Characterisation of *Candida albicans, Actinomyces naeslundii* and *Streptococcus mutans* interaction and its role in promoting oral carcinogenesis

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A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

April 2016

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ABSTRACT

*Candida albicans* has been widely reported in the aetiology of oral cancer. However, the role of its interaction with members of the oral microbiome, such as *Actinomyces naeslundii* and *Streptococcus mutans*, in promoting oral carcinogenesis is still under investigation. The overall hypothesis of the present study is that polymicrobial biofilms of *C. albicans*, *A. naeslundii* and *S. mutans* are involved in oral carcinogenesis with the specific hypotheses as following: 1) Auto-aggregation and co-aggregation of *C. albicans* is strain-dependent; 2) Polymicrobial biofilm formation is *C. albicans* strain- and medium-dependent; 3) Polymicrobial interactions within biofilms grown in flow-cells affects *C. albicans* biofilm formation; and 4) Oral epithelial cells have an enhanced malignant phenotype when grown in the presence of polymicrobial biofilm effluent.

The present study showed that *C. albicans* was able to auto-aggregate and co-aggregate with *A. naeslundii* and/or *S. mutans* during planktonic growth. Co-aggregation was shown to be variable between the eight strains of *C. albicans* with *A. naeslundii* and *S. mutans* found to co-aggregate on both yeast and hyphae of *C. albicans*. The static biofilm study showed that *C. albicans* formed yeast when grown in 25% artificial saliva medium (ASM) and hyphae when grown in RPMI-1640. Variability in biomass and metabolic activity was observed when *C. albicans* strains were grown as mono-cultured and polymicrobial biofilms. In addition, ASM-grown *C. albicans*, which predominantly forms yeast, was also able to form both mono-cultured and polymicrobial biofilms in a flow-cell environment. Overall the biomass of polymicrobial biofilms was found to be low relative to mono-cultured biofilms, indicating antagonistic interactions between species. The present study showed that biofilm effluent collected from flow-cell grown biofilms was able to promote oral
carcinogenesis by increasing the adhesion of H357 cells (oral squamous cell carcinoma cell line) to extracellular matrix molecules. Furthermore, the expression of pro-inflammatory cytokines from H357 was found to increase when grown in conditioned media suggesting that the biofilm effluent might have a role in the promotion of oral carcinogenesis.

In conclusion, polymicrobial interactions of \textit{C. albicans} \textit{A. naeslundii} and \textit{S. mutans} promote oral carcinogenesis, thus supporting the hypothesis that polymicrobial biofilms of \textit{C. albicans}, \textit{A. naeslundii} and \textit{S. mutans} are involved in oral cancer by promoting carcinogenesis. Moreover, this carcinogenesis promoting activity of polymicrobial biofilms is more likely to be \textit{C. albicans} strain-specific.
DECLARATION

This is to certify that:

i. The thesis comprises only my original work towards the PhD except where indicated

ii. Due acknowledgement has been made in the text to all other material used

iii. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices as approved by the Research Higher Degrees Committee

Mohd Hafiz Arzmi

29th April 2016
THE PREFACE

This PhD thesis comprises of seven chapters that determine the role of polymicrobial interaction of *C. albicans*, *A. naeslundii* and *S. mutans* in promoting oral cancer. The study was carried out under the supervision of Professor Michael McCullough, Professor Stuart Dashper, Associate Professor Nicola Cirillo and Associate Professor Neil O’Brien-Simpson. This study was done in collaboration with Professor Eric Reynolds (Chapter 3 and 4), Deanne Catmull (Chapter 5), Dr. Tanya D’Cruze (Chapter 5) and Dr. Jason Lenzo (Chapter 6). This is my original work with all the experiments have been done myself. The analysis of data was carried out with the contribution of collaborators. I have also contributed with more than 80% of the published journals in this thesis.

The editorial assistance from Dr. Catherine Butler who is a knowledgeable person in the academic discipline of the thesis has been very helpful during the preparation of the thesis. Furthermore, two chapters of this thesis that have been published in FEMS Yeast Research (Chapter 3) and Medical Mycology (Chapter 4), were also thoroughly reviewed by the editors and reviewers of the journals.

Finally, I would like to acknowledge Ministry of Higher Education, Malaysia, International Islamic University Malaysia (IIUM), Oral Health Cooperative Research Centre (OHCRC), Melbourne Dental School, The University of Melbourne and International Association of Dental Research (IADR), Australia for the scholarship and research funding for this work.
ACKNOWLEDGEMENT

I would like to thank to all my supervisors, Professor Michael McCullough, Professor Stuart Dashper, Associate Professor Nicola Cirillo and Associate Professor Neil O’Brien Simpson for all the input, knowledge and help in the completion of my research and thesis. I also would like to thank Professor Eric Reynolds for his advice and contribution especially in the publication of journals.

I would like to thank Deanne Catmull and Dr. Tanya D’Cruze for the assistant in flow-cell biofilm experiment and Dr. Jason Lenzo particularly in flow cytometry and Bio-Plex assays.

My gratitude to my parents, Arzmi Mansor and Safiah Abdul Aziz, my mother in law, Norhayati Abdul Aziz and my younger brother, Muhammad Hazwan Arzmi who have been giving a lot of supports and doa’ throughout my study.

Thanks also to my beloved wife, Nurul ‘Izzah Zulkifli, my beloved daughters, Iffah Humaira Mohd Hafiz, Iffah Huriyya Mohd Hafiz and Iffah Huwayna Mohd Hafiz for being very patient while Abi was so tired and moody. Thank you for everything that you have sacrificed.

Finally, I would like to thank to all my colleagues, especially Dr. Ali Alnuaimi, Dr. Antonio Celentano, Dr. Catherine Butler and Dr. Tami Yap for being very supportive.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASM</td>
<td>Artificial saliva medium</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CV</td>
<td>Crystal violet</td>
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<tr>
<td>EPS</td>
<td>Extracellular polysaccharides</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridisation</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factors</td>
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<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>IADR</td>
<td>International Association of Dental Research</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<td>min</td>
<td>Minute</td>
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<td>mL</td>
<td>Milliliter</td>
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<td>mm</td>
<td>Millimeter</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>rpm</td>
<td>Revolution per minute</td>
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RPMI  Roswell Park Memorial Institute medium
s     Second
SD    Standard deviation
SDA   Sabouraud’s dextrose agar
TEMED N,N,N,N-tetramethylethylenediamine
TNF   Tumour necrosis factor
v/v   volume/volume
WHO   World Health Organization
XTT   Tetrazolium salt, 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide
TABLE OF CONTENTS

ABSTRACT .................................................................................................................................1
DECLARATION ..........................................................................................................................3
THE PREFACE ..........................................................................................................................4
ACKNOWLEDGEMENT ..............................................................................................................5
ABBREVIATIONS ....................................................................................................................6
TABLE OF CONTENTS .............................................................................................................8
LIST OF TABLES .......................................................................................................................14
LIST OF FIGURES ...................................................................................................................17
CONFERENCES AND PUBLICATIONS .................................................................................20
LITERATURE REVIEW ............................................................................................................21
1.1 General Introduction ..........................................................................................................22
1.2 Oral Microorganisms .........................................................................................................22
    1.2.1 Candida species ..........................................................................................................23
        1.2.1.1 Virulence factors of Candida species .................................................................23
        1.2.1.2 Prevalence of C. albicans in the oral cavity ......................................................27
        1.2.1.3 Growth requirements .........................................................................................28
        1.2.1.4 Clinical manifestation .........................................................................................29
    1.2.2 Actinomyces species ..................................................................................................33
    1.2.3 Streptococcus species ...............................................................................................33
1.3 Intra-kingdom and inter-kingdom interaction of Candida spp. ....................................35
    1.3.1 C. albicans and non-albicans Candida spp. ..........................................................40
3.4 Results....................................................................................................................85

3.4.1 Morphology of *C. albicans* in RPMI-1640 and 25% ASM.........................85

3.4.2 Auto-aggregation ...............................................................................................85

3.4.3 Inter-kingdom co-aggregation .........................................................................88

3.4.4 Scanning Electron Microscopy analyses .........................................................88

3.5 Discussion.............................................................................................................92

3.6 Conclusion ............................................................................................................95

POLYMICROBIAL BIOFILM FORMATION BY *CANDIDA ALBICANS*,

*ACTINOMYCES NAESLUNDII AND STREPTOCOCCUS MUTANS IS CANDIDA ALBICANS* STRAIN AND MEDIUM DEPENDENT .........................................................96

4.1 Abstract ................................................................................................................97

4.2 Introduction ..........................................................................................................98

4.3 Materials and methods .....................................................................................101

4.4 Results ...............................................................................................................103

4.4.1 Morphology of *C. albicans* biofilms in RPMI-1640 and 25% ASM...........103

4.4.2 Effect of microbial interaction and medium on biofilm biomass ...............103

4.4.3 Effect of microbial interaction and medium on metabolic activity ..........107

4.5 Discussion .........................................................................................................111

4.6 Conclusion .......................................................................................................115

POLYMICROBIAL BIOFILM FORMATION BY *CANDIDA ALBICANS*,

*ACTINOMYCES NAESLUNDII AND STREPTOCOCCUS MUTANS IN A FLOW ENVIRONMENT* .....................................................................................................................116

5.1 Abstract .........................................................................................................117
5.2 Introduction ........................................................................................................... 119
5.3 Materials and methods ......................................................................................... 122
5.4 Results .................................................................................................................. 123
  5.4.1 Mono-cultured biofilms of C. albicans, A. naeslundii and S. mutans ....... 123
  5.4.2 Polymicrobial biofilms of C. albicans, A. naeslundii and S. mutans........ 123
  5.4.3 Effect of polymicrobial interaction on C. albicans, A. naeslundii and S.
         mutans biofilms .................................................................................................. 124
5.5 Discussion ............................................................................................................. 131
5.6 Conclusion ............................................................................................................ 135

BIOFILM EFFLUENT OF CANDIDA ALBICANS, ACTINOMYCES NAESLUNDII
AND STREPTOCOCCUS MUTANS AFFECT THE ADHESION, EPITHELIAL
MESENCHYMYAL TRANSITION AND CYTOKINE EXPRESSION OF NORMAL
AND MALIGNANT ORAL KERATINOCYTES .............................................................. 136

6.1 Abstract ................................................................................................................. 137
6.2 Introduction .......................................................................................................... 139
6.3 Materials and methods ......................................................................................... 145
6.4 Results .................................................................................................................. 146
  Part I: Adhesion assay ............................................................................................ 146
    6.4.1 Adhesion of OKF6 to ECM ........................................................................ 146
    6.4.2 Adhesion of H357 to ECM ....................................................................... 146
    6.4.3 Comparison of adhesion to ECM between OKF6 and H357............... 147
  Part II: Epithelial-mesenchymal transition (EMT) ................................................ 151
    6.4.4 Percentage of cells expressing E-cadherin and vimentin....................... 151
6.4.5 Mean fluorescence intensity (MFI).................................................154

Part III: Cytokine assay......................................................................................158

6.4.6 Expression of cytokines by OKF6 and H357 .....................................158

6.4.6.1 Interleukin 2 (IL-2) ...........................................................................163
6.4.6.2 Interleukin 4 (IL-4) ...........................................................................165
6.4.6.3 Interleukin 6 (IL-6) ...........................................................................167
6.4.6.4 Interleukin 8 (IL-8) ...........................................................................169
6.4.6.5 Interleukin 10 (IL-10) ......................................................................171
6.4.6.6 Granulocyte-macrophage colony-stimulating factor (GM-CSF) ...173
6.4.6.7 Interferon gamma (IFN-γ) .................................................................175
6.4.6.8 Tumour necrosis factor alpha (TNF-α) ............................................176
6.4.6.9 Overall.................................................................................................177

6.5 Discussion.....................................................................................................181

6.6 Conclusion.....................................................................................................186

DISCUSSION AND CONCLUSION ................................................................187

7.1 Discussion.....................................................................................................188

7.2 Conclusion and future studies.................................................................196

REFERENCES .................................................................................................197

APPENDICES ....................................................................................................245
Table 1.1 Microorganisms isolated from oral cavity .................................................................36
Table 1.2 Interaction of *C. albicans* with various important species of microorganisms in the oral cavity ..........................................................................................38
Table 1.3 Summary of cytokines that possibly have a role in the progression of OSCC ..........................................................59
Table 3.1 Auto and co-aggregation scores of pairs of eight strains of RPMI-grown (hyphal form) and ASM-grown (yeast form) *C. albicans*, *A. naeslundii* and *S. mutans*. Percentage aggregation as measured by OD$_{620nm}$ change over 1 h .........................................................................................90
Table 4.1 Static biofilm biomass scores of eight strains of RPMI-grown and ASM-grown *C. albicans*, *A. naeslundii* (An) and *S. mutans* (Sm) as measured by OD$_{620nm}$ after 72 h incubation .........................................................................................106
Table 4.2 Static biofilm metabolic activity scores of eight strains of RPMI-grown and ASM-grown *C. albicans*, *A. naeslundii* (An) and *S. mutans* (Sm) as measured by OD$_{450nm}$-OD$_{620nm}$ after 72 h incubation .........................................................................................09
Table 4.3 Mono-culture metabolic activity per biofilm biomass (XTT/CV) scores of eight strains of RPMI-grown (hyphal form) and ASM-grown (yeast form) *C. albicans*, *A. naeslundii* (An) and *S. mutans* (Sm) ........................................110
Table 5.1 Total biomass ($\mu$m$^3$ $\mu$m$^{-2}$) of ASM-grown *C. albicans*, *A. naeslundii* and *S. mutans* after 24 h incubation in a flow-cell (3 mL h$^{-1}$) at 37 °C in mono-cultured biofilm and polymicrobial biofilms ........................................................................129
Table 5.2 Surface roughness, average and maximum thickness and percentage surface colonisation of ASM-grown *C. albicans*, *A. naeslundii* and *S. mutans* after 24 h incubation in a flow-cell (3 mL h$^{-1}$) at 37 °C ...........................................130
Table 6.1A Adhesion of OKF6 and H357 in 80% serum free medium (SFM) containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM) and polymicrobial (TRI) biofilm effluents

Table 6.1B Fold change of OKF6 and H357 adhesion when incubated with *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM) and polymicrobial (TRI) biofilm effluents compared to non-effluent (NE)

Table 6.2A Percentage positive of OKF6 and H357 cells treated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM) and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h

Table 6.2B Percentage difference of positive OKF6 and H357 cells expressing vimentin and E-cadherin between 2 h and 24 h incubated in NE, ALC3, AN, SM and TRI effluents at 37 °C, 5% CO₂ for 2 h and 24 h

Table 6.3A Mean fluorescence intensity (MFI) of vimentin and E-cadherin of OKF6 and H357 cells treated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM) and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h

Table 6.3B Percentage difference of mean fluorescence intensity (MFI) of OKF6 and H357 cells expressing vimentin and E-cadherin between 2 h and 24 h incubated in NE, ALC3, AN, SM and TRI effluents at 37 °C, 5% CO₂ for 2 h and 24 h

Table 6.4A Cytokines expressed by OKF6 (pg mL⁻¹) incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3),
**A. naeslundii** (AN), **S. mutans** (SM) and polymicrobial (TRI) biofilm effluent at 37 °C, 5% CO₂ for 2 h.........................................................159

Table 6.4B Cytokines expressed by OKF6 (pg mL⁻¹) incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), **C. albicans** (ALC3), **A. naeslundii** (AN), **S. mutans** (SM) and polymicrobial (TRI) biofilm effluent at 37 °C, 5% CO₂ for 24 h..............................................................160

Table 6.4C Cytokines expressed by H357 (pg mL⁻¹) incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), **C. albicans** (ALC3), **A. naeslundii** (AN), **S. mutans** (SM) and polymicrobial (TRI) biofilm effluent at 37 °C, 5% CO₂ for 2 h..............................................................161

Table 6.4D Cytokines expressed by H357 (pg mL⁻¹) incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), **C. albicans** (ALC3), **A. naeslundii** (AN), **S. mutans** (SM) and polymicrobial (TRI) biofilm effluent at 37 °C, 5% CO₂ for 24 h..............................................................162

Table 6.5A Percentage difference of cytokines expressed by OKF6 incubated in NE, ALC3, AN, SM and TRI effluents at 37 °C, 5% CO₂ between 2 h and 24 h incubation..................................................................................................................179

Table 6.5B Percentage difference of cytokines expressed by H357 incubated in NE, ALC3, AN, SM and TRI effluents at 37 °C, 5% CO₂ between 2 h and 24 h incubation..................................................................................................................180
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Flow-cell system</td>
<td>71</td>
</tr>
<tr>
<td>3.1</td>
<td>Gram-stained of <em>C. albicans</em> cultures observed under light microscopy at 1000x magnification</td>
<td>86</td>
</tr>
<tr>
<td>3.2</td>
<td>Percentage auto-aggregation in RPMI-1640 (A) and 25% ASM (B) grown <em>C. albicans</em> after 1 h incubation in co-aggregation buffer</td>
<td>87</td>
</tr>
<tr>
<td>3.3</td>
<td>SEM of <em>C. albicans</em> (strain ALT4) auto-aggregation (A &amp; E), inter-kingdom interaction with <em>A. naeslundii</em> (B &amp; F), <em>S. mutans</em> (C &amp; G) and both bacteria (D &amp; H). <em>C. albicans</em> was grown in RPMI-1640 (A, B, C &amp; D) and 25% ASM (E, F, G &amp; H)</td>
<td>91</td>
</tr>
<tr>
<td>4.1</td>
<td>Gram-stained biofilms of <em>C. albicans</em> strain ALC3 observed under light microscope at 200x magnification after 72 h incubation at 37 °C in 24-well plate at 90 rpm</td>
<td>105</td>
</tr>
<tr>
<td>5.1A</td>
<td>Representative CLSM image of mono-cultured <em>C. albicans</em> as observed using a 63x objective at 512 x 512 pixels magnification</td>
<td>125</td>
</tr>
<tr>
<td>5.1B</td>
<td>Representative CLSM image of mono-cultured <em>A. naeslundii</em> as observed using 63x objective at 512 x 512 pixels magnification</td>
<td>126</td>
</tr>
<tr>
<td>5.1C</td>
<td>Representative CLSM image of mono-cultured <em>S. mutans</em> as observed using a 63x objective at 512 x 512 pixels magnification</td>
<td>127</td>
</tr>
<tr>
<td>5.1D</td>
<td>Representative CLSM image of polymicrobial biofilms as observed using a 63x objective at 512 x 512 pixels magnification (Red: <em>C. albicans</em>; Green: <em>A. naeslundii</em>; Blue: <em>S. mutans</em>)</td>
<td>128</td>
</tr>
<tr>
<td>6.1</td>
<td>Fold change of OKF6 and H357 adhesion when incubated with <em>C. albicans</em> (ALC3), <em>A. naeslundii</em> (AN), <em>S. mutans</em> (SM) and polymicrobial (TRI) biofilm effluents compared to non-effluent (NE)</td>
<td>150</td>
</tr>
</tbody>
</table>
Figure 6.2A Fold change of IL-2 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change were the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE..........................164

Figure 6.2B Fold change of IL-4 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change were the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE..........................166

Figure 6.2C Fold change of IL-6 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change were the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE..........................168

Figure 6.2D Fold change of IL-8 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change were the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE..........................170
Figure 6.2E Fold change of IL-10 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change were the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE...............................172

Figure 6.2F Fold change of GM-CSF expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change were the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE..............174

Figure 6.2G Fold change of TNF-α expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluent at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change were the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE...............................178
CONFERENCES AND PUBLICATIONS


CHAPTER 1

LITERATURE REVIEW
1.1 General Introduction

The oral cavity contains a multitude of microorganisms, including monera and fungi (Dewhirst et al., 2010). They live in an ecological shared space, a community in the human oral cavity (Nobbs and Jenkinson, 2015). The soft mucosal surfaces of the tongue, gingival sulci, cheeks, lips, palate and tonsils, and the hard surfaces of teeth provide convenient habitat niches for more than 700 oral microbial species including Candida species, Actinomyces species and Streptococcus species (Kolenbrander et al., 2010; Chandra et al., 2016).

The oral microbiome can exist in both planktonic and biofilm (plaque) forms (Kolenbrander et al., 2010). An oral biofilm is defined as a community of microorganisms that attach to surfaces in the oral cavity and are encapsulated within extracellular polymeric substances (EPS), derived from both the microorganisms and the oral environment (Filoche et al., 2010). It is suggested that cell-cell signalling mechanisms between members of polymicrobial biofilms contributes to the successful colonisation of bacteria in periodontal pockets (Yamada et al., 2005). For example, Neisseria, an aerobic bacterium, has been found to metabolise the surrounding oxygen and release carbon dioxide intensely, thus contributing to the survival of obligate anaerobic bacteria such as Porphyromonas gingivalis (Kolenbrander et al., 2006; Marsh, 2015).

1.2 Oral Microorganisms

It has been shown that more than 2000 bacterial taxa exist in the oral cavity with a large number of opportunistic pathogens found to be involved in periodontal and systemic diseases (Dewhirst et al., 2010; Warinner et al., 2014). These oral microorganisms are classified into different kingdoms including monera (bacteria),
fungi (yeast) and protozoa. Previously, these microorganisms were classified according to their morphology, fermentation of simple sugar and chemical analyses, however, with the advance of DNA analysis technology, classification is now based upon comparative 16S rRNA gene sequencing (Chaffin, 2008).

1.2.1 Candida species

Candida spp. belong to the Eukaryota domain and are known as imperfect fungi within the family of Cryptococcaceae (Odds, 1979). These unicellular yeasts possess globose, ellipsoidal and occasionally triangular microscopic morphology with the sizes of the blastopores, hyphae and pseudohyphae varying between candidal species (Kurtzman et al., 2011). There are at least seven Candida spp. that are significant in human pathogenesis including C. albicans, C. kefyr, C. glabrata, C. krusei, C. parapsilosis, C. dubliniensis and C. stellatoidea (Sida et al., 2016). C. albicans has been reported to be the most prevalent in the oral cavity (Akdeniz et al., 2002; Nejad et al., 2013). Some Candida spp. such as C. albicans and C. dubliniensis, have the ability to form septate hyphae (Samaranayake, 2006). A few Candida spp., such as C. krusei, have been shown to form pseudohyphae (Arzmi et al., 2012). The cell wall of Candida spp. has been shown to possess β-glucan, mannoprotein and chitin that can assist in adhesion to oral surfaces and co-aggregation with other bacteria, both in planktonic and biofilm growth (Chaffin, 2008).

1.2.1.1 Virulence factors of Candida species

Virulence factors of Candida spp. vary between species (Haynes, 2001). At least seven significant virulence factors of Candida spp. have been reported, including the ability to undergo phenotypic switching, cell surface hydrophobicity, production
of hydrolytic enzymes, dimorphism, candidalysin, quorum sensing and biofilm formation (Williams et al., 2011; Kragelund et al., 2016; Sida et al., 2016).

Phenotypic switching is one of the most important virulence factors of C. albicans, C. glabrata, C. dubliniensis and C. krusei (Anderson et al., 1987; Soll, 1992; Jones, et al., 1994; Lachke et al., 2000; Lachke et al., 2002; Vargas, et al., 2004; Arzmi et al., 2012; Kragelund et al., 2016). The significance of the switching strategy has similarities to human immune function and aims to counter threats in the host’s environment.

Rapid phenotypic switching has been shown to enhance the survivability of Candida spp. as an adaptive response to the stressful environment of the oral cavity (Hellstein et al., 1993; Haynes, 2001). In a suppressed environment, it is postulated that Candida spp. employs two mechanisms; mitotic recombination and subsequently, phenotypic switching. A direct consequence of mitotic recombination is the loss of heterozygosity throughout the entire genome. This deletion of the genome does, however, affect the viability of Candida spp., especially in situations of multiple modified conditions (Vargas et al., 2004). On the other hand, phenotypic switching is a mechanism of adaptation that avoids alteration of the candidal genome. This spares the alteration expression of genes, such as those involved in adhesion, and resistance of Candida spp. to phagocytosis by polymorphonuclear leukocytes. Thus, heterozygosity of the entire genome of Candida spp. is conserved (Marsh et al., 2009).

In addition, cell surface hydrophobicity, another important virulence factor, allows adherence of Candida spp. to host surfaces. The hydrophobicity among Candida spp. varies, likely due to a difference in hydrophobic-associated surface protein expression. There is a positive correlation between hydrophobicity and
interaction of *Candida* spp. with itself, endothelial cells and inert surfaces (Razak *et al*., 2006; Silva-Dias *et al*. 2015).

The production of hydrolytic enzymes, such as aspartyl proteinase, phospholipases, lipases, phosphomonoesterase and hexosaminidase has been reported to contribute to the pathogenicity of *Candida* spp. in oral candidosis (Williams *et al*., 2011). Aspartyl proteinase, coded by the secreted aspartyl proteinase (SAP) genes, is considered central in the development of candidal infections. *C. albicans* and *C. krusei* derived aspartyl proteinases have the ability to penetrate the host cell, thus causing candidal infection (Samaranayake and Ferguson, 1994).

Another important hydrolytic enzyme in host invasion is phospholipase. There are 4 types of phospholipase (type A, B, C and D). *C. albicans* produces phospholipase A and C (Samaranayake and Ferguson, 1994). Phospholipase A can attack cell membranes and is found on the cell surface, especially at bud formation sites. Furthermore, the enzyme activity is enhanced when the hyphae are in direct contact with host tissue (Williams *et al*., 2011).

Dimorphism is another important virulence factor of *Candida* spp. It is defined as the ability of the microorganism to switch between yeast and hyphal morphology depending on the environment and requirement. This is modulated by gene expression within the cell (Nantel *et al*., 2002). A hypha (plural, hyphae) is a long branching filamentous structure of fungus that consists of one or more cells surrounded by tubular chitin-made cell walls. It is classified as the main mode of vegetative fungal growth. Hyphae prefer to grow together in a compact tuft or mycelium, particularly during the germination phase. In addition, this configuration has also been shown to assist in the adhesion and colonisation of the host tissue by *C*. 
albicans (Tronchin et al., 1988; Hawser and Douglas, 1994; San Millan et al., 1996; Madigan et al., 2012).

The ability of Candida spp. to produce quorum sensing molecules has recently been categorised as a virulence factor of this microorganism. It has been reported that quorum sensing molecules are also involved in the colonisation of C. albicans in the oral cavity (Shih and Huang, 2002). For example, Farnesol, secreted by C. albicans, can freely pass across membranes and has been reported to be a biofilm-limiting agent which inhibits the conversion of yeast to hyphal form in biofilm once its threshold is reached (Ramage et al., 2002). Via these mechanisms, the number of microorganisms within the consortia is optimised and overpopulation, which increases competition for nutrients, oxygen, water and sites, is avoided. It has been suggested that one of the factors that determines dissemination of Candida spp. infection within the oral cavity is the secretion of Farnesol (Ramage et al., 2002). Detached Candida spp. cells will adhere on the substratum where no Farnesol is detected (Hornby et al., 2001; Ramage et al., 2002).

Candidalysin is newly discovered cytolytic peptide toxin that has been classified as a virulence factor of C. albicans. This toxin is secreted by C. albicans hyphae and has been shown to disrupt epithelial membranes, activate epithelial immunity and trigger a danger response signalling pathway (Hofer, 2016). A mutant strain of C. albicans lacking the candidalysin did not activate or damage epithelial cells and was also shown to exhibit less tissue damage and neutrophil infiltration in the tongues of mice than the wild-type, thus supporting the important role of candidalysin as a virulence factor in the oral cavity (Moyes et al., 2016).
The ability to form biofilm on the oral surfaces is an important virulence factor of *Candida* spp. in the oral cavity. *Candida* spp. adhesion initiates colonisation of both hard and soft tissue surfaces in the oral cavity (Höfs *et al.*, 2016). In general, this adhesion occurs via non-specific and specific interactions. Non-specific interactions consist of physicochemical forces such as Van der Waals forces, electrostatic forces and acid-base interactions. Specific interactions involve protein mediation between *Candida* spp. and the substratum (Van Oss, 1995).

Following adhesion, the attached cells grow and form a structured community known as a biofilm. An oral biofilm or dental plaque is defined as a thin layer comprised of various microbial communities encapsulated within EPS and attached to a hard, soft or prosthetic surface (Holmes *et al.*, 2002; Samaranayake *et al.*, 2002). The details of biofilm development will be further discussed in Section 1.4.

### 1.2.1.2 Prevalence of *C. albicans* in the oral cavity

*C. albicans* is a commensal yeast that inhabits the epidermis, vagina, gastrointestinal tract, nails and oral cavity (Williams *et al.*, 2011; Chandra *et al.*, 2016). It is estimated that 70% of people with a healthy oral cavity have either a transient or permanent residence of *C. albicans* (Mitchell, 2007; Thein *et al.*, 2007). Several factors that promote the conversion of *C. albicans* from commensal to opportunistic microorganism in patients are impaired salivary gland function, a high carbohydrate diet, tobacco smoking, drug abuse and use of broad-spectrum antibiotics. This is commonly seen in the immunocompromised, including those using long-term corticosteroids and common endocrine disorders such as diabetes mellitus (Williams *et al.*, 2011). It has also been associated with oral mucosal presentations and diseases such as oral cancer and may play a role in symptoms of oral burning (Samaranayake, 2006; Scardina *et al.*, 2007; Williams *et al.*, 2011; Cavalcanti *et al.*, 2016).
C. albicans infection can be local and/or systemic. Systemic infection of C. albicans can be very severe and lead to fatality with mortality rates up to 60% (Leroy et al., 2009). The treatment of the infection is difficult and its role may sometimes only be determined by post-mortem. In the oral cavity, C. albicans has been found to colonise mucosal surfaces including buccal and labial mucosa, dorsum or lateral borders of tongue, hard and soft palate regions, as well as tooth surfaces and denture-bearing areas (Harriott and Noverr, 2011). Virulence factors that contribute to the successful colonisation of C. albicans in the oral cavity are previously discussed in Section 1.2.1.1.

1.2.1.3 Growth requirements

There are many factors involved in the growth of Candida spp. in the oral cavity. However, the two main factors are nutrients and host temperature.

The role of nutrients as a growth requirement has been widely discussed. Candida is a chemoheterotrophic organism that requires carbon and nitrogen for growth (Marsh et al., 2009). Carbohydrates are the most readily utilised form of carbons in both oxidative and non-oxidative pathways. Thus, the presence of carbohydrates influences the colonisation of Candida spp. in the oral cavity. Certain carbohydrates, such as sucrose and glucose, have been shown to increase the adhesion potential of C. albicans on to hard and soft oral surfaces (Samaranayake et al., 1986; Jin et al., 2004). Glucose is an acid promoter that leads to the reduction of pH in the oral environment, which activates acid proteinase and phospholipase enzymes, and enhances the adherence capability of Candida spp. (Jin et al., 2004). Additionally, the production of mannoprotein surface layer in the environment where glucose is present has been shown to assist the adherence capability of Candida spp. (Modrzewska and Kurnatowski, 2015; Demirezen et al., 2016).
Furthermore, body temperature has also been shown to influence the growth of *Candida* spp. *Candida* spp. has been shown to grow at the optimal temperature of 37 °C (Singh *et al.*, 2002). This is also the optimal temperature for various pathogenic microorganisms in the oral cavity, such as *S. mutans* and *Actinomyces* spp. (MacFarlane and Samaranayake, 2014). Any alteration in normal body temperature may influence the competitiveness of organisms within the normal microbiome, thus enhancing the growth of opportunistic *Candida* spp. Many experimental assays are conducted at 37 °C and this is generally accepted as the standard incubation temperature for candidal species (Marsh *et al.*, 2009).

### 1.2.1.4 Clinical manifestation

Various classifications of candidosis and *Candida* spp. related disease have been proposed (Meurman, 2016). The earliest classification was acute pseudomembranous candidosis (oral thrush), acute atrophic candidosis, chronic atrophic candidosis and chronic hyperplastic candidosis (Scully *et al.*, 1994). Oral candidosis has also been described as primary oral candidosis, a candidal infection confined to the oral and perioral tissue, and secondary oral candidosis, where the oral presentation is a manifestation of systemic infection (Samaranayake *et al.*, 1990). Some keratinised primary lesions super-infected by *Candida* spp. have now been classified under the category of primary oral candidosis such as leukoplakia, lichen planus and lupus erythematosus (Axéll *et al.*, 1997). Furthermore, in later classifications, the terminology of ‘acute’ and ‘chronic’ have been removed and thought to have little bearing on the causality and treatment of the condition (McCullough and Savage, 2005).

Primary oral candidosis has been divided into three major clinical variants, pseudomembranous, erythematous and hyperplastic. Furthermore, there are a further
four lesions that have been classified as primary oral candidosis that are denture-associated erythematous stomatitis, chronic hyperplastic, angular cheilitis and median rhomboid glossitis.

Pseudomembranous candidosis (oral thrush) is a common disease of neonates and elderly debilitated persons at rates of 5 to 10% (Samaranayake et al., 2009). The lack of microbiota in the oral cavity of infants allows Candida spp. to flourish. In elderly people, the disease can be caused by debilitation, xerostomia or atrophy of the host immune system (Reichart et al., 2000). The disease is characterised by the presence of white curd-like patches on the tongue, labial mucosal, cheeks, palate and lips, that consist of dead mucosal cells, hyphal white plaques, blastospores, inflammatory cells, fibrin and desquamated epithelial cells. The patches can be easily removed leaving an erythematous background (Samaranayake et al., 2009). Oral thrush can disseminate to the surfaces of oesophagus and pharynx, which leads to feeding difficulties especially in infants (Klein and Klein, 1985).

The presentation of erythematous candidosis has previously been described in association with the usage of broad-spectrum antibiotic therapy including tetracycline (Williams et al., 2011). The surfaces affected may include the buccal mucosal, palate and tongue. The mucosa may appear erythematous and atrophic (Millsop and Fazel, 2016). The condition can be caused by the overgrowth of commensal Candida spp. due to the usage of broad-spectrum antibiotic therapy. This may inhibit the growth of other antagonistic microorganisms to Candida spp., thus increasing the survival of the yeast within the oral environment (Ito et al., 2015).

Denture-associated erythematous stomatitis (DAES) presents an area of erythematous mucosa corresponding to the fitting surface of a prosthesis (Williams et al., 2011). It is suggested to occur in up to 75% of denture wearers and is typically
asymptomatic (Webb et al., 1998b). Nocturnal denture wear as well as poor denture hygiene are major predisposing factors (Budtz-Jørgensen, 1975; Williams et al., 2011). These habits, including the accompanying limited flow of saliva over the area, may allow an established biofilm that includes the overgrowth of Candida spp. Biofilms on the oral prosthesis have been shown to be critical in the development of DAES (Manfredi et al., 2013). Increasing surface roughness of dentures has been shown to correlate with Candida spp. biofilm development (Lamfon et al., 2005). Additionally, frictional irritation due to denture surface roughness may damage normal mucosa if the denture is ill-fitting, which may allow the infiltration of Candida spp. (Williams et al., 2011).

Another primary oral candidosis is hyperplastic candidosis (Candida leukoplakia). The clinical presentation of this disease may include areas of keratosis, particularly in the post commissure region of the buccal mucosa (Scardina et al., 2007; Millsop and Fazel, 2016). The risk factors of the disease include impaired salivary gland function, drugs, dentures, high carbohydrate diet, diabetes mellitus, Cushing’s syndrome, malignancies and immunosuppressive conditions of patients (Scardina et al., 2007). Hyperplastic candidosis usually develops as isolated white patches intraorally and may be hard to differentiate between other causes of oral diseases such as leukoplakia (Lamey and Samaranayake, 1988). The patches cannot be removed with scraping and histopathologically will include hyphal elements (Walker et al., 1990; de Lima et al., 2015). Marsh and Martin (1992) demonstrated the production of nitrosamines in the saliva of patients that had been infected by C. albicans. Nitrosamines are known carcinogens and the role of Candida spp. in the development of oral cancer continues to be hypothesised, particularly in relation to hyperplastic candidosis.
Angular cheilitis, which is classified as primary oral candidosis, may affect individuals of any age. However, it is commonly seen in elderly patients (Samaranayake and Holmstrup, 1989). Clinically, it presents as erythema and ulceration of the angles of the mouth (Millsop and Fazel, 2016). Angular cheilitis has also been reported to be associated with concomitant intraoral candidosis and worn dentures (Budtz-Jørgensen et al., 1990; Farah et al., 2010).

Finally, median rhomboid glossitis (MRG) is characterised by a rhomboidal shaped area of papillary atrophy of the mid-posterior dorsum of the tongue, anterior to the circumvallate papillae. There may additionally be a corresponding lesion on the palate. It is generally asymptomatic, symmetric and a flat/smooth lesion, although it can be lobulated or nodular (Manfredi et al., 2013). Previously, Candida spp. were suggested to be aetiological agents of MRG due to candidal hyphae observed penetrating the superficial layers of the epithelium histopathologically (Scully et al., 1994). However, the role of Candida spp. as causative agents in this disease remains undetermined (Manfredi et al., 2013).

Chronic mucocutaneous candidosis is a rare disease that occurs in young children and elderly males and is classified as a secondary oral candidosis (Williams et al., 2011). The infection may be associated with disorders of T-cell production. This type of candidosis commonly affects the oral cavity; it is usually infiltrated with Candida spp. in thick granulomatous plaques (Farah et al., 2000; Liu and Hua, 2007). It is important to understand that the removal of the candidosis may lead to other infections by opportunistic bacteria including Pseudomonas spp. and Staphylococcus spp. Chronic mucocutaneous candidosis may become a lifetime superinfection, however the systemic imidazole antifungal agent ketonazole may aid in combating the disease (Marsh and Martin, 1992).
1.2.2 Actinomyces species

*Actinomyces* spp. are facultative anaerobic Gram-positive rod bacteria that can be easily isolated from the oral cavity (Schaal and Yassin, 2015). The bacteria have been classified as part of the oral microbiome with some species categorised as primary colonisers, such as *A. naeslundii*, that form fimbriae to assist in attachment to oral surfaces (Kolenbrander *et al.*, 2010).

*A. naeslundii* is the most common isolate of *Actinomyces* spp. in the oral cavity and has been considered to be an early oral coloniser (Arai *et al.*, 2015). The genospecies of the bacterium is subdivided into genospecies 1 and 2. Genospecies 2 is now classified as *A. oris* (Yamane *et al.*, 2013). Thus, *A. naeslundii* denotes what was previously designated as genospecies 1. Some *A. naeslundii* strains are able to produce urease, which modulates pH in the oral biofilm, as well as neuraminidase, which can modify receptors in the acquired-pellicle to aid bacterial adhesion (Yaling *et al.*, 2008). Additionally, fimbriae that are present on the surface of *A. naeslundii* contribute to colonisation of the oral cavity through cell-to-cell and cell-to-surface adhesion, thus aiding in the formation of polymicrobial biofilms (Kolenbrander *et al.*, 2010).

1.2.3 Streptococcus species

The genus *Streptococcus* is comprised of facultative anaerobic Gram-positive cocci bacteria. The nomenclature of *Streptococcus* spp. is in reference to their coccal morphology in contrast to short rods or cocco-bacilli (Parks *et al.*, 2015). *Streptococcus* spp. have a tenacious binding ability for enamel surfaces and are pathogens with high cariogenic potential. They form voluminous amounts of EPS that facilitate the formation of oral biofilms (Samaranayake, 2006). The majority of *Streptococcus* spp. are α-haemolytic on blood agar and referred to as the viridans-
group, that are clustered into four main groups: the mitis-group, salivarius-group, anginosus-group and mutans-group (mutans streptococci) (Marsh et al., 2009).

The mutans-group of streptococci remains of great interest due to its role in the aetiology of dental caries. Mutans streptococci include nine serotypes (a-h, and k) which are determined by specific carbohydrate antigens present on the cell wall of the bacteria. The major serotypes usually isolated from the human host are serotypes c, e, f and k (Nakano et al., 2013).

*S. mutans* is the most common mutans-group isolate and is epidemiologically implicated as the primary pathogen of enamel caries (Leme et al., 2006). It possesses a cell wall with carbohydrate antigens, lipoteichoic acid and lipoproteins. Antigen I/II is an example of an antigenic protein of *S. mutans* that is involved in the initial adherence to the salivary pellicles on oral surfaces (Chuzeville et al., 2015). *S. mutans* also produces polysaccharides that are comprised of fructan, glucan and mutan metabolised from sucrose (Koo et al., 2010). These polysaccharides, in association with *S. mutans*, help the formation and maturation of dental plaque (oral biofilm) (Leme et al. 2006). Additionally *S. mutans* is able to communicate with other members of the same group by releasing diffusible signalling molecules that lead to the transfer of genes and provides a convenient condition for the growth of other bacteria in the oral biofilm matrix (Marsh et al., 2009).

Even though infection by *S. mutans* is scarce on oral mucosal surfaces, reports have shown a significant proliferation of the bacteria in disrupted mucosa and in association with modified host defences such as in neutropenic patients (Tunkel and Sepkowitz, 2002). Table 1.1 shows a range of other microorganisms that can be isolated from the oral cavity.
1.3 Intra-kingdom and inter-kingdom interaction of *Candida* spp.

*Candida* spp. have been reported to be involved in both intra-kingdom and inter-kingdom interactions (Morales and Hogan, 2010). Intra-kingdom interaction is defined as communication between microorganisms of the same kingdom (such as yeast to yeast) whereas inter-kingdom interaction is the communication between at least two different kingdoms (such as yeast to bacteria). These interactions can occur during planktonic growth and within the biofilm (Marsh *et al.*, 2009).
<table>
<thead>
<tr>
<th>Kingdom Bacteria</th>
<th>Gram-positive cocci</th>
<th>Gram-negative cocci</th>
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<td><strong>Gram-positive cocci</strong></td>
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<td>Streptococcus anginosus</td>
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<td>Moraxella spp.</td>
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<td>S. gordonii</td>
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<td>Neisseria mucosa</td>
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<td>S. mitis</td>
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<td>N. sicca</td>
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<td>S. mutans</td>
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<td>N. subflava</td>
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<td>S. oralis</td>
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<td>Veillonella parvula</td>
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<td>S. salivarius</td>
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<td>V. dispar</td>
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<td>S. sanguinis</td>
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<td>V. atypica</td>
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<td>Staphylococcus spp.</td>
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<td><strong>Gram-positive rod</strong></td>
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<td>Actinomyces israelii</td>
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<td>A. meyeri</td>
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<td>A. naeslundii genospecies 1</td>
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<td>A. naeslundii genospecies 2</td>
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<td>A. odontolyticus</td>
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<td>Bifidobacterium dentium</td>
<td>Actinobacillus actinomycetemcomitans</td>
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<td>B. denticolens</td>
<td>Bacteroides capillosus</td>
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<td>Eubacterium minutum</td>
<td>B. forsythus</td>
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<td>E. nodatum</td>
<td>B. fragilis</td>
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<td>Lactobacillus acidophilus</td>
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<td>L. casei</td>
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<td>L. oris</td>
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<td>L. salivarius</td>
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<td><strong>Kingdom Fungi (yeast)</strong></td>
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<td>Candida albicans</td>
<td>Entamoeba gingivalis</td>
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<td>C. glabrata</td>
<td>Giardia lamblia</td>
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<td>C. glliermondi</td>
<td>Trichonomas tenax</td>
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<td><strong>Gram-negative rod</strong></td>
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Table 1.1 Microorganisms isolated from the oral cavity (Marsh et al., 2009).
Interactions of *Candida* spp. with bacteria have been reported to occur via co-aggregation and co-adhesion (Kolenbrander *et al.*, 2010). Co-aggregation and co-adhesion are two important interactions in the oral cavity (Thein *et al.*, 2007). Co-aggregation is defined as the association of genetically distinct microorganisms such as bacterium and yeast during planktonic growth (Gibbons and Nygaard, 1970; Bos *et al.*, 1996; Kolenbrander, 2000; Kolenbrander *et al.*, 2002; Rickard *et al.*, 2003; Al-Ahmad *et al.*, 2007; Ledder *et al.*, 2008). This mechanism may involve the interaction of proteins on the yeast surfaces and carbohydrate-containing molecules on the bacterial surfaces (Arzmi *et al.*, 2015).

*Candida* spp. co-adhesion is defined as the interaction between microorganisms in planktonic growth with those adhered to an oral surface (Bos *et al.*, 1996; Kolenbrander, 2000; Kolenbrander *et al.*, 2002; Rickard *et al.*, 2003; Al-Ahmad *et al.*, 2007). This is significant for non-primary colonisers in biofilm development, as *C. albicans* has been reported to co-adhere with *S. gordonii* which is a primary coloniser. *S. gordonii* has been shown to produce high molecular mass cell surface polypeptides, encoded by *cshA* and *cshB* that assist in the co-adhesion of the bacterium to the yeast (Holmes *et al.*, 1995). Many separate studies have investigated the interactions between *C. albicans* and bacteria in the oral cavity and these are summarised in Table 1.2. The following discussion will outline the key important factors in the interaction between intra-kingdom *Candida* spp., between *Candida* spp. and *Streptococcus* spp., *Candida* spp. and *Actinomyces* spp., *Candida* spp. and *Pseudomonas* spp., *Candida* spp. and *Porphyromonas* spp., and finally *Candida* spp. and *Prevotella* spp.
<table>
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<tr>
<th>References</th>
<th>Microorganisms</th>
<th>Interaction</th>
<th>Results</th>
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<tbody>
<tr>
<td>Purohit et al., 1977</td>
<td><em>C. albicans</em> and</td>
<td>Antagonistic</td>
<td><em>L. acidophilus</em> inhibited growth of <em>C. albicans</em>.</td>
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<td><em>L. acidophilus</em></td>
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<td>Collins and Hardt, 1980</td>
<td><em>C. albicans</em> and</td>
<td>Antagonistic</td>
<td><em>L. acidophilus</em> produced peroxidase and induced growth retardation of <em>C. albicans</em>.</td>
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<td><em>L. acidophilus</em></td>
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<td>Makrides and MacFarlane, 1982</td>
<td><em>C. albicans</em> and</td>
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<td>Adherence of <em>C. albicans</em> to epithelial cells was increased in the presence of fimbriae on <em>E. coli</em>.</td>
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<td><em>E. coli</em></td>
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<td>Bagg and Silverwood, 1986</td>
<td><em>C. albicans</em> and</td>
<td>Synergistic</td>
<td><em>C. albicans</em> co-aggregated <em>S. sanguinis</em>.</td>
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<td><em>S. sanguinis</em></td>
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<td>Verran and Motteram, 1987</td>
<td><em>C. albicans</em> and</td>
<td>Synergistic</td>
<td>Adherence of <em>C. albicans</em> to acrylic increased after pre-incubation with <em>S. sanguinis</em> / <em>S. salivarius</em>.</td>
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<td><em>S. sanguinis</em> /</td>
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<td><em>S. salivarius</em></td>
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<td><em>C. albicans</em> and</td>
<td>Synergistic</td>
<td>Adherence of <em>C. albicans</em> increased with <em>S. mutans</em> co-culture.</td>
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<td>Jenkinson et al., 1990</td>
<td><em>C. albicans</em> and</td>
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<td>Co-aggregation occurred but inhibited after heat and protease treatments.</td>
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<td><em>S. sanguinis</em> /</td>
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<td><em>S. gordonii</em></td>
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<td>Nair and Samaranayake, 1996a; 1996b</td>
<td><em>C. albicans</em> and</td>
<td>Antagonistic</td>
<td>Treatment of <em>C. albicans</em> with <em>S. sanguinis</em> decreased its adherence to acrylic surfaces.</td>
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<td><em>S. sanguinis</em> /</td>
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<td><em>S. salivarius</em> reduced adherence of <em>C. albicans</em> to human buccal epithelial cells.</td>
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<td><em>S. salivarius</em></td>
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Table 1.2 Interaction of *C. albicans* with various important species of microorganisms in the oral cavity.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Strains</th>
<th>Interaction</th>
<th>Summary</th>
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<tbody>
<tr>
<td>Nair and Samaranayake, 1996a; 1996b</td>
<td>C. albicans and E. coli</td>
<td>Synergistic</td>
<td>Resistance of C. albicans was increased.</td>
</tr>
<tr>
<td></td>
<td>C. albicans and P. gingivalis</td>
<td>Antagonistic</td>
<td>Adherence of C. albicans to acrylic and human buccal epithelial cells was suppressed.</td>
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<tr>
<td></td>
<td>C. krusei and P. gingivalis</td>
<td>Synergistic</td>
<td>Exposure of C. krusei to P. gingivalis increased its adherence to an acrylic surface.</td>
</tr>
<tr>
<td>Busscher et al., 1997</td>
<td>C. albicans and S. thermophilus</td>
<td>Synergistic</td>
<td>S. thermophilus mediated adhesion of C. albicans to silicone rubber.</td>
</tr>
<tr>
<td>Nair et al., 2001</td>
<td>C. albicans and L. casei</td>
<td>Synergistic</td>
<td>Germ tube of C. albicans was stimulated.</td>
</tr>
<tr>
<td></td>
<td>C. albicans and P. intermedia</td>
<td>Antagonistic</td>
<td>Germ tube formation of C. albicans was suppressed.</td>
</tr>
<tr>
<td>Adam et al., 2002</td>
<td>C. albicans and Staphylococcus spp.</td>
<td>Synergistic</td>
<td>Staphylococcus spp. adhered to C. albicans and induced hyphal formation.</td>
</tr>
<tr>
<td>Hogan and Kolter, 2002</td>
<td>C. albicans and P. aeruginosa</td>
<td>Antagonistic</td>
<td>P. aeruginosa formed a dense biofilm and adsorbed nutrients from the hyphae of C. albicans before killing.</td>
</tr>
</tbody>
</table>

(Continued)
1.3.1 *C. albicans and non-albicans Candida spp.*

Although *C. albicans* is the most significant *Candida* sp. in pathogenesis, *non-albicans Candida* (NAC) species can additionally play a role. The occurrence of NAC species such as *C. dubliniensis*, *C. glabrata* and *C. tropicalis* has increased in awareness among medical practitioners (Reichart *et al.*, 2005). Surprisingly, in some conditions, the carriage of NAC can outnumber that of *C. albicans* (Joshi *et al.*, 1991; Tzar *et al.*, 2015). With regards to a mixed biofilm of *Candida* spp., research has shown a competitive interaction between *C. albicans* and NAC in a polystyrene tube model (El-Azizi *et al.*, 2004). Furthermore, interaction of *C. albicans* with *C. krusei* has been shown to vary depending on the nutrients supplied during the experiments (Thein *et al.*, 2007).

1.3.2 *Candida spp. and Actinomyces spp.*

*Actinomyces* spp. are Gram-positive bacteria that have been recognised as an important human pathogen in the oral cavity (Schaal and Yassin, 2015; Steininger C and Willinger, 2016). These microorganisms have been previously reported to be involved in root surface caries and gingivitis (Shen *et al.*, 2005). *Candida* spp. and *Actinomyces* spp. have been found to co-aggregate in varying degrees with co-aggregation of *C. albicans* with *A. naeslundii* reported as being *C. albicans* strain-dependent (Grimaudo *et al.*, 1996; Arzmi *et al.*, 2015). This phenomenon has been supported by subsequent findings that identified the interaction of protein complexes on fungal surfaces with carbohydrate-containing moieties on *A. naeslundii* surfaces (Grimaudo *et al.*, 1996). Even though co-aggregation of *C. albicans* and *Actinomyces* spp. has been widely reported (Bagg and Silverwood 1986; Grimaudo *et al.*, 1996; Arzmi *et al.*, 2015), the adhesion of *C. albicans* to polymethylmethacrylate (PMMA) in a flow-cell system when grown in TNMC buffer (1 mM Tris-HCl, 0.15 mM NaCl,
1 mM MgCl₂, 1 mM CaCl₂ in 1 L) has been shown to decrease when pre-cultured with *A. naeslundii* T14V-J1 (Millsap *et al.*, 2000). Chapters 3 and 4 will discuss in more detail the interaction of *C. albicans* with *A. naeslundii* during planktonic and biofilm growth, respectively.

1.3.3 *Candida* spp. and *Streptococcus* spp.

*Streptococcus* spp. form part of the human oral microbiome, with the mutans-group being the most well-known for their high cariogenic potential (Klein *et al.*, 2015). Co-aggregation of *C. albicans* with *S. sanguinis*, and *C. albicans* with *S. mutans* have been reported, suggesting that the interaction may be due to the presence of streptococcal cell surface proteins that co-aggregate with cell surface carbohydrates of *C. albicans* (Bagg and Silverwood, 1986; Arzmi *et al.*, 2015). A similar finding has been reported where protein-carbohydrate interactions have been shown to exist between *C. albicans* with *S. sanguinis, S. anginosus, S. oralis* and *S. gordonii* (Jenkinson *et al.*, 1990). This mutualistic interaction is important to microorganisms in dental plaque as it enhances the development of food chains using metabolic by-products such as glycoproteins (Marsh, 1994). Chapters 3 and 4 will discuss in more detail the interaction of *C. albicans* with *S. mutans* during planktonic and biofilm growth, respectively. Chapters 3 and 4 will discuss in more detail the interaction of *C. albicans* with *S. mutans* in planktonic and biofilm, respectively.

1.3.4 *Candida* spp. and *Pseudomonas* spp.

*Pseudomonas* spp. are Gram-negative bacilli that belong to the genus *Enterobacteriacea*. These saprophytic aerobic bacteria possess virulence factors including extracellular proteases, exotoxins and endotoxins (Samaranayake, 2006). Studies have found that *P. aeruginosa* has the ability to suppress the growth of *C. albicans* (Grillot *et al.*, 1994; Kerr, 1994). The bacterium has been shown to form a
dense biofilm on *C. albicans* and receive nutrients from the candidal hyphae, which later killed the fungus (Hogan and Kolter, 2002). *P. aeruginosa* has also been found to inhibit *C. albicans* biofilm formation by synthesising anti-candidal agents such as extracellular bacterial glycocalyx, pyrrolnitrin and 3-oxo-C12-homoserine lactone (Hogan *et al.*, 2004; Thein *et al.*, 2006).

**1.3.5 Candida spp. and Porphyromonas spp.**

*Porphyromonas* spp. are Gram-negative coccobacilli that can be isolated from dental plaque. These anaerobic, black-pigmented bacteria are classified as part of the normal oral microbiome (Wade, 2013). *P. gingivalis* is highlighted as the most important among these species due to its frequent isolation from sub-gingival sites and identification as a major periodontal pathogen (Thein *et al.*, 2007). For instance, *P. gingivalis* has been shown to produce proteases that destroy haemolysin, haeme-sequestering proteins, collagens, immunoglobulins and complements (Popova *et al.*, 2013; Antipa *et al.*, 2015). The presence of fimbriae on the cell surface of the bacteria mediates adhesion to the host cell while the capsule has been shown to resist phagocytosis, which contributes to the virulence of *P. gingivalis* in periodontal disease (Samaranayake, 2006). *P. gingivalis* has been found to suppress adhesion of *C. albicans* to acrylic surfaces (Thein *et al.*, 2006). This is supported by a subsequent study that observed a significant inhibition of germ-tube formation of *C. albicans* by *P. gingivalis* (Nair *et al.*, 2001). The suppression of the adhesion of *C. albicans* to denture acrylic surfaces by *P. gingivalis* suggests antagonistic interactions between the microorganisms which may also occur in the oral cavity (Nair and Samaranayake, 1996b). It is suggested that metabolites of *P. gingivalis* and other anaerobic bacteria may inhibit the colonisation of *C. albicans* in the gingival crevicular area (Thein *et al.*, 2006). Further study is required to identify the specific inhibitors.
1.3.6 *Candida* spp. and *Prevotella* spp.

*Prevotella* spp. are Gram-negative bacilli found in the human oral cavity. These species, in particular *P. intermedia*, have been associated with the development of periodontal diseases (Nair *et al.*, 2001). Furthermore, *P. intermedia* has been shown to suppress the development of germ-tube formation in *C. albicans* thus affecting adhesion to the host cell (Nair *et al.*, 2001). It is suggested that metabolites of *P. intermedia* and other anaerobic bacteria may inhibit the colonisation of *C. albicans* in the gingival crevicular area (Thein *et al.*, 2006). Further study is required to identify the specific inhibitors, which have not been previously reported.

1.4 Oral biofilms

The oral microbiome exists as both planktonic cells and oral biofilm (Kolenbrander *et al.*, 2010). An oral biofilm is defined as a community of microorganisms that attach to oral surfaces and is encapsulated within EPS of microbial and salivary origin (Donlan and Costerton, 2002). It is suggested that cell-cell signalling mechanisms between members of a polymicrobial biofilm contribute to the successful colonisation of bacteria. For example, *Neisseria* spp. have been found to metabolise surrounding oxygen in the periodontal pocket releasing carbon dioxide that contributes to the survival of obligate anaerobic bacteria (Kolenbrander *et al.*, 2006; Marsh, 2015).

Biofilms have been reported to increase the resistance up to 1000-fold of opportunistic oral microorganisms, such as *C. albicans*, to antimicrobial agents including fluconazole, thus increasing their pathogenicity towards the host (Stewart and Corteston, 2001; Tobudic *et al.*, 2012). It has been suggested that the EPS encapsulating biofilms are a barrier limiting the penetration of antimicrobial agents,
subsequently reducing the susceptibility of the targeted microorganisms. Furthermore, several enzymes produced by microorganisms within the biofilm have been reported to neutralise active antimicrobial compounds, further reducing susceptibility of targeted pathogens (Gilbert et al., 2002). Nutrient limitation has been shown to occur within biofilms in the basal region that reduced microbial growth and changed cell surface composition (Nadell et al., 2016; Ng et al., 2016). The susceptibility to antimicrobial agents is thus modulated by biochemical pathways associated with actively growing microorganisms (Mah and O'Toole, 2001, Donlan and Costerton, 2002). Finally, slower growing microorganisms could represent ‘persister cells’ (LaFleur et al., 2006; Lewis, 2007; Lewis, 2010). It has been suggested that persister cells possess a resistant phenotype which reduces their susceptibility to antimicrobial agents (LaFleur et al., 2006).

1.4.1 Importance of oral fluids in the oral biofilm development

The oral cavity contains oral fluids such as saliva and gingival crevicular fluid. These fluids are important in the first line of host defence as well as allowing the proliferation of oral biofilms due to their nutrient content.

Saliva is an exocrine secretion comprised of water, proteins and electrolytes, such as sodium, phosphate, calcium, magnesium, bicarbonate, phosphate and chloride (Berkovitz et al., 2002). Saliva is produced by the major and minor salivary glands. The major salivary glands consist of paired parotid, submandibular and sublingual glands. The minor salivary glands are found throughout the lower lip, tongue, palate, cheeks and pharynx (de Almeida et al., 2008).

The major roles of saliva include maintaining the integrity of teeth, a buffering agent, and cleaning, protecting and lubricating the oral cavity (Dawes, 2008). Saliva has been shown to maintain the physicochemical integrity of tooth enamel by
modulating remineralisation and demineralisation with calcium, fluoride and phosphate and this has been reported to be the main factor controlling the stability of enamel hydroxyapatite (de Almeida et al., 2008). Saliva also is an effective buffer that neutralises potentially damaging acids of dietary and biofilm origin. The composition of secretions from each gland differs while biochemical compounds, including phosphates, bicarbonate and peptides, found in normal whole saliva, result in a mean pH of 6.75 to 7.25 that prevents enamel demineralisation (Dawes, 2008). The viscosity of saliva has been shown to provide mechanical cleansing of planktonic microorganisms and food debris, thus limiting the nutrient intake by the oral biofilm (de Almeida et al., 2008).

Saliva forms a seromucosal covering that lubricates and protects the oral surfaces from microbial infection (Stack and Papas, 2001; Nagler, 2004). Research has shown that saliva forms a thin film approximately 70 μm to 100 μm deep over all external surfaces in the oral cavity, known as the salivary pellicle (Dawes, 2008). Mucins, which are proteins with a high carbohydrate content, have been reported to provide lubrication and protection to oral epithelial cells from dehydration, while other salivary proteins are also involved in host defence mechanisms, such as aggregation of exogenous microorganisms, thus facilitating clearance during swallowing or expectoration (de Almeida et al., 2008).

Gingival crevicular fluid (GCF) is the exudate originating from plasma that passes through the gingiva. This fluid influences the development of biofilms in the oral cavity (Wade, 2013). GCF has been shown to assist the development of subgingival plaque around and below the gingival margin (Marsh and Devine, 2011). GCF contains a higher total protein content than saliva that may be converted to nutrients such as peptides, amino acids and carbohydrates by bacterial enzymes.
(Marcotte and Lavoie, 1998). In addition, GCF also contains antibodies that are both non-specific and specific for a variety of periodontal disease important microorganisms (Barros et al., 2016).

1.4.2 Importance of salivary flow rate in oral biofilm development

Circadian rhythms can affect salivary flow and composition, both of which are important for diminishing colonisation by microorganisms (Edgar, 1992; Humphrey and Williamson, 2001). The circadian rhythm results in the lowest flow rate occurring during sleep (de Almeida et al., 2008; Dawes, 2008). Throughout the year, the lowest flow rates are observed in summer whilst the highest flow rates are in winter (Edgar, 1990). It has been suggested that this variability affects the distribution of proteins such as mucin and agglutinin that serve as the receptors for microorganisms on oral surfaces and thus assists the formation of oral biofilms (Willet et al., 1991; Holmes et al., 2002). For example, the total concentration of proteins in resting whole saliva is estimated at 220 μg mL\(^{-1}\) whereas in stimulated saliva, it is approximately 280 μg mL\(^{-1}\) (Marsh et al., 2009). Furthermore, parotid saliva alone has been shown to support the adherence of S. mutans to hydroxyapatite beads (Carlen et al., 1996).

Flow rates also differ between individuals of various backgrounds. On average, flow rate of un-stimulated saliva is 0.3 mL min\(^{-1}\) during waking hours with an average of 300 mL for 16 hours when waking, and may drop to nearly zero during sleeping hours (Edgar, 1990). Un-stimulated salivary flow rate is considered normal above 0.1 mL min\(^{-1}\) whereas for stimulated saliva, the minimum volume accepted as normal increases to 0.2 mL min\(^{-1}\) (Humphrey and Williamson, 2001). Any un-stimulated flow rate that is below 0.1 mL min\(^{-1}\) is considered as hypo-salivation (Edgar, 1990; Humphrey and Williamson, 2001). Meanwhile, stimulated saliva can
be up to 7 mL min\(^{-1}\) and has been reported to be 80% to 90% of the average salivary production (Edgar, 1990).

Low flow rate has been shown to reduce the protective function of saliva in the oral cavity, thus increasing the incidence of caries and the colonisation of microorganisms such as *Candida* spp., *Actinomyces* spp. and *Streptococcus* spp. (Loesche, 1986; Humphrey and Williamson, 2001; Holmes *et al*., 2002; Kolenbrander *et al*., 2010; Pandey *et al*., 2015).

## 1.5 Epidemiology of oral cancer

In 2008, 12.4 million new cases and 7.6 million deaths due to cancer were reported worldwide (Ariyawardana and Johnson, 2013). Of these, 263,000 cases of lip and oral cavity cancer, and 135,000 pharyngeal cases were reported representing 2.1% and 1.1% of all new cancers, respectively (Ferlay *et al*., 2010). Oral cancer has been classified as the sixth most common cancer in the world (Warnakulasuriya, 2009) and globally ranks as the eighth most common cancer in males and the 13\(^{th}\) in females (Parkin *et al*., 2005). It is estimated that the incidence of oral cancer is around 275,000 cases with two-thirds of these reported in developing countries (Ferlay *et al*., 2004).

The highest incidences of oral cancer have been reported in South-east Asia (Taiwan), South Asia (India and Pakistan), Western Europe (e.g. France), Eastern Europe (e.g. Slovakia, Slovenia and Hungary), parts of Latin America and the Caribbean (e.g. Puerto Rico and Brazil) and Pacific regions (Papua New Guinea and Melanesia) (Banoczy and Squier, 2004; Wünsch-Filho and de Camagro, 2001). In the United States, oral cancer accounts for nearly 2.3% of all cancers and has a relatively low five-year survival rate with almost 30,000 new oral cancer cases diagnosed every
year with 8,000 associated deaths (Silverman, 2001; Greenlee et al., 2001). Even though surgical advances have improved the life quality for patients, the overall mortalities remain unchanged (Casiglia and SB, 2001; Casto et al., 2009).

There were a total of 60,826 new cases of lip, oral cavity and oropharyngeal cancer diagnosed between 1992 and 2008 in Australia. This corresponds to 2.9% of the total cancer load and 1.6% of all cancer deaths in Australia (Farah et al., 2014). In 2009, oral cancer was classified as the eighth most diagnosed cancer in Victoria (Alnuaimi et al., 2014). The most common site of new oral cancer cases was found to be the lip, followed by the tongue (Sugerman and Savage, 2002; Farah et al., 2014). Even though the incidence in Australia has been reducing in the past three decades, no significant change was observed in the mortality rate (Ariyawardana and Johnson, 2013; Farah et al., 2014).

In Malaysia, oral cancer was found to be the 20th most common cancer for females and 28th for males in 2006, with squamous cell carcinoma reported to be the most common type of oral cancer (Omar et al., 2006; Ashazila et al., 2011; Helen-Ng et al., 2012). In regard to ethnicity, individuals of Indian descent were found to have the highest prevalence at 4.0%, followed by the indigenous people of Sabah and Sarawak at 2.5% and the lowest prevalence was among those of Chinese descent at 0.5% (Zain et al., 1997; Zain; 2001).

1.6 Risk factors for oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) has been classified as the most common type of oral cancer (Ferlay et al., 2004), accounting for greater than 90% of malignancies originating from the oral cavity (Casiglia and SB, 2001). It has been
reported that the average five-year survival rate following a diagnosis of oral cancer is less than 50% (Zakrzewska, 1999).

OSCC is associated with a number of aetiological factors, including the use of tobacco and heavy alcohol consumption. Other factors suggested to have a role in OSCC formation include areca nut/betel quid chewing, dietary intake and microbial infections (Warnakulasuriya et al., 2005).

1.6.1 Tobacco

Asia, Australia and the Far East are the largest tobacco consumers followed by Americans, Eastern Europe and Western Europe (Petti, 2009). Global data on smoking prevalence showed that almost one billion men in the world smoke with 35% of those found in developed countries and 50% found in developing countries (Petti, 2009). Meanwhile, approximately 250 million women in the world are daily smokers with 22% found in developed and 9% found in developing countries (Petti, 2009). Even though cigarette smoking amongst women has been shown to be declining in some developed countries such as Australia and Canada, several countries in Southern, Central and Eastern Europe have shown increased or stagnant rates (Mackay and Eriksen, 2002; Petti, 2009).

Tobacco smoking is a strong independent risk factor for oral cancer (Johnson, 2001; Winn, 2001; Vineis et al., 2004; Warnakulasuriya et al., 2005; Vallecillo Capilla et al., 2007; Hirota et al., 2008; Pelucchi et al., 2008, Stucken et al., 2010). It has been estimated that 43% to 60% of oral cancers are attributable to tobacco smoking (Sasco et al., 2004) with the risk of developing oral cancer proportional to the number of cigarettes smoked and duration of smoking. Pipe and cigar smokers are at a higher risk for oral cancer development when compared to cigarette smokers (Franceschi et al., 1990; Talamini et al., 2000; Lubin et al., 2009). The most
important carcinogens are tobacco-specific nitrosamines such as 4-(methylnitrosamino-1-(3-pyridyl)-1-butanone, N-nitrosonornicotine, benzo[a]pyrene, and aromatic amines (Hecht, 2003).

1.6.2 Alcohol

According to the World Health Organisation (WHO) report in 2014, it was estimated that per year, an average of 6.2 L of pure alcohol was consumed by individuals aged 15 years or older, with the highest consumption levels reported in the developed world, particularly in the European region and the Americas (World Health Organisation, 2014). In Australia, cancer has been reported to be attributable to the consumption of alcohol. It is estimated that 3,208 cancers, which is equivalent to 2.8% of all cancers occurring in Australian adults, may be due to alcohol consumption (Pandeya et al., 2015).

This is due to the breakdown of ethanol to the carcinogen acetaldehyde, which has an established role in carcinogenesis (Schlecht et al., 2001; Huang et al., 2003; Seitz et al., 2004; Boccia et al., 2009; Ogden, 2009; Warnakulasuriya 2009). In animal studies, ethanol has been shown to have a synergistic effect when combined with other carcinogens such as nitrosamines (Hooper et al., 2009). Furthermore, the effect of both alcohol consumption and tobacco smoking has been reported to have a multiplicative effect on carcinogenesis when compared to each factor in isolation. (Pelucchi et al., 2008; Hashibe et al., 2009).

1.6.3 Betel quid

_Piper betle_ is a plant belonging to the _Piperaceae_, which originated from South East Asia including India, Sri Lanka and Bangladesh (Hoque et al., 2012). The betel leaf itself is known as Sireh (Malay), Paan (Urdu and Hindi), Vetrilai (Tamil) and Ikmo (Tagalog) (Datta et al., 2011). _P. betle_ is an evergreen plant with glossy
heart-shaped leaves and white catkins. Usually, the leaf is chewed together with areca nut, lime and gambier leaves (Johnson et al., 2011). The nut gives the reddish colour to the saliva and thus darkens the teeth.

In South-East Asia and the Pacific Islands, 600 million people have been reported to practice betel quid chewing, similar to the activity in South America of chewing coca leaves or tobacco (Gupta and Ray, 2004). It is estimated that 10% to 20% of the world’s population, corresponding to 600 to 1200 million people, use betel quid (Gupta and Ray, 2004). It is classified as the fourth most frequently consumed psychoactive substance after nicotine, ethanol and caffeine (Pickwell et al., 1994; Norton, 1998; Gupta and Ray, 2004).

Betel quid has been classified as an oral carcinogen in humans by the International Agency for Research on Cancer, with evidence for a dose-response relationship (Petti, 2009). It has been reported that betel quid chewing may produce carcinogenic nitrosamines such as 3-methylnitrosopropionitrile (Petti, 2009). Frequent and long-term betel quid consumption has been shown to increase oral cancer risk. However, the risk of low to moderate betel quid chewing remains unclear (Subapriya et al., 2007; Petti, 2009).

Even though betel quid has been classified as an oral carcinogen in humans, it is culturally believed that the leaves can be a treatment for various diseases including bad breath, headache, boils, conjunctivitis, itches, mastitis, mastoiditis and ringworm (Chopra et al., 1956). The essential oil of P. betle was reported to contain antibacterial, antiprotozoan and antifungal properties (Indu and Ng, 2002). Research has shown that the plant may produce bacteriostatic and fungistatic effects against Salmonella typhi, Escherichia coli and C. albicans respectively (Guha and Jain, 1997; Indu and Ng, 2002). P. betle was found to be effective as an anti-dermatophyte
against *C. albicans*, *Microsporum gypseum* and *Trichosporon beigelii* and phytopathogens such as *Sclerotium rolfsii*, *Alternaria solani* and *Phytophthora infestans* (Rahman *et al.*, 2005). Crude aqueous extracts of *P. betle* have been reported to reduce the cell surface hydrophobicity of *S. sanguinis*, *S. mitis* and *Actinomyces* spp. (Razak *et al.*, 2006).

### 1.6.4 Dietary and genetic factors

Thirty to forty per cent of global cancer cases are attributable to unhealthy diet, obesity and lack of physical activity and 10% to 15% of cases are associated with low fruit and vegetable intake (Popkin, 2007). It has been suggested that the antioxidant and anti-carcinogenic properties that exist in plants, such as vitamin A, C and E, carotenoids, flavonoids, phytosterols, folates and fibres, play an essential role in counterbalancing the detrimental effects of other carcinogenic substances, such as betel quid chewing, alcohol consumption and tobacco smoking (Serdula *et al.*, 1996; Agudo *et al.*, 1999).

### 1.6.5 Microbial infection

Since the 1960’s, *Candida* spp. have been suggested to be associated with oral leukoplakic lesions (Cawson, 1969a). *Candida* spp. have been recognised as an independent risk factor in the development of oral carcinoma (Cawson, 1969b). An aetiological role for *Candida* spp. in the progression of oral mucosal keratoses to carcinoma was first suggested by Cawson in 1966 (Cawson, 1969a). Researchers have found that the majority of non-homogenous leukoplakias that are most often invaded by *C. albicans*, have higher malignant transformation potential than the homogenous types (Renstrup, 1970; Banoczy and Sugar; 1972, Cawson and Binnie, 1980; Pindborg, 1980; Axéll *et al.*, 1984).
Candida spp., when associated with dysplasia, may represent a secondary infection of a pre-existing altered epithelium (Barrett et al., 1998; Kragelund et al., 2016). Furthermore, C. albicans was more commonly isolated from oral biofilms on OSCC sites when compared to the control sites (Nagy et al., 1998). There is additionally a correlation between oral yeast carriage and the presence of oral epithelial dysplasia (McCullough et al., 2002). Despite many clinical and experimental conclusions describing an association between C. albicans and malignant transformation, the exact role of the yeast in the development of dysplastic changes remains unclear (Supriya et al., 2016).

C. albicans produces C. albicans alcohol dehydrogenases (CaADH), the enzyme that converts alcohol to acetaldehyde, which may play a role in oral carcinogenesis. Bakri et al. (2010) showed that CaADH1 could utilise the pro-carcinogenic substrate ethanol, using reversible conversion mechanisms, to produce highly carcinogenic acetaldehyde. Acetaldehyde, a carcinogenic substrate, alters epithelial cells, thus potentiating the formation of OSCC (Väkeväinen et al., 2002; Salaspuro, 2003; Kurkivuori et al., 2007; Alnuaimi et al., 2015).

1.7 Cytokines and carcinogenesis

Inflammation induced by pathogens may be involved in carcinogenesis, particularly after the classification of Helicobacter pylori as a class-1 carcinogen in humans by the WHO International Agency for Research on Cancer (IARC) (Peek and Blaser, 2002; Björkholm et al., 2003; Correa and Houghton, 2007). One factor that may induce inflammation is the increase of pro-inflammatory cytokines and growth factors due to microbial infection (Fantini and Pallone, 2008).
Cytokines are soluble proteins released by cells and are both autocrine and paracrine in nature, and facilitate communication between cells (Lázár-Molnár et al., 2000). Cytokine signals are received at the cell surface, not only as single messages, but also in complex, subtle, synergistic and antagonistic combinations that coordinate processes, including the stimulation of haematopoiesis, orchestration of directed leukocyte migration (chemokinesis), activation of various inflammatory cells, stimulation of lymphocyte development and maturation, and processes related to the immune response (Budhu and Wang, 2006). However some circumstances, such as failure to resolve an injury, might provoke excessive immune cell infiltration that then leads to persistent cytokine production. As a result, the host may respond to the persistent cytokine expression by enhancing cancer formation and progression (Budhu and Wang, 2006).

1.7.1 Clinical significance of cytokines in carcinogenesis

Among cytokines synthesised by human epithelial cells, IL-6, IL-8, GM-CSF and TNF-α have been widely studied (Kitadai et al., 2000; Riedel et al., 2005; Kishimoto, 2006; Duffy et al., 2008; Lederle et al., 2011). It is suggested that these cytokines may be involved in the progression of oral carcinoma.

IL-6 is a pleiotropic cytokine involved in the acute phase of inflammation and is a major inducer of C-reactive protein (Kishimoto, 2006). This cytokine has been the major mediator linking inflammation to cancers together with TNF-α (Kundu and Surh, 2008; Balkwill and Mantovani, 2012). IL-6 has been reported to promote malignant growth of skin squamous cell carcinoma by regulating a complex cytokine and protease network (Lederle et al., 2011). IL-6 is one of the main chemokines present in serum samples of head and neck cancer patients and elevated IL-6 levels can independently predict tumour recurrence, poor survival, and tumour metastasis.
IL-6 in serum has been reported to be higher in patients with OSCC compared to controls and has been proposed as an additional marker in early detection of oral cancer (Jablonska et al., 1997; Dawes and Dong, 1995; Wu et al., 2015; Rao et al., 2016).

Even though much research has been performed to understand the role of IL-6 in carcinogenesis, very little is known about the direct role of this cytokine in head and neck tumour metastasis and epithelial mesenchymal transition (EMT), which is characterised by the loss of cell-cell junctions and cell polarity (Yadav et al., 2011).

IL-8 is an important cytokine that can be isolated from lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (Oppenheim et al., 1991). Research has shown that the cytokine is produced by various cells including endothelial cells, fibroblasts, lymphocytes, neutrophils, keratinocytes, epithelial cells, hepatocytes and lung macrophages (Kunkel et al., 1991; Kondo et al., 1993; Bersinger et al. 2011). In addition, IL-8 can be produced by a variety of tumours, both constitutively and in response to cytokines (Kolář et al. 2012). This cytokine has also been detected in surgical specimens, fresh cultured cell lines and well-defined cell lines of head and neck squamous cell carcinoma (Chen et al., 1999; Cohen et al., 1995).

Two receptors for IL-8, IL-8RA (CXCR1) and IL-8RB (CXCR2), have been identified on human neutrophils, both of which are members of the seven transmembrane domain family of G-protein-associated receptors (Oppenheim et al., 1991). Both of these receptors bind IL-8 with high affinity, but IL-8RA is more specific for IL-8 whereas IL-8RB will also bind with similar affinity to other CXC chemokines that possess a specific N-terminal motif. IL-8RA is expressed on a variety of cells including neutrophils, T cells, monocytes and fibroblasts, whereas the
expression of IL-8RB is rather more restricted. Interestingly, these receptors of IL-8 have also been identified on tumour cells including squamous cell carcinoma (SCC) (Cohen et al., 1995; Wang et al., 1996; Reiland et al., 1999; Brew et al., 2000). IL-8 stimulates many physiopathological functions in various tumour cells. Tumour cells such as prostate carcinoma (Reiland et al., 1999; Inoue et al., 2000), melanoma (Luca et al., 1997), breast carcinoma (Youngs et al., 1997) and gastric carcinoma (Kitadai et al., 2000) cell lines respond chemotactically to IL-8. Furthermore, high expression of IL-8 in cancerous liver tissue has been shown to associate with a higher frequency of portal vein, venous, and bile duct invasion in hepatocellular carcinoma (HCC) patients with surgical resection and may therefore be important in invasion and metastasis (Akiba et al., 2001).

IL-18 is suggested to have a role in carcinogenesis. This cytokine has been shown to be synthesised as a 24 kDa inactive precursor in cells such as macrophages, dendritic cells, Kupffer cells and some tumour cells (Sugawara, 2000, Cho et al., 2002). The inactive cytokine is cleaved by the IL-1β converting enzyme (caspase-1) in the cytoplasm and then secreted as an 18 kDa active protein, which can induce the formation of interferon gamma (IFN-γ) (Ushio et al., 1996; Sugawara, 2000; Gracie et al., 2003). Enhanced IL-18 expression is positively correlated with the pathogenesis of malignant skin tumours (Park et al., 2001). It has also been shown that this cytokine regulates hepatic melanoma metastasis by increasing the adherence of melanoma cells and the expression of vascular cell adhesion molecule-1 (Vidal-Vanaclocha et al., 2000). Furthermore, IL-18 secreted by the B16 murine melanoma cell line has been shown to be involved in the immune escape of murine melanoma cells (Cho et al., 2000). Therefore, it is suggested that IL-18 may be important in the formation of OSCC.
Granulocyte macrophage colony-stimulating factor (GM-CSF) has been shown to be important in carcinogenesis. It is termed a growth factor since it supports the colonisation of granulocyte, macrophage, erythroid, megakaryocyte and eosinophil progenitor cell lines (Burgess and Metcalf, 1980). GM-CSF is a 127 amino acid monomer with a mass ranging from 14 to 35 kDa depending on the amount of glycosylation in vivo (Cantrell et al., 1985). On mature haematopoietic cells, GM-CSF activates the effector functions of granulocytes, monocytes/macrophages and eosinophils (Morrissey et al., 1987). It is produced and released by various cell lines in response to immune and/or inflammatory stimuli, including activated T cells (Kharkevitch et al., 1994), B cells (Pistoia et al., 1993), mast cells (Levi-Schaffer et al., 1998), endothelial cells (Sieff et al., 1987) and fibroblasts (Koeffler et al., 1987). Recently, GM-CSF has been reported to have a functional role on non-haematopoietic cells by inducing human endothelial cells to migrate and proliferate (Budhu and Wang, 2006). Interestingly, GM-CSF can also stimulate the proliferation of a number of tumour cell lines, including breast cancer cell lines (Park et al., 2007). Cancer cells have been shown to produce GM-CSF (Burgdorf et al., 2009). Therefore, increased expression of this cytokine can be used as a biomarker in the detection of OSCC.

Since 1987, tumour necrosis factor (TNF)-α has been reported to be involved in breast cancer with TNF mRNA and protein detected in malignant and stromal cells in human biopsies (Spriggs et al., 1987). Malignant cell-derived TNF has been reported to enhance the growth and spread of syngeneic-, xenogeneic- and carcinogenic-induced tumours of the bowel, pancreas, skin and ovary (Kundu and Surh, 2008; Balkwill, 2009). Research has found that TNF-α in HCC patients was higher in metastatic liver carcinoma than the healthy individual (Nakazaki, 1992).
Furthermore, higher levels of TNF-α were found in the tissue surrounding HCC and hepatic metastasis than in the tumour (Bortolami et al., 2002). This pro-inflammatory and pro-angiogenic cytokine has also been shown to increase from OSCC tumour cells together with IL-1, IL-6 and IL-8 compared to normal cells (SahebJamee et al., 2008). The mechanisms of the increase of TNF-α during tumour growth are not fully defined. In an ovarian cancer model, TNF-α was an important component of the malignant cell-autonomous network of inflammatory cytokines, including chemokines stromal cell-derived factor 1 (SDF1) and C-C chemokine ligand 2 (CCL2), which have been suggested to aid in the proliferation and survival of malignant cells, stimulating angiogenesis and metastasis (Kulbe et al., 2007).

Other pro-inflammatory cytokines involved in carcinogenesis are IL-4, IL-10 and interferon-gamma (IFN-γ). IL-4 has been reported as both a pro- and anti-inflammatory cytokine (Brieland et al., 2001; Hosoyama et al., 2011). A study using a mammary carcinoma model has shown that Th2-derived IL-4 is responsible for the promotion of metastasis (DeNardo et al., 2009). IL-4 has also been reported to accelerate tumour cell growth from rhabdomyosarcoma (RMS) tissue, suggesting that the cytokine could potentially be a growth factor acting on tumour cells directly or indirectly through tumour associated macrophages (TAMs) (Hosoyama et al., 2011).

In addition, IL-10 has been shown to promote tumour progression together with regulatory B cells during squamous carcinogenesis (Schioppa et al., 2011). IL-10 was found to inhibit major histocompatibility complex type II dependent antigen presentation, activation of type I helper T cells and autologous T cell specific tumour lysis from melanoma cells, suggesting that a mechanism of escape of tumour cells from the human immune system may have been activated through secretion of the cytokine (Tartour and Fridman, 1998). Furthermore, interferon-gamma (IFN-γ) was
found to synergistically act with TNF-α to induce IL-8 production from a human gastric cancer cell line, which has been reported to be involved in cancer cell metastasis (Yasumoto et al., 1992). Table 1.3 summarises the functions of inflammatory cytokines that might be involved in oral carcinogenesis.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>Pro-inflammatory, pro-metastasis and accelerates tumour growth from rhabdomyosarcoma tissue.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Pro-inflammatory, neovascularisation, pro-metastasis, B cell activation and acute phase response.</td>
</tr>
<tr>
<td>IL-8</td>
<td>Neo-vascularisation, pro-metastasis, increases cell migration and activation.</td>
</tr>
<tr>
<td>IL-10</td>
<td>Promotes tumour progression during squamous carcinogenesis.</td>
</tr>
<tr>
<td>IL-18</td>
<td>Up-regulates inflammatory mediators and innate immune responses. Up-regulates Th1 response that assists clonal expansion of cytotoxic T-lymphocytes (CTL) and Th2 response that increases antibody production.</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Pro-inflammatory, pro-atherogenic and anti-inflammatory. Up-regulates inflammatory mediators and innate immune responses.</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Pro-inflammatory, anti-inflammatory, pro-metastasis</td>
</tr>
</tbody>
</table>

Table 1.3 Summary of cytokines that possibly have a role in the progression of OSCC.
1.8 Hypotheses

The overall hypothesis is that polymicrobial biofilms of *C. albicans*, *A. naeslundii* and *S. mutans* are involved in oral carcinogenesis. The null hypothesis is that polymicrobial biofilms of *C. albicans*, *A. naeslundii* and *S. mutans* have no role in oral carcinogenesis.

The specific hypothesis of the first study is that the auto-aggregation and co-aggregation of *C. albicans* is strain-dependent. The null hypothesis is that auto-aggregation and co-aggregation of *C. albicans* is not strain-dependent. The hypothesis of the second study is that polymicrobial biofilm formation is *C. albicans* strain- and medium-dependent. The null hypothesis is that polymicrobial biofilm formation is not *C. albicans* strain- and medium-dependent. The hypothesis of the third study is that the polymicrobial interactions within biofilms grown in a flow-cell affects *C. albicans* biofilm formation. The null hypothesis is that polymicrobial interactions in flow-cell biofilms do not affect *C. albicans* biofilm formation. Finally, the hypothesis of the fourth study is that oral epithelial cells have an enhanced malignant phenotype when grown in the presence of polymicrobial biofilm effluent. The null hypothesis is that the presence of polymicrobial biofilms does not enhance the malignant potential of oral epithelial cells.
1.9 Aims

The aim of the aggregation study was to assess the auto-aggregation and co-aggregation of *C. albicans*, *A. naeslundii* and *S. mutans* during planktonic growth. The aim of the static biofilm study was to assess the effect of mono- and co-culture of *C. albicans*, *A. naeslundii* and *S. mutans* on static biofilm formation. The aim of the flow-cell biofilm study was to assess polymicrobial interactions within biofilms of *C. albicans*, *A. naeslundii* and *S. mutans* in a flow-cell environment. Finally, the aim of the adhesion, epithelial-mesenchymal-transition and cytokine studies was to assess the malignant phenotype of oral epithelial cells when grown in medium containing biofilm effluent of mono-cultured and polymicrobial *C. albicans*, *A. naeslundii* and *S. mutans*. The malignant phenotype was measured by adherence to extracellular matrix molecules, epithelial to mesenchymal transition and cytokine expression. Oral epithelial cell lines derived from both normal oral epithelium (OKF6) and oral squamous cell carcinoma (H357) were used to assess the interaction.
CHAPTER 2

MATERIALS AND METHODS
2.1 Growth of microorganisms

*C. albicans* American Type Culture Collection (ATCC) 32354 (ALT1), ATCC MYA-2876 (ALT2), ATCC 90234 (ALT3), ATCC 18804 (ALT4), genotype A isolated from AIDS patient (ALC1), genotype B isolated from AIDS patient (ALC2), oral cancer isolate 1 (ALC3) and oral cancer isolate 2 (ALC4) were used in this study. *C. albicans* strains were sub-cultured on Sabouraud’s dextrose agar (SDA) (Difco, USA) and incubated at 37 °C aerobically for 24 h.

To grow bacteria, stock cultures of *A. naeslundii* (NCTC 10301) and *S. mutans* (Ingbritt), provided by the Oral Health Cooperative Research Centre, Melbourne Dental School, The University of Melbourne, were revived by sub-culturing onto blood agar (40 g L⁻¹ blood agar base and 100 mL L⁻¹ defibrinated horse blood; Microbiology Medium Preparation Unit, Australia) and Todd-Hewitt yeast extract (THYE) agar (36.4 g L⁻¹ Todd-Hewitt broth, 8 g L⁻¹ yeast extract and 15 g L⁻¹ Bacto agar), respectively. The agar plates were incubated at 37 °C for 48 h.

2.2 Aggregation assay

A semi-quantitative spectrophotometric assay based on that outlined by Ledder et al. (2008) and Nagaoka et al. (2008) was used to analyse the aggregation of the microorganisms. Initially, 24 h cultures of *C. albicans* grown aerobically in RPMI-1640 or 25% ASM (0.625 g L⁻¹ type II porcine gastric mucin, 0.5 g L⁻¹ bacteriological peptone, 0.5 g L⁻¹ tryptone, 0.25 g L⁻¹ yeast extract, 0.088 g L⁻¹ NaCl, 0.05 g L⁻¹ KCl, 0.05 g L⁻¹ CaCl₂ and 0.25 mg mL⁻¹ haemin, pH 7.0 supplemented with 2.5 mM DTT and 0.5 g L⁻¹ sucrose) to stationary phase were harvested by centrifugation at 12,000 g for 5 min and washed twice using co-aggregation buffer (0.1 mM CaCl₂, 0.1 mM MgCl₂, 150 mM NaCl, 3.1 mM NaN₃ dissolved in 1 mM Tris buffer and adjusted to pH 7.0). The supernatant was discarded and the pellet was
re-suspended in co-aggregation buffer. A similar protocol was repeated for *S. mutans* and *A. naeslundii*, except these microorganisms were grown in heart infusion broth (HIB) to stationary phase.

To determine auto-aggregation, *C. albicans*, *A. naeslundii* and *S. mutans* were standardised in co-aggregation buffer to give a final cell density of $10^6$ cells mL$^{-1}$, $10^7$ cells mL$^{-1}$ and $10^8$ cells mL$^{-1}$ respectively in separate sterile 2 mL Eppendorf tubes, equivalent to an optical density of 0.5 at 620 nm wavelength (OD$_{620nm}$). The number of cells was enumerated using the colony forming unit (cfu) counting method on SDA (*C. albicans*), blood agar (*A. naeslundii*) and THYE agar (*S. mutans*) during optimisation to confirm the cell density at absorbance OD$_{620nm}$ of 0.5. Different cell densities were chosen to mimic the variability of the microorganisms in the oral cavity (Lamfon *et al.*, 2005). Each suspension was mixed thoroughly using a vortex mixer for 30 s and the OD$_{620nm}$ at time (t) = 0 h was measured. The inoculum was incubated at room temperature for 1 h to allow aggregation and the OD$_{620nm}$ was recorded. Sterile co-aggregation buffer was used as a standard blank. Percentage aggregation was calculated using the following equation:

$$\% \text{ Auto-aggregation} =$$

$$\left(\frac{[\text{OD}_{620nm} \text{ (t = 0 h)} - \text{OD}_{620nm} \text{ (t = 1 h)}]}{\text{OD}_{620nm} \text{ (t = 0 h)}}\right) \times 100$$

Percentage auto-aggregation was calculated for classification of auto-aggregation; 1) high (more than 40%), 2) intermediate (30% to 40%) and 3) low auto-aggregation (less than 30%).

A similar protocol was repeated for the study of co-aggregation by inoculating *C. albicans*, *A. naeslundii* or/and *S. mutans* (inter-kingdom), and *A. naeslundii* and *S. mutans* (intra-kingdom) into a sterile 2 mL Eppendorf tube with the same cell density
used for auto-aggregation. The suspension was mixed thoroughly using a vortex mixer and the OD$_{620\text{nm}}$ at $t = 0$ h recorded. The suspension was incubated at room temperature for 1 h followed by the measurement of optical density at OD$_{620\text{nm}}$. The OD$_{620\text{nm}}$ at time ($t$) = 0 h of dual-culture and tri-culture were 1.0 and 1.5, respectively.

Percentage co-aggregation was assessed using the following equation:

$$\% \ Co-aggregation = \left(\frac{\text{OD}_{620\text{nm}} (t = 0 \text{ h}) - \text{OD}_{620\text{nm}} (t = 1 \text{ h})}{\text{OD}_{620\text{nm}} (t = 0 \text{ h})}\right) \times 100$$

2.3 Scanning Electron Microscopy (SEM) imaging

The 0 h and 1 h suspensions (100 $\mu$L sample) of a selected representative $C. albicans$ strain, $A. naeslundii$ (NCTC 10301) and $S. mutans$ (Ingbrit), prepared as above, were transferred onto cover slips and fixed with 1% osmium tetroxide (OsO$_4$) vapour. The specimens were dehydrated thoroughly in a freeze-drying system, sputter coated with palladium gold to a thickness of approximately 20 nm and observed using a scanning electron microscope (XL 30 Series, Philips, Japan).

2.4 Static biofilm formation

A quantitative assay based on that outlined by Yamada et al. (2005) and Alnuaimi et al. (2013) was used to analyse static biofilm formation by the microorganisms. To study intra-kingdom biofilms, streak diluted cultures of $C. albicans$, $A. naeslundii$ and $S. mutans$ were grown on SDA, blood agar and THYE agar respectively, for 24 h at 37 °C and several single colonies were resuspended in RPMI-1640 (Alnuaimi et al., 2013) or 25% ASM, and standardised to give a final cell density of 10$^6$ cells mL$^{-1}$, 10$^7$ cells mL$^{-1}$ and 10$^8$ cells mL$^{-1}$ respectively in separate sterile 2 mL Eppendorf tubes, equivalent to an absorbance of 0.5 at 620 nm wavelength (OD$_{620\text{nm}}$). The suspensions were mixed thoroughly using a vortex mixer for 30 s. Subsequently, 200 $\mu$L of each suspension, resulting in 2 x 10$^5$ cells ($C.$
albicans), 2 x 10^6 cells (A. naeslundii) and 2 x 10^7 cells (S. mutans) of initial inoculum, was pipetted into each well of a sterile 96-well plate (Nunc, Denmark). The number of cells mono- and co-cultured was optimised using the cfu counting method on SDA (C. albicans), blood agar (A. naeslundii) and THYE agar (S. mutans) to confirm the ratio of the cells in the inocula. Different cell densities were chosen to mimic the variability of the microorganisms in the oral cavity (Lamfon et al., 2005). Finally, the 96-well plate was incubated in an orbital shaker at 90 rpm for 72 h at 37 °C (Alyos, Thermo Fisher Scientific, Australia) to mimic the dynamic oral environment (Alnuaimi et al., 2013). The medium was replenished aseptically every 24 h.

A similar protocol was used to study inter-kingdom biofilm formation by inoculating C. albicans, A. naeslundii or/and S. mutans into a sterile 2 mL Eppendorf tube with a similar cell density as in the intra-kingdom assay resulting in 2 x 10^5 cells (C. albicans), 2 x 10^6 cells (A. naeslundii) and 2 x 10^7 cells (S. mutans) for each combination per well. The number of cells was confirmed using the cfu counting method. The suspension was mixed thoroughly using a vortex mixer and 200 µL of the suspension was pipetted into a sterile 96-well plate. The plate was incubated aerobically for 72 h at 37 °C in an orbital shaker at 90 rpm and the medium was replenished aseptically every 24 h. The resultant 72 h biofilm was assessed by the crystal violet assay (Section 2.6) and the XTT reduction assay (Section 2.7).

### 2.5 Gram stain

Gram stain was performed on C. albicans ALC3 strain following growth in RPMI-1640 and 25% ASM for 72 h at 37 °C for the determination of morphology. Initially, 1 mL of suspension of RPMI-1640 or ASM-grown C. albicans containing 2 x 10^5 cells was pipetted into each well of a 12-well plate and incubated at 37 °C in an
orbital shaker at 90 rpm. The medium was replenished aseptically every 24 h incubation. Following incubation, the supernatant was discarded and each well was washed carefully with phosphate buffered saline (PBS) (Sigma-Aldrich, USA) twice to remove non-adherent cells. Later, Gram staining was performed by adding 1 mL of methanol to each well for fixation and incubated for 15 min at 25 °C. The supernatant was then discarded and the plate was air-dried for 45 min. 1 mL of 0.1% (w/v) crystal violet (CV) solution was added into each well and incubated for a further 1 min at 25 °C. Subsequently, the plate was washed gently twice under running distilled water. 1 mL of 70% (v/v) ethanol was pipetted to de-stain for 10 sec and washed immediately under running water. 1 mL of 1% (w/v) safranin was pipetted and left for 1 min prior to final washing. The plate was air-dried and observed under the light microscope (CH Series, Olympus, Australia) (Madigan et al., 2012). A similar protocol was repeated for *A. naeslundii* and *S. mutans* to confirm each species prior to experiment.

### 2.6 Crystal violet (CV) assay

Crystal violet (CV) assays were performed according to the protocol outlined by Alnuaimi *et al.* (2013). Initially, the biofilm in each well of a 96-well plate was washed twice with sterile PBS to remove non-adherent cells. 200 µL of methanol was added to each well for fixation and incubated for 15 min at 25 °C. The supernatant was then discarded and the plate was air-dried for 45 min. 200 µL of 0.1% (w/v) CV solution was added into each well and incubated for a further 20 min at 25 °C. The plate was washed gently twice using running distilled water and 200 µL of 33% (v/v) acetic acid added to de-stain the biofilm. The plate was incubated for 5 min at room temperature. A 100 µL aliquot of this solution was transferred to a new sterile 96-
well plate and the absorbance was measured at OD_{620nm} using a microtiter plate reader (Victor³, Perkin-Elmer, Australia).

### 2.7 XTT reduction assay

An XTT reduction assay was performed according to the protocol provided by the manufacturer (Sigma-Aldrich, USA). Briefly, the biofilm-coated wells were washed twice with sterile PBS to remove non-adherent cells. Subsequently, 160 µL of sterile PBS and 40 µL of 4% XTT salt containing 1% phenazine methosulphate (Sigma-Aldrich, USA) were pipetted into each well to give a final volume of 200 µL. The plate was incubated at 37 °C for 3 h in the dark. Following incubation, 100 µL of the suspension was transferred into a new sterile 96-well plate and the absorbance at OD_{450nm} and OD_{620nm} wavelengths were measured using a microtiter plate reader. Measurement at the reference wavelength of OD_{620nm} was subtracted from OD_{450nm} to account for background fluorescence.

### 2.8 Flow-cell preparation

A single-track flow-cell (40 mm long, 16 mm wide and 2 mm deep) milled into a high-density polyethylene block was used to examine biofilm formation (Zhu et al., 2013; Department of Engineering, University of Melbourne, Australia). A standard-sized 24 mm x 60 mm uncoated glass coverslip (Menzer-Glaser, Germany) served as the substratum and was secured to the flow-cell using a silicone adhesive (GE Silicones, General Electric Company, Waterford, NY). Sodium hypochlorite with 0.5% available chlorine was pumped through the system at a flow rate of 3 mL h⁻¹ overnight to ensure sterility. Subsequently, overnight rinsing with sterile milliQ water delivered at the same flow rate was performed to remove the bleach. The flow-
cell system was then treated with 25% ASM (Section 2.2) for 2 h at 37 °C to condition the glass surface with medium prior to inoculation.

2.9 Flow-cell biofilm formation

A quantitative assay based upon that outlined by Zhu et al. (2013) was used to examine flow-cell biofilm formation. *C. albicans* ALC3 was chosen for use in the flow-cell biofilm study as the strain was isolated from a patient with OSCC (Section 2.1). To study intra-kingdom biofilms, *C. albicans* was grown in 25% ASM, while *A. naeslundii* and *S. mutans* were grown in HIB to stationary phase, washed twice, resuspended in separate sterile 15 mL tubes in 25% ASM and standardised to give a final cell density of $10^6$ cells mL$^{-1}$, $10^7$ cells mL$^{-1}$ and $10^8$ cells mL$^{-1}$ respectively. The suspension was mixed thoroughly using a vortex mixer for 30 s. Subsequently, 1 mL of this suspension was added with 2 mL of 25% ASM, resulting in $1 \times 10^6$ cells (*C. albicans*), $1 \times 10^7$ cells (*A. naeslundii*) and $1 \times 10^8$ cells (*S. mutans*), of initial inoculum which was injected into the system aseptically. These final cell densities have been chosen to replicate the microbial composition of the previous co-aggregation (Chapter 3; Arzmi et al., 2015) and static biofilms studies (Chapter 4). Finally, the system was incubated statically and inverted to allow cells to attach to the glass substratum for 1 h prior to constant flow (3 mL h$^{-1}$) of 25% ASM for 23 h to give a total of 24 h incubation at 37 °C. The experiment was conducted in three different flow-cells with each flow-cell representing one biological replicate (three in total).

A similar protocol was used in the study of polymicrobial biofilms formation by inoculating *C. albicans*, *A. naeslundii* and *S. mutans* into a sterile 15 mL tube at the same cell density as in the intra-kingdom for each combination per flow-cell. The
suspension was mixed thoroughly using a vortex mixer and 3 mL of the suspension, resulting in $1 \times 10^6$ cells (C. albicans), $1 \times 10^7$ cells (A. naeslundii) and $1 \times 10^8$ cells (S. mutans), was injected into the system aseptically with a subsequent 1 h static incubation prior to constant flow (3 mL h$^{-1}$) of 25% ASM for 23 h to give a total of 24 h incubation at 37 °C. The experiment was conducted in three different flow-cells representing three biological replicates.

The effluent was collected in a sterile 250 mL Schott bottle (Schott, Australia) on ice, followed by filter sterilisation and storage at -80 °C prior to use.

**2.10 Flow-cell gel acrylamide preparation**

At the completion of the 24 h incubation period, the biofilms were rinsed *in situ* with sterile PBS (Sigma-Aldrich, Australia) to remove non-adherent cells. Subsequently, the biofilms were fixed with 50% ethanol for 1 h at room temperature followed by washing with PBS for 30 min. The fixed biofilms were embedded in 20% acrylamide with 0.02% of ammonium persulfate and 0.8% of $N,N,N,N$-tetramethylethylenediamine (TEMED), and incubated at room temperature for 30 min to solidify the gel. The coverslip was then removed and the biofilm embedded in the polymerised acrylamide slab was stored in PBS at 4 °C prior to fluorescent *in situ* hybridisation (FISH) staining.
2.11 Fluorescent In Situ Hybridisation (FISH) staining

An 8 mm$^2$ portion of the polymerised acrylamide slab was excised and placed into a 4-well Nunclon Surface multidish plate (ThermoFisher Scientific, Australia). Customised specific probes (Life Technologies, USA) were then added at a final concentration of 15 μM for *C. albicans* and *S. mutans* probes, and 30 μM for *A. naeslundii* probes in the presence of 15% formamide in hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% SDS adjusted to pH 7.3) (Zainal-Abidin *et al.*, 2012). Species-specific probe GCC AAG GCT TAT ACT CGC T with the 5’ end labelled with Alexa Fluor 555 was used for the detection of *C. albicans*, CGG TTA TCC AGA AGG GG with the 5’ end labelled with Alexa Fluor 488 was used in the detection of *A. naeslundii* and ACT CCA GAC TTT CCT GAC with the 5’ end labelled with Alexa Fluor 647 was used for *S. mutans* detection (Life Technologies, USA).
2.12 Confocal Laser Scanning Microscopy (CLSM) and image analysis

Fluorescently labelled biofilms were visualised by CLSM (LSM 510 Meta, Carl Zeiss, Germany) with an inverted stage as described by Dashper et al. (2013) and Zhu et al. (2013). Horizontal (xy) optodigital sections were taken through the depth of the biofilm (z) every 2 µm for mono-cultured C. albicans biofilm and every 1 µm for mono-cultured A. naeslundii, S. mutans and polymicrobial biofilms. Each stack was imaged using a 63x objective at 512 x 512 pixels, with each frame at 0.28 µm (x) x 0.28 µm (y). To determine reproducibility, 5 image stacks in random positions were obtained at wavelengths of 555 nm (C. albicans), 488 nm (A. naeslundii) and 647 nm (S. mutans) for each channel from each of three biological replicates. All images were analysed with COMSTAT software to determine the biometric parameters of the biofilms including roughness coefficient, biofilm biomass, average thickness, maximum thickness and percentage surface colonisation (Heydorn et al., 2000). Three-dimensional reconstructed images were produced using Zeiss LSM image browser software (Carl Zeiss, Germany).

Roughness coefficient (Ra) is calculated based on the thickness distribution of the biofilm that provides a measurement of the variable thickness of the biofilm, which is an indicator of biofilm heterogeneity (Murga et al., 1995; Heydorn et al., 2000).

The biofilm biomass is defined as the number of biomass pixels in all images of a stack multiplied by the voxel size [(pixel size) \times (pixel size) \times (pixel size)] and divided by the substratum area of the image stack (Heydorn et al., 2000). The resulting value is biomass volume divided by substratum area (µm\(^3\) µm\(^{-2}\)). Bio-volume represents the overall volume of the biofilm, and also provides an estimate of the biomass in the biofilm.
Average biofilm thickness is a measurement of the spatial size of the biofilm whereas maximum thickness is the thickness over a given location, ignoring pores and voids inside the biofilm (Heydorn et al., 2000). The percentage of substratum coverage is the fraction of the area occupied by biomass in each image of a stack. The substratum coverage is the area coverage in the first image of the stack, which reflects the efficiency of microbial colonisation on the substratum (Heydorn et al., 2000).

The percentage proportion of *C. albicans*, *A. naeslundii* and *S. mutans* in polymicrobial biofilms was calculated using the following equation:

\[
\% \text{ Proportion} = \frac{\text{Biomass of specific microorganism}}{\text{Total biomass of microorganisms}} \times 100
\]

2.13 Cell lines and culture

OKF6, a normal human oral epithelial cell line provided by the Oral Health Cooperative Research Centre (OHCRC), The University of Melbourne, Australia was grown in 12 mL of keratinocyte serum-free medium (k-SFM) (Invitrogen, Australia) containing 25 μg mL⁻¹ bovine pituitary extract and 0.2 ng mL⁻¹ human recombinant epidermal growth factor 1-53, supplemented with 0.4 mM CaCl₂ in a 75 cm² flask (Dalley et al., 2014). H357 (Sigma-Aldrich, Australia), a cancerous cell line isolated from the tongue of a patient with OSCC, was grown in 12 mL of Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 (Sigma, Australia) containing 10% (v/v) fetal bovine serum, 0.6 μg mL⁻¹ L-glutamine (Sigma, Australia), and 0.5 μg mL⁻¹ hydrocortisone (Sigma-Aldrich, Australia) in a 75 cm² flask (Minter et al., 2003). Both OKF6 and H357 were incubated at 37 °C, 5% CO₂ for five to seven days to
reach 80% confluence. The medium was replenished every two days for the optimum growth of the cells. Cells were detached from the flask using 0.25% trypsin/EDTA (Sigma-Aldrich, Australia) for the adhesion assay, and 10 mM of EDTA in PBS (Sigma-Aldrich, Australia) for the EMT and Bio-Plex assays, for approximately 5 min at 37 °C, 5% CO₂. The concentration of cells was finally standardised in a prepared test cell growth medium (Section 2.14) prior to adhesion (Section 2.15), EMT and cytokine assays (Section 2.16).

2.14 Preparation of test cell growth media

Optimisation of test cell growth media has been conducted at three different concentrations of serum free medium (SFM) containing biofilm effluent (100% SFM, 80% SFM and 50% SFM). To assess cell viability, 10 μL of the seeded suspension in a 12-well plate (Corning, NY) as described in Section 2.16, was immediately collected and mixed with 10 μL of 0.4% trypan blue dye (Bio-Rad, CA) followed by quantification using a TC10 automated cell counter (Bio-Rad, CA). 80% SFM (v/v) was selected in the present study based on the optimisation that exhibited approximately 95% of cells were viable.

To prepare test cell growth media, SFM (DMEM/F12 and k-SFM for H357 and OKF6 respectively) was diluted in the biofilm effluent (Section 2.9) of (1) *C. albicans* (ALC3), (2) *A. naeslundii* (AN), (3) *S. mutans* (SM) and poly-microbial (TRI) biofilms, and non-effluent 25% ASM (NE), to give a final concentration of 80% (v/v) SFM (Steele and Fidel, 2002). Subsequently, each solution was mixed thoroughly and warmed in a water bath at 37 °C for 5 min prior to adhesion, EMT and Bio-Plex assays.
2.15 Cell-extracellular matrix (ECM) adhesion assay

Cell-extracellular matrix (ECM) adhesion assays were carried out using the CytoSelect 48-well Cell Adhesion Assay ECM Array kit (Cell Biolabs, USA). The kit provides five types of ECM protein (fibronectin, collagen I, collagen IV, laminin and fibrinogen) coated wells and eight wells of bovine serum albumin coated substratum that serves as the negative control. The protocol provided by the manufacturer was followed. Initially, 150 μL of the cell line suspension was standardised to 1.5 x 10^4 cells in test cell growth media and added into each well of the 48-well plate and incubated at 37 °C, 5% CO₂ for 90 min. The supernatant was discarded from each well followed by four times washing with 250 μL of PBS (Sigma-Aldrich, Australia) containing 2 mM CaCl₂ and 2 mM MgCl₂. The suspension was then aspirated and 200 μL of Cell Stain Solution (Cell Biolabs, USA) was added followed by 10 min incubation at room temperature. The stain solution was removed and each well was washed four times with 500 μL of sterile milliQ water. The plate was air-dried at room temperature for 1 h and 200 μL of Extraction Solution (Cell Biolabs, USA) was added into each well followed by incubation at room temperature for 10 min on an orbital shaker at 45 rpm (Alyos, Thermo Fisher Scientific, Australia). Finally, 150 μL of the extracted samples was transferred into a 96-well plate and measured at 570 nm wavelength (OD₅₇₀nm) using an automated plate reader (Victor³, Perkin-Elmer, Australia).

Fold change was calculated using the following equation:

\[
\text{Fold change} = \frac{\text{Adhesion of cells incubated in biofilm effluent}}{\text{adhesion of cells in NE}}.
\]
2.16 Preparation of cell suspensions for EMT and Bio-Plex assays

To prepare cell suspensions, OKF6 and H357 were standardised in DMEM/F12 and k-SFM respectively, to a concentration of $1 \times 10^6$ cells mL$^{-1}$. 500 μL of cell suspension, equivalent to $5 \times 10^5$ cells, was seeded into the wells of a 12-well plate (Corning, NY) followed by incubation at 37 °C, 5% CO$_2$ (Thermo-Fisher, Australia) for 24 h to reach approximately 80% confluence. The cells were seeded in triplicate in two different 12-well plates for each cell line. Following incubation, 500 μL of each prepared 80% (v/v) SFM test cell growth medium was added to different wells and incubated for 2 h and 24 h at 37 °C, 5% CO$_2$ prior to EMT and Bio-Plex assays.

2.17 EMT assay using flow cytometry

Expression of E-cadherin and vimentin was determined by staining with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human E-cadherin (67A4) and phycoerythrin (PE)-conjugated mouse anti-human vimentin (SPM576) (Novus Biologicals, CO, USA) as per manufacturer’s instructions. Initially, the supernatant (conditioned media) was collected from wells (Section 2.16) for Bio-plex assays (Section 2.18) and the cells were incubated with antibodies in FACS buffer (PBS, 2% BSA, 2 mM EDTA) for 20 min on ice before washing once in PBS by centrifugation for 5 min at 720 g. The supernatant was removed and the cells were re-suspended in FACS buffer. Cells were read on a LSR Fortessa X-20 (Becton Dickinson, Australia). A typical forward and side scatter gate was set to exclude dead cells and aggregates and a total of $1 \times 10^5$ events in the gate were collected. Flow cytometry data was analysed using FlowJo analysis software (FlowJo, OR, USA).
percentages of cells expressing vimentin and E-cadherin, and mean fluorescence intensity (MFI) have been measured for both OKF6 and H357 cell lines.

Percentage difference of cells expressing EMT markers between 2 h and 24 h was calculated using the following equation:

\[
\% \text{ Difference of cells expressing markers} = \frac{(\text{Cell expressing markers at 24 h} - \text{cell expressing markers at 2 h})}{\text{cell expressing markers at 2 h}} \times 100
\]

Whereas, percentage difference of EMT markers expressed by cells between 2 h and 24 h was calculated using the following equation:

\[
\% \text{ Difference of MFI} = \frac{(\text{MFI at 24 h} - \text{MFI at 2 h})}{\text{MFI at 2 h}} \times 100
\]

2.18 Bio-Plex assays

To quantify the amount of cytokines secreted by epithelial cells in response to biofilm effluent, the conditioned medium was collected and analysed using the Bio-Plex protein array system and Bio-Rad cytokine multi-plex panel (Bio-Rad). The method of Bio-Plex analysis was based on Luminex technology and simultaneously measures IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF and IFN-γ. In brief, anti-cytokine/chemokine antibody-conjugated beads were added to individual wells of a 96-well filter plate and adhered using vacuum filtration. The wells were washed and 50 µL of pre-diluted standards or samples were added and the filter plate was adjusted to shake at 300 rpm. Thereafter, the filter plate was washed and 25 µL of pre-diluted multiplex biotin-conjugated antibodies were added. After washing, 50 µL of pre-
diluted streptavidin-conjugated PE was added, followed by additional washing and the addition of 125 µL of Bio-Plex buffer to each well. The filter plate was analysed using the Bio-Plex protein array system and the concentration of each cytokine and chemokine was determined using Bio-Plex protein array system on a Bio-Plex 200 system (Biorad).

Fold change was calculated using the following equation:

\[
\text{Fold change} = \frac{\text{Cytokines expressed by cells incubated in biofilm effluent}}{\text{cytokines expressed by cells incubated in NE}}
\]

Percentage difference of cytokines expressed by cells between 2 h and 24 h was calculated using the following equation:

\[
\% \text{ Difference of cytokines expression} = \frac{[(\text{Cytokines expressed at 24 h} - \text{cytokines expressed at 2 h}) / \text{cytokines expressed at 2 h}]}{100}
\]

For IFN-γ, less than the lowest detectable measure (LLD) was standardised at 0.4 pg mL\(^{-1}\), as this was the lowest detected cytokine using the Bio-Plex.
CHAPTER 3

CO-AGGREGATION OF CANDIDA ALBICANS, ACTINOMYCES NAESLUNDII AND STREPTOCOCCUS MUTANS IS CANDIDA ALBICANS STRAIN-DEPENDENT
3.1 Abstract

Microbial interactions are necessarily associated with the development of polymicrobial oral biofilms. The aim of this study was to determine the co-aggregation of eight strains of *C. albicans* with *A. naeslundii* and *S. mutans*. In auto-aggregation assays, *C. albicans* strains were grown in either RPMI-1640 or 25% artificial saliva medium (ASM) whereas bacteria were grown in heart infusion broth (HIB). *C. albicans*, *A. naeslundii* and *S. mutans* were suspended to give $10^6$ cells mL$^{-1}$, $10^7$ cells mL$^{-1}$ and $10^8$ cells mL$^{-1}$, respectively, in co-aggregation buffer followed by a 1 h incubation. The absorbance difference at 620 nm ($\Delta$Abs) between 0 h and 1 h was recorded. To study co-aggregation, the same protocol was used, except combinations of microorganisms were incubated together. The mean $\Delta$Abs% of auto-aggregation of the majority of RPMI-1640-grown *C. albicans* was higher than in ASM-grown. Co-aggregation of *C. albicans* with *A. naeslundii* and/or *S. mutans* was variable among *C. albicans* strains. Scanning electron microscopy (SEM) images showed that *A. naeslundii* and *S. mutans* co-aggregated with *C. albicans* in dual- and tri-culture. In conclusion, the co-aggregation of *C. albicans*, *A. naeslundii* and *S. mutans* is *C. albicans* strain-dependent.
3.2 Introduction

Auto-aggregation is defined as the adherence ability of microorganisms belonging to the same species (Boris et al., 1997), while co-aggregation is the ability of genetically distinct microorganisms to adhere to each other (Ledder et al., 2008). Both auto-aggregation and co-aggregation have been classified as important mechanisms in the development of oral biofilms and postulated to provide protective mechanisms to the microbial inhabitants against shear forces that occur within the oral cavity (Handley et al., 2001). Aggregation contributes to the integration of new microbial species into biofilms, facilitating the exchange of genes and metabolic products that in turn supports survival of microorganisms against variable environmental conditions (Gibbons and Nygaard, 1970; Bos et al., 1996; Kolenbrander, 2000; Kolenbrander et al., 2002; Rickard et al., 2003; Al-Ahmad et al., 2007; Ledder et al., 2008).

Furthermore, co-aggregation has been shown to improve the colonisation of oral epithelial cells by C. albicans, as pre-incubation of buccal epithelial cells with fimbriated strains of E. coli or Klebsiella pneumoniae increases the adherence and subsequent attachment of C. albicans (Bagg and Silverwood, 1986). Pre-adherence of S. sanguinis and S. gordonii to the hard surfaces of the oral cavity provides adhesion sites for C. albicans, which supports the importance of polymicrobial inter-kingdom interactions in the oral cavity (Jenkinson et al., 1990; Bamford et al., 2009; Shirliff et al., 2009).

The oral microbiome comprises a wide variety of microorganisms such as yeasts (C. albicans) and bacteria (Actinomyces spp. and Streptococcus spp.). Candida spp. that belong to kingdom fungi, especially C. albicans, have been found to colonise approximately 40% to 50% of healthy oral cavities (Manfredi et al., 2013). The
number increases in immunocompromised patients with diseases such as AIDS and diabetes (Grimaudo et al., 1996; Thein et al., 2009). The human oral microbiome is also comprised of over 600 prevalent taxa at species level although only half of these have been cultured in the laboratory (Dewhirst et al., 2010). Among the important oral bacteria, A. naeslundii is an early oral coloniser that can constitute up to 27% of supragingival dental plaque (Nyvad and Kilian, 1987; Li et al., 2004). The ability of this species to co-aggregate with other oral microorganisms has been well recognised (Grimaudo et al., 1996; Li et al., 2001). S. mutans, an acidogenic and aciduric gram-positive oral bacterium, is widely regarded as a causative agent of dental caries (Peters et al., 2012).

The majority of in vitro studies of oral microbial co-aggregation have assessed dual-species oral bacteria interactions (Grimaudo et al., 1996; Cisar et al., 1979; Eke et al., 1989; Umemoto et al., 1999; Handley et al., 2001; Foster and Kolenbrander, 2004; Shen et al., 2005; Rosen and Sela, 2006; Ledder et al., 2008), and information of inter-kingdom interactions is limited. Further, as yet, no study utilising ASM for the growth of C. albicans has been undertaken to assess inter-kingdom co-aggregation. This is clinically relevant as C. albicans grows as yeast in 25% ASM and as hyphae in RPMI-1640, and this dimorphism has a role in the virulence of the species (Arzmi et al., 2012; Arzmi et al., 2014). The yeast form of C. albicans can adhere to the host cell surfaces by the expression of adhesins, which trigger yeast-to-hyphae transition, followed by the expression of invasins by the hyphal form that mediate the uptake of the fungus by the host cell through endocytosis (Kim and Sudbery, 2011; Gow et al., 2011; Mayer et al., 2013). In addition, research has also found that S. salivarius strain K12 preferred to co-aggregate to the hyphal region of C. albicans than the yeast after 3 h incubation in RPMI-1640 during planktonic
growth (Ishijima et al., 2012). A similar interaction was also observed between S. gordonii and C. albicans in which more bacteria co-aggregated at the hyphal region of the yeast (Bamford et al., 2009).

The aim of the present study was to determine the co-aggregation of C. albicans, A. naeslundii and S. mutans with the hypotheses that auto-aggregation and co-aggregation are C. albicans strain-dependent.
3.3 Materials and methods

*C. albicans* strains were grown on Sabouraud’s dextrose agar (SDA) (Difco, USA) and incubated at 37 °C aerobically for 24 h, whereas, *A. naeslundii* (NCTC 10301) and *S. mutans* (Ingbritt), were revived by sub-culturing onto blood agar (Difco, USA) and Todd-Hewitt yeast extract agar (Difco, USA), respectively. The agar plates were incubated at 37 °C for 48 h. To study auto-aggregation and co-aggregation, a semi-quantitative spectrophotometric assay based on that outlined by Ledder et al. (2008) and Nagaoka et al. (2008) was used to analyse the aggregation of the microorganisms (Section 2.2). To verify the co-aggregation of microorganisms, SEM was conducted for 0 h and 1 h suspensions (Section 2.3). All experiments were run in triplicate (three biological replicates) with each replicate comprised of three technical replicates. All data were statistically analysed using SPSS software version 22.0 using independent t-test to compare between the auto-aggregation of *C. albicans* in RPMI-1640 and 25% ASM. The analyses were considered statistically significant when $P < 0.05$. 
3.4 Results

3.4.1 Morphology of *C. albicans* in RPMI-1640 and 25% ASM

*C. albicans* was shown to be predominantly in the hyphal form when grown in RPMI-1640 medium after 24 h incubation whereas the yeast form was the most observed in 25% ASM after the same period of incubation (Figure 3.1).

3.4.2 Auto-aggregation

Variation in auto-aggregation of RPMI-1640 grown *C. albicans* strains (hyphal growth) was observed with a group of four strains (ALT3, ALT4, ALC1 and ALC3) exhibiting high auto-aggregation (over 40%), two strains (ALT1 and ALC4) exhibiting intermediate auto-aggregation (30% to 40%), and two strains (ALT2 and ALC2) exhibiting low auto-aggregation (Table 3.1; Figure 3.2A). The auto-aggregation values of *A. naeslundii* and *S. mutans* were also classified as low with 11.4% and 7.4%, respectively (Table 3.1).

Four strains of ASM-grown *C. albicans* (ALT2, ALT3, ALC1 and ALC4) (yeast growth) exhibiting intermediate auto-aggregation while the remainder strains (ALT1, ALT4, ALC2 and ALC3) were classified as exhibiting low auto-aggregation (Table 3.1; Figure 3.2B).

There were four strains of *C. albicans* that exhibited significantly more auto-aggregation when grown in RPMI-1640 (hyphal growth) (ALT1, ALT4, ALC1 and ALC3) compared to 25% ASM (yeast growth) (*P* < 0.05). Two strains (ALT2 and ALC2) showed significantly more auto-aggregation when grown in 25% ASM than RPMI-1640 (*P* < 0.05) and two strains (ALT3 and ALC4) exhibited no difference in auto-aggregation regardless of the media type (Figure 3.2).
Figure 3.1 Gram-stained of *C. albicans* cultures observed under light microscopy at 1000x magnification.

Left: *C. albicans* (ALT4) grown in RPMI-1640 after 24 h incubation at 37 °C; >75% of *C. albicans* cells were present in hyphal form in this medium.
Right: *C. albicans* (ALT4) grown in 25% ASM after 24 h incubation at 37 °C; 100% of *C. albicans* displaying yeast morphology in this medium.
Figure 3.2 Percentage auto-aggregation in RPMI-1640 (A) and 25% ASM (B) grown C. albicans after 1 h incubation in co-aggregation buffer.

The study was conducted in three biological replicates with each replicate consisted of three technical replicates. Data were analysed using independent t-test and considered as significantly different when $P < 0.05$. * indicates significantly more auto-aggregation between the two growth media.
3.4.3 Inter-kingdom co-aggregation

All strains of RPMI-grown *C. albicans* (hyphal growth) were found to co-aggregate with *A. naeslundii* ranging from 9.9 ± 0.5% (ALT3) to 26.2 ± 0.4% (ALC3). Co-aggregation of RPMI-grown *C. albicans* with *A. naeslundii* and *S. mutans* were also observed for all strains of the yeast ranging from 2.2 ± 0.3% (ALT3) to 17.0 ± 0.6% (ALC1). Our study showed that ASM-grown *C. albicans* strains (yeast form) co-aggregated with *A. naeslundii* ranging from 9.6 ± 0.7% (ALT2) to 23.0 ± 0.1% (ALC3). ASM-grown *C. albicans* strains were observed to co-aggregate *S. mutans* ranging from 9.9 ± 0.2% (ALT3) to 28.1 ± 0.1% (ALT4) (Table 3.1). Co-aggregation of ASM-grown *C. albicans* with *A. naeslundii* and *S. mutans* were observed in all strains of the yeast ranging from 12.9 ± 0.4% (ALT2) to 25.8 ± 0.5% (ALT1) (Table 3.1).

3.4.4 Scanning Electron Microscopy analyses

SEM analysis of RPMI-grown *C. albicans* ALT4 strain exhibited auto-aggregation in co-aggregation buffer after 1 h incubation (Figure 3.3A). Co-aggregation was observed between *C. albicans* and *A. naeslundii* (Figure 3.3B). In addition, an SEM image also revealed that *S. mutans* co-aggregated with *C. albicans* mostly at the hyphal region of the yeast (Figure 3.3C). The co-aggregation of RPMI-grown ALT4 *C. albicans* with *A. naeslundii* and *S. mutans* showed that *A. naeslundii* and *S. mutans* were partially aggregating with *C. albicans* at the hyphal region. *A. naeslundii* was also observed to co-aggregate with *S. mutans* (Figure 3.3D).

SEM analysis showed that ASM-grown *C. albicans* ALT4 strain (yeast growth) had auto-aggregation (Figure 3.3E) and *A. naeslundii* was found to co-aggregate on the yeast surface after 1 h incubation (Figure 3.3F). Co-incubation of ALT4 *C. albicans* with *S. mutans* revealed that there was inter-kingdom co-
aggregation between the two microorganisms with clumps of bacteria attached to the yeast surface of ALT4 *C. albicans* (Figure 3.3G). In addition, an SEM image of the interaction between ASM-grown ALT4 *C. albicans* with both bacterial species showed that *A. naeslundii* and *S. mutans* co-aggregated on the surface of the yeast. Finally, the image also revealed that *S. mutans* cells were co-aggregating with *A. naeslundii* after 1 h incubation (Figure 3.3H).

Taken together, the data demonstrate that the auto-aggregation and inter-kingdom co-aggregation of *C. albicans*, *A. naeslundii* and *S. mutans* are *C. albicans* strain-dependent.
Table 3.1 Auto and co-aggregation scores of pairs of 8 strains of RPMI-grown (hyphal form) and ASM-grown (yeast form) *C. albicans*, *A. naeslundii* and *S. mutans*. Percentage aggregation as measured by OD$_{620\text{nm}}$ change over 1 h (see materials and methods section). Data are means from three separate experiments (SD are given in parenthesis). The study was conducted in three biological replicates with each replicate consisted of three technical replicates. *Auto-aggregation scores representative of interaction between cells from the same culture. # *A. naeslundii* and *S. mutans* were grown in BHI respectively.

<table>
<thead>
<tr>
<th>Strains</th>
<th>RPMI-1640</th>
<th>25% ASM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Auto-aggregation</td>
<td>An</td>
</tr>
<tr>
<td>ALT1</td>
<td>24.6 (0.4) 18.2 (0.1) 5.4 (0.1)</td>
<td>17.6 (0.2) 17.8 (0.2) 25.8 (0.5)</td>
</tr>
<tr>
<td>ALT2</td>
<td>17.6 (0.4) 16.4 (0.1) 13.7 (0.3)</td>
<td>9.6 (0.7) 24.8 (0.5) 12.9 (0.4)</td>
</tr>
<tr>
<td>ALT3</td>
<td>9.9 (0.5) 15.4 (0.4) 2.2 (0.3)</td>
<td>14.6 (0.4) 9.9 (0.2) 23.3 (0.2)</td>
</tr>
<tr>
<td>ALT4</td>
<td>17.7 (0.5) 17.3 (0.5) 10.9 (0.1)</td>
<td>14.8 (0.1) 28.1 (0.1) 22.0 (0.2)</td>
</tr>
<tr>
<td>ALC1</td>
<td>18.7 (0.4) 20.0 (0.2) 17.0 (0.6)</td>
<td>15.5 (0.2) 10.3 (0.5) 16.7 (0.3)</td>
</tr>
<tr>
<td>ALC2</td>
<td>19.7 (0.1) 12.3 (0.2) 8.1 (0.2)</td>
<td>20.5 (0.3) 10.5 (0.1) 21.9 (0.3)</td>
</tr>
<tr>
<td>ALC3</td>
<td>26.2 (0.4) 19.5 (0.2) 13.7 (0.3)</td>
<td>23.0 (0.1) 19.3 (0.5) 17.3 (0.3)</td>
</tr>
<tr>
<td>ALC4</td>
<td>18.3 (0.2) 22.7 (0.4) 15.5 (0.2)</td>
<td>14.6 (0.5) 22.0 (0.2) 21.7 (0.3)</td>
</tr>
<tr>
<td>An#</td>
<td>9.6 (1.1)</td>
<td>*11.4 (0.7)</td>
</tr>
<tr>
<td>Sm#</td>
<td>9.6 (1.1)</td>
<td>*7.4 (0.6)</td>
</tr>
</tbody>
</table>
Figure 3.3 SEM of *C. albicans* (strain ALT4) auto-aggregation (A & E), inter-kingdom interaction with *A. naeslundii* (B & F), *S. mutans* (C & G) and both bacteria (D & H). *C. albicans* was grown in RPMI-1640 (A, B, C & D) and 25% ASM (E, F, G & H). Magnification is as shown on each image (6500x and 10000x).
3.5 Discussion

Co-aggregation is a mechanism that induces the development of a complex architecture of oral biofilms, which assists the attachment of secondary colonisers such as *S. mutans* (Kolenbrander, 2000; Min and Rickard, 2009).

We have shown that inter-kingdom co-aggregation was strain-dependent. The co-aggregation of the majority of RPMI-grown (hyphal growth) *C. albicans* strains, when grown with *S. mutans* and *A. naeslundii* either alone or in combination, resulted in variable co-aggregation. The observed variability of co-aggregation in *C. albicans* may be attributable to the different abundances of specific molecules that are important in adhesion and quorum sensing (e.g. Farnesol) from different strains, which have been suggested to have a role in inter-kingdom interactions of *C. albicans* and bacteria (Morales and Hogan, 2010). Furthermore, the variability of co-aggregation observed in ASM-grown *C. albicans* (yeast growth) supports our hypothesis that the co-aggregation of *C. albicans* to *A. naeslundii* and *S. mutans* is highly dependent on the individual yeast strain.

Variability of co-aggregation was observed when ASM-grown *C. albicans* strains were co-incubated with *S. mutans* and *A. naeslundii*. This variability suggests that *S. mutans* might have induced the formation of binding sites on the yeast surface that allow the co-aggregation of *A. naeslundii* to ASM-grown *C. albicans* when co-cultured. Previous study has shown that *C. albicans* that has been co-cultured with *S. mutans* increased the binding site of the yeast (Webb *et al.*, 1998a; Calderone *et al.*, 2000; Falsetta *et al.*, 2014). These results support our hypothesis that co-aggregation is highly dependent on the *C. albicans* strain. It cannot be related to the production of glucan by *S. mutans* glucosyltransferases as no sucrose was present, however it may
be that specific proteins are induced on the surface of *C. albicans* due to the interaction with *S. mutans* that promotes further interaction with *A. naeslundii* (Holmes *et al.*, 1995; Koo *et al.*, 2010; Falsetta *et al.*, 2014). Further research is necessary to assess this hypothetical possibility.

It can be postulated that the observed variability in co-aggregation may be related to that specific strain’s ability to produce both non-specific (adhesins) and specific (lectin-saccharide) cell surface receptors (Kolenbrander and Williams, 1981; McIntire *et al.*, 1982; Rickard *et al.*, 2003; Rosen and Sela, 2006; Ledder *et al.*, 2008). The specific co-aggregation between *C. albicans* and *A. naeslundii* is due to the presence of mannose-containing adhesin protein on the yeast cell surface (Grimaudo *et al.*, 1996). This same study also showed variation in the co-aggregation of *A. naeslundii* with four different yeast strains, which supports the present study. However, the study does not include the co-aggregation ability of *C. albicans*’ hyphae and the tri-cultured polymicrobial interaction, which has been conducted in the present study. Furthermore, other research has shown significant strain variation of the cell wall biogenesis in *C. albicans*, that may have a role in the observed variation in aggregation ability (Ragni *et al.*, 2011). Further analysis of the cell wall structure of a range of *C. albicans* strains is necessary to fully elucidate the mechanism of this observed variability.

The sum of auto-aggregation of *C. albicans*, *A. naeslundii* and *S. mutans* cannot be combined to produce expected co-aggregation value in order to determine the increase or decrease co-aggregation when co-incubated. This is due to the total number of cells present in each Eppendorf tube is significantly higher for the coaggregation studies, which will dramatically alter the dynamics of cell-cell interactions. Thus, the co-aggregation data has been used to compare the relative
coaggregation capabilities of each \textit{C. albicans} strain.

It has previously been suggested that, due to the limitation of nutrients present in RPMI-1640, growth in this media induces yeast-hyphae transition leading to predominant hyphal growth (Urban \textit{et al.}, 2006). Our light microscope images confirmed this with greater than 75\% of \textit{C. albicans} cells growing in hyphal form in RPMI-1640. No previous study has assessed the form of growth at SEM level when \textit{C. albicans} is grown in 25\% ASM. The present study is the first to observe \textit{C. albicans} cellular morphology in 25\% ASM using SEM which confirmed that in this media, \textit{C. albicans} does not grow in hyphal form.

Future assessment of co-aggregation of \textit{C. albicans}, \textit{A. naeslundii} and \textit{S. mutans} requires animal studies to assess oral biological factors, such as salivary flow and immunological components that exist in the oral cavity, which may influence aggregation. These \textit{in vivo} studies of co-aggregation are likely to enhance our understanding of the mutual interaction of microorganisms in the oral cavity, a process likely to be critical in chronic infection and potentially oral carcinogenesis.
3.6 Conclusion

In conclusion, auto-aggregation and inter-kingdom co-aggregation of *C. albicans* have been shown to be strain-dependent and this is likely to be important in polymicrobial oral biofilm formation.
CHAPTER 4

POLYMICROBIAL BIOFILM FORMATION BY CANDIDA ALBICANS,
ACTINOMYCES NAESLUNDII AND STREPTOCOCCUS MUTANS IS
CANDIDA ALBICANS STRAIN AND MEDIUM DEPENDENT
4.1 Abstract

Oral biofilms comprise of extracellular polysaccharides and polymicrobial microorganisms. The aim of this study was to determine the effect of polymicrobial interactions of *C. albicans*, *A. naeslundii* and *S. mutans* on biofilm formation with the hypotheses that biofilm biomass and metabolic activity are both *C. albicans* strain and medium dependent. To study monospecific biofilms, *C. albicans*, *A. naeslundii* and *S. mutans* were inoculated into 25% artificial saliva medium (ASM) and RPMI-1640 in separate vials, whereas to study polymicrobial biofilm formation, the inoculum containing microorganisms was prepared in the same vial prior inoculation into a 96-well plate followed by 72 h incubation. Finally, biofilm biomass and metabolic activity were measured using crystal violet (CV) and XTT assays, respectively. Our results showed variability of mono-cultured and polymicrobial biofilm biomass between *C. albicans* strains and medium. Based on cut-offs, out of 32, seven RPMI-grown biofilms had high biofilm biomass (HBB), whereas, in ASM-grown biofilms, 14 out of 32 were HBB. Of the 32 biofilms, 21 were high metabolic activity (HMA), whereas in 25% ASM, there was no biofilm had exhibiting HMA. Significant differences were observed between 25% ASM and RPMI-grown biofilms with respect to metabolic activity (*P* < 0.01). In conclusion, biofilm biomass and metabolic activity were both *C. albicans* strain and growth medium dependent.
4.2 Introduction

The oral cavity is a habitat for various microorganisms including yeast and bacteria (Morales and Hogan, 2010). This oral microbiome provides a balanced oral environment however perturbation of this homeostasis may lead to the development of dysbiosis and oral disease (Atanasova and Yilmaz, 2015).

*Candida* spp., *Actinomyces* spp. and *Streptococcus* spp. are common inhabitants of the human oral cavity (Wade, 2013; O'Donnell *et al*., 2015; Kolenbrander *et al*., 2010). *Candida* spp. have been found to colonise approximately 50% of healthy human oral cavities (Manfredi *et al*., 2013). *C. albicans* is the most frequently isolated *Candida* spp. from the oral cavity, especially in immunocompromised patients with diseases such as AIDS and diabetes (Thein *et al*., 2006; Nobile and Johnson, 2015). *Actinomyces* spp. And *Streptococcus* spp. are the normal components of the human oral microbiome, with some species associated with dental caries initiation and development (Wade, 2013). *A. naeslundii* is categorised as an early oral coloniser that can constitute up to 27% of supragingival dental plaque (Arai *et al*., 2015; Cheaib *et al*., 2015; Cavalcanti *et al*., 2016). *Streptococcus mutans* is an acidogenic and aciduric Gram-positive oral bacterium that is widely regarded as a pathogen that initiates dental caries in association with other oral bacteria (Peters *et al*., 2012; Wade, 2013).

Dimorphism is an important virulence factor of *C. albicans*. It is defined as the ability of *Candida* spp. to change morphology between yeast and hyphal forms (Arzmi *et al*., 2012; Arzmi *et al*., 2014). *C. albicans* is predominantly in the yeast form during early colonisation of the oral cavity, however, subsequent invasion of oral epithelial cells is predominantly by the hyphal form. The yeast form of *C. albicans* can adhere to host cell surfaces by the expression of adhesins, which trigger
yeast-to-hyphae transition, followed by the expression of invasins by the hyphal form that mediate the uptake of the fungus by the host cell through induced endocytosis (Gow et al., 2011; Kim and Sudbery, 2011; Mayer et al., 2013).

The majority of in vitro studies of biofilms have been with mono-cultured and dual-cultured oral microorganisms (Cisar et al., 1979; Eke et al., 1989; Umemoto et al., 1999; Handley et al., 2001; Foster and Kolenbrander, 2004; Shen et al., 2005; Ledder et al., 2008; Zhu et al., 2013; Dutton et al., 2014), and information from tri-culture polymicrobial biofilms remains limited (Zainal-Abidin et al., 2012; Dashper et al., 2014; Cavalcanti et al., 2016). As yet, no study utilising artificial saliva medium for the growth of C. albicans has been undertaken to assess polymicrobial biofilms. This is clinically relevant as C. albicans grows as yeast in 25% ASM and as hyphae in RPMI-1640.

Crystal violet (CV) and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(penylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assays are two methods for biofilm quantification. CV assay measures the microbial biofilm biomass where the dye interacts with negatively charged molecules present on the surface of the microorganisms and extracellular polysaccharide (Cheaib et al., 2015). The XTT assay is a colorimetric-based assay of cell metabolic activity using tetrazolium hydroxide (McCluskey et al., 2005): Tetrazolium hydroxide is an active compound that is converted to formazan by the activity of dehydrogenases involved in the metabolic pathways of microbial cells (Peeters et al., 2008). Succinate dehydrogenases of prokaryotic cells and mitochondrial dehydrogenases of eukaryotic cells are examples of dehydrogenase activity that can be detected by XTT (McCluskey et al., 2005; Moffa et al., 2016).
The aims of the present study were to determine the effect of interactions of *C. albicans*, *A. naeslundii* and *S. mutans* on the formation of polymicrobial biofilms and to assess this interaction when biofilms were grown in 25% ASM for predominantly yeast growth and in RPMI-1640 for predominantly hyphal growth. We hypothesized that this polymicrobial biofilm formation is *C. albicans* strain- and growth medium-dependent.
4.3 Materials and methods

A quantitative assay based on that outlined by Yamada et al. (2005) and Alnuaimi et al. (2013) was used to analyse static intra-kingdom and inter-kingdom biofilms formation by C. albicans, A. naeslundii and S. mutans (Section 2.4). Gram staining was performed on C. albicans ALC3 strain for the determination of morphology (Section 2.5). Crystal violet (CV) assay was performed according to the protocol outlined by Alnuaimi et al. (2013) to assess biofilm biomass (Section 2.6) and XTT reduction assay was performed according to the protocol provided by the manufacturer (Sigma-Aldrich, USA) to assess metabolic activity of biofilm (Section 2.7).

All biofilms containing C. albicans were divided into terciles according to biofilm biomass and metabolic activity for CV and XTT assays, respectively. This division provided the cut-offs to classify strains as high, moderate and low biofilm biomass (HBB, MBB and LBB); and high, moderate and low metabolic activity (HMA, MMA and LMA) (Marcos-Zambrano et al., 2014).

All experiments were run in triplicate (three biological replicates) with each replicate comprised of three technical replicates. Using SPSS software version 22.0, all data were statistically analysed by applying chi-square test to compare between the categories (high, medium and low) for each assay. Since comparison was made between independent groups that were not normally distributed, thus, non-parametric chi-square was chosen for the statistical analysis. Meanwhile, to compare between ATCC and clinical strains biofilm biomass, two-tailed independent t-test analysis was conducted as the data were normally distributed. Comparison between a group of ATCC isolates (ALT1, ALT2, ALT3 and ALT4) and a group of clinical isolates (ALC1, ALC2, ALC3, ALC4) of C. albicans was analysed using two-tailed
independent t-test since the data were normally distributed. Finally, multiple comparisons between mono-cultured with polymicrobial biofilms were analysed using ANOVA post hoc Tukey test since the equal variances were assumed as analysed using Levene’s test.
4.4 Results

4.4.1 Morphology of C. albicans biofilms in RPMI-1640 and 25% ASM

C. albicans biofilm growth was predominantly in the hyphal form when grown in RPMI-1640, and in the yeast form when grown in 25% ASM after 24 h incubation as observed by Gram staining (Figure 4.1).

4.4.2 Effect of microbial interaction and medium on biofilm biomass

Biofilm biomass was categorised into terciles using the following CV measurement cut-offs: LBB < 2.280, MBB 2.280-2.535, HBB > 2.535. None of mono-cultured C. albicans was categorised as HBB, however, when co-cultured with A. naeslundii three C. albicans strains (ALT1, ALT2 and ALT3) were categorised as HBB (Table 4.1). Only ALT1 was categorised as HBB when co-cultured with S. mutans in RPMI-1640 whereas in tri-cultured biofilms, three strains of C. albicans (ALT1, ALT2 and ALT3) were categorised HBB (Table 4.1).

None of mono-cultured ASM-grown C. albicans exhibited HBB, however, in the presence of A. naeslundii, seven strains of C. albicans were classified as HBB (Table 4.1). Interaction of C. albicans with S. mutans showed that two strains (ALT1 and ALT3) were HBB, while in tri-cultured biofilms, five C. albicans strains (ALT1, ALT2, ALT3, ALT4 and ALC2) were classified as HBB (Table 4.1).

Analyses of all 32 biofilms for biomass showed that there were seven biofilms classified as HBB (21.9%), 12 MBB (37.5%) and 13 LBB (40.6%) when the biofilms were grown in RPMI-1640 (hyphal growth). Biofilms grown in 25% ASM (yeast form) showed 14 biofilms categorised as HBB (43.8%), ten MBB (31.3%) and eight LBB (25.0%). There were more biofilms with HBB when grown in 25% ASM (yeast form) than RPMI-1640 (hyphal form), however, this did not reach statistical significance ($P > 0.05$).
Five RPMI-grown biofilms (hyphal form) had significantly increased biomass when *C. albicans* strains were co-cultured with *A. naeslundii* (ATCC: ALT1, ALT4; Clinical: ALC1, ALC2 and ALC4) compared with mono-cultured *C. albicans* biofilm (*P* < 0.05). Further, co-culture of *C. albicans* with *S. mutans* increased biomass of six biofilms (ATCC: ALT1, ALT4; Clinical: ALC1, ALC2, ALC3 and ALC4) significantly (*P* < 0.05). Five biofilms (ATCC: ALT1, ALT4; Clinical: ALC1, ALC2 and ALC4) increased biomass significantly when *C. albicans* was co-cultured with both *A. naeslundii* and *S. mutans* when compared with the mono-cultured biofilm of *C. albicans* (*P* < 0.05; Table 4.1).

Two ASM-grown biofilm (ATCC: ALT1 and ALT2; yeast form) had a significantly increased biomass when *C. albicans* was co-cultured with *A. naeslundii* compared with the mono-cultured *C. albicans* biofilm (*P* < 0.05). One biofilm (ATCC: ALT1) showed a significant increase (*P* < 0.05) and one (ATCC: ALT2) a significant decrease (*P* < 0.05) in biomass when *C. albicans* was co-cultured with *S. mutans*. There was one strain (ATCC: ALT1) that showed a significant increase in biomass when *C. albicans* was co-cultured with both *A. naeslundii* and *S. mutans* compared with mono-cultured *C. albicans* biofilm (*P* < 0.05; Table 4.1).
Figure 4.1 Gram-stained biofilms of *C. albicans* strain ALC3 observed under light microscope at 200x magnification after 72 h incubation at 37 °C in 24-well plate at 90 rpm.

A: ASM-grown *C. albicans* biofilm; B: RPMI-grown *C. albicans* biofilm.
Table 4.1 Biofilm biomass scores of eight strains of RPMI-grown and ASM-grown *C. albicans*, *A. naeslundii* (An) and *S. mutans* (Sm) as measured by OD$_{620}$nm after 72 h incubation. Data are means from three biological replicates with each replicate consisted of three technical replicates (SD are given in parenthesis). Significant difference (*P* < 0.05) observed between dual-cultured *C. albicans-An* (*), *C. albicans-Sm* (#) or tri-cultured (I) to mono-cultured *C. albicans* biofilms grown in the same medium.

<table>
<thead>
<tr>
<th>Strains</th>
<th>RPMI-1640</th>
<th>25% ASM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono</td>
<td>An</td>
</tr>
<tr>
<td>ALT1</td>
<td>2.389 (0.019)</td>
<td><em>2.681 (0.016)</em></td>
</tr>
<tr>
<td>ALT2</td>
<td>2.501 (0.326)</td>
<td>2.658 (0.094)</td>
</tr>
<tr>
<td>ALT3</td>
<td>2.408 (0.211)</td>
<td>2.666 (0.064)</td>
</tr>
<tr>
<td>ALT4</td>
<td>1.554 (0.170)</td>
<td><em>2.492 (0.129)</em></td>
</tr>
<tr>
<td>ALC1</td>
<td>1.762 (0.205)</td>
<td><em>2.259 (0.267)</em></td>
</tr>
<tr>
<td>ALC2</td>
<td>1.594 (0.083)</td>
<td><em>2.268 (0.410)</em></td>
</tr>
<tr>
<td>ALC3</td>
<td>0.722 (0.270)</td>
<td>1.722 (0.359)</td>
</tr>
<tr>
<td>ALC4</td>
<td>1.445 (0.127)</td>
<td><em>1.965 (0.128)</em></td>
</tr>
<tr>
<td>An</td>
<td>0.073 (0.002)</td>
<td>0.073 (0.002)</td>
</tr>
<tr>
<td>Sm</td>
<td>0.066 (0.001)</td>
<td>0.066 (0.001)</td>
</tr>
</tbody>
</table>

- Low biofilm biomass (LBB)
- Moderate biofilm biomass (MBB)
- High biofilm biomass (HBB)
4.4.3 Effect of microbial interaction and medium on metabolic activity

Biofilm metabolic activity based on the XTT assay was divided into terciles and categorised based on the following cut-offs: LMA < 0.120, MMA 0.120-0.550, HMA > 0.550. RPMI-1640 mono-cultured growth resulted in six strains of *C. albicans* (ALT1, ALT2, ALT4, ALC1, ALC2 and ALC4) categorised with HMA (Table 4.2). Seven *C. albicans* strains (ALT1, ALT2, ALT3, ALT4, ALC1, ALC2 and ALC4) when co-cultured with *A. naeslundii* in RPMI-1640 had HMA. Only two strains of *C. albicans* (ALT1 and ALT2) had HMA when co-cultured with *S. mutans* in RPMI-1640. Six *C. albicans* strains (ALT1, ALT2, ALT3, ALT4, ALC2 and ALC4) were categorised as having HMA when co-cultured in RPMI-1640 with both *A. naeslundii* and *S. mutans* (Table 4.2).

25% ASM mono-cultured growth resulted in all *C. albicans* strains being categorised with LMA (Table 4.2), however, in the presence of *A. naeslundii*, all strains had MMA. Interaction of *C. albicans* with *S. mutans* showed that all *C. albicans* strains remained with LMA whereas, in the presence of both *A. naeslundii* and *S. mutans*, there were three strains having MMA (ALT1, ALT4 and ALC2) and five strains with LMA (ALT2, ALT3, ALC1, ALC3 and ALC4) (Table 4.2).

Analyses of all 32 biofilms showed that there were 21 biofilms of RPMI-grown biofilms (hyphal growth) categorised as having HMA (65.6%) and 11 with MMA (34.4%). In addition, there were 11 ASM-grown biofilms (yeast growth) categorised as having MMA (34.4%) and 21 with LMA (65.6%). Thus, statistically significant higher metabolic activity was observed when biofilms were grown in RPMI-1640 (*P* < 0.01).

Only *C. albicans* strains ALT3 when co-cultured with *A. naeslundii* showed an increased activity when grown in RPMI-1640 when compared with mono-cultured
C. albicans. Furthermore, there were four C. albicans strains (ALT4, ALC1, ALC2 and ALC4) that exhibited a decrease in metabolic activity when co-incubated with S. mutans compared with the mono-cultured biofilm of C. albicans. There was only one biofilm (ALC1) that showed decreased bioactivity when C. albicans was co-cultured with both A. naeslundii and S. mutans compared with mono-cultured C. albicans (Table 4.2).

Three RPMI-grown biofilms (ATCC: ALT3; Clinical: ALC2 and ALC4; hyphal form) exhibited significant increase activity when C. albicans was co-cultured with A. naeslundii in comparison with the mono-cultured C. albicans biofilm (P < 0.05). Four biofilms (ATCC: ALT4; Clinical: ALC1, ALC2 and ALC4) showed significant decrease metabolic activity when C. albicans was co-cultured with S. mutans. Whereas, one biofilm (Clinical: ALC1) displayed a significant decrease activity when C. albicans was co-cultured with both A. naeslundii and S. mutans when compared with mono-cultured C. albicans (P < 0.05; Table 4.2).

Finally, based on metabolic activity per unit biomass in mono-cultured biofilms, ALT4 and ALC3 were found to be the most active C. albicans strains when grown in 25% ASM and ALT2 was the least active when grown in the same medium. Whereas, in RPMI-1640, ALC3 was found to be the most active while ALT3 was the least (Table 4.3).
Table 4.2 Static biofilm metabolic activity scores of eight strains of RPMI-grown and ASM-grown *C. albicans*, *A. naeslundii* (An) and *S. mutans* (Sm) as measured by OD$_{450nm-620nm}$ after 72 h incubation. Data are means from three biological replicates with each replicate consisted of three technical replicates (SD are given in parenthesis). Significant difference (*P* < 0.05) observed between dual-cultured *C. albicans*-An (*), *C. albicans*-Sm (#) or tri-cultured (φ) to mono-cultured *C. albicans* biofilms grown in the same medium.

<table>
<thead>
<tr>
<th>Strains</th>
<th>RPMI-1640</th>
<th>25% ASM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono</td>
<td>An</td>
</tr>
<tr>
<td>ALT1</td>
<td>0.780 (0.022)</td>
<td>0.819 (0.021)</td>
</tr>
<tr>
<td>ALT2</td>
<td>0.788 (0.012)</td>
<td>0.811 (0.029)</td>
</tr>
<tr>
<td>ALT3</td>
<td>0.395 (0.074)</td>
<td>*0.610 (0.120)</td>
</tr>
<tr>
<td>ALT4</td>
<td>0.738 (0.0130)</td>
<td>0.665 (0.035)</td>
</tr>
<tr>
<td>ALC1</td>
<td>0.641 (0.033)</td>
<td>0.645 (0.058)</td>
</tr>
<tr>
<td>ALC2</td>
<td>0.610 (0.034)</td>
<td>*0.726 (0.003)</td>
</tr>
<tr>
<td>ALC3</td>
<td>0.525 (0.030)</td>
<td>0.522 (0.004)</td>
</tr>
<tr>
<td>ALC4</td>
<td>0.557 (0.024)</td>
<td>*0.638 (0.019)</td>
</tr>
</tbody>
</table>

| An | Mono | 0.050 (0.002) | 0.002 (0.001) | 0.147 (0.028) | 0.011 (0.002) |
| Sm | 0.001 (0.001) | 0.002 (0.001) | 0.002 (0.002) | 0.011 (0.002) |

Legend:
- **Low metabolic activity (LMA)**
- **Moderate metabolic activity (MMA)**
- **High metabolic activity (HMA)**
Table 4.3 Mono-culture metabolic activity per biofilm biomass (XTT/CV) scores of 8 strains of RPMI-grown (hyphal form) and ASM-grown (yeast form) *C. albicans*, *A. naeslundii* (An) and *S. mutans* (Sm). Data are means from three biological replicates with each replicate consisted of three technical replicates (SD are given in parenthesis).

<table>
<thead>
<tr>
<th>Media</th>
<th>ALT1 (SD)</th>
<th>ALT2 (SD)</th>
<th>ALT3 (SD)</th>
<th>ALT4 (SD)</th>
<th>ALC1 (SD)</th>
<th>ALC2 (SD)</th>
<th>ALC3 (SD)</th>
<th>ALC4 (SD)</th>
<th>An (SD)</th>
<th>Sm (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>0.326 (0.007)</td>
<td>0.319 (0.046)</td>
<td>0.166 (0.044)</td>
<td>0.476 (0.075)</td>
<td>0.366 (0.027)</td>
<td>0.382 (0.009)</td>
<td>0.807 (0.221)</td>
<td>0.388 (0.044)</td>
<td>0.681 (0.035)</td>
<td>0.022 (0.016)</td>
</tr>
<tr>
<td>25% ASM</td>
<td>0.008 (0.000)</td>
<td>0.005 (0.001)</td>
<td>0.007 (0.002)</td>
<td>0.051 (0.016)</td>
<td>0.011 (0.002)</td>
<td>0.010 (0.000)</td>
<td>0.051 (0.032)</td>
<td>0.022 (0.006)</td>
<td>0.087 (0.019)</td>
<td>0.003 (0.002)</td>
</tr>
</tbody>
</table>
4.5 Discussion

To our knowledge, this is the first study to evaluate the effect of microbial interactions of yeast growth and hyphal growth of *C. albicans*, *A. naeslundii* and *S. mutans* on the formation of static biofilms *in vitro*. The results of the present study clearly demonstrate that both biofilm biomass and metabolic activity are *C. albicans* strain and growth medium dependent.

The present study has shown a variation of biofilm biomass and metabolic activity between strains of *C. albicans*. Overall, when grown as mono-cultured the majority of clinical strains had a significantly lower biofilm biomass than the ATCC reference strains when grown in RPMI (hyphal form). However, a significant increase of biomass was observed in all clinical strains that did not occur in ATCC strains (ALT2 and ALT3) when grown in polymicrobial biofilms. Furthermore, biofilms that were formed by clinical isolates of *C. albicans* have shown lower biofilm biomass compared with the reference strains *C. albicans* (Alnuaimi et al., 2013). Furthermore, the metabolic activity has been shown to vary among *C. albicans* strains; however, the morphology of *C. albicans* in this previous study was unknown (Alnuaimi et al., 2013). Strain variability of *C. albicans* has been shown in the oral cavity of different individuals (Hellstein et al., 1993; Kleinegger et al., 1996). In addition, *C. albicans* strains isolated from HIV-infected patients produce higher levels of aspartic proteinases (SAPs), compared with strains isolated from uninfected patients (de Bernardis et al., 1996). SAP is a putative virulence factor that is able to affect *C. albicans* biofilm formation in the oral cavity together with phenotypic switching, morphogenesis and quorum sensing (Morales and Hogan, 2010; Arzmi et al., 2012). Thus, the results from the present study may indicate a symbiotic
interaction between clinical \textit{C. albicans} and oral microorganisms that may lead to the increase of colonisation in the oral cavity of diseased patients.

The metabolic activity of biofilms was shown to be growth media dependent, with the majority of ASM-grown \textit{C. albicans} biofilms having lower metabolic activity than those grown in RPMI-1640, particularly mono-cultured biofilms (Table 4.2, Table 4.3). It is postulated that RPMI-1640, which contains limited nutrients, induces stress in \textit{C. albicans}, thus promoting hyphal formation. This does not occur when the yeast is grown in 25\% ASM that is rich in nutrients. Interestingly, previous studies based on the growth rate have shown that \textit{C. albicans} with low metabolic activity are more invasive and associated with disease, while conversely those with high activity are non-invasive (Baillie and Douglas, 1998; Silva et al., 2011; Tobudic et al., 2012). Furthermore, low metabolic activity has been shown to reduce the antifungal susceptibility of \textit{C. albicans} within the biofilm, which could be due to minimal absorption of antifungal agents such as amphotericin B, thus affecting inactivation kinetics (Mah and O’Toole, 2001).

The metabolic activity of all \textit{C. albicans} strains that were grown in 25\% ASM increased in the presence of \textit{A. naeslundii} in dual-cultured biofilms. However, a decrease of metabolic activity was observed in tri-cultured biofilms when compared to the dual-cultured biofilms of \textit{C. albicans} and \textit{A. naeslundii}, suggesting that these microorganisms may be interacting metabolically. It is postulated that in the presence of \textit{A. naeslundii}, \textit{C. albicans} may increase mitochondrial dehydrogenase activity that in turn, increased the activity of succinate dehydrogenases of \textit{A. naeslundii}. In addition, \textit{S. mutans} has been shown to reduce the metabolic activity in tri-cultured biofilms compared with the dual-cultured \textit{C. albicans-A. naeslundii} biofilms, suggesting that the antagonistic metabolic interaction between \textit{A. naeslundii} and \textit{S.
mutans, demonstrated in the present study (Table 4.2), may have affected overall metabolic activity of the consortia. *C. albicans* and *A. naeslundii* have been shown to synthesize mitochondrial and succinate dehydrogenases, respectively, that were reported to be detectable by XTT (McCluskey et al., 2005; Moffa et al., 2016). Even though *S. mutans* has been found to synthesize an NADH-dependent lactate dehydrogenase; the present study revealed that enzyme activity was not detected with XTT suggesting that the assay is not suitable for the study of *S. mutans* metabolic activity. Furthermore, it is also postulated that the decrease of metabolic activity in tri-cultured biofilms compared to dual-cultured could be due to the severe nutrient limitation. The metabolic activity may be higher at earlier time points; however, several aspects need to be considered such as the strain and morphology of *C. albicans*. Even though there are more biofilms when grown as mono-cultured, however, the competition of nutrient in co-cultured may have induced *C. albicans* to produce hyphae, which require more energy. Since the present study was measuring XTT at on 72 h, therefore, further research to determine the metabolic activity at different time point including 24 h is highly recommended.

In the present study, the biofilm biomass was shown to vary with microbial interactions (mono-cultured *C. albicans*, dual-cultured *C. albicans* and *A. naeslundii*, dual-cultured *C. albicans* and *S. mutans*, tri-cultured *C. albicans*, *A. naeslundii* and *S. mutans*). The majority of RPMI-1640 grown *C. albicans* (hyphal form) biofilm biomass were observed to increase in the presence of bacteria compared with mono-cultured *C. albicans*. *A. naeslundii* and *S. mutans* have been shown to bind to *C. albicans* through its mannose-containing surface protein (Rickard et al., 2003; Ledder et al., 2008; Dutton et al., 2014; Falsetta et al., 2014; Sztajer et al., 2014; Nobile and Johnson, 2015). This interaction has been reported to induce the formation of
extracellular polysaccharide, thus promoting the adherence of the late colonisers to form a complex polymicrobial biofilm potentially enhancing biofilm biomass (Wade, 2013; Cheaïb et al., 2015; Nobile and Johnson, 2015; Cavalcanti et al., 2016).

The present study found that the ATCC strains form excellent mono-cultured biofilms in both 25% ASM and RPMI-1640 such that addition of A. naeslundii or S. mutans resulted in no additional biomass in the majority of biofilms. However, the clinical strains that were poor biofilm formers in RPMI-1640 were observed to increase biofilm biomass significantly when A. naeslundii or S. mutans was co-inoculated (Table 4.1). This result indicates that the choice of isolates in the study of the interaction between oral yeast and oral bacteria in biofilms is critical. The C. albicans ATCC strains assessed in the present study would appear to have lost either the ability, or need, to interact with oral bacteria (Harriott and Noverr, 2011), thus investigations using only ATCC strains of C. albicans are likely to not reflect the true interactions that are occurring in the oral cavity.

We have demonstrated that C. albicans predominantly in the yeast form when grown as a biofilm in 25% ASM, whereas, RPMI-grown C. albicans biofilms were predominated by the hyphal form (Figure 4.1). These results support previous work that showed the proportion of yeast and hyphal cells of C. albicans present in the biofilm is dependent upon the nutrient source, where nitrogen-based medium allowed for more yeast growth and biofilms grown in RPMI-1640 with high salts, amino acids and D-glucose, showed more hyphal growth (Chandra et al., 2001).
4.6 Conclusion

Biofilm biomass and metabolic activity have been shown to be both *C. albicans* strain and medium dependent. This is likely to have significance in the development of polymicrobial oral biofilms *in vivo*.
CHAPTER 5

POLYMICROBIAL BIOFILM FORMATION BY CANDIDA ALBICANS, ACTINOMYCES NAESLUNDII AND STREPTOCOCCUS MUTANS IN A FLOW ENVIRONMENT
5.1 Abstract

*C. albicans*, *A. naeslundii* and *S. mutans* have been shown to exist as polymicrobial biofilms in the oral cavity. The aim of this study was to determine the effect of polymicrobial interactions of OSCC-isolated *C. albicans* (ALC3), *A. naeslundii* and *S. mutans* on biofilm formation in a flow environment. To study mono-cultured biofilm formation, *C. albicans*, *A. naeslundii* and *S. mutans* were inoculated in 25% artificial saliva medium (ASM), and standardised to a final density of $10^6$ cells mL$^{-1}$, $10^7$ cells mL$^{-1}$ and $10^8$ cells mL$^{-1}$ respectively in separate 15 mL tubes. Cell suspensions (3 mL) were inoculated into a flow-cell system prior to commencement of a constant medium flow rate of 3 mL h$^{-1}$ for 24 h at 37 °C. To study polymicrobial biofilm formation, the same protocol was repeated, except that the inoculum that was standardised to the same cell density as used in the mono-cultured biofilm assay, was prepared in the same vial. The biofilms were fixed with 50% ethanol, embedded in 20% gel acrylamide, stained by fluorescent *in situ* hybridisation (FISH) using DNA species-specific probes, imaged using confocal scanning laser microscopy (CSLM) and analysed using COMSTAT software. The biomass of *C. albicans* and *S. mutans* in polymicrobial biofilms exhibited significant decreases ($P < 0.05$) compared to mono-cultured biofilms. The roughness coefficient of polymicrobial biofilms exhibited a significant increase compared to mono-cultured *C. albicans* ($P < 0.05$), however, a significant decrease was observed when compared to mono-cultured *A. naeslundii* ($P < 0.05$). Significant increases of average thickness and maximum thickness of polymicrobial biofilms were observed when compared to mono-cultured *C. albicans* ($P < 0.05$) and *A. naeslundii* ($P < 0.05$), however, significant decreases of the parameters were observed in polymicrobial biofilms when compared to mono-cultured *S. mutans* biofilm ($P < 0.05$). A significant increase of
surface colonisation was observed in polymicrobial biofilms when compared to mono-cultured *A. naeslundii* (*P* < 0.05) and *S. mutans* biofilms (*P* < 0.05), however, a significant decrease was observed when compared to mono-cultured *C. albicans* biofilm. In conclusion, *C. albicans*, *A. naeslundii* and *S. mutans* formed polymicrobial biofilms. The inclusion of *A. naeslundii* in these biofilms resulted in a decrease in both *C. albicans* and *S. mutans*. This may mean that *A. naeslundii* can be potentially used as a probiotic to control *C. albicans* and *S. mutans* colonisation.
5.2 Introduction

Oral microorganisms, including *C. albicans*, *A. naeslundii* and *S. mutans*, have been shown to exist as components of complex polymicrobial biofilms in the oral cavity (Kolenbrander et al., 2002; El-Azizi et al., 2004; Foster and Kolenbrander, 2004; Kolenbrander et al., 2010). However, slight changes in the microenvironment such as microbial interactions, nutrient supply and shear forces may affect the dynamic structure of polymicrobial biofilms (Morales and Hogan, 2010; Kolenbrander et al., 2010; Diaz et al., 2012; Zhu et al. 2013; Marsh et al., 2016). Synergies and antagonisms between microorganisms such as the interactions between *C. albicans*, *A. naeslundii* and *S. mutans* have been previously shown during planktonic growth (Chapter 3) that have been suggested to affect the dynamic structure of oral biofilms (Arzmi et al., 2015). Furthermore, the different nutrient composition of the medium used to grow polymicrobial biofilms, such as 25% ASM and RPMI-1640, was shown to affect *C. albicans* morphology, biomass and metabolic activity in static biofilms (Chapter 4). In addition, mucin containing ASM has been shown to provide binding sites for the attachment of early colonisers to the substratum (de Repentigny et al., 2000; Derrien et al., 2010) whereas sucrose containing media has been reported to induce the synthesis of glucosyltransferases (Gtfs) from *S. mutans* that assist in the formation of polymicrobial biofilms (Falsetta et al., 2014).

*C. albicans*, *A. naeslundii* and *S. mutans* are important members of the oral microbiome (Nobbs and Jenkinson, 2015; Höfs et al., 2016). *C. albicans* is the most prevalent opportunistic and pathogenic fungus that can cause oral candidosis (Kim and Sudbery, 2011). The yeast has also been found to associate with leukoplakic lesions and is recognised as an independent risk factor for oral carcinoma (Cawson, 1969a). Transition of yeast to hyphae is usually related to the ability of *C. albicans* to
colonise oral surfaces. Yeast cells are predominantly found to colonise the surface of oral substrata, whereas hyphal cells are frequently found during invasive colonisation of mucosal cells (Finkel and Mitchell, 2011; Banerjee et al., 2013). *A. naeslundii* has been categorised among the pioneer colonisers that may constitute up to 27% of supragingival dental plaque (Nyvad and Kilian, 1987; Li et al., 2004), whereas *S. mutans* is a Gram-positive, facultative anaerobic bacterium that utilises a broad spectrum of sugars and excretes organic acids that leads to the increase of acidity in plaque inducing dental caries (Takahashi and Nyvad, 2011; Burne et al., 2012). *S. mutans* is also known as one of the most important members of the oral microbiome that supports the structure of mature oral biofilms (Sztajer et al., 2014).

The microbial balance in the oral cavity can be disrupted by various factors including a high carbohydrate diet, which can lead to *C. albicans* infection (Williams et al. 2011). Thus, a balance has to be maintained in order to limit colonisation and proliferation of opportunistic pathogens or pathobionts in the oral cavity. The idea of microbial homeostasis led to the discovery of prebiotics, which are nutritional supplements that beneficially affect the host by improving the microbial balance of the intestine (Fuller, 1989). Later, probiotics were discovered which have been defined as live microorganisms that provide health benefits to the host when administered in adequate amounts (Salminen et al., 1998). Even though probiotics have been suggested to provide benefits to human health, side effects may be associated when bacteria are consumed.

According to a 2002 report released by the World Health Organisation (WHO), there are four types of side effects including systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer (Doron and Snydman, 2015). The use of a probiotic may in some ways
be beneficial in changing the flora but this in turn may result in an imbalance of the normal microbiome and lead to the colonisation of opportunistic microorganisms (dysbiosis). Even though infection caused by the consumption of probiotics is rare, septicaemia and endocarditis caused by Lactobicilli spp. has been reported with the majority of infection cases due to the patient’s normal microbiome (Marteau and Shanahan, 2003). Thus, the use of any probiotic must be rigorously assessed prior to use and consequently stringently controlled.

In 1995 synbiotics were discovered; they are a mixture of probiotics and prebiotics that provide benefits to the host by improving the survival and implantation of dietary supplements containing live microbes, by selectively stimulating the growth and/or by activating one or a limited number of health-promoting bacteria (Lilly and Stillwell, 1965; Kojima et al., 2016).

The aim of the present study was to determine the effect of polymicrobial interactions of C. albicans, A. naeslundii and S. mutans on biofilm formation in a flow environment; with the hypotheses that C. albicans, A. naeslundii and S. mutans form polymicrobial biofilms and that polymicrobial interactions of C. albicans, A. naeslundii and S. mutans affect colonisation of oral microorganisms.
5.3 Materials and methods

*C. albicans* isolated from an oral cancer patient (ALC3) was sub-cultured on Sabouraud’s dextrose agar (SDA) (Difco, USA) and incubated at 37 °C aerobically for 24 h. Stock cultures of *A. naeslundii* (NCTC 10301) and *S. mutans* (Ingbritt), were revived by sub-culturing onto blood agar (Difco, USA) and Todd-Hewitt yeast extract agar (Difco, USA) respectively. The agar plates were incubated at 37 °C for 48 h (Section 2.1). Following that, biofilms were developed in a flow-cell system for 24 h at 37 °C (Section 2.8; Section 2.9), embedded in gel acrylamide (Section 2.10), labelled using Fluorescence In Situ Hybridisation (FISH) (Section 2.11), visualised by CLSM (LSM 510 Meta, Carl Zeiss, Germany; Section 2.12) and analysed using COMSTAT to determine the roughness coefficient, biofilm biomass, average thickness and maximum thickness and percentage surface colonisation (Heydorn *et al.*, 2000; Section 2.12).

The biometric data were statistically analysed using SPSS software version 22.0 by applying ANOVA with a post hoc Tukey test to compare biometric parameters of *C. albicans*, *A. naeslundii* and *S. mutans* in mono-cultured and polymicrobial biofilms between replicates. The biometric data were statistically analysed using SPSS software version 22.0. An independent *t*-test was applied to compare the biometric parameters between mono-cultured and polymicrobial biofilms except for the mono-cultured *A. naeslundii*, which was analysed using the Mann-Whitney test due to the wide standard deviation (non-parametric data). Statistical analyses were considered significant when *P* < 0.05.
5.4 Results

5.4.1 Mono-cultured biofilms of *C. albicans*, *A. naeslundii* and *S. mutans*

The present study showed that *C. albicans*, *A. naeslundii* and *S. mutans* were able to form biofilms in a flow environment when grown in mono-culture after 24 h incubation (Figure 5.1A, Figure 5.1B, Figure 5.1C). Of all three mono-cultured biofilms, *S. mutans* had the largest biomass \((27.70 \pm 2.83 \, \mu m^3 \, \mu m^{-2})\), average thickness \((32.03 \pm 1.96 \, \mu m)\) and maximum thickness \((35.13 \pm 2.87 \, \mu m)\). Conversely, the mono-cultured *A. naeslundii* biofilm exhibited the smallest biomass \((0.85 \pm 0.68 \, \mu m^3 \, \mu m^{-2})\) and average thickness \((4.26 \pm 2.98 \, \mu m)\), being 32-times and 8-times lower than that of *S. mutans* respectively (Table 5.1; Table 5.2). In addition, the mono-cultured *C. albicans* biofilm had the smallest maximum thickness \((15.53 \pm 4.47 \, \mu m)\) being 2-times lower than for *S. mutans* (Table 5.2).

5.4.2 Polymicrobial biofilms of *C. albicans*, *A. naeslundii* and *S. mutans*

*C. albicans*, *A. naeslundii* and *S. mutans* formed biofilms in a flow environment when grown as polymicrobial biofilms after 24 h incubation based on the average values over three biological replicates (Figure 5.1D). The total biomass of polymicrobial biofilms was \(2.017 \pm 0.088 \, \mu m^3 \, \mu m^{-2}\) with the biomasses of *C. albicans*, *A. naeslundii* and *S. mutans* that formed polymicrobial biofilms being \(0.94 \pm 0.24 \, \mu m^3 \, \mu m^{-2}\) (46.8%), \(0.66 \pm 0.81 \, \mu m^3 \, \mu m^{-2}\) (32.8%) and \(0.41 \pm 0.27 \, \mu m^3 \, \mu m^{-2}\) (20.4%) respectively (Table 5.1). The roughness coefficient, average thickness, maximum thickness and percentage colonisation of polymicrobial biofilms was \(0.78 \pm 0.40 \, Ra\), \(8.54 \pm 6.62 \, \mu m\), \(22.47 \pm 4.47 \, \mu m\) and 13.07%, respectively (Table 5.2). In addition, the maximum thickness of the polymicrobial biofilms was \(22.40 \pm 4.50 \, \mu m\).
5.4.3 Effect of polymicrobial interaction on *C. albicans*, *A. naeslundii* and *S. mutans* biofilms

A variation in the biometric parameters of biofilms was observed when comparisons were made between mono-cultured *C. albicans*, *A. naeslundii* and *S. mutans* with polymicrobial biofilms (Table 5.2). The present study showed that mono-cultured *S. mutans* biofilms exhibited statistically significant larger average thickness (32.03 ± 1.96 µm), maximum thickness (35.15 ± 2.87 µm) and surface colonisation (29.94%) compared to the polymicrobial biofilms (8.54 ± 6.62 µm, 22.47 ± 4.47 µm and 13.07%, respectively; *P* < 0.05; Table 5.2).
Figure 5.1A Representative CLSM image of mono-cultured *C. albicans* as observed using a 63x objective at 512 x 512 pixels magnification. Biofilm was developed on ASM-coated glass substratum in a flow-cell system for 24 h (3 mL h⁻¹) at 37 °C to form biofilm. The biofilm was embedded in gel acrylamide, labelled using FISH technique and visualised by CLSM (LSM 510 Meta, Carl Zeiss, Germany.)
Figure 5.1B Representative CLSM image of mono-cultured *A. naeslundii* as observed using a 63x objective at 512 x 512 pixels magnification.
Figure 5.1C Representative CLSM image of mono-cultured *S. mutans* as observed using a 63x objective at 512 x 512 pixels magnification.
Figure 5.1D Representative CLSM image of polymicrobial biofilms as observed using a 63x objective at 512 x 512 pixels magnification (Red: *C. albicans*; Green: *A. naeslundii*; Blue: *S. mutans*).
Table 5.1 Total biomass (µm$^3$ µm$^{-2}$) of ASM-grown *C. albicans*, *A. naeslundii* and *S. mutans* after 24 h incubation in a flow-cell (3 mL h$^{-1}$) at 37 °C in mono-cultured biofilm and polymicrobial biofilms.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Mono-cultured biofilm (µm$^3$ µm$^{-2}$)</th>
<th>Polymicrobial biofilms (µm$^3$ µm$^{-2}$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>4.43 (1.21)</td>
<td>0.94 (0.24)</td>
<td><em>P &lt; 0.05</em>#</td>
</tr>
</tbody>
</table>
| *A. naeslundii*| 0.85 (0.68)                              | 0.66 (0.81)                               | *P < 0.05* |*
| *S. mutans*    | 27.70 (2.83)                             | 0.41 (0.27)                               | *P < 0.05*# |

Data are means from three separate experiments (SD are given in parenthesis). Data were analysed using independent t-test# and Mann-Whitney* to compare between mono-cultured and polymicrobial biofilms of specific microorganisms. Data were considered as significantly different when $P < 0.05$. 

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129
Table 5.2 Surface roughness, average and maximum thickness and percentage surface colonisation of ASM-grown *C. albicans*, *A. naeslundii* and *S. mutans* after 24 h incubation in a flow-cell (3 mL h\(^{-1}\)) at 37 °C.

<table>
<thead>
<tr>
<th></th>
<th><em>C. albicans</em> mono-cultured biofilm</th>
<th><em>A. naeslundii</em> mono-cultured biofilm</th>
<th><em>S. mutans</em> mono-cultured biofilm</th>
<th>Polymicrobial biofilms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>Roughness coefficient (Ra)</td>
<td>0.55 (0.17)</td>
<td>1.24 (0.43)</td>
<td>0.12 (0.05)</td>
<td>0.78 (0.40)</td>
</tr>
<tr>
<td></td>
<td><em>P</em> &gt; 0.05</td>
<td><em>P</em> &lt; 0.05</td>
<td><em>P</em> &gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Average thickness (µm)</td>
<td>4.59 (0.73)</td>
<td>4.26 (2.98)</td>
<td>32.03 (1.96)</td>
<td>8.54 (6.62)</td>
</tr>
<tr>
<td></td>
<td><em>P</em> &lt; 0.05</td>
<td><em>P</em> &lt; 0.05</td>
<td><em>P</em> &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Maximum thickness (µm)</td>
<td>8.98 (2.05)</td>
<td>15.53 (4.47)</td>
<td>35.13 (2.87)</td>
<td>22.47 (4.47)</td>
</tr>
<tr>
<td></td>
<td><em>P</em> &lt; 0.05</td>
<td><em>P</em> &lt; 0.05</td>
<td><em>P</em> &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Surface colonisation (%)</td>
<td>45.63 (25.25)</td>
<td>3.66 (2.43)</td>
<td>29.94 (3.74)</td>
<td>13.07 (4.01)</td>
</tr>
<tr>
<td></td>
<td><em>P</em> &lt; 0.05</td>
<td><em>P</em> &lt; 0.05</td>
<td><em>P</em> &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Data are means from three separate experiments (SD are given in parenthesis). Data were analysed using independent t-test* to compare between mono-cultured and polymicrobial biofilms of the same biometric (e.g. ‘Roughness coefficient’ of mono-cultured *C. albicans* biofilm was compared with the ‘roughness coefficient’ of polymicrobial biofilms). Green showed significantly higher and red showed significantly lower in mono-cultured biofilms compared to polymicrobial biofilms. Data were considered as significantly different when *P* < 0.05.
5.5 Discussion

To our knowledge, this is the first study to evaluate polymicrobial biofilm formation by *C. albicans*, *A. naeslundii* and *S. mutans* in a flow-cell environment using an artificial saliva medium. The present study measured five different biometric parameters; the roughness coefficient, biofilm biomass, average biofilm thickness, maximum biofilm thickness and percentage surface colonisation of the cells in the biofilm.

The present study showed that *C. albicans*, *A. naeslundii* and *S. mutans* formed biofilms on artificial saliva-coated substratum when grown as a mono-cultured and polymicrobial biofilm in a flow-cell environment, thus supporting the hypothesis that *C. albicans*, *A. naeslundii* and *S. mutans* form biofilm in a flow environment. In this system, *A. naeslundii* was a very poor biofilm former, whilst *S. mutans* produced a robust biofilm when grown as a mono-cultured biofilm. Studies using gamma-irradiated Stovall flow-cell systems (40 mm long, 4 mm wide and 1 mm deep) have shown that *A. naeslundii* and *S. mutans* form mono-cultured biofilms when grown in sucrose containing tryptic soy broth and ASM respectively (Dashper *et al.*, 2013; Blanc *et al.*, 2014; Arai *et al.*, 2015). Furthermore, *C. albicans* and *S. mutans* have been shown to form biofilms both mono-cultured and co-cultured in a flow environment using saliva supplemented with HIB and PBS in a flow-cell track (40 mm long, 3 mm wide and 2 mm deep) (Diaz *et al.*, 2012). An *in vitro* study using a flow-cell system was shown to simulate the conditions encountered by microorganisms in the oral cavity such as shear stress rates due to salivary flow (Sánchez-Vargas *et al.*, 2013). A more robust biofilm formed by *S. mutans* in the flow environment is suggested due to the ability of the bacterium to utilise sucrose.
from 25% ASM subsequently forming extracellular polysaccharides (EPS) through glucosyltransferases (Koo et al., 2010; Bowen and Koo, 2011; Ren et al., 2016). This did not occur with *A. naeslundii* in the present study, where poor biofilm forming ability was observed. It could be that the EPS which are formed in the flow environment promote the adherence of *S. mutans*, thus enhancing the development of a mono-cultured *S. mutans* biofilm. Further study such as the quantification of EPS is required to support this hypothesis.

The biomass of the polymicrobial biofilms was significantly reduced compared to the biomass of *C. albicans* and *S. mutans* mono-cultured biofilms. Furthermore, the percentage surface colonisation in the polymicrobial biofilms was significantly lower than for mono-cultured *C. albicans* and *S. mutans* biofilms, but not for *A. naeslundii*. These findings support our hypothesis that polymicrobial interactions affected microbial colonisation in a flow environment. Even though *in vitro* studies have shown that mutualistic interactions between *C. albicans* and *S. mutans* occur through adhesins (non-specific) and lectin-saccharide cell surface receptors (specific) bindings (McIntire et al., 1982; Rickard et al., 2003; Rosen and Sela, 2006; Ledder et al., 2008), antagonism between the two species has also been reported (Thein et al., 2006). *C. albicans* has been shown to decrease adherence when co-cultured with *S. mutans* on acrylic sheets in Gibbons and Nygaard culture medium (Barbieri et al., 2007). Furthermore, the quorum-sensing molecule Farnesol that is synthesised by *C. albicans* during biofilm formation has been reported to disrupt the membrane of *S. mutans*, as well as the accumulation of polysaccharide contents of streptococcal biofilms (Koo et al., 2003; Jabra-Rizk et al., 2006).

Our study has shown that the biomass of both *C. albicans* and *S. mutans* in polymicrobial biofilms was significantly decreased more than 50% compared to the
mono-cultured biofilms. Furthermore, a negative effect was also observed in the surface colonisation of *C. albicans* and *S. mutans* which exhibited a significant decrease in polymicrobial biofilms. It would appear that *A. naeslundii* may have some potential as a probiotic to inhibit the colonisation of *C. albicans* in the oral cavity. Antagonism has been reported between *C. albicans* and *A. naeslundii* (Millsap *et al.*, 1999; Thein *et al.*, 2006). *A. naeslundii* T14V-J1 has been shown to suppress the adhesion of *C. albicans* ATCC 10261 when grown in a flow-cell chamber (Millsap *et al.*, 2000). The metabolic products of *A. naeslundii* have been reported to both inhibit and stimulate the biofilm formation of *C. albicans*, depending on the experimental methods employed (Gutiérrez and Benito, 2004; Thein *et al.*, 2006). In addition, antagonism between *S. mutans* and *A. naeslundii* has been widely reported due to the production of H$_2$O$_2$ and bacteriocins by *Streptococcus* spp. (Jakubovics *et al.*, 2008; Avila *et al.*, 2009; Zhu and Kreth, 2012). Although antagonism between *A. naeslundii* and *S. mutans* has been reported, co-aggregation assays have shown that both species can grow in close proximity (Zhu and Kreth, 2012; Arzmi *et al.*, 2015). It may be that the cell densities of the microorganisms and *C. albicans* morphology in polymicrobial biofilms influenced the interaction between *C. albicans*, *A. naeslundii* and *S. mutans*. A previous study has shown that polymicrobial biofilms that were generated from an inoculum of 10$^7$ cells mL$^{-1}$ of *C. albicans* (hyphal growth) and 10$^8$ cells mL$^{-1}$ of bacteria (*Streptococcus oralis* and *Actinomyces oris*) in a static biofilm system exhibited synergism between microorganisms (Cavalcanti *et al.*, 2016). In contrast, antagonism between *C. albicans* and Actinomyces israelii has also been reported in static polymicrobial biofilms (Thein *et al.* 2006).
In the present study, we observed that all three microorganisms had similar maximum thickness in polymicrobial biofilms. However, there was significant variation when grown in mono-culture with *S. mutans* having the greatest maximum thickness. These findings support the hypothesis of the present study that polymicrobial interactions affect colonisation of oral microorganisms. *A. naeslundii* is an early oral coloniser that binds to proline-rich proteins of the salivary pellicle (Kolenbrander *et al.*, 2010). In the presence of high sucrose in 25% ASM, *S. mutans* has been reported to produce large amounts of glucosyltransferases that aid the attachment of *C. albicans* and *A. naeslundii* through glucan binding proteins (Koo *et al.*, 2010). Gtfs are associated with the production of EPS, the prime building blocks of dental plaque (Koo *et al.*, 2010), and this is most likely the principle contributor to the extracellular component of the polymicrobial biofilm. Complex polymicrobial biofilms in the oral cavity are associated with a number of disease states, such as oral candidosis, dental caries and periodontal diseases (Harriott and Noverr, 2011).

Based on the static biofilm study of eight strains of *C. albicans*, it has been shown that the biofilm formation is *C. albicans* strain-dependent (chapter 4). However, a similar claim is inappropriate for *A. naeslundii* and *S. mutans* since there were only *A. naeslundii* (NCTC 10301) and *S. mutans* (Ingbritt) have been used in the study. It is suggested that different strain of *A. naeslundii* and *S. mutans* may form different biofilm biometric parameters when grown in flow-cell system. Previous study has shown that 44 genotypes of *S. mutans* were producing different range of biofilm due to the variable amount of glucosyltransferases (Gtfs), particularly GtfB and GtfC expressed by *S. mutans* (Mattos-Graner *et al.*, 2004). Furthermore, *A. naeslundii* genospecies 2 has been the most frequent isolated from adults compared to genospecies 1 (Paddick *et al.*, 2003). Therefore, it can be postulated that biofilm
formation may also be *A. naeslundii* and *S. mutans* strain-dependent. Further research is required to support this hypothesis.

**5.6 Conclusion**

In conclusion, *C. albicans*, *A. naeslundii* and *S. mutans* formed polymicrobial biofilms in a flow environment. The overall biomass of the polymicrobial biofilm was low relative to mono-cultured biofilms indicating significant antagonistic interactions between these species. This was shown to affect the surface roughness, biofilm thickness and surface colonisation in a flow-cell environment. Furthermore, *A. naeslundii* may have some potential as a probiotic to control *C. albicans* and *S. mutans* overgrowth, providing a dynamic balance between *C. albicans*, *A. naeslundii* and *S. mutans*. Thus, these interactions are likely to play significant roles in the pathogenicity of oral microorganisms, plaque formation, dysbiosis and oral diseases (Kolenbrander, 2000; Sbordone and Bortolaia, 2003; Min and Rickard, 2009; Morales and Hogan, 2010).
CHAPTER 6

BIOFILM EFFLUENT OF CANDIDA ALBICANS, ACTINOMYCES NAESLUNII AND STREPTOCOCCUS MUTANS AFFECT THE ADHESION, EPITHELIAL MESENCHYMAL TRANSITION AND CYTOKINE EXPRESSION OF NORMAL AND MALIGNANT ORAL KERATINOCYTES
6.1 Abstract

Microbial infections, including those caused by *C. albicans*, *S. mutans* and *A. naeslundii* have been suggested to play a role in carcinogenesis. Malignant tumours such as carcinomas are characterised by the ability of tumour cells to invade the underlying connective tissues followed by migration to form metastases at distant sites. In this context, epithelial to mesenchymal transition (EMT) has been shown to assist in cell migration through extracellular matrix (ECM), by inducing the formation of the mesenchymal phenotype of epithelial cells, which is important for metastasis. This pro-invasive phenotype associates with an altered integrin-ECM adhesion and inflammation induced by pathogens has been suggested to be involved in determining characteristics of the tumour microenvironment. In the present study, we assessed the ability of microbial biofilm effluent obtained from *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM) and poly-microbial (TRI) biofilms, to induce a pro-invasive phenotype in oral epithelial cells. To study EMT, OKF6 (normal oral epithelial cell) and H357 (oral squamous cell carcinoma) cell lines were incubated for 2 h and 24 h at 37 °C, 5% CO₂, in test cell growth media (80% serum free medium). The cells were collected and flow cytometry was undertaken for the detection of vimentin and E-cadherin. CytoSelect 48-well Cell Adhesion Assay ECM Array kit was used to study the adhesion of OKF6 and H357 to ECM components. Simultaneously, the conditioned medium was collected and the presence of cytokines was detected using Bio-Plex. The present study showed that the incubation of H357 in ALC3 effluent significantly increased the adhesion of these malignant cells to collagen IV and laminin I when compared to control non-effluent (NE) (*P* < 0.05). Furthermore, a significant decrease of vimentin was observed after 24 h incubation when incubated with ALC3 compared to NE (*P* < 0.05). ALC3 effluent was also found to
significantly increase the expression of IL-10 and GM-CSF from H357 after 2 h incubation ($P < 0.05$), and IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF and TNF-α after 24 h compared to NE ($P < 0.05$). Finally, an increase of the majority of pro-inflammatory cytokines in H357 incubated with ALC3 and TRI effluent was observed after 24 h incubation compared to 2 h. Overall, the majority of H357 incubated in biofilm effluent increased adhesion to ECM components, and significantly increased the expression of inflammatory cytokines. For OKF6, the majority of cells showed significantly decreased adhesion to ECM components ($P < 0.05$) and exhibited no change in cytokine expression when compared to NE. Taken together these results demonstrated that the adhesion of OKF6 and H357 to ECM, EMT and cytokine expression, are biofilm effluent-dependent. Furthermore, the biofilm effluent affect on the adhesion, EMT and cytokine expression from OKF6 and H357 showed an enhanced malignant phenotype when grown in the presence of polymicrobial biofilm effluent; this may act as a promoter of oral cancer but most likely not as an initiator.
6.2 Introduction

Cancer is the leading cause of death in developed countries and the second in the developing countries (Jemal et al., 2011). In 2008, about 12.7 million cancer cases were reported worldwide, and of these, 263,900 were oral cancer (Warnakulasuriya, 2009; Jemal et al., 2011). The highest oral cancer rates are found in South-Central Asia, and Central and Eastern Europe whereas the lowest cases are in Africa, Central America and Eastern Asia (Jemal et al., 2011). In countries such as Pakistan, Bangladesh, India and Sri Lanka, oral cancer is the most common cancer and accounts for up to 30% of all diagnosed cancers (Warnakulasuriya, 2009). In Malaysia, oral cancer has been the 20th most common cancer in females and 28th for males with the highest rates being reported for Indian ethnicity followed by Malay and Chinese (Ashazila et al., 2011).

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of malignancies originating from the oral mucosa (Casiglia and SB, 2001; Johnson et al., 2011). It has been reported that the average 5-year survival rate for oral cancer is less than 50% (Zakrzewska, 1999). These poor figures largely reflect the tumour stage at presentation as well as the development of loco-regional recurrences and distant metastases. Hence, the acquisition of a metastatic phenotype is of paramount importance in determining oral cancer progression and prognosis. The risk factors that lead to OSCC include tobacco smoking, heavy alcohol consumption, poor oral hygiene, unhealthy diets, and microbial infections (Hooper et al., 2009; Chocolatewala et al., 2010; Meurman, 2010; Rajeev et al., 2012; Khajuria and Metgud, 2015).

Microbial infections by yeasts such as C. albicans, bacteria and viruses have been widely suggested to have a causal role in oral cancer (Rodriguez et al, 2007;
Scheper et al., 2008; Hooper et al., 2009; Chocolatewala et al., 2010; Meurman, 2010; Rajeev et al., 2012; Marttila et al., 2013; Khajuria and Metgud, 2015). The aetiological role of Candida spp. in oral mucosal keratoses progression to carcinoma has been suggested since 1966 with the majority of non-homogenous leukoplakias invaded by C. albicans and these have higher malignant transformation potential than the homogenous leukoplakia (Cawson, 1969a; Cawson, 1969b; Meurman, 2010). Furthermore, oral yeast carriage has also been found to correlate with the presence of oral epithelial dysplasia, which supports the role of microbial infections in oral carcinogenesis (McCullough et al., 2002).

Streptococcus spp. and Actinomyces spp. have been postulated to be involved in oral carcinogenesis. An in vitro study on S. mutans has shown that this bacterium is able to synthesise the alcohol dehydrogenase enzyme that converts alcohol to carcinogenic acetaldehyde, widely reported to be involved in oral carcinogenesis (Kurkivuori et al., 2007; Hooper et al., 2009). Furthermore, study of the oral microbiome of patients with OSCC has shown increased numbers of Actinomyces spp., including A. naeslundii, compared to the individuals with a healthy oral cavity (Nagy et al., 1998; Pushalkar et al., 2011).

Carcinoma is characterised by the ability of the malignant cell to invade the underlying connective tissues followed by migration to form metastases at distant sites (Lyons and Jones, 2007). These processes require the alteration of cell to cell and cell to extracellular matrix (ECM) interactions that involves adhesion molecules such as collagen, laminin, fibronectin and fibrinogen (Ahmed et al., 2005; Lyons and Jones, 2007). These cell adhesion-proteins have been shown to promote the attachment and migration of cancerous cells to surrounding ECM and are believed to be involved in tumour cell survival, metastasis and angiogenesis (Fabricius et al.,
Integrins, which are in contact with complementary ECM molecules, regulate normal cell behavior and alterations of integrin-mediated cell adhesion machinery have been implicated in oral carcinogenesis (Rathinam and Alahari, 2010; Fabricius et al., 2011).

Epithelial-mesenchymal transition (EMT) is a mechanism of alteration of cell-to-cell and cell-to-ECM interaction that allows the movement of the epithelial cells to the surrounding environment (Radisky, 2005). This mechanism has been shown to assist in cell migration through ECM by inducing the formation of the mesenchymal phenotype of the epithelial cell. In normal conditions, epithelial cell structure is maintained by cell-to-cell interactions such as cadherin-based adherent junctions and desmosomes, whereas mesenchymal cells are mostly without direct contact or defined cell polarity, but have distinct cell-to-ECM interactions and cytoskeletal structures (Radisky, 2005). An inappropriate utilisation of EMT may occur in the formation of OSCC and metastasis of malignant cells (Kang and Massagué, 2004; Yang et al., 2004; Radisky, 2005). Furthermore, EMT has also been reported to be involved in the increase of resistance of malignant cells to apoptosis regulator molecules (Maestro et al., 1999; Vega et al., 2004).

Vimentin and E-cadherin have been widely used as markers for EMT (Hugo et al., 2007). Vimentin is a protein that belongs to type III intermediate filaments (IF). IFs are expressed by nearly all eukaryotic cells and are composed of proteins that provide mechanical strength to the structure of tissues (Cooper, 2000). The expression of vimentin has been shown to induce the changes in cell shape, motility and adhesion of epithelial cells to mesenchymal (Mendez et al., 2010). During EMT, epithelial cells lose adhesion to neighbouring cells and change shape to be elongated and flat, a common morphology of mesenchymal cells. During this process, vimentin
is expressed, and this correlates with both mesenchymal shape and enhanced motility (Mendez et al., 2010). Further, vimentin has been shown to be expressed in vivo during tumorigenesis and metastasis in prostate cancer and metastatic breast carcinoma (Lang et al., 2002). Therefore, the increased amount of vimentin from malignant oral tissue is an indicator of malignancy (Lang et al., 2002; Hugo et al., 2007; Nijkamp et al., 2011).

Cadherins have been shown to mediate cell-to-cell binding that is critical in maintaining tissue structure and morphology (Gumbiner, 2005). E-cadherin is a glycoprotein that establishes homophilic interactions with E-cadherin-adjacent-molecules expressed by neighbouring cells to produce the core of epithelial adherence junction (Nagafuchi et al., 1987; Gumbiner, 2005). Functional loss of E-cadherin in epithelial cells has been considered as a marker for EMT during tumour progression (Onder et al., 2008; Nijkamp et al., 2011; Yadav et al., 2011). Cells expressing E-cadherin have been reported to be silenced by a number of different mechanisms including transcriptional repression (Bolós et al., 2003), histone deacetylation (Peinado et al., 2004), down-regulation of gene expression through promoter hypermethylation (Hasegawa et al., 2002) and somatic mutation (Berx et al., 1995).

Inflammation induced by pathogens has been shown to be involved in carcinogenesis, particularly after the classification of Helicobacter pylori as a class-1 carcinogen in humans by the World Health Organization (WHO) International Agency for Research on Cancer (IARC) (Peek and Blaser, 2002, Björkholm et al., 2003, Correa and Houghton, 2007). One of the factors that leads to inflammation is the increase of pro-inflammatory cytokines due to microbial infection of oral mucosa (Fantini and Pallone, 2008). Cytokines are signalling molecules that regulate the differentiation, proliferation and many other important functions of human
inflammatory cells. Cytokines are important in host defence and their release from
infected tissues has been shown to activate effector immune cells (leukocytes),
subsequently activating a cascade of specific defence mechanisms towards pathogens.
The cytokines that have been shown to be involved in inflammation include
interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-18, tumour necrosis factor (TNF)-α, IFN-γ
and GM-CSF (Dongari-Bagtzoglou et al., 1999; Rouabhia et al., 2002, Schaller et al.,
2002, Steele and Fidel, 2002, Dongari-Bagtzoglou and Kashleva, 2003a, Dongari-

Despite considerable clinical and experimental evidence describing the
association between C. albicans and malignant transformation, no study has been
carried out to investigate the effect of C. albicans, S. mutans and A. naeslundii biofilm
effluent on the adhesion of normal and malignant oral keratinocytes. Furthermore, the
paracrine regulation of EMT from C. albicans alone or in the context of polymicrobial
biofilms has never been investigated. In particular, no study has been conducted
regarding the effect of effluent from polymicrobial biofilms formed by the yeast C.
albicans, S. mutans and A. naeslundii on the expression of vimentin and E-cadherin in
the normal oral epithelial cell OKF6 and the OSCC cell line H357. These cell lines
were selected in the present study to determine the role of effluent from biofilms
formed by C. albicans, S. mutans and A. naeslundii to: (1) affect the adhesion of
normal and OSCC cell lines to ECM molecules (fibronectin, collagen I, collagen IV,
laminin I, and fibrinogen), (2) to affect EMT of normal and OSCC cell lines as
indicated by the expression of vimentin and E-cadherin, and (3) to affect cytokine
expression (IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF and IFN-γ) of normal and
OSCC cell lines. Collectively, we hypothesised that the biofilm effluent affects the
adhesion and EMT of OKF6 and H357, and the expression of pro-inflammatory
cytokines from the cell lines, which enhances their malignant phenotype when grown in the presence of polymicrobial biofilm effluent and may have a role in oral carcinogenesis.
6.3 Materials and methods

OKF6, a normal human oral epithelial cell and H357, cell isolated from the tongue of patient with OSCC, were grown as described in Section 2.13. Subsequently, Cell-ECM adhesion was conducted (Section 2.15) to determine the adhesion of OKF6 and H357 to extracellular proteins of fibronectin, collagen I collagen IV, laminin and fibrinogen when treated with test cell growth media (Section 2.14) using CytoSelect 48-well Cell Adhesion Assay ECM Array kit (Cell Biolabs, USA). The epithelial-mesenchymal transition (EMT) assay was conducted to determine the expression of E-cadherin and vimentin (Section 2.17), whereas the expression of pro-inflammatory cytokines from OKF6 and H357 which have been grown in biofilm effluent was assessed with Bio-plex assays (Section 2.18). Test cell growth medium was prepared using SFM (DMEM/F12 and k-SFM for H357 and OKF6 respectively), that was diluted in biofilm effluent (Section 2.9) of (1) C. albicans (ALC3), (2) A. naeslundii (AN), (3) S. mutans (SM) and poly-microbial (TRI), and non-effluent 25% ASM (NE), to give a final concentration of 80% (v/v) SFM (Steele and Fidel, 2002). NE is the control to determine the role of biofilm effluent on cell-ECM adhesion, EMT and the expression of cytokines from OKF6 and H357.

All data were statistically analysed using SPSS Statistic software version 22.0 using ANOVA post hoc Dunnett’s test to compare the adhesion, EMT and cytokine expression of cells incubated with biofilm effluent to control (NE), and to compare between two time points for each assay. ANOVA was chosen due to the data being normally distributed and the post hoc Dunnett’s test was chosen due to the presence of the control group (NE). Results were considered as statistically significant when $P < 0.05$. 
6.4 Results

Part I: Adhesion assay

6.4.1 Adhesion of OKF6 to ECM

Incubation with ALC3 effluent caused a significant decrease in OKF6 adhesion to fibronectin, collagen I, collagen IV and laminin I compared to incubation with NE \((P < 0.05; \text{Table 6.1A})\). Incubation with AN effluent caused a significant decrease of adhesion to fibronectin, collagen I and laminin \((P < 0.05)\), whereas incubation with SM effluent showed decrease of adhesion to collagen IV and fibrinogen significantly when compared to the incubation in NE \((P < 0.05; \text{Table 6.1A})\). Furthermore, incubation of OKF6 with TRI caused a significant decrease of adhesion to collagen I and fibrinogen when compared to the incubation in NE \((P < 0.05)\). There was only one situation where OKF6 showed significantly enhanced adhesion compared to NE, and that was when incubated with \(S. \text{mutans}\) effluent where adhesion to fibronectin was increased \((P < 0.05; \text{Table 6.1A})\).

6.4.2 Adhesion of H357 to ECM

Incubation with ALC3 effluent caused a significant decrease of H357 adhesion to fibronectin and fibrinogen \((P < 0.05)\). However, significant increases were observed in the adhesion to collagen IV and laminin I, when compared to the incubation in NE \((P < 0.05; \text{Table 6.1A})\). Incubation of H357 with AN effluent caused a significant decrease of adhesion to fibronectin. However, a significant increase of adhesion to fibrinogen was observed compared to the incubation with NE \((P < 0.05; \text{Table 6.1A})\). The adhesion of H357 to collagen I, collagen IV and laminin I were increased significantly \((P < 0.05)\) when incubated with SM effluent, whereas a significant decrease of adhesion to fibronectin and fibrinogen was observed compared
to incubation with NE ($P < 0.05$; Table 6.1A). Incubation with TRI effluent caused a significant increase of H357 adhesion to fibronectin, collagen I and laminin I ($P < 0.05$). However, the adhesion of H357 to fibrinogen was observed to decrease significantly compared to incubation of H357 with NE ($P < 0.05$; Table 6.1A).

### 6.4.3 Comparison of adhesion to ECM between OKF6 and H357

The data demonstrated that the majority of biofilm effluent decreased the adhesion of OKF6 to ECM components, as incubation in all biofilm effluents showed decreased adhesion of OKF6 to collagen I, collagen IV and fibrinogen when compared to NE (Table 6.1B; Figure 6.1A). Meanwhile, the majority of microbial effluent increased the adhesion of H357 to ECM components, while incubation in all biofilm effluents showed increased adhesion of H357 to laminin I when compared to NE (Table 6.2; Figure 6.1B). Of note, the large fold changes of increased adhesion of H357 cells to collagen IV observed after incubation with biofilm effluent from ALC3 (4.58-fold) and SM (2.07-fold). Furthermore, very large enhanced adhesion to laminin I was observed when H357 was incubated with ALC3 (15.07-fold), SM (6.54-fold) and TRI (10.69-fold) effluents (Table 6.1B; Figure 6.1B).

Collectively, the results of adhesion assays showed that microbial biofilm effluent influenced cell-ECM interaction and this was dependent on the pathogen present in the biofilm and the cell used in the analysis (Table 6.1B; Figure 6.1). Effluent increased adhesion to ECM components more commonly for the oral cancer cell line (H357) compared with the normal oral epithelial cell line (OKF6), and this was most pronounced for adhesion to collagen IV and laminin I.
Table 6.1A Adhesion of OKF6 and H357 in 80% serum free medium (SFM) containing 20% of non-effluent ASM (NE), C. albicans (ALC3), A. naeslundii (AN), S. mutans (SM), and polymicrobial (TRI) biofilm effluents.

<table>
<thead>
<tr>
<th>ECM molecules</th>
<th>OKF6</th>
<th>H357</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
<td>ALC3</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.154 (0.005)</td>
<td>0.080 (0.004)</td>
</tr>
<tr>
<td>Collagen I</td>
<td>0.165 (0.001)</td>
<td>0.109 (0.009)</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>0.176 (0.027)</td>
<td>0.133 (0.004)</td>
</tr>
<tr>
<td>Laminin I</td>
<td>0.041 (0.001)</td>
<td>-0.008 (0.009)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.180 (0.011)</td>
<td>0.099 (0.022)</td>
</tr>
</tbody>
</table>

Data is the optical density measured by spectrophotometer at wavelength OD$_{570\text{nm}}$. Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data is considered as significantly different when $P < 0.05$. 

<table>
<thead>
<tr>
<th>Color</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Green</strong></td>
<td>Significantly higher compared to NE</td>
</tr>
<tr>
<td><strong>Gold</strong></td>
<td>No-significant difference compared to NE</td>
</tr>
<tr>
<td><strong>Pink</strong></td>
<td>Significantly lower compared to NE</td>
</tr>
</tbody>
</table>

148
Table 6.1B Fold change of OKF6 and H357 adhesion when incubated with *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents compared to non-effluent (NE).

| ECM Molecules | OKF6      |  |  |  | H357      |  |  |  |
|---------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
|               | ALC3      | AN        | SM        | TRI       | ALC3      | AN        | SM        | TRI       |
| Fibronectin   | 0.52 (0.01) | 0.79 (0.08) | 1.15 (0.03) | 0.97 (0.01) | 0.53 (0.06) | 0.68 (0.03) | 0.74 (0.03) | 1.21 (0.00) |
| Collagen I    | 0.66 (0.06) | 0.84 (0.00) | 0.95 (0.00) | 0.64 (0.01) | 0.84 (0.03) | 0.39 (0.04) | 1.31 (0.10) | 1.51 (0.05) |
| Collagen IV   | 0.77 (0.10) | 0.99 (0.21) | 0.78 (0.01) | 0.91 (0.00) | 4.58 (0.04) | 0.57 (0.01) | 2.07 (0.11) | 1.04 (0.11) |
| Laminin I     | -0.20* (0.22) | -0.20* (0.03) | 0.54 (0.02) | 1.53 (0.04) | 15.07 (0.08) | 1.31 (0.00) | 6.54 (0.04) | 10.69 (0.09) |
| Fibrinogen    | 0.55 (0.11) | 0.81 (0.05) | 0.56 (0.03) | 0.54 (0.02) | 0.23 (0.00) | 1.35 (0.01) | 0.45 (0.01) | 0.49 (0.00) |

Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. * Cells adhered to laminin I were less than those adhered to the control substrata (Bovine serum albumin, BSA).
Figure 6.1 Fold change of OKF6 and H357 adhesion when incubated with *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM) and polymicrobial (TRI) biofilm effluents compared to non-effluent (NE). Bars are the SD. The study was conducted in three biological replicates with each replicate consisting of three technical replicates.
Part II: Epithelial-mesenchymal transition (EMT)

6.4.4 Percentage of cells expressing E-cadherin and vimentin

Incubation of OKF6 with ALC3, AN and SM effluent caused a significant increase of cells expressing vimentin compared to NE after 24 h ($P < 0.05$; Table 6.2A). Incubation of the cell line with all biofilm effluents caused a significant increase of cells expressing E-cadherin after 24 h when compared to NE ($P < 0.05$; Table 6.2A).

The same set of experiments was subsequently performed on OSCC cells (H357). Incubation of H357 with all effluents caused a significant decrease of cell expressing vimentin compared to NE after 24 h incubation ($P < 0.05$; Table 6.2A). A significant increase of cells expressing E-cadherin was observed between 2h and 24 h incubation when H357 was incubated with SM compared to NE ($P < 0.05$; Table 6.2A).

All biofilm effluents and NE decreased OKF6 cells expressing vimentin after 24 h incubation compared to 2 h (-1.5% to -32.4%; Table 6.2B). An increase of cells expressing E-cadherin was observed after 24 h when incubated in the majority of biofilm effluents (ALC3, AN and SM) (12.5% to 23.0%; Table 6.2B).

The incubation of H357 in NE was found to increase cells expressing vimentin (44.9%) and decrease cells expressing E-cadherin (-12.7%) after 24 h incubation compared to 2 h (Table 6.2B). Over this time period, the majority of microbial effluents (ALC3, AN and SM) reduced H357 cells expressing vimentin (-21.5% to -23.6%) as well as cells expressing E-cadherin (-12.7% to -29.2%; Table 6.2B).
Table 6.2A Percentage positive of OKF6 and H357 cells treated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluent at 37 °C, 5% CO₂ for 2 h and 24 h.

<table>
<thead>
<tr>
<th>Hours</th>
<th>EMT markers</th>
<th>OKF6</th>
<th>H357</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NE</td>
<td>ALC3</td>
</tr>
<tr>
<td>2</td>
<td>Vimentin</td>
<td>62.1 (1.8)</td>
<td>61.1 (0.3)</td>
</tr>
<tr>
<td></td>
<td>E-cadherin</td>
<td>65.5 (1.3)</td>
<td>61.9 (0.6)</td>
</tr>
<tr>
<td>24</td>
<td>Vimentin</td>
<td>42.2 (2.4)</td>
<td>52.4 (2.5)</td>
</tr>
<tr>
<td></td>
<td>E-cadherin</td>
<td>54.6 (1.1)</td>
<td>71.5 (5.7)</td>
</tr>
</tbody>
</table>

Significantly higher compared to NE
No-significant difference compared to NE
Significantly lower compared to NE

Data is percentage positive of cells expressing EMT markers as measured by flow cytometry. Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data was considered as significantly different when $P < 0.05$. 
<table>
<thead>
<tr>
<th>EMT markers</th>
<th>OKF6 (%)</th>
<th>H357 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
<td>ALC3</td>
</tr>
<tr>
<td>Vimentin</td>
<td>-32.0 (4.1)</td>
<td>-14.2 (4.4)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>-16.6 (3.2)</td>
<td>15.6 (10.3)</td>
</tr>
</tbody>
</table>

Significantly higher compared to NE
No-significant difference compared to NE
Significantly lower compared to NE

Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Negative results indicated a decrease of positive cells expressing EMT markers, whereas positive results indicated an increase of positive cells after 24 h incubation. Data was considered as significantly different when $P < 0.05$. 

153
6.4.5 Mean fluorescence intensity (MFI)

Mean fluorescence intensity (MFI) showed that the incubation of OKF6 in SM effluent caused a significant increase of vimentin compared to NE after 24 h incubation ($P < 0.05$; Table 6.3A). In addition, no biofilm effluent was observed to cause a significant difference of E-cadherin expressed by OKF6 compared to NE ($P > 0.05$; Table 6.3A).

Incubation of the malignant cell line (H357) with ALC3, AN and SM effluent was found to decrease the MFI of vimentin significantly after 24 h compared to incubation with NE ($P < 0.05$; Table 6.3A). There was no significant difference of E-cadherin between H357 incubated with biofilm effluent compared to incubation with NE ($P > 0.05$; Table 6.3A).

All biofilm effluents and NE decreased expression of vimentin by OKF6 after 24 h incubation compared to 2 h ($-16.9\%$ to $-53.1\%$; Table 6.3B). An increase of E-cadherin was observed after 24 h when incubated in the majority of biofilm effluents (ALC3, AN and SM; $2.0\%$ to $11.3\%$; Table 6.3B). Furthermore, H357 when incubated in NE was found to have increased expression of vimentin ($31.7\%$) and decreased expression of E-cadherin ($-18.1\%$) after 24 h incubation compared to 2 h (Table 6.3B). All biofilm effluents were found to reduce the expression of vimentin by H357 ($-30.2\%$ to $-58.1\%$; Table 6.3B). Furthermore, all biofilm effluents and NE were observed to decrease E-cadherin ($-18.1\%$ to $-42.2\%$; Table 6.3B).

Collectively, the data demonstrated that the expression of vimentin and E-cadherin was regulated in a cell type, time-dependent and biofilm effluent-specific manner. The expression of vimentin by the normal epithelial cell line, OKF6, was profoundly decreased after 24 h incubation in NE ($32.0\%$) (Table 6.2B) and MFI
Although this decrease was observed when OKF6 was incubated with mono-cultured biofilm effluent, this decrease was less pronounced than for the control medium ($P < 0.05$). Furthermore, there was an increase in both the number of cells expressing E-cadherin as well as MFI when OKF6 cells were incubated with mono-cultured biofilm effluent (Table 6.2B and 6.3B).

Interestingly, a paradoxical effect was observed with the EMT of H357 cells, with the majority of biofilm effluents resulting in an enhanced malignant phenotype as observed by the decreased expression of E-cadherin, while at the same time having a diminished malignant phenotype, as observed by the decreased expression of vimentin.
Table 6.3A Mean fluorescence intensity (MFI) of vimentin and E-cadherin of OKF6 and H357 cells treated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h.

<table>
<thead>
<tr>
<th>Hours</th>
<th>EMT markers</th>
<th>OKF6</th>
<th>H357</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
<td>ALC3</td>
<td>AN</td>
</tr>
<tr>
<td>2</td>
<td>Vimentin</td>
<td>1554.3 (134.1)</td>
<td>1666.3 (86.0)</td>
</tr>
<tr>
<td></td>
<td>E-cadherin</td>
<td>184.3 (6.4)</td>
<td>175.3 (5.9)</td>
</tr>
<tr>
<td>24</td>
<td>Vimentin</td>
<td>805.3 (84.7)</td>
<td>1103.7 (115.6)</td>
</tr>
<tr>
<td></td>
<td>E-cadherin</td>
<td>166.0 (44.2)</td>
<td>178.7 (17.2)</td>
</tr>
</tbody>
</table>

- Significantly higher compared to NE
- No-significant difference compared to NE
- Significantly lower compared to NE

Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data was considered as significantly different when *P* < 0.05.
Table 6.3B Percentage difference of mean fluorescence intensity (MFI) of OKF6 and H357 cells expressing vimentin and E-cadherin between 2 h and 24 h incubated in NE, ALC3, AN, SM, and TRI effluents at 37 °C, 5% CO₂ for 2 h and 24 h.

<table>
<thead>
<tr>
<th>EMT markers</th>
<th>OKF6 (%)</th>
<th>H357 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
<td>ALC3</td>
</tr>
<tr>
<td>Vimentin</td>
<td>-48.2%</td>
<td>-33.6%</td>
</tr>
<tr>
<td></td>
<td>(3.1)</td>
<td>(8.4)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>-10.1%</td>
<td>2.0%</td>
</tr>
<tr>
<td></td>
<td>(22.7)</td>
<td>(11.5)</td>
</tr>
</tbody>
</table>

Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Negative results indicated a decreased percentage of MFI, whereas positive results indicated an increased percentage of MFI after 24 h incubation. Data was considered as significantly different when $P < 0.05$. 

<table>
<thead>
<tr>
<th>Significantly higher compared to NE</th>
<th>No-significant difference compared to NE</th>
<th>Significantly lower compared to NE</th>
</tr>
</thead>
</table>
Part III: Cytokine assay

6.4.6 Expression of cytokines by OKF6 and H357

IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF and TNF-α were expressed constitutively in OKF6 and H357, and were secreted at different concentrations when grown in different cell types and effluents after 2 h and 24 h. In contrast, IFN-γ was detected only in OKF6 incubated in NE, SM and TRI effluents for 24 h, and in H357 incubated in AN, SM and TRI for 2 h, and all effluents incubated for 24 h (Table 6.4A and 6.5B).
Table 6.4A Cytokines expressed by OKF6 (pg mL^{-1}) incubated with 80% serum free medium containing 20% non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO$_2$ for 2 h.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>NE (pg mL$^{-1}$)</th>
<th>ALC3</th>
<th>AN</th>
<th>SM</th>
<th>TRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>0.11 (0.04)</td>
<td>0.16 (0.05)</td>
<td>0.17 (0.04)</td>
<td>0.21 (0.00)</td>
<td>0.18 (0.05)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.04 (0.03)</td>
<td>0.05 (0.02)</td>
<td>0.03 (0.02)</td>
<td>0.05 (0.03)</td>
<td>0.03 (0.01)</td>
</tr>
<tr>
<td>IL-6</td>
<td>7.25 (1.77)</td>
<td>3.98 (1.29)</td>
<td>6.05 (3.99)</td>
<td>13.75 (3.43)</td>
<td>9.61 (2.83)</td>
</tr>
<tr>
<td>IL-8</td>
<td>50.64 (8.06)</td>
<td>36.27 (3.84)</td>
<td>42.09 (8.70)</td>
<td>71.10 (13.82)</td>
<td>54.13 (14.14)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.68 (0.05)</td>
<td>0.67 (0.04)</td>
<td>0.65 (0.10)</td>
<td>0.68 (0.05)</td>
<td>0.59 (0.04)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>7.09 (0.25)</td>
<td>7.04 (0.10)</td>
<td>6.44 (0.33)</td>
<td>7.25 (0.34)</td>
<td>6.28 (0.34)</td>
</tr>
<tr>
<td>IFN-$\gamma$</td>
<td>LLD</td>
<td>LLD</td>
<td>LLD</td>
<td>LLD</td>
<td>LLD</td>
</tr>
<tr>
<td>TNF-$\alpha$</td>
<td>1.27 (0.21)</td>
<td>1.41 (0.12)</td>
<td>1.34 (0.12)</td>
<td>1.41 (0.12)</td>
<td>1.34 (0.12)</td>
</tr>
</tbody>
</table>

Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data was considered as significantly different when *P* < 0.05. LLD = Less than lowest detectable measure. For IFN-$\gamma$, this was 0.4 pg mL$^{-1}$.
Table 6.4B Cytokines expressed by OKF6 (pg mL$^{-1}$) incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluent s at 37 °C, 5% CO$_2$ for 24 h.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>OKF6 (pg mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.33 (0.09)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.10 (0.03)</td>
</tr>
<tr>
<td>IL-6</td>
<td>36.64 (10.79)</td>
</tr>
<tr>
<td>IL-8</td>
<td>212.05 (13.92)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.74 (0.01)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>10.93 (1.78)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.85 (0.78)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.90 (0.22)</td>
</tr>
</tbody>
</table>

Data was the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data was considered as significantly different when $P < 0.05$. LLD = Less than lowest detectable measure. For IFN-γ, this was 0.4 pg mL$^{-1}$. 
Table 6.4C Cytokines expressed by H357 (pg mL$^{-1}$) incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), C. albicans (ALC3), A. naeslundii (AN), S. mutans (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO$_2$ for 2 h.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>H357 (pg mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.16 (0.05)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.06 (0.02)</td>
</tr>
<tr>
<td>IL-6</td>
<td>12.21 (2.25)</td>
</tr>
<tr>
<td>IL-8</td>
<td>21.89 (4.07)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.58 (0.02)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>6.47 (0.24)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>LLD</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.27 (0.21)</td>
</tr>
</tbody>
</table>

Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data was considered as significantly different when $P < 0.05$. LLD = Less than lowest detectable measure. For IFN-γ, this was 0.4 pg mL$^{-1}$. 

![Data Table](#)
Table 6.4D Cytokines expressed by H357 (pg mL\(^{-1}\)) incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), \textit{C. albicans} (ALC3), \textit{A. naeslundii} (AN), \textit{S. mutans} (SM), and polymicrobial (TRI) biofilm effluent at 37 °C, 5% CO\(_2\) for 24 h.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>H357 (pg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.21 (0.08)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.05 (0.02)</td>
</tr>
<tr>
<td>IL-6</td>
<td>39.09 (13.88)</td>
</tr>
<tr>
<td>IL-8</td>
<td>13.59 (2.90)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.58 (0.04)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>6.82 (0.59)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>LLD</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.27 (0.00)</td>
</tr>
</tbody>
</table>

Data was the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data was considered as significantly different when \( P < 0.05 \). LLD = Less than lowest detectable measure. For IFN-γ, this was 0.4 pg mL\(^{-1}\).
6.4.6.1 Interleukin 2 (IL-2)

After 2 h, only OKF6 incubated with SM effluent caused a significant increase (2.24-fold) of IL-2 expression when compared to NE ($P < 0.05$; Table 6.4A; Figure 6.2A). There was no significant difference of IL-2 expression in OKF6 after 24 h incubation in all suspension when compared to NE ($P > 0.05$; Table 6.4B). An increase of IL-2 was observed when OKF6 was incubated in biofilm effluent (58.7% to 157.3%) after 24 h incubation compared to 2 h where no significant difference was observed when compared to NE ($P > 0.05$; Table 6.5A).

After 2 h, H357 incubated with AN and SM effluents showed a significant increase of IL-2 (3.89-fold and 4.64-fold respectively), compared to NE ($P < 0.05$; Table 6.4C; Figure 6.2A). After 24 h, H357 incubated in ALC3 (6.65-fold), AN (7.11-fold), SM (10.65-fold) and TRI (4.29-fold) effluents showed significantly increased expression of IL-2 compared to NE ($P < 0.05$; Table 6.4D; Figure 6.2A). Increased expression of IL-2 was observed when H357 was incubated in biofilm effluent (110.5% to 451.6%) after 24 h incubation compared to 2 h, with the largest two increases observed when incubated in ALC3 (451.6%) and TRI (342.1%) effluents compared to NE ($P < 0.05$; Table 6.5B).
Figure 6.2A Fold change of IL-2 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold changes were the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE. Bars are the SD. The study was conducted in three biological replicates with each replicate consisting of three technical replicates.
6.4.6.2 **Interleukin 4 (IL-4)**

There was no statistically significant change of IL-4 expression in OKF6 after 2 h and 24 h incubation with all biofilm effluent compared to NE ($P > 0.05$; Table 6.4A; Table 6.4B). Increased expression of IL-4 was observed when OKF6 was incubated in biofilm effluent (78.6% to 283.3%) after 24 h incubation compared to 2 h with no significant difference observed when compared to NE ($P > 0.05$; Table 6.5A).

After 2 h, H357 incubated in AN (1.96-fold) and SM (2.49-fold) effluent showed significantly increased expression of IL-4 compared to NE ($P < 0.05$; Table 6.4C; Figure 6.2B). After 24 h, H357 incubated in ALC3 (4.50-fold), AN (4.92-fold), SM (4.48-fold) and TRI (3.05-fold) effluents showed significant increases in IL-4 compared to NE ($P < 0.05$; Figure 6.6B). Increased expression of IL-4 was observed when H357 was incubated in biofilm effluent (50.6% to 282.1%) after 24 h incubation compared to 2 h with significant increases observed when incubated in ALC3 and TRI effluent compared to NE ($P < 0.05$; Table 6.5B).
Figure 6.2B Fold change of IL-4 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), C. albicans (ALC3), A. naeslundii (AN), S. mutans (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold changes are the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE. Bars are the SD. The study was conducted in three biological replicates with each replicate consisting of three technical replicates.
6.4.6.3 Interleukin 6 (IL-6)

After 2 h, only OKF6 incubated with SM effluent caused significantly increased (1.99-fold) IL-6 expression when compared to NE ($P < 0.05$; Table 6.4A; Figure 6.2C). Meanwhile, after 24 h incubation, OKF6 cells incubated in TRI effluent was shown to significantly increase expression of IL-6 when compared to NE (2.28-fold; $P < 0.05$; Table 6.4B; Figure 6.2C). Increased expression of IL-6 was observed when OKF6 was incubated in biofilm effluent (3.98% to 13.75%) after 24 h incubation compared to 2 h with no significant difference observed when compared to NE ($P > 0.05$; Table 6.5A).

After 2 h, H357 incubated in AN (15.76-fold), SM (19.87-fold) and TRI (3.30-fold) effluent showed significantly increased expression of IL-6 compared to NE ($P < 0.05$; Table 6.4C; Figure 6.2C). After 24 h, H357 grown in ALC3 (13.86-fold), AN (14.79-fold), SM (25.78-fold) and TRI (7.78-fold) effluents had significantly increased expression of IL-6 compared to NE ($P < 0.05$; Table 6.4D; Figure 6.2C). Increased expression of IL-6 was observed when H357 was incubated in biofilm effluents (166.2% to 3169.6%) after 24 h incubation, compared to 2 h where significant increases were observed when incubated in ALC3 (3169.6%) and TRI (611.1%) effluents compared to NE ($P < 0.05$; Table 6.5B).
Figure 6.2C Fold change of IL-6 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO$_2$ for 2 h and 24 h. Fold changes are the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE. Bars are the SD. The study was conducted in three biological replicates with each replicate consisting of three technical replicates.
6.4.6.4 Interleukin 8 (IL-8)

After 2 h, OKF6 incubated with ALC3 effluent caused a significant decrease (0.72-fold) of IL-8 expression when compared to NE ($P < 0.05$; Table 6.4A; Figure 6.2D). After 24 h, TRI effluent was shown to significantly increase cells expressing IL-8 (1.91-fold) compared to cells that were grown in NE ($P < 0.05$; Table 6.4B; Figure 6.2D). Increased expression of IL-8 was observed when OKF6 was incubated in biofilm effluent (224.7% to 662.6%) after 24 h incubation compared to 2 h where only TRI effluent (662.6%) showed a significant increase when compared to NE ($P < 0.05$; Table 6.5A).

After 2 h, H357 incubated in AN (2.79-fold) and SM (2.07-fold) effluent showed significantly increased expression of IL-8 compared to NE ($P < 0.05$; Table 6.4C; Figure 6.2D). After 24 h, H357 incubated in ALC3 (19.45-fold), AN (21.70-fold), SM (10.71-fold) and TRI (5.60-fold) effluent was shown to increase IL-8 significantly compared to NE ($P < 0.05$; Table 6.4D; Figure 6.2D). Increased expression of IL-8 was observed when H357 was incubated in biofilm effluent (100.2% to 783.9%) after 24 h incubation compared to 2 h with significant increases observed when incubated in all biofilm effluents compared to NE ($P < 0.05$; Table 6.5B).
Figure 6.2D Fold change of IL-8 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), C. albicans (ALC3), A. naeslundii (AN), S. mutans (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change is the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE. Bars are the SD. The study was conducted in three biological replicates with each replicate consisting of three technical replicates.
6.4.6.5 Interleukin 10 (IL-10)

There was no significant difference of OKF6 expressing IL-10 when incubated in all biofilm effluents compared to NE after 2 h and 24 h ($P > 0.05$; Table 6.4A; Table 6.4B). Increased expression of IL-10 was observed when OKF6 was incubated in biofilm effluent (8.3% to 18.4%) after 24 h incubation compared to 2 h where no biofilm effluents showed a significant difference when compared to NE ($P > 0.05$; Table 6.5A).

ALC3 (1.15-fold), AN (1.20-fold), SM (1.23-fold) and TRI (1.17-fold) effluents have been shown to increase H357 expressing IL-10 significantly when compared to NE after 2 h incubation ($P < 0.05$; Table 6.4C; Figure 6.2E). After 24 h incubation, H357 in ALC3 (1.28-fold), AN (1.27-fold) and TRI (1.21-fold) effluents have been shown to increase expression of IL-10 significantly when compared to NE ($P < 0.05$; Table 6.4D; Figure 6.2E). Increased expression of IL-10 was observed when H357 was incubated in ALC3, AN and TRI effluents (3.5% to 10.9%) after 24 h incubation compared to 2 h and a 15.1% decrease for OKF6 cells grown in SM. No biofilm effluent showed significant differences when compared to NE ($P > 0.05$; Table 6.5B).
Figure 6.2E Fold change of IL-10 expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change is the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE. Bars are the SD. The study was conducted in three biological replicates with each replicate consisting of three technical replicates.
6.4.6.6 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

After 2 h incubation, OKF6 grown in TRI effluent (0.89-fold) exhibited significantly decreased expression of GM-CSF when compared to NE ($P < 0.05$; Table 6.4A; Figure 6.2F). After 24 h incubation, OKF6 incubated in TRI effluent (1.40-fold) exhibited significantly increased expression of GM-CSF compared to NE ($P < 0.05$; Table 6.4B; Figure 6.2F). An increase of GM-CSF was observed when OKF6 was incubated in biofilm effluent (54.5% to 141.4%) after 24 h incubation compared to 2 h, where TRI effluent (141.1%) showed a significant increase when compared to NE ($P < 0.05$; Table 6.5A).

After 2 h incubation, H357 incubated in ALC3 (1.17-fold), AN (1.31-fold) and SM (1.29-fold) were shown to increase GM-CSF significantly compared to cells incubated in NE ($P < 0.05$; Table 6.4C; Figure 6.2F). Incubation of H357 for 24 h in biofilm effluent of ALC3 (1.72-fold), AN (1.71-fold), SM (1.68-fold) and TRI (1.51-fold) have been shown to increase expression of GM-CSF significantly compared to cells that were incubated in NE ($P < 0.05$; Table 6.4D; Figure 6.2F). Increased expression of GM-CSF was observed when H357 was incubated in all biofilm effluents (37.7% to 54.7%) after 24 h incubation compared to 2 h, where ALC3 (54.7%) and TRI (45.4%) effluent showed a significant difference when compared to NE ($P < 0.05$; Table 6.5B).
Figure 6.2F Fold change of GM-CSF expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change is the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE. Bars are the SD. The study was conducted in three biological replicates with each replicate consisting of three technical replicates.
6.4.6.7 Interferon gamma (IFN-γ)

IFN-γ expressed by OKF6 cells was not detected after 2 h incubation (Table 6.4A). However, after 24 h incubation only OKF6 cells that were incubated in NE, SM and TRI effluents were detected expressing IFN-γ (Table 6.4B). Increased expression of IFN-γ was observed when OKF6 was incubated in biofilm effluent (0.0% to 739.2%) after 24 h incubation compared to 2 h with only TRI (739.2%) effluent showing a significant increase when compared to NE ($P < 0.05$; Table 6.5A).

After 2 h incubation, IFN-γ was only detected when H357 was incubated in AN, SM and TRI effluents, whereas after 24 h, IFN-γ was only detected when H357 was incubated in ALC3, AN, SM and TRI effluents. Increased expression of IFN-γ was observed when H357 was incubated in biofilm effluent (192.0% to 2785.8%) after 24 h incubation compared to 2 h where all biofilm effluents showed significant increases when compared to NE ($P < 0.05$; Table 6.5B).
6.4.6.8 Tumour necrosis factor alpha (TNF-α)

There was no change of TNF-α expressed by OKF6 after 2 h and 24 h incubation in all suspensions compared to NE ($P > 0.05$; Table 6.4A; Table 6.4B). Increased expression of TNF-α was observed when OKF6 was incubated in all biofilm effluents (15.8% to 78.7%) after 24 h incubation compared to 2 h with no biofilm effluent showing significant difference when compared to NE ($P > 0.05$; Table 6.5A).

After 2 h, H357 incubated in AN (2.44-fold) and SM (2.49-fold) effluents exhibited increased expression of TNF-α compared to NE significantly ($P < 0.05$; Table 6.4C; Figure 6.2G). In addition, H357 incubated in ALC3 (5.47-fold), AN (4.75-fold), SM (6.13-fold) and TRI (2.47-fold) effluent was shown to increase expression of TNF-α compared to NE significantly after 24 h ($P < 0.05$; Table 6.4D; Figure 6.2G). Increased expression of TNF-α was observed when H357 was incubated in biofilm effluent (94.6% to 483.0%) after 24 h incubation compared to 2 h where all biofilm effluents showed a significant increase when compared to NE ($P < 0.05$; Table 6.5B).
6.4.6.9 Overall

Taken together, these results demonstrate that the expression of IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF and IFN-γ were expressed in a cell type, time-dependent and biofilm effluent-specific manner where the majority of biofilm effluents induced the expression of cytokines in the malignant cell line (H357) and not in the normal epithelial cell line (OKF6). Furthermore, the effluent of \textit{C. albicans} (ALC3) and polymicrobial biofilms (TRI) was shown to significantly increase cytokine production by H357 cells after 24 h compared to incubation in artificial saliva (NE).
Figure 6.2G Fold change of TNF-α expression by OKF6 (left) and H357 (right) cells incubated with 80% serum free medium containing 20% of non-effluent ASM (NE), *C. albicans* (ALC3), *A. naeslundii* (AN), *S. mutans* (SM), and polymicrobial (TRI) biofilm effluents at 37 °C, 5% CO₂ for 2 h and 24 h. Fold change is the ratio of mean cytokine expression at 2 h and 24 h of biofilm effluents (ALC3, AN, SM and TRI), to NE. Bars are the SD. The study was conducted in three biological replicates with each replicate consisting of three technical replicates.
Table 6.5A Percentage difference of cytokines expressed by OKF6 incubated in NE, ALC3, AN, SM, and TRI effluents at 37 °C, 5% CO₂ between 2 h and 24 h incubation.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>OKF6 (%)</th>
<th>NE</th>
<th>ALC3</th>
<th>AN</th>
<th>SM</th>
<th>TRI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>288.0(299.0)</td>
<td>129.1(82.9)</td>
<td>112.2(110.5)</td>
<td>58.7(22.0)</td>
<td>157.3(70.7)</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td></td>
<td>225.0(288.3)</td>
<td>78.6(6.2)</td>
<td>280.0(113.8)</td>
<td>178.6(138.9)</td>
<td>283.3(146.5)</td>
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<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td>415.4(137.5)</td>
<td>570.8(279.7)</td>
<td>633.2(633.5)</td>
<td>218.8(57.2)</td>
<td>800.9(380.2)</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td></td>
<td>325.6(67.8)</td>
<td>408.5(73.7)</td>
<td>332.2(162.7)</td>
<td>224.7(145.3)</td>
<td>662.6(135.6)</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td></td>
<td>8.7(8.1)</td>
<td>12.4(3.4)</td>
<td>8.3(17.2)</td>
<td>14.7(10.6)</td>
<td>18.4(9.5)</td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td></td>
<td>53.8(21.3)</td>
<td>54.5(1.9)</td>
<td>83.5(35.4)</td>
<td>60.3(5.4)</td>
<td>141.4(23.2)</td>
</tr>
<tr>
<td>*<em>IFN-γ</em></td>
<td></td>
<td>112.5(194.9)</td>
<td>0.0(0.0)</td>
<td>214.2(370.9)</td>
<td>225.0(194.9)</td>
<td>739.2(167.4)</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td>51.6(20.4)</td>
<td>15.8(16.6)</td>
<td>26.0(8.5)</td>
<td>41.5(26.4)</td>
<td>78.7(31.0)</td>
</tr>
</tbody>
</table>

Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data was considered as significantly different when P < 0.05. Positive results indicated increased percentage of cytokines synthesised by OKF6 between 2 h and 24 h incubation. *The measure of LLD (0.4 pg mL⁻¹) was used to assess percentage difference.
Table 6.5B Percentage difference of cytokines expressed by H357 incubated in NE, ALC3, AN, SM, and TRI effluents at 37 °C, 5% CO₂ between 2 h and 24 h incubation.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th></th>
<th>H357 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
<td>ALC3</td>
</tr>
<tr>
<td>IL-2</td>
<td>46.3</td>
<td>(77.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(59.4)</td>
</tr>
<tr>
<td>IL-4</td>
<td>-3.6</td>
<td>(68.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(67.6)</td>
</tr>
<tr>
<td>IL-6</td>
<td>243.0</td>
<td>(187.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1166.9)</td>
</tr>
<tr>
<td>IL-8</td>
<td>-35.1</td>
<td>(25.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(196.3)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.0</td>
<td>(6.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.6)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5.4</td>
<td>(6.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.2)</td>
</tr>
<tr>
<td>IFN-γ*</td>
<td>0.0</td>
<td>(0.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(434.5)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.9</td>
<td>(17.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(62.8)</td>
</tr>
</tbody>
</table>

- **Green**: Significantly higher compared to NE
- **Yellow**: No-significant difference compared to NE
- **Pink**: Significantly lower compared to NE

Data is the mean of three separate experiments (SD are given in parentheses). The study was conducted in three biological replicates with each replicate consisting of three technical replicates. Data was considered as significantly different when $P < 0.05$. Negative results indicated a decrease of cytokines expressed by H357, whereas positive results indicated an increased percentage between 2 h and 24 h incubation. *The measure of LLD (0.4 pg mL⁻¹) was used to assess percentage difference.
6.5 Discussion

Carcinomas are characterised by the ability of malignant cells to invade underlying connective tissue and to migrate, forming metastasis at distant sites (Lyons and Jones, 2007). One of the important properties of cells in the progression of oral cancer is the ability to adhere to extracellular matrix (ECM). To our knowledge, this is the first study undertaken to elucidate the role of biofilm effluent of *C. albicans*, *A. naeslundii* and *S. mutans* in the adhesion to ECM molecules, regulation of epithelial-mesenchymal transition (EMT) and the expression of pro-inflammatory cytokines by OKF6 (normal epithelial cell line) and H357 (OSCC cell line).

We have shown distinct variation in the ability of OKF6 and H357 to adhere to ECM components when incubated in biofilm effluent, with H357 exhibiting increased adhesion to ECM molecules, particularly collagen IV and laminin I. This was most pronounced when H357 was incubated with *C. albicans* biofilm effluent. Further, increased adhesion was also observed when H357 was incubated in polymicrobial biofilm effluent compared to NE, thus supporting the hypothesis that the adhesion of cells to ECM molecules is biofilm effluent-dependent and the polymicrobial biofilm effluent increased the malignant phenotype, suggesting that biofilm effluent is an oral cancer promoter rather than initiator. The variability of adhesion of OKF6 and H357 cells to ECM components has been suggested to be due to the alteration of cells’ integrins following interaction with biofilm effluent (Lyons and Jones, 2007). Integrins are known as the largest family of cell adhesion molecules that consist of multiple combinations of α- and β-subunits (Van Waes and Carey, 1992; Lyons and Jones, 2007). Proteins synthesised by microorganisms such as proteases from *C. albicans* (Karkowska-Kuleta *et al.*, 2009) and Bcl-2 family proteins from bacteria (Khajuria and Metgud, 2015) have been shown to induce
alteration of integrins of epithelial cells. Furthermore, the α1-subunit of integrins that preferentially binds to collagen IV, and the α3-subunit that acts as a receptor for laminins, have been shown to change during interaction with the proteins of microorganisms (Lyons and Jones, 2007). The results of the present study support these previous findings, as biofilm effluent, particularly ALC3, induced enhanced adhesion of H357 to collagen IV and laminin I and polymicrobial biofilm effluent enhanced adhesion of the OSCC cells to fibronectin, collagen IV and laminin I. It is important to note that the oral environment comprises a large range of polymicrobial organisms, including C. albicans, A. naeslundii and S. mutans that may alter the integrins of malignant cells. Subsequent triggering of a spectrum of signals involved in the process of growth and proliferation may promote oral carcinogenesis in a paracrine fashion (Carter et al., 1990; Ono et al., 1999; Shinohara et al., 1999; Meurman, 2010).

The EMT assay of the present study revealed a paradoxical effect with both an enhanced and a diminished malignant phenotype being observed concurrently. These results support the hypothesis that EMT is biofilm effluent-dependent. EMT is indicated by the increase of vimentin and the decrease of E-cadherin expression from epithelial cells (Lang et al., 2002; Hugo et al., 2007; Onder et al., 2008; Nijkamp et al., 2011; Yadav et al., 2011). The presence of a stimulator, such as a cell wall extract of the oral bacterium Fusobacterium nucleatum has been shown to increase the expression of vimentin and decrease the expression of E-cadherin in OSCC cell lines HN008 and HN5 (Krisanaprakornkit and Iamaroon, 2012). EMT has been reported to be involved in the increased resistance of malignant cells to apoptosis regulator molecules (Maestro et al., 1999; Vega et al., 2004) indicating the important
role of EMT in metastasis of malignant cells (Kang and Massagué, 2004; Yang et al., 2004; Radisky, 2005).

The result in the present study of an increase in E-cadherin expression by OKF6 cells after 24 h incubation with mono-cultured biofilm effluent is likely indicative of adhesion, colonisation, internalisation and potentially invasion. An increase of E-cadherin expression has been suggested to be a strategy of colonisation of *C. albicans*, *A. naeslundii* and *S. mutans* to oral epithelial cells (Delva and Kowalczyk, 2009). Furthermore, cadherins have been reported to form a route for the internalisation of bacteria and yeast into the epithelial cells during oral thrush (Phan et al., 2005; Delva and Kowalcyzyk, 2009). In addition, Als3 protein synthesised by *C. albicans* has been thought to mimic cadherin-cadherin binding, thus initiating the invasion of the yeast to the oral epithelial cells (Phan et al., 2005). The observed increase in expression of E-cadherin by the H357 cell line at an early time point (2 h) in the present study may well be indicative of this early colonisation and invasion. This however did not persist at later (24 h) time points in this cell line. Interestingly, the normal cell line OKF6 showed an increase in the expression of E-cadherin at this later stage (24 h), a finding likely to indicate the time required for normal cells to allow for internalisation or invasion.

The present study has shown significant variability of cytokine expression by OKF6 and H357 when incubated with the effluent from different biofilms. These results support the hypotheses that cytokine expression by OKF6 and H357 is biofilm effluent-dependent and that *C. albicans*, *A. naeslundii*, *S. mutans* and polymicrobial biofilm effluents increase the malignant phenotype and can act as an oral cancer promoter. The variability of cytokines expressed by the cells may represent the homeostatic mechanism of innate immunity at mucosal tissues responding to the
presence of biofilm effluent (Steele and Fidel, 2002). Furthermore, oral epithelial cells increased synthesis of IL-6, IL-8, IL-10 and TNF-α when incubated with precultured C. albicans medium compared to medium alone (Steele and Fidel, 2002). Similarly, incubation of endothelial cells with C. albicans conditioned medium has been shown to increase the expression of IL-6 and IL-8 after 12 h incubation compared to 8 h (Filler et al., 1996). These results are consistent with our finding, where H357 was observed to increase the same pro-inflammatory cytokines after 24 h incubation with ALC3 and polymicrobial biofilm effluents.

The increase of cytokines expressed by H357 is thought to be due to the presence of proteins glycosylated with N- or O-linked mannosyl residues, β-glucans and chitins from the C. albicans cell wall, as well as the presence of SAPs (Dongari-Bagtzoglou and Kashleva, 2003a; Mostefaoui et al., 2004; Schaller et al., 2005). These proteins have been previously shown to increase the expression of IL-6, IL-8, IL-10, GM-CSF, TNF-α and IFN-γ from epithelial cells and human mono-nuclear cells that were incubated in C. albicans conditioned medium compared to NE (Dongari-Bagtzoglou and Kashleva, 2003a; Mostefaoui et al., 2004; Schaller et al., 2005; Netea et al., 2006). The increased expression of pro-inflammatory cytokines, particularly IL-6, IL-8 and GM-CSF are important in inflammation as well as tumorigenesis of malignant cells (Kitadai et al., 2000). IL-6 has been reported to have an anti-apoptotic effect on malignant cells (Burgdorf et al., 2009). In addition, direct autocrine tumour promoting effects of IL-6 have been demonstrated in multiple myeloma by both increasing proliferation and preventing apoptosis (Thaler et al., 1994; Frassanito et al., 2001). GM-CSF has been previously shown to be a tumour cell stimulator (Burgdorf et al., 2009), and IL-8 has been reported to be involved in carcinogenesis by inducing angiogenesis (Lin and Karin, 2007; Fantini and Pallone,
The expression of IL-8 by human carcinoma cells has been shown to directly correlate with tumour vascularity and disease progression (Kitadai et al., 2000). Thus the results of the present study clearly demonstrate the role of microbial effluent in promoting oral carcinogenesis.

The present study has shown that malignant oral epithelial cells (H357) were observed to increase the expression of many more cytokines than normal epithelial cells (OKF6) when incubated with biofilm effluent. The tumour microenvironment is rich with cytokines and other inflammatory mediators that have been shown to influence the growth of cancer cells (Balkwill and Mantovani, 2001; Balkwill, 2004; Seruga et al., 2008). Furthermore, high expression of pro-inflammatory cytokines such as TNF-α in various human cancers, such as breast, prostate, bladder and leukaemia, has also been reported suggesting an important role of cytokines in oral cancer progression (Kundu and Surh, 2008). Further, several preclinical studies have shown a significant increase of TNF-α in gastric lesions and inflamed colonic mucosa in patients with Helicobacter pylori infection (Noach et al., 1994; Noguchi et al., 1998). The results of the present study indicate that, via the enhanced expression of cytokines by malignant oral epithelial cells, oral microbial biofilms and in particular those containing C. albicans, could potentially act as promoters of oral cancer progression. Previous study has shown that Candida spp. particularly C. albicans were isolated from 30% of patients with cancerous lesion and 32% of patients with precancerous lesion between 2007 and 2009 in Naples, Italy (Gallè et al., 2013). It is suggested that many cancer promoters exist in the oral cavity which C. albicans infection could be one of them.
6.6 Conclusion

Biofilm effluent promoted oral carcinogenesis by increasing the adhesion of an oral squamous cell carcinoma cell line to extracellular matrix molecules and the increase of pro-inflammatory cytokine expression. This tumour growth promoting effect of oral microbial biofilms may be occurring at either the early stages in oral carcinogenesis or perhaps as an enhancement of later tumour progression. Nevertheless, the oral microbial biofilm promotion of oral cancer has profound clinical implications and requires further elucidation of the exact mechanism by which it occurs, as well as confirmation of its occurrence in vivo.
CHAPTER 7

DISCUSSION AND CONCLUSION
7.1 Discussion

Cancer has been the leading cause of death in developed countries and second in the developing countries (Jemal et al., 2011), with oral squamous cell carcinoma (OSCC) accounting for more than 90% of malignancies originating from the oral mucosa (Casiglia and SB, 2001; Johnson et al., 2011). The risk factors that lead to OSCC include heavy alcohol consumption, tobacco smoking, unhealthy diet, poor oral hygiene and microbial infections (Hooper et al., 2009; Chocolatewala et al., 2010; Meurman, 2010; Rajeev et al., 2012; Khajuria and Metgud, 2015).

Yeast and bacterial infections have been widely suggested to have a causal role in oral cancer (Meurman, 2010; Rajeev et al., 2012; Khajuria and Metgud, 2015). Yeast such as *C. albicans* carriage has been found to correlate with the presence of oral epithelial dysplasia (McCullough et al., 2002). Bacteria such as *S. mutans* have been shown to synthesise alcohol dehydrogenase. This enzyme is reported to convert alcohol to carcinogenic acetaldehyde (Kurkivuori et al., 2007; Hooper et al., 2009). In addition, *A. naeslundii* has been shown to colonise the oral cavity of cancer patients more than in healthy individuals (Nagy et al., 1998; Pushalkar et al., 2011).

The promotion of oral carcinogenesis by microorganisms begins from the interaction of the oral microbiome (Kolenbrander, 2000; Min and Rickard, 2009). To assess polymicrobial interactions, a co-aggregation study of eight strains of *C. albicans* with *A. naeslundii* and *S. mutans* was conducted (Section 2.2). The present study has shown that co-aggregation was *C. albicans* strain-dependent with the majority of the yeast grown in RPMI-1640 (hyphal growth). When co-incubated with *S. mutans* and *A. naeslundii* either alone or in combination, variable co-aggregation resulted (Chapter 3). Variability of co-aggregation was also observed from ASM-grown *C. albicans* (yeast growth) strains that were co-incubated with *S. mutans* and
A. naeslundii. It can be postulated that the observed variability in co-aggregation may be related to that specific strain’s ability to produce both non-specific (adhesins) and specific (lectin-saccharide) cell surface receptors (Kolenbrander and Williams, 1981; McIntire et al., 1982; Grimaudo, 1996; Rickard et al., 2003; Rosen and Sela, 2006; Ledder et al., 2008). The observed variability of co-aggregation in C. albicans may also be attributable to different strains having different abundances of specific molecules such as Farnesol, that have been suggested to have a role in polymicrobial interactions of C. albicans to oral bacteria (Morales and Hogan, 2010). The findings of the present study supported the hypothesis that auto-aggregation and co-aggregation are C. albicans strain-dependent, and rejected the null hypothesis that auto-aggregation and co-aggregation are not C. albicans strain-dependent.

Oral microorganisms are required to develop polymicrobial biofilms on the oral substrata in order to potentially promote oral carcinogenesis (Chapter 4). To determine the effect of polymicrobial interaction of C. albicans, A. naeslundii and S. mutans to biofilm formation, the biofilm biomass and metabolic activity were assessed using crystal violet and XTT assays, respectively (Section 2.4). The present study has shown a variation of biofilm biomass and metabolic activity between C. albicans strains based on the classification proposed by Marcos-Zambrano et al. (2014). Strain variability of C. albicans is present in the oral cavity of different individuals (Hellstein et al., 1993; Kleinegger et al., 1996). C. albicans strains isolated from HIV-infected patients are reported to produce higher levels of aspartic proteinases (SAPs), that are important in the formation of C. albicans biofilm, compared to the strains isolated from uninfected patients (Morales and Hogan, 2010; Arzmi et al., 2012).
The biofilm biomass (Chapter 4 and Chapter 5) and metabolic activity were shown to vary with microbial interactions (Chapter 4) and be morphology-dependent (Chapter 4). The biofilm biomass in static biofilms of the majority of RPMI-1640 grown *C. albicans* (hyphal form) was observed to increase in the presence of bacteria compared with mono-cultured *C. albicans*. *A. naeslundii* and *S. mutans* have been shown to bind to *C. albicans* through its mannose-containing surface protein (Kolenbrander and Williams, 1981; McIntire et al., 1982; Grimaudo et al., 1996; Rickard et al., 2003; Rosen and Sela, 2006; Ledder et al., 2008). This interaction has been reported to induce the formation of extracellular polysaccharide, thus promoting the adherence of the late colonisers to form a complex of polymicrobial biofilm (Nyvad and Kilian, 1987; Grimaudo et al., 1996; Li et al., 2004). The variability of metabolic activity in polymicrobial biofilms suggests that these microorganisms may be interacting metabolically (Chapter 4). It is postulated that in the presence of *A. naeslundii*, *C. albicans* increased mitochondrial dehydrogenase activity that in turn increased the activity of succinate dehydrogenases of *A. naeslundii*.

The present study showed that *S. mutans* decreased the overall metabolic activity in tri-cultured polymicrobial biofilms compared with the dual-cultured polymicrobial *C. albicans-A. naeslundii* biofilms. Furthermore, ASM-grown *C. albicans* biofilms were observed to have lower metabolic activity than those grown in RPMI-1640 (Chapter 4), particularly mono-cultured biofilms. *Candida* spp. with low metabolic activity are reported to be more invasive and associated with disease, while conversely those with high activity are non-invasive (Kuhn et al., 2003; Tobudic et al., 2012), and this may have a role in promoting oral carcinogenesis. These findings on static biofilms of *C. albicans*, *A. naeslundii* and *S. mutans* supported our specific hypotheses that polymicrobial biofilm formation is *C. albicans* strain- and
morphology-dependent, thus rejecting the null hypothesis that polymicrobial biofilm formation is not \textit{C. albicans} strain- and morphology-dependent.

The oral cavity has a constant salivary flow with the oral substrata coated with saliva (Sánchez-Vargas \textit{et al.}, 2013; Marsh \textit{et al.}, 2016). These characteristics of the oral environment have been shown to limit the colonisation of oral microorganisms (de Almeida \textit{et al.}, 2008; Marsh \textit{et al.}, 2016) however by-products secreted by the oral microbiome from the biofilm consortium to the oral cavity may have a role in promoting oral cancer. Thus, the study of biofilms of \textit{C. albicans}, \textit{A. naeslundii} and \textit{S. mutans} was conducted in a flow-cell system to determine the effect of the polymicrobial interaction of \textit{C. albicans}, \textit{A. naeslundii} and \textit{S. mutans} on biofilm formation in a flow environment (Section 2.8 to Section 2.11). Simultaneously, effluent from these biofilms was collected for further assessment (Chapter 6). The present study showed that \textit{C. albicans}, \textit{A. naeslundii} and \textit{S. mutans} were able to form polymicrobial biofilms on ASM-coated substrata, with the biofilm biomass of \textit{C. albicans} in the polymicrobial biofilms significantly decreased compared to the monocultured biofilms (Chapter 5). These results were converse to that observed in static biofilms (Chapter 4), indicating the important role of salivary flow in the oral cavity. Furthermore, the biomass assessed by the crystal violet assay (Chapter 4) included both extracellular polysaccharides and microorganisms, while the biomass assessed by fluorescence \textit{in situ} hybridisation (Chapter 5) determined biomass specifically for the microorganisms within the biofilms. Mutualistic and antagonistic interactions have been reported between \textit{C. albicans} with \textit{S. mutans} (McIntire \textit{et al.}, 1982; Rickard \textit{et al.}, 2003; Rosen and Sela, 2006; Thein \textit{et al.}, 2006; Ledder \textit{et al.}, 2008), and \textit{C. albicans} with \textit{A. naeslundii} (Millsap \textit{et al.}, 1999; Thein \textit{et al.}, 2006). \textit{C. albicans} has been shown to decrease adherence when co-cultured with \textit{S. mutans} on
acrylic sheets in Gibbons and Nygaard culture medium (Barbieri et al., 2007). Quorum-sensing molecule such as Farnesol synthesised by C. albicans during biofilm formation has been reported to disrupt the membrane of S. mutans, as well as the accumulation and polysaccharide contents of biofilms of the streptococci (Koo et al., 2003; Jabra-Rizk et al., 2006). In addition, the metabolic products of A. naeslundii have been reported to both inhibit and stimulate the biofilm formation of C. albicans depending on the experimental methods employed (Gutiérrez and Benito, 2004; Thein et al., 2006).

The present study has shown that the average thickness and maximum thickness of polymicrobial biofilms was significantly increased when compared to the mono-cultured C. albicans but not when compared to S. mutans. The increase of the thickness is suggested to be due to the increase of extracellular polysaccharide in the biofilm consortium when surrounded by 25% ASM containing sucrose (Koo et al., 2010). Extracellular polysaccharides have been shown to provide attachment sites for C. albicans that is critical for the colonisation of the microorganism to the oral substrata (Harriott and Noverr, 2011). Therefore, the results of the present study supported our third specific hypotheses that C. albicans, A. naeslundii and S. mutans form polymicrobial biofilms, and that polymicrobial interactions affect colonisation of oral microorganisms in a flow-cell environment. We therefore reject the null hypotheses that C. albicans, A. naeslundii and S. mutans do not form polymicrobial biofilms and that polymicrobial interactions do not affect colonisation of oral microorganisms in a flow-cell environment.

Polymicrobial biofilms have been shown to produce high amounts of extracellular polysaccharides with various by-products in the oral cavity due to interaction with microbial colonies and the oral environment (Koo et al., 2003; Jabra-
Rizk et al., 2006). However the role of biofilm effluent, from *C. albicans*, *A. naeslundii* and *S. mutans* grown as mono-cultured and polymicrobial biofilms, on oral carcinogenesis remains unknown (Chapter 6). To assess the ability of microbial biofilm effluent in promoting oral carcinogenesis, biofilm effluent obtained from *C. albicans*, *A. naeslundii*, *S. mutans* and polymicrobial biofilms was assessed on both normal epithelial cells (OKF6) and oral squamous cell carcinoma epithelial cells (H357) (Section 2.13). An array of assays was conducted including: 1) an adhesion assay to assess the ability of the cell lines to adhere to major important extracellular matrix (ECM) molecules using the CytoSelect 48-well Cell Adhesion Assay ECM Array kit (Section 2.15); 2) an epithelial to mesenchymal transition (EMT) assay to assess the phenotypic changes of the cell lines by the detection of vimentin and E-cadherin expression using flow cytometry (Section 2.17); and 3) the Bio-Plex to assess the expression of pro-inflammatory cytokines from biofilm effluent-treated cells (Section 2.18). The present study showed that the OSCC cell line, H357, when incubated with *C. albicans* biofilm effluent increased adhesion to ECM molecules, particularly collagen IV and laminin I (Chapter 6). Furthermore, an increased adhesion to fibronectin, collagen IV and laminin I was also exhibited when H357 was incubated in polymicrobial biofilm effluent (TRI). The increased adhesion of H357 cells to ECM components is likely to be due to the alteration of cell integrins while interacting with the biofilm effluent (Lyons and Jones, 2007). Integrins are known as the largest family of cell adhesion molecules that consist of multiple combinations of α- and β-subunits (Van Waes and Carey, 1992; Lyons and Jones, 2007). Proteases of *C. albicans* (Karkowska-Kuleta et al., 2009) have been shown to induce alteration of integrins, particularly the α1-subunit of epithelial cell integrins that preferentially bind to collagen IV and the α3-subunit that acts as a receptor for laminins (Lyons and
which are important in the development of oral cancer. The oral environment comprises more diverse polymicrobial biofilms than that assessed in the present study, and these may alter integrins of malignant cells more than observed in the present study, thus promoting oral carcinogenesis in a paracrine fashion (Carter et al., 1990; Ono et al., 1999; Shinohara et al., 1999; Meurman, 2010).

An increase of E-cadherin expression by OKF6 and H357 cells when incubated with biofilm effluent is likely indicative of adhesion, colonisation, internalisation and potentially invasion. An increase of E-cadherin expression has been suggested to be a strategy of colonisation of C. albicans, A. naeslundii and S. mutans to oral epithelial cells (Delva and Kowalczyk, 2009). Furthermore, cadherins have been reported to form a route for the internalisation of pathogens into epithelial cells during oral thrush (Phan et al., 2005; Delva and Kowalcyzyk, 2009). In addition, Als3 protein synthesised by C. albicans has been thought to mimic cadherin-cadherin binding, thus initiating the invasion of yeast into oral epithelial cells (Phan et al., 2005). These findings indicate the strategy of C. albicans colonisation to the surface and the subsurface of oral epithelial cells and is likely to promote carcinogenesis as indicated by the expression of pro-inflammatory cytokines (Section 6.4.3).

We observed an increase of pro-inflammatory cytokine expression by oral cancer cells, H357 when incubated with C. albicans and polymicrobial effluent. This may be due to the presence of proteins glycosylated with N- or O-linked mannosyl residues, β-glucans and chitins from the C. albicans cell wall, as well as the presence of SAPs (Dongari-Bagtzoglou and Kashleva, 2003a; Mostefaoui et al., 2004; Schaller et al., 2005; Netea et al., 2006). The increase of pro-inflammatory cytokines, particularly IL-6, IL-8 and GM-CSF are important in inflammation as well as...
tumorigenesis of malignant cells (Kitadai et al., 2000). IL-6 has been reported to have an anti-apoptotic effect on malignant cells (Thaler et al., 1994; Frassanito et al., 2001; Burgdorf et al., 2009). GM-CSF has been previously shown to be a tumour cell stimulator (Burgdorf et al., 2009), whereas IL-8 has been reported to be involved in carcinogenesis by inducing angiogenesis (Lin and Karin, 2007; Fantini and Pallone, 2008). Cytokines are also known to be secreted by the oral epithelial cells in order to prevent carcinogenesis and as an action to overcome microbial colonisation. However, research has also shown that the over-secretion of pro-inflammatory cytokines may induce carcinogenesis. In the present study, we have found a significant increase of pro-inflammatory cytokines synthesised by the OSCC cell line compared to the normal epithelial cell line, indicating that biofilm effluent from C. albicans grown as both mono-cultured and polymicrobial biofilms is promoting oral cancer, but not inducing cancer (Budhu and Wang, 2006; Fantini and Pallone, 2008).

Thus, these findings on adhesion, EMT and cytokine expression assays supports our hypothesis that oral epithelial cells have an enhanced malignant phenotype, promoting oral carcinogenesis, when grown in the presence of polymicrobial biofilms. Thus the null hypothesis that the presence of polymicrobial biofilms does not enhance the malignant potential of oral epithelial cells was rejected.
7.2 Conclusion and future studies

The findings of the present study supported the overall hypothesis that polymicrobial biofilms of *C. albicans*, *A. naeslundii* and *S. mutans* are involved in oral cancer by promoting carcinogenesis. Moreover, this carcinogenesis promoting activity of polymicrobial biofilms is likely to be *C. albicans* strain-specific. This tumour growth promoting effect of oral microbial biofilms may occur at either the early stages in oral carcinogenesis or perhaps as an enhancement of later tumour progression. Nevertheless, the oral microbial biofilm promotion of oral cancer has profound clinical implications and requires further elucidation of the exact mechanism by which this occurs, as well as *in vivo* confirmation of its occurrence.

Future *in vivo* studies of co-aggregation, biofilm formation of *C. albicans*, *A. naeslundii* and *S. mutans*, and the role of biofilms in the expression of pro-inflammatory cytokines are required to assess oral biological factors, such as salivary flow and immunological components that may influence oral cancer promotion. These *in vivo* studies will enhance our understanding of the interaction of microorganisms in the oral cavity, a process likely to be critical in chronic infection and potentially oral carcinogenesis. An assessment of the by-products secreted in biofilm effluent are also required in order to understand what specific proteins lead to the promotion of oral carcinogenesis. This has the potential for the development of agents that counteract these proteins and then aid in the prevention of oral cancer.
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APPENDICES
EDITORIAL

GAING MORE INSIGHT INTO THE DETERMINANTS OF CANDIDA SPECIES PATHOGENICITY IN THE ORAL CAVITY

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Received June 26, 2013 – Accepted March 17, 2014

Candida infection (candidiasis) is potentially life threatening and can occur in almost all anatomical sites, including the mouth. Candida species are in fact the most common fungal pathogens isolated from the oral cavity and frequently cause superficial infections such as oral candidiasis and denture-associated erythematous stomatitis. Whilst systemic dissemination of Candida from intraoral foci is rare and largely due to severe deficits of the host immune defenses, the development of localized oral candidiasis is most commonly related to a variety of non-immune determinants such as Candida virulence factors and permissive oral microenvironment. In particular, phenotypic switching and dental biofilm have emerged as major determinants for the pathogenicity of Candida and are currently the subject of intense research. An understanding of the molecular aspects underlying the biological behavior of Candida will be the key to the development of effective preventive as well as therapeutic measures for invasive and oral candidiasis.

Candida inhabits various parts of the human body including the epidermis, vagina, gastro-intestinal tract, nails and oral cavity (1). The diseases caused by Candida became common in the late 19th and 20th centuries and its prevalence is still increasing worldwide as a result of multiple factors which can facilitate the conversion of its commensal level to the parasitic level (2). According to Scardina et al. (3), the risk factors that enhance the severity of a candidal infection can be found widely in patients with impaired salivary gland, drug abusers, immunocompromised, high carbohydrate diet, smoking habits and Cushing’s syndrome. Candidal infection can occur in almost all human organs. However, it is the systemic infection that can be much more severe and may lead to mortality. According to Leroy et al. (4), the mortality rate due to systemic infection of Candida is up to 60% and still increasing. The treatment

Key words: oral candidiasis, virulence factors, phenotypic switching, dental biofilm
of candidal infection can be difficult and most of the diagnoses can only be achieved by autopsy. With the current incidence in Europe on the rise, there have been reports of a 5-fold increase in candidemia in the last ten years (5).

*Candida* has been identified as the common member of the oral microflora and estimated to be present in approximately 40-60% of the general population. It can be present either as transient or permanent colonizer in the oral cavity (6). It is also recognised as an opportunistic microorganism that has the ability to cause oral diseases, such as oral candidiasis (7).

The most common oral condition caused by *Candida* is oral candidiasis (8). In a most recent study, candidal infection was also associated with oral cancer, burning mouth syndrome, endodontic diseases and taste disorder (1). *Candida albicans* is the main causative agent of oropharyngeal candidiasis. Researchers have, however, found that non-albicans species also contributed significantly to the development of oral candidiasis (9). Cases due to non-albicans species are increasing in number and this has raised great concern to society.

*Candida* is identified to colonise several types of host cells including epithelial, endothelial and phagocytic cells. In the oral cavity, *Candida* prefers to colonise several surfaces including the buccal and labial mucosa, dorsum or lateral borders of tongue, hard and soft palate regions, tooth surfaces and denture-bearing areas (10). This colonising ability is contributed by factors including the ability of oral *Candida* to produce specific enzymes such as agglutinin-like proteins and integrin-like proteins that lead to the formation of biofilm on oral surfaces. In addition, other factors that influence the colonisation of *Candida* are the reduction of salivary flow, low salivary pH, trauma, carbohydrate-rich diets and epithelial loss (11).

Here we distinguish between two categories of pathogenic determinants: extrinsic determinant, i.e. those provided by the host, which are permissive for growth and survival of *Candida*; and intrinsic determinants, i.e. those related to the characteristics of *Candida* species. Oral biofilm, which *stricto sensu* belongs to the first group, has been considered as an intrinsic determinant in that it relies on the ability of *Candida* to interact with the oral microflora.

**GROWTH REQUIREMENT OF CANDIDA SPECIES**

**Availability of nutrients**

*Candida* is a chemoheterotrophic organism that requires carbon and nitrogen for growth. According to Madigan and Martinko (12), the mutual interaction of carbon and nitrogen is important in the metabolism of microorganisms. Carbohydrates are the most readily utilised form of carbon in both oxidative and non-oxidative pathway. Thus, the presence of carbohydrates influences the colonisation of *Candida* in the oral cavity. Certain carbohydrates, such as sucrose and glucose, have been shown to increase the adhesion potential of *Candida albicans* towards hard and soft surfaces of the oral cavity. Glucose is an acid promoter that leads to the reduction of pH in the oral environment and as a consequence, activates acid proteinases and phospholipases, which enhance the adherence capability of *Candida*. In addition, the production of mannoprotein surface layer in an environment where glucose is present has been shown to assist the adherence capability of *Candida* including *C. krusei* in the oral cavity (7).

*Candida* has a nitrogen content of around 10% of their dry weight (7). The source of nitrogen is usually provided by organic compounds which can be easily found in the oral environment. Nitrogen is also determined as the main stimulatory factor in yeast extract as it encourages bio-stimulation of microbial growth.

**Influence of oral fluids**

Saliva provides moisture and helps in lubricating the oral cavity. Furthermore, it also provides indigenous organic constituents including antimicrobial factors such as lysozyme, lactoferrin, calprotectin, lactoperoxidase, cystatins, histatins, VEGF and SLPI and chromogranin A which inhibit the growth of oral pathogens (13). The presence of cytokines, such as IL-17 and immunoglobulins, in saliva are also beneficial to the oral cavity as they inhibit the dissemination of oral microorganisms especially *Candida* species (14).

Saliva also introduces the formation of a thin film approximately 0.1 mm deep over all external surfaces in the oral cavity. The major role of the whole saliva is to maintain the integrity of teeth by
clearing off food debris and buffering the potential damaging acids produced by oral biofilm or dental plaque. The chemical composition of secretions from each gland is different. Bicarbonate, phosphates and peptides are examples of buffering agents in the saliva that give normal saliva a mean pH of 6.75 to 7.25 (7).

The flow rate of saliva is under the influence of circadian rhythms where the lowest flow rate has often been recorded during sleeping. Low flow rate of saliva reduces the protective function of saliva and increases the colonisation and development of microorganisms including Candida. Salivary composition is also affected by circadian rhythms, for example the total concentration of protein in whole saliva during resting time is estimated at 220 mg/100 mL, whereas the total protein in stimulated saliva is estimated at 280 mg/10 mL. The difference in the amount of protein may affect the distribution of the normal microflora in the oral cavity, as some proteins are known to serve as receptors in the colonisation of microorganisms to the saliva-coated surfaces of the teeth (7). Proteins and glycoproteins such as mucin in the saliva act as the primary source of nutrients for resident microflora including Candida. Salivary composition is also affected by circadian rhythms, for example the total concentration of protein in whole saliva during resting time is estimated at 220 mg/100 mL, whereas the total protein in stimulated saliva is estimated at 280 mg/10 mL. The difference in the amount of protein may affect the distribution of the normal microflora in the oral cavity, as some proteins are known to serve as receptors in the colonisation of microorganisms to the saliva-coated surfaces of the teeth (7).

In addition to saliva, the gingival crevicular fluid (GCF) in the oral cavity can also influence the colonisation of oral Candida species. The flow of GCF is slow at healthy sites but increases drastically at areas with gingivitis by 147% and up to 30-fold at areas with advanced periodontal diseases. GCF also has a role in the development of subgingival plaque around and below the gingival margin. Moreover, it contains higher total protein compared to saliva which is capable of providing nutrient to several commensal microorganisms in the oral cavity (7). Among the host defence components in GCF are IgG and neutrophils which are directed specifically against important periodontal microorganisms and inhibit the colonisation by the action of opsonisation or activation of complement cascade (15).

Role of body temperature

The optimum growth temperature for Candida species including C. albicans has been shown to range from 30°C to 37°C (16). This range of temperature is also the optimum temperature of various pathogenic microorganisms in the oral cavity. Any alteration in the normal body temperature may influence the competitiveness among the normal microflora to survive which will then enhance the development of opportunistic microorganisms such as Candida. Nonetheless, many experimental assays were conducted at 37°C and this is generally accepted as the standard incubation temperature for Candida species (7).

Intrinsic pathogenic determinants of Candida species

The virulent factors of each different Candida species are not similar and can be a competitive factor between each different species. Among the important virulent factors of Candida species are phenotypic switching, adhesion (both to extracellular matrix and dental biofilm), cell surface hydrophobicity, and enzyme production.

Phenotypic switching

Two mechanisms are postulated to be involved in the ability of Candida to survive and adapt in a suppressed environment. The first is by undergoing mitotic recombination and the second is by carrying out phenotypic switching. A direct consequence of mitotic recombination is the loss of heterozygosity throughout the entire genome. This deletion of genome, however, affects the viability of Candida especially in the multiple changing conditions (17). Phenotypic switching, on the other hand is a phenomenon that occurs as a result of changes in the growth environment. A severely suppressed growth condition may lead to high frequency switching in candidal cells (Fig. 1). This adaptation is associated with the alteration of gene expression which eventually may lead to alteration of adheriveness, susceptibility and the resistance of candidal cells to phagocytosis and polymorphonucleur (PMN) leukocyte. This mechanism of action does not involve deletion of any candidal genome, thus, the heterozygosity of the entire genomic are well maintained (7).

Phenotypic switching in Candida albicans was first defined in 1985 as the capacity to undergo
spontaneous, reversible transitions between a set number of colony morphologies (18). This phenomenon is now recognized as an important technique for the survival of *Candida* within an environment such as the oral cavity. This mechanism enables *Candida* to adapt in a suppressed environment and to develop as dominant in the host. *Candida* can undergo reversibly high frequency of phenotypic switching which increases the survivability of the pathogen (17).

Phenotypic switching is identified as one of the important virulent factors in *C. albicans* (17) *C. glabrata* (19) and *C. krusei* (20). The significance of the switching strategy is in a way similar to the human immunity function whereby it is aimed to counter threats in the host’s environment. Therefore, scientists have suggested that phenotypic switching mechanism does enhance the survivability of *Candida* by rapidly changing its phenotype as an adaptive response to the suppressed environment (21).

Phenotypic switching may influence the normal physiological growth of *Candida* species such as *C. albicans* (17). Under the smooth white and wrinkle phenotypes, *C. albicans* has been shown to exhibit faster growing colonies compared to when it is in the form of heavy myceliated with ring phenotype. In addition, phenotypic switching is also discovered to be able to alter the adhesive properties of *Candida*. Findings by our group (20) demonstrated...
that the adherence ability of second generation switched *C. krusei* was increased significantly in flow cell supplemented with unstimulated saliva. Furthermore, this virulence attribute may also induce the formation of tube and pseudohyphae in *Candida*, which enhances the adherence capacity of the candidal strains (19).

**Adhesion: key role of the extracellular polymeric matrix**

The adherence ability of *Candida* is an important factor in the initiation of oral candidiasis. Adherence can occur either on the hard tissue surfaces, such as teeth and palatal surface, or on soft surfaces, such as the buccal and lingual surfaces (22). Characteristics of *Candida* that contribute to the adherence on these surfaces include the formation of pseudohyphae and extracellular matrix.

A single filament hypha (plural, hyphae) is a long branching filamentous structure of fungus which can be found easily in the developmental phase of *Candida* (12). It is classified as the main mode of vegetative fungal growth and consists of one or more cells that are surrounded by tubular cell walls made of chitin. Hyphae usually grow together to form compact tufts which are known as mycelium. Hyphae formation is usually referred to the germination phase of fungi. However, it is also involved in the colonisation of the target host. Pseudohyphae are distinguished from true hyphae by their method of
growth, which lacks cytoplasmic connection between the cells. The pseudohyphae of Candida are usually found to possess incomplete budding blastoconidia whereby cells remain attached to the mother cells after division. C. albicans and C. krusei have been recognised to develop pseudohyphae which adhere to the monolayer of human epithelial cells and hard surfaces (20).

In many cases, extracellular polymeric substance (EPS) matrix is also produced by oral microorganisms once they are adhered to the oral surfaces. EPS matrix is a network of non-living mass which provides support to cells including Candida (Fig. 2). The presence of EPS matrix, which has a slimy texture, provides a significant role to support attachment and proliferation of the cells (23). Furthermore, the synthesis of EPS has also been found to increase significantly when exposed to liquid flow (24). This anchorage property assists the colonisation of Candida to hard tissue surfaces and thus, contributes to the formation of biofilm. When in a biofilm, the resistance of candidal species towards various antifungal agents, including amphotericin B, voriconazole and ketoconazole, has been found to increase up to 1000-fold compared to planktonic stage (24, 25). Mitchell et al. (26) recently found that the presence of matrix B-1,3 glucan in EPS matrix sequesters antifungal drug which then increases the resistance to fluconazole.

**Dental biofilms**

Biofilm production is considered a potential virulence factor of some Candida species (27). Dental biofilm is defined as a thin layer comprising of various communities of microorganisms including bacteria, fungi and yeast that are attached to oral surfaces and on the surface of prosthesis, including dental acrylic surfaces and human epithelial cells. Microorganisms in the biofilm are enclosed in a matrix of extracellular polymeric substance (EPS). This biofilm provides protection to the microorganisms and facilitates the interaction among each other with the contribution of enzymes such as catalase and superoxidase dismutase (11, 28). The development of biofilm is dependent on the dietary, salivary and oral environmental factors that interact with the microorganisms within the community of the biofilm.

The formation of biofilm has been shown to reduce the susceptibility of microorganism to antimicrobial agents, which may then lead to the increase in pathogenicity (11). This phenomenon is suggested to occur due to the restriction of the antimicrobial agents to penetrate the matrix of the biofilm which then reduces the susceptibility of the target microorganism (29). Furthermore, the presence of transcription factor Efg1 in C. albicans biofilm has also been reported to induce the tolerance toward miconazole, caspofungin and amphotericin B (30).

The development of dental biofilm involves several stages which are the acquired pellicle formation on the oral surface; adhesion, reversible and irreversible interactions between the pellicle and the colonising microbes; co-aggregation between microorganisms; and detachment of microbes from the oral surfaces. These sequences of events may eventually form a structural and functional organised microbial community that, if allowed to accumulate, may enhance the potential of periodontal disease and dental caries (28). Specifically, co-aggregation or co-adhesion has been suggested to involve Candida in the late stage of oral biofilm formation. This is a process of microbial adhesion involving the late colonisers on to the early colonisers of dental biofilm. It is a phenomenon of cell-to-cell recognition of genetically distinct partner cell types (31). The co-aggregation can be facilitated either through intragenerics such as the interaction between S. sanguis and Actinomyces sp. or intergenerics such as the interaction between Streptococcus sp. or Actinomyces sp. and Prevotella sp. C. krusei has been found to be involved in co-aggregation with S. mutans, S. sanguis and S. salivaruis in the presence of sucrose. C. albicans has also been reported to have high coaggregation with S. sanguinis, S. oralis and S. gordonii (32). Protein such as lectin is usually involved in co-aggregation. This carbohydrate-binding protein attaches to the carbohydrate-binding protein receptors of other cells which then contribute to the increased thickness of the dental biofilm.

Once a climax community is achieved in the biofilm, detachment of some microbes may occur in the final stage of the oral biofilm development. The microorganism is released from the matrix of the biofilm to the fluid surrounding the biofilm, a
process which has been reported to be facilitated by several enzymes such as proteases, fluid shear stress, multivalent cross-linking cations and microbial growth status (33). This process of detachment will, however, help the microorganism colonise other surfaces in the oral cavity. An example of a microorganism involved in the detachment process from the oral biofilm is *Prevotella loescheii* which produces proteases that hydrolyse adhesion-associated fimbriae which is important in its co-aggregation with *S. oralis* (31). Furthermore, the detachment stage can also be initiated due to the presence of certain quorum sensing molecules such as farnesol, which has been found to be related to biofilm-self-limitation. The level of farnesol increases proportionally to the number of *Candida* cells until threshold where the molecule starts to suppress the yeast-to-mycelium conversion of newly budding cells. As a result, the adherence is reduced within the architecture of biofilm, and releasing yeast forms *Candida* during the dissemination stage (34).

**Cell surface hydrophobicity**

The virulence factor of *C. krusei* can also be observed from the cell surface hydrophobicity characteristic. This factor is classified as one of the most important adherence mechanisms in the colonisation of the host surface, as well as in denture-related candidiasis (22). In fact, one of the key properties contributing to the initial adherence to the solid surfaces of acrylic dentures are hydrophobic interactions, and this feature has salient clinical implications for prevention and therapy of denture-related candidiasis. Various experimental approaches have been used to examine the mechanisms of hydrophobic interactions between *Candida* species and solid surfaces. The hydrophobic nature of the denture surface has been cited as a factor in the development of new bactericidal materials (35).

*C. krusei* is more hydrophobic compared to other medically important *Candida* species (22). *C. krusei* was reported to possess the same hydrophobicity level as *C. glabrata* and *C. Tropicalis*, but is more hydrophobic compared to *C. albicans* and *C. parapsilosis*. Super-hydrophilic surfaces have been reported to accept few bacterial or fungal cells (35) and could be a potent method for the reduction of the adherence of relatively hydrophobic fungal cells, particularly the hyphal form of *C. albicans* which causes denture stomatitis and related infections.

**Enzymatic activity**

Hydrolitic enzymes of *Candida* have been reported to contribute to its pathogenicity in causing oral diseases such as oral candidiasis. The enzymes include aspartyl proteinase, phospholipases, lipases, phosphomonooesterase and hexosaminidase (1). Among these enzymes, aspartyl proteinase has attracted most interest and is widely considered to be central in the development of candidal infection. Aspartyl proteinase is a hydrolytic enzyme which is secreted by the transcription and translation of sphingolipid activator protein (SAP) gene. This enzyme has the ability to invade host and also contributes as a defence system of yeast. Examples of candidal species possessing this enzyme are *C. albicans* and *C. krusei* (22).

Another important hydrolitic enzyme is phospholipase which is identified as an enzyme that invades the host tissue. This enzyme activity has been observed in many fungal pathogens including *Candida*. There are 4 types of phospholipases, namely A, B, C and D. Phospholipase A and C can be found in *C. albicans*; however, there is no evidence that shows the presence of phospholipase B and D in candidal species (22). Phospholipase A can attack cell membranes and can be easily found on the cell surface especially at the sites of bud formation. Hence, the enzyme activity can be enhanced when the hyphae are in direct contact with the host tissue (1).

**CONCLUSIONS**

Candidiasis is an ubiquitous infectious disease and its incidence has been increasing over the last few years, not only in immunocompromised patients, thus becoming a public health problem. Knowledge of factors that affect the virulence of the *Candida* strains is essential, and the oral cavity provides an ideal environment to study not only the intrinsic characteristics of *Candida*, but also their interactions in a complex environment such as the oral biofilm. An understanding of the molecular aspects underlying the biological behavior of Candida will be the key to the development of effective preventive as well as
therapeutic measures for invasive and oral candidiasis.

REFERENCES


RESEARCH ARTICLE

Coaggregation of Candida albicans, Actinomyces naeslundii and Streptococcus mutans is Candida albicans strain dependent

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One sentence summary: Coaggregation between Candida albicans, Actinomyces naeslundii and Streptococcus mutans.

Editor: Richard Calderone

ABSTRACT

Microbial interactions are necessarily associated with the development of polymicrobial oral biofilms. The objective of this study was to determine the coaggregation of eight strains of Candida albicans with Actinomyces naeslundii and Streptococcus mutans. In autoaggregation assays, C. albicans strains were grown in RPMI-1640 and artificial saliva medium (ASM) whereas bacteria were grown in heart infusion broth. C. albicans, A. naeslundii and S. mutans were suspended to give 10⁶, 10⁷ and 10⁸ cells mL⁻¹ respectively, in coaggregation buffer followed by a 1 h incubation. The absorbance difference at 620 nm (ΔAbs) between 0 h and 1 h was recorded. To study coaggregation, the same protocol was used, except combinations of microorganisms were incubated together. The mean ΔAbs% of autoaggregation of the majority of RPMI-1640-grown C. albicans was higher than in ASM grown. Coaggregation of C. albicans with A. naeslundii and/or S. mutans was variable among C. albicans strains. Scanning electron microscopy images showed that A. naeslundii and S. mutans coaggregated with C. albicans in dual- and triculture. In conclusion, the coaggregation of C. albicans, A. naeslundii and S. mutans is C. albicans strain dependent.

Keywords: aggregation; yeast form; hyphal form

INTRODUCTION

Autoaggregation is defined as the adherence ability of microorganisms belonging to the same species (Boris, Suarez and Barbés 1997), while coaggregation is the ability of genetically distinct microorganisms to adhere to each other (Ledder et al. 2008). Both autoaggregation and coaggregation have been classified as important mechanisms in the development of oral biofilms and postulated to provide protective mechanisms to the microbial inhabitants against shear forces that occur within the oral cavity. Aggregation contributes to the integration of new microbial species into biofilms, facilitating the exchange of genes and metabolic products that in turn support survival of microorganisms against variable environmental conditions (Gibbon and Nygaard 1970; Bos, Van-der-Mei and Busscher 1996; Kolenbrander 2000; Kolenbrander et al. 2002; Rickard et al. 2003; Al-Ahmad et al. 2007; Ledder et al. 2008).

Furthermore, coaggregation has been shown to improve the colonization of oral epithelial cells by C. albicans, as preincubation of buccal epithelial cells with fimbriated strains of...
Escherichia coli or Klebsiella pneumoniae increases the adherence and subsequent attachment of C. albicans (Bagg and Silverwood 1986). Preadherence of Streptococcus sanguinis and S. gordonii to the hard surfaces of the oral cavity provides adhesion sites for C. albicans, which supports the importance of interkingdom interactions in the oral cavity (jenkinson, Lala, Shepherd 1990; Bamford et al. 2009; Shirilff, Peters and Jabra-Rizk 2009).

The oral microbiota comprises a wide variety of microorganisms such as yeasts (C. albicans) and bacteria (Actinomycetes spp. and streptococci). Candida spp. that belong to kingdom fungi, especially C. albicans, have been found to colonize approximately 40–50% of healthy oral cavities (Manfredi et al. 2013). The number increases in immunocompromised patients with diseases such as AIDS and diabetes (Grimaudo, Nesbitt and Clark 1996; Thein et al. 2009). The human oral microbiome also comprises of over 600 prevalent taxa at species level although only half of these have been cultured in the laboratory (Dewhirst et al. 2010). Among the important oral bacteria, A. naeslundii is an early oral colonizer that can constitute up to 27% of supragingival dental plaque (Nywad and Kilian 1987; Li et al. 2004). The ability of this species to coaggregate with other oral microorganisms has been well recognized (Grimaudo, Nesbitt and Clark 1996; Li et al. 2001). Streptococcus mutans, an acidogenic and aciduric gram-positive bacterial species, is widely recognized as a causative agent of dental caries (Peters et al. 2012).

The majority of in vitro studies of oral microbial coaggregation have assessed species-specific oral bacteria interactions (Cisar, Kolenbrander and McIntire 1979; Handley et al. 1985; Eke, Rotimi and Laughon 1989; Umemoto et al. 1999; Foster and Kolenbrander 2004; Shen, Samaranyake and Yip 2005; Rosen and Sela 2006; Ledder et al. 2008), and information of interkingdom interactions is further, as yet, no study utilizing artificial saliva medium (ASM) for the growth of C. albicans has been undertaken to assess interkingdom coaggregation. This is clinically relevant as C. albicans grows as yeast in ASM and as hyphae in RPMI-1640, and this dimorphism has a role in the virulence of the species (Arzmi et al. 2012, 2014). The yeast form of C. albicans can adhere to the host cell surfaces by the expression of adhesins, which trigger yeast-to-hyphae transition, followed by the expression of invasins by the hyphal form that mediate the uptake of the fungus by the host cell through endocytosis (Molero et al. 1998; Gow et al. 2011; Sudbery 2011; Mayer, Wilson and Hube 2013). In addition, research has also found that S. salivarius strain K12 preferred to coaggregate to the hyphal region of C. albicans than the yeast after 3 h incubation in RPMI-1640 at planktonic phase (Ishijima et al. 2012). A similar interaction was also observed between S. gordonii and C. albicans in which more bacteria coaggregated at the hyphal region of the yeast (Bamford et al. 2009).

The aim of the present study was to determine the coaggregation of C. albicans, A. naeslundii and S. mutans with the hypothesises that autoaggregation and coaggregation are C. albicans strain dependent.

**MATERIALS AND METHODS**

**Growth of microorganisms**

C. albicans American Type Cell Culture (ATCC) 32354 (ALT1), ATCC MYA-2876 (ALT2), ATCC 90234 (ALT3), ATCC 18804 (ALT4), genotype A isolated from AIDS patient (ALC1), genotype B isolated from AIDS patient (ALC2), oral cancer isolate 1 (ALC3) and oral cancer isolate 2 (ALC4) were used in this study. C. albicans strains were subcultured on Sabouraud’s dextrose agar (Difco, USA) and incubated at 37°C aerobically for 24 h.

To grow bacteria, stock cultures of A. naeslundii (NCTC 10301) and S. mutans (Ingbritt), provided by Oral Health Cooperative Research Centre, Melbourne Dental School, The University of Melbourne, were revived by subculturing onto blood agar (Difco, USA) and Todd-Hewitt yeast extract agar (Difco, USA), respectively. The agar plates were incubated at 37°C for 48 h.

**Aggregation assay**

A semiquantitative spectrophotometric assay based on that outlined by Ledder et al. (2008) and Nagaoka et al. (2008) was used to analyse the aggregation of the microorganisms. Initially, 24-h cultures of C. albicans grown aerobically in RPMI-1640 or 25% ASM (0.625 g L\(^{-1}\) type II porcine gastric mucin, 0.5 g L\(^{-1}\) bacteriological peptone, 0.5 g L\(^{-1}\) tryptone, 0.25 g L\(^{-1}\) yeast extract, 0.088 g L\(^{-1}\) NaCl, 0.05 g L\(^{-1}\) KCl, 0.05 g L\(^{-1}\) CaCl\(_2\) and 0.25 mg mL\(^{-1}\) haemin, pH 7.0 supplemented with 2.5 mM DTT and 0.5 L L\(^{-1}\) sucrose) to stationary phase were harvested by centrifuging at 12 000 g for 5 min and washed twice using coaggregation buffer (0.1 mM CaCl\(_2\), 0.1 mM MgCl\(_2\), 150 mM NaCl, 3.1 mM NaN\(_3\) dissolved in 1 mM Tris buffer and adjusted to pH 7.0). The supernatant was discarded and the pellet resuspended in coaggregation buffer. A similar protocol was repeated for S. mutans and A. naeslundii except these microorganisms were grown in heart infusion broth (HIB) to stationary phase.

To determine autoaggregation, C. albicans, A. naeslundii and S. mutans were standardized in coaggregation buffer to give a final cell density of 10\(^6\), 10\(^7\) and 10\(^8\) cells mL\(^{-1}\), respectively, in separate sterile 2 mL Eppendorf tubes that were equivalent to 1h). In addition, research has also found that S. salivarius strain K12 preferred to coaggregate to the hyphal region of C. albicans than the yeast after 3 h incubation in RPMI-1640 at planktonic phase (Ishijima et al. 2012). A similar interaction was also observed between S. gordonii and C. albicans in which more bacteria coaggregated at the hyphal region of the yeast (Bamford et al. 2009).

The aim of the present study was to determine the coaggregation of C. albicans, A. naeslundii and S. mutans with the hypothesises that autoaggregation and coaggregation are C. albicans strain dependent.

\[ \text{% Auto-aggregation} = \left( \frac{(\text{OD}_{620\text{nm}}(t = 0\text{h}) - \text{OD}_{620\text{nm}}(t = 10\text{h}))}{\text{OD}_{620\text{nm}}(t = 0\text{h})} \right) \times 100 \]

Percentage autoaggregation was calculated for classification of autoaggregation; (1) high (more than 40%), (2) intermediate (30–40%) and (3) low autoaggregation (less than 30%). A similar protocol was repeated for the study of coaggregation by inoculating C. albicans, A. naeslundii or/and S. mutans (interkingdom), and A. naeslundii and S. mutans (intrakingdom) into a sterile 2 mL Eppendorf tube with the same cell density as in the autoaggregation. The suspension was mixed thoroughly using a vortex mixer for 30 s and the OD\(_{620\text{nm}}\) at time (t) = 0 h was recorded. The inoculum was incubated at room temperature for 1 h to allow aggregation and the OD\(_{620\text{nm}}\) was recorded. Sterile coaggregation buffer was used as the blank. Percentage aggregation was calculated using the following equation:

\[ \text{% Co-aggregation} = \left( \frac{(\text{OD}_{620\text{nm}}(t = 0\text{h}) - \text{OD}_{620\text{nm}}(t = 1\text{h}))}{\text{OD}_{620\text{nm}}(t = 0\text{h})} \right) \times 100 \]
Scanning electron microscopy (SEM) imaging

The 0 h and 1 h suspensions (100 μL sample) of a selected representative C. albicans strain, ALT4, A. naeslundii (NCTC 10301) and S. mutans (Ingbritt), prepared as above, were transferred onto cover slips and fixed with 1% osmium tetra-oxyde (OsO₄) vapour. The specimens were dehydrated thoroughly in a freeze-drying system, sputter coated with palladium gold to a thickness of approximately 20 nm and observed using a scanning electron microscope (XL 30 Series, Philips, Japan).

Statistical analysis

All data were statistically analysed using SPSS software version 22.0 using independent t-test and considered statistically significant when P < 0.05.

RESULTS

Morphology of C. albicans in RPMI-1640 and ASM

C. albicans was shown to be predominantly in the hyphal form when grown in RPMI-1640 medium after 24 h incubation whereas the yeast form was the most observed in ASM after the same period of incubation (Fig. 1).

Autoaggregation

Variation in autoaggregation of RPMI-1640 grown C. albicans strains (hyphal growth) was observed with a group of four strains (ALT3, ALT4, ALC1 and ALC3) exhibiting high autoaggregation (over 40%), two strains (ALT1 and ALC4) exhibiting intermediate autoaggregation (30–40%) and two strains (ALT2 and ALC2) exhibiting low autoaggregation (Table 1, Fig. 2A). The autoaggregation values of A. naeslundii and S. mutans were also classified as low with 11.4 and 7.4%, respectively (Table 1).

Four strains of ASM-grown C. albicans (ALT2, ALT3, ALC1 and ALC4) (yeast growth) exhibiting intermediate autoaggregation while the remainder strains (ALT1, ALT4, ALC2 and ALC3) were classified as exhibiting low autoaggregation (Table 1, Fig. 2B).

There were four strains of C. albicans that exhibited significantly more autoaggregation when grown in RPMI-1640 (hyphal growth) (ALT1, ALT4, ALC1 and ALC3) compared to ASM (yeast growth) (P < 0.05). Two strains (ALT2 and ALC2) showed significantly more autoaggregation when grown in ASM than RPMI-1640 (P < 0.05) and two strains (ALT3 and ALC4) exhibited no difference in autoaggregation regardless of the media type (Fig. 2).

Interkingdom coaggregation

All strains of RPMI-grown C. albicans (hyphal growth) were found to coaggregate with A. naeslundii ranging from 9.9 ± 0.5% (ALT3) to 26.2 ± 0.4% (ALC3). Coaggregation of RPMI-grown C. albicans with A. naeslundii and S. mutans was also observed for all strains of the yeast ranging from 2.2 ± 0.3% (ALT3) to 17.0 ± 0.6% (ALC1). Our study showed that ASM-grown C. albicans strains (yeast form) coaggregated with A. naeslundii ranging from 9.6 ± 0.7% (ALT2) to 23.0 ± 0.1% (ALC3). ASM-grown C. albicans strains were observed to coaggregate S. mutans ranging from 9.9 ± 0.2% (ALT3) to 28.1 ± 0.1% (ALT4) (Table 1). Coaggregation of ASM-grown C. albicans with A. naeslundii and S. mutans were observed in all strains of the yeast ranging from 12.9 ± 0.4% (ALT2) to 25.8 ± 0.5% (ALT1) (Table 1).

SEM analyses

SEM analysis of RPMI-grown C. albicans ALT4 strain exhibited autoaggregation in coaggregation buffer after 1 h incubation (Fig. 3A). Coaggregation was observed between C. albicans and A. naeslundii (Fig. 3B). In addition, an SEM image also revealed that S. mutans coaggregated with C. albicans mostly at the hyphal region of the yeast (Fig. 3C). The coaggregation of RPMI-grown ALT4 C. albicans with A. naeslundii and S. mutans showed that A. naeslundii and S. mutans were partially aggregating with C. albicans at the hyphal region. A. naeslundii was also observed to coaggregate with S. mutans (Fig. 3D).

SEM analysis showed that ASM-grown C. albicans ALT4 strain (yeast growth) had autoaggregation (Fig. 3E) and A. naeslundii was found to coaggregate on the yeast surface after 1 h incubation (Fig. 3F). Coincubation of ALT4 C. albicans with S. mutans revealed that there was interkingdom coaggregation between the two microorganisms with clumps of bacteria attached to the yeast surface of ALT4 C. albicans (Fig. 3G). In addition, an SEM image of the interaction between ASM-grown ALT4 C. albicans with both bacterial species showed that A. naeslundii and S. mutans coaggregated on the surface of the yeast. Finally, the image also revealed that S. mutans cells were coaggregating with A. naeslundii after 1 h incubation (Fig. 3H).

Taken together, the data demonstrate that the autoaggregation and interkingdom coaggregation of C. albicans, A. naeslundii and S. mutans are C. albicans strain dependent.
**DISCUSSION**

Coaggregation is a mechanism that induces the development of a complex architecture of oral biofilms, which assists the attachment of secondary colonizers such as *S. mutans* (Kolenbrander 2000; Min and Rickard 2009).

We have shown that interkingdom coaggregation was strain dependent. The coaggregation of the majority of RPMI-grown (hyphal growth) *C. albicans* strains, when grown with *S. mutans* and *A. naeslundii* either alone or in combination, resulted in variable coaggregation. The observed variability of coaggregation in *C. albicans* may be attributable to the different abundances of specific molecules that are important in adhesion and quorum sensing (e.g. farnesol) from different strains, which have been suggested to have a role in interkingdom interactions of *C. albicans* and bacteria (Morales and Hogan 2010). Furthermore, the variability of coaggregation observed in ASM-grown *C. albicans* (yeast growth) supports our hypothesis that the coaggregation of *C. albicans* to *A. naeslundii* and *S. mutans* is highly dependent on the individual yeast strain.

We have observed variability of coaggregation when ASM-grown *C. albicans* strains were coincubated with *S. mutans* and *A. naeslundii*. This variability suggests that *S. mutans* might have induced the formation of binding sites on the yeast surface that allow the coaggregation of *A. naeslundii* to ASM-grown *C. albicans* when cocultured. These results support our hypothesis that coaggregation is highly dependent on the individual yeast strain. It cannot be related to the production of glucan by *S. mutans* glucosyltransferases as no sucrose was present; however, it may be that specific proteins are induced on the surface of *C. albicans* due to the interaction with *S. mutans* that promotes further interaction with *A. naeslundii* (Holmes, Gopal and Jenkinson 1995; Koo et al. 2010; Falsetta et al. 2014). Further research is necessary to assess this hypothetical possibility.

It can be postulated that the observed variability in coaggregation may be related to that specific strain’s ability to produce both non-specific (adhesins) and specific (lectin-saccharide) cell surface receptors (Kolenbrander and Williams 1981; McIntyre, Crosby and Vatter 1982; Rickard et al. 2003; Rosen and Sela 2006; Ledder et al. 2008). Previous studies have shown that the specific coaggregation between *C. albicans* and *A. naeslundii* is due to the presence of mannose-containing adhesin protein on the yeast cell surface (Grimaudo, Nesbitt and Clark 1996). This same study also showed variation in the coaggregation of *A. naeslundii* with four different yeast strains which supports the present study. Furthermore, other research has shown significant strain variation of the cell wall biogenesis in *C. albicans*, that may have a role in the observed variation in aggregation ability (Ragni et al. 2011). Further analysis of the cell wall structure of a range of *C. albicans* strains is necessary to fully elucidate the mechanism of this observed variability.

It has previously been suggested that, due to the limitation of nutrients present in RPMI-1640, growth in this media induces yeast–hyphae transition leading to predominant hyphal growth (Urban et al. 2006). Our light microscope images confirmed this with greater than 75% of *C. albicans* cells growing in hyphal form in RPMI-1640. No previous study has assessed the form of growth at SEM level when *C. albicans* is grown in ASM. The present study
Figure 2. Percentage autoaggregation in RPMI-1640 (A) and ASM (B) grown C. albicans after 1 h incubation in coaggregation buffer. Data were analysed using independent t-test and considered as significantly different when $P < 0.05$. Asterisk indicates significantly more autoaggregation between the two growth media.

Figure 3. SEM of C. albicans autoaggregation (A and E), interkingdom interaction with A. nae slundii (B and F), S. mutans (C and G) and both bacteria (D and H). C. albicans was grown in RPMI-1640 (A–D) and ASM (E–H). Magnification is as shown on each image (6500× and 10 000×).

is the first to observe C. albicans cellular morphology in ASM using SEM imaging and we have shown that, similar to the light microscope observations, in this media C. albicans does not grow in hyphal form.

Future assessment of coaggregation of C. albicans, A. nae slundii and S. mutans requires animal studies to assess oral biological factors, such as salivary flow and immunological components that exist in the oral cavity, which may influence aggregation. These in vivo studies of coaggregation are likely to enhance our understanding of the mutual interaction of microorganisms in the oral cavity, a process likely to be critical in chronic infection and potentially oral carcinogenesis.

**CONCLUSION**

In conclusion, autoaggregation and interkingdom coaggregation of C. albicans have been shown to be strain dependent and this is likely to be important in polymicrobial oral biofilm formation.
FUNDING
This work was funded by Oral Health Cooperative Research Centre (OHCRC) and the Melbourne Dental School.

Conflict of interest. None declared.

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Polymicrobial biofilm formation by *Candida albicans*, *Actinomyces naeslundii*, and *Streptococcus mutans* is *Candida albicans* strain and medium dependent

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Received 27 January 2016; Revised 24 April 2016; Accepted 26 April 2016

Abstract

Oral biofilms comprise of extracellular polysaccharides and polymicrobial microorganisms. The objective of this study was to determine the effect of polymicrobial interactions of *Candida albicans*, *Actinomyces naeslundii*, and *Streptococcus mutans* on biofilm formation with the hypotheses that biofilm biomass and metabolic activity are both *C. albicans* strain and growth medium dependent. To study monospecific biofilms, *C. albicans*, *A. naeslundii*, and *S. mutans* were inoculated into artificial saliva medium (ASM) and RPMI-1640 in separate vials, whereas to study polymicrobial biofilm formation, the inoculum containing microorganisms was prepared in the same vial prior inoculation into a 96-well plate followed by 72 hours incubation. Finally, biofilm biomass and metabolic activity were measured using crystal violet and XTT assays, respectively. Our results showed variability of monospecies and polymicrobial biofilm biomass between *C. albicans* strains and growth medium. Based on cut-offs, out of 32, seven RPMI-grown biofilms had high biofilm biomass (HBB), whereas, in ASM-grown biofilms, 14 out of 32 were HBB. Of the 32 biofilms grown in RPMI-1640, 21 were high metabolic activity (HMA), whereas in ASM, there was no biofilm had HMA. Significant differences were observed between ASM and RPMI-grown biofilms with respect to metabolic activity (*P* < .01). In conclusion, biofilm biomass and metabolic activity were both *C. albicans* strain and growth medium dependent.

Key words: Polymicrobial biofilm, crystal violet assay, XTT assay.

Introduction

The oral cavity is a habitat for various microorganisms including yeast and bacteria.\(^1\) This oral microbiome provides a balanced oral environment however perturbation of this homeostasis may lead to the development of dysbiosis and oral disease.\(^2\)
Candida species, Actinomyces species and streptococci are common inhabitants of the human oral cavity.\textsuperscript{3–5} Candida spp. have been found to colonise approximately 50% of healthy human oral cavities.\textsuperscript{6} Candida albicans is the most frequently isolated Candida spp. from the oral cavity, especially in immunocompromised patients with diseases such as AIDS and diabetes.\textsuperscript{7,8} Many actinomycetes and streptococci are normal components of the human oral microbiota, with some species associated with dental caries initiation and development.\textsuperscript{4} Actinomyces naeslundii is categorized as an early oral coloniser that can constitute up to 27% of supragingival dental plaque.\textsuperscript{9–10} Streptococcus mutans is an acidogenic and aciduric Gram-positive oral bacterium that is widely regarded as a pathogen that initiates dental caries in association with other oral bacteria.\textsuperscript{4,11}

Dimorphism is an important virulence factor of C. albicans. It is defined as the ability of Candida spp. to change morphology between yeast and hyphal forms.\textsuperscript{12,13} C. albicans is predominantly in the yeast form during early colonisation of the oral cavity, however, subsequent invasion of oral epithelial cells is predominantly by the hyphal form. The yeast form of C. albicans can adhere to host cell surfaces by the expression of adhesins, which trigger yeast-to-hyphae transition, followed by the expression of invasins by the hyphal form that mediate the uptake of the fungus by the host cell through induced endocytosis.\textsuperscript{14–16}

The majority of in vitro studies of biofilms have been with monospecies and dual-species oral microorganisms,\textsuperscript{17–25} and information from triculture polymicrobial biofilms remains limited.\textsuperscript{26–28} As yet, no study utilising artificial saliva medium (ASM) for the growth of C. albicans has been undertaken to assess polymicrobial biofilms. This is clinically relevant as C. albicans grows as yeast in ASM and as hyphae in RPMI-1640.

Crystal violet (CV) and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium hydroxide (XTT) assays are two methods for biofilm quantification. CV assay measures the microbial biofilm biomass where the dye interacts with negatively charged molecules present on the surface of the microorganisms and extracellular polysaccharide.\textsuperscript{10} The XTT assay is a colorimetric-based assay of cell metabolic activity using tetrazolium hydroxide.\textsuperscript{29} Tetrazolium hydroxide is an active compound that is converted to formazan by the activity of dehydrogenases involved in the metabolic pathways of microbial cells.\textsuperscript{30} Succinate dehydrogenases of prokaryotic cells and mitochondrial dehydrogenases of eukaryotic cells are examples of dehydrogenase activity that can be detected by XTT.\textsuperscript{29,31}

The aims of the present study were to determine the effect of interactions of C. albicans, A. naeslundii and S. mutans on the formation of polymicrobial biofilms and to assess this interaction when biofilms were grown in ASM for predominantly yeast growth and in RPMI-1640 for predominantly hyphal growth. We hypothesized that this polymicrobial biofilm formation is C. albicans strain- and growth medium-dependent.

Materials and methods

Growth of microorganisms

C. albicans American Type Cell Culture (ATCC) 32354 (ALT1), ATCC MYA-2876 (ALT2), ATCC 90234 (ALT3), ATCC 18804 (ALT4), a genotype A strain isolated from oral infections in a human immunodeficiency virus (HIV) positive patient (ALC1), a genotype B strain isolated from oral infections in an HIV positive patient (ALC2), a strain isolated from oral cancer patient number one (ALC3), and a strain isolated from oral cancer patient number two (ALC4) were used in the present study.\textsuperscript{32,33} C. albicans strains were subcultured on Sabauraud’s dextrose agar (SDA) (Difco, USA) and incubated at 37°C aerobically for 24 hours.

Bacteria were grown from stock cultures of A. naeslundii (NCTC 10301) and S. mutans (Ingbrit), provided by the Oral Health Cooperative Research Centre, Melbourne Dental School, The University of Melbourne, and were revived by subculturing onto blood agar (40 g/l blood agar base and 100 ml/l defibrinated horse blood) and Todd-Hewitt yeast extract (THYE) agar (36.4 g/l Todd-Hewitt broth, 8 g/l yeast extract and 15 g/l Bacto agar), respectively. The agar plates were incubated at 37°C for 24 hours.

Static biofilm formation

A quantitative assay based on that outlined by Yamada et al.\textsuperscript{34} and Alnuaimi et al.\textsuperscript{32} was used to analyze static biofilm formation by the microorganisms. To study monospecies biofilm, streak diluted cultures of C. albicans, A. naeslundii and S. mutans were grown on SDA, blood agar and THYE agar respectively, for 24 hours at 37°C and several single colonies were resuspended in RPMI-1640\textsuperscript{32} or 25% ASM (0.625 g/l type II porcine gastric mucin, 0.5 g/l bacteriological peptone, 0.5 g/l tryptone, 0.25 g/l yeast extract, 0.088 g/l NaCl, 0.05 g/l KCl, 0.05 g/l CaCl\textsubscript{2} and 0.25 mg/ml haemin, pH 7.0 supplemented with 2.5 mM DTT and 0.5 g/l sucrose), and standardized to give a final cell density of 10\textsuperscript{6} cells/ml, 10\textsuperscript{7} cells/ml and 10\textsuperscript{8} cells/ml, respectively, in a separate sterile 2-ml Eppendorf tubes that equivalent to an absorbance of 0.5 at 620 nm wavelength (OD\textsubscript{620nm}). The suspensions were mixed thoroughly using a vortex mixer for 30 seconds. Subsequently, 200 µl of each suspension containing 2 × 10\textsuperscript{5} cells (C. albicans), 2 × 10\textsuperscript{6} cells (A. naeslundii) and 2 × 10\textsuperscript{7} cells (S. mutans) of
initial inoculum was pipetted into each well of sterile 96-well plate (Nunc, Denmark). Finally, the plate was incubated in an orbital shaker at 90 rpm for 72 hours at 37°C (Alyos, Thermo Fisher Scientific, Australia) to mimic the dynamic of oral environment. The medium was replenished aseptically every 24 hours.

A similar protocol was used to study polymicrobial biofilm formation by inoculating C. albicans, A. naeslundii or/and S. mutans into a sterile 2-ml Eppendorf tube with a similar cell density as in the monospecies assay resulting in 2 × 10⁵ cells (C. albicans), 2 × 10⁶ cells (A. naeslundii), and 2 × 10⁷ cells (S. mutans) for each combination per well. The suspension was mixed thoroughly using a vortex mixer and 200 µl of the suspension was pipetted into sterile 96-well plate. The plate was incubated aerobically for 72 hours at 37°C in an orbital shaker at 90 rpm and the medium was replenished aseptically every 24 hours.

**Gram stain**

Gram stain was performed on C. albicans ALC3 strain following growth in RPMI-1640 and ASM for 72 hours at 37°C for the determination of morphology. Initially, 1 ml of suspension of RPMI-1640 or ASM-grown C. albicans containing 2 × 10⁵ cells was pipetted into each well of 12-well plate and incubated at 37°C in an orbital shaker at 90 rpm. The medium was replenished aseptically every 24 hours of incubation. Following incubation, the supernatant was discarded and each well was washed carefully with phosphate buffered saline (PBS) (Sigma-Aldrich, USA) twice to remove non-adherent cells. Later, Gram staining was performed and the sample was observed under a light microscope (CH Series, Olympus, Australia).

**Crystal violet assay**

Crystal violet (CV) assay was performed according to the protocol outlined by Alnuaimi et al. Initially, the biofilm in each well of 96-well plate was washed twice with sterile PBS to remove nonadherent cells. In sum, 200 µl of methanol was added to each well for fixation and incubated for 15 minutes at 25°C. The supernatant was then discarded and the plate was air-dried for 45 minutes. And 200 µl of 0.1% (w/v) CV solution was added into each well and incubated for a further 20 minutes at 25°C. The plate was washed gently twice using running distilled water, and 200 µl of 33% (v/v) acetic acid was added to de-stain the biofilm. The plate was incubated for five minutes at room temperature. A 100 µl aliquot of this solution was transferred to a new sterile 96-well plate and the absorbance was measured at OD₆₂₀ nm using a microtiter plate reader (Victor³, Perkin-Elmer, Australia).

**XTT reduction assay**

XTT reduction assay was performed according to the protocol provided by the manufacturer (Sigma-Aldrich, USA). Briefly, the biofilm-coated wells were washed twice with sterile PBS to remove non-adherent cells. Subsequently, 160 µl of sterile PBS and 40 µl of 4% XTT salt containing 1% phenazine methosulphate (Sigma-Aldrich, USA) were pipetted into each well to give a final volume of 200 µl. The plate was incubated at 37°C for three hours in the dark. Following incubation, 100 µl of the suspension was transferred into a new sterile 96-well plate and the absorbance at OD₄₅₀ nm and OD₆₂₀ nm wavelengths were measured using a microtiter plate reader. Measurement of absorbance at the reference wavelength of OD₆₂₀ nm was subtracted from OD₄₅₀ nm to remove background absorbance.

**Statistical analysis**

All biofilms containing C. albicans were divided into terciles according to biofilm biomass and metabolic activity for CV and XTT assays, respectively. This method of dividing a population of C. albicans containing biofilms into high, moderate and low has been previously used to assess both biofilm biomass and biofilm metabolic activity. This division provided the cut-offs to classify strains as high, moderate and low biofilm biomass (HBB, MBB and LBB); and high, moderate and low metabolic activity (HMA, MMA, and LMA). Using SPSS software version 22.0, all data were statistically analyzed by applying chi-square test to compare between the categories for each assay and two-tailed t-test to compare between ATCC and clinical strains biofilm biomass. Comparison between a group of ATCC isolates (ALT1, ALT2, ALT3, and ALT4) and a group of clinical isolates (ALC1, ALC2, ALC3, ALC4) of C. albicans was analyzed using two-tailed t-test. Multiple comparisons between monospecies with polymicrobial biofilms such as between monospecies C. albicans ALT1 with dual-species C. albicans ALT1-A. naeslundii, C. albicans ALT1-S. mutans, and trispecies, were compared using ANOVA post hoc Tukey test.

**Results**

**Morphology of C. albicans biofilms in RPMI-1640 and ASM**

C. albicans biofilm growth was predominantly in the hyphal form when grown in RPMI-1640, and in the yeast form when grown in ASM after 24 hours incubation as observed by Gram staining (Figure 1).
Figure 1. Gram-stained biofilms of *Candida albicans* strain ALC3 observed under light microscope at 200x magnification after 72 hours incubation at 37°C in 24-well plate at 90 rpm. A: Artificial saliva medium (ASM)-grown *Candida albicans* biofilm; B: RPMI-grown *Candida albicans* biofilm.

Effect of microbial interaction and growth medium on biofilm biomass

Biofilm biomass was categorized into terciles using the following CV measurement cut-offs: LBB < 2.280, MBB 2.280-2.535, HBB > 2.535. None of monocultured *C. albicans* was categorized as HBB, however, when co-cultured with *A. naeslundii* three *C. albicans* strains (ALT1, ALT2, and ALT3) were categorized as HBB (Table 1). Only ALT1 was categorized as HBB when co-cultured with *S. mutans* in RPMI-1640 whereas in tricultured biofilms, three strains of *C. albicans* (ALT1, ALT2, and ALT3) were categorized as HBB (Table 1).

None of ASM monocultured *C. albicans* exhibited HBB, however, in the presence of *A. naeslundii*, seven strains of *C. albicans* were classified as HBB (Table 1). Interaction of *C. albicans* with *S. mutans* showed that two strains (ALT1 and ALT3) were HBB, while in tricultured biofilms, five *C. albicans* strains (ALT1, ALT2, ALT3, ALT4, and ALC2) were classified as HBB (Table 1).

Table 1. Static biofilm biomass scores of 8 strains of RPMI-grown (hyphal form) and artificial saliva medium (ASM)-grown (yeast form) *Candida albicans*, *Actinomyces naeslundii* (An), and *Streptococcus mutans* (Sm).

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Strains</th>
<th>RPMI-1640</th>
<th>ASM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mono</td>
<td>An</td>
</tr>
<tr>
<td>ALT1</td>
<td>(0.326)</td>
<td>2.501</td>
<td>2.656</td>
</tr>
<tr>
<td>ALCC</td>
<td>(0.064)</td>
<td>2.408</td>
<td>2.666</td>
</tr>
<tr>
<td>ALT3</td>
<td>(0.129)</td>
<td>1.554</td>
<td>2.492</td>
</tr>
<tr>
<td>ALT4</td>
<td>(0.155)</td>
<td>1.762</td>
<td>2.259</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.226)</td>
<td>(0.121)</td>
</tr>
<tr>
<td>ALC1</td>
<td>(0.267)</td>
<td>1.594</td>
<td>2.268</td>
</tr>
<tr>
<td>ALC2</td>
<td>(0.012)</td>
<td>1.722</td>
<td>2.215</td>
</tr>
<tr>
<td>ALC3</td>
<td>(0.267)</td>
<td>1.445</td>
<td>1.965</td>
</tr>
<tr>
<td>Bacteria</td>
<td>(0.002)</td>
<td>0.066</td>
<td>0.149</td>
</tr>
</tbody>
</table>

Readings are absorbance measured at OD_{520 nm}. Data are means from three separate experiments (SD are given in parenthesis). Significant difference (P<0.05) observed between dual-species *C. albicans-A. naeslundii* (*), *C. albicans-S. mutans* (#) or tri-species (ø) to mono-species *C. albicans* biofilms grown in the same medium.
Table 2. Static biofilm metabolic activity scores of RPMI-grown (hyphal form) and artificial saliva medium (ASM)-grown (yeast form) Candida albicans, Actinomyces naeslundii (An) and Streptococcus mutans (Sm).

<table>
<thead>
<tr>
<th>Strains</th>
<th>RPMI-1640</th>
<th>ASM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono</td>
<td>An</td>
</tr>
<tr>
<td>ALT1</td>
<td>0.780</td>
<td>0.819</td>
</tr>
<tr>
<td></td>
<td>(0.022)</td>
<td>(0.021)</td>
</tr>
<tr>
<td>ALT2</td>
<td>0.788</td>
<td>0.811</td>
</tr>
<tr>
<td></td>
<td>(0.012)</td>
<td>(0.029)</td>
</tr>
<tr>
<td>ALT3</td>
<td>0.395</td>
<td>*0.610</td>
</tr>
<tr>
<td></td>
<td>(0.074)</td>
<td>(0.120)</td>
</tr>
<tr>
<td>ALT4</td>
<td>0.738</td>
<td>0.665</td>
</tr>
<tr>
<td></td>
<td>(0.130)</td>
<td>(0.035)</td>
</tr>
<tr>
<td>ALC1</td>
<td>0.641</td>
<td>0.645</td>
</tr>
<tr>
<td></td>
<td>(0.033)</td>
<td>(0.058)</td>
</tr>
<tr>
<td>ALC2</td>
<td>0.610</td>
<td>*0.726</td>
</tr>
<tr>
<td></td>
<td>(0.034)</td>
<td>(0.003)</td>
</tr>
<tr>
<td>ALC3</td>
<td>0.525</td>
<td>0.522</td>
</tr>
<tr>
<td></td>
<td>(0.030)</td>
<td>(0.004)</td>
</tr>
<tr>
<td>ALC4</td>
<td>0.557</td>
<td>*0.638</td>
</tr>
<tr>
<td></td>
<td>(0.024)</td>
<td>(0.019)</td>
</tr>
</tbody>
</table>

Analyses of all 32 biofilms for biomass showed that there were seven biofilms classified as HBB (21.9%), 12 MBB (37.5%), and 13 LBB (40.6%) when the biofilms were grown in RPMI-1640 (hyphal growth). Biofilms grown in ASM (yeast form) showed 14 biofilms categorized as HBB (43.8%), ten MBB (31.3%), and eight LBB (25.0%). There were more biofilms with HBB when grown in ASM (yeast form) than RPMI-1640 (hyphal form), however, this did not reach statistical significance ($P > .05$).

Five RPMI-grown biofilms (hyphal form) had significantly increased biomass when C. albicans strains were co-cultured with A. naeslundii (ATCC: ALT1, ALT4; Clinical: ALC1, ALC2, and ALC4) compared with monocultured C. albicans biofilm ($P < .05$). Further, co-culture of C. albicans with S. mutans increased biomass of six biofilms (ATCC: ALT1, ALT4; Clinical: ALC1, ALC2, ALC3 and ALC4) significantly ($P < .05$). Five biofilms (ATCC: ALT1, ALT4; Clinical: ALC1, ALC2, and ALC4) increased biomass significantly when C. albicans was co-cultured with both A. naeslundii and S. mutans when compared with the monocultured biofilm of C. albicans ($P < .05$; Table 1).

Two ASM-grown biofilm (ATCC: ALT1 and ALT2; yeast form) had a significantly increased biomass when C. albicans was co-cultured with A. naeslundii compared with the monocultured C. albicans biofilm ($P < .05$). One biofilm (ATCC: ALT1) showed a significant increase ($P < .05$) and one (ATCC: ALT2) a significant decrease ($P < .05$) in biomass when C. albicans was co-cultured with S. mutans. There was one strain (ATCC: ALT1) that showed a significant increase in biomass when C. albicans was co-cultured with both A. naeslundii and S. mutans compared with monocultured C. albicans biofilm ($P < .05$; Table 1).

Effect of microbial interaction and growth medium on metabolic activity

Biofilm metabolic activity based on the XTT assay was divided into terciles and categorized based on the following cut-offs: LMA $< 0.120$, MMA $0.120-0.550$, HMA $> 0.550$. RPMI-1640 monocultured growth resulted in six strains of C. albicans (ALT1, ALT2, ALT4, ALC1, ALC2, and ALC4) categorized with HMA (Table 2). Seven C. albicans strains (ALT1, ALT2, ALT3, ALT4, ALC1, ALC2, and ALC4) when co-cultured with A. naeslundii in RPMI-1640 had HMA. Only two strains of C. albicans (ALT1 and ALT2) had HMA when co-cultured with S. mutans in RPMI-1640. Six C. albicans strains (ALT1, ALT2, ALT3, ALT4, ALC2, and ALC4) were categorized as having HMA when co-cultured in RPMI-1640 with both A. naeslundii and S. mutans (Table 2).
ASM monocultured growth resulted in all *C. albicans* strains being categorized with LMA (Table 2), however, in the presence of *A. naeslundii*, all strains had MMA. Interaction of *C. albicans* with *S. mutans* showed that all *C. albicans* strains remained with LMA whereas, in the presence of both *A. naeslundii* and *S. mutans*, there were three strains having MMA (ALT1, ALT4, and ALC2) and five strains with LMA (ALT2, ALT3, ALC1, ALC3, and ALC4) (Table 2).

Analyses of all 32 biofilms showed that there were 21 biofilms of RPMI-grown biofilms (hyphal growth) categorized as having HMA (65.6%) and 11 with MMA (34.4%). In addition, there were 11 ASM-grown biofilms (yeast growth) categorized as having MMA (34.4%) and 21 with LMA (65.6%). Thus, statistically significant higher metabolic activity was observed when biofilms were grown in RPMI-1640 (*P* < .01).

Only *C. albicans* strains ALT3 when co-cultured with *A. naeslundii* showed an increased activity when grown in RPMI-1640 when compared with monospecies *C. albicans*. Furthermore, there were four *C. albicans* strains (ALT4, ALC1, ALC2, and ALC4) that exhibited a decrease in metabolic activity when co-incubated with *S. mutans* compared with the monocultured biofilm of *C. albicans*. There was only one biofilm (ALC1) that showed decreased bioactivity when *C. albicans* was co-cultured with both *A. naeslundii* and *S. mutans* compared with monocultured *C. albicans* (Table 2).

Three RPMI-grown biofilms (ATCC: ALT3; Clinical: ALC2 and ALC4; hyphal form) exhibited significant increased activity when *C. albicans* was co-cultured with *A. naeslundii* in comparison with the monocultured *C. albicans* biofilm (*P* < .05). Four biofilms (ATCC: ALT4; Clinical: ALC1, ALC2, and ALC4) showed significant decreased metabolic activity when *C. albicans* was co-cultured with *S. mutans*. Whereas, one biofilm (Clinical: ALC1) displayed a significant decreased activity when *C. albicans* was co-cultured with both *A. naeslundii* and *S. mutans* when compared with monocultured *C. albicans* (*P* < .05; Table 2).

Finally, based on metabolic activity per unit biomass in monospecies biofilms, ALT4 and ALC3 were found to be the most active *C. albicans* strains when grown in ASM and ALT2 was the least active when grown in the same medium. Whereas, in RPMI-1640, ALC3 was found to be the most active while ALT3 was the least (Table 3).

### Discussion

To our knowledge, this is the first study to evaluate the effect of microbial interactions of yeast growth and hyphal growth of *C. albicans*, *A. naeslundii*, and *S. mutans* on the formation of static biofilms *in vitro*. The results of the present study clearly demonstrate that both biofilm biomass and metabolic activity are *C. albicans* strain and growth medium dependent.

The present study has shown a variation of biofilm biomass and metabolic activity between strains of *C. albicans*. Overall, when grown as monospecies the majority of clinical strains had a significantly lower biofilm biomass than the ATCC reference strains. However, a significant increase of biomass was observed in all clinical strains that did not occur in ATCC strains (ALT2 and ALT3) when grown in polymicrobial biofilms. Previous research also showed that biofilms formed by clinical isolates of *C. albicans* exhibited lower biofilm biomass compared with the reference strains *C. albicans*. Furthermore, the metabolic activity has been shown to vary among *C. albicans* strains; however, the morphology of *C. albicans* in this previous study was unknown. Strain variability of *C. albicans* has been shown in the oral cavity of different individuals.

Previous research has shown that *C. albicans* strains isolated from HIV-infected patients produce higher levels of aspartic proteinases (SAPs), compared with strains isolated from uninfected patients. SAP is a putative virulence factor that is able to affect *C. albicans* biofilm formation in the oral cavity together with phenotypic switching, morphogenesis and quorum sensing. Thus, the results from the present study may indicate a symbiotic interaction between clinical *C. albicans* and oral microorganisms that may lead to the increase of colonisation in the oral cavity of diseased patients. The metabolic activity of biofilms was shown to be growth media dependent, with the majority of removal of both *C. albicans* and ALC1 to ALC4 for clinical strains), *Actinomyces naeslundii* (An) and *Streptococcus mutans* (Sm).

**Table 3.** Monospecies biofilm metabolic activity over biomass (XTT assay/crystal violet assay) scores of eight strains of RPMI-grown (hyphal form) and artificial saliva medium (ASM) - grown (yeast form) *Candida albicans* (ALT1 to ALT4 for ATCC strains and ALC1 to ALC4 for clinical strains), *Actinomyces naeslundii* (An) and *Streptococcus mutans* (Sm).

<table>
<thead>
<tr>
<th>Media</th>
<th>ALT1</th>
<th>ALT2</th>
<th>ALT3</th>
<th>ALT4</th>
<th>ALC1</th>
<th>ALC2</th>
<th>ALC3</th>
<th>ALC4</th>
<th>An</th>
<th>Sm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>0.326</td>
<td>0.319</td>
<td>0.166</td>
<td>0.476</td>
<td>0.366</td>
<td>0.382</td>
<td>0.807</td>
<td>0.388</td>
<td>0.681</td>
<td>0.022</td>
</tr>
<tr>
<td>(0.007)</td>
<td>(0.046)</td>
<td>(0.044)</td>
<td>(0.075)</td>
<td>(0.027)</td>
<td>(0.009)</td>
<td>(0.022)</td>
<td>(0.044)</td>
<td>(0.035)</td>
<td>(0.016)</td>
<td></td>
</tr>
<tr>
<td>ASM</td>
<td>0.008</td>
<td>0.005</td>
<td>0.007</td>
<td>0.051</td>
<td>0.011</td>
<td>0.010</td>
<td>0.051</td>
<td>0.022</td>
<td>0.087</td>
<td>0.003</td>
</tr>
<tr>
<td>(0.000)</td>
<td>(0.001)</td>
<td>(0.002)</td>
<td>(0.016)</td>
<td>(0.002)</td>
<td>(0.000)</td>
<td>(0.002)</td>
<td>(0.006)</td>
<td>(0.019)</td>
<td>(0.002)</td>
<td></td>
</tr>
</tbody>
</table>

Data are means from three separate experiments (SD are given in parentheses).
ASM-grown *C. albicans* biofilms having lower metabolic activity than those grown in RPMI-1640, particularly monospecies biofilms (Table 2, Table 3). It is postulated that RPMI-1640, which contains limited nutrients, induces stress in *C. albicans*, thus promoting hyphal formation. This does not occur when the yeast is grown in ASM that is rich in nutrients. Interestingly, previous studies have shown that *Candida* spp., with low metabolic activity are more invasive and associated with disease, while conversely those with high activity are non-invasive. Furthermore, low metabolic activity has been shown to reduce the antifungal susceptibility of *C. albicans* within the biofilm, which could be due to minimal absorption of antifungal agents such as amphotericin B, thus affecting inactivation kinetics.

The metabolic activity of all *C. albicans* strains that were grown in ASM increased in the presence of *A. naeslundii* in dual-cultured biofilms. However, a decrease of metabolic activity was observed in trispecies biofilms when compared to the dual-cultured biofilms of *C. albicans* and *A. naeslundii*, suggesting that these microorganisms may be interacting metabolically. It is postulated that in the presence of *A. naeslundii*, *C. albicans* may increase mitochondrial dehydrogenase activity that in turn, increased the activity of succinate dehydrogenases of *A. naeslundii*. In addition, *S. mutans* has been shown to reduce the metabolic activity in trispecies biofilms compared with the dual-cultured *C. albicans*-A. *naeslundii* biofilms, suggesting that the antagonistic metabolic interaction between *A. naeslundii* and *S. mutans*, demonstrated in the present study (Table 2), may have affected overall metabolic activity of the consortia. *C. albicans* and *A. naeslundii* have been shown to synthesize mitochondrial and succinate dehydrogenases, respectively, that were reported to be detectable by XTT. Even though *S. mutans* has been found to synthesize an NADH-dependent lactate dehydrogenase; the present study revealed that enzyme activity was not detected with XTT suggesting that the assay is not suitable for the study of *S. mutans* metabolic activity.

In the present study, the biofilm biomass was shown to vary with microbial interactions (monocultured *C. albicans*, dual-cultured *C. albicans* and *A. naeslundii*, dual-cultured *C. albicans* and *S. mutans*, tricultured *C. albicans*, *A. naeslundii*, and *S. mutans*). The majority of RPMI-1640 grown *C. albicans* (hyphal form) biofilm biomass was observed to increase in the presence of bacteria compared with monocultured *C. albicans*. Previous research has shown that *A. naeslundii* and *S. mutans* bind to *C. albicans* through its mannose-containing surface protein. This interaction has been reported to induce the formation of extracellular polysaccharide, thus promoting the adherence of the late colonisers to form a complex polymicrobial biofilm potentially enhancing biofilm biomass. Previous studies have also demonstrated that oral biofilms are composed of various microorganisms indicating the important role of polymicrobial interactions in plaque biofilm development, dysbiosis, and oral disease.

The present study found that the ATCC strains form excellent monocultured biofilms in both ASM and RPMI-1640 such that addition of *A. naeslundii* or *S. mutans* resulted in no additional biomass in the majority of biofilms. However, the clinical strains that were poor biofilm formers in RPMI-1640 were observed to increase biofilm biomass significantly when *A. naeslundii* or *S. mutans* was co-inoculated (Table 1). This result indicates that the choice of isolates in the study of the interaction between oral yeast and oral bacteria in biofilms is critical. The *C. albicans* ATCC strains assessed in the present study would appear to have lost either the ability, or need, to interact with oral bacteria; thus, investigations using only ATCC strains of *C. albicans* are likely to not reflect the true interactions that are occurring in the oral cavity.

We have demonstrated that *C. albicans* predominantly in the yeast form when grown as a biofilm in ASM, whereas RPMI-grown *C. albicans* biofilms were predominated by the hyphal form (Fig. 1). These results support previous work that showed the proportion of yeast and hyphal cells of *C. albicans* present in the biofilm is dependent upon the nutrient source, where nitrogen-based medium allowed for more yeast growth and biofilms grown in RPMI-1640 with high salts, amino acids and D-glucose, showed more hyphal growth.

### Conclusion

Biofilm biomass and metabolic activity have been shown to be both *C. albicans* strain and growth medium dependent. This is likely to have significance in the development of polymicrobial oral biofilms in vivo.

### Acknowledgments

This work was funded by the Oral Health Cooperative Research Centre (OHCRC) and Melbourne Dental School, The University of Melbourne.

### Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

### References


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Characterisation of Candida albicans, Actinomyces naeslundii and Streptococcus mutans interaction and its role in promoting oral carcinogenesis

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