Yeast Species in the Oral Cavities of Older People

Nurulhuda M. Thiyahuddin
BDS (Malaya)

A thesis submitted in partial fulfilment of requirements for the degree of Doctor of Clinical Dentistry (Special Needs)

Department of Oral Diagnostic and Surgical Sciences
Faculty of Dentistry
University of Otago
Dunedin
New Zealand

August 2019
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contents</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>6</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>8</td>
</tr>
<tr>
<td>Oral and Poster Presentations</td>
<td>10</td>
</tr>
<tr>
<td>List of Tables</td>
<td>11</td>
</tr>
<tr>
<td>List of Figures</td>
<td>13</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>14</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>17</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>17</td>
</tr>
<tr>
<td>1.2 Literature Review</td>
<td>20</td>
</tr>
<tr>
<td>1.2.1 Older Population</td>
<td>20</td>
</tr>
<tr>
<td>1.2.2 Candida</td>
<td>21</td>
</tr>
<tr>
<td>1.2.3 Candida albicans</td>
<td>22</td>
</tr>
<tr>
<td>1.2.3.1 Characteristics of Candida albicans</td>
<td>23</td>
</tr>
<tr>
<td>1.2.3.2 Factors associated with antifungal resistance and virulence in Candida albicans</td>
<td>25</td>
</tr>
<tr>
<td>Adhesion</td>
<td>25</td>
</tr>
<tr>
<td>Biofilm formation</td>
<td>26</td>
</tr>
<tr>
<td>Enzyme secretion</td>
<td>27</td>
</tr>
<tr>
<td>Sugar sensing</td>
<td>28</td>
</tr>
<tr>
<td>1.2.4 Candidiasis</td>
<td>28</td>
</tr>
<tr>
<td>1.2.4.1 Factors predisposing to oral candidiasis</td>
<td>30</td>
</tr>
<tr>
<td>Prostheses</td>
<td>30</td>
</tr>
<tr>
<td>Drug therapy</td>
<td>31</td>
</tr>
<tr>
<td>Hyposalivation</td>
<td>31</td>
</tr>
</tbody>
</table>
2.2.2 Sample Collection and Analysis

2.2.2.1 Clinical examination

2.2.2.2 Collection of oral samples

Whole saliva

Mucosal Swabs

Mucosal Smears

2.2.2.3 Cytology

2.2.3 Laboratory Procedures

2.2.3.1 Yeast identification, quantification and storage

2.2.3.2 DNA analysis

Polymerase Chain Reaction amplification

DNA gel electrophoresis

PCR product clean up

DNA sequencing

MLST analysis

2.2.3 Statistical Analysis

Chapter 3: Results

3.1 Overview

3.2 Study Participants

3.2.1 Demographics

3.2.2 Denture Use

3.2.3 Hyposalivation

3.2.4 Cytology Examination

3.2.5 Medication

3.3 Microbiological Analysis

3.3.1 Yeast Colonisation

3.3.2 Yeast species distribution colonizing the oral cavity
3.3.3 Multi Locus Sequence Typing of 87 Candida albicans isolates .........................68

3.3.3.1 Diploid Sequence Typing. ........................................................................... 68
3.3.3.2 Candida albicans microevolution ................................................................... 75
3.3.3.3 The relatedness of Candida albicans strains ............................................... 80
3.3.3.4 Hybrid strains between C. albicans and C. dublinsiensis ............................. 82

Chapter 4: Discussion .................................................................................................. 83

4.1 Introduction ............................................................................................................ 83

4.2 Sampling Methods ............................................................................................... 83

4.3 Study Participants ............................................................................................... 84

4.4 Yeast Colonization .............................................................................................. 85

4.4.1 Dentures .......................................................................................................... 86
4.4.2 Salivary Flow .................................................................................................... 86
4.4.3 Medication ........................................................................................................ 88
4.4.4 Hyphae-positive smears .................................................................................. 88
4.4.5 Species variation .............................................................................................. 89

4.5 Presumptive identification of Candida species with CHROMagar™ Candida Agar ....90

4.6 Smear Test ............................................................................................................ 90

4.7 Precise Identification of Yeast Species .................................................................. 91

4.8 Multi Locus Sequence Typing ............................................................................. 92

4.8.1 Candida albicans strains .................................................................................. 92
4.8.2 Microevolution of C. albicans strains ............................................................... 93
4.8.3 Relatedness of strains ...................................................................................... 94
4.8.4 Hybrid strains .................................................................................................. 94

4.9 Future Studies ...................................................................................................... 94

4.10 Conclusions ....................................................................................................... 95

References ............................................................................................................... 96
Appendix I: Ethical Approval.................................................................116
Appendix II: Ngai Tahu Consultation Approval ....................................118
Appendix III: Patient Information Sheet.............................................120
Appendix IV: Consent Form.................................................................125
Appendix V: Laboratory Form...............................................................127
Appendix VI: Abstract for American Academy of Oral & Maxillofacial Pathology and
International Association of Oral Pathology Congress 2018 ..................128
Appendix VII: Abstract for Malaysian International Dental Exhibition and Conference ......129
Appendix VIII: Abstract for Australian Society of Special Needs in Dentistry Walkabout
Conference 2019 ‘Caring Together’ .....................................................131
Appendix IX: Abstract for University of Otago Student Research Symposium ........133
Appendix X: FASTA file of all Candida albicans/Candida dubliniensis chimera strains ........135
Appendix XI: Research Publication.......................................................148
Abstract

Introduction:
Oral candidiasis is prevalent among older people due to predisposing factors such as impaired immune defences, medications and denture use. An increasing number of older people live in rest home facilities and it is unclear how this institutionalised living affects the quantity and type of fungi colonizing these people’s oral cavities.

Objective:
The aim of this study was to investigate the presence and abundance of yeast species and *Candida albicans* strains in saliva and from the oral mucosal swabs and smears of older people living in rest homes and those living at home.

Methods:
Smears and swabs of the palate and tongue, and saliva samples were taken from participants residing in rest homes (RH; n = 25) and older people living in their own homes (OH; n = 25). Yeast in samples were quantified and presumptively identified by culturing on CHROMagar *Candida* agar. Sequencing of the ITS2 region of rDNA was carried out to confirm yeast species. Multilocus sequence typing (MLST) of seven housekeeping genes was used to compare *Candida albicans* strains.

Results:
A higher proportion of RH residents had *Candida* hyphae present in smears compared to OH participants (35% vs. 30%) although this difference was not statistically significant (*p* = 0.74). RH residents had, on average, 23 times as many yeast per mL saliva as OH participants (*p* = 0.01). Seven yeast species were identified in OH samples and only five in RH samples, with *C. albicans* and *C. glabrata* being the most common species isolated from both participant groups. MLST analysis of 87 *C. albicans* strains revealed 34 different diploid sequence types (DST). Participants with normal saliva flow tended to
have one *C. albicans* strain at all three sample sites (palate, tongue, saliva). Participants with low salivary flow rates also appeared to have mostly the same *C. albicans* strain at all three sample sites. For these strains, however, there was evidence of gene variation including loss of heterozygosity and aneuploidy which may be an indication of strain adaptation to the altered oral environment. Nine strains, found in four rest home participants, appeared to be *C. albicans/Candida dubliniensis* chimeras (hybrid strains).

Conclusion:
The results indicate that communal living for those who reside in an age care facility has an impact on the abundance of yeast species and the prevalence of chimera strains. This may be due to morbidities which led to the need for residential care and/or related to the rest home environment.
Acknowledgement

In the name of Allah, most gracious, most merciful.

My humble gratitude to God Almighty for bestowing countless blessings which I have enjoyed throughout my life. Thank you for the gift of a wonderfully supportive family; Haizal, Irfan and Sofea, with whom I have shared my frustrations, tears and joys especially in past three years of my life as a student. This journey would not have been possible without your unconditional support, thank you.

I would like to express my greatest appreciation to Professor Richard Cannon for his support and wisdom. As a new researcher, I had a lot to learn and you are always happy to impart valuable and constructive suggestions throughout the process of this study.

My special thanks to Dr Erwin Lamping for his enthusiastic encouragement and useful critiques. The high expectations that you demand from me and all of your students are out of the love and respect for science and the aspiration to achieve a high standard of work.

I am particularly grateful to Professor Alison Rich for her guidance and knowledge, always encouraging me to look at the bigger picture. You played a big part in my initial decision of taking up this course, and you have made it possible for me to do so. For that, I’m utterly grateful.

To Dr Graeme Ting, who is also my clinical supervisor, thank you for teaching me that you can stand your ground while still maintaining grace and kindness. I am ever grateful for your kind advice, enthusiasm (when I wasn’t) and wisdom.
Working and learning from all of you has enriched me in so many ways.

I wish to acknowledge the help provided by Mrs Maureen Simpson, Ms Angela Anderson, Dr Rajni Wilson, Ms Sharla Kennedy and Ms Lynda Horne in the initial stages of this research in terms of acquiring instruments and materials.

Thank you to all my research participants, whom I’ve had the pleasure of meeting, for being part of this study. I wish to acknowledge the New Zealand Ministry of Health for granting the financial support for this research and the University of Otago for granting me staff financial assistance to pursue this course.

I truly appreciate the help of Mrs Nazatulaziah Zainal, Mrs Sandra Mason and the many others who have contributed to this research and my DClinDent course.
Oral and Poster Presentations

1) American Academy of Oral & Maxillofacial Pathology and International Association of Oral Pathology Congress 2018 Poster Competition

24\textsuperscript{th} to 28\textsuperscript{th} June 2018 in Vancouver, Canada

2) Malaysian International Dental Exhibition and Conference Oral Presentation

14\textsuperscript{th} July 2019 in Kuala Lumpur, Malaysia

3) Australian Society of Special Needs in Dentistry Walkabout Conference

2019 ‘Caring Together’ Oral Presentation

19\textsuperscript{th} July 2019 in Melbourne, Australia

4) University of Otago Student Research Symposium Oral Presentation

4\textsuperscript{th} August 2019 in University of Otago, Dunedin, New Zealand
List of Tables

Table 1. ITS primers used for yeast identification ........................................... 53
Table 2. Gene fragments and primers used for *C. albicans* MLST analysis......... 53
Table 3. Distribution of yeast species colonizing of the oral cavities of OH residents. 58
Table 4. Distribution of yeast species in the oral cavities of participants living in the Montecillo RH.......................................................................................... 59
Table 5. Distribution of yeast species in the oral cavities of participants living in LSOP RH ........................................................................................................... 59
Table 6. Demographics, clinical features, and colonization status of 20 participants living in their OH compared with 20 RH participants. ........................................ 60
Table 7. Comparison of saliva yeast presence, yeast colonization level and PAS positive smears between denture wearers and non-denture wearers............... 61
Table 8. Distribution of PAS-positive smears and high, low or no yeast cell counts in saliva samples of denture versus non-denture wearers with either low (L) or normal (N) salivary flow rates. ................................................................. 62
Table 9. Comparison of saliva yeast presence, yeast colonization level and PAS-positive smears between participants with low and normal salivary flow.......... 62
Table 10. Species distribution in 70 yeast-positive oral samples from 13 OH and 16 RH participants .................................................................................................. 68
Table 11. MLST housekeeping gene information................................................. 69
Table 12. DSTs of 36 *C. albicans* strains isolated from saliva, tongue or palate of 13 (52%) participants living in their OHs that were colonized with *C. albicans*. ............ 70
Table 13. DSTs of 27 *C. albicans* strains isolated from saliva, tongue or palate samples of 6 (46%) participants living in the Montecillo rest home that were colonized with *C. albicans* ......................................................................................... 71
Table 14. DSTs of 24 *C. albicans* strains isolated from saliva, tongue or palate samples of 10 (91%) participants living in LSOP RH that were colonized with *C. albicans*. .... 72

Table 15. Diploid Sequence Types (DSTs) of 87 *C. albicans* isolates. .................. 74

Table 16. Microevolution of *C. albicans* strains in participants with normal saliva flow. ................................................................. 75

Table 17. Microevolution of *C. albicans* strains in participants with low saliva flow . 77

Table 18. Microevolution of 15 *C. albicans* isolates from the saliva, tongue and palate samples of two participants, one with a low (CL1) and the other one with normal saliva flow rate (MN7). ................................................................. 78
List of Figures

Figure 1. Percentage of participants colonised by yeast at each oral site. .................. 64

Figure 2. Quantity of yeast at oral sites in participants.................................................. 65

Figure 3. Species distribution of yeast colonizing the oral cavities of 13 (65%) OH and
16 (80%) RH participants.............................................................. 66

Figure 4. Sequence alignment of the 74 nucleotide positions within the seven
housekeeping genes (H1-H7) at which SNPs occurred in the 87 C. albicans isolates... 73

Figure 5. Phylogenetic tree of the 3535 bp concatenated housekeeping gene sequences
of 87 C. albicans isolates isolated from the indicated participants. ......................... 81
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>ALS</td>
<td>Agglutinin-like sequence (adhesin gene family)</td>
</tr>
<tr>
<td>APECED</td>
<td>Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy</td>
</tr>
<tr>
<td>CDR</td>
<td><em>Candida</em> drug resistance (gene encoding transporter)</td>
</tr>
<tr>
<td>CfU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>Chr</td>
<td>Chromosome</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DST</td>
<td>Diploid sequence type</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide Triphosphate</td>
</tr>
<tr>
<td>e.g</td>
<td>Example</td>
</tr>
<tr>
<td>EED</td>
<td>Epithelial escape and dissemination gene</td>
</tr>
<tr>
<td>et al.</td>
<td>et alia (and others)</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GAL</td>
<td>Gene involved in galactose metabolism</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl glucosamine</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HGT</td>
<td>Glucose transporter gene</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HWp</td>
<td>Adhesin gene family</td>
</tr>
<tr>
<td>IFF/HYR</td>
<td>Adhesin gene family</td>
</tr>
</tbody>
</table>
ITS  Internal transcribed space
Kb  1000 base pairs of DNA
Lip  Lipase
LOH  Loss of heterozygosity
LSOP  Little Sisters of The Poor and Scare Heart Rest Home
MDR  Multi drug resistance (drug efflux gene family)
mg  Milligram
min  Minute
ml  Millilitre
MLST  Multi Locus Sequence Typing
Ng  Nanogram
NZDA  New Zealand Dental Association
OH  Own home
p value  Probability value
PAS  Periodic acid–Schiff
PCR  Polymerase chain reaction
PL  Phospholipases
RH  Rest home
rpm  Revolutions per minute
rRNA  Ribosomal ribonucleic acid
s  Second
Sap  Secreted aspartic proteinases
SNP  Single nucleotide polymorphism
TEM  Transmission electron microscopy
TLR  Toll-like receptor
<table>
<thead>
<tr>
<th>w/v</th>
<th>Ratio of weight to volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPD</td>
<td>Yeast extract Peptone Dextrose</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Introduction

The oral cavity is colonized by hundreds of different microorganisms that form complex microbial communities. Most of these communities are present in biofilms on various oral surfaces. The immune system of healthy individuals maintains stable biofilm communities that help prevent oral infections. Impaired immune defences can cause an imbalance of the microbiota, or dysbiosis, and lead to bacterial, viral or fungal infections. *Candida* species are a normal component of the oral microbiota (Cannon & Chaffin, 1999b) that are carried in the mouths of about 40%-60% of healthy individuals (Zegarelli, 1993). *C. albicans* is the most prevalent species (Cannon, Holmes, Mason, & Monk, 1995)(Meurman et al., 2011) and non-*albicans* *Candida* species, for example, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* may be found in mouths of healthy individuals (Odds, 1988). Other species such as *Candida guilliermondii*, *Candida kefyr*, *Candida rugosa*, *Candida dubliniensis*, and *Candida famata* have also been reported, but less frequently (López-Martínez, 2010)(Miceli, Díaz, & Lee, 2011).

Oral candidiasis is the most common type of fungal infection of the oral cavity and *C. albicans* is the most frequent cause as it is a commensal microorganism (Nobile & Johnson, 2015). A provisional diagnosis of candidiasis can be made based on the medical history of the individual and oral examination of presenting signs and symptoms. This diagnosis can be confirmed histologically by demonstrating the presence of hyphae in mucosal smears (Guarner & Brandt, 2011). Patients with oral candidiasis can be treated with antifungal agents such as polyenes (e.g. amphotericin B and nystatin) and azole
derivatives (e.g. fluconazole, ketoconazole, clotrimazole, and miconazole). It has been reported that 20% of patients with oral candidiasis experience recurrence of infection, and around 30% of the recurrences were caused by *Candida* strains different from the first episode of infection (Gallè et al., 2011). *Candida* species differ in their susceptibility to antifungal agents. *C. glabrata* and *C. krusei* show reduced sensitivity to azoles (Meurman et al., 2011; Sanguinetti, Posteraro, & Lass-Flörl, 2015) and *Candida lusitaniae* can develop resistance to amphotericin B (Atkinson, Lewis, & Kontoyiannis, 2008). In addition, *C. parapsilosis* has been reported to cause outbreaks of candidemia in immunocompromised patients in hospital settings (Marconi et al., 2016) where it was thought to be transmitted by the hands of health care workers.

Factors that predispose to candidiasis include systemic factors caused by drug therapy (e.g. broad-spectrum antibiotics, corticosteroids) (Barbe, 2018), reduced saliva flow, chronic disease processes, malnutrition, immunosuppression, and neoplasia in addition to local factors including wearing dentures overnight, ill-fitting dentures and poor prosthesis hygiene (Razak et al., 2014). In the oral cavity, components of the innate immune system, such as salivary flow and biological components within saliva, play a major role in preventing infections (Humphrey & Williamson, 2001). Factors that reduce saliva flow predispose to infections such as candidiasis. In studies of people with xerostomia (dry mouth), it has been shown that there is an inverse correlation between salivary flow rate and the number of *Candida* cells in oral samples (Shinozaki et al., 2012) (Nadig et al., 2017). There was also an inverse correlation between the salivary flow rate and the severity of oral candidiasis (Shinozaki et al., 2012). Xerostomia can be induced by a large number of medications and older people often are prescribed multiple medications. Denture wearing increases with age and dentures obstruct saliva flow across mucosal surfaces. This reduces the clearance of microbes and creates environments that
promote fungal growth. Therefore, it is not surprising that older people are more likely than younger people to have oral mucosal lesions, including candidiasis (Lynge Pedersen, Nauntofte, Smidt, & Torp, 2015).

Populations worldwide are ageing. The World Health Organization (WHO) estimates that by 2050 the proportion of the population that is ‘older’ (above the age of 60) will have increased from 12% in 2015 to 22%, that is, from 900 million to 2 billion people (WHO factsheet/Ageing and health, 2018). Studies have shown that elderly patients living in institutional care facilities such as nursing homes and hospitals are more susceptible to oral and systemic Candida infections (Dimopoulos, Flevari, Theodorakopoulou, Velegraki, & Armaganidis, 2013), mainly due to comorbidities, indwelling devices, and common care facility processes, for example diet and oral care practices. It is important to know the types of Candida species colonizing the older community especially individuals in institutionalized settings as there is a variety of resistant species which may be a threat to health. What is concerning is the emergence of non-albicans Candida strains with lower antifungal susceptibility in self-caring, nursing home residents (Meurman et al., 2011). The presence of resistant Candida species could pose a problem for older people if they become immune compromised and develop candidiasis.

As many older people now live in residential aged care facilities, the aim of this study was to compare the Candida species colonizing the oral cavities of people living in rest homes with those living in their own homes.
1.2 Literature Review

1.2.1 Older Population

An older person is someone who has passed middle age and is approaching old age. The New Zealand population is ageing, 14.2% of the population are above the age of 65, and this number is expected to increase to 26.7% (about 1 in 4 people) by 2063 (Statistics New Zealand, 2014). This reflects the ageing trend of the world population. The United Nations estimated that the number of people above the age of 60 will double from 962 million in 2017 to 2.1 billion in 2050 (UN-DESA, 2017). The increased proportion of older people in the population is due to declining birth rates, increasing longevity and improvements in survival from accidents and disease.

The process of ageing is often associated with a decline in physical, sensory or cognitive capacity. The older person may be affected by chronic diseases that require them to take medications, undergo treatment like chemotherapy or radiotherapy which contribute to ongoing disability, variable levels of frailty, dependency and a reduction in quality of life. There is a dynamic and complex relationship between poor oral health and frailty. Poor oral health may affect nutritional intake which can lead to lethargy, frailty, illness and other health issues.

Half of the older people living in New Zealand reside in residential care (Broad et al 2015), which was about 32,000 people in 2013 (Statistics New Zealand, 2014). Residential care refers to long-term care (hospital-based, dementia unit, or rest home) given to individuals who live in a residential setting rather than in their own home. Often members of this population do not routinely engage in oral health care practices which
would assist in reducing oral disease and maintaining oral health (M. B. Smith, Thomson, & Gribben, 2012).

Specific physiological characteristics of older populations (e.g. use of multiple medications and dry mouth) make them more susceptible to colonization and infection by *Candida* species (Watanabe, 2014)(Dimopoulos, Flevari, Theodorakopoulou, Velegrak 2013). Dry mouth is more common among older people, as is a higher prevalence of yeast carriage in individuals with dry mouth (Thomson, 2015)(Hou, 2012)(Meurman et al., 2011). Studies have shown that older patients living in institutional care facilities are more susceptible to oral and systemic *Candida* infections (Grimoud et al., 2005)(Dimopoulos, Flevari, Theodorakopoulou, Velegraki, & Armaganidis, 2013). Superficial oral *Candida* infections which are not well managed in elderly or immunosuppressed patients, have the potential to spread.

### 1.2.2 Candida

*Candida* is a genus of yeast found in the oral cavity, gastrointestinal tract and genitourinary tract and on the skin where they are usually benign but can become pathogenic. The taxonomy of *Candida* is a complex subject as, historically, the genus *Candida* was defined as fungi able to form hyphae or pseudo-hyphae and reproduce by mitotic division (asexually). Analysis has since found that *Candida* species actually represent a polyphyletic group derived from more than one common evolutionary ancestor that are able to reproduce by both sexual and asexual modes. Many species have retained the ‘*Candida*’ name even when proven to have a sexual form, for example,
Candida tropicalis and Candida krusei. Thus, the genus name ‘Candida’ may be misleading as Candida species can be very different from one another.

Candida belongs to the Saccharomycotina sub-phylum which is a subdivision of the Ascomycota phylum within the Kingdom Fungi. The Saccharomycotina sub-phylum comprises an estimated 807 species of eleven distinct clades (Hittinger et al., 2015). Most of the important ‘Candida’ species belong to four major clades:

i) Candida CTG (217 species; including C. albicans, C. dubliniensis, C. tropicalis, C. parapsilosis, C. guilliermondii, C. lusitaniae, C. famata, and C. rugosa) that translate CTG codons as serine instead of the usual leucine.

ii) Saccharomycetaceae (~129 species) including C. kefyr and the post Whole Genome Duplication (WGD) species C. glabrata and S. cerevisiae

iii) Pichiaceae (~134 species) including C. krusei and C. inconspicua

iv) Phaffomycetaceae (~92 species) including C. norvegensis

A clade is a group of organisms originating from a common ancestor, and their lineal descendants and represents a branch in the phylogenetic tree. As the population evolves, the branch splits into smaller branches reflecting evolutionary history. Species within the same clade do share some features, for example, species in the Candida CTG clade have a distinctive codon usage. However, each species is unique and genetically distinct from each other.

1.2.3 Candida albicans

Worldwide, invasive candidiasis affects more than 250,000 people every year, causing the deaths of more than 50,000 and C. albicans accounts for half the isolates detected (Kullberg & Arendrup, 2015). C. albicans is an opportunistic pathogen residing
harmlessly on the body as a commensal. Fungal proliferation is kept under control by the host immune system and a protective bacterial microbiome of the gut and other mucosal surfaces (Cheng, Joosten, Kullberg, & Netea, 2012a). *C. albicans* is able to thrive in different parts of the body (gut, vagina, oral mucosa, skin) without causing disease (Nobile & Johnson, 2015). It is a very flexible and adaptable organism in its capacity for survival in and on the human body.

1.2.3.1 Characteristics of *Candida albicans*

*C. albicans* is a polymorphic organism, as it is able to grow as ovoid, unicellular, yeast cells (sometimes referred to as blastospores), filamentous hyphae, pseudo-hyphae with constricted septa and thick-walled chlamydospores (Odds, 1988). However, novel cell morphotypes, opaque (a/α), grey and gastrointestinal induced transition (GUT) cell types, have recently been reported, further complicating our understanding of host-*Candida* interactions of commensalism and disease (Noble, Gianetti, & Witchley, 2017). The morphological changes between these growth forms are stimulated by environmental conditions such as temperature, pH, concentration of CO₂ and glucose. Yeasts propagate by mitotic division in favourable growth conditions forming clonal populations of haploid, diploid and/or polyploid cells (Dujon, 2010). Some species, such as *Saccharomyces cerevisiae*, have the ability to undergo a complete sexual cycle. *C. albicans* has the genes necessary for most stages of a sexual cycle but is thought to only undergo sexual reproduction rarely.

The *C. albicans* genome consists of a 16 megabase (haploid) genome organized in 8 mostly heterozygous diploid chromosomes (Selmecki, Forche, & Berman, 2010). It reproduces by asexual clonal division, as well as mating between cells of the opposite
mating type and undergoes a parasexual cycle. In this cycle, diploids mate forming tetraploids which then undergo sequential chromosome loss, producing near-diploid progeny that are often trisomic and/or homozygous for one or more whole chromosomes. The *C. albicans* genome has a high level of plasticity; it is capable of undergoing genomic changes like gross chromosomal rearrangements, aneuploidy, and loss of heterozygosity (LOH) under stressful conditions like extreme temperatures, nutrient deficiency and exposure to chemotherapeutic or antifungal drugs (Forche et al., 2011). LOH is a genetic event during mitotic division resulting in daughter cells that are homozygous in one or more alleles. This may be due to mitotic recombination, gene conversion or deletion. The range of LOH may be short tracts (≤10 kb), long tracts (>10 kb) or whole chromosomes (Forche et al., 2011). Aneuploidy is the presence of an abnormal number of chromosomes – instead of two copies of each chromosome, there may be only one copy or three or more copies of particular chromosomes. Aneuploidy can occur in a cell when there is LOH of a whole chromosome. Aneuploidy is generally disadvantageous as it often uncovers recessive detrimental mutations. However, some aneuploidies provide a fitness advantage where cells that retain the more beneficial allele after LOH may exhibit a growth advantage under stressful conditions over cells that do not undergo LOH (Wertheimer, Stone, & Berman, 2016).

Yeast cell growth is favoured by relatively low temperatures (~30°C), acidic pH and the absence of serum. Growth of yeast cells allows for the dissemination of the organism to other sites. The transition of yeast to hyphal growth is triggered by many nutritional and environmental factors, including the presence of serum, neutral pH, relatively high temperatures (37°C), nutrient starvation, hypoxia and high concentrations of CO₂ (Lu,
Su, & Liu, 2014). The hyphal form is crucial in the infection process, as it enables tissue penetration and escape from immune cells. The morphological plasticity of *C. albicans* is a critical factor in its pathogenicity both superficially and systemically.

Phenotypic switching in *C. albicans* is referred to the spontaneous, reversible, white to opaque cell-type switching system (Noble et al., 2017). The switch changes the cell morphology (size and shape of cells), giving the organism the ability to form hyphae, improve adhesion, change its membrane composition, differ the range of secretory products, reduce sensitivity to neutrophils and oxidants, antigenicity and drug susceptibility (Soll, 2014). White cells are round or ovoid and form smooth, domed colonies. They secrete a chemoattractant for leukocytes, express genes involved in fermentative metabolism, they are not competent for mating and are more virulent in systemic candidiasis compared to the larger opaque cells. Opaque cells are bean-shaped, the surface is covered with pimples and they form flat translucent colonies. The cells are resistant to neutrophil engulfment, express genes involved in oxidative metabolism and mate more efficiently than white cells (Bommanavar, Gugwad, & Malik, 2017).

1.2.3.2 Factors associated with antifungal resistance and virulence in Candida albicans

Adhesion

*Candida* species have the ability to adhere tightly to different surfaces, either biological or inert abiotic surfaces. Adhesion is the important first step in the attachment and proliferation of cells and the establishment of fungal infections. *C. albicans* adhesins are cell wall proteins, the three major groups are encoded by the *ALS*, *HWP*, and *IFF/HYR* gene families. The ability of conformational activation of adhesins, differential gene expression patterns, and allelic variability of these gene families, make *C. albicans* highly
adaptable in its adhesion to different surfaces (Cavalheiro & Teixeira, 2018). *Candida* adhesion to other *Candida* cells or other microbes leads to the formation of biofilms of either multi- or mono-species communities (de Groot, Bader, de Boer, Weig, & Chauhan, 2013).

**Biofilm formation**

*C. albicans* biofilms are composed of cells in different morphologies encased in an extracellular matrix. These biofilms have been found on endothelial cells, epithelial mucosal tissues, urinary and central venous catheters, pacemakers, mechanical heart valves, joint prostheses, contact lenses, and dentures. Biofilms have the potential to seed disseminated bloodstream infections and lead to invasive systemic infections of tissues and organs. Biofilm formation comprises four stages: (a) adherence to a surface (usually by yeast cells); (b) proliferation forming a basal layer of cells; (c) growth of pseudohyphae and hyphae along with production of extracellular matrix material; and (d) slow dispersal of yeast-form cells from the biofilm to seed new sites (Nobile & Johnson, 2015). Complex interspecies interactions occur within polymicrobial biofilms which pose a significant clinical challenge as they are resistant to conventional antimicrobial therapy, the host immune system, and other environmental interventions. Factors which contribute to the antifungal resistance of biofilms are the extracellular matrix which acts as a barrier to drug diffusion, alterations in gene expression during biofilm formation (e.g. upregulation of *CDR* and *MDR* genes encoding azole drug transporters) and the presence of biofilm persister cells which display resistance to fluconazole (Cavalheiro & Teixeira, 2018). The cell wall of *C. albicans* is a dynamic structure which provides protection against physical and chemical insult, is responsible for the different shapes displayed by *C. albicans* and plays an important role in interactions with host tissues. The cell wall is
mainly composed of proteins, glucans, and chitin. The inner layer of the cell wall appears translucent under transmission electron microscopy (TEM) and is composed of a chitin and polysaccharide matrix. The outer layer is enriched with mannoprotein and appears denser under TEM and has perpendicularly aligned fibrils that are mostly mannoproteins (Chaffin, 2008). The ability of *C. albicans* to alter its cell wall architecture and composition determines the cell morphology and enables its evasion of the host immune system (Chaffin, 2008). The main structural component of the *C. albicans* cell wall is $\beta$-1,3-glucan (Ene et al., 2012). The glucan composition of the wall determines the organism’s sensitivity to environmental stress and antifungals (da Silva Dantas et al., 2016). Cell wall polysaccharide interacts with pattern recognition receptors on host cells stimulating an immune response. Altering the expression and presentation of critical polysaccharide pathogen-associated molecular patterns (PAMPs) reduces the detection of *C. albicans* by neutrophils (Chaffin, 2008)(da Silva Dantas et al., 2016).

**Enzyme secretion**

*C. albicans* secretes hydrolytic enzymes which possess a range of functions including degradation of host connective tissues, nutrient acquisition and evasion of the host immune system (da Silva Dantas et al., 2016). The hydrolytic enzymes are secreted aspartic proteinases (Saps), secreted phospholipases (PLs) and lipases (Lips). The *C. albicans* Sap family comprises 10 proteins, each functioning optimally at different pH, temperatures, and acting on different carbon and nitrogen sources (Schaller, Borelli, Korting, & Hube, 2005).
Sugar sensing

Nutrients in the form of glucose, fructose, mannose and N-acetyl glucosamine (GlcNAc) are important for Candida growth (Lu, Su, & Liu, 2014). *C. albicans* has a unique sugar sensing and signalling system involving the glucose transporter gene family consisting of 20 high-affinity glucose transporters. Each transporter/sensor is unique to a specific type of sugar and concentration level. *C. albicans* HGT9, HXT10, HGT12, and HGT17 are strongly expressed at low glucose concentrations (0.2%) and repressed at 5% glucose while other *HGT* genes are expressed in 2% glucose. *C. albicans* GAL1, GAL7, GAL10 are involved in sensing galactose and Hgt4 is the sensor for fructose and mannose. *HGT12* and *HGT7* encode transporters for fructose and mannose (Van Ende, Wijnants, & Van Dijck, 2019). GlcNAc is transported into the cells and metabolized into fructose-6-phosphate, which is further metabolized to pyruvate. Studies suggest that GlcNAc induces galactose absorption by *C. albicans* (Chaffin, 2008). *C. albicans* is capable of utilising different carbon sources simultaneously in the presence of glucose making it adaptable to dynamic changes within the host (Van Ende et al., 2019).

1.2.4 Candidiasis

Candidiasis is the term for fungal infections caused by *Candida*. Candidiasis may occur in the oral cavity, in the gastrointestinal tract, on the skin, or in the genitourinary tract. It can manifest as a topical or systemic disease. Oral candidiasis is the most common type of opportunistic infection of the oral cavity, and *C. albicans* is the most frequent cause of this condition (Nobile & Johnson, 2015).

Colonization by *Candida* depends on the entry of cells into the oral cavity, attachment and growth, tissue penetration and cell removal/clearance from the oral cavity (Cannon
and that rates of replication at least match the rate of clearance. Clearance of *Candida* from the oral cavity is achieved by swallowing and oral hygiene. Colonization by *Candida* does not signify oral disease. Candidiasis occurs when replication exceeds clearance and there is the presence of tissue damage.

*Candida* is acquired by manual transmission, saliva transfer or contaminated food and drink (Fanello, Bouchara, Jousset, Delbos, & LeFlohic, 2001). To colonize the oral cavity, the *Candida* cells utilise cell wall adhesins to adhere to the host surfaces (Cavalheiro & Teixeira, 2018). Adhesins are molecules which enable binding of *Candida* cells to oral epithelial cells, saliva molecules, teeth, other organisms, and dentures (de Groot et al., 2013). The adhesins found on the cell wall of *C. albicans* are highly specific in promoting adhesion to different types of surface, in aggregation, biofilm formation, and modulating host immune responses. ALS-like genes have been found in non-*albicans* *Candida* *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii* while IFF/HYR gene families have been identified in CTG-clade *Candida* species. *C. glabrata* contains Epa adhesins which bind to epithelial and endothelial cells, and innate immune cells (de Groot, Bader, de Boer, Weig, & Chauhan, 2013).

Glucose functions as the building block for the synthesis of other biomolecules as well as for energy production via fermentation or respiration. Glucose may also be a ligand for receptors or a substrate for transporters (Van Ende, Wijnants, & Van Dijck, 2019). In a nutrient-rich environment, *C. albicans* maintains its yeast form. *C. albicans* exhibit increased resistance to azole antifungal drugs and osmotic stress in the presence of increased glucose concentrations (0.01–1%) due to upregulation of stress resistance genes (Ene et al., 2012). Patients with diabetes are reported to be more susceptible to
systemic Candida infection as C. albicans cells respond to glucose in the bloodstream by increasing their resistance to oxidative and cationic stresses (Rodaki et al., 2009). Candidiasis is also more prevalent in patients with poor oral/denture hygiene and dry mouth as the latter reduces saliva diluting oral sugars (Billings, Dye, Iafolla, Grisius, & Alevizos, 2017). Candida competes for glucose with other organisms in the oral cavity and proliferate in the absence of bacteria as seen in long term antibiotic use (Cannon & Chaffin, 1999).

After colonization, the next step in the pathophysiology of candidiasis is host tissue invasion. C. albicans hyphae invade the epithelial via active tissue penetration (epithelial attachment, tissue invasion, and tissue damage)(Cannon et al., 1995). C. albicans can also spread passively via induced endocytosis, whereby viable and dead C. albicans cells are engulfed by epithelial cells. Within the epithelial cells, viable C. albicans cells form hyphae, which can pierce the cells via the action of the EED1 gene (epithelial escape and dissemination 1). C. albicans is also able to downregulate epithelial TLR4 expression, increasing the vulnerability of epithelial cells to C. albicans infection (Cheng, Joosten, Kullberg, & Netea, 2012b).

1.2.4.1 Factors predisposing to oral candidiasis

Prostheses

Prostheses with poor retention or stability cause irritation which can lead to local microscopic breaches of the oral mucosa, allowing the organism access into the host tissues (Farah, Amos, Leeson, & Porter, 2018). Poor denture hygiene allows biofilm development which can act as a reservoir of Candida cells. Due to the poor salivary flow adjacent to the fitting surface of the denture, this region has a low oxygen tension and
low pH, favouring yeast proliferation. A higher percentage of denture wearers (64.2%) had *Candida* carriage compared to non-denture wearers (19.2%) (Pereira Lyon et al., 2006). Worn-down dentures cause reduced vertical dimension which increases the wrinkling/creasing of the commissures of the lips. Stagnation of saliva in this region reduces clearance and is a predisposing factor for angular cheilitis.

**Drug therapy**

Broad-spectrum antimicrobial therapy alters the oral microbiota (Cannon & Chaffin, 1999a). This dysbiosis promotes the proliferation and growth of *Candida*. Excessive use of antimicrobial mouthwash may induce resistance in C. albicans strain (Edlind, Smith, & Edlind, 2005). Aerosolised corticosteroids (as in steroid inhalers), as well as topical and systemic corticosteroids, cause local immunosuppression making the host tissue susceptible to *Candida* invasion (Kennedy et al., 2000).

**Hyposalivation**

Xerostomia, which is the sensation of dry mouth, may be caused by hyposalivation or altered taste sensation. Hyposalivation (salivary gland hypofunction) is the production of less than 2 ml per min of unstimulated whole saliva (Longman et al., 1997). Patients with hyposalivation have an increased rate of oral mucosal candidiasis symptoms and higher *Candida* carriage compared to controls (Shinozaki et al., 2012), with colony counts of more than 400 cfu/ml (Nadig et al., 2017). Saliva in the oral cavity is useful for lubrication, buffering action, clearance, maintenance of tooth and mucosal integrity, antibacterial and antifungal activity, taste and digestion (Humphrey & Williamson, 2001). Hyposalivation may be due to many factors including physical damage to salivary glands such as trauma or surgery. Radiotherapy often used as primary therapy in head
and neck cancer or as an adjunct to surgery or combined with chemotherapy can contribute to hyposalivation (Mercadante, Al Hamad, Lodi, Porter, & Fedele, 2017). Radiation causes an acute inflammatory reaction followed by fibrosis of salivary glands, reduced blood flow and loss of acinar cells resulting in decreased salivary flow. Radiation-induced salivary gland dysfunction is dependent on the radiation dose (more than 26G) and field (Hegarty & Hodgson, 2008). Another main cause of hyposalivation is xerogenic medication, such as anticholinergic drugs, antihistamines, sedatives, hypnotics, and beta-blockers, especially when taken concurrently (Hegarty & Hodgson, 2008). Diseases can affect salivary gland function and reduce salivary flow, for example, sialadenitis due to Sjogren’s syndrome, hepatitis C and HIV infection and sialadenosis in cases of diabetes mellitus, alcoholism and eating disorders (Farah, Lynch, & McCullough, 2010).

**Dietary Factors**

*Candida* can utilise glucose, sucrose, galactose, fructose, and mannose for growth and proliferation. Diets rich in glucose and galactose improve *Candida* biofilm formation on acrylic denture surfaces (Nikawa, Nishimura, Hamada, Kumagai, & Samaranayake, 1997). The presence of high glucose concentrations in the oral cavity is a contributing factor to increasing *Candida* load. Nutritional deficiency in iron, folate, vitamin B₁₂, and vitamin C reduces host defences and results in the loss of mucosal integrity (Bhattacharya, Misra, & Hussain, 2016). This condition increases the host’s vulnerability to invasion of tissues by *Candida*. 
Malignant Disease

Haematologic malignant disease such as leukaemia, lymphoma and myeloma alter the production and function of white blood cells debilitating the host immune system. Patients with these malignant conditions as well as other types of cancer usually undergo cytotoxic chemotherapy or radiotherapy or a combination of both. These treatment modalities impair the host immune system by reducing the number of innate immune cells and components of the adaptive immune system, putting the patient at risk of infection (Teoh & Pavelka, 2016). Cancer therapies reduce cell proliferation and turnover in connective tissue. Radiation initially causes inflammation of the mucosal tissue, followed by fibrosis, hypo-vascularity and tissue ischaemia which reduce the ability of the tissue to heal and resist Candida infection (Squier & Kremer, 2001).

Endocrine Disorders

Patients with endocrine disorders may present with immune cell deficiency, where surveillance for recognition and destruction of Candida cells is inefficient, increasing the risk of infection. Patients with diabetes show a neutrophil defect and impaired phagocytic activity of lymphocytes and monocytes (Lecube, Pachón, Petriz, Hernández, & Simó, 2011) making them more susceptible to Candida infection. Lack of insulin or blocked insulin action prevents glucose from entering cells and it accumulates in tissues, blood, and urine instead. Glucose is utilised by Candida for growth, adhesion, biofilm formation and antifungal resistance (Rodaki et al., 2009). Patients with Addison’s Disease (adrenocortical hypofunction) will be on long term corticosteroid therapy to replace missing hormones. As steroids suppress the immune system, there is increased susceptibility to infections. Hypothyroidism on its own, or as part of Autoimmune
Polyendocrinopathy Type 1 (APECED) syndrome, may present with persistent candidiasis (Humbert et al., 2018).

**Immunodeficiencies**

Over 60% of HIV-infected patients, and more than 80% of patients diagnosed with AIDS present with pseudomembranous candidiasis and erythematous candidiasis (Farah et al., 2010). Patients with severe immunodeficiency syndromes, for example, DiGeorge syndrome, hereditary myeloperoxidase deficiency and Chediak-Higashi syndrome often present with candidiasis (Farah et al., 2010). Patients on long-term steroid therapy for the management of asthma, connective tissue disorders (e.g. rheumatoid arthritis, systemic lupus erythematosus), gastrointestinal disorders (e.g. ulcerative colitis, Chron’s disease), mucocutaneous disease (pemphigus), post organ transplant or renal disorders (nephrotic syndrome) are immunosuppressed and at higher risk of *Candida* infection.

1.2.4.2 Clinical manifestations of Candida infection of the mouth:

**Pseudomembranous Candidiasis**

This is the most common type of candidiasis and it occurs on the soft palate, oropharynx, buccal mucosa, tongue and gingiva (Niimi, Firth & Cannon 2010). It presents as non-adherent creamy or yellow plaques which are easily removed exposing an erythematous base which bleeds easily. Patients may experience pain, burning sensation, altered (sour) taste sensation, and bleeding. This condition may be acute (recent onset, not more than 3 months) or chronic (lesion present for more than 3 months). Chronic pseudomembranous candidiasis is commonly seen in immunocompromised patients for example patients undergoing chemotherapy, poorly controlled diabetes, leukaemia, and HIV/AIDS (Williams & Lewis, 2011).
Erythematous Candidiasis

Erythematous candidiasis presents as a well-demarcated erythematous area, or depapillated area of the tongue. The acute type is associated with antibiotic and/or corticosteroid use (Williams & Lewis, 2011). Chronic erythematous candidiasis, also known as denture-induced stomatitis, usually presents as an erythematous region of the fitting surface of a denture (Zegarelli, 1993). The Newton (1962) classification of this condition is as follows:

- **Type I** Localised inflammation
- **Type II** Diffused erythema involving part of, or entire, denture-bearing area
- **Type III** Inflammation, erythema and granular or papillary type of tissue hyperplasia

This is commonly seen in patients with poor denture hygiene who routinely leave their dentures in their mouth overnight.

Plaque-like/nodular Candidiasis

This type of presentation is also known as chronic hyperplastic candidiasis. It mainly presents as chronic, irregular, well-demarcated, slightly raised, adherent white plaques. It may be homogenous or speckled and usually found in the post-commissural buccal mucosa. This is a potentially malignant lesion, with malignant transformation seen in up to 60% of cases (Bakri, Hussaini, Holmes, Cannon, & Rich, 2010). Some lesions of this nature do resolve with antifungal therapy. Close monitoring is necessary for persistent lesions due to its propensity to undergo malignant transformation.
Angular cheilitis

This type of candidiasis presents as inflamed, crusting and cracking of the commissure at the corners of the mouth. This may be caused by local factors like reduced facial height and poor muscle tone. Systemically, it may be due to vitamin or mineral deficiency (iron, folate, vitamin B₁₂) as seen in patients with anaemia (Coronado-Castellote & Jiménez-Soriano, 2013). There is commonly a bacterial component (for example Staphylococcus aureus) associated with this lesion (Zegarelli, 1993). Treatment for this type of candidiasis is the management of the underlying cause and use of topical antifungals and antibiotics.

Median Rhomboid Glossitis

This is a symmetrical, depapillated, erythematous, rhombus/diamond/elliptical-shaped lesion on the posterior dorsal surface of the tongue anterior to the circumvallate papilla. This is due to atrophy of filiform papilla in the area and is a form of chronic fungal infection. It is usually asymptomatic, however some patients experience a burning sensation on taking certain types of food (Radfar, 2015).

Chronic Mucocutaneous Candidiasis

This condition presents as recurrent and progressive Candida infections of the skin, nails, oral cavity and genital mucosa. It is associated with defects in acquired cellular immunity, with T-cell and Th17 deficiency. The disease may be associated with endocrine and autoimmune disorders of hereditary origin, for example APECED syndrome (Humbert et al., 2018). Infections are usually superficial, and systemic candidiasis is rarely observed. The treatment consists of management of the underlying
cause - therapy for the infectious disease, endocrinopathies and specific treatment for the immunological deficiency.

1.2.4.3 Diagnosis of Candidiasis

A diagnosis of candidiasis is made mainly from the clinical and medical history as well as an oral examination of presenting signs and symptoms. The clinical diagnosis may be confirmed with microbiological and/or cytological or histological investigation. Microbiological investigations may be carried out to establish a differential diagnosis and to determine possible resistance to antifungal drugs. The diagnosis of candidiasis can be confirmed cytologically by confirming the presence of hyphae in mucosal smears or histologically with biopsies stained with Haematoxylin and Eosin (H&E), Grocott-Gomori's Stain (GMS) or Periodic acid–Schiff (PAS) (Guarner & Brandt, 2011). *C. glabrata*, which can cause candidiasis, is unable to form hyphae or pseudo-hyphae and so is not identified morphologically.

*Candida* species may be identified using CHROmagar *Candida* or Chromagar ID, which use chromogenic chemicals in the agar to colour growing yeast colonies. These agars can be used to isolate and differentiate certain *Candida* species and quantify the presence of yeast by counting colony forming units (cfus). *Candida* species are presumptively identified according to the colony colour. This diagnostic method is easily accessible and simple to use (Coronado-Castellote & Jiménez-Soriano, 2013). The yeast species and strain can be confirmed with DNA sequencing techniques like Multi Locus Sequence Typing (MLST) as used in this study. The internal regions of seven selected housekeeping genes are sequenced to identify inter-strain genetic relatedness. This method is highly discriminatory and reproducible for the characterization of *C. albicans*
Other yeast species may be identified through sequencing the internal transcribed space (ITS) regions ITS1 and ITS2 of the small subunit rRNA (Hoggard et al., 2018). *Candida* carriage can be investigated by plating a known volume of an oral rinse or whole saliva on Sabouraud dextrose agar. Patients with clinical signs of *Candida* infection had been found to have greater than 400 cfu per ml of saliva, compared to carriers of *C. albicans* that had less than 400 cfu per ml saliva (Epstein, Pearsall, & Truelove, 1980). Fluorescent *in situ* hybridization (FISH) can be used to detect yeast in blood samples (Guarner & Brandt, 2011) and multiplex tandem PCR has the potential for earlier diagnosis of candidemia from serum and plasma samples (Lau et al., 2010).

It is important to determine the underlying cause of the disease, as candidiasis rarely occurs without predisposing factors. Poor denture retention, stability, hygiene, and low vertical dimension may be contributing factors. Patients with angular cheilitis may require a full blood count, and measurement of iron and folate levels. Patients with chronic mucocutaneous candidiasis, HIV-positive individuals and others with systemic disease will require blood tests for haematological and immunological investigation. Hyposalivation affects buffering in the oral cavity and reduces the clearance of *Candida*. An unstimulated saliva flow test may be conducted to detect hyposalivation, 0.3 ml/min is the normal flow rate, level of less than 0.2 ml/min is regarded as salivary hypofunction (Longman et al., 1997) while values below 0.1 ml/min are considered extremely low (Humphrey & Williamson, 2001).
Candida infections may have similar clinical presentations to other oral conditions. For example, traumatic lesions, chemical burns, lichen planus, mucositis, erythema multiforme, systemic lupus erythematosus, and syphilis may all have intra-oral appearances that mimic Candida infection (Farah et al., 2010)(Millsop & Fazel, 2016). Therefore, it is extremely important that clinicians make the correct diagnosis in order to provide appropriate treatment.

1.2.4.3 Treatment of candidiasis

Treating the underlying cause is the best long-term management strategy in dealing with candidiasis. This will address the root cause and minimise the use of antifungal drugs.

Patients who have been taking long-term antibiotics may experience a reduction in candidiasis with the cessation of antibiotics. The cessation of antibiotics allows re-growth of oral bacteria, restoring natural microbial balance (Cannon & Chaffin, 1999). Candidiasis caused by the use of steroid inhalers due to effect of the immunosuppressant agent on the mucosa may be controlled by the use of a spacer for inhaled corticosteroid delivery (Pramod & Jayasree, 2013).

Chronic erythematous candidiasis can be managed by addressing the condition of the patient’s dentures. Denture hygiene should be evaluated and improved by daily cleansing and soaking the dentures twice a week for about half an hour in white vinegar (diluted 1:20), 0.1% hypochlorite solution, or chlorhexidine gluconate (2% suspension), and then
rinsing them and allowing them to air dry (Farah et al., 2010)(Millsop & Fazel, 2016). Dentures should not be worn overnight to encourage salivary flow to cleanse the denture fitting surface.

Angular cheilitis caused by a lowered facial height may be corrected by increasing the patient’s vertical dimension. This may be done by rebasing or remaking the dentures to the correct height or increasing the height of posterior teeth in cases of attrition. Patients suffering from iron and or vitamin B deficiency, need to be investigated to determine the cause of the deficiency.

Minimising the availability of sugars in the oral cavity slows down the proliferation of Candida. This can be achieved by reducing the intake of sugary foods and beverages. Effective removal of oral sugars must be carried out by brushing teeth, gums, and tongue twice a day as well as interdental cleaning by using floss or interdental brushes.

Hyposalivation can be managed by stimulation and substitution. Saliva flow may be stimulated by:

2) Salivary gland massage, repeated direct stimulation through the feedback system increases salivary flow (Ono & Uchiyama, 2017).
3) Using a cholinergic agonist such as pilocarpine which is a direct acting cholinergic parasympathomimetic agent. It stimulates muscarinic receptors in secretory glands (Mercadante et al., 2017).
Adverse reactions to pilocarpine are nausea, dyspepsia, hypertension, palpitation, headache, visual disturbances, pruritus, and urinary urgency. It provides an additive action when used with other cholinergic and anticholinesterase drugs (e.g. donepezil, galantamine, rivastigmine). There is the risk of bradycardia when used with beta blockers (such as atenolol, bisoprolol, metoprolol). The recommended dose for pilocarpine hydrochloride 4% is 5 mg 3-4 times a day (up to 30 mg per day), swish in mouth and swallow.

Saliva substitutes may be used such as:
1) Methylcellulose (1%): 1-2 sprays at the back of the mouth and tongue as required
2) Oralube: 1-2 sprays at the back of mouth and tongue as required.
3) GC Dry Mouth Gel: Apply gel on the tongue and spread on oral mucosa as needed.

Oral candidiasis may be treated with antifungal agents either topically or systemically depending on the severity of the infection. Antifungals may be divided into five classes: polyenes, azoles, echinocandins, allylamines, and other agents, according to the mechanism of action.

Polyene antifungal agents such as nystatin and amphotericin B bind to ergosterol molecules in the plasma membrane of Candida cells. This forms aqueous pores which alter the membrane permeability, resulting in leakage of intracellular ions and cell death (Ghannoum & Rice, 1999). Polyenes interact to some degree with cholesterol-containing membranes, making it relatively toxic to host cells (Perfect, 2017). Nephrotoxicity is one of the main side effects of amphotericin B. Newer lipid formulations of this drug have
been developed to reduce renal dysfunction. These drugs are not absorbed when taken orally. They may be used topically for oral, oropharyngeal and perioral lesions, otherwise they are given intravenously for systemic fungal infection (The New Zealand Formulary, 2019).

Azole antifungal agents inhibit the fungal cytochrome P450 enzyme lanosterol 14α-demethylase which reduces the ergosterol content of membranes, and thus inhibits growth and replication (Chen & Sorrell, 2007). First generation azoles possess imidazole rings (containing 2 nitrogen atoms) and include clotrimazole, miconazole, and ketoconazole. Second generation azoles possess triazole rings (containing 3 nitrogen atoms) and include fluconazole and itraconazole. Third generation triazoles include voriconazole and posaconazole. Newer azoles have improved antifungal activity, safety, pharmacokinetics, and formulations; however, they still inhibit mammalian cytochrome P450 enzyme, allowing multiple drug interactions (Perfect, 2017). Hepatotoxicity, drug interactions, interference with QT (heart beat) interval and peripheral neuropathy are adverse reactions associated with azole drugs (Mourad & Perfect, 2018).

Echinocandin antifungals such as caspofungin and micafungin block 1,3-β-glucan synthase, preventing cell wall synthesis. This is highly specific to the fungal cell wall; echinocandins are less toxic to host tissues which do not have cell walls. Caspofungin is active against *Aspergillus* and *Candida* species while micafungin is indicated for azole-resistant fungal infections (The New Zealand Formulary, 2019). Infusion-related adverse reactions have been noted due to histamine release causing symptoms such as rash, hypotension, bronchospasm, and angioedema (Mourad & Perfect, 2018). Hepatotoxicity has been reported with micafungin use (The New Zealand Formulary, 2019).
Echinocandin drugs are only available for intravenous transfusion and are used for invasive candidiasis and candidemia (Perfect, 2017).

Allylamine antifungal agents inhibit early steps of ergosterol biosynthesis and increase membrane permeability resulting in disruption of the cellular function of Candida (Ghannoum & Rice, 1999). Terbinafine has shown good inhibition of C. albicans, azole-resistant Candida and Cryptococcus neoformans (Ghannoum & Rice, 1999) (The New Zealand Formulary, 2019).

Flucytosine interferes with pyrimidine metabolism, RNA, DNA, and protein synthesis in the fungal cell (Ghannoum & Rice, 1999). It is used in combination with a polyene for cryptococcal meningitis and severe systemic candidiasis. Flucytosine is not used as monotherapy due to the rapid development of fungal drug resistance (Mourad & Perfect, 2018). Leukopenia, thrombocytopenia, and hepatotoxicity have been reported with this drug use (The New Zealand Formulary, 2019).

1.3 Clinical relevance

Understanding the distribution of yeast species and strains among the older population is important in implementing strategies for disease prevention. As the proportion of older people in the population increases, the focus should be aimed towards improving oral health care. This is because older people are living longer with multiple medical comorbidities and risks for oral candidiasis. Prevention of oral diseases leads to better overall health and quality of life.
1.4 Objective of this study:
To investigate and compare the prevalence of *Candida* species and *C. albicans* strains in the oral cavities of older people living in institutional age-care facilities and those living at home.

1.5 Aims of the investigation:
To investigate the presence and abundance of *Candida* species in saliva and from swabs and smears of people living at an institutional rest home and those living at home.
To detect and identify different types of *C. albicans* strains using Multi Locus Sequence Typing (MLST) in the two population groups.

1.6 Hypothesis
There are significant differences in the prevalence of *Candida* species and *C. albicans* strains between older people living in an institutional care facility and those living at home.

1.7 Significance of this research
Upon completion of this study, we will have a better understanding of the prevalent *Candida* species and strains in the mouths of older people. We will also be able to determine if communal living, for example living in close proximity to one another, sharing the same caregivers and food handlers for those who reside in age-care facilities has an impact on this prevalence. A review of oral hygiene and cross-contamination protocols may be needed to improve the overall healthcare of the elderly.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 List of Materials

i) Agarose gel 1.5%

Preparation:

2.1 g agarose powder UltraPure™ (ThermoFisher Scientific, Christchurch New Zealand)

140 ml Tris Acetate EDTA (TAE) Buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3)

0.5 µl Ethidium Bromide (EtBr 2 mg/ml)

Put room temperature buffer (TAE) into a 500ml flask. Place a magnetic stir bar into the flask. Place the flask on a magnetic stirrer and add the agarose powder as the solution mixes, to prevent the formation of agarose clumps. Remove the stir bar. Cover the flask opening with plastic wrap, pierce a small hole for ventilation. Place the flask in the microwave oven and heat until bubbles appear. Remove the flask carefully and swirl gently to resuspend any agarose particles. Reheat the solution until it comes to a boil, and all the agarose particles are dissolved. Remove the flask carefully and swirl gently to fully mix the solution. Place the flask under running water while gently swirling the contents and let cool to 50–60°C. Add EtBr and swirl to get an even mix before pouring the solution into 2 large casting trays. Ethidium Bromide is a fluorescent intercalating agent that binds to DNA, allowing DNA visualization under UV light. It is a potentially mutagenic compound and should be handled according to the material safety data sheet.
ii) CHROMagar™ *Candida* agar plates (Becton Dickinson & Co [BD], Franklin Lakes, NJ, USA)
Supplier: Fort Richards Ltd, Auckland, New Zealand

iii) ExoSAP-IT™ (ThermoFisher Scientific)
Supplier: Life Technologies NZ Ltd, Christchurch, New Zealand

iv) KOD FX Neo DNA Polymerase (Toyobo, Osaka, Japan)
Supplier: Biotech Lab Inc, Tokyo, Japan

v) Yeast extract Peptone Dextrose (YPD) Agar
Preparation:
10 g yeast extract
20 g peptone Sigma-Aldrich (St. Louis, MO, USA)
20 g glucose
20 g agar
700 ml distilled water
Place water with magnetic stir bar into a beaker onto a magnetic stirrer and begin stirring at medium speed. Add yeast extract, peptone and glucose until all powders have dissolved. Add agar when other ingredients are dissolved. Remove the stir bar and pour solution into a graduated cylinder and add distilled water to make 1 liter. Place solution into screw cap glass bottle. Autoclave at 121°C for 20 minutes. Place in water bath at 46°C for one hour then pour into sterile, labeled petri dishes. Allow to cool and set.
vi) Yeast extract Peptone Dextrose (YPD) liquid medium

Preparation:

5 g yeast extract,
10 g glucose,
10 g peptone Sigma-Aldrich

Distilled water

Mix all the above ingredients with distilled water to make 500 ml in a beaker with a magnetic stir bar. Place on a magnetic stirrer and mix well. Pipet 10 ml portions of the medium into glass universal bottles, cover and autoclave at 121°C for 15 min.

vii) Yeast extract Peptone Dextrose (YPD) glycerol medium

Preparation:

5 g yeast extract,
10 g glucose,
10 g peptone Sigma-Aldrich

125 g glycerol

Distilled water

Mix all the above ingredients with distilled water to make 500 ml in a beaker with a magnetic stir bar. Place on a magnetic stirrer and mix well. Pipet 10 ml portions of the medium into glass universal bottles, cover and autoclave at 121°C for 15 min.
2.2 Methods

2.2.1 Study Participants

2.2.1.1 Ethical Approval

Ethical approval for this study was obtained from the University of Otago Human Ethics Committee (Ethics Committee reference number H17/081) prior to recruitment of participants. Consultation was undertaken with the Ngāi Tahu Research Consultation Committee.

2.2.1.2 Participant Selection

This pilot cross-sectional study was a convenience sampling of two groups of older people; people living at rest home facilities (n = 25) and people living in their own homes (n = 25). The term “Rest Home” is unique to New Zealand and is the name for a long-term residential nursing facility or nursing home. These facilities cater for older people with varying levels of dependency. Rest home participants came from Montecillo Veterans Home and Hospital and Little Sisters of the Poor, both located in Dunedin, New Zealand. Participants for the control group were members of the community recruited using posters circulated via Age Concern, Bowling Clubs Dunedin and by personal contact.

2.2.1.3 Inclusion criteria

Participants had to be aged 65 or older and healthy (no sign of disease), or have well-controlled systemic disease, with sufficient mouth opening to allow intraoral access and possess the ability to consent to participate.
2.2.1.4 Exclusion criteria

Individuals who had used antibiotics or antifungals in the past two months or were terminally ill or current tobacco smokers were excluded from participating in the study.

The selection criteria were provided to the rest homes and names of suitable candidates were supplied by the Nurse Manager. Participants were given the information sheet (Appendix III) and had ample opportunity to ask questions and voice any concerns. Consent forms (Appendix IV and V) were signed, or verbal consent was given by patients with physical disability. This process was carried out at the participant’s bedside (rest home) or in their own home (control).

2.2.2 Sample Collection and Analysis

2.2.2.1 Clinical examination

A clinical examination was carried out by the researcher, involving direct observation of the condition of the oral mucosa using cheek retractors a dental mirror. Participants who exhibited signs of mucosal inflammation and had hyphae present in the smear test were prescribed antifungal agents. This was either miconazole nitrate oral gel 2% (applied to affected site and placed on fitting surface of denture 4 times a day for 2 weeks) or amphotericin B 10g lozenges (1 lozenge dissolved in mouth 4 times a day for 2 weeks) or both. Denture wearers were advised to remove dentures overnight and denture hygiene instructions were given. The rest home in-house medical doctor and the nurse manager were involved in this process to provide participant support. Participants who required urgent dental attention were referred to the Urgent Care Unit of the Faculty of Dentistry, University of Otago, for immediate treatment.
2.2.2.2 Collection of oral samples

**Whole saliva**

Whole unstimulated saliva was collected from participants and the rate of production measured in order to identify salivary gland hypofunction (a rate of \(<0.2\text{ml/min}\) (Longman et al., 1997)). Participants were requested to allow their saliva to pool in their mouths for one minute then expectorate it into a glass measuring cup. This was repeated five times. The total volume of saliva produced was recorded and the saliva transferred to a sterile 15 ml Falcon tube. Patients who were unable to generate saliva rinsed their mouths with 2 ml sterile water to enable subsequent analysis of the yeast present in their mouths.

**Mucosal Swabs**

Participants’ hard palates and dorsal surface of tongues were swabbed with sterile cotton buds, then the cotton buds were placed in 2 ml sterile water contained in a 15 ml Falcon tube. Saliva and mucosal swabs were kept on ice and transported to the Faculty of Dentistry Molecular Biosciences Laboratory for further analysis.

**Mucosal Smears**

Smears of the palate and tongue were taken by gently rubbing a wooden spatula on the dorsal surface of the tongue and hard palate, avoiding the area that had just been swabbed, but immediately adjacent to it. The material obtained on the spatula was smeared onto a glass slide and the slide immersed in a Coplin jar filled with 95\% ethanol. Glass slides were labeled with the participant’s name, date of birth and site from which the smear was taken. The Coplin jars were transported to the University of Otago Faculty of Dentistry Oral Pathology Centre where they were stained with Periodic acid-Schiff (PAS). PAS
detects glycogen in fungal walls which stains pink to red/purple and is well differentiated from host tissues (Guarner & Brandt, 2011).

### 2.2.2.3 Cytology

PAS-stained slides were examined by an Oral Pathology Consultant to determine the presence or absence of hyphae and/or yeast.

### 2.2.3 Laboratory Procedures

#### 2.2.3.1 Yeast identification, quantification and storage

Falcon tubes containing saliva and the 2 ml suspensions of palate and tongue swabs were vortexed for 30s. Portions (100 µl) of the whole saliva and suspensions were spread on CHROMagar™ Candida agar plates (Becton Dickinson & Co [BD], Franklin Lakes, NJ, USA), and incubated at 37°C for 48 h. CHROMagar™ Candida agar contains chromogenic β-glucosaminidase substrates that report yeast hexosaminidase and alkaline phosphatase activity. The reaction of these substrates with species-specific enzymes gives colonies different colours: green (*C. albicans*), purple (*C. glabrata*), pink (*C. krusei*) and white (*C. parapsilosis*) (Ghelardi et al., 2008). *Candida* species were presumptively identified according to colony colour based on the manufacturer’s instructions, and colony forming units (cfu) per ml sample were determined. The number of colonies on plates were multiplied by 10 to get the cfu/ml sample. When the numbers of cfu were too high to count accurately, portions of saliva and swab suspensions were serially diluted 10, 100 or 1000 times and the number of cfu multiplied accordingly in order to accurately quantify the cfu per 1 ml sample. Five colonies presumptively identified as *C. albicans* were picked from each site (saliva, tongue and palate) with a
sterile loop, and the cells were suspended into 10 ml sterile Yeast Extract–Peptone–Dextrose (YPD) liquid medium. From sites that had other yeast species present, three additional colonies of each species were picked. The cell suspensions were incubated by shaking (200 rpm) overnight at 30°C in a rotary incubator (Multitron II Infors HT, Bottmingen Switzerland). Cells were harvested by centrifugation at 5000 rpm (3184 x g) for 3 min using a Centrifuge 5804 Eppendorf Darmstadt, Germany. Cells were then resuspended in 1 ml sterile YPD containing 25% (w/v) glycerol as a cryo-protectant. After allowing glycerol to diffuse into the cells for ~30 min the cell suspension was transferred into screw capped 1.5 ml Eppendorf tubes and stored at -80°C.

2.2.3.2 DNA analysis

Polymerase Chain Reaction amplification

Yeast species were confirmed by colony polymerase chain reaction (PCR) amplification and sequencing of the rDNA internal transcribed spacer region ITS2 (Hoggard et al., 2018) (Lott, Kuykendall, & Reiss, 1993). Colony PCR was carried out using KOD FX Neo DNA Polymerase (Toyobo, Osaka, Japan) and dNTP. Each 20 µl PCR reaction contained:

0.8 µl sterile water;
10 µl 2 x PCR Buffer for KOD FX Neo;
4 µl dNTPs (2 mM);
2 µl ITS3 forward primer: (Table 1) (3.2 µM);
2 µl ITS4 reverse primer (Table 1) (3.2 µM);
1 µl of template cell suspensions in 20 µl sterile water of each yeast colony; and
0.2 µl KOD FX Neo (1.0 U/µl).
Table 1. ITS primers used for yeast identification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>rRNA operon binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS 3 (forward)</td>
<td>5’-GCATCGATGAAGAAGACGCAGC-3’</td>
<td>5.8S</td>
</tr>
<tr>
<td>ITS 4 (reverse)</td>
<td>5’-TCCTCCGCTTATTGATATGC-3’</td>
<td>Large subunit</td>
</tr>
</tbody>
</table>

(Op De Beeck et al, 2014)

The PCR cycle conditions were:

1) Initial denaturation at 98°C for 30 seconds
2) Denaturation at 98°C for 10 seconds
3) Annealing at 55°C for 10 seconds
4) Elongation at 68°C for 40 seconds (step 2 to 4 were repeated for 44 cycles)
5) Final elongation step at 68°C for 1 minute

*C. albicans* strain typing was carried out by PCR amplification of fragments of the seven housekeeping genes *AAT1a, ACC1, ADP1, MP1b, SYA1, VPS13*, and *ZWF1b* (Table 2) (Bougnoux et al., 2003) using the protocol above.

Table 2. Gene fragments and primers used for *C. albicans* MLST analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Product</th>
<th>Primers</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AAT1a</em></td>
<td>Aspartate aminotransferase</td>
<td>Fwd5’-ACTCAAGCTAGATTAGTTGGC-3’ Rev 5’-CAGCAACATGATGGCCC-3’</td>
<td>478</td>
</tr>
<tr>
<td><em>ACC1</em></td>
<td>Acetyl-CoA carboxylase</td>
<td>Fwd 5’-GCAAGAGAAATTTTTCATCAATG-3’ Rev 5’-TTTATCAACATCATTCAAGTG-3’</td>
<td>519</td>
</tr>
<tr>
<td><em>ADP1</em></td>
<td>ATP-dependent permease</td>
<td>Fwd 5’-GAGCCAAAGTGAATGATGTTG-3’ Rev 5’-TTGATCAACAAACCGATAAT-3’</td>
<td>537</td>
</tr>
</tbody>
</table>
**DNA gel electrophoresis**

The presence of DNA fragments of correct size and their concentrations were determined by subjecting 1 µl of the 20 µl PCR amplified DNA sample mixed with 5 µl of 1x Bromophenol Blue Loading Dye (3’,3”,5’,5”-Tetrabromophenolsulfophthalein sodium salt and glycerol) to electrophoresis (100 V for 28 min) through an agarose gel (1.5% w/v) containing ethidium bromide (EtBr). EtBr staining was achieved by adding 0.5 µl of a 2 mg/ml EtBr stock into 140 ml of 1.5% agarose gel mixture.

**PCR product clean up**

Unincorporated DNA oligomer primers were removed from PCR amplified DNA fragments with ExoSAP-IT™ (ThermoFisher Scientific, Christchurch, New Zealand) according to the manufacturer’s instructions. PCR samples were diluted to give 50ng of 0.3-0.7kb DNA fragments in 10 µl. ExoSAP-IT™ (0.1 µl) was added to the PCR sample. Samples were incubated at 37°C for 15 min to remove all unincorporated primers after which the temperature was increased to 80°C for another 15 min to inactivate the enzyme that would otherwise interfere with DNA sequencing of the treated samples.
**DNA sequencing**

DNA sequencing of the PCR amplified internal transcribed spacer region ITS2 was carried out to identify yeast species using ITS3 forward primer and performed by the Genetic Analysis Service, Department of Anatomy, University of Otago. The 5 µl sequence reaction DNA samples that were sent for sequencing ideally contained 1 µl (i.e. ~5ng-7ng) ExoSAP-IT™-treated PCR products, 1µl ITS3 forward primer and 3 µl sterile distilled water.

*C. albicans* strains were typed by sequencing the PCR amplified *AAT1a, ACC1, ADP1, MP1b, SYA1, VPS13,* and *ZWF1b* gene fragments using the respective forward primers (Table 2) and the sequencing protocol described above.

**Multi Locus Sequence Typing (MLST) analysis**

Yeast species were confirmed by visualizing the DNA sequencing chromatograms with FinchTV (version 1.5.0) and further analyzed with UniPro UGENE (version 1.29.0) and compared with the blast software program to the publicly available nucleotide database collection at [https://www.ncbi.nlm.nih.gov/BLAST/](https://www.ncbi.nlm.nih.gov/BLAST/). (Last accessed 2/8/2019)

The diploid sequence type (DST) of individual *C. albicans* strains was determined by comparing concatenations of the seven housekeeping gene fragment sequences with the publicly available MLST database ([https://pubmlst.org/calbicans/](https://pubmlst.org/calbicans/)). Allelic variation was determined by the presence of single nucleotide polymorphisms (SNPs) (double peaks) in the chromatogram. The allelic variation of *C. albicans* strains between participants and within a participant was recorded.

The concatenated sequence of all the genes were uploaded to the Phylemon 2.0, an online platform for phylogeny and molecular evolution analysis.
2.2.3 Statistical analysis

The t-test was used to compare parametrically distributed data. The Mann-Whitney U test was used to compare non-parametrically distributed data. Differences were considered significant if $p < 0.05$. For binary dependent variables, SPSS software (version 25, IBM Corporation) was used for cross-tabulations, with Chi-square tests used to determine statistical significance (at the 0.05 level).
Chapter 3: Results

3.1 Overview

This pilot cross-sectional study was a convenience sampling of two groups of older people: people living at rest home (RH) facilities (n = 25) and people living in their own homes (OH) (n = 25). Enrolling participants from aged care facilities in Dunedin proved to be very challenging as many facilities cited over-commitment to various other research projects carried out by the University of Otago. The two RHs that agreed to participate were Montecillo Veterans Home and Hospital (Montecillo; n=13) and Little Sisters of the Poor (LSOP) Sacred Heart Home and Hospital (n=12). The list of eligibility criteria (see section 2.2.1.3 and 2.2.1.4) was given to the RH Nurse Managers who identified suitable candidates. The RH residents were approached individually and given the Participant Information Sheet (Appendix III) and the opportunity to ask questions about the study. Residents who were vision impaired had the information sheet read out to them. Residents who agreed to participate signed the Consent Form (Appendix IV) as well as the Laboratory Form (Appendix V) consenting to the smear results to be used for research. Participants in the control group were members of the public recruited from posters circulated via Age Concern (26 Bath St, Dunedin, 9016), Bowling Clubs Dunedin and personal contact. People who were interested and eligible to participate were visited in their OHs. The participants were given ample opportunity to ask questions about the study. OH participants were given both Consent and Laboratory forms to sign before the study commenced.
3.2 Study participants

A total of 25 people from each group (RH and OH) participated in this study. Each group consisted of 17 women and 8 men. Tables 3-5 depict the distribution of the various yeast species found in saliva, tongue and palate samples from the oral cavities of OH (Table 3), Montecillo (Table 4) and LSOP (Table 5) participants. The tables also include the participants age and whether they were denture wearers or had yeast hyphae detected in the oral smear samples.

Table 3. Distribution of yeast species colonizing of the oral cavities of OH residents (empty cells mean no yeast colonies were detected).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Denture</th>
<th>Hyphae</th>
<th>Saliva cfu (10^3/mL)</th>
<th>Tongue cfu (10^3/swab)</th>
<th>Palate cfu (10^3/swab)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Non-albicans</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
<td>Cg   Cl  Cp  Cgm</td>
<td>Ca</td>
</tr>
<tr>
<td>CL1*</td>
<td>67</td>
<td>✓</td>
<td>✓</td>
<td>74.00</td>
<td>107.00</td>
<td>1.06</td>
</tr>
<tr>
<td>CL2</td>
<td>72</td>
<td></td>
<td></td>
<td>0.21</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>CL3</td>
<td>76</td>
<td>✓</td>
<td>✓</td>
<td>2.54</td>
<td>0.01</td>
<td>0.78</td>
</tr>
<tr>
<td>CL4</td>
<td>86</td>
<td>✓</td>
<td>✓</td>
<td>9.16</td>
<td>1.06</td>
<td>0.06</td>
</tr>
<tr>
<td>CL5</td>
<td>85</td>
<td>✓</td>
<td>✓</td>
<td>0.99</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>CL6</td>
<td>92</td>
<td>✓</td>
<td></td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL7</td>
<td>84</td>
<td>✓</td>
<td>✓</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL8</td>
<td>88</td>
<td>✓</td>
<td>✓</td>
<td>3.32</td>
<td>1.08</td>
<td>3.76</td>
</tr>
<tr>
<td>CL9</td>
<td>88</td>
<td>✓</td>
<td>✓</td>
<td>0.23</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>CN1*</td>
<td>66</td>
<td></td>
<td></td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN2</td>
<td>71</td>
<td>✓</td>
<td></td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN3*</td>
<td>67</td>
<td></td>
<td></td>
<td>0.10</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>CN4</td>
<td>83</td>
<td>✓</td>
<td></td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN5</td>
<td>74</td>
<td></td>
<td></td>
<td>1.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN6*</td>
<td>75</td>
<td></td>
<td></td>
<td>5.54</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>CN7</td>
<td>78</td>
<td>✓</td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN8</td>
<td>82</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN9</td>
<td>82</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN10</td>
<td>92</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN11</td>
<td>85</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN12</td>
<td>83</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN13</td>
<td>81</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN14</td>
<td>85</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN15</td>
<td>83</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CN16</td>
<td>84</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

a C = participants living in their own homes; L = participants with low salivary flow rate (<0.2 ml/min; top); N = participants with normal salivary flow rate (≥0.2 ml/min; bottom).

b Ca = Candida albicans, Cg = Candida glabrata, Cl = Candida lusitaniae, Cp = Candida parapsilosis, Cgm = Candida guilliermondii, Yl = Yarrowia lipolitica, Pf = Pichia fermentans.

* These participants were excluded from the analysis presented in Table 6.
Table 4. Distribution of yeast species in the oral cavities of participants living in the Montecillo RH (empty cells mean no yeast colonies were detected).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Denture</th>
<th>Hyphae</th>
<th>Saliva cfu (10^3 / mL)</th>
<th>Tongue cfu (10^3 / swab)</th>
<th>Palate cfu (10^3 / swab)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ca^b Non-albicans^b</td>
<td>Ca^b Non-albicans^b</td>
<td>Ca^b Non-albicans^b</td>
</tr>
<tr>
<td>ML1^*</td>
<td>94</td>
<td>✓</td>
<td>✓</td>
<td>3.00 1040.00 0.38 16.48</td>
<td>0.20 12.24</td>
<td>13.78 0.32</td>
</tr>
<tr>
<td>ML2 94</td>
<td>✓</td>
<td>✓</td>
<td>18.00 1.00 2.64 0.28</td>
<td>0.16 0.08</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>ML3 74</td>
<td>✓</td>
<td>✓</td>
<td>2.20</td>
<td>1.50 2.04 0.08</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>ML4* 95</td>
<td>✓</td>
<td>✓</td>
<td>0.55</td>
<td>7.45 14.70 0.06</td>
<td>0.16 0.34</td>
<td></td>
</tr>
<tr>
<td>ML5 75</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>1.92 0.50 0.14</td>
<td>0.14 0.24</td>
<td></td>
</tr>
<tr>
<td>ML6 92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN1 91</td>
<td>✓</td>
<td>✓</td>
<td>0.59</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN2 94</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>0.41 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN3 94</td>
<td>✓</td>
<td>✓</td>
<td>4.76 0.05</td>
<td>0.50 4.92</td>
<td>0.18 2.44</td>
<td></td>
</tr>
<tr>
<td>MN4 89</td>
<td>✓</td>
<td>✓</td>
<td>0.02</td>
<td>1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN5 84</td>
<td>✓</td>
<td>✓</td>
<td>0.41</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN6 82</td>
<td></td>
<td></td>
<td>1.04</td>
<td>0.62</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>MN7^* 98</td>
<td>✓</td>
<td>✓</td>
<td>2816</td>
<td>8.48</td>
<td>5.90</td>
<td>0.26 0.28</td>
</tr>
<tr>
<td>ML1^* 94</td>
<td>✓</td>
<td>✓</td>
<td>3.00 1040.00 0.38 16.48</td>
<td>0.20 12.24</td>
<td>13.78 0.32</td>
<td></td>
</tr>
<tr>
<td>ML2 94</td>
<td>✓</td>
<td>✓</td>
<td>18.00 1.00 2.64 0.28</td>
<td>0.16 0.08</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>ML3 74</td>
<td>✓</td>
<td>✓</td>
<td>2.20</td>
<td>1.50 2.04 0.08</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>ML4^* 95</td>
<td>✓</td>
<td>✓</td>
<td>0.55</td>
<td>7.45 14.70 0.06</td>
<td>0.16 0.34</td>
<td></td>
</tr>
<tr>
<td>ML5 75</td>
<td>✓</td>
<td>✓</td>
<td>1.92 0.50 0.14</td>
<td>0.14 0.24</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>ML6 92</td>
<td></td>
<td></td>
<td>1.04</td>
<td>0.62</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

^a M = participants living in Montecillo rest home; L = participants with low salivary flow rate (<0.2 ml/min; top); N = participants with normal salivary flow rate (≥0.2 ml/min; bottom).

^b Ca = Candida albicans, Cg = Candida glabrata, Cd = Candida dubliniensis, Sc = Saccharomyces cerevisiae, Ct = Candida tropicalis, Ch = Candida albicans/Candida dubliniensis chimera.

* These participants were excluded from the analysis presented in Table 6.

Table 5 Distribution of yeast species in the oral cavities of participants living in LSOP RH (empty cells mean no yeast colonies were detected).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Denture</th>
<th>Hyphae</th>
<th>Saliva cfu (10^3 / mL)</th>
<th>Tongue cfu (10^3 / swab)</th>
<th>Palate cfu (10^3 / swab)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ca^b Non-albicans^b</td>
<td>Ca^b Non-albicans^b</td>
<td>Ca^b Non-albicans^b</td>
</tr>
<tr>
<td>SL1 91</td>
<td>✓</td>
<td>✓</td>
<td>1.11</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL2 82</td>
<td>✓</td>
<td>✓</td>
<td>24.00</td>
<td>2.26</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>SL3* 94</td>
<td>✓</td>
<td>✓</td>
<td>2816</td>
<td>8.48</td>
<td>5.90</td>
<td></td>
</tr>
<tr>
<td>SL4 81</td>
<td>✓</td>
<td>✓</td>
<td>9.00</td>
<td>3.00 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL5 81</td>
<td></td>
<td>✓</td>
<td>0.45</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL6* 102</td>
<td>✓</td>
<td>✓</td>
<td>0.12</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL7 89</td>
<td>✓</td>
<td>✓</td>
<td>39.00</td>
<td>1.00 0.74</td>
<td>0.12 0.28</td>
<td></td>
</tr>
<tr>
<td>SL8 83</td>
<td>✓</td>
<td>✓</td>
<td>0.37</td>
<td>0.67 0.06</td>
<td>0.06 0.14</td>
<td></td>
</tr>
<tr>
<td>SL9 73</td>
<td></td>
<td>✓</td>
<td>2.31</td>
<td>4.58</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>SN1 91</td>
<td>✓</td>
<td>✓</td>
<td>17.00</td>
<td>36.00 1.32</td>
<td>1.34 0.38</td>
<td>0.62</td>
</tr>
<tr>
<td>SN2 88</td>
<td></td>
<td></td>
<td>472.00</td>
<td>1.90</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>SN3 87</td>
<td>✓</td>
<td>✓</td>
<td>1.51</td>
<td>0.98</td>
<td>0.14</td>
<td></td>
</tr>
</tbody>
</table>

^a S = participants living in Little Sisters of the Poor rest home; L = participants with low salivary flow rate (<0.2 ml/min; top); N = participants with normal salivary flow rate (≥0.2 ml/min; bottom).

^b Ca = Candida albicans, Cg = Candida glabrata, Pa = Pichia anomala, Sc = Saccharomyces cerevisiae, Ch = Candida albicans/Candida dubliniensis chimera.

* These participants were excluded from the analysis presented in Table 6.
In order to match the participants from the two groups in terms of gender and age to reduce bias in the comparison, 20 participants from each group were selected without reference to any other participant characteristics. Participants excluded from the analysis presented in Table 6 are marked with asterisks in Tables 3 - 5.

Table 6. Demographics, clinical features, and colonization status of 20 participants living in their OH compared with 20 RH participants.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OH (n = 20)</th>
<th>RH (n = 20)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>83.1 (71-92)</td>
<td>86.0 (72-94)</td>
<td>0.50 a</td>
</tr>
<tr>
<td>Proportion with dentures</td>
<td>55%</td>
<td>70%</td>
<td>0.33 b</td>
</tr>
<tr>
<td>Mean salivary flow rate [ml/min] (range)</td>
<td>0.25 (0-0.7)</td>
<td>0.23 (0-0.6)</td>
<td>0.35 a</td>
</tr>
<tr>
<td>Salivary gland hypofunction (&lt;0.2 ml/min)</td>
<td>40%</td>
<td>55%</td>
<td>0.34 b</td>
</tr>
<tr>
<td>Proportion with hyphae in smears (PAS-positive)</td>
<td>30%</td>
<td>35%</td>
<td>0.74 b</td>
</tr>
<tr>
<td>Denture wearer</td>
<td>67%</td>
<td>86%</td>
<td></td>
</tr>
<tr>
<td>Low saliva flow rate</td>
<td>67%</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td>Mean number of medications (range)</td>
<td>5.4 (0-11)</td>
<td>6.6 (1-12)</td>
<td>0.25 a</td>
</tr>
<tr>
<td>Proportion with saliva colonized by yeast</td>
<td>65%</td>
<td>80%</td>
<td>0.29 b</td>
</tr>
<tr>
<td>Proportion with tongue colonized by yeast</td>
<td>35%</td>
<td>80%</td>
<td>0.004 b</td>
</tr>
<tr>
<td>Proportion with palate colonized by yeast</td>
<td>25%</td>
<td>65%</td>
<td>0.01 c</td>
</tr>
<tr>
<td>Level of yeast colonization of saliva (cfu/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1,410</td>
<td>32,240</td>
<td>0.01 c</td>
</tr>
<tr>
<td>Median (range)</td>
<td>50 (0-9,160)</td>
<td>2,260 (0-4.72x10^5)</td>
<td></td>
</tr>
<tr>
<td>Level of yeast colonization of tongue (cfu/swab)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>271</td>
<td>2,400</td>
<td>0.001 c</td>
</tr>
<tr>
<td>Median (range)</td>
<td>0 (0-2,200)</td>
<td>1,970 (0-14,760)</td>
<td></td>
</tr>
<tr>
<td>Level of yeast colonization of palate (cfu/swab)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>606</td>
<td>1,150</td>
<td>0.03 c</td>
</tr>
<tr>
<td>Median (range)</td>
<td>0 (0-10,240)</td>
<td>200 (0-14,100)</td>
<td></td>
</tr>
</tbody>
</table>

a t-test
b Chi-square test
c Mann-Whitney U test
p values in bold are significant (<0.05).

3.2.1 Demographics

On average, RH participants were 2.9 years older than those living in their OHs, but this difference was not statistically significant (t-test = 0.50). The mean age of OH and RH
participants was 83.1 (range: 71-92) and 86.0 (range: 72-94) years, respectively. Thirteen subjects of each group (65%) were women and 7 (35%) were men.

3.2.2 Denture Use

The denture use status was recorded because it is a risk factor for candidiasis (C. Farah et al., 2010). About half (55%) of OH and 70% of RH participants wore dentures, which was not significantly different (chi-square test = 0.33; Tables 6 and 8). However, significantly ($p = 0.04$; chi-square test) more (84%; 21/25) denture wearing participants presented with yeast-positive saliva than non-denture wearing participants (53%; 8/15; Table 7). 17 of the 25 denture wearers had high yeast colonization levels (> 400 cfu/ml saliva (Nadig et al., 2017)), whereas only 5 of the 15 non-denture wearers had high levels of yeast ($p = 0.03$; Table 7). Perhaps somewhat surprisingly, four out of five were from LSOP while non-denture wearers in Montecillo and OH generally did not have high yeast carriage.

<table>
<thead>
<tr>
<th></th>
<th>Denture wearing participants</th>
<th>Non-denture wearing participants</th>
<th>p value $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast positive saliva</td>
<td>84%</td>
<td>53%</td>
<td>0.04</td>
</tr>
<tr>
<td>Highly colonized (&gt;400cfu/ml)</td>
<td>68%</td>
<td>33%</td>
<td>0.03</td>
</tr>
<tr>
<td>PAS-positive smear</td>
<td>40%</td>
<td>20%</td>
<td>0.19</td>
</tr>
</tbody>
</table>

$^a$ Chi-square test

$p$ values in bold are significant (<0.05)
Table 8. Distribution of PAS-positive smears and high, low or no yeast cell counts in saliva samples of denture versus non-denture wearers with either low (L) or normal (N) salivary flow rates.

<table>
<thead>
<tr>
<th>Denture Saliva flow</th>
<th>OH (n = 20)</th>
<th>RH (n = 20)</th>
<th>Montecillo (n = 10)</th>
<th>LSOP (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nr</td>
<td>PAS Yeast cells count Higha Lowb No</td>
<td>Nr</td>
<td>PAS Yeast cells count High Low No</td>
</tr>
<tr>
<td>✓</td>
<td>L</td>
<td>7</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>✓</td>
<td>N</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>L</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>N</td>
<td>8</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>6</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

a >400 cfu/ml
b 30-230 cfu/ml

3.2.3 Hyposalivation

Salivary gland hypofunction (defined as an unstimulated whole salivary flow rate < 0.2 ml/min) (Longman et al., 1997) was present in 55% of RH participants (Table 6 and 8). This was also seen in 40% participants living in their OH. This difference was not statistically significant (p = 0.34; chi-square test; Table 6). A greater proportion of participants with low salivary flow presented with yeast colonisation compared to people with normal salivary flow (95% vs 52% p = 0.002) (Table 9). A greater proportion of participants with low salivary flow were highly colonized with yeast and present with more PAS-positive smears compared to those with normal salivary flow, but the differences were not statistically significant (Table 8 and 9).

Table 9. Comparison of saliva yeast presence, yeast colonization level and PAS positive smears between participants with low and normal salivary flow.

<table>
<thead>
<tr>
<th></th>
<th>Low salivary flow (&lt;0.2ml/min)</th>
<th>Normal salivary flow (≥0.2ml/min)</th>
<th>p valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast positive saliva</td>
<td>95%</td>
<td>52%</td>
<td>0.002</td>
</tr>
<tr>
<td>Highly colonized (&gt;400cfu/ml)</td>
<td>68%</td>
<td>38%</td>
<td>0.06</td>
</tr>
<tr>
<td>PAS-positive smear</td>
<td>47%</td>
<td>19%</td>
<td>0.06</td>
</tr>
</tbody>
</table>

a Chi-square test
3.2.4 Cytology Examination

Six (30%) OH participants were PAS-positive, with hyphae detected in smears taken from their palate and/or tongue. Four of them (67%) were denture wearers and had low salivary flow. Seven (35%) participants living in RHs had PAS-positive smears. Six of them (86%) were denture wearers and five (71%) had low salivary flow (Table 6).

3.2.5 Medication

Many participants in both cohorts were taking a number of medications. Participants living in their OHs were taking between 0 and 11 medications daily with a mean of 5.4. RH participants were taking between 1 and 12 medications daily with a mean of 6.6. This difference was not statistically significant ($p = 0.25$; $t$-test). Participants with low salivary flow rate ($<0.2$ ml/min) were taking a mean of 7.4 medications daily, which was significantly more than participants with normal salivary flow rate (4.8 medications daily, $p = 0.005$; $t$-test).

3.3 Microbiological Analysis

3.3.1 Yeast Colonisation

Yeast colonies were presumptively identified based on their colour on CHROMagar™ Candida agar plates. Saliva was the oral site most frequently colonised with yeast, followed by the tongue and the least colonised site was the palate (Figure 1). A higher proportion of RH residents carried yeast in their oral cavity (80% saliva, 80% tongue, and 65% palate) compared to people living at home (65% saliva, 35% tongue, and 25% palate). This difference was significant for the tongue and palate colonisation: saliva.
Comparing results for the 20 matched OH and RH participants, saliva of participants positive for yeast living in RHs had a higher (40,305 cfu/ml) mean yeast carriage (range: 410-472,000 cfu/ml; Tables 4 and 5) than OH participants (2,171 cfu/ml; range: 20-9,160 cfu/ml; Table 3). However, 7 OH and 3 RH participants, of which all but one (ML6; Table 4) had normal saliva flow, had no detectable yeast in their saliva. Including these participants in the calculations reduced their mean yeast saliva levels to 32,240 cfu/ml (median: 2260 cfu/ml) and 1,410 cfu/ml (median: 50 cfu/ml), respectively. Participants in the two rest homes showed different levels of Candida carriage. LSOP participants’ saliva contained, on average, a significantly higher concentration of yeast (61,042 cfu/ml) compared to Montecillo participants (3,446 cfu/ml) and this contributed to the increased mean yeast concentration for RH participants.

The mean cfu/swab of the tongue samples from OH participants was 271 (range: 0-2,200) compared to 2,400 (range: 0-14,760) of the RH participants. The mean cfu/swab of the
palate samples from OH participants was 606 (range: 0-10,240) compared to 1,150 (range: 0-14,100) of the RH participants (Figure 2). Thus, RH participants had significantly higher amounts of yeast present in each sample type compared to people living in their OH.

![Figure 2] Figure 2 Quantity of yeast at oral sites in participants. Level of yeast colonization at each site in each group (cfu/mL of saliva samples, cfu/swab for tongue and palate swabs ± standard deviation).

Participants with PAS-positive smears had a higher number of yeast present in their mouths, 11 of the 13 PAS-positive participants had more than 400 cfu/ml saliva, consistent with previous observations (Epstein et al., 1980).

Most participants with low salivary flow (<0.2 ml/min) (Longman et al., 1997) were yeast-positive (18 out of 19 participants, 95%). However, only 11 out of 21 (52%) participants with normal salivary flow were yeast-positive (Table 9). Interestingly, most
of those 11 participants with normal saliva flow had only *C. albicans* isolates, only four had other yeast species present.

### 3.3.2 Yeast species distribution colonizing the oral cavity

*C. albicans* was the most frequently isolated species by far from the oral cavity from both participant groups. Seven yeast species were identified in individuals living in their OH: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. lusitaniae*, *C. guilliermondii*, *Pichia fermentans* and *Yarrowia lipolytica* (Figure 3). Yeast species identified in individuals living in RH were *C. albicans*, *C. glabrata*, *Saccharomyces cerevisiae*, *C. dubliniensis*, *C. tropicalis* and *C. albicans/C. dubliniensis* chimera strains.

![Species distribution of yeast colonizing the oral cavities of 13 (65%) OH and 16 (80%) RH participants.](image)

*Figure 3* Species distribution of yeast colonizing the oral cavities of 13 (65%) OH and 16 (80%) RH participants.
From the 120 oral samples taken from both groups of participants, 25 OH and 45 RH samples were yeast positive. Of these 70 yeast-positive samples, 44 contained one, 20 contained two and only six contained three yeast species (Table 10). The majority (68) of them contained either *C. albicans* or *C. albicans/C. dubliensis* chimera strains (62) or *C. glabrata* (22). Only two samples (saliva of participants CL6 and CL7) contained neither of these three yeast species: saliva of participant CL6 contained a few (40 cfu/ml) *C. lusitaniae* cells and saliva of participant CL7 contained a few (60 cfu/ml) *C. parapsilosis* cells; tongue and palate samples of these two participants were yeast free (Table 3). The six oral samples (i.e. saliva, tongue and palate of participants CL9 and ML5) lacking any *C. albicans* or *C. albicans/C. dubliensis* chimera cells had a high number of *C. glabrata* colonies instead. The three oral samples of the PAS-negative participant CL9 contained only *C. glabrata* (Table 3). However, the tongue and palate samples of the PAS-positive participant ML5 contained low counts of a second yeast species, *C. tropicalis*, which probably account for the hyphae observed in the PAS-positive smears of those two oral samples. The PAS-positive participant SL3, not part of the selected group of 20 RH participants, was the only exception. That participant had very high number of only one yeast species, that was not *C. albicans*, a *C. albicans/C. dubliensis* chimera or *C. glabrata* but *Pichia anomala* (Table 5). This interesting observation remains to be further investigated because, to the best of our knowledge, *P. anomala*, a relative of *C. krusei*, does not form hyphae although participant SL3 was PAS-positive. Thus, it is possible that we simply failed to detect *C. albicans* in participant SL3. *C. albicans* and *C. glabrata* were most frequently encountered together in the same sample; half (10 of 20) of all samples with two and five of six samples with three yeast species had *C. albicans* and *C. glabrata*. The only participant with four yeast species (*C.
albicans, C. glabrata, C. parapsilosis and P. fermentans) was an OH resident with low saliva flow (CL8; Table 3).

Table 10. Species distribution in 70 yeast-positive oral samples from 13 OH and 16 RH participants (6 from Montecillo and 10 from LSOP) with one (left), two (centre) or a maximum of three (right) different yeast species

<table>
<thead>
<tr>
<th>Group</th>
<th>One species (44)</th>
<th>Two species (20)p</th>
<th>Three species (6)p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca Ch Cg Cp Cl</td>
<td>Ca Cg Ch Sc Cg Ca</td>
<td>Ca Yl</td>
</tr>
<tr>
<td></td>
<td>Cg Ca Ch Sc Sc Ch Ct Cl</td>
<td>Cgui Ca</td>
<td>Ca Cg Cp Ca Sc Ch</td>
</tr>
<tr>
<td>OH</td>
<td>14 3 1 1</td>
<td>1 1 1</td>
<td>1 1 1</td>
</tr>
<tr>
<td>Montecillo</td>
<td>4 4 1</td>
<td>2 1 2 1</td>
<td>1 1 1</td>
</tr>
<tr>
<td>LSOP</td>
<td>16 5 3 1</td>
<td>1 1 1</td>
<td>1 1 1</td>
</tr>
<tr>
<td>Total</td>
<td>34 4 4 1 1</td>
<td>7 3 1 1</td>
<td>1 1 1</td>
</tr>
</tbody>
</table>

*a* Ca = C. albicans, Ch = C. albicans/C. dubliniensis chimera, Cg = C. glabrata, Cp = C. parapsilosis, Cl = C. lusitaniae, Ct = C. tropicalis, Cgui = C. guilliermondii, Sc = S. cerevisiae, Yl = Y. lipolytica, Pf = P. fermentas.

*b* Species are listed according to their abundance with those above being more abundant than the species below.

### 3.3.3 Multi Locus Sequence Typing of 87 Candida albicans isolates

#### 3.3.3.1 Diploid Sequence Typing.

A selection of *C. albicans* strains from the participants was subjected to MLST. DNA oligomer primers for PCR amplification of the seven housekeeping genes and details of the seven amplicons that were used to determine the allele types and the DSTs are listed in Table 11. Frequently alleles of genes were found to differ by one or more single nucleotide polymorphisms (SNPs). The combination of allele types for the seven genes in a particular strain resulted in a specific DST. Generally, one to three strains from *C. albicans*-positive individuals were typed, but for two individuals, one with low saliva flow (CL1) and one with normal saliva flow (MN7), 15 strains were typed: 5 from saliva, 5 from the tongue, and 5 from the palate. In total, 87 *C. albicans* strains were typed and
the DST for each strain determined. The 609 MLSTs of the seven genes of 36 *C. albicans* isolates from the 13 yeast-positive OH residents, 27 *C. albicans* isolates from the six yeast-positive Montecillo and 24 *C. albicans* isolates from the 10 yeast-positive LSOP participants are provided in Tables 12, 13 and 14, respectively. The allele types for each strain were recorded and are presented in Tables 12, 13 and 14, NM indicates a no match to any allele sequence type in the database. With these results 34 different DSTs could be distinguished for the 87 *C. albicans* isolates. The 34 unique DST were numbered for result analysis (Table 15).

---

**Table 11. MLST housekeeping gene information.**

<table>
<thead>
<tr>
<th>Gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primer (5’ – 3’)</th>
<th>Size&lt;sup&gt;b&lt;/sup&gt; (bp)</th>
<th>ORF&lt;sup&gt;b&lt;/sup&gt; (bp)</th>
<th>Chr&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Position (bp)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAT1a (H1)</td>
<td>ACTCAAGCTAGATTTTTTGGC CAGCAACATGAGTTCGCC</td>
<td>478</td>
<td>1314</td>
<td>II</td>
<td>1,077,700-1,079,013</td>
</tr>
<tr>
<td>ACC1 (H2)</td>
<td>GCAAGAGAAATTTAAAAATTCAGATGTTCAATCAATG</td>
<td>519</td>
<td>6816</td>
<td>R</td>
<td>155,196-162,011</td>
</tr>
<tr>
<td>ADP1 (H3)</td>
<td>GAGCCAAGTATGAATGATTTTG TTGATCAAACAAAAACCGATAAT</td>
<td>537</td>
<td>3117</td>
<td>R</td>
<td>1,248,944-1,245,828</td>
</tr>
<tr>
<td>MPIb (H4)</td>
<td>ACCAGAAATGGCCATTGC GCAGCCATGCAATTAT</td>
<td>486</td>
<td>1326</td>
<td>II</td>
<td>1,972,394-1,973,719</td>
</tr>
<tr>
<td>SYA1 (H5)</td>
<td>AGAAGATTTGTTGCTTGTTACTG GTTACCTTTACCACAAGCTTT</td>
<td>746</td>
<td>2910</td>
<td>VI</td>
<td>804,157-807,107</td>
</tr>
<tr>
<td>VPS13 (H6)</td>
<td>TCGTGGAGAGATAATCGACTT ACGATGGATCCAGCTTCC</td>
<td>741</td>
<td>9252</td>
<td>IV</td>
<td>1,341,803-1,351,054</td>
</tr>
<tr>
<td>ZWF1b (H7)</td>
<td>GATCCATTTTGATCCCTGAAGC GCCATTGATAAGTACCTGAG</td>
<td>702</td>
<td>1524</td>
<td>I</td>
<td>1,954,783-1,953,260</td>
</tr>
</tbody>
</table>

<sup>a</sup> The seven housekeeping genes AAT1a to ZWF1b (H1-H7) are listed alphabetically.

<sup>b</sup> Amplicon size and size of the entire ORF.

<sup>c</sup> Chromosomal location.
Table 12. DSTs of 36 C. albicans strains isolated from saliva, tongue or palate of 13 (52%) participants living in their OHs that were colonized with C. albicans (66% or 6 of 9 participants with low and 44% or 7 of 16 participants with normal saliva flow).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Allele types for MLST genes a</th>
<th>DST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAT1a</td>
<td>ACC1</td>
</tr>
<tr>
<td>CL1S1</td>
<td>NM b</td>
<td>3</td>
</tr>
<tr>
<td>CL1S2</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL1S3</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL1S4</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>CL1S5</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL1T1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL1T2</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL1T3</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL1T4</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL1T5</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>CL1P1</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>CL1P2</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>CL1P3</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL1P4</td>
<td>178</td>
<td>3</td>
</tr>
<tr>
<td>CL1P5</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL2S4</td>
<td>133</td>
<td>26</td>
</tr>
<tr>
<td>CL2T1</td>
<td>133</td>
<td>26</td>
</tr>
<tr>
<td>CL3S4</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL3T4</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL3P1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CL4S5</td>
<td>143</td>
<td>14</td>
</tr>
<tr>
<td>CL4T1</td>
<td>143</td>
<td>14</td>
</tr>
<tr>
<td>CL4P1</td>
<td>143</td>
<td>14</td>
</tr>
<tr>
<td>CL5S2</td>
<td>139</td>
<td>8</td>
</tr>
<tr>
<td>CL8S4</td>
<td>NM</td>
<td>5</td>
</tr>
<tr>
<td>CL8T1</td>
<td>NM</td>
<td>5</td>
</tr>
<tr>
<td>CL8P1</td>
<td>NM</td>
<td>5</td>
</tr>
<tr>
<td>CN2S1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CN3S1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CN8S1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>CN9S1</td>
<td>143</td>
<td>14</td>
</tr>
<tr>
<td>CN9P1</td>
<td>143</td>
<td>14</td>
</tr>
<tr>
<td>CN12S1</td>
<td>NM</td>
<td>2</td>
</tr>
<tr>
<td>CN14S4</td>
<td>NM</td>
<td>5</td>
</tr>
<tr>
<td>CN14T1</td>
<td>NM</td>
<td>5</td>
</tr>
<tr>
<td>CN15S1</td>
<td>NM</td>
<td>3</td>
</tr>
</tbody>
</table>

a Number of allele type in database
b No match with database allele types.
Table 13. DSTs of 27 C. albicans strains isolated from saliva, tongue or palate samples of 6 (46%) participants living in the Montecillo rest home that were colonized with C. albicans (66% or 4 of 6 participants with low and 29% or 2 of 7 participants with normal salivary flow)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Allele types for MLST genes (^a)</th>
<th>DST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAT1a</td>
<td>ACC1</td>
</tr>
<tr>
<td>ML1S3</td>
<td>NM (^b)</td>
<td>5</td>
</tr>
<tr>
<td>ML1T1</td>
<td>NM</td>
<td>5</td>
</tr>
<tr>
<td>ML1P1</td>
<td>NM</td>
<td>5</td>
</tr>
<tr>
<td>ML2S4</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>ML2T1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>ML2P4</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>ML3S5</td>
<td>NM</td>
<td>4</td>
</tr>
<tr>
<td>ML3T1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>ML4S3</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>ML4T4</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>ML4P1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>MN4S5</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>MN7S1</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7S2</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7S3</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7S4</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7S5</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7T1</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7T2</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7T3</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7T4</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7T5</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7P1</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7P2</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7P3</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7P4</td>
<td>NM</td>
<td>23</td>
</tr>
<tr>
<td>MN7P5</td>
<td>NM</td>
<td>23</td>
</tr>
</tbody>
</table>

\(^a\) Number of allele type in database

\(^b\) No match with database allele types.
Table 14. DSTs of 24 C. albicans strains isolated from saliva, tongue or palate samples of 10 (91%) participants living in LSOP RH that were colonized with C. albicans (88% or 7 of 8 participants with low and 100% or 3 of 3 participants with normal saliva flow).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Allele types for MLST genes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAT1a</td>
<td>ACC1</td>
</tr>
<tr>
<td>SL1S1</td>
<td>NM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>SL2S2</td>
<td>NM</td>
<td>4</td>
</tr>
<tr>
<td>SL2T1</td>
<td>NM</td>
<td>4</td>
</tr>
<tr>
<td>SL2P1</td>
<td>NM</td>
<td>4</td>
</tr>
<tr>
<td>SL4S4</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>SL4P2</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>SL5S1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SL6S5</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>SL7S2</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SL7T1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SL7P1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SL8S5</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>SL8T1</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>SL8P1</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>SL9S5</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>SN1S1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SN1T1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SN1P1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SN2S1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SN2T1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SN2P1</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SN3S3</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SN3T4</td>
<td>NM</td>
<td>3</td>
</tr>
<tr>
<td>SN3P1</td>
<td>NM</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of allele type in database

<sup>b</sup> No match with database allele types.

There were 74 positions in the housekeeping genes (H1 – H7; 3535 bp) for the 87 C. albicans isolates at which SNPs were found (Figure 4).
Sequence alignment of the 74 nucleotide positions within the seven housekeeping genes (H1-H7) at which SNPs occurred in the 87 C. albicans isolates. Thirty-four different DSTs were distinguished. Housekeeping gene H1 had 9, H2 5, H3 13, H4 10, H5 11, H6 15 and H7 11 positions at which nucleotides differed (SNP).
The sequences of the 609 individual genes were submitted to the C. albicans MLST database and 34 different DSTs were identified for the 87 C. albicans isolates (Figure 4, Table 15). All of these DST were unique to the study strains: none of the DST were in the database.

Table 15. Diploid Sequence Types (DSTs) of 87 C. albicans isolates.

<table>
<thead>
<tr>
<th>DST №</th>
<th>Isolates</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>CL1S4, CL1T5, CL1P1, CL1P2, CL1P5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>CL1S3, CL1S5, CL1T2, CL1T3, CL1P3</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>CL1S2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>CL1P4</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>SL4S4, SL4P2, SL9S5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>ML4S3</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>ML4T4</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>MN4S5</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>ML4P1</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>SL7P1, SN3P1, ML2S4</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>CL1S1, CL1T1, CL1T4</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>CN15S1</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>CN2S1</td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td>CN8S1, SL5S1, SL7S2, SL7T1, SN1S1, SN1T1, SN1P1, SN3S3, SN3T4, ML2T1, ML2P4, ML3T1</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>CN3S1, SL1S1</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>CL3S4</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>CL3T4, CL3P1</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>CL2S4</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>CL2T1</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>SL8S5, SL8T1,</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>SL8P1</td>
</tr>
<tr>
<td>22</td>
<td>3</td>
<td>SL2S2, SL2T1, SL2P1</td>
</tr>
<tr>
<td>23</td>
<td>1</td>
<td>ML3S5</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>CL4S5, CL4T1, CL4P1, CN9S1, CN9P1</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>SL6S5</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>CL5S2</td>
</tr>
<tr>
<td>27</td>
<td>3</td>
<td>ML1S3, ML1T1, ML1P1</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>CL8S4</td>
</tr>
<tr>
<td>29</td>
<td>2</td>
<td>CL8T1, CL8P1</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>CN14S4, CN14T1</td>
</tr>
<tr>
<td>31</td>
<td>1</td>
<td>CN12S1</td>
</tr>
<tr>
<td>32</td>
<td>2</td>
<td>MN7T3, MN7T5</td>
</tr>
<tr>
<td>33</td>
<td>3</td>
<td>SN2S1, SN2T1, SN2P1</td>
</tr>
<tr>
<td>34</td>
<td>13</td>
<td>MN7S1, MN7S2, MN7S3, MN7S4, MN7S5, MN7T1, MN7T2, MN7T4, MN7P1, MN7P2, MN7P3, MN7P4, MN7P5</td>
</tr>
</tbody>
</table>
3.3.3.2 Candida albicans microevolution

The genetic variation of *C. albicans* strains within the same participant and between participants was investigated. This was done by identifying SNPs (which appear as double peaks on the sequencing chromatograms) for each of the housekeeping genes for a particular strain and seeing whether this heterozygosity (D = diploid) was present at the positions in those genes in other strains or whether those strains had one or other of the two SNP bases (A haplotype or B haplotype). Thus, for each gene in each strain there could be particular haplotypes (A or B) where both alleles were the same, or they could contain SNP(s) meaning that the two alleles were different (D). If, for genes in particular strains, several SNPs all lost their heterozygosity and became equivalent to an A or B allele, this indicated that a region of one allele had become duplicated in daughter cells, due to crossing over during DNA replication, a process called “loss of heterozygosity” (LOH). The analysis of the allele types in strains are presented in Tables 16-18 below.

*Table 16. Microevolution of *C. albicans* strains in participants with normal saliva flow (only those participants who had more than one *C. albicans* isolate sequenced are included).*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Isolates†</th>
<th>Genea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAT1a</td>
<td>ACC1</td>
</tr>
<tr>
<td></td>
<td>Chr2</td>
<td>ChrR</td>
</tr>
<tr>
<td>CN9</td>
<td>S1</td>
<td>B2</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>B2</td>
</tr>
<tr>
<td>CN14</td>
<td>S4</td>
<td>D3</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D3</td>
</tr>
<tr>
<td>SN1</td>
<td>S1</td>
<td>D1</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D1</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D1</td>
</tr>
<tr>
<td>SN2</td>
<td>S1</td>
<td>D3</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D3</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D3</td>
</tr>
<tr>
<td>SN3</td>
<td>S3</td>
<td>D1</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>D1</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D1</td>
</tr>
</tbody>
</table>

† S, T and P followed by a number (1 to 5) are strain names for the saliva, tongue or palate isolates.

Haploid sequence types on blue background indicate LOH (loss of heterozygosity) and (T) indicates triploid sequences with 1:2 SNP ratios due to duplication of one chromosome. A, B and D with a number indicate the various haploid (A or B allele) or diploid (D) sequence types.
In Tables 16-18, the number following the letters A, B or D indicates the level of similarity between each gene sequence for example; D2 is most similar to D1, while D8 is the least similar to D1. Participants with normal salivary flow did not show much genetic change (Table 16). Only one participant, SN3, had aneuploidies (unusual number of chromosomes – usually three instead of two) in Chr 1 and Chr 6 in SN3T4, while SN3P1 had the loss of the A allele in Chr 6. Due to these changes, participant SN3 was colonised with 3 versions of the same strain with 2 DSTs: DST14 for SN3S3 and SN3T4 and DST10 for SN3P1 (Table 15).
Table 17. Microevolution of *C. albicans* strains in participants with low saliva flow (only those participants who had more than one *C. albicans* isolate sequenced are included).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Isolates†</th>
<th>Gene§</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AAT1a Chr2</td>
<td>ACC1 ChrR</td>
<td>ADP1 ChrR</td>
<td>MP1b Chr2</td>
<td>SYA1 Chr4</td>
<td>VPS13 Chr6</td>
<td>ZWF1b Chr1</td>
<td></td>
</tr>
<tr>
<td>CL2</td>
<td>S4</td>
<td>B1</td>
<td>D1</td>
<td>D2</td>
<td>B1</td>
<td>D1</td>
<td>D1</td>
<td>B2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>B1</td>
<td>D1</td>
<td>D2</td>
<td>B1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td></td>
</tr>
<tr>
<td>CL3</td>
<td>T4</td>
<td>D1</td>
<td>A1</td>
<td>A2</td>
<td>D1</td>
<td>D2</td>
<td>D2</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>D1</td>
<td>D2</td>
<td></td>
</tr>
<tr>
<td>CL4</td>
<td>S5</td>
<td>B2</td>
<td>D2</td>
<td>D3</td>
<td>D2</td>
<td>D3</td>
<td>D3</td>
<td>D3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>B2</td>
<td>D2</td>
<td>D3</td>
<td>D2</td>
<td>D3</td>
<td>D3</td>
<td>D3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>B2</td>
<td>D2</td>
<td>D3</td>
<td>D2</td>
<td>D3</td>
<td>D3</td>
<td>D3</td>
<td></td>
</tr>
<tr>
<td>CL8</td>
<td>S4</td>
<td>D3</td>
<td>D3</td>
<td>B2</td>
<td>D4</td>
<td>B1</td>
<td>B1</td>
<td>D5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D3</td>
<td>D3</td>
<td>D4</td>
<td>D5</td>
<td>B1</td>
<td>B1</td>
<td>D5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D3</td>
<td>D3</td>
<td>D4</td>
<td>D5</td>
<td>B1</td>
<td>B1</td>
<td>D5</td>
<td></td>
</tr>
<tr>
<td>SL2</td>
<td>S2</td>
<td>D4</td>
<td>D4</td>
<td>D5</td>
<td>B1</td>
<td>D5</td>
<td>B2</td>
<td>B3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D4</td>
<td>D4</td>
<td>D5</td>
<td>B1</td>
<td>D5</td>
<td>B2</td>
<td>B3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D4</td>
<td>D4</td>
<td>D5</td>
<td>B1</td>
<td>D5</td>
<td>B2</td>
<td>B3</td>
<td></td>
</tr>
<tr>
<td>SL4</td>
<td>S4</td>
<td>A1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>D6</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>D6</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td>SL7</td>
<td>S2</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>D6</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>D6</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>B3</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td>SL8</td>
<td>S5</td>
<td>B3</td>
<td>D6</td>
<td>D6</td>
<td>D6</td>
<td>D7</td>
<td>B2</td>
<td>B3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>B3</td>
<td>D6</td>
<td>D6</td>
<td>D6</td>
<td>D7</td>
<td>B2</td>
<td>B3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>B3</td>
<td>D6</td>
<td>D6</td>
<td>B2</td>
<td>D2</td>
<td>B2</td>
<td>B3</td>
<td></td>
</tr>
<tr>
<td>ML1</td>
<td>S3</td>
<td>D5</td>
<td>D3</td>
<td>D7</td>
<td>D7</td>
<td>D8</td>
<td>D8</td>
<td>A1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D5</td>
<td>D3</td>
<td>D7</td>
<td>D7</td>
<td>D8</td>
<td>D8</td>
<td>A1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D5</td>
<td>D3</td>
<td>D7</td>
<td>D7</td>
<td>D8</td>
<td>D8</td>
<td>A1</td>
<td></td>
</tr>
<tr>
<td>ML2</td>
<td>S4</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>B3</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>D6</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>D6</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td>ML3</td>
<td>S5</td>
<td>D4</td>
<td>D4</td>
<td>D5</td>
<td>B1</td>
<td>D5</td>
<td>B2</td>
<td>D8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>D6</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>D1</td>
<td>A1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
<td>B3</td>
<td>D1</td>
<td></td>
</tr>
</tbody>
</table>

† S, T and P followed by a number (1 to 5) are strain names for the saliva, tongue or palate isolates.

§ Haploid sequence types on blue background indicate LOH (loss of heterozygosity) and (T) indicates triploid sequences with 1:2 SNP ratios due to duplication of one chromosome. A, B and D with a number indicate the various haploid (A or B allele) or diploid (D) sequence types.

** Partial LOH (i.e. only the 3' half of H4 (MP1b) of CL8S4 experienced LOH).

However, more participants with low salivary flow had strains with evidence of microevolution (8 out of 12 participants; Table 17). Most of the genetic changes involved LOH, thus increasing the number of variations of the same *C. albicans* strain within the same participant.
Table 1. Microevolution of 15 C. albicans isolates from the saliva, tongue and palate samples of two participants, one with a low (CL1) and the other one with normal saliva flow rate (MN7).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Isolates</th>
<th>Gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AAT1&lt;sub&gt;a&lt;/sub&gt; Chr2</th>
<th>ACC&lt;sub&gt;1&lt;/sub&gt; ChrR</th>
<th>ADP1 ChrR</th>
<th>MP1b Chr2</th>
<th>SYA1 Chr4</th>
<th>VPS13 Chr6</th>
<th>ZWF1b Chr1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL1</td>
<td></td>
<td></td>
<td>D1</td>
<td>A1</td>
<td>A1 (T)</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>D1 (T)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
</tr>
<tr>
<td>T1</td>
<td></td>
<td></td>
<td>D1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
<td>D1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
</tr>
<tr>
<td>T3</td>
<td></td>
<td></td>
<td>D1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
</tr>
<tr>
<td>T4</td>
<td></td>
<td></td>
<td>D1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
</tr>
<tr>
<td>T5</td>
<td></td>
<td></td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>D1</td>
</tr>
<tr>
<td>P1</td>
<td></td>
<td></td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>D1</td>
</tr>
<tr>
<td>P2</td>
<td></td>
<td></td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td></td>
<td>D1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
</tr>
<tr>
<td>P4</td>
<td></td>
<td></td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>B1</td>
</tr>
<tr>
<td>P5</td>
<td></td>
<td></td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>D1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D7</td>
<td>D4</td>
<td>A1</td>
<td>D7</td>
<td>D6</td>
<td>D7</td>
<td>D6</td>
</tr>
<tr>
<td>MN7</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>D7</td>
<td>D7</td>
<td>D7</td>
<td>D6</td>
</tr>
<tr>
<td>T1</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>D7</td>
<td>D7</td>
<td>D7</td>
<td>D6</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>D7</td>
<td>D7</td>
<td>D7</td>
<td>D6</td>
</tr>
<tr>
<td>T3</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>D7</td>
<td>D7</td>
<td>D7</td>
<td>A2</td>
</tr>
<tr>
<td>T4</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>D7</td>
<td>D7</td>
<td>D7</td>
<td>D6</td>
</tr>
<tr>
<td>T5</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>D7</td>
<td>D7</td>
<td>D7</td>
<td>A2</td>
</tr>
<tr>
<td>P1</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>B1</td>
<td>D7</td>
<td>D6</td>
<td>D6</td>
</tr>
<tr>
<td>P2</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>B1</td>
<td>D7</td>
<td>D6</td>
<td>D6</td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>B1</td>
<td>D7</td>
<td>D6</td>
<td>D6</td>
</tr>
<tr>
<td>P4</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>B1</td>
<td>D7</td>
<td>D6</td>
<td>D6</td>
</tr>
<tr>
<td>P5</td>
<td></td>
<td></td>
<td>B1</td>
<td>D7</td>
<td>D4</td>
<td>B1</td>
<td>D7</td>
<td>D6</td>
<td>D6</td>
</tr>
</tbody>
</table>

† S, T and P followed by a number (1 to 5) are strain names for the saliva, tongue or palate isolates.

<sup>a</sup> Haploid sequence types on blue background indicate LOH (loss of heterozygosity) and (T) indicates triploid sequences with 1:2 SNP ratios due to duplication of one chromosome. A, B and D with a number indicate the various haploid (A or B allele) or diploid (D) sequence types.

In participant CL1, there were 6 different versions of the same strain with 5 different DSTs and one possible aneuploid strain. CL1T1 and CL1T4 isolates of DST11 (Table 15) were quite possibly closest to the diploid C. albicans strain type from which all 5 other DSTs evolved over time in participant CL1. CL1S1 DST11 (Table 15) quite possibly evolved from CL1T1 or CL1T4 by duplicating one copy each of ChrR and Chr1.
(chromosomal duplication does not change the DST). DST3 (CL1S2) and DST1 (CL1S4, -T5, -P1, -P2, -P5; Table 15) probably evolved from DST11 (i.e. CL1T1 or -T4) by LOH (loss of B allele) in H7 (ZWF1b) and H1 (AAT1a), respectively. DST2 (CL1S3, -S5, -T2, -T3, -P3; Table 15) most likely evolved from DST3 (CL1S2) by LOH of H3 and H4 (loss of B alleles) on ChrR and Chr2, respectively. DST4 *C. albicans* cells of participant CL1 (CL1P4; Table 15) most likely evolved from DST1 by LOH of H7 (loss of A allele). Thus, one *C. albicans* isolate (S1) of participant CL1 experienced aneuploidies in Chr1 and ChrR, another isolate (S2) experienced LOH of H7, five isolates (S4, T5, P1, P2, P5) are derivatives of a cell that experienced LOH of H1, five isolates (S3, S5, T2, T3, P3) are derivatives of a cell that experienced an additional two LOH events of H3 and H4 of a cell that shared its ancestry with isolate S2, and one isolate (P4) experienced an additional LOH event in H7 from an isolate that shared its ancestry with S4. Thus, all 15 *C. albicans* isolates of CL1 are probably offspring of a single DST11 cell that has undergone significant microevolution (i.e. one aneuploidy and four separate LOH events (i.e. one in H1, H3 and H4 and two in H7) over a period of time.

Hardly any microevolution was detected in 15 *C. albicans* isolates characterized from another participant (MN7) with a normal saliva flow rate (Table 18). Similar to CL1, all 15 *C. albicans* isolates of participant MN7 were clearly derived from a single ancestor in their distant past. Thirteen isolates were identical (DST34; Table 15), and two isolates (MN7T3, -T5; Table 18) were offspring of the same DST34 cell that had experienced a single LOH event in Chr6 in the recent past.
3.3.3.3 The relatedness of Candida albicans strains

The phylogenetic relatedness of the 87 *C. albicans* isolates is depicted in Figure 5. The phylogenetic tree shows the genetic similarity between all *C. albicans* strains sequenced. The strains from RH and OH participants appear to be randomly distributed across all branches of the phylogenetic tree and so are not closely related.
Figure 5 Phylogenetic tree of the 3535 bp concatenated housekeeping gene sequences of 87 C. albicans isolates isolated from the indicated participants. The tree revealed no geographical clustering of strains isolated from LSOP (blue) or Montecillo (magenta) RHs compared to the control participants living in their OHs (green).
3.3.3.4 Hybrid strains between C. albicans and C. dubliniensis

Quite to our surprise, nine “C. albicans” strains isolated from four RH participants (three from Montecillo and one from LSOP) appeared to in fact be C albicans/C. dubliniensis hybrid strains (ML3P1, MN2S4, -T4, -P1, MN5S1, -T1, -P1 and SL4T1, -P1). Two of these participants had low salivary flow rate, the other two had normal salivary flow rate. Four of the nine hybrid strains were isolated from the palate, three from the tongue and two from saliva samples. The strains within the same participant were very similar or identical to each other (see Appendix X). However, the hybrid strains between the four participants were quite different, suggesting four separate hybridization events.
Chapter 4: Discussion

4.1 Introduction

The focus of this study was the oral health of older people. The objective was to investigate if living conditions would have an impact on the yeast colonization in the oral cavities of the older people. Evaluation of the methodological approaches, the results in relation to relevant literature, limitations of the study and future directions will be discussed in this chapter.

4.2 Sampling methods

A cross sectional study was the preferred type of study for a pilot descriptive project such as this with no interventions. As such, it is an observational study at a specific point in time which analyses data from a population, or a subgroup(s) of the population. Ideally, all rest home participants should be from one establishment as this would remove any variable factor due to different processes being used at different rest homes. Unfortunately, the largest rest home in Dunedin declined to participate in this study. The other rest homes had a lower capacity, thus the need for two rest homes to provide sufficient participants, 13 from Montecillo Rest Home and 12 from Little Sisters of the Poor.

The results indicated that participants in both rest homes had higher levels of yeast colonization than participants living at home. Although participants from both rest homes had high levels of colonization, participants from Little Sisters of the Poor had, on average, significantly higher levels of colonization. Despite this difference in colonization levels, strain typing revealed that isolates from each rest home were
dispersed across the phylogenetic tree indicating that having two different rest homes did not influence the results of this study greatly. However, future studies should aim to involve participants from a single rest home.

4.3 Study participants

The number of participants recruited from each population group was 25. To remove age as a confounding factor, 20 participants from each group were selected that were matched as best as possible for age and gender, without taking any other factor into consideration. This selection resulted in an RH group with mean age of 86.0 compared to 83.1 for those living in their OH, this difference was not statistically significant. This mean age for RH participants corresponds with the Statistics New Zealand data from 2013 which states that 72% of RH residents were above the age of 80 (Statistics New Zealand, 2013) and that 50% of New Zealanders live in RHs by the age of 85 (Broad et al., 2015). Older people are encouraged to remain independent and to continue living in their OHs for as long as possible. So, although we were able to get older community dwelling participants age-matched to those in RHs we had to exclude younger OH participants and older RH participants to achieve the matching. Thirteen participants of each group (65%) were female and 7 (35%) were male, this reflects the fact that more than two-thirds of people in residential care for older people are female, 68.1% (Statistics New Zealand, 2013). To get a better understanding of the yeast species variation and distribution in the two populations, data from all 25 participants from each group were used in the MLST DNA sequencing analysis.
4.4 Yeast colonization

Saliva was the most highly colonized oral site sampled, followed by the tongue and the palate was the least colonized. Compared to the tongue and palate, saliva was the most frequently colonized site as well. This may be due to the salivary function of oral clearance (Humphrey & Williamson, 2001), whereby yeast colonizing other parts of the oral cavity are washed off into saliva. Saliva, therefore, may be used as an indicator of the state of colonization in the oral cavity in the study of yeast species variety. The level of yeast colonization for each site was much higher in RH participants, even though there were no significant differences in terms of age, salivary flow, denture use, and number of medications taken by participants in the two groups. This may be because participants in RH are frailer and require assistance in activities of daily living including oral hygiene care, while participants in OH are more independent and more able to care for themselves. This study indicates that there is a need for good oral care in RHs. The New Zealand Dental Association (NZDA) in January 2011 published ‘Healthy Mouth, Healthy Ageing: Oral Health Guide for Caregivers of Older People’ which is free to download from the Ministry of Health website (https://www.health.govt.nz/publication/healthy-mouth-healthy-ageing). The NZDA has also claimed to have carried out training for 4000 caregivers to date. However, there is currently no framework to evaluate any form of best practice protocol for oral hygiene care in RHs. Dietary factor may contribute to a much higher yeast colonization in RH participants. Older people living in rest homes have meals prepared for them but have limited input or choice on the types of food served (Abbey, Wright, & Capra, 2015). Some older people face complex nutritional problems which arise during the progressive decline in cognitive, behavioural and physical functions. Some rest homes resort to food
high in carbohydrate and sugar to encourage food intake, however, research on this is limited.

4.4.1 Dentures

A high number of participants were denture wearers; 70% of RH participants and 55% who were living at home (this difference is not significant). This corresponds with the NZ Older People Oral Health Survey 2012 which found 70% of edentulous people wore dentures (Smith, Thomson, & Gribben, 2012). A significantly larger proportion (84%) of denture wearing participants presented with yeast positive saliva while yeast was only present in 53% of non-denture wearing participants. This is consistent with a study by Pereira Lyon et al., 2006, which showed a higher percentage of denture wearers had Candida carriage compared to non-denture wearers.

Denture use is a risk factor to candidiasis (C. S. Farah et al., 2018). Poor denture hygiene, overnight denture use, dentures with poor retention and/or stability and worn-down dentures which reduce vertical dimension are factors that increase risk of candidiasis. Dentures may also provide a surface that promotes Candida adherence.

4.4.2 Salivary Flow

Salivary function studies may be carried out by:

i) Sialometry: measuring salivary flow rates
   - whole unstimulated saliva: the collection of saliva at rest, closely relates with symptoms of salivary gland hypofunction
   - stimulated saliva: the collection of saliva after stimulation (chewing, sucking or eating)
- selective saliva from specific glands: the collection of stimulated saliva from the duct opening of parotid, submandibular and sublingual gland using specialised collection apparatus

ii) Ultrasonography
- able to detect changes to major salivary glands during inflammation
- non-invasive, high specificity and moderate sensitivity

iii) Sialography
- introduction of radio-opaque dye into the salivary duct
- able to detect dilation or duct obstruction
- non-specific with risks of discomfort and infection

iv) Salivary scintiscanning
- examines all major salivary glands simultaneously
- small radiation hazard
- not always available and expensive

v) Salivary gland biopsy

(Scully & Scully, 2013)

The whole unstimulated saliva flow rate test was chosen for this study because it is a simple, generally acceptable and non-invasive method of investigating overall salivary output.

There was no significant difference between RH and OH participants in terms of their mean salivary flow rate (0.23 ml/min vs 0.25 ml/min, respectively). However, this study confirmed an association between low salivary flow rate and increased yeast colonization in the oral cavity and hyphae-positive smears as previously reported (Nadig et al., 2017) (Shinozaki et al., 2012). Older people in general suffer from reduced salivary flow. As
discussed in Chapter 1 (1.2.4.3) it is important to manage hyposalivation in all patients with low salivary flow. This reduces the risk of developing candidiasis as well as realising the many benefits of a moist mouth.

4.4.3 Medication

There was no significant difference in the numbers of medications taken by RH participants and OH participants. However, participants with low salivary flow rate were taking, on average, more medications per day than those with normal salivary flow rate. Hyposalivation due to medication has been reported for older populations by numerous authors (Smidt, Torpet, Nauntofte, Heegaard, & Pedersen, 2011)(Lynge Pedersen et al., 2015). As people age, their health declines, and there is a corresponding increase in the number of medications prescribed to manage their conditions (Barbe, 2018). Clinicians prescribing these medications should develop a proactive approach to manage the common side effects of these medications, such as reduced salivary function.

4.4.4 Hyphae-positive smears

A third of participants (13) had hyphae-positive smears. There was no significant difference in the prevalence of hyphae-positive smears between participants living at home to those living in RHs. Most participants with hyphae-positive smears were denture wearers and had a low salivary flow rate (<0.2 ml/min). Hyphae-positive smears are indicative of candidiasis as hyphae are required to penetrate host tissues. These results are consistent with denture wearing, and low salivary flow, increasing the risk of candidiasis. Almost all participants with hyphae-positive smears had high concentrations of yeast in their saliva (>400 cfu/ml), this confirms the report by Epstein, Pearsall, & Truelove, 1980.
4.4.5 Species variation

*C. albicans* was the species found most frequently in participant samples from both groups followed by *C. glabrata*. Interestingly, many other yeast species, some of which are not commonly associated with the oral cavity were identified. Participants living in their OH had *C. parapsilosis, C. lusitaniae, C. guilliermondii, Pichia fermentans* and *Yarrowia lipolytica* in addition to *C. albicans* and *C. glabrata* in their oral cavities. While *Saccharomyces cerevisiae, C. dubliniensis, C. tropicalis* and *C. albicans/C. dubliniensis* chimera strains were the other species identified in RH participant samples. It is interesting that *C. albicans* and *C. glabrata* were the only two yeast species found in both groups; the rest were found in either the OH or the RH participants. This may be due to the small size of this study population, and low incidence of the non-*albicans* non-*glabrata* species. It may also be because *C. albicans* has successful commensal traits like adherence to buccal epithelial cells, biofilm formation, production of hydrolytic enzymes and haemolytic activity compared to other species (Deorukhkar, Saini, & Mathew, 2014). Many of the yeast species found in this study have been reported as having either intrinsic or acquired resistance to antifungals for example *C. albicans, C. glabrata, C. parapsilosis, C. lusitaniae, C. guilliermondii, C. tropicalis*, and *Y. lipolytica* (Atkinson et al., 2008)(Papon, Courdavault, Clastre, & Bennett, 2013)(Meurman et al., 2011)(Zhao et al., 2015). It is not known if any of the yeast species and *C albicans* strains identified in this study are resistant to antifungal agents.
4.5 Presumptive identification of *Candida* species with CHROMagar™ *Candida* Agar

CHROMagar™ *Candida* agar is a simple and reliable tool to quantify yeast colonization. It is also valuable for presumptively identifying different yeast species. Growth on CHROMagar™ *Candida* medium results in colonies with distinctive colours which, according to the manufacturer’s manual for identification, are *C. albicans* green, *C. tropicalis* blue, and *C. krusei* pale rose. In this study we have found in some instances two shades of green, or two distinctively different sizes of green colonies present on the same plate. Our further investigation of green colonies by rDNA sequencing, revealed that there was no species difference between different shades or sizes of colonies of the same colour within the same participant. However, we were not able to distinguish between *C. albicans* and *C. dubliniensis* or a hybrid strain of these two species with this method, as they all formed green colonies.

4.6 Smear Test

The smear test with PAS staining is a simple and effective method to identify fungal hyphae indicative of fungal invasion into host tissues. A PAS-positive smear indicates to the clinician that there is active fungal infection and that investigations are required to determine any predisposing factors and that antifungal medication is required. A smear allows a direct diagnosis of candidiasis but is limited to hyphae-forming fungi such as *C. albicans* and *C. tropicalis*. Many fungi, such as *C. glabrata*, are not capable of forming hyphae but can still cause candidiasis. The smear test is unable to distinguish species type based on the morphology of organism observed. A positive smear test may need to be reclassified as ‘hyphae positive’. A negative smear test (no hyphae observed) which has
yeast-like cells present may need further investigation. Plating swab suspension or saliva on CHROMagar plates may identify non-hyphae forming fungi, and high numbers of CFU (≥400/ml) indicates active fungal infection. A truly negative smear test (no hyphae or yeast-like cells observed) indicates no fungal infection. In this study, DNA analysis indicated that C. albicans was the yeast most commonly found in samples. Hyphae-positive smears was found in about one third of the participants. All participants with PAS-positive smears were C. albicans-positive except for two participants; ML5 (had C tropicalis present which can form hyphae) and MN5 (had S cerevisiae which forms pseudo-hyphae, and also a chimera strain - a mix of C albicans and C dubliniensis). Thus, the smear test is not an absolute diagnostic tool for candidiasis but, as C. albicans is the most common cause of candidiasis, it is a useful clinical tool. We also observed a PAS-positive smear in SL3 which only had Pichia anomala (a non-hyphae forming organism) growth on CHROMagar™ Candida agar. C. albicans may have been present in the smear (as detected with PAS) and swab. However, the C. albicans growth on CHROMagar™ Candida agar may have been inhibited by Pichia anomala toxins (Mathews, Conti, Witek-Janusek, & Polonelli, 1998).

4.7 Precise identification of yeast species

Non-albicans species isolates presumptively identified from growth on CHROMagar™ Candida agar were subjected to DNA sequencing of the internal transcribed spacer (ITS) region in the ribosomal RNA (rRNA) operon. This ITS region has been accepted as the formal fungal barcode (Op De Beeck et al., 2014)(Hoggard Michael et al., 2018) with a large database of sequences available at https://www.ncbi.nlm.nih.gov/BLAST. This study has provided a rare glimpse into the diverse yeast species present in the oral cavity. It is also interesting to note that most people have only one fungal species present in their
mouth at any one time. When two species are found together, they are usually *C. albicans* and *C. glabrata*. They are both successful commensals and pathogens in humans, yet they are different to one another in terms of phylogeny, genetics and phenotype (Brunke & Hube, 2013). It is not known if these *Candida* species being present in the same niche makes them less susceptible to antifungal agents.

4.8 Multi Locus Sequence Typing (MLST)

MLST using the DNA sequences of seven housekeeping genes was a very useful tool to compare the *C. albicans* strains present in the oral cavities of individuals. It is however time consuming, resource intensive, and requires a high level of skill and knowledge in the field of microbiology. The value of this technique is that MLST data from different studies worldwide are centralized in public databases, allowing the comparison of strains from all over the world. The PubMLST database website is currently recognized as the most complete repository for MLST data (Muñoz et al., 2019).

4.8.1 *Candida albicans* strains

A total of 87 *C. albicans* strains were isolated from the participants in this study. Many of the strains were identical according to the MLST Diploid Sequence Type (DST) and there were 34 different DSTs. None of the 34 DSTs was a match to the strains contained in the database at https://pubmlst.org/calbicans. This database does not represent all global isolates because the data are voluntarily submitted by researchers aware of the database. It is, however, a scientifically valuable dataset as it is the largest repository of *C. albicans* strain types (Muñoz et al., 2019). The database was last updated on 9/10/2018 and contains a total of 4,392 isolates. In the past, the database was updated 3-4 times a
year. All 34 DSTs from this study have been submitted to be added to the database but, at the time of printing this thesis, we have yet to receive a confirmation that the DST have been entered. When they are entered, as they are not already present in the database, they will be assigned unique DST numbers.

4.8.2 Microevolution of *Candida albicans* strains

The genetic diversity of *C. albicans* is evident from this study. An interesting finding was a link between salivary flow rate and microevolution. Participants with low salivary flow rates had *C. albicans* strains in their oral cavities with more genetic changes like aneuploid gene alleles, loss of heterozygosity (either partial or full) and chromosomal duplications compared to strains from people with normal salivary flow rates. One explanation would be that *C. albicans* is constantly changing in people with low salivary flow rates to better adapt to that environment. We noted up to five variations of the same strain within one site in one participant. Participant CL1 (low salivary flow) had 6 different versions of the same strain with 5 different DSTs while MN7 (normal salivary flow) had 2 different versions of the same strain with 2 DSTs. Most participants had only been colonized by one *C. albicans* strain in their oral cavity. The strains in participants colonized by more than one DST appear to have originated from the same ancestor strain which had undergone microevolution. This study has given us a first glimpse into the possible changes to the genetic code by comparing seven housekeeping gene sequences of up to five independent isolates from the same location in the oral cavity of an individual. We have also been able to compare the changes relative to strains in other locations in the oral cavity.
4.8.3 Relatedness of strains

*C. albicans* strains from OH and RH participants were spread throughout the phylogenetic tree of strains. Living in close proximity to one another, sharing the same caregivers and food handlers, appeared to have no effect on the relatedness of the *C. albicans* strains present in the oral cavities of RH participants. This strain diversity disproves one of the hypotheses (1.6) of this study.

4.8.4 Hybrid strains

Nine strains that appeared to be hybrids of *C. albicans* and *C. dubliniensis* were identified in four RH participants. Studies have shown that these strains are able to mate and produce stable hybrid in a laboratory setting (Pujol et al., 2004)(Chakraborty et al., 2013). Little is known about these strains in nature as they have never been reported. This may indicate that sexual reproduction occurs more frequently as a propagation method than commonly believed.

4.9 Future studies

The susceptibility of all yeast species identified in this study against antifungal agents needs to be investigated. The identification of resistant strains and prevalence (if any) would be vital information to ensure early detection of these resistant strains and contribute towards global surveillance efforts. In this study, nucleotide polymorphism was noted more frequently in participants with low salivary flow rate. It remains to be determined if these changes in the genetic code has affected the amino acid sequence of
protein (Smith, 2008), potentially increasing the pathogenicity of \textit{C. albicans}. The chimera strains identified in this study require confirmation and further investigation. It would be interesting to know why these strains were only found in RH participants and their susceptibility level to antifungal agents.

4.10 Conclusions

This study has shown that older people living in RH are more likely to be colonized by yeast, have a higher degree of yeast colonization as well as the prevalence of chimera strains compared to those living in their OH. Regardless of living conditions, older people wearing dentures, and those with low salivary flow, are at higher risk of yeast colonization and candidiasis. \textit{C. albicans} and \textit{C. glabrata} are the most common yeasts found in the oral cavity. \textit{C. albicans} is associated with the majority of PAS-positive smears. Saliva may be used as an indicator for species variety in the oral cavity. \textit{C. albicans} strains undergo more genetic changes in people with low salivary flow. Preventive care is the best way forward to maintain oral health of older people, some suggested strategies are as follows:

i) Clinicians should be encouraged to include baseline salivary flow rate as part of the initial dental consultation for all older people and medically compromised patients. Information gathered will allow hyposalivation strategies (if needed) to be incorporated into the treatment plan.

ii) Devise care plans encouraging best practice of oral health care in residential aged care facilities that can be implemented nationwide.
References:


of pathogenic yeast species. *Clinical Microbiology and Infection* 14(2);141-7
https://doi.org/10.1111/j.1469-0691.2007.01872.x


Retrieved February 20, 2019.


Appendix I: Ethical Approval

Professor R Cannon
Sir John Walsh Research Institute
Department of Oral Diagnostic and Surgical Sciences
Faculty of Dentistry

9 August 2017

Dear Professor Cannon,

I am again writing to you concerning your proposal entitled "Candida species and strains in the oral cavities of the elderly: a comparison between people in home-based care and in an aged care facility", Ethics Committee reference number H17/081.

Thank you for your e-mail of 8th August 2017 with response attached addressing the issues raised by the Committee.

On the basis of this response, I am pleased to confirm that the proposal now has full ethical approval to proceed.

The standard conditions of approval for all human research projects reviewed and approved by the Committee are the following:

Conduct the research project strictly in accordance with the research proposal submitted and granted ethics approval, including any amendments required to be made to the proposal by the Human Research Ethics Committee.

Inform the Human Research Ethics Committee immediately of anything which may warrant review of ethics approval of the research project, including: serious or unexpected adverse effects on participants; unforeseen events that might affect continued ethical acceptability of the project; and a written report about these matters must be submitted to the Academic Committees Office by no later than the next working day after recognition of an adverse occurrence/event. Please note that in cases of adverse events an incident report should also be made to the Health and Safety Office:

http://www.otago.ac.nz/healthandsafety/index.html

Advise the Committee in writing as soon as practicable if the research project is discontinued.

Make no change to the project as approved in its entirety by the Committee, including any wording in any document approved as part of the project, without prior written approval of the Committee for any change. If you are applying for an amendment to your approved research, please email your request to the Academic Committees Office:
Approval is for up to three years from the date of this letter. If this project has not been completed within three years from the date of this letter, re-approval or an extension of approval must be requested. If the nature, consent, location, procedures or personnel of your approved application change, please advise me in writing.

The Human Ethics Committee (Health) asks for a Final Report to be provided upon completion of the study. The Final Report template can be found on the Human Ethics Web Page [http://www.otago.ac.nz/council/committees/committees/HumanEthicsCommittees.html](http://www.otago.ac.nz/council/committees/committees/HumanEthicsCommittees.html)

Yours sincerely,

[Signature]

Mr Gary Witte  
**Manager, Academic Committees**  
Tel: 479 8256  
Email: gary.witte@otago.ac.nz

cc. Professor R D Cannon  Director  Sir John Walsh Research Institute
Appendix II: Ngai Tahu Consultation Approval

NGĀI TAHU RESEARCH CONSULTATION COMMITTEE
TE KOMITI RAKAHAU KI KAI TAHU

Tuesday, 04 July 2017.

Professor Richard Cannon,
Sir John Walsh Research Institute,
DUNEDIN.

Te Tiaki Koe Professor Richard Cannon,

Candida species and strains in the oral cavity of the elderly: a comparison between people in home-based care and in an aged-care facility.

The Ngai Tahu Research Consultation Committee (the committee) met on Tuesday, 04 July 2017 to discuss your research proposition.

By way of introduction, this response from The Committee is provided as part of the Memorandum of Understanding between Te Runanga o Ngai Tahu and the University. In the statement of principles of the memorandum it states “Ngai Tahu acknowledges that the consultation process outlined in this policy provides no power of veto by Ngai Tahu to research undertaken at the University of Otago”. As such, this response is not “approval” or “mandate” for the research, rather it is a mandated response from a Ngai Tahu appointed committee. This process is part of a number of requirements for researchers to undertake and does not cover other issues relating to ethics, including methodology they are separate requirements with other committees, for example the Human Ethics Committee, etc.

Within the context of the Policy for Research Consultation with Māori, the Committee bases its consultation on that defined by Justice McGechan:

"Consultation does not mean negotiation or agreement. It means: setting out a proposal not fully decided upon; adequately informing a party about relevant information upon which the proposal is based; listening to what the others have to say with an open mind (in that there is room to be persuaded against the proposal); undertaking that task in a genuine and not cosmetic manner. Reaching a decision that may or may not alter the original proposal."

The Committee considers the research to be of importance to Māori health.

As this study involves human participants, the Committee strongly encourage that ethnicity data be collected as part of the research project. That is the questions on self-identified ethnicity and descent, these questions are contained in the latest census.

The Committee suggests researchers consider the Southern District Health Board’s Tikaka Best Practice document, in particular patient engagement. The document also covers the collection, storage and disposal of blood and tissue samples. This document is available on the Southern District Health Board website.

The Committee suggests dissemination of the findings to relevant Māori health organisations, for example the National Māori Organisation for Dental Health, Oranga Niho and to

The Ngai Tahu Research Consultation Committee has membership from:

Te Runanga o Ōrākau Incorporated
Kiai Hiapo Nīnara ki Pokotekiti
Te Runanga o Maniapoto
Ngāi Tahu Research Consultation Committee
Te Komiti Rakahau ki Kai Tahu

Professor John Broughton and Malcolm Dacker, who are involved in Māori Dental Health, University of Otago.

We wish you every success in your research and the committee also requests a copy of the research findings.

This letter of suggestion, recommendation and advice is current for an 18 month period from Tuesday, 04 July 2017 to 4 January 2019.

Nīihau noa, nā

Mark Brunton
Kaiwhakahaere Rangahau Māori
Research Manager Māori
Research Division
Te Whare Wānanga o Otago
Ph: +64 3 479 8738
Email: mark.brunton@otago.ac.nz
Web: www.otago.ac.nz

The Ngāi Tahu Research Consultation Committee has membership from:

Te Rūnanga o Ōhau Incorporated
Kia Hīpo Rūnaka ki Pukatea
Te Rūnanga o Moutahi
Appendix III: Patient Information Sheet

| Study title: | Candida species and strains in the oral cavity of the elderly: a comparison between people in home-based care and in an aged care facility |
| Principal investigator: | Name: Nurulhuda Mohd Thiyahuddin  
Department: Oral Diagnostic and Surgical Sciences  
Position: DClinDent Special Needs Candidate |
| Contact phone number: | 03 4797025 |

Introduction

Fungal infection of the mouth is very common in the older people and people with symptoms of dry mouth. The natural ageing process and effects of certain medications reduces saliva production which causes dry mouth. Fungal infection is also common in people who wear dentures.

Fungus naturally present in the mouth is Candida which comes in many different species and strains. Candida is usually harmless. However, when infection occurs the mouth becomes sore. This infection is then treated with antifungal medication.
Studies have shown that some *Candida* species are immune to certain antifungal drugs. We wish to find out which *Candida* species and strains can be found in the mouth of elderly participants.

Thank you for showing an interest in this project. Please read this information sheet carefully. Take time to consider and, if you wish, talk with relatives or friends, before deciding whether or not to participate.

If you decide to participate we thank you. If you decide not to take part there will be no disadvantage to you and we thank you for considering our request.

**What is the aim of this research project?**

The aim of this project is to investigate and compare *Candida* species and strains between participants who live at home and those who live in an age care facility. The outcome of this study will contribute towards a better understanding of the risk of candida infection and better inform antifungal treatment protocols in elderly patients.

**Who is funding this project?**

This study is funded by the Ministry of Health.

**Who are we seeking to participate in the project?**

We need to sample two groups of elderly people:

1) people living at an age care facility; and

2) people living at home who may, or may not, be patients of the Special Care Unit, Faculty of Dentistry, University of Otago.
Enrolment of participants will be by voluntary participation after informed consent is obtained.

Participants of this study must be:
- Age 65 and above
- Generally well or have well controlled medical conditions
- Able to cooperate with the procedures
- Able to give consent

People who cannot participate are:
- People who had used antibiotic/antifungal in the past 2 months
- Terminally ill
- Smokers

If you participate, what will you be asked to do?

We will examine your mouth.

You will need to collect saliva in your mouth for one minute then spit into a container. This will be repeated 5 times. If you are not able to do this, you will need to rinse your mouth with bottled water and spit into a container.

A tissue swab will be done by gently rubbing your palate and tongue with a cotton bud. A tissue smear will be done by gently scraping your palate and tongue with a wooden spatula.

The procedure will be done once and will take less than 15 minutes

Is there any risk of discomfort or harm from participation?

There will be no harm or discomfort to the participant. However, there is a risk that you might gag during the swab and smear taking procedure. Please inform us beforehand if you have a strong gag reflex. There is also a small risk of mucosal tear during smear taking. This procedure will be done gently and quickly to reduce these risk.
What specimens, data or information will be collected, and how will they be used?

The investigator will collect information on your age, gender, medical history and if you wear dentures.

The saliva and swab taken will be tested for presence of different types of *Candida* species and strains. The remainder of the sample will be stored securely until the completion of the project. The cotton bud will be disposed and the rest of the sample will then be destroyed by sterilisation.

The smear taken will be spread onto a glass slide and stained. This will then be examined under a microscope. The remainder of the sample and the wooden spatula will be disposed.

As samples of human tissue will be taken in this study, there may be cultural issues regarding tissue storage and disposal. We suggest that you involve your family/whanau at every stage of this research. We acknowledge that you have the full authority to choose to participate in this study or not. If you would like to participate, you will first need to fill in a consent form.

The data collected will be securely stored and only the people mentioned below are able to gain access to it. All personal information will be destroyed immediately at the end of the project except that as required by the University research policy. Raw health data will be retained in secure storage for ten years after which they will be destroyed.

What about anonymity and confidentiality?
The result of this study will be reported and may be published. However, no material which could personally identify you will be used in the report.

**If you agree to participate, can you withdraw later?**

Your participation is entirely voluntary (your choice).

You may withdraw from participation in the project at any time and without any disadvantage to yourself.

**Any questions?**

If you have any questions now or in the future, please feel free to contact either:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nurul Thiyahuddin</th>
<th>Contact phone number:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>DClinDent Candidate</td>
<td>03 4797025</td>
</tr>
<tr>
<td>Department</td>
<td>Oral Diagnostic and Surgical Sciences</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Richard Cannon</th>
<th>Contact phone number:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>Director</td>
<td>03 4797081</td>
</tr>
<tr>
<td>Department</td>
<td>Sir John Walsh Research Institute</td>
<td></td>
</tr>
</tbody>
</table>

*This study has been approved by the University of Otago Human Ethics Committee (Health). If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (phone +64 3 479 8256 or email gary.witte@otago.ac.nz). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.*
Appendix IV: Consent Form

Candida species and strains in the oral cavity of the elderly: a comparison between people in home-based care and in an aged care facility

Consent form for participant

Following signature and return to the research team this form will be stored in a secure place for ten years.

Name of participant:…………………………………………..

I have read the Information Sheet concerning this study and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at stage.

1. I confirm that I meet the criteria for participation which are explained in the Information Sheet.

2. I know that my participation in the project is entirely voluntary, and that I am free to withdraw from the project at any time without disadvantage.

3. I know that as a participant I will have to provide my medical records, have my saliva collected and mouth tissues swabbed and scraped.
4. I understand the nature and size of the risks of discomfort or harm which are explained in the Information Sheet.

5. I know that when the project is completed all personal identifying information will be removed from the paper records and electronic files which represent the data from the project, and that these will be placed in secure storage and kept for at least ten years.

6. I understand that the results of the project may be published and be available in the University of Otago Library, and that any personal identifying information will remain confidential between myself and the researchers during the study, and will not appear in any spoken or written report of the study.

7. I know that there is no remuneration offered for this study, and that no commercial use will be made of the data.

8. I understand that at the end of the study, any remaining samples will be disposed of using standard disposal methods.

Signature of participant: ___________________________ Date: _____________

Name of person taking consent: ______________________ Date: _____________
Appendix V: Laboratory Form

<table>
<thead>
<tr>
<th>PATIENT'S SURNAME</th>
<th>GIVEN NAMES</th>
<th>ADDRESS</th>
<th>DOB</th>
<th>MFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>REQUESTING CLINICIAN'S NAME</td>
<td>NMC/MBRN NUMBER</td>
<td>NZ Number</td>
<td>Copies to</td>
<td></td>
</tr>
</tbody>
</table>

PLEASE PRINT CLINICAL DETAILS

SPECIMEN COLLECTION
TIME
DATE / /

URGENT

- Biopsy Incisional
- Biopsy Excisional
- Excision of lesion
- Resection of lesion
- Excision of recurrent lesion

 Clinician's Provisional Diagnosis

- Radiographs/photos attached
- Radiographs/photos emailed
- No. of containers sent

Clinician Signature / / Date

Patient Consent

Oral Pathology Centre, as part of the Faculty of Dentistry, is involved in teaching, research and continuing education for the dental profession. We request your consent to use your specimen for these purposes, once the diagnosis has been made. You will not be identified from the material used.

Patient's signature as consent / / Date
Appendix VI: Abstract for American Academy of Oral & Maxillofacial Pathology and International Association of Oral Pathology Congress 2018

Poster Competition 24th to 28th June 2018 in Vancouver, Canada.

*Candida* species and strains in the oral cavities of the elderly: a comparison between people in home-based care and in aged-care facilities

Mohd Thiyahuddin N*, Cannon RD*, Lamping E*, Rich AM*

*Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand

Oral candidiasis is prevalent in the elderly population due to medical conditions, use of systemic medications and the presence of oral appliances such as dentures. It is uncertain whether residing in an aged-care facility contribute to *Candida* carriage and has an impact on the *Candida* species or strains colonizing elderly patients.

**Objective:** The aim of this study was to investigate the presence and abundance of *Candida* species and strains in saliva and from the oral mucosal swabs and smears of people living in institutional rest homes and those living at home.

**Method:** Smears of the palate and tongue were taken from participants (rest home n = 25 and control = 25) for histological analysis using PAS staining to identify the presence of hyphae. Unstimulated whole saliva and material obtained from swabs of palate and tongue were spread on CHROMagar plates and incubated at 37°C for 48 hours. *Candida* species were identified by their colour, and colony forming units (CFU) were counted.

**Findings:** A higher proportion of elderly people living in rest homes had PAS-**positive** hyphae present in smears (9/25, 38%) compared to those living in their own home (7/25, 28%). CFU were present in 17/25 (68%) palatal swabs, 20/25 (80%) tongue swabs and in saliva from 21/25 (84%) of elders living in rest homes compared with 6/25 (24%) palatal swabs, 8/25 (32%) tongue swabs and from saliva in 16/25 (64%) of elderly living in their own home.

**Conclusion:** The results indicate that elderly people living in aged-care facilities are more likely to have *Candida* hyphae detected on PAS-stained smears and have a higher *Candida* carriage rate compared to elderly living at home. This may be due to the presence of co-morbidities which led to the need for residential care and/or may be related to the rest home environment.
Appendix VII: Abstract for Malaysian International Dental Exhibition and Conference

Oral Presentation, 14th July 2019 in Kuala Lumpur, Malaysia.

2019 Malaysia-International Dental Exhibition and Conference (MIDEC)

Presenter’s full name: Nurulhuda Mohd. Thiyahuddin

Name of Institution: Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, New Zealand

Email address: nurul.thiyahuddin@otago.ac.nz

Contact number: 0064 34797026

Preferred mode of presentation: Oral (Window)

I hereby certify that this abstract has never been published or presented.

YEAST SPECIES IN THE ORAL CAVITIES OF OLDER PEOPLE:
A COMPARISON BETWEEN PEOPLE LIVING IN THEIR OWN HOMES TO THOSE IN REST HOMES.

Details of all authors:

1) Nurulhuda Mohd. Thiyahuddin
Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand; nurul.thiyahuddin@otago.ac.nz

2) Dr Erwin Lamping
Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand; erwin.lamping@otago.ac.nz

3) Professor Alison Rich
Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand; alison.rich@otago.ac.nz

4) Professor Richard Cannon
Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand; richard.cannon@otago.ac.nz
Abstract:

Aim: Oral candidiasis is prevalent among older people due to predisposing factors such as impaired immune defenses, use of xerogenic medications and dentures. An increasing number of older people live in rest home facilities and it is unclear how this institutionalized living affects the quantity and type of fungi colonizing these people’s oral cavities.

Material and Methods: Smears and swabs of the palate and tongue and saliva samples were taken from participants residing in rest homes (RH; n = 20) and older people living in their own homes (OH; n = 20). Yeast in samples were quantified and identified by culturing on CHROMagar *Candida* and sequencing the ITS2 region of rDNA.

Results: A higher proportion of RH residents had *Candida* hyphae present in smears compared to OH participants (35% vs 30%). RH residents had, on average, 23 times as many yeasts per ml saliva as OH participants. Seven yeast species were identified in OH samples and only five in RH samples, with *Candida albicans* and *Candida glabrata* being the most common species isolated from both participant groups.

Conclusion: The results indicate that older people living in aged-care facilities were more likely to have candidiasis and have a higher yeast carriage rate than similarly aged people living at home. This may be due to morbidities which led to the need for residential care and/or related to the rest home environment.
Appendix VIII: Abstract for Australian Society of Special Needs in Dentistry Walkabout Conference 2019 ‘Caring Together’

Oral Presentation, 19th July 2019 in Melbourne, Australia.

Name of presenter: Nurul Thiyahuddin

Title/degree: DClinDent Special Needs candidate

Title of abstract: Yeast species in the oral cavities of older people

List of authors/affiliations:

NM Thiyahuddin, E Lamping, A Rich, RD Cannon

Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago

Abstract:

Oral candidiasis is prevalent among older people due to predisposing factors such as impaired immune defences, medications and denture use. An increasing number of older people live in rest home facilities and it is unclear how this institutionalised living affects the quantity and type of fungi colonizing these people’s oral cavities. Smears and swabs of the palate and tongue, and saliva samples were taken from participants residing in rest homes (RH; n = 25) and older people living in their own homes (OH; n = 25). Yeast in samples were quantified and presumptively identified by culturing on CHROMagar Candida agar. Sequencing of the ITS2 region of rDNA was carried out to confirm yeast species. Multilocus sequence typing (MLST) of 7 housekeeping genes was used to
compare *Candida albicans* strains. A higher proportion of RH residents had Candida hyphae present in smears compared to OH participants (35% vs. 30%) although this difference was not statistically significant (p = 0.74). RH residents had, on average, 23 times as many yeast per mL saliva as OH participants (p = 0.01). *C. albicans* and *C. glabrata* being the most common species isolated from both participant groups. All *C. albicans* strains were similar within the same participant but very different between participants. *C. albicans/Candida dubliniensis* chimeras (hybrid strains) were found in 4 rest home participants. The results indicate that communal living for those who reside in an age care facility has an impact on the abundance of yeast species and the prevalence of chimera strains. This may be due to morbidities which led to the need for residential care and/or related to the rest home environment.
Appendix IX: Abstract for University of Otago Student Research Symposium

Oral Presentation, 4th August 2019 in, Dunedin, New Zealand

First name: Nurul
Last name: Thiyahuddin
Campus: Dunedin
Email Address: nurul.thiyahuddin@otago.ac.nz
Type of presentation: Oral
Name of Presenter: Nurul Thiyahuddin
Name of all authors and their departments:
Prof Richard Cannon (SJWRI),
Dr Erwin Lamping (SJWRI),
Prof Alison Rich (SJWRI)
Title of Presentation: Yeast Species in the Oral Cavities of Older People: A Comparison between People Living in Their Own Homes and Those in Rest Homes
Keywords: oral candidiasis; older people; elderly; rest home; yeast carriage; Candida albicans

Abstract: Oral candidiasis is prevalent among older people due to predisposing factors such as impaired immune defenses, medications and denture use. An increasing number of older people live in rest home facilities and it is unclear how this institutionalized living affects the quantity and type of fungi colonizing these people’s oral cavities. Smears and swabs of the palate and tongue and saliva samples were taken from participants residing in rest homes (RH; n = 20) and older people living in their own...
homes (OH; n = 20). Yeast in samples were quantified and identified by culturing on CHROMagar Candida and sequencing the ITS2 region of rDNA. A higher proportion of RH residents had hyphae present in smears compared to OH participants (35% vs. 30%) although this difference was not statistically significant (p = 0.74). RH residents had, on average, 23 times as many yeast per mL saliva as OH participants (p = 0.01). Seven yeast species were identified in OH samples and only five in RH samples, with *Candida albicans* and *Candida glabrata* being the most common species isolated from both participant groups. The results indicate that older people living in aged-care facilities were more likely to have candidiasis and have a higher yeast carriage rate than similarly aged people living at home. This may be due to morbidities which led to the need for residential care and/or related to the rest home environment.
Appendix X: FASTA file of all *Candida albicans*/*Candida dubliniensis* chimera strains

The FASTA file below depicts the result of DNA sequencing using Multi Locus Sequence Typing of seven house keeping genes; H1-H7 for *C. albicans*/*C. dubliniensis* chimera strains ML3P1, MN2S4, MN2T4, MN2P1, MN5S1, MN5T1, MN5P1 and SL4T1. Each single nucleotide polymorphism (SNP) was colour coded blue (A or reference allele) and green (B or alternative allele). The colour turquoise indicates diploid sequence which appear as double peaks in the sequencing chromatogram.

>ML3P1-H1
TGATATTCCATTGGCTCCACCAGACAAGATTTTGGGTATTTCTGAAGCTTAT
AACAAAGATTTTGGGTATTTCTGAAGCTTAT
GATAATTCCGTTAAAATGTTTAACAAATTATTTTTTTCTCAGTTAAAAGCTGAAGAA
TTTTTATTGGGTAAAGAAACTGAAAAGGAATATACTGCCATTGTTGCTC
AAAATTTCATCAATTGGAAAAATTTCATTCATTAATAAAG
ATGCAATGTAACAAATTATGTGATAGTGAATTTGATTGAGAAAATTTCAT
GGTAGAATTGTTACTGCTCAAAA
CTATCTCTGGTACTGGATCACTTAGAGTTATC
GCTGATTCT
TTATTCTAACAAGAAATTTTTGTTTCAAAAACAACTTGGGCTAATCATGGTT
GCTGGTCTTCAA

>ML3P1-H2 – 1B:2A SNP ratio – aneuploid or triploid
CATATTGAGGGTCATCTGTTGTTCAAACCTCTTTATGTTGAAATTTTTGCTA
AACATAGAGAAACAAATTTGGAAAATTATCTGAGGTGTTGAATCTCCAAAC
ATATTGTCTTGGATGTTGCGCAAAATTTCAATTCAAACAGGACCACTGTTGCT
TGCCATTGCTGCCCCTGAAGTTATGTCAGACCTACTGGCTTATTGAT
TTGGTTAGTAGAATATCTGATATGACAGACTTCTATTGTTGAATGGG
AAATTCAAGTGGCATAATGGGAGC
YGGCTGGTGTGTAAGGATCGTCAACAG
GCTGCTGCTGCCGGTGGCGAATATCGACATCTGAACTACAGCTCTTCT
GTGCTGATTTGGACCTTTGTTGAGTTCTTTAACCAGGATCCCACAAAGA
CTGGGTTTTTAGCTCCAGCAAGACACTTGGATG

>ML3P1-H3
CAGGGTGAAGGTATTTTATTCT
AAAGCTCAAGGATACTCTCCCTCCCGAT
AATCCCAATGTTACCCCTTGTTAATTTTGGTAAATATTGGCTGTTG
TTTATTTTTACGTTGTTCCCTTCTTGAAGATAATATTAGTGAAGTC
GCCCTTGTTCAAGAAGTAATGGGCAACGGTPGATTCTAGGATGGAAC
AGACATCTGGACGCTTACGCTCTTGAAGTAAATATGAGGCTTTGCACT
CTGGGTAAGTAGTAGAATATCATGATGAAGACACTGCTATTGTTGAATGGG
AAATTCAAGTGGCATAATGGGAGC
YGGCTGGTGTGTAAGGATCGTCAACAG
GCTGCTGCTGCCGGTGGCGAATATCGACATCTGAACTACAGCTCTTCT
GTGCTGATTTGGACCTTTGTTGAGTTCTTTAACCAGGATCCCACAAAGA
CTGGGTTTTTAGCTCCAGCAAGACACTTGGATG
ATATTTTGCTGGTAAGACAAAGATGGTAAAAATCGATGGATCAATTTTATG
TGACCGGAAAATCCAAATAGCCAAAACACTACAGTCTATTATCGGGGT
>ML3P1-H4 – 1B:3A SNP ratio – aneuploid or tetraploid
TTGGCTAAAACACTTGTCCTGGAAATTGGAATTTATTGGGKCAAG
AATTAGGTGACGAATTTCGTATTGAATTTACACACAGAGGGATGGGAA GTCAAGATGATGTTAAACATAGAAAAATTTGGCAAAAAGTTGTTGGAAT
TAAAGAAACTCAGTATTGATTATATGACAAATTTCAAAAAACGAGTTGTGA T
AAAGAACAACGACAGAACCTCAAGTGTCAAAGGATTATTTTACTGATTAC
CGAGTATTAACAAAGGATAAAAACACAAACATTTCCTAATGACATCGGATTAT
TTTGTGAGTGTCTCTATTGAAACCCAGTGGGTGTTTGAACAAGGGGGAAGCAATGGTTCTTACGATGATTTATG
>ML3P1-H5 – ~1 part Candida dubliniensis and 3 parts Candida albicans
>ML3P1-H6
TGAGCTATACATGAAACCAAGACGTGGTGAAGGATCCATCGAATTCCAGA
AGAGTTTCAGTTTCAAGAAGAGGCTTAGTCAACATACGCTCC
GAGGTGATCTTTCAACATATTTCCAGATTTCTGATTATGAGCTTCTCGAAAT
CCAGAAGGTTTGTCTATTGGAAGAGATGATGCAAATTTTGCTG
GGTTTGAGCTTTTTGATTGTGGATATCGAATTGCTTGGATA
TGAAATACAAACCTTTGGGAAAGGACGACAAATTTACTGCT
ATGCTGAGGTACATATTGAGCATTATAATAATATTCAATTATTGACAGTAC
ATCTGGGAGCCATTTGGAAGATGTGGCCAAATAGCAGTTATGTGCAAAAT
ATCCCCACACCCAGGCCTAATTATAGGTACATTCTAGACAGGT
AGCAATAGTGAACCTTACATCCAAAGGCAGGTAGTCTGATCTACAGATTC
CGATTGATTACTTCCAGAGAAGATTTAAGCAAGGAGGTTGAAGATACCC
ATACGTATAATGAGACTGTITGAGATTTTGGAAGTGGATATGAC
AAAGTAACGAAAACAAACTGGAATTCAAATCATGGGATTCTAAACCTTG
GAGCTTTGAGGACTG
>ML3P1-H7 – mix of mostly Candida albicans and a bit Candida dubliniensis
>MN2S4-H1 - Candida dubliniensis retrotransposon
>MN2S4-H2 – Candida albicans
CATATTGGAGCTATCCTGTTTCAAACCTCTTATGTTGAATAATTTTGT
AACATAGAAGACAAATTTGGGAAATTATTCGTAAGGTGGTGTGATTCCAAAC
ATATTTTTTTTGTATGTGCAACATATTTCCTTAATACCAAGACATGGGTT
TGCCATTGCTGCGCCGCTGAAGATTATATGTCAGACGTCATTACACGGCTTATG
TGGGTGAATTGATATCTATCTTATGGAAGACAGCATTATTATTTGGAAGATGGA
AATTCCAAGGCTTAAATATGGAAGCTGTTGGAACAGATGCTCAACAGG
CTGCTGCTGCGTCGTGCAAGATGTAGACGTTCTACATCGATGGTAAGAACAAGGATGGTATGATTAATGGATCGATTTA
TGTTAATGGAAATCCAATAGACCCAAAACATTACAGATTTATCGGGGT
>MN2S4-H3 – Candida dubliniensis
TCAGGTGATGTATCCATAAAAGGTAATACACGTTACACTCCACAGAG
AACAAAGGTGATTTGGAGTAAATTTGGGTTGGAATGTTGTTGG
TTTGGTTTTTGGCATTTGTTCCTCTCCTTCTTGAAGAATATTAGTGCGTCGCT
GTTCAGAAAATTTGGGTATGACGCACTTTGAGATGGCATTGACTG
YCTCATGAA
TCAAAATTTTGAGGCTTACACACATTGTCGTTTGGAAGATATAAAGGTAGAGT
TGCTGTGGATCGAACAAATTGGAATGACTTGGTTGTGAAATACCAAGAG
AGAATGXYTTGGCAAGATTTGGGCGGGTGGTGCTGCTGAATACCTATT
GGATATTGGGCAGGAACAAAGGAGATGTGTATGATTTATTGAGATCTTATGGAATGGAACATGCTTATTAC
TGTTAATGGGAAATCCAATAGACCCAAAACATACAGATCTTATCGGGGT
>MN2S4-H4 – Candida dubliniensis

136
TTGGCTAAAAACTTTGACTACGTTCTCTGAATTAACGAAATTATTGGTCAG
AATTGGGTTAGTAAATTTGTTAGTTGTAATTAACACTACAGCAAAATTGGA
GTCAAGATGTTAACAATAGAAAATTGTTCAGATGAAATATTGATTTAGTTAT
TTGGTGGATGTCTTCTTGAAACGCTTGGCTGGAACAAAGGGGAAGCA
TAATTTACACACGCAAAGAGCCCTATCGATATATTATGGTGAATTAGTA
>TMN2S4-H5 – Candida dubliniensis
TGAATTTGAAACAAAGAATCAGATGCTGCTGCAAGTTACCATTTTGAGTTGTC
AAGGAATCTAAATCCAAAGGAATAGGGGTGCGTTAAAGAAATTATCATT
TCCGTATGTTGAAACAAGGTAATACGTTGAAAACTTCAAAATAGTGGAATAAA
TCAATCAGAGATATTGAGGCCTAGACAAAGAGGAACAAAGAAGAC
ATTGATGTACAAATGGGTTGATAAGATAAAATGTTCTATTTA
TTGGTTTCTCATGTTTCAATTACTGGTAATGCAAGGCAATTACGTA
TAACTTGTACAAAAGCAAGAAGAAATCAATTATTGTTGAGTTACCG
GCGAAACCCTGATGCTCATAATGGTGATTGTATGAGCTGTAAGGCTATG
CCAAGGGAATATGCAACGTAGTTAGCTAAAGGCTGTTACCTGGAACATTG
GTGGTAAACGTGGTG
>TMN2S4-H6 – impossible to read sequence
>MN2S4-H7 – Candida dubliniensis
GCTTTTGATAATATTGATGTTAATTGATGTTATATTGGGTCAATATACTAAAT
CAGAAGATGGTACAAACCAGGTTATTATGATAAACAGGTGAATGTCTCTG
ATTCTAAAGCTGTTACTTATGCTGCTTTTAAAGTAAACATTCTAAATAGAG
ATGGGATGGTGTTCCTAATTGTGTTTACGTTGCTGTAAGGCTTAGAAGATG
AAATGAAAAAATACAGAATTAGTTATATTATTCATATTCAATATAGAGCAAT
TATTTAAAAATTATTCAAAAATCCCGGGATTTCTACTGAAACTTTA
CTGATATTAGATTTAATCTCTGACTCTGATTTCAAAAAAGATTTTGAGATTCC
AGAAGCTTATGAAGCTTTAATTAGAGATTGTTATATTGGAATATCATTCTAA
TTGGTGAAGATGTTAATTGGAATTTTGCTGTTAAGGATATTTTAGCTCAATTAT
TAATAAGCAGTTGGAAGATCCTGCTTAAATAATAATGAAATACATATTATCTTCT
ATGGTTCAAAAGGCTTCAAAAGATTTAGATTTAAGAGAATAATATTGAAAGAATCATGGTT
ATATATTATGGTACCTCG
>TMN2T4-H1 – ~20% Candida dubliniensis retrotransposon
TGATATCCATTTGGCCACCCAGACAAATTTTTGGTATTCTGAAGCTTAT
AACAAGATCTCATAACCTCAAAGATTTGAGTTGCTTATCAGATAATTTCTG
ATTGAAATTTGGAAGATAGGAACTGAAGGAAATATTATCTGACATGGTTCCC
AAAAATTCTACCATCAAGTTAAAAATTCTTATTTCAAAATCTTCTAAATAAG
ATGGCAATGTTAAAACATTATATTGATGAGTTAGAATGGTACTCCTGCAAAA
CCATCTCTGATCTGATACITTAGATTATTGGTATTTTGATGGTACAATG
TTTATATTTTGGTACCTTCCAACAAACACTTGCTGCTTACATCGTGTTACATTCTCTGAA
>TMN2T4-H2 – Candida albicans
CATATTTTGAGTCATCATGTTTCAACACTCTTATGGAATTATTTGGCTA
AACATAGAAGAACAATATTGAGGAAATTACTCGTGAGTGTGTTGATACCAAC
ATATTGTGTGTTGAGTTGGCAATTTTCTTATCAATCCAGACCCATGGGT
TGCCATGCTGCCTGCTGAAGTTATGTGAGCTCATAACGGTCTTATTGAT
TTGGTACAAATTGAAATATCATGTTAATAGCAGACACTTCTGATGGAATAGGA
AATTCAAGTTGGCTAATATGGGAGC
C
GCTGGTGTAAACGATGCTCAACAGG
CTGCTGCTGC
GGTGGCGATGATTCGACATCT
ATGAAACATGCAGCTTCTGT
GTCTGATTTGACCTTTGTTGTTGATTCTAAAACCGAGCATTCCACAAGAACT
GGTGTTTTAGCTCCAGCAAGACACTTGGATG

> MN2T4-H2 – a second experiment amplified 2 parts Candida albicans and 1 part Candida dubliniensis
CATATTTTGAGRITCATCTGTGTTCAAAAACCTK
CATATGGGAATATTTGGCTAATATGGGAGC
CTGCTGCTGC
GGTGGCGATGATTCGACATCT
ATGAAACATGCAGCTTCTGT
GTCTGATTTGACCTTTGTTGTTGATTCTAAAACCGAGCATTCCACAAGAACT
GGTGTTTTAGCTCCAGCAAGACACTTGGATG

>Candida dubliniensis
> MN2T4-H3 – Candida dubliniensis
TCAGTGTTGATCTCCATAAAAAGTGAAATACCGGTTAATCTACATATCCAGGAG
AATCCAAATCTCATCCTTTGTAATTTTGTTGATATAATTGGGTCTTGTGTG
TTTGTTTTTGGGACAGTTGTCTCTCTCTGGAATAATATTGGTATCTGGCCTT
GGTCAAGAAAAATGGGTGATGCGATTGATTCAGCCTAAGACCTACATGCGG
TTGCTGTTGCTGACAAMTTTGGAATGTGCTTGGTATTTGGTGAATAACCGG
AATGGGTTGATGATTAACATAGAAAATTGTTACAAAAAGTGTTTGGTAAAT
TAATGAACACCAATGAAGATATTATAAAGCAACAACTACTAAATCTGG
AAGAATGYYTTGGCAAAATTTGGGTTGTTGCTGAAAAACTACGTTCAT
GGATATTGTGCCGGTAAAGAAAGATTGGAATTGTGATACGTTGCTGAAAAACCTAC
TTGCTGTTGCTGACAAMTTTGGAATGTGCTTGGTATTTGGTGAATAACCGG
AATGGGTTGATGATTAACATAGAAAATTGTTACAAAAAGTGTTTGGTAAAT
TAATGAACACCAATGAAGATATTATAAAGCAACAACTACTAAATCTGG
AAGAATGYYTTGGCAAAATTTGGGTTGTTGCTGAAAAACTACGTTCAT

>TGATTTTGGGACAGTTGTCTCTCTCTGGAATAATATTGGTATCTGGCCTT
GGTCAAGAAAAATGGGTGATGCGATTGATTCAGCCTAAGACCTACATGCGG
TTGCTGTTGCTGACAAMTTTGGAATGTGCTTGGTATTTGGTGAATAACCGG
AATGGGTTGATGATTAACATAGAAAATTGTTACAAAAAGTGTTTGGTAAAT
TAATGAACACCAATGAAGATATTATAAAGCAACAACTACTAAATCTGG
AAGAATGYYTTGGCAAAATTTGGGTTGTTGCTGAAAAACTACGTTCAT

> MN2T4-H4 - Candida dubliniensis – 2nd experiment also amplified ~10% Candida albicans
TTGGCTAAAAACCTTGACTACAGTTTCGTAATTTAACGGAAATATTGGTCGAAG
AATTGGTTGATGAAATTTGGTTAGTGTTGATTA
ACTACAGCAGAAATTTGGGA
GTCAAGATGTGATGTTAACAATAGAAAATTTGGTTACAAAAGTGTGGTTGAATAT
TAATGGGAAACCAATGAAGATATTATAAAGCAACAAACTACTAAATCTGG
AAGAACAAAGGAGGCAAAATTCGATTTGGTGAATAACCGG
AATGGGTTGATGATTAACATAGAAAATTGTTACAAAAAGTGTTTGGTAAAT
TAATGAACACCAATGAAGATATTATAAAGCAACAACTACTAAATCTGG
AAGAATGYYTTGGCAAAATTTGGGTTGTTGCTGAAAAACTACGTTCAT

>TGATTTTGGGACAGTTGTCTCTCTCTGGAATAATATTGGTATCTGGCCTT
GGTCAAGAAAAATGGGTGATGCGATTGATTCAGCCTAAGACCTACATGCGG
TTGCTGTTGCTGACAAMTTTGGAATGTGCTTGGTATTTGGTGAATAACCGG
AATGGGTTGATGATTAACATAGAAAATTGTTACAAAAAGTGTTTGGTAAAT
TAATGAACACCAATGAAGATATTATAAAGCAACAACTACTAAATCTGG
AAGAATGYYTTGGCAAAATTTGGGTTGTTGCTGAAAAACTACGTTCAT

> MN2T4-H5 – Candida dubliniensis
TGAAATTGAACAAAGAATCGATGCTGTCCAAGTTACCTTTTTATTGGGTGTTTGCT
AACAAATCAATTGGAATTTAGGGGTCTTTGGAATTTTGAGTATAGTTTTGATAAT
TCCATCHGTGTTAAACAAAGGTTAAGGCTTTACAAAAGTGTGGTTGTTGTTGTT
TTGCTGTTGCTGACAAMTTTGGAATGTGCTTGGTATTTGGTGAATAACCGG
AATGGGTTGATGATTAACATAGAAAATTGTTACAAAAAGTGTTTGGTAAAT
TAATGAACACCAATGAAGATATTATAAAGCAACAACTACTAAATCTGG
AAGAATGYYTTGGCAAAATTTGGGTTGTTGCTGAAAAACTACGTTCAT

>TGATTTTGGGACAGTTGTCTCTCTCTGGAATAATATTGGTATCTGGCCTT
GGTCAAGAAAAATGGGTGATGCGATTGATTCAGCCTAAGACCTACATGCGG
TTGCTGTTGCTGACAAMTTTGGAATGTGCTTGGTATTTGGTGAATAACCGG
AATGGGTTGATGATTAACATAGAAAATTGTTACAAAAAGTGTTTGGTAAAT
TAATGAACACCAATGAAGATATTATAAAGCAACAACTACTAAATCTGG
AAGAATGYYTTGGCAAAATTTGGGTTGTTGCTGAAAAACTACGTTCAT

> MN2T4-H6 – Candida dubliniensis ~20% bad background sequence
CGACTTTGACATGAAATCGATGCTGTCCAAGTTACCTTTTTATTGGGTGTTTGCT
AACAAATCAATTGGAATTTAGGGGTCTTTGGAATTTTGAGTATAGTTTTGATAAT
TCCATCHGTGTTAAACAAAGGTTAAGGCTTTACAAAAGTGTGGTTGTTGTTGTT
TTGCTGTTGCTGACAAMTTTGGAATGTGCTTGGTATTTGGTGAATAACCGG
AATGGGTTGATGATTAACATAGAAAATTGTTACAAAAAGTGTTTGGTAAAT
TAATGAACACCAATGAAGATATTATAAAGCAACAACTACTAAATCTGG
AAGAATGYYTTGGCAAAATTTGGGTTGTTGCTGAAAAACTACGTTCAT

> MN2T4-H6 – Candida dubliniensis ~20% bad background sequence
CCACCAGGTGGTTGCTATTTGAAAAAGGTAAGTTGCAATCGCATTCTTGGT
GGTTGAGAATTTTGTTTGAGTTTGTGTGCTTGCTTGAATAT
GCAATACATTTTTGGAACCGCAATCCATGGTGCCACAGATCT
TGCTGGAATTTGCTAATTTGAAATATATTATTTTCAATTATG
TCTTGGGAACCTTTGTTGGGAAAGCTGGCAATACGTTAGTCAAA
GCTGGCAGACCACCAGACTTATTATAGTGAAAAATATTTCCAGAACAAG
GCTCAGATGACGTCGATTTGCTGATTGCCATTAGACTCAGATTTCA
GACTTTTGACGTTTATTTCCAGATATTCTGAGATGGACTCCCTCAGAATT
CCAGAAGGTGTGCTATTGTGAAGAGATGAATGCCAATTTTGGT
GGTTTGAGAGTTTTGATTGGTGATGTATCTGAGTTGCCTGTCCTTGATA
TGAAAATCAAACCTTTTGAAGCACGAGCAATTAACTGGTC
>MN2T4-H6 – 2nd experiment amplified a Candida albicans allele
TGAGCTATAACATGAAGCGACCAAGTGTTGAGAGATGATCACTGAATTCAGA
AGAGTTTCGATTTTCTAAAGGAAAGGCTCTTCAAACATACGCTCC
GACTGTCTTTCAACATTTCCAGATATTCTGAGATGGACTCCCTCAGAATT
CCAGAAGGTGTGCTATTGTGAAGAGATGAATGCCAATTTTGGT
GGTTTGAGAGTTTTGATTGGTGATGTATCTGAGTTGCCTGTCCTTGATA
TGAAAATCAAACCTTTTGAAGCACGAGCAATTAACTGGTC
ACGCTGGAATTTGCAATTATAGGCAATATTAAATATATTCAATTATG
CAGGACACCAACCAACCTCAATTGAGAGCTGAAATGCAATT
AGGTTTGACGTTTATTTCCAGATATTCTGAGATGGACTCCCTCAGAATT
CCAGAAGGTGTGCTATTGTGAAGAGATGAATGCCAATTTTGGT
GGTTTGAGAGTTTTGATTGGTGATGTATCTGAGTTGCCTGTCCTTGATA
TGAAAATCAAACCTTTTGAAGCACGAGCAATTAACTGGTC
>MN2T4-H7 – Candida dubliniensis
GCTTTTGATAAATATGGATTGAATTGATATTCTGCTCATAATACTAAAT
CAGAAAGATGGTACAAACCAGGTTATTAGTAGGATAAAAAACGGTGAAATCCTG
ATTCTAAAGCTGGTAACTTATTGGCTTTTAGAGTAAACCATATAATGAAAG
ATGGGAGAGGCTTCATGGAATTTGCTGATTGCCATTAGACTCAGATTTCA
GACTTTTGACGTTTATTTCCAGATATTCTGAGATGGACTCCCTCAGAATT
CCAGAAGGTGTGCTATTGTGAAGAGATGAATGCCAATTTTGGT
GGTTTGAGAGTTTTGATTGGTGATGTATCTGAGTTGCCTGTCCTTGATA
TGAAAATCAAACCTTTTGAAGCACGAGCAATTAACTGGTC
>MN2P1-H1 - ~20% Candida dubliniensis retrotransposon
TGATATTCCATTGGCCACACCAGACCAACATTGGATTTTCTGAAAGTTAT
AACAAAGATCTACACCTAAACAAATCTCATTGGGTGTTGTGCTTATAGA
GATAATCTGAGGTAAACATTGTTATTTTCCAAATTGGTAAAAAAGCCTGAAAGA
ATTCTATTGGGAAAGAAGACTGAAAAGATAACTGGCATTGGTGTCTCC
AAAAATTTCCAATCTATTTGGGAAATTTCATTTTCCAAATCATTAAATG
ATGCCAAGATTGAAACATTATGATGAGTTTGATAGGAATTTCTGCTCAAA
CCATCTCTGTTGACTGAGCTACTGATATTAGTATTAGCTGAGTTATGGT
TATCTTAAACAGAAAAATTTGGCTCACAACCAGATGTCGATTTGTTG
>
AGAAGCTTATGAAGCTTTAATTAGAGATTGTTATTTAGGTAATCATTCTAAT
TTTGTTAGAGATGATGAATTGGATGT
TTCTTGGAAATTATTTACTCCATTAT
TAAAAGCAGTTGAAGATCCTGCTAATAAAATAGAATTACAATATTATCTT
ATGGTTCAAAAGGTTPCCCAAAAGATTATTTGGAAGATCATGGTT
ATATATTATGTTGATCCGGT

>MN5S1-H1 - mix of Candida albicans H1 and Candida dubliniensis retrotransposon
>CATATTTTGGAGCTATCCTGTGTTCAACACCTCTKAATGGTTGAATTATTTTGCTA
AACATAGAAGAAATTGGAATTATCTGGAGAGTTGTGTTGATCCAAAC
YATTGTTTTTTGATGTTGGCACAATTTTATCTAATCCAGAGYCCATGGGT
TGCCATTGCTGCMGCTGAAGTKTATGTCAGACGTCATACCGTTATTTGTA
TTGGGTAATATTGAAATATCATGAAAYGACAGCCTTCTTTATGGTAGG
AAATTCAAGTTCATARTATGGGAGCAGCTGGAAGTAGCTCAAACAGGTCTCGT
YGGTTGAATGAYTCCATACATGATGAAACACTGGCWTCT
CAGTGTCTATGTCACCTXTTGGTGATTTC

>MN5S1-H2 - 1:1 SNP ratios for C albicans and C dubliniensis
CATATTTTGGAGCTATCCTGTGTTCAACACCTCTKAATGGTTGAATTATTTTGCTA
AACATAGAAGAAATTGGAATTATCTGGAGAGTTGTGTTGATCCAAAC
YATTGTTTTTTGATGTTGGCACAATTTTATCTAATCCAGAGYCCATGGGT
TGCCATTGCTGCMGCTGAAGTKTATGTCAGACGTCATACCGTTATTTGTA
TTGGGTAATATTGAAATATCATGAAAYGACAGCCTTCTTTATGGTAGG
AAATTCAAGTTCATARTATGGGAGCAGCTGGAAGTAGCTCAAACAGGTCTCGT
YGGTTGAATGAYTCCATACATGATGAAACACTGGCWTCT
CAGTGTCTATGTCACCTXTTGGTGATTTC

>MN5S1-H3 – Candida dubliniensis
TCAGGTGAGTGATGACTCATCAATAAAAGTGAAATTACACGTACACTATGCCAG
ATCCAAAGTGCTCCATCTTGGTAATTTGTTGATATTTTGGGCTTGTGGTG
TTTGTGGTTTTGCGATCTCTCTCTTGGAGAAATATTAGTGAGCTGCGCTT
GCTCAAGAAAAATGGGTATAGGCGCCATGCTGATCATCGAACATCCAACTG
TGCTGAGTGTCCAAATTGGAATGAGCTTCTTTGATTTGTGTTGAAACCAAGAG
AGAATGTTGCAATAATGTTGGGCTGCTTGCTGTCGAAAATACGTATTAT
GGATATTGTTGCGTAAAGAAGATGTTGATATTGAGAGTAGCTCAAACCC

>MN5S1-H4 - Candida dubliniensis
TTGGCTCAAACCTTTGGACTACAGTTCCTGGAATTAACAGAAAATATTGGTAGCAG
AATTTGTTGATGAAATTGTGTTGATATTTAAGACAGTACCAGGAAATTGGA
GCTAAGATGATGTTAAATAGAAATGTGGTATGGATATAT
TAAGAAGAACTCCITAAGAGATATTAAAGAAGAAACTACAAATACCTGG
AAAAGAGAGAAATGGGAACTCTAAAGGTGTTATAGGGATAGCTGAAATCCAA
CAGAGTTAATACAAATTGGAATGAGCTTCTTTGATTTGTGTTGAAACCAAGAG
AGAATGTTGCAATAATGTTGGGCTGCTTGCTGTCGAAAATACGTATTAT
GGATATTGTTGCGTAAAGAAGATGTTGATATTGAGAGTAGCTCAAACCC

>MN5S1-H5 - Candida dubliniensis
TGAGCTTACATGAAAGCACCAGAGTTGTTGATGTTTTCCTTGGAAATTTTACTCAATTAT
TTCCGATAGTTGAAACAAAGATGATATACTGAAAATTTTGGAAATTTAGGATAA
TCAATCCAAAGATGAAATGCTGACTCAGACAAAAAGAGAAACAAAGAAGACAT
TGGATGTTAACAATTTGTTGGAATGATAAAAGAAATGTCTTCATCAATTAT
TTGCTATCAACTTTGCGAATTTACTCGTAAATGCAAACAGGCAATTTGGAAGTCA
TTAACTCTGATACAAAGAAAAGCAAACTCATAATTTTATTATGTCAGCAGCG
GCCAAACGATATAAAGCTGCTCAGATGTTGATTTGTGTTGAAAGATTTGAGTCA
CCAAAGGGAATATGCTGACCTGACTGATATTGACGAGGACG

>MN5S1-H6 - three of six have 1:2 SNP ratio – tri- or tetraploid
TGAGCTTACATGAAAGCACCAGAGTTGTTGATGTTTTCCTTGGAAATTTTACTCAATTAT
TTCCGATAGTTGAAACAAAGATGATATACTGAAAATTTTGGAAATTTAGGATAA
TCAATCCAAAGATGAAATGCTGACTCAGACAAAAAGAGAAACAAAGAAGACAT
TGGATGTTAACAATTTGTTGGAATGATAAAAGAAATGTCTTCATCAATTAT
TTGCTATCAACTTTGCGAATTTACTCGTAAATGCAAACAGGCAATTTGGAAGTCA
TTAACTCTGATACAAAGAAAAGCAAACTCATAATTTTATTATGTCAGCAGCG
GCCAAACGATATAAAGCTGCTCAGATGTTGATTTGTGTTGAAAGATTTGAGTCA
CCAAAGGGAATATGCTGACCTGACTGATATTGACGAGGACG

>MN5S1-H6 - three of six have 1:2 SNP ratio – tri- or tetraploid
TGAGCTTACATGAAAGCACCAGAGTTGTTGATGTTTTCCTTGGAAATTTTACTCAATTAT
TTCCGATAGTTGAAACAAAGATGATATACTGAAAATTTTGGAAATTTAGGATAA
TCAATCCAAAGATGAAATGCTGACTCAGACAAAAAGAGAAACAAAGAAGACAT
TGGATGTTAACAATTTGTTGGAATGATAAAAGAAATGTCTTCATCAATTAT
TTGCTATCAACTTTGCGAATTTACTCGTAAATGCAAACAGGCAATTTGGAAGTCA
TTAACTCTGATACAAAGAAAAGCAAACTCATAATTTTATTATGTCAGCAGCG
GCCAAACGATATAAAGCTGCTCAGATGTTGATTTGTGTTGAAAGATTTGAGTCA
CCAAAGGGAATATGCTGACCTGACTGATATTGACGAGGACG

141
CCAGAAGGTGTGGCTATTGTGAAAGGTGAAGAGATGAATGCCAATTTTGGTTGGTTTGAG
R
TTTGTTTTGATTGGTGATGTATCAGAATTGCCTGTCCTTGATA
TGAAAATCAACCTTTTGAAGCACGAGCAATTAACTGAGCTGAC
AGTCAAGTGCTATATGAGCAATATATAAATATTTCAATTATGAATCTGC
CTTCCGACACCACAAGCCTCAATTAGTAGAGGTGATTTCTAGACAGGT
TC
AGGATCTTA
ATGCTGAGGTACATATTGAGCATTATATAAATATATTCAATTATGCACGATC
ATCTTGGGAGCCATTTGGAATTTGGCCAATACGAGCTATGTACA
AAATCCCAAGAATGAAATAGTTATATAGAATATGCTGGAAATTTGGAATGAGTC
AAAGWAATCCGAAACCACACTTGGAAATCAATCATGGGATTTACCT
GGAGCTTTGAGAATCT

> MN5S1-H7 - Candida dubliniensis
GCTTTTGATAATATTGATTTAATGATGTTATATTGGCTAAATATACTAAATCAGAAGATGGTAC
C
AAACCAGGTTATTTAGATGATAAAACGGTGAAACTG
ATTCTAAAGCTGTACTTATGCTGTTTTAGATGAAACATTCAATAATGAAAG
ATGGGATGGTGTTCCAATTTTGTTACGTGGTTGAAGCTTATGGAAGATG
AAAGTGGAAATTAGAATTCAATTAAACCTGGAAATGATTTACCT
TTAAGAGCTTTGAGAGATCTTGCTAATAAAAGATTATAGAATATGCTGGAAATTTGGAATGAGTC
AAAGWAATCCGAAACCACACTTGGAAATCAATCATGGGATTTACCT

> MN5T1-H1 - mix of Candida albicans H1 and Candida dubliniensis retrotransponson
> MN5T1-H2 - 2:1 SNP ratios for C albicans and C dubliniensis
CATATTTTGAGRCTCATTGTTGTTCAACACCTCK
TATGGTGAATTTTTTGCTAAACATAGAGATACCAGGTTACACTATCCCAGAG
AATCCAAAGTTCACTCTTTGGTGTGTTTGTGTTTTGGCAGTTGTCTCTCTTGAGAAATATTAGTGAGTCGGCCTT
GTTCAAGAAAAATGGGTATGAGCCATTGGAATTCAATAGACCCAAAACATTACAGATCTATTATCGGGT

> MN5T1-H3 - Candida dubliniensis
TCAGGTAGGTGATCCATCACATATCCCAAGAGATACCATTTTTGGAATACATTGACAGGTGAGGAGTTAACAACTCA
AATCCAAAGTTCACTCTTTGGTGTGTTTGTGTTTTGGCAGTTGTCTCTCTTGAGAAATATTAGTGAGTCGGCCTT
GTTCAAGAAAAATGGGTATGAGCCATTGGAATTCAATAGACCCAAAACATTACAGATCTATTATCGGGT

> MN5T1-H4 - Candida dubliniensis
TTGGCTAAAACTTTTGACTACAGTTCTGTGAAATACAGGAATTTGGTGCTAACAGGATCTTTTGGTGTGTTTGTGTTTTGGCAGTTGTCTCTCTTGAGAAATATTAGTGAGTCGGCCTT
GTTCAAGAAAAATGGGTATGAGCCATTGGAATTCAATAGACCCAAAACATTACAGATCTATTATCGGGT

142
TAATGAACACCAATGAAGATATTATATAGGAAAGCACAACACTCTAAAATTACTTG
AAAGAACAGAAAGGGAACCTCAAGTGTTTAAAAACATTGATTCTAGATTAC
TGGTTGAGATGCTCTTGGTAACACGTGTTGCTAACAAAGGGGAAGGCRA
TGTGTTTACAAAGCAAGACCCTCTCAGATATTATGGAATTATGTTGATATTAG

>MN5T1-H5 - Candida dubliniensis and ~10% Candida albicans
TGAATTTGAAACAAAAATCAGTGTGCTGCTGGAATTTTACCATTGTGTTGTC
AAGGATCTAAATCAGAATTTGGTGGTCGTTAAGGAATTATCATTCAATT
TCCGTTAGTGAAGAAAAGAGATATTACGAAATTTGCAAAATTTGGAATTAA
TCAAATCAAGGATAATTGGAAGGCTAGACAAAAAGAGGAAACAAAGAGAC
ATTGAGTGTAGTTAAATTTGGAAGTATAGGAAGAAATTTTGCTCAATT
TTGTTCACTTTGCTATTTGCAAATATCTGTAAGCTGACTAAGCAATATT
GCAAAACGATAAAGTTTGCAGATATTGTGAGAAGACTGCTGAAAGCCTG
GTTTGGCAATTATTAGGTAATATTGGTATACAGAATTGTTTCAATTCTTGTA
GAAAATCAAAAACTTTTTGAAAGCCACGGCAATTAACCTGGTCACGGATCTTA
ATGCTGTAGTACATATTTGAGCATTATATAACATATTTCAATTATGACAGTC
ACCTTGAGGACCCCTATGGAACAGATTTTGAAGAAGGTGCAATTGCAAATTG
TTTGTCTGCAGTTCAATTTGCAGATATTGGTATACAGAATTGTTTCAATTCTTG
GATAGCTGAAACCAACACTTTGAAAGCACAACACTCTAAAATTACTTG
AAAGAACAGAAAGGGAACCTCAAGTGTTTAAAAACATTGATTCTAGATTAC
TGGTTGAGATGCTCTTGGTAACACGTGTTGCTAACAAAGGGGAAGGCRA
TGTGTTTACAAAGCAAGACCCTCTCAGATATTATGGAATTATGTTGATATTAG

>MN5T1-H6 – three unique SNPs all in the 1st position have ~1:2 ratios with the
typical SNP twice as high – tri or tetraploid
TGAGCTTACATGAACACCAACAGTTGTTAGAGGATCATCGAATTCAGA
AGATTTGCAATTTTCAAAAAGAGGCTCTTCACAAYACGCTCC
GAGTGTACCTTTCAACATATTGGTTAGATTTGATACAGTCCCTCAGAAT
CCAGAAGGTTGCAACATTGCAAGGTGTAAGATGCAATGCAATTGTTG
GTTGAGCATTGTTCCTTTGATTTGGAAGTTCATATGAAAGTGAATTAG

>MN5T1-H7 – Candida dubliniensis
GCTTTTGATATAATTTGAGTTAGTTATTTTGCAATATATATCAAT
CAGAAAGTGTGACCAACCCGTTATTTGTTGAAATATAGCAAAGGTGGAAT
ATTCTAAGGCTTTATTGACTCTTATTGCAAGTAAACATCTTAAATAGGAAG
ATGGGATGTTGATCCTACCAATTTGTGTAAGAATGTCAGATATAATGGA
AAATTTGAAATAGAATTCAATTTTAAACCTTGGTAACAAAGGAATTCTA
GAAATCCAAAGAATGAAATGTGTTAATGAAACAAACAGATATCAAT
TATTTAAAAATATTTTCAACGTTCCAGTTTCTGAATCTTCAAT
CTGATTTGAGATTACATTGCTACTGTTATTTGCAAGATTTTTGGATTC
AGAAGGCTTTATGGAACATTTTATGTTTATGGAATAATTATTACCATTAT
TAAAACGATTTGGAAGATCTCCTACAAATATAAATGAAATCAATATTTTCA
ATGTTCCAACAGCTAAAGATTTAAGAAATATTGGAAGAATCATGTT
ATATATTGATGATACCT

>MN5P1-H1 - ~20% Candida dubliniensis retrotransposon
TGATATTCCATGGGTCGACACGAGAATTTGTTGGTAATTCTGAAGCATTAT
AACAAATGATTTCAACCCCTAACAAACTTCAAATTTGGGTTGCTATAGA
GATAATTYGGTAACAAAATTATTATTTTCCCCATCGTAAAAAGCTGAAAGA
ATTTTATTTGGGTAAAGAAACTGAAAAGGAATATACTGCCATTGTTGTTCC
AAAAATTTCCAATCAATTGTGAAAAATTTCATTTTCAACAATTCTAATAAAG
ATGCCAATGGTAAACAATTAATTGATGATGGTAGAATTGTTACTGCTCAAA
CCATCTCTGGTACTGGGACTTTAGAGTTAT
Y
GCTGATT
T
Y
TTGAAT
C
GTTT
Y
TATTCTAAAAGAAATTTTTGCTTCCAAAACCAACTTGGCTAATATCGTT
GCTGTTTCAAAA

>MN5P1-H2 - 2:1 SNP ratios for *C. albicans* and *C. dubliniensis*
CATATTTTGAGRTCACTCTGGTTCTAAACCTCOKTATGGGAAAATTTTGCTA
AACATAGACAATTTGGATTTATTTTCCGTRGGTTGATTTCCCAAAC
AYATGTTTTTGTATGTTGKCAACATTYTRATCAATTCCAAYCATGTTG
TGCCATTGGCTGMCCTGAAGTKTATGGCAACGGCTACATTATGTAGT
TTGGGTAAATTAATATCATATGTTAAAYGACAGCTCCTAATTTGGAAGT
AAATTCAAGTTRCTARRATAGGGGAGDCTGGWGTAAAYGATGCTCAACA
GGCTGCTCYTGCYYGTTGGYAGTAYTCCATCWTGAAATCACTGWCCT
CWTGKTCTGATTCCACTTTTGTGTTGATTCYAAAACCGAGCATWCYACAA
GAACCTGGTGKYTTAGCTCCYMCAGACACTTGGGATG

>MN5P1-H3 – Candida dubliniensis
TCAGGTGATGTAATCCATAAAAGTGAAATCCAGTTACACTATCCACAG
AATCCAAAATTTCCACTTCTTGTAATTTTGTTGATTTGGGCTTTGTTGG
TTTGTTTTTGGCAGTTTGCCTCTCTCTTGGAAGAAATATTAGTGAGTCGGCCT
GTTCAGAAGAAAATGGGTATGAGCCATTGGATTCAGATCCTAG

>MN5P1-H4 - Candida dubliniensis
TTGGCTAAAACCTTGACTACAGTTCTCGAATTAACAGAATAATTGTCGAAG
ATTGGTTGATGAATTTGGTTAGTGGTATTAARACTACCAGCAGAAATTTGGAAG
GTCAAGATGTTGTTAAACATAGAAATTTGTATTAAAAGTGTTTGGTAAAT
TAATGAAACCCAATGGAAGATATTTATAAAAGCAACAAACACTACTAAATTTG
AAAAGACAGAAGGGAACCCTCAAGTGTTTTAAAAACATTGATTCTAGATAC
CAGAGTTATACAAACAACAGGCAATTTCCATGATATTTGGAATTAT
TTTGTGAGTGCCTCTTTGTGTAACACCACTGTGCTTGGAAACAGGGGGAAG
GTAAGCCTAACAGCCAAAGACCTCCTACATGCAATATTAGTGTTGATATATTGA

>MN5P1-H5 - Candida dubliniensis and ~15% Candida albicans
TGAATTTGAAACAGAATGCTAGTGCTGCGAATTTTATTGTTGTTTGC
AAGGAATCTAAATCCAGGAAATAGGATTGCTTAAAAAGAAATTTATCATT
TTCCGTTATGGGATAAAACAGTTAATCTGAAAAATTCGAAAAATTGGATAAA
TCAATCAGAAATTTGGAAAGGCTAGACAAAGAGGAAGAAAACAGAG
ATTGAGTGATTAAACATATTGGGAAATGATAAAGAAATTTGCTCATATT
TTGGTCTCTAGTTCACATTACTGCTAATTCGAAGCCAAATTTGGAAGCA
TTAACACTGATCAGAAGGCAAAATCTAAATTTATTATTGTTGANG
GCCTAGAACAAAAAGGTGCTGCTGATGTTACAACTGAACTGCTAC
GAGTTGACTTCAAAATTTGCTTCCAAAAACCAACTTGGGCTAATTGAG

>MN5P1-H6 - 1:2 SNP ratios – aneuploid or tri- or tetraploid
TGAGCTATACATGAGACAAACCAGKTTTTGTTAGAGGAATRACATCGAATTGCAG
AGAGTTTACGTTTTTCAAAAGAATTCCAAAGAAGGCTTTTCAAAATACGTCC
GAGGTACATTCTCAACTATTTCCAGATATCTGATTATTGAGRCATCTCCGAATT
TCAAAACTTTGAGCTACAAACATTGCTGTTTTGAAGATATAAGTGAGGTT
TGCTGGAGTGTCCACAAGTTTGTGAAATGAGCTTGTTTGTGAAAACCCAG
AGAAATTGTTGCATTATGGGAGGTTCCGGTGCTGTTGAAACCTACGTATATT
GGATATTGTTGCAATATTGAAAAGAAATGGGATAGTATTTTGAATTAATTTG
TAATAGAACTAAGAGATATATTATGAAAGAGCTACTAAATTACTTG
AAAGAACAGAAGGGAACCTCAAGGTGTTGAAACATGTGAGTTATCT
AGAGTTTAAATCAGAAGAAGCCTGAAAAATTTTGAATTAGAATTCAATTTG
CCAGAAACGAAATGTGTTGAAATGAAAACAGTGCATTTTTGCATATATAT
TCTGGGCATAACTCTTCTGAAATACGGAAATTTGTTGTTTGCT
AAGGAACTCAAATCAGGAAAGTTGGTTGCTTGAAGAATATATCAATT
TCCGTTATGGATATAAACAGATTAAGCTAAAATCTAATTTGATGAAACGCT
AAGAATTTGATGTTAAGTATGTTATATTG
CAGAAGATGGTACCAACCAGGTTATTTAGATGATAAAAC
RGTGAATCCTGATTTAAAGCTGTTACTTATGCTCTTTAGTAAACATTCATAATGAG
AAAGAATTGAAATTAGAATTCAATTTAAACCTGTTGCTAAAGGAATGTTTAAA
GAAATTCAAAGAAATGAATTAGTTATTAGAATACAACCAAATGAAGCAATT
TATTTAAAAATTAATCACTGGGATTTCTACTGAAACTTCTTTAA
CTGATTTAGATTTAACTTATGCTACTCGTTATTCAAAAGATTTTGGATTCC
AGAAGCTTATGAAGCTTTAATTAGGAGGTTATTTAGGTAATCATTCTAAT

>SL4T1-H4 – Candida dubliniensis
TTGGCTAAAACCTTCTCAGTTACTAGTCTCCTGAAATACGGAAATTTGTTGTTGCT
AAGGAACTCAAATCAGGAAAGTTGGTTGCTTGAAGAATATATCAATT
TCCGTTATGGATATAAACAGATTAAGCTAAAATCTAATTTGATGAAACGCT
AAGAATTTGATGTTAAGTATGTTATATTG
CAGAAGATGGTACCAACCAGGTTATTTAGATGATAAAAC
RGTGAATCCTGATTTAAAGCTGTTACTTATGCTCTTTAGTAAACATTCATAATGAG
AAAGAATTGAAATTAGAATTCAATTTAAACCTGTTGCTAAAGGAATGTTTAAA
GAAATTCAAAGAAATGAATTAGTTATTAGAATACAACCAAATGAAGCAATT
TATTTAAAAATTAATCACTGGGATTTCTACTGAAACTTCTTTAA
CTGATTTAGATTTAACTTATGCTACTCGTTATTCAAAAGATTTTGGATTCC
AGAAGCTTATGAAGCTTTAATTAGGAGGTTATTTAGGTAATCATTCTAAT

>SL4T1-H5 – Candida dubliniensis plus ~10% Candida albicans
TGAATTGGAAACAGAATGAGGATGGTACCAACCAGGTTATTTAGATGATAAAAC
RGTGAATCCTGATTTAAAGCTGTTACTTATGCTCTTTAGTAAACATTCATAATGAG
AAAGAATTGAAATTAGAATTCAATTTAAACCTGTTGCTAAAGGAATGTTTAAA
GAAATTCAAAGAAATGAATTAGTTATTAGAATACAACCAAATGAAGCAATT
TATTTAAAAATTAATCACTGGGATTTCTACTGAAACTTCTTTAA
CTGATTTAGATTTAACTTATGCTACTCGTTATTCAAAAGATTTTGGATTCC
AGAAGCTTATGAAGCTTTAATTAGGAGGTTATTTAGGTAATCATTCTAAT

>SL4T1-H6
TGAGCTTATACATGAAGCACCAGCAAGCTTTTGAGAGGATGATCGAATTCAG
AGAGTTTTCAATTTCAAAGACTTCAATATCAGATTTCCAGGCTACAT
TCCTCAGAATTCCAGAAGTGTGTTGAAATGAAAACAGTGCATTTTTGCATATATAT
TCTGGGCATAACTCTTCTGAAATACGGAAATTTGTTGTTGCT
AAGGAACTCAAATCAGGAAAGTTGGTTGCTTGAAGAATATATCAATT
TCCGTTATGGATATAAACAGATTAAGCTAAAATCTAATTTGATGAAACGCT
AAGAATTTGATGTTAAGTATGTTATATTG
CAGAAGATGGTACCAACCAGGTTATTTAGATGATAAAAC
RGTGAATCCTGATTTAAAGCTGTTACTTATGCTCTTTAGTAAACATTCATAATGAG
AAAGAATTGAAATTAGAATTCAATTTAAACCTGTTGCTAAAGGAATGTTTAAA
GAAATTCAAAGAAATGAATTAGTTATTAGAATACAACCAAATGAAGCAATT
TATTTAAAAATTAATCACTGGGATTTCTACTGAAACTTCTTTAA
CTGATTTAGATTTAACTTATGCTACTCGTTATTCAAAAGATTTTGGATTCC
AGAAGCTTATGAAGCTTTAATTAGGAGGTTATTTAGGTAATCATTCTAAT

>SL4T1-H7 – Candida dubliniensis
GCTTTTGAAAATATTGTGTTAATGATGTTATTTGGGCTAAATATACTAAAT
CAGAAGATGGTACCAACCAGGTTTTATTAGATGATAAAAC
RGTGAATCCTGCTTTTATGAGTAAACATTCAATATGAAAG
ATGAGGATTGGTCTAACATTTTCCTGCTGTTGAAACGCTTATGAAATT
AAGTGGAAAATAGAATTCTAATTGAAACCTGTTGCTAAGGAATGTTTAAA
GAAATTCAAAAGAATGTGTTGAAATGAAAACAGTGCATTTTTGCATATATAT
TCTGGGCATAACTCTTCTGAAATACGGAAATTTGTTGTTGCT
AAGGAACTCAAATCAGGAAAGTTGGTTGCTTGAAGAATATATCAATT
TCCGTTATGGATATAAACAGATTAAGCTAAAATCTAATTTGATGAAACGCT
AAGAATTTGATGTTAAGTATGTTATATTG
CAGAAGATGGTACCAACCAGGTTATTTAGATGATAAAAC
RGTGAATCCTGATTTAAAGCTGTTACTTATGCTCTTTAGTAAACATTCATAATGAG
AAAGAATTGAAATTAGAATTCAATTTAAACCTGTTGCTAAAGGAATGTTTAAA
GAAATTCAAAGAAATGAATTAGTTATTAGAATACAACCAAATGAAGCAATT
TATTTAAAAATTAATCACTGGGATTTCTACTGAAACTTCTTTAA
CTGATTTAGATTTAACTTATGCTACTCGTTATTCAAAAGATTTTGGATTCC
AGAAGCTTATGAAGCTTTAATTAGGAGGTTATTTAGGTAATCATTCTAAT
TTTGTAGAGATGATGAAATGGGATTCTTTGGAAAAATTATTTACTCCATTAT
AAAAGCTAGTTGAAGATTCTGCTATATCAATAGAAATTACAATATTCCTT
ATGGTCAAAGGTTCTCAAAAAGATTTAAGAAAATATTTGAAAGATCATGTT
ATATATTTAGTGATCCAGGT
Appendix XI: Research Publication

Yeast Species in the Oral Cavities of Older People: A Comparison between People Living in Their Own Homes and Those in Rest Homes

Nurulhuda Mohd Thiyahuddin, Erwin Lamping, Alison M. Rich and Richard D. Cannon

Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin 9054, New Zealand; nurul.thiyahuddin@otago.ac.nz (N.M.T.); erwin.lamping@otago.ac.nz (E.L.); alison.rich@otago.ac.nz (A.M.R.)
* Correspondence: richard.cannon@otago.ac.nz; Tel.: +64-3-479-7081

Received: 26 February 2019; Accepted: 9 April 2019; Published: 12 April 2019

Abstract: Oral candidiasis is prevalent among older people due to predisposing factors such as impaired immune defenses, medications and denture use. An increasing number of older people live in rest home facilities and it is unclear how this institutionalized living affects the quantity and type of fungi colonizing these people’s oral cavities. Smears and swabs of the palate and tongue and saliva samples were taken from participants residing in rest homes (RH, n = 20) and older people living in their own homes (OH, n = 20). Yeast in samples were quantified and identified by culturing on CHROMagar Candida and sequencing the ITS2 region of rDNA. A higher proportion of RH residents had Candida hyphae present in smears compared to OH participants (35% vs. 30%) although this difference was not statistically significant (p = 0.74). RH residents had, on average, 23 times as many yeast per mL saliva as OH participants (p = 0.01). Seven yeast species were identified in OH samples and only five in RH samples, with Candida albicans and Candida glabrata being the most common species isolated from both participant groups. The results indicate that older people living in aged-care facilities were more likely to have candidiasis and have a higher yeast carriage rate than similarly aged people living at home. This may be due to morbidities which led to the need for residential care and/or related to the rest home environment.

Keywords: oral candidiasis; older people; elderly; rest home; yeast carriage; Candida albicans

1. Introduction

The oral cavity is home to many hundreds of species of microorganisms. The majority of these microbes are present in biofilms on the various oral surfaces. The immune systems of healthy individuals maintain stable biofilm communities that help prevent oral infections. When immune defenses are impaired this can cause dysbiosis of the microbiota and lead to bacterial, viral or fungal infections.

Candida species are a normal component of the oral microbiota [1] that are carried in the mouths of about 40-60% of healthy individuals [2]. While Candida albicans is the most prevalent species [3,4], non-albicans Candida species that can be found in healthy individuals include Candida glabrata, Candida tropicalis, Candida parapsilosis, and Candida krusei [5]. Other species such as Candida guilliermondii, Candida kefyr, Candida rugosa, Candida dubliniensis and Candida famata have also been reported, but less frequently [6,7]. Oral candidiasis is the most common type of fungal infection of the oral cavity and may present as pseudomembranous candidiasis, erythematous candidiasis, plaque-like/nodular candidiasis, denture-associated candidiasis, angular cheilitis, median rhomboid glossitis and chronic mucocutaneous candidiasis [8]. Due to its prevalence as a commensal microbe, C. albicans is the most frequent cause of oral candidiasis [9]. A preliminary clinical diagnosis of candidiasis can be made based on the medical history of the individual, their symptoms and an oral examination. This diagnosis can
be confirmed histologically by the presence of hyphae in mucosal smears. Patients with oral candidiasis can be treated with antifungal agents such as polyenes (e.g., amphotericin B and nystatin) and azole derivatives (e.g., fluconazole, ketoconazole, clotrimazole and micazole). It has been reported that 20% of patients with oral candidiasis experience recurrence of infection, and around 30% of the recurrences were caused by Candida strains different from the first episode of infection [10]. Candida species differ in their susceptibility to antifungal agents and C. glabrata and C. krusei show reduced susceptibility to azoles [4,11] and Candida lusitaniae can develop resistance to amphotericin B [12]. In addition, C. parapsilosis has been reported to cause outbreaks of candidemia in immunocompromised patients in hospital settings [13] where it is thought to be transmitted by the hands of health care workers. Therefore, in institutionalized settings it is important to know what species of Candida are colonizing individuals.

Colonization of the oral cavity by Candida depends on the entry of yeast cells, attachment and growth, and cell removal from the oral cavity—the rate of yeast replication must at least match the rate of clearance [1]. Key factors that predispose for Candida colonization include systemic factors caused by drug therapy (e.g., broad spectrum antibiotics, corticosteroids), reduced saliva flow, chronic disease processes, malnutrition, immunosuppression and neoplasia while local factors include wearing dentures overnight, ill-fitting dentures and poor prosthetic hygiene [14]. In the oral cavity, components of the innate immune system, such as salivary flow and biological components within saliva, play a major role in preventing infections. Factors that reduce salivary flow predispose to infections such as candidiasis. In studies of people with xerostomia (dry mouth) it has been shown that there is an inverse correlation between salivary flow rate and the number of Candida cells in oral samples [15,16]. There was also an inverse correlation between the salivary flow rate and the severity of oral candidiasis [15]. The proportion of the population receiving medications increases with age and older people are often prescribed multiple medications, many of which reduce saliva flow rates. Denture wearing also increases with age and dentures obstruct saliva flow across mucosal surfaces reducing the clearance of microbes and creating environments that promote fungal growth. Therefore, it is not surprising that older people are more likely than younger people to have oral mucosal lesions, including candidiasis [17].

Around the world, populations are ageing. The WHO estimates that by 2050 the proportion of the population that is ‘older’ (in this case above the age of 60) will have increased from 12% in 2015 to 22%, that is, from 900 million to 2 billion people [18]. Studies have shown elderly patients living in institutional care facilities, such as nursing homes and hospitals, are more susceptible to oral and systemic Candida infections [19], mainly due to comorbidities, in-dwelling devices and common care facility processes. What is concerning is the emergence of non-albicans Candida strains with lower antifungal susceptibility in self-caring, nursing home residents [4]. C. glabrata and C. krusei strains showed reduced susceptibility to azoles and C. rugosa and C. krusei strains were less susceptible to fluconazole than other yeast. The presence of Candida strains with reduced susceptibility to antifungal agents could pose a problem for older people if they become immune compromised and develop candidiasis.

A significant number of older people live in residential aged care facilities [20], which in New Zealand was approximately 32,000 people in 2013 [21]. The objective of this study was to compare the Candida species colonizing the oral cavities of people living in rest homes with those living in their own homes.

2. Materials and Methods

2.1. Participants

This pilot cross-sectional study was a convenience sampling of two groups of older people; people living at rest home facilities (n = 20) and people living in their own homes (n = 20). Rest home participants came from either Montecillo Veterans Home and Hospital, or Little Sisters of the Poor,
both located in Dunedin, New Zealand. Participants for the control group were members of the public recruited from posters circulated via Age Concern, Bowling Clubs Dunedin and personal contact. Inclusion criteria were that participants had to be above the age of 65, healthy or have well controlled systemic disease, have sufficient mouth opening to allow intraoral access and able to give consent. Participants were excluded from taking part in this study if they had used antibiotics or antifungals in the past 2 months, were terminally ill or were current smokers. Ethical approval for this study was obtained from the University of Otago Human Ethics Committee (approval number H17/081).

2.2. Clinical Examination

A clinical examination was carried out by direct observation of the condition of the oral mucosa. Participants who exhibited mucosal inflammation and had hyphae present in the smear test were prescribed antifungal agents. If they wore dentures they were advised to leave them out at night and denture hygiene instructions were given. The rest home in-house medical doctor and nurse manager were involved in this process to further support the participants.

2.3. Collection of Oral Samples

Unstimulated saliva was collected from participants. Participants were requested to allow their saliva to pool in their mouths for one minute then expectorate it into a glass measuring cup. This was done 5 times. The total volume of saliva produced was recorded and the saliva transferred to a sterile 15 mL Falcon tube. Patients who were unable to generate saliva rinsed their mouths with 2 mL of sterile water. This was to enable analysis of salivary gland function and the yeast present in saliva. Participants’ palates and tongues were swabbed with sterile cotton buds and each swab was placed in 2 mL sterile water in a sterile 15 mL Falcon tube.

Smears of the palate and tongue were taken by gently rubbing a wooden spatula on the dorsal surface of the tongue and hard palate, avoiding the area that had just been swabbed, but immediately adjacent to it. The material obtained on the spatula was smeared onto a glass slide and the slides immersed in Coplin jars filled with 95% ethanol. The Coplin jars were transported to the University of Otago Faculty of Dentistry Oral Pathology Centre where they were stained with periodic acid-Schiff (PAS).

2.4. Analysis of Samples

Saliva and mucosal swabs were transported on ice to the Faculty of Dentistry Molecular Biosciences Laboratory for analysis and storage (4 °C). Whole saliva samples and suspensions of palate and tongue swabs were vortexed for 30 s and portions (100 μL) were spread on CHROMagar Candida agar plates (Becton Dickinson & Co., Franklin Lakes, NJ, USA) which were incubated at 37 °C for 48 h. Candida species were presumptively identified according to colony color, and colony forming units (cfu) per ml sample were determined. When the numbers of cfu on plates were too many to count, portions of the stored yeast suspensions were diluted in sterile water 100- or 1000-fold and plated and incubated as described above. The numbers of colonies on plates were converted to cfu/mL saliva by multiplying by the dilution factor and then by 10 (as 100 μL was plated). Yeast species were confirmed by colony polymerase chain reaction (PCR) amplification and sequencing of the rDNA internal transcribed spacer region ITS2 [22]. Colony PCR was carried out using KOD FX Neo DNA Polymerase (Toyobo, Osaka, Japan), where each 20 μL PCR reaction contained 0.6 μL sterile water, 10 μL 2× PCR Buffer for KOD FX Neo, 4 μL dNTPs (2 mM), 2 μL each of forward and reverse primers ITS1(F) 5'-GCATCGATGAAGAACGCAGC and ITS2(R) 5'- TCCCTCCGCTATTGATATGC [23] (3.2 μM), 1 μL of template cells (suspended in 20 μL sterile water) and 0.4 μL KOD FX Neo (1.0 U/μL). The PCR cycle conditions were: initial denaturation at 98 °C for 30 s (1 cycle); followed by 44 cycles of the following steps: denaturation at 98 °C for 10 s; annealing at 55 °C for 10 s; elongation at 68 °C for 40 s; and final elongation step at 68 °C for 1 min.
The presence of PCR products was confirmed by running 1 µL of colony PCR sample with 5 µL of 1x loading dye on agarose gel (1.5% w/v) through electrophoresis at 100 V for 28 min with ethidium bromide staining. DNA oligomer primers were removed from colony PCR products with ExoSAP-IT™ (ThermoFisher Scientific, Christchurch, New Zealand) according to the manufacturer’s instructions. DNA sequencing was carried out using primer ITS3(F) by the Genetic Analysis Service, Anatomy Department, University of Otago. Results were analysed with the computer programs FinchTV (version 1.5.0) and UniPro UGENE (version 1.29.0) and the sequences compared with the publicly available database at https://www.ncbi.nlm.nih.gov/BLAST/.

PAS-stained slides were examined by a consultant oral pathologist to determine the presence or absence of fungal hyphae.

2.5. Statistical Analysis

The t-test was used to compare parametrically distributed data. The Mann-Whitney U test was used to compare non-parametrically distributed data. Differences were considered significant if p < 0.05. For binary dependent variables, SPSS software (version 25, IBM Corporation) was used for cross-tabulations, with Chi-square tests used to determine statistical significance (at the 0.05 level).

3. Results

3.1. Participant Demographics

The participants in the two groups (those who lived in their own homes, and those who lived in rest homes) were sex-matched and although the participants from rest homes were, on average, 2.9 years older than those who lived in their own homes, this difference was not statistically significant (Table 1).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Participant Location</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>83.1 (71-92)</td>
<td>86.0 (72-94)</td>
</tr>
<tr>
<td>Proportion with dentures</td>
<td>55%</td>
<td>70%</td>
</tr>
<tr>
<td>Mean salivary flow rate [mL/min] (range)</td>
<td>0.25 (0-0.7)</td>
<td>0.23 (0-0.6)</td>
</tr>
<tr>
<td>Proportion with hyphae in sputum</td>
<td>30%</td>
<td>33%</td>
</tr>
<tr>
<td>Mean number of medications (range)</td>
<td>5.4 (0-11)</td>
<td>6.6 (1-12)</td>
</tr>
<tr>
<td>Proportion with saliva colonized by yeast</td>
<td>65%</td>
<td>87%</td>
</tr>
<tr>
<td>Proportion with tongue colonized by yeast</td>
<td>35%</td>
<td>80%</td>
</tr>
<tr>
<td>Proportion with palate colonized by yeast</td>
<td>25%</td>
<td>65%</td>
</tr>
<tr>
<td>Level of yeast colonization of saliva (cfu/mL)</td>
<td>1410 (50-4100)</td>
<td>32,240 (0-4.72 x 10^7)</td>
</tr>
<tr>
<td>Level of yeast colonization of tongue (cfu/swab)</td>
<td>271</td>
<td>240</td>
</tr>
<tr>
<td>Level of yeast colonization of palate (cfu/swab)</td>
<td>Median (range)</td>
<td>606</td>
</tr>
</tbody>
</table>

* t-test; a Chi-square test; b Mann-Whitney U test. p values in bold typeface are significant (<0.05).
The participants from rest homes were more likely to wear dentures (full or partial) than those living in their own homes, and they had, on average, a slightly lower salivary flow rate, although this difference was not statistically significant (Table 1). Fifty-five percent of the rest home participants had salivary gland hypofunction (defined as an unstimulated whole salivary flow rate < 0.2 mL/min [24]) as did 40% of those living at home; this difference was not statistically significant (p = 0.34; chi-square test). Rest home participants were more likely to be diagnosed with candidiasis (hyphae present in smears of the palate and tongue; Table 1) than participants living in their own homes (difference not statistically significant; p = 0.74 Table 1).

The participants were taking a variety of, and usually multiple, medications. The number of medications being taken by people living in their own homes ranged from 0 to 11 with a mean of 5.4 medications (Table 1). Rest home participants were taking, on average, more medications (6.6, range: 1–12), but this difference was not statistically significant (p = 0.25; t-test). If, however, all participants were categorized as either having a normal salivary flow rate or a low salivary flow rate (<0.2 mL/min), those with an abnormally low flow rate were taking, on average, significantly more medications than those with a normal flow rate (7.4 vs. 4.8; p = 0.005, t-test).

3.2. Colonization with Yeast

Eighty percent of the rest home participants were colonized by yeast at one or more of the oral sites sampled (saliva, tongue, or palate) as were 65% of participants living in their own homes. Colonization rates at the three sites varied for both groups of participants (Figure 1).

![Figure 1. Presence of yeast at oral sites in participants. Percentage of participants colonized by yeast at each oral site.](image)

Of the oral samples, saliva samples were most commonly yeast-positive followed by tongue swabs and then palate swabs. Each oral site was more often colonized by yeast in participants from rest homes. This increased colonization of rest home participants was statistically significant for all three sites (saliva p = 0.01, tongue p = 0.001, palate p = 0.03; Table 1). Eighty-four percent of those wearing dentures had saliva that contained yeast whereas saliva was yeast-positive in only 53% of those without dentures. This difference was statistically significant (p = 0.04; chi-square test). The numbers of yeast in each sample differed considerably and were higher in those participants with PAS-positive smears. Of the 13 participants with PAS-positive smears, 11 had yeast detected in either the tongue or palate swab samples. Yeast were also detected in either the tongue or palate swab samples in a further 11 participants who were not PAS-positive. For those people colonized with yeast, the mean number of cfu per mL of saliva was 40,305 (median: 4425; range: 410-472,000) for participants from rest homes, whereas the mean for participants living in their own homes was only 2171 (median: 230; range:
20–9160). As several of the participants living in their own homes had no detectable yeast in their saliva the mean colonization level for the group was 1410 cfu/mL (median: 50 cfu/mL) whereas it was 32,240 cfu/mL (median: 2260 cfu/mL) for the rest home group (Table 1, Figure 2). The numbers of yeast in each type of samples from participants from rest homes were significantly higher than those from people living in their own homes (Table 1).

![Figure 2. Quantity of yeast at oral sites in participants. Level of yeast colonization at each site in each group (cfu/mL of saliva samples, cfu/swab for tongue and palate swabs ± standard deviation).](image)

3.3. Species of Colonizing Yeast

Yeast were identified by sequencing their rDNA internal transcribed spacer region ITS2. The yeast species most frequently isolated in each sample type from both participant groups was *C. albicans* followed by *C. glabrata*. Seven yeast species were identified in samples from individuals living in their own homes: *C. albicans, C. glabrata, C. parapsilosis, C. lusitaniae, C. guilliermondii, Pichia fermentans* and *Yarrowia lipolytica* (Figure 3). Only five yeast species (*C. albicans, C. glabrata, Saccharomyces cerevisiae, C. dubliniensis and C. tropicalis*) could be identified in rest-home participants, even though more samples were yeast-positive.

![Figure 3. Species of yeast colonizing participants.](image)
Of the 60 oral samples taken from the participants living in their own homes, 25 were positive for yeast, whereas 45 of the 60 residential home samples contained yeast. Of the total 70 oral samples that were yeast-positive, 42 contained only one species of yeast and 28 had more than one species. The species that were found together most frequently were *C. albicans* and *C. glabrata*. The largest number of species found in the oral cavity of a participant (who lived in their own home) was four (Figure 4).

![Bar chart showing number of yeast species colonizing individual participants.](image)

**Figure 4.** Number of yeast species colonizing individual participants.

4. Discussion

In this study the types, and quantities, of yeast colonizing the oral surfaces of older people living in rest homes were compared with those in people living in their own homes. Three oral sites were sampled to make these measurements, the tongue, the palate, and whole unstimulated saliva. The tongue was sampled as it has been shown to be one of the most frequently colonized oral sites [23], and the palate is also often colonized in patients with full upper dentures [5]. We found that in both groups of participants the tongue was more frequently colonized than the palate, and that yeast were most frequently found in saliva; all individuals with yeast on their tongues or palate also had yeast in saliva, and for some individuals yeast were only detected in their saliva. This is probably because if yeast colonize oral surfaces, they are likely to be washed off to some extent by saliva. Thus, for a study of the variety of fungi in people’s mouths, yeast in saliva can be used as a proxy for oral colonization and saliva may act as a reservoir for colonization of other oral sites. It is difficult to assign a concentration of yeast in saliva, on swabs, or in smears that might indicate candidiasis as best factors are important in disease progression. The presence of hyphae in smears is a good indication of clinically-significant disease as the hyphal form can penetrate tissues. However, many *Candida* species, such as *C. glabrata*, do not form hyphae making the diagnosis of candidiasis from the inspection of smears more difficult. The fact that yeast were cultured from more people than had PAS-positive smears may reflect the greater sensitivity of culturing yeast from swabs or saliva samples, but this positive culture does not give the clinician an indication of the disease status.

There are a number of factors that are known to favor oral colonization with yeast. These include reduced salivary flow rates [16,26] and wearing dentures [27]. In the present study we found that people living in their own homes who had a low unstimulated salivary flow rate (<0.2 mL/min) had, on average, a greater concentration of yeast in their saliva than people with a higher flow rate (2675 cfu/mL compared to 568 cfu/mL, p = 0.009; Mann–Whitney U test). This was not the case for rest-home residents. Although a larger proportion of rest-home residents who had a normal salivary flow rate were yeast-negative compared to those with reduced flow rates (33% versus 9%) their mean
colonization level was higher than that for residents with a reduced salivary flow rate due to two residents with high salivary yeast concentrations and normal flow rates. Unstimulated saliva flow rates reduce with age [28] and older people are often prescribed medications which are known to be xerogenic (cause dry mouth). Due to the number and variety of medications our participants were prescribed, it was not possible to control for medications between the groups. There was no significant difference in the mean number of medications taken by the two groups of participants, but there was a significant association between low salivary flow rate and a higher number of medications. Participants in both groups who wore dentures had greater concentrations of yeast in their saliva than those participants who did not wear dentures, but the differences were not statistically significant.

CHROMagar Candida is a useful medium for the presumptive identification of C. albicans from biological samples and for distinguishing other fungal species. Combining this primary screen with rDNA ITS2 sequencing of isolates enabled precise identification of the predominant fungal species present in oral samples without the detection of extremely minor components of the mycoflora as is often the case with direct next generation sequencing of biological samples. As has been shown previously [4,5,15], the most common yeast species found in the mouths of participants was C. albicans. The second most frequently isolated species was C. glabrata. This may reflect the increased prevalence of this species in clinical samples that has been reported in recent years [29]. It is of concern that of the 10 yeast species detected in participants, seven (C. albicans, C. glabrata, C. parapsilosis, C. lusitaniae, C. guillermondii, C. tropicalis, and Y. lipolytica) have been reported as being potentially resistant to antifungals [12,29-33]. It was beyond the scope of the present project to measure the antifungal susceptibility of all the fungal isolates, but this would provide an indication of whether the older adults sampled were at risk from a fungal infection that would be difficult to treat.

As populations age, an increasing number of older people are being cared for in rest homes, yet there are few studies on the microbial colonization of people living in these institutions. One comparison of older people in institutionalized long-term care with those not in such care found that oral colonization with yeast was more frequent in the institutionalized individuals [34]. This study did not, however, identify the colonizing yeast. We found that oral sites were more frequently colonized by yeast in rest-home participants than in people living in their own home and that the mean concentration of yeast in the saliva of rest-home participants was 23 times higher than that in non-rest home participants (the median was 45 times higher). This may indicate poorer oral hygiene in the rest home participants which may be due to the reason they are in rest homes—they are less capable of independent living. The increased numbers of opportunistic fungal pathogens in rest home residents suggests that the rest homes would be well advised to pay greater attention to the oral hygiene of residents.

It was interesting to note that a greater variety of yeast species was detected in people living in their own homes (7 species) compared to those living in rest homes (5 species) with only two species (C. albicans and C. glabrata) common to both groups. A possible explanation is that communal living and shared care assistants result in the sharing of yeast between rest home residents. This possibility could be investigated further by MLST (multilocus sequence typing) analysis of the predominant species in both groups, C. albicans, to determine the relatedness of strain types in the two groups, as we have done previously for people wearing dentures [35]. Alternatively, a higher proportion of people in rest homes may have morbidities that favor yeast growth, and yeast species particularly well-adapted to the oral environment in these people may out-compete other species. This could lead to high numbers of a single yeast species in such individuals. Species with the potential to be drug resistant were present in both the rest home residents (C. albicans, C. glabrata, C. dubliniensis, and C. tropicalis) and people living in their own homes (C. albicans, C. glabrata, C. parapsilosis, C. lusitaniae, C. guillermondii, and Y. lipolytica) emphasizing the need for all older people to keep in good health and maintain good oral hygiene.

This study has some limitations. One is the small sample size. While a few studies report the colonization of older people living in institutionalized care by yeast [34,36], there are no quantitative analyses of the different yeast present. Thus, it was not possible to perform a sample-size estimation
for this study, and a pilot investigation was undertaken. Also, participants were recruited from two
rest homes which introduced a potential confounder. Despite the small sample size, the present study
revealed some important statistically significant differences between the oral colonization of people
living in rest homes and those living in their own homes. The results from this study, therefore, provide
a justification, and basis, for examining the oral fungal colonization of larger samples of older people
from a single rest home and older people living in their own homes.

**Author Contributions:** Conceptualization, R.D.C., N.M.T. and A.M.R.; methodology, E.L., A.M.R. and R.D.C.;
investigation, N.M.T.; data curation, N.M.T.; writing—original draft preparation, N.M.T.; writing—review and
acquisition, N.M.T., E.L., A.M.R. and R.D.C.

**Funding:** This research was funded by a New Zealand Ministry of Health Oral Health Research Grant, (grant
number RFR15).

**Acknowledgments:** We would like to thank Murray Thomson (Faculty of Dentistry, University of Otago) for
his assistance with statistical analysis, and Trudy Mine (Faculty of Dentistry, University of Otago) for assistance
with the figures.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the
study, in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision
to publish the results.

**References**

   [CrossRef]


