malaysia). The presence of precipitation indicated a positive result.	(Nayak et al. 2009)
Flavonoid	Magnesium powder (Sigma-Aldrich, USA) and a few drops of fuming HCl (Merck, USA) were added to 0.1 mL of sample. Orange, pink and red to purple colours were apparent when flavones, flavonols and/or xanthones were present, respectively. If zinc was used (Sigma-Aldrich, USA) instead of magnesium, a deep red colour was apparent if flavanonols were present while a weak pink colour was apparent if flavanones or flavonols were present.	(Silva et al. 1998)
Terpenoid	A 0.1 mL of sample was added to 0.9 mL of chloroform (R&M Chemicals, Malaysia) and 1 mL of acetic anhydride (Sigma-Aldrich, USA) before adding 2 mL of 98% H_2SO_4 to the mixture. Formation of a reddish brown colour indicated the presence of terpenoids.	(Kumar et al. 2009)
Sterol/steroid	A 0.1 mL of sample was added to 0.9 mL of chloroform before adding 1 mL of 98% H_2SO_4 (Salkowski method). A positive result was indicated by the formation of two phases with a yellow/green fluorescent colour appearing in the upper layer.	(Kumar et al. 2009)

4.3 Results

The results of the attachment and biofilm assays are presented in Figure 4.1. The attachment and biofilm formation by the untreated cells of both strains were significantly ($p<0.05$) greater on HA than on glass. Bacterial attachment to glass was reduced significantly ($p<0.05$) by the extract of oolong tea, for *S. mutans* ATCC 25175 by 0.6 log CFU/cm^2 and *S. mutans* ATCC 35668 by 0.3 log CFU/cm^2 and by the extract of black tea for *S. mutans* ATCC 35668 by 0.3 log CFU/cm^2. The attachment of both strains of *S. mutans* to HA slides was reduced significantly ($p<0.05$) by all the tea extracts tested, by 0.3-0.7 log CFU/cm^2 depending on the tea extract and the strain [Figure 4.1(A) and (B)]. Biofilm formation by both strains of *S. mutans* on glass was only reduced significantly ($p<0.05$) by the extract of pu-erh tea, for *S. mutans* ATCC 25175 by 1 log CFU/cm^2 and *S. mutans* ATCC 35668 by 1.1 log CFU/cm^2. However, biofilm formation on HA was significantly ($p<0.05$) reduced by extracts of both pu-erh and chrysanthemum tea for both strains by 1.1-2.6 log CFU/cm^2, and by extract of black tea for *S. mutans* ATCC 25175 by 1.2 log CFU/cm^2 [Figure 4.1(C) and (D)]. Notably, biofilm formation by both strains on HA was significantly ($p<0.05$) enhanced by extract of oolong tea. The nested ANOVA conducted to compare the inhibitory effects of the tea extracts on attachment and biofilm formation, using the *S. mutans* strain and surface type as the independent variables, indicated that the extract of oolong tea was the most effective inhibitor of attachment ($p<0.05$) and extract of pu-erh tea was the most effective inhibitor of biofilm formation ($p<0.05$). For this reason, the effect of oolong tea extracts on attachment and pu-erh tea extracts on biofilm formation were selected for further study.

The SEM images in Figure 4.2 show the appearance of attached cells of *S. mutans* with and without treatment with the extract of oolong tea. It was observed that cells treated with the extract of oolong tea had coatings on the cell surface [Figure 4.2(C), (D), (G), and (H)], which were not present on the control cells [Figure 4.2(A), (B), (E), and (F)].

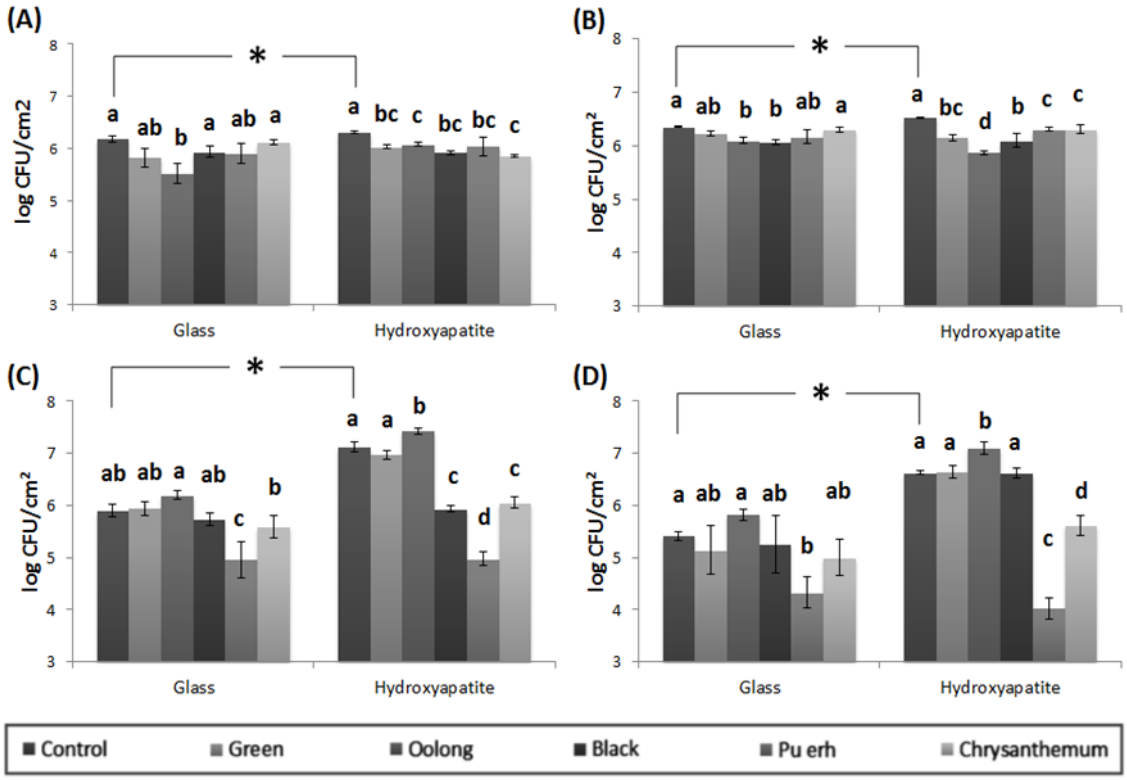


Figure 4.1 Effect of tea extracts on the attachment and biofilm formation by two strains of *S. mutans* on glass and HA. Enumeration of the effects of different tea extracts on the attachment of *S. mutans* ATCC 25175 (A) and *S. mutans* ATCC 35668 (B) to glass slides and HA, and on biofilm formation by *S. mutans* ATCC 25175 (C) and *S. mutans* ATCC 35668 (D) on glass and HA (log CFU/cm^2, n=3). Values labelled with the same letter are not significantly different ($p>0.05$) among the treatments on the same surface. *indicates that the attachment or biofilm forming ability of the untreated cells of *S. mutans* ATCC 25178 were significantly different from those of *S. mutans* ATCC 35668 ($p<0.05$).

The SEM images in Figures 4.3 and 4.4 show the appearance of *S. mutans* biofilms with and without treatment with the extract of pu-erh tea. For *S. mutans* ATCC 25175, it can be seen [Figure 4.3(C) and (D)] that on the HA substratum treatment with the extract of pu-erh tea resulted in coated cells of larger size with rougher cell surfaces. The cell density was also substantially lower compared to biofilm cells from the control [Figure 4.3(A) and (B)]. The distribution of biofilm cells on a flat (glass) surface differed in treatments with the extract of pu-erh tea. Specifically, the untreated cells formed chains

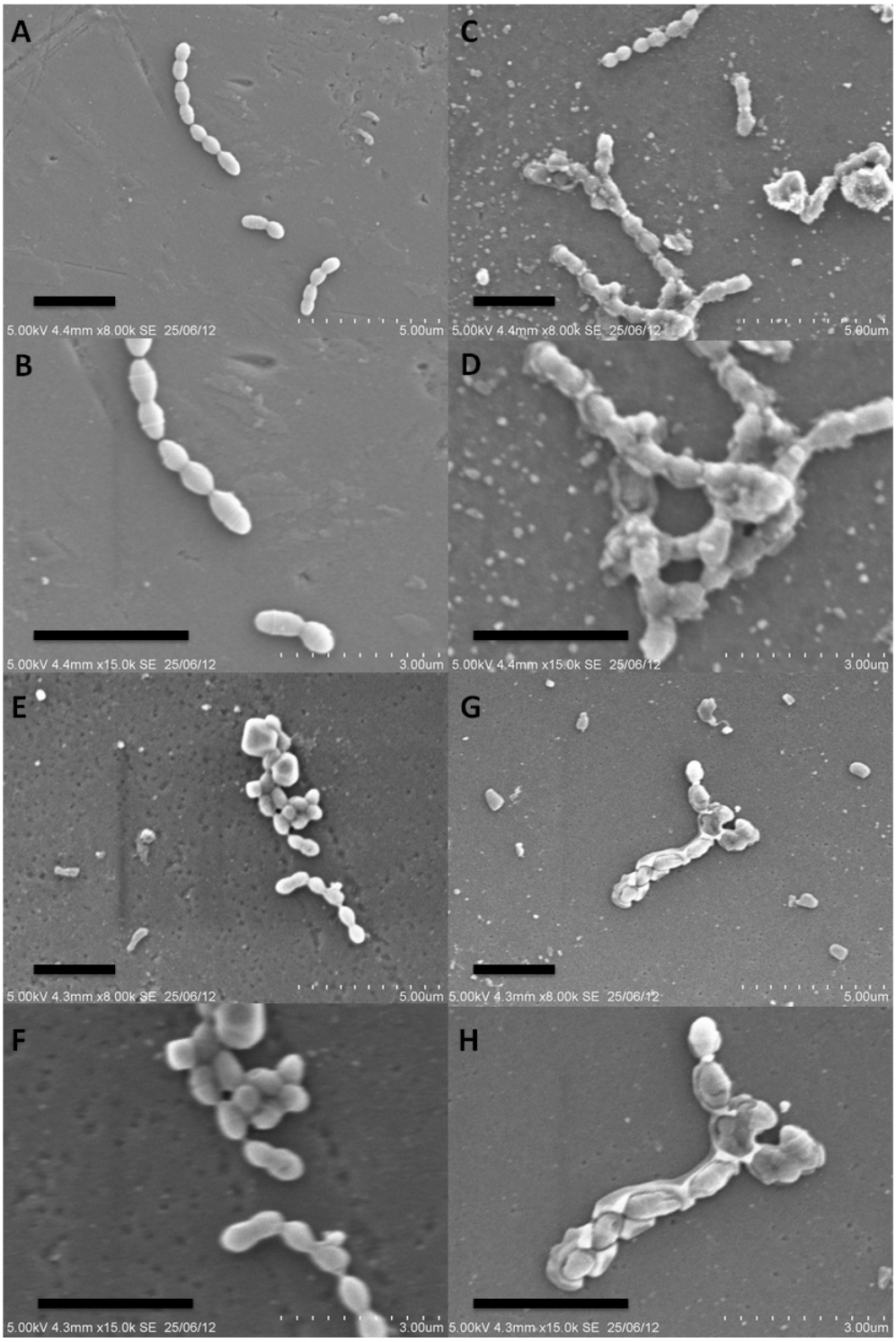


Figure 4.2 SEM images showing the effect of oolong tea extracts on *S. mutans* attached to a glass surface. A, B = untreated attached cells of *S. mutans* ATCC 25175; C, D= oolong tea extract treated attached cells of *S. mutans* ATCC 25175; E, F = untreated attached cells of *S. mutans* ATCC 35668; G, H = oolong tea extract treated attached cells of *S. mutans* ATCC 35668. Scale bars = 3 μm.

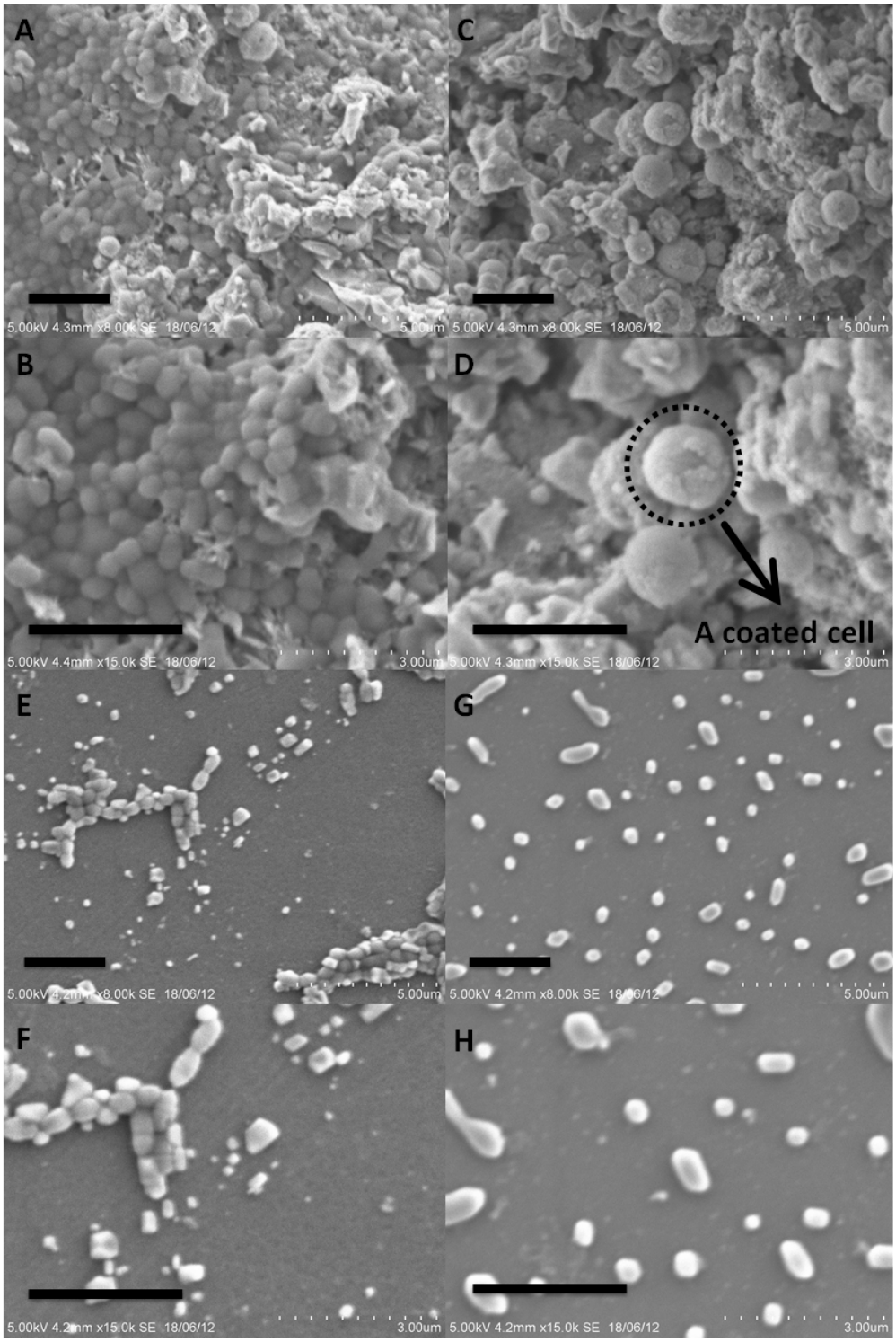


Figure 4.3 SEM images showing the effect of pu-erh tea extracts on biofilms of *S. mutans* ATCC 25175. A, B = cells grown without pu-erh tea extracts on HA; C, D= cells grown with pu-erh tea extracts on HA; E, F = cells grown without pu-erh tea extracts on glass; G, H = cells grown on with pu-erh tea extracts on glass. Scale bars = 3 μm.

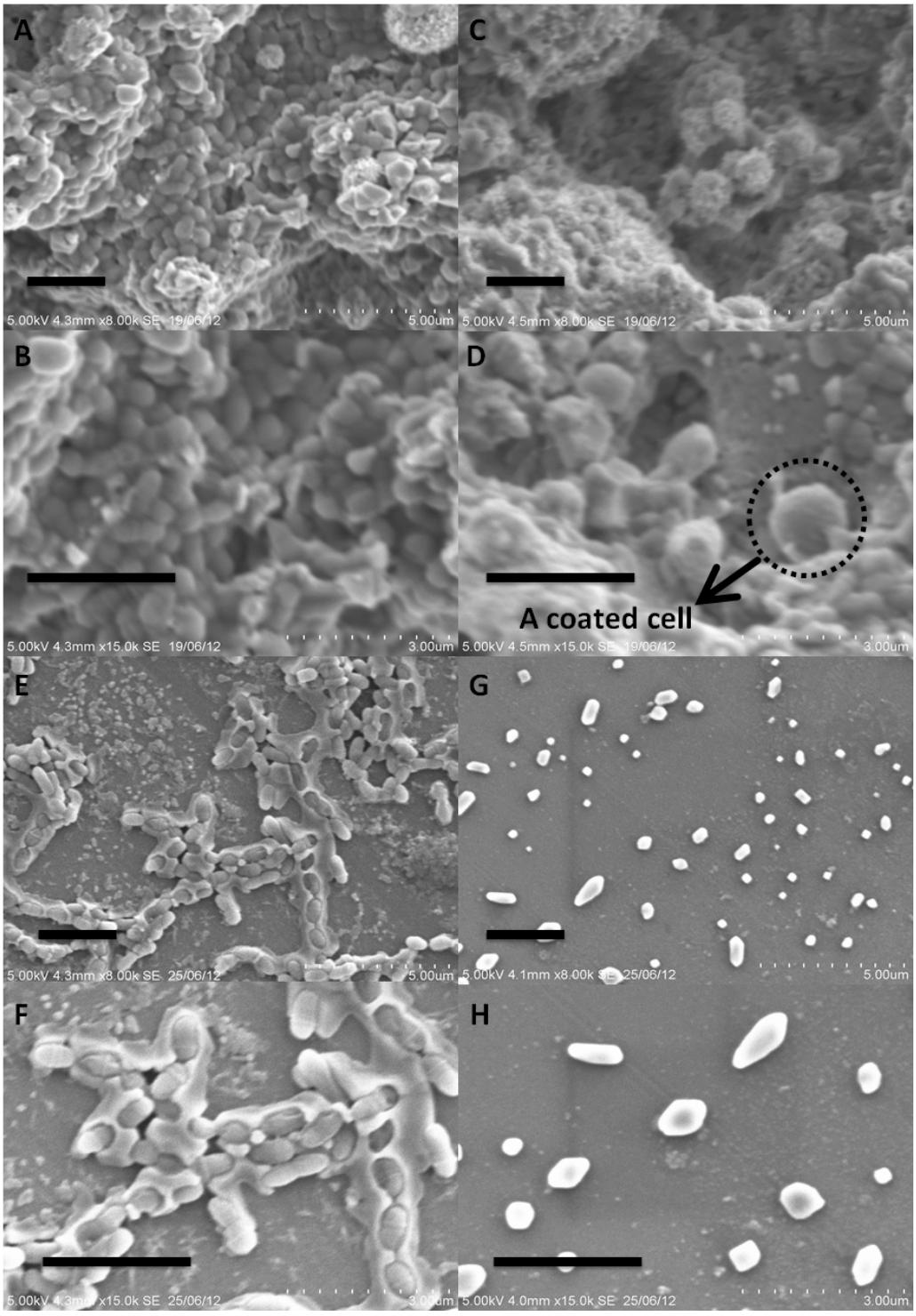


Figure 4.4 SEM images showing the effect of pu-erh tea extracts on biofilms of *S. mutans* ATCC 35668. A, B = cells grown without pu-erh tea extracts on HA; C, D = cells grown with pu-erh tea extracts on HA; E, F = cells grown without pu-erh tea extracts on glass; G, H = cells grown with pu-erh tea extracts on glass. Scale bars = 3 μm.

and clusters on the surface [Figure 4.3(E) and (F)], while the treated cells appeared individually [Figure 4.3(G) and (H)]. Notably, the large and rough cells seen on the HA surface were not present on the flat glass surface and only the coated cells, which had similar sizes to the control cells, were present. Similar observations were found for *S. mutans* ATCC 35668 (Figure 4.4). It was also noted that the control cells of *S. mutans* ATCC 35668 [Figures 4.2(E) and (F), and 4.4(E) and (F)] appeared to be surrounded by a larger quantity of an unidentified coating compared to the control cells of *S. mutans* ATCC 25175 [Figures 4.2(A) and (B), and 4.3(E) and (F)].

The coatings, which were believed to comprise components from the oolong and pu-erh teas, were extracted with acetone and analysed using phytochemical screening methods. The results (Table 4.2) show that the coatings on cells treated with extracts from both oolong and pu-erh teas (both strains of *S. mutans*) contained sterols/steroids, tannins, flavonoids and glycosides. Notably, the untreated cells also contained glycosides. In addition, the colour response of the flavonoid qualitative test suggested that the flavonoids present were flavonol/flavanone in all cases (Table 4.3).

The results from the total phenolic and the total tannic quantitative tests (Figure 4.5) showed that the coatings contained ~21-27% of simple phenolic compounds in all cases (3.19-4.84 μg GAE/10^9 cells), and 73-79% of those were tannins (8.51-18.43 μg GAE/10^9 cells). The results of the flavonoid quantitative tests are presented in Table 4.4. The flavonoid content of coatings was 2.68-6.08 μg QE/10^9 cells. It was noted that the total phenolic content, the total tannin content and the total flavonoid content in the surface coatings of *S. mutans* ATCC 25175 treated with extracts of both the oolong and the pu-erh teas were significantly higher ($p<0.05$) than those of the coatings from *S. mutans* ATCC 35668 cells given the same exposures.

Table 4.2 The results of the phytochemical screening tests on cell coatings.

	S. mutans ATCC 25175			*S. mutans* ATCC 35668		
	Control	Oolong (attachment)	Pu-erh (biofilm)	Control	Oolong (attachment)	Pu-erh (biofilm)
Terpenoid	—	—	—	—	—	—
Sterol/steroid	—	+	+	—	+	+
Flavonoid	—	+	+	—	+	+
Tannin	—	+	+	—	+	+
Saponin	—	—	—	—	—	—
Glycoside	+	+	+	+	+	+
Alkaloid	—	—	—	—	—	—

Note: — = a negative reading; + = a positive reading; n=3, using three independently grown cultures. The results for the replicates were consistent.

Table 4.3 The results of the flavonoid qualitative screening tests on cell coatings.

	S. mutans ATCC 25175			*S. mutans* ATCC 35668		
	Control	Oolong (attachment)	Pu-erh (biofilm)	Control	Oolong (attachment)	Pu-erh (biofilm)
Flavone/xanthone	—	—	—	—	—	—
Flavanonol	—	—	—	—	—	—
Flavonol/flavanone	—	+	+	—	+	+

Note: — = a negative reading; + = a positive reading; n=3, using three independently grown cultures. The results for the replicates were consistent.

Table 4.4 The results of flavonoid quantitative tests on cell coatings.

	S. mutans ATCC 25175			*S. mutans* ATCC 35668		
	Control	Oolong (attachment)	Pu-erh (biofilm)	Control	Oolong (attachment)	Pu-erh (biofilm)
Total flavonoid content	0.38±0.04	6.08±0.96	5.47±0.72	0.17±0.08	4.41±0.27	2.68±0.34

Note: All results are presented as means ± SD (μg QE/10^9 cells, n=3).

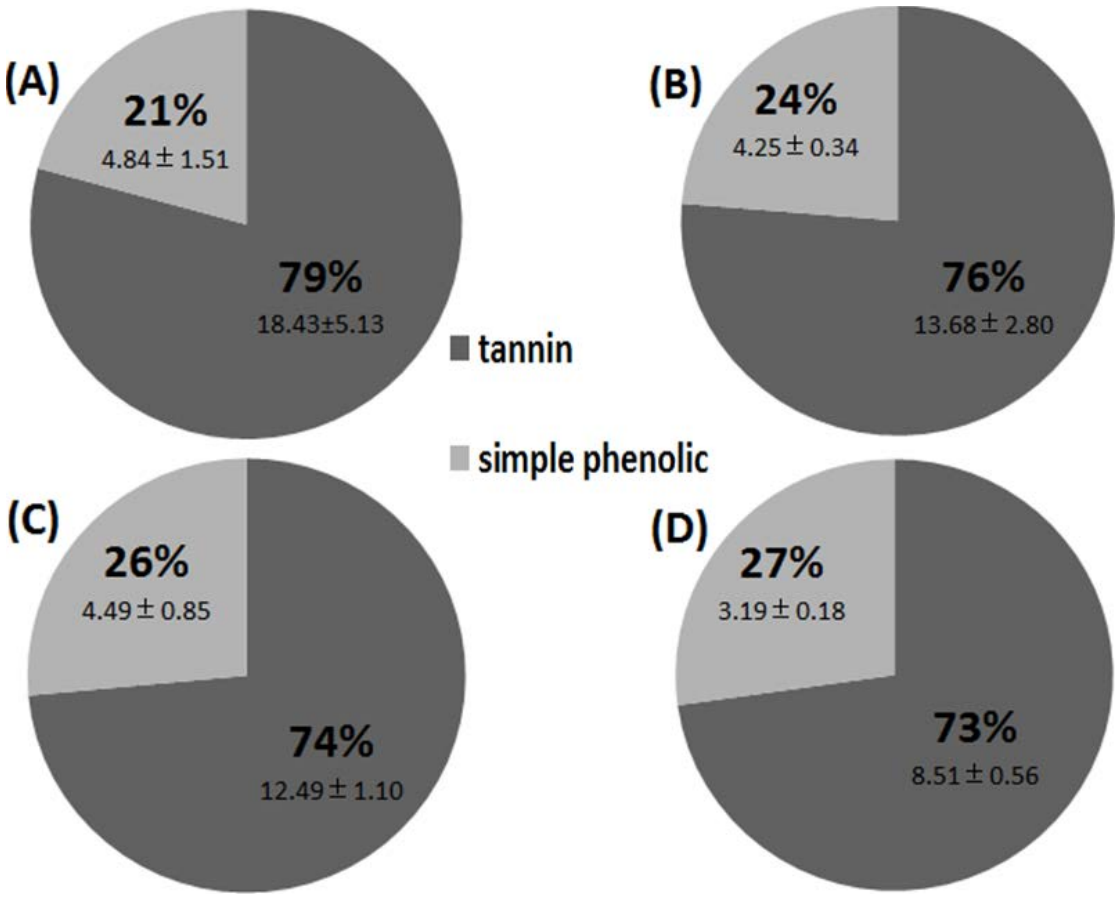


Figure 4.5 Quantification (μg GAE/10^9 cells, mean ± SD, n=3) and ratios (%) of tannins and simple phenolics in the cell surface coatings. (A) Oolong tea extract treated cells of *S. mutans* ATCC 25175, (B) cells of *S. mutans* ATCC 25175 grown with pu-erh tea extracts, (C) oolong tea extract treated cells of *S. mutans* ATCC 35668, and (D) cells of *S. mutans* ATCC 35668 grown with pu-erh tea extracts.

4.4 Discussion

The result of the attachment assay shows that a greater number of tea extracts had an inhibitory effect on attachment to HA than to glass for the two *S. mutans* strains used in this study (Figure 4.1). This could result from the super hydrophilic nature of HA which has a water contact angle of 8.11° ± 1.35°. According to the interfacial thermodynamic theory (van Oss et al., 1985), this hydrophilic nature will result in weaker hydrophobic interactions between the surface and the cells of *S. mutans*, which have been reported to be highly hydrophobic (Nostro et al., 2004), thereby resulting in lower attachment forces. By contrast, the number of cells attached and in the biofilm was higher on HA than on glass for both strains of *S. mutans* (Figure 4.1). This result may be attributed to the roughness of the HA surface, which provides a larger surface area for the attachment of bacterial cells. Although extracts from oolong tea were determined to be the best inhibitor of attachment, this extract was also found to promote biofilm formation on HA [Figure 4.1(C) and (D)], this may be because it induces cells of *S. mutans* to auto-aggregate (Matsumoto et al., 1999). The results from the current study also showed that extracts from green tea did not inhibit the attachment of cells or reduce the formation of a biofilm to any appreciable extent for either strain of *S. mutans*. This finding suggests that monomeric tea phenolics alone, which are high in green teas but lower in fermented teas due to polymerization, might not contribute to inhibiting attachment and biofilm formation (Friedman, 2007). The inhibitory effect of tea extracts on cell attachment and biofilm formation observed in the current study may therefore be a result of large molecules in the extracts or the synergistic effect of polymeric and monomeric tea phenolics.

SEM (Figures 4.2-4.4) demonstrated that cells of *S. mutans* were coated by components from the tea extracts. This suggests that bacterial cell surface components can bind to polyphenolic compounds, especially large molecule phenolics (tannins) which have a high affinity for proteins and carbohydrates (McMahon et al., 2000).

The biofilm cells coated with extract of pu-erh tea [Figures 4.3(C) and (D), and 4.4(C) and (D)] had larger sizes and rougher surfaces than untreated cells [Figures 4.3(A) and (B), and 4.4(A) and (B)]. However, the enlarged cells were present on the HA (rough) slides but not on the glass (flat) slides [Figures 4.3(G) and (H), and 4.4(G) and (H)], suggesting that the enlarged cells cannot form a biofilm on the smoother surface. This could be attributed to the roughness of the HA surface which may affect the initial attachment of the bacterial cells (Goulter et al., 2011) and subsequently also influence the formation of a biofilm. The increased size of the cells may also alter the steric arrangement between them, altering initial attachment and resulting in cells not attaching in clumps or chains (Clark and Gibbons, 1977). The SEM images also show that treatment with the extract from pu-erh tea altered the distribution of cells in the biofilm formed on glass. Cells arranged in chains and clusters [Figures 4.3(E) and (F), and 4.4(E) and (F)] were replaced by cells attached individually [Figures 4.3(G) and (H), and 4.4(G) and (H)], suggesting that extract of pu-erh tea may alter cellular aggregation. As outlined above, tea coatings that can affect cell surface properties and therefore attachment may also alter cell to cell interactions. It is also possible that components of tea might coat the surface of the substratum, especially HA (tea is known to stain tooth surfaces), as the substratum slides were incubated with the cell suspension in the presence of tea extract during the attachment and biofilm assays. Any coating on the surface of the substratum might also affect cellular attachment and biofilm formation. This approach simulates what is likely to happen in the mouth if bacteria are exposed to tea, and further research is required to establish what effect tea has in terms of coating the surface of the substratum.

Glycosides, sterols/steroids, flavonoids and tannins were found in the coatings covering the cell surfaces (Table 4.2). Glycosides were also present in the controls and are probably components of the cells themselves rather than arising from the coatings. The presence of sterols/steroids was determined by the Salkowski method, which indicated

the presence of indolic compounds (Glickmann and Dessaux, 1995). The positive results observed could be due to the presence of tea indoles which are usually components of the flavour or the pigment compounds of tea (Kubo and Morimitsu, 1995). The flavonoid components of the coating were identified as flavonol or flavanone (Table 4.3). Tea does not contain flavanones but it does contain three flavonols, quercetin, kaempferol and myricetin (Wang & Helliwell, 2001). Quercetin and kaempferol are highly water insoluble and since experiments were conducted in a water-based system (PBS), myricetin was the most likely flavonol present in the cell coatings. However, since there is no screening method for flavon-3-ols, which are a major phenolic constituent in low-degree fermented tea (such as oolong), and flavon-3-ols have been reported to be able to bind to various types of proteins (Mori et al., 2010), it is also reasonable to assume that flavon-3-ols were also part of the flavonoid content in the coatings of cells treated with extract of oolong tea. In the qualitative and quantitative phytochemical screening tests, tannins are regarded as large molecule (MW>500) polyphenolic compounds. Dimers or polymers of flavon-3-ols, namely theaflavin and thearubigin, are the most common tannins in tea products (Graham, 1992), and were assumed to be the major tannin component in the cell coatings.

The phenolic constituents found in the coatings on the bacterial cells were mostly hydrophilic but they still contained hydrophobic moieties, such as aromatic rings, which may interact with hydrophobic components of the cell membrane (Nalina and Rahim, 2007). Therefore, it is suggested that the hydrophilic moieties (such as hydroxyl groups) of the phenolic compounds may be exposed on the cell surface and ‘neutralize’ cell surface hydrophobicity. This would result in an enhanced interaction between the cells and the liquid (water-based) medium, but a reduced interaction between the cells and the surface during attachment to a substratum. In addition, coating of the cell surface may block surface proteins and structures that interact with the surface of the substratum.

The ratio of tannins to simple phenolics in each of the coatings was approximately 3:1 (Figure 4.5) suggesting that larger molecule polyphenols bind more readily to surface components of the *S. mutans* cell than smaller compounds. In general, tannins have a higher affinity for proteins (Rawel et al, 2006) and carbohydrates (Shahidi and Naczk, 2004) compared to simple phenolics. It has been suggested that the proteins on the surface of *S. mutans* play an important role in the interactions between bacterial cells and polyphenols (Matsumoto et al., 1999). The preferred binding sites of phenolic compounds are usually hydroxyl groups and this can facilitate interactions with proteins, particularly tryptophan residues, either via covalent bonds or non-covalent bindings such as hydrogen bonds and electrostatic attraction (Rawel et al., 2006). Tannins have more hydroxyl residues than simple phenolics and therefore may exhibit higher binding efficiencies to surface components of *S. mutans* cells. A study by Matsumoto et al. (1999) also indicated that it was the polymeric polyphenols in oolong tea that were responsible for the alteration in the surface properties and the inhibition of attachment of cells of *S. mutans*.

The total flavonoid, total tannin and total phenolic contents (Figure 4.5 and Table 4.4) in the surface coatings of *S. mutans* ATCC 25178 were approximately one third higher ($p<0.05$) than those of *S. mutans* ATCC 35668, suggesting that *S. mutans* ATCC 25178 cells bind to tea components more readily. This could be due to a higher amount of an unidentified substance (possibly extracellular polysaccharide) surrounding the cells of *S. mutans* ATCC 35668. This substance might occupy or block some of the binding sites on the cell surface thereby reducing the amount of components that can bind from the tea extracts. This feature of the data also highlights the importance of strain variation in investigating mechanisms to prevent bacterial adhesion to surfaces.

Additional studies are required to simulate the situation in the oral cavity more realistically. For example, it would be useful to investigate the effect of tea on wild type

S. mutans, isolated from the human oral cavity which may behave differently from laboratory strains. In addition, the effect of tea extracts on cells broken off from a pre-existing biofilm and planktonically grown cells may also be different. The concentrations of tea extracts used in this study [which were at sub-minimum inhibitory concentration (MIC) levels] were lower than those in a cup of freshly brewed tea, which may reach a dose that is inhibitory or lethal to cells of *S. mutans*, especially in the case of green and oolong tea (Xu et al., 2012). It is therefore likely that immediately after tea consumption, cells of *S. mutans* in the oral cavity could be killed or inhibited. The concentration of tea will subsequently be diluted gradually by saliva to sub-MIC level, at which point the attachment of cells or biofilm formation may be suppressed. Furthermore, tea may have concomitant effects on dental surfaces, which could also influence the potential for attachment by bacterial cells and subsequent biofilm formation.

4.5 Conclusions

In summary, components in the extract of oolong tea reduced the attachment of cells of *S. mutans* and components in the extract of pu-erh tea reduced biofilm formation. Specifically, it is suggested that flavonoids, tannins and indolic compounds coat the surfaces of cells, probably altering cell surface properties and thereby affecting the interactions between bacterial cells and the surface of the substratum.

Chapter 5

Modeling of Bacterial Attachment: Predictability and Stochasticity

The work presented in this chapter represents the following publication submitted for peer review:

Wang, Y., Lee, S.M., Gentle I.R. and Dykes G.A. (Submitted) A mathematical approach for modeling the physicochemical process of bacterial attachment to abiotic surfaces. *Environmental Science & Technology*.

Chapter 5a XDLVO Study of Attachment of Oral Bacteria to Hard Surfaces

5a.1 Introduction

Mechanistic mathematical models are often used to predict bacterial attachment to abiotic surfaces (van Oss et al., 1986; Chen et al., 2011; Warning et al., 2013). The most commonly used model reported in the literature is the extended XDLVO theory (Roosjen et al., 2006; Bayoudha et al., 2009; Chia et al., 2011; Nguyen et al., 2011a) which establishes and uses the energy profiles of Lifshitz-van der Waals (LW), electrostatic (EL) and short range Lewis acid-base (AB) (hydrogen bonding) interactions between bacterial cells and the substratum surface. The outputs of the XDLVO model provide some understanding of the mechanism of bacterial attachment in many cases (Jucker et al., 1997; Bos et al., 1999; Hermansson, 1999).

The experimental results of the determination of oral bacterial attachment (*S. mutans* ATCC 25175, *S. mutans* ATCC 35668, *S. salivarius* ATCC 13419, *S. mitis* ATCC 49456 and *A. naeslundii* ATCC 51655) to the hard surfaces (glass, stainless steel and hydroxyapatite) obtained from Chapter 2a (Figure 2a.2) were compared to those predicted by the XDLVO theory by establishing the attachment interaction energies. The results for the attachment of *A. naeslundii* ATCC 51655 were also examined by XDLVO even though cell counts were determined differently as Chia et al. (2011) determined that results obtained from the two methods (direct counting under a microscope and spread plating after stomaching; Chapter 2a) were not significantly different. In order to calculate the interaction energies, zeta potentials and contact angles of all strains and hard surfaces were determined.

5a.2 Methods and materials

5a.2.1 Zeta potential measurements

The ZPs of bacteria were obtained from Chapter 2a. The ZPs of the hard surfaces were obtained from the literature (Reynolds and Wong, 1983; Chia et al., 2011).

5a.2.2 Contact angle measurements

Contact angle measurements were performed on the hard surfaces and on lawns of the bacteria as previously described by Nguyen et al. (2011a). A bacterial lawn was prepared by filtering 20 mL of bacterial suspension (containing approximately 10^8 CFU/mL) through a membrane filter (pore size: 0.22 μm, pore diameter: 25 mm; Millipore) using negative pressure. The cell-filter was air-dried for 30 min, attached to a glass slide using double sided tape and further dried using a freeze drier (FDU-2100, Eyela, Japan) overnight. A 2 μL drop of liquid was placed onto a hard surface or bacterial lawn using a 10 μL syringe fitted with a needle gage (Ramé-Hart Inc., USA) and the contact angles were measured using a goniometer (Model 250, Ramé-Hart Inc.) with the aid of DROPimage software (Ramé-Hart Inc.). For each material, 10 drops of liquid were measured on each of three independently prepared surfaces and the mean values were reported in this study.

The contact angles were used to calculate the energy characteristics of the bacteria and materials using Young's equation given by Bos et al. (1999).

$$\cos\theta = -1 + \frac{2\sqrt{\gamma_s^{LW}\gamma_l^{LW}}}{\gamma_l} + \frac{2\sqrt{\gamma_s^{+}\gamma_l^{-}}}{\gamma_l} + \frac{2\sqrt{\gamma_s^{-}\gamma_l^{+}}}{\gamma_l} \qquad (5a.1)$$

Where γ^{LW} is the LW component of surface tension (mJ/m^2), γ^{+} and γ^{-} are respectively the electron acceptor and donor parameters of AB component of surface tension

(mJ/m^2), θ is the contact angle and γ_l is the surface tension (mJ/m^2) of the liquid. The subscripts *s* and *l* stand for the solid surface and the liquid used in the measurement respectively.

The contact angles of three liquids [water, 1-bromonaphthalene (Sigma-Aldrich) and formamide (Sigma-Aldrich)] with known surface tension components (Bos et al., 1999) were measured in order to calculate the surface tension components of the hard surfaces and the bacteria.

5a.2.3 XDLVO calculation

According to XDLVO theory, the overall interaction energy between a cell and a surface [$\Delta G^{XDLVO}(l)$] as a function of the separation distance (l) between them, is the sum of LW [$\Delta G^{LW}(l)$], AB [$\Delta G^{AB}(l)$] and EL [$\Delta G^{EL}(l)$] interaction energies (van Oss, 1989).

$$\Delta G^{XDLVO}(l) = \Delta G^{LW}(l) + \Delta G^{AB}(l) + \Delta G^{EL}(l) \qquad (5a.2)$$

The energy values for the interaction components [$\Delta G^{LW}(l)$, $\Delta G^{AB}(l)$ and $\Delta G^{EL}(l)$] are given by Bos et al. (1999). The separation distance dependent LW interaction energy is obtained from:

$$\Delta G^{LW}(l) = -\frac{A}{6}\left[\frac{2r(1+r)}{l(1+2r)}\right] - ln\left(\frac{1+2r}{l}\right) \qquad (5a.3)$$

where r is the radius of the bacterium (0.75 μm) and A is the Hamaker constant, which can be determined by:

$$\Delta G_{adh}^{LW} = -\frac{A}{12\pi l_0^2} = -2\left(\sqrt{\gamma_b^{LW}} - \sqrt{\gamma_l^{LW}}\right)\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_l^{LW}}\right) \qquad (5a.4)$$

where ΔG_{adh}^{LW} is the LW component of the free energy of adhesion at contact and l_0 is the minimum separation distance (0.157 nm). The subscript *b* stands for bacteria. The separation distance dependent AB interaction energy can be calculated by:

$$\Delta G^{AB}(l) = 2\pi r \Delta G_{adh}^{AB} \lambda \exp\left(\frac{l_0 - l}{\lambda}\right) \quad (5a.5)$$

where λ is the characteristic decay length of AB interaction in the liquid medium (13 nm for hydrophobic bacteria) (Nguyen et al., 2011a), ΔG_{adh}^{AB} is the AB component of the free energy interaction at contact, which can be calculated by:

$$\Delta G_{adh}^{AB} = 2\left[\left(\sqrt{\gamma_b^+} - \sqrt{\gamma_s^+}\right)\left(\sqrt{\gamma_b^-} - \sqrt{\gamma_s^-}\right) - \left(\sqrt{\gamma_b^+} - \sqrt{\gamma_l^+}\right)\left(\sqrt{\gamma_b^-} - \sqrt{\gamma_l^-}\right) - \left(\sqrt{\gamma_s^+} - \sqrt{\gamma_l^+}\right)\left(\sqrt{\gamma_s^-} - \sqrt{\gamma_l^-}\right)\right] \quad (5a.6)$$

The separation distance dependent EL interaction energy can be calculated by:

$$\Delta G^{EL}(l) = \pi\varepsilon\varepsilon_0 r\left(\xi_b^2 + \xi_s^2\right)\left\{\frac{2\xi_b^2\xi_s^2}{\xi_b^2 + \xi_s^2} ln\left[\frac{1 + exp(-\kappa l)}{1 - exp(-\kappa l)}\right] + ln\left[1 - exp(-2\kappa l)\right]\right\} \quad (5a.7)$$

where ε_0 denotes the dielectric permittivity of vacuum (8.854×10^{-12} J m^{-1} V^{-2}) and ε denotes the relative permittivity of the liquid medium (78.2 for water) (Bos et al., 1999), ξ is the ZP in the surrounding liquid medium and κ is the inverse Debye layer thickness [$3.28 \times 10^9\sqrt{I}$ m^{-1}, where I is the ionic strength of the electrolyte in terms of molarity (i.e. 150 mM)] (Bayoudh et al., 2009).

5a.3 Results

5a.3.1 Interaction energy profiles

Table 5a.1 shows the contact angles, surface tensions and ZPs of the bacteria and the hard surfaces examined in this study. Results of water contact angle measurements represent the hydrophobicity of the substratum surfaces and the bacteria. Stainless steel was the most hydrophobic surface among the three and hydroxyapatite was the most hydrophilic one. The hydrophobicity values of the bacteria obtain using CAM were compared with those obtained using BATH assays (Chapter 2a) using regression analysis. No clear correlation was observed from a linear or a Spearman rank regression ($p>0.05$).

Based on the result of CAM and zeta potential measurements the pairwise interaction energies between the bacteria and hard surfaces were calculated and shown in Table 5a.2. A negative value indicates an attractive force between the cell surface and the substratum surface. *S. mutans* ATCC 35668 had the highest interaction energies with all surfaces among all strains at approximately -3000 to -5000 $\times 10^{-18}$ J while *A. naeslundii* ATCC 51655 had the lowest energies at approximately -2000 to -3000 $\times 10^{-18}$ J. Notably, The energies AB interactions were in general greater than those of LW and EL interactions by four to five orders of magnitude.

The profiles of change in total interaction energy as a function of separation distance between bacterial cells and substratum surfaces are shown in Figure 5a.1. There was only one deep energy minima without an energy barrier observed from each of the profiles, indicating that the attraction force dramatically increased with a reducing separation distance in all cases.

Table 5a.1 Contact angles, zeta potentials and surface tension energies of the five oral bacteria and three hard surfaces.

Bacterial and substratum surface	Contact angle (°)[a]			Surface tension (mJ/m^2)			ZP (mV)
	θ_B	θ_F	θ_W	γ^{LW}	γ^+	γ^-	
S. mutans ATCC 25175	18.0±3.2	44.2±3.1	39.3±4.2	42.3	0.26	1.66	-4.1±2.3
S. mutans ATCC 35668	36. 8±4.4	44.0±2.1	57.8±4.4	36.0	0.34	0.10	-4.4±1.8
S. salivarius ATCC 13419	19.0±2.0	32.4±3.9	34.3±3.1	42.0	0.03	4.16	-9.6±0.9
S. mitis ATCC 49456	16.0±1.3	54.8±8.3	32.4±4.0	42.7	2.41	0.47	-10.6±0.4
A. naeslundii ATCC 51655	28.5±5.0	39.3±6.5	28.2±3.2	41.7	0.12	5.25	-11.9±1.4
Glass	39.6±3.1	39.4±0.7	48.9±1.0	34.8	0.54	1.54	-23.6[b]
Stainless steel	25.6±2.0	84.6±2.8	97.2±3.3	40.2	4.26	41.91	-25.0[b]
Hydroxyapatite	4.8±0.5	36.7±1.7	8.1±1.4	44.2	0.42	6.01	-9.1[b]

[a] θ_B, θ_F and θ_W represent contact angle measurements of 1-bromonaphthalene, formamide and water respectively.

[b] The ZP of glass and stainless steel were reported by Chia et al. (2011) and the ZP of hydroxyapatite was published by Reynolds and Wong (1983).

Table 5a.2 Lifshitz van der Waals (ΔG^{LW}), Lewis acid-base (ΔG^{AB}), electrostatic (ΔG^{EL}) and total (ΔG^{XDLVO}) interaction energies (10^{-18} J) between five oral bacteria and three hard surfaces based on the XDLVO theory at the minimum separation distance (0.157 nm).

Strain	Glass				Stainless steel				Hydroxyapatite			
	ΔG^{LW}	ΔG^{AB}	ΔG^{EL}	ΔG^{XDLVO}	ΔG^{LW}	ΔG^{AB}	ΔG^{EL}	ΔG^{XDLVO}	ΔG^{LW}	ΔG^{AB}	ΔG^{EL}	ΔG^{XDLVO}
S. mutans ATCC 25175	-0.02	-4188	-0.32	-4189	-0.01	-3198	-0.40	-3199	-0.01	-4168	0.10	-4168
S. mutans ATCC 35668	-0.02	-5157	-0.27	-5158	-0.02	-4057	-0.35	-4057	-0.01	-5158	-0.12	-5158
S. salivarius ATCC 13419	-0.02	-3662	0.53	-3661	-0.01	-2572	0.51	-2572	-0.01	-3582	0.34	-3582
S. mitis ATCC 49456	-0.02	-3792	0.68	-3791	-0.01	-3374	0.67	-3373	-0.01	-3932	0.38	-3932
A. naeslundii ATCC 51655	-0.02	-3224	0.86	-3223	-0.01	-2297	0.86	-2297	-0.01	-3169	0.41	-3169

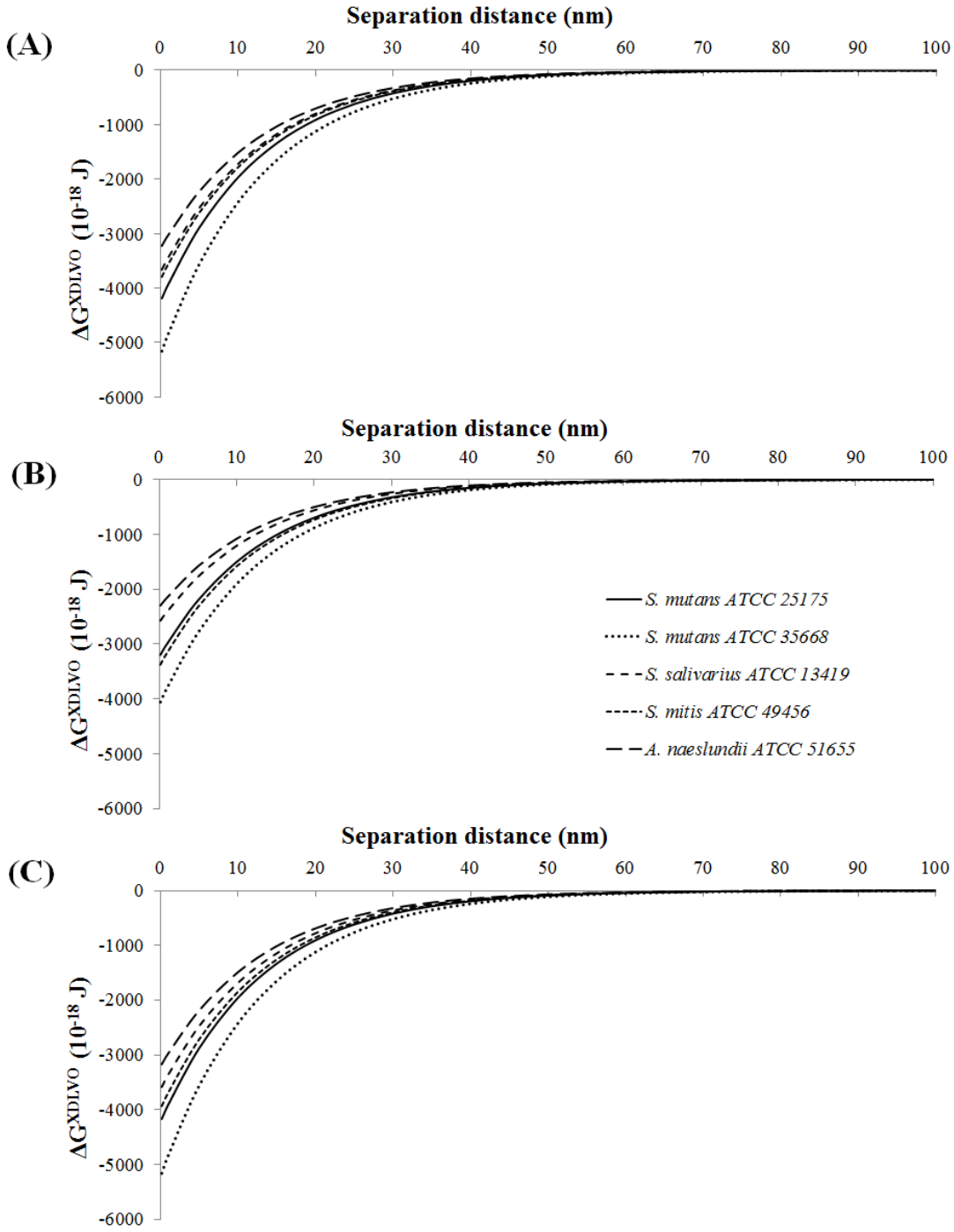


Figure 5a.1 Changes in the total interaction energies as a function of distance between five oral bacteria and three hard surfaces [(A) glass, (B) stainless steel and (C) hydroxyapatite] calculated using the XDLVO theory.

5a.3.2 Predictability of attachment by XDLVO

A scatterplot of bacterial attachment against XDLVO interaction energy at the minimum separation distance is shown in Figure 5a.2. The XDLVO theory failed to predict the attachment of *S. mitis* ATCC 49456 to all surfaces and also the attachment of all strains to stainless steel. Interestingly, the interaction energies between hydroxyapatite and the bacterial cells were similar to those between glass and the bacterial cells, but the attachment of the bacteria to hydroxyapatite was significantly greater than to glass ($p<0.05$).

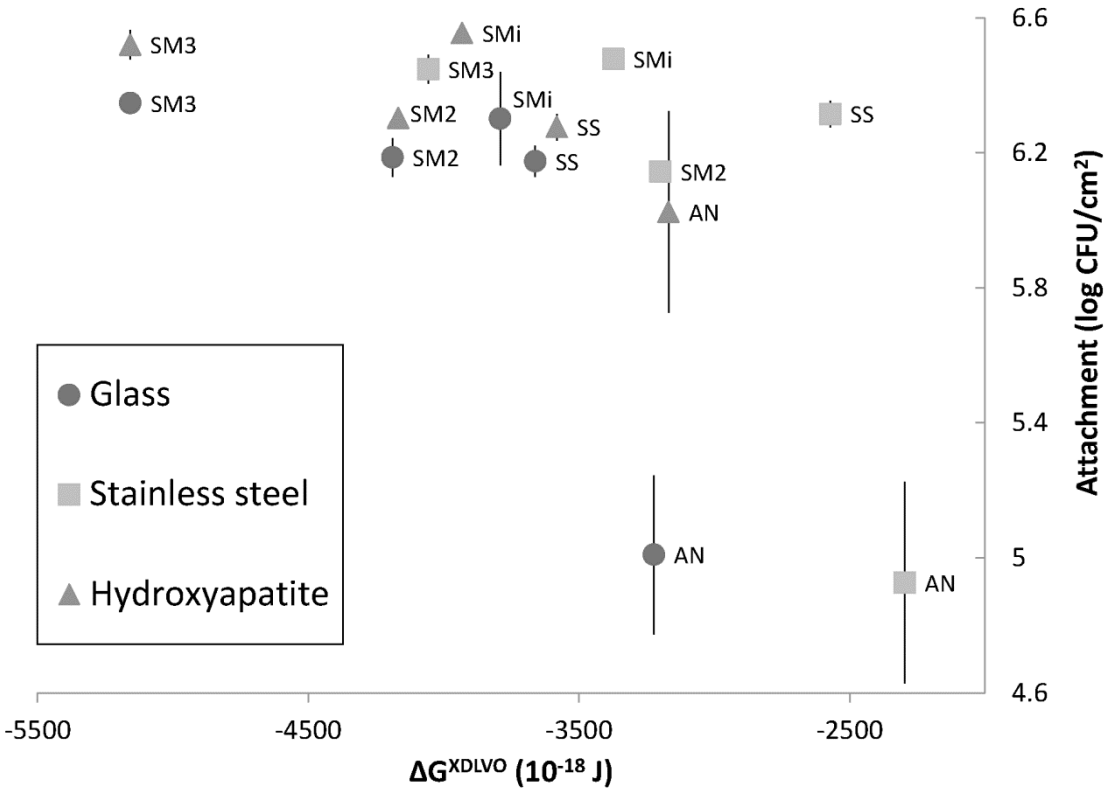


Figure 5a.2 Scatterplot of attachment of oral bacteria against overall attachment interaction energy (ΔG^{XDLVO}). SM2: *S. mutans* ATCC 25175; SM3: *S. mutans* ATCC 35668; SS: *S. salivarius* ATCC 13419; SMi: *S. mitis* ATCC 49456; and AN: *A. naeslundii* ATCC 51655.

5a.4 Discussion

Although both BATH assay and CAM measure the hydrophobicity nature of the surface of bacterial cell and interpret it in different ways, the results should theoretically be correlated to each other. This was however not the case in the present study. The discrepancies between the hydrophobicity results obtained from the two methods might be due to the difficulty in measuring the contact angle of a drop of liquid on a bacterial lawn as bacterial lawns can absorb liquid droplets that are placed on them. Also, the accuracy of CAM highly depends on the wetness of bacterial lawns which greatly affects the surface tension energies calculated by Young's equation (Drumm et al., 1989). In BATH assays, electrostatic interactions between the hydrocarbon and bacterial cells may have an influence on the resulted hydrophobicity value, which can be eliminated by adjusting the pH of the suspending buffer solution to the bacterial isoelectric point (pI value). In addition, these methods estimate cell surface hydrophobicity by quantifying the bulk hydrophobicity of a massive amount of cells which can greatly be affected by other interactions such as cell to cell interactions (Nguyen et al., 2011a). These discrepancies between methods can result in difficulties in study the mechanisms of bacterial attachment.

The interaction energy data shown in Table 5a.2 indicate that AB interaction energy dominated the overall energy in the case of attachment of all bacteria to all surfaces examined. Hydrogen bonding could therefore be the key interaction contributing to the attachment of the oral bacterial strains in the system used. It was suggested in the literature that Streptococci in general possess relatively more ligand-receptor sites available for hydrogen bond formation on their cell surfaces than many other bacteria do (Chen et al., 2011). For instance, a *Streptococcus* cell attached to stainless steel through 60 short-range bonds (Mei et al., 2009), and only 12 short-range bonds were formed between an *Escherichia coli* cell and a silicon nitride AFM tip (Abu-Lail and Camesano, 2006). The greatness of an AB interaction energy over other interaction energies usually result in an extremely deep energy minima and the absence of an

energy barrier (Hermansson, 1999), which has been shown in the present study.

The XDLVO theory failed to predict the attachment of *S. mitis* ATCC 49456 suggesting that in cases of *S. mitis* there were non-physiochemical factors, such as surface appendages and outer membrane proteins, affecting attachment (Murray et al., 1986; Goulter et al., 2009). The XDLVO also failed to predict attachment to stainless steel suggesting that that this surface has some factors contributing to attachment other than the parameters considered by XDLVO. For example, stainless steel is a conductive surface on which charge can transfer to and from bacterial cells resulting in a change of electric potential and in turn affecting attachment (Poortinga et al., 1999). The failure to predict attachment may also be due to the stochasticity of the process under some conditions. For example Chia et al. (2011) indicated that stochasticity depends on the magnitude of bacterial cell surface and the substratum surface properties and suggested that attachment is predictable only when values of cell surface properties are significantly different between different bacterial strains. In addition, biological variability plays a role in bacterial attachment and consequently affects the predictions by XDLVO which are solely deterministic without taking noises into account. It was apparent that the attachment of all strains to hydroxyapatite was greater than to glass and this could be due to the roughness of the hydroxyapatite surface (Figure 2a.4), a feature that is not considered by XDLVO. The XDLVO theory was originally designed to predict attachment and aggregation in colloidal systems and it is therefore not an optimal model for bacterial attachment systems even though it is widely used for them. An empirical model which accounts for biological variability (using probability distributions) and more physicochemical parameters (such as substratum surface roughness) should be developed in order to predict bacterial attachment in a better way. In addition, bacterial and substratum models used to study bacterial attachment in future research should include large ranges of values of physicochemical parameters, in order to gain better understanding on the feature of stochasticity.

5a.5 Conclusion

The XDLVO study reveals that the key mechanism for the attachment of the oral strains to the hard surfaces could be AB interactions. However, XDLVO failed to fully predict the attachment due to inaccuracy of CAM and the lack of consideration of other key parameters such as substratum surface roughness and biological variability. This leads to a need to develop a new approach to model bacterial attachment to hard surfaces accounting more physicochemical parameters and using probability distributions.

Declaration for Thesis Chapter 5b

Declaration by candidate

In the case of Chapter 5b, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Sui Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Ian Gentle	IG was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 6% of writing and the review of the publication.	8%

Candidate's Signature	[redacted]	**Date** June 12, 2013

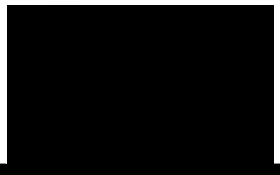
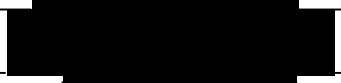
Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University, Sunwasy Campus**

Signature 1		**Date** June 14, 2013
Signature 2		**Date** June 13, 2013
Signature 3		**Date** June 13, 2013

Chapter 5b Mathematical Modeling of the Physicochemical Process of Bacterial Attachment

5b.1 Introduction

Bacterial attachment to surfaces which can directly or indirectly result in biofouling is of concern in many spheres such as medicine (Goulter et al., 2009), food safety (Rivas et al., 2007; Warning and Datta, 2013), engineering (Li and Logan, 2004; Warning and Datta, 2013) and the environment (Warning and Datta, 2013). Understanding the mechanism of bacterial attachment and controlling and minimizing it is therefore important. The mechanical process of bacterial attachment has been extensively studied but is still not well understood (Goulter et al., 2009; Warning and Datta, 2013).

Previous researchers investigating the mechanisms of bacterial interaction with hard abiotic surfaces have generally attempted to correlate one or more potential physicochemical factors, such as bacterial surface hydrophobicity, charge or substratum surface properties, with the numbers of bacteria attaching to a surface (Liu et al., 2004; Rivas et al., 2007; Salerno et al., 2009). Mathematical models have been developed as part of these studies and applied in mechanistic studies (Warning and Datta, 2013).

Mechanistic models, such as the thermodynamic theory, Derjaguin-Landau-Verwey-Overbeek (DLVO) theory and extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theory (van Oss et al., 1986; Chen et al., 2011), that linearly relate bacterial attachment to different physicochemical interactions are widely used for predicting bacterial attachment to hard abiotic surfaces but they have not been able to fully explain the process in many cases (Chen et al., 2011; Chia et al., 2011; Nugyen et al., 2011; Warning and Datta, 2013) due to specific limitations. For example, thermodynamic theory only takes hydrophobic interaction between bacteria

and substratum surfaces into account but does not consider electrostatic interactions (van Oss, 1989). The XDLVO theory on the other hand accounts for Lifshitz-van der Waals, electrostatic and Lewis acid-base interactions, but it assumes that substratum surfaces are perfectly smooth and uniform (Salerno et al., 2009) and bacterial cells are uniform in size (Chia et al., 2011), which is usually not the case. Of more fundamental concern is that mechanistic models are generally deterministic and do not take biological variability into account. The development of empirical models and semi-empirical models is an approach often used to represent bacterial attachment. Many of these models, however, only consider one or two variable parameters without controlling other factors or considering interactions between the parameters (Medilanski et al., 2002; Li and Logan, 2004; Kang and Choi, 2005; Salerno et al., 2009; Tang et al., 2009). In addition, the parameters used in these models are often varied across small ranges limiting the value of these models (Salerno et al., 2009; Tang et al., 2009).

In this study an empirical model was constructed based on six surface parameters: bacterial surface hydrophobicity, charge, cell size, substratum surface hydrophobicity, charge and roughness. The parameters were varied across a large range and interactions between the parameters were taken into consideration. The outputs of the empirical model were then substituted into a normal approximated binomial distribution model to build a semi-empirical model which probabilistically better represents bacterial attachment to hard abiotic surfaces.

5b.2 Materials and methods

5b.2.1 Model description

A semi-empirical approach for modeling bacterial attachment to abiotic surfaces was established in this study. It involves a combination of an empirical model and a normal approximated binomial probability distribution model and therefore can both deterministically and probabilistically predict attachment.

The empirical model uses hydrophobic interactions, electrostatic interactions, bacterial cell size and substratum surface roughness as predictors and has the probability of attachment of an individual cell as a deterministic response output. This model is based on a polynomial linear regression expressed as:

$$p(\%) = \frac{x}{n} = a \times \text{Hydrophobic interaction} + \text{b} \times \text{Electrostatic interaction} + \text{c} \times \text{Substratum surface roughness} + \text{d} \times \text{Cell size} + \text{e} \quad (5\text{b}.1)$$

where p is the probability of attachment of an individual bacterial cell (%), x is the number of bacterial cells attached to a surface, n is the total number of bacterial cells in the system, a, b, c and d are coefficients for the predictors and e is a constant.
In order to establish a semi-continuous empirical model, two bacteria with different surface hydrophobicity, charge, and cell size were mixed at different ratios to create gradient values of these properties. Similarly, two substratum surface materials with different hydrophobicity, charge and roughness were mixed at different ratios for the same purpose. Pairwise assays of bacterial attachment for the different ratios of bacteria to different ratios of substratum surface materials were conducted. In this model bacterial cells were considered as colloidal particles regardless of the species and biological properties (such as outer membrane components and surface appendages). The hydrophobicity of, and charge on, bacteria and substratum surfaces as well as substratum surface roughness and cell size were quantified as bulk properties as described below.

To extend the value of this model the response of the empirical model (p) was substituted into a binomial distribution model as the dichotomous random variable and used to plot a probability distribution curve. This could be used to predict the probability of attachment to a surface of a given number of bacterial cells [equation (5b.2)] or bacterial cells within a given interval [equation (5b.3)] as follows:

$$f(x') = P = \frac{n!}{x'!(n-x')!} p^{x'}(1-p)^{n-x'} \qquad (0 \le x' \le n) \qquad (5b.2)$$

where *P* is the probability of *x'* number of bacterial cells attaching to a surface and *x'* is discrete random variable (in this case the number of bacterial cells).
or:

$$\int_{x_0}^{x_1} f'(x')dx' = P' = \int_{x_0}^{x_1} \left[\frac{n!}{x'!(n-x')!} p^{x'}(1-p)^{n-x'} \right] dx' \quad (x_0 \le x' \le x_1) \qquad (5b.3)$$

where *P'* is the probability of a given interval (x_0 to x_1) of bacterial cells attaching to a surface.

The values of *n* and *x'* are generally too high for a binomial distribution plot because a large total number results in a curve covering a small range of possible outcomes of discrete random variables (i.e. a small range of possible numbers of attached bacterial cells). In such cases predictions are unlikely to be accurate. Bacterial attachment to surfaces generally involves large numbers of cells in the system. For this reason predictions are not specific for each cell but for groups of cells. In the distribution model, *n* and *x'* values were therefore substituted by a unit number [1 unit = 1 million (10^6) CFU] for practical purposes. This approach resulted in the distribution model becoming a normal approximated binomial distribution and its response is the probability of *x'* unit(s) of bacterial cells attaching to a surface (*P*) or the probability of an interval of units [x_0 unit(s) to x_1 unit(s)] of bacterial cells attaching to a surface (*P'*).

5b.2.2 Experimental methods

5b.2.2.1 Bacterial culture and growth conditions

Streptococcus salivarius ATCC 13419 (relatively hydrophobic, negatively charged, relatively larger in size; Table 5b.1) and *Escherichia coli* ATCC 700728 (relatively hydrophilic, positively charged, relatively smaller in size; Table 5b.1) were obtained from the American Type Culture Collection (Manassas, USA) and maintained on Tryptic Soy Agar (TSA; Merck, USA) at 4°C. For all experiments both strains were grown in Tryptic Soy Broth (TSB; Merck) at 37°C for 24 h with shaking at 150 rpm.

5b.2.2.2 Preparation of bacterial suspensions

A 20 mL aliquot of each TSB culture was centrifuged at 7669 g at 4°C for 15 min. The pellet was gently washed with 150 mM phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl, pH 7.4; 1st BASE, Singapore) and resuspended in 20 mL PBS at a cell density of approximately 2.5×10^8 CFU/mL for *S. salivarius* and 3.5×10^9 CFU/mL for *E. coli*. Bacterial suspensions were prepared by mixing the two bacteria resuspended in PBS at a ratio of 10:0, 8:2, 6:4, 4:6, 2:8 and 0:10 (CFU:CFU) to a final cell density of 1×10^7 CFU/mL.

5b.2.2.3 Cell surface hydrophobicity (CSH) measurement

Cell surface hydrophobicity was measured as a bulk property using the Bacterial Attachment to Hydrocarbon (BATH) assay (Rosenberg et al., 1980). Briefly, a 3 mL aliquot of bacterial suspension was mixed with 1 mL of hexane (as the hydrocarbon; Sigma-Aldrich, USA) and vortexed for 2 min. The mixture was allowed to separate for 1 h. The OD_{550} of the aqueous phase was measured before (A_0) and after (A) addition of hexane. The hydrophobicity was expressed as $\%\ Binding\ to\ hexane = (1 - A/A_0) \times 100\%$.

5b.2.2.4 Cell surface charge measurement

Bacterial cell surface charge was measured as previously described (Chia et al., 2011). A 1 mL aliquot of bacterial suspension (pH 7.4) was used in a zetasizer (Nano ZS-ZEN3600; Malvern Instruments Ltd., UK) for charge measurement. Cell surface charge was expressed as zeta potential (ζ_b; mV).

5b.2.2.5 Cell size measurement

Bacterial cell size was measured with a zetasizer using the same method for cell surface charge measurement described above. Cell size was expressed as Z-Average (d.nm).

5b.2.2.6 Substratum surface preparation

Porous poly(butyl methacrylate-*co*-ethyl dimethacrylate) (BMA-*co*-EDMA; superhydrophobic, negatively charged, relatively rough; Table 5b.1) (Levkin et al., 2009) and aluminum sec-butoxide (ASB; superhydrophilic, positively charged, relatively smooth; Table 5b.1) (Jing et al., 2005) were used as substratum surface materials. The BMA-*co*-EDMA was prepared by mixing BMA (24% wt.; Merck), EDMA (16% wt.; Merck), 1-decanol (40% wt.; Merck), cyclohexanol (20% wt.; Merck) and 2, 2-dimethoxy-2-phenylacetophenone (DMPAP; as a photoinitiator; 1% wt. with respect to the monomers; Sigma-Aldrich). The ASB coating was prepared by mixing ASB (Acros Organics, USA), 2-propanol (R&M Chemicals, Malaysia) and ethyl acetoacetate (EAcAc; Acros Organics) at a ratio of 1:5:1 (mol:mol). The BMA-*co*-EDMA and ASB were mixed at ratios of 10:0, 8:2, 6:4, 4:6, 2:8 and 0:10 (vol:vol) before being coated onto glass slides (75 × 20 mm).

To achieve covalent coating of the BMA-*co*-EDMA/ASB mixtures onto glass surfaces, glass slides were first functionalized with 3-(trimethoxysilyl) propyl methacrylate (Sigma-Aldrich) (Levkin et al., 2009). Briefly, the slides were washed with distilled

water, immersed in 1 M NaOH (R&M Chemicals) for 1 h, rinsed with distilled water, immersed in 0.2 M HCl (R&M Chemicals), rinsed again with distilled water and dried under nitrogen gas. After the cleaning process a few drops of 20% (vol/vol) 3-(trimethoxysilyl) propyl methacrylate in ethanol (J. Kollin Chemicals, UK) and adjusted to pH 5 using acetic acid (R&M Chemicals) were placed on a glass slide and covered by a second glass slide. Another few drops of the solution were placed on the second slide and covered by a third glass slide. These stacks of glass slides were incubated with the solution for 30 min and treated with the solution again for another 30 min. The functionalized slides were washed in acetone (Sigma-Aldrich) and dried under nitrogen gas.

After functionalization the glass slides were coated with 3 mL of the BMA-*co*-EDMA/ASB mixtures, stacked and irradiated with UV light for 16 h using a UV collimated light source fitted with a 20 W UV-C lamp (254 nm; G20T10; Sankyo Denki, Japan). The resultant slides were carefully separated using a surgical blade, immersed in methanol (Systerm, UK) for 30 min and boiled in distilled water for 30 s. All substratum surfaces were autoclaved prior to the attachment assays.

5b.2.2.7 Substratum surface hydrophobicity measurement

Substratum surface hydrophobicity was measured by determining the water contact angle with surfaces using a goniometer (Model 250, Ramé-Hart Inc.) as previously described by Chia et al. (2011). A 2 µL drop of distilled water was placed on a substratum surface using a 10 µL syringe (Ramé-Hart Inc., USA) fitted with a needle gauge and the contact angles were measured using DROPimage software (Ramé-Hart Inc.). For each surface, 10 drops of water were measured on each of three independently prepared surfaces. Substratum surface hydrophobicity was expressed as average water contact angle (θ; °).

5b.2.2.8 Substratum surface charge measurement

Substratum surface charge (ξ_s; mV) was measured using a nanoparticle size and zeta potential analyzer (DelsaNano C; Beckman Coulter, USA) using 1 mM KCl (Sigma-Aldrich) as the electrolyte.

5b.2.2.9 Substratum surface roughness measurement

Substratum surface roughness was measured using an atomic force microscope (AFM; Veeco Instruments, Inc., Canada). Images for roughness (5 × 5 μm) were obtained using silicon nitride cantilevers (Budget Sensors, Bulgaria) with a spring constant of 0.06 N/m at a scan rate of 2 Hz and analyzed using Nanoscope software (version 5, Digital Instruments, Canada). Surface roughness was reported as root mean square (*RMS*; nm).

5b.2.2.10 Scanning electron microscopy (SEM) study

The substratum surfaces were visualized using a scanning electron microscope (S-3400N; Hitachi, Japan) at 2,000 times magnification.

5b.2.2.11 Attachment assay

An attachment assay was performed for each bacterial suspension to each substratum surface as previously described (Hood and Zottola, 1997). Briefly, a substratum surface slide (75 × 20 mm) was immersed in 50 mL bacterial suspension and incubated for 30 min without shaking. The slide was then removed from the suspension, gently rinsed three times with PBS and stained by 0.1% (wt/vol) crystal violet (Sigma-Aldrich). The number of attached cells was counted using a microscope (BX51; Olympus, Japan) and a total of 50 fields were counted for each slide. The ability to attach was expressed as *Attachment probability of an individual cell (p; %) = attached cell number / total cell number in the suspension (10^7 CFU/mL × 50 mL).*

5b.2.2.12 Model testing

The model was tested against experimental data derived from a previously study (Chapter 2a: Figure 2a.2). The attachment of four Streptococcal strains (*Streptococcus mutans* ATCC 25175 and 35668, *Streptococcus salivarius* ATCC 13419 and *Streptococcus mitis* ATCC 49456) to glass and stainless steel and their surface properties were measured using the same methods as described above. The probability of attachment of an individual bacterial cell in each case was calculated using the empirical model and was used to plot the distribution curve. The results of the numbers of bacteria attaching obtained from experiments were indicated on the theoretical distributions.

5b.2.3 Statistical analysis

All assays were performed in triplicate with independently grown cultures and all results are expressed as mean ± standard deviation unless otherwise stated. In order to normalize the data, cell surface hydrophobicity (CSH; %) data and probability of attachment (*p*; %) data were arcsine transformed (using radians), substratum surface water contact angles (θ; °) were *cos* (θ/2) transformed and then normalized using arcsine (using radians). Surface roughness (RMS; nm) and cell size (Z-Average; d.nm) data were logit transformed in order to assess the role of their magnitudes in attachment. Therefore the predictors were:

1. hydrophobic interaction [$\sqrt{arcsin(\%CSH)\times arcsin(\cos\frac{\theta}{2})}$];

2. electrostatic interaction ($-\frac{|\xi_b\xi_s|}{\xi_b\xi_s}\sqrt{|\xi_b\xi_s|}$);

3. substratum surface roughness ($log_{10}RMS$) and

4. cell size ($log_{10}Z\text{-}Average$).

The dependent variable was probability of attachment of an individual cell [*arcsin(p)*].

The correlation between each predictor and the dependent variable was examined using curve estimation. The reciprocities between the properties were examined by comparing regression slopes and covering area as described below. All predictors were entered into the empirical model in a stepwise fashion with a 95% confidence level based on changes in the F value (those with a confidence level of below 90% were removed). The interval of each bacterial or substratum surface property in which bacterial attachment was stochastic was determined by dividing the standard error of the estimation by the unstandardized coefficient of each predictor. All regressions were performed using SPSS software (PASW Statistics 18; SPSS Inc.). Probability distribution plots were performed using MINITAB software (MINITAB 15.1; Minitab Inc., USA).

5b.3 Results

5b.3.1 Experimental results

The results of bacterial and substratum surface property measurements are presented in Table 5b.1. Stepwise gradients for each of the bacterial and substratum surface properties were created. Cell and substratum surface hydrophobicity ranged from 18 to 84% and from 6 to 161° water contact angle (Appendix IV), respectively. Bacterial and substratum surface charge ranged from -10.1 to 2.3 mV and from -31.7 to 26.9 mV (Appendix IV), respectively. Substratum surface roughness and bacterial cell size ranged from 36 to 333 nm and from 3418 to 5162 d.nm, respectively. Table 5b.2 shows the individual probability of attachment of bacteria for all combinations of bacteria and surfaces. Microscopic observations demonstrated that no microbial aggregates were apparent between the two species. *S. salivarius* appeared as short chains and *E. coli* appeared as pairs or individual cells. All cells were clearly separated and countable (images not shown). It was also observed that the ratios of *S. salivarius* and *E. coli* cells attached to the substratum surfaces did not correlate with their initial ratios in the cell suspensions. The probability of bacterial attachment to the 2:8 BMA-*co*-EDMA : ASB substratum surface ratio were the highest as compared to attachment to other surfaces.

Cell mixtures with a higher ratio of *S. salivarius* as compared to *E. coli* had a higher probability of attaching to all surfaces except in the case of the attachment of 10:0 *S. salivarius* : *E. coli* bacterial ratio to the 10:0 BMA-*co*-EDMA : ASB ratio surface. Figure 5b.1 shows the SEM and AFM images of the substratum surfaces. The roughness of the surfaces decreased with a decreasing ratio of BMA-*co*-EDMA : ASB.

5b.3.2 Computational outputs

Figure 5b.2 shows the correlation between each predictor and the dependent variable. The correlation between hydrophobic interaction and attachment for each case was positively linear ($p<0.05$) with an R^2 ranging from 0.37 to 0.73. The overall correlation (considering all cases) had an R^2 of 0.63 ($p<0.05$). The correlation between electrostatic interaction and attachment for each case was also positively linear ($p<0.05$) with an R^2 ranging from 0.23 to 0.68. Electrostatic interaction and attachment were not correlated when considering all cases ($p<0.05$). The correlations between substratum surface roughness and attachment were negatively exponential ($p<0.05$) with R^2s ranging from 0.58 to 0.86. The overall correlation had an R^2 of 0.19 ($p<0.05$). However, using $exp(-log_{10}RMS)$ as a predictor instead of $log_{10}RMS$ did not improve the quality (R^2 and F) of the overall empirical model. The correlations between cell size and attachment were positively linear ($p<0.05$) with R^2s ranging from 0.17 to 0.83. The overall R^2 was 0.45 ($p<0.05$).

Table 5b.1 Surface hydrophobicity, charge and cell size of bacterial mixtures and surface hydrophobicity, charge and roughness of substratum material mixtures.

Ratios of bacterial and substratum material mixtures		Hydrophobicity [a]	Charge (mV)	Roughness (RMS; nm) [b]	Cell size (d.nm)
S. salivarius : *E. coli* [c]	10:0	84±5.2%	-10.1±1.0	-	5162±459
	8:2	67±2.6%	-8.4±0.9	-	4504±498
	6:4	59±1.6%	-7.2±0.5	-	4293±165
	4:6	55±1.9%	-6.5±0.6	-	4066±219
	2:8	45±4.1%	-5.4±0.5	-	3847±367
	0:10	18±3.4%	2.3±1.0	-	3418±204
BMA-*co*-EDMA : ASB [d]	10:0	161±1.1°	-31.7±0.2	333	-
	8:2	133±2.7°	-27.4±0.6	97	-
	6:4	101±1.3°	-24.5±0.1	77	-
	4:6	74±0.6°	-15.6±0.4	65	-
	2:8	51±1.4°	22.9±0.3	36	-
	0:10	6±1.0°	26.9±0.1	37	-

[a] Bacterial surface hydrophobicity was expressed as % binding to hexane while substratum surface hydrophobicity was expressed as θ (°).

[b] Substratum surface roughness was obtained from one reading only.

[c] The ratios of *S. salivarius* and *E. coli* were based on colony forming unit (CFU).

[d] The ratios of BMA-*co*-EDMA and ASB were based on volume.

Table 5b.2 Pairwise probability (%) of attachment of an individual cell in different bacterial and substratum ratios.

Ratios of bacterial mixture (*S. salivarius* : *E. coli*) [b]	Ratios of substratum surface mixture (BMA-*co*-EDMA : ASB) [a]					
	10:0	8:2	6:4	4:6	2:8	0:10
10:0	0.46±0.29	4.21±0.42	5.55±0.51	4.58±0.44	5.94±1.20	4.88±0.53
8:2	2.32±0.90	2.63±0.46	3.73±0.67	4.58±0.62	5.17±0.52	3.89±0.38
6:4	1.38±0.42	2.16±0.31	2.79±0.58	2.73±0.62	4.89±0.23	3.17±0.25
4:6	0.78±0.09	1.33±0.29	1.80±0.33	2.15±0.37	5.94±1.20	2.49±0.07
2:8	0.50±0.13	0.90±0.10	0.93±0.13	1.61±0.22	2.22±0.28	1.84±0.38
0:10	0.03±0.01	0.07±0.01	0.10±0.02	0.22±0.02	0.63±0.19	0.29±0.07

[a] The ratios of BMA-*co*-EDMA to ASB were based on volume.

[b] The ratios of *S. salivarius* to *E. coli* were based on colony forming unit (CFU).

It was also found that substratum surface hydrophobicity had an impact on bacterial surface hydrophobicity with respect to its ability to affect attachment. Figure 5b.3A shows the regression plots of bacterial surface hydrophobicity against attachment to different surfaces. The regression lines were significantly different (ANCOVA; $p<0.05$) with different slopes and different areas between the x axis [from *arcsin(0)* to *arcsin(100%)*] and each line, suggesting that bacterial surface hydrophobicity affected attachment differently on different surfaces. The regressions of the slopes and areas were plotted against substratum surface hydrophobicity (Figure 5b.3B and C) and were found to be quadratic (parabola; $y = ax^2 + bx + c$). The vertices ($-b/2a$) of the parabolas were 61.0° for the slope plot, suggesting that bacterial surface hydrophobicity has its strongest effect on attachment to substratum surfaces with this water contact angle, and 52.5° for the area plot, suggesting that substratum surfaces with this water contact angle are most favorable for bacterial attachment. Overall this suggests that substratum surfaces with a water contact angle of between 50 to 60° are highly susceptible to

bacterial colonization. Using the same method, substratum surface hydrophobicity was also found to be affected by bacterial surface hydrophobicity. The higher the bacterial surface hydrophobicity the stronger the effect of substratum surface hydrophobicity (R^2=0.94), and the greater the probability of bacterial attachment (R^2=0.95; calculations not shown). Interestingly, reciprocity was also observed between substratum surface roughness and cell size. It was found that the effect of surface roughness was stronger on the attachment of both smaller and larger cells as compared to cells with a medium size and was lowest at a cell size of 4270.7 d.nm (R^2=0.87; calculations not shown). In addition, cell size was found to have a stronger effect on attachment to smoother surfaces (R^2=0.76; calculations not shown). However, a similar relationship was not found between bacterial surface charge and substratum surface charge.

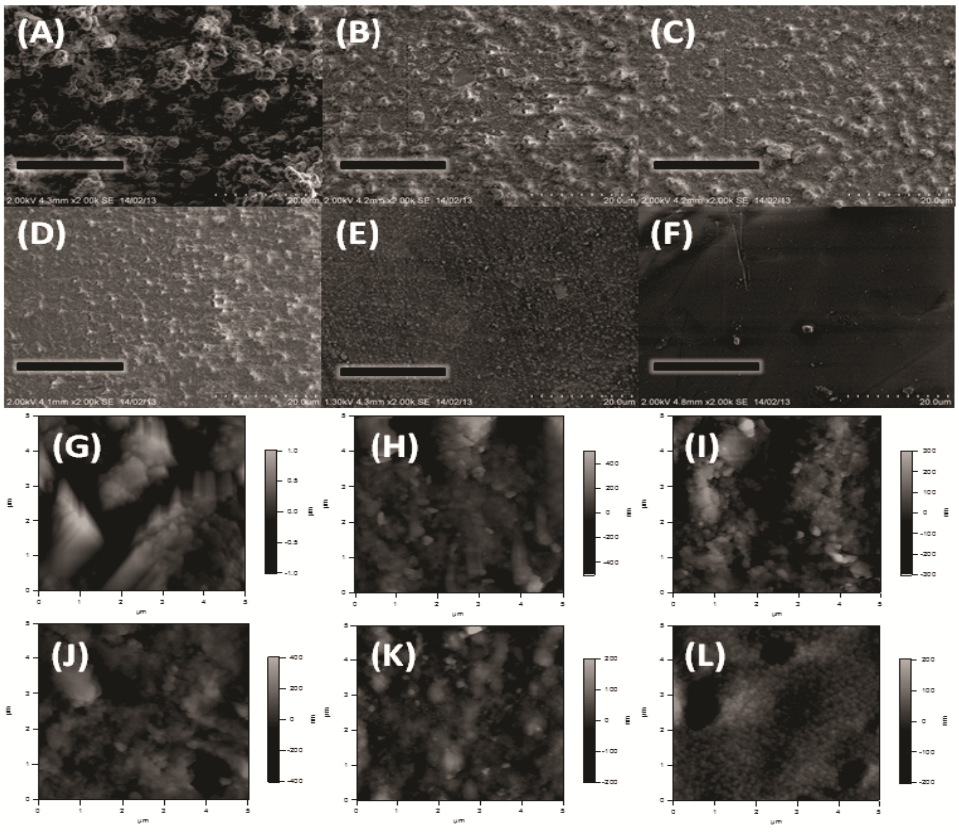


Figure 5b.1 Scanning electron microscopy images (scale bar: 20 μm) and atomic force microscopy images (5 × 5 μm) of the substratum surfaces with the ratios of BMA-*co*-EDMA to ASB at 10:0 (A, G), 8:2 (B, H), 6:4 (C, I), 4:6 (D, J), 2:8 (E, K) and 0:10 (F, L).

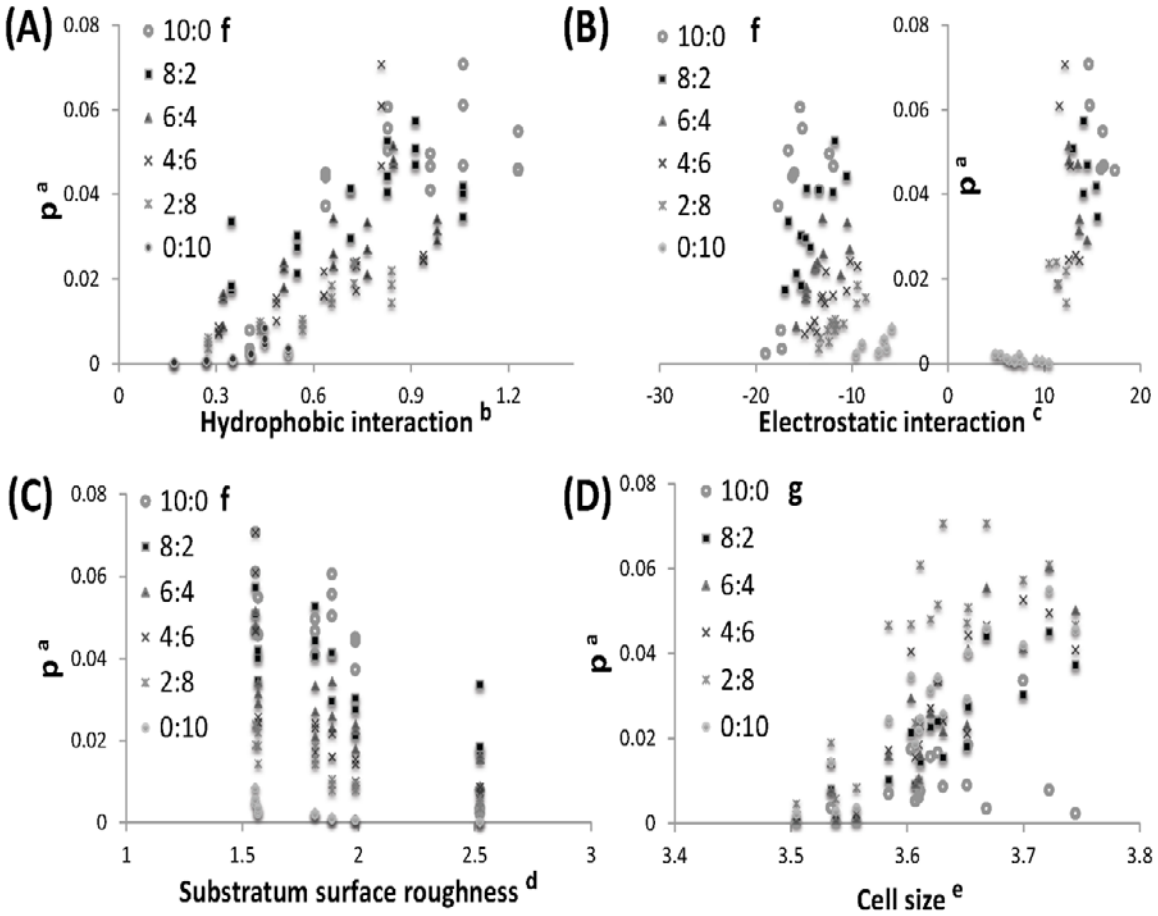


Figure 5b.2 Scatterplots of each predictor against attachment. (A) hydrophobic interaction; (B) electrostatic interaction; (C) substratum surface roughness; (D) cell size. The values of predictors and the dependent variable were based on transformed data: [a] p: *arcsin (% probability of attachment of individual cell)*; [b] hydrophobic interaction: $\sqrt{arcsin(\%CSH)\times arcsin(\cos\frac{\theta}{2})}$; [c] electrostatic interaction: $-\frac{|\xi_b\xi_s|}{\xi_b\xi_s}\sqrt{|\xi_b\xi_s|}$; [d] substratum surface roughness: $log_{10}RMS$; [e] cell size: log_{10}*Z-Average*. [f] The cases in (A), (B) and (C) were grouped based on bacterial ratios, [g] those in (D) were grouped based on substratum surface material ratios.

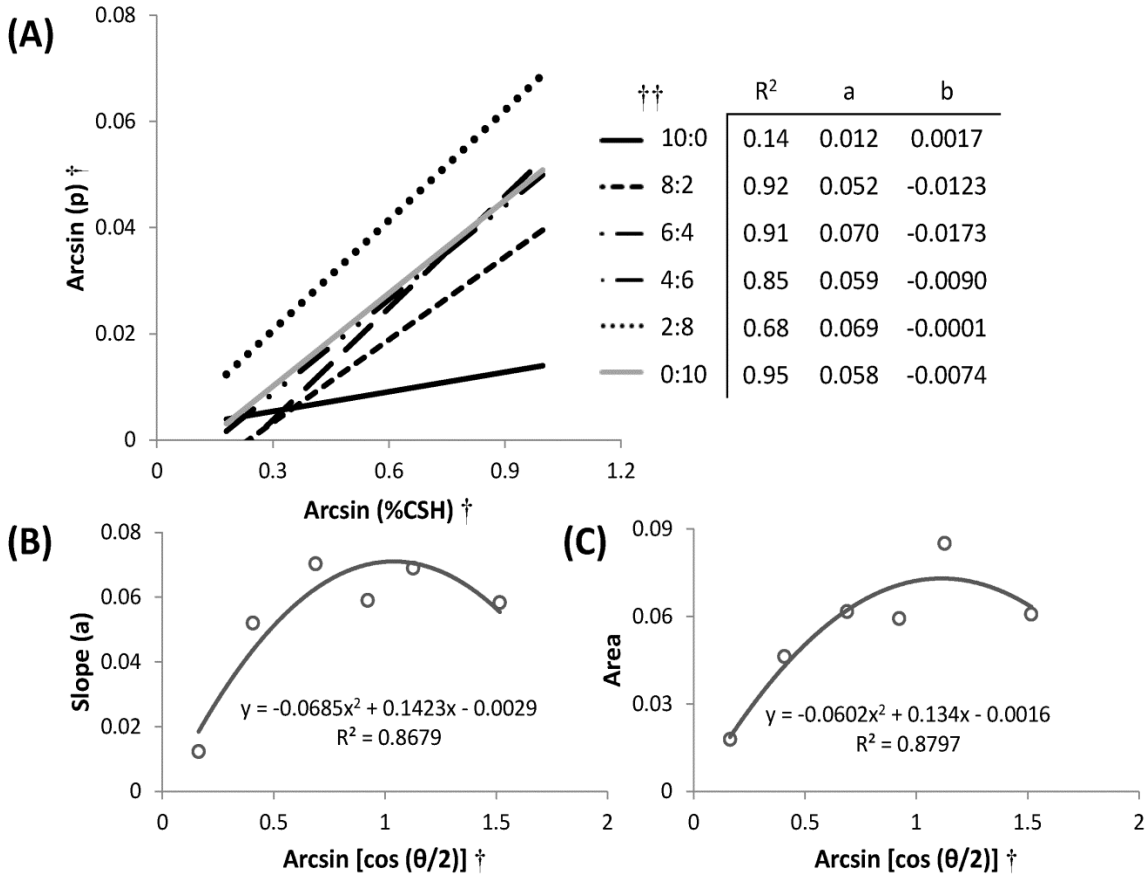


Figure 5b.3 The influence of substratum surface hydrophobicity on the correlation between bacterial cell surface hydrophobicity and attachment. (A) Linear regressions of bacterial surface hydrophobicity against attachment on different surfaces (expressed as $y = ax + b$); (B) The quadratic regression of substratum surface hydrophobicity against the slopes (a) of linear regressions (expressed as $y = ax^2 + bx + c$); (C) The quadratic regression of substratum surface hydrophobicity against the areas between the x axis [from *arcsin(0)* to *arcsin(100%)*] and each linear regression line, obtained from $Area = \int_{-\frac{a}{b}(\geq 0)}^{arcsin\ 100\%} (ax + b)dx$ (expressed as $y = ax^2 + bx + c$). † Values of attachment were expressed as *arcsin(%individual cell attachment)*, values of bacterial surface hydrophobicity were expressed as *arcsin(%CSH)* and values of substratum surface hydrophobicity were expressed as *arcsin[cos(θ/2)]*. †† The cases in (A) were grouped based on substratum surface material ratios.

All predictors entered into the empirical model had significant F value changes ($p<0.05$) and the empirical model was established with an R^2 of 0.814 and a standard error of the estimation of 0.0082 (0.82% in attachment probability) (Table 5b.3). Table 5b.4 shows the coefficient of each predictor in the regression equation which were used to establish the equation:

$$p = \sin\{[0.038\sqrt{\frac{\pi}{180}\arcsin(\%CSH)\times\frac{\pi}{180}\arcsin(\cos\frac{\theta}{2})} + 5.15\times10^{-5} \times(-\frac{|\xi_b\xi_s|}{\xi_b\xi_s})\sqrt{|\xi_b\xi_s|} - 0.003\log_{10}RMS + 0.12\log_{10}\text{Z - Average} - 0.431]\times\frac{A}{15}\times\frac{180}{\pi} \quad (5b.4)$$

where A is the substratum surface area (cm^2) and is divided by the area of the substratum surfaces used in this study (15 cm^2). The $\frac{\pi}{180}$ and $\frac{180}{\pi}$ in the equation serve as conversion factors between radians and angles (^{o}).

The standardized coefficients (Table 5b.4) represent the significance of the predictors in the response. Hydrophobic interaction had the highest absolute standardized coefficient (0.535) indicating that it played the most important role in attachment as compared to the other predictors. Electrostatic interaction (0.034) by contrast had the lowest impact. Notably, the coefficient for surface roughness was negative (-0.048) meaning that it had a negative effect on attachment. This suggests that he smoother the substratum surface is the more probable bacterial attachment will occur, a finding that is in accord with the individual correlation between surface roughness and attachment shown in Figure 5b.2.

Table 5b.3 The R^2, standard error of the estimation and change statistics of each predictor in the empirical model.

Model [a]	R^2	Std. Err. of the Estimate	Change Statistics		
			R^2 Change	F Change	p (F Change)
Predictor 1	0.649	0.0115	0.649	117.610	<0.001
Predictor 1+2	0.667	0.0110	0.018	5.442	0.004
Predictor 1+2+3	0.725	0.0099	0.058	14.790	<0.001
Predictor 1+2+3+4	**0.814**	**0.0082**	0.089	28.695	<0.001

[a] Predictor 1: hydrophobic interaction [$\sqrt{arcsin(\%CSH)\times arcsin(cos\frac{\theta}{2})}$]; 2: electrostatic interaction ($-\frac{|\xi_b\xi_s|}{\xi_b\xi_s}\sqrt{|\xi_b\xi_s|}$); 3: substratum surface roughness ($log_{10}RMS$); 4: cell size ($log_{10}Z\text{-}Average$). All predictors were entered into the empirical model in a stepwise fashion at a 95% confidence level. The numbers in bold face are the R^2 and standard error of the estimation of the overall model.

Table 5b.4 Standardized and unstandardized coefficients for each predictor in the empirical model.

Predictor	Unstandardized Coefficient	Standardized Coefficient (Beta)
Hydrophobic interaction	0.038	0.535
Electrostatic interaction	5.15×10^{-5}	0.034
Substratum surface roughness	-0.003	-0.048
Cell size	0.120	0.304
(Constant)	-0.431	-

The standard error of the estimation (0.0082) indicates the prediction accuracy of the model and can be used to determine under which conditions bacterial attachment is stochastic. The range of values for each property (Figure 5b.4) under which attachment is stochastic was determined by dividing the standard error by the unstandardized coefficient of the corresponding predictor and back transforming the result to raw values for the property. Each of the intervals indicates how far apart the values of a property should be to result in significantly different bacterial attachment. If the value of a property in one case falls within the interval of that in another case, the resultant bacterial attachment is considered not to be differentiable and therefore bacterial attachment in this interval is deemed to be stochastic (or unpredictable) using this model. The range of values of bacterial surface hydrophobicity for which attachment is stochastic narrowed with increasing hydrophobicity, indicating that the attachment of relatively hydrophilic bacteria is likely to be more stochastic than for hydrophobic ones. For example, if all other properties stay the same, in order to attach in significantly different numbers to a surface than bacteria with 20% hydrophobicity, the hydrophobicity of other bacteria must be 11.3% higher or 11.5% lower. For bacteria with 80% hydrophobicity, on the other hand, the hydrophobicity of other bacteria need only be 6.4% higher or 7.5% lower to attach in significantly different numbers to a surface. The reason that the interval changes with changing values of the property is that the data transformation from the raw value to the predictor is based on non-linear transformations such as arcsine, cosine and logit. The size of substratum surface hydrophobicity interval remains constant (13.4° water contact angle for either the upper or the lower interval) because the non-linearity of the cosine transformation was compromised by the arcsine transformation. The size of the intervals associated with charge (net charge for both bacteria and substratum surface, and for both cases of co- and counter-charge) and surface roughness were wide due to their low impacts on attachment (reflected as low standardized coefficient values). This suggests that the difference in charge or roughness in different scenarios need to be extremely large to allow differences in attachment of bacteria to surfaces to be significant. Bacterial

attachment is therefore highly stochastic when considering only electrostatic interaction or surface roughness. The interval of cell size, however, widened with an increasing Z-Average value, indicating that the attachment of smaller cells is relatively more predictable.

5b.3.3 Model testing

The results of testing the model for its ability to deterministically and probabilistically predict the attachment of four Streptococcal strains to glass and stainless steel are presented in Figure 5b.5. All of the experimental results fell into the probability distribution curve suggesting that the model probabilistically predicted attachment in all cases. The experimental results of the attachment to stainless steel were farther from the vertexes (deterministic prediction results) of the curves than those to glass, suggesting the deterministic predictions were more accurate on glass than on stainless steel.

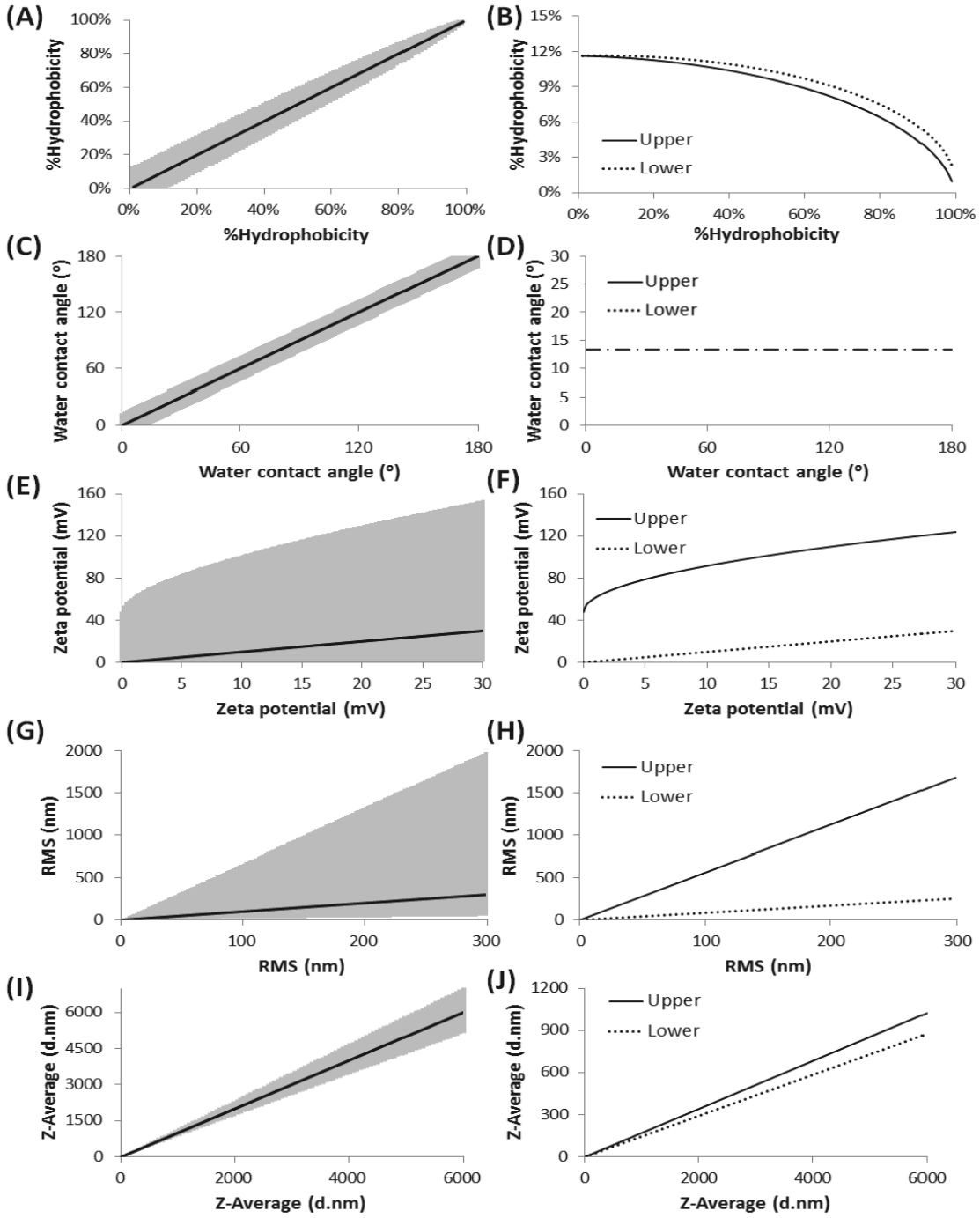


Figure 5b.4 Range of values of bacterial and substratum surface properties in which bacterial attachment to surfaces is stochastic (first letter) and the size of the each range (upper zone and lower zone; second letter). Properties: bacterial surface hydrophobicity (A, B); substratum surface hydrophobicity (C, D); net charge (for both bacteria and substratum surface, and for both cases of co- and counter- charged; E, F); substratum surface roughness (G, H); and cell size (I, J). The shaded zones indicate the interval in which bacterial attachment to surfaces is stochastic.

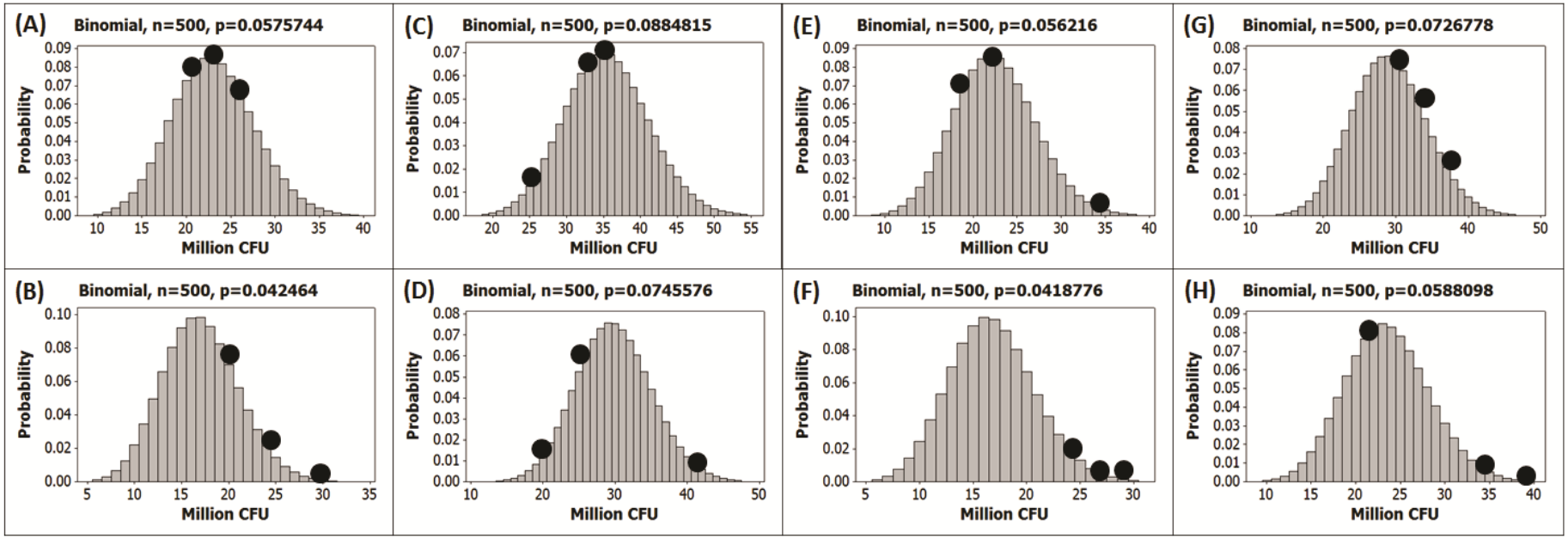


Figure 5b.5 Deterministic and probabilistic model testing (binomial distribution plots) using experimental data of the attachment to glass (first letter) and stainless steel (second letter) of *Streptococcus mutans* ATCC 25175 (A, B), *Streptococcus mutans* ATCC 35668 (C, D), *Streptococcus salivarius* ATCC 13419 (E, F) and *Streptococcus mitis* ATCC 49456 (G, H). The distribution curves are the probabilistic prediction results and the vertices of the curves are the deterministic prediction results, n is the total unit number of the cells in the bacterial suspension [1 unit = 1 million (10^6) CFU] and p is the probability of attachment of an individual cell (unit). The black spots are the attachment results obtained from experiments (in triplicate).

5b.4 Discussion

All bacterial and substratum surface properties included in this study were found to have significant influence on attachment of bacteria to surfaces but with different levels of impact. Hydrophobic interactions, which were found to be the strongest predictor in the empirical model, are generally recognized to play an important role in bacterial attachment. Bacterial surface hydrophobicity and substratum surface hydrophobicity have been repeatedly proven to positively correlate with attachment (Liu et al., 2004; Boks et al., 2008; Goulter et al., 2009; Salerno et al., 2009). In addition to Lifshitz-van der Waals forces, hydrophobic interactions are also influenced by Lewis acid-base forces (hydrogen bonding) (van Oss et al., 1986), which have been reported to dominate the overall free energy in bacterial attachment systems in many cases based on XDLVO theory (Kang and Choi, 2005; Chen et al., 2011). This may explain the significance of the hydrophobic interaction found in this study. Electrostatic interactions are also generally considered to be an important factor for bacterial attachment to surfaces. Arguments regarding its significance have been raised (Kang and Choi, 2005; Salerno et al., 2009; Warning and Datta, 2013) and some suggest its importance in attachment may be overrated (Poortinga et al., 2005), a conclusion which would concur with the computational results of the present study. Specifically, it has been suggested that bacterial cells are ion-penetrable resulting in a lower density of charge on the outer surface of a cell than the charge a cell as a whole carries (Poortinga et al., 2005). In addition, the electrostatic effect may be distinct only when other interactions are weak, for example, the attachment of hydrophilic but highly charged bacteria could be relatively more correlated to electrostatic interactions. This feature may explain the finding of some studies in which charge appears strongly correlated with attachment and functional properties of uniformly hydrophobic bacteria (Rivas et al., 2005). The effect of charge could be strain/species and substratum surface dependent and masked by other properties or interactions. Substratum surface roughness was found to negatively affect attachment which is in accord with the findings of other studies (Goulter et al., 2011). In

contrast with the work of Medilanski et al. (2002) who found that bacteria attach in a high number to both smoother and rougher surfaces and that minimal attachment occurs at a medium surface roughness. A large range of surface roughness should therefore be screened with respect to bacterial attachment in order to obtain a better understanding of this process. In addition to this, roughness may also be associated with other substratum surface properties since it can, for instance, increase the effect of surface tension and thereby affect surface hydrophobicity (Quirynen et al., 1990). The contribution of roughness to attachment therefore is likely to be more complex than most models take into account. In the current study cell size was also indicated to have a positive effect on attachment and it has been suggested to be due to the fact that size may affect the steric arrangement between the cells (Clark and Gibbons, 1977) and therefore result in different spatial distributions (e.g. formation of chains or clumps on the substratum surface).

The reciprocity found between bacterial and substratum surface hydrophobicity reveals that higher bacterial surface hydrophobicity was correlated with a higher probability of bacterial attachment to surfaces which also increased the effect of substratum surface hydrophobicity on bacterial attachment, which is generally accepted in the literature (Zita and Hermansson, 1997; Rad et al., 1998; Li and Logan, 2004; Liu et al., 2004). This reciprocity indicates that substratum surfaces with a medium hydrophobicity are more favorable for bacterial attachment than those with high or low hydrophobicity. This phenomenon results in bacterial surface hydrophobicity having a greater effect on attachment than other parameters. This phenomenon suggests that hydrophobic interactions in an attachment system are not fully energy-dependent (i.e. higher surface tension energy does not necessarily result in greater attachment). It was also found that substratum surface roughness and cell size are not independent in terms of their contributions to attachment. This is in agreement with the finding of Medilanski et al. (2002) who suggested that the optimum roughness of the substratum surface for attachment depends on the size and shape of the cells. The reciprocity between

roughness and cell size was however not explained by the model.

The range of values of individual properties in which bacterial attachment to a surface were stochastic indicates the levels of the predictability and stochasticity of bacterial attachment. The range of values in which bacterial attachment was stochastic for bacterial surface hydrophobicity decreased with increasing values of hydrophobicity, suggesting that the attachment of hydrophilic bacteria is more stochastic than that of hydrophobic ones. This leads to a hypothesis that correlations between bacterial surface hydrophobicity and attachment are more likely to be found for relatively hydrophobic bacteria, a feature which has been observed by some authors (Grivet et al., 2000; Roosjen et al., 2006; Goulter et al., 2009). According to this hypothesis correlation between substratum surface hydrophobicity and attachment can be found on both hydrophobic and hydrophilic surfaces as the range of values remained constant. The range of values for substratum surface roughness and cell size, however, increased with increasing value of these properties, suggesting that attachment scenarios involving large cells or rough surfaces are less predictable. This finding is not unreasonable as rougher surfaces and larger cells could have more complicated surface energy profiles and spatial distribution as mentioned above. The wide range of values for charge suggests that predictions for bacterial attachment to surfaces based on charges could be difficult. These results may act as a guide for future research examining the relationship between these properties and bacterial attachment and specifically provide insight into the selection of reasonable ranges of the properties that can respond in a predictable manner.

Probability distribution analysis has previously been used in bacterial attachment systems by Chen et al. (2011) A Poisson distribution was applied to estimate the probability of the formation of a hydrogen bond between a bacterial cell surface and a substratum surface, and the probability of the formation of a given number of hydrogen bonds on the same bacterial cell surface. In our study, a probability distribution was

used in a less specific way (i.e. to estimate the probability of bacterial attachment). This approach is therefore useful in biological systems with respect to the occurrence of an event or a specific interaction in an event as it provides a probabilistic analysis based on a deterministic analysis.

The testing of the model demonstrated that all experimental results fell within the binomial distribution curves but not all of them were exactly on the vertices, suggesting that biological variability exists in attachment systems which also contribute to the stochastic nature of such systems. This finding illustrates the importance of probabilistic prediction which is more useful than the deterministic prediction from mechanistic models such as thermodynamic theory and XDLVO theory (Chia et al., 2011; Nguyen et al., 2011a). It was also observed that the predictions for the attachment of bacteria to stainless steel were less accurate than those to glass, suggesting that there are other factors in the case of stainless steel, in addition to the predictors used in the model, that affect bacterial attachment. For example, stainless steel is a conductive surface which allows charge transfer to and from bacterial cells resulting in a change of electric potential and in turn affecting attachment (Nguyen et al., 2011a). This model should not be extensively used for prediction purposes because no model can all-inclusively explain bacterial attachment due to the complexity of the process (Goulter et al., 2009; Warning and Datta, 2013). The model can and should be used to gain a fundamental understanding about the role of the properties included in it for bacterial attachment.

All mathematical models of the type described here have limitations. The current model, for example, is only valid for bacterial attachment in water-based systems and onto uniform surfaces (i.e. abiotic surfaces). In addition, the model was based on the results obtained from experiments performed at a population scale and therefore provides a better qualitative understanding of attachment at the macroscopic level. It would be useful to include experiments at the scale of the individual bacterium in future models and this may help to explain attachment in a more practical way.

5b.5 Conclusions

In summary, a semi-empirical approach that deterministically and probabilistically represents the process of bacterial attachment was established in this study. The approach revealed that hydrophobic interactions play the most important role in bacterial attachment to surfaces while electrostatic interactions have little effect on attachment. It also suggests that bacterial attachment is stochastic under some conditions associated with particular ranges of predictor values.

Chapter 6

General Discussion and Conclusions

6.1 Major findings and contributions of this study

The primary aims of this project were to investigate the effects of tea extracts on oral bacteria with respect to their cell surface properties, attachment and biofilm formation on surfaces, and to study the predictability and stochasticity of bacterial attachment to hard surfaces using the XDLVO theory and a new mathematical approach.

Based on the results obtained from this project, the following conclusions can be drawn: The tea extracts can alter the cell surface properties of the five oral bacterial strains used in this project and can inhibit their attachment and biofilm formation on different abiotic surfaces (Chapter 2a). In addition, a reduction in bacterial surface hydrophobicity was the key mechanism of the effect of the tea extracts in inhibiting attachment of Streptococcal strains (Chapter 2a). Furthermore the XDLVO theory revealed that hydrogen bonding is a key feature of hydrophobic interactions that dominated the overall interaction in the attachment systems (Chapter 5a). It was also established that the inhibitory effects of oolong tea extract on the attachment of *S. mutans* strains and pu-erh tea extracts on the biofilm formation of *S. mutans* were due to the coating of cell surfaces by flavonoids, tannins and indolic compounds (Chapter 4). It is therefore reasonable to conclude that tea components can coat the bacterial cells and occupy the ligand receptor sites on cell surfaces that can form hydrogen bonds with substratum surfaces. This in turn may interfere with the formation of hydrogen bonds and reduces the hydrophobicity of cells resulting in an inhibition of attachment. Pu-erh and chrysanthemum tea extracts were found to effectively inhibit the attachment of Streptococci to cultured human gingival cells (Chapter 3). The mechanism of this inhibition was not investigated in this project but this is an interesting finding worthy of future research. In total, findings contribute to the literature of the effects of tea on oral health with respect to the prevention of oral bacterial colonization of surfaces. Based on the conclusions above, it can reasonably be suggested what a cup of tea may do in a real oral environment with respect to bacterial attachment. After tea consumption some of

the oral bacteria may be killed or inhibited when tea components are at high concentrations. Subsequently attachment and biofilm formation by the remaining cells on dental hard and soft tissues may be suppressed when the concentrations of tea components are diluted by saliva (Xu et al., 2012). In addition to the bactericidal effects of tea, the ability of tea to inhibit oral bacterial attachment and biofilm formation introduces another benefit of the supplementation of tea components in mouthwashes and toothpastes. These effects on attachment and biofilm formation need to be further confirmed by *in vivo* studies in the oral cavity.

Cell surface properties, attachment and biofilm formation by oral bacterial can be affected by various oral conditions such saliva and dietary sucrose *in vitro* (Chapter 2b and Chapter 2c). It is therefore reasonable to assume that the inhibitory effects of tea extracts on attachment and biofilm formation can also be influenced by these conditions in the oral cavity (Kolenbrander and London, 1993). This indicates that the effects of tea on oral bacterial properties and behaviors should be studied in the presence of different components found in the oral cavity.

The XDLVO theory was not effective in predicting the attachment of oral bacterial to hard surfaces because it is solely deterministic and does not consider some of the key parameters affecting attachment (Chapter 5a). The newly developed empirical model which takes into account substratum surface roughness and cell size with the aid of a normal approximated binomial distribution can more accurately predict attachment of the type used in this project. The empirical model showed that hydrophobic interactions are the most important physicochemical mechanism of bacterial attachment. It also defined the level of predictability and stochasticity of bacterial attachment by determining the range of values of each cell/substratum property within which bacterial attachment to a hard surface is random (Chapter 5b). This new mathematical modeling approach suggests directions for future research. Specifically they could provide a guide for the selection of reasonable ranges of the properties that can respond in a predictable

manner. Mathematical models can never be universally valid because ideal systems do not exist. They should therefore not be extensively used for prediction purposes but should be used to study the mechanisms of bacterial attachment (Bos et al., 1999).

6.2 Future directions

This project provides some level of understanding with respect to the physicochemical process of bacterial attachment and in particular the attachment of oral bacterial to surface and the ability of tea to inhibit. Below are listed potential areas of future research that could be conducted in order to gain a better understanding of these processes and to help develop strategies to control and minimize bacterial attachment.

6.2.1 Identification of the tea components coated on cell surfaces

The tea components coated onto cell surfaces of *S. mutans* and that inhibited their attachment and biofilm formation were identified as flavonoids, tannins and indolic compounds using phytochemical screening methods (Chapter 4). It is important to identify the specific compounds involved in future research. The compounds extracted from the coated cell surfaces could be isolated and purified using column chromatography and high-performance liquid chromatography (HPLC) systems equipped with semi-preparative column. They could subsequently be identified using high-performance liquid chromatography-mass spectrometry (HPLC-MS) and nuclear magnetic resonance (MNR) spectroscopy techniques (Manojlović et al., 2011).

6.2.2 Bacterial attachment to biotic surfaces

Substantial inhibitory effects of pu-erh and chrysanthemum tea extracts on the attachment of oral Streptococci to cultured human gingival fibroblasts were observed (Chapter 3) in this project. The mechanisms behind this effect were not investigated. In

future research the mechanisms of this effect should be established since the reductions in attachment observed were substantial (a reduction up to 4 log CFU/well). The effect of tea extracts on the attachment of oral bacteria to other oral soft tissues should be studied in order to understand the broader picture of the beneficial effects of tea on oral health.

6.2.3 Study the effects of tea in a simulated oral environment

As suggested in Chapter 2b and 2c conditions such as the presence of saliva and dietary sucrose that alter bacterial cell surface properties and affect their colonization of hard surfaces may also influence the inhibitory effects of tea. It would therefore be appropriate to study the effects of tea on attachment and biofilm formation by oral bacteria on surfaces in a simulated oral environment in future research. For example, the presence of saliva as a suspending fluid, sucrose as a nutrient source for bacteria and periodical flow condition using a flow chamber could be included in such studies (Busscher and van der Mei, 1995).

6.2.4 In vivo studies

The inhibitory effects of tea on attachment of oral bacteria and their biofilm formation were apparent and reproducibly observed in *in vitro* studies. These effects need to be further confirmed by *in situ* studies using extracted teeth or by *in vivo* studies using animal models.

6.2.5 Probabilistic modeling and modeling for the biological process of bacterial attachment

A mathematical approach to predict bacterial attachment using probability distributions was successfully applied in this study (Chapter 5b). Future modeling of bacterial attachment should use probability analysis and should take more parameters into

account (Chen et al., 2011). In addition, future models should include, where possible, biological interactions and factors such as biological cell surface properties or biotic substratum surfaces in order to represent both the physicochemical and the biological processes of bacterial attachment.

6.2.6 Method standardization and advanced techniques

The lack of process control and standardization of methods to study bacterial attachment and bacterial/substratum properties results in difficulties in studying the mechanisms of bacterial attachment and poor predictions by mechanistic models. This in turn results in difficulties in the development of strategies to control attachment. Standardization of methods should be implemented across laboratories and advanced techniques such as AFM and chemical force microscopy (CFM) should be used to avoid bulk property and population behavior measurements and thereby eliminate noise (Nguyen et al., 2011a).

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Appendix I Detachment Assays

The following appendix contains the results obtained from the detachment assays of oral bacteria from hard surfaces (Chapter 2a).

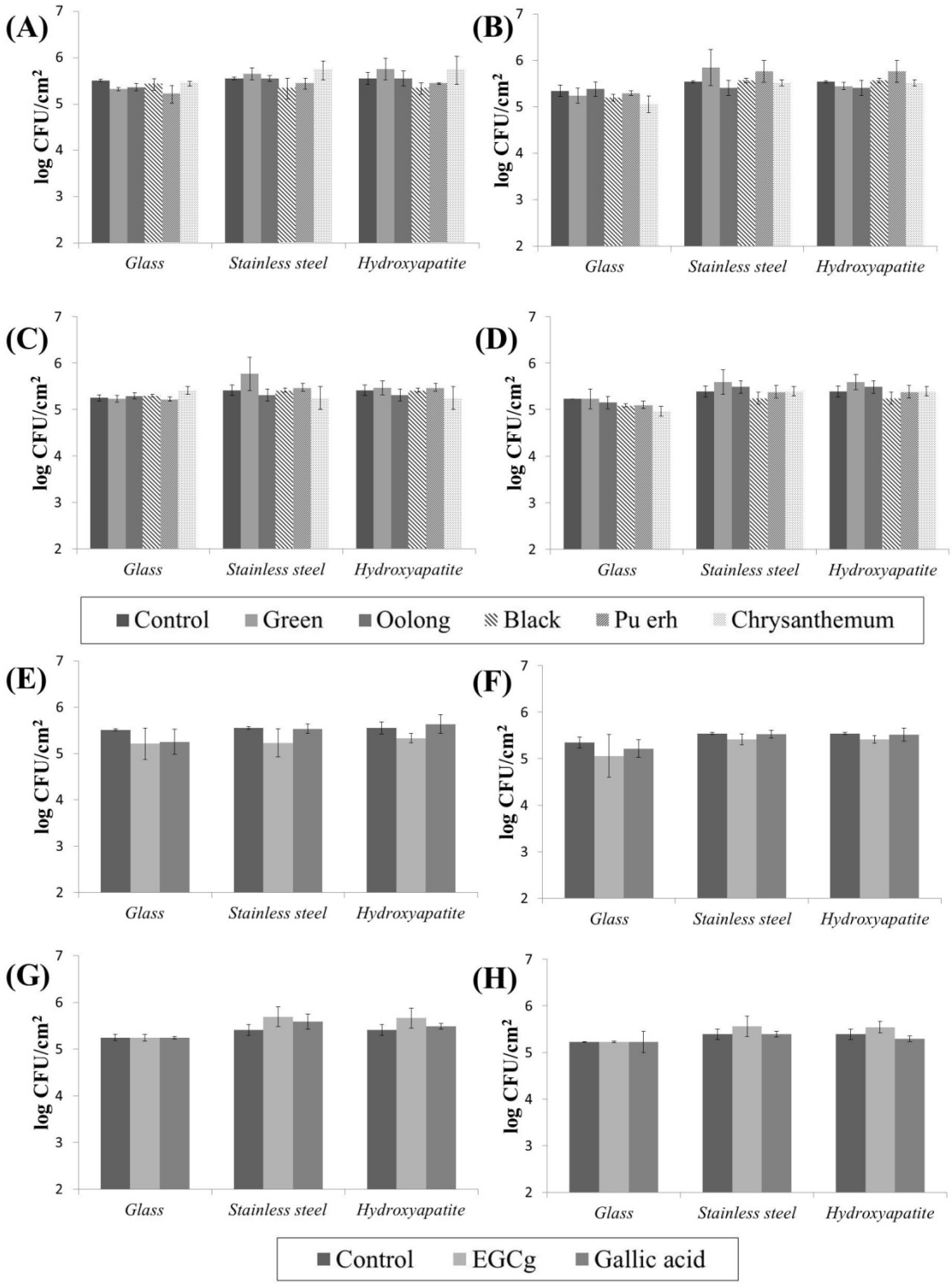


Figure A.1 Effect of tea extracts (first letter) and tea components (second letter) on bacterial detachment from different surfaces. (A) (E) *S. mutans* ATCC 25175, (B) (F) *S. mutans* ATCC 35668, (C) (G) *S. salivarius* ATCC 13419 and (D) (H) *S. mitis* ATCC 49456.

Appendix II Method Validation of the Biofilm Formation Assays

The following appendix contains the results obtained from the method validation for the biofilm formation assays (Chapter 2a, 2b and 4).

In order to validate the method for biofilm formation assays (2a.2.5.5, 2b.2.7 and 4.2.4), a survival test and a detachment ability test was carried out to ensure that sonication can effectively detach biofilm cells without killing them.

Survival tests

A Falcon tube containing 40 mL of bacterial cells suspension was sonicated using a water bath sonication under the same conditions as stated in Chapter 2a, 2b and 4 (2a.2.5.5, 2b.2.7 and 4.2.4). An aliquot of the suspension was taken out from the tube at 5 and 10 min, serially diluted, spread plated on TSA and incubated at 37^{o}C for 48 h before enumeration. The results show that sonication up to 10 min did not kill the cells (Figure A.2).

Detachment ability tests

A hard surface slide with a biofilm grown on top was placed in a Falcon tube containing 40 mL of PBS and sonicated for 10 min under the same conditions. An aliquot of the PBS was serially diluted, spread plated on TSA and incubated at 37^{o}C for 48 h before enumeration. The slide was then transferred into a stomacher bag containing 50 mL of PBS and pummeled in a stomacher for 10 min under the same conditions as stated in Chapter 2a, 2b and 4 (2a.2.5.5, 2b.2.7 and 4.2.4) to remove the remaining cells. An aliquot of the homogenate was serially diluted, spread plated on TSA and incubated at 37^{o}C for 48 h before enumeration. The cell counts before stomaching (PRE) were compared to those after stomaching (POST). The results show that the cell number on the slide was reduced by sonication by up to 2 log CFU/cm^{2} in all cases, indicating that sonication can remove approximately 99% of the biofilm cells from the slide (Figure A.3).

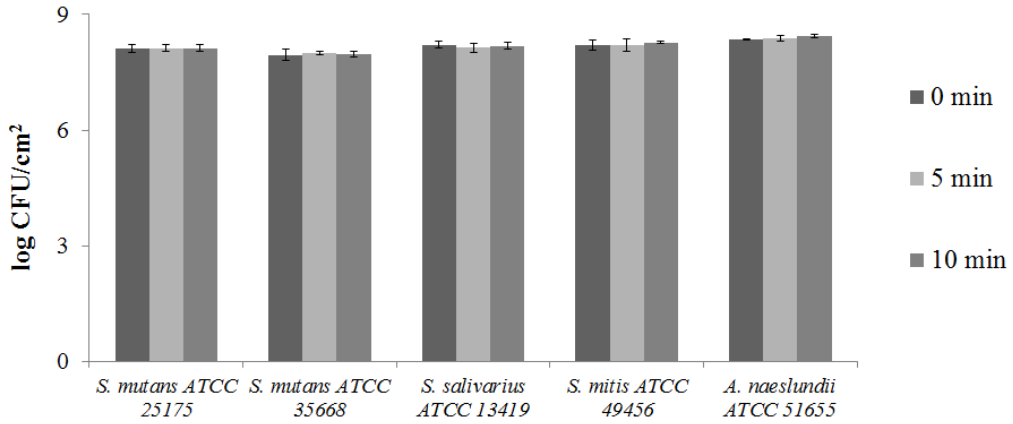


Figure A.2 Survival test of five oral bacteria after 5 and 10 min sonication.

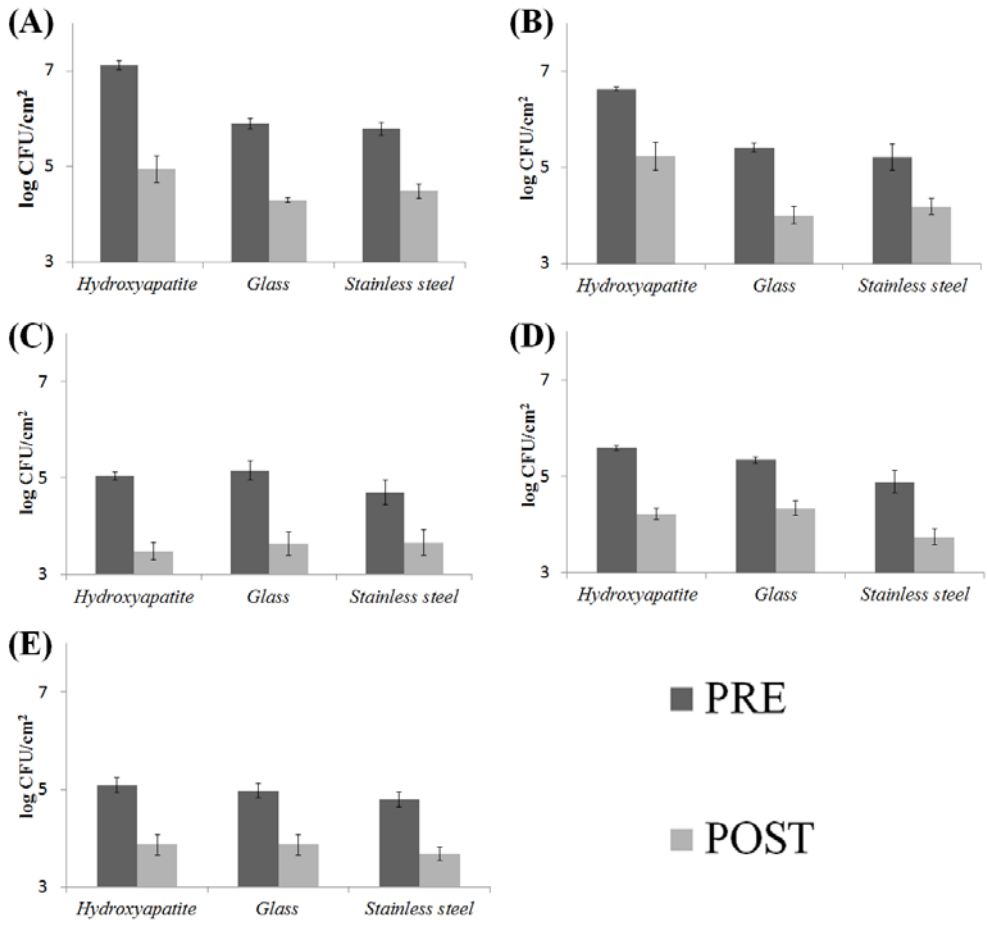


Figure A.3 The ability of sonication (10 min) to detach biofilm cells of five oral bacteria from three hard surfaces. (A) *S. mutans* ATCC 25175, (B) *S. mutans* ATCC 35668, (C) *S. salivarius* ATCC 13419, (D) *S. mitis* ATCC 49456 and (E) *A. naeslundii* ATCC 51655.

Appendix III Cytotoxicity Assays

The following appendix contains the results obtained from the cytotoxicity assays of tea extracts and tea components against HGF-1 cell line and the image of HGF-1cells (Chapter 3).

Table A.1 Cytotoxicity of five tea extracts and two tea components against HGF-1 cells.

	Green	Oolong	Black	Pu-erh	Chrysanthemum	EGCg	Gallic acid
MIC (mg/mL)	5.000	5.000	>5.000	>5.000	>5.000	0.125	0.125

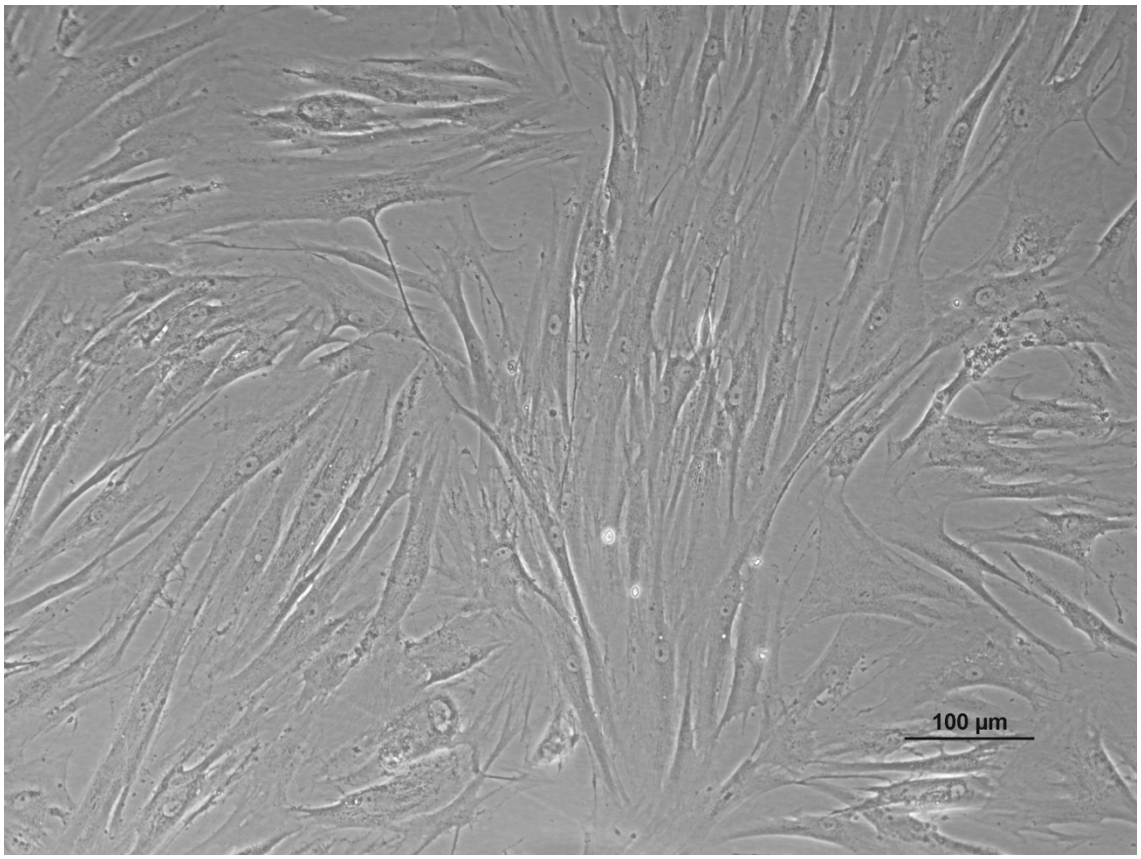


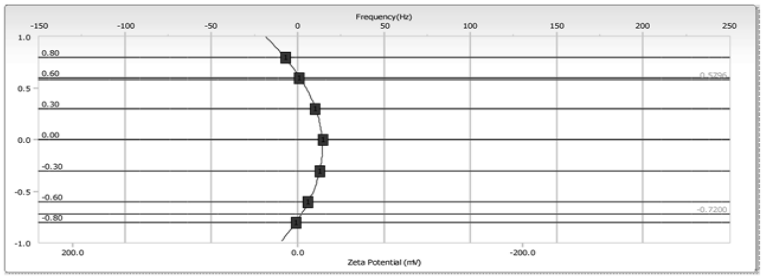
Figure A.4 Immortalized human gingival fibroblast-1 (HGF-1 ATCC CRL-2014) cell line.

Appendix IV Hard Surface Zeta Potential and Water Contact Angle Measurements

The following appendix contains the results and images obtained from the zeta potential measurements and the water contact angle measurements for the hard surfaces for the mathematical modeling (Chapter 5b).

(A)

EOS Plot



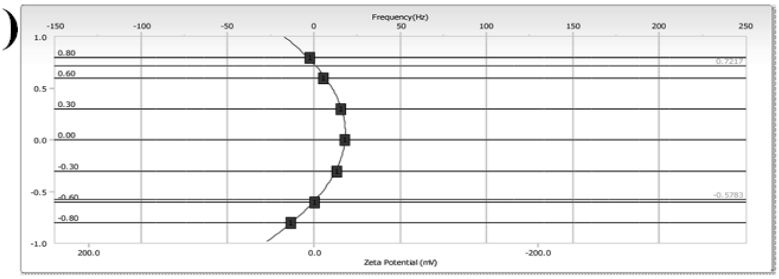
Measurement Results

Zeta Potential	: -3.52	(mV)	Doppler shift	: 2.29	(Hz)
Mobility	: -2.743e-005	(cm²/Vs)	Base Frequency	: 121.5	(Hz)
Conductivity	: 0.1281	(mS/cm)	Conversion Equation	: Smoluchowski	

Zeta Potential of Cell			Diluent Properties		
Flat Surface of Sample	: -31.88	(mV)	Diluent Name	: WATER	
Lower Surface	: -19.50	(mV)	Temperature	: 25.0	(°C)
Cell Condition			Refractive Index	: 1.3328	
Cell Type	: Flat Surface Cell		Viscosity	: 0.8878	(cP)
Avg. Electric Field	: -17.30	(V/cm)	Dielectric Constant	: 78.3	
Avg. Current	: -0.11	(mA)			

(B)

EOS Plot



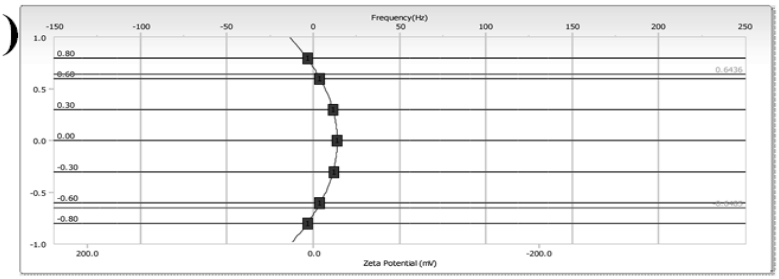
Measurement Results

Zeta Potential	: -1.59	(mV)	Doppler shift	: 1.03	(Hz)
Mobility	: -1.235e-005	(cm²/Vs)	Base Frequency	: 120.4	(Hz)
Conductivity	: 0.1347	(mS/cm)	Conversion Equation	: Smoluchowski	

Zeta Potential of Cell			Diluent Properties		
Flat Surface of Sample	: -27.66	(mV)	Diluent Name	: WATER	
Lower Surface	: -45.73	(mV)	Temperature	: 24.8	(°C)
Cell Condition			Refractive Index	: 1.3328	
Cell Type	: Flat Surface Cell		Viscosity	: 0.8919	(cP)
Avg. Electric Field	: -17.31	(V/cm)	Dielectric Constant	: 78.4	
Avg. Current	: -0.12	(mA)			

(C)

EOS Plot



Measurement Results

Zeta Potential	: -3.97	(mV)	Doppler shift	: 2.60	(Hz)
Mobility	: -3.106e-005	(cm²/Vs)	Base Frequency	: 121.7	(Hz)
Conductivity	: 0.1360	(mS/cm)	Conversion Equation	: Smoluchowski	

Zeta Potential of Cell			Diluent Properties		
Flat Surface of Sample	: -24.44	(mV)	Diluent Name	: WATER	
Lower Surface	: -24.04	(mV)	Temperature	: 25.1	(°C)
Cell Condition			Refractive Index	: 1.3328	
Cell Type	: Flat Surface Cell		Viscosity	: 0.8858	(cP)
Avg. Electric Field	: -17.31	(V/cm)	Dielectric Constant	: 78.3	
Avg. Current	: -0.12	(mA)			

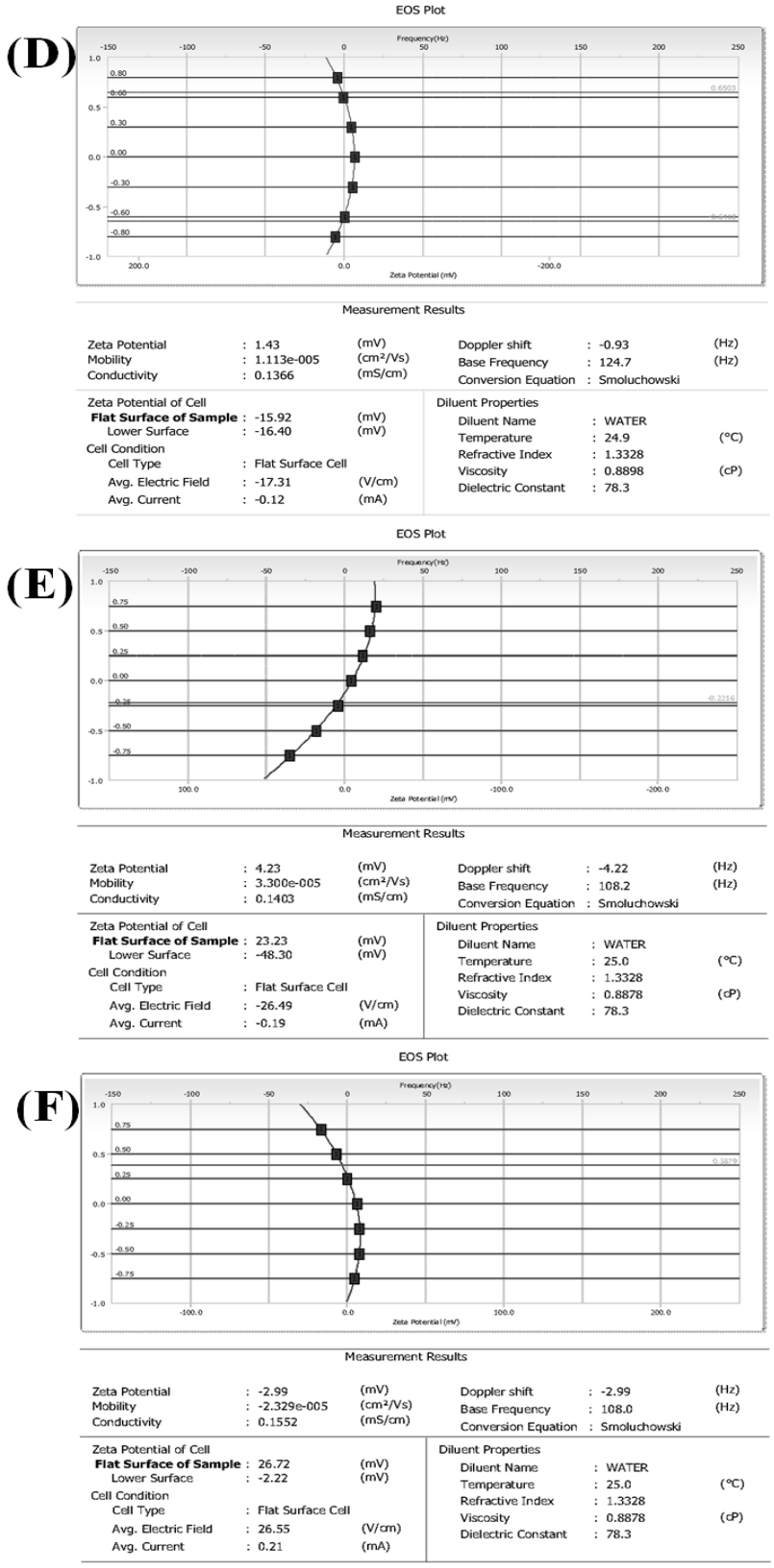


Figure A.5 Electro-osmotic stream (EOS) plot of the substratum surfaces for mathematical modeling. Ratios of BMA-*co*-EDMA to ASB at 10:0 (A), 8:2 (B), 6:4 (C), 4:6 (D), 2:8 (E) and 0:10 (F).

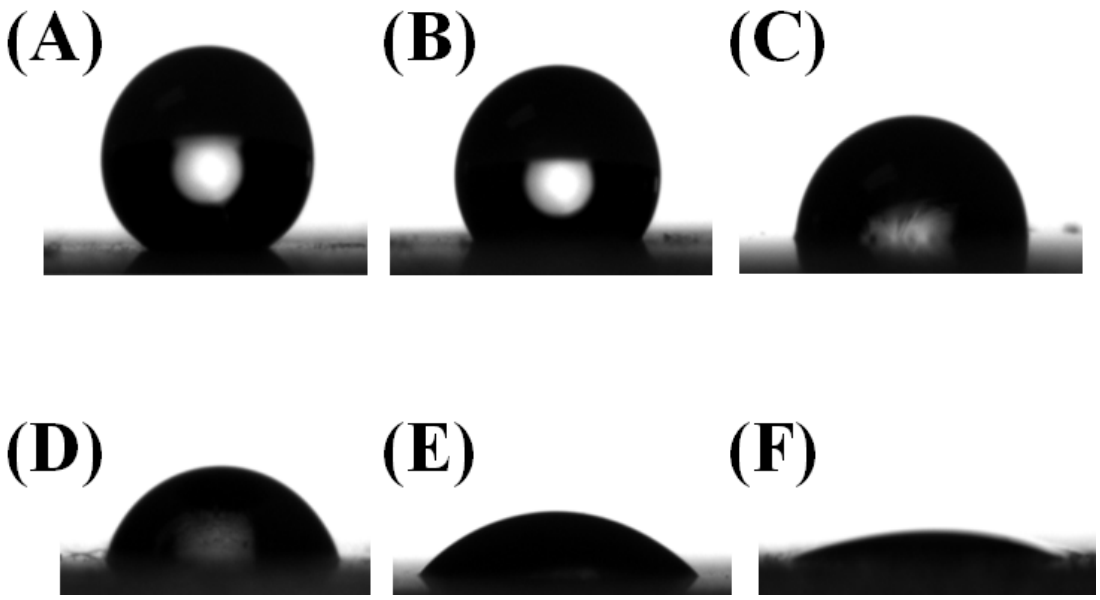


Figure A.6 Water contact angles of the substratum surfaces for mathematical modeling. Ratios of BMA-*co*-EDMA to ASB at 10:0 (A), 8:2 (B), 6:4 (C), 4:6 (D), 2:8 (E) and 0:10 (F).

Appendix V Publications Related to This Thesis

Biofouling: The Journal of Bioadhesion and Biofilm Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gbif20

Potential mechanisms for the effects of tea extracts on the attachment, biofilm formation and cell size of Streptococcus mutans

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Potential mechanisms for the effects of tea extracts on the attachment, biofilm formation and cell size of *Streptococcus mutans*

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(*Received 31 October 2012; final version received 24 January 2013*)

Tea can inhibit the attachment of *Streptococcus mutans* to surfaces and subsequent biofilm formation. Five commercial tea extracts were screened for their ability to inhibit attachment and biofilm formation by two strains of *S. mutans* on glass and hydroxyapatite surfaces. The mechanisms of these effects were investigated using scanning electron microscopy (SEM) and phytochemical screening. The results indicated that extracts of oolong tea most effectively inhibited attachment and extracts of pu-erh tea most effectively inhibited biofilm formation. SEM images showed that the *S. mutans* cells treated with extracts of oolong tea, or grown in medium containing extracts of pu-erh tea, were coated with tea components and were larger with more rounded shapes. The coatings on the cells consisted of flavonoids, tannins and indolic compounds. The ratio of tannins to simple phenolics in each of the coating samples was ~3:1. This study suggests potential mechanisms by which tea components may inhibit the attachment and subsequent biofilm formation of *S. mutans* on tooth surfaces, such as modification of cell surface properties and blocking of the activity of proteins and the structures used by the bacteria to interact with surfaces.

Keywords: dental caries; tea; bacterial attachment; *Streptococcus mutans*; biofilms; scanning electron microscopy

Introduction

Attachment and biofilm formation on tooth surfaces are regarded as virulence factors for oral bacteria. These processes initiate a buildup of dental plaque and eventually cause dental caries (Marsh 1994). The virulence of *Streptococcus mutans*, which is the major aetiological agent responsible for dental caries (Aas et al. 2008), has been studied widely, including its ability to attach and form biofilms (Hamilton-Miller 2001).

Tea (*Camellia sinensis*) is the second most popular drink worldwide after water. Commercial tea products are classified into four types based on their degree of fermentation. These types are green tea (non-fermented), oolong tea (partially fermented), black tea (fully fermented) and pu-erh tea (post-fermented) (Peterson et al. 2005). The fermentation process condenses tea components (such as epicatechins) into dimers (theaflavins) and polymers (thearubigins). The degree of fermentation therefore influences the polymerisation level of tea phenolics (Peterson et al. 2005). Tea has been demonstrated to benefit oral health through anti-cariogenic activities (Otake et al. 1991). These activities include direct bactericidal effects against oral bacteria (Rasheed & Haider 1998), the inhibition of bacterial adhesion to tooth surfaces (Tagashira et al. 1997), the inhibition of glycosyltransferase activity and biosynthesis of glucan (Hada et al. 1989) and the inhibition of human and bacterial amylase with an associated limitation on sugar availability for bacterial metabolism (Zhang & Kashket 2000).

Studies on *S. mutans* indicate that tea can reduce the attachment of the bacterium to hydroxyapatite (HA), which is a calcium mineral often used as a model of tooth surfaces. Otake et al. (1991) showed that green tea could inhibit the attachment of *S. mutans* to saliva-coated HA, and that this inhibition was due to the interactions between the tea components and the bacterial cells. Matsumoto et al. (1999) found that both small and large molecules from oolong tea could bind to the surface proteins of *S. mutans* and via this mechanism could reduce the cell surface hydrophobicity, induce auto-aggregation and prevent bacterial attachment to HA. These studies, while indicating the potential of tea in inhibiting attachment of *S. mutans* to teeth, did not examine the outcomes for biofilm formation. Furthermore, these studies only examined the inhibition of attachment of *S. mutans* to HA and not other model tooth surfaces, and did not study the mechanisms responsible for the inhibition to any great extent.

In the current study, extracts of a range of commercial tea products were screened for the ability to inhibit attachment and biofilm formation by two strains of *S. mutans* on glass and HA substrata. Some inhibitory effects were established and the potential mechanisms behind these were investigated using scanning electron microscopy (SEM) and phytochemical screening.

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Materials and methods

Bacterial cultures and growth conditions

Two strains of *S. mutans* (ATCC 25175 and ATCC 35668) were used in this study and were obtained from the American-Type Culture Collection (Manassas, USA). Both of the strains were maintained on *Mitis salivarius* agar (Difco, USA) at 4 °C. Experimental cultures were grown in tryptic soy broth (TSB) (Merck, USA) at 37 °C for 24 h with shaking at 150 rpm, unless otherwise stated.

Preparation of tea extracts

Extracts of five commercial tea products namely green tea, oolong tea, black tea, pu-erh tea and chrysanthemum tea (Ten Ren Tea Co. Ltd, Taiwan) were prepared by mixing 1 g of tea product with 20 ml of 90% (v/v) acetone (Sigma-Aldrich, USA) for 2 h (Perva-Uzunalić et al. 2006). The resulting extracts were evaporated under vacuum at 40 °C, freeze dried and stored at −20 °C for further use.

Stock solutions of the tea extracts were prepared by dissolving 100 mg of the extract in 10 ml of phosphate-buffered saline (PBS) (2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl, pH 7.4, 1st BASE, Singapore) containing 1% (v/v) methanol (Systerm, UK) and filter sterilising though a 0.2 μm filter (Millipore, USA).

Preparation of hard surfaces

Glass slides were painted with 40% (w/v) bonding adhesive (60% limestone, 30% kaolin, 8% ethylene glycol, 1% SiO_2 and 1% TiO_2 [all wt], Over Sea Plaster Supply and Construction Sdn Bhd, Malaysia) and partially air dried for 3 min. The slides were then coated with a paste consisting of 10% cement (56% CaO, 40% SiO_2, 3% Al_2O_3·-Fe_2O_3 and 1% MgO [all wt], Over Sea Plaster Supply and Construction Sdn Bhd, Malaysia), 40% (w) HA powder (Sigma-Aldrich, USA) and 50% (w) distilled water. Slides were then air dried for 16 h and sterilised by autoclaving.

Glass slides (5 × 5 mm) were used to model the smoothness of the surface of teeth (Forssten et al. 2010), and HA slides (5 × 5 mm) were used as a model for the chemical composition of teeth (Apella et al. 2008). The model surfaces were degreased by soaking in acetone for 30 min, rinsed in distilled water and sterilised by autoclaving.

Attachment and biofilm formation assays

The attachment assays were carried out as described previously (Goulter et al. 2011) with modifications. Briefly, bacterial cells were pelleted by centrifuging 20 ml of TSB cultures at 7669 *g* for 15 min at 4 °C. The cell pellets were washed gently with PBS and resuspended in the tea extract solutions at 1.25 mg ml^{-1} (containing ∼10^7 CFU ml^{-1}). PBS without the addition of bacterial cells was used as a null control. Preliminary antimicrobial susceptibility testing had established that the concentration of tea extract used did not kill or inhibit the bacteria (data not shown). Glass or HA slides were incubated in the cell suspensions at 37 °C for 30 min without shaking. The slides were then removed from the suspensions and gently rinsed three times with PBS to remove any loosely attached bacterial cells. The attached cells on the glass substratum were stained with 0.1% (w/v) crystal violet (Sigma-Aldrich), on HA substratum 0.1% (w/v) acridine orange (Sigma-Aldrich) was used. The number of cells on the respective substrata was determined using light or epifluorescence microscopy (BX51; Olympus, Japan). A total of 50 fields of view were counted for each slide and the number of attached cells was expressed as log CFU cm^{-2}.

To study the formation of biofilms, 10 ml of filter sterilized tea extract (with a final concentration of 1.25 mg ml^{-1}) and the attachment substratum (glass or HA) were added to 10 ml of autoclaved TSB (at double the normal concentration). These flasks were then inoculated with 0.1 ml of a 24 h old TSB culture and incubated at 37 °C for 3 days without shaking. The same volume of TSB without tea extract was used as a null control. The biofilm formed was quantified using a method adapted from Cassat et al. (2007). Briefly, after incubation, slides were washed with PBS (×3) and transferred into Falcon tubes (TPP®, Switzerland) containing 50 ml of PBS. To detach the cells from the biofilm, the tubes were sonicated for 10 min using a water bath sonicator (LC-130H; ELMA, Germany) at room temperature with a frequency of 35 kHz. An aliquot of the PBS was serially diluted, spread plated on tryptic soy agar (Merck, USA) and incubated at 37 °C for 24 h before enumeration. The method was verified and results indicated that sonication detaches 95–99% of the biofilm cells without killing them (data not shown).

SEM study

The experimental and control slides, with attached cells or biofilms, were washed in PBS, air dried and fixed with 4% (v/v) glutaraldehyde (Sigma-Aldrich, USA) in PBS. The fixed slides were washed a second time in PBS and air dried, followed by snap freezing with liquid nitrogen and freeze drying. The slides were then platinum-sputtered using a sputter coater (Q150RS; Quorum, UK) prior to examination with a SEM (S-3400N; Hitachi, Japan).

Extraction of tea components associated with bacterial surfaces

A 1 l TSB culture of *S. mutans* (ATCC 25175 or ATCC 35668) was treated with tea extracts under the same conditions of exposure as those used for the attachment or biofilm assays. After incubation, cells were pelleted at 12,090 *g* for 15 min at 4 °C using a large volume centrifuge (6930; Kubota, Japan). To ensure cells were clean, the pellet was resuspended in 500 ml of PBS before being centrifuged for a second time. The resulting pellets were resuspended in 1 l of acetone and incubated for

24 h at 40 °C with magnetic stirring at 700 rpm. The suspension was then filtered through 0.2 µm bottle top filters (Corning, USA). The filtrate was evaporated under vacuum at 40 °C and freeze dried. Controls containing untreated cells were prepared in the same way. The dried product was dissolved in 2 ml of methanol (Systerm, UK) prior to phytochemical screening.

Phytochemical screening

Phytochemical screening tests were conducted qualitatively for major phytoconstituents. The methods are shown in Table 1. Total phenolic, total tannin and total flavonoid contents were determined quantitatively as indicated below.

Total phenolic and total tannin contents were measured using the Folin–Ciocalteau method before and after treatment with polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, USA), which has a high affinity for tannins. Briefly, to determine total phenolic content, 20 µl of the sample were added to 100 µl of 10% (v/v) Folin–Ciocalteau's reagent (R&M Chemicals, Malaysia) and 80 µl of 7.5% (w/v) Na_2CO_3 (R&M Chemicals, Malaysia) in a well of a microtitre plate (Jet Biofil, China). The plate was placed in the dark for 30 min before the absorbance was measured at 765 nm. Gallic acid (Sigma-Aldrich, USA) was used to produce a standard curve. To determine total tannin content, about 0.5 ml of the sample was first mixed with 50 mg of PVPP and 0.5 ml of distilled water. The mixture was vortexed, incubated at 4 °C for 15 min and subsequently vortexed again prior to centrifuging at 1409 *g* for 10 min in order to remove tannins. The non-tannin phenolics in the supernatant were then quantified using the Folin–Ciocalteau method described above. The difference between the total phenolic content and the simple phenolic content is the measure of tannins. Both the total phenolic and the total tannin contents were expressed as µg of gallic acid equivalent (GAE) 10^9 cells^{-1} (Kaur & Kapoor 2001).

The total flavonoid content was determined using the aluminium chloride colorimetric method. A 50 µl aliquot of the sample was added to 10 µl of 10% (w/v) $AlCl_3$ (Bendosen, Malaysia), 10 µl of 1 M CH_3CO_2K (R&M Chemicals, Malaysia) and 130 µl of distilled water in a well of a microtitre plate. The plate was incubated at room temperature for 30 min before the absorbance was measured at 435 nm. The

Table 1. Methods for qualitative phytochemical screening.

Phytochemical	Method	Reference
Alkaloid	100 µl of the sample were added to 0.5 ml of 2 M HCl (Merck, USA) and 0.5 ml of Dragendroff reagent (R&M Chemicals, Malaysia). The mixture was then centrifuged at 1409*g* for 5 min. The presence of an orange brown precipitate indicated a positive result	Kumar et al. (2009)
Saponin	100 µl of the sample were boiled with 0.9 ml of distilled water for 15 min. The solution was then cooled, mixed vigorously and left to stand for 3 min. The formation of foam indicated a positive result	Kaur and Arora (2009)
Glycoside	A mixture of 100 µl of the sample and 100 µl of 10% (w/v) ethanolic α-naphthol solution (Sigma-Aldrich, USA) was added to 0.5 ml of 98% H_2SO_4 (Merck, USA). The appearance of a purple ring at the interface indicated the presence of glycosides	Silva et al. (1998)
Tannin	A mixture of 100 µl of the sample and 200 µl of 2% (w/v) NaCl solution (R&M Chemicals, Malaysia) was centrifuged at 1409*g* for 5 min. The supernatant was mixed with 0.5 ml of 1% (w/v) gelatin solution (R&M Chemicals, Malaysia). The presence of a precipitate indicated a positive result	Nayak et al. (2009)
Flavonoid	Magnesium powder (Sigma-Aldrich, USA) and a few drops of fuming HCl (Merck, USA) were added to 0.1 ml of sample. Orange, pink and red to purple colours were apparent when flavones, flavonols and/or xanthones were present, respectively. If zinc was used (Sigma-Aldrich, USA) instead of magnesium, a deep red colour was apparent if flavanonols were present while a weak pink colour was apparent if flavanones or flavonols were present	Silva et al. (1998)
Terpenoid	100 µl of the sample were added to 0.9 ml of chloroform (R&M Chemicals, Malaysia) and 1 ml of acetic anhydride (Sigma-Aldrich, USA) before adding 2 ml of 98% H_2SO_4 to the mixture. The formation of a reddish brown colour indicated the presence of terpenoids	Kumar et al. (2009)
Sterol/steroid	100 µl of the sample were added to 0.9 ml of chloroform before adding 1 ml of 98% H_2SO_4 (Salkowski method). A positive result was indicated by the formation of two phases, with a yellow/green fluorescent colour appearing in the upper layer	Kumar et al. (2009)

blank was prepared using distilled water in place of aluminium chloride and the standard curve was obtained using quercetin as the substrate (Sigma-Aldrich, USA). The total flavonoid content was expressed as μg quercetin equivalent (QE) 10^9 cells^{-1} (Chang et al. 2002).

Statistical analysis

A one-way ANOVA with *post hoc* Tukey's comparison was performed to compare the attachment and biofilm formation between control and treatments with tea extract, and to compare the quantities of phytochemicals in the cell surface coatings between the two strains of *S. mutans*. A nested ANOVA was performed to determine which tea extracts were most effective at inhibiting attachment and biofilm formation. All analysis was conducted using SPSS software (PASW Statistics 18; SPSS Inc.) at a 95% confidence level. All experiments were performed in triplicate with independently grown cultures and all values were expressed as means ± SD.

Results

The results of the attachment and biofilm assays are presented in Figure 1. The attachment and biofilm formation by the untreated cells of both strains were significantly ($p < 0.05$) greater on HA than on glass. Bacterial attachment to glass was reduced significantly ($p < 0.05$) by the extract of oolong tea, for *S. mutans* ATCC 25175 by 0.6 log CFU cm^{-2} and *S. mutans* ATCC 35668 by 0.3 log CFU cm^{-2} and by the extract of black tea for *S. mutans* ATCC 35668 by 0.3 log CFU cm^{-2}. The attachment of both strains of *S. mutans* to HA slides was reduced significantly ($p < 0.05$) by all the tea extracts tested, by 0.3–0.7 log CFU cm^{-2} depending on the tea extract and the strain (Figure 1(A) and (B)). Biofilm formation by both strains of *S. mutans* on glass was only reduced significantly ($p < 0.05$) by the extract of pu-erh tea, for *S. mutans* ATCC 25175 by 1 log CFU cm^{-2} and *S. mutans* ATCC 35668 by 1.1 log CFU cm^{-2}. However, biofilm formation on HA was significantly ($p < 0.05$) reduced by extracts of both pu-erh and chrysanthemum tea for both strains by 1.1–2.6 log CFU cm^{-2}, and by extract of black tea for *S. mutans* ATCC 25175 by 1.2 log CFU cm^{-2} (Figure 1(C) and (D)). Notably, biofilm formation by both strains on HA was significantly ($p < 0.05$) enhanced by extract of oolong tea. The nested ANOVA conducted to compare the inhibitory effects of

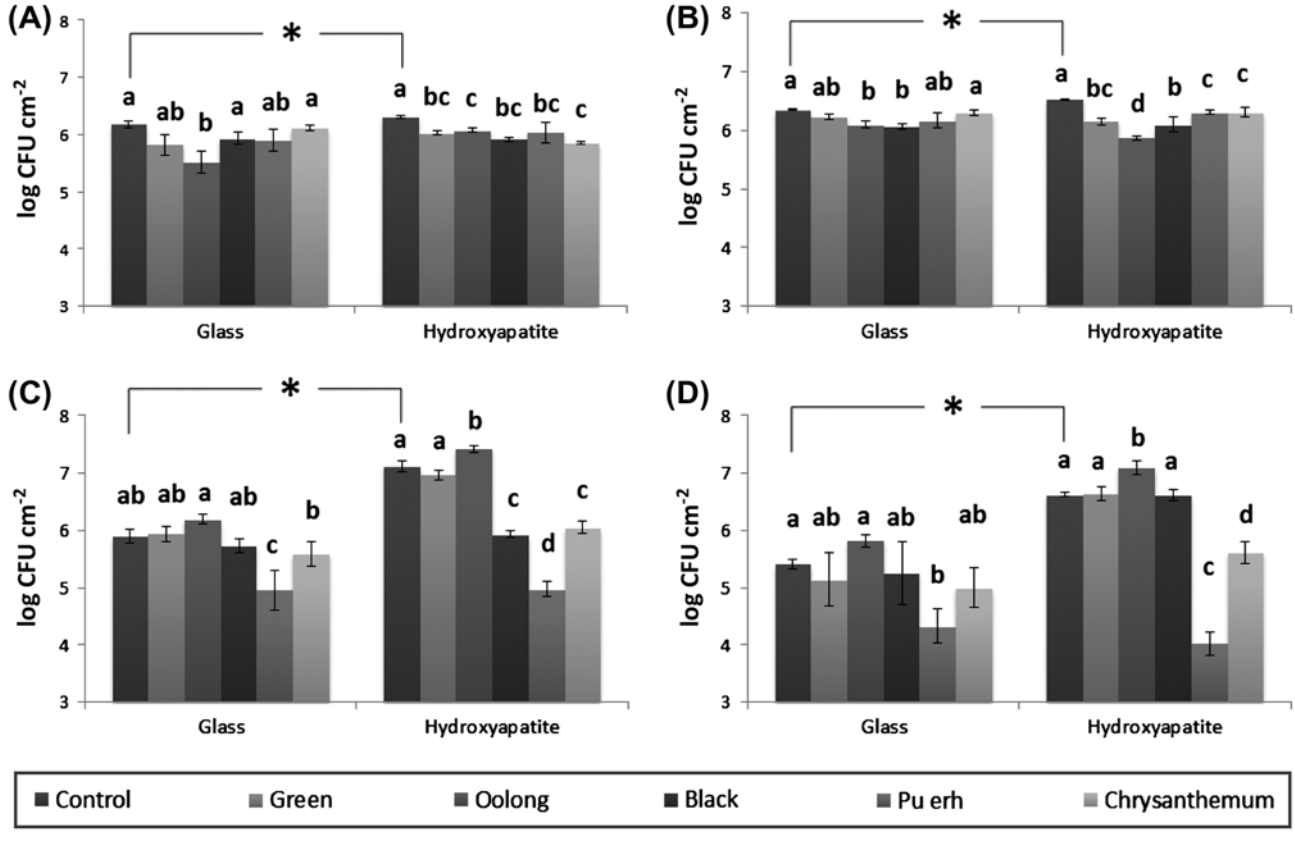

Figure 1. Enumeration of the effects of different tea extracts on the attachment of *S. mutans* ATCC 25175 (A) and *S. mutans* ATCC 35668 (B) to glass slides and HA, and on biofilm formation by *S. mutans* ATCC 25175 (C) and *S. mutans* ATCC 35668 (D) on glass and HA (log CFU cm^{-2}, $n = 3$). Values labelled with the same letter are not significantly different ($p > 0.05$) among the treatments on the same surface. *indicates that the attachment or biofilm forming ability of the untreated cells of *S. mutans* ATCC 25178 were significantly different from those of *S. mutans* ATCC 35668 ($p < 0.05$).

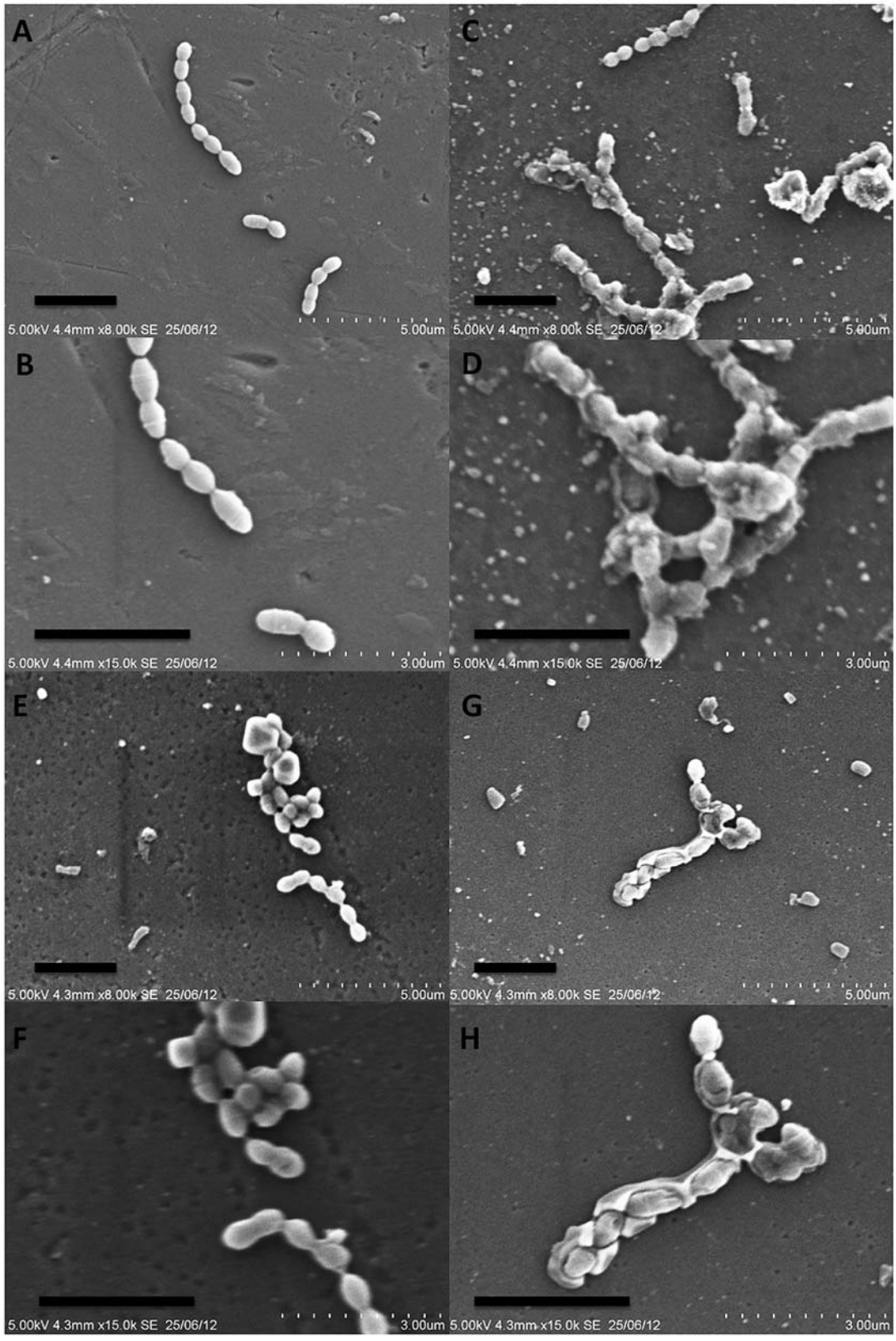

Figure 2. SEM images showing the effect of oolong tea extracts on *S. mutans* attached to a glass surface. A, B = untreated attached cells of *S. mutans* ATCC 25175; C, D = oolong tea extract treated attached cells of *S. mutans* ATCC 25175; E, F = untreated attached cells of *S. mutans* ATCC 35668; G, H = oolong tea extract treated attached cells of *S. mutans* ATCC 35668. Scale bars = 3 μm.

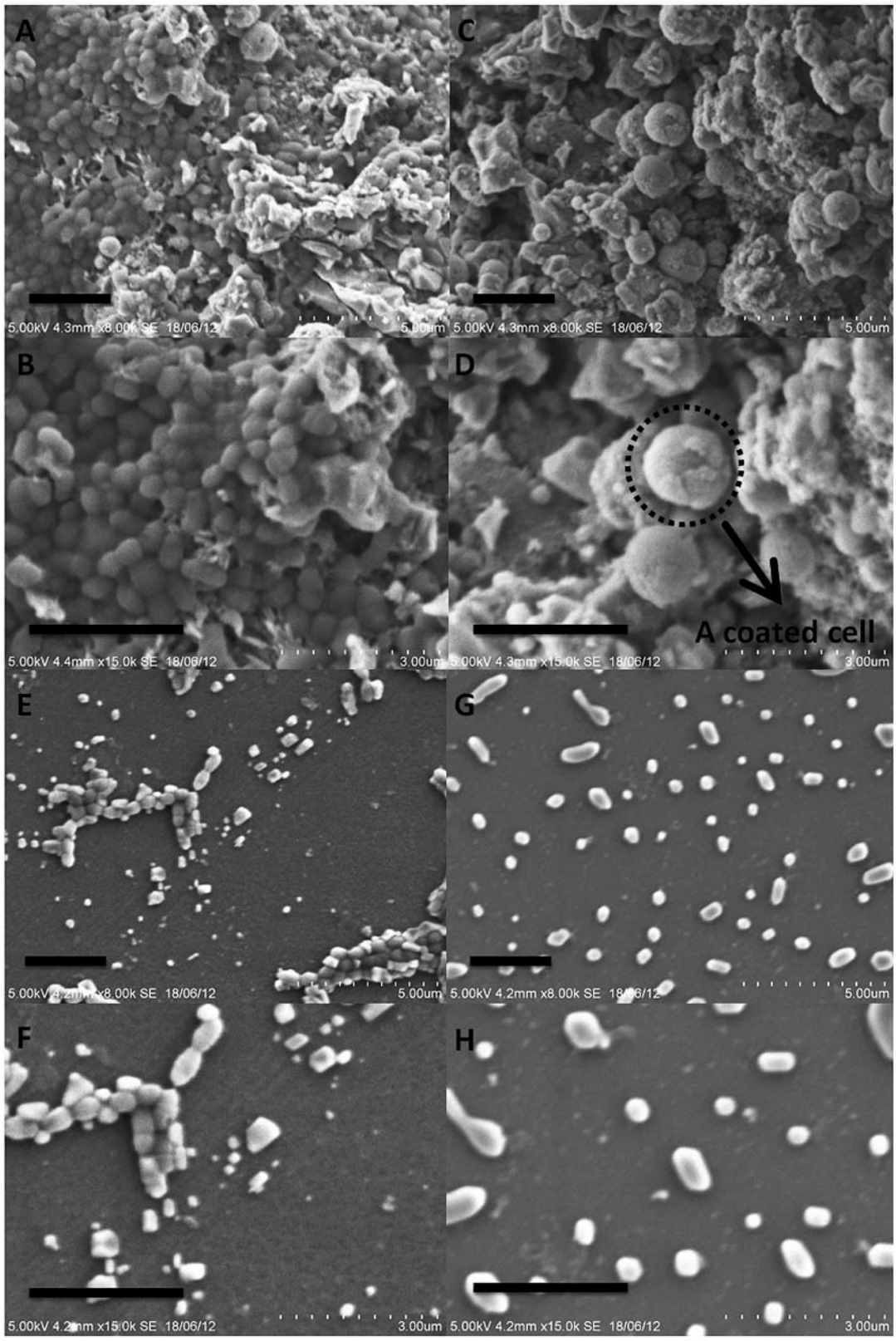

Figure 3. SEM images showing the effect of pu-erh tea extracts on biofilms of *S. mutans* ATCC 25175. A, B = cells grown without pu-erh tea extracts on HA; C, D = cells grown with pu-erh tea extracts on HA; E, F = cells grown without pu-erh tea extracts on glass; G, H = cells grown on with pu-erh tea extracts on glass. Scale bars = 3 μm.

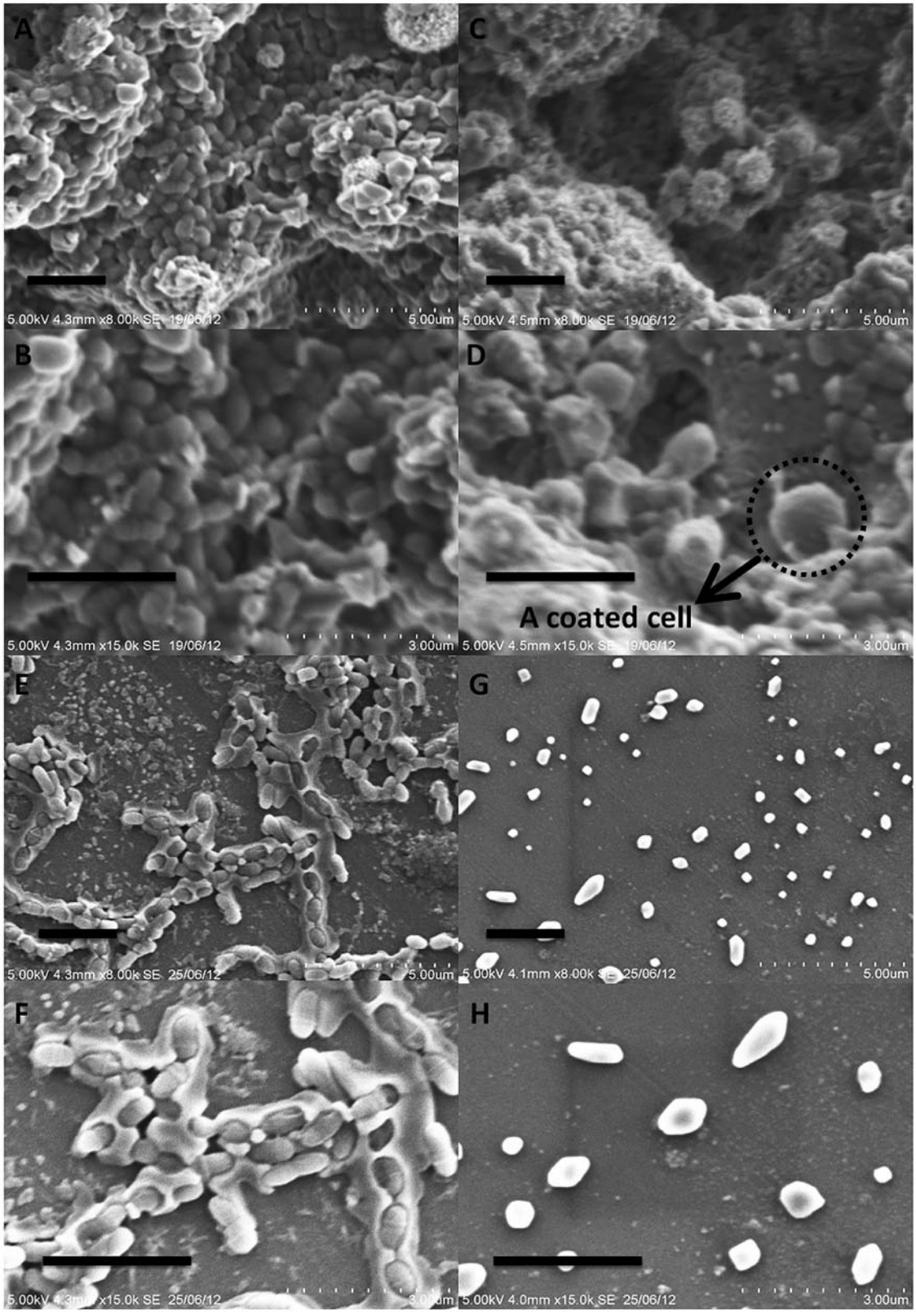

Figure 4. SEM images showing the effect of pu-erh tea extracts on biofilms of *S. mutans* ATCC 35668. A, B = cells grown without pu-erh tea extracts on HA; C, D = cells grown with pu-erh tea extracts on HA; E, F = cells grown without pu-erh tea extracts on glass; G, H = cells grown with pu-erh tea extracts on glass. Scale bars = 3 μm.

Table 2. The results of the phytochemical screening tests on cell coatings.

	S. mutans ATCC 25175			*S. mutans* ATCC 35668		
	Control	Oolong (attachment)	Pu-erh (biofilm)	Control	Oolong (attachment)	Pu-erh (biofilm)
Terpenoid	–	–	–	–	–	–
Sterol/steroid	–	+	+	–	+	+
Flavonoid	–	+	+	–	+	+
Tannin	–	+	+	–	+	+
Saponin	–	–	–	–	–	–
Glycoside	+	+	+	+	+	+
Alkaloid	–	–	–	–	–	–

Note: – = a negative reading; + = a positive reading; $n = 3$, using three independently grown cultures. The results for the replicates were consistent.

the tea extracts on attachment and biofilm formation, using the *S. mutans* strain and surface type as the independent variables, indicated that the extract of oolong tea was the most effective inhibitor of attachment ($p < 0.05$) and extract of pu-erh tea was the most effective inhibitor of biofilm formation ($p < 0.05$). For this reason, the effect of oolong tea extracts on attachment and pu-erh tea extracts on biofilm formation were selected for further study.

The SEM images in Figure 2 show the appearance of attached cells of *S. mutans* with and without treatment with the extract of oolong tea. It was observed that cells treated with the extract of oolong tea had coatings on the cell surface (Figure 2(C), (D), (G), and (H)), which were not present on the control cells (Figure 2(A), (B), (E), and (F)).

The SEM images in Figures 3 and 4 show the appearance of *S. mutans* biofilms with and without treatment with the extract of pu-erh tea. For *S. mutans* ATCC 25175, it can be seen (Figure 3(C) and (D)) that on the HA substratum treatment with the extract of pu-erh tea resulted in coated cells of larger size with rougher cell surfaces. The cell density was also substantially lower compared to biofilm cells from the control (Figure 3(A) and (B)). The distribution of biofilm cells on a flat (glass) surface differed in treatments with the extract of pu-erh tea. Specifically, the untreated cells formed chains and clusters on the surface (Figure 3(E) and (F)), while the treated cells appeared individually (Figure 3(G) and (H)). Notably, the large and rough cells seen on the HA surface were not present on the flat glass surface and only the coated cells, which had similar sizes to the control cells, were present. Similar observations were found for *S. mutans* ATCC 35668 (Figure 4). It was also noted that the control cells of *S. mutans* ATCC 35668 (Figures 2(E) and (F), and 4(E) and (F)) appeared to be surrounded by a larger quantity of an unidentified coating compared to the control cells of *S. mutans* ATCC 25175 (Figures 2(A) and (B), and 3(E) and (F)).

The coatings, which were believed to comprise components from the oolong and pu-erh teas, were extracted with acetone and analysed using phytochemical screening methods. The results (Table 2) show that the coatings on cells treated with extracts from both oolong and pu-erh teas (both strains of *S. mutans*) contained sterols/steroids, tannins, flavonoids and glycosides. Notably, the untreated cells also contained glycosides. In addition, the colour response of the flavonoid qualitative test suggested that the flavonoids present were flavonol/flavanone in all cases (Table 3).

The results from the total phenolic and the total tannic quantitative tests (Figure 5) showed that the coatings contained ~21–27% of simple phenolic compounds in all cases (3.19–4.84 μg GAE 10^9 cells^{-1}), and 73–79% of those were tannins (8.51–18.43 μg GAE 10^9 cells^{-1}). The results of the flavonoid quantitative tests are presented in Table 4. The flavonoid content of coatings was 2.68–6.08 μg QE 10^9 cells^{-1}. It was noted that the total phenolic content, the total tannin content and the total flavonoid content in the surface coatings of *S. mutans* ATCC 25175 treated with extracts of both the oolong and the pu-erh teas were significantly higher ($p < 0.05$) than those of the coatings from *S. mutans* ATCC 35668 cells given the same exposures.

Table 3. The results of the flavonoid qualitative screening tests on cell coatings.

	S. mutans ATCC 25175			*S. mutans* ATCC 35668		
	Control	Oolong (attachment)	Pu-erh (biofilm)	Control	Oolong (attachment)	Pu-erh (biofilm)
Flavone/xanthone	–	–	–	–	–	–
Flavanonol	–	–	–	–	–	–
Flavonol/flavanone	–	+	+	–	+	+

Note: – = a negative reading; + = a positive reading; $n = 3$, using three independently grown cultures. The results for the replicates were consistent.

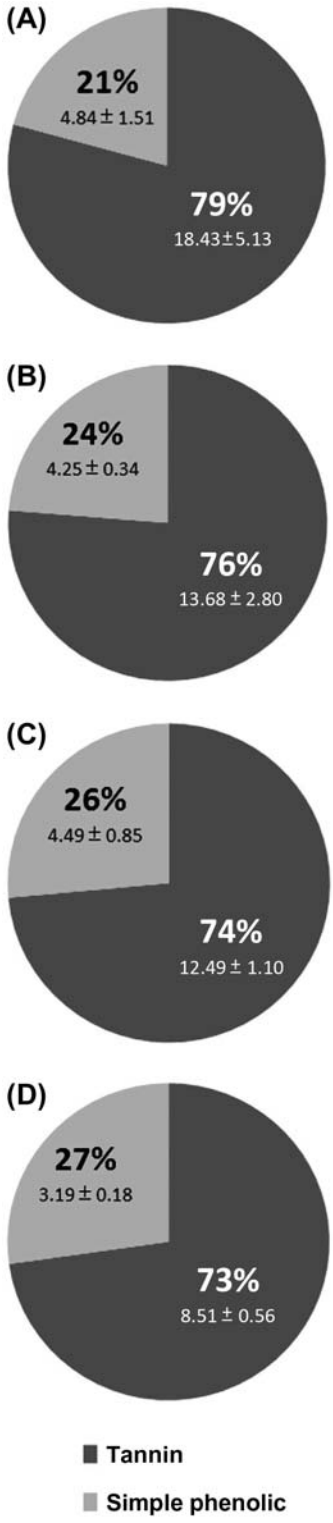

Figure 5. Quantification (μg GAE 10^9 cells^{-1}, mean ± SD, $n = 3$) and ratios (%) of tannins and simple phenolics in the cell surface coatings of (A) oolong tea extract treated cells of *S. mutans* ATCC 25175, (B) cells of *S. mutans* ATCC 25175 grown with pu-erh tea extracts, (C) oolong tea extract treated cells of *S. mutans* ATCC 35668, and (D) cells of *S. mutans* ATCC 35668 grown with pu-erh tea extracts.

Discussion

The result of the attachment assay shows that a greater number of tea extracts had an inhibitory effect on attachment to HA than to glass for the two *S. mutans* strains used in this study (Figure 1). This could result from the super hydrophilic nature of HA which has a water contact angle of 8.11° ± 1.35°. According to the interfacial thermodynamic theory (van Oss et al. 1985), this hydrophilic nature will result in weaker hydrophobic interactions between the surface and the cells of *S. mutans*, which have been reported to be highly hydrophobic (Nostro et al. 2004), thereby resulting in lower attachment forces. By contrast, the number of cells attached and in the biofilm was higher on HA than on glass for both strains of *S. mutans* (Figure 1). This result may be attributed to the roughness of the HA surface, which provides a larger surface area for the attachment of bacterial cells. Although extracts from oolong tea were determined to be the best inhibitor of attachment, this extract was also found to promote biofilm formation on HA (Figure 1(C) and (D)), this may be because it induces cells of *S. mutans* to auto-aggregate (Matsumoto et al. 1999). The results from the current study also showed that extracts from green tea did not inhibit the attachment of cells or reduce the formation of a biofilm to any appreciable extent for either strain of *S. mutans*. This finding suggests that monomeric tea phenolics alone, which are high in green teas but lower in fermented teas due to polymerisation, might not contribute to inhibiting attachment and biofilm formation (Friedman 2007). The inhibitory effect of tea extracts on cell attachment and biofilm formation observed in the current study may therefore be a result of large molecules in the extracts or the synergistic effect of polymeric and monomeric tea phenolics.

SEM (Figures 2–4) demonstrated that cells of *S. mutans* were coated by components from the tea extracts. This suggests that bacterial cell surface components can bind to polyphenolic compounds, especially large molecule phenolics (tannins) which have a high affinity for proteins and carbohydrates (McMahon et al. 2000).

The biofilm cells coated with extract of pu-erh tea (Figures 3(C) and (D), and 4(C) and (D)) had larger sizes and rougher surfaces than untreated cells (Figures 3 (A) and (B), and 4(A) and (B)). However, the enlarged cells were present on the HA (rough) slides but not on the glass (flat) slides (Figures 3(G) and (H), and 4(G) and (H)), suggesting that the enlarged cells cannot form a biofilm on the smoother surface. This could be attributed to the roughness of the HA surface which may affect the initial attachment of the bacterial cells (Goulter et al. 2011) and subsequently also influence the formation of a biofilm. The increased size of the cells may

Table 4. The results of flavonoid quantitative tests on cell coatings.

	S. mutans ATCC 25175			*S. mutans* ATCC 35668		
	Control	Oolong (attachment)	Pu-erh (biofilm)	Control	Oolong (attachment)	Pu-erh (biofilm)
Total flavonoid content (μg QE 10^{-9} cells)	0.38 ± 0.04	6.08 ± 0.96	5.47 ± 0.72	0.17 ± 0.08	4.41 ± 0.27	2.68 ± 0.34

Note: All results are presented as means ± SD ($n = 3$).

also alter the steric arrangement between them, altering initial attachment and resulting in cells not attaching in clumps or chains (Clark & Gibbons 1977). The SEM images also show that treatment with the extract from pu-erh tea altered the distribution of cells in the biofilm formed on glass. Cells arranged in chains and clusters (Figures 3(E) and (F), and 4(E) and (F)) were replaced by cells attached individually (Figures 3(G) and (H), and 4(G) and (H)), suggesting that extract of pu-erh tea may alter cellular aggregation. As outlined above, tea coatings that can affect cell surface properties and therefore attachment may also alter cell to cell interactions. It is also possible that components of tea might coat the surface of the substratum, especially HA (tea is known to stain tooth surfaces), as the substratum slides were incubated with the cell suspension in the presence of tea extract during the attachment and biofilm assays. Any coating on the surface of the substratum might also affect cellular attachment and biofilm formation. This approach simulates what is likely to happen in the mouth if bacteria are exposed to tea, and further research is required to establish what effect tea has in terms of coating the surface of the substratum.

Glycosides, sterols/steroids, flavonoids and tannins were found in the coatings covering the cell surfaces (Table 2). Glycosides were also present in the controls and are probably components of the cells themselves rather than arising from the coatings. The presence of sterols/steroids was determined by the Salkowski method, which indicated the presence of indolic compounds (Glickmann & Dessaux 1995). The positive results observed could be due to the presence of tea indoles which are usually components of the flavour or the pigment compounds of tea (Kubo & Morimitsu 1995). The flavonoid components of the coating were identified as flavonol or flavanone (Table 3). Tea does not contain flavanones but it does contain three flavonols, quercetin, kaempferol and myricetin (Wang & Helliwell 2001). Quercetin and kaempferol are highly water insoluble and since experiments were conducted in a water-based system (PBS), myricetin was the most likely flavonol present in the cell coatings. However, since there is no screening method for flavon-3-ols, which are a major phenolic constituent in low-degree fermented tea (such as oolong), and flavon-3-ols have been reported to be able to bind to various types of proteins (Mori et al. 2010), it is also reasonable to assume that flavon-3-ols were also part of the flavonoid content in the coatings of cells treated with extract of oolong tea. In the qualitative and quantitative phytochemical screening tests, tannins are regarded as large molecule (MW > 500) polyphenolic compounds. Dimers or polymers of flavon-3-ols, namely theaflavin and thearubigin, are the most common tannins in tea products (Graham 1992), and were assumed to be the major tannin component in the cell coatings.

The phenolic constituents found in the coatings on the bacterial cells were mostly hydrophilic but they still contained hydrophobic moieties, such as aromatic rings, which may interact with hydrophobic components of the cell membrane (Nalina & Rahim 2007). Therefore, it is suggested that the hydrophilic moieties (such as hydroxyl groups) of the phenolic compounds may be exposed on the cell surface and 'neutralize' cell surface hydrophobicity. This would result in an enhanced interaction between the cells and the liquid (water-based) medium, but a reduced interaction between the cells and the surface during attachment to a substratum. In addition, coating of the cell surface may block surface proteins and structures that interact with the surface of the substratum.

The ratio of tannins to simple phenolics in each of the coatings was approximately 3:1 (Figure 5) suggesting that larger molecule polyphenols bind more readily to surface components of the *S. mutans* cell than smaller compounds. In general, tannins have a higher affinity for proteins (Rawel et al 2006) and carbohydrates (Shahidi & Naczk 2004) compared to simple phenolics. It has been suggested that the proteins on the surface of *S. mutans* play an important role in the interactions between bacterial cells and polyphenols (Matsumoto et al. 1999). The preferred binding sites of phenolic compounds are usually hydroxyl groups and this can facilitate interactions with proteins, particularly tryptophan residues, either via covalent bonds or non-covalent bindings such as hydrogen bonds and electrostatic attraction (Rawel et al 2006). Tannins have more hydroxyl residues than simple phenolics and therefore may exhibit higher binding efficiencies to surface components of *S. mutans* cells. A study by Matsumoto et al. (1999) also indicated that it was the polymeric polyphenols in oolong tea that were responsible for the alteration in the

surface properties and the inhibition of attachment of cells of *S. mutans*.

The total flavonoid, total tannin and total phenolic contents (Figure 5 and Table 4) in the surface coatings of *S. mutans* ATCC 25178 were approximately one-third higher ($p < 0.05$) than those of *S. mutans* ATCC 35668, suggesting that *S. mutans* ATCC 25178 cells bind to tea components more readily. This could be due to a higher amount of an unidentified substance (possibly extracellular polysaccharide) surrounding the cells of *S. mutans* ATCC 35668. This substance might occupy or block some of the binding sites on the cell surface thereby reducing the amount of components that can bind from the tea extracts. This feature of the data also highlights the importance of strain variation in investigating mechanisms to prevent bacterial adhesion to surfaces.

Additional studies are required to simulate the situation in the oral cavity more realistically. For example, it would be useful to investigate the effect of tea on wild type *S. mutans*, isolated from the human oral cavity which may behave differently from laboratory strains. The concentrations of tea extracts used in this study (which were at sub-minimum inhibitory concentration [MIC] levels) were lower than those in a cup of freshly brewed tea, which may reach a dose that is inhibitory or lethal to cells of *S. mutans*, especially in the case of green and oolong tea (Xu et al. 2012). It is therefore likely that immediately after tea consumption, cells of *S. mutans* in the oral cavity could be killed or inhibited. The concentration of tea will subsequently be diluted gradually by saliva to sub-MIC level, at which point the attachment of cells or biofilm formation may be suppressed. Furthermore, tea may have concomitant effects on dental surfaces, which could also influence the potential for attachment by bacterial cells and subsequent biofilm formation.

In summary, components in the extract of oolong tea reduced the attachment of cells of *S mutans* and components in the extract of pu-erh tea reduced biofilm formation. Specifically, it is suggested that flavonoids, tannins and indolic compounds coat the surfaces of cells, probably altering cell surface properties and thereby affecting the interactions between bacterial cells and the surface of the substratum.

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BMC Research Notes

SHORT REPORT Open Access

Inhibition of attachment of oral bacteria to immortalized human gingival fibroblasts (HGF-1) by tea extracts and tea components

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Abstract

Background: Tea has been suggested to promote oral health by inhibiting bacterial attachment to the oral cavity. Most studies have focused on prevention of bacterial attachment to hard surfaces such as enamel.

Findings: This study investigated the effect of five commercial tea (green, oolong, black, pu-erh and chrysanthemum) extracts and tea components (epigallocatechin gallate and gallic acid) on the attachment of five oral pathogens (*Streptococcus mutans* ATCC 25175, *Streptococcus mutans* ATCC 35668, *Streptococcus mitis* ATCC 49456, *Streptococcus salivarius* ATCC 13419 and *Actinomyces naeslundii* ATCC 51655) to the HGF-1 gingival cell line. Extracts of two of the teas (pu-erh and chrysanthemum) significantly ($p < 0.05$) reduced attachment of all the *Streptococcus* strains by up to 4 log CFU/well but effects of other teas and components were small.

Conclusions: Pu-erh and chrysanthemum tea may have the potential to reduce attachment of oral pathogens to gingival tissue and improve the health of oral soft tissues.

Keywords: Tea, Oral bacteria, Attachment, Gingival cells, Pu-erh, Chrysanthemum

Findings

Introduction

Oral Streptococci such as *Streptococcus mutans* are pathogens commonly associated with dental plaque and the formation of caries [1]. In order to initiate disease these bacteria must attach to components of the oral cavity such as the enamel, tongue, saliva or gums [2].

Plant extracts and phytochemicals can inhibit bacterial attachment to abiotic and biotic surfaces by altering cell surface properties including hydrophobicity, surface charge and the presence of structures such as flagella [3-5]. Tea is one such potential attachment inhibitor [6]. Non-fermented teas or partially-fermented teas, such as green tea and oolong tea, have strong bactericidal activity and may inhibit bacterial attachment to some elements of the gastrointestinal tract [7-11]. Fully fermented teas, such as black tea and pu-erh tea, have less effective bactericidal activity but may inhibit attachment of bacteria to dental plaque [12].

Previous studies investigating bacterial attachment and inhibition by phytochemicals to components of the oral cavity have focused on attachment to hard surfaces such as enamel [10,13,14]. Attachment of bacteria to soft tissues in the mouth can also initiate disease and for this reason we investigated the effects of tea extracts and tea components on attachment of oral pathogenic bacteria to an immortalized line of connective gingival fibroblasts *in vitro*.

Materials and methods

Bacteria and growth conditions

Five strains of bacteria, namely *Streptococcus mutans* (ATCC 25175), *Streptococcus mutans* (ATCC 35668), *Streptococcus mitis* (ATCC 49456), *Streptococcus salivarius* (ATCC 13419) and *Actinomyces naeslundii* (ATCC 51655), were selected for this study and obtained from the American Type Culture Collection (Manassas, USA). All bacteria were maintained on Mitis Salivarius Agar (MSA; Difco, USA) at 4°C and grown in Tryptic Soy Broth (TSB; Merck, USA) at 37°C for 24 h with shaking at 150 rpm for all experiments. Bacterial suspensions were prepared by centrifuging 20 mL of TSB cultures at 7669 × g and 4°C for 15 min, washing the resultant pellet gently with

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phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl, pH 7.4; 1st BASE, Singapore) and resuspending it in 20 mL PBS, tea extract solutions or tea component solutions prepared as described below.

Preparation of tea extracts and tea components

Commercial green tea, oolong tea, black tea, pu-erh tea and chrysanthemum tea (Ten Ren Tea Co. Ltd., Taiwan) extracts were prepared using 90% acetone (Sigma Aldrich, USA) at the ratio of 1:20 (wt/vol) for 2 h. The resultant extracts were evaporated under vacuum at 40°C, freeze dried and stored at −20°C until further use. Using this method reportedly allows for extraction of more than 95% of the phenolic compounds in tea, including catechins, myricetin, quercetin and kaempherol [15]. Epigallocatechin gallate (EGCg; 95% [vt/vt]; Sigma-Aldrich) and gallic acid (Sigma-Aldrich) were also used as they are major phenolic components of teas. Specifically, EGCg constitutes approximately 10% of the dry weight of green tea and its level decreases with increasing degree of fermentation [16]. Levels of gallic acid, on the other hand, increase with fermentation and constitute approximately 0.5% dry weight of black tea [17]. The stock solutions for all experiments were prepared by dissolving 100 mg of tea extracts or tea component in 10 mL PBS containing 1% (vol/vol) methanol (Systerm, UK) and the resultant solutions were filter sterilized though a 0.2 μm filter (Millipore, USA).

Determination of total phenolic, total tannin and total flavonoid content

Total phenolic and total tannin contents of the tea extracts were determined using the Folin-Ciocalteau colorimetric method [18]. To determine the total phenolic content, a 15 μL tea extract solution (1 mg/mL) was added to 80 μL of 7.5% (wt/vol) sodium carbonate (R&M Chemicals, Malaysia) and 75 μL of 10% (vol/vol) Folin-Ciocalteau reagent (R&M Chemicals) in a well of a microtitre plate (Jet Biofil, China). The plate was incubated in the dark for 30 min before measuring the absorbance at 765 nm. To determine the total tannin content, 0.5 mL of the sample solution was mixed with 0.5 mL of distilled water and 50 mg of poly(vinylpolypyrrolidone) (PVPP; Sigma-Aldrich) which has a high affinity to tannins. The mixture was vortexed, incubated at 4°C for 15 min and vortexed again prior to centrifuging at 1409 g for 10 min in order to remove tannins. The supernatant containing non-tannin phenolics was then quantified using the Folin-Ciocalteau method described above. The difference between the total phenolic content and the non-tannin phenolic content is the measure of tannins. A standard curve was plotted using gallic acid, and the total phenolic and total tannin contents were expressed as μg gallic acid equivalent (GAE) / mg.

Total flavonoid content was measured using the aluminum chloride colorimetric method [19]. A 50 μL of tea extract sample dissolved in methanol (1 mg/mL) was added to 10 μL of 10% (wt/vol) aluminum chloride (Bendosen, Malaysia), 10 μL of 1 M potassium acetate (R&M Chemicals) and 80 μL of distilled water in the wells of a microtitre plate. The plate was incubated at room temperature for 30 min before the absorbance was measured at 435 nm. The blank was prepared using distilled water in place of aluminum chloride. A standard curve was plotted using quercetin (Sigma-Aldrich) and the total flavonoid content was expressed as μg quercetin equivalent (QE) / mg.

Cell culture

Immortalized human gingival fibroblast-1 HGF-1 (ATCC CRL-2014) were obtained from the American Type Culture Collection and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 4 mM L-glutamine (Sigma-Aldrich) and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich). No antibiotic supplement was used. Cells were incubated at 37°C in 5% CO_2 atmosphere, fed every 48 h and routinely sub-cultured every 5 - days with a split ratio of 1:3 using 1 × trypsin-EDTA (0.05%; Sigma-Aldrich) for 3 min at 37°C.

Bacterial attachment assay

Bacterial attachment assays were as described by Mellor, Goulter, Chia, and Dykes [20] with some modifications. Briefly, monolayers of HGF-1 cells were grown in 24-well tissue culture plates (Jet Biofil) to a density of 1.8 (± 0.2) × 10^5 cells per well (approximately 100% coverage). Prior to the attachment assay the culture medium in each well was removed and the cell monolayer was washed with PBS. The monolayer was incubated at 37°C for 30 min with 2 mL aliquots of tea extracts or tea components (PBS as control) containing suspended bacteria (~1 × 10^7 CFU/mL). The concentrations of the tea extracts and compounds used to suspend bacteria were previously determined by antimicrobial susceptibility assays and cytotoxicity assays not kill or inhibit the bacteria or the HGF-1 cells at the concentrations used in this study. After incubation the supernatant in each well was removed and the wells were washed three times with 2 mL PBS. The monolayer with bacteria attached was then detached by incubating with 400 μL 0.3 × trypsin-EDTA (at which concentration trypsin does not kill or inhibit the bacteria) at 37°C for 5 min. The detached bacteria were then serial diluted, spread plated on Tryptic Soy Agar (TSA; Merck) and quantified after 24 h incubation. The ability of bacteria to attach to wells without HGF-1 cells was also determined in order to ensure that the bacteria attached to HGF-1 cells but not to

the plastic material of the plate. The numbers of bacteria attached to the cell line was expressed as log CFU/well.

Statistical analysis

A one way ANOVA (Tukey's comparison) was performed on all data sets using MINITAB software (MINITAB 15.1; Minitab Inc., USA) at a 95% confidence level. All assays were performed in triplicate with independently grown cultures.

Results and discussion

The results of total phenolic, total tannin and total flavonoid content assays are presented in Table 1. The total phenolic and total flavonoid content decreased and total tannins increased, with an increasing degree of fermentation from green tea to oolong tea to black tea to pu-erh tea. These differences between teas are probably due to the polymerization of flavonoids (especially flavon-3-ols) into large molecule polyphenols (tannins) which occur during the fermentation process [16]. Chrysanthemum tea, which is a blend of black tea and dried chrysanthemum, had similar levels of total phenolic and total tannin to pu-erh tea and similar levels of flavonoids to green tea. This suggests that dried chrysanthemum is rich in flavonoids.

Baseline data for attachment of the bacterial strains to the cell line and empty wells are shown in Table 2. Bacterial attachment to the cell line was ~2 log higher ($p < 0.05$) than that to the plastic in the wells indicating that 90% to 99% of bacteria were attached to the cell line and validating the assay.

The results of assays investigating the effect of the tea extracts and tea components on bacterial attachment to the cell line are presented in Figure 1. All strains exhibited a similar ability to attach to the cell line except for *Streptococcus salivarius* ATCC 13419 which attached in significantly lower numbers as compared to *Streptococcus mitis* ATCC 49456 ($p < 0.05$). Green tea extracts, oolong tea extracts and black tea extracts inhibited the attachment of *Streptococcus mitis* ATCC49456 by between ~1 and ~2 log CFU/well (90-99% attachment inhibition; $p < 0.05$), but had no effect on the other strains ($p > 0.05$). Pu-erh tea extracts and chrysanthemum tea extracts, on the other hand, reduced the attachment of all *Streptococcus* strains to cells by between ~2 and ~4 log CFU/well (99–99.99% attachment inhibition; $p < 0.05$). The attachment of *Actinomyces naeslundii* ATCC 51665 to cells was not affected by any of the tea extracts tested ($p > 0.05$). Of particular note is that the extract of chrysanthemum tea, which, as mentioned above, is a blend of black tea and dried chrysanthemum, had a greater ($p < 0.05$) effect on inhibiting attachment than the black tea extract alone. This suggests that the active compounds in the chrysanthemum tea extract were contributed by the chrysanthemum components and not the black tea components of the mix. Pu-erh tea and chrysanthemum tea extracts, which were found to contain relatively higher levels of tannin, had a greater effect ($p < 0.05$) than the non-fermented or partially-fermented tea extracts on *Streptococcus* strains, suggesting that Streptococci may be more sensitive to polymeric flavonoids or other large molecule polyphenols with respect to their attachment to HGF-1.

Non-fermented or partially-fermented teas, such as green tea and oolong tea, have been previously shown to inhibit the attachment of *Streptococcus mutans* to collagen and tooth surfaces [12]. As indicated, in our study extracts of these teas only slightly inhibited the attachment of one bacterial strain to the gingival cell line. In addition, EGCg and gallic acid were found to have no significant effect ($p > 0.05$) on the ability of all strains to attach to the cell line. This finding suggests a possible reason for the relative ineffectiveness of the lower degree fermented tea extracts (green tea and oolong tea), which are rich in these compounds, in inhibiting adhesion. Fibronectin (Fn) is located on the outer surface of the HGF-1 plasma membrane and acts as a receptor protein for oral bacteria such as *Treponema denticola* [21]. *Streptococcus mutans* and *Streptococcus salivarius* have wall-associated protein A (wapA) in their outer membrane that allows them to bind collagen and a wide range of extracellular matrix molecules including type I collagen, laminin, keratin and Fn [22,23]. Tea catechins,

Table 1 Total phenolic, total tannin and total flavonoid contents of the tea extracts

	Total phenolic content (μg GAE / mg)	Total tannin content (μg GAE / mg)	Total flavonoid content (μg QE / mg)
Green tea	527 ± 34 (a)	149 ± 26 (a)	7.30 ± 0.68 (a)
Oolong tea	469 ± 28 (a, b)	161 ± 35 (a, b)	4.89 ± 0.14 (b)
Black tea	411 ± 20 (b, c)	241 ± 19 (b, c)	2.97 ± 0.59 (c)
Pu-erh tea	349 ± 35 (c)	305 ± 34 (c)	1.68 ± 0.68 (c)
Chrysanthemum tea	376 ± 13 (c)	280 ± 8 (c)	7.61 ± 0.42 (a)

All results are presented as the means followed by SDs. Values labeled with the same letter are not significantly different ($p > 0.05$) among the tea extract samples. Tukey's comparisons were conducted separately for each assay.

Table 2 Baseline data for bacterial attachment to the HGF-1 cell line and empty wells

	Mean±SD attachment (log CFU/well)				
	Streptococcus mutans **ATCC25175**	***Streptococcus mutans*** **ATCC35668**	***Streptococcus salivarius*** **ATCC13419**	***Streptococcus mitis*** **ATCC49456**	***Actinomyces naeslundii*** **ATCC51655**
Attachment to cell line	4.67 ± 0.25	4.75 ± 0.35	3.68 ± 0.77	5.11 ± 0.14	4.68 ± 0.34
Attachment to empty wells	2.37 ± 0.18	2.38 ± 0.27	2.14 ± 0.49	2.34 ± 0.47	2.19 ± 0.08

such as EGCg, have been reported to impair the adhesion promoting ability of Fn [24], and inhibit the interactions between Fn and attaching cells by binding to the Fn receptor integrin β1 [25]. These catechins should theoretically inhibit attachment but this was not the case in our study.

This study suggests that the mechanisms of inhibition of attachment of oral pathogens to gingival cells by tea or tea extracts may be different than that of inhibition to other components of the oral cavity. Based on this *in vitro* study extracts of pu-erh tea and chrysanthemum tea, in particular, may have the potential to reduce attachment of oral pathogens to gingival tissue and improve the health of oral soft tissues but this finding needs to be confirmed by *in vivo* studies. In order to further assess the situation in the oral cavity testing fresh brewed teas (hot water extracts) for adhesion inhibitory effect is required. The experimental setup used in this study could also be used to evaluate the effect of tea on the adhesion of other oral pathogenic microorganism, such as *Candida albicans*, which have been reported to adhere to human buccal epithelial cells and cause oral candidosis [26].

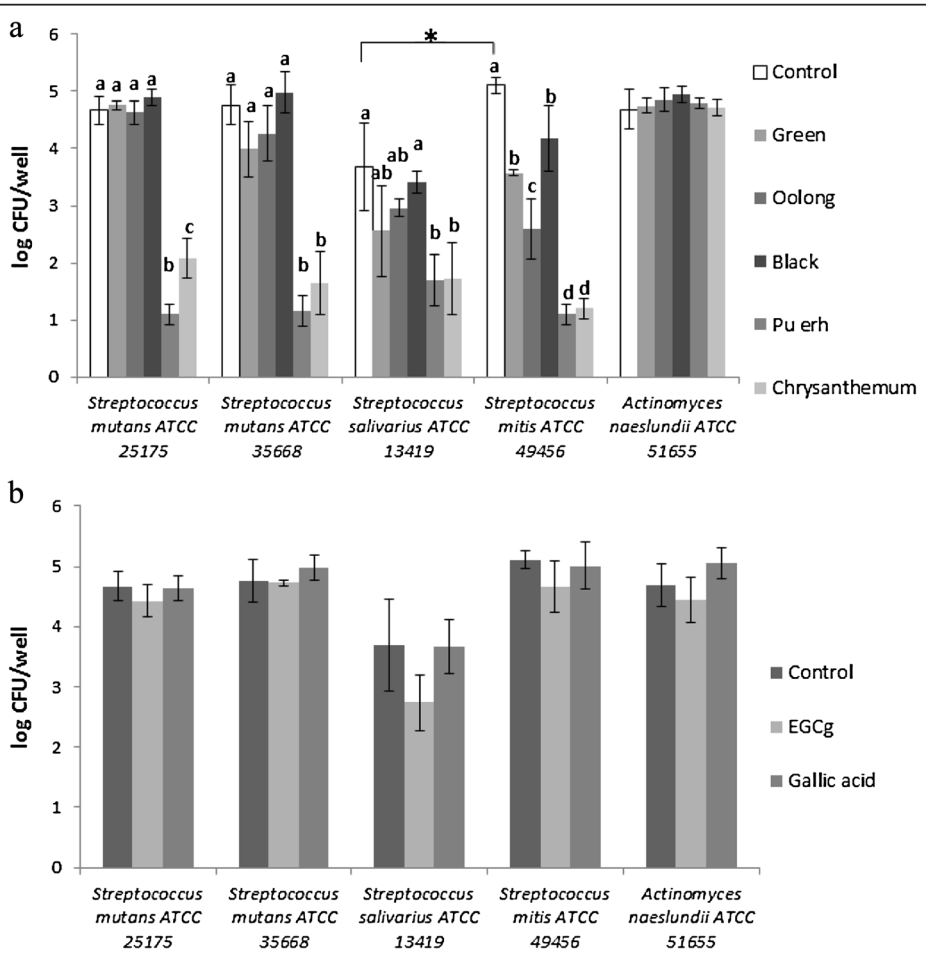

Figure 1 Effects of extracts and compounds on oral bacterial attachment. Effect of tea extracts (**a**) and EGCg and gallic acid (**b**) on oral bacterial attachment to the HGF-1 gingival cell line (log CFU/cm^2, n = 3). Values labeled with the same letter are not significantly different ($p > 0.05$) among the treatments within a strain. Tukey's comparisons were conducted separately for each strain. The * symbol indicates that the attachment of *Streptococcus salivarius* was significantly different from that of *Streptococcus mitis* ($p < 0.05$).

Abbreviations

HGF-1: Human gingival fibroblast-1; CFU: Colony forming unit; ATCC: American type culture collection; PBS: Phosphate buffered saline; EGCg: Epigallocatechin gallate; TSA: Tryptic soy agar; TSB: Tryptic soy broth; PVPP: Poly(vinylpolypyrrolidone); GAE: Gallic acid equivalent; QE: Quercetin equivalent; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; ANOVA: Analysis of variance; Fn: Fibronectin; WapA: Wall-associated protein A; SD: Standard deviation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YW carried out the cell culture, attachment study, statistical analysis and drafted the manuscript. FFLC participated in the cell culture and reviewed the manuscript. SML reviewed and revised the manuscript for intellectual content. GAD contributed to the conception and design of the study and reviewed and revised the manuscript. All authors read and approved the final manuscript.

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