



REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree	Year	Name of Author
PhD	2005	LAMFON, HANADI A.

COPYRIGHT

This is a thesis accepted for a Higher Degree of the University of London. It is an unpublished typescript and the copyright is held by the author. All persons consulting the thesis must read and abide by the Copyright Declaration below.

COPYRIGHT DECLARATION

I recognise that the copyright of the above-described thesis rests with the author and that no quotation from it or information derived from it may be published without the prior written consent of the author.

LOANS

Theses may not be lent to individuals, but the Senate House Library may lend a copy to approved libraries within the United Kingdom, for consultation solely on the premises of those libraries. Application should be made to: Inter-Library Loans, Senate House Library, Senate House, Malet Street, London WC1E 7HU.

REPRODUCTION

University of London theses may not be reproduced without explicit written permission from the Senate House Library. Enquiries should be addressed to the Theses Section of the Library. Regulations concerning reproduction vary according to the date of acceptance of the thesis and are listed below as guidelines.

- A. Before 1962. Permission granted only upon the prior written consent of the author. (The Senate House Library will provide addresses where possible).
- B. 1962 - 1974. In many cases the author has agreed to permit copying upon completion of a Copyright Declaration.
- C. 1975 - 1988. Most theses may be copied upon completion of a Copyright Declaration.
- D. 1989 onwards. Most theses may be copied.

This thesis comes within category D.

This copy has been deposited in the Library of UCL

This copy has been deposited in the Senate House Library, Senate House, Malet Street, London WC1E 7HU.

**Investigation into the formation and
susceptibility of *Candida albicans* biofilms and
denture plaque *in vitro*.**

Submitted in fulfilment of the conditions governing candidates for the
degree of

DOCTOR OF PHILOSOPHY
UNIVERSITY OF LONDON

Hanadi Lamfon

BDS MSc MFD RCSI (Ireland)

Oral Medicine

Eastman Dental Institute, UCL

February 2005

UMI Number: U592251

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U592251

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Microbial biofilms are wide-spread in nature and the organisms within them are often able to cause acute and chronic human diseases. The nature of biofilms is complex and the organisms behave differently from their planktonic counterparts. Many studies have been carried out on bacterial biofilms related to oral diseases, but little work has focused upon the potential clinical role of fungal and mixed fungal and bacterial biofilms.

The aims of this project were to develop an *in vitro* model to investigate the development and structure of fungal biofilms and mixed fungal and bacterial biofilms associated with Denture related Stomatitis (DRS) and the effectiveness of antimicrobial agents against them. Single species *Candida albicans* and denture plaque derived from patients with DRS was grown in biofilms on dental materials in a Constant Depth Film Fermentor.

Initial colonisation (2 – 6 h) of *C. albicans* was dependent upon the material and the roughness of the surface on which the biofilm formed. Higher numbers of yeast cells were present on enamel compared to dentine or acrylic whilst the highest numbers of cells were found on the roughest surfaces during early biofilm development. Confocal microscopy revealed that the hyphal form was associated with biofilm development and structure.

Candida albicans grown in the biofilm phase of growth was more resistant to fluconazole, miconazole and chlorhexidine than when grown planktonically, although drug resistance varied between agents and biofilm growth phases. Microcosm biofilms containing similar proportions of genera to those of DRS lesions were developed. Further, the delivery of DRS-associated treatments was mimicked and it was demonstrated that a combination of miconazole and

chlorhexidine was able to reduce the total bacterial count by 3 log₁₀ and the number of *Candida* species by over 2 log₁₀.

It can be concluded from this work that the initial phase of biofilm formation has a significant role to play in the development of biofilms on various oral surfaces and their subsequent susceptibility. Further, the development of an in vitro denture plaque model allowed the determination of the likely clinical effectiveness of treatment regimes.

Index of contents

Title	1
Abstract	2
Index of contents	4
Declaration	9
Acknowledgements	10
List of figures	11
List of tables	16
Abbreviations	18
Publications and presentations related to this thesis	20
CHAPTER 1	
Introduction	22
1.1 Fungi	23
1.1.1 <i>Candida albicans</i>	24
1.1.2 Virulence of <i>Candida albicans</i>	27
1.1.2.1 Germ tubes formation and adherence of <i>C. albicans</i>	27
1.1.2.2 Production of enzymes	29
1.1.2.3 The cell wall	30
1.1.2.4 Phenotypic switching	30
1.1.3 Genotypic subgroups of <i>Candida albicans</i>	31
1.1.4 <i>Candida</i> and oral diseases	32
1.1.4.1 Predisposing factors for oral candidosis	33
1.1.4.2 Classification of oral candidosis	35
1.1.4.2.1 Pseudomembraneous candidosis or thrush	36
1.1.4.2.2 Atrophic (erythematous) candidosis	37
1.1.4.2.3 Hyperplastic candidosis (<i>Candida</i> leukoplakia)	37
1.1.4.2.4 Denture related stomatitis	38
1.1.4.2.5 Angular cheilitis (angular stomatitis, perleche)	38
1.1.4.2.6 Median rhomboid glossitis	39
1.1.4.2.7 Chronic mucocutaneous candidosis	39
1.1.4.3 Isolation and identification of <i>Candida</i> species	40
1.1.4.3.1 Smear	40

1.1.4.3.2 Swab	41
1.1.4.3.3 Imprint culture	41
1.1.4.3.4 Salivary culture	41
1.1.4.3.5 Oral rinse	41
1.1.4.3.6 Impression culture	42
1.1.4.3.7 Biopsy	42
1.1.4.4 Treatment of oral candidosis	45
1.1.4.4.1 Polyenes	46
1.1.4.4.2 Azoles	47
1.1.4.4.3 DNA analogues	49
1.1.4.4.4 Others	49
1.2 The oral environment in health and disease	50
1.3 Biofilms	50
1.3.1 Defining a biofilm	51
1.3.2 The formation of a biofilm	53
1.3.3 Communication within a biofilm	55
1.3.4 Biofilm structure	57
1.3.4.1 Extracellular polysaccharides	58
1.3.5 Biofilm resistance	59
1.3.5.1 Delayed penetration of the antimicrobial agents	60
1.3.5.2 Altered growth rate of biofilm organisms	61
1.3.5.3 Unique biofilm physiology	63
1.3.5.4 Persisters	63
1.3.6 Biofilm detachment and death	64
1.3.7 Biofilm related diseases	65
1.3.8 Biofilm-based models	68
1.4 Candidal biofilms	71
1.4.1 Structure of <i>Candida albicans</i> biofilms	71
1.4.1.1 The nature of contact surface	73
1.4.1.2 Growth factors	73
1.4.1.3 <i>Candida</i> morphogenesis	74
1.4.1.4 <i>Candida</i> species involved	74
1.4.2 Resistance of <i>Candida albicans</i> biofilms	75

1.4.2.1 Delayed penetration of the antimicrobial agents	75
1.4.2.2 Altered growth rate of biofilm organisms	77
1.4.2.3 Efflux pumps	77
1.4.2.4 Unique biofilm physiology	78
1.5 Denture related stomatitis	79
1.5.1 Clinical features	79
1.5.2 Classification	79
1.5.3 Causes	80
1.5.3.1 Denture plaque and denture related stomatitis	81
1.5.4 Association of <i>Candida albicans</i> with DRS	83
1.5.5 Diagnosis	84
1.5.6 Treatment	84
1.6 Aim of the research	89
CHAPTER 2	
Materials and methods	90
2.1 Preparation of substrata	91
2.1.1 Denture acrylic	91
2.1.2 Enamel and dentine	92
2.2 Organisms used in the study	93
2.2.1 Ethical approval	93
2.2.2 Clinical isolates of <i>Candida albicans</i>	93
2.2.3 Mixed-species clinical samples	93
2.3 Nutrient source	95
2.4 Constant Depth Film Fermentor	96
2.4.1 Assembly of CDFE	96
2.4.2 Inoculation of the CDFE and continuous media supply	100
2.4.2.1 Single-species	100
2.4.2.2 Mixed-species	100
2.4.3 Operating conditions	103
2.4.4 Sample removal	103
2.4.5 Analytical procedures	103
2.4.5.1 Single-species	103

2.4.5.2 Mixed-species	104
2.5 Susceptibility testing	107
2.5.1 Susceptibility testing for antifungal agents	107
2.5.2 Susceptibility testing for antimicrobial agent	108
2.6 Scanning Electron Microscopy	109
2.7 Confocal Laser Scanning Microscopy	111
2.8 Surface characteristics	112
2.9 Identification of <i>Candida</i> species	113
2.9.1 CHROMagar	113
2.9.2 API® Candida Kit	114
2.9.3 CornMeal agar	115

CHAPTER 3

The characterisation of an <i>in vitro</i> model system to grow biofilms of <i>Candida albicans</i> on relevant oral surfaces	116
3.1 Introduction	117
3.2 Materials and methods	119
3.3 Results	120
3.4 Discussion	138

CHAPTER 4

The effect of surface roughness and strain variability on the biofilm forming ability of <i>Candida albicans</i>	143
4.1 Introduction	144
4.2 Materials and methods	146
4.3 Results	147
4.4 Discussion	163

CHAPTER 5

The susceptibility of <i>Candida albicans</i> biofilms to antifungal and antimicrobial agents	167
5.1 Introduction	168
5.2 Materials and methods	170

5.3 Results	175
5.4 Discussion	185
CHAPTER 6	
Composition of <i>in vitro</i> denture plaque biofilms and susceptibility to antifungals	189
6.1 Introduction	190
6.2 Materials and methods	192
6.3 Results	194
6.4 Discussion	205
CHAPTER 7	
Conclusions and suggestions for further work	210
References	214
Publications arising from this thesis	260

Declaration

I hereby certify that the findings reported in this thesis result entirely from my own work. The scanning electron microscopy sample preparation was carried out by Ms N. Mordan (Eastman Dental Institute, UCL). The confocal scanning electron microscopy work was carried out in collaboration with the School of Anatomy, UCL. Colleagues who helped in various aspects of the work are listed in the acknowledgments. The work has not previously been submitted, in part or in full, for a degree or diploma for this or any other University or examination board.

Hanadi Lamfon

Oral Medicine

Eastman Dental Institute, UCL

University of London

256, Gray's Inn Road

London WC1X 8LD

February 2005

Acknowledgements

Firstly, I would like to thank God who has given me the strength and health to complete my thesis.

I am extremely grateful to my supervisors, Dr Jonathan Pratten and Professor Stephen Porter for their helpful guidance and support and without whom the completion of this thesis would not have been possible.

I am also grateful to Graham Palmer who helped me in the preparation of some materials, Zubaidah Al-Karrawi for her technical assistance and kindness, and all my friends and colleagues at the Eastman Dental Institute for their help and friendship.

I would also like to thank Umm-Alqura University for their generous funding of my studies.

Special thanks go to my parents for their encouragement and love and to my husband for his warm support.

List of figures

1.1	<i>C. albicans</i> showing yeast, hyphae and budding forms.	25
1.2	Figure showing the different forms of <i>C. albicans</i> with the possible morphological transition between forms.	26
1.3	Diagrammatic representation of stages associated with biofilm formation.	54
1.4	Diagrammatic representation of the mushroom-like microcolonies that constitute the structural element of biofilm.	58
2.1	The Constant Depth Film Fermentor.	96
2.2	Schematic horizontal section through the fermentor.	98
2.3	Schematic vertical section through the fermentor with insert indicating one of the pans in greater detail.	99
2.4	Schematic diagram indicating inoculation of the CDFF.	101
2.5	Schematic diagram showing continuous media supply to the CDFF.	102
2.6	Scanning electron microscope photograph of the surface of self-cured acrylic disks polished with different surface roughness.	110
2.7	Proscan image showing roughness readings as generated by Scantron software.	112
2.8	API <i>Candida</i> kit strip showing the identification of <i>Candida albicans</i> .	114
3.1a	Viable counts of <i>C. albicans</i> biofilms grown on PTFE over a period of 384 h.	123
3.1b	The percentage of yeast and hyphae of <i>C. albicans</i> biofilms grown on PTFE for a period of 384 h.	125
3.2a	Viable counts of <i>C. albicans</i> biofilms grown on self and heat-cured	126

- acrylic for a period of 72 h.
- 3.2b The percentage of yeast and hyphae of *C. albicans* biofilms grown on self-cured acrylic for a period of 72 h. 128
- 3.2c The percentage of yeast and hyphae of *C. albicans* biofilms grown on heat-cured acrylic for a period of 72 h. 128
- 3.3a Viable counts of *C. albicans* biofilms grown on three different surfaces over a period of 96 h using artificial saliva as a nutrient medium. 129
- 3.3b The percentage of yeast and hyphae of *C. albicans* biofilms grown on acrylic surface over a period of 96 h using artificial saliva as a nutrient medium. 131
- 3.3c The percentage of yeast and hyphae of *C. albicans* biofilms grown on enamel surface over a period of 96 h using artificial saliva as a nutrient medium. 131
- 3.3d The percentage of yeast and hyphae of *C. albicans* biofilms grown on dentine surface over a period of 96 h using artificial saliva a nutrient medium. 132
- 3.4 Overnight suspensions of *C. albicans* grown in: YPD, artificial saliva, artificial saliva without Lab-lemco, artificial saliva without mucin and artificial saliva without Lab-lemco and without mucin. 133
- 3.5a Viable counts of *C. albicans* biofilms grown on three different surfaces over a period of 96 h using YPD as a nutrient medium. 134
- 3.5b The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on acrylic surface over a period of 96 h using YPD as a nutrient medium. 136

- 3.5c The percentage of yeast and hyphae of *C. albicans* biofilms grown on enamel surface over a period of 96 h using YPD as a nutrient medium. 136
- 3.5d The percentage of yeast and hyphae of *C. albicans* biofilms grown on dentine surface over a period of 96 h using YPD as a nutrient medium. 137
- 4.1 The percentage of yeast and hyphal cells of *C. albicans* biofilms grown on different surface roughness of self and heat-cured acrylic ($R_a = 2.5 \mu\text{m}$, $R_a = 3.6 \mu\text{m}$ and $R_a = 9.3 \mu\text{m}$) over 2 h. 153
- 4.2 The viable counts of two genotype A *C. albicans* biofilms grown on acrylic over 72 h. 154
- 4.3 The number of yeast and hyphal cell counts of high adherence *C. albicans* biofilms genotype A grown on different surface roughness of denture acrylic over 72 h. 155
- 4.4 The number of yeast and hyphal cell counts of low adherence genotype A *C. albicans* biofilms grown on acrylic with different surface roughness over 72 h. 156
- 4.5 The viable counts of two genotype B *C. albicans* biofilms grown on acrylic over 72 h. 157
- 4.6 The number of yeast and hyphal cell counts of high adherence genotype B *C. albicans* biofilms grown on acrylic with different surface roughness over 72 h. 158
- 4.7 The number of yeast and hyphal cell counts of low adherence genotype B *C. albicans* biofilms grown on acrylic with different surface roughness over 72 h. 159

4.8	CLSM images showing <i>C. albicans</i> biofilms formation on denture acrylic of various surface roughness after 6 h growth in the CDFF.	160
4.9	CLSM images showing <i>C. albicans</i> biofilms formation on denture acrylic of various surface roughness after 24 h growth in the CDFF.	161
4.10	CLSM images showing <i>C. albicans</i> biofilms formation on denture acrylic of various surface roughness after 72 h growth in the CDFF.	162
5.1	Almar blue containing multiwell plate used to test the antifungal susceptibility of <i>C. albicans</i> . Wells arranged within columns contain different concentrations of fluconazole and each row contains a different isolate.	174
5.2	Growth of genotype A <i>C. albicans</i> biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 2 µg/ml of fluconazole for 1, 24 and 48 h.	177
5.3	Growth of genotype A <i>C. albicans</i> biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 256 µg/ml of fluconazole for 1, 24 and 48 h.	178
5.4	Growth of genotype B <i>C. albicans</i> biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 256 µg/ml of fluconazole for 1, 24 and 48 h.	178
5.5	Growth of genotype A <i>C. albicans</i> biofilms grown on denture acrylic for 2, 6, 24, 48 and 72 h and exposed to 256 µg/ml of fluconazole for; 1, 24 and 48 h.	179
5.6	Growth of genotype A <i>C. albicans</i> biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 256 µg/ml of miconazole for 1, 24 and 48 h.	180

- 5.7 Growth of genotype B *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 256 µg/ml of miconazole for 1, 24 and 48 h. 180
- 5.8 Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 2, 6, 24, 48 and 72 h and exposed to 256 µg/ml miconazole for; 1, 24 and 48 h. 181
- 5.9 Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 2.5 % of chlorhexidine for 1, 5 and 15 minutes. 182
- 5.10 Growth of genotype B *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 2.5 % of chlorhexidine for 1, 5 and 15 minutes. 182
- 5.11 Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 2, 6, 24, 48 and 72 h and exposed to 0.019 % chlorhexidine for; 1, 5 and 15 minutes. 183
- 6.1 Viable count of microorganisms present in a mixed-species biofilm after growth in CDFD for 504 h. 201
- 6.2 Viable counts of *Candida* spp. of the 72 h biofilm in different concentrations of fluconazole or miconazole. 202
- 6.3 Viable counts of *Candida* spp. of the 72 h biofilm in different concentrations of chlorhexidine. 202
- 6.4 Viable counts of *Candida* species, aerobic and anaerobic bacteria of the 72 h biofilm in different concentrations of chlorhexidine. 203
- 6.5 Number of microorganisms of a mixed-species biofilm prior to pulsing with miconazole and chlorhexidine and after 240 h pulsing. 204

List of tables

1.1	Classification of oral candidosis as proposed by Axell and co-workers (1997).	36
1.2	Table showing some of molecular methods for identification of <i>Candida</i> species.	44
1.3	Antifungal agents used to treat oral candidosis.	46
1.4	Table indicating biofilm related human infections.	67
2.1	Composition of artificial saliva.	95
2.2	Various non-selective and selective agars used for culturing organisms during the study.	105
2.3	Composition of CFATA from (Zylber and Jordan 1982).	106
2.4	Identification of <i>Candida</i> spp. using the CHROMagar method.	113
3.1	Composition of YPD broth.	116
3.2	Mean \pm the standard deviation of yeast and hyphal cell counts of <i>C. albicans</i> biofilms grown on PTFE over 384 h.	124
3.3	Mean \pm the standard deviation of yeast and hyphal cell counts of <i>C. albicans</i> biofilms grown on self and heat-cured acrylic over 72 h.	127
3.4	Mean \pm the standard deviation of yeast and hyphal cell counts of <i>C. albicans</i> biofilms grown on acrylic, enamel and dentine over 96 h using artificial saliva as a nutrient medium.	130
3.5	Mean \pm the standard deviation of yeast and hyphal cell counts of <i>C. albicans</i> biofilms grown on acrylic, enamel and dentine over 96 h using YPD as a nutrient medium.	135
4.1	Mean \pm the standard deviation of viable counts of <i>C. albicans</i> biofilms grown on self and heat-cured acrylic with different surface roughness	151

	($R_a = 2.5 \mu\text{m}$, $R_a = 3.6 \mu\text{m}$ and $R_a = 9.3 \mu\text{m}$) over 2 h.	
4.2	Mean \pm the standard deviation of yeast and hyphal cell counts of <i>C. albicans</i> biofilms grown on self and heat-cured acrylic with different surface roughness ($R_a = 2.5 \mu\text{m}$, $R_a = 3.6 \mu\text{m}$ and $R_a = 9.3 \mu\text{m}$) over 2 h.	152
5.1	Different concentrations of fluconazole or miconazole used against <i>C. albicans</i> biofilms related to the planktonic MIC.	172
5.2	Different concentrations of chlorhexidine used against <i>C. albicans</i> biofilms related to the planktonic MIC.	173
5.3	Concentration of fluconazole, miconazole and chlorhexidine required to inhibit growth of <i>C. albicans</i> .	184
6.1	Identification of different <i>Candida</i> spp. of control patients.	197
6.2	Identification of different <i>Candida</i> spp. of DRS patients.	198
6.3	The range in number (CFU) of selected genera from patients with and without DRS compared to the numbers obtained <i>in vitro</i> (72 – 240 h).	199
6.4	The range (%) in compositions of genera present on the mucosa and denture surfaces of patients suffering from DRS and from the CDFF.	200

Abbreviations

°C	Degrees Celsius
CAMM	Cell Adhesion Measurement Module
CBA	Columbia Blood agar
CDFE	Constant Depth Film Fermentor
CFATA	Cadmium Fluoride Acriflavin Tellurite agar
CFU	Colony Forming Units
CLSM	Confocal Laser Scanning Microscopy
DRS	Denture related Stomatitis
dH ₂ O	Distilled water
EPS	Extracellular polysaccharide
FAA	Fastidious Anaerobes agar
g	gram
HIV	Human Immunodeficiency Virus
h	hours
L	litre
log	logarithmic
MIC	Minimum Inhibitory Concentration
µg	microgram
µl	microlitre
ml	millilitre
µm	micrometre
mm	millimetre
MSA	Mitis Salivarius agar

NCCLS	National Committee for Clinical Laboratory Standards
PBS	Phosphate-buffered saline
PMMA	Polymethylmethacrylate
PTFE	Polytetrafluoroethylene
RA	Rogosa agar
R _a	Average surface roughness
r.p.m	revolutions per minute
SAP	Secreted Aspartyl Proteinases
SDA	Sabouraud's Dextrose Agar
SEM	Scanning Electron Microscopy
spp.	species
V	Voltage
VA	Veillonella agar
YPD	Yeast extract, peptone and dextrose

Publications and presentations related to this thesis

Peer Reviewed Papers

Lamfon H, Porter SR, McCullough M, Pratten J (2003). Formation of *Candida albicans* biofilms on non-shedding oral surfaces. *Eur. J. Oral Sci.* **111**, 465-471.

Lamfon H, Porter SR, McCullough M, Pratten J (2004). The susceptibility of *Candida albicans* biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study. *J. Antimicrob. Chemother.* **53**, 383-385.

Lamfon H, Al-Karaawi Z, Porter SR, McCullough M, Pratten J (2005). Composition of *in vitro* denture plaque biofilms and susceptibility to antifungals. *FEMS Microbiol Lett.* **242**, 345-351.

Chapters in Books

Pratten J, **Lamfon H**, Porter SR, McCullough M (2003). Formation and susceptibility of *Candida albicans* biofilms. *In Biofilm Communities: Order from Chaos?* (Eds McBain A, Allison D, Brading M, Rickard A, Verran J, Walker J) pp. 299-308. BioLine: Cardiff. Antony Rowe Ltd, Chippenham, U.K.

Conference Proceedings

Lamfon H, Porter SR, McCullough M, Pratten J (2002). Formation of *Candida albicans* biofilms on non-shedding oral surfaces. (poster presentation). Annual Meeting of the British Society for Oral Medicine, Leeds, 9th – 10th May.

Lamfon H, Pratten J, McCullough M, Porter SR (2002). Susceptibility of *Candida albicans* biofilms to fluconazole. (oral presentation). The 6th Biennial Congress of the European Association of Oral Medicine, Lisbon, Portugal, 3rd – 5th October.

Lamfon H, Porter SR, McCullough M, Pratten J (2003). *In vitro* evaluation of miconazole and chlorhexidine for the treatment of denture-associated stomatitis. (poster presentation). Annual Meeting of the British Society for Oral Medicine, Dundee, 9th – 10th May.

Lamfon H, Porter SR, McCullough M, Pratten J (2002). Composition and susceptibility of microcosm denture plaque. (poster presentation) 7th Biennial Congress of the European Association of Oral Medicine, Berlin, Germany, 23rd – 25th October.

Chapter 1

Introduction

1.1 FUNGI

Fungi are found throughout nature and survive by absorbing their nutrients from living or dead organisms and organic matter. There are groups of species which are parasites of plants or animals, as well as humans. The frequency of life-threatening fungal infections in humans is rising world-wide, for example, they represent 10 % of all nosocomial septicemia (Wey *et al.*, 1988; Beck-Sague and Jarvis, 1993) and are considered as the fourth leading cause of bloodstream infections in the United States (Collin *et al.*, 1999). The most important fungal infections of the oral cavity are caused by yeast species belonging to genus *Candida* (Sullivan *et al.*, 2004).

Candida species (spp.) reside harmlessly in the mouths of healthy individuals (Abu-Elteen and Abu-Alteen, 1998). However, if the balance of the normal oral microflora is disrupted or the immune defences are compromised, *Candida* spp. can give rise to clinical disease (Powderly *et al.*, 1999; Naglik *et al.*, 2003). *Candida albicans* is by far the most common oral species (Ellepola and Samaranayake, 2000c; Akpan and Morgan, 2002) and is present in up to 60 % of healthy individuals (Kleinegger *et al.*, 2001).

A large number of non-*albicans* *Candida* spp. can also be found in the mouth including *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. dubliniensis*, *C. lusitaniae*, and *C. guilliermondii* (Epstein and Polsky, 1998; Collin *et al.*, 1999; Al-Karaawi *et al.*, 2002). Some of these non-*albicans* *Candida* spp. have the potential to give rise to clinical disease, particularly in immunocompromised persons. Indeed it has been suggested that the incidence of infections caused by non-*albicans* *Candida* spp. is increasing (Krcmery and Barnes, 2002). However, in the absence of lesions, the isolation of *Candida* spp. from the mouth does not represent evidence of oral candidosis (Cannon *et al.*, 1995) i.e. clinical illness, and indeed healthy individuals

harbour those non-*albicans Candida* spp. without developing illness (Al-Karaawi *et al.*, 2002).

1.1.1 *Candida albicans*

Candida albicans is a dimorphic yeast, however, this organism also has the ability to adapt itself to a range of morphologies thus it can be considered as a polymorphic yeast as reviewed by Chaffin *et al.*, 1998. *C. albicans* does not have a reproductive cycle, but reproduces by multilateral budding (Farah *et al.*, 2000). Macroscopically, *Candida* spp. colonies appear as a cream or yellowish colour with a variable texture depending upon species (Eggimann *et al.*, 2003). Microscopically (Figure 1.1), *C. albicans* can grow as round or oval unicellular yeast cells (blastospore), pseudohyphae (elongated yeast cells with branching filaments with constrictions where they joined) and hyphae (Bagg *et al.*, 1999a). Hyphae are thread-like tubes containing the fungal cytoplasm and its organelles are divided by walls known as septa. Hyphae develop as a result of germination of blastospores and are characterised by the presence of parallel walls with the absence of constrictions (Kobayashi and Cutler, 1998). In addition, under specific environmental conditions, *C. albicans* can produce chlamyospores which are round thick walled structures produced at the termini of hyphae. However, the functions of chlamyospores are unclear (Sonneborn *et al.*, 1999, Sullivan *et al.*, 2004).

Approximately 50 – 60 % of humans carry *Candida* spp. in their mouth (Samaranayake and Lamey, 1988). Colonization rates increase with illness and can be as high as 70 – 80 % in severely infected patients as reviewed by Fidel *et al.*, 1999. The prevalence also increases with the introduction of oral prostheses. For example, the prevalence of *Candida* spp. can increase in denture wearers to up to

values of 60 – 100 % (Pires *et al.*, 2002).

Local overgrowth may clinically manifest as candidal infection of the mouth, and this gives rise to symptoms such as local mucosal discomfort or altered taste (Akpan and Morgan, 2002).

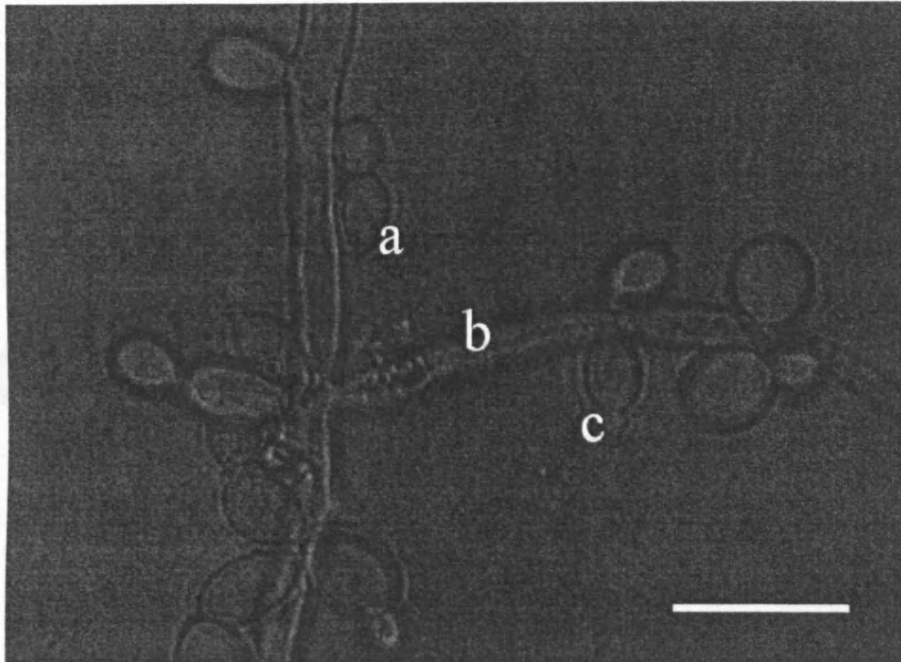


Figure 1.1: *C. albicans* showing yeast (a), hyphae (b) and budding (c) forms. (bar = 10 μm).

The reversible conversion between the yeast cells to either hyphal or pseudohyphal growth (Calderone and Fonzi, 2001; Gow *et al.*, 2002) is via the formation of germ tube (Figure 1.2). Germ tubes resist phagocytic killing and facilitate yeast adherence to epithelial cells by promoting aggregation of yeast cells and bridging of adjacent hyphal elements, hence bringing organisms into immediate contact with the epithelium (Ellepola and Samaranayake, 2000a). This morphological transition between forms depends upon certain environmental conditions such as temperature,

pH and the availability of nutrients (Corner and Magee, 1997) and are stimulated by the growth in the host and invasion (Gow *et al.*, 2002). The hyphal form of *C. albicans* has long been regarded as essential for tissue invasion, but there is evidence to suggest that the yeast form may also be able to initiate host invasion or damage (Ray and Payne, 1988).

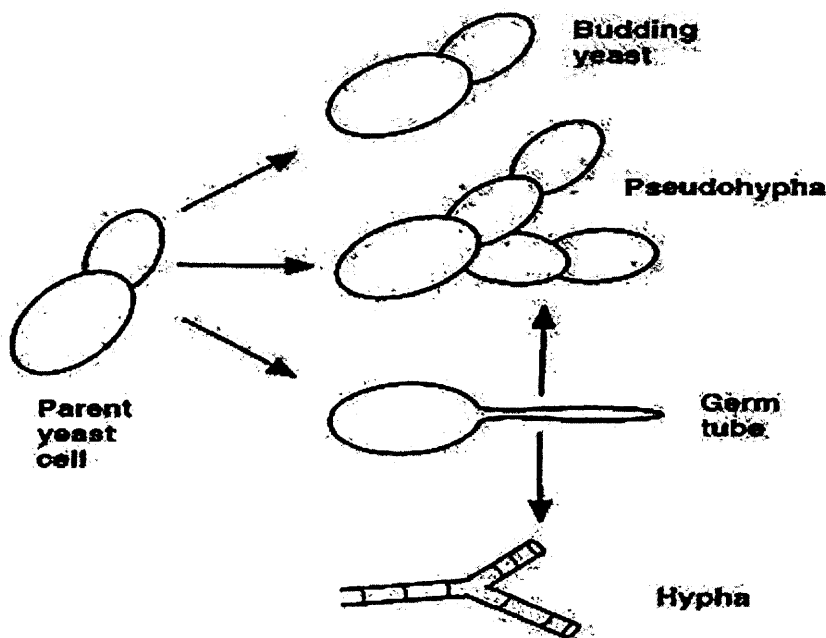


Figure 1.2: Figure showing the different forms of *C. albicans* with the possible morphological transition between forms.

Thigmotropism is a recognized property of *C. albicans* grown on surfaces and within solid substrates. It can be defined as the ability to sense and respond to changes in surface contours and enables invasive growth (Davies *et al.*, 1999). The direction of hyphal growth responds thigmotropically to invade surface contours and follow grooves and ridges (Gow *et al.*, 1994; Watts *et al.*, 1998). Nikawa and co-workers suggested that hyphal induction by thigmotropic reaction is important in the formation of candidal biofilm on saliva- or serum-coated acrylic surfaces (Nikawa *et*

al., 1998b).

1.1.2 Virulence of *Candida albicans*

Candida albicans possesses several virulence factors that promote colonization or invasion by adhering to the host cells and release a variety of virulence factors that encourage successful colonization or invasion of host tissues (Calderone and Braun, 1991; Haynes, 2001). These virulence factors of *C. albicans* include; the ability to adhere, the production of enzymes (e.g. proteinase), and the production of germ tubes (Kretschmar *et al.*, 1999; Haynes, 2001; Hube and Naglik, 2001). Other factors which also contribute to this virulence, include phenotypic switching, the cell wall and yeast morphogenesis (Calderone and Braun, 1991; Calderone and Fonzi, 2001). However, these virulence factors which cause clinical infections may vary upon the stage and site of infection, the type of infection and the nature of host response (Hube and Naglik, 2001).

1.1.2.1 Germ tubes formation and adherence of C. albicans

As previously stated, *C. albicans* can germinate forming a germ tube and subsequently produce filaments which may appear as hyphae or pseudohyphae (Kobayashi and Cutler, 1998). This yeast to hyphae transition is considered to be a virulence factor that enables *C. albicans* to invade human tissues (Brown and Gow, 1999; Hazan *et al.*, 2002).

The germ tubes may improve fungal adherence to host cells and facilitate host penetration and later tissue damage (Kretschmar *et al.*, 1999; Bernhardt *et al.*, 2001). The ability of *C. albicans* to adhere more effectively to host epithelium than other species may reflect the ability of the former to produce germ tubes. With regards to

the mouth, *C. albicans* can easily adhere to oral epithelial cells and colonize oral surfaces such as the dorsum of the tongue and buccal mucosa. It can also be found in supra and subgingival plaque and within plaque associated with intra-oral appliances (Arendorf and Walker, 1980; Bagg *et al.*, 1999a). *C. albicans* can even invade human dental hard tissues such as dentine (Kinirons, 1983; Damm *et al.*, 1988; Sen *et al.*, 1997) as well as human buccal epithelial cells (Bailey *et al.*, 1995). *C. albicans* can also bind to salivary components including mucin (Hoffman and Haidaris, 1993). Thus *C. albicans* utilizes a variety of different mechanisms to aid adherence to the oral tissues and hence invasion particularly of the soft tissues.

C. albicans can adhere to non-host surfaces such as resin-composite restorative dental materials (Maza *et al.*, 2002) and acrylic (Verran *et al.*, 1991). It has been shown that the increase of acrylic surface roughness can increase retention of microbial cells (Verran *et al.*, 1991; Verran and Maryan, 1997; Radford *et al.*, 1998b). Thus even if *C. albicans* does not adhere well to oral tissues, it can still exert a local action by its retention to dental restorative material or oral prostheses.

The ability of different *Candida* spp. to adhere to a surface varies. Such variation in adherence may underline the differences in virulence ability among *Candida* spp. (i.e. their ability to colonize mucosal surfaces). For example, *C. albicans* and *C. tropicalis* can adhere *in vitro* to host cells to a greater extent than *C. krusei* or *C. guilliermondii* (McCullough *et al.*, 1996; Biasoli *et al.*, 2002). Different strains of *C. albicans* also demonstrate differences in their ability to adhere to surfaces and hence strains with increased adherence have a greater ability to cause infection as reviewed by Calderone and Braun, 1991. In addition, *C. albicans* isolates which adhere strongly to buccal epithelial cells have the highest relative proteinase activity and are the most pathogenic (Ghannoum and Abu-Elteen, 1986).

1.1.2.2 Production of enzymes

The complex pathogenesis of *C. albicans* is aided by the production of a range of extracellular enzymes that facilitate adherence and tissue damage. The two most significant hydrolytic enzymes produced by *C. albicans* are Secreted Aspartyl Proteinases (SAPs) and phospholipases. These enzymes are able to resist host immune defence mechanism and aid in penetration of host tissues (Calderone and Fonzi, 2001; Haynes, 2001).

Proteinases are by far the most prominent virulence factor (Naglik *et al.*, 2003). These enzymes constitute a family of isoenzymes encoded by at least nine (Schaller *et al.*, 1999a) or ten (Hube and Naglik, 2001; Naglik *et al.*, 2004) related SAP genes that are implicated in the virulence of *C. albicans*. Different SAP genes are crucial for mucosal and systemic infections and are involved in adherence of *C. albicans*, tissue damage and evasion of host immune responses (Hube and Naglik, 2001). The production of these genes is a highly regulated process (Naglik *et al.*, 2004) and their expression has been shown to be regulated differentially (Schaller *et al.*, 1999b). The distinct role of SAP genes is dependent upon the type and on the progress of the infection (Staib *et al.*, 2000). These enzymes are capable of digesting host proteins for nutritional purposes and degrading IgA and complement proteins to avoid and resist antimicrobial attack (McCullough *et al.*, 1996; Staib *et al.*, 2000). They are also capable of distorting host cell surface structures and intercellular substances (Hube and Naglik, 2001).

Phospholipases are also associated with the virulence of *C. albicans* (Ibrahim *et al.*, 1995). These enzymes are involved in the early steps of host invasion and have the ability to degrade phospholipids and facilitate yeast penetration (Ghannoum, 2000). Phospholipase-producing strains adhere well to epithelial cells (Fidel *et al.*, 1999).

Lipases are concentrated at the tip of fungal hyphae, facilitating active invasion (Scully *et al.*, 1994).

1.1.2.3 The cell wall

The cell wall of the yeast mediates the initial physical interaction between the fungi and the environment (Chaffin *et al.*, 1998). It protects the organism and promotes the colonization and growth of the cells (Calderone and Braun, 1991; McCullough *et al.*, 1996).

The cell wall contributes to the pathogenicity of *C. albicans* by aiding in the adhesion of the organism to the host tissue. It also acts as a permeability barrier against host immune defences and antifungal agents and maintaining the characteristic shape of the fungi (McCullough *et al.*, 1996; Chaffin *et al.*, 1998).

1.1.2.4 Phenotypic switching

C. albicans frequently exhibits variant colony forms and has the ability to switch reversibly at high frequency among variant phenotypes (Scully *et al.*, 1994; Calderone and Fonzi, 2001). High frequency phenotypic switching is a phenomenon of cells emanating from a single progenitor that express different phenotypes which results in high rate variant colony morphology (Soll, 1992). For example, smooth colony-forming yeast may produce colonies with a rough surface (smooth to rough switching). This switching can be triggered by low doses of ultra violet radiation as reviewed by Calderone and Fonzi, 2001.

Another phenotypic switching system known as white-opaque transition is recognised by colonies changing from white to opaque with characteristic differences in cell shape, size and colour (Slutsky *et al.*, 1987). The system is

reversible with high frequencies and contribute to the plasticity of the organism (Calderone and Fonzi, 2001).

It has been postulated that the switching mechanism of *Candida* spp. can potentiate its pathogenic features by altering the functional activity and can affect cellular physiology and morphology (Soll, 1992). Switching can also help the yeast in rapid adaptation to the environment (Radford *et al.*, 1998a; Calderone and Fonzi, 2001) hence facilitating the invasion of different environments. It can elude the immune system by altering its surface antigenicity and by escaping from the action of antifungals (Scully *et al.*, 1994).

Thus colonisation of the oral cavity by *Candida* spp. is enhanced by specific adherence interactions with oral surfaces that resist host clearance action. Subsequent tissue invasion is facilitated by hyphal formation, contact sensing and the secretion of hydrolytic enzymes (Cannon *et al.*, 1995). It is the host defence mechanism that determines *Candida* clearance, colonisation and subsequently disease formation (Cannon and Chaffin, 1999). Understanding of adherence mechanisms may lead to specific preventive treatments for individuals predisposed to candidosis.

1.1.3 Genotypic sub-groups of *Candida albicans*

Several investigations have demonstrated distinct genotypic sub-groups of *C. albicans* which may have varying pathogenicity. The identification of these genotypically distinct subgroups (A or B) depends upon the presence or absence of the 25S rDNA transposable intron (Al-Karaawi *et al.*, 2002). Genotype A *C. albicans* is the most common of these genotypes. However, there has been an

increase in the incidence overtime of other genotypic subgroups of *C. albicans* such as genotype B and C (McCullough *et al.*, 1999b). All of these genotypic sub-groups of *C. albicans* have been found to be less susceptible to antifungal agents compared to other *Candida* species (McCullough *et al.*, 1999a).

1.1.4 *Candida* and oral diseases

Clinical illness caused by *Candida* spp. have been described as “disease of the diseased” as it usually arises when host immunity is reduced (Marsh and Martin, 1999a). This genus of organism can give rise to a wide spectrum of disorders in humans particularly in immunocompromised individuals, these varying from superficial mucosal lesions to, albeit rarely, life-threatening disseminated invasive infections (Webb *et al.*, 1998a; Haynes, 2001; Sullivan *et al.*, 2004). The spectrum of such diseases is perhaps exemplified by the candidal infection associated with Human Immunodeficiency Virus (HIV) disease in which oral candidosis is a frequent manifestation and most common opportunistic infection seen in these immunocompromised individuals (Powderly *et al.*, 1999; Reichart, 2003). The prevalence of oral candidosis in HIV patients ranges between 9 % to over 90 % depending on the stage of HIV disease and the study population (Greenspan, 1994a). In immunocompromised patients, infections can spread through the bloodstream leading to severe infection with significant morbidity and mortality (Akpan and Morgan, 2002).

1.1.4.1 Predisposing factors for oral candidosis

Although the transition from commensalism to disease is associated with the virulence of the organism, host factors are important in the development of candidal infections. As mentioned earlier, immunosuppression is the most likely predisposing factor of oral candidal diseases; however, a wide range of other local and systemic factors can also increase oral carriage of yeast and can predispose to clinical disease. A number of local features can enhance candidal growth and resultant disease in the oral cavity. For example, the presence of dental prostheses (Abu-Elteen and Abu-Alteen, 1998; Coelho *et al.*, 2004) and xerostomia (e.g. Sjogren's syndrome) (Leung *et al.*, 2000; Radfar *et al.*, 2003; Porter *et al.*, 2004) are common and can lead to clinically manifested disease. Tobacco smoking (Crockett *et al.*, 1992; Rindum *et al.*, 1994), especially in those wearing dentures (Abu-Elteen and Abu-Alteen, 1998), can also predispose to oral candidosis. Furthermore, low salivary pH may increase the oral carriage of *Candida* species (Arendorf and Walker, 1980; Bercier *et al.*, 1999).

In particular, the wearing of dental prostheses is associated with an increase in oral colonization by *Candida* species. Dentures can produce a local environment that favour fungal growth (Muzyka and Glick, 1995; Budtz-Jorgensen *et al.*, 1996). Additionally, ill-fitting denture appliances and poor denture hygiene can cause local trauma and increase the risk of mucosal penetration and colonization (Darwazeh *et al.*, 2001). Individuals wearing orthodontic appliances may be liable to candidal infection of the mouth (Bialasiewicz *et al.*, 1993). An increase in *Candida* colonization has also been observed in children with dental caries (Sziegoleit *et al.*, 1999).

Oral mucosal diseases such as leukoplakia and lichen planus may increase the oral carriage of yeasts (Krogh *et al.*, 1987; Lipperheide *et al.*, 1996). In addition, patients with moderate or severe epithelial dysplasia, oral squamous cell carcinoma, median rhomboid glossitis and squamous papillomas have been reported to have increased carriage of *Candida* spp. in the mouth (Barrett *et al.*, 1998; McCullough *et al.*, 2002). However, it remains unclear how yeast infection influences the development and the progression of dysplasia (McCullough *et al.*, 2002).

Several systemic causes are important predisposing factors for candidal infection. Immunosuppressive disease (Patton *et al.*, 2002; Samaranayake *et al.*, 2002; Kerdpon *et al.*, 2004), diabetes mellitus (Dorocka-Bobkowska *et al.*, 1996; Guggenheimer *et al.*, 2000; Manfredi *et al.*, 2004), long-term use of immunosuppressant regimes and corticosteroid therapy (Kennedy *et al.*, 2000) all can enhance candidal infection of the mouth.

Leukaemias and other diseases of bone marrow white cell production and anti-tumour chemotherapy and sometimes radiotherapy can increase the risk of oral candidal disease (Samaranayake *et al.*, 1984; Jobbins *et al.*, 1992). Bone marrow transplant recipients are also at risk of developing candidal infection (Tollema *et al.*, 1999). Down's syndrome and DiGeorge syndrome may increase oral carriage and clinical disease of *C. albicans* as a consequence of the associated immunosuppression (Carlstedt *et al.*, 1996; Atkinson *et al.*, 2000). Nutritional factors such as deficiencies of iron, folic acid, vitamins, and diets rich in carbohydrates (Samaranayake, 1986) can also predispose to oral candidosis.

1.1.4.2 Classification of oral candidosis

Because of the variable clinical manifestations of the disease and the multifactorial aetiology, the classification of oral candidal infections is fraught with difficulties (Farah *et al.*, 2000; Ellepola and Samaranayake, 2000c). Over the years a number of classification systems have been suggested. A revised reclassification of oral candidosis (Table 1.1) was proposed in 1997 (Axell *et al.*, 1997). Primary oral candidosis, is confined to the oral or peri-oral tissues, while secondary oral candidosis is a manifestation of a more generalized mucocutaneous disease as categorised by Axell *et al.*, (1997). However, a number of rare candidal infections do not strictly fall into either of these two categories - cheilo-candidosis and chronic multifocal candidosis. The former gives rise to a chronic ulcerative granulating lesion at the vermilion border of the lower lip (Samaranayake and Lamey, 1988). Multifocal candidosis is not considered to be a specific form of candidosis, but may be used to describe the presence of erythematous candidosis, or *Candida*-associated or *Candida*-superinfected lesions either intra-orally or peri-orally (Axell *et al.*, 1997).

Table 1.1: Classification of oral candidosis as proposed by Axell and co-workers (1997).

Primary oral candidosis	Secondary oral candidosis
<i>Acute forms</i>	<i>Oral manifestations of systemic mucocutaneous candidosis (as a result of diseases such as thymic aplasia and candidosis endocrinopathy syndrome).</i>
Pseudomembraneous	
Erythematous	
<i>Chronic forms</i>	
Hyperplastic	
Nodular	
Plaque like	
Erythematous	
Pseudomembraneous	
<i>Candida-associated lesions</i>	
Denture stomatitis	
Angular chelitis	
Median rhomboid glossitis	
<i>Keratinized primary lesions</i>	
<i>superinfected with candida</i>	
Leukoplakia	
Lichen planus	
Lupus erythematosus	

1.1.4.2.1 Pseudomembraneous candidosis or thrush

Pseudomembraneous candidosis can give rise to soft, friable, whitish creamy plaques resembling milk curd. These are typically found on the hard and soft palates and posterior buccal mucosa that can be scraped off to leave an underlying erythematous and sometimes bleeding base (Scully *et al.*, 1994; Akpan and Morgan,

2002). Thrush is usually painless (Reichart *et al.*, 2000). The plaque consists of fibrin, keratin, necrotic tissues, desquamated epithelial cells; it is penetrated by bacteria and fungal hyphae or sometimes blastospores as well as inflammatory cells (Farah *et al.*, 2000; McIntyre, 2001). Thrush commonly arises with HIV disease, age, diabetes mellitus, leukaemia, xerostomia, and recurrent use of topical and systemic broad spectrum antibiotics (Scully *et al.*, 1994; Akpan and Morgan, 2002).

1.1.4.2.2 Atrophic (erythematous) candidosis

This usually manifests as a painless erythematous area of the buccal mucosa and dorsum of the tongue (Ellepola and Samaranayake, 2000c). The palate may be simultaneously affected giving rise to a “contact” or “kissing” lesion (Reichart *et al.*, 2000). Erythematous candidosis may arise *de novo* or as a result of shedding of the pseudomembrane of thrush (Ellepola and Samaranayake, 2000a). Erythematous candidosis is commonly associated with recurrent broad spectrum antibiotic or corticosteroid therapies and HIV disease (Scully *et al.*, 1994).

1.1.4.2.3 Hyperplastic candidosis (*Candida* leukoplakia)

Hyperplastic candidosis presents as an adherent white patch usually at the commissures of the mouth (Sitheeque and Samaranayake, 2003) and, less commonly, at other oral mucosal surfaces (Akpan and Morgan, 2002). The lesion reveals a hyperplastic reactions of epithelial and connective tissues (Axell *et al.*, 1997). Hyperplastic candidosis may also manifest as homogeneous or speckled lesions (nodular) (Ellepola and Samaranayake, 2000a). Chronic hyperplastic candidosis typically arises as a consequence of tobacco smoking (Epstein and Polsky, 1998), although much less common risk factors were also involved which

include; HIV disease and other immunosuppressive states (Lynch, 1994; Sitheeque and Samaranayake, 2003). Unlike pseudomembranous candidosis, chronic hyperplastic candidosis is characterised by oral epithelial hyperplasia and sometimes dysplasia (Ellepola and Samaranayake, 2000a) and because of the latter, the disease is considered to be potentially malignant (McIntyre, 2001). There does, however, remain considerable controversy as to whether this lesion is potentially malignant, and if *Candida* does indeed give rise to the dysplasia.

1.1.4.2.4 Denture related stomatitis

Denture related stomatitis (DRS) is considered as a *Candida*-associated lesion (McIntyre, 2001). It is a chronic inflammatory condition that manifests as erythematous areas of the denture bearing mucosa, either localised or generalised (Muzyka and Glick, 1995) that affect mainly the mucosa under maxillary denture (Farah *et al.*, 2000). Lesions are usually asymptomatic, although occasional patients report soreness and burning or tingling sensation beneath the denture, and may also be associated with angular cheilitis (Farah *et al.*, 2000; Ellepola and Samaranayake, 2000a). Denture related stomatitis is discussed in greater detail in section 1.5.

1.1.4.2.5 Angular cheilitis (angular stomatitis, perleche)

Angular cheilitis manifests as erythematous cracking or fissures of one or both angles of the mouth (Lynch, 1994), and in some patients may extend to facial folds. Angular cheilitis is thought to be caused by accumulation of saliva containing *Candida*, occasionally streptococci and *Staphylococcus aureus* at the corners of the mouth (Lamey and Samaranayake, 1988; Akpan and Morgan, 2002). It can be seen with other forms of oral candidosis, mainly chronic atrophic candidosis (Russotto,

1980; Wilson, 1998). Angular cheilitis typically arises due to decreased vertical dimension of the denture occlusion due to underlying bone resorption or wear of the occlusal surface of old dentures (Penhall, 1980; Ellepola and Samaranayake, 2000a; McIntyre, 2001). Less common contributing factors may include deficiency of iron and/or vitamins B6 and B12 or folic acid (Akpan and Morgan, 2002; Blanck *et al.*, 2002) and poorly controlled diabetes mellitus (McIntyre, 2001). Angular cheilitis can also be a feature of HIV (Reichart, 2003; Ranganathan *et al.*, 2004).

1.1.4.2.6 Median rhomboid glossitis

Median rhomboid glossitis manifests as a painless chronic symmetrical area on the tongue anterior to circumvallate papillae. The filiform papillae are depapillated giving rise to a rhomboid or elliptical area of redness of the dorsum of the tongue (Scully *et al.*, 1994; Sherman *et al.*, 2002), although occasionally the affected mucosa can have a hyperplastic exophytic or lobuated appearance (Scully *et al.*, 1994). Median rhomboid glossitis can arise in HIV disease, xerostomia, long term tobacco smoking and sometimes may be a feature of long term use of topical corticosteroids (Kolokotronis *et al.*, 1994; Akpan and Morgan, 2002). It may also a feature of erythematous candidosis as the “kissing” palatal lesion (Reichart *et al.*, 2000), as mentioned earlier.

1.1.4.2.7 Chronic mucocutaneous candidosis

This is a term given to a group of rare diseases characterised by recurrent and/or persistent *Candida* infection of mucocutaneous surfaces. Affected patients can have any of the aforementioned types of candidal infections of the mouth and respond poorly to topical treatment (Scully *et al.*, 1994; Ellepola and Samaranayake, 2000a).

The underlying defect is related to the impairment of immune defences with a failure of the patient's T-lymphocytes to produce cytokines that are essential for expression of cell-mediated immunity to *Candida* (Kirkpatrick, 2001). Chronic mucocutaneous candidosis may be classified as familial, diffuse, candidosis endocrinopathy syndrome and candidosis thymoma syndrome (Scully *et al.*, 1994).

1.1.4.3 Isolation and identification of Candida species

Diagnosis of superficial fungal infections is through their characteristic clinical signs, however, a variety of laboratory investigations may occasionally be required (Muzyka and Glick, 1995), particularly when the clinical presentation is severe, atypical, non-responsive to therapy and/or in a potentially immunocompromised host. Several sampling methods are available for the diagnosis of oral candidosis include; the use of a smear, swab from a suspected oral site, imprint culture, collection of whole saliva, oral rinse and biopsy. The indication and the use of these sampling is dependant on the type of oral candidal infection present (Lamey and Samaranayake, 1988).

1.1.4.3.1 Smear

This method is based upon a smear being taken by scraping the lesion by blunt instrument and smearing directly onto a glass slide. The specimen is then dried, fixed and stained with periodic acid-Schiff (PAS) or Gram stain. This method is widely used and allows a quick diagnosis, and can demonstrate candidal hyphae (Farah *et al.*, 2000), but has a low sensitivity (Sitheeque and Samaranayake, 2003).

1.1.4.3.2 Swab

In this technique, a sample is obtained by rubbing a sterile cotton-tipped swab over the area before inoculating the specimen onto Sabouraud's dextrose agar (SDA) and incubating for 48 – 72 h, after which time the growth of fungal colonies is evaluated (Sherman *et al.*, 2002). This method is simple and widely used, but may be limited by the ability to select a sample site (Sitheeque and Samaranayake, 2003).

1.1.4.3.3 Imprint culture

This method utilizes sterile plastic foam-pads previously dipped in Sabouraud's broth directly before use. The pad is applied to the site for 1 minute and then pressed on Sabouraud's agar for incubation and culture (Scully *et al.*, 1994). This is generally a reliable method which can target the infected site and distinguish between infected and carrier states, although the selection of a sample site can be difficult if clinical signs are absent (Williams and Lewis, 2000).

1.1.4.3.4 Salivary culture

In this method, subjects expectorate approximately 2 ml of saliva into a sterile container. The sample is then cultured on Sabouraud's agar. This method is as useful as the imprint culture technique, but it is not suitable for patients with xerostomia, or when a site of infection cannot be identified (Sitheeque and Samaranayake, 2003).

1.1.4.3.5 Oral rinse

The oral rinse method is simple and entails the subject rinsing the mouth with 2 ml of sterile phosphate-buffered saline (PBS) or distilled water for 1 minute and then expectorating the rinse into a sterile container. The solution is then vortexed and

cultured on SDA for 24 to 48 hours at 37°C. The Colony Forming Unit (CFU) of *Candida* can then be determined (Williams and Lewis, 2000). High candidal counts corresponds with high fungal loads in the mucus membrane areas (McIntyre, 2001). This technique allows accurate detection of oral yeasts, but cannot identify the possible sites of candidal disease (Williams and Lewis, 2000; Sitheequ and Samaranayake, 2003).

1.1.4.3.6 Impression culture

Impression culture involves the use of alginate impressions of the maxilla and the mandible and then casted in agar prepared with Sabouraud's broth and incubated. This technique allows distribution of yeasts on oral surfaces to be determined (Sitheequ and Samaranayake, 2003).

1.1.4.3.7 Biopsy

This is an invasive method and inappropriate for regular use. However, it is helpful and accurate method to confirm diagnosis of oral lesions such as in chronic hyperplastic candidosis (Williams and Lewis, 2000; Farah *et al.*, 2000).

Relevant haematological and serological investigations should also be performed, when appropriate, in order to identify any undiagnosed diseases such as diabetes mellitus (McIntyre, 2001). Additionally, correct identification of different *Candida* spp. has become essential for clinical management as well as for epidemiological studies. After isolation of *Candida* from the mouth using one of the previous methods, different systems are available to identify *Candida* species. It is possible to discriminate *Candida* spp. by either phenotypic methods such as CHROMagar, germ

tube testing, CornMeal agar and carbohydrate assimilation tests (API system) or by genotypic method including the investigation of gene transcription to identify specific genes required for microbial pathogenesis and survival in the host. The molecular methods provide definitive differentiation of species to characterize the genetic difference between different *Candida* species. Some of these methods are indicated in Table 1.2.

Table 1.2: Table showing some of molecular methods for identification of *Candida* species.

Molecular methods	Identification at subspecies level	Routine screening	Time consuming / expensive	Reproducibility / sensitivity	References
Restriction enzyme analysis	+	-	+	+	Pincus <i>et al.</i> , 1999
DNA typing	+	-	+	+	Lupetti <i>et al.</i> , 1995
Restriction fragment length polymorphism (RFLP)	+	+	+	+	Deak <i>et al.</i> , 2004
Randomly amplified polymorphic DNA (RAPD)	-	+	-	-	Reiss <i>et al.</i> , 1998; Bautista-Munoz <i>et al.</i> , 2003
rRNA sequencing analysis	-	-	-	-	Reiss <i>et al.</i> , 2000

1.1.4.4 Treatment of oral candidosis

Prevention and elimination of local factors that predispose to oral candidosis may reduce the incidence of candidal infections (Sherman *et al.*, 2002). Treatment of systemic disease that may predispose to oral yeast colonization and growth, such as diabetes mellitus, will play a major role in reducing or preventing candidal colonization. Management of candidosis can be accomplished with topical applications of antifungal agents, and if this is not controlled, treatment should include systemic medications (Epstein, 1990).

Several antifungal agents are available for the treatment of oral candidal infections.

The most common antifungal drugs in current use are listed in Table 1.3.

Table 1.3: Antifungal agents used to treat oral candidosis.

Class	Agent
Polyenes	Nystatin
	Amphotericin B
Azoles	Imidazoles:
	Clotrimazole
	Miconazole
	Ketoconazole
	Triazoles:
	Fluconazole
	Itraconazole
DNA analogues	5-fluorocytosine
Others	Chlorhexidine gluconate

1.1.4.4.1 Polyenes

Nystatin can inhibit the biosynthesis of ergosterol in the fungal cell membrane. This inhibition of ergosterol biosynthesis alters the permeability of the yeast cell membrane, resulting in leakage of cell constituents and death (Muzyka and Glick, 1995; Ellepola and Samaranayake, 2000c). Nystatin has both a fungicidal and fungistatic action, depending upon the concentration administered (Ellepola and Samaranayake, 2000c). Nystatin is only available for topical application (Budtz-Jorgensen and Lombardi, 1996) due to being too toxic for systemic application (Scully *et al.*, 1994).

The mechanism of action of amphotericin B is similar to that of nystatin. Amphotericin B inhibits fungi via interaction with ergosterol, resulting in a loss of membrane-selective permeability. It can exert either a fungistatic or fungicidal effect depending upon the drug concentration (Ellepola and Samaranayake, 2000c). Amphotericin B is the broadest spectrum among all antifungal agents and is commonly used against systemic fungal infections (Cornely *et al.*, 2003). It can be used topically for the treatment of oral candidal infections and available as; lozenges ointments, suspensions and creams (Budtz-Jorgensen and Lombardi, 1996). It can also be given intravenously for the treatment of systemic mycoses, but there is a risk of nephrotoxicities (Muzyka and Glick, 1995; Epstein and Polsky, 1998).

1.1.4.4.2 Azoles

Most azoles are fungistatic (Epstein, 1990) and work by interfering with the synthesis of ergosterol fungal cell membrane and alter membrane-associated function (Ellepola and Samaranayake, 2000c).

Clotrimazole can be used for the treatment of oral candidosis especially in immunocompromised individuals such as HIV-infected patients (Greenspan, 1994b; Rex *et al.*, 2000). Clotrimazole is available as a topical preparation (Sherman *et al.*, 2002).

Miconazole acts against fungal infections such as *Candida* spp. and some Gram-positive bacteria such as *Staphylococcus aureus* (Ellepola and Samaranayake, 2000c). Miconazole is generally used topically (Scully *et al.*, 1994) as the systemic use of miconazole is superceded by the availability of other less toxic azoles. Miconazole formulated into a lacquer has been shown to be effective in the treatment of DRS (Budtz-Jorgensen and Carlino, 1994; Konsberg and Axell, 1994).

Ketoconazole has a broad spectrum of activity. It is generally reserved for severe infections such as chronic mucocutaneous candidosis or HIV-related candidal infections (Epstein, 1990). However, the associated side effects such as gastrointestinal intolerance, hepatotoxicity and nephrotoxicity have limited its clinical application (Muzyka and Glick, 1995; Akpan and Morgan, 2002).

Fluconazole has a broad spectrum activity against most strains of *C. albicans*, but is less active against non-*albicans Candida* species, particularly *C. krusei* and *C. glabrata* (Budtz-Jorgensen and Lombardi, 1996; Ellepola and Samaranayake, 2000c). Fluconazole has fewer serious side effects compared to ketoconazole and is prescribed when a long-term therapy is required (Sherman *et al.*, 2002). It is well absorbed by the gastrointestinal tract (Akpan and Morgan, 2002) and can be administered systemically (orally or intravenously) (Scully *et al.*, 1994). Fluconazole is widely used as prophylaxis against fungal infection in bone marrow transplant recipients for 75 days following transplantation (Marr *et al.*, 2000). An administration of fluconazole orally to patients with DRS produces a pronounced reduction of erythema and soreness (Budtz-Jorgensen *et al.*, 1988).

Itraconazole is a triazole drug that has a broader spectrum of activity than fluconazole that includes non-*albicans Candida* spp. and is suitable for oral and intravenous administrations (Cornely *et al.*, 2003). It is well-absorbed after oral administration (Ellepola and Samaranayake, 2000c). Itraconazole may be a useful agent in the management of some azole-resistant *Candida* in patients with HIV infection and oropharyngeal candidosis (Ruhnke *et al.*, 1994).

1.1.4.4.3 DNA analogues

Flucytosine (5-fluorocytosine) can interfere with nucleic acid synthesis of the yeast cells (Scully *et al.*, 1994). It is useful as oral therapy for the treatment of deep systemic candidal infections and is mainly given in combination with other antifungal agents (particularly with amphotericin B) due to the rapid development of resistance among clinical yeast species (Pallasch, 2002).

1.1.4.4.4 Others

Chlorhexidine gluconate has also been proposed as an adjunctive therapeutic supplement for prevention and topical treatment of some types of oral candidosis such as pseudomembranous candidosis and DRS or as a denture disinfectant (Ellepola and Samaranayake, 2000c). It has antimicrobial activity and ability to inhibit the adherence of *Candida* spp. to the denture-acrylic and oral mucosal surfaces as reviewed by Budtz-Jorgensen and Lombardi, 1996.

A number of new generation antifungal agents have been proposed. These include voriconazole, posaconazole and ravuconazole, liposomal nystatin and echinocandins (caspofungin, micafungin) (Cornely *et al.*, 2003). Voriconazole is a broad spectrum azole that can be administered orally or intravenously (Richardson and Kokki, 1998). Voriconazole and caspofungin can be used for the treatment of invasive candidosis and have a good activity against *Aspergillus* (Bachmann *et al.*, 2002; Ostrosky-Zeichner *et al.*, 2003)

1.2 THE ORAL ENVIRONMENT IN HEALTH AND DISEASE

Many human surfaces are available for bacterial attachment (skin, respiratory, intestinal mucosa, oral cavity etc.). However, as the majority of these surfaces are continually being shed (along with the bacteria attached to them) and renewed, the opportunities for bacterial attachment are more limited (Wilson, 2001). The oral cavity is similar to other parts of the digestive tract in having a resident microflora, which develops naturally with a characteristic composition (Marsh, 1994). These organisms establish a harmonious relationship with the host. However, when this balance is disturbed a variety of diseases can occur at different sites within the mouth (Bagg *et al.*, 1999b).

Microbial adherence to an oral surface is an essential step for colonisation leading to subsequent tissue invasion and the ability to cause disease (Dorocka-Bobkowska *et al.*, 2003). There are different distinct microbial habitats within the mouth include the lips, cheek, palate, tongue (mucosal surfaces) and teeth (hard non-shedding surfaces). These habitats provides different ecological conditions which enable the growth of populations that are specific to that niche (Marsh and Martin, 1999b).

1.3 BIOFILMS

The first detailed description of microbial attachment to surfaces appeared in the literature 60 years ago when Claude Zobell first noted the preference of marine bacteria for growth on surfaces (Zobell, 1943). However, it was not until the late 1970's that the term 'biofilm' made its first appearance in the scientific literature when William Costerton put forward a general theory of biofilm predominance (Costerton *et al.*, 1978).

The almost universal association between microorganisms and surfaces is now widely accepted. Advances in microscopic technology have led to an appreciation that bacterial biofilms are composed of microcolonies attached to a surface, on which bacteria and other microorganisms are able to develop into organised communities (Costerton *et al.*, 1999). Biofilm formation can alter the local environmental conditions quite considerably and this has important ecological consequences as, for example, observed in the mouth. The resulting community will, in turn, alter the conditions within the growing biofilm and create environments suitable for other types of bacteria. As such, vast numbers of micro-habitats will be generated within the biofilm allowing the survival of species with different nutritional and physico-chemical requirements (Wilson, 2001).

1.3.1 Defining a biofilm

Bacteriologists have studied most aspects of bacterial structures and behaviour using cells that have been grown in suspension in a liquid medium. The organisms grown in this manner are termed planktonic organisms, however; in most natural environments they become associated with a surface, and this is now considered the 'normal' microbial lifestyle. Cells in biofilm expressed markedly different protein profiles when compared to their planktonic counterparts (Sauer *et al.*, 2002). Growing in this form is an efficient means of persisting in a localised area protected from the surrounding environment (Watnick and Kolter, 2000).

Biofilms have been defined in a number of ways, but it is difficult to come up with a definition of the term that is satisfactory to all researchers in the field. In 1978, Costerton and co-workers observed that communities of attached bacteria in aquatic systems were found to be enclosed in a polysaccharide matrix which mediated

adhesion (Costerton *et al.*, 1978). Further, in 1987 and 1995, the group stated that biofilms consist of single cells and microcolonies, all embedded in a highly hydrated, predominantly anionic exopolymer matrix emphasizing that biofilm could adhere to surfaces and interfaces to each other (Costerton *et al.*, 1987; Costerton *et al.*, 1995). Finally, a biofilm as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface was proposed as a definition in 1999 (Costerton *et al.*, 1999).

Wilson (2001) described the main features of a biofilm as; a) a three dimensional structure containing one or more bacterial species; b) it forms at interfaces – solid/liquid, liquid/air; solid/air; c) it shows spatial heterogeneity due to physio-chemical and chemical gradients that develop within it; d) biofilm is often permeated by water channels and; e) the organisms within the biofilm exhibit a marked resistance to antimicrobial agents and host defence systems compared to planktonic microbes. Biofilms are therefore cellular communities with an ordered structure and circulatory system. They display different physiologies within different regions, have a form of intercellular communication and can resist noxious chemicals and other threats from their surrounding environment. Others have defined a biofilm as a population of cells growing on a surface and enclosed in an expolysaccharides matrix (Lewis, 2001) or as communities of microorganisms that are attached to a surface (O'Toole *et al.*, 2000). Donlan stated that “biofilm is a process whereby microorganisms irreversibly attached to and grow on a surface and produce extracellular polymers that facilitate attachment and matrix formation, resulting in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription” (Donlan, 2001a).

1.3.2 The formation of a biofilm

In natural environments, bacteria rarely adhere to the substratum itself, invariably this is coated with a layer of adsorbed molecules known as a “conditioning film” and it is to this film that the organism usually adheres. In the oral cavity, this conditioning film (up to 1.0 μm thick) is known as the salivary pellicle and is formed mainly from glycoproteins and other salivary molecules (Wilson, 2001).

Several phases or stages (Figure 1.3) have been recognised in the formation of biofilm (Sauer *et al.*, 2002; Stoodley *et al.*, 2002). Biofilm formation begins with the attachment of single cells to a surface-the substratum. Firstly, in the reversible step, cells may require motility or may have random contact with a surface. This is followed by the attachment of the cells to the substratum by adherence through surface appendages or by the production of extracellular polymeric substances. The attached cells then may grow into microcolonies depending upon the availability of nutrients on the surface itself or from the liquid phase above the substratum. In a hydrodynamic environment the development of the microcolonies depends on cell-cell binding interactions. These microcolonies start to grow and form a mature biofilm in which the biofilm structure with its distribution of biomass. The presence of fluid-filled voids illustrates the heterogeneity of the biofilm and the rigid properties of the developed structure. The stability of the biofilm is protected by cell-cell interactions and by the extracellular polymeric substance (EPS) both surrounding and integrated into the biomass of the biofilm. Finally, the biofilm may partially detach, releasing cells that may move away to other locations where a new cycle can begin (Molin and Tolker-Nielsen, 2003).

In order to progress from the reversible attachment stage to the irreversible adhesion phase of biofilm formation, the attached cells up-regulate genes that produce

enzymes concerned with the synthesis of EPS which cement their adhesion to the surface and to other bacterial cells. The resultant biofilm structure has different cell envelope proteins from those found in planktonic cells. Therefore, once bacterial cells adhered to the surface, they express different phenotypes from their planktonic counterparts (Costerton, 1999; Sauer *et al.*, 2002). The features of the substrata may have a considerable effect on the rate and extent of attachment by microorganisms (Donlan, 2001a). For example, rougher surfaces which are preferentially colonised, provide niches for bacteria as they provide an increased surface area (Lytle *et al.*, 1989).

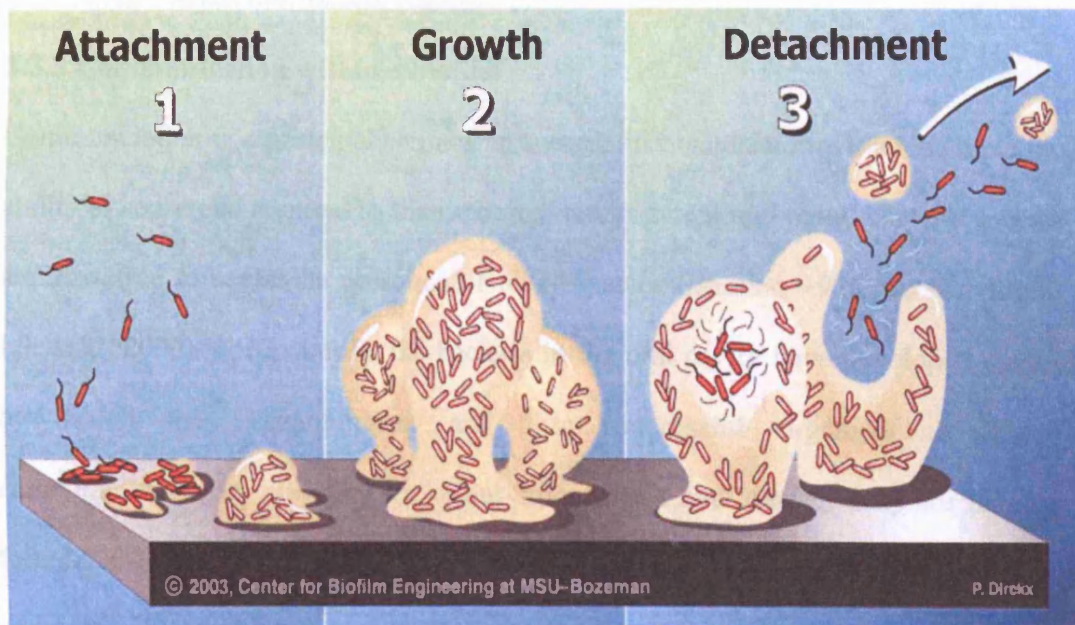


Figure 1.3: Diagrammatic representation of stages associated with biofilm formation. Figure is reproduced from the Center for Biofilm Engineering at MSU-Bozeman.

Biofilm formation on medical device may be composed of a single-species or multi-species biofilms (Donlan, 2001b). These surfaces can be colonised by both bacteria and yeasts (Marrie and Costerton, 1984; Reid *et al.*, 1992; Hawser and Douglas, 1994). Microorganisms on catheter surfaces show two forms: the sessile form, in which the organisms embedded in the biofilm and a planktonic form in which the organisms disseminate over the surface. Microbial colonisation and biofilm formation can occur within 24 h following the insertion of a device (Raad, 1998). The formation of microbial biofilm on biomaterials also involves sequential steps. These include the absorption of the host conditioning film to the device surface and the adhesion of microorganisms anchored by EPS and growth (Reid, 1999).

1.3.3 Communication within a biofilm

Communication is a principal element in successful organisations. Bacteria have the ability to sense and respond to their external environment and many bacteria species are also able to sense the presence of other species (Bassler, 1999; de Kievit and Iglewski, 2000). For example, the bacteria in the oral cavity have developed means by which they communicate and form successful organisations. These communications are essential for initial colonisation and subsequently for biofilm formation (Kolenbrander *et al.*, 2002). Most oral bacteria can physically interact with and adhere to other bacteria and all display specific recognition patterns with their respective partner cells. Many bacteria using a quorum-sensing system to regulate their physiological properties include; virulence, biofilm formation and motility. This mechanism works through the production of signals that can be detected by neighbouring cells (Cvitkovitch *et al.*, 2003).

Colonisation is successful if the adherent cells grow and contribute metabolically in the oral bacterial environment (Whittaker *et al.*, 1996). Metabolic interactions may occur through the breakdown of a substrate by the extracellular enzymatic activity of one organism that creates biologically available substrates for different organisms or when the excretion of a metabolite by one organism can be used by a different organism as a nutrient source (Kolenbrander *et al.*, 2002).

Bacterial coaggregation is a common phenomenon in a variety of multi-species biofilm communities. It is a process by which genetically distinct bacteria attached to one another via specific molecules. Such interactions are able to influence the development of complex multi-species biofilms (Rickard *et al.*, 2003). The multiplication of the organisms attached to the already adherent cells is a phenomenon known as co-adhesion (Wilson, 2001). This interaction enables cell communication through signalling and genetic exchange within a biofilm and also can signal other biofilm members, leading to metabolic communication that defined the microbial community and its spatial organisation of the community (Kolenbrander, 2000).

Several interactions occur between *Candida* spp. and other microorganisms which facilitate fungal survival in mixed-species oral communities and play an important role in colonisation by *C. albicans* of hard and soft oral surfaces (Jenkinson *et al.*, 1990; Holmes *et al.*, 1995; Jabra-Rizk *et al.*, 1999).

Oral biofilms are an excellent example of species complexity within a biofilm as the total species diversity in the oral cavity has been postulated to be more than 500 species (Paster *et al.*, 2001; Foster and Kolenbrander, 2004). Antagonistic and beneficial interactions between this complex microbial community will exert a homeostatic stable effect (climax community) (Wilson, 2001).

1.3.4 Biofilm structure

Biofilms are dynamic with respect to structure and composition (Palmer and White, 1997). They display great differences in their structure and thickness. In humans, for example, biofilms which form on tooth surfaces are relatively thick, while biofilms which form on implanted medical devices tend to be thinner (Wilson, 2001). The use of Confocal Laser Scanning Microscopy (CLSM) has enabled the examination of biofilms in their native hydrated state and allowed an accurate estimation of biofilm structure and dimensions (Baillie and Douglas, 1999b). Complex structures such as mushroom-like stacks of micro-colonies (Figure 1.4) containing bacteria embedded in extracellular matrix and separated by water channels (Costerton *et al.*, 2003) are known to exist in some environments. This biofilm structure forms under; low nutrient concentration, high hydrodynamic shear stress and absence of mechanical, abrasive and compressive force (Wilson, 2001). It also influenced by growth rate, cell signalling, motility, EPS production and physical growth environment (Stoodley *et al.*, 2002). Additionally, the chemical composition and physical structure of the substratum, liquid flow rate, and the nature of the organism will also affect the final structure (Wilson, 1996).

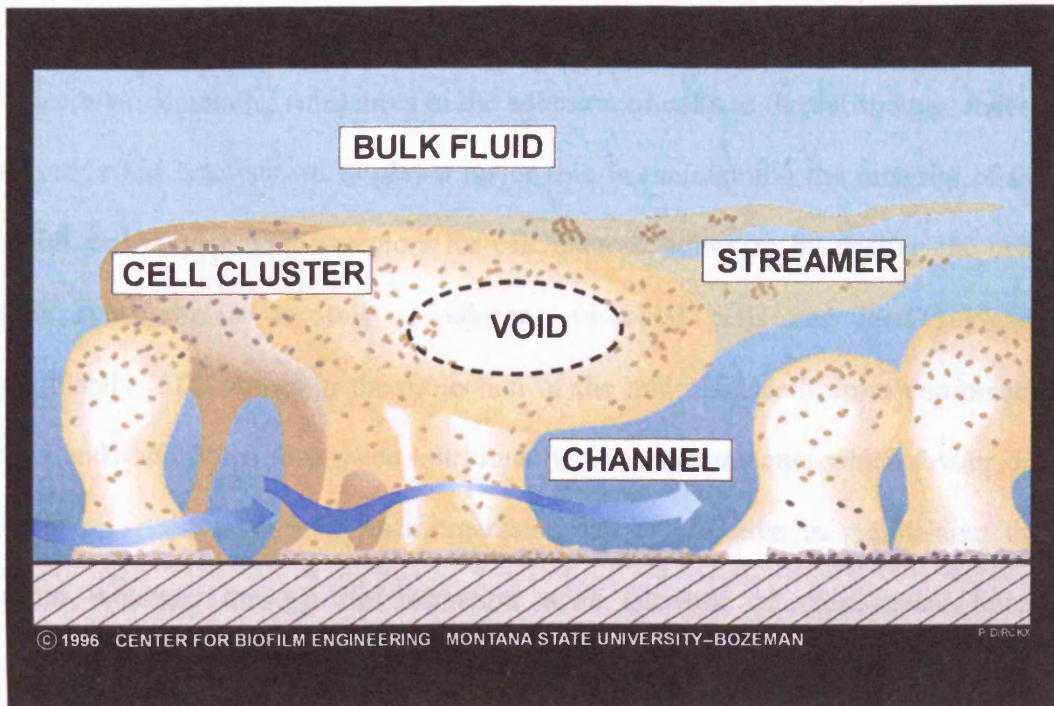


Figure 1.4: Diagrammatic representation of the mushroom-like microcolonies that constitute the structural element of biofilm. Figure is reproduced from the Center for Biofilm Engineering MSU-Bozeman.

1.3.4.1 Extracellular polysaccharides

The key elements in biofilm structure are bacteria, the extracellular matrix and water. The synthesis of extracellular matrix molecules occurs during the colonisation stage that follows the initial adhesion of bacteria (Wilson 2001). Bacterial biofilms are embedded in a thick, highly hydrated anionic matrix (Costerton *et al.*, 1987). The slow bacterial growth observed in most biofilms would be expected to enhance production of EPS which varies greatly in composition and hence in chemical and physical properties. Extracellular matrix consists mainly of polysaccharides and a wide range of other molecules, including lectins, proteins and lipids (Sutherland, 2001). It is a major component of the biofilm and may comprise 50 – 95 % of the dry

weight (Sutherland, 1999). Zobell (1943) suggested the involvement of the extracellular cementing substances in the adhesion of cells to the substratum, indeed the matrix has been shown to play a major role in maintaining the integrity of the biofilm and may provide receptors for cell induced adhesion by coating the cells which synthesise it as well as adjacent microbial cells and inert surfaces. Additionally, EPS assists in the protection of the microbial cells from detrimental compounds and helps to provide nutritionally rich environments which favour the growth of specific types of microorganisms and is effective in maintaining the biofilm structure through the formation of a network of cross-linked linear macromolecules (Sutherland, 1999). It may interact with antimicrobial agents and prevent access of the compounds or reduce their concentration (Sutherland, 2001). Freeman and Lock (1995) have suggested that the EPS could act as a buffer against changes in the available organic substrates. Understanding the chemical and structural characteristics of the biofilm matrix may help to understand the structure and function of the biofilms.

1.3.5 Biofilm resistance

Biofilms are especially difficult to eradicate and are a source of many recalcitrant infections. Infections due to microbial biofilms are a major cause of morbidity and mortality and treatment of such infections is difficult. Due to resistance to antimicrobial agents, biofilms may act as a source of persistent infection and may harbour pathogenic organisms (Donlan, 2001a). Pathogenic yeast such as *Candida albicans* can also form recalcitrant biofilms (refer to section 1.4.2). Resistance can be defined as the ability of a microorganism to grow in the presence of an elevated level of antimicrobial (Lewis, 2001). There are two main mechanisms of bacterial

resistance to biocides, intrinsic (as in the natural biofilm growth mode) or acquired (acquisition of resistance plasmid or transposons) (Russell, 1995; Donlan, 2001a). Roberts and co-workers showed that bacteria in a biofilm may transfer resistance genes (Roberts *et al.*, 1999).

Biofilms form highly organised structured communities and their formation follows an ordered sequence of events (Schembri *et al.*, 2003). Biofilms formed in a protected environment may shed individual cells into the surrounding tissues and causing acute illness that resist treatment (Davies, 2003). Costerton and colleagues published a review article that was the first to articulate the general phenomenon of biofilm resistance to antimicrobial agents compared to their planktonic counterparts (Costerton *et al.*, 1987). It has been shown that there is up to a 1000-fold decrease in susceptibility of biofilms compared to planktonic cells (Nichols *et al.*, 1989). The reasons for the differing susceptibilities of organisms when grow as a biofilm and those grown in suspension are a subject of continuing debate. It is clear that the mechanism of biofilm resistance to antimicrobial agents is extremely complex and several mechanisms have been proposed to account for the extraordinary resistance of biofilm to antimicrobial agents as detailed below.

1.3.5.1 Delayed penetration of the antimicrobial agents

In order to inactivate the encased cells, antimicrobial agents must diffuse through the biofilm matrix. It has been supposed that EPS might exclude or limit the access of drugs to organisms within the biofilm (Douglas, 2003). EPS may act as a barrier against the penetration of the agent either by influencing the rate of transport or the reaction of antimicrobial agents with the matrix (Costerton *et al.*, 1987; Donlan and Costerton, 2002). This EPS is negatively charged and acts as an ion-exchange resin

that is capable of binding a positively charged antimicrobial molecule and preventing it reaching the embedded biofilm cells (Anwar *et al.*, 1992). Reaction sink (neutraliser) is another mechanism by which a matrix may play a role in biofilm resistance. This is augmented by the retention of extracellular products and drug-inactivating enzymes or degenerative enzymes that are able to break down certain antibacterial agents (Morton *et al.*, 1998; Gilbert *et al.*, 2002).

However, there appears to be some debate over whether the EPS hinders the diffusion of antibacterial agents into biofilms (Morton *et al.*, 1998; Mah and O'Toole, 2001). Anderl and co-workers showed that an antibiotic (ciprofloxacin) was able to penetrate the biofilm quickly, yet was unable to kill the bacteria (Anderl *et al.*, 2000). It has also been shown that EPS has a minimal effect against the penetration of some other antibiotics (tobramycin and cefsulodin) (Nichols *et al.*, 1988; Nichols *et al.*, 1989). This suggests that other mechanisms may contribute to the resistance of these cells.

Hence, the biofilm matrix does represent a barrier that delays the penetration of certain compounds of antimicrobial agents, but it may not form an impenetrable barrier to diffusion and does not appear to be the sole explanation for the increased resistance of biofilm (Mah and O'Toole, 2001).

1.3.5.2 Altered growth rate of biofilm organisms

Depending on their location within the biofilm, different cells are metabolically active or inactive. For example, cells in the outer layer of biofilms tend to be more viable and more metabolically active and surround an internal core of nonviable bacteria (Hope and Wilson, 2003). Their cell envelope is more permeable to nutrients such as oxygen and these have better ability to discharge metabolic waste

products (Anwar *et al.*, 1992). In contrast, cells that are deeply situated within a biofilm, have reduced growth rates due to nutrient limitation and therefore exist in a slow-growing or starved state (Brown *et al.*, 1988). Additionally, the accumulation of the metabolic waste products may cause unfavourable growth conditions and the cells may become dormant (Anwar *et al.*, 1992).

The slow bacterial growth rate is strictly proportional to the decrease in antibiotic-induced killing effect and hence the increase in biofilm resistance (Evans *et al.*, 1991; Duguid *et al.*, 1992). In fact, some antibiotics, such as penicillin, kill only growing cells (Lewis, 2001).

The encapsulated community of a biofilm are at a lower risk from environmental stresses (Anwar *et al.*, 1992). Under these circumstances, the embedded biofilm cells may have sufficient time to switch on the expression of antibiotic-resistant factors (Giwerzman *et al.*, 1991). This may contribute to the inherent resistance to antibiotics that are active in planktonic bacteria. Cells situated at the bulk water interface of biofilms are killed more rapidly than cells found more deeply within clusters and which remain unaffected by the agent (Davies, 2003). These deeply situated bacteria can potentially form a nidus for continued dissemination of the infection. Additionally, biofilm associated cells grow more slowly compared to planktonic cells (Williams *et al.*, 1997; Donlan and Costerton, 2002) and the production of EPS is also higher for attached bacteria (Evans *et al.*, 1994). This will provide an increase in EPS production within the slow growing centre of biofilms and consequently alter the distribution and cell density throughout the matrix and confer a spatial structural organisation upon the community to provide customised microniches at various point within a biofilm (Costerton *et al.*, 1994). Hence, antimicrobial agents are either taken up more slowly (Donlan and Costerton, 2002)

or possibly the cells are less permeable to the agents or have altered the antibiotic target sites (Anwar *et al.*, 1992).

1.3.5.3 Unique biofilm physiology

Biofilm bacteria are physiologically distinct from their planktonic counterparts. As a consequence of attachment, bacteria express unique genes that possibly enhance resistance to antimicrobial treatments and host immune defence responses. These biofilm-specific genes are different from genes expressed in the free floating cells (Becker *et al.*, 2001; Schembri *et al.*, 2003). Hence, the activated genes modified biofilm susceptibility toward antimicrobials (Gilbert *et al.*, 1997). To enhance the effectiveness of the existing antibiotics, these genes need to be identified and could become possible targets for chemotherapeutic treatment and disrupt the multicellular structure of biofilms (Stewart and Costerton, 2001).

1.3.5.4 Persisters

A recent and novel hypothesis for biofilm resistance relates to the presence of persistent cells (persisters). A persister is a hypothetical cell state in which microorganisms are protected from all types of antimicrobial agents in a similar manner to a dormant, spore-like cell. The physiological state of these cells allows them to survive during antibiotic treatment. It is suggested they are present in relatively small numbers but are able to 'reseed' the biofilm in the event of, for example, a chemical challenge. At this point they are able to grow rapidly in the presence of nutrients released from their lysed community partners and survive in the post-treatment phase. It has been suggested that they are formed in higher numbers in biofilms, because of nutrient limitation, than they do in a planktonic culture

(Gilbert *et al.*, 2002).

Thus the biofilm EPS can delay the penetration of some antimicrobial agents, while the reduced growth rate and nutrient limitation within a biofilm can also influence the physiology and the sensitivity of bacteria. However, multiple mechanisms are required for overall antimicrobial resistance (Mah and O'Toole, 2001) and the intrinsic phenotypic resistance of cells within a biofilm may be a primary factor. Together with cell-cell communication and organised structure, a biofilm can act as a community that can control physiological processes and responses via gene expression (Morton *et al.*, 1998).

1.3.6 Biofilm detachment and death

Detachment from biofilms is an important consideration in the dissemination of infection. Bloodstream and urinary tract infections could result from these detached bacteria (Donlan and Costerton, 2002). Detachment is a general term used to describe the release of cells from the biofilm or substratum, either individually or in groups (Stoodley *et al.*, 2002). Salivary flow may also contribute to the detachment of cells from the oral biofilm (Kolenbrander, 2000). It has been shown that single cells and small clusters in mixed-species biofilms detached more frequently than the larger clusters (Stoodley *et al.*, 2001). The detached section can either re-adhere to the surface and could comprise an effective means of colonising a large area of substratum (Wilson, 2001) or may return to the planktonic mode of growth (Stoodley *et al.*, 2002).

Death of organisms comprising biofilms has been also observed in monoculture and mixed-species biofilms. Death in monoculture biofilms can be defined as cessation

of cellular activity, whereas in mixed-species population it can be assessed in a term of loss of a particular member of the species in the community as a consequence of environmental changes. This may involve a change in the proportion of species or their function. Importantly, the loss may cause a shift to different population (Palmer and White, 1997).

1.3.7 Biofilm related diseases

Biofilms are responsible for a number of diseases in humans (Table 1.4) and detached cells can cause systemic infections, depending on the response of the host immune system (Donlan, 2001a).

Microorganisms may adhere to a wide range of surfaces including inert, non-living materials or living tissues (Donlan, 2001b). Advances in medical and surgical techniques have resulted in the use of a wide range of medical devices implanted into the body which provide non-shedding surfaces on which a biofilm can form. Thus medical implants such as catheters, urethral stents, cardiac pacemakers can be colonised by microorganisms and subsequently form a biofilm. These surfaces are not well protected by host defences and can offer a focal point for organisms to grow (Reid, 1999). Millions of these devices are in use each year and cause frequent vascular-catheter-related bloodstream infections (Raad, 1998). As well as bacteria, species of yeast are also able to form a biofilms on the surface of these catheter materials (Hawser and Douglas, 1994). The associated sepsis of these biofilms is one of the most serious complications of intravenous therapy and removal of these devices becomes necessary to cure the patient from the infection thus increasing the cost and trauma to the patient (Khardori and Yassien, 1995; Mah and O'Toole, 2001).

The structure of biofilms has the highest impact on the outcome of chronic bacterial infections such as infective endocarditis, where individual microcolonies break off and detach, causing a serious risk of infective emboli in the capillary bed (Donlan and Costerton, 2002). Advances in technology have decreased the problems associated with biomaterial related infections, however, there is still a need to overcome these problems and there is limited evidence to suggest that bacterial colonisation on devices can be prevented over the long time (Reid, 1999).

Table 1.4: Table indicating biofilm related human infections.

Infections caused by biofilms	Specific example	Reference
Implanted medical devices related infections	Prosthetic valve endocarditis	Parsek and Singh, 2003; Kojic and Darouiche, 2004
	Central venous catheter	Donlan, 2001b; Kojic and Darouiche, 2004
	Intravascular catheter	Raad, 1998
	Urinary catheter	Choong and Whitfield, 2000; Trautner and Darouiche, 2004
	Joint prostheses	Kojic and Darouiche, 2004
Lung disease	Cystic fibrosis	Costerton, 2001; Parsek and Singh, 2003
Ear infections	Otitis media	Post, 2001; Donlan and Costerton, 2002
Oral infections	Caries and periodontal diseases	Marsh and Bradshaw, 1995; Rosan and Lamont, 2000; Donlan and Costerton, 2002

1.3.8 Biofilm-based models

Traditionally microbiologists have studied organisms in broth culture in their planktonic phase. However, in order to study biofilm communities in the laboratory, researchers have had to devise methods to study organisms in this phase of growth. One of the most studied *in vitro* biofilms are those representing dental plaque. Researchers have studied dental plaque grown in the laboratory for a number of years in artificial mouth models. Interestingly, many of the biofilm models available today are derived from these earlier systems. An artificial mouth was used to grow dental plaque *in vitro* with a specific focus on dental caries research (Pigman *et al.*, 1952). The models were specifically constructed to mimic the situation in the oral cavity. Dental plaque formation was studied on an extracted mounted human teeth inoculated with saliva and then supplied with a mucin-containing artificial saliva as reviewed by Spratt and Pratten, 2003. The model has been used to study root surface caries and enamel caries and the effect of fluoride (Shu, 1998).

There are a wide range of available biofilm model systems including; the Robbins device, Rotatorque and the Cell Adhesion Measurement Module (CAMM). However, many of these models tend not to be used to study oral biofilms (Wimpenny, 1997).

The difficulties associated with *in vivo* studies of dental plaque include; the complexity and heterogeneity of plaque structure, problems related to access and sampling and ethical issues. Additionally, high variability of *in vivo* experimental protocols and the results are difficult to interpret (Sissons, 1997; Spratt and Pratten, 2003).

In order to be able to explain plaque ecology and behaviour, these models must be realistic and reflect the behaviour that is under investigations (Sissons, 1997). There

are a number of biofilm-based models that have been used, or could be used to study oral biofilms (Wilson, 1996; Wimpenny, 1997). These different biofilm systems have strengths and limitations and hence compromise between the reality of the *in vivo* ecosystem and the simplicity and controllability of the model needing to be evaluated (Sissons, 1997).

The flow cell is often the system of choice for short-term adhesion and colonisation studies, as it enables the non-destructive observation of biofilm development in real time (Busscher and van der Mei, 1995). It permits adherence of early colonizing bacteria to a saliva-conditioned solid substratum and can be examined directly by CLSM without disruption of the growing biofilm (Foster *et al.*, 2004). The Flow cell can also be used for the development of multi-species oral bacterial biofilms (Foster and Kolenbrander, 2004). Additionally, it can be employed as a rapid assessment of antimicrobial efficacy against bacterial biofilms (Foster *et al.*, 2004). It consists of a transparent chamber of fixed depth through which the growth medium passes. When mounted on a microscope stage, this system allows observation of biofilm development inside the chamber (Spratt and Pratten, 2003). Sterile saliva is used as a sole nutrient source and is also used to coat the glass before inoculation with bacteria. Bacteria will adhere to the saliva conditioning film leading to biofilm formation (Kolenbrander *et al.*, 2002).

Chemostat-based systems have also been used to model oral biofilm formation (Wilson, 1996; Wimpenny, 1997; Sissons, 1997). A large number of replicate samples and variety of experimental surfaces, such as hydroxyapatite disks, can be submerged in the fluid phase. Multi-species oral bacterial biofilms have been also produced using chemostats (Bradshaw *et al.*, 1996). However, a disadvantage of this system is that biofilm thickness can not be defined and it is not consistent with the *in*

vivo situation where only a thin film of fluid is present on oral surfaces unlike the large fluid mass in a chemostat (Spratt and Pratten, 2003).

When using an antimicrobial agent over a period of time, data must be comparable and reproducible from one experiment to another. In order to study biofilm development and susceptibility, a Constant Depth Film Fermentor (CDFF) can be used (for more details refer to section 2.4). It is a steady-state device (Wimpenny, 1997) that allows the production of biofilms under conditions similar to those found in the oral cavity. Biofilm is grown on solid substrata with a thin liquid film of nutrients supplied. The scraper blade can control the thickness of formed biofilm by removing excess biofilm. This imitates the continuous removal of the upper most layers of the biofilm due to shear forces present in the oral cavity which exist through eating and tongue movement (Wilson, 1996). The system is a versatile biofilm model and has been used in many investigations concerning the formation of oral biofilms and their susceptibility to antimicrobial agents. For example, it has been used in the study of microbial microleakage around the dental restorations (Matharu *et al.*, 2001), oral malodour generation (Pratten *et al.*, 2003), and the composition of dental plaque on orthodontic bonding materials (Badawi *et al.*, 2003).

Wilson (1996) stated several advantages of the CDFF to generate oral biofilms; these include: a) the ability to produce many replicates (up to 90) in one single experiment, b) allows pulsing of the nutrients or antimicrobial agents, c) at various intervals, it allows biofilm sampling (with replicates), d) mixed plaque samples or pure cultures can be used for inoculation (continuously or intermittently), e) it can be autoclaved and temperature-controlled, f) a different substrata can be investigated, and finally g) it can be used to investigate the effect of antimicrobial agents on biofilms.

1.4 CANDIDAL BIOFILMS

In contrast to the extensive literature describing bacterial biofilms, relatively little attention has been paid to fungal biofilms. *C. albicans* biofilms share several properties with bacterial biofilms, including their structural heterogeneity, the presence of EPS and their associated resistance to antifungal agents. However, *Candida* spp. differ from bacteria in several important characteristics which are relevant to biofilms. These include; the dimorphic or polymorphic forms of *Candida*, the larger size of *Candida*, the chemical composition and overall architecture of its cell surface compared to bacteria and finally, the presence of hyphal forms that complicate biofilm treatment (Baillie and Douglas, 1999a).

The increase use of transplantation procedures and chronic indwelling devices, which can act as substrata for biofilm growth and subsequent infections, have increase in the prevalence of fungal diseases, *C. albicans* being the most commonly associated fungus (Chandra *et al.*, 2001a).

Other non-*albicans Candida* spp. also form biofilms of clinical importance. For example, candidemia due to *C. parapsilosis* infections is associated with the use central venous catheter (Shin *et al.*, 2002). *C. dubliensis*, closely related to *C. albicans*, also produces a biofilm with the defining characteristic of high matrix content and antifungal resistance (Ramage *et al.*, 2001).

1.4.1 Structure of *Candida albicans* biofilms

Microorganisms appear to share common basic phases during biofilm formation. *C. albicans* biofilms form distinct organised developmental structures through three phases; early, intermediate and maturation (Chandra *et al.*, 2001a). Following attachment of fungal cells to a surface, a three dimensional structure is formed that is

composed of cells surrounded by expolymeric matrix (Kumamoto, 2002). *C. albicans* biofilms are typically composed of a layer of yeast cells adhering to the surface with a layer of filamentous cells above it (hyphal form) (Hawser and Douglas, 1994) all surrounded by exopolymeric matrix (Baillie and Douglas, 1999b; Chandra *et al.*, 2001a). Previous studies on biofilm formation by Scanning Electron Microscopy (SEM) demonstrated that at early stages of biofilm formation, yeast-phase cells of *C. albicans* adhered to the surface of catheter disks and during the subsequent 48 h, a dense biofilm consisting of yeast cells, germ tubes, pseudohyphae and hyphae was observed. Extracellular polymeric material was visible on the surface of some of these morphological forms (Hawser and Douglas, 1994).

As in their bacterial counterparts, expolysaccharide material is also found in *C. albicans* biofilms. Baillie and Douglas isolated the EPS of *C. albicans* biofilms and compared its composition to that produced by planktonic cells. They found that EPS from both cultures contained carbohydrate, protein and other components. However, the biofilm EPS contained significantly less total carbohydrate and protein than the planktonic EPS with a higher glucose proportion, and galactose, which was unique to the biofilm. These results suggest that *C. albicans*, unlike many bacteria, may produce biofilm-specific EPS (Baillie and Douglas, 2000).

It has been shown that the synthesis of EPS in biofilms is not extensive under static conditions, however, EPS synthesis increases markedly when the developing biofilm are subjected to a liquid flow under gentle shaking (Hawser *et al.*, 1998).

Generally, the overall organisation of biofilms formed by *C. albicans* is similar to that of bacterial biofilm. The detailed structure of *C. albicans* biofilms are highly dependent upon the conditions under which the biofilm formed (Kumamoto, 2002).

Indeed numerous experimental parameters can influence the formation and the

structure of *C. albicans* biofilm such as; nature of contact surface, growth factors, *Candida* morphogenesis, and the *Candida* spp. involved. These will be discussed separately.

1.4.1.1 *The nature of contact surface*

The nature of contact surface has been shown to influence biofilm formation of *C. albicans*. For example, *C. albicans* biofilms formed on acrylic denture material such as polymethylmethacrylate (PMMA), are much thinner than biofilms formed on catheter material such as silicon elastomer (Chandra *et al.*, 2001a). Hawser and Douglas also reported that *in vitro* formation of *C. albicans* biofilm was increased when grown on latex or silicone elastomer compared with polyvinyl chloride, but decreased on polyurethane or silicone (Hawser and Douglas, 1994). Results to-date have indicated that the structure of *C. albicans* biofilm architecture depends upon highly specific contact-induced gene expression (Douglas, 2003).

1.4.1.2 *Growth factors*

C. albicans biofilm grown on PMMA denture material in one medium, for example, YNB medium have been shown to be composed predominantly of yeast cells form, whereas biofilm grown in another medium (e.g RPMI) were composed predominantly of filamentous form. These results suggest that biofilm growth is not morphology specific as both the yeast and hyphal forms of *C. albicans* were able to form a biofilm (Chandra *et al.*, 2001a).

1.4.1.3 *Candida morphogenesis*

One distinguishing feature of *C. albicans* is its ability to survive in a number of morphological forms which can affect biofilm formation. Baillie and Douglas have investigated the role of morphogenesis in the structure of *C. albicans* biofilm on catheter using SEM. In this study, they compared a biofilm produced by wild-type strains of *C. albicans* with those formed by two morphological mutants, incapable of yeast and hyphal growth, respectively. They found that the wild-type strains produced a distinct two-layered biofilm, a thin basal yeast layer and a thick hyphal layer. The hyphae-negative mutant produced only a basal layer, whereas the yeast-negative mutant produced only the thicker hyphal outer layer which was easily detached from the surface. This suggests that dimorphism can affect biofilm formation and that the basal yeast layer has an important function in anchoring the biofilm to the surface. However, as both mutants were capable of forming a biofilm, dimorphism is not an absolute prerequisite for biofilm formation, albeit of different thickness, and that dimorphism might be necessary for the development of a spatially organised structure (Baillie and Douglas, 1999b). More recently, Ramage and co-workers have demonstrated that *C. albicans* mutants lacking a regulator protein (Efg1) of hyphal morphogenesis, failed to produce a filament-containing layer which was observed in wild type *C. albicans* biofilms. This demonstrated that filamentation is required for normal biofilm development (Ramage *et al.*, 2002c).

1.4.1.4 *Candida species involved*

Studies on different *Candida* spp. have shown that biofilm growth varied between species. An *in vitro* study on biofilm formation of different *Candida* spp. demonstrated that biofilm formation by *C. parapsilosis*, *C. pseudotropicalis* and *C.*

glabrata produced significantly less biofilm compared to *C. albicans* (Hawser and Douglas, 1994). Recently, another study by Kuhn and co-workers, confirmed these findings in that *C. albicans* produced more biofilm than biofilms produced by *C. parapsilosis*, *C. glabrata* and *C. tropicalis* and additionally *C. albicans* was able to form a more complex biofilm than *C. parapsilosis* biofilms, which were composed of only clumped blastospores (Kuhn *et al.*, 2002a).

1.4.2 Resistance of *Candida albicans* biofilms

As observed in bacterial biofilms, *Candida* biofilms are known to be resistant to antifungal agents (Hawser and Douglas, 1995). The mechanisms by which *Candida* biofilms resist the action of antifungal agents are not known. Wroblewska and co-worker concluded that frequent use of triazoles in prophylaxis of fungal infections, especially in immunocompromised patients, may be responsible for the increasing rate of *C. albicans* biofilm resistance (Wroblewska *et al.*, 2002).

Because the structure of *C. albicans* biofilm varies in response to environmental conditions, it is thought that the mechanism of drug resistance of these biofilms could also vary (Kumamoto, 2002). The possible mechanisms for bacterial biofilm resistance (refer to section 1.3.5) may also play a role in the increased resistance of *Candida* biofilm to antifungal agents. In this section, the resistance related to *Candida* biofilms is discussed.

1.4.2.1 Delayed penetration of the antimicrobial agents

Developing *C. albicans* biofilm are associated with the presence of extracellular material (Chandra *et al.*, 2001a), which could act as a physical barrier against antibiotics and contribute to drug resistance as in bacterial biofilms (Gilbert *et al.*,

1997). However, it is unclear whether *C. albicans* biofilm resistance is due to the production of extracellular material or due to genetic biochemical alterations in fungal cells.

Most studies of bacterial biofilm matrix indicates that the matrix does not form a major barrier to drug diffusion, although for certain compounds penetration can be delayed (Mah and O'Toole, 2001). To assess the possible role of the matrix material in the resistance of *C. albicans* biofilms to antifungal agents, susceptibility profiles of *C. albicans* biofilms incubated statically (i.e. containing relatively little matrix) were compared with those biofilms incubated in gentle shaking (which produced more matrix material). Both biofilms grown with or without shaking did not exhibit significant differences in susceptibility to any of drug tested. These results indicate that the matrix does not constitute a significant barrier to antifungal drugs, and thus extensive matrix production may not enhance biofilm resistance (Baillie and Douglas, 2000). However, other studies with biofilms produced under flow conditions showed that re-suspended cells (which had lost most of their matrix) were 20 % less resistance to amphotericin B than intact biofilms suggesting that the biofilm matrix might play a minor role in excluding antifungal agents from the biofilm (Baillie and Douglas, 1998a; Baillie and Douglas, 1998b). Ramage and co-workers also demonstrated that intact biofilms were not affected by high concentration of fluconazole. Additionally, sessile cells (1 h adherence) can resist fluconazole at the same concentrations as of intact biofilms, indicating that sessile resistance was not entirely related to biofilm ultrastructure and matrix material. They also showed that re-suspended biofilm cells were resistance to fluconazole (MICs increased 64 – 1000 times compared to planktonic cells), but were up to 8 times less resistance to fluconazole than intact biofilms. This suggests that the matrix may

contribute to overall resistance, but is not considered as a sole determinant of biofilm resistance (Ramage *et al.*, 2002a).

1.4.2.2 Altered growth rate of biofilm organisms

Biofilms cells are thought to grow slowly because of the limited availability of nutrients particularly at the base of the biofilm. This is frequently associated with changes in composition of the cell surface which may in turn affect the susceptibility of biofilms to antimicrobial agents (Douglas, 2003). To investigate this possibility, Baillie and Douglas have examined the importance of growth rate of *C. albicans* biofilms on antifungal resistance. They used a perfused biofilm fermentor to generate *C. albicans* biofilms at different growth rates. The susceptibility of these biofilm cells was compared with planktonic organisms grown at the same rates in a chemostat. They found that biofilms were resistance to amphotericin B at all growth rates tested, whereas planktonic cells were resistant only at a low growth rates (Baillie and Douglas, 1998a). A separate study (Baillie and Douglas, 1998b), using a cylindrical cellulose filter, have demonstrated that at the same low growth rate, glucose-limited and iron-limited biofilms were equally resistance to amphotericin B. These results suggest that growth rate does not have a major influence on the susceptibilities of a biofilm to the drug.

1.4.2.3 Efflux pumps

The role of efflux pumps in the drug resistance of mature *C. albicans* biofilm has been studied previously. Ramage and co-workers investigated the effect of efflux pumps in relation to resistance of *C. albicans* biofilm to fluconazole and demonstrated that the expression of genes encoding efflux pumps were up-regulated

during biofilm formation, suggesting a possible role in biofilm resistance. However, the mutant strains deficient in transporters for efflux pumps were also resistant to high concentrations of fluconazole when grown as a biofilm, but hypersusceptible to fluconazole in planktonic (Ramage *et al.*, 2002a).

1.4.2.4 Unique biofilm physiology

Since drug resistance of *Candida* biofilms can not be solely attributed to a matrix barrier effect or to a low growth rate, it seems that the biofilm mode of growth on a surface in which a contact induced gene expression occurs may be the mechanism by which drug resistance is acquired (Baillie and Douglas, 2000). A study by Bailey and co-workers investigated the key events which occur following adherence of *C. albicans* to human buccal epithelial cells. They found that following the adherence of *C. albicans* to epithelial cells, synthesis of new specific proteins took place by *C. albicans* indicating possible signalling events (Bailey *et al.*, 1995). This observation may explain the events that involve *C. albicans*-host interactions. Hence, drug resistant may arise because of the expression of specific, surface-induced contact genes (Kumamoto, 2002).

1.5 DENTURE RELATED STOMATITIS

1.5.1 Clinical features

Denture related stomatitis or chronic atrophic candidosis is the commonest clinical form of oral candidosis associated with denture wearers, orthodontic appliances and obturators for cleft palate (Webb *et al.*, 1998b). The prevalence of DRS has been reported to vary from 11 – 67 % in complete denture wearers as reviewed by Arendorf and Walker (1987) and a more recent study showed the prevalence of DRS in the denture wearing group was 26.8 % (Fenlon *et al.*, 1998). The reason of this large reported variation is probably multifactorial including the choice of study population, the diagnostic criteria and the date of the study (Radford *et al.*, 1999). Denture related stomatitis typically affects the palatal mucosa beneath the fitting surface of partial or complete dentures while the lower mucosa beneath the lower denture is seldom affected (Wilson, 1998; Bagg *et al.*, 1999a). The condition is more common in females than males (Davenport, 1970; Pires *et al.*, 2002) and can be accompanied with other forms of candidosis particularly angular cheilitis, median rhomboid glossitis and chronic hyperplastic candidosis (Webb *et al.*, 1998b). DRS is usually asymptomatic, although patients may complain of mucosal bleeding and swelling, burning sensation, halitosis, taste changes and dryness of the mouth (Arendorf and Walker, 1987).

1.5.2 Classification

The condition can be classified according to Newton classification based on the clinical appearance of the inflamed mucosa under the maxillary complete denture (Newton, 1962). Type I is localized simple inflammation or a pin-point hyperaemia. Type II is the most common type and is characterised by erythematous or

generalized inflammation presenting as more diffuse erythema or oedema involving a part of, or the entire, denture-covering area of the palatal mucosa. Finally, Type III is a granular type (inflammatory papillary hyperplasia) hyperplastic reaction result in nodular lesion that commonly involving the central part of the hard palate and the alveolar ridge. All three types may be found simultaneously and in varying combinations. It was thought that the papillary type of the condition is due to the presence of the prostheses, however, a similar condition has been reported in patients with HIV infections (Reichart *et al.*, 1994), suggesting that the presence of a denture is not the prime aetiological factor for this condition (Ellepola and Samaranayake, 2000a). Although the Newton classification is well reported, its exact role in aiding the treatment of DRS is questionable, as it does not provide useful information regarding the aetiology and hence the management of DRS.

1.5.3 Causes

Denture related stomatitis is now assumed to be multifactorial (Wilson, 1998; Ramage *et al.*, 2004). The condition is principally due to a microbial infection caused by poor oral and denture hygiene (Budtz-Jorgensen *et al.*, 1996; Sakki *et al.*, 1997; Pires *et al.*, 2002) and continuous wearing of denture while sleep (Aguirre *et al.*, 1996; Jeganathan *et al.*, 1997; Barbeau *et al.*, 2003).

Rarely, a range of other predisposing factors may cause the condition including; depressed host defences such as HIV disease (Reichart *et al.*, 1994), diabetes mellitus (Dorocka-Bobkowska *et al.*, 1996), smoking (Cumming *et al.*, 1990; Barbeau *et al.*, 2003), neoplastic diseases, chemotherapy, radiotherapy (Dorko *et al.*, 2001) and frequent use of antibiotics or corticosteroids (Budtz-Jorgensen, 1990).

The majority of patients with DRS, however, do not have any systemic diseases likely to predispose to such condition. In addition, some of the suggested associations are disputed, for example, while diabetes mellitus is suggested to predispose to DRS, some of these patients are known to carry some oral *Candida*, but do not have features of DRS. Hence, the amount of oral yeast may be more strongly influenced by local oral factors, such as the presence of dentures, than systemic aspects of diabetes mellitus (Manfredi *et al.*, 2002). Thus local factors are much more likely to predispose to DRS such as denture trauma caused by an ill-fitting denture, incorrect jaw relationships or occlusal errors (Budtz-Jorgensen, 1990) or wearing a denture during sleep (Aguirre *et al.*, 1996; Jeganathan *et al.*, 1997; Barbeau *et al.*, 2003).

1.5.3.1 Denture plaque and denture related stomatitis

The microbial plaque that forms on the fitting surface of the denture (denture plaque) is of great importance. It is a dense microbial layer comprising of microorganisms and their metabolites, containing more than 10^{11} organisms per gram in wet weight (Nikawa *et al.*, 1998a). Denture plaque forms on the denture of both healthy individuals and patients with DRS (Barbeau *et al.*, 2003). It is more evident on the denture fitting surfaces than on other denture surfaces (Keng and Lim, 1996).

Microbiological studies have shown that the composition of microbial flora of denture plaque in DRS is similar to that of dental plaque, but with a greater proportion of *Candida* species (Theilade *et al.*, 1983; Budtz-Jorgensen and Theilade, 1983). The microenvironment present underneath the denture favours the formation of denture plaque which is formed in the space between the mucosa and the fitting surface of the denture. This protected environment allows the formation of a plaque

which is very different from, for example, supra-gingival plaque both in terms of structure and microbial composition. Factors which influence the compositional structure include low shear forces and the creation of different environmental gradients through the denture plaque which harbours more obligatory anaerobic bacteria. Hence, in the relatively stagnant area on the denture fitting surface, plaque tends to be more acidogenic (favouring streptococci) and *Candida* species (Verran, 1988).

Radford and Radford have investigated the structure of denture plaque using SEM and revealed that denture plaque mainly consists of coccoid and rod-shaped bacteria with a few yeast cells (Radford and Radford, 1993). Recently, Ramage and co-workers (2004) have examined two denture samples retrieved from patients with DRS using SEM. These samples revealed the presence of *Candida* biofilms consisting of an intricate network of yeast cells and hyphae (Ramage *et al.*, 2004).

Studies have indicated that *C. albicans* is more likely to be present on the fitting surface of upper dentures than the palatal mucosa in patients with DRS (Davenport, 1970). The presence of close denture contact upon the oral mucosa alters the local environmental conditions as the cleansing action of the tongue on the palate is absent and the salivary flow containing antimicrobial factors is prevented from contacting the microorganisms (McIntyre, 2001). Additionally, the denture acts as a reservoir of a mixed-species biofilm and creates an acidic and anaerobic environment (Muzyka and Glick, 1995; Budtz-Jorgensen *et al.*, 1996). It has been suggested that this unique microenvironment provided by the denture may promote *C. albicans* proliferation and facilitate the disease progression without the need for any other predisposing factors (Konsberg and Axell, 1994; Maver-Biscanin *et al.*, 2004).

1.5.4 Association of *Candida albicans* with DRS

Candida albicans has been widely associated with the aetiology of DRS (Arendorf and Walker, 1979). It is generally considered that *C. albicans* is the causative agent of this condition and it is the most frequently isolated species in such patients (75 – 92 %) (Martin-Mazuelos *et al.*, 1997; McMullan-Vogel *et al.*, 1999). However, it has been suggested that a combination of *C. albicans* and other microorganisms is more likely to be responsible for DRS (Kulak *et al.*, 1997). For example, several genera of bacteria have been isolated from DRS patients including (e.g. *Streptococcus*, *Veillonella*, *Lactobacillus*, *Prevotella* and *Actinomyces* spp.) (Koopmans *et al.*, 1988). Additionally, some other non-*albicans* *Candida* spp. have been identified including; *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. kefyr*, *C. lusitaniae*, *C. freyschusii*, *C. zeylanoides* and *Saccharomyces cerevisiae* (McMullan-Vogel *et al.*, 1999; Dorko *et al.*, 2001).

The attachment of *C. albicans* to oral surfaces is a critical event in the colonisation of the oral cavity and in the development of oral diseases. Adhesion of *C. albicans* to the fitting surface of the denture is an important step in the pathogenesis of DRS (Budtz-Jorgensen and Lombardi, 1996). The secreted acid proteinase is likely to be involved in the pathogenesis of this condition, however, because of the decreased salivary flow and low pH under the denture, the secretion of the acid proteinase may not be required (McMullan-Vogel *et al.*, 1999). It has been shown that the presence of yeasts on the denture is associated with the extent of inflammation (Barbeau *et al.*, 2003). Budtz-Jorgensen and co-workers showed a significant correlation between the high density of yeasts in 7 day old denture plaque in patients with DRS and a positive clinical response to antifungal treatment indicating that yeasts of denture plaque may play an important role as a pathogen in DRS (Budtz-Jorgensen *et al.*,

1983).

Aguirre and co-workers have investigated the cytological changes of the oral mucosa in patients with DRS. They found that the smears from DRS patients presented a higher amount of cytological cellular material, fungal cells and inflammatory cells compared to the control group (Aguirre *et al.*, 1996).

1.5.5 Diagnosis

Diagnosis of DRS can usually be established from a clinical examination alone. The erythema and oedema of the denture bearing areas is confined to the area covered by the denture. White plaque (oral thrush) may sometimes also be associated (Wilson, 1998). The microbiological demonstration of *C. albicans* on the denture or underlying mucosa may also help to confirm the diagnosis (Barbeau *et al.*, 2003), however, it must be appreciated that this organism will often also arise in the mouth of persons without DRS.

1.5.6 Treatment

Although DRS is usually asymptomatic and the patient usually unaware of the condition, there is a risk that with time papillary hyperplasia of the palatal mucosa will arise which may require surgical treatment (Wilson, 1998). The management of DRS requires both removal of the source of candidal infection and the elimination of the infection from the oral tissues (McIntyre, 2001). The existing denture should be examined for any inaccuracies and any obvious faults which need to be corrected such as ill-fitting denture or vertical dimension of occlusion. Construction of a new denture may be required if the existing appliance is difficult to treat (Farah *et al.*, 2000). This should be considered after the resolution of DRS (Wilson, 1998).

Because of the build-up of denture plaque which is considered as a primary factor in patients with DRS, there is a need to improve oral and denture hygiene (Keng and Lim, 1996; Kulak-Ozkan *et al.*, 2002) and when relevant, advise the patient not to wear the denture while a sleep (Farah *et al.*, 2000). Regular denture hygiene instruction and patient motivation are fundamental to maintain adequate oral hygiene and to improve DRS (Pires *et al.*, 2002). The dentures have to be soaked in disinfecting agent such as 0.2 % chlorhexidine overnight (Budtz-Jorgensen and Lombardi, 1996; Farah *et al.*, 2000). Lal and co-workers have investigated the use of chlorhexidine gluconate as a mouth rinse and as a denture soak for the treatment of DRS for 24 days. The mouth rinse (0.12%) was used twice daily and the denture soaked overnight. They found that chlorhexidine completely eliminated *C. albicans* on the acrylic denture surface and significantly reduced palatal inflammation. However, after termination of treatment, *C. albicans* recolonised the denture surface and palatal inflammation recurred. They suggested that continued daily used of an effective over-the-counter antifungal denture soak and mouth rinses would be desirable and that treatment of the DRS should include antimicrobial topical application to both denture and mucosa (Lal *et al.*, 1992). Barasch and co-workers have also used chlorhexidine as a mouth rinses (0.12 %) for HIV-infected children who have oral candidosis. They suggested that topical chlorhexidine rinses can treat and prevent oral fungal disease produced by *C. albicans* (Barasch *et al.*, 2004). Chlorhexidine gluconate at 0.2 % concentration can also be used as a mouth rinse and a 2 % suspension is used as an overnight denture disinfectant (Ellepola and Samaranayake, 2000b). Additionally, beside chemical denture cleansing, mechanical plaque control of the denture fitting surface such as denture brushing should be accompanied (Jeganathan *et al.*, 1997).

Topical treatment with antifungal agents such as nystatin, amphotericin B, miconazole, and ketoconazole has produced a significant reduction of inflammation and oral symptoms of DRS (Budtz-Jorgensen, 1990). DRS is most commonly treated with polyene antifungal agents such as nystatin which is applied as a thin layer of cream to the fitting surface of the denture and simultaneously to the denture bearing mucosa (Sherman *et al.*, 2002; Diaz-Arnold and Marek, 2002). Topical treatment with nystatin or amphotericin B suspensions or lozenges are also useful in eliminating the yeast from the mucosal surfaces. The patient should remove the denture while undertaking the treatment and dissolve antifungal tablets four times daily (Farah *et al.*, 2000; Sherman *et al.*, 2002). Epstein and co-workers have showed that treatment of oral candidosis with topical nystatin (mouth rinse) four times daily for two weeks resulted in a significant reduction in the number of *Candida* in saliva with a marked improvement in the disease. However, the condition recurred rapidly after cessation of treatment (Epstein *et al.*, 1981). Egusa and co-workers showed that the *in vitro* exposure of denture acrylic to sub-therapeutic concentrations of polyenes (nystatin and amphotericin B) can suppress the adhesion of *Candida* species (Egusa *et al.*, 2000).

A number of studies have been carried out to test the efficacy of denture lining materials that contain antifungal agents for the treatment of DRS (Douglas and Walker, 1973; Thomas and Nutt, 1978). Thomas and Nutt have showed that when tissue conditioner (Visco-gel) was combined with nystatin powder, a successful inhibition of the growth of *C. albicans*, *C. krusei* and *C. tropicalis* was observed. However, there was a lack of inhibition with Visco-gel alone or a Visco-gel/amphotericin B combination (Thomas and Nutt, 1978).

The use of fluconazole as a mouth rinse was also investigated and shown to be useful in the management of patients with oral candidiasis (Epstein *et al.*, 2002). Another option for treatment of DRS is to use a ketoconazole lozenges or miconazole gel applied to the denture fitting surface while the denture is being worn. The inflammation is usually cleared within 7 – 14 days (Farah *et al.*, 2000) with the associated angular cheilitis if present (Budtz-Jorgensen and Lombardi, 1996).

A sustained drug-delivery device releasing miconazole can also be used to treat some cases of DRS which had previously resisted antifungal therapy (Lamb and Douglas, 1988). Miconazole has shown to be effective in the treatment of DRS with an observed reduction of yeast scores and palatal erythema. The agent can be formulated into a lacquer and applied to the denture fitting surface after thorough cleansing and drying the denture (Konsberg and Axell, 1994; Budtz-Jorgensen and Carlino, 1994; Ellepola and Samaranayake, 2000b).

Topical treatment is often safe, satisfactory and less expensive (Farah *et al.*, 2000), however, if all these measures failed to resolve the condition, systemic antifungal therapy can be prescribed (Webb *et al.*, 1998c). This treatment can be considered for immunocompromised patients and with those with poor compliance (McIntyre, 2001) or when candidal infection respond poorly to topical antifungal agents (Sherman *et al.*, 2002).

Oral administration of fluconazole in patients with DRS produced a pronounced reduction of yeasts in the oral cavity with decreased inflammation (Budtz-Jorgensen *et al.*, 1988), however, a high rate of clinical relapse and recurrence after therapy was reported (Budtz-Jorgensen *et al.*, 1988; Bissell *et al.*, 1993).

Itraconazole in the form of solution or capsules have also shown to be effective in the treatment of DRS (Cross *et al.*, 2000). However, there may be a strain persistence

and subsequent recolonization of the oral cavity by other less sensitive *Candida* spp. (Cross *et al.*, 2004). Cross and co-workers have compared the effect of two antifungal agents (fluconazole and itraconazole) for the treatment of DRS and found that both drugs reduce palatal inflammation in patients with DRS (Cross *et al.*, 1998).

The most important therapeutic measure in the prevention of DRS is to instruct the patient in efficient oral and denture hygiene measures. This may include methods such as mechanical cleansing of the denture and daily use of denture soaking agents. These measures are critical for the elimination of infection and in preventing recurrence'

1.6 AIM OF THE RESEARCH

Many studies have been carried out on bacterial biofilms and their association with oral diseases. However, in comparison, little work has been focused on oral fungal biofilms. Better understanding of the process of biofilm formation may impact clinical decision-making and provide a clear picture of the limitations of conventional therapies for the treatment of fungal infections. The aim of this project was to develop an *in vitro* model, previously used to grow bacterial biofilms, to investigate biofilm development, structure and effectiveness of antifungal and antimicrobial agents. Furthermore, to study mixed fungal and bacterial biofilms which are associated with denture related stomatitis.

Chapter 2

Materials and methods

2.1 PREPARATION OF SUBSTRATA

2.1.1 Denture acrylic

Acrylic resin was employed as a biofilm substratum for the growth of *C. albicans* in several sections of the study. Both self and heat-cured polymethylmethacrylate were used (Dentsply Limited, Weybridge, Swindon, U.K.). Cylinders of the materials, with a diameter of 5 mm, suitable in size to be placed into the polytetrafluoroethylene (PTFE) pans of the fermentor, were constructed as described below.

For heat-cured acrylic disks, a filling wax replica of each cylinder was initially constructed (Kemdent Associated Dental Products Ltd, Swindon, U.K.). The wax replica was then embedded in plaster and stone and boiled out. The acrylic polymer and monomer were mixed using the manufacture's instructions. The plaster was then coated with sodium alginate as a separator and the resultant space was filled with heat-cured acrylic, which was then polymerized by heating in a water bath for 7 h at 70 °C and a further 3 h at 100 °C before slowly cooling to minimize the stress within the acrylic. The heat-cured acrylic cylinders were then sectioned using a lathe (Boxford AUD, Halifax, U.K.) to a final depth of 1 mm.

The self-cured acrylic (Dentsply) was prepared according to manufacturer's instructions and placed directly into the PTFE moulds to achieve disks of 5 mm diameter and 1 mm depth. Acrylic was mixed and applied to the CDFD pan mould in a semi-liquid phase to facilitate loading. To decrease free monomer, the curing of self-cured acrylic was carried out under pressure using a hydro-flask (DB Orthodontics Ltd, Skipton, U.K.) to decrease porosity and to complete the curing cycle. Additionally, the disks were stored in water to leach out excess monomer present.

To facilitate polishing the acrylic disks were fitted onto the plugs in such a manner as the top of the disks exceeded the height of the CDFP pans. The pans were subsequently inverted and prepared to a specific surface roughness using different grades of silicon carbide grit on a Knuth Rotor (Struers Limited, Glasgow, U.K.). This was achieved by manual grinding of the surface with water used as a lubricant. The cutting was in one direction with constant pressure applied. The papers used were grit numbers of 1000, 500 and 80 corresponding to particle sizes of 16 - 20 μm , 28 - 33 μm and 180 - 230 μm respectively. The polishing of the materials did not significantly affect the thickness of the disk.

2.1.2 Enamel and dentine

Disks of enamel and dentine of appropriate size for insertion into PTFE pans of the fermentor were prepared. Extracted permanent teeth were stored in 37 % formaldehyde (Sigma, Poole, U.K.) before being sterilized by autoclaving (121°C for 60 minutes). The teeth were then washed with distilled water before handling. The enamel or dentine surfaces of the teeth were then prepared to the same roughness using silicon carbide grit number 1000 as described for acrylic disks (the R_a value of the materials was confirmed as 2.5 μm , however this was not confirmed by SEM). The teeth were then cut into 5 mm diameter cylinders using a trepanning tool on a Metal Worker Lathe/Drill (Clarke International, London, U.K.) before being sectioned using a Rotary Diamond Saw (Testbourne Limited, Basingstoke, U.K.).

2.2 ORGANISMS USED IN THE STUDY

2.2.1 Ethical approval

Ethical approval for the study was granted by the joint University College of London/University College London Hospitals Trust Committee on the ethics of Human Research. The purpose and nature of the project was explained to each participating patient. A written consent form was signed by each patient.

2.2.2 Clinical isolates of *Candida albicans*

Clinical samples were obtained from patients attending the Eastman Dental Hospital, University College London Hospitals (UCLH), as part of a separate study (Al-Karaawi *et al.*, 2002). All of the patients underwent a clinical oral examination. Mouth swills, using 10 ml of sterile distilled water were collected from each patient and 1 ml was cultured on Sabouraud Dextrose Agar (Oxoid Ltd, Basingstoke, U.K.) to assess the number of yeast cells present. All of the isolates were defined to species level by both phenotypic and molecular methods (Al-Karaawi *et al.*, 2002). Six strains of defined species of *C. albicans* were used for the present study, three strains with genotype A and three strains with genotype B.

2.2.3 Mixed-species clinical samples

After receiving an informed consent from each patient, samples were taken from 10 individuals (mean age 70 years; 2 males and 8 females), attending the Oral Medicine and Prosthetic Dentistry clinics at the Eastman Dental Hospital, UCLH. All 10 patients were wearing upper full or partially removable dentures, 5 had clinical signs of DRS (refer to section 1.5) and showed Type 1 Newton classification while the remaining 5 had no clinical signs of the disease. None of the patients had a history of

systemic disease likely to predispose to oral candidosis and none were taking any systemic or local radiation or using antimicrobial agents for the management of DRS.

Two samples were obtained from each subject using a calcium alginate swab (Technical Service Consultants, Heywood, U.K.) on the mucosa beneath the denture and a separate swab was taken to sample the denture fitting surface. Each swab was then immediately placed into 4 ml Calgon-Ringer's solution (Oxoid) in a sterile bijou containing 5 sterile glass beads (Sigma, Poole, U.K.).

The sample was immediately vortex-mixed for one minute or until the calcium alginate swab had dissolved. Serial dilutions of the suspension was carried out in PBS (Oxoid) were then prepared to a final dilution of 1 : 10⁶. Stock samples of each specimen were then stored at -70 °C in 1 ml of LB Broth (Sigma) storage medium containing 50 % glycerol (BDH, Poole, U.K.) until required for use as an inoculum for the Constant Depth Film Fermentor (refer to section 2.4.2.2).

2.3 NUTRIENT SOURCE

Artificial saliva as developed by Russell and Coulter (1975) was employed as a nutrient source in all experiments. The composition of the artificial saliva is listed per one litre of distilled water and detailed in Table 2.1.

Table 2.1: Composition of artificial saliva.

Constituents	Quantity (g/L dH ₂ O)	Supplier
'Lab-lemco' powder*	1	Oxoid
Yeast Extract*	2	Oxoid
Proteose Peptone*	5	Oxoid
Mucin Type III: Partially Purified from Porcine Stomach	2.5	Sigma
Sodium chloride	0.35	Sigma
Calcium chloride	0.2	Sigma
Potassium chloride	0.2	Sigma

*Contains average % w/w: Total nitrogen: 11.63 %, Amino nitrogen: 3.36 % and NaCl: 4.66 %.

2.4 CONSTANT DEPTH FILM FERMENTOR

The Constant Depth Film Fermentor (CDFF; Figure 2.1) used throughout this study was a modified version of the fermentor described by Peters and Wimpenny (1988) and engineered by John Parry-Jones Engineering (Cardiff, U.K.).

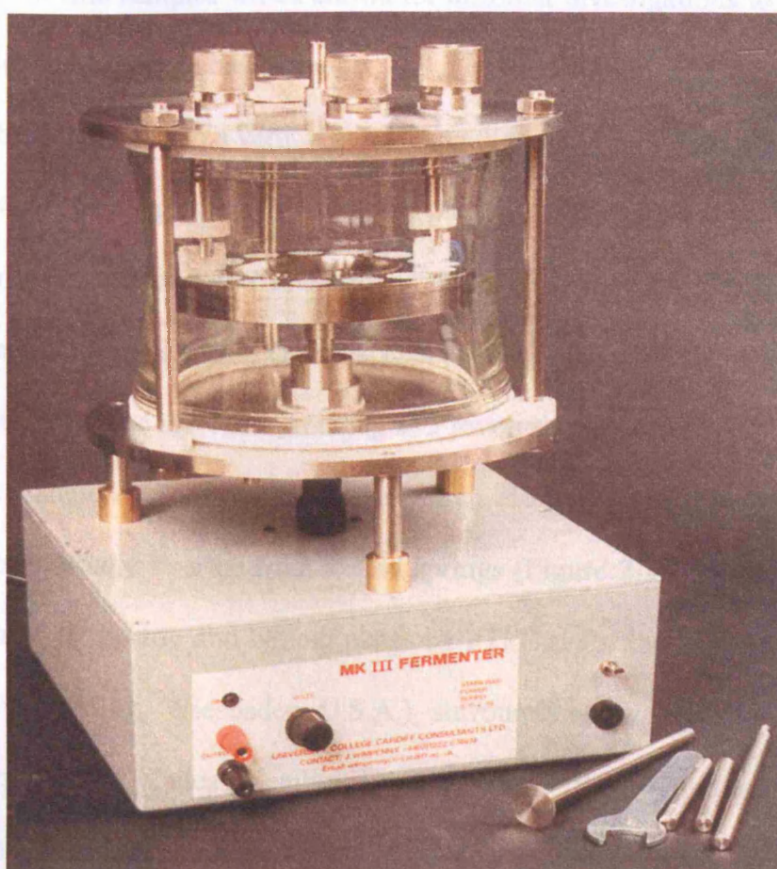


Figure 2.1: The Constant Depth Film Fermentor.

2.4.1 Assembly of CDFF

The CDFF consists of a stainless steel turntable which holds fifteen PTFE pans flush around its rim (Figure 2.2). Each pan is inserted and held flush with the turntable by silicone rubber 'O' rings. Each of the pans contains a central threaded hole into which a tool is inserted for removal and replacement and 5 - 6 cylindrical holes, 5

mm in diameter, which contain PTFE plugs. Biofilms can be grown either on the PTFE plugs or on disks of various materials (e.g. acrylic, enamel and dentine) supported by these plugs. The disks sit on the PTFE plugs of the same diameter and can be accurately recessed using a template 300 μm . The removal of each film pan produces 5 biofilm samples which allows for different investigations to be performed and statistical variations to be determined.

The turntable is attached to a vertical central spindle running through the base plate via an autoclavable bearing assembly. The base plate is supported by three stainless steel legs to accommodate the detachable gearbox motor (RS, Corby, U.K.). The gearbox is driven by a 15 V power supply (Farnell, Leeds, U.K.) housed in the support in which the fermentor sits. The steel disk, rotating at 3 revolutions per minute (r.p.m.), passes beneath two angled PTFE scraper blades which are forced onto the surface of the steel disk by pressure exerted from loaded springs (Figure 2.3). A borosilicate glass section, sealed to the top and bottom plates by PTFE seals and high vacuum silicon grease (Dow Corning, Wiesbaden, U.S.A.), surrounds and completely encloses the turntable and scraper blades assembly. The top plate is fitted with a number of inlets for medium and supplements as well as a sample port and air inlet. The air inlet has two Whatman Hepa-vent air filters (0.2 μm) (Arbor Technologies, Ann Arbor, MI, U.S.A.) attached. The bottom plate contains a medium outlet port.

As with all fermentation work, there is a large amount of tubing required to supply nutrient medium and other liquids to and from the fermentor. Silicone tubing (Versilic, Charny, France) was used for the CDFP for several reasons: it is flexible, durable, non-toxic, autoclavable and can be visually inspected for contamination. Separate silicone pump tubing (Watson-Marlow Ltd., Falmouth, U.K.) was required for use with the peristaltic pumps and, where required, quick-disconnect fittings

(Nalgene, Hereford, U.K.) were used. The CDFP was sterilised by autoclaving (steam under pressure) at 121 °C for 15 minutes.

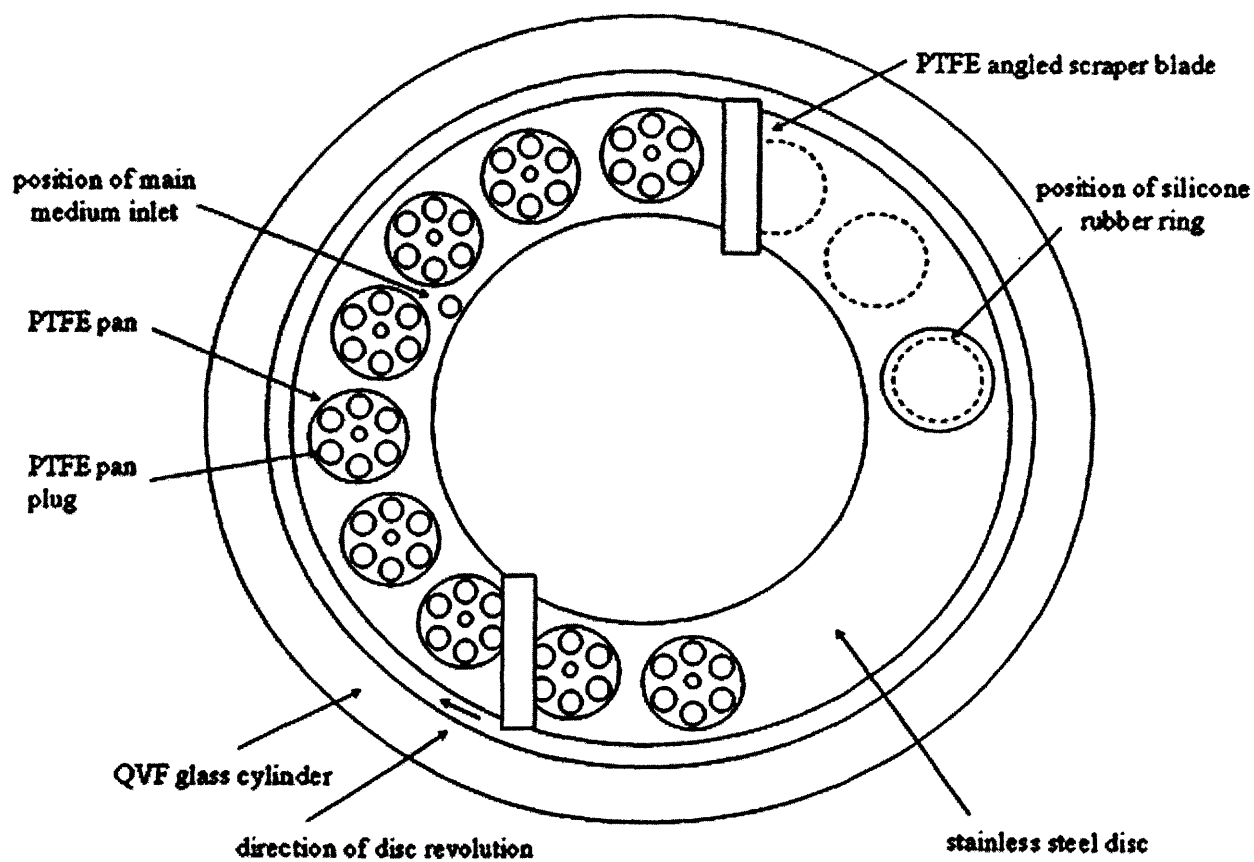


Figure 2.2: Schematic horizontal section through the fermentor.

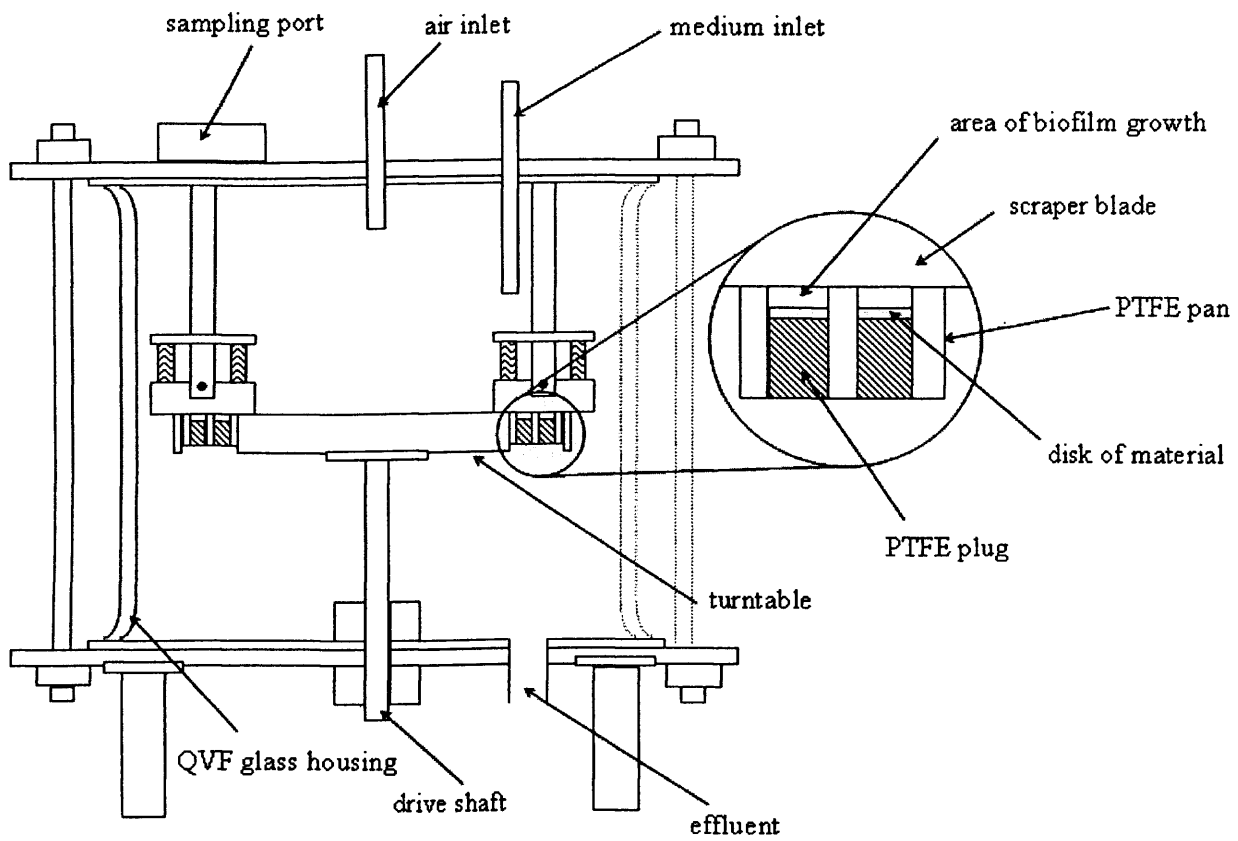


Figure 2.3: Schematic vertical section through the fermentor with insert indicating one of the pans in greater detail.

2.4.2 Inoculation of the CDFFF and continuous media supply

2.4.2.1 *Single-species*

A 10 ml overnight culture of *Candida albicans* in artificial saliva was added to 500 ml of sterile artificial saliva, mixed and then pumped into the CDFFF for 8 h (Figure 2.4). The inoculum was continuously mixed by using a magnetic stirrer. After this time, the inoculum flask was disconnected and the CDFFF was fed from a medium reservoir of sterile artificial saliva, the waste being collected in an effluent bottle (Figure 2.5). The artificial saliva was delivered at a rate of 0.72 liter/day, the mean salivary flow rate in humans (Guyton, 1992).

2.4.2.2 *Mixed-species*

200 µl from each DRS patient sample, (refer to section 2.2.3) (5 mucosa samples and 5 denture samples) was pooled under aseptic conditions to provide an inoculum of 2 ml. This was added to 500 ml of artificial saliva (Figure 2.4 and 2.5), and pumped into the CDFFF inoculated for 8 h before being attached to the sterile medium reservoir.

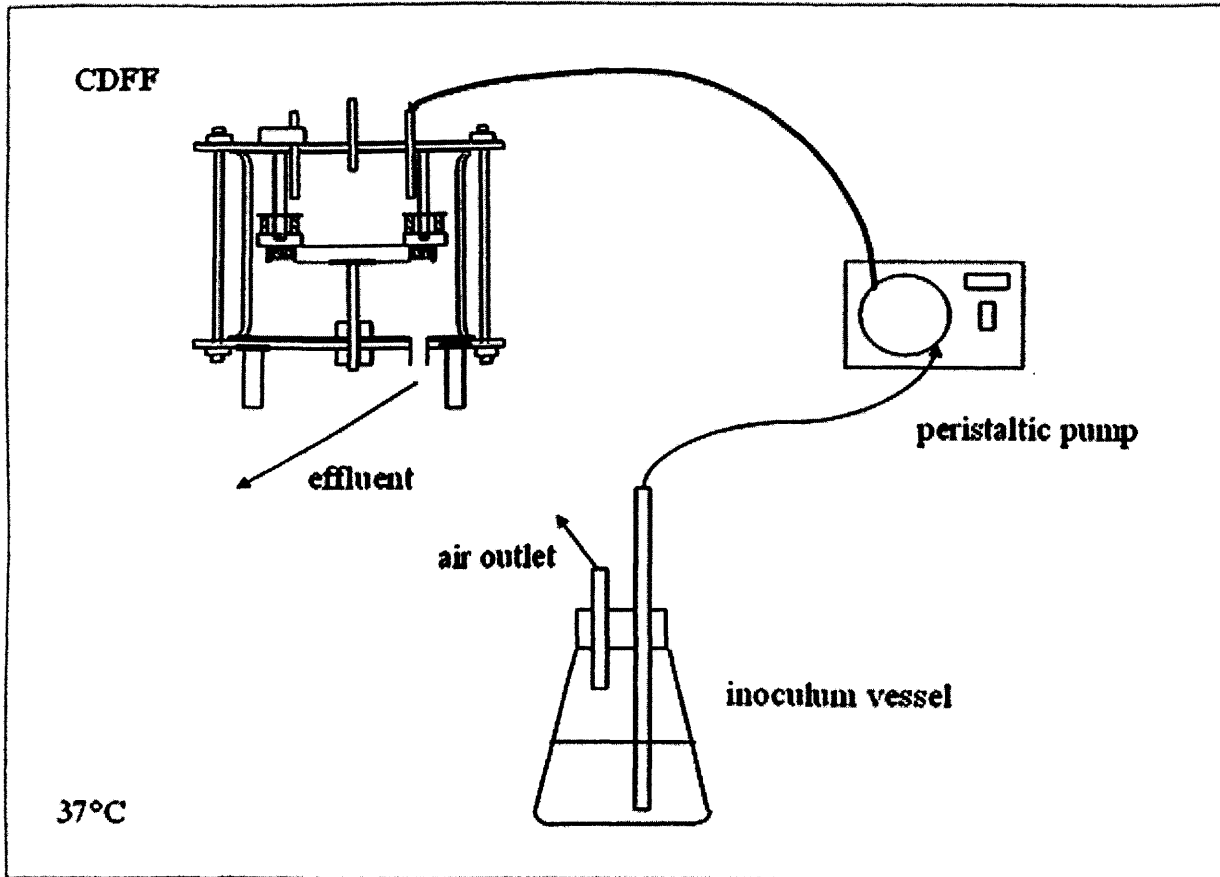


Figure 2.4: Schematic diagram indicating inoculation of the CDFF.

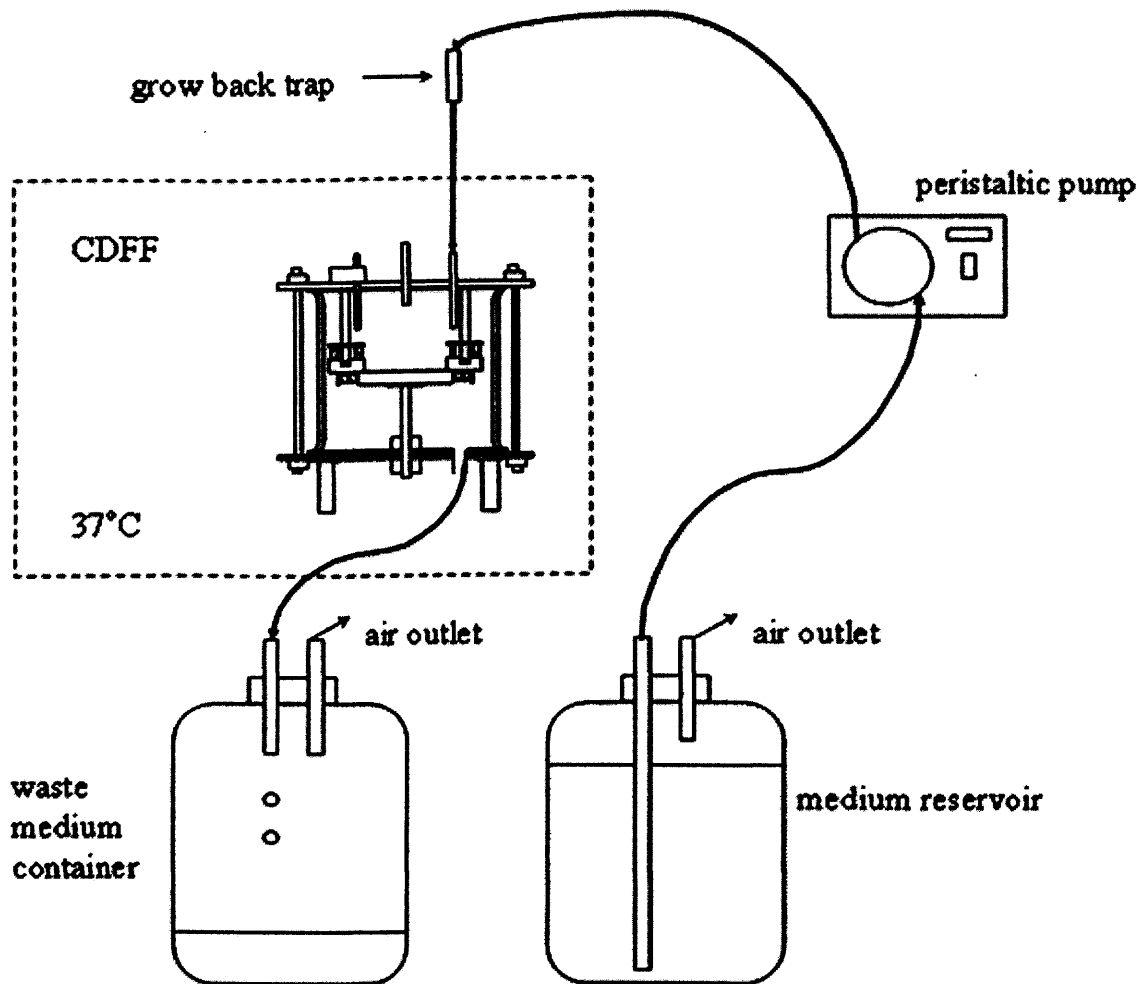


Figure 2.5: Schematic diagram showing continuous media supply to the CDF.

2.4.3 Operating conditions

The CDFE was housed in a 37 °C incubator during all experiments and was operated aerobically.

2.4.4 Sample removal

At various time intervals, the turntable of the CDFE was stopped by reducing the voltage on the power supply to zero, allowing the PTFE pans to be removed from the sampling port. In order to allow aseptic removal of the pans, the sample port area was first sterilized by flaming using a portable bunsen burner. The PTFE pan was then removed from the CDFE and the sampling port quickly replaced. The disks were removed from the pans using pre-sterilised forceps and placed into an appropriate vessel.

2.4.5 Analytical procedures

All of the experiments were carried out in duplicate and statistical analysis was performed using the student's t-test (two-sample assuming equal variances) to determine *p*-values.

2.4.5.1 *Single species*

For the single-species biofilms CFU per biofilm, hyphae and yeast counts were determined. Pans were removed from the CDFE at various time intervals and 3 disks were aseptically removed and each placed into 1 ml PBS before being vortexed for 1 minute to disrupt the biofilm, and a serial dilution was carried on and used to inoculate a SDA. The number of yeast and hyphal cells were determined using a haemocytometer (Hawksley, London, U.K.). Five aliquots from each sample were

counted under a light microscope with a 40 x long working distance objective (Leitz Wetzlar, Germany).

2.4.5.2 Mixed-species

Serial dilutions were carried out and plated onto a range of non-selective and selective agars in order to ascertain the predominant bacterial and candidal genera present (Table 2.2). Preliminary identification (colony morphology and Gram stain) was made of all types of colonies on each medium in order to confirm the selectivity of the media for the required genera/groups. Colonies isolated from the selective agars were considered to be 'genera-group' organisms. Any bacteria that were not of the expected genera were excluded from these data.

Table 2.2: Various non-selective and selective agars used for culturing organisms during the study. *CFATA was prepared as detailed below (Table 2.3).

Media used	Abbreviation	Selectivity	Supplier
Columbia Blood Agar	CBA	Non-Selective	Oxoid
Fastidious Anaerobes Agar	FAA	Non-Selective	Bioconnections, Leeds, U.K.
Sabouraud's Dextrose Agar	SDA	<i>Candida</i> species	Oxoid
Mitis Salivarius Agar	MSA	<i>Streptococcus</i> species	Difco Laboratories, Detroit, MI
Rogosa Agar	RA	<i>Lactobacillus</i> species	Oxoid
Veillonella Agar	VA	<i>Veillonella</i> species	Difco
Cadmium Fluoride Acriflavin Tellurite Agar*	CFATA	<i>Actinomyces</i> species	See Table 2.3

SDA and CBA were incubated aerobically at 37°C for 24 h, while all other agars were incubated anaerobically for 48 – 72 h at 37°C.

Table 2.3: Composition of CFATA (Zylber and Jordan, 1982).

Constituents	Quantity per L in dH₂O	Supplier
Tryptone soya broth powder	30 g	Oxoid
Agar technical powder	15 g	Oxoid
Glucose	5 g	BDH
Horse blood	50 ml	E and O Laboratories, Bonnybridge, U.K.
Cadmium sulphate	13 mg	Sigma
Sodium fluoride	85 mg	Sigma
Neutral acriflavin	1.2 mg	Sigma
Potassium tellurite	2.5 mg	Sigma
Basic fuschin	1.25 mg	Sigma

2.5 SUSCEPTIBILITY TESTING

The antifungal agents used in this study were fluconazole (Pfizer Ltd, Tadworth, U.K.) and miconazole (Johnson and Johnson, Beerse, Belgium). Additionally, an antimicrobial agent, chlorhexidine digluconate (Sigma, Poole, U.K.), was also used.

2.5.1 Susceptibility testing for antifungal agents

Antifungal susceptibility testing to determine the Minimum Inhibitory Concentration (MIC) of planktonic cells for fluconazole or miconazole against planktonic cells was employed using the National Committee for Clinical Laboratory Standards (NCCLS) M27-A microdilution method (NCCLS M27-A, 1997). Alamar blue (Trek Diagnostic Systems Ltd, East Grinstead, U.K.) was employed for accurate assessment of growth endpoints. The medium used for dilution was 10.4 gm RPMI 1640 (Sigma-Aldrich, Dorset, U.K.) medium buffered with 34.35 gm MOPS (N-morpholino propanesulfonic acid, Sigma-Aldrich) in 1 L of distilled water to give a final molarity of 0.165 and adjusted to pH 7.0 with NaOH (BDH Merck, Poole, U.K.).

The antifungal agents (fluconazole and miconazole) were diluted in RPMI to give a concentration ranging from 0.125 - 1024 µg/ml. These microdilutions were loaded in sterile disposable microtitration multiwell plates (Nunclon™ Δ Delta, BDH Merck, U.K.) as described in NCCLS M27-A (1997).

Yeast cells were then prepared after 24 h culture on SDA. The yeast cells were suspended in sterile 0.85 % saline and the mixture was adjusted by spectrophotometer (at 530 nm wavelength) to give a final transmission between 0.08 – 1. The yeast cell suspension was further diluted in RPMI to give a final concentration 1×10^6 to 5×10^6 cells/ml. The cell suspension was then loaded to the

multiwell plates that contained the test concentrations of agents. The yeast cell suspension was then incubated at 35°C and the results were observed at 24 and 48 h for the presence or absence of visible growth with the aid of a reading mirror and compared with the control well. Additionally, Alamar blue determined the endpoint when the colours change from blue to pink.

The antifungal susceptibility of biofilms were determined by placing a biofilm disks for 1, 24 and 48 h in a multiwell plate that contain different concentrations of the test agents according to NCCLS broth microdilution method as described previously (Hawser and Douglas, 1995).

2.5.2 Susceptibility testing for antimicrobial agent

The same protocol of MIC determination was used for chlorhexidine. Chlorhexidine was diluted in sterile water (instead of RPMI) with a concentration ranging from 0.000019 - 20 % which were also loaded in microtitration multiwell plates. The yeast cell suspension was then incubated in microtitre plates at 35 °C for 5 minutes. A 20 µl sample from each well was then placed into SDA and incubated 37 °C for 24 h. The results were observed after 24 h for the presence or absence of visible growth compared with the control.

The antifungal susceptibility of biofilms were determined by placing a biofilm disks for 1, 5 and 15 minutes in a multiwell plate that contain different concentrations of chlorhexidine.

2.6 SCANNING ELECTRON MICROSCOPY

Cylinders for electron microscopy were fixed in 3 % (v/v) gluteraldehyde buffered with sodium cacodylate (0.1 M) at 4°C overnight, and then post-fixed in osmium tetroxide (1 % v/v) for 2 h before being dehydrated in a graded series of alcohols (20 %, 50 %, 70 %, 90 % ethanol, 15 minutes each; 100 % ethanol, 3 x 10 minutes). They were placed in hexamethyldisilazane for 5 minutes, removed to filter paper and allowed to dry. The dried specimens were mounted onto aluminium stereoscan stubs with either conductive carbon cement (Leit-C, Neubauer, Munster, Germany) or rapid set Araldite (Devcon Ltd, Shannon, Ireland). The dried, mounted specimens were coated with a thin metallic layer of gold / palladium in a Polaron E5000 sputter coater (BioRad, Hercules, CA, U.S.A.) and viewed in a Cambridge Stereoscan 90B Electron Microscope (Cambridge Inst., Cambridge, U.K.) (Figure 2.6a-c).

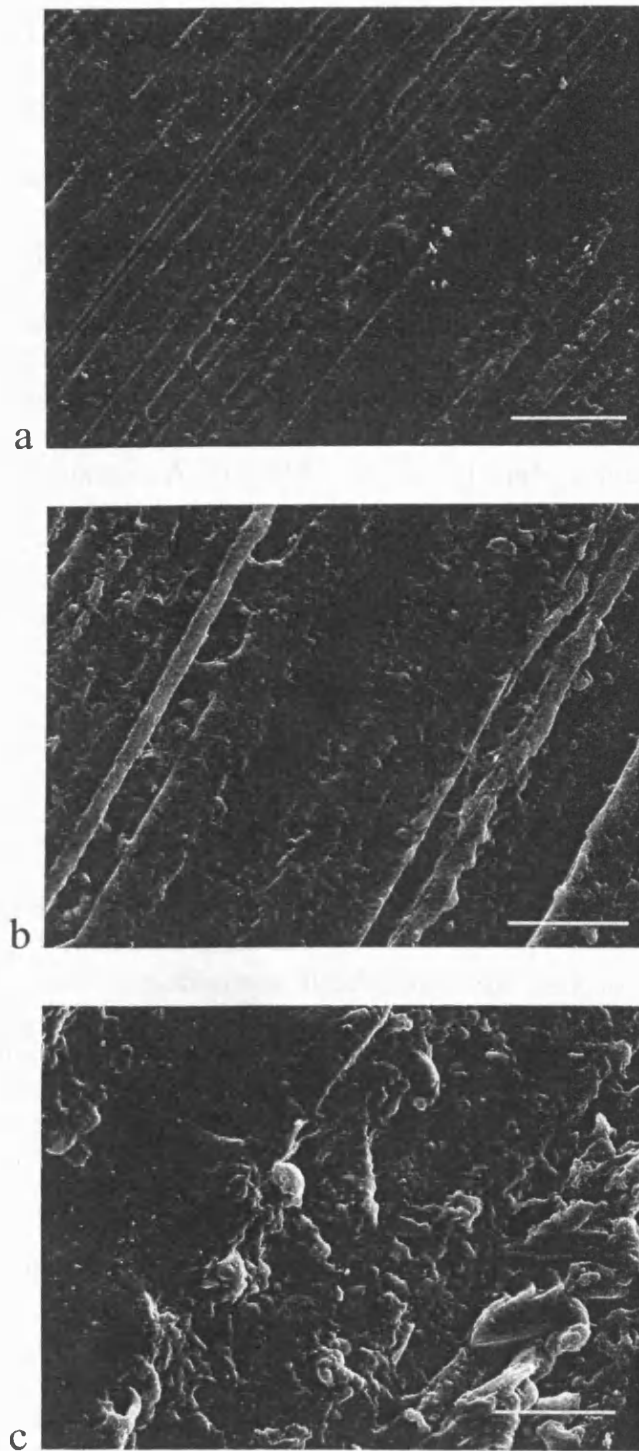


Figure 2.6: Scanning electron microscope photograph of the surface of self-cured acrylic disks polished to different surface roughness; a) $R_a = 2.5 \mu\text{m}$, b) $R_a = 3.6 \mu\text{m}$ and c) $R_a = 9.3 \mu\text{m}$. Bar = $200 \mu\text{m}$.

2.7 CONFOCAL LASER SCANNING MICROSCOPY

Confocal microscopy uses focused laser light to illuminate a specimen and gather the emitted fluorescent light through a pin-hole aperture. This results in images with a very narrow focal plane that excludes most of the light from outside the plane of focus. In this study, CLSM was used in conjunction with fluorescent dyes, LIVE/DEAD[®] Yeast Viability Kit (Molecular Probes, Oregon, U.S.A.). It is a two component stain, component A: Fun-1[™] Cell stain (10 mM solution in DMSO) and component B: Calcofluor[™] White M2R Fluorescent brightener 28; Tinopal LPW (5 mM in water). In the normal viewing of the stain images, the viable cells fluoresce green while the non-viable cells fluoresce red.

Single-species biofilm disks were placed into 10 ml of distilled water containing 2 μ l of component A: Fun-1[™] Cell stain. This labels the cell walls of yeast and fungi regardless of the cell's metabolic state.

The suspension of stained biofilm was incubated in the dark to allow the stain to develop for 15 minutes. Scans of the biofilms were taken using a Leica DMLFS fixed stage microscope with a Leica TCS SP confocal laser scan-head (Leica, Milton Keynes, U.K.). The objectives used were an infinity corrected 20 X PL APO with numerical aperture 0.6 and 5 X PL FLUOTAR with numerical aperture 0.12. The dry lens was used so not to disturb the biofilm. CLSM was used to obtain a series of optical sections through the depth of the biofilm. The Leica confocal software was employed to produce maximum-intensity projections from the Z-series. Two to three biofilm images were taken according to the representative organisms. The resulting collections of confocal optical were saved on CD as TIF files.

2.8 SURFACE CHARACTERISTICS

A Proscan 1000 scanning laser profilometer (Scantron Industrial Products Limited, Taunton, U.K.) was used to measure the average surface roughness (R_a) of the different surface finish of the substrata (Figure 2.7). Using this technique the R_a values for ten evenly distributed lines across the surface (5 in the X direction and 5 in the Y direction) were obtained and the mean for these ten values were calculated. Measurements were carried out in triplicate and the average R_a was determined.

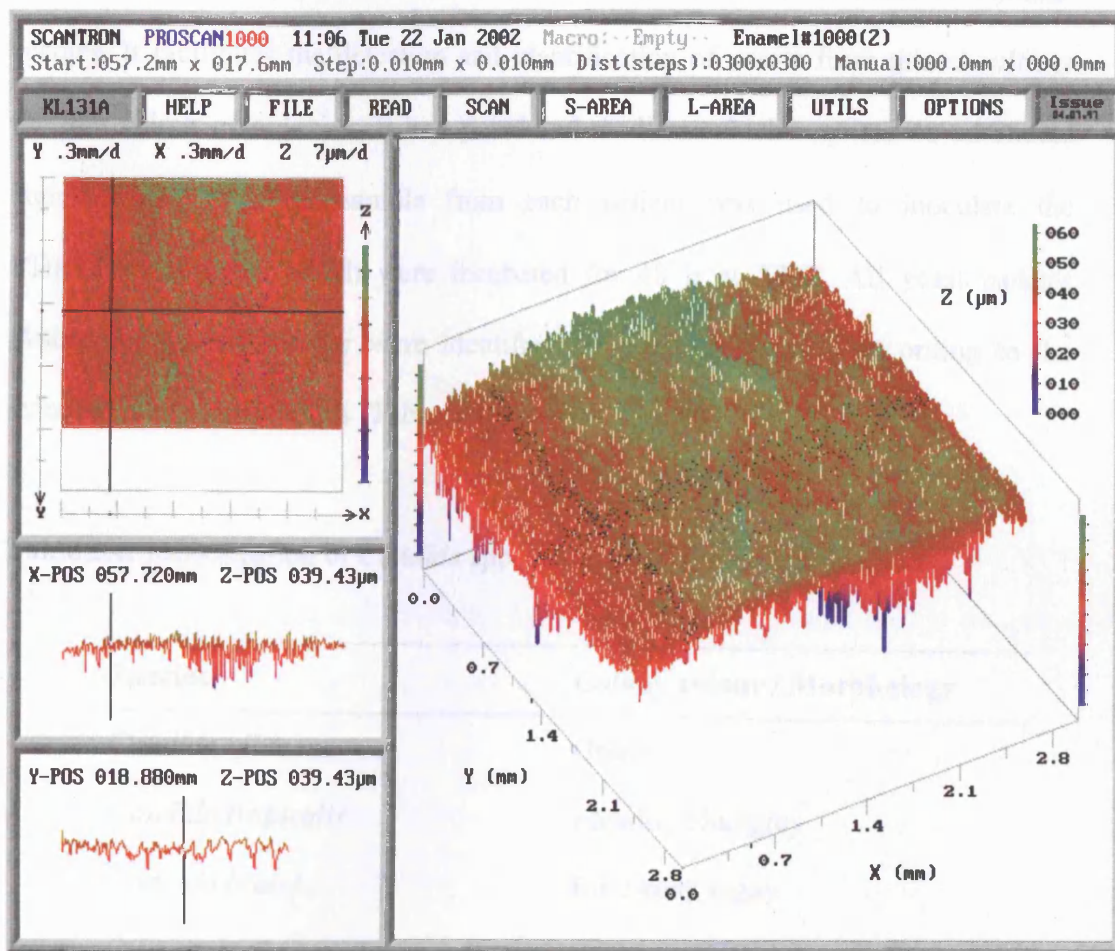


Figure 2.7: Proscan image showing roughness readings as generated by Scantron software.

2.9 IDENTIFICATION OF *CANDIDA* SPECIES

Candida spp. were defined to the species level by using several different methods including, CHROMagar (M-Tech Diagnostics Ltd, Warrington, U.K.), API[®] Candida kit (BioM'erieux, Basingstoke, U.K.) and CornMeal Agar (Oxoid).

2.9.1 CHROMagar

CHROMagar is a differential cultural medium that allows selective isolation of yeasts and additionally allows identification to species level for some of *Candida* species. It facilitates the detection and identification of yeasts from mixed cultures through colour of colonies. It is a powdered medium which requires no additional supplements. A 20 µl sample from each patient was used to inoculate the CHROMagar plates which were incubated for 48 h at 37°C. All yeast isolates observed on CHROMagar were identified by colony colouring according to the manufacturer's instructions (Table 2.4).

Table 2.4: Identification of *Candida* spp. using the CHROMagar method.

Species	Colony colour / Morphology
<i>Candida albicans</i>	Green
<i>Candida tropicalis</i>	Metallic blue/grey
<i>Candida krusei</i>	Rose-pink fuzzy
Other spp.	White to pink

2.9.2 API[®] Candida Kit

This is a standardised kit system for the identification of yeasts in 18 – 24 h. The API Candida system (bioMérieux, Marcy-l'Etoile, France) works by spontaneous colour change during incubation. The microorganisms were first isolated on SDA medium to ensure pure colonies. The ampule of API NaCl 0.85 % Medium (2 ml) was opened carefully and inoculated with one, or several, well isolated identical pure colonies using a swab until the solution became turbid. Immediately after preparation the yeast suspension was homogenized and used. This suspension was distributed into the tubes avoiding bubble formation. Some of the tests needed to be covered with mineral oil (underlined test) immediately after inoculating the strip. The strip was then incubated for 18 – 24 h at 37 °C in aerobic conditions. After this time, the results of the reaction were obtained (Figure 2.8) by referring to the reading table and recorded as + or – on the result sheet. The reaction revealed spontaneous colour changes and the results obtained visually and coded as a numerical profile on the result sheet. The tests separated into groups of three and subsequently assigned. By adding together the numbers corresponding to positive reactions with each group, a 4-digit numerical profile was obtained. The identification of *Candida* spp. was then obtained by using APILAB Plus V 3.3.3 software.



Figure 2.8: API *Candida* kit strip showing the identification of *Candida albicans*.

2.9.3 CornMeal agar

CornMeal agar (Oxoid) was used to study the morphology of yeasts. Colonies of *Candida* spp. were streaked out from a pure colony and a microscope slide coverslip placed on to the top of the streaked culture. The agar with the top slide was incubated for 24 – 48 h at 25 – 28°C. The agar plate was then placed directly onto the microscope stage and viewed using x 40 long working distance objective (Olympus microscope, Southall, U.K.).

Chapter 3

**The characterisation of an *in vitro* model system to grow biofilms of
Candida albicans on relevant oral surfaces**

3.1 INTRODUCTION

Candida albicans is the most common fungal pathogen isolated from the human oral cavity. One of the most important virulence factors of this species is its ability to adhere to a surface. Adherence is considered as a first step of the infection process by *Candida* spp. (Haynes, 2001) and a prerequisite for initial colonisation. This adherence mechanism, exhibited by *C. albicans*, contributes to the persistence of this pathogen within the host and enables it to colonise many oral niches (Cannon *et al.*, 1995).

Changes on the surface of the *Candida albicans* may be brought about by yeast/host interactions. For example, adherence of *C. albicans* to human buccal epithelial cells induces the synthesis of new proteins and the expression of signal proteins (Bailey *et al.*, 1995). Additionally, Tronchin and co-workers demonstrated specific proteins that are involved in the process of germ tube adherence (Tronchin *et al.*, 1988).

The mouth provides a large number of diverse surfaces onto which *C. albicans* can adhere. Examples include soft shedding surfaces of the buccal mucosa and hard non-shedding surfaces such as teeth and oral prostheses. These surfaces are rapidly bathed in saliva and adsorb salivary proteins. This acquired pellicle of proteins may promote or inhibit binding and yeast growth, however, clean enamel has been shown to reduce attachment of *C. albicans* (Cannon *et al.*, 1995).

The availability of a well-characterised, reproducible laboratory model is essential for understanding the nature of *C. albicans* biofilms, and facilitates *in vitro* performing studies related to biofilm formation, structure and antifungal properties. Several artificial biofilm systems have been developed to grow *C. albicans*. These are generally adhesion assays where the organisms are allowed to adhere to various materials such as catheters (Hawser and Douglas, 1994), acrylic (Samaranayake and

MacFarlane, 1980), denture soft lining materials (Nikawa *et al.*, 1993; Radford *et al.*, 1998b; Bulad *et al.*, 2004), or resin-composite restorative material (Maza *et al.*, 2002).

In particular, adherence of *Candida* spp. to acrylic surfaces has previously been studied (Segal *et al.*, 1988; Verran and Maryan, 1997; Radford *et al.*, 1998a). The factors implicated in the adhesion of *C. albicans* to acrylic surfaces are complex (Samaranayake *et al.*, 1980), and the degree of biofilm activity formation on acrylic surfaces has been shown to be affected by both the dietary sugar, nature of the pellicle and the *Candida* isolates (Nikawa *et al.*, 1997; Nikawa *et al.*, 2000). These findings have important clinical implications as any material inserted in the oral cavity is instantaneously coated by saliva or crevicular exudates (Nikawa *et al.*, 1997).

Hence, when developing a model to grow organisms in a similar manner to the *in vivo* scenario, it is important to take in account the nature of the substratum, the growth medium and the formation of a suitable pellicle. The initial aim of this work was to develop an *in vitro* model to establish the conditions necessary for the development of *C. albicans* biofilm formation on a series of orally relevant substrata. The establishment of such a model will thus permit studies of the features that may influence biofilm formation related to DRS.

3.2 MATERIALS AND METHODS

The aim of the first experiment was to grow *Candida albicans* to a steady state in the CDFE with PTFE used as the substratum. In later experiments, acrylic resin was used together with enamel and dentine prepared to the appropriate size for insertion into CDFE as described previously (refer to section 2.1.1 and 2.1.2). A clinical isolate of *C. albicans* genotype A, defined to the species level by both phenotypic and genotypic methods (refer to section 2.2.2), was cultured in 10 ml of artificial saliva overnight and was added to 500 ml of sterile artificial saliva before being pumped into the CDFE. The CDFE was incubated at 37°C and operated aerobically. Samples were removed at various time intervals up to a maximum of 384 h.

YPD broth was used in one experiment as a nutrient medium, the composition of which is listed in Table 3.1.

Table 3.1: Composition of YPD broth.

Constituents	Quantity in g per L in dH ₂ O	Supplier
Yeast extract	10	Oxoid
Peptone	20	Oxoid
Dextrose	20	Sigma

The CFU, hyphae and yeast counts were determined (refer to section 2.4.5.1) and representative images taken using a light microscope (400 x magnification) (Leitz Wetzlar, Germany).

3.3. RESULTS

Biofilm growth of *C. albicans* was carried out on a range of substrata using artificial saliva as a general growth medium. The viable counts of *C. albicans* biofilms on PTFE are shown in Figure 3.1a. The number of CFU increased over time from 24 h to a maximum at 144 h. The viable counts then reduced by approximately 1 log₁₀ between 168 and 336 h. Table 3.2 shows the number (mean) of yeast and hyphal cells of *C. albicans* biofilm from the same experiment. The yeast cell counts increased from the time of the inoculum and reached a maximum at 216 h, before reducing until the end of the experiment at 384 h. There were fewer numbers of hyphal cells compared to yeast cells over the whole period of the experiment. Between 24 and 48 h, the percentage of hyphae represented 0.6 % of the total cell count, this proportion increased until 312 h when 20 % of the population were in the hyphal form (Figure 3.1b).

The development of *C. albicans* biofilms on self and heat-cured acrylic were investigated over a period of 72 h (Figure 3.2a). The CFU of the biofilms increased over time for both types of acrylic denture material. There was no significant difference ($p > 0.05$) between the self and heat-cured acrylic during the course of the experiment. The yeast and hyphal cell counts (Table 3.3) indicated that the heat-cured acrylic had a higher number of yeast cells attached than the self-cured acrylic; however, this was not significant ($p > 0.05$). Interestingly, during the early development of the biofilm (2 h), there was no hyphal growth on either of the acrylic surfaces. At 6 h, hyphae were observed on the heat-cured acrylic, but none were detected on self-cured. After this time, the heat-cured acrylic showed higher hyphal cell counts than self-cured acrylic, however, these differences were also not significant ($p > 0.05$). The percentage of hyphae on both acrylic (Figure 3.2b and c)

were similar between 24 and 72 h.

Biofilm growth over 96 h was determined on acrylic, enamel and dentine surfaces using artificial saliva as nutrient medium. Figure 3.3a shows the viable counts during biofilm development. The numbers of CFU comprising the biofilm grown on enamel was significantly higher ($p < 0.05$) than acrylic and dentine at early stages of biofilm formation (6 h). Growth on the acrylic surfaces was generally seen to be the lowest, except at 6 and 48 h, when the dentine had the lowest CFU. The total number of yeast and hyphal cells observed from the three surfaces are shown in Table 3.4. The yeast cell counts present on the enamel were significantly higher ($p < 0.05$) than the other surfaces at 6 h. After this time, the number of yeast cells on the enamel continued to remain higher than on the other materials until the end of the experiment at 96 h, although only at 48 h was this significant ($p < 0.05$). In comparison, the numbers of yeast cells on the acrylic surface was always less than on the other two materials. There were fewer hyphal cells compared to yeast cells on all of the surfaces at all of the time periods. At 6 h the highest numbers of hyphal cells detected were on the dentine surfaces and this continued to increase on both dentine and acrylic surfaces over time. In contrast, the number of hyphae present on the enamel surfaces increased from 6 h to 48 h by approximately $2 \log_{10}$. At 72 h the numbers dropped, which also corresponded to a fall in the number of yeast cells and hence the total cells comprising the biofilm, before the numbers recovered at the end of the experiment (96 h). The percentage of yeast and hyphal cells are shown in Figures 3.3b-d. The highest percentage of hyphal cells on acrylic (4.8 %) (Figure 3.3b) and dentine (12.6 %) (Figure 3.3d) were observed at 6 h. In contrast, at 6 h the percentage of hyphae present on enamel (Figure 3.3c) was at the lowest level during the course of the experiment (0.6 %).

In order to determine the effect of the inoculum on *C. albicans* biofilm growth, the morphology of planktonically grown cells (Figure 3.4) was determined in YPD, artificial saliva and in artificial saliva with or without Lab-lemco or mucin. Figure 3.4a shows *C. albicans* grown in YPD where only the yeast form was observed. In contrast, the growth of *C. albicans* in artificial saliva (Figure 3.4b) showed both the yeast and the hyphal forms of *C. albicans*. The same diversity was also demonstrated when artificial saliva was used without Lab-lemco (Figure 3.4c), without mucin (Figure 3.4d) and without both (Figure 3.4e).

YPD was used as a nutrient medium for *C. albicans* biofilm grown on acrylic, enamel and dentine over 96 h (Figure 3.5 a). The CFU on the enamel surface was higher than acrylic and dentine at 6 and 24 h, although the differences were not significant ($p > 0.05$). However, at 48 and 72 h biofilms grown on dentine contained the highest number of CFU compared to the other surfaces. Acrylic surfaces always showed the lowest CFU compared to enamel and dentine. Enamel showed a higher number of yeast cells present at 6 ($p < 0.05$) and 24 h (Table 3.5). At 48 h, the number of yeast cells dropped, but recovered again at 72 and 96 h. Acrylic surfaces again showed the lowest number of yeast cells compared to enamel and dentine over 96 h. Enamel also contained a higher number of hyphal cells than the other surfaces at 6 ($p < 0.05$) and 24 h. Figures 3.5b-d show the percentage of yeast and hyphal cells of all three surfaces. The percentage of hyphal cells increased over time and reached a maximum of approximately 20 % at the end of the experiment (96 h).

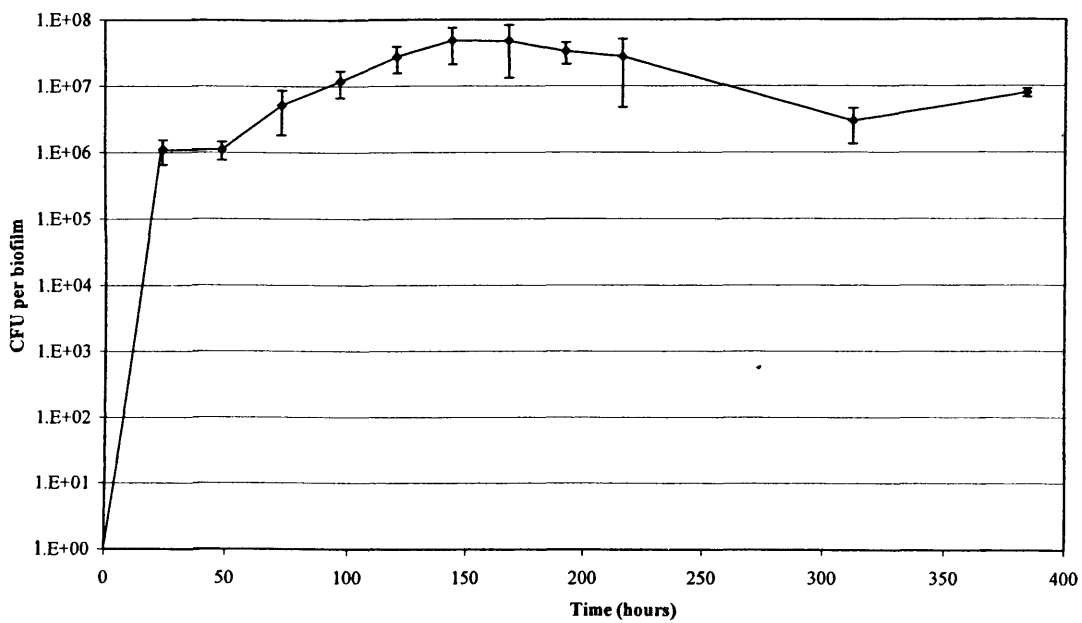


Figure 3.1a: Viable counts of *C. albicans* biofilms grown on PTFE over a period of 384 h. Error bars represent standard deviations (n = 6).

Table 3.2: Mean \pm the standard deviation of yeast and hyphal cell counts of *C. albicans* biofilms grown on PTFE over 384 h (n = 6).

Biofilm age	Yeast cell counts	Hyphal cell counts
24 h	$4.6 \times 10^5 \pm 1.9 \times 10^5$	$2.7 \times 10^3 \pm 5.8 \times 10^2$
48 h	$4.9 \times 10^5 \pm 2.2 \times 10^5$	$3 \times 10^3 \pm 1.7 \times 10^3$
72 h	$1.3 \times 10^6 \pm 9.7 \times 10^5$	$8.4 \times 10^4 \pm 7.1 \times 10^4$
96 h	$3 \times 10^6 \pm 1.1 \times 10^6$	$1.2 \times 10^5 \pm 3.8 \times 10^4$
120 h	$5.5 \times 10^6 \pm 1.9 \times 10^6$	$2.4 \times 10^5 \pm 4 \times 10^4$
144 h	$7.5 \times 10^6 \pm 4.3 \times 10^6$	$3.1 \times 10^5 \pm 2.4 \times 10^5$
168 h	$5.9 \times 10^6 \pm 2.5 \times 10^6$	$4 \times 10^5 \pm 1 \times 10^5$
192 h	$6.5 \times 10^6 \pm 7.9 \times 10^5$	$6.7 \times 10^5 \pm 7 \times 10^4$
216 h	$8.6 \times 10^6 \pm 7.9 \times 10^6$	$4.5 \times 10^5 \pm 3.2 \times 10^5$
312 h	$1.8 \times 10^6 \pm 1.1 \times 10^6$	$4.6 \times 10^5 \pm 3.2 \times 10^5$
384 h	$1.1 \times 10^6 \pm 3.1 \times 10^5$	$1.4 \times 10^5 \pm 9 \times 10^4$

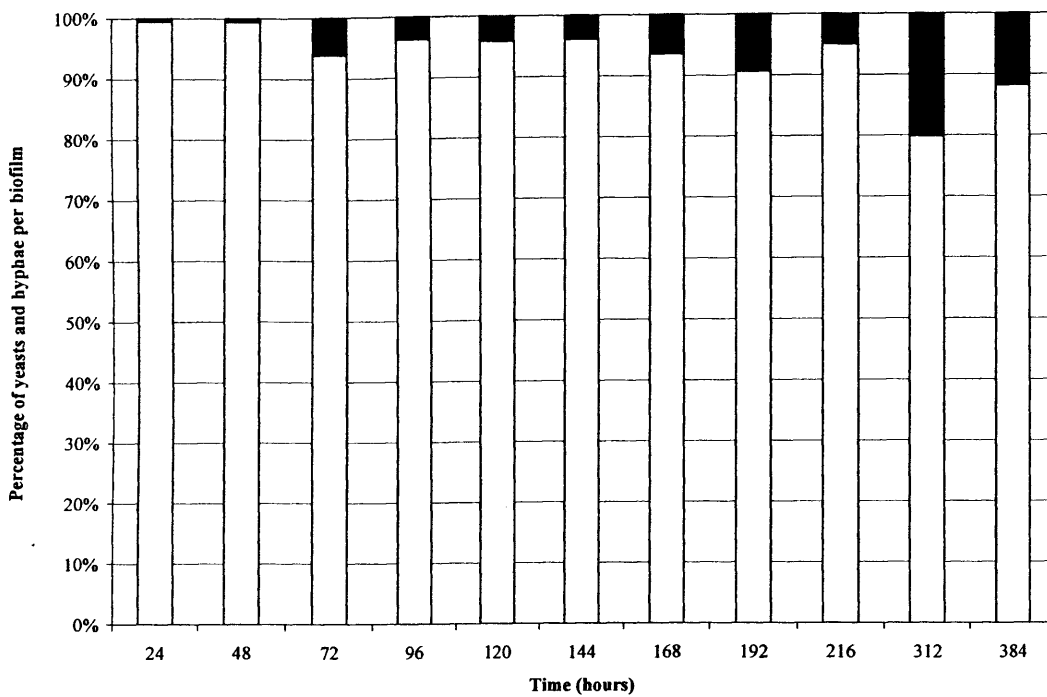


Figure 3.1b: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on PTFE for a period of 384 h. (Shown is the average percentage n = 6).

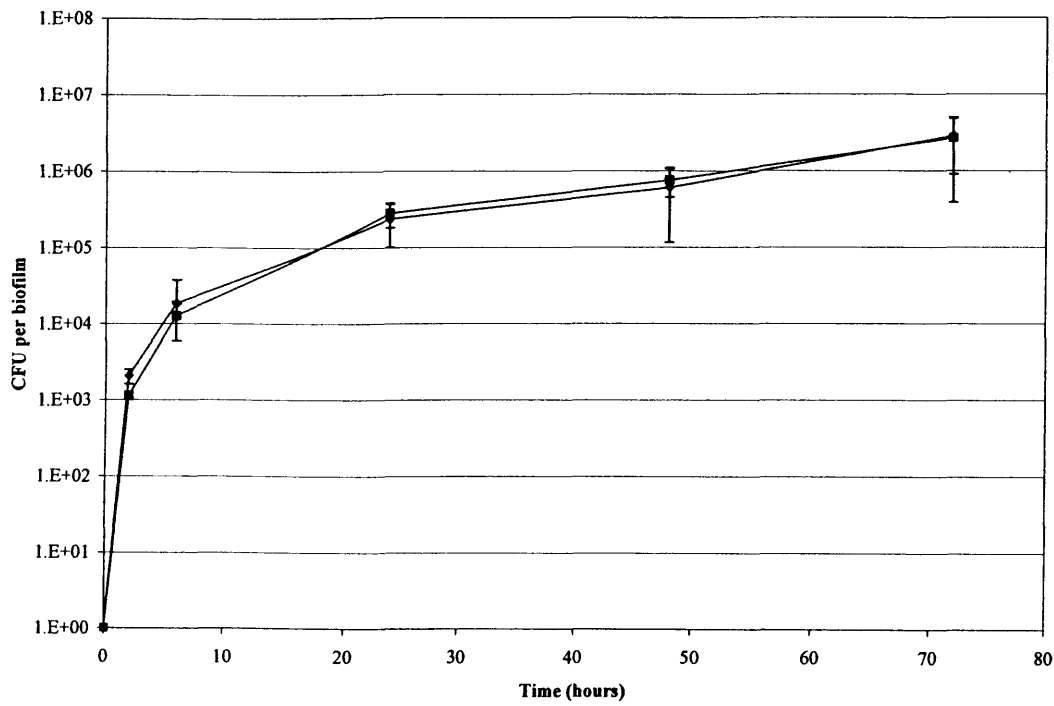


Figure 3.2a: Viable counts of *C. albicans* biofilm grown on self (filled diamonds) and heat-cured (filled squares) acrylic for a period of 72 h. Error bars represent standard deviations (n = 6).

Table 3.3: Mean \pm the standard deviation of yeast and hyphal cell counts of *C. albicans* biofilms grown on self and heat-cured acrylic over 72 h.

Biofilm age	self-cured acrylic		Heat-cured acrylic	
	Yeast cell counts	Hyphal cell counts	Yeast cell counts	Hyphal cell counts
2 h	2.3x10 ³ \pm 5.8x10 ²	0	4x10 ³ \pm 1x10 ³	0
6 h	1.2x10 ⁴ \pm 6x10 ³	0	1.7x10 ⁴ \pm 4.7x10 ³	1x10 ³ \pm 1x10 ³
24 h	7.2x10 ⁴ \pm 1.4x10 ⁴	7x10 ³ \pm 4.4x10 ³	9.4x10 ⁴ \pm 4.5x10 ⁴	7.3x10 ³ \pm 5.5x10 ³
48 h	2.4x10 ⁵ \pm 2.6x10 ⁵	2.6x10 ⁴ \pm 1.2x10 ⁴	3x10 ⁵ \pm 1.1x10 ⁵	2.8x10 ⁴ \pm 3.6x10 ³
72 h	7.4x10 ⁵ \pm 4.2x10 ⁵	3.4x10 ⁴ \pm 1.9x10 ⁴	8.5x10 ⁵ \pm 6.8x10 ⁵	4.3x10 ⁴ \pm 2.3x10 ⁴

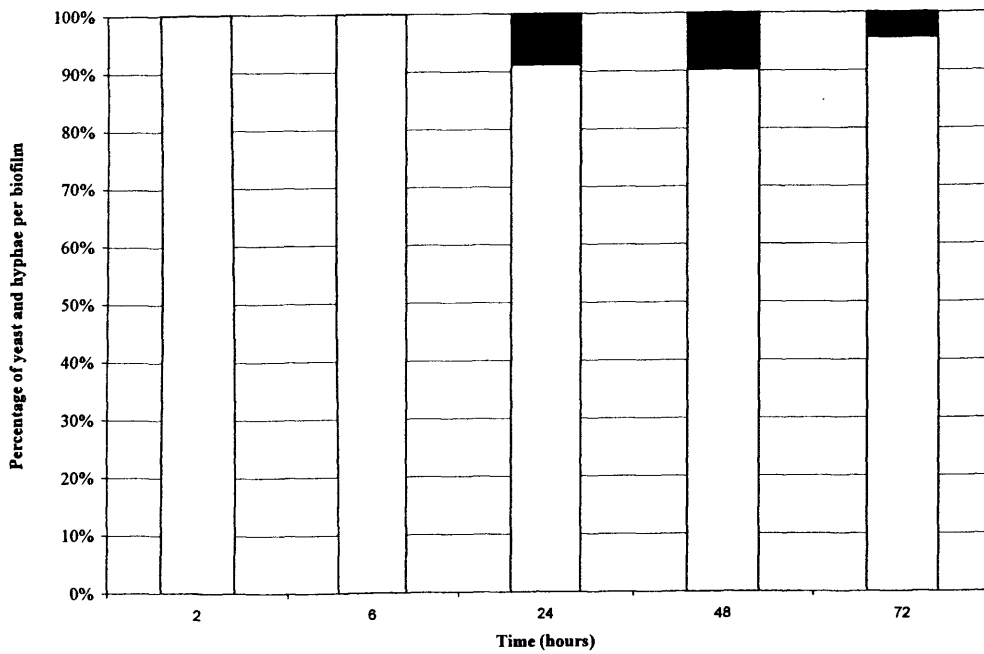


Figure 3.2b: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on self-cured acrylic for a period of 72 h. (Shown is the average percentage n = 6).

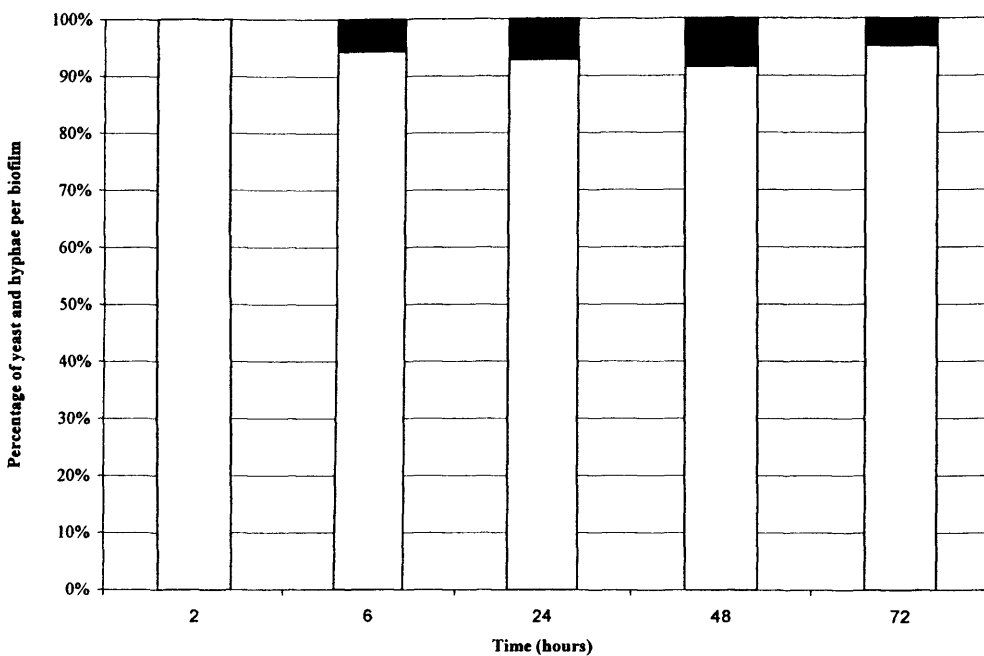


Figure 3.2c: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on heat-cured acrylic for a period of 72 h. (Shown is the average percentage n = 6).

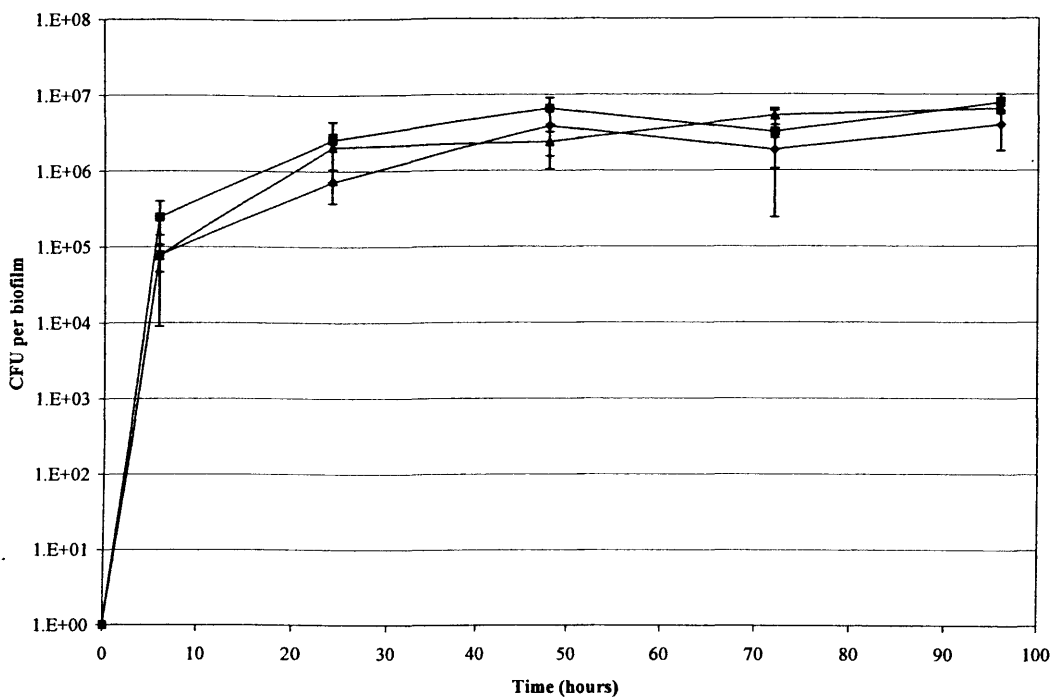


Figure 3.3a: Viable counts of *C. albicans* biofilms grown on three different surfaces over a period of 96 h using artificial saliva as a nutrient medium. Filled diamonds - acrylic, filled squares - enamel, filled triangles - dentine. Error bars represent standard deviations (n = 6).

Table 3.4: Mean \pm the standard deviation of yeast and hyphal cell counts of *C. albicans* biofilms grown on acrylic, enamel and dentine over 96 h using artificial saliva as a nutrient medium (n = 6).

Biofilm age	Acrylic		Enamel		Dentine	
	Yeast cell counts	Hyphal cell counts	Yeast cell counts	Hyphal cell counts	Yeast cell counts	Hyphal cell counts
6 h	3.3×10^4 $\pm 7.6 \times 10^3$	1.7×10^3 $\pm 5.8 \times 10^2$	8.3×10^5 $\pm 7 \times 10^5$	5×10^3 $\pm 2 \times 10^3$	5.8×10^4 $\pm 2.5 \times 10^4$	8.3×10^3 $\pm 4 \times 10^3$
24 h	4.3×10^5 $\pm 1.3 \times 10^5$	3×10^3 $\pm 2 \times 10^3$	1.3×10^6 $\pm 4.2 \times 10^5$	2.9×10^4 $\pm 7.1 \times 10^3$	8×10^5 $\pm 2 \times 10^5$	1×10^4 $\pm 1.5 \times 10^3$
48 h	1×10^6 $\pm 6.6 \times 10^5$	8.7×10^3 $\pm 1.5 \times 10^3$	9×10^6 $\pm 1.2 \times 10^7$	2.9×10^5 $\pm 2.3 \times 10^5$	1.2×10^6 $\pm 2.6 \times 10^5$	1.7×10^4 $\pm 1.2 \times 10^4$
72 h	5.3×10^5 $\pm 3.7 \times 10^4$	1.2×10^4 $\pm 1.2 \times 10^4$	1.3×10^6 $\pm 4.4 \times 10^5$	3.7×10^4 $\pm 1.7 \times 10^4$	1.2×10^6 $\pm 5 \times 10^5$	2.6×10^4 $\pm 1.2 \times 10^4$
96 h	7.9×10^5 $\pm 3.5 \times 10^5$	2.3×10^4 $\pm 8.5 \times 10^3$	3.3×10^6 $\pm 3.8 \times 10^5$	1.5×10^5 $\pm 5.8 \times 10^4$	1.9×10^6 $\pm 1.1 \times 10^6$	1.4×10^5 $\pm 1.2 \times 10^5$

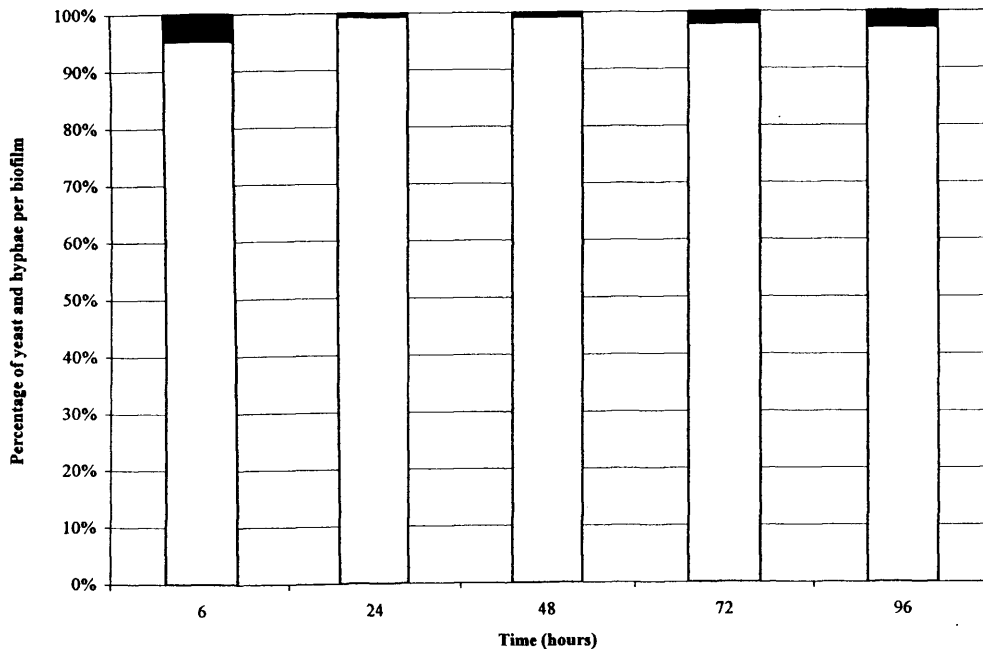


Figure 3.3b: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on acrylic surface over a period of 96 h using artificial saliva as a nutrient medium. (Shown is the average percentage n = 6).

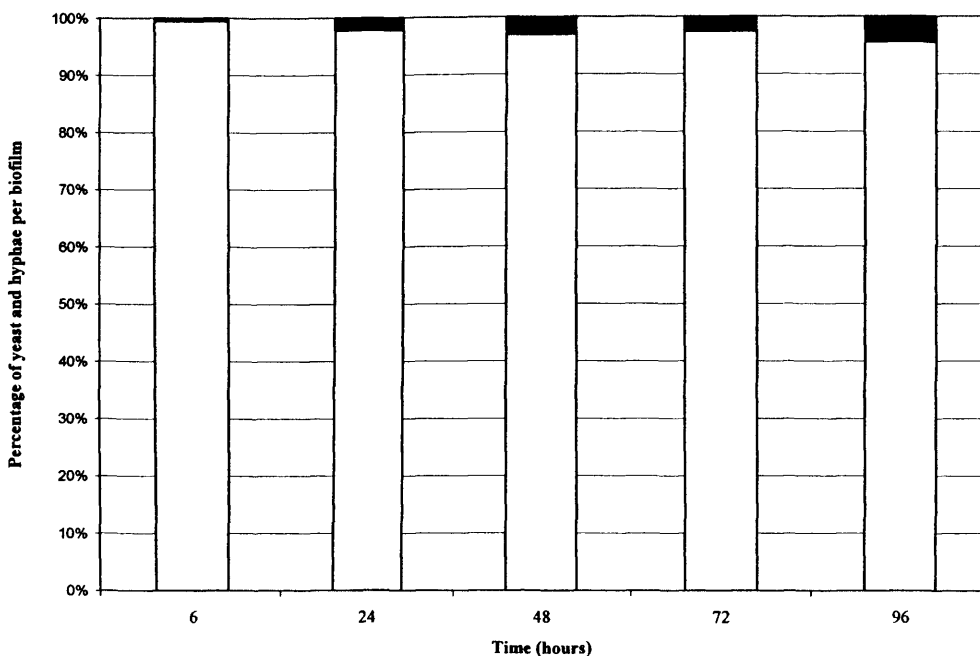


Figure 3.3c: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on enamel surface over a period of 96 h using artificial saliva as a nutrient medium. (Shown is the average percentage n = 6).

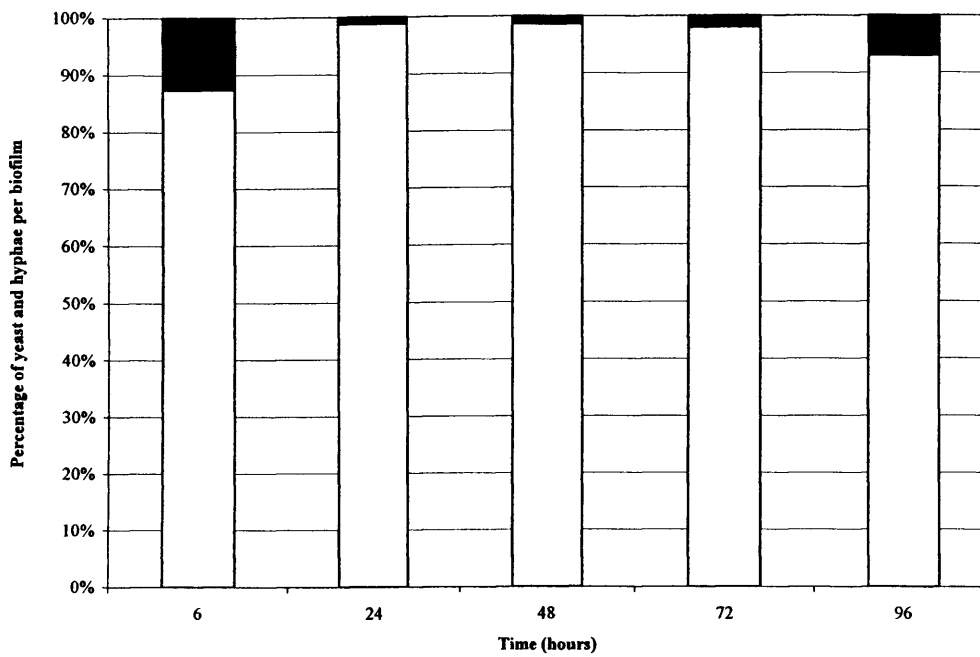


Figure 3.3d: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on dentine surface over a period of 96 h using artificial saliva a nutrient medium. (Shown is the average percentage n = 6).

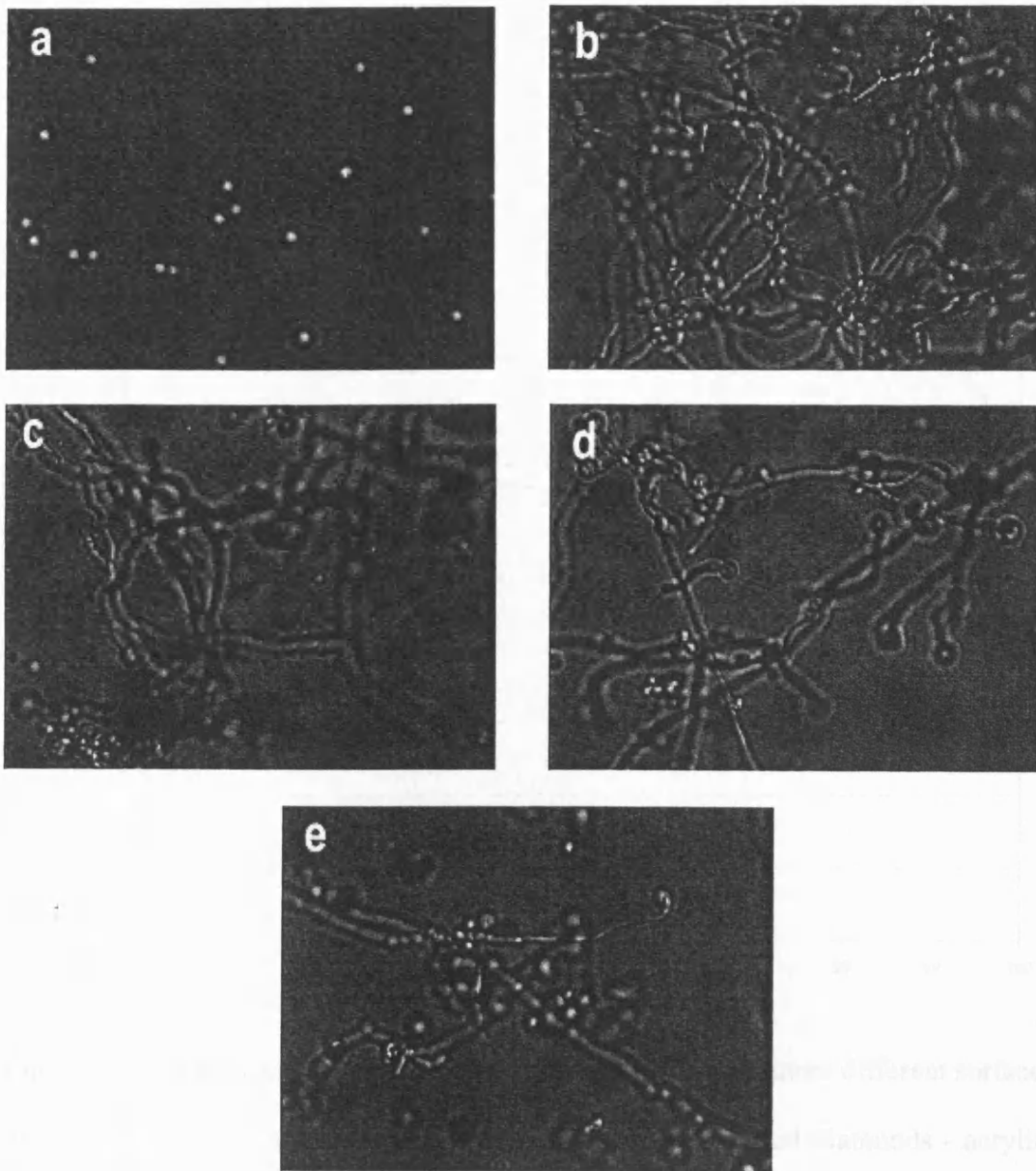


Figure 3.4: Overnight suspensions of *C. albicans* grown in: a) YPD, b) artificial saliva, c) artificial saliva without Lab-lemco, d) artificial saliva without mucin and, e) artificial saliva without Lab-lemco and without mucin (x 400 magnification).

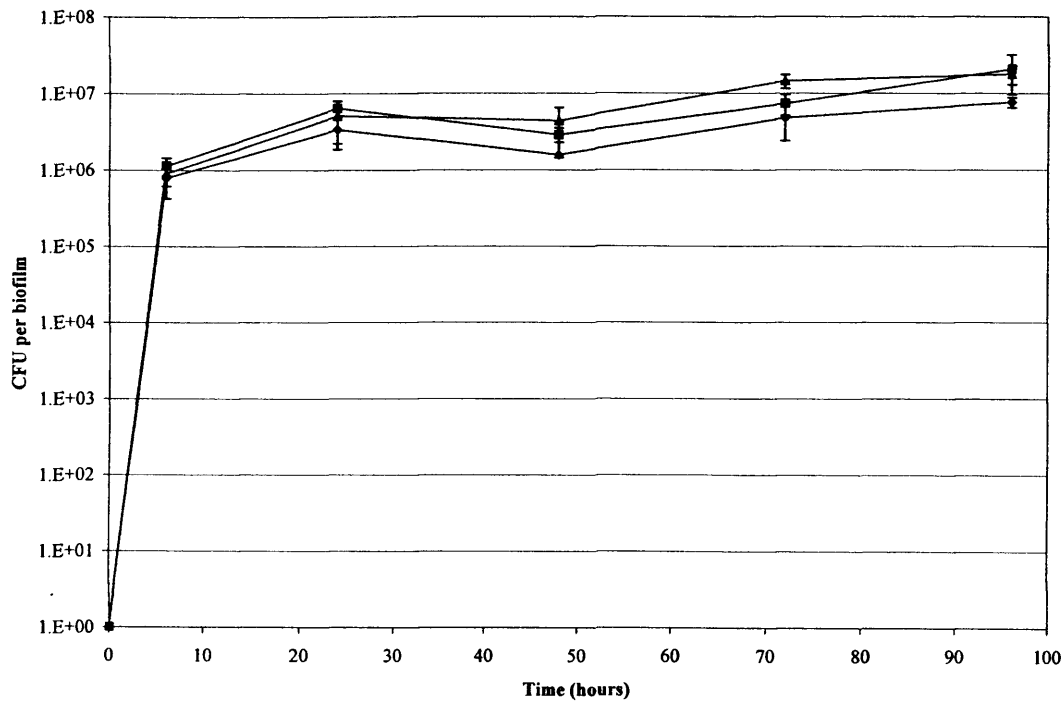


Figure 3.5a: Viable counts of *C. albicans* biofilms grown on three different surfaces over a period of 96 h using YPD as a nutrient medium. Filled diamonds - acrylic, filled squares – enamel, filled triangles – dentine. Error bars represent standard deviations (n = 6).

Table 3.5: Mean \pm the standard deviation of yeast and hyphal cell counts of *C. albicans* biofilms grown on acrylic, enamel and dentine over 96 h using YPD as a nutrient medium (n = 6).

Biofilm age	Acrylic		Enamel		Dentine	
	Yeast cell counts	Hyphal cell counts	Yeast cell counts	Hyphal cell counts	Yeast cell counts	Hyphal cell counts
6 h	1.3x10 ⁵ \pm 4.4x10 ⁴	4x10 ³ \pm 2.6x10 ³	3.5x10 ⁶ \pm 4x10 ⁶	3x10 ⁴ \pm 1x10 ⁴	5x10 ⁵ \pm 2.4x10 ⁴	1.7x10 ⁴ \pm 8.5x10 ³
24 h	8.5x10 ⁵ \pm 6.3x10 ⁵	1.3x10 ⁴ \pm 7.6x10 ³	2x10 ⁶ \pm 6.6x10 ⁵	5.3x10 ⁴ \pm 2.4x10 ⁴	1.1x10 ⁶ \pm 5.3x10 ⁵	2.6x10 ⁴ \pm 1x10 ⁴
48 h	2.9x10 ⁵ \pm 1.6x10 ⁵	1x10 ⁴ \pm 5x10 ³	1.4x10 ⁶ \pm 1.3x10 ⁶	1.3x10 ⁵ \pm 5x10 ⁴	2.3x10 ⁶ \pm 1.4x10 ⁶	1.4x10 ⁵ \pm 8.1x10 ⁴
72 h	1.3x10 ⁶ \pm 5.6x10 ⁵	1.7x10 ⁵ \pm 3x10 ⁴	3x10 ⁶ \pm 3.2x10 ⁵	3.2x10 ⁵ \pm 3.1x10 ⁴	4.8x10 ⁶ \pm 1x10 ⁶	2.7x10 ⁵ \pm 2.5x10 ⁴
96 h	1.6x10 ⁶ \pm 6.1x10 ⁵	3.8x10 ⁵ \pm 1x10 ⁵	5.8x10 ⁶ \pm 3.2x10 ⁶	1.5x10 ⁶ \pm 7.6x10 ⁵	8.1x10 ⁶ \pm 4.1x10 ⁶	1.9x10 ⁶ \pm 8.1x10 ⁵

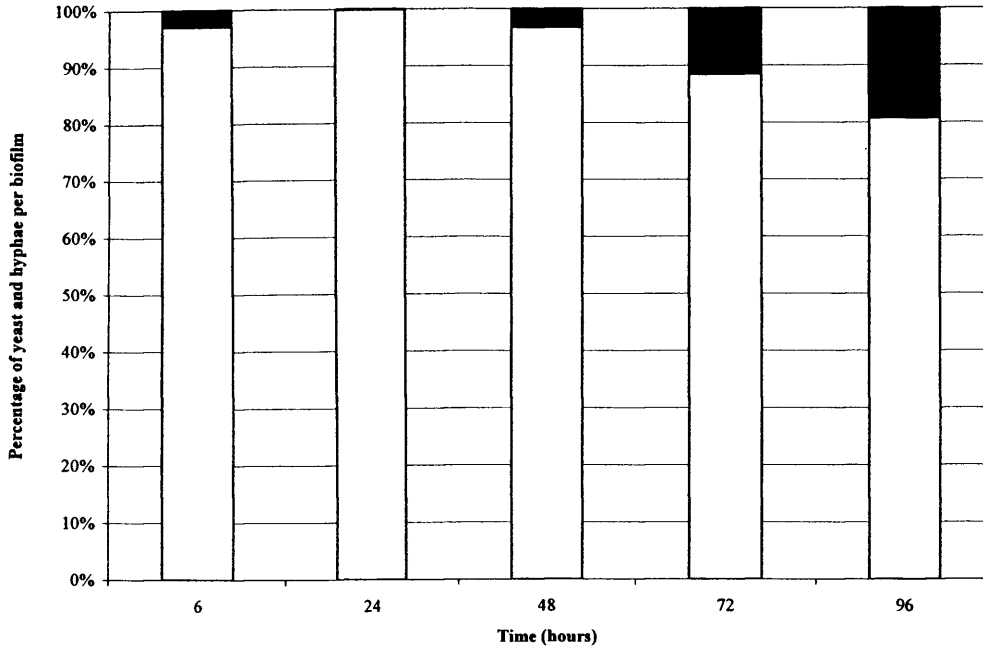


Figure 3.5b: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on acrylic surface over a period of 96 h using YPD as a nutrient medium. (Shown is the average percentage n = 6).

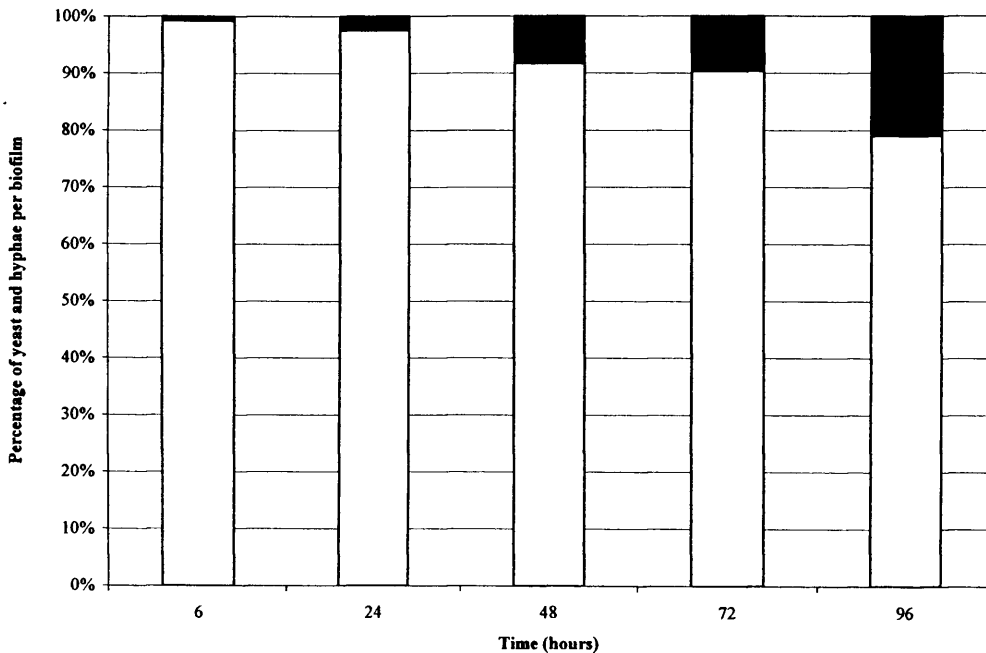


Figure 3.5c: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on enamel surface over a period of 96 h using YPD as a nutrient medium. (Shown is the average percentage n = 6).

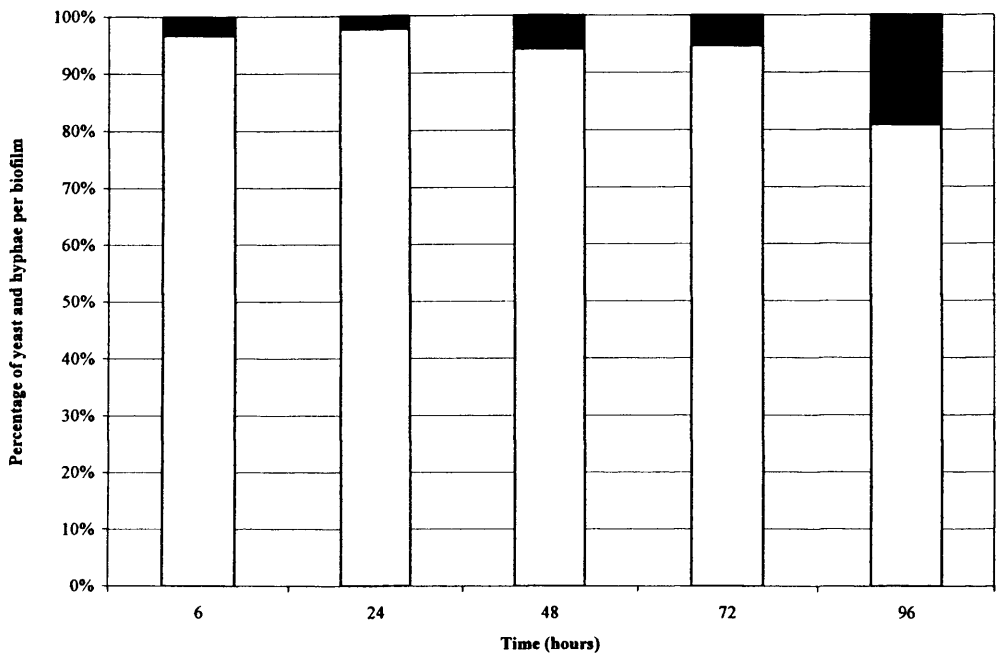


Figure 3.5d: The percentage of yeast (white) and hyphae (black) of *C. albicans* biofilms grown on dentine surface over a period of 96 h using YPD as a nutrient medium. (Shown is the average percentage n = 6).

3.4 DISCUSSION

Infections caused by *Candida* spp. are increasing as the numbers of immunocompromised patients in the community increase. Adherence of *C. albicans* is thought to be an essential event in the pathogenesis of candidosis and is considered as a virulence factor (Haynes, 2001). The aim of this study was to establish the growth of *C. albicans in vitro* as a biofilm likely to represent the *in vivo* scenario.

The model used, the Constant Depth Film Fermentor, is particularly suited to studies of biofilms of oral microbes in that it provides an environment similar to that found in the oral cavity (Morgan and Wilson, 2001). Biofilms are able to grow on a solid substratum with nutrients being provided in a thin film of liquid over the surface, which is continually replenished. The action of the scraper blades simulates the continuous removal of the outermost layers of dental plaque due to the shear forces present in the mouth (Wilson, 1999). By growing biofilms in the CDFF over an extended period of 384 h, it was possible to determine that a stable population was achievable. A biofilm will always be spatially heterogeneous, but biofilms grown in the CDFF have been shown to become quasi-steady state (Wimpenny, 1999). The results from previous studies (Pratten *et al.*, 1998a) have suggested that the base of the biofilm may be occupied by deteriorating or lysing cells. Cells undergoing lysis will generate substrates enabling re-growth. The observation of a decrease in viable counts after 150 h before increasing later in the experiment may be explained by this cycle of re-growth, even within a simple single-species biofilm. The ability to produce biofilms in a steady state is an important aspect of the model and provides the opportunity for the reproducible testing of antimicrobials which will be discussed further in later chapters.

No synthetic solution can be a complete substitute for natural saliva, however, due to the constraints of sterilisation and the need of large volume of human saliva, the nutrient medium carried out in the present study (apart from one run with YPD) was artificial saliva formulated by Russell and Coulter (1975). This synthetic substitute was needed to reproduce features of saliva which are considered important in cultural studies of dental plaque while remaining relatively inexpensive and simple to produce (Pratten *et al.*, 1998c). The artificial saliva used in this study provides a suitable growth medium and an adequate conditioning film on the surfaces used, hence both the yeast and hyphal forms of *C. albicans* were able to grow and form a structured biofilm. The use of mucin (containing glycoproteins) does mimic the protein pellicle present on oral surfaces and can act as receptors for adhesion molecules. Glycoproteins which are found in human saliva have been shown previously to form a salivary pellicle in bacterial biofilm studies (Pratten *et al.*, 1998b; Mulligan *et al.*, 2003). The salivary pellicle is known to have an important function in the formation of dental plaque. Through this pellicle, saliva exerts a profound influence on microbial adhesion to oral surfaces. However, its role in the formation of denture plaque is less well documented as there is considerable variation in results from different studies (Radford *et al.*, 1999). For example, it has been shown that mixed human saliva reduces candidal adhesion to acrylic surfaces and denture base materials (Samaranayake *et al.*, 1980; Radford *et al.*, 1998b) and can reduce microbial adhesion to silicon rubber (Busscher *et al.*, 1997), while other studies reported enhanced adhesion of *C. albicans* to saliva-coated acrylic surfaces and denture lining materials (Edgerton *et al.*, 1993; Nikawa *et al.*, 1993). Nikawa and co-workers have investigated the effect of a serum pellicle on the formation of *Candida* biofilm formation on acrylic surfaces. They found a significant increase in

biofilm growth of *Candida* spp. on protein coated acrylics. They concluded that the increase in serum content in the proteinaceous pellicle promoted biofilm formation of most *Candida* spp. (Nikawa *et al.*, 2000). Indeed the results of the present study showed that artificial saliva permits the growth of both forms of *C. albicans* confirming previous studies (Edgerton *et al.*, 1993; Nikawa *et al.*, 1993).

There have only been few studies carried out on the differences between self and heat-cured acrylic and the adhesion of *C. albicans* to these materials. In 1972, Davenport showed that there was no difference in surface roughness of heat and self-cured acrylic resin. This is in accordance with the results of the current study in which the numbers of *C. albicans* grown on self and heat-cured acrylic resin were not significantly different. It was suggested that the fine texture and absence of porosity of the denture surface would not permit plaque retention through penetration of surface defects or mechanical attachment to surface irregularities (Davenport, 1972).

In addition to acrylic, there are several other hard tissues present in the mouth available for colonisation such as enamel, dentine and cementum. Hyphae are able to penetrate into cracks, follow the ridges and migrate into dentinal tubules (Sen *et al.*, 1997). Limited data exist on the formation of *C. albicans* biofilms on dental hard tissues and few clinical reports clearly show fungal invasion of dentinal tubules. Hodson and Craig studied dental plaque and carious teeth in 100 children for the incidence of *C. albicans*. Their investigation included histological examination of 30 extracted teeth, which included a few cases in which fungal organisms had entered dentinal tubules (Hodson and Craig, 1972). Later, in 1983, Kinirons reported *Candidal* invasion of dentinal tubules in a patient with severe hypodontia (Kinirons, 1983). Another example of dentinal invasion was made by Damm and co-workers

that presented two cases of dentinal candidiasis that occurred in two cancer patients, one with acute myelogenous leukaemia and the second with carcinoma (Damm *et al.*, 1988). In the present study, tooth surfaces (enamel and dentine) were shown to have more growth of *C. albicans* than acrylic. Thus dental hard tissues can present a reservoir for disseminating of candidal infections. Although a useful tool for comparing two or more different surfaces in terms of their profile, R_a values are limited as they do not give an indication of the true nature of the roughness e.g. whether the surface contains 'spikes' or 'scratches'.

Traditionally, Yeast extract, Peptone and Dextrose broth is used as a growth medium to grow *C. albicans*. A planktonic overnight growth of *C. albicans* in YPD in the current study allowed only the growth of the yeast form of *C. albicans*. These results were also shown by Imanishi and co-workers (2004). In contrast, when *C. albicans* was grown in YPD in the presence of a surface, both the yeast and hyphal form were observed eventually. The process by which morphogenesis in *C. albicans* is regulated is poorly understood at the molecular level. Hawser and Douglas have observed by SEM that the adherence of *C. albicans* blastoconidia to catheters is followed by conversion to hyphae. These events indicate that a contact-induced regulation of gene expression may be occurring (Hawser and Douglas, 1994). Hyphal cells may sense and respond to changes in surface contours, follow grooves and ridges and invade the surface (Gow *et al.*, 1994; Watts *et al.*, 1998). It is therefore likely that during biofilm formation, hyphal emergence, particularly thigmotropic reaction, a well known characteristic for cells that grow in close contact to the surface, may be involved.

The effect of different surfaces (acrylic, enamel and dentine) was also investigated in the CDFP over 96 h using YPD as a nutrient medium. Variation in the number of

organisms attached was observed between the different materials used. In comparison to artificial saliva, YPD growth medium showed higher viable counts of *C. albicans* per biofilm when grown on acrylic, enamel and dentine. Samaranayake and MacFarlane (1980) showed that sucrose enhanced the adhesion of *C. albicans* to acrylic suggesting that the soft, carbohydrate-rich diets consumed frequently by denture wearers could induce yeast to colonise and adhere more tenaciously to denture surfaces. Additionally, diets rich in glucose or galactose may modify biofilm formation of *Candida* spp. on denture acrylic surfaces (Nikawa *et al.*, 1997). The increased adherence of yeast, possibly induced by sucrose, to dentures could play an important role in the pathogenesis of the candidal diseases affecting denture wearers. It can be concluded from this series of experiments that the type of surface upon which *C. albicans* is grown and the growth media used affect the initial formation and development of *C. albicans* biofilms. The next chapter will focus on the effect of one of the surface properties (surface roughness) on the formation of *C. albicans* biofilms.

Chapter 4

**The effect of surface roughness and strain variability on the biofilm
forming ability of *Candida albicans***

4.1 INTRODUCTION

The roughness of a surface is considered an important aspect in the ability of dental plaque to form, and may contribute to microbial colonisation and plaque maturation on the surface (Quirynen and Bollen, 1995). Surface roughness has previously been shown to affect the adhesion of microorganisms to catheters (Tebbs *et al.*, 1994) and enamel surfaces (Gurgan *et al.*, 1997).

Roughness of materials used in dentistry influences microbial retention and adhesion. The increase in surface roughness can increase retention of microbial cells to acrylic surfaces. Indeed this has been reported for *C. albicans* (Verran *et al.*, 1991; Verran and Maryan, 1997; Radford *et al.*, 1998b). Surface roughness can provide niches in which microorganisms are protected from shear forces and oral hygiene measures as bacteria can be irreversibly attached easily and, more frequently, in these sites (Quirynen and Bollen, 1995). This in turn may lead to the importance of surface roughness in plaque formation and mucosal inflammation.

Dentures may function as a reservoir of infection (Davenport, 1970, Budtz-Jorgensen *et al.*, 1996) as these enhance denture plaque attachment which may in turn predispose to DRS. Both the type of denture acrylic and its surface roughness can affect the early stages of biofilm formation (Morgan and Wilson, 2001). The presence of surface irregularity and roughness in the denture may increase the likelihood of microorganisms retention on the surface after cleaning the denture. Thus smooth denture surfaces are more desirable than rougher surface as this will facilitate denture cleaning and hygiene (Verran and Maryan, 1997). However, adjustment of the denture base is frequently required in the clinic and this may leave unpolished areas, especially if they involve the denture fitting surface. Hence these areas may enhance plaque accumulation and infection.

Candida albicans remains the most common fungal commensal of the mouth. In addition, it is recognised to be one of the most pathogenic of *Candida* species. It exhibits a number of pathogenic mechanisms that contribute to its virulence including the cell wall (McCullough *et al.*, 1996), the ability of phenotypic switching (Soll, 1992; Calderone and Fonzi, 2001), the ability to form germ tubes, to adhere to host cells, and to secrete proteinases and phospholipases (Ibrahim *et al.*, 1995; Kretschmar *et al.*, 1999; Haynes, 2001; Naglik *et al.*, 2003). This has been previously reviewed in detail (refer to section 1.1.2). An association of these are important contributory factors for pathogenicity of *C. albicans* (Ghannoum and Abu-Elteen, 1986; Abu-Elteen *et al.*, 2001). Different strains of *C. albicans* show variations in their virulence factors (Al-Karaawi *et al.*, 2002 and 2004)

The structural organisation of biofilms is a key characteristic which defines biofilm growth. The advent of modern imaging techniques such as CLSM allow the researcher to examine a biofilm in a fully hydrated living state (Baillie and Douglas, 1999b). The study of bacterial biofilm structure using CLSM is well documented; however, there are few studies on the structure of fungal biofilms using CLSM.

The aim of these experiments was to investigate the effect of surface roughness on biofilm formation using different strains of *C. albicans* with high and low adherence properties which have been determined from previous non-biofilm related studies.

4.2 MATERIALS AND METHODS

In order to investigate the ability of various strains of *C. albicans* to adhere to materials with different surface roughness with relation to the oral cavity, the CDFD was employed as a model system. Both self and heat-cured acrylic were used as substrata for biofilm growth as described previously (refer to section 2.1.1).

Clinical isolates (refer to section 2.2.2) of defined species of high and low adherence genotype A and B (total of four strains) *C. albicans* were cultured overnight in 10 ml of artificial saliva to be used as inoculum and added to 500 ml of sterile artificial saliva and pumped into the CDFD as described previously (refer to section 2.4.2.1 and 2.4.3). Samples were then removed at 2 h for both acrylic type and at various time intervals up to maximum 72 h for the other experiments.

The CFU, hyphae and yeast counts were determined (refer to section 2.4.5.1) and images of *C. albicans* biofilms were taken for different surface roughness using CLSM with a fluorescent stain (refer to section 2.7). Five to six images were taken and the most representative of these were presented in the results section. A scanning laser profilometer (Proscan 1000) was used to accurately measure the surface roughness of the substrata as described previously (refer to section 2.8). The surface roughness was expressed as R_a , this value is the arithmetic mean deviation of the surface height from the mean line through the profile. Changes in surface topography were also confirmed by SEM (refer to section 2.6).

4.3 RESULTS

To assess the contribution of surface roughness on the formation of *C. albicans* biofilms, self and heat-cured acrylic were prepared and polished with abrasive paper and used as a substratum for biofilm growth over a 2 h period. Table 4.1 shows the viable counts of *C. albicans* biofilms on both types of acrylic with different surface roughness. At 2 h, the CFU per biofilm on self-cured acrylic was higher than heat-cured acrylic for all the surface roughness, although none of the differences were significant ($p > 0.05$). The yeast and hyphal cell counts from this experiment are presented in Table 4.2. Again there were no significant differences ($p > 0.05$) between the two types of acrylic. Interestingly, there were no hyphae on both types of acrylic on the smoothest surface ($R_a = 2.5 \mu\text{m}$), hence, the percentage of hyphal cells (Figure 4.1) increased with increasing surface roughness for both acrylic types. The self and heat-cured acrylic contained the highest hyphal cells at $R_a = 3.6 \mu\text{m}$ with 12.5 % and 14.3 % respectively.

C. albicans genotypes A and B, each one with different adherence properties were additionally investigated over a 72 h period. Figure 4.2 represents the viable counts of two strains from genotype A with high and low adherence properties over 72 h on acrylic. At 2 h biofilm growth, the high adherence strain showed the higher CFU per biofilm on the roughest surface ($R_a = 9.3$). After 4 h, the viable counts were lower for all surface roughness compared to the low adherence strain. Towards the end of the experiment at 48 and 72 h, the high adherence strain contained the highest CFU per biofilm for all of the surface roughness with a maximum growth of 1.2×10^7 CFU per biofilm. Figure 4.3 shows the number of yeast cells (a) and hyphal cells (b) on different surface roughness over 72 h for high adherence genotype A. At 6 h there were significant differences between the number of yeast cells present and the

roughness of the surface ($p < 0.05$), the highest number of yeast cells were found on the roughest surface while fewer yeast cells were detected on the smoothest surface (Figure 4.3a). After 6 h there was no correlation between the surface roughness and the number of yeast cells present. The number of hyphae present on each surface (Figure 4.3b) was related to the surface roughness at 6 and 24 h with the highest number of hyphae on the roughest surfaces ($p < 0.05$). As the biofilm developed there was no significant difference ($p > 0.05$) between the numbers of hyphae on each of the different surfaces.

Figure 4.4 shows the yeast cell counts (a) and hyphal cell counts (b) of low adherence genotype A biofilms grown on the different surface roughness. The yeast and hyphal cell counts were increased over time (2 – 72 h) at all different surface roughness in which yeast cell counts were always higher than the hyphal cell counts. During initial biofilm formation, the highest numbers of yeast cells were found on the roughest surface while fewer yeast cells were detected on the smoothest surface (Figure 4.4a). The number of hyphae present on each surface was also related to the surface roughness (Figure 4.4b).

C. albicans genotype B strain was also investigated over the same time period (Figure 4.5). During early biofilm formation (2 and 6 h), the high adherence strain of genotype B were higher than the low adherence strain at all surface roughness ($p < 0.05$), however, at 24 h biofilm, there was no difference between the two strains at all surface roughness. The same results were obtained for low and high adherence genotype A (Figure 4.2). In contrast, the low adherence genotype B strain was higher than the high adherence strain until the end of the experiment (72 h). Figure 4.6 showed the yeast cell counts (a) and hyphal cell counts (b) of high adherence strain genotype B. There were increased number of yeast and hyphal cell counts over time

(2 – 72 h) at all different surface roughness in which yeast cell counts were always higher than the hyphal cell counts (same results obtained by genotype A). At initial biofilm formation (6 h), the highest numbers of yeast cells were found on the roughest surface while fewer yeast cells were detected on the smoothest surface (Figure 4.6a). The number of hyphae present on each surface was also related to the surface roughness (Figure 4.6b). The same results were also obtained of the yeast and hyphal cells counts of the low adherence strain genotype B (Figure 4.7a and b). The biofilms were also examined by CLSM. Using this technique, it could be seen that the structure of the biofilms was also dependent upon the surface roughness. Figure 4.8a-c shows the initial attachment of *C. albicans* biofilm on various surface roughness after 6 h. Abrasions are visible on the surfaces in these figures from the bottom left to the top right where the material has been machined with the silicon carbide grit. At this time point (6 h), on all the surfaces, the images are dominated by the yeast form of the *Candida*. Interestingly, a germ tube form is visible (Figure 4.8b). After 24 h biofilm growth (Figure 4.9), the smoothest surface ($R_a = 2.5 \mu\text{m}$) shows biofilm formation in micro-colonies with fewer hyphal cells visible (Figure 4.9a). There seem to be more hyphal forms visible on the roughest surface, while the formation appears to be in alignment with the etchings (Figure 4.9b-c). At 72 h, mature biofilms were present on the surfaces (Figure 4.10a-c). On the smoother surface the biofilm has formed in discrete, well defined stacks with a large surface area not colonised and the hyphal form of the yeast was associated with the base of the biofilm (Figure 4.10a). The roughest surfaces showed that the hyphal cells are also established towards the base of the biofilm (Figure 4.10b-c). In these basal layers the yeast form seems to be positively associated with the hyphal forms. Additionally, there are some areas with little or no colonisation and channels and

voids are apparent within the biofilm.

Table 4.1: Mean \pm the standard deviation of viable counts of *C. albicans* biofilms grown on self and heat-cured acrylic with different surface roughness ($R_a = 2.5 \mu\text{m}$, $R_a = 3.6 \mu\text{m}$ and $R_a = 9.3 \mu\text{m}$) over 2 h.

Surface roughness (R_a)	Self-cured acrylic	Heat-cured acrylic
$R_a = 2.5 \mu\text{m}$	2.1×10^3	1.1×10^3
	$\pm 4.5 \times 10^2$	$\pm 1.3 \times 10^2$
$R_a = 3.6 \mu\text{m}$	3.5×10^3	3.1×10^3
	$\pm 3 \times 10^2$	$\pm 6.8 \times 10^2$
$R_a = 9.3 \mu\text{m}$	2.6×10^3	2.4×10^3
	$\pm 8.7 \times 10^2$	$\pm 7.2 \times 10^2$

Table 4.2: Mean \pm the standard deviation of yeast and hyphal cell counts of *C. albicans* biofilms grown on self and heat-cured acrylic with different surface roughness ($R_a = 2.5 \mu\text{m}$, $R_a = 3.6 \mu\text{m}$ and $R_a = 9.3 \mu\text{m}$) over 2 h.

Average surface roughness (R_a)	Self-cured acrylic		Heat-cured acrylic	
	Yeast cell counts	Hyphal cell counts	Yeast cell counts	Hyphal cell counts
$R_a = 2.5 \mu\text{m}$	2.3×10^3	0	4×10^3	0
	$\pm 5.8 \times 10^2$	0	$\pm 1 \times 10^3$	0
$R_a = 3.6 \mu\text{m}$	7×10^3	1×10^3	8×10^3	1.3×10^3
	$\pm 4 \times 10^3$	0	$\pm 3.5 \times 10^3$	$\pm 5.8 \times 10^2$
$R_a = 9.3 \mu\text{m}$	1.3×10^4	1.3×10^3	1.4×10^4	1.4×10^3
	$\pm 8 \times 10^3$	$\pm 5.8 \times 10^2$	$\pm 5.5 \times 10^3$	$\pm 1.1 \times 10^3$

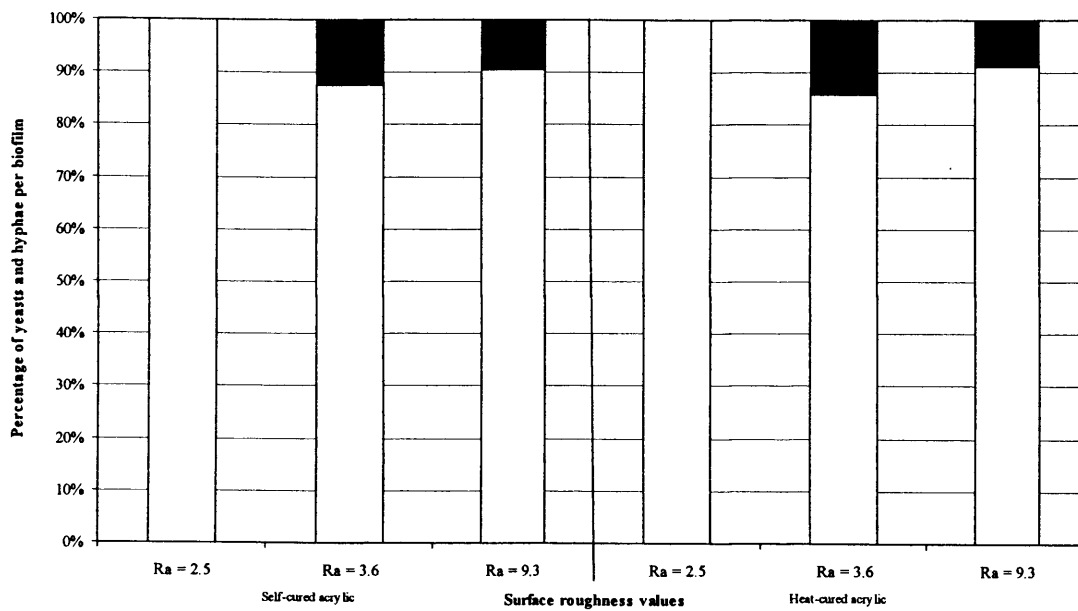


Figure 4.1: The percentage of yeast (white) and hyphal cells (black) of *C. albicans* biofilms grown on different surface roughness of self and heat-cured acrylic ($R_a = 2.5 \mu\text{m}$, $R_a = 3.6 \mu\text{m}$ and $R_a = 9.3 \mu\text{m}$) over 2 h.

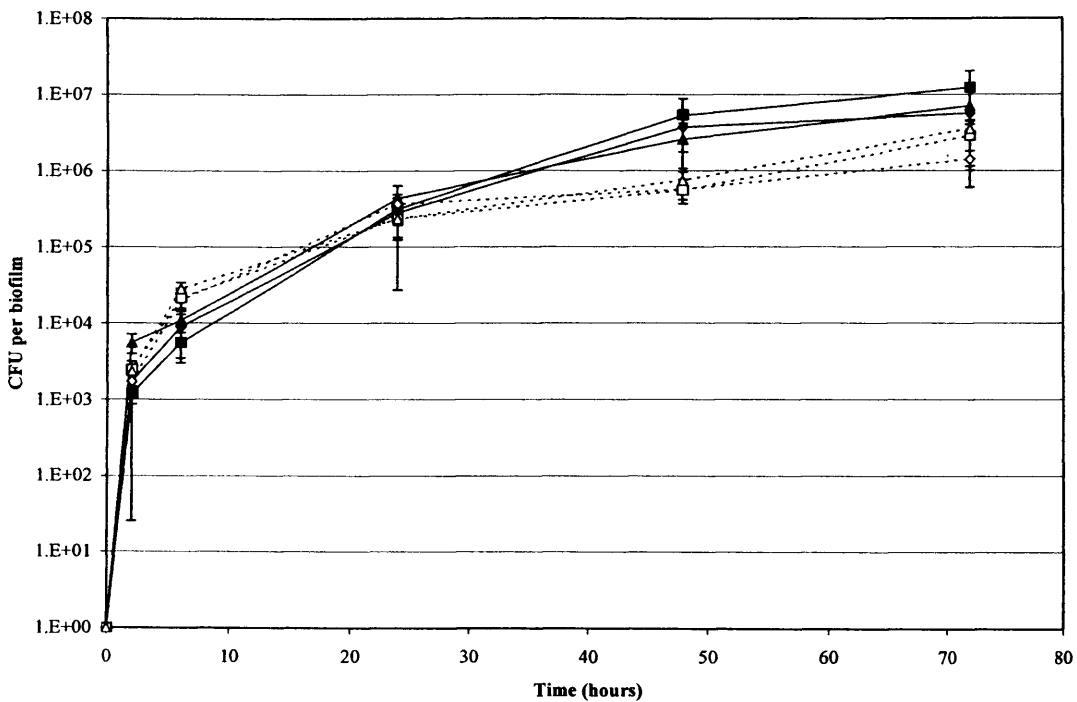


Figure 4.2: The viable counts of two genotype A *C. albicans* biofilms grown on acrylic over 72 h. High adherence strain with black line (Filled diamond: $R_a = 2.5 \mu\text{m}$, filled square: $R_a = 3.6 \mu\text{m}$ and filled triangle: $R_a = 9.3 \mu\text{m}$). Low adherence strain with dotted line (open diamond: $R_a = 2.5 \mu\text{m}$, open square: $R_a = 3.6 \mu\text{m}$ and open triangle: $R_a = 9.3 \mu\text{m}$). Error bars represent standard deviations.

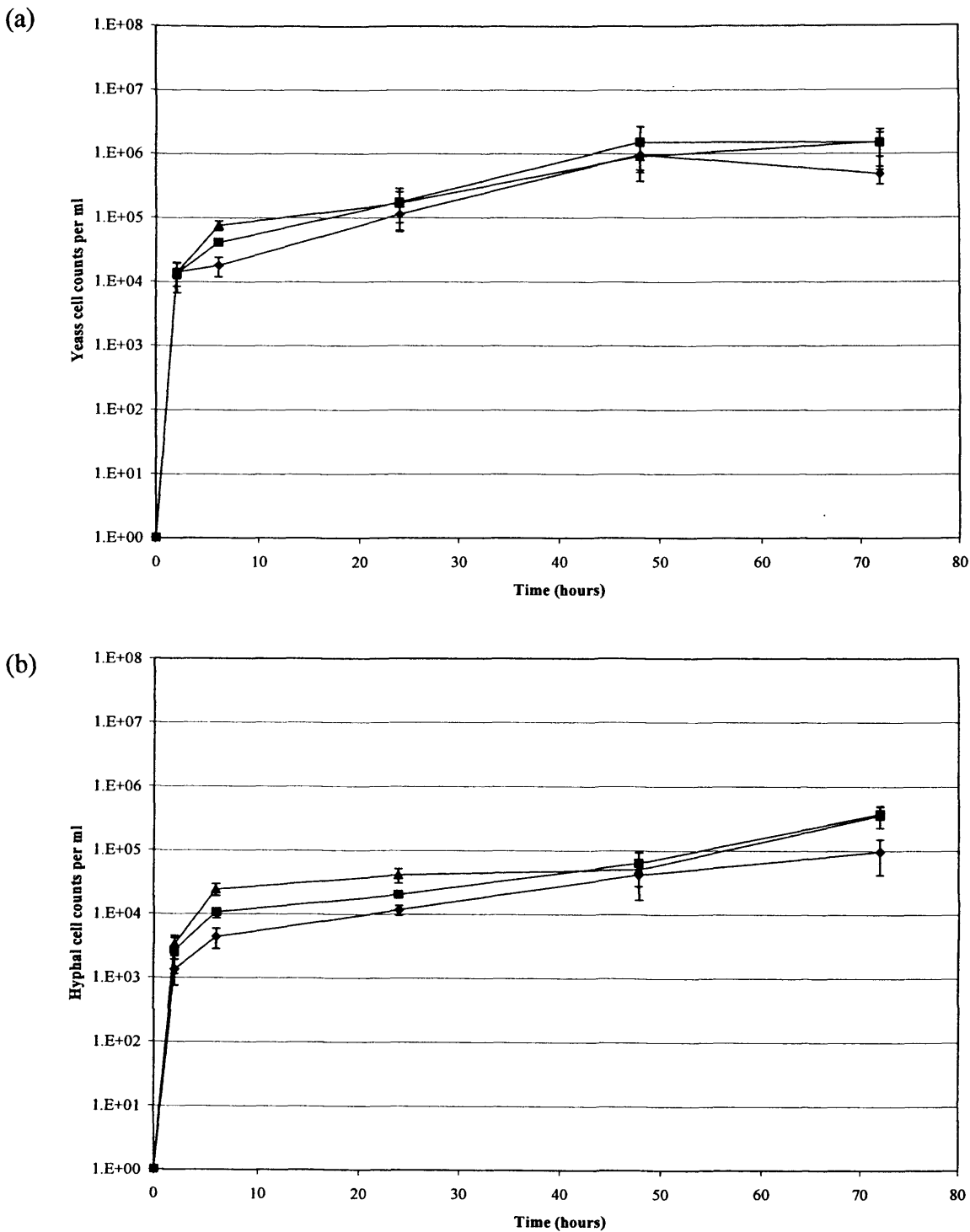


Figure 4.3: The number of yeast (a) and hyphal (b) cell counts of high adherence *C. albicans* biofilms genotype A grown on different surface roughness of denture acrylic over 72 h (Filled diamond: $R_a = 2.5 \mu\text{m}$, filled square: $R_a = 3.6 \mu\text{m}$ and filled triangle: $R_a = 9.3 \mu\text{m}$). Error bars represent standard deviations.

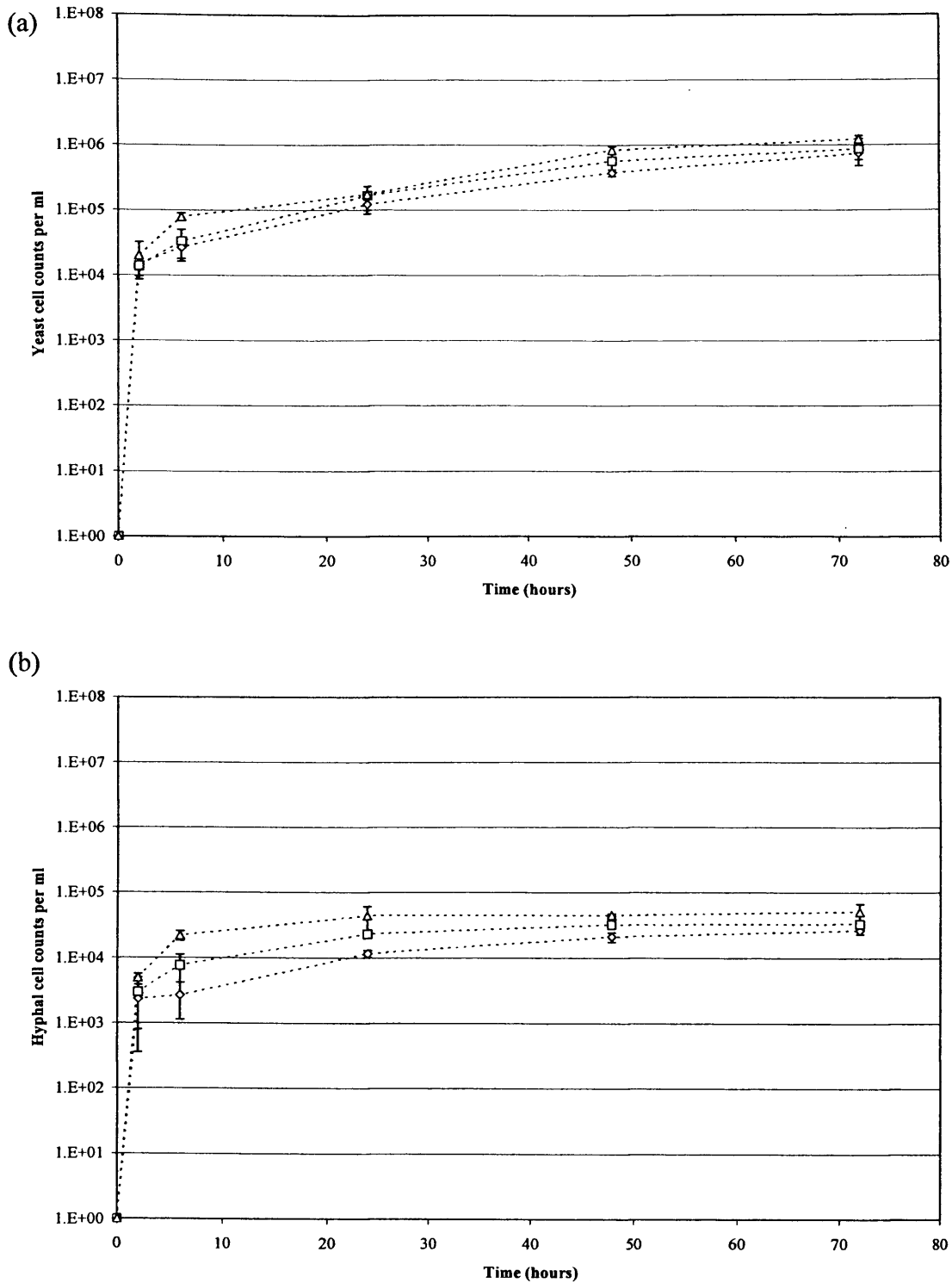


Figure 4.4: The number of yeast (a) and hyphal (b) cell counts of low adherence genotype A *C. albicans* biofilms grown on acrylic with different surface roughness over 72 h (open diamond: $R_a = 2.5 \mu\text{m}$, open square: $R_a = 3.6 \mu\text{m}$ and open triangle: $R_a = 9.3 \mu\text{m}$). Error bars represent standard deviations.

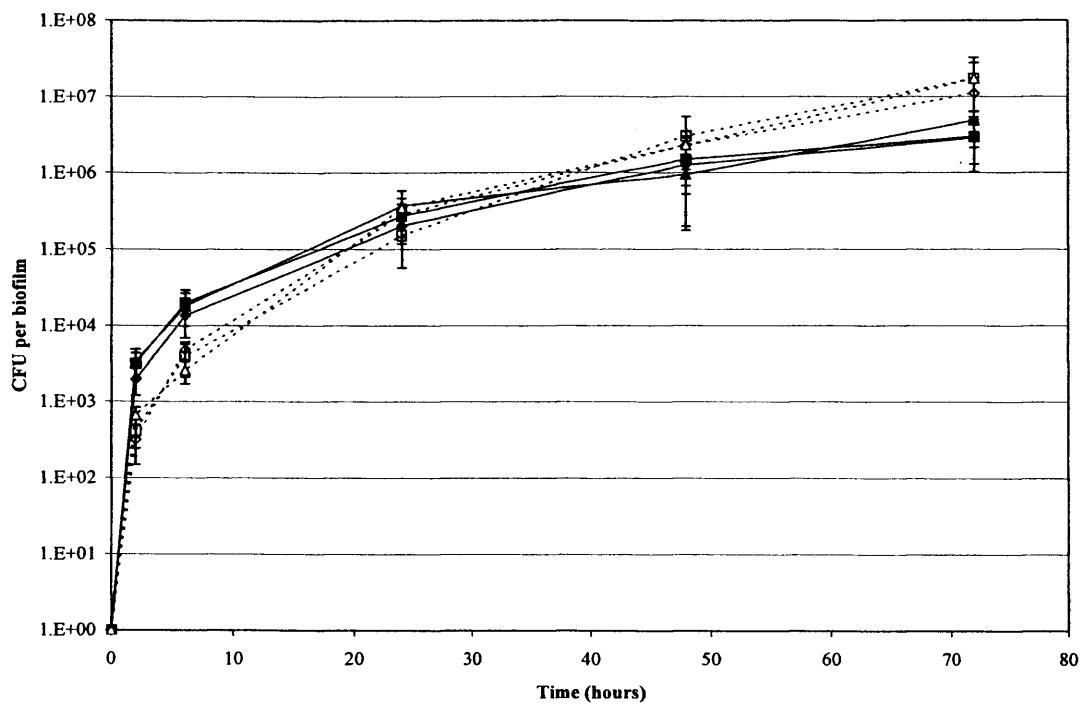


Figure 4.5: The viable counts of two genotype B *C. albicans* biofilms grown on acrylic over 72 h. High adherence strain with black line (Filled diamond: $R_a = 2.5 \mu\text{m}$, filled square: $R_a = 3.6 \mu\text{m}$ and filled triangle: $R_a = 9.3 \mu\text{m}$). Low adherence strain with dotted line (open diamond: $R_a = 2.5 \mu\text{m}$, open square: $R_a = 3.6 \mu\text{m}$ and open triangle: $R_a = 9.3 \mu\text{m}$). Error bars represent standard deviations.

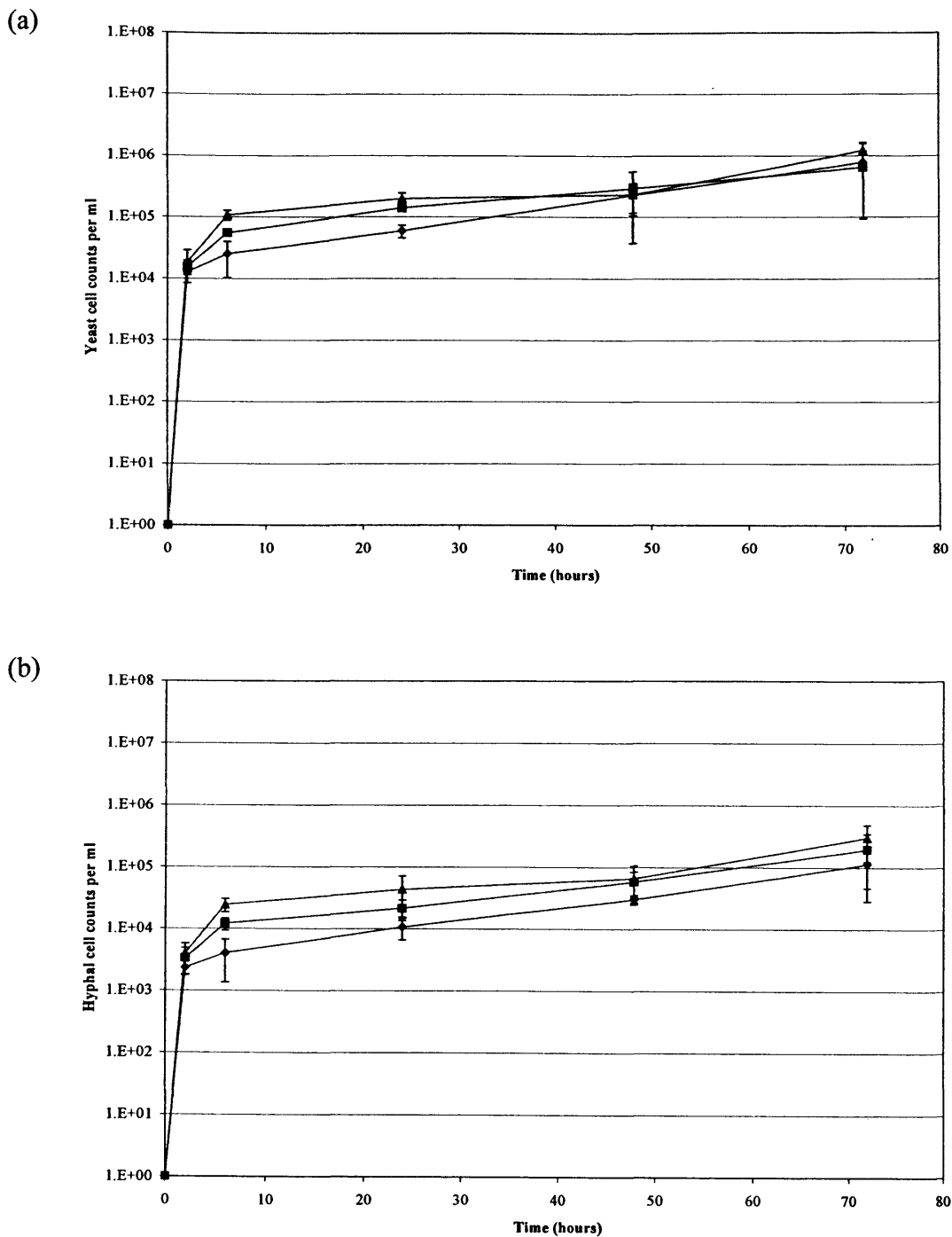


Figure 4.6: The number of yeast (a) and hyphal (b) cell counts of high adherence genotype B *C. albicans* biofilms grown on acrylic with different surface roughness over 72 h (Filled diamond: $R_a = 2.5 \mu\text{m}$, filled square: $R_a = 3.6 \mu\text{m}$ and filled triangle: $R_a = 9.3 \mu\text{m}$). Error bars represent standard deviations.

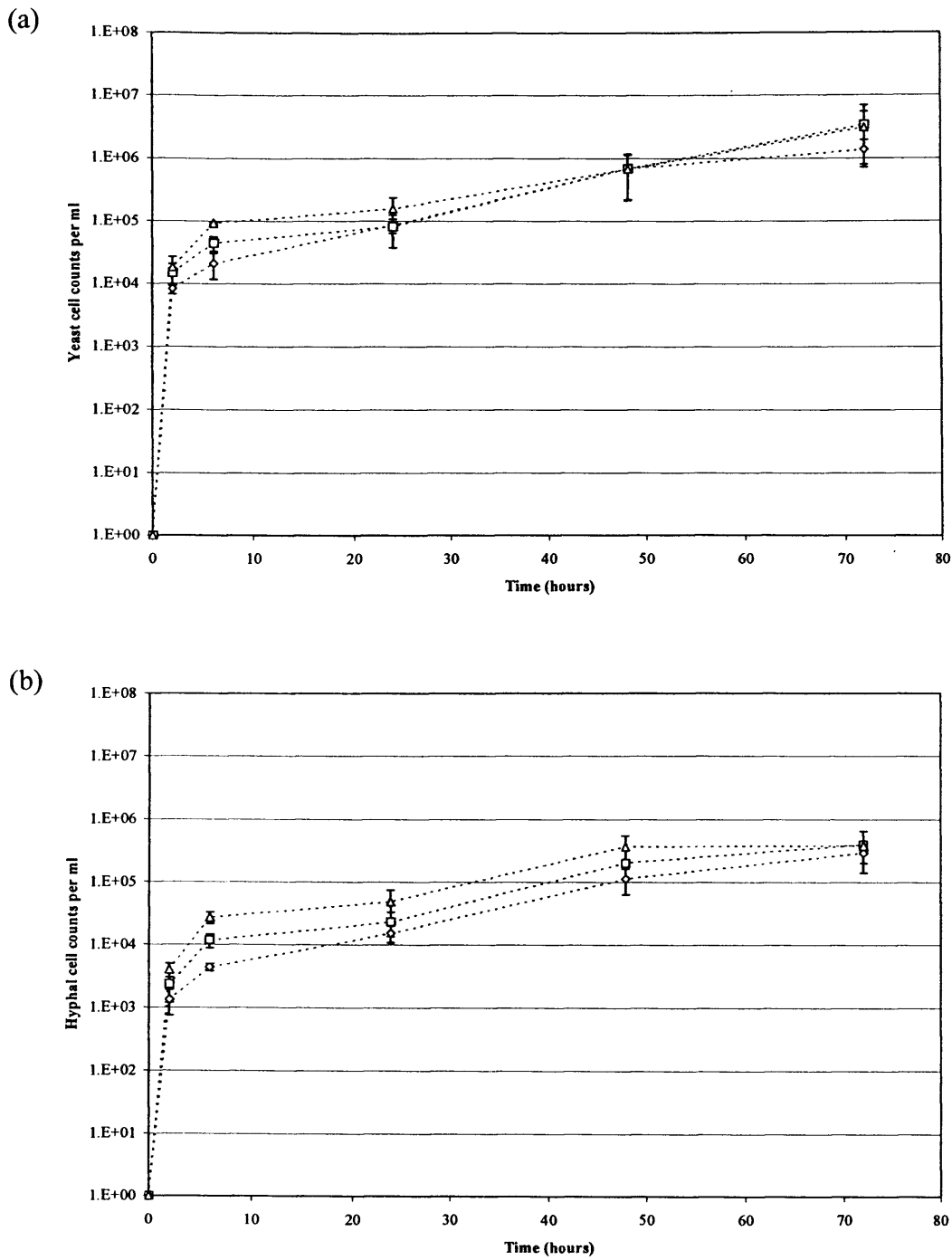


Figure 4.7: The number of yeast (a) and hyphal (b) cell counts of low adherence genotype B *C. albicans* biofilms grown on acrylic with different surface roughness over 72 h (open diamond: $R_a = 2.5 \mu\text{m}$, open square: $R_a = 3.6 \mu\text{m}$ and open triangle: $R_a = 9.3 \mu\text{m}$). Error bars represent standard deviations.

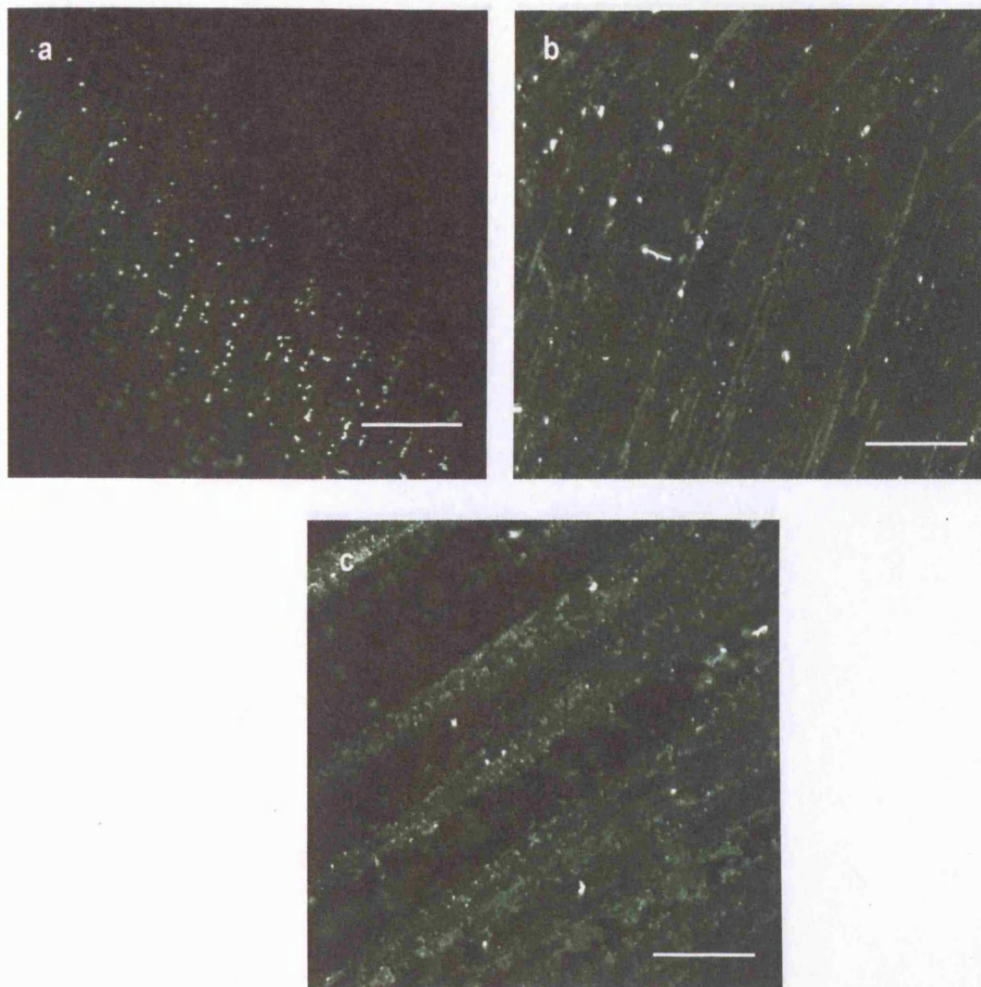


Figure 4.8: CLSM images showing *C. albicans* biofilms formation on denture acrylic of various surface roughness after 6 h growth in the CDFF. a) $R_a = 2.5 \mu\text{m}$, b) $R_a = 3.6 \mu\text{m}$ and c) $R_a = 9.3 \mu\text{m}$. Bar = 200 μm .

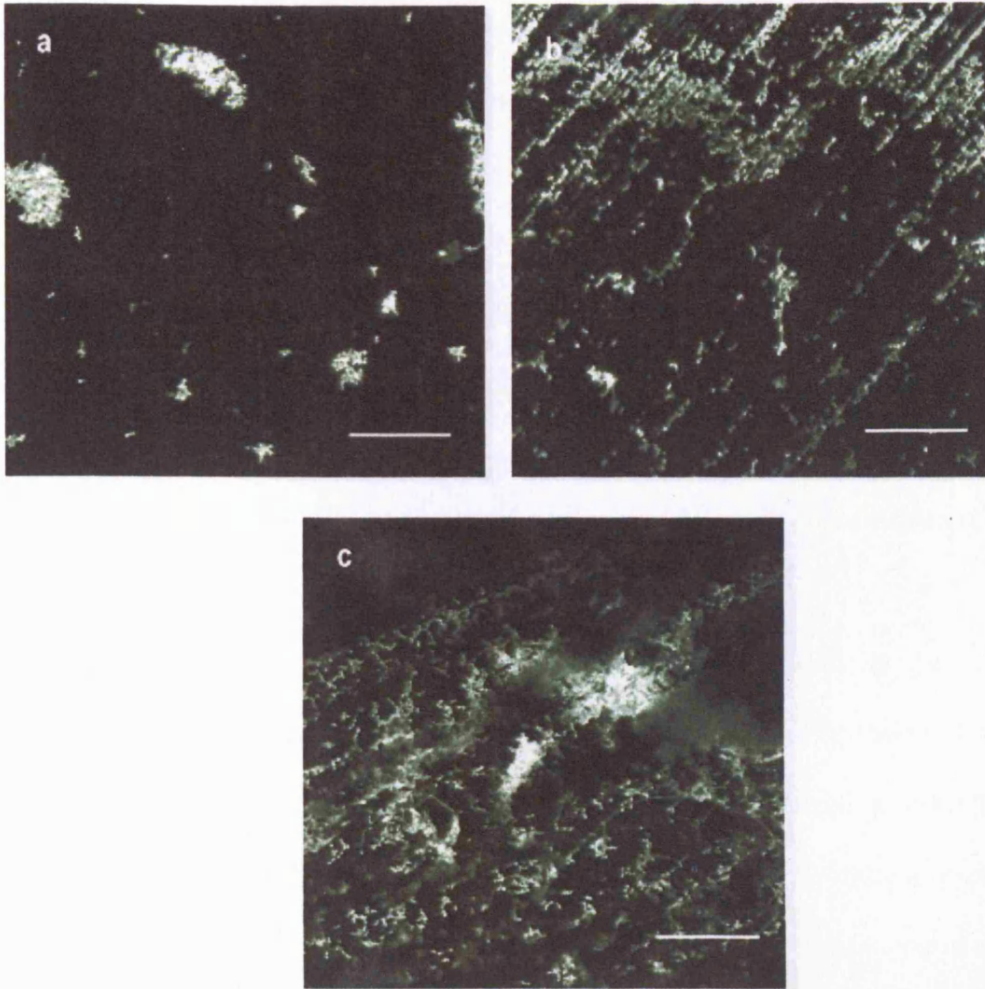


Figure 4.9: CLSM images showing *C. albicans* biofilm formation on denture acrylic of various surface roughness after 24 h growth in the CDFF. a) $R_a = 2.5 \mu\text{m}$, b) $R_a = 3.6 \mu\text{m}$ and c) $R_a = 9.3 \mu\text{m}$. Bar = 200 μm .

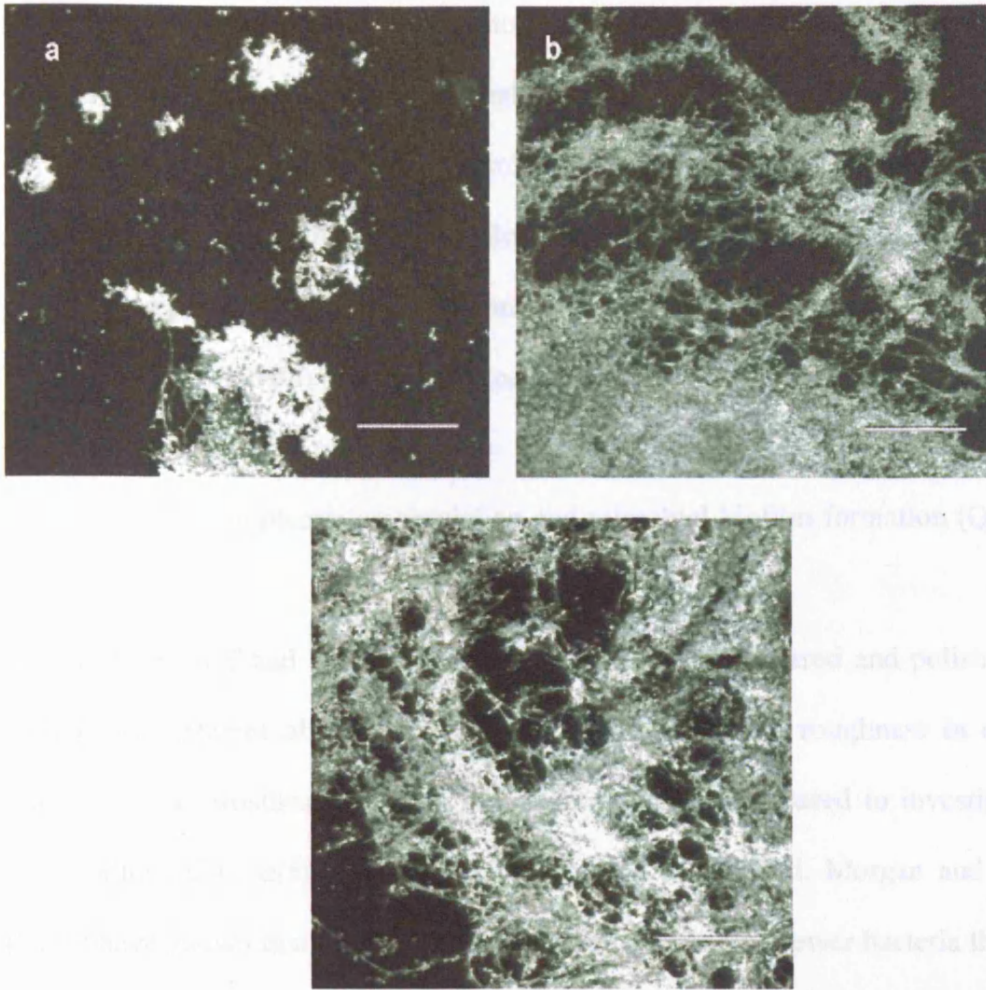


Figure 4.10: CLSM images showing *C. albicans* biofilms formation on denture acrylic of various surface roughness after 72 h growth in the CDFF. a) $R_a = 2.5 \mu\text{m}$, b) $R_a = 3.6 \mu\text{m}$ and c) $R_a = 9.3 \mu\text{m}$. Bar = 200 μm .

4.4 DISCUSSION

Surface roughness is a significant factor that facilitates attachment and retention of microorganisms to surfaces. It is thought that this is due to the greater surface area (Lytle *et al.*, 1989) available for microbial adhesion and the provision of protected sites for colonisation (Quirynen and Bollen, 1995). The effect of surface roughness has been reported previously (Verran *et al.*, 1991; Verran and Maryan, 1997; Radford *et al.*, 1998b) with an increase in surface roughness causing increased retention of cells on acrylic surfaces. Surface roughness can be considered as an important factor in plaque accumulation and microbial biofilm formation (Quirynen *et al.*, 1990).

In this study, self and heat-cured denture acrylic were prepared and polished by a number of different abrasive papers with different surface roughness in order to mimic an oral prosthesis *in vivo*. These were subsequently used to investigate the colonisation of *C. albicans* in the CDFD over a 2 h period. Morgan and Wilson (2001) have shown that heat-cured acrylic was colonised by fewer bacteria than self-cured acrylic after 2 h with increased colonization for both types of acrylic with increase surface roughness. However, after 4 h, while the effect of the type of acrylic on colonisation was evident, this was not the case for the surface roughness effect. Hence, after this time, the increase in surface roughness had no effect on the number of organisms attached to either acrylic. The results of the current study demonstrated no significant difference ($p > 0.05$) in *C. albicans* biofilm formation at 2 h for either self or heat-cured acrylic in both CFU and yeast and hyphal cell counts at any surface roughness. This is in accordance with previous studies by Davenport who showed no difference in surface roughness of heat and self-cured acrylic resin (Davenport, 1972).

It has been shown that *C. albicans* can preferentially adhere to rough surfaces compared to smooth with greater adherence to the machined soft lining materials compared with acrylic (Radford *et al.*, 1998b). The results of the current study are in line with the previous study (Verran *et al.*, 1991) in which *C. albicans* adhered significantly in higher numbers to roughened acrylic than to smooth acrylic. Verran and Maryan also showed a significantly higher number of cells were observed on the rough surfaces than on smooth surfaces, with more *C. albicans* cells on rough silicon than rough acrylic surfaces (Verran and Maryan, 1997). Ramage and co-workers (2004) visualised *Candida* biofilms from two denture samples and showed that yeast and hyphal cells of these biofilms were embedded into cracks and imperfections of the biomaterial (Ramage *et al.*, 2004). This observation indicated that surface roughness and cracks within the acrylic support attachment and colonization of *Candida*. These investigations may reveal the importance of surface imperfection that facilitates retention of cells.

In the present study, two different *C. albicans* genotypes (A and B) each with high and low adherence properties were used to study *C. albicans* biofilm formation on self-cured acrylic with different surface roughness in the CDFD for 72 h. Ghannoum and Abu-Elteen emphasized the role played by some specific properties of certain strains of *C. albicans* in the pathogenesis of candidosis. For example, those isolates which adhered most strongly to buccal epithelial cells were found to be the most pathogenic organisms (Ghannoum and Abu-Elteen, 1986). The strains used in the current study were previously isolated and several factors associated with virulence were assessed in a separate non-biofilm study. The results of the current study showed that surface roughness has an effect on the ability of hyphae to adhere to the acrylic denture during the first 24 h of biofilm formation. Interestingly, no

differences were seen in the ability of the organisms to form biofilms. Certainly, it is clear from these results that any differences in variability for high and low adherence strains were not seen in the subsequent growth and formation of more complex biofilms.

CLSM biofilm images were displayed in three-dimensional (3-D) projections and showed the structure of *C. albicans* biofilm on different surface roughness on denture acrylic. Previous studies which have evaluated the structure of *C. albicans* biofilms by CLSM (Chandra *et al.*, 2001a) showed that *C. albicans* has a highly heterogeneous architecture composed of cellular and acellular elements and consists of three phases of organised growth structure through early, intermediate and maturation phases. Similar phases have been also reported for bacterial biofilm formation (O'Toole *et al.*, 2000). Other studies using scanning electron and light microscopy on catheter disks have shown that biofilm consist of a thin, basal yeast layer and a thicker, but more open, hyphal layer (Baillie and Douglas, 1999b). Under the conditions of this study, it has been shown that a developmental process of biofilm formation with early colonisation by the yeast form and subsequent colonization by the hyphal form may be important in biofilm structure and formation. Chandra and co-workers showed that the proportions of yeast and hyphal cells present in the biofilm were dependent upon the nutrient source. For example, biofilms grown in a nitrogen based medium contained mainly the yeast form, whereas filaments predominated in RPMI-grown biofilms (Chandra *et al.*, 2001a). In the current study, where biofilms were supplied with artificial saliva in an environment similar to the *in vivo* situation, it appears that the presence of hyphal cells at the substratum interface may be integral to the formation and development of *Candida albicans* biofilm structure.

Previous studies showed that the adhesion of *Staphylococcus epidermidis* was reduced when a PMMA surfaces with various surface roughness were coated with bovine serum albumin, whereas the adhesion of *Pseudomonas aeruginosa* unchanged (Taylor *et al.*, 1998). Radford and co-workers showed that when denture base materials were pre-coated with saliva, the adhesion of *C. albicans* is reduced and also the effect of surface roughness is diminished (Radford *et al.*, 1998b). Hence, although surface roughness can affect bacterial adhesion, the presence of salivary pellicle may mask or modify these effects. In the present study mucin was included in the nutrient source (artificial saliva) to provide a pellicle similar to that which is found in the mouth. This may diminish the effect of different surface materials (i.e. self and heat-cured acrylic) as demonstrated in the present study as well as surface roughness on the different surfaces.

In conclusion, the experimental results reported in this chapter showed that surface roughness had a significant effect on the initial adhesion of *C. albicans*, however, further biofilm development was unaffected by either surface roughness or strain of the organism. Additionally, by using CLSM to investigate biofilm development on surfaces with different roughness, under these *in vitro* conditions, the organisation of biofilms may centre upon the roughness of the surface and the hyphal form of the organism. Clinically, surfaces that promote plaque development should be handled in such a manner to produce smooth surfaces. This will reduce the ability of *C. albicans*, a commonly opportunistic pathogen associated with DRS, to adhere and colonise denture surfaces.

Such structurally organised biofilms are known to be more resistant to antimicrobial agents. Hence the next chapter will focus upon the susceptibility of *C. albicans* biofilms to antimicrobial and antifungal agents used in the treatment of DRS.

Chapter 5

The susceptibility of *Candida albicans* biofilms to antifungal and antimicrobial agents

5.1 INTRODUCTION

Biofilms are a protected niche of microorganisms that can create a source of persistent infection due to their resistance to antibiotic treatment. They can form on a variety of implanted medical devices resulting in biofilm-associated infections that constitute a significant public health problem (Costerton *et al.*, 1999). Biofilm grown *Candida* species, like their bacterial counterparts, are highly resistant to antimicrobials (Hawser and Douglas, 1995).

Biofilm formation is critical in the development of DRS, in which *Candida* spp. biofilms play a key role (Ramage *et al.*, 2004). It has been noted that the symptoms of DRS often return shortly after treatment (Budtz-Jorgensen *et al.*, 1988; Bissell *et al.*, 1993), suggesting that *C. albicans* biofilms associated with DRS are resistant to the action of antifungal drugs (Ramage *et al.*, 2004).

Many studies have demonstrated drug resistance of *Candida* spp. biofilms. These include the growth of *Candida* biofilms on different surfaces including, cylindrical cellulose filter (Baillie and Douglas, 1998b; Baillie and Douglas, 1999b), polystyrene (Ramage *et al.*, 2001), silicon elastomer (Chandra *et al.*, 2001a), polyurethane (Lewis *et al.*, 2002) and denture acrylic (Chandra *et al.*, 2001b).

The mechanisms by which *C. albicans* biofilms resist the action of antifungal agents are, as yet, not known, however, various mechanisms have been proposed to explain the recalcitrance resistance of *C. albicans* biofilms to antimicrobial agents (Douglas, 2003). It has been suggested that drug resistance may arise as a result of surface-induced gene expression (Baillie and Douglas, 2000) and may also depend on the phase of biofilm growth (Mukherjee *et al.*, 2003). These have been described previously in more detail (refer to section 1.4.2).

In order to study biofilm perturbation, results must be comparable and reproducible from experiment to experiment, hence in this study, CDFF was used in order to grow replicate biofilms over time in a steady-state system.

The aim of this chapter was to assess the *in vitro* susceptibility of *C. albicans* biofilms to fluconazole, miconazole and chlorhexidine.

5.2 MATERIALS AND METHODS

Previously characterized clinical isolates of *Candida albicans* genotype A and B (refer to section 2.2.2) were used as the inoculum for the susceptibility experiments. The nutrient source in all experiments was an artificial saliva (refer to section 2.3). Self-cured acrylic denture disks were used as substrata and prepared to the same surface roughness ($R_a = 2.5 \mu\text{m}$) using silicon carbide grit as described previously (refer to section 2.1.1).

Antifungal agents commonly used to treat DRS were tested against *C. albicans* biofilms. Firstly, the planktonic MIC of fluconazole, miconazole and chlorhexidine was determined for *C. albicans* genotype A and B, as described previously (refer to section 2.5.1 and 2.5.2). The MIC of fluconazole and miconazole was read as the lowest concentration that prevented a colour change from blue to pink. Figure 5.1 shows examples of the MIC for some of the isolates utilising Alamar blue to indicate a colour change and *C. albicans* growth from those isolates. The MIC for chlorhexidine was assessed by the lowest concentration of the agent that prevented the growth of *C. albicans* on SDA.

Using the previously determined planktonic MIC as a reference, different concentrations of fluconazole, miconazole (Table 5.1) and chlorhexidine (Table 5.2) that correspond to times planktonic MIC (X MIC) were subsequently used against both genotypes of *C. albicans* biofilms.

Initially, biofilms of *C. albicans* genotype A and B were grown on the acrylic disks in the CDFP for 72 h before being removed and treated with fluconazole (0.125, 0.25, 0.5, 1 and 2 $\mu\text{g/ml}$) for 1, 24 and 48 h. Subsequently, higher concentrations of fluconazole or miconazole were used (0.25, 2, 16, 64 and 256 $\mu\text{g/ml}$). Chlorhexidine was also used (0.019, 0.15, 0.3, 1.25 and 2.5 %) against these biofilms for 1, 5 and 15

minutes.

In separate experiment, the susceptibility of biofilms of different ages (2, 6, 24, 48 and 72 h) was also investigated. These biofilms were treated for 1, 24 and 48 h in fluconazole or miconazole (256 µg/ml) or in chlorhexidine (0.019 %) for 1, 5 and 15 minutes. The biofilms were then removed from the antifungal agents and placed in 1 ml of PBS and vortexed vigorously for 1 minute to remove any organisms from the surface and plated onto SDA. Plates were then incubated at 37 °C for 24 h. Subsequently, the post-biofilm MIC of any remaining colonies present on the agar was determined. This is achieved using NCCLS susceptibility testing using the same protocol as described previously (refer to section 2.5).

Table 5.1: Different concentrations of fluconazole or miconazole used against *C. albicans* biofilms related to the planktonic MIC.

Times planktonic MIC	Fluconazole or miconazole concentration ($\mu\text{g/ml}$)
0.5 X MIC	0.125
MIC	0.25
2 X MIC	0.5
4 X MIC	1
8 X MIC	2
64 X MIC	16
256 X MIC	64
1024 X MIC	256

Table 5.2: Different concentrations of chlorhexidine used against *C. albicans* biofilms related to the planktonic MIC.

Times planktonic MIC	Chlorhexidine concentrations (%)
0.5 X MIC	0.019
MIC	0.04
4 X MIC	0.15
8 X MIC	0.3
32 X MIC	1.25
64 X MIC	2.5

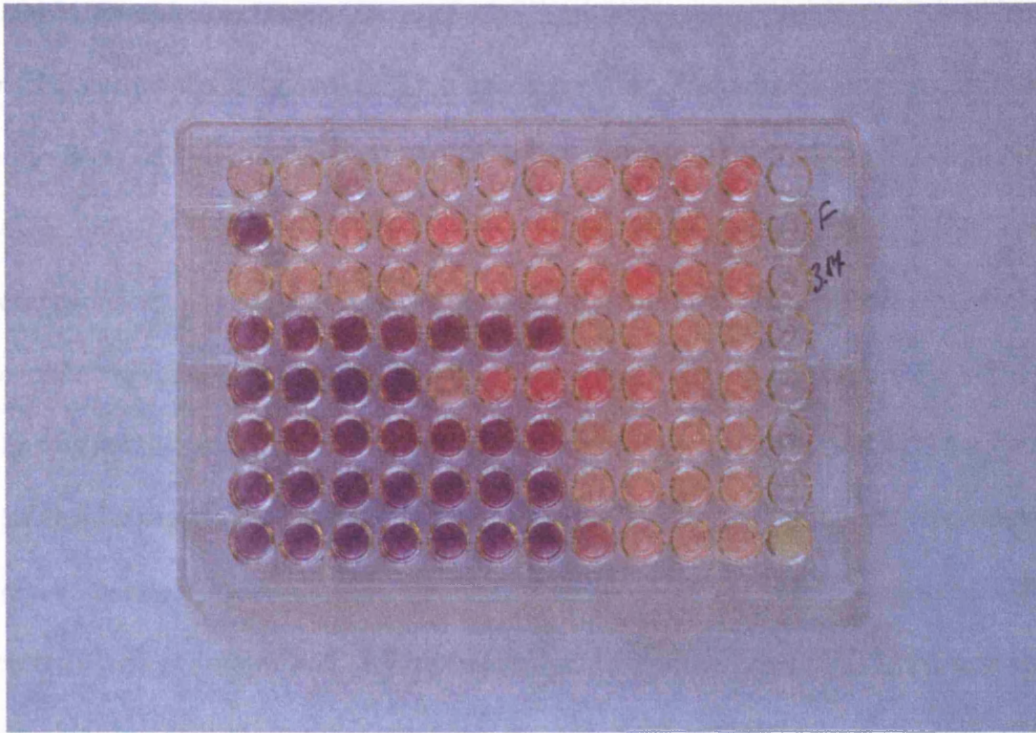


Figure 5.1: Almar blue containing multiwell plate used to test the antifungal susceptibility of *C. albicans*. Wells arranged within columns contain different concentrations of fluconazole and each row contains a different isolate.

5.3 RESULTS

The susceptibility of *C. albicans* biofilms to fluconazole, miconazole and chlorhexidine was investigated using CDFF. Initial studies showed that the planktonic MIC of *C. albicans* genotype A and B was 0.25 µg/ml for fluconazole and miconazole and 0.04 % for chlorhexidine.

Figure 5.2 shows the exposure of 72 h genotype A *C. albicans* biofilms to different concentrations of fluconazole (0.125, 0.25, 0.5, 1 and 2 µg/ml) for 1, 24 and 48 h. The results show that *C. albicans* biofilms were resistant to the action of fluconazole at all concentrations used up to 8 X MIC (2 µg/ml). Accordingly, higher concentrations of fluconazole were used against biofilms for the second experiment (Figure 5.3). These biofilms were still found to be resistant to fluconazole up to 1024 X MIC (256 µg/ml). Similar results were also observed for genotype B (Figure 5.4). Indeed there was no difference between the values obtained for biofilms of each genotype. The susceptibility of genotype A *C. albicans* biofilms of various ages (2 – 72 h) was also investigated by exposing these biofilms to sub-biofilm MIC concentration (256 µg/ml) of fluconazole for 1, 24 and 48 h (Figure 5.5a-c). At an early biofilm age (2 h), *C. albicans* biofilms were susceptible to fluconazole, however, the degree of susceptibility decreased with an increase in biofilm age. At no time point were there any significant differences ($p > 0.05$) between any of the control and test biofilms. However, compared to the control biofilms, a decrease of approximately 1 log₁₀ ($p < 0.05$) was observed during early biofilm formation at 2 and 6 h and at 2 h when exposed to fluconazole for 24 h (Figure 5.5b) and 48 h (Figure 5.5c) respectively.

The same series of experiments were also carried out for miconazole (Figure 5.6, 5.7 and 5.8). It was also demonstrated that 72 h biofilms of *C. albicans* genotype A and B were resistant to miconazole up to 1024 X MIC (256 µg/ml). Again no difference was

observed between the two genotypes. When genotype A *C. albicans* biofilms of various ages were exposed to (256 µg/ml) miconazole (Figure 5.8a-c), a significant decrease ($p < 0.05$) in CFU was observed during early biofilm formation at 2 and 6 h compared to the control biofilms.

Figure 5.9 shows the susceptibility of genotype A *C. albicans* biofilms to various concentrations (0.019, 0.15, 0.3, 1.25 and 2.5 %) of chlorhexidine for 1, 5 and 15 minutes. The results show that chlorhexidine was effective at inhibiting biofilm growth at a concentration of 0.3 %, however, significant reductions ($p < 0.05$) of over 2 log₁₀ CFU was also observed when the biofilm was exposed to 0.15 % for 5 and 15 minutes, compared to a 1 minute exposure. In contrast, when *C. albicans* genotype B biofilms were treated under the same conditions (Figure 5.10), there was total inhibition of growth at a concentration of 0.15 %. When biofilms of various ages were exposed to sub-MIC (0.019 %) concentration of chlorhexidine for 1, 5 and 15 minutes (Figure 5.11a-c), a decrease in CFU of *C. albicans* were observed during early biofilm formation (2 – 6 h). When the exposure time was extended to 15 minutes, greater reductions in viable counts were observed at the early time periods. When the MIC of post biofilm organisms were determined, the results were found to be the same as those obtained by planktonic cells prior to treatment with antifungals.

In summary, the results have shown that *C. albicans* biofilms were highly resistant to ($\leq 1024 \times$ MIC) fluconazole and miconazole compared to the same cells grown in suspension. However, in contrast, chlorhexidine inhibited the growth of *C. albicans* biofilms at concentration of up to 8 x MIC (Table 5.3). When biofilms of different age were exposed to sub-MIC levels of antifungal agents, both miconazole and chlorhexidine were effective in inhibiting growth of early biofilm formation (2 and 6 h).

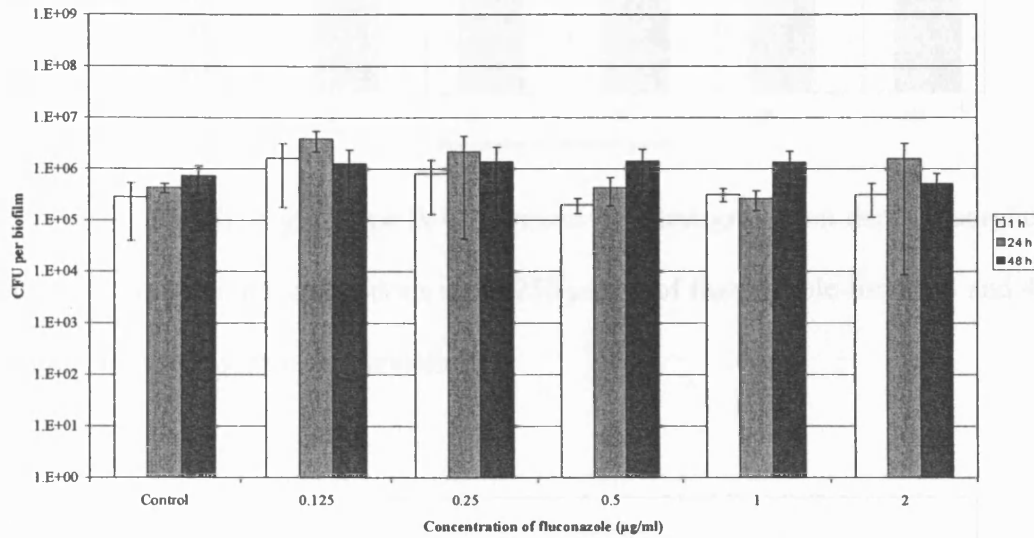


Figure 5.2: Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 2 µg/ml of fluconazole for 1, 24 and 48 h. Error bars represent standard deviations.

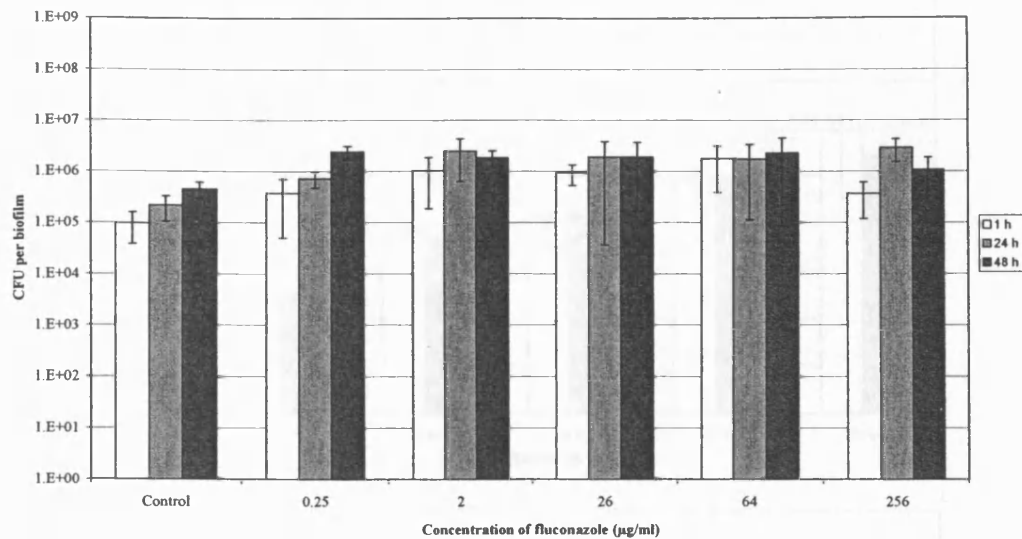


Figure 5.3: Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 256 µg/ml of fluconazole for 1, 24 and 48 h. Error bars represent standard deviations.

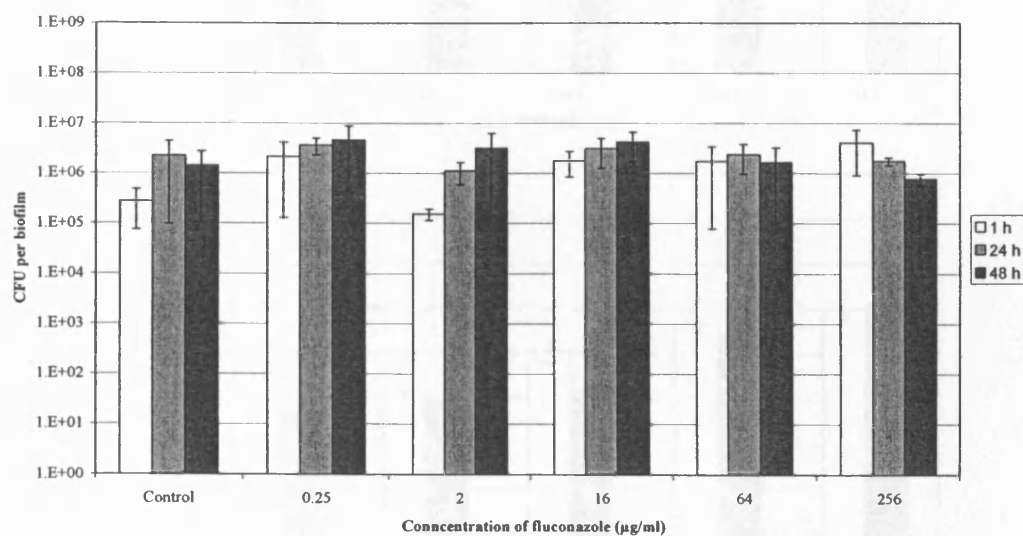


Figure 5.4: Growth of genotype B *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 256 µg/ml of fluconazole for 1, 24 and 48 h. Error bars represent standard deviations.

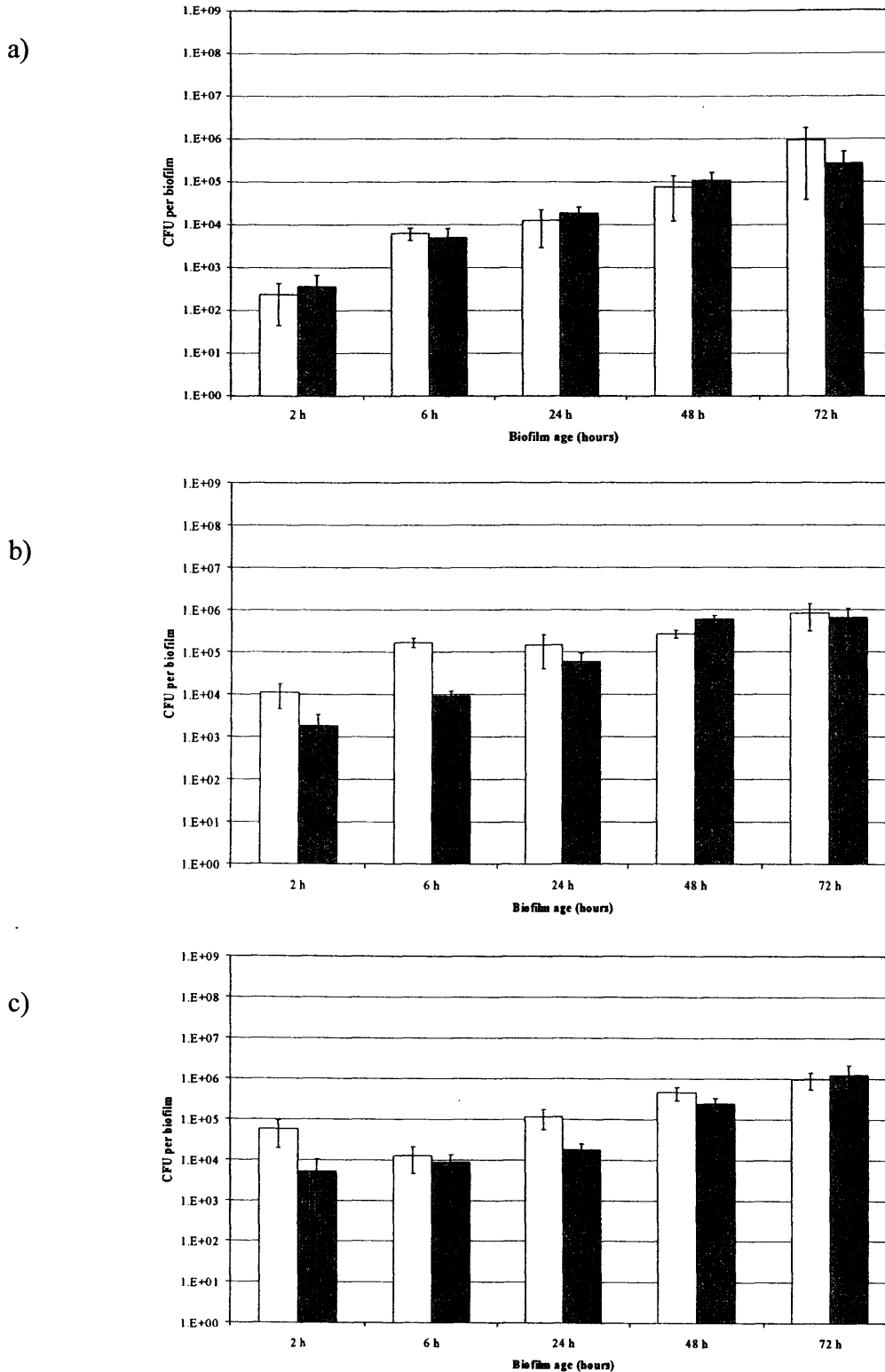


Figure 5.5: Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 2, 6, 24, 48 and 72 h and exposed to 256 $\mu\text{g/ml}$ of fluconazole for; a) 1 h, b) 24 h and c) 48 h. Open bars = control and filled bars = exposure to fluconazole. Error bars represent standard deviations.

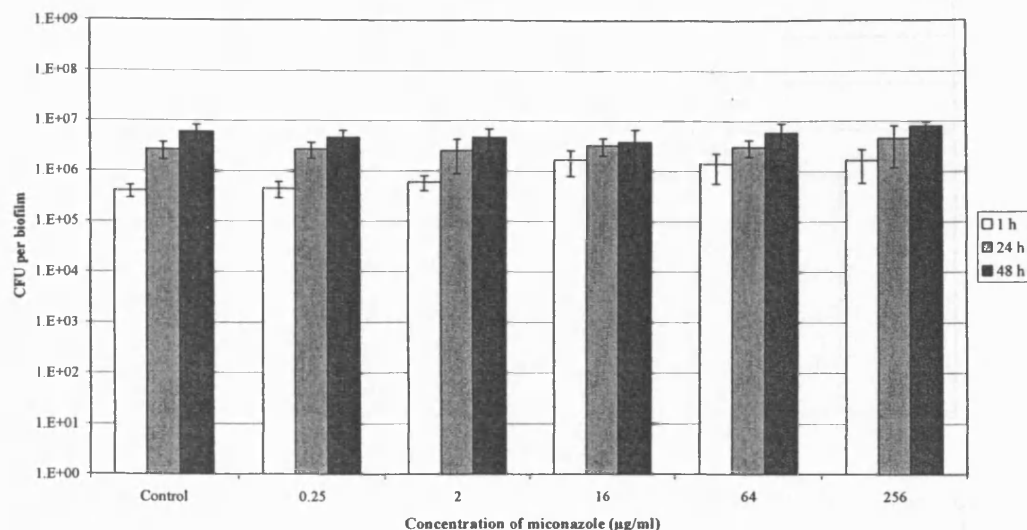


Figure 5.6: Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 256 µg/ml of miconazole for 1, 24 and 48 h. Error bars represent standard deviations.

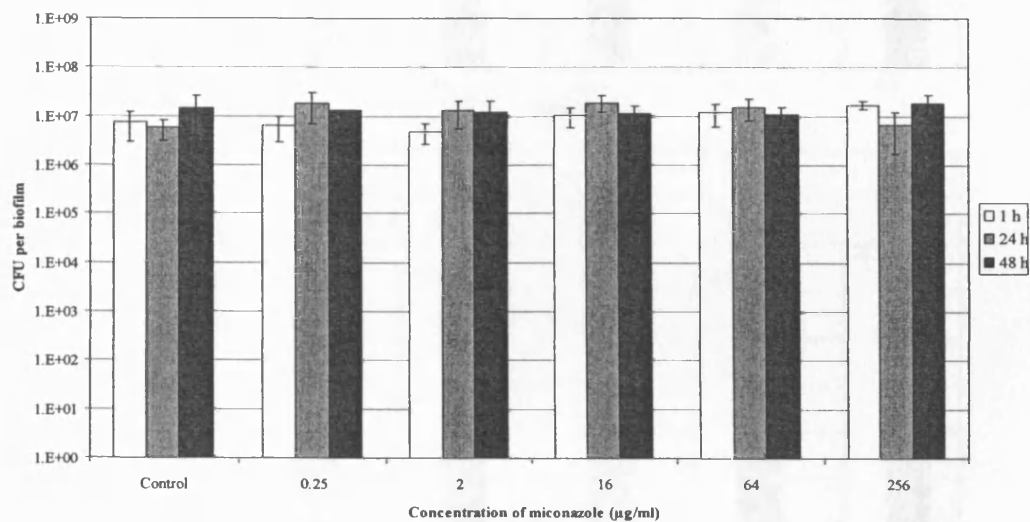


Figure 5.7: Growth of genotype B *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 256 µg/ml of miconazole for 1, 24 and 48 h. Error bars represent standard deviations.

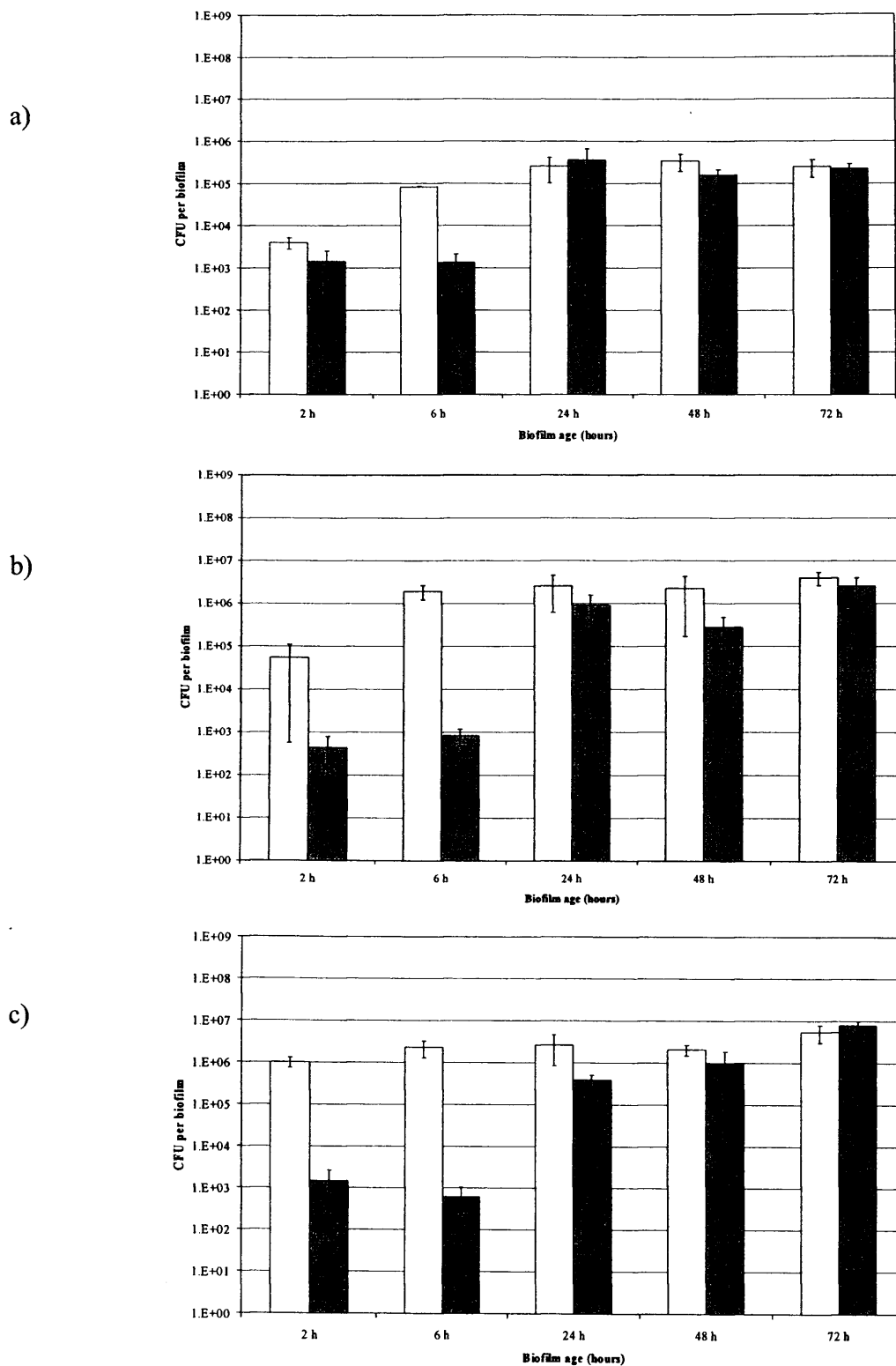


Figure 5.8: Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 2, 6, 24, 48 and 72 h and exposed to 256 $\mu\text{g/ml}$ miconazole for; a) 1 h, b) 24 h and c) 48 h. Open bars = control and filled bars = exposure to miconazole. Error bars represent standard deviations.

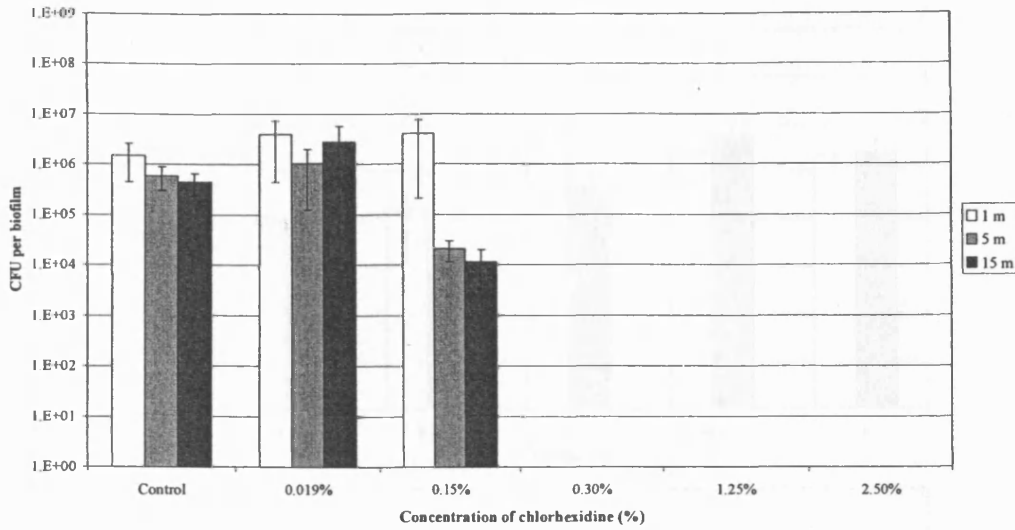


Figure 5.9: Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 2.5 % of chlorhexidine for 1, 5 and 15 minutes. Error bars represent standard deviations.

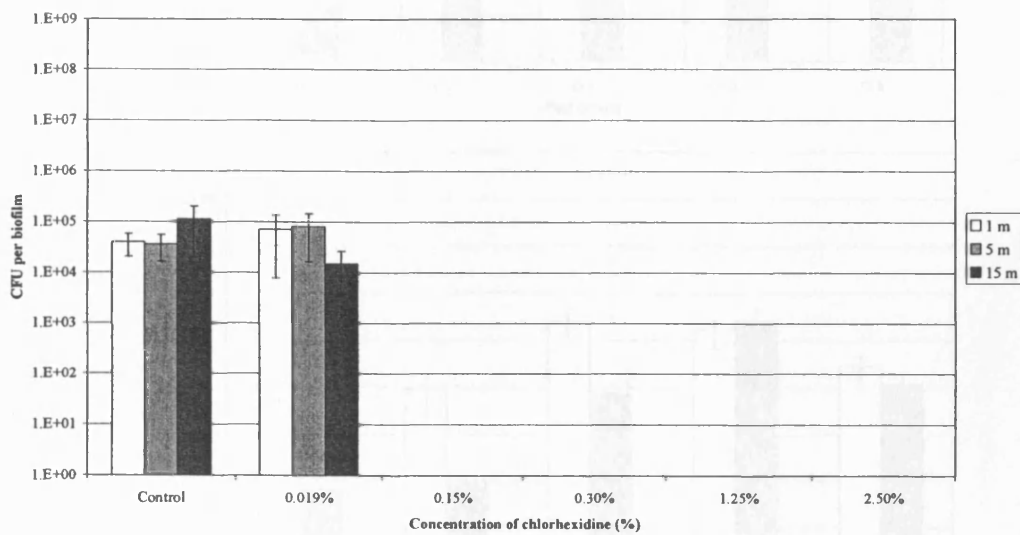


Figure 5.10: Growth of genotype B *C. albicans* biofilms grown on denture acrylic for 72 h and exposed to concentrations up to 2.5 % of chlorhexidine for 1, 5 and 15 minutes. Error bars represent standard deviations.

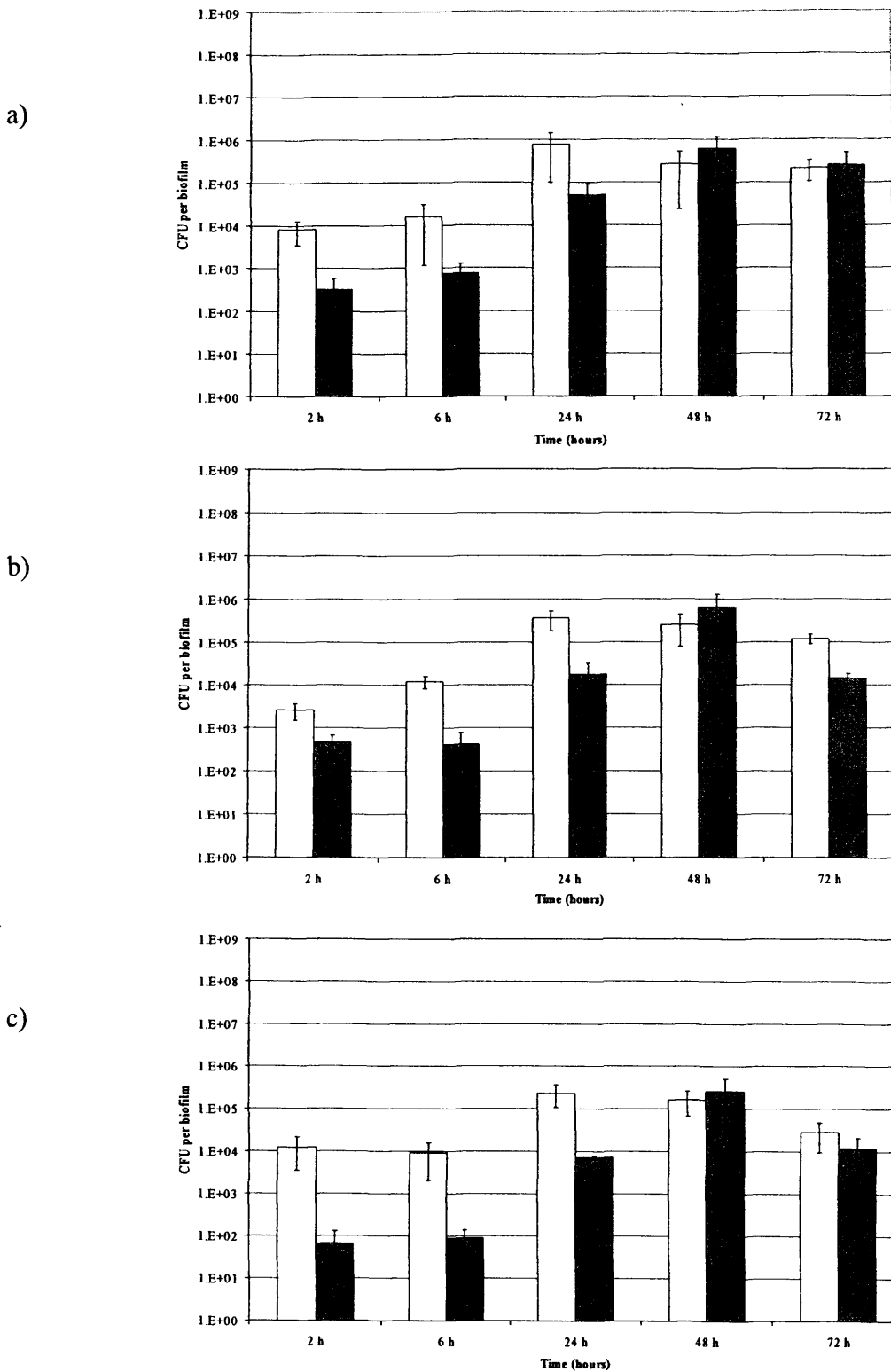


Figure 5.11: Growth of genotype A *C. albicans* biofilms grown on denture acrylic for 2, 6, 24, 48 and 72 h and exposed to 0.019 % chlorhexidine for; a) 1 minute, b) 5 minutes and c) 15 minutes. Open bars = control and filled bars = exposure to chlorhexidine. Error bars represent standard deviations.

Table 5.3: Concentration of fluconazole, miconazole and chlorhexidine required to inhibit growth of *C. albicans*.

Agent	Planktonic	72 h biofilms	x MIC
Fluconazole ($\mu\text{g/ml}$)	0.25	>256	>1024
Miconazole ($\mu\text{g/ml}$)	0.25	>256	>1024
Chlorhexidine digluconate (%)	0.04	0.3	8

5.4: DISCUSSION

Biofilm resistance is a phenomenon consistently expressed across microbial biofilm systems (Hawser and Douglas, 1995; Donlan and Costerton, 2002; Gilbert *et al.*, 2002). Such resistance may explain the persistence of many infections (Costerton *et al.*, 1999) and is likely to be of great clinical relevance (refer to section 1.3.7).

The mechanisms contributing to increased antifungal resistance of candidal biofilms have not been defined. Several possibilities have been investigated, including effect of growth rate (Baillie and Douglas, 1998a), nutrient limitation (Baillie and Douglas, 1998b), the protective nature of exopolymer matrix (Baillie and Douglas, 2000), multidrug efflux pumps (Ramage *et al.*, 2002a) and the differential expression of candidal biofilm genes that are not expressed when in the planktonic phase of growth (Chandra *et al.*, 2001a). Additionally, Baillie and Douglas have also shown that biofilms formed on two different types of catheter material possess different susceptibilities to amphotericin B, suggesting that drug resistance may be related to the expression of surface-induced genes (Baillie and Douglas, 2000).

Previous studies have showed that *C. albicans* biofilms grown on denture acrylic exhibits resistance to antifungal agents (amphotericin B, nystatin, fluconazole and chlorhexidine) used to treat DRS (Chandra *et al.*, 2001b). The results of the present study are in agreement with earlier studies in that *C. albicans* biofilms were highly resistant to the antifungal agents (fluconazole and miconazole) and an antimicrobial agent (chlorhexidine), although this agent was able to inhibit biofilm growth at lower concentrations. It has been shown previously that fluconazole was ineffective against candidal biofilms (Ramage *et al.*, 2002b). Indeed Kuhn and co-workers showed that *Candida* biofilms formed on silicon elastomer rapidly (within 6 h) exhibited fluconazole resistance compared to planktonic cells (Kuhn *et al.*, 2002a). Hawser and

Douglas also showed that *C. albicans* biofilm (48 h) formed on catheter material were more resistance than planktonic cells to the action of five clinically important antifungal agents (amphotericin B, flucytosine, fluconazole, itraconazole, and ketokonazole) (Hawser and Douglas, 1995).

It has been shown previously using planktonic cells that strains of *C. albicans* genotype A, B and C have different susceptibilities to flucytosine (McCullough *et al.*, 1999a). The results of the present work have also highlighted differences between the susceptibilities of two genotypes when exposed to chlorhexidine. However, exposure to fluconazole or miconazole showed no such differences.

When re-suspended cells were grown on solid media and then the MIC tested, these were no longer resistant to the three agents thus demonstrating that the phenotype was reversible, a phenomenon which has previously been demonstrated (Baillie and Douglas, 1998a). Kuhn and co-workers have investigated the effect of pre-incubation of initial culture of *C. albicans* with sub-MIC levels of antifungal drugs. They showed a decrease in the ability of *C. albicans* cells to form a biofilm (Kuhn *et al.*, 2002b). In the present study *C. albicans* biofilms of different ages were also exposed to sub-MIC level of antifungal agents and the results show more susceptibility of early biofilm formation than mature biofilm thus indicating the importance of this formation phase. Biofilm resistance mechanisms are complex and multifactors may be involved (Ramage *et al.*, 2002a). Although the exact mechanism of biofilm resistance is not known, a possible resistance mechanism may be related to a slow growth rate within biofilms that delay the uptake of drugs (Donlan and Costerton, 2002; Douglas, 2003), and increased EPS production. Previously, it has been shown that mature biofilms contain more EPS material than younger biofilms (Chandra *et al.*, 2001a). This material may preclude drug penetration (Gilbert *et al.*, 1997; Douglas, 2003). In the

current study, early biofilms were more susceptible to drugs compared to a mature biofilms, and biofilm resistance increased with increasing biofilm age. This would expect that cells within young biofilms have a higher growth rate and contain less EPS material. However, a previous study has shown that low growth rate did not have a major influence on the susceptibilities of a biofilm to the drug (Baillie and Douglas, 1998a). In addition, it has been demonstrated that biofilm matrix does not constitute a significant barrier to antifungal drugs indicating that drug resistance is unrelated to the extent of matrix formation (Baillie and Douglas, 2000). Thus the matrix may contribute to overall resistance, but is not considered as a sole determinant of resistance (Ramage *et al.*, 2002a) and that it may play only a minor role in biofilm resistance (Baillie and Douglas, 1998a).

Chandra and co-workers have evaluated the structure of *C. albicans* biofilms and indicated three phases of growth; early, intermediate and mature phases extending over 72 h. They showed that mature biofilms were highly resistant to fluconazole, amphotericin B, nystatin and chlorhexidine and this resistance developed over time, coincident with biofilm maturation (Chandra *et al.*, 2001a). Hence, the current study was carried out over the same time period to encompass these phases.

Recently, it has been reported that newly developed antifungal agents were effective against *Candida* biofilms. For example, caspofungin displayed ≥ 99 % killing of *C. albicans* cells within a biofilm (Ramage *et al.*, 2002b). Kuhn and co-workers have shown that *Candida* biofilms were resistance to two new triazoles (voriconazole and ravuconazole), however, lipid formulations of amphotericin B and echinocandins appeared to have activity against *Candida* biofilm (Kuhn *et al.*, 2002b). Bachmann and co-workers also showed that caspofungin can affect cellular morphology and metabolic status of cells within the biofilm and displayed a potent *in vitro* activity

against *C. albicans* biofilms (Bachmann *et al.*, 2002). These drugs have been recently introduced and all of the experiments were carried out *in vitro* using biofilm or animal models. Hence, the efficacy of these drugs with its pharmacokinetic and toxicological properties needs further investigation before these new antifungals can be used for the treatment of oral candidosis *in vivo*.

To understand biofilm development, data must be reproducible from experiment to experiment. One approach to reproducibility is to develop constant depth reactors where surface growth is periodically removed to maintain a constant geometry. Such a device, which employs a mechanical scraper bar, is the CDFF which was designed to investigate the growth of dental plaque organisms. Although previous studies have indicated differences in susceptibilities between antifungal compounds over time (Chandra *et al.*, 2001a), none have demonstrated this using an *in vitro* model designed to study oral biofilms. Indeed, although this model has been used extensively for studying the susceptibility of bacterial biofilms this work describes its first application for studying the susceptibility of fungal biofilms.

In conclusion, the results of this study shown that *C. albicans* biofilms are highly resistant to both antifungal and antimicrobial agents and this resistance depends upon the age of biofilm and on the type of the agent used. Interestingly, there were large differences in the degree of susceptibility of *C. albicans* biofilms between fluconazole, miconazole and chlorhexidine with the latter being the most effective. The observed differences for biofilms of different ages gives some indication as to the mechanism involved in reduced biofilm susceptibility.

Chapter 6

**Composition of *in vitro* denture plaque biofilms and susceptibility to
antifungals**

6.1 INTRODUCTION

Previous chapters have focused upon single species *C. albicans* biofilms. However, in order to study the microflora associated with DRS in which a combination of *C. albicans* and other microorganisms is to be expected (Kulak *et al.*, 1997), a mixed species biofilm will be investigated in this chapter.

Most biofilms formed in the oral cavity are composed of a mixture of microorganisms. This adds to the inter and intra-species interactions and to the complexity of the mixed-species biofilm present. *C. albicans* remains the most commonly isolated fungal species from the mixed bacterial-fungal biofilm of DRS patients (Martin-Mazuelos *et al.*, 1997; McMullan-Vogel *et al.*, 1999). The formation of *Candida* biofilms on dentures may assist the survival of fungal cells and contribute to the progression of the disease process in patients with DRS (Ramage *et al.*, 2004).

Although the primary causative organism in DRS is *C. albicans*, other oral bacteria (Koopmans *et al.*, 1988) and non-*albicans Candida* species (McMullan-Vogel *et al.*, 1999; Dorko *et al.*, 2001) are also present. Adhesion interactions between *C. albicans* and oral bacteria are generally thought to play a critical role in microbial colonisation of denture acrylic and consequently DRS (Millsap *et al.*, 1999). For example, oral bacteria play a complex role in regulating the adhesion of *C. albicans* to acrylic denture surfaces (Samaranayake *et al.*, 1980) and possibly enhance fungal attachment via co-aggregation and synergistic interactions with the fungi (Sato *et al.*, 1997).

Dentures are considered as an important predisposing factor for DRS (Budtz-Jorgensen, 1990). Denture plaque can function as a biofilm and represent a protective reservoir for oral microbes (Budtz-Jorgensen *et al.*, 1996; Chandra *et al.*,

2001b) especially in ill-fitting dentures and in patients with poor oral hygiene (Ellepola and Samaranayake, 1998). In an examination of 50 denture stomatitis patients, it was found that greater numbers of *Candida* spp. were recovered from smears prepared from the fitting surface of upper dentures than from those on the palatal mucosa. It was concluded that treatment intended to reduce the number of *Candida* spp. present should be directed to the denture rather than the mucosa covering the denture (Davenport, 1970). However, removal of these biofilms from dentures is difficult due to the inherent resistance of biofilms to antimicrobial agents (Gilbert *et al.*, 1997).

Traditional treatment of candidal infections is bimodal: firstly to remove the source and secondly to eliminate the infection from tissues (McIntyre, 2001). These procedures include oral and denture hygiene improvement, discontinuation of nocturnal denture wear, the use of topical or systemic antifungal agents and eventual denture replacement (Webb *et al.*, 1998c; Pires *et al.*, 2002). Fundamentally, patients must maintain adequate oral and denture hygiene in order to reduce the likelihood of DRS (Pires *et al.*, 2002). For more detailed of DRS treatment, refer to section 1.5.6.

Several studies have investigated the susceptibility of *C. albicans* single-species biofilms to antifungal agents (Chandra *et al.*, 2001a). However, there are no data concerning antimicrobial susceptibility of fungi within a microflora more likely to be associated with DRS. Hence, the aim of the present study was to determine the composition of the *in vivo* and *in vitro* denture plaque population in which *Candida* spp. are maintained and the susceptibility of this organism when it is part of mixed fungal and bacterial biofilms *in vitro*.

6.2 MATERIALS AND METHODS

Samples were obtained from 10 individuals attending the Eastman Dental Hospital, UCLH, as described previously (refer to section 2.2.3). Ethical approval for this study was granted by the joint University College of London/University College London Hospital Trust Committee on the ethics of Human Research (refer to section 2.2.1). Serial dilutions were plated onto a range of non-selective and selective agars (refer to section 2.4.5.2). Images of *Candida* spp. grown on SDA of both control and DRS patients were visualised microscopically (x 400 magnification). *Candida* spp. which were recovered from the denture and mucosa samples were further identified to the species level using different methods including, CHROMagar, API[®] Candida kit and CornMeal Agar as described previously (refer to section 2.9).

Denture acrylic (self-cured) disks were used as the substrata for biofilm production. All the disks were prepared to the same surface roughness ($R_a = 2.5 \mu\text{m}$) using silicon carbide grit on a Knuth Rotor as described previously (refer to section 2.1.1). The R_a values for denture acrylic among patients wearing dentures vary considerably; however, the value used is within the range expected for the fitting surface of denture acrylic. Artificial saliva was used as nutrient source in these experiments (refer to section 2.3).

A 2 ml mixture consisting of denture and mucosa samples from each DRS patient was used as an inoculum for the CDFE experiments as described previously (refer to section 2.4.2.2). Biofilms were allowed to grow on acrylic disks in the CDFE for 504 h. At various times interval (2, 6, 24, 48, 72, 96, 336 and 504 h), pans were aseptically removed from the CDFE and the predominant bacterial and candidal genera were determined. Biofilms from the same experiment were tested against fluconazole, miconazole and chlorhexidine. Disks, containing the biofilms, were removed from

the CDFP after 72 h growth and placed in fluconazole or miconazole each at concentrations of 0.25, 2, 16, 64, and 256 $\mu\text{g/ml}$ and chlorhexidine digluconate at concentrations of 0.019, 0.15, 0.3, 1.25, and 2.5 %. The biofilms were then treated for 24 h in fluconazole or miconazole or for 15 minutes in chlorhexidine at 37°C. The biofilms were then vortexed vigorously for 1 minute to remove any cells from the surface and plated onto SDA for all antifungal agents and onto CBA and FAA for chlorhexidine sample.

In a separate experiment, the microbial genera from patients suffering from DRS were compared to those from 72 and 240 h biofilms obtained from CDFP prior to pulsing of the antifungal agents. After 240 h biofilm growth, the susceptibility of biofilms was determined against miconazole gel solutions (Daktarin[®] gel, Johnson and Johnson, Beerse, Belgium; dissolved in dH₂O to give a final concentration of 48 $\mu\text{g/ml}$) and chlorhexidine (0.2 %) being pulsed into the CDFP for 5 minutes, twice daily for 168 h. All of the CDFP experiments were carried out in duplicate. Statistical analysis was performed using the student's t-test (two-sample) to determine p-values.

6.3 RESULTS

The aim of this part of the study was to produce denture plaque biofilms in an *in vitro* model. Firstly, bacteria and *Candida* genera recovered from patient samples were identified from control subjects and from those patients with DRS (Table 6.3). The number of *Candida* recovered from the denture fitting surface was higher than those originating from the mucosal surface. Through the visualization of colony morphology on SDA, 4 different isolates were observed from the control group (Table 6.1), while 6 isolates were seen in the DRS group (Table 6.2). An attempt was made to further identify *Candida* to the species level using three different identification methods (CHROMagar, API[®] Candida kit and CornMeal Agar). *C. albicans* was the most predominant species identified from both groups i.e. the mucosa and denture samples. However, using the CHROMagar and API[®] Candida tests, identification of one species was not always possible.

Table 6.3 shows the range in CFU of selected genera within the patient samples and the mixed-species biofilms grown from these samples. There was a large range in microbial numbers between the control group and those patients with denture related stomatitis. Hence, there were no significant differences between any of the two patient groups ($p > 0.05$) or the sample sites ($p > 0.05$). Interestingly, 50 % of the sites from the control group contained no *Candida* species (range 0 - 3.7×10^5 CFU per sample), but all the DRS patients harboured this genus at both sites (range 8.2×10^3 - 2.8×10^6 CFU per sample). *Candida* spp. were always isolated *in vitro* from the CDFF with a range of 2.7×10^6 - 1×10^7 CFU per biofilm.

The percentage range of genera in DRS patients and in the CDFF is shown in Table 6.4. The *Streptococcus* spp. and *Actinomyces* spp. predominated with up to 76 % and 55 % of the population represented by these genera respectively. The proportions of

Candida spp. *in vitro* and *in vivo* were similar with a smaller percentage range observed in the model system.

The growth of *in vitro* mixed-species biofilms derived from DRS patients sample is shown in Figure 6.1. The results show that all microbial genera originally recovered from the DRS patients were able to grow in the model system. There was a steady increase in both bacterial and fungal numbers until 96 h with a total count of approximately 2×10^8 CFU/biofilm. After this time biofilm growth was stable except for the *Lactobacillus* spp. which dropped dramatically at 336 h before recovering towards the end of the experiment. The susceptibility of *Candida* spp. within this mixed-species biofilms (72 h) were tested against fluconazole or miconazole as detailed in Figure 6.2. This genus was resistant to both antifungal agents at a concentration of $\leq 256 \mu\text{g/ml}$. Chlorhexidine was ineffective at inhibiting growth when used in a concentration of up to 0.3 % (Figure 6.3). However, growth of the *Candida* spp. was significantly reduced ($p < 0.05$) by approximately $4 \log_{10}$ in 1.25 % chlorhexidine and no growth was observed in 2.5 % chlorhexidine concentration. Figure 6.4 shows the effect of different concentrations of chlorhexidine on both the aerobic and anaerobic bacterial species compared to *Candida* spp. It can be seen that the total aerobic and anaerobic populations were resistance to chlorhexidine at concentrations of up to 0.3 %. When the mixed-species biofilms were exposed to 1.25 % concentration of chlorhexidine, a significant reduction ($p < 0.05$) of approximately $3 \log_{10}$ was observed. However, these bacteria were still resistance to 2.5 % concentration of chlorhexidine despite a significant reduction ($p < 0.05$) of approximately $2 \log_{10}$ at this higher concentration.

The susceptibility of the microcosm denture plaque was also tested by pulsing in miconazole and chlorhexidine to represent a treatment regime for 168 h (Figure 6.5).

The total anaerobic count was unaffected by the initial pulse, but over a period of 96 h the total count was reduced by approximately 3.5 \log_{10} ($p < 0.05$). After this time, between 144h and 168 h post-pulsing, there was an increase in the total viable counts by over 1 \log_{10} . The number of *Candida* spp. was also reduced by over 2 \log_{10} (a 99.9 % kill) until after 120 h where again an increase in numbers was observed.

Table 6.1: Identification of different *Candida* spp. of control patients.

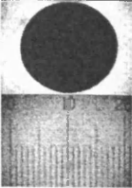
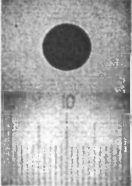
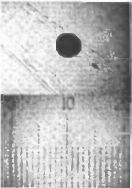
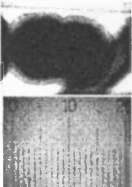
Description	Image on SDA	Mucosa	Denture	Identification by CHROMagar	Identification by API [®] Candida Kit	Identification by CornMeal agar
Large convex circular white		+	+	<i>C. albicans</i> / <i>C. tropicalis</i> / others	<i>C. albicans</i>	<i>C. albicans</i>
Medium convex circular white		+	+	<i>C. albicans</i> / <i>C. tropicalis</i>	<i>C. albicans</i> / <i>C. tropicalis</i>	<i>C. albicans</i>
Small convex circular white		+	+	<i>C. albicans</i> / <i>C. tropicalis</i> / <i>C. krusei</i>	<i>C. albicans</i> / <i>C. keyfer</i>	Not identified
Hazy raised transparent		+	+	<i>C. krusei</i>	Not identified	Not identified

Table 6.2: Identification of different *Candida* spp. of DRS patients.

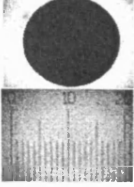
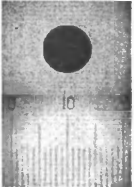

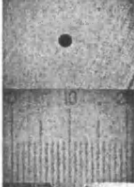


Description	Image on SDA	Mucosa	Denture	Identification by CHROMagar	Identification by API [®] Candida Kit	Identification by CornMeal agar
Large convex circular white		+	+	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>
Medium convex circular white		+	+	<i>C. albicans</i> / <i>C. tropicalis</i>	<i>C. albicans</i> / <i>C. tropicalis</i> / <i>C. famata</i>	<i>C. albicans</i>
Small convex circular white		+	+	<i>C. albicans</i> / <i>C. tropicalis</i> / <i>C. krusei</i>	<i>C. albicans</i> / <i>C. keyfer</i> / <i>C.</i> <i>famata</i>	Not identified
Very small convex circular white		+	+	Others	<i>C. kefyr</i> / <i>Saccharo-</i> <i>cerevisiae</i>	Not identified
Irregular convex shape large		+	-	<i>C. albicans</i> / <i>C. tropicalis</i> / <i>C. krusei</i>	<i>C. albicans</i>	<i>C. albicans</i>
Irregular convex shape small		+	-	<i>C. albicans</i> / <i>C. tropicalis</i> / <i>C. krusei</i>	<i>C. albicans</i> / <i>C. famata</i>	Not identified

Table 6.3: The range in number (CFU) of selected genera from patients with and without DRS compared to the numbers obtained *in vitro* (72 – 240 h).

	Control: mucosa n = 5	Control: Denture n = 5	DRS: Mucosa n = 5	DRS: Denture N = 5	Microcosm denture plaque n = 12
Total aerobes	2.6x10 ⁵ – 8.2x10 ⁶	2.2x10 ⁵ – 1.8x10 ⁶	8.3x10 ⁵ – 1.3x10 ⁷	7.4x10 ⁵ – 2.2x10 ⁷	7.8x10 ⁷ – 5.1x10 ⁸
Total anaerobes	7.2x10 ⁵ – 1.2x10 ⁸	4.5x10 ⁵ – 7.4x10 ⁷	1.7x10 ⁶ – 7.2x10 ⁶	5.7x10 ⁵ – 7.7x10 ⁷	1.6x10 ⁸ – 3.3x10 ⁸
<i>Actinomyces</i> spp.	3.4x10 ⁵ – 5.3x10 ⁶	4.8x10 ⁵ – 1.4x10 ⁷	2.9x10 ⁵ – 2.8x10 ⁶	2x10 ⁵ – 3.5x10 ⁷	4.7x10 ⁷ – 8.8x10 ⁷
<i>Candida</i> spp.	0 – 2.4x10 ⁴	0 – 3.7x10 ⁵	8.2x10 ³ – 6.5x10 ⁵	1.5x10 ⁴ – 2.8x10 ⁶	2.7x10 ⁶ – 1x10 ⁷
<i>Lactobacillus</i> spp.	0 – 7.6x10 ⁵	0 – 4.8x10 ⁷	0 – 3.4x10 ⁴	0 – 6.6x10 ³	3.9x10 ⁵ – 5.4x10 ⁵
<i>Streptococcus</i> spp.	2.2x10 ⁵ – 9.1x10 ⁵	2x10 ⁴ – 8.9x10 ⁶	3.6x10 ⁵ – 2.1x10 ⁶	3.7x10 ⁵ – 2.8x10 ⁷	5.2x10 ⁷ – 2.5x10 ⁸
<i>Veillonella</i> spp.	4.9x10 ⁴ – 1.4x10 ⁶	1.2x10 ⁴ – 1.1x10 ⁸	0 – 2.9x10 ⁵	0 – 1.2x10 ⁷	1x10 ⁷ – 1.3x10 ⁷

Table 6.4: The range (%) in compositions of genera present on the mucosa and denture surfaces of patients suffering from DRS and from the CDFP (combined data from 72 and 240 h). Data represent the range of each type of organism expressed as a percentage of the total anaerobic viable count.

	% range in DRS patients	% range in microcosm denture plaque
<i>Actinomyces</i> spp.	17 – 45	14 – 55
<i>Candida</i> spp.	0.5 – 9	2 – 3
<i>Lactobacillus</i> spp.	0 – 0.5	0.2 – 0.3
<i>Streptococcus</i> spp.	21 – 65	33 – 76
<i>Veillonella</i> spp.	0 – 15	3 – 8

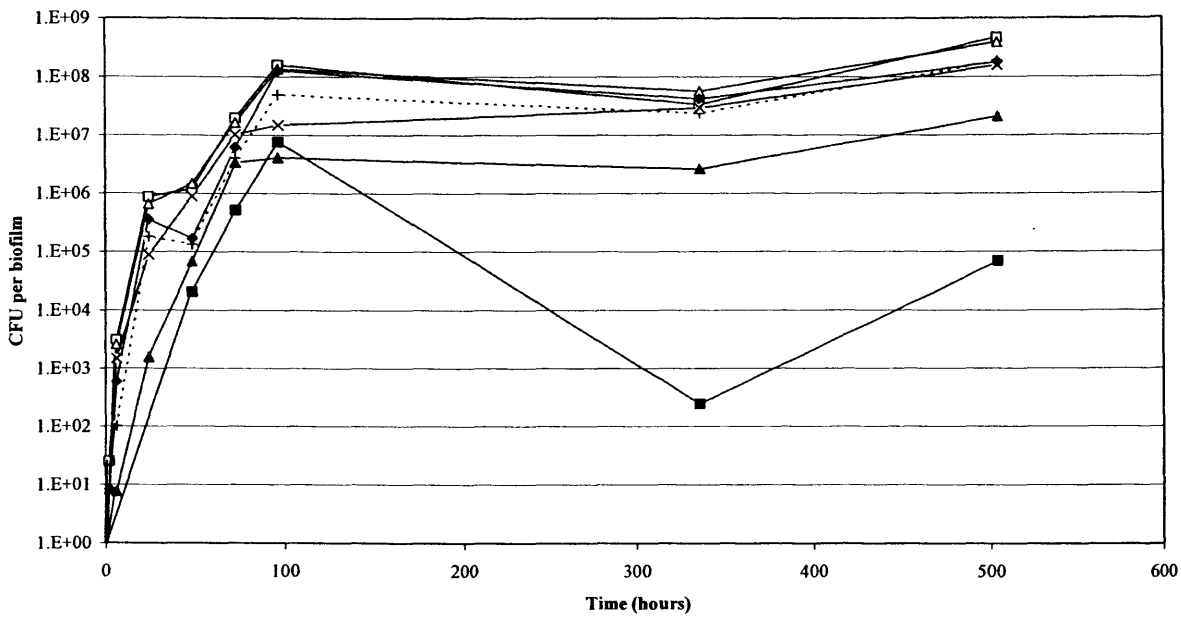


Figure 6.1: Viable count of microorganisms present in a mixed-species biofilm after growth in CDFP for 504 h. Symbols represent: aerobic count (open squares), anaerobic count (open triangles), *Actinomyces* spp. (filled diamonds), *Candida* spp. (dotted line), *Lactobacillus* spp. (filled squares), *Streptococcus* spp. (crosses) and *Veillonella* spp. (filled triangles).

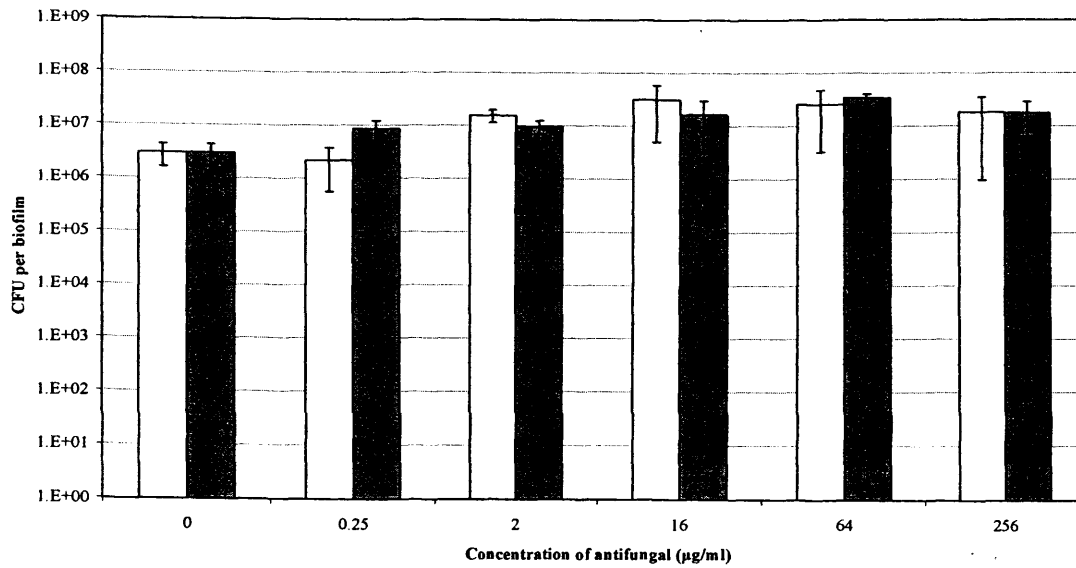


Figure 6.2: Viable counts of *Candida* spp. of the 72 h biofilm in different concentrations of fluconazole (open bars) or miconazole (filled bars). Error bars represent standard deviations.

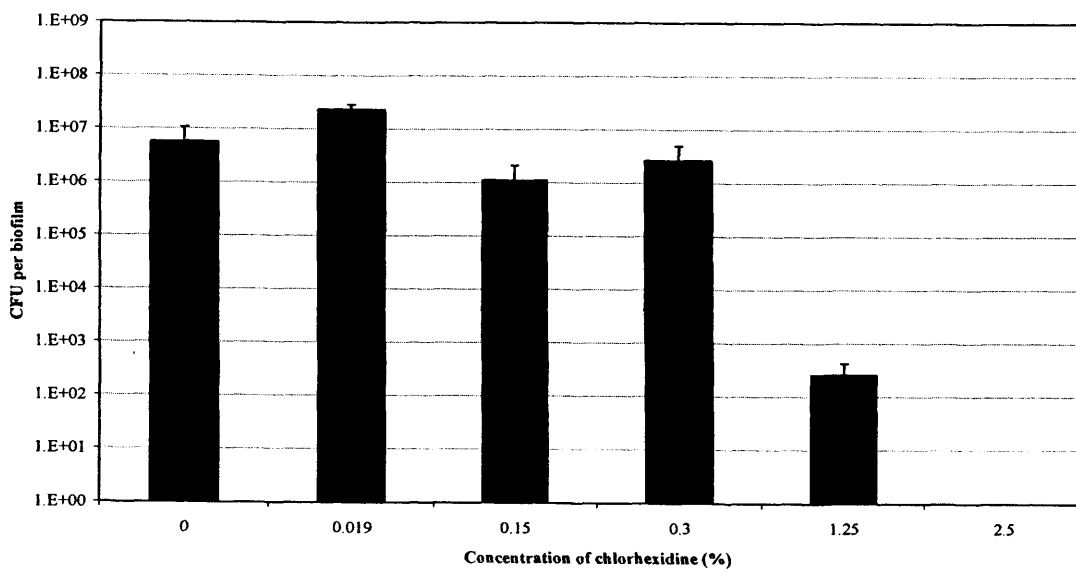


Figure 6.3: Viable counts of *Candida* spp. of the 72 h biofilm in different concentrations of chlorhexidine. Error bars represent standard deviations.

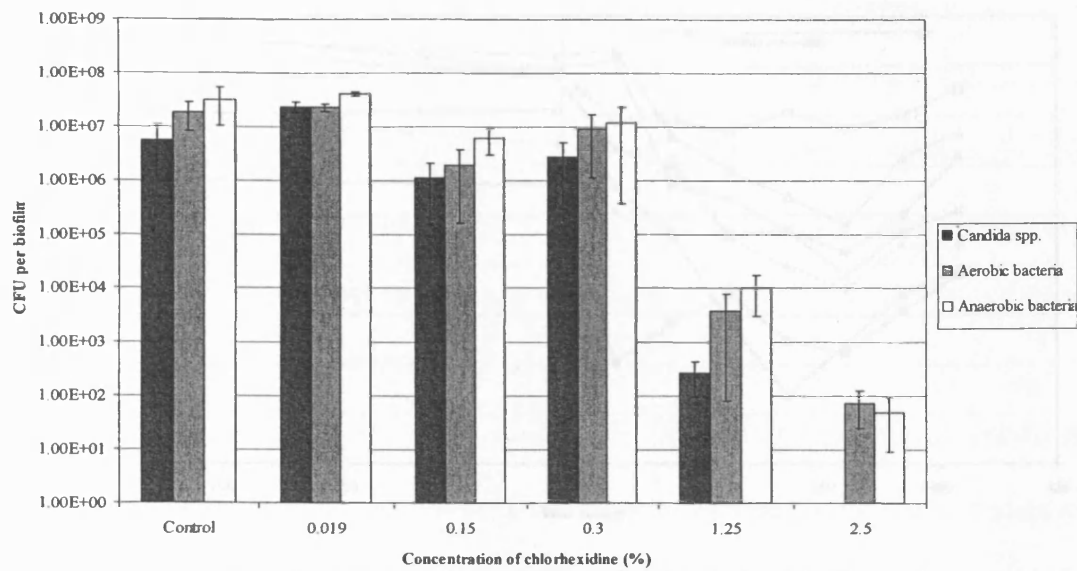


Figure 6.4: Viable counts of *Candida* species, aerobic and anaerobic bacteria of the 72 h biofilm in different concentrations of chlorhexidine. Error bars represent standard deviations.

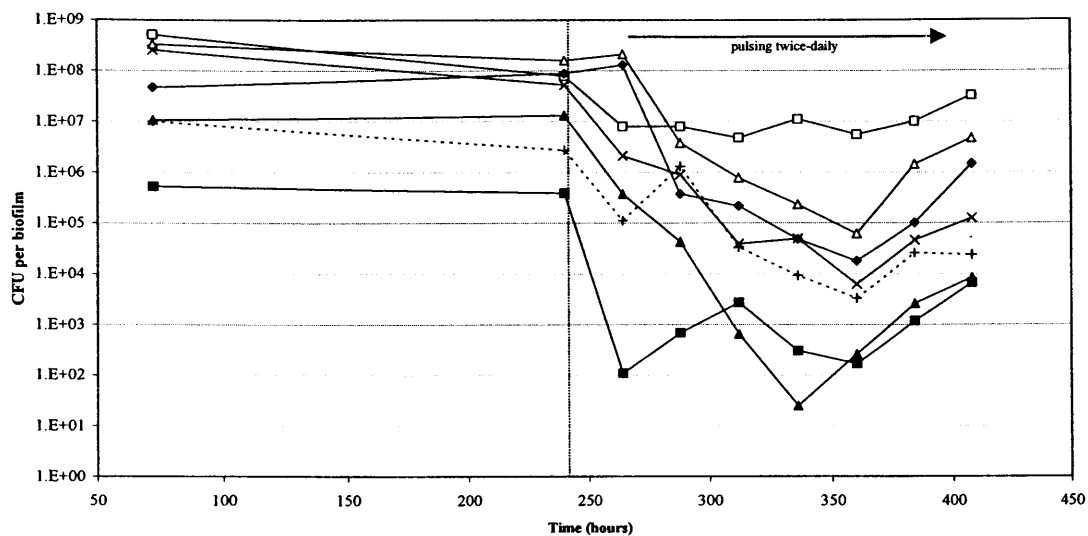


Figure 6.5: Number of microorganisms of a mixed-species biofilm prior to pulsing with miconazole and chlorhexidine and after 240 h pulsing. Further pulsing was carried out twice-daily. Symbols represent: aerobic count (open squares), anaerobic count (open triangles), *Actinomyces* spp. (filled diamonds), *Candida* spp. (dotted line), *Lactobacillus* spp. (filled squares), *Streptococcus* spp. (crosses) and *Veillonella* spp. (filled triangles).

6.4 DISCUSSION

Denture related stomatitis is a chronic infection which associated with denture wearers (Webb *et al.*, 1998b). However, there have been relatively few studies on such mixed bacterial-fungal biofilms generated *in vitro*.

Adherence of *C. albicans* to the acrylic surface is considered as the first step in the pathogenesis of DRS (Ellepola and Samaranayake, 1998). The denture acts as a reservoir for dissemination of fungal cells and continuous infection of the mucosa despite the presence of salivary antimicrobial factors (Budtz-Jorgensen *et al.*, 1996).

Additionally, the surface of the denture usually has micropits and imperfections that can harbour microorganisms (Ramage *et al.*, 2004) and may obscure mechanical or chemical removal of these organisms.

Different microbial species have frequently been reported and associated with DRS. The clinical samples from the present study were evaluated for the presence of several key oral microbial genera including *Candida* spp., *Actinomyces* spp., *Lactobacillus* spp., *Streptococcus* spp., and *Veillonella* spp. *Candida* spp. were recovered from all of the DRS patients compared to only half from the control samples, a result also observed by previous workers (Cross *et al.*, 2004).

The predominant organisms were streptococci, an observation which has also been reported previously (Koopmans *et al.*, 1988). The development of multi-species biofilm communities is influenced by co-aggregation (Foster and Kolenbrander, 2004) and bacteria are often associated with *Candida* spp. in mixed-species biofilms *in vivo* (Douglas, 2003). There is also evidence that co-aggregation of oral streptococci enhances fungal colonisation on denture surfaces thereby increasing *C. albicans* attachment (Verran and Motteram, 1987). Nair and Samaranayake also shown that adhesion of yeasts to acrylic surfaces is modulated by the pre-existing

bacterial flora on these surfaces (Nair and Samaranayake, 1996). Thus it is possible that this interaction of bacteria with *C. albicans* can influence colonization of the yeast to soft and hard surfaces in the oral cavity (Jenkinson *et al.*, 1990).

The increase of immunocompromised patients has resulted in the emergence of non-*albicans Candida* spp. that are associated with DRS (Egusa *et al.*, 2000; McIntyre, 2001). Several studies have demonstrated different *Candida* spp. isolated from patients with DRS (Cross *et al.*, 1998; McMullan-Vogel *et al.*, 1999; Dorko *et al.*, 2001; Pires *et al.*, 2002). However, other studies have show that only *C. albicans* were isolated from the palatal and denture surfaces of study patients (Lal *et al.*, 1992). The results of the present study have shown that the number of *Candida* recovered from the denture fitting surface was higher than those originating from mucosal surface. This observation was consistent with previous findings (Davenport, 1970; Budtz-Jorgensen *et al.*, 1996) and supports the opinion that the infection originates from the area underneath the denture in which the acid and anaerobic bacteria beneath the close-fitting denture favour yeast proliferation (Budtz-Jorgensen, 1990).

The ideal method of identification of microorganisms should be highly reproducible and allow rapid examination of a large number of isolates with a minimum cost. The results of this work showed that there was a variation in the identification of *Candida* spp. using the tests employed. For example, although CHROMagar is a convenient method for identification of different *Candida* species, it can be unreliable and may underestimate the presence of some *Candida* spp. particularly on secondary culture (Tintelnot *et al.*, 2000) indicating that the use of traditional identification techniques may be unsuitable in characterising, to species level,

Candida spp. from DRS patients. Further work should employ molecular techniques in identifying species present (Al-Karaawi *et al.*, 2002).

Due to the site specificity of plaque related diseases and the heterogeneous nature of dental plaque there are many problems associated with microbiological sampling from the oral cavity. Additionally, there are ethical problems with carrying out *in vivo* studies related to antibiotics, antimicrobials and antifungals. Testing of such agents against denture biofilms has thus required the use of relevant *in vitro* models. Drake and co-workers have developed an *in vitro* bacteria-yeast colonization model to assess the efficacy of denture soaking agents against the microorganisms (Drake *et al.*, 1992). In the present study, the Constant Depth Film Fermentor was used to model *in vitro* growth of DRS by growing a mixed-species biofilm on denture acrylic disks which produced microcosm plaques of similar proportions to those seen in the patient samples. The development of *in vitro* models may help in studying the oral microflora implicated in DRS. Previous studies using this model system have also demonstrated the ability to grow oral microcosms representing dental plaque derived from saliva (Pratten *et al.*, 1998b) and malodorous tongue plaque (Pratten *et al.*, 2003). The results of the current study have shown that mixed microbial populations derived from clinical isolates recovered from patients with DRS were able to form biofilms *in vitro* using the CDFE. However, the growth of some species, such as lactobacilli, was variable through the course of the experiment. This may be explained by the microbial interactions between different genera within the mixed-species biofilm. Such interactions within dental plaque include nutrition competition, dictating the prevalence of particular species over the others, or as a secondary feeder in which the metabolic product of one organism provide a main source of nutrients for another (Marsh, 1995). These interactions help different microbial

species to survive in environments where they may not otherwise persist.

Despite the use of antifungal drugs to treat DRS, the persistence of denture plaque on the denture fitting surfaces remains a problem. Chandra and co-workers (Chandra *et al.*, 2001b) showed that *C. albicans* biofilms, grown *in vitro*, were highly resistant to the antifungal agents (fluconazole, nystatin, amphotericin B and chlorhexidine) used to treat DRS. The results of the present study also showed that *Candida* spp. within mixed-species biofilms exhibit resistance to antifungal agents. As seen in the single-species work, there were large differences in the degree of susceptibility to fluconazole, miconazole and chlorhexidine. When *Candida* spp. were part of mixed-species biofilm, they were still able to grow in concentrations of antifungal agent comparable to concentrations used against *C. albicans* single-species biofilms (refer to chapter 5). Interestingly, bacterial species were resistance to chlorhexidine at concentrations of up to 2.5 % compared to *Candida* spp. which were killed at this concentration.

The clinical effectiveness of chlorhexidine in DRS has been known since the 1970's. Budtz-Jorgensen and Loe have investigated the use of 2 % chlorhexidine gluconate as a denture disinfectant. They showed a significant amelioration of inflamed tissues beneath the denture (Budtz-Jorgensen and Loe, 1972). Spiechowicz and co-workers also showed that chlorhexidine gluconate was totally effective in preventing attachment and growth of *C. albicans* on acrylic resin (Spiechowicz *et al.*, 1990).

When the antimicrobial and antifungal agents were used in combination, although relatively effective over short periods of time, the biofilms became increasingly resistant to the dual-therapy. It appears that the presence of *C. albicans* may modulate the action of antibiotics while bacteria can affect antifungal activity in mixed fungal-bacterial biofilms thus increasing the resistance of a mixed-species

biofilm. For example, interactions between different matrix polymers, or indeed the viscosity of the biofilm matrix, produced by different species may reduce the penetration of antifungal agents (Adam *et al.*, 2002).

In the clinical scenario the effect of fluconazole alone and fluconazole and chlorhexidine applied to the fitting surface of the denture has been evaluated. It was found that treatment of DRS for 2 weeks with fluconazole tablets once a day in conjunction with 2 % concentration of chlorhexidine solution applied to the inner surface of the denture twice daily resulted in a greater improvement of palatal inflammation with a decrease in candidal colonization than the use of fluconazole alone or fitting new dentures without medication (Kulak *et al.*, 1994; Arikan *et al.*, 1995). Although relapse was observed after treatment, the incidence of recurrence in those patients treated with fluconazole alone was higher than that seen after treatment with fluconazole in addition to chlorhexidine (Kulak *et al.*, 1994).

In this study a model of denture plaque which contains *Candida* spp was developed. *In vivo* the heterogeneity of such biofilms may have an impact on the ability of an infection to respond to antimicrobial therapy (El Azizi *et al.*, 2004). Hence, a better understanding of denture plaque biofilms may help to the development of new therapeutic approaches for the treatment of DRS.

Chapter 7

Conclusions and suggestions for further work

Although there has been a reduction in the population of edentulous people in developed countries, this would not necessarily reflect a reduction in DRS in the population group wearing complete dentures (Radford, et al., 1999). Dentures provide a suitable environment for growth and colonization of microorganisms. The yeast *Candida albicans* is frequently encountered as a harmless commensal in the oral cavity (Akpan and Morgan, 2002). However, oral candidosis is a common opportunistic infection seen most commonly in compromised patients. Additionally, oral yeast may be more strongly influenced by the presence of local oral factors, such as the presence of dentures.

Denture related stomatitis is the commonest form of oral candidosis. It is associated with wearing of dentures in which *Candida albicans* is the most common organism isolated (Wilson, 1998). It has been suggested that a combination of this yeast and oral bacterial species are likely to be responsible for the disease. Together, these organisms can form denture plaque biofilm (Keng and Lim, 1996; Kulak-Ozkan *et al.*, 2002) which is difficult to treat because of the inherent resistance of these biofilms to antifungal agents (Ramage *et al.*, 2004). Hence, despite the use of antifungal drugs to treat DRS, the recurrence of this disease remains a problem (Budtz-Jorgensen *et al.*, 1988; Bissell *et al.*, 1993).

The initial aim of this work was to set up an *in vitro* model to establish the conditions suitable for the development of *C. albicans* biofilm formation and evaluating the effectiveness of antimicrobial agents against candidal biofilms. A CDFP was used to grow the biofilms in order to mimic the *in vivo* scenario; the biofilms were grown in an aerobic environment on relevant oral surfaces, with nutrients supplied by artificial saliva. The results of the studies showed that the model produced a large number of reproducible biofilms of *C. albicans*. By

establishing the growth of *C. albicans* biofilms *in vitro*, it allowed the study of various factors which may be important in the disease process to be investigated.

The first of these parameters was to grow *C. albicans* biofilm on relevant oral surfaces (acrylic, enamel and dentine) and acrylic with different surface roughness. The results showed that the type of surface and the growth media used affected the initial formation and development of *C. albicans* biofilms. Although there was no difference between biofilms formed on self and heat cured acrylic, growth of *C. albicans* biofilm on the dental hard tissues (enamel and dentine) was different when compared to biofilm formed on self-cured acrylic. This indicates that attention should also be concentrated on dental hard tissues, and not only on the denture acrylic, as these surfaces may harbour this organism and can present an additional reservoir for candidal infections.

The use of artificial saliva in this work provided an appropriate growth medium in which both the yeast and hyphal forms of *C. albicans* were able to form by providing a conditioning film and consequently salivary pellicle formation on the surfaces and thus help to initiate biofilm formation. The use of YPD (commonly used for the growth of *C. albicans*) enhanced the initial growth of *C. albicans*. This may be due to the sugar content of YPD that has been shown to improve the adhesion of *C. albicans* to the surface (Nikawa *et al.*, 1997). However, artificial saliva and YPD can produce characteristic biofilms containing both the yeast and hyphal forms of *C. albicans*.

The effect of surface roughness has been reported previously (Verran *et al.*, 1991; Verran and Maryan, 1997; Radford *et al.*, 1998b). However, the present work evaluates the effect of surface roughness on the formation of *C. albicans* biofilms using CDF. Denture acrylic were prepared and polished with different surface

roughness in order to mimic an oral prosthesis *in vivo*. The results showed that surface roughness had a significant effect on the initial biofilm formation of *C. albicans*, however, this was not true for the type of acrylic used. When different genotypes of *C. albicans*, with different adherence properties were used, biofilm development was unaffected by the strain of organism, regardless of its virulence, highlighting the differences between adhesion assays and biofilm formation.

Confocal laser scanning microscopy was used to investigate biofilm development on the various surfaces. It proved to be a convenient method of observing the structure of the biofilms. The use of this technique to investigate biofilm structure allowed the study of the spatial arrangement of the biofilm. The results showed that surface roughness had a significant effect on the initial formation of *C. albicans* biofilms. Previous study (Howser and Douglas, 1994) has shown that adhesion of *C. albicans* blastoconidia to a surface is followed by its conversion to hyphae, indicating a contact-induced regulation of gene expression. The use of CLSM in this study to investigate biofilm development on different surface roughness revealed that the hyphal form was associated with biofilm development and structure. It is therefore hyphal emergence by thigmotropic reaction that may be involved during biofilm formation by sensing surface changes (Watts *et al.*, 1998).

To study biofilm treatment, all experiments must be reproducible using a steady state model. Indeed the CDFP was originally designed for this purpose. Hence, the model was used to assess the *in vitro* susceptibility of *C. albicans* biofilms against the commonly used antifungal agents (fluconazole, miconazole and chlorhexidine) used to treat DRS *in vivo*. Although this model has been used broadly to assess the susceptibility of bacterial biofilms, this work demonstrated its first use for fungal biofilms. The results of this work confirm earlier study of *C. albicans* biofilm

resistance (Chandra *et al.*, 2001b) in which these biofilms were highly resistant to both antifungal and antimicrobial agents. Large differences in the degree of susceptibility of *C. albicans* biofilms were observed between fluconazole, miconazole and chlorhexidine with the latter being the most effective. This may be due to the fungicidal effect of this agent compared to the fungistatic action of the azole-group of antifungals. There are several suggested explanations for reduced susceptibilities of biofilms, as described in the introduction, including reduced growth rate of organisms comprising biofilms; the production of EPS by the organisms forming a barrier to antimicrobial agents and that bacteria in biofilms are genetically distinct from their planktonic counterparts.

Differences in susceptibility were also observed for biofilms of different ages. It is well documented that cells within young biofilms, which have a higher growth rate are more susceptible than mature biofilms (which have a low growth rate and contain more EPS material). However, it has been shown that the low growth rate does not have a major influence on the resistance of *C. albicans* biofilms to antifungal drugs and the matrix material may play only a minor role in biofilm resistance (Baillie and Douglas, 1998a). The results of the present study indicate that biofilms in the early phases of growth were more susceptible to the drugs; however, these biofilms (or indeed single cells attached to a surface) were still far more resistant than their planktonic counterparts. Hence, a combination of effects including both the growth rate and EPS may play only a minor role in the susceptibility of these younger biofilms and it is more likely that surface-associated changes to the cell are more important.

These results highlight the importance of early detection of biofilm formation associated with DRS which can be achieved via prevention of denture biofilm

formation through frequent denture hygiene before the establishment of a more resistance mature biofilm.

Although *C. albicans* is considered the primary causative organism in DRS, oral bacteria (Koopmans *et al.*, 1988) and other, non-*albicans Candida* species, (McMullan-Vogel *et al.*, 1999; Dorko *et al.*, 2001) are also present. Hence, the final aim of the study was to determine the composition and susceptibility of multi-species (i.e. fungi and bacteria) denture plaque associated with DRS. The development of *in vitro* models will assist to study the oral microflora implicated in DRS. Bacterial-fungal interactions play a critical role in colonization of these microorganisms on denture acrylic and the development of DRS (Millsap *et al.*, 1999). Clinical samples recovered from patients with DRS were used as an inoculum to form *in vitro* biofilms which mimicked the microflora present in these patients. The results demonstrated that *C. albicans* was the most prominent fungal spp. identified from the patient samples and that this organism, when present in a mixed-species biofilm, exhibited increased resistance to antifungal agents. It has also been shown that bacterial species were resistant up to a concentration of 2.5 % chlorhexidine compared to *Candida* species. These remaining bacteria may be beneficial and constitute a normal microflora, however, they may enhance the growth of other species. Additionally, when a combined pulse of miconazole and chlorhexidine was applied to a mixed-species biofilm, the biofilms became increasingly resistant to the dual-therapy. Beside the already mentioned mechanisms of biofilm resistance, a more recent suggestion is the presence of some persistent cells within a biofilm. These cells are able to survive and proliferate under antimicrobial administration thus bringing about considerable recalcitrance upon the biofilm environment (Gilbert *et al.*, 2002). Further understanding and realization of

the nature of these cells may help to understand the mechanisms involved in biofilm resistance. It has been shown that the symptoms of DRS often return soon after treatment (Budtz-Jorgensen *et al.*, 1988; Bissell *et al.*, 1993). It may be that both a combination of biofilm resistance by, for example, persister cells and re-colonisation by commensal organisms are important factors in the development of an oral microflora which is able to cause inflammation and recurrence of the disease.

The present study used a biofilm-based model to assess the formation and the susceptibility of *C. albicans* biofilm against denture plaque microorganisms under conditions similar to those which would exist *in vivo*. The CDFF proved to be a convenient and versatile means of generating large numbers (up to 75 replicates) of candidal biofilms. The model is particularly suited to studies of oral biofilms in that it provides an environment similar to that found in the oral cavity. The biofilms were grown on a solid substratum with nutrients being provided in a thin film of liquid, continually replenished, trickling over the surface of the biofilm. The removal of the surfaces of the biofilms by the scraper blade simulates the continuous removal of the outermost layers of plaque by chewing and tongue movements. Most studies, however, have employed single-species biofilms rather than mixed fungal and bacterial biofilms characteristic of DRS. The CDFF was used to grow single and multi-species biofilms under conditions similar to those prevailing in the oral cavity. In terms of the microcosm plaques the composition of the various species within these biofilms was similar to that found in DRS. The results of susceptibility studies of the present work revealed differences between antifungal and antimicrobial agents in their effectiveness against microcosm denture plaque generated in the CDFF. The screening of potential anti-plaque and antimicrobial agents for use in preventing and/or treating plaque-related diseases using this approach is of considerable value. It may be able to reduce

the need for large numbers of expensive clinical trials by selecting compounds, and the concentrations at which to use them, prior to expensive clinical trials.

In summary, oral candidosis in the form of DRS is a chronic inflammation associated with denture wearers. Dentists should accept the responsibility of providing complete care of the denture patient. Insertion of dentures should not be considered as a final stage of treatment, but dentists should continue maintaining and caring for the health of oral tissues. Early diagnosis and treatment of this disease should be undertaken and post-insertion of denture hygiene instruction should be given routinely to motivate the patient. As the denture can act as a reservoir harbouring a microbial flora, time should be taken to remove these biofilms by plaque control and the use of antifungal agents.

Future work

The present work has established that the CDFF is a reliable *in vitro* system to undertake studies of the structure and antifungal susceptibility of biofilms of *Candida albicans*. This system may thus provide a means of investigating other perhaps more clinically significant fungal infections such as pseudomembraneous candidosis (thrush). In particular, there is a need to establish the biological events that lead to a reduced antifungal sensitivity of pseudomembraneous candidosis in immunocompromised patients.

It has been established, by using the CDFF, that both the surface type and its roughness can affect biofilm formation and structure as well as other environmental conditions. Hence, other biofilms of *C. albicans* associated with non-orally related surfaces could be investigated, for example, arterial catheters.

All of the present work was undertaken growing biofilms on a solid substratum with nutrients being provided over the surface of the biofilm. The results of this study (and others) has shown that the mucosa which is covered by the denture also harbour this mixed microbial plaque. Hence, the use of a mucosal surface as a substratum to form biofilms may produce interesting results especially with regard to cell invasion and susceptibility. In order to carry out such studies a different model, such as a flow cell, which permits the direct visualisation of attachment and growth over short periods of time, may be required for the co-culture of mucosal cells and yeasts. Additionally, by using this technique it would be possible to evaluate growth on shedding versus non-shedding surfaces, for example, by evaluating attachment onto cells under various shear rates.

References

- Abu-Elteen KH, Abu-Alteen RM (1998). The prevalence of *Candida albicans* populations in the mouths of complete denture wearers. *New Microbiol.* **21**, 41-48.
- Abu-Elteen KH, Elkarmi AZ, Hamad M (2001). Characterization of phenotype-based pathogenic determinants of various *Candida albicans* strains in Jordan. *Jpn. J. Infect. Dis.* **54**, 229-236.
- Adam B, Baillie GS, Douglas LJ (2002). Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *J. Med. Microbiol.* **51**, 344-349.
- Aguirre JM, Verdugo F, Zamacona JM, Quindos G, Ponton J (1996). Cytological changes in oral mucosa in denture stomatitis. *Gerodontology* **13**, 63-67.
- Akpan A, Morgan R (2002). Oral candidiasis. *Postgrad. Med. J.* **78**, 455-459.
- Al-Karaawi ZM (2004). Molecular characterization of *Candida* species in healthy patients and bone marrow transplant recipients. PhD thesis.
- Al-Karaawi ZM, Manfredi M, Waugh AC, McCullough MJ, Jorge J, Scully C, Porter SR (2002). Molecular characterization of *Candida* spp. isolated from the oral cavities of patients from diverse clinical settings. *Oral Microbiol. Immunol.* **17**, 44-49.
- Anderl JN, Franklin MJ, Stewart PS (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* **44**, 1818-1824.
- Anwar H, Strap JL, Costerton JW (1992). Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrob. Agents*

Chemother. **36**, 1347-1351.

Arendorf TM, Walker DM (1979). Oral candidal populations in health and disease.

Br. Dent. J. **147**, 267-272.

Arendorf TM, Walker DM (1980). The prevalence and intra-oral distribution of

Candida albicans in man. *Arch. Oral Biol.* **25**, 1-10.

Arendorf TM, Walker DM (1987). Denture stomatitis: a review. *J. Oral Rehabil.* **14**,

217-227.

Arikan A, Kulak Y, Kadir T (1995). Comparison of different treatment methods for

localized and generalized simple denture stomatitis. *J. Oral Rehabil.* **22**, 365-

369.

Atkinson JC, O'Connell A, Aframian D (2000). Oral manifestations of primary

immunological diseases. *J. Am. Dent. Assoc.* **131**, 345-356.

Axell T, Samaranayake LP, Reichart PA, Olsen I (1997). A proposal for

reclassification of oral candidosis. *Oral Surg. Oral Med. Oral Pathol. Oral*

Radiol. Endod. **84**, 111-112.

Bachmann SP, VandeWalle K, Ramage G, Patterson TF, Wickes BL, Graybill JR,

Lopez-Ribot JL (2002). *In vitro* activity of caspofungin against *Candida albicans*

biofilms. *Antimicrob. Agents Chemother.* **46**, 3591-3596.

Badawi H, Evans RD, Wilson M, Ready D, Noar JH, Pratten J (2003). The effect of

orthodontic bonding materials on dental plaque accumulation and composition *in*

vitro. *Biomaterials* **24**, 3345-3350.

- Bagg J, MacFarlane TW, Poxton IR, Miller CH, Smith AJ (1999a). Oral fungal infections. *In* Essentials of microbiology for dental students. pp. 289-299. Oxford University Press, New York, U.S.A.
- Bagg J, MacFarlane TW, Poxton IR, Miller CH, Smith AJ (1999b). The oral microflora and dental plaque. *In* Essentials of microbiology for dental students. pp. 229-239. Oxford University Press, New York, U.S.A.
- Bailey A, Wadsworth E, Calderone R (1995). Adherence of *Candida albicans* to human buccal epithelial cells: host-induced protein synthesis and signaling events. *Infect. Immun.* **63**, 569-572.
- Baillie GS, Douglas LJ (1998a). Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. *Antimicrob. Agents Chemother.* **42**, 1900-1905.
- Baillie GS, Douglas LJ (1998b). Iron-limited biofilms of *Candida albicans* and their susceptibility to amphotericin B. *Antimicrob. Agents Chemother.* **42**, 2146-2149.
- Baillie GS, Douglas LJ (1999a). *Candida* biofilms and their susceptibility to antifungal agents. *Methods Enzymol.* **310**, 644-656.
- Baillie GS, Douglas LJ (1999b). Role of dimorphism in the development of *Candida albicans* biofilms. *J. Med. Microbiol.* **48**, 671-679.
- Baillie GS, Douglas LJ (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J. Antimicrob. Chemother.* **46**, 397-403.
- Barasch A, Safford MM, Dapkute-Marcus I, Fine DH (2004). Efficacy of

- chlorhexidine gluconate rinse for treatment and prevention of oral candidiasis in HIV-infected children: a pilot study. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **97**, 204-207.
- Barbeau J, Seguin J, Goulet JP, de Koninck L, Avon SL, Lalonde B, Rompre P, Deslauriers N (2003). Reassessing the presence of *Candida albicans* in denture-related stomatitis. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **95**, 51-59.
- Barrett AW, Kingsmill VJ, Speight PM (1998). The frequency of fungal infection in biopsies of oral mucosal lesions. *Oral Dis.* **4**, 26-31.
- Bassler BL (1999). How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr. Opin. Microbiol.* **2**, 582-587.
- Bautista-Munoz C, Boldo XM, Villa-Tanaca L, Hernandez-Rodriguez C (2003). Identification of *Candida* spp. by randomly amplified polymorphic DNA analysis and differentiation between *Candida albicans* and *Candida dubliniensis* by direct PCR methods. *J. Clin. Microbiol.* **41**, 414-420.
- Beck-Sague C, Jarvis WR (1993). Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System. *J. Infect. Dis.* **167**, 1247-1251.
- Becker P, Hufnagle W, Peters G, Herrmann M (2001). Detection of differential gene expression in biofilm-forming versus planktonic populations of *Staphylococcus aureus* using micro-representational-difference analysis. *Appl. Environ. Microbiol.* **67**, 2958-2965.

- Bercier JG, al Hashimi I, Haghghat N, Rees TD, Oppenheim FG (1999). Salivary histatins in patients with recurrent oral candidiasis. *J. Oral Pathol. Med.* **28**, 26-29.
- Bernhardt J, Herman D, Sheridan M, Calderone R (2001). Adherence and invasion studies of *Candida albicans* strains, using *in vitro* models of esophageal candidiasis. *J. Infect. Dis.* **184**, 1170-1175.
- Bialasiewicz D, Kurnatowska A, Smiech-Slomkowska G (1993). Characteristics of fungi and attempts of their elimination from the oral cavity in children treated with orthodontic appliances. *Med. Dosw. Mikrobiol.* **45**, 389-392.
- Biasoli MS, Tosello ME, Magaro HM (2002). Adherence of *Candida* strains isolated from the human gastrointestinal tract. *Mycoses* **45**, 465-469.
- Bissell V, Felix DH, Wray D (1993). Comparative trial of fluconazole and amphotericin in the treatment of denture stomatitis. *Oral Surg. Oral Med. Oral Pathol.* **76**, 35-39.
- Blanck HM, Bowman BA, Serdula MK, Khan LK, Kohn W, Woodruff BA (2002). Angular stomatitis and riboflavin status among adolescent Bhutanese refugees living in southeastern Nepal. *Am. J. Clin. Nutr.* **76**, 430-435.
- Bradshaw DJ, Marsh PD, Schilling KM, Cummins D (1996). A modified chemostat system to study the ecology of oral biofilms. *J. Appl. Bacteriol.* **80**, 124-130.
- Brown AJ, Gow NA (1999). Regulatory networks controlling *Candida albicans* morphogenesis. *Trends Microbiol.* **7**, 333-338.
- Brown MR, Allison DG, Gilbert P (1988). Resistance of bacterial biofilms to

antibiotics: a growth-rate related effect? *J. Antimicrob. Chemother.* **22**, 777-780.

Budtz-Jorgensen E, Mojon P, Banon-Clement JM, Baehni P (1996). Oral candidosis in long-term hospital care: comparison of edentulous and dentate subjects. *Oral Dis.* **2**, 285-290.

Budtz-Jorgensen E (1990). Etiology, pathogenesis, therapy, and prophylaxis of oral yeast infections. *Acta Odontol. Scand.* **48**, 61-69.

Budtz-Jorgensen E, Carlino P (1994). A miconazole lacquer in the treatment of *Candida*-associated denture stomatitis. *Mycoses* **37**, 131-135.

Budtz-Jorgensen E, Holmstrup P, Krogh P (1988). Fluconazole in the treatment of *Candida*-associated denture stomatitis. *Antimicrob. Agents Chemother.* **32**, 1859-1863.

Budtz-Jorgensen E, Loe H (1972). Chlorhexidine as a denture disinfectant in the treatment of denture stomatitis. *Scand. J. Dent. Res.* **80**, 457-464.

Budtz-Jorgensen E, Lombardi T (1996). Antifungal therapy in the oral cavity. *Periodontol. 2000.* **10**, 89-106.

Budtz-Jorgensen E, Theilade E (1983). Regional variations in viable bacterial and yeast counts of 1-week-old denture plaque in denture-induced stomatitis. *Scand. J. Dent. Res.* **91**, 288-295.

Budtz-Jorgensen E, Theilade E, Theilade J (1983). Quantitative relationship between yeast and bacteria in denture-induced stomatitis. *Scand. J. Dent. Res.* **91**, 134-142.

- Bulad K, Taylor RL, Verran J, McCord JF (2004). Colonization and penetration of denture soft lining materials by *Candida albicans*. *Dent. Mater.* **20**, 167-175.
- Busscher HJ, Geertsema-Doornbusch GI, van der Mei HC (1997). Adhesion to silicone rubber of yeasts and bacteria isolated from voice prostheses: influence of salivary conditioning films. *J. Biomed. Mater. Res.* **34**, 201-209.
- Busscher HJ, van der Mei HC (1995). Use of flow chamber devices and image analysis methods to study microbial adhesion. *Methods Enzymol.* **253**, 455-477.
- Calderone RA, Braun PC (1991). Adherence and receptor relationships of *Candida albicans*. *Microbiol. Rev.* **55**, 1-20.
- Calderone RA, Fonzi WA (2001). Virulence factors of *Candida albicans*. *Trends Microbiol.* **9**, 327-335.
- Cannon RD, Chaffin WL (1999). Oral colonization by *Candida albicans*. *Crit. Rev. Oral Biol. Med.* **10**, 359-383.
- Cannon RD, Holmes AR, Mason AB, Monk BC (1995). Oral *Candida*: clearance, colonization, or candidiasis? *J. Dent. Res.* **74**, 1152-1161.
- Carlstedt K, Krekmanova L, Dahllof G, Ericsson B, Braathen G, Modeer T (1996). Oral carriage of *Candida* species in children and adolescents with Down's syndrome. *Int. J. Paediatr. Dent.* **6**, 95-100.
- Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D, Martinez JP (1998). Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol. Mol. Biol. Rev.* **62**, 130-180.

- Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA (2001a). Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. *J. Bacteriol.* **183**, 5385-5394.
- Chandra J, Mukherjee PK, Leidich SD, Faddoul FF, Hoyer LL, Douglas LJ, Ghannoum MA (2001b). Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. *J. Dent. Res.* **80**, 903-908.
- Choong S, Whitfield H (2000). Biofilms and their role in infections in urology. *BJU Int.* **86**, 935-941.
- Coelho CM, Sousa YT, Dare AM (2004). Denture-related oral mucosal lesions in a Brazilian school of dentistry. *J. Oral Rehabil.* **31**, 135-139.
- Collin B, Clancy CJ, Nguyen MH (1999). Antifungal resistance in non- *albicans* *Candida* species. *Drug Resist. Updat.* **2**, 9-14.
- Cornely OA, Bohme A, Buchheidt D, Glasmacher A, Kahl C, Karthaus M, Kern W, Kruger W, Maschmeyer G, Ritter J, Salwender HJ, Sandherr M, Schiel X, Schuttrumpf S, Sieniawski M, Silling G, Ullmann AJ, Wolf HH (2003). Prophylaxis of invasive fungal infections in patients with hematological malignancies and solid tumors--guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). *Ann. Hematol.* **82**, 186-200.
- Corner BE, Magee PT (1997). *Candida* pathogenesis: Unravelling the threads of infection. *Curr. Biol.* **7**, 691-694.
- Costerton JW (1999). Introduction to biofilm. *Int. J. Antimicrob. Agents* **11**, 217-

221.

Costerton JW (2001). Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends Microbiol.* **9**, 50-52.

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, DRSgupta M, Marrie TJ (1987). Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol.* **41**, 435-464.

Costerton JW, Geesey GG, Cheng KJ (1978). How bacteria stick. *Sci. Am.* **238**, 86-95.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995). Microbial biofilms. *Annu. Rev. Microbiol.* **49**, 711-745.

Costerton JW, Lewandowski Z, DeBeer D, Caldwell D, Korber D, James G (1994). Biofilms, the customized microniche. *J. Bacteriol.* **176**, 2137-2142.

Costerton JW, Stewart PS, Greenberg EP (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-1322.

Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G (2003). The application of biofilm science to the study and control of chronic bacterial infections. *J. Clin. Invest.* **112**, 1466-1477.

Crockett DN, O'Grady JF, Reade PC (1992). *Candida* species and *Candida albicans* morphotypes in erythematous candidiasis. *Oral Surg. Oral Med. Oral Pathol.* **73**, 559-563.

Cross LJ, Bagg J, Aitchison TC (2000). Efficacy of the cyclodextrin liquid

- preparation of itraconazole in treatment of denture stomatitis: comparison with itraconazole capsules. *Antimicrob. Agents Chemother.* **44**, 425-427.
- Cross LJ, Bagg J, Wray D, Aitchison T (1998). A comparison of fluconazole and itraconazole in the management of denture stomatitis: a pilot study. *J. Dent.* **26**, 657-664.
- Cross LJ, Williams DW, Sweeney CP, Jackson MS, Lewis MA, Bagg J (2004). Evaluation of the recurrence of denture stomatitis and *Candida* colonization in a small group of patients who received itraconazole. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **97**, 351-358.
- Cumming CG, Wight C, Blackwell CL, Wray D (1990). Denture stomatitis in the elderly. *Oral Microbiol. Immunol.* **5**, 82-85.
- Cvitkovitch DG, Li YH, Ellen RP (2003). Quorum sensing and biofilm formation in Streptococcal infections. *J. Clin. Invest.* **112**, 1626-1632.
- Damm DD, Neville BW, Geissler RH Jr., White DK, Drummond JF, Ferretti GA (1988). Dentinal candidiasis in cancer patients. *Oral Surg. Oral Med. Oral Pathol.* **65**, 56-60.
- Darwazeh AM, Al Refai S, Al Mojaiwel S (2001). Isolation of *Candida* species from the oral cavity and fingertips of complete denture wearers. *J. Prosthet. Dent.* **86**, 420-423.
- Davenport JC (1970). The oral distribution of *Candida* in denture stomatitis. *Br. Dent. J.* **129**, 151-156.
- Davenport JC (1972). The denture surface. *Br. Dent. J.* **133**, 101-105.

- Davies D (2003). Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discov.* **2**, 114-122.
- Davies JM, Stacey AJ, Gilligan CA (1999). *Candida albicans* hyphal invasion: thigmotropism or chemotropism? *FEMS Microbiol. Lett.* **171**, 245-249.
- de Kievit TR, Iglewski BH (2000). Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.* **68**, 4839-4849.
- Deak R, Bodai L, Aarts HJ, Maraz A (2004). Development of a novel, simple and rapid molecular identification system for clinical *Candida* species. *Med. Mycol.* **42**, 311-318.
- Diaz-Arnold AM, Marek CA (2002). The impact of saliva on patient care: A literature review. *J. Prosthet. Dent.* **88**, 337-343.
- Donlan RM (2001a). Biofilm formation: a clinically relevant microbiological process. *Clin. Infect. Dis.* **33**, 1387-1392.
- Donlan RM (2001b). Biofilms and device-associated infections. *Emerg. Infect. Dis.* **7**, 277-281.
- Donlan RM, Costerton JW (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **15**, 167-193.
- Dorko E, Jenca A, Pilipcinec E, Danko J, Svicky E, Tkacikova L (2001). *Candida*-associated denture stomatitis. *Folia Microbiol.* **46**, 443-446.
- Dorocka-Bobkowska B, Budtz-Jorgensen E, Wloch S (1996). Non-insulin-dependent diabetes mellitus as a risk factor for denture stomatitis. *J. Oral Pathol. Med.* **25**,

411-415.

Dorocka-Bobkowska B, Konopka K, Duzgunes N (2003). Influence of antifungal polyenes on the adhesion of *Candida albicans* and *Candida glabrata* to human epithelial cells *in vitro*. *Arch. Oral Biol.* **48**, 805-814.

Douglas LJ (2003). *Candida* biofilms and their role in infection. *Trends Microbiol.* **11**, 30-36.

Douglas WH, Walker DM (1973). Nystatin in denture liners--an alternative treatment of denture stomatitis. *Br. Dent. J.* **135**, 55-59.

Drake D, Wells J, Ettinger R (1992). Efficacy of denture cleansing agents in an *in vitro* bacteria-yeast colonization model. *Int. J. Prosthodont.* **5**, 214-220.

Duguid IG, Evans E, Brown MR, Gilbert P (1992). Effect of biofilm culture upon the susceptibility of *Staphylococcus epidermidis* to tobramycin. *J. Antimicrob. Chemother.* **30**, 803-810.

Edgerton M, Scannapieco FA, Reddy MS, Levine MJ (1993). Human submandibular-sublingual saliva promotes adhesion of *Candida albicans* to polymethylmethacrylate. *Infect. Immun.* **61**, 2644-2652.

Eggimann P, Garbino J, Pittet D (2003). Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *Lancet Infect. Dis.* **3**, 685-702.

Egusa H, Ellepola AN, Nikawa H, Hamada T, Samaranayake LP (2000). Exposure to subtherapeutic concentrations of polyene antifungals suppresses the adherence of *Candida* species to denture acrylic. *Chemotherapy* **46**, 267-274.

- El Azizi MA, Starks SE, Khardori N (2004). Interactions of *Candida albicans* with other *Candida* spp. and bacteria in the biofilms. *J. Appl. Microbiol.* **96**, 1067-1073.
- Ellepola AN, Samaranayake LP (1998). Adhesion of oral *Candida albicans* isolates to denture acrylic following limited exposure to antifungal agents. *Arch. Oral Biol.* **43**, 999-1007.
- Ellepola AN, Samaranayake LP (2000a). Antimycotic agents in oral candidosis: an overview: 1. Clinical variants. *Dent. Update.* **27**, 111-116.
- Ellepola AN, Samaranayake LP (2000b). Antimycotic agents in oral candidosis: an overview: 2. Treatment of oral candidosis. *Dent. Update.* **27**, 165-174.
- Ellepola AN, Samaranayake LP (2000c). Oral candidal infections and antimycotics. *Crit. Rev. Oral Biol. Med.* **11**, 172-198.
- Epstein JB (1990). Antifungal therapy in oropharyngeal mycotic infections. *Oral Surg. Oral Med. Oral Pathol.* **69**, 32-41.
- Epstein JB, Gorsky M, Caldwell J (2002). Fluconazole mouthrinses for oral candidiasis in postirradiation, transplant, and other patients. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **93**, 671-675.
- Epstein JB, Pearsall NN, Truelove EL (1981). Oral candidiasis: effects of antifungal therapy upon clinical signs and symptoms, salivary antibody, and mucosal adherence of *Candida albicans*. *Oral Surg. Oral Med. Oral Pathol.* **51**, 32-36.
- Epstein JB, Polsky B (1998). Oropharyngeal candidiasis: a review of its clinical spectrum and current therapies. *Clin. Ther.* **20**, 40-57.

- Evans DJ, Allison DG, Brown MR, Gilbert P (1991). Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. *J. Antimicrob. Chemother.* **27**, 177-184.
- Evans E, Brown MR, Gilbert P (1994). Iron chelator, exopolysaccharide and protease production in *Staphylococcus epidermidis*: a comparative study of the effects of specific growth rate in biofilm and planktonic culture. *Microbiology* **140**, 153-157.
- Farah CS, Ashman RB, Challacombe SJ (2000). Oral candidosis. *Clin. Dermatol.* **18**, 553-562.
- Fenlon MR, Sherriff M, Walter JD (1998). Factors associated with the presence of denture related stomatitis in complete denture wearers: a preliminary investigation. *Eur. J. Prosthodont. Res. Dent.* **6**, 145-147.
- Fidel PL Jr., Vazquez JA, Sobel JD (1999). *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin. Microbiol. Rev.* **12**, 80-96.
- Foster JS, Kolenbrander PE (2004). Development of a multispecies oral bacterial community in a saliva-conditioned flow cell. *Appl. Environ. Microbiol.* **70**, 4340-4348.
- Foster JS, Pan PC, Kolenbrander PE (2004). Effects of antimicrobial agents on oral biofilms in a saliva-conditioned flowcell. *Biofilms* **1**, 3-10.
- Freeman C, Lock MA (1995). The biofilm polysaccharide matrix: A buffer against changing organic substrate supply. *Limnol. Oceanogr.* **40**, 273-278.

- Ghannoum M, Abu-Elteen K (1986). Correlative relationship between proteinase production, adherence and pathogenicity of various strains of *Candida albicans*. *J. Med. Vet. Mycol.* **24**, 407-413.
- Ghannoum MA (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clin. Microbiol. Rev.* **13**, 122-143.
- Gilbert P, Allison DG, McBain AJ (2002). Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *Symp Ser Soc Appl Microbiol.* **92**, 98-110.
- Gilbert P, DRS J, Foley I (1997). Biofilm susceptibility to antimicrobials. *Adv. Dent. Res.* **11**, 160-167.
- Giwerzman B, Jensen ET, Hoiby N, Kharazmi A, Costerton JW (1991). Induction of beta-lactamase production in *Pseudomonas aeruginosa* biofilm. *Antimicrob. Agents Chemother.* **35**, 1008-1010.
- Gow NA, Brown AJ, Odds FC (2002). Fungal morphogenesis and host invasion. *Curr. Opin. Microbiol.* **5**, 366-371.
- Gow NA, Perera TH, Sherwood-Higham J, Gooday GW, Gregory DW, Marshall D (1994). Investigation of touch-sensitive responses by hyphae of the human pathogenic fungus *Candida albicans*. *Scanning Microsc.* **8**, 705-710.
- Greenspan D (1994a). Treatment of oral candidiasis in HIV infection. *Oral Surg. Oral Med. Oral Pathol.* **78**, 211-215.
- Greenspan D (1994b). Treatment of oropharyngeal candidiasis in HIV-positive patients. *J. Am. Acad. Dermatol.* **31**, 51-55.

- Guggenheimer J, Moore PA, Rossie K, Myers D, Mongelluzzo MB, Block HM, Weyant R, Orchard T (2000). Insulin-dependent diabetes mellitus and oral soft tissue pathologies: II. Prevalence and characteristics of *Candida* and candidal lesions. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **89**, 570-576.
- Gurgan S, Bolay S, Alacam R (1997). *In vitro* adherence of bacteria to bleached or unbleached enamel surfaces. *J. Oral Rehabil.* **24**, 624-627.
- Guyton AC (1992). Secretory functions of the alimentary tract. *In Human physiology and mechanisms of disease.* pp. 711. Saunders, Philadelphia, U.S.A.
- Hawser SP, Baillie GS, Douglas LJ (1998). Production of extracellular matrix by *Candida albicans* biofilms. *J. Med. Microbiol.* **47**, 253-256.
- Hawser SP, Douglas LJ (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infect. Immun.* **62**, 915-921.
- Hawser SP, Douglas LJ (1995). Resistance of *Candida albicans* biofilms to antifungal agents *in vitro*. *Antimicrob. Agents Chemother.* **39**, 2128-2131.
- Haynes K (2001). Virulence in *Candida* species. *Trends Microbiol.* **9**, 591-596.
- Hazan I, Sepulveda-Becerra M, Liu H (2002). Hyphal elongation is regulated independently of cell cycle in *Candida albicans*. *Mol. Biol. Cell* **13**, 134-145.
- Hodson JJ, Craig GT (1972). The incidence of *Candida albicans* in the plaques of teeth of children. *Dent. Pract. Dent. Rec.* **22**, 296-301.
- Hoffman MP, Haidaris CG (1993). Analysis of *Candida albicans* adhesion to salivary mucin. *Infect. Immun.* **61**, 1940-1949.

- Holmes AR, Gopal PK, Jenkinson HF (1995). Adherence of *Candida albicans* to a cell surface polysaccharide receptor on *Streptococcus gordonii*. *Infect. Immun.* **63**, 1827-1834.
- Hope CK, Wilson M (2003). Measuring the thickness of an outer layer of viable bacteria in an oral biofilm by viability mapping. *J Microbiol. Methods* **54**, 403-410.
- Hube B, Naglik J (2001). *Candida albicans* proteinases: resolving the mystery of a gene family. *Microbiology* **147**, 1997-2005.
- Ibrahim AS, Mirbod F, Filler SG, Banno Y, Cole GT, Kitajima Y, Edwards JE Jr., Nozawa Y, Ghannoum MA (1995). Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infect. Immun.* **63**, 1993-1998.
- Imanishi Y, Yokoyama K, Nishimura K (2004). Inductions of germ tube and hyphal formations are controlled by mRNA synthesis inhibitor in *Candida albicans*. *Jpn. J. Med. Mycol.* **45**, 113-119.
- Jabra-Rizk MA, Falkler WA Jr., Merz WG, Kelley JI, Baqui AA, Meiller TF (1999). Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum*. *J. Clin. Microbiol.* **37**, 1464-1468.
- Jeganathan S, Payne JA, Thean HP (1997). Denture stomatitis in an elderly edentulous Asian population. *J. Oral Rehabil.* **24**, 468-472.
- Jenkinson HF, Lala HC, Shepherd MG (1990). Coaggregation of *Streptococcus sanguis* and other streptococci with *Candida albicans*. *Infect. Immun.* **58**, 1429-1436.

- Jobbins J, Bagg J, Parsons K, Finlay I, Addy M, Newcombe RG (1992). Oral carriage of yeasts, coliforms and staphylococci in patients with advanced malignant disease. *J. Oral Pathol. Med.* **21**, 305-308.
- Keng SB, Lim M (1996). Denture plaque distribution and the effectiveness of a perborate-containing denture cleanser. *Quintessence. Int.* **27**, 341-345.
- Kennedy WA, Laurier C, Gautrin D, Ghezzi H, Pare M, Malo JL, Contandriopoulos AP (2000). Occurrence and risk factors of oral candidiasis treated with oral antifungals in seniors using inhaled steroids. *J. Clin. Epidemiol.* **53**, 696-701.
- Kerdpon D, Pongsiriwet S, Pangsomboon K, Iamaroon A, Kampoo K, Sretrirutchai S, Geater A, Robison V (2004). Oral manifestations of HIV infection in relation to clinical and CD4 immunological status in northern and southern Thai patients. *Oral Dis.* **10**, 138-144.
- Khadori N, Yassien M (1995). Biofilms in device-related infections. *J. Ind. Microbiol.* **15**, 141-147.
- Kinirons MJ (1983). Candidal invasion of dentine complicating hypodontia. *Br. Dent. J.* **154**, 400-401.
- Kirkpatrick CH (2001). Chronic mucocutaneous candidiasis. *Pediatr. Infect. Dis. J.* **20**, 197-206.
- Kleinegger CL, Stoeckel DC, Kurago ZB (2001). A comparison of salivary calprotectin levels in subjects with and without oral candidiasis. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **92**, 62-67.
- Kobayashi SD, Cutler JE (1998). *Candida albicans* hyphal formation and virulence:

is there a clearly defined role? *Trends Microbiol.* **6**, 92-94.

Kojic EM, Darouiche RO (2004). *Candida* infections of medical devices. *Clin. Microbiol. Rev.* **17**, 255-267.

Kolenbrander PE (2000). Oral microbial communities: biofilms, interactions, and genetic systems. *Annu. Rev. Microbiol.* **54**, 413-437.

Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer RJ Jr. (2002). Communication among oral bacteria. *Microbiol. Mol. Biol. Rev.* **66**, 486-505.

Kolokotronis A, Kioses V, Antoniadis D, Mandraveli K, Doutsos I, Papanayotou P (1994). Median rhomboid glossitis. An oral manifestation in patients infected with HIV. *Oral Surg. Oral Med. Oral Pathol.* **78**, 36-40.

Konsberg R, Axell T (1994). Treatment of *Candida*-infected denture stomatitis with a miconazole lacquer. *Oral Surg. Oral Med. Oral Pathol.* **78**, 306-311.

Koopmans AS, Kippuw N, de Graaff J (1988). Bacterial involvement in denture-induced stomatitis. *J. Dent. Res.* **67**, 1246-1250.

Krcmery V, Barnes AJ (2002). Non-*albicans* *Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. *J. Hosp. Infect.* **50**, 243-260.

Kretschmar M, Hube B, Bertsch T, Sanglard D, Merker R, Schroder M, Hof H, Nichterlein T (1999). Germ tubes and proteinase activity contribute to virulence of *Candida albicans* in murine peritonitis. *Infect. Immun.* **67**, 6637-6642.

Krogh P, Holmstrup P, Thorn JJ, Vedtofte P, Pindborg JJ (1987). Yeast species and

- biotypes associated with oral leukoplakia and lichen planus. *Oral Surg. Oral Med. Oral Pathol.* **63**, 48-54.
- Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA (2002a). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect. Immun.* **70**, 878-888.
- Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA (2002b). Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob. Agents Chemother.* **46**, 1773-1780.
- Kulak Y, Arikan A, Delibalta N (1994). Comparison of three different treatment methods for generalized denture stomatitis. *J. Prosthet. Dent.* **72**, 283-288.
- Kulak Y, Arikan A, Kazazoglu E (1997). Existence of *Candida albicans* and microorganisms in denture stomatitis patients. *J. Oral Rehabil.* **24**, 788-790.
- Kulak-Ozkan Y, Kazazoglu E, Arikan A (2002). Oral hygiene habits, denture cleanliness, presence of yeasts and stomatitis in elderly people. *J. Oral Rehabil.* **29**, 300-304.
- Kumamoto CA (2002). *Candida* biofilms. *Curr. Opin. Microbiol.* **5**, 608-611.
- Lal K, Santarpia RP, III, Pollock JJ, Renner RP (1992). Assessment of antimicrobial treatment of denture stomatitis using an *in vivo* replica model system: therapeutic efficacy of an oral rinse. *J. Prosthet. Dent.* **67**, 72-77.
- Lamb DJ, Douglas CW (1988). Treatment of denture stomatitis by a sustained drug-delivery device: a preliminary study. *J. Dent.* **16**, 219-221.

- Lamey PJ, Samaranayake LP (1988). Oral candidosis: 2. Diagnosis and management. *Dent. Update*. **15**, 328-331.
- Leung WK, DRSsanayake RS, Yau JY, Jin LJ, Yam WC, Samaranayake LP (2000). Oral colonization, phenotypic, and genotypic profiles of *Candida* species in irradiated, dentate, xerostomic nasopharyngeal carcinoma survivors. *J. Clin. Microbiol.* **38**, 2219-2226.
- Lewis K (2001). Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* **45**, 999-1007.
- Lewis RE, Kontoyiannis DP, Darouiche RO, Raad II, Prince RA (2002). Antifungal activity of amphotericin B, fluconazole, and voriconazole in an *in vitro* model of *Candida* catheter-related bloodstream infection. *Antimicrob. Agents Chemother.* **46**, 3499-3505.
- Lipperheide V, Quindos G, Jimenez Y, Ponton J, Bagan-Sebastian JV, Aguirre JM (1996). *Candida* biotypes in patients with oral leukoplakia and lichen planus. *Candida* biotypes in leukoplakia and lichen planus. *Mycopathologia* **134**, 75-82.
- Lupetti A, Guzzi G, Paladini A, Swart K, Campa M, Senesi S (1995). Molecular typing of *Candida albicans* in oral candidiasis: karyotype epidemiology with human immunodeficiency virus-seropositive patients in comparison with that with healthy carriers. *J. Clin. Microbiol.* **33**, 1238-1242.
- Lynch DP (1994). Oral candidiasis. History, classification, and clinical presentation. *Oral Surg. Oral Med. Oral Pathol.* **78**, 189-193.
- Lytle MS, Adams JC, Dickman DG, Bressler WR (1989). Use of nutrient response

- techniques to assess the effectiveness of chlorination of rapid sand filter gravel. *Appl. Environ. Microbiol.* **55**, 29-32.
- Mah TF, O'Toole GA (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **9**, 34-39.
- Manfredi M, McCullough MJ, Al-Karaawi ZM, Hurel SJ, Porter SR (2002). The isolation, identification and molecular analysis of *Candida* spp. isolated from the oral cavities of patients with diabetes mellitus. *Oral Microbiol. Immunol.* **17**, 181-185.
- Manfredi M, McCullough MJ, Vescovi P, Al-Kaarawi ZM, Porter SR (2004). Update on diabetes mellitus and related oral diseases. *Oral Dis.* **10**, 187-200.
- Marr KA, Seidel K, Slavin MA, Bowden RA, Schoch HG, Flowers ME, Corey L, Boeckh M (2000). Prolonged fluconazole prophylaxis is associated with persistent protection against candidiasis-related death in allogeneic marrow transplant recipients: long-term follow-up of a randomized, placebo-controlled trial. *Blood* **96**, 2055-2061.
- Marrie TJ, Costerton JW (1984). Scanning and transmission electron microscopy of in situ bacterial colonization of intravenous and intraarterial catheters. *J. Clin. Microbiol.* **19**, 687-693.
- Marsh P, Martin M (1999a). Oral fungal infections. *In Oral microbiology*. pp. 153-162. Wright, Oxford, U.K.
- Marsh P, Martin M (1999b). The mouth as microbial habitat. *In Oral microbiology*. pp. 5-16. Wright, Oxford, U.K.

- Marsh PD (1994). Microbial ecology of dental plaque and its significance in health and disease. *Adv. Dent. Res.* **8**, 263-271.
- Marsh PD (1995). Dental plaque. In *Microbial biofilms*. (Eds. Lappin-Scott HM and Costerton JW) pp. 282-300. Cambridge University Press, Cambridge, U.K.
- Marsh PD, Bradshaw DJ (1995). Dental plaque as a biofilm. *J. Ind. Microbiol.* **15**, 169-175.
- Martin-Mazuelos E, Aller AI, Romero MJ, Rodriguez AA, Gutierrez MJ, Bernal S, Montero O (1997). Response to fluconazole and itraconazole of *Candida* spp. in denture stomatitis. *Mycoses* **40**, 283-289.
- Matharu S, Spratt DA, Pratten J, Ng YL, Mordan N, Wilson M, Gulabivala K (2001). A new *in vitro* model for the study of microbial microleakage around dental restorations: a preliminary qualitative evaluation. *Int. Endod. J.* **34**, 547-553.
- Maver-Biscanin M, Mravak-Stipetic M, Jerolimov V, Biscanin A (2004). Fungicidal effect of diode laser irradiation in patients with denture stomatitis. *Lasers Surg. Med.* **35**, 259-262.
- Maza JL, Elguezabal N, Prado C, Ellacuria J, Soler I, Ponton J (2002). *Candida albicans* adherence to resin-composite restorative dental material: influence of whole human saliva. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **94**, 589-592.
- McCullough M, Jaber M, Barrett AW, Bain L, Speight PM, Porter SR (2002). Oral yeast carriage correlates with presence of oral epithelial dysplasia. *Oral Oncol.*

38, 391-393.

McCullough MJ, Clemons KV, Stevens DA (1999a). Molecular and phenotypic characterization of genotypic *Candida albicans* subgroups and comparison with *Candida dubliniensis* and *Candida stellatoidea*. *J. Clin. Microbiol.* **37**, 417-421.

McCullough MJ, Clemons KV, Stevens DA (1999b). Molecular epidemiology of the global and temporal diversity of *Candida albicans*. *Clin. Infect. Dis.* **29**, 1220-1225.

McCullough MJ, Ross BC, Reade PC (1996). *Candida albicans*: a review of its history, taxonomy, epidemiology, virulence attributes, and methods of strain differentiation. *Int. J. Oral Maxillofac. Surg.* **25**, 136-144.

McIntyre GT (2001). Oral candidosis. *Dent. Update.* **28**, 132-139.

McMullan-Vogel CG, Jude HD, Ollert MW, Vogel CW (1999). Serotype distribution and secretory acid proteinase activity of *Candida albicans* isolated from the oral mucosa of patients with denture stomatitis. *Oral Microbiol. Immunol.* **14**, 183-189.

Millsap KW, Bos R, van der Mei HC, Busscher HJ (1999). Adhesion and surface-aggregation of *Candida albicans* from saliva on acrylic surfaces with adhering bacteria as studied in a parallel plate flow chamber. *Antonie Van Leeuwenhoek* **75**, 351-359.

Molin S, Tolker-Nielsen T (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr. Opin. Biotechnol.* **14**, 255-261.

- Morgan TD, Wilson M (2001). The effects of surface roughness and type of denture acrylic on biofilm formation by *Streptococcus oralis* in a constant depth film fermentor. *J. Appl. Microbiol.* **91**, 47-53.
- Morton LHG, Greenway DLA, Gaylarde CC, Surman SB (1998). Consideration of some implications of the resistance of biofilms to biocides. *Int. Biodeter. Biodegr.* **41**, 247-259.
- Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA (2003). Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect. Immun.* **71**, 4333-4340.
- Mulligan AM, Wilson M, Knowles JC (2003). Effect of increasing silver content in phosphate-based glasses on biofilms of *Streptococcus sanguis*. *J Biomed. Mater. Res.* **67A**, 401-412.
- Muzyka BC, Glick M (1995). A review of oral fungal infections and appropriate therapy. *J. Am. Dent. Assoc.* **126**, 63-72.
- Naglik J, Albrecht A, Bader O, Hube B (2004). *Candida albicans* proteinases and host/pathogen interactions. *Cell Microbiol.* **6**, 915-926.
- Naglik JR, Challacombe SJ, Hube B (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev.* **67**, 400-428.
- Nair RG, Samaranayake LP (1996). The effect of oral commensal bacteria on candidal adhesion to denture acrylic surfaces. An *in vitro* study. *APMIS* **104**, 339-349.

- National Committee for Clinical Laboratory Standards M27-A (1997). Reference method for dilution antifungal susceptibility testing of yeasts approved standard. Vol. 17. Wayne, Pennsylvania, U.S.A.
- Newton AV (1962). Denture sore mouth. *Br. Dent. J.* **112**, 357-360.
- Nichols WW, Dorrington SM, Slack MP, Walmsley HL (1988). Inhibition of tobramycin diffusion by binding to alginate. *Antimicrob. Agents Chemother.* **32**, 518-523.
- Nichols WW, Evans MJ, Slack MP, Walmsley HL (1989). The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. *J. Gen. Microbiol.* **135**, 1291-1303.
- Nikawa H, Hamada T, Yamamoto T (1998a). Denture plaque--past and recent concerns. *J. Dent.* **26**, 299-304.
- Nikawa H, Hayashi S, Nikawa Y, Hamada T, Samaranayake LP (1993). Interactions between denture lining material, protein pellicles and *Candida albicans*. *Arch. Oral Biol.* **38**, 631-634.
- Nikawa H, Nishimura H, Hamada T, Kumagai H, Samaranayake LP (1997). Effects of dietary sugars and, saliva and serum on *Candida* biofilm formation on acrylic surfaces. *Mycopathologia* **139**, 87-91.
- Nikawa H, Nishimura H, Hamada T, Makihira S, Samaranayake LP (1998b). Relationship between thigmotropism and *Candida* biofilm formation *in vitro*. *Mycopathologia* **144**, 125-129.
- Nikawa H, Nishimura H, Makihira S, Hamada T, Sadamori S, Samaranayake LP

- (2000). Effect of serum concentration on *Candida* biofilm formation on acrylic surfaces. *Mycoses* **43**, 139-143.
- O'Toole G, Kaplan HB, Kolter R (2000). Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **54**, 49-79.
- Ostrosky-Zeichner L, Oude Lashof AM, Kullberg BJ, Rex JH (2003). Voriconazole salvage treatment of invasive candidiasis. *Eur. J. Clin. Microbiol. Infect. Dis.* **22**, 651-655.
- Pallasch TJ (2002). Antifungal and antiviral chemotherapy. *Periodontol.* **2000**, **28**, 240-255.
- Palmer RJ Jr., White DC (1997). Developmental biology of biofilms: implications for treatment and control. *Trends Microbiol.* **5**, 435-440.
- Parsek MR, Singh PK (2003). Bacterial biofilms: an emerging link to disease pathogenesis. *Annu. Rev. Microbiol.* **57**, 677-701.
- Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE (2001). Bacterial diversity in human subgingival plaque. *J. Bacteriol.* **183**, 3770-3783.
- Patton LL, Phelan JA, Ramos-Gomez FJ, Nittayananta W, Shiboski CH, Mbuguye TL (2002). Prevalence and classification of HIV-associated oral lesions. *Oral Dis.* **8**, 98-109.
- Penhall B (1980). Preventive measures to control further bone loss and soft tissue damage in denture wearing. *Aust. Dent. J.* **25**, 319-324.

- Peters AC, Wimpenny JWT (1988). A constant-depth laboratory model film fermentor. *Biotechnol. Bioeng.* **32**, 263-270.
- Pigman W, Elliott HC Jr., Laffre RO (1952). An artificial mouth for caries research. *J. Dent. Res.* **31**, 627-633.
- Pincus DH, Coleman DC, Pruitt WR, Padhye AA, Salkin IF, Geimer M, Bassel A, Sullivan DJ, Clarke M, Hearn V (1999). Rapid identification of *Candida dubliniensis* with commercial yeast identification systems. *J. Clin. Microbiol.* **37**, 3533-3539.
- Pires FR, Santos EB, Bonan PR, De Almeida OP, Lopes MA (2002). Denture stomatitis and salivary *Candida* in Brazilian edentulous patients. *J. Oral Rehabil.* **29**, 1115-1119.
- Porter SR, Scully C, Hegarty AM (2004). An update of the etiology and management of xerostomia. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **97**, 28-46.
- Post JC (2001). Direct evidence of bacterial biofilms in otitis media. *Laryngoscope* **111**, 2083-2094.
- Powderly WG, Gallant JE, Ghannoum MA, Mayer KH, Navarro EE, Perfect JR (1999). Oropharyngeal candidiasis in patients with HIV: suggested guidelines for therapy. *AIDS Res. Hum. Retroviruses* **15**, 1619-1623.
- Pratten J, Barnett P, Wilson M (1998a). Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria. *Appl. Environ. Microbiol.* **64**, 3515-3519.

- Pratten J, Pasu M, Jackson G, Flanagan A, Wilson M (2003). Modelling oral malodour in a longitudinal study. *Arch. Oral Biol.* **48**, 737-743.
- Pratten J, Smith AW, Wilson M (1998b). Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine. *J. Antimicrob. Chemother.* **42**, 453-459.
- Pratten J, Wills K, Barnett P, Wilson M (1998c). *In vitro* studies of the effect of antiseptic-containing mouthwashes on the formation and viability of *Streptococcus sanguis* biofilms. *J. Appl. Microbiol.* **84**, 1149-1155.
- Quirynen M, Bollen CM (1995). The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *J. Clin. Periodontol.* **22**, 1-14.
- Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Darius PL, van Steenberghe D (1990). The influence of surface free energy and surface roughness on early plaque formation. An *in vivo* study in man. *J. Clin. Periodontol.* **17**, 138-144.
- Raad I (1998). Intravascular-catheter-related infections. *Lancet* **351**, 893-898.
- Radfar L, Shea Y, Fischer SH, Sankar V, Leakan RA, Baum BJ, Pillemer SR (2003). Fungal load and candidiasis in Sjogren's syndrome. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **96**, 283-287.
- Radford DR, Challacombe SJ, Walter JD (1998a). Adherence of phenotypically switched *Candida albicans* to denture base materials. *Int. J. Prosthodont.* **11**, 75-81.
- Radford DR, Challacombe SJ, Walter JD (1999). Denture plaque and adherence of

- Candida albicans* to denture-base materials *in vivo* and *in vitro*. *Crit. Rev. Oral Biol. Med.* **10**, 99-116.
- Radford DR, Radford JR (1993). A SEM study of denture plaque and oral mucosa of denture-related stomatitis. *J. Dent.* **21**, 87-93.
- Radford DR, Sweet SP, Challacombe SJ, Walter JD (1998b). Adherence of *Candida albicans* to denture-base materials with different surface finishes. *J. Dent.* **26**, 577-583.
- Ramage G, Bachmann S, Patterson TF, Wickes BL, Lopez-Ribot JL (2002a). Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J. Antimicrob. Chemother.* **49**, 973-980.
- Ramage G, Tomsett K, Wickes BL, Lopez-Ribot JL, Redding SW (2004). Denture stomatitis: A role for *Candida* biofilms. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **98**, 53-59.
- Ramage G, Vande WK, Wickes BL, Lopez-Ribot JL (2001). Biofilm formation by *Candida dubliniensis*. *J. Clin. Microbiol.* **39**, 3234-3240.
- Ramage G, VandeWalle K, Bachmann SP, Wickes BL, Lopez-Ribot JL (2002b). *In vitro* pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. *Antimicrob. Agents Chemother.* **46**, 3634-3636.
- Ramage G, VandeWalle K, Lopez-Ribot JL, Wickes BL (2002c). The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol. Lett.* **214**, 95-

100.

- Ranganathan K, Umadevi M, Saraswathi TR, Kumarasamy N, Solomon S, Johnson N (2004). Oral lesions and conditions associated with human immunodeficiency virus infection in 1000 South Indian patients. *Ann. Acad. Med. Singapore* **33**, 37-42.
- Ray TL, Payne CD (1988). Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. *Infect. Immun.* **56**, 1942-1949.
- Reichart PA (2003). Oral manifestations in HIV infection: fungal and bacterial infections, Kaposi's sarcoma. *Med. Microbiol Immunol.* **192**, 165-169.
- Reichart PA, Samaranayake LP, Philipsen HP (2000). Pathology and clinical correlates in oral candidiasis and its variants: a review. *Oral Dis.* **6**, 85-91.
- Reichart PA, Schmidt-Westhausen A, Samaranayake LP, Philipsen HP (1994). *Candida*-associated palatal papillary hyperplasia in HIV infection. *J. Oral Pathol. Med.* **23**, 403-405.
- Reid G (1999). Biofilms in infectious disease and on medical devices. *Int. J. Antimicrob. Agents* **11**, 223-226.
- Reid G, Denstedt JD, Kang YS, Lam D, Nause C (1992). Microbial adhesion and biofilm formation on ureteral stents *in vitro* and *in vivo*. *J. Urol.* **148**, 1592-1594.
- Reiss E, Obayashi T, Orle K, Yoshida M, Zancope-Oliveira RM (2000). Non-culture based diagnostic tests for mycotic infections. *Med. Mycol.* **38**, 147-159.

- Reiss E, Tanaka K, Bruker G, Chazalet V, Coleman D, Debeaupuis JP, Hanazawa R, Latge JP, Lortholary J, Makimura K, Morrison CJ, Murayama SY, Naoe S, Paris S, Sarfati J, Shibuya K, Sullivan D, Uchida K, Yamaguchi H (1998). Molecular diagnosis and epidemiology of fungal infections. *Med. Mycol.* **36**, 249-257.
- Rex JH, Walsh TJ, Sobel JD, Filler SG, Pappas PG, Dismukes WE, Edwards JE (2000). Practice guidelines for the treatment of candidiasis. Infectious Diseases Society of America. *Clin. Infect. Dis.* **30**, 662-678.
- Richardson MD, Kokki MH (1998). Antifungal therapy in 'bone marrow failure'. *Br. J. Haematol.* **100**, 619-628.
- Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol.* **11**, 94-100.
- Rindum JL, Stenderup A, Holmstrup P (1994). Identification of *Candida albicans* types related to healthy and pathological oral mucosa. *J. Oral Pathol. Med.* **23**, 406-412.
- Roberts AP, Pratten J, Wilson M, Mullany P (1999). Transfer of a conjugative transposon, Tn5397 in a model oral biofilm. *FEMS Microbiol. Lett.* **177**, 63-66.
- Rosan B, Lamont RJ (2000). Dental plaque formation. *Microbes. Infect.* **2**, 1599-1607.
- Ruhnke M, Eigler A, Tennagen I, Geiseler B, Engelmann E, Trautmann M (1994). Emergence of fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus

- infection. *J. Clin. Microbiol.* **32**, 2092-2098.
- Russell AD (1995). Mechanisms of bacterial resistance to biocides. *Int. Biodeter. Biodegr.* **36**, 247-265.
- Russell C, Coulter WA (1975). Continuous monitoring of pH and Eh in bacterial plaque grown on a tooth in an artificial mouth. *Appl. Microbiol.* **29**, 141-144.
- Russotto SB (1980). The role of *Candida albicans* in the pathogenesis of angular cheilosis. *J. Prosthet. Dent.* **44**, 243-246.
- Sakki TK, Knuuttila ML, Laara E, Anttila SS (1997). The association of yeasts and denture stomatitis with behavioral and biologic factors. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **84**, 624-629.
- Samaranayake LP (1986). Nutritional factors and oral candidosis. *J. Oral Pathol.* **15**, 61-65.
- Samaranayake LP, Calman KC, Ferguson MM, Kaye SB, MacFarlane TW, Main B, Welsh J, Willox J (1984). The oral carriage of yeasts and coliforms in patients on cytotoxic therapy. *J. Oral Pathol.* **13**, 390-393.
- Samaranayake LP, Fidel PL, Naglik JR, Sweet SP, Teanpaisan R, Coogan MM, Blignaut E, Wanzala P (2002). Fungal infections associated with HIV infection. *Oral Dis.* **8**, 151-160.
- Samaranayake LP, Lamey PJ (1988). Oral candidosis: 1. Clinicopathological aspects. *Dent. Update.* **15**, 227-231.
- Samaranayake LP, MacFarlane TW (1980). An *in-vitro* study of the adherence of

- Candida albicans* to acrylic surfaces. *Arch. Oral Biol.* **25**, 603-609.
- Samaranayake LP, McCourtie J, MacFarlane TW (1980). Factors affecting the *in vitro* adherence of *Candida albicans* to acrylic surfaces. *Arch. Oral Biol.* **25**, 611-615.
- Sato M, Tsuchiya H, Akagiri M, Takagi N, Iinuma M (1997). Growth inhibition of oral bacteria related to denture stomatitis by anti-candidal chalcones. *Aust. Dent. J.* **42**, 343-346.
- Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J. Bacteriol.* **184**, 1140-1154.
- Schaller M, Hube B, Ollert MW, Schafer W, Borg-von Zepelin M, Thoma-Greber E, Korting HC (1999a). *In vivo* expression and localization of *Candida albicans* secreted aspartyl proteinases during oral candidiasis in HIV-infected patients. *J. Invest. Dermatol.* **112**, 383-386.
- Schaller M, Korting HC, Schafer W, Bastert J, Chen W, Hube B (1999b). Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol. Microbiol.* **34**, 169-180.
- Schembri MA, Kjaergaard K, Klemm P (2003). Global gene expression in *Escherichia coli* biofilms. *Mol. Microbiol.* **48**, 253-267.
- Scully C, el Kabir M, Samaranayake LP (1994). *Candida* and oral candidosis: a review. *Crit. Rev. Oral Biol. Med.* **5**, 125-157.
- Segal E, Lehrman O, Dayan D (1988). Adherence *in vitro* of various *Candida*

- species* to acrylic surfaces. *Oral Surg. Oral Med. Oral Pathol.* **66**, 670-673.
- Sen BH, Safavi KE, Spangberg LS (1997). Colonization of *Candida albicans* on cleaned human dental hard tissues. *Arch. Oral Biol.* **42**, 513-520.
- Sherman RG, Prusinski L, Ravenel MC, Joralmon RA (2002). Oral candidosis. *Quintessence. Int.* **33**, 521-532.
- Shin JH, Kee SJ, Shin MG, Kim SH, Shin DH, Lee SK, Suh SP, Ryang DW (2002). Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. *J. Clin. Microbiol.* **40**, 1244-1248.
- Shu M (1998). Study of root caries in an artificial mouth. *N. Z. Dent. J.* **94**, 62-64.
- Sissons CH (1997). Artificial dental plaque biofilm model systems. *Adv. Dent. Res.* **11**, 110-126.
- Sitheequ MA, Samaranayake LP (2003). Chronic hyperplastic candidosis / candidiasis (candidal leukoplakia). *Crit. Rev. Oral Biol. Med.* **14**, 253-267.
- Slutsky B, Staebell M, Anderson J, Risen L, Pfaller M, Soll DR (1987). "White-opaque transition": a second high-frequency switching system in *Candida albicans*. *J. Bacteriol.* **169**, 189-197.
- Soll DR (1992). High-frequency switching in *Candida albicans*. *Clin. Microbiol. Rev.* **5**, 183-203.
- Sonneborn A, Bockmuhl DP, Ernst JF (1999). Chlamydospore formation in *Candida albicans* requires the Efg1p morphogenetic regulator. *Infect. Immun.* **67**, 5514-

5517.

Spiechowicz E, Santarpia RP, Pollock JJ, Renner RP (1990). *In vitro* study on the inhibiting effect of different agents on the growth of *Candida albicans* on acrylic resin surfaces. *Quintessence. Int.* **21**, 35-40.

Spratt DA, Pratten J (2003). Biofilms and the oral cavity. *Rev. Environ. Sci. Bio/Technol.* **2**, 109-120.

Staib P, Kretschmar M, Nichterlein T, Hof H, Morschhauser J (2000). Differential activation of a *Candida albicans* virulence gene family during infection. *Proc. Natl. Acad. Sci. USA* **97**, 6102-6107.

Stewart PS, Costerton JW (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135-138.

Stoodley P, Sauer K, Davies DG, Costerton JW (2002). Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* **56**, 187-209.

Stoodley P, Wilson S, Hall-Stoodley L, Boyle JD, Lappin-Scott HM, Costerton JW (2001). Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl. Environ. Microbiol.* **67**, 5608-5613.

Sullivan DJ, Moran GP, Pinjon E, Al Mosaid A, Stokes C, Vaughan C, Coleman DC (2004). Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*. *FEMS Yeast Res.* **4**, 369-376.

Sutherland IW (1999). Biofilm matrix polymers - Role in adhesion. *In* Dental plaque revisited. (Eds. Newman HN and Wilson M) pp. 49-62. BioLine, Cardiff, U.K.

- Sutherland IW (2001). Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* **147**, 3-9.
- Sziegoleit F, Sziegoleit A, Wetzell WE (1999). Effect of dental treatment and/or local application of amphotericin B to carious teeth on oral colonization by *Candida*. *Med. Mycol.* **37**, 345-350.
- Taylor RL, Verran J, Lees GC, Ward AJP (1998). The influence of substratum topography on bacterial adhesion to polymethyl methacrylate. *J. Mat. Sci. Mater. Med.* **9**, 17-22.
- Tebbs SE, Sawyer A, Elliott TS (1994). Influence of surface morphology on *in vitro* bacterial adherence to central venous catheters. *Br. J. Anaesth.* **72**, 587-591.
- Theilade E, Budtz-Jorgensen E, Theilade J (1983). Predominant cultivable microflora of plaque on removable dentures in patients with healthy oral mucosa. *Arch. Oral Biol.* **28**, 675-680.
- Thomas CJ, Nutt GM (1978). The *in vitro* fungicidal properties of Visco-gel, alone and combined with nystatin and amphotericin B. *J. Oral Rehabil.* **5**, 167-172.
- Tintelnot K, Haase G, Seibold M, Bergmann F, Staemmler M, Franz T, Naumann D (2000). Evaluation of phenotypic markers for selection and identification of *Candida dubliniensis*. *J. Clin. Microbiol.* **38**, 1599-1608.
- Tollemar J, Gross N, Dolgiras N, Jarstrand C, Ringden O, Hammarstrom L (1999). Fungal prophylaxis by reduction of fungal colonization by oral administration of bovine anti-*Candida* antibodies in bone marrow transplant recipients. *Bone Marrow Transplant* **23**, 283-290.

- Trautner BW, Darouiche RO (2004). Role of biofilm in catheter-associated urinary tract infection. *Am. J. Infect. Control* **32**, 177-183.
- Tronchin G, Bouchara JP, Robert R, Senet JM (1988). Adherence of *Candida albicans* germ tubes to plastic: ultrastructural and molecular studies of fibrillar adhesins. *Infect. Immun.* **56**, 1987-1993.
- Verran J. (1988). Preliminary studies on denture plaque microbiology and acidogenicity. *Microb. Ecol. Health Dis.* **1**, 51-55.
- Verran J, Lees G, Shakespeare AP (1991). The effect of surface roughness on the adhesion of *Candida albicans* to acrylic. *Biofouling* **3**, 183-192.
- Verran J, Maryan CJ (1997). Retention of *Candida albicans* on acrylic resin and silicone of different surface topography. *J. Prosthet. Dent.* **77**, 535-539.
- Verran J, Motteram KL (1987). The effect of adherent oral streptococci on the subsequent adherence of *Candida albicans* to acrylic *in vitro*. *J. Dent.* **15**, 73-76.
- Watnick P, Kolter R (2000). Biofilm, city of microbes. *J. Bacteriol.* **182**, 2675-2679.
- Watts HJ, Very AA, Perera TH, Davies JM, Gow NA (1998). Thigmotropism and stretch-activated channels in the pathogenic fungus *Candida albicans*. *Microbiology* **144**, 689-695.
- Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW (1998a). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 1. Factors influencing distribution of *Candida* species in the oral cavity. *Aust. Dent. J.* **43**, 45-50.

- Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW (1998b). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 2. Oral diseases caused by *Candida* species. *Aust. Dent. J.* **43**, 160-166.
- Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW (1998c). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 3. Treatment of oral candidosis. *Aust. Dent. J.* **43**, 244-249.
- Wey SB, Mori M, Pfaller MA, Woolson RF, Wenzel RP (1988). Hospital-acquired candidemia. The attributable mortality and excess length of stay. *Arch. Intern. Med.* **148**, 2642-2645.
- Whittaker CJ, Klier CM, Kolenbrander PE (1996). Mechanisms of adhesion by oral bacteria. *Annu. Rev. Microbiol.* **50**, 513-552.
- Williams DW, Lewis MA (2000). Isolation and identification of *Candida* from the oral cavity. *Oral Dis.* **6**, 3-11.
- Williams I, Venables WA, Lloyd D, Paul F, Critchley I (1997). The effects of adherence to silicone surfaces on antibiotic susceptibility in *Staphylococcus aureus*. *Microbiology* **143**, 2407-2413.
- Wilson J (1998). The aetiology, diagnosis and management of denture stomatitis. *Br. Dent. J.* **185**, 380-384.
- Wilson M (1996). Susceptibility of oral bacterial biofilms to antimicrobial agents. *J. Med. Microbiol.* **44**, 79-87.
- Wilson M (1999). Use of constant depth film fermentor in studies of biofilms of oral bacteria. *Methods Enzymol.* **310**, 264-279.

- Wilson M (2001). Bacterial biofilms and human disease. *Sci. Prog.* **84**, 235-254.
- Wimpenny J (1999). Laboratory models of biofilm. *In* Dental plaque revisited. (Eds. Newman HN and Wilson M) pp. 89-110. BioLine, Cardiff, U.K.
- Wimpenny JW (1997). The validity of models. *Adv. Dent. Res.* **11**, 150-159.
- Wroblewska MM, Swoboda-Kopec E, Rokosz A, Krawczyk E, Marchel H, Luczak M (2002). Epidemiology of clinical isolates of *Candida albicans* and their susceptibility to triazoles. *Int. J. Antimicrob. Agents* **20**, 472-475.
- Zobell CE (1943). The effect of solid surfaces upon bacterial activity. *J. Bacteriol.* **46**, 39-56.
- Zylber LJ, Jordan HV (1982). Development of a selective medium for detection and enumeration of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaque. *J. Clin. Microbiol.* **15**, 253-259.

Publications arising from this thesis

Formation of *Candida albicans* biofilms on non-shedding oral surfaces

Hanadi Lamfon¹, Stephen R. Porter¹,
Michael McCullough²,
Jonathan Pratten¹

Lamfon H, Porter SR, McCullough M, Pratten J. Formation of Candida albicans biofilms on non-shedding oral surfaces. Eur J Oral Sci 2003; 111: 465–471. © Eur J Oral Sci, 2003

Susceptibility of *Candida albicans* biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study

Hanadi Lamfon¹, Stephen R. Porter¹, Michael McCullough² and Jonathan Pratten^{1*}

H. Lamfon *et al.*

Susceptibility of *Candida albicans* biofilms



Composition of in vitro denture plaque biofilms and susceptibility to antifungals

Hanadi Lamfon ^a, Zubaida Al-Karaawi ^a, Michael McCullough ^b,
Stephen R. Porter ^a, Jonathan Pratten ^{a,*}

FORMATION AND SUSCEPTIBILITY OF *CANDIDA ALBICANS* BIOFILMS

J. Pratten, H. Lamfon, S.R. Porter and M. McCullough

J. Praten, H. Lamfon, S.R. Porter & M. McCullough

FORMATION AND SUSCEPTIBILITY OF CANDIDA ALBICANS BIOFILMS

J. Pratten, H. Lamfon, S.R. Porter & M. McCullough

FORMATION AND SUSCEPTIBILITY OF CANDIDA ALBICANS BIOFILMS



FORMATION AND SUSCEPTIBILITY OF CANDIDA ALBICANS BIOFILMS

J. Pratten, H. Lamfon, S.R. Porter & M. McCullough