A HYDROGEL IN COMBINATION WITH ESSENTIAL OILS FOR ORAL THERAPY

A thesis submitted in partial fulfilment of the requirements
of the Manchester Metropolitan University
for the degree of Doctor of Philosophy

School of Healthcare Science
Faculty of Science and Engineering

Elisa Serra

December 2018

DECLARATION AND STATEMENTS

Declaration

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed……(candidate) Date…….

Statement 1

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

Signed……(candidate) Date…….

Statement 2

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

Signed……(candidate) Date…….

Statement 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for interlibrary loan, and for the title and summary to be made available to outside organisations.

Signed……(candidate) Date…….

ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude to Dr Sladjana Malic for her constant support. Without her advice and encouragement, I would have never completed the thesis on time. My sincere thanks for reading through the dissertation when she was on annual leave. I would like to extend my appreciation to the other two members of the team, Prof Dr Joanna Verran and Dr Araida Hidalgo-Bastida.

My sincere gratitude goes to Prof Alastair Sloan, who gave me the possibility to spend four months at Cardiff University, and to Prof David Williams. I am grateful to them for their supervision and scientific advice. A special thank goes to Dr Amr Alraies and Dr Wayne Nishio Ayre for their constant and essential help with the *ex vivo* model.
I would like to express my gratefulness to Prof Thomas Wilkinson for giving me the possibility to work in his lab at Swansea University. Even if I stayed there for a short time, I learned a lot.

I would like to extend my gratitude to all the people that helped me in the lab. Among all of them, I would like to give my sincere thanks to the lab technicians Paul, Glenn and Lee that taught an engineer how to work in a microbiology, cell, and chemistry lab, respectively. In addition, I am deeply grateful to Rhiannon Parkhouse for proofreading the thesis.

I would like to thank all my friends in Manchester, Zurich, and Milan. My sincere gratitude goes to Robertinho for always being there and for letting me feel at home. Thanks to the Moss Side volleyball team, for both the trainings and the nights out. My sincere gratitude goes to Zalessio, Marco, and Monica. Despite the distance, their support was essential.

Last and the most important, thanks to my family and brother. Even if I do not live anymore with them, they are always on my mind and in my heart.

TABLE OF CONTENTS

CHAPTER 2: ANTIMICROBIAL PROPERTIES OF ESSENTIAL OILS AND BIOCIDES

CHAPTER 3: EVALUATION OF THE CYTOTOXICITY AND IMMUNE RESPONSE TO ANTIMICROBIALS

CHAPTER 4: DEVELOPMENT OF A METHYLCELLULOSE HYDROGEL WITH *MELISSA OFFICINALIS* ESSENTIAL OIL

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ACP	Acyl carrier protein
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AMU	Atomic mass unit
ATP	Adenosine triphosphate
B2M	Beta2-microglobulin
BOP	Bleeding on probing
BrdU	Bromodeoxyuridine
C3	Complement component 3
CAL	Clinical attachment level
CDA	Chlorhexidine diacetate
CDFF	Constant depth film fermenter
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CHC	Chlorhexidine hydrochloride
CHX	Chlorhexidine
CLRs	C-type lectin receptors
CLSM	Confocal laser scanning microscopy
CP	Chemically pure
Ct	Cycle threshold
CS	Chitosan
CYC1	Cytochrome C1
DCM	Dichloromethane
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAA	Fastidious anaerobe agar
FAB	Fastidious anaerobe broth
F-ATPase	Phosphorylation factor-ATPase
FBS	Fetal bovine serum
FDA	Food and drug administration
FIC	Fraction inhibitory concentration
FTIR	Fourier transform infrared spectroscopy
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCF	Gingival crevicular flow
GC-MS	Gas chromatography-mass spectrometry
GI	Gingival index
GP	Glycerophosphate
H&E	Haematoxylin and eosin
HEMA	2-hydroxyethyl methacrylate
HTCC	Chitosan quaternary ammonium salt
HIV	Human immunodeficiency virus
HPFL	Human periodontal ligament fibroblasts
IC50	Half maximal inhibitory concentration 50

IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
INT	Iodonitrotetrazolium
LPS	Lipopolysaccharide
LVR	Linear viscoelastic region
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MDR	Multidrug efflux pump
MIC	Minimum inhibitory concentration
MLC	Minimal lethal concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NCBI	National center for biotechnology information
NHS	National health service
PAMPs	Pathogen-associated molecular patterns
PAS	Periodic acid schiff
PBI	Papillary bleeding index
PBS	Phosphate buffered saline
PDD	Probing pocket depth
PEM/THFM	Poly(ethylmethacrylate)/tetrahydrourfuryl methacrylate
PEO	Poly(ethylene oxide)
PGA	Poly(glycolic acid)
PHEMA	Poly(2-hydroxyethyl methacrylate)
PI	Plaque index
PIA	Polysaccharide intercellular adhesin
PJI	Prosthetic joint infection
PL	Phospholipases
PLA	Poly(lactic acid)
PM-ATPase	Plasma membrane-ATPase
PMMA	Poly(methyl methacrylate)
PMNs	Polymorphonuclear neutrophils
PPO	Poly(phenylene oxide)
PRRs	Pattern recognition receptors
PSS	Plaque staining score
PVA	Poly(vinyl alcohol)
RGD	Arginylglycylaspartic acid
RHE	Reconstituted human epithelium
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real time-polymerase chain reaction
SAP	Secreted aspartic proteinase
SBI	Sulcus bleeding Index
SCC	Squamous cell carcinoma
SD	Standard deviation
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SEM	Standard error of the mean
SEM	Scanning electron microscope

SHED cells	Stem cells from exfoliated deciduous teeth
SRP	Scaling and root planing
TGFβ	Transforming growth factor beta
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor alpha
TTO	Tea tree oil
UBC	Ubiquitin C
UV	Ultraviolet
WHO	World Health Organisation
XRCT	X-ray computed tomography

LIST OF UNITS

°C	Degree Celsius
µm	Micrometre
µl	Microlitre
bp	Base pair
eV	Electronvolt
g	Gram
h	Hour
Hz	Hertz
l	Litre
m	Metre
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
ng	Nanogram
nm	Nanometre
Pa	Pascal
ppm	Parts per million
rpm	Revolutions per minute
sec	Second
v/v	Volume per unit volume
w/v	Weight per unit volume
w/w	Weight per unit weight

LIST OF SYMBOLS

$\dot{\gamma}$	Shear rate
δ	Phase lag
ε	Strain
ε_0	Strain amplitude
η	Viscosity
ρ	Density
σ	Shear
σ_0	Shear amplitude
ω	Angular frequency
CH_3	Methyl group
CO_2	Carbon dioxide
$\cos$	Cosine
E	Young's modulus
E_h	Redox potential
G'	Storage modulus
G''	Loss modulus
H	Hydrogen
K	Potassium
M_e	Mean length
n	Number
NaOCl	Sodium hypochlorite
O	Oxygen
OH	Hydroxyls group
S	Swelling
Sin	Sine
Tan	Tangent
V	Volume
W	Weight
$	Dollar
€	Euro
%	Percentage
£	Pound

PREFACE

The work presented in this thesis was undertaken at Manchester Metropolitan University, UK. The experiments on blood cells (Chapter 3) were carried out at Swansea University, UK. The *ex vivo* rodent mandible model (Chapter 5) was developed at the School of Dentistry, Cardiff University, UK.

Chapter 2 and Chapter 3 were partly published in:

Serra, E., Hidalgo-Bastida, L. A., Verran, J., Williams, D. and Malic, S. (2018) 'Antifungal activity of commercial essential oils and biocides against *Candida albicans*'. *Pathogens,* 7(1) pp. 1-12 (see Appendix 1).

ABSTRACT

Oral diseases such as periodontal diseases and oral candidiasis are significant health problems in humans. Periodontal disease is a common disease of the oral cavity and the major cause of tooth loss in adults. Four clinical forms of primary oral candidiasis are recognised, and the management of such infections is limited due to the low number of antifungal drugs available, their relatively high toxicity and the emergence of antifungal resistance. The use of hydrogels in delivery of biocides has been explored due to their biocompatibility, ease with which they can be charged with drugs, and potential to confer mechanical and structural properties similar to biological tissue. This can be used both for anaerobic bacteria and fungal (i.e. *Candida*) infections to treat a wider spectrum of oral diseases.

The aim of this study was to develop a novel antimicrobial therapy for oral diseases by utilising a hydrogel in combination with *Melissa officinalis* essential oil.

A range of essential oils and biocides has been tested for their antifungal properties mainly against *Candida albicans* in a planktonic and biofilm growth form and against bacterial species associated with periodontal disease (in the planktonic growth form). The cytotoxicity of the compounds that showed the best antimicrobial properties were tested and the chosen essential oil was incorporated into a methylcellulose hydrogel. Finally, an *ex vivo* rodent mandible model to mimic oral candidiasis was developed.

Antimicrobial screening showed *Melissa officinalis* to be the most successful essential oil relating to antimicrobial properties and cytotoxicity.
The infection of the rodent mandible showed *C. albicans* invasion of the gingiva and the release of pro-inflammatory cytokines. The application of *Melissa officinalis* oil significantly decreased the CFU/ml and the pro-inflammatory response. One percent (1% (v/v)) and 2% (v/v) *Melissa officinalis* oil was successfully incorporated into 10% (w/w) and 12% (w/w) methylcellulose hydrogel. Rheology revealed that the hydrogel was injectable and gellified in two minutes at 37 °C. The drug release was a function of the *Melissa officinalis* concentration and the loaded hydrogel successfully inhibited *Candida* growth *in vitro.*

A 3D *ex vivo* rodent mandible model to mimic oral candidiasis was developed and used to test the antifungal properties of *Melissa officinalis* oil. Moreover, a potentially injectable methylcellulose hydrogel loaded with *Melissa officinalis* oil was synthesised. This hydrogel was shown to elicit antifungal properties *in vitro*.

In conclusion, the study showed that essential oils were antimicrobial and that methylcellulose hydrogels could be used as drug delivery systems.

OVERVIEW OF THE THESIS STRUCTURE

The focus of the present study was to develop a novel antimicrobial therapy for oral diseases by utilising a hydrogel in combination with *Melissa officinalis* essential oil.

In the first instance, a range of essential oils and biocides has been tested for their antimicrobial properties against *Candida albicans* (2 strains) in planktonic and biofilm growth form and against four bacterial species associated with periodontal disease (in planktonic form). A simple microtiter plate assay was used to determine the antimicrobial properties of the tested agents (Chapter 2).

The cytotoxicity of the compounds that showed the best antimicrobial properties was tested on mouse fibroblasts (Chapter 3). Having established the most suitable antimicrobial agent (*Melissa officinalis* essential oil), the pro- and anti-inflammatory host response to *Melissa officinalis* was evaluated using human blood cells harvested from three healthy individuals (Chapter 3).

The second part of this thesis focused on the development of a methylcellulose hydrogel with *Melissa officinalis* essential oil (Chapter 4). A 10% (w/w) or 12% (w/w) methylcellulose hydrogel loaded with 1% (v/v) and 2% (v/v) *Melissa officinalis* oil was synthesised, then the rheological properties, the essential oil leaching and the antimicrobial potential against a *C. albicans* strain were evaluated (Chapter 4).

In the final part of the study, an *ex vivo* mandible rodent model to mimic oral candidiasis was developed. This approach involved dissecting the mandible of 28-day-old male Wistar rats and infecting it with *C. albicans*. *Candida* growth was monitored through histological examination after incubation for 24 and 48 hours with and without 1% (v/v) *Melissa officinalis* oil (Chapter 5).

Chapter 1

Literature review

1.1 Biofilms

Biofilms are three-dimensional structures attached to a surface, a human tissue or an artificial surface (e.g. denture), in which communities of bacteria and/or fungi are embedded in an extracellular matrix (Figure 1.1) (Cortés et al., 2011). The extracellular matrix is mainly composed of polysaccharides, proteins, nucleic acids and lipids synthesised by the bacteria, as well as molecules taken up from the oral environment. The extracellular matrix acts as a scaffold allowing the adhesion and the cohesion of microorganisms and promotes cell-cell signalling (Flemming and Wingender, 2010). Cell-cell signalling is based on the production of small molecules, such as acyl-homoserine and peptides by Gram-negative and Gram-positive bacteria, respectively. These small molecules allow bacteria to adapt to various environments and to regulate and co-ordinate their gene expression (Davies, 2003). In addition, the close proximity of bacteria promotes horizontal gene transfer, which is one of the mechanisms involved in biofilm antibiotic resistance (Davies, 2003). The extracellular matrix also acts as a nutrient source, providing carbon, nitrogen and phosphorous compounds. In addition, it is highly hydrated allowing the survival of bacteria even in water-deficient environments (Flemming and Wingender, 2010). Moreover, it is a physical barrier that limits the efficacy of phagocytosis during the immune response (Flemming and Wingender, 2010).

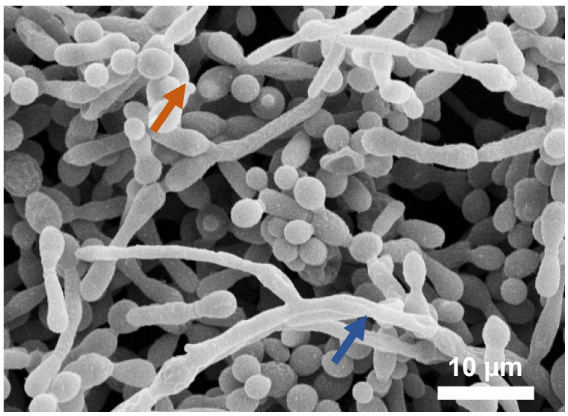


Figure 1.1 - Scanning electron microscope (SEM) image of a *C. albicans* biofilm. Orange arrow indicates the yeast form, blue arrow indicates the hyphal element (Ramage et al., 2005)

Significant knowledge on biofilms was acquired with the use of micro-electrodes, chemical probes and confocal laser scanning microscopy (CLSM). The latter allows the biofilm structure to be studied without manipulating the sample. In the past it was thought that biofilms were compact structures, however CLSM highlighted the presence of an open architecture with channels and voids that allow nutrient diffusion. Moreover, a horizontal and vertical gradient in key parameters (e.g. oxygen, pH, redox potential) was observed, explaining the coexistence of micro-colonies within the biofilm that need different conditions to survive (Davies, 2003; Flemming et al., 2016).

1.1.1 Dental plaque

The oral microflora is composed by a wide range of bacteria, yeasts, viruses and mycoplasmas. More than 772 microbial species have been identified in the mouth, but only 70% were successfully cultured outside the oral environment (Verma et al., 2018). The vast oral microbial population reflects the complexity of the oral environment, which is characterised by species with different features. Physiologically, a harmonious relationship exists between the oral microflora and

the host. Oral microorganisms naturally form oral biofilms, also named dental plaques, in areas such as stagnant regions of the teeth and areas between the teeth and gingival crevice. Even if the dental plaque is part of the host defences protecting the sites from pathogenic colonisers, the harmonious relationship can become pathogenic and lead to oral diseases, such as dental caries and periodontal diseases (Marsh and Lewis, 2009; Seneviratne et al., 2011). Several causes can shift this relationship to become pathogenic such as the presence of bacteria at sites normally not accessible to them (e.g. blood stream), poor oral hygiene, the prescription of antibiotics or a carbohydrate rich diet (Marsh, 1994; Marsh and Lewis, 2009).

1.1.2 Mechanisms of dental plaque formation

Biofilm formation is a multistage process that can be subdivided into several phases (Figure 1.2):

I. Acquired pellicle formation

   A few seconds after a clean tooth comes in contact with the oral environment, proteins, glycoproteins and lipids contained in saliva are adsorbed onto the enamel. After a few hours, the adsorption and de-adsorption processes reach an equilibrium. The conditioning of the tooth surface is a crucial step in the formation of the biofilm because bacteria do not interact directly with the naked surface. Once molecules bind to the surface they can undergo conformational modifications, such as the exposure of some receptors, allowing the binding of primary colonisers (Liljemark and Bloomquist, 1996; Marsh and Lewis, 2009).

II. Primary bacterial adhesion

Planktonic microorganisms contained in saliva move to the surface randomly (e.g. flow) or directly (e.g. chemotaxis and motility). Once the microorganisms are close enough to the surface (<1 nm), physical forces develop between the acquired pellicle and the cell membrane. These interactions are weak, long range and non-specific and include i) attractive forces such as hydrophobic and van der Waals forces and ii) electrostatic forces which are mainly repulsive since both the pellicle and the membrane are negatively charged. Once the sum of the attractive forces exceeds the repulsive charges, microorganisms adhere to the surface (Dunne, 2002; Marsh and Lewis, 2009; Gupta et al., 2016).

III. Secondary bacterial adhesion

The specific binding between adhesins present on the membrane and complementary receptors on the tooth allow the formation of strong and short-range interactions. Once bacteria are covalently attached to the surface, they can only be removed physically or chemically. Early colonisers are aerobic bacteria, mainly streptococci (e.g. *Streptococcus sanguinis, Streptococcus oralis and Streptococcus mitis*) and *Actinomyces* spp., while anaerobic bacteria are rarely detected at this stage (Marsh and Lewis, 2009; Silva et al., 2012). Once attached, bacteria proliferate and form micro-colonies embedded in an extracellular matrix composed of proteins and glycoproteins produced by bacteria or taken up from the saliva.

IV. Biofilm maturation

Over time a shift in the microflora plaque is observed and an increased evidence of Gram-positive bacilli is present. These bacteria can interact with microorganisms present in the oral cavity that cannot directly bind to the pellicle. Moreover, their fermentation products can be a primary nutrient source for other bacteria (Marsh and Lewis, 2009). In parallel, they cause changes in the oral environment (e.g. redox potential, pH and oxygen level) creating the ideal conditions for the recruitment of anaerobic bacteria (Seneviratne et al., 2011).

Anaerobic bacteria would not naturally co-aggregate with the pioneers, but *Fusobacterium nucleatum* acts as a “bridge” between early colonisers and later ones. Once the mature plaque is formed, a heterogeneity in terms of bacteria species and environmental conditions is observed. Gradients in nutrients, toxic products, oxygen and pH are present both vertically and horizontally, allowing the presence of microenvironments mainly composed by certain species into the same plaque (Sbordone and Bortolaia, 2003; Marsh and Lewis, 2009).

V. Detachment from the surface

The last stage of the biofilm formation is the detachment of bacteria from the surface. This process can be either active (e.g. enzymes synthesised by bacteria break the bindings) or caused by the shear stress. Once detached bacteria can colonise other sites in the body (Marsh and Lewis, 2009).

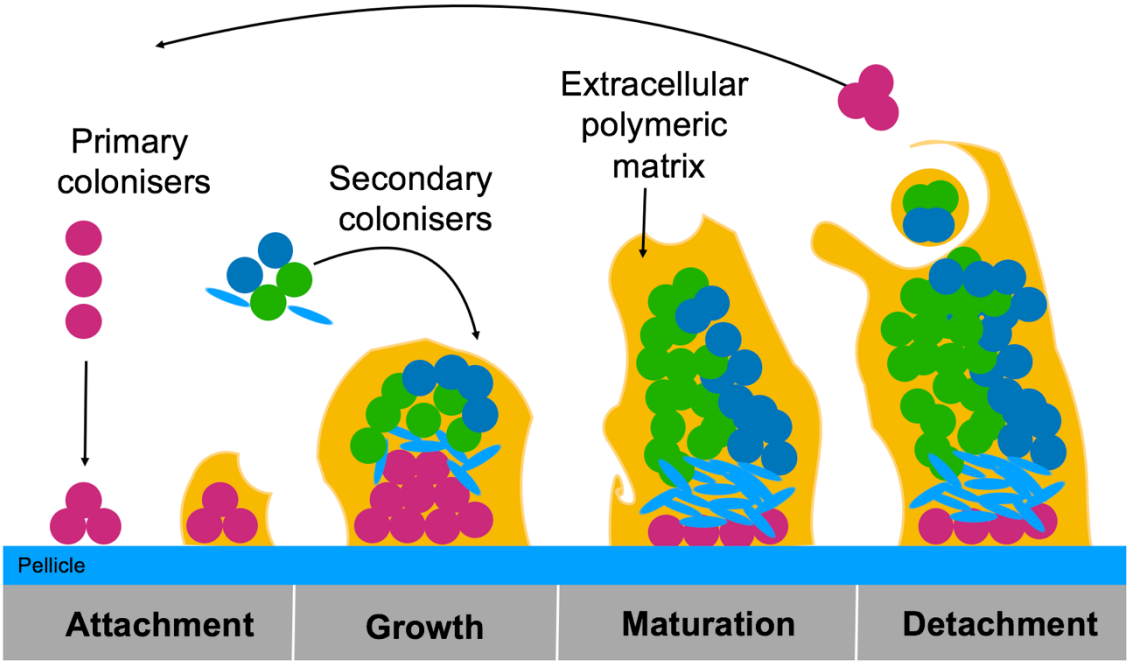


Figure 1.2 - Schematic representation of the stages involved in dental plaque formation. The pellicle forms on the tooth surface and primary colonisers adhere to it. Once the attachment becomes irreversible, secondary colonisers can attach to early colonisers. The biofilm grows, and a mature biofilm is formed. Lastly, bacteria can detach and colonise new surfaces. Figure adapted from Stoodley and Dirckx (2003)

1.1.3 Biofilm resistance to antimicrobials

Bacteria in biofilms are between 10 to 1000 times more resistant to antibiotics than in the planktonic form (Davies, 2003; Macià et al., 2014). The mechanisms responsible for this resistance may be one or more of the following:

I. Reduced penetration of the antimicrobial agents into the biofilm matrix

   For a long time, the extracellular matrix was thought to prevent the penetration of drugs into the biofilm. More recently it was shown that it is not a physical barrier, however it can still act as a chemical barrier against certain antibiotics. Indeed, being negatively charged it can interact with the positively charged antibiotics delaying their penetration or inactivating them (Olsen, 2015).

II. Slow growth rate and metabolism

As mentioned above, biofilms are characterised by a heterogeneous population and a gradient of oxygen and nutrients. The lack of nutrients leads to the presence of a population of dormant or slow-growing cells that are less susceptible to antibiotics (Olsen, 2015).

Indeed, antibiotics generally act against bacteria in growth phase, targeting some crucial processes such as the replication, transcription, translation and the cell wall synthesis (Olsen, 2015). These dormant bacteria which are less susceptible to antibiotics are called 'persister cells' and are usually found in the deepest layers of the biofilm, in which the nutrients and gas exchanges are limited. Being able to resist antibiotics, "persistent cells" guarantee the continuous progression of the infection (Harrison et al., 2005; Roberts and Stewart, 2005).

III. Expression of a biofilm phenotype to resist antibiotics

Bacteria within biofilms show a different phenotype, named by Costerton et al. (1999) as the "biofilm phenotype". Because of different gene expression, the target or the efficiency of the antibiotics can be altered (Marsh and Lewis, 2009). In parallel, bacteria can overexpress genes associated with antibiotic resistance, such as the multidrug efflux pump (MDR) (Davies, 2003).

1.2 Oral diseases

Despite the latter part of the 20th century experiencing an improvement in both general and oral health, dental caries and periodontal diseases still remain the major global oral health problem (Petersen, 2003). Besides significant effects on individual and social life due to pain, difficultly in eating and chewing and damaged teeth, oral diseases have an economic impact. It has been estimated

that the expenditure on oral diseases amounted to $422 billion in 2010. Direct treatment costs were estimated at $298 billion yearly, corresponding to 4.6% of the global health expenditure, while indirect costs such as those due to sick-days were $144 billion yearly (Listl et al., 2015). Taking into account the 27 states of the European Union, the direct costs were estimated at €72.96 billion while the indirect ones at €37.56 billion (Patel, 2012). In addition, without an efficient prevention and solution to the burden of oral diseases, the direct costs are expected to increase up to €93 billion in 2020, exceeding those for cancer, heart diseases, stroke and dementia (Patel, 2012). Concerning the economic impact of dental care in the UK, the NHS spends around £3.4 billion per year, while the private market is around £2.3 billion per year. As in the rest of Europe, these costs are expected to increase (NHS England, 2014; Claxton et al., 2016).

The distribution of oral diseases varies both intercountry and intracountry. Oral diseases are a function of socio-economic status, educational level and the environment. Generally, people with a lower income and education, and living in rural areas show a poorer periodontal status. Reports also highlighted the influence of the ethnicity and religion on periodontal health, such as in the US where black people have a risk of periodontal destruction three times higher than white people (Petersen and Ogawa, 2012).

The intercountry variations are mainly due to the different incomes that increase accessibility to the health care system for habitants from high-income countries compared to those from low- and middle-income countries. For example, the dentist population ratio in Africa is 1:150000 or more, whilst it is estimated at 1:2000 in high-income countries. Besides the accessibility to the health care

system, oral diseases are also related to the lifestyle and the presence of systemic chronic diseases (Petersen and Ogawa, 2012).

Despite a recent positive trend largely due to an improvement of self-care practise and behavioural changes, dental caries is still the most common chronic disease affecting from 60 to 90% of children and almost 100% of adults (Udina and Gulenko, 2018). Concerning periodontal diseases, the percentage of people affected by severe periodontal diseases accounts for 10-15% of the global population, whilst half suffers from gingivitis bleeding, gingivitis bleeding with calculus or shallow periodontal pocket. However, the increase of life expectancy (i.e. teeth are retained for longer) and of diabetes (a recognised risk factor) are expected to add to the burden of periodontal diseases (Burt, 2005; Petersen and Ogawa, 2012).

1.2.1 Dental caries

Dental caries is a multifactorial disease that is caused by the concomitant presence of cariogenic bacteria, fermentable carbohydrates and a tooth surface. In addition, other factors such as the oral hygiene, the shape of the tooth, the diet and the saliva can contribute to the development of the disease (Figure 1.3) (Mathur and Dhillon, 2018).

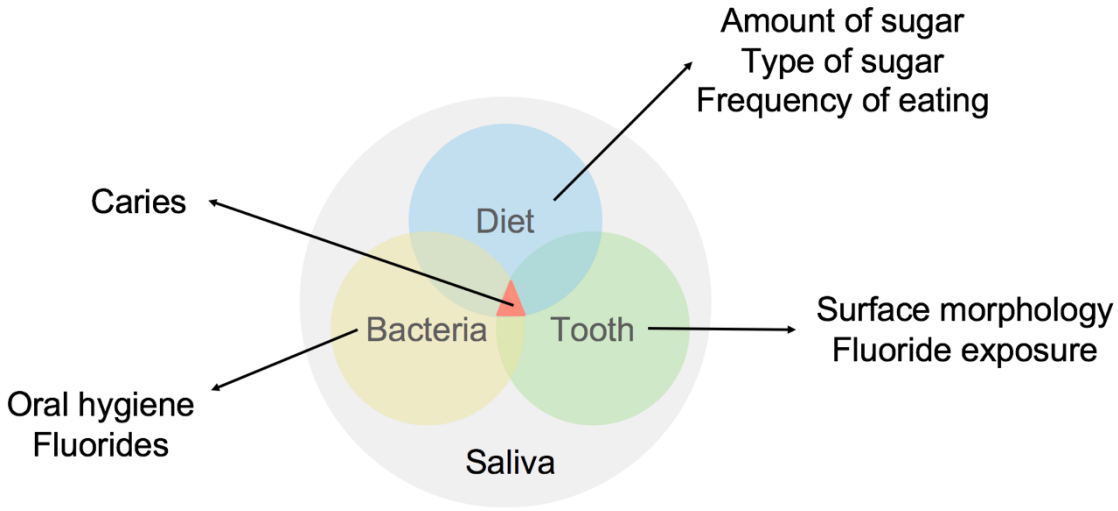


Figure 1.3 - Aetiology of dental caries. Figure adapted from Mathur and Dhillon (2018)

Dental caries is caused by an ecological imbalance within dental plaque that leads to the destruction of the hard tissues of the teeth. In physiological conditions, the surface of the teeth is covered by dental plaque. The consortium of bacteria present in the dental plaque comprises cariogenic bacteria which are weakly competitive and represent only a small portion of the total population. However, if the production of acid products due to the fermentation of carbohydrates increases, a decrease in pH is observed. The fall of the pH below 5.5 causes the release of calcium and phosphate from the tooth surface and the promotion of the growth of carcinogenic bacteria, such as *lactobacilli* and *Streptococci mutans*, that further increase the production of acid compounds. The result is a disequilibrium between remineralisation and demineralisation that leads to the demineralisation of the enamel subsurface (Figure 1.4). If the process is not arrested, small lesions called “white spots” can be clinically detected and at the last stage the cavitation occurs (Moynihan and Petersen, 2004; Marsh and Lewis, 2009).

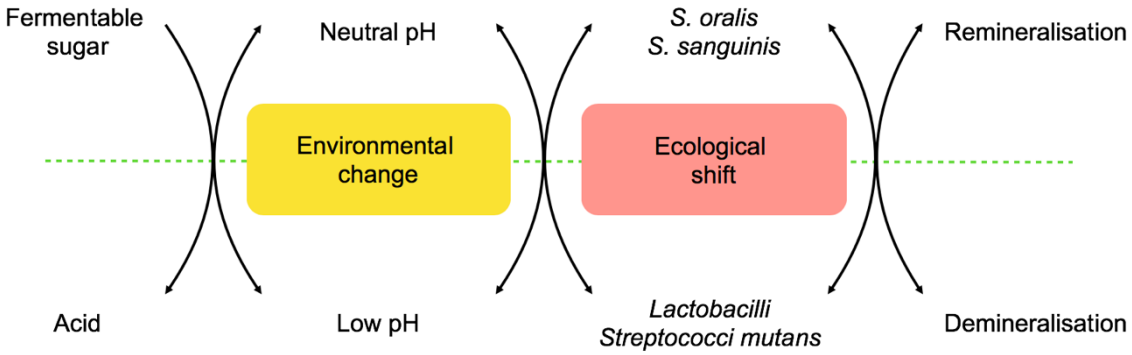


Figure 1.4 - Schematic representation of the "ecological plaque hypothesis" for dental caries. The fermentation of carbohydrates causes the production of acid products that decrease the pH. Low pH favours the growth of acid-tolerating bacteria, such as *Streptococci mutans* and *lactobacilli*, that further increase the production of acid compounds. This results in the demineralisation of the tooth (Marsh and Lewis, 2009)

The progression of the disease can be delayed by fluoride, which promotes the diffusion of calcium and phosphate into the tooth, and by saliva that deposits minerals where the demineralisation process occurred (Selwitz et al., 2007).

Since teeth are more susceptible during eruption, dental caries mainly affect the deciduous dentition of young children and the permanent dentition of adolescents. However, increased life expectancy and the consequently longer retention of the teeth in the mouth are causing a burden of the disease even among elderly. Indeed, in the presence of gum recession the tooth root, which is less mineralised than the crown, is exposed and susceptible to decay. This form of caries, named root caries, is characterised by the penetration of bacteria into the deepest tissues even at the early stage of the infection (Moynihan and Petersen, 2004).

1.2.1.1 Pathogenic mechanisms in dental caries

Pathogenic determinants include the ability of cariogenic bacteria to adhere to the teeth surfaces and form dental plaque. The adhesion of *Streptococcus*

mutans can be both sucrose-independent or sucrose-dependent. The sucrose-independent mechanism seems to be responsible for the initial adhesion to the acquired enamel pellicle, while the sucrose-dependent mechanism is responsible for the formation of mature dental plaque (Krzysciak et al., 2014; Banas, 2017). In addition, the ability of *S. mutans* to synthesise glucans from sucrose increases the efficacy of colonisation and adhesion (Banas, 2017). Moreover, cariogenic bacteria, especially *S. mutans,* are acidogenic and produce acid products (e.g. formate, acetate, ethanol and lactate) (Marsh and Lewis, 2009; Banas, 2017). Acid products cause a further reduction of the pH in the dental plaque promoting both the demineralisation process and the increase of the proportion of *S. mutans* or acid-tolerant and acid-genic bacteria (Banas, 2017).

Furthermore, cariogenic bacteria are acid-tolerant, so both the glycolytic activities and growth are preserved in inhibitory conditions (i.e. low pH). The ability to survive in extreme environmental conditions is due to their capacity to maintain the intracellular pH to a healthy level by retaining protons intracellularly and by increasing the activity of the F-ATPase pump and the membrane permeability to acid products (Marsh and Lewis, 2009; Banas, 2017).

1.2.2 Periodontal diseases

Periodontal diseases can be divided into gingivitis and periodontitis. Gingivitis is an inflammation of the gingiva caused by the bacterial plaque populating the tissues, while periodontitis is an advanced inflammation process that causes the destruction of one or more supporting structures of the tooth (i.e. alveolar bone, periodontal ligament, root cementum and gingiva), eventually leading to tooth loss (Figure 1.5) (The American Academy of Periodontology, 1999; Tonetti et al., 2013). Periodontitis develops from previous gingivitis, although not all gingivitis

turns into periodontitis. In addition to these two chronic forms, periodontal diseases include an aggressive form named juvenile periodontitis that mainly affects young people. Acute forms include the necrotising ulcerative gingivitis, which destroys the gingival tissue, and the necrotising ulcerative periodontitis in which the bone around the teeth is infected or exposed (Petersen and Ogawa, 2012).

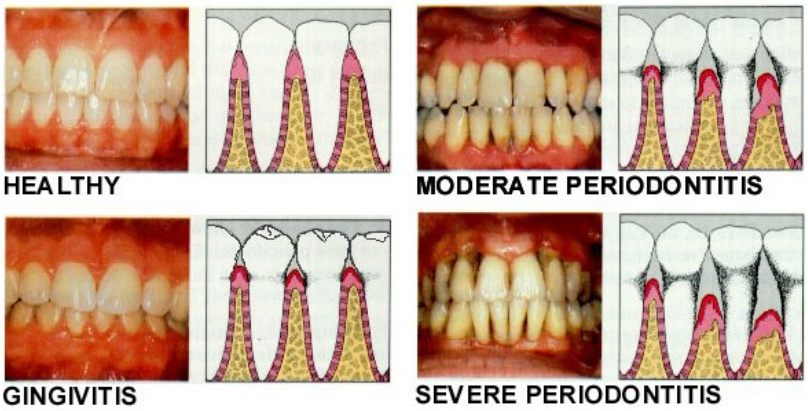


Figure 1.5 - Progression of periodontal disease from healthy to severe periodontitis (Gromadzkim, 2017)

The main aetiology of periodontal diseases is the oral microflora and in particular the pathogenic dental plaque associated with it. The application of new technologies such as DNA sequencing within the field of oral microbiology allowed the identification of more than 770 species in the oral cavity (Verma et al., 2018). Although it is commonly accepted that periodontal diseases are an inflammatory process, several hypothesis concerning the aetiology have been formulated over time. Between the 1930s and 1970s, a “nonspecific plaque hypothesis” according to which periodontal diseases are due to the overall mass of the bacteria accumulated on the tissues and specific bacteria do not have a leading role was accepted (Theilade, 1986; Marsh, 1994).

This theory was surpassed in the following years by a “specific plaque hypothesis” (Loesche, 1976; Marsh, 1994). The specific plaque hypothesis was based on the observation that some bacteria (e.g. *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Campylobacter rectus, Eubacterium nodatum, Peptostreptococcus micros, Staphylococcus intermedius* and *Treponema* spp.*)* seem to play a pivotal role in the initiation and progression of the disease. However, it did not explain how it was possible to have periodontitis in the absence of these bacteria and how their presence did not necessarily lead to periodontal diseases.

To overcome these limitations , “an ecological plaque hypothesis” that includes both, the “nonspecific plaque hypothesis” and the “specific plaque hypothesis”, is mainly accepted (Marsh, 1991). According to this hypothesis (the “ecological plaque hypothesis”), periodontal diseases are due to a perturbation of the oral environment that causes a shift from a commensal to a pathogenic relationship with the host. Therefore, pathogenic organisms can be part of the oral microbiota without causing the disease because at physiological conditions they are weakly competitive and represent only a small portion of the total community. However, once a perturbation occurs (e.g. increase in the pH and oxygen tension), these bacteria become prevalent and stronger and play a key role in the development of periodontal diseases (Figure 1.6) (Marsh, 1991; Marsh, 1994; Marsh and Lewis, 2009).

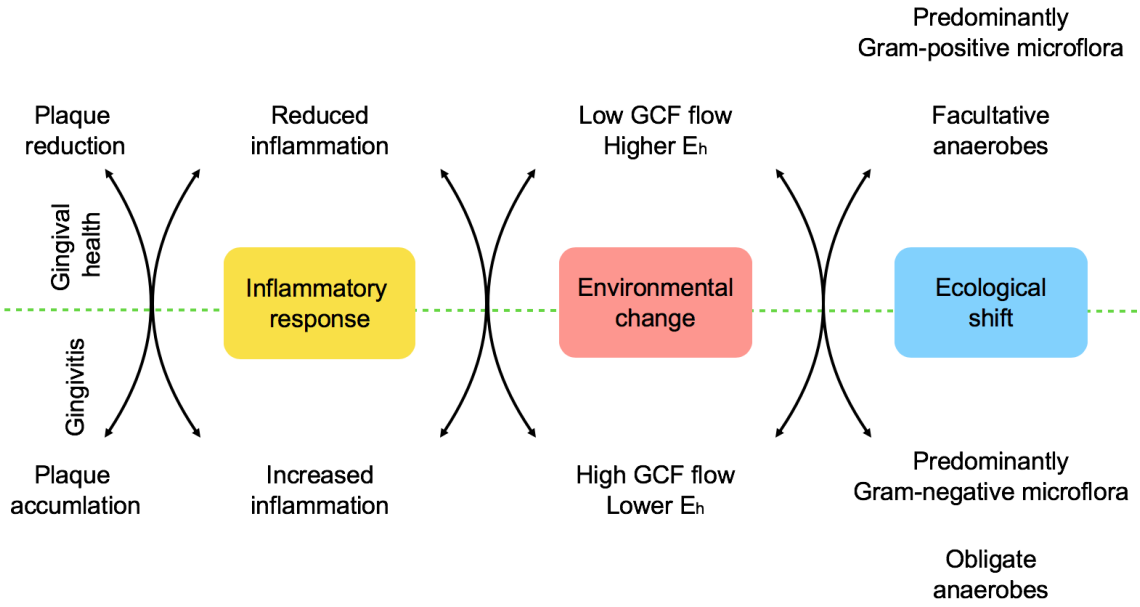


Figure 1.6 - Schematic representation of the "ecological plaque hypothesis" for periodontal diseases. Plaque accumulation causes host inflammatory responses. This leads to changes in the environmental conditions (e.g. redox potential (E_h) and gingival crevicular flow (GCF)) that promote the growth of anaerobic Gram-negative bacteria (Marsh and Lewis, 2009)

1.2.2.1 Pathogenic mechanisms in periodontal diseases

The main feature of periodontal diseases is the damage of the supporting tissues of the teeth. Tissues can be damaged directly by virulence factors produced by pathogenic bacteria or indirectly by the host inflammatory response (Figure 1.7).

Mechanisms of direct pathogenicity include:

- Colonisation

The adhesins present on the membrane of periodontal pathogens interact with complementary receptors allowing the binding of bacteria to the host tissues. Receptors can be located on the gingival epithelial cells, on the root surface or on early colonisers (Marsh and Lewis, 2009).

- Evasion and inactivation of the host defences

Bacteria can interfere with phagocytosis by decreasing the number of neutrophils or by interfering with host mechanisms involved with the opsonisation, phagocytosis and bacterial killing (The American Academy of Periodontology, 1999; Hajishengallis, 2015). *Aggregatibacter actinomycetemcomitans* and *Campylobacter rectus* produce leukotoxins that lyse neutrophils, monocytes and a sub-population of lymphocytes (Marsh and Lewis, 2009; Gholizadeh et al., 2017). *Porphyromonas gingivalis* secretes proteolytic enzymes that can degrade antibodies or complement proteins, preventing the accumulation of these molecules on the bacterial surface and, as a consequence, delaying the recruitment of neutrophils and the onset of phagocytosis (The American Academy of Periodontology, 1999; Sochalska and Potempa, 2017). In addition, bacteria can synthesise factors that suppress the host immune response avoiding the production of protective antibodies. Lastly, bacteria can penetrate into the tissues to evade the action of neutrophils or can resist host defences thanks to a polysaccharidic capsule, such as the one of *P. gingivalis* (The American Academy of Periodontology, 1999).

- Tissue-damaging enzymes and metabolites

The supporting tissues of the teeth can be directly damaged by some enzymes and metabolites produced by bacteria. For example, *P. gingivalis* can produce collagenase, hyaluronidase, chondroitin sulphatase and glycylprolyl peptidase that degrade the tissue matrix molecules (The American Academy of Periodontology, 1999; Eley and Cox, 2003). In addition, cytotoxic metabolic by-products such as ammonia, amines and butyric and propionic acids, can be released in the oral environment. Lastly, some molecules synthesised by

periodontal pathogens (e.g. lipopolysaccharides (LPS) and lipoteichoic acid) can cause bone resorption (Marsh and Lewis, 2009; Hienz et al., 2015).

Indirect pathogenicity is due to the host inflammatory processes that begin immediately after the bacterial infection and can cause tissue damage and bone resorption. In particular, during the cell mediated response the activated macrophages release cytokines (e.g. IL-1, IL-6, TNF) that cause collagenase release, bone resorption and tissue damage (The American Academy of Periodontology, 1999; Hasan and Palmer, 2014), whilst in the humoral immunity the immunoglobulins activate the complement cascade resulting in the release of prostaglandins that cause bone resorption (Marsh and Lewis, 2009; Hasan and Palmer, 2014).

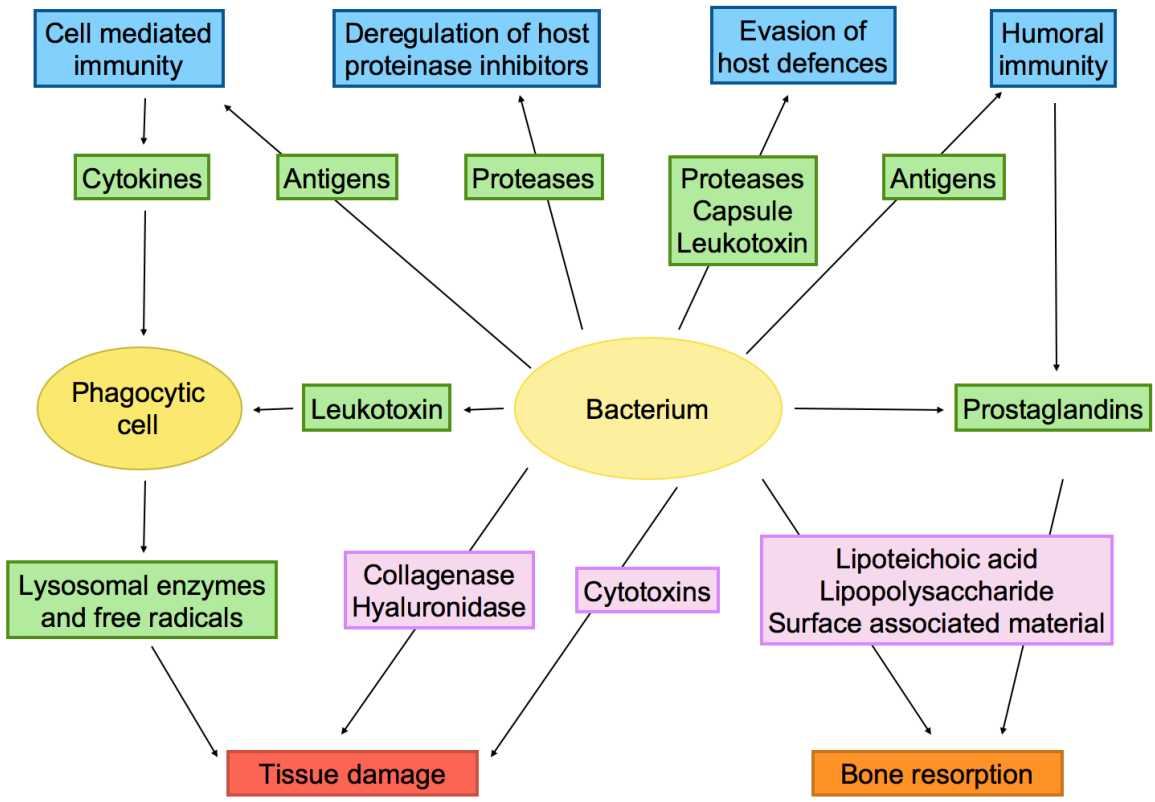


Figure 1.7 - Diagram that illustrates the direct and indirect mechanisms by which dental plaque damages the host tissues (Marsh and Lewis, 2009)

1.2.3 Oral candidiasis

Candida species are commensal microorganisms of the oral cavity, mainly found on the posterior part of the tongue and on the oral mucosa. *Candida* is an opportunistic pathogen and can cause diseases due to alterations of the oral environment, leading to the most common human fungal infection, named oral candidiasis. Even though more than 17 *Candida* species can cause human infections, oral candidiasis is mainly caused by *C. albicans* (Sardi et al., 2013). *Candida* exists in the planktonic and biofilm form. Oral candidiasis identifies four main oral diseases associated with *Candida*: pseudomembranous candidiasis, acute atrophic candidiasis, chronic hyperplastic candidiasis, and chronic atrophic candidiasis

1.2.3.1 Pseudomembranous candidiasis

Pseudomembranous candidiasis is characterised by the formation of a white pseudomembrane on the mucosa, on hard and soft palate, and on periodontal tissues (Figure 1.8-A). The pseudomembrane composed by *Candida* species, fibrin and desquamated epithelial cells can be removed by gentle scraping (Akpan and Morgan, 2002). This infection is acute and caused by host predisposition: it is mainly diffused in babies because their immune system is not fully developed, and among the elderly because of a poor diet or immunosuppression (e.g. human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS)). Furthermore, it is found in people that use steroid inhalers against asthma. It is postulated that steroids supress the cellular immunity and phagocytosis, promoting *Candida* growth (Muzyka, 2005).

In general, it is sufficient to elicit the predisposing host factors to go back to the healthy condition. Immunosuppressed patients can also show a more severe

form of pseudomembranous candidiasis, named chronic pseudomembranous candidiasis. In this pathogenic status, antifungal therapies successfully eradicate pseudomembranes for a limited amount of time. In worst cases, chronic pseudomembranous candidiasis causes the colonisation of the oesophagus inducing chest pain and difficulties in swallowing (Williams and Lewis, 2011).

1.2.3.2 Acute atrophic candidiasis

Acute atrophic candidiasis is characterised by the presence of painful reddened patches on the oral mucosal, typically on the dorsum of the tongue (Figure 1.8-B) (Marsh and Lewis, 2009). This form is associated with the prescription of antibiotics that, being active against bacteria, decrease the competitive pressure promoting *Candida* growth. Generally, it is sufficient to interrupt the antibiotic treatment to go back to the healthy level of *Candida*. Even in this form, the concomitant presence of immunosuppression can lead to a chronic status (Williams and Lewis, 2011).

1.2.3.3 Chronic hyperplastic candidiasis

Chronic hyperplastic candidiasis is an asymptomatic form generally associated with smoking. It is characterised by the formation of white bilateral patches in the buccal commissure regions (Figure 1.8-C). In this form, *Candida* hyphae are able to penetrate into the epithelial layer and inflammatory cells within the lamina. For this reason, it is generally characterised by changes in the thickness of the epithelial layer (Williams & Lewis 2011). Because *Candida* invades the deepest tissues, it cannot be removed simply by scraping, and a biopsy is necessary to detect it. Moreover, some studies highlighted a possible link between chronic

hyperplastic candidiasis and oral cancer (Williams et al. 2001.; Sitheeque 2003; Ramirez-garcia et al. 2014)

1.2.3.4 Chronic atrophic candidiasis

Chronic atrophic candidiasis, also known as denture stomatitis, is the most common oral disease associated with *Candida* and affects up to 65% of people wearing dentures (Figure 1.8-D) (Williams & Lewis 2011). The main cause of chronic atrophic candidiasis is the denture that creates an ideal environment for *Candida* growth in the upper fitting surface (Salerno et al., 2011a). Indeed, *Candida* is able to adhere to the acrylic of the denture and, even if poorly attached, is retained at the site because of the limited saliva flow. It has been observed that more colonies are present if the surface is rough and that denture base cracks are ideal sites for colonisation, protecting the microorganisms from shear stresses even during the denture cleaning (Williams & Lewis 2011). Moreover, if dentures do not properly fit, the friction between the prosthesis and the epithelium causes damage to the mucosal barrier, facilitating the invasion of *Candida* into the deeper tissues.

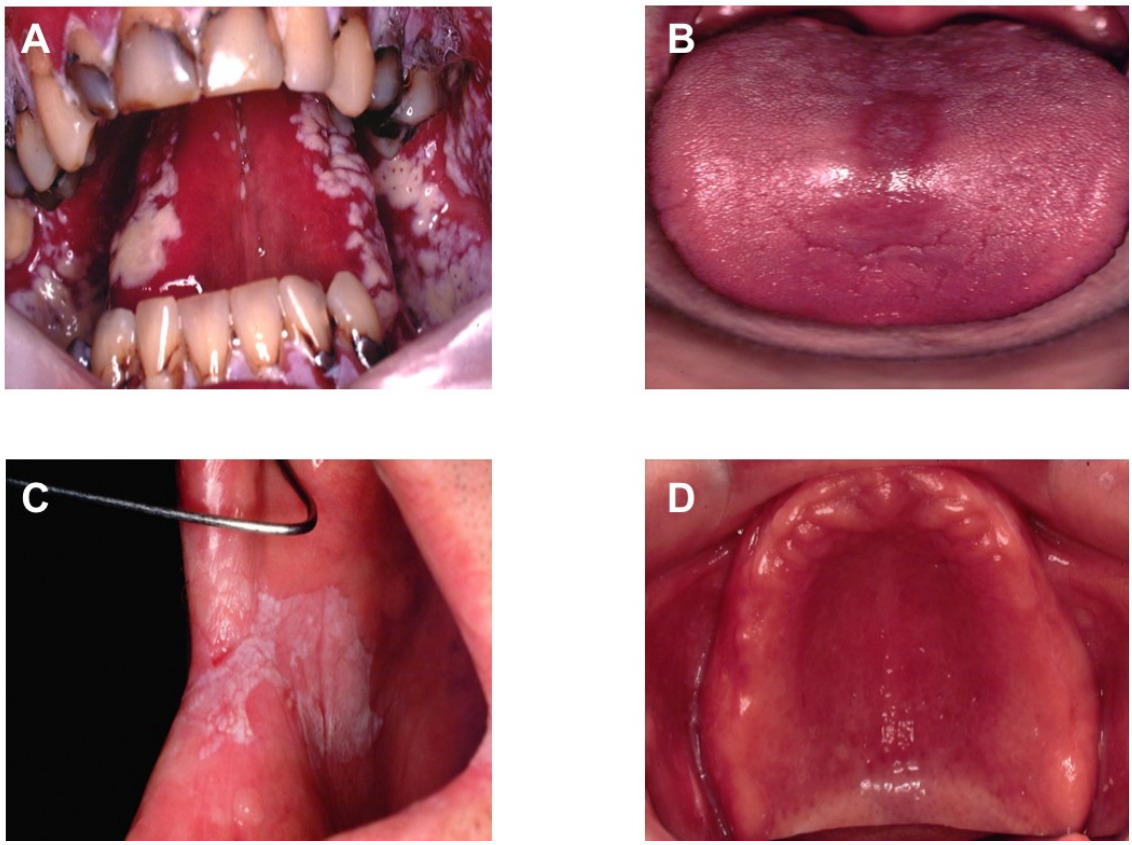


Figure 1.8 - Oral candidiasis infections: (A) pseudomembranous candidiasis, (B) acute atrophic candidiasis, (C) chronic hyperplastic candidiasis and (D) chronic atrophic candidiasis. Images courtesy of the School of Dentistry, Cardiff University

1.2.3.5 *C. albicans* virulence factors

Even if *C. albicans* is a commensal microorganism of the oral cavity, changes in the environment can induce the expression of virulence genes (Figure 1.9). *Candida albicans* pathogenic factors include the presence of adhesins on the cell membrane that allow the binding to the host tissues or to an artificial surface. Several adhesins (e.g. Als1p, Ala1p, Hwp1p, Int1p and Mnt1p) can bind to complementary receptors present on the host cells or to extracellular matrix proteins (e.g. fibronectin, laminin, collagen and fibrinogen). Once *Candida* adheres, it cannot be removed by the salivary flow or swallowing (Calderone and Fonzi, 2001; Mayer et al., 2013). Another virulence factor of *Candida* is its morphogenesis, which is its ability to transform from the unicellular yeast form to

the pseudohyphal and hyphal form. The filamentous form promotes the invasion of the epithelium and increases the resistance to phagocytosis by host immune cells (Marsh and Lewis, 2009; Huang, 2012).

In addition, *C. albicans* is able to respond to stimuli by phenotypic switching which causes a change in gene expression affecting the adhesive properties, the drug susceptibly and the resistance to phagocytosis (Calderone and Fonzi, 2001; Marsh and Lewis, 2009; Huang, 2012). For example, in the so-called "white-opaque transition cells", *C. albicans* switches from a smooth, white colony phenotype containing round budding cells to an opaque colony phenotype containing large, elongated, asymmetrical budding cells which show a different virulence (Soll, 2002).

Lastly, *C. albicans* secretes hydrolytic enzymes, such as the secreted aspartyl proteinases (SAP) and the phospholipases (PL), that destruct the host tissues. Phospholipases are enzymes that hydrolyse phospholipids into fatty acids and contribute to the host cellular lysis and to the exposure of adhesive receptors (Haynes, 2001; Marsh and Lewis, 2009). Four classes of phospholipases have been identified (PLA, PLB, PLC and PLD), even if the only one required for virulence in an animal model was found to be PLB (Calderone and Fonzi, 2001). The secreted aspartyl proteinases (SAP) include at least nine proteins that are characterised by activity in an acid environment (Marsh and Lewis, 2009). The role of SAP as virulence factors needs further investigation. To date it is believed they degrade the host extracellular matrix proteins and it was shown that deletion of genes encoding for these proteins attenuated the virulence (Calderone and Fonzi, 2001; Sardi et al., 2013).

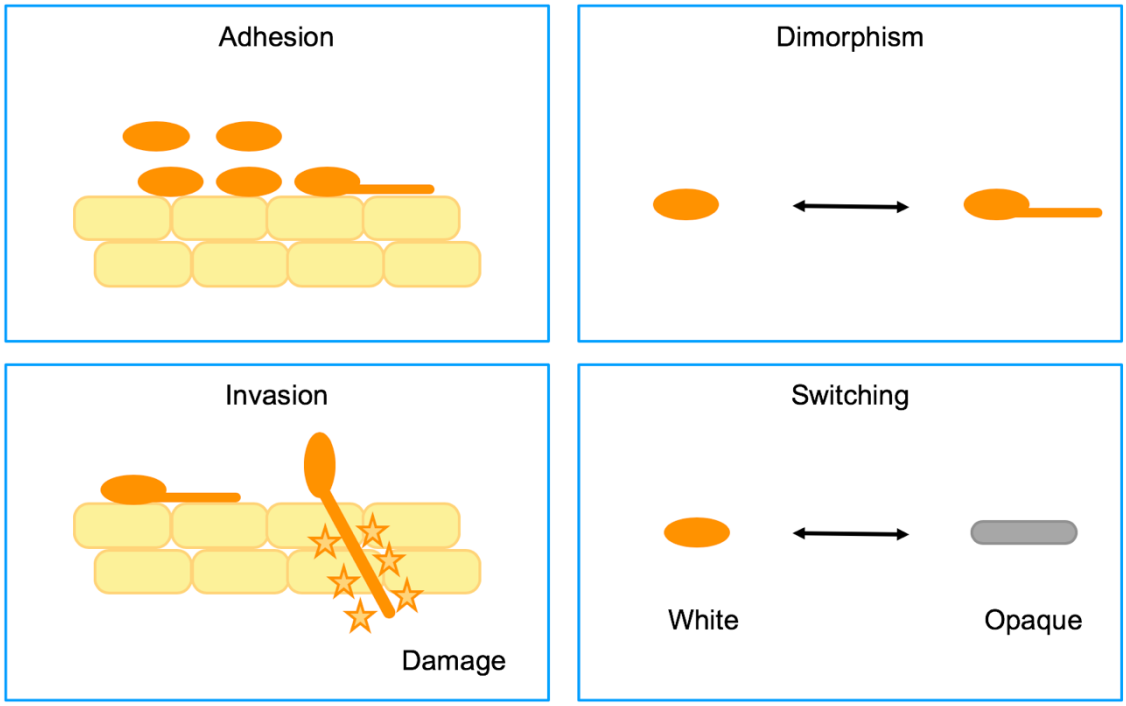


Figure 1.9 - Illustration of the pathogenicity mechanisms of *C. albicans*. Figure adapted from Mayer et al. (2013)

1.3 Risk factors

1.3.1 Dental caries

Several factors have been related to the formation of dental caries. The main ones can be grouped into physical and biological, behavioural and socioenvironmental.

The most important physical and biological risk factors include:

- Salivary composition and flow: saliva allows the removal of bacterial by-products produced during the fermentation and acts as a natural buffer. Moreover, it contains antimicrobials and prevents bacterial adhesion to the enamel surface (Krol, 2003)
- High count of *Streptococcus mutans*: *Streptococcus mutans* has a crucial role in the development of dental caries because of its ability to adhere to the tooth surface and to survive at low pH (Krol, 2003; Harris et al., 2004)

- Gingival recession: gingival recession causes the retention of the plaque in the area and the exposure of the root canal which is more sensitive to demineralisation (Selwitz et al., 2007)
- Variation in tooth enamel: hypoplasia of the enamel can promote the retention and colonisation of bacteria increasing the risk of dental caries (Krol, 2003; Harris et al., 2004)

Behavioural factors include:

- Poor oral hygiene: a good oral hygiene is fundamental to control the formation of dental plaque
- Diet: the greater and more frequent the uptake of carbohydrates, the greater the risk of dental caries, although the use of fluoride toothpaste can limit this correlation (Anderson, 2002). The British Nutrition Foundation defined extrinsic sugars as the most important dietary cause of dental caries (Arens, 1999)
- Low exposure to fluorides: fluorides increase the resistance of the enamel to demineralisation and promote remineralisation, inhibiting the fall of the pH below the critical value (Moynihan and Petersen, 2004).

  Socioenvironmental factors show a higher incidence of dental caries among poor and less educated people, ethnic minority groups and people with several risky lifestyle factors (Selwitz et al., 2007)

1.3.2 Periodontal diseases

Besides the oral microflora, the main risk factors for periodontal diseases include tobacco use, alcohol consumption, oral hygiene and systemic diseases (Petersen and Ogawa, 2012). In terms of oral hygiene, a major awareness of the importance of regular tooth brushing and of the use of fluorinate toothpastes is present.

However, as mentioned in Section 1.2, oral hygiene is still strongly related to the educational level (i.e. practical hygiene is more frequent among educated people) and varies between developed countries and middle- low- income countries. Smoking is an important risk factor for chronic diseases, especially when it is combined with alcohol consumption. A correlation between number of cigarettes and the extent and severity of periodontal diseases has been found (AlJehani, 2014). Besides causing attachment loss, smoking affects the healing process and causes a higher reabsorption of alveolar bone. In addition, the vasoconstriction caused by cigarettes decreases the oxygen tension and creates suitable conditions for the growth of anaerobic bacteria (Petersen and Ogawa, 2012). These main risks factors are shared with the most important chronic diseases such as cardiovascular diseases, cancer, chronic obstructive pulmonary disease and type 2 diabetes (Genco and Borgnakke, 2013). The strongest correlation between periodontal diseases and chronic diseases has been observed with diabetes mellitus of whom periodontal diseases are the sixth complication. This relation is particularly important considering that diabetes is expected to increase by 50% by 2030 (Petersen and Ogawa, 2012).

1.3.3 Oral candidiasis

The shift from a commensal to a pathogenic form of *Candida* can be due to systemic or local factors. Generally, local factors alter the oral environment promoting the overgrowth of *Candida*, while systemic factors have an impact on the host defence system (Vasconcellos et al., 2014).

The most important local factors are:

- Diet: since carbohydrates are the primary energy source of *Candida*, a carbohydrate-rich diet favours proliferation and formation of biofilms (Santana et al., 2013)
- Trauma: tissue damage can promote the penetration of *Candida* into the oral epithelium and increase its permeability to toxins and soluble factors (Salerno et al., 2011b)
- Saliva: both a low and a high saliva flow have been identified as risk factors. On one hand, a high flow is desirable because it cleans the surface and being rich in antimicrobial and anticandidal molecules controls *Candida* growth. On the other, saliva contains some proteins such as mucines that act as adhesion receptors for proteins present on the cell membrane (Salerno et al., 2011b)
- pH and oxygen level: low pH and high oxygen tension reduces the growth of some bacteria promoting proliferation of *Candida* species (Webb, 1998; Gleiznys et al., 2015)
- Smoking: cigarettes affect the oral microbiota and decrease saliva flow promoting *Candida* overgrowth (Vasconcellos et al., 2014)
- Dentures and permeability of the acrylic resins: dentures are the main cause of chronic atrophic candidiasis. The reduced salivary flow and the environmental conditions following implantation (e.g. low pH and oxygen level), create the ideal scenario for *Candida* growth. The irregular surface of the acrylic promotes a strong adhesion of *Candida* and bacteria. Once attached, they are difficult to eliminate both mechanically and chemically. Moreover, denture's age can lead to a poor fit, plaque accumulation and increased surface roughness (Williams and Lewis, 2011)

Systemic factors include:

- Diabetes mellitus: high glucose levels and low salivary flow are typically observed in diabetic patients. As previously discussed, these parameters create the ideal conditions for the overgrowth of *Candida*. Indeed, it has been observed that dentures of diabetic patients show more colonies than those of healthy patients (Soysa et al., 2008; Salerno et al., 2011b; Sanjeeta, 2014)
- Drugs: drugs such as broad-spectrum antibiotics and immunosuppressive therapy change the oral microbiota decreasing the competitive pressure and promoting *Candida* growth. In general, once the treatment is suspended *Candida* shifts to a commensal form (Akpan and Morgan, 2002)
- Immunological disorders: patients with an altered immunological system are more susceptible to infections (Gleiznys et al., 2015)
- Lack of nutritional factors: deficiency of vitamin B12 and vitamin C reduces host defences and causes the disintegration of the oral mucosa, facilitating the migration and colonisation of *Candida* (Gleiznys et al., 2015)

1.4 Current therapies

1.4.1 Prevention and treatment for dental caries

Treatments for dental caries depend on the severity of the infection and aim to restore the tooth. In early stage, dental caries can be removed, and the tooth filled with adhesive materials, while if the infection spreads into the pulp it is necessary to extract the tooth. However, restoration without prevention has a limited durability, so it is also important to prevent the formation of caries. Prevention treatments target the risk factors and include a good oral hygiene that allows early biofilms to be removed, the use of fluorides (self-administered through toothpaste or clinically administered by the application of gels), the

application of dental sealants, a low sugar diet and the control of the saliva flow (Krol, 2003; Selwitz et al., 2007).

1.4.2 Prevention and treatment for periodontal diseases

Both the prevention of periodontal diseases and the long-term efficacy of periodontal therapies depend on the ability of patients to maintain a good plaque removal. In most of the cases, tooth brushing combined with monthly dental control is sufficient to control the formation of the plaque and avoid the generation and progression of periodontal diseases (Watt and Petersen, 2012). Recently, a major awareness of the importance of oral hygiene rose with school-health programs and educating patients on proper tooth brushing (Watt and Petersen, 2012). At the same time, improvements on both the tooth brushings and the toothpastes have been achieved by the introduction of power tooth brushings and fluoride toothpastes to the market (Plessas, 2014). Moreover, new formulations containing some chemical compounds, such as triclosan and chlorhexidine, or natural compounds have been created to further reduce the plaque formation (Drisko, 2001).

However, a good oral hygiene is not always sufficient to control periodontal diseases because some risk factors, many of which are shared with chronic diseases, make some people more predisposed than others. Therefore, prevention programs should also work on patients' behaviour by reducing alcohol consumption and smoking and promoting a healthy diet (Petersen, 2003).

Current periodontal therapies aim to mechanically eliminate the source of inflammation. This goal is obtained firstly by controlling the oral health through oral hygiene, and secondly by removing the bacterial plaque and the infected

tissues (Plessas, 2014). The plaque elimination can be accomplished mechanically, eventually combining the procedure with an antimicrobial treatment (Drisko, 2001). Non-surgical mechanical procedures consist on the elimination of bacteria and endotoxins using a manual or power-driven scaler and include scaling, which eliminates the supragingival plaque, and root debridement that eliminates the subgingival calculus (Drisko, 2001). Most of the time scaling and root planing are sufficient to arrest gingival infections and, together with oral hygiene, restore and maintain a healthy periodontum. However, in some cases these procedures are not efficient. Non-surgical procedures show low efficacy in diabetic patients and smokers and in the presence of severe periodontitis because they do not reach the bacteria in the deepest layers (Labriola et al., 2005; Plessas, 2014). For this reason, in case of severe periodontitis (i.e. pocket deeper than 6 mm), surgical therapies are preferred. These include i) gingivectomy in which the suprabony pocket is excised, ii) flap debridement and iii) modified Widman flap that remove the inflamed pocket wall and expose the alveolar bone and root tooth allowing a better access for scaling and root planing, and iv) the excisional new attachment procedure that aims to promote new connective tissue attachment to root surfaces (Figure 1.10) (Wang and Greenwell, 2001; Deas et al., 2016).

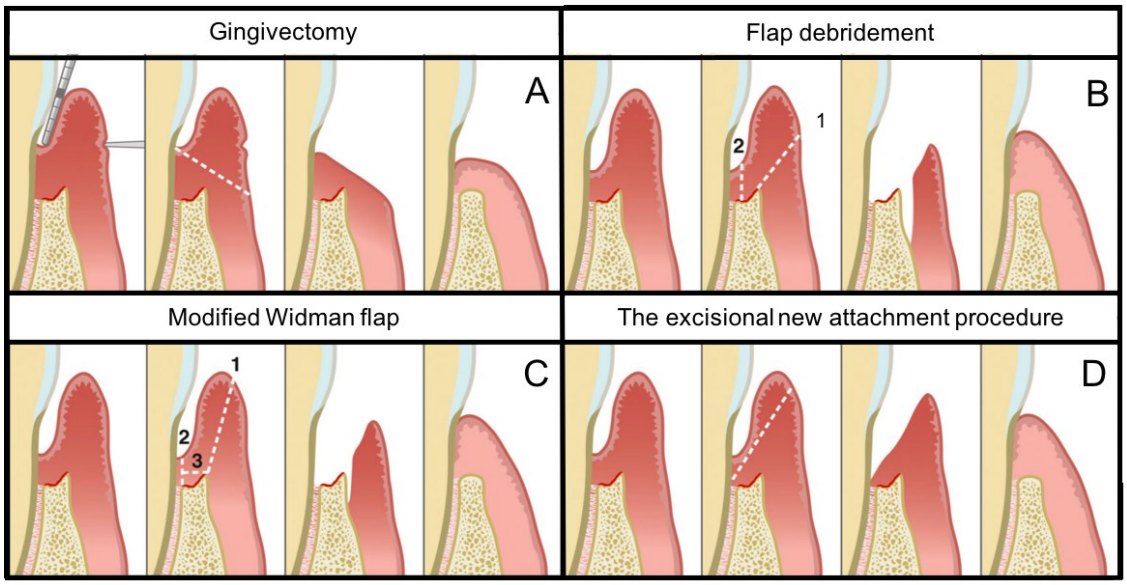


Figure 1.10 - Surgical procedure for severe periodontitis (Deas et al., 2016)

Other therapies, such as the local delivery of antimicrobial compounds, have been investigated to enhance the efficacy of the mechanical treatments. Some studies reported modest depth reduction of the pocket when the mechanical treatment was accompanied by the delivery of antimicrobials such as tetracycline and chlorhexidine (Aurer and Plančak, 2004). In case of aggressive and severe periodontitis that do not have benefits of the non-surgical mechanical treatments, systemic antibiotics have also been used. The main advantage of systemic antibiotics is their ability to act against bacteria that colonise the deepest tissues, which cannot be reached by other therapies. However, the use of systemic antibiotics has to take into account the side effect of antibiotics (i.e. the spreading of resistance) and the fact that periodontal diseases involve a consortium of bacteria, so it is not possible to target all the bacteria with a single compound (Aurer and Plančak, 2004). Other therapies that can be used in addition to mechanical treatments include photodynamic therapy, based on the use of a low-power laser that targets the bacteria treated with a photosensitiser at a specific wavelength, and pocket irrigation with antimicrobials. However, the efficacy of

these therapies alone and in combination with the root planing and scaling is still debated (Plessas, 2014).

Despite all reported therapies eliminating bacterial plaques and repairing tissues, they do not restore the periodontium to a normal functional state (Laurenti and Abdallah, 2015). For this reason, periodontal regenerative procedures to restore the normal functionality of the periodontum have recently been developed. These therapies include i) hard and soft tissue grafts that act as scaffolds promoting bone regeneration (osteoconductive potential) and can contain bone-forming cells (osteogenic potential) or bone-inductive molecules (osteoinduction potential), ii) biomodifications of the root tooth surface with matrix protein, iii) guided tissue regeneration with the use of a physical barrier that promotes the repopulation of the damaged root tooth by cells, and iv) the delivery of growth factors such as bone morphogenetic proteins and platelet-derived growth factors (Figure 1.11). However, the success of these procedures is still variable and unpredictable (Ramseier et al., 2012).

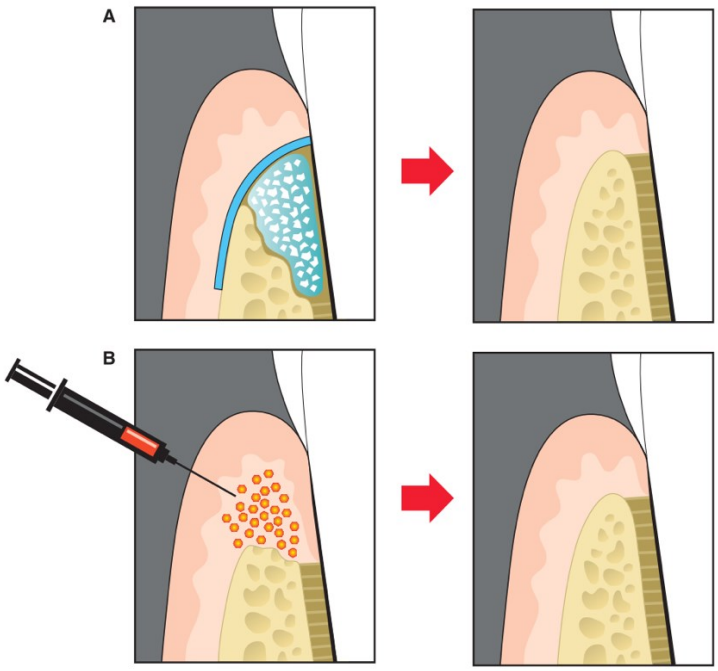


Figure 1.11 - Periodontal regenerative procedures. A) Application of a graft material and growth factors into the intrabony defect covered by a bioresorbable membrane. B) Application of gene vectors for the transduction of growth factors producing target cells (Deas et al., 2016)

1.4.3 Prevention and treatment for oral candidiasis

Treatments for oral candidiasis involve the use of topical or systemic antifungals, and denture cleansers in case of denture stomatitis. Concerning the latter aspect, patients should clean the denture both chemically and physically. Indeed, even if the physical cleaning (i.e. brushing) can limit the biofilm formation on the prosthesis, it can be difficult to properly remove the plaque in the presence of cracks or roughness. For this reason, it is important to accomplish a chemical treatment of the denture, soaking the prosthesis in a chemical denture cleaner such as a disinfectant (Gleiznys et al., 2015).

Antifungal agents against *Candida* can be grouped according to the target. The most common are the polyenes and the azoles. The former includes nystatin and amphotericin B, which are fungicidal: they are able to bind the sterol ergosterol causing leakages in the cell membrane. The latter include fluconazole and

miconazole which are fungistatic and inhibit the enzyme lanosterol demethylase involved in the synthesis of the ergosterol (Williams and Lewis, 2011).

1.4.4 Challenges: antibiotic and antifungal resistance

The production of penicillin in 1941 marked the beginning of the “golden age” of antibiotic discovery. However, since 1980s the development of new antibiotics has dramatically decreased, and those recently introduced on the market belong to pre-existing classes (Oldfield and Feng, 2014). The lack of new antibiotics, together with the inappropriate and extensive exposure to these drugs, led to the problem of antibiotic resistance, classified by the World Health Organisation (WHO) as a major public health threat for the future. Dentists’ prescriptions of antibiotics account for 7% and in the recent years an increase in resistance within the periodontal microbiota has been recorded (Sweeney et al., 2004).

Amoxicillin resistance has been found in *Veillonella* spp. and *Prevotella denticola* isolated from root canals (Lana et al., 2001; Sweeney et al., 2004) and at least one *Prevotella* spp. strain producing β-lactamase was found in 53.2% of patients and 39.4% of the periodontal pockets investigated by Fosse et al. (1999).

High levels of resistance of α-haemolytic streptococci to penicillin have been also recorded. Moreover, interspecies gene resistant transfer between *Streptococcus pneumoniae* and other α-haemolytic streptococci was found (Reichmann et al., 1997; Sweeney et al., 2004).

Resistance to metronidazole can be conferred by mutations to the enzymes that reduce metronidazole to its active form, or by mutations that decrease the uptake of the drug. All the anaerobic strains tested by Roche and Yoshimori (1997) did not show resistance to metronidazole. However, Madinier et al. (1999)

investigated 50 strains of *A. actinomycetemcomitans* showing that 76% were not sensitive to the drug (Sweeney et al., 2004).

Regarding antifungal resistance, fluconazole-resistance species were mainly found in HIV-AIDS patients with oropharyngeal or oesophageal candidiasis (Kanafani and Perfect, 2008). In a study of Law et al. (1994) up to one-third of patients with advanced AIDS showed fluconazole-resistant *C. albicans* strains. However, the introduction of antiretroviral therapies decreased the frequency of fluconazole resistance species in patients with HIV (Masiá and Gutiérrez, 2002). In general, the rate of azole-resistance is low (1-2% in *C. albicans*), even if resistance of *Candida glabrata* increased from 7% in 2001 to 12% in 2004 (Pfaller et al., 2006; Kanafani and Perfect, 2008).

Concerning resistance to amphotericin B, it is rare among *Candida* species even if some *Candida* spp. (e.g. *C. glabrata* and *Candida krusei*) show a lower susceptibility to the antifungal than *C. albicans* (Sanglard and Odds, 2002).

Compared to antibiotics, antifungals on the market are limited because the interest in *Candida* infections is recent and *Candida* is a eukaryotic organism with some features common with human cells (Williams and Lewis, 2011). Therefore, it is more difficult to discover compounds that are fungicidal but not cytotoxic. For this reason, antifungals commercially available show a relatively high toxicity (Boros-Majewska et al., 2014). In parallel, the rise of resistance of *Candida* to the antifungals available on the market led to a new interest in the antifungal properties of natural and chemical compounds.

1.5 Biocides

1.5.1 Chlorhexidine

Chlorhexidine (CHX) is an antiseptic agent commonly used in hospitals for hand sanitation, disinfection of surgical environments, instrumental sterilisation and wounds and skin disinfection (Cordenonsi et al., 2013). Besides these applications, CHX has an antimicrobial activity against a wide range of oral pathogens since it is able to bind to the negatively charged cell membrane and alter the osmotic equilibrium (Greenstein et al., 1986). Typical MICs values range from 0.003% (v/v) to 0.025% (v/v) and from 0.003% (v/v) to 0.005% (v/v) against *C. albicans* and periodontal bacteria, respectively (Barkvoll and Attramadal, 1989; Oosterwaal et al., 1989; Amorim et al., 2004; Solmaz and Korachi, 2013). Schiott et al. (1970) observed that twice daily brushing with 0.2% CHX caused a reduction of up to 95% in the number of bacteria counted in the saliva. Since then, interest in CHX as a topical antiseptic to be used in oral therapy increased and different local delivery systems (e.g. gel, mouthwash and varnish) were studied. Pietruska et al. (2006) compared the efficacy of mechanical scaling combined with 0.2% of CHX rinse twice a day, 1% CHX gel applied to the periodontal pocket at one-week intervals with surgical dressing, or 1% CHX gel applied to the periodontal pocket at one-week intervals without surgical dressing. A significant improvement in clinical parameters (e.g. plaque index (PI), sulcus bleeding index (SBI) and gingival index (GI)) was found in all the groups and the greatest difference between the baseline and the follow-up examination was observed in the group with the surgical dressing. In particular, the scaling could be a sufficient treatment for periodontal diseases and the efficacy of the gel depends on the achievement at the site of the inhibitory concentration and on the ability to keep this concentration over time (Pietruska et al., 2006).

A xanthan hydrogel containing 0.1% chlorhexidine digluconate, delivered in the first day, and 1.5% of chlorhexidine dihydrochloride, delivered in the following 6-9 days, was investigated by Matesanz et al. (2013). Although the antimicrobial potential of this hydrogel was limited, significant achievements in the bleeding on probing (BOP) and in the proportion of shallow pockets (1-3 mm) were observed compared to the scaling procedure alone. Ji et al. (2010) studied the potential of a CS-HTCC/GP-0.1% CHX thermosensitive hydrogel and were able to control the concentration of drug delivery and release rate adjusting the initial concentration of CHX and the concentration of the α,β-glycerophosphate (GP), respectively.

Besides some well-known advantages of gel use, such as the possibility to keep the CHX concentration for a longer period and the possibility to reach the remote sites, gels could also limit the side effects of CHX such as the discoloration on the tooth surface and the bitter taste (Pietruska et al., 2006). Concerning the use of CHX as a treatment for denture stomatitis, CHX was added to denture lining materials and denture acrylic resin. Bertolini et al. (2014) investigated two different resins-based denture soft lining materials charged with chlorhexidine diacetate (CDA) and chlorhexidine hydrochloride (CHC). Both the release and the diameter of the zone inhibition of CDA were a function of the initial concentration, while CHC did not show any antifungal activity once incorporated into the resins, meaning that it was disabled during the preparation or it was not able to diffuse through the resin. Salim et al. (2012) examined the suitability of a self-cured poly(ethyl methacrylate)/tetrahydro-furfuryl methacrylate (PEM/THFM) as CHX delivery system. They found that the leaching kinetic was characterised by a high initial elution followed by a slower and constant release that lasted up to 28 days. Even if CHX is still considered as the gold standard antiseptic

treatment and it is one of the most used agents for mouth irrigation, there is an important issue regarding toxicity and staining. Indeed, brown staining of the oral mucosal, teeth and acrylic dentures have been reported and several studies have shown a toxic effect even at low concentrations (Ellepola and Samaranayake, 2001). Tu et al. (2015) demonstrated that CHX has a cytotoxic effect on stem cells from exfoliated deciduous teeth (SHED cells) in a dose and time manner: 0.1% CHX inhibited the 90% of the viable cells and only concentrations lower than 0.001% (v/v) did not affect proliferation. Cabral and Fernandes (2007) investigated toxicity for human alveolar bone cells, reporting that concentrations higher than 0.005% (v/v) changed the cell shape and the cells' attachment to the substrate.

1.5.2 Triclosan

Triclosan is an organic compound that shows *in vitro* and *in vivo* antimicrobial properties against a wide range of bacteria and certain fungi (Dann and Hontela, 2011). It inhibits the enzyme enoyl-acyl carrier protein reductase, that has a fundamental role in the fatty acids synthase cycle, causing growth inhibition or bacterial lysis (Saunders et al., 2000). Being non-ionic, it can be easily added to toothpastes to increase the antimicrobial properties. The risk of resistance that can follow the widespread use of products containing triclosan has been investigated in a 5 year study by Cullinan et al. (2014). Similar MIC values were reported between samples from the group brushing with 0.3% (w/v) triclosan-dentifrice and the control, meaning that a long exposure to triclosan did not affect the MICs. Moreover, no growth was observed in both groups at a concentration of 0.3% (w/v) of triclosan, normally used within dentifrices. Different solutions have been proposed to enhance the antimicrobial properties of triclosan such as

the combination with other antimicrobial dentifrice-compatible agents (e.g. zinc) or the combination with copolymers to increase its permanence in the mouth (Finney et al., 2003). Several clinical trials and *in vitro* studies have been performed to investigate the efficacy of a triclosan/copolymer toothpaste compared to a negative control toothpaste. Rosling et al. (1997) in a 36 months study found that the addition of triclosan decreased the total number of bacteria counted in adults susceptible to destructive periodontitis. Interestingly, the number of samples containing bacteria associated with periodontal diseases (e.g. *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans*) decreased, while the number of bacterial species associated with oral health (e.g. *Actinomyces naeslundii, Capnocytophaga* and different streptococci) was constant or increased. In addition, the frequency of deep periodontal pockets and the number of sites exhibiting additional probing attachment and bone loss improved, meaning that besides its antimicrobial properties the triclosan/copolymer toothpaste could self-prevent recurrent periodontitis. Similar results were obtained in a 5 year study by Cullinan et al. (2003) that showed that the use of a triclosan/copolymer dentifrice slowed down the formation of the periodontal pocket in subjects with pre-existing attachment loss (probing pocket depth (PDD) > 3.5 mm), while it did not have a significant effect in those with PDD > 2mm. The antimicrobial properties of a triclosan/copolymer dentifrice were also tested by Fine et al. (2006) that found that brushing with triclosan resulted in a 90% reduction of anaerobes at the site. Besides proven antimicrobial efficacy, triclosan has anti-inflammatory properties. Gaffar et al. (1995) found that triclosan inhibited the cyclo-oxygenase and lipoxygenase, decreasing the ability of fibroblasts to produce inflammatory cytokines and inflammatory mediators. In a recent work, Wallet et al. (2012) investigated how triclosan altered the

inflammatory response in different cells of the oral mucosal: human monocytic cells (THP1), human periodontal ligament fibroblasts (HPFL) and human oral epithelial cells. They discovered that the treatment with triclosan caused a dose dependent inhibition of LPS-induced cytokines of THP1 and HPFL, while it did not affect the LPS-induced cytokines of the epithelial cells. However, the pre-treatment of the oral epithelial cells with triclosan inhibited the secretion of LPS-induced cytokines (Wallet et al., 2012).

While several studies reported on the use of triclosan as treatment for periodontal diseases (Cullinan et al., 2003), a limited knowledge on its activity against *C. albicans* is available. Higgins et al. (2012) found that triclosan was fungicidal against azole-susceptible *C. albicans* at a concentration of 16 mg/l but it had an antagonistic activity with fluconazole at sub-inhibitory concentrations.

Besides being used as an ingredient in dental products, triclosan is found in antibacterial soaps, deodorant soaps, dishwashing liquids, cosmetics products kitchen utensils, toys, bedding, clothes, fabrics, and rubbish bags. This wide spectrum of application led to an accumulation of triclosan in the environment (e.g. in the soil and water) that can cause microbial resistance and effect the ecosystem and the human health (Dhillon et al., 2015).

1.6 Essential oils

Essential oils are natural products produced by aromatic plants and mainly composed of terpenes and terpenoids and other molecules (e.g. acids, alcohols, aldehydes, aliphatic hydrocarbons, acyclic esters or lactones) (Nazzaro et al., 2013). The composition and the ratio of the components depend on the plant's origins and influence the antimicrobial properties of the essential oils. Being hydrophobic, they interact with the cell membrane changing its permeability and

causing leaching of intracellular components (e.g. radicals, proteins, calcium ions) and the inactivation of enzymatic mechanisms (Bakkali et al., 2008). Moreover, they can penetrate into the cell and interact with the mitochondrial membrane causing cell death (Nazzaro et al., 2013).

Several essential oils have been reported to have an antifungal or antibacterial activity. Among these, twelve essential oils that showed an activity against oral pathogens, *C. albicans* or periodontal bacteria, were selected. The characteristics of each oil are described in detail in the following sections. In addition, linalool, a compound commonly found in the essential oils and approved by the Food and Drug Administration (FDA), and E-cinnamaldehyde, a terpene with good antimicrobial activity, are discussed.

1.6.1 Basil

Basil (*Ocimum basilicum)* is a culinary and medicinal herb used in folk medicine against headaches, intestinal worms, kidney disorders and as an antispasmodic agent. Its antimicrobial activity is associated with the two main compounds, linalool and methyl chavicol (Cardoso et al., 2016). Basil affects the production of ergosterol, the capsule size of *Candida* and the biofilm formation (Cardoso et al., 2016). Typical minimum inhibitory concentrations (MICs) for *C. albicans* reported are 0.5% (v/v) (Hammer et al., 1999), 0.312% (v/v) (Szweda et al., 2015) and 0.14% (v/v) (Cardoso et al., 2016). In addition, Kraivaphan et al. (2013) reported an antibacterial activity against *P. gingivalis* with minimum bactericidal concentrations (MBCs) of 0.4% (v/v) and 1.6% (v/v) against planktonic and biofilm growth mode, respectively.

1.6.2 Bergamot

Bergamot (*Citrus bergamia*) is obtained from the peel of the fruit and is mainly composed of a volatile fraction (93–96%), whose principal components are limonene (40%), linalool (8%) and linalyl acetate (28%) (Romano et al., 2005). It has anti-inflammatory properties and an antifungal activity with MICs ranging from 1% (v/v) to 10% (v/v) (Hammer et al., 1999; Romano et al., 2005).

1.6.3 Cinnamon

Cinnamon (*Cinnamomum zeylanicum)* is a tropical evergreen tree that grows in Sri Lanka, India and Madagascar (Unlu et al., 2010). Several studies reported antimicrobial and antioxidant properties of both the essential oil and the single compounds (e.g. E-cinnamaldehyde). However, the chemical composition of the oil obtained from the bark, leaf, root and fruit of cinnamon can be significantly different (Paranagama et al., 2002). Unlu et al. (2010) studied the antimicrobial potential of the essential oil from the bark against 21 bacteria and four *Candida* species and found a strong antimicrobial activity against all the microorganisms tested with MICs between 0.004% (v/v) and 0.1% (v/v). Concerning the antimicrobial potential against oral bacteria, cinnamon oil was shown to be active against two cariogenic bacteria, *S. mutans* and *Lactobacillus plantarum*, both in the planktonic and in the biofilm form (Filoche et al., 2005). Moreover, a non-antagonistic antimicrobial effect was observed when cinnamon was added to CHX. The blend resulted in a 10-fold reduction in the CHX concentration needed to achieve the same level of inhibition of CHX alone (Filoche et al., 2005). Concerning the cytotoxicity of the bark oil, experiments on fibroblasts highlighted a toxicity at concentrations higher than 0.001% (v/v) (Unlu et al., 2010).

1.6.4 Citronella

Citronella oil (*Cymbopogon nardus)* is mainly composed of citronellal, geraniol and citronellol which are well known for their antiseptic properties. Besides being an antiseptic, it is reported to have anti-candidal properties at concentrations of 0.25% (v/v) (Hammer et al., 1998) and between 0.004% (v/v) and 0.014% (v/v) (Trindade et al., 2015). In addition, it inhibited the attachment of *C. albicans* to dental implants and cover screws with results similar to Nystatin (Trindade et al., 2015). Moreover, Ocheng et al. (2015) found that citronella oil inhibited the growth of *A. actinomycetemcomitans* and *P. gingivalis* at a concentration of 0.01% (v/v).

1.6.5 Geranium

Geranium oil (*Pelargonium graveolens)* is used in aromatherapy for the treatment of acne, eczema, haemorrhoids, inflammation and for improving circulation (Zore et al., 2010). The main compounds are geraniol, geranyl acetate and citronellol. Zore et al. (2010) explored the antifungal activity of these components against sensitive and non-sensitive fluconazole *C. albicans* strains. Geraniol and geranyl acetate were fungicidal at 0.064% (v/v), while citronellol was fungistatic at 0.256% (v/v). Cytotoxicity evaluation on HeLa cells revealed that the MICs of geraniol and geranyl acetate were not toxic, while the MIC of citronellol killed 4% of cells. Budzyńska et al. (2014) examined the mechanisms of action of geranium in term of expression of virulence factors, germ tube formation, morphology and cell attachment. Both a one hour pre-treatment at MIC (0.097% (v/v)) or a constant exposure at sub-MICs reduced the expression of virulence factors, the formation of germ tubes, the penetration of *Candida* into the agar and the

attachment to epithelial cells. To the best of the author's knowledge, no data on the antibacterial activity against periodontal pathogens is available.

1.6.6 Lavender

Lavender oil (*Lavender angustifolia)* is mostly produced in the Balkans. The main components are non-terpenes, oxides and linalyl. Besides being an anti-depressant, anti-stress, anti-inflammatory and antiseptic, it showed antimicrobial properties against fungi and bacteria (Thosar et al., 2013). Typical MICs against *Candida* were 1.12% (v/v) (Giordani et al., 2004), 0.8% (v/v) (Thosar et al., 2013), 0.25% (v/v) and 0.5% (v/v) (Hammer et al., 1999). Concerning the activity of lavender against oral pathogens, Takarada et al. (2004) observed that lavender inhibited the growth of Gram-negative bacteria with MIC ranging from 0.25% (v/v) to 0.5% (v/v), while it did not affect the growth of oral streptococci. Moreover, it did not kill bacteria meaning that it is bacteriostatic. Low MICs (0.4% (v/v)) against oral anaerobic bacteria were reported by Gursoy et al. (2009). Concerning the cytotoxicity, experiments conducted on human umbilical vein endothelial cells demonstrated that the viability was almost not affected by the presence of the oil, even at high concentrations (0.5% (v/v)) (Takarada et al., 2004).

1.6.7 Melissa

Melissa (*Melissa officinalis*) is a herb used to give fragrance to food and beverage products. It is utilised as a medical plant since different therapeutic effects (e.g. energiser, anticonvulsant, tranquiliser and digestive) have been attributed to the essential oil (Babpour et al., 2009). Besides these functions, it has antimicrobial and antioxidant properties. Concerning its efficacy against oral bacteria, melissa was found to inhibit six out of nine strains of *P. gingivalis* and *Prevotella* spp. at

concentration lower than 0.2% (v/v) (Iauk et al., 2003). Abdellatif et al. (2014) reported an antifungal activity of the oil at concentration of 0.3% (v/v). By contrast, Mimica-Dukic et al. (2004) found that melissa inhibited *Candida* growth at concentrations of 3% (v/v), while it was fungicidal at 6% (v/v).

1.6.8 Myrtle

Myrtle oil (*Myrtus communis)* has anti-inflammatory, antioxidant and antimicrobial properties against both Gram-negative and Gram-positive bacteria. Sulieman (2009) investigated its potential use as a root irrigant finding that concentrations of 35% (v/v) of *Myrtus communis* alcoholic extract solution had an antimicrobial efficacy comparable to that of 0.2% of CHX or 5.25% sodium hypochlorite (NaOCl). Moreover, clinical trials showed that the bacterial count after 3 weeks was not significantly different from the one found with common irrigants (CHX and NaOCl), while it was significant lower from the one obtained with a saline solution. Considering the efficacy of myrtle against bacteria involved with periodontal diseases, Hedayati et al. (2013) reported a MIC50 value and MIC90 against *P. gingivalis* of 0.1% (v/v) and 0.8% (v/v) respectively. Lower values were obtained in another study in which the MICs were calculated using the agar dilution method that gave values ranging from 0.025% (v/v) to 0.2% (v/v) (Gursoy et al., 2009). Concerning antifungal activity, Fani et al. (2014) examined the potential of the oil against oral pathogens, including *C. albicans,* isolated from patients with denture stomatitis. They found that *C. albicans* was sensitive to Turkish myrtle at concentrations of 0.004% (v/v). Mahboubi and Bidgoli (2010) reported a synergistic activity with amphotericin and MICs and minimal lethal concentrations (MLCs) of 0.8% (v/v) - 1.6% (v/v) and 1.6% (v/v) - 3.2% (v/v)

respectively. Cannas et al. (2013) analysed the essential oil obtained from an Italian myrtle and found a higher antifungal activity with MIC of 0.0002% (v/v).

1.6.9 Peppermint

Peppermint oil (*Mentha piperita*) is obtained by distillation of mint leaves. The main components are menthol (38-48%) and menthones (20-30%). It is widely used in food and pharmaceutical industries because of its antiseptic, antibacterial and antiviral properties. It has been reported to inhibit the growth of *C. albicans* at a concentration of 0.05% (v/v) (Thosar et al., 2013) and it showed an anti-biofilm activity, although the inhibition was lower than 50% (Sandasi et al., 2011). Concerning its efficacy against bacteria involved with periodontal diseases, Shapiro et al. (1994) obtained MICs lower than 0.2% (v/v) on obligate anaerobes and lower than 0.6% (v/v) on facultative anaerobes. In addition Shapiro et al. (1994) observed a synergetic effect between peppermint and tea tree oil against *A. actinomycetemcomita*ns and *P. gingivalis*.

1.6.10 Sage

Sage oil (*Salvia officinalis)* is widely used in folk medicine and as flavouring of food products. In a study carried out by Shapiro et al. (1994) the MICs of several essential oils against anaerobic oral bacteria were investigated and sage showed the highest antimicrobial properties together with tea tree oil and peppermint oil. Gursoy et al. (2009) reported MICs of 0.8% (v/v) against oral pathogens, which were higher than those found for *Satureja hortenis* and *Juniperus communis*. In addition, sage essential oil had antifungal properties with typical MICs of 0.3% (v/v) (Sookto et al., 2013) and 1.45% (v/v) (Nacsa-farkas et al., 2014). As stated

by Sookto et al. (2013), sage oil has also anti-adhesive properties inhibiting the adherence of *C. albicans* on poly(methyl methacrylate) (PMMA) resin surface.

1.6.11 Spearmint

Spearmint oil (*Mentha piperita*) has been investigated mostly as a food protective but it also has anti-candidal activity at a concentration of 0.12% (v/v) (Hammer et al., 1999).

1.6.12 Tea tree oil

Tea tree oil (TTO; *Melaleuca alternifolia*) is derived from the paper bark tea tree. It is utilised by Aborigines to treat abrasions, cuts, colds and influenza. Nowadays, it is used as a natural additive in cosmetics and medicine (Soukoulis and Hirsch, 2004). Concerning oral health, TTO is one of the components commonly added to toothpastes to enhance its antimicrobial properties (Hammer et al., 1998). The main components of TTO are terpinen-4-ol and 1,8-cineole. The former has anti-inflammatory and anti-bacterial properties and the latter is also able to penetrate into the human skin becoming an undesirable allergen (Pazyar et al., 2013). For this reason, TTO can cause adverse reactions such as skin irritation, systemic hypersensitivity reaction, allergic contact or systemic dermatitis. Soukoulis and Hirsch (2004) in an eight week study investigated the effect on clinically relevant parameters (i.e. gingival index (GI), papillary bleeding index (PBI) and plaque staining score (PSS)) of a 2.5% TTO-gel compared to a positive control (0.2% CHX-gel) and a negative control (placebo gel). They observed that the 2.5% TTO-gel caused both a reduction of the GI and of the PBI. However, these improvements were not associated with a decrease of the PSS, meaning that the mode of action of the TTOs was more anti-inflammatory

than anti-bacterial. Similar results in terms of GI were obtained in a 6 months study by Kolambkar et al. (2012) that investigated the use of a TTO-gel as a treatment in combination with mechanical debriment. The authors also observed an improvement in the plaque index (PI) similar to that obtained with the placebo gel, meaning that in this case a good oral hygiene was sufficient to avoid formation of the plaque after the scaling and root planing (SRP). By contrast, significant differences were observed on the clinical attachment level (CAL) and on the concentration of Pentraxin-3, a protein produced during the inflammatory process, confirming the anti-inflammatory properties of TTO. Concerning antifungal activity against *C. albicans*, typical MICs range from 0.04% (v/v) to 0.5% (v/v) (Hammer et al., 1999). de Campos Rasteiro et al. (2014) stated a minimum biofilm eradication concentration (MBEC) value of 12.5% (v/v) and evaluated its efficacy as treatment for oral candidiasis in infected mice. They observed that 12.5% (v/v) of TTO caused a reduction in colony forming unit (CFU) of 5.33 $\log_{10}$ compared to a reduction of 0.24 $\log_{10}$ in the control, and they noticed significantly fewer epithelial lesions.

1.6.13 E-cinnamaldehyde

E-cinnamaldehyde is an organic compound found in different species of the genus of Cinnamomum and the major compound of the essential oil of cinnamon bark. E-cinnamaldehyde has antifungal properties being able to interact with the fungal membrane. Taguchi et al. (2013) found that at concentrations lower than 0.001% (v/v) E-cinnamaldehyde did not affect the viability and the metabolic activity of *C. albicans* but it inhibited mycelial growth. By contrast, concentrations higher than 0.004% (v/v) were fungicidal causing changes in the cell membrane.

Quale et al. (1996) reported an antifungal activity against fluconazole resistant *Candida* species at concentrations between 0.003% (v/v) and 0.02% (v/v).

1.6.14 Linalool

Linalool (3,7-dimethyl-1,6-octadien-3-ol) is a terpene alcohol and one of the main components of most essential oils. It is commonly used in hygiene products and as a disinfectant and food additive, since it is recognised as safe by the FDA (Hsu et al., 2013). It has anti-candidal activity at concentrations from 0.09% (v/v) to 0.29% (v/v) (Khan et al., 2010) and between 0.14% (v/v) and 0.5% (v/v) (Hsu et al., 2013). In particular, it is able to inhibit ergosterol synthesis causing changes in the membrane conformation and the leakage of internal components (Khan et al., 2010). Moreover, it interferes with both the formation of biofilms and pre-formed biofilms. Indeed, it inhibits the formation of germ tubes of hyphae which are fundamental for obtaining a strongly attached and compact biofilm (Hsu et al., 2013). Besides its antifungal potential, linalool showed an antibacterial activity against periodontal pathogens with MICs and MBECs between 0.01% (v/v) and 0.2% (v/v) (Park et al., 2012). Concerning the cytotoxicity, Prashar et al. (2004) showed that concentrations lower than 0.044% (v/v) did not affect the viability of endothelial cells and fibroblasts.

Table 1.1 summarises some MICs of essential oils against *C. albicans* reported in the literature.

Table 1.1 - MICs of essential oils against *C. albicans*

Common name	Plant species	MIC [% (v/v)]	References
Basil	*Ocimum basilicum*	0.5, 0.312, 0.14	(Hammer et al., 1999; Szweda et al., 2015; Cardoso et al., 2016)
Bergamot	*Citrus bergamia*	1 - 10	(Hammer et al., 1999; Romano et al., 2005)
Cinnamon	*Cinnamomum zeylanicum*	0.04 - 0.1, 0.03	(Ferhout et al., 1999; Unlu et al., 2010)
Citronella	*Cymbopogon nardus*	0.25, 0.004 - 0.014	(Hammer et al., 1999; Trindade et al., 2015)
Geranium	*Pelargonium graveolese*	0.097	(Budzyńska et al., 2014)
Lavandula	*Lavandula angustifolia*	1.12, 0.8, 0.25, 0.5	(Hammer et al., 1999; Giordani et al., 2004; Thosar et al., 2013)
Melissa	*Melissa officinalis*	0.3, 3	(Mimica-Dukic et al., 2004; Abdellatif et al., 2014)
Myrtus	*Myrtus communis*	0.8 - 1.6, 0.005, 0.0002	(Mahboubi and Bidgoli, 2010; Cannas et al., 2013; Fani et al., 2014)
Peppermint	*Mentha piperita*	0.05	(Thosar et al., 2013)
Sage	*Salvia officinalis*	0.3, 1.4	(Sookto et al., 2013; Nacsa-farkas et al., 2014)
Spearmint	*Mentha spicata*	0.12	(Chao et al.)
Tee tree oil	*Melaleuca alternifolia*	0.04 - 0.5	(Hammer et al., 1999)

1.7 Hydrogels

As stated by Langer and Vacanti (1993) “Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function”. Therefore, the aim of periodontal disease treatment is to restore the alveolar bone, the tooth-associated cementum and the periodontal ligament. Tissue restoration is obtained with scaffolds combined with cells and eventually growth factors (Laurenti and Abdallah, 2015). Cells modulated by growth factors synthesise new extracellular matrix and differentiate, while the scaffold acts as a support and guides the regeneration. Scaffolds should be biocompatible to avoid an immune host reaction and biodegradable to avoid the need for a second surgical intervention to remove it. Moreover, it should allow cell attachment and proliferation and have mechanical and structural properties similar to the biological tissues (Laurenti and Abdallah, 2015). Therefore, a suitable candidate to be used in tissue engineering is a hydrogel. Hydrogels are a 3D-network composed of cross-linked polymeric chains that form an insoluble polymer (Slaughter et al., 2009). Because of the high affinity with water and of the chemical and physical bonds between polymeric chains, hydrogels swell in water without dissolving. Swollen hydrogels have some properties in common with living tissues such as the rubbery consistency and the low interfacial tension that minimises the adsorption of proteins and cells and the consequent risk of immune reactions (Bhattarai et al., 2010). Hydrogels have been widely used in tissue and regenerative medicine, mainly as drug and/or cell delivery systems and as pre-formed or injectable scaffolds (Lee and Mooney, 2001; Slaughter et al., 2009; Kim et al., 2014; Pal et al., 2017). Natural and synthetic polymers can

be used in hydrogel production. The advantages and disadvantages of these types of hydrogels are summarised in Table 1.2.

Table 1.2 - Advantages and disadvantages of natural and synthetic polymers used in tissue engineering applications (Tanzi, 2010; O'Brien, 2011)

	Natural polymers	Synthetic polymers
Advantages	Structure similar to the natural extracellular matrix	Control of kinetics of degradation
	Biocompatible	Good mechanical properties
	Biologically active	Tailored architecture
	Promotion cell adhesion and growth	No risk of disease transmission
	Biodegradable	
Disadvantages	Variability depending on the source	Risk of tissue necrosis because of a decrease in local pH during degradation
	Poor mechanical properties	Reduced biological activity
	Risk of disease transmission	Risk of rejection
	Difficulties in sterilisation and processing	
	Rapid enzymatic degradation	

1.7.1 Synthetic polymers

Synthetic biomaterials are used in scaffold production for their biocompatibility and biodegradability. Compared to natural polymers, synthetics can be fabricated in large scale with a tailored architecture and have higher mechanical stretch that generally results in low degradation rates. The main drawback is the risk of

rejection and of tissue necrosis due to the lowering of the local pH during the degradation process (O'Brien, 2011). Synthetic polymers used in hydrogel production include: poly(lactic acid), poly(glycolic acid), poly(ethylene oxide), poly(ethylene glycol), poly(2-hydroxyethyl methacrylate), and poly(vinyl alcohol).

1.7.1.1 Poly(lactic acid) and poly(glycolic acid) and their copolymers

Poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) are among the most studied polymers because of their biocompatibility (Figure 1.12).

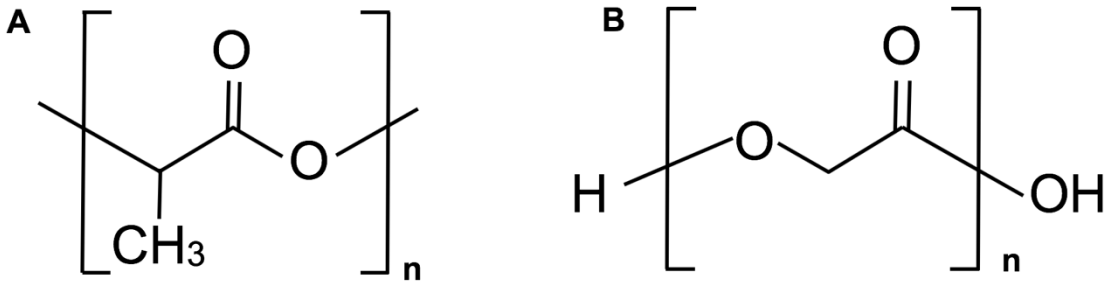


Figure 1.12 - Repetitive unit of poly(lactic acid) (A) and poly(glycolic acid) (B)

Their clinical use has been approved by the US FDA. Because of their mechanical strength and biodegradability, they are mainly used for bone and cartilage regeneration and nervous and cardiovascular tissue engineering (Figure 1.13) (Wang et al., 2010; Narayanan et al., 2016; Santoro et al., 2016). The degradation primarily occurs by hydrolysis but even an enzymatic degradation can take place *in vivo.* The hydrolytic degradation releases carbon dioxide near the site of implantation resulting in a pH decrease. The lowering of the local pH is the main drawback of these polymers causing both an inflammation process and an increased rate of degradation that can lead to the loss of the mechanical properties before the tissue is regenerated. Copolymers PLA-PGA have been

shown to slow down the degradation process (Felix Lanao et al., 2013; Naahidi et al., 2017; Sun et al., 2017).

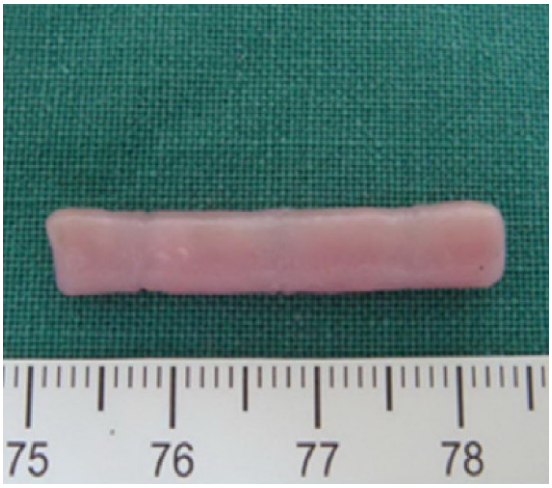


Figure 1.13 - Small diameter elastic blood vessel obtained by culturing PGA and smooth muscle cells in a pulsatile bioreactor for 8 days. Scale in centimetres (Wang et al., 2010)

1.7.1.2 Poly(ethylene oxide) and poly(ethylene glycol)

Poly(ethylene oxide) (PEO) is a hydrophilic, biocompatible polymer approved by the FDA (Figure 1.14)(Naahidi et al., 2017).

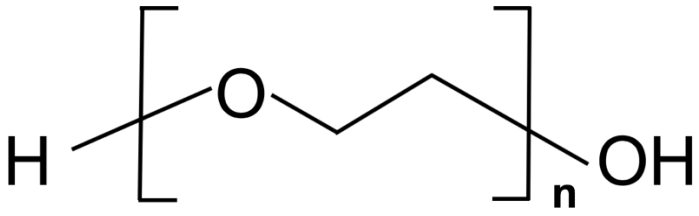


Figure 1.14 - Repetitive unit of poly(ethylene oxide)

Being inert, it has a low immunogenicity and does not interact with proteins and cells (Lee and Mooney, 2001; Naahidi et al., 2017). For these reasons, it has been successfully used to encapsulate cells that do not have to interact with the polymer (Elisseeff et al., 2000; Bryant and Anseth, 2001), but if necessary it could

be modified with the arginylglycylaspartic acid (RGD) sequence to promote cell adhesion (Slaughter et al., 2009). The short version of PEO (i.e. low molecular weight) is poly(ethylene glycol) (PEG) which has the same main features of PEO. Both these polymers can photopolymerise and have adjustable architecture and mechanical properties (Naahidi et al., 2017).

Pluronic, a series of triblock copolymer of PEO and PPO (PEO–PPO–PEO) (poloxamer), is one of the most studied copolymers. Pluronic forms a thermo-reversible gel without cross-linking reagents and is widely used for drug delivery (Park et al., 2009; Diniz et al., 2015). Pluronic's lack of degradability limits its clinical application, therefore copolymers such as PLA-PEO-PLA that degrade and have a temperature dependent reversible sol-gel transition at body temperature have also been synthesised (Kim et al., 2014).

1.7.1.3 Poly(2-hydroxyethyl methacrylate)

Poly(2-hydroxyethyl methacrylate) (PHEMA) is obtained by free radical polymerisation from hydroxyethyl methacrylate (HEMA) (Figure 1.15).

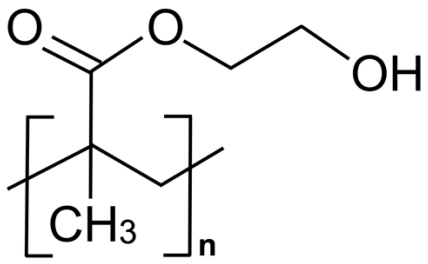


Figure 1.15 - Repetitive unit of poly(2-hydroxyethyl methacrylate)

It is biologically inert and therefore prevents protein adsorption and cell adhesion. It is mainly used in ophthalmology (e.g. artificial cornea of keratoprothesis) and as a drug delivery system (Figure 1.16) (Gulsen and Chauhan, 2005; Slaughter

et al., 2009; Longitudinal et al., 2015; Maity et al., 2016). PHEMA is not degradable at physiological conditions but a dextran-modified PHEMA hydrogel that undergoes enzymatic degradation was successfully synthesised (Meyvis et al., 2000; Lee and Mooney, 2001).

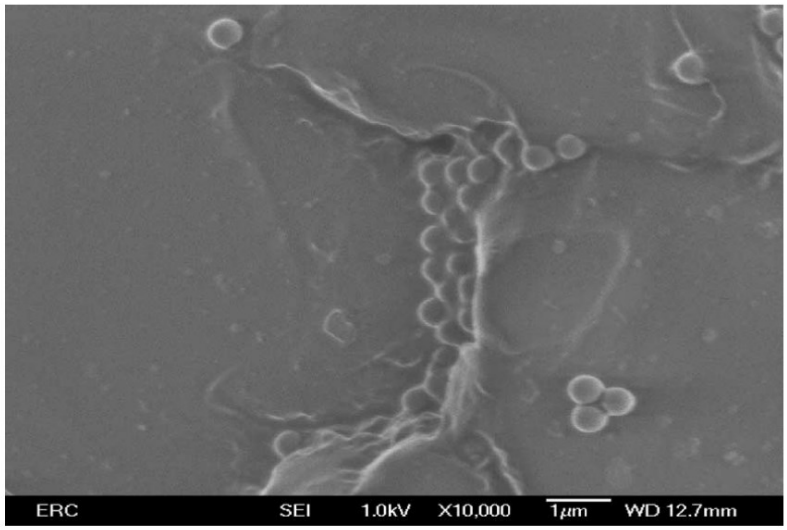


Figure 1.16 - SEM image of a HEMA hydrogel loaded with nanoparticles formulated with an ophthalmic drug. The drug was released from the hydrogel for a period of over 8 days (Gulsen and Chauhan, 2005)

1.7.1.4 Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA) is obtained by hydrolysis of poly(vinyl acetate) (Figure 1.17).

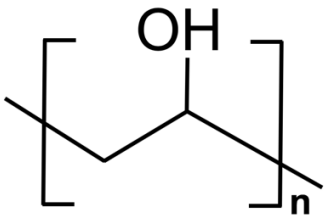


Figure 1.17 - Repetitive unit of poly(vinyl alcohol)

The degree of hydrolysis and the molecular weight determine the hydrophilicity and solubility of PVA. PVA hydrogels can be crosslinked physically, or chemically with glutaraldehyde. Being elastic, PVA can induce cell orientation and matrix synthesis by transmitting the mechanical stimuli to cells. Moreover, it is stronger than other synthetic polymers and it has a low coefficient fraction and structural properties similar to natural cartilage (Figure 1.18) (Oka et al., 2000; Slaughter et al., 2009; Ng et al., 2014). Being non-degradable at physiologically conditions, PVA hydrogels are mainly used as long-term scaffolds (Lee and Mooney, 2001), even if biodegradable copolymers such as the PEG-PVA have also been produced.

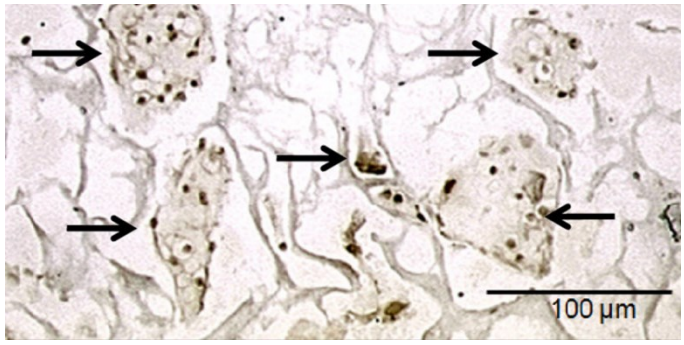


Figure 1.18 - Chondrocytes seeded throughout the PVA scaffold. Cells were viable and proliferate (arrows, BrdU immunohistochemistry) over 1 week in culture (Ng et al., 2014)

1.7.2 Natural polymers

Natural biomaterials are widely used in scaffold production because of their high biocompatibility and degradability. Their degradation can occur enzymatically and by acid-hydrolysis and is a function of the weight and degree of crosslinking of the polymer. Being natural, they interact well with cells allowing their attachment and proliferation. The main disadvantages are a high variability in the properties even within the same batch, a risk of transmission of infections and difficulties in

the production and sterilisation processes (O'Brien, 2011). Natural polymers used in the hydrogel synthesis include: collagen, hyaluronate, fibrin, alginate, chitosan, and methylcellulose.

1.7.2.1 Collagen

Collagen is the main constituent of the extracellular matrix and the most widely used natural polymer. Although it can be physically crosslinked, it is mainly used in the chemically crosslinked form which increases the mechanical properties and reduces degradation (Lee and Mooney, 2001). Collagen is used as a delivery device for cells and growth factors and in tissue engineering applications such as the regeneration of the spinal and vocal cord, cartilage, wound skin, ophthalmology and artificial blood vessel (Rho et al., 2006; Glowacki and Mizuno, 2008; Slaughter et al., 2009; Hesse et al., 2011; Kontturi et al., 2014; Yuan et al., 2014). Copolymers such as collagen-hyaluronate that increase the deposition of the extracellular matrix have also been synthesised (Slaughter et al., 2009).

1.7.2.2 Hyaluronate

Hyaluronate is one of the glycosaminoglycans constituting the extracellular matrix. Hyaluronate hydrogels can be formed by covalent crosslinking of hydrazide derivatives or by radical polymerisation of glycidyl methacrylate. It is naturally and rapidly degraded by hyaluronidase, an enzyme contained in cells and serum (Lee and Mooney, 2001). Besides the fast degradation, the main disadvantages are the low mechanical properties, the risk of a host immune response and the risk of disease transmission (Lee and Mooney, 2001). Because of its high viscoelasticity and space filling properties, it is used in ophthalmic

surgery, soft tissue augmentation, wound healing, artificial skin and osteoarthritis treatment (Slaughter et al., 2009; Tripodo et al., 2015; Kim et al., 2017).

1.7.2.3 Fibrin

Fibrin is a protein present in the blood and can be used to produce autologous scaffolds (i.e. scaffold obtained from the cells of the patient) by enzymatic polymerisation in the presence of thrombin. Fibrin is degraded enzymatically, and the by-products are nontoxic and do not elicit an inflammatory reaction. The degradation process can be controlled by apronitin, a proteinase inhibitor (Lee and Mooney, 2001). Fibrin hydrogels are used to deliver growth factors and cells, whereas copolymers with hyaluronate have been used to deliver chondrocytes, smooth muscle cells and skeletal cells (Figure 1.19) (Linnes et al., 2007; Ahmed et al., 2008; Slaughter et al., 2009). The main limitation of a fibrin hydrogel is the low mechanical properties.

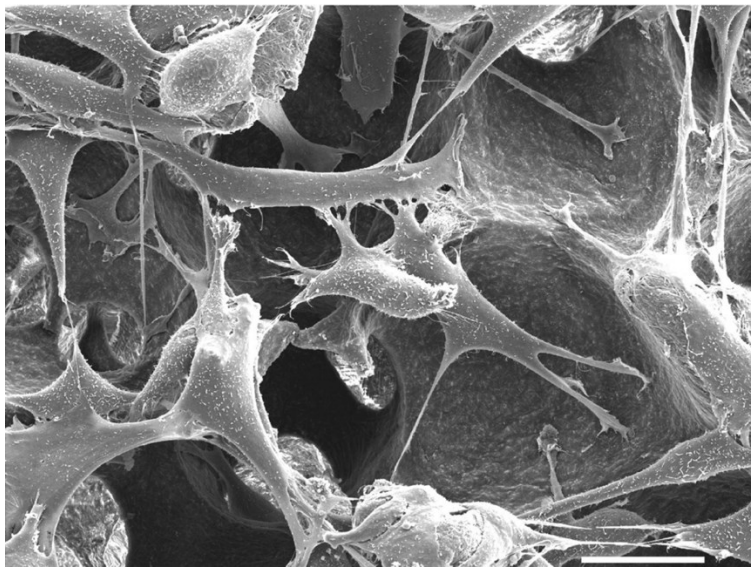


Figure 1.19 - SEM image of NIH 3T3 cells cultured on fibrin scaffold at 1000X magnification. Cells are attached and spread on the fibrin matrix (Linnes et al., 2007). Scale bar = 20μm

1.7.2.4 Alginate

Alginate is obtained from brown algae and is biocompatible, has low toxicity and is cheap. Since it gelifies in the presence of divalent cations, alginate hydrogels can be simply obtained by injecting alginate with an ionic solution. The properties of the resultant hydrogel are a function of the reaction temperature and of the multivalent ions (Slaughter et al., 2009). Applications of alginate hydrogels include wound dressing and cell and drug delivery (Figure 1.20) (Tan and Takeuchi, 2007; Kolambkar et al., 2012; Lee and Mooney, 2012). The main limitations are that its degradation is unpredictable and uncontrollable without a chemical crosslinker, and that it only has weak interactions with cells and protein. Therefore, the surface needs to be functionalised with sequences that promote adhesion (e.g. RGD) (Lee and Mooney, 2001).

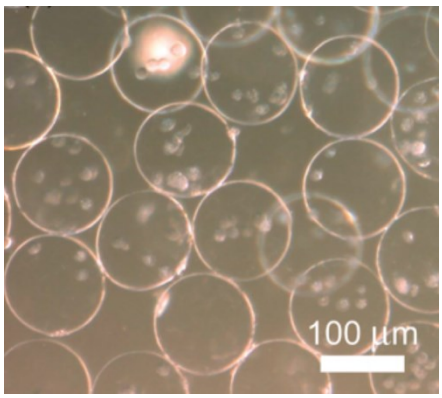


Figure 1.20 - Jurkat cells encapsulated into alginate hydrogel beads (Tan and Takeuchi, 2007)

1.7.2.5 Chitosan

Chitosan is a natural polymer with a structure similar to glycosaminoglycans. It is biocompatible with low toxicity and is enzymatically degraded *in vivo* by chitosanase and lysozyme. Being insoluble, it is difficult to create a chitosan hydrogel at natural conditions and therefore, derivatives have been synthesised

to enhance its solubility. The addition of sugar (e.g. fructose and galactose) showed an improvement of interaction with cells, while proteins such as collagen, albumin and gelatin showed an improvement in neural regeneration (Lee and Mooney, 2001). Chitosan is used in a wide range of tissue engineering applications from wound dressing to orthopaedic application and as a drug delivery system (Khor and Lim, 2003). In addition, chitosan has shown antimicrobial properties against a wide range of microorganisms, including *Candida* and *P. gingivalis* (Ikinci et al., 2002; Peña et al., 2013; Costa et al., 2014).

1.7.2.6 Methylcellulose

Cellulose is the most abundant polysaccharide in nature. It consists of linear chains of (1 -> 4) linked D-glucose units. The crystalline structure created by the intramolecular hydrogen bonds determines the insolubility of cellulose in water. For this reason, derivatives of water soluble cellulose (e.g. methylcellulose, carboxymethylcellulose and hydroxypropylmethylcellulose) have been synthesised (Nasatto et al., 2015b). Because of its reversible thermal behaviour that made it suitable for tissue engineering and cell sheet engineering, methylcellulose is one of the most widely used derivatives (Thirumala et al., 2013; Nasatto et al., 2015b). Indeed, by increasing the temperature, a decrease in viscosity is observed, until a critical temperature is reached and a thermoreversibile hydrogel is formed. Two phases characterise the gelation process: a “clear loose gel” is formed at relative low temperatures (around 50°C) because of the hydrophobic interaction between the methylated glucose zone, whilst when the temperature is increased above 60°C phase separation leads to the formation of a “turbid strong gel” (Li et al., 2001; Nasatto et al., 2015b). In

addition, the thermal behaviour of methylcellulose can be controlled by polymer concentration, molecular weight and the use of additives such as salts, alcohols and surfactants (Nasatto et al., 2015b). Further details on methylcellulose are provided in Chapter 4.

1.7.3 Hydrogel characterisation

1.7.3.1 Morphology

SEM provides information on the morphology of the hydrogel surface. The hydrogel is scanned with an electron beam and the secondary electrons, produced by the interaction between the beam and the atoms, give information about the surface. The sample is required to be dry and conductive so, after being frozen in liquid nitrogen and lyophilised, has to be coated with a conductive material (e.g. platinum, palladium, gold). SEM also allows the study of the adhesion, proliferation and organisation of the cells seeded or encapsulated in the hydrogel (Gulrez et al., 2011).

1.7.3.2 Molecular characterisation

The chemical composition of a hydrogel can be identified using Fourier transform infrared spectroscopy (FTIR). This analytic technique is based on the principle that chemical bonds, excited at a certain wavelength, absorb at a frequency that is typical of the type of chemical bound (Gulrez et al., 2011).

1.7.3.3 Gelation

Thermosensitive hydrogels gellify with an increase in temperature. The gelation kinetics can be investigated by the test tube inverting method or by the rheological

properties of the hydrogel. In the test tube inverting method the solution is kept for one minute at the desired temperature and then inverted for 30 seconds (Ji et al., 2010). The gelation point is defined as the condition in which no flow occurs over 30 seconds. In rheological analysis the gel point is defined as the temperature in which the storage modulus (G') and the loss modulus (G'') crossover (Zuidema et al., 2014).

1.7.3.4 Swelling

The swelling kinetic is measured by monitoring the changes in weight of the hydrogel over time. The hydrogel is firstly dried and weighed and then immersed into a phosphate buffered saline (PBS) solution and removed at specific time intervals. The PBS in excess is removed from the surface with a filter paper and the hydrogel is weighed. The swelling is expressed by the following formula (1.1):

$$S = \frac{W_t - W_0}{W_t} * 100 \qquad (1.1)$$

where S is the swelling and W_0 and W_t are the weights of the hydrogel in the dried state and in a swelled state after a specific time (t), respectively (Gulrez et al., 2011).

1.7.3.5 Degradation

The degradation kinetic is measured by monitoring weight loss overtime. After weighing the sample in the dry state, the hydrogel is incubated in PBS. At specific

time intervals, the hydrogel is removed from the solution, washed with distilled water and dried. The degradation is measured by the following equation (1.2):

$$Weight\ loss\ (\%) = \frac{W_0 - W_d}{W_0} * 100 \quad (1.2)$$

where W_0 and W_d are the weights of the hydrogel in the dried state and after a specific time (t), respectively (Moshaverinia et al., 2012).

1.7.3.6 Porosity and pore size

Porosity and pore size are important parameters that should be taken into account to allow both cell penetration and diffusion of nutrients and metabolic waste. The porosity can be evaluated with different techniques such as gas adsorption, liquid displacement and mercury porosimetry (Lawrence and Jiang, 2017). For example, in the liquid displacement method the porosity is quantitatively determined after having immersed the hydrogel for 48 hours in ethanol with the following equation (1.3):

$$Porosity\ = \frac{W_2 - W_1}{\rho V_1} \quad (1.3)$$

with V_1: initial volume of the scaffold, W_1 and W_2: weights of the scaffold before and after the immersion in ethanol, and ρ: density of the ethanol (Sindhura Reddy et al., 2014).

The pore size can be investigated by imaging methods such as SEM and x-ray computed tomography (XRCT) that provide information not only on the pore size

but also on the porosity, number of open pores and interconnectivity (Lawrence and Jiang, 2017).

1.7.3.7 *In vitro* drug release

The drug release is monitored by tracking the concentration of the drug realised from the hydrogel over time. The concentration of drug in the withdrawn sample is analysed by ultraviolet (UV) spectroscopy, absorbance or gas chromatography–mass spectrometry (GC-MS). Once the drug concentration is calculated, different models (e.g. Zero-Order Model, First-Order Model, Higuchi Model and Korsmeyer-Peppas Model) can be used to evaluate the drug release kinetic (Lobo and Costa, 2001; Ranjha and Qureshi, 2014).

1.8 *Ex vivo* models

When a new treatment is developed, a crucial step is the evaluation of the therapy on a cell model. *In vitro* cell models or human and animal *in vivo* models can be used. *In vitro* cell models use a single cell type or two cell types in case of co-cultures and 3D-organoid cultures (Sloan and Lynch, 2012). Despite being cheap and relatively easy, these models have an important limitation due to the inability to reproduce the *in vivo* cellular organisation. To overcome this limit, *in vivo* models were introduced. They are a gold standard, but they still have some drawbacks: the experiments are expensive, and some ethical issues might arise from the number of animals required. Moreover, clear data are difficult to obtain because of the intrinsic systemic influences (Sloan and Lynch, 2012). For these reasons, *ex vivo* models were also developed. These models maintain the natural arrangement of cells and tissues, while removing systemic influences.

Furthermore, more than one experiment can be carried out on the same animal, reducing the number of animals needed and costs (Sloan et al., 2016).

In 1998 Sloan et al. developed an *ex vivo* culture of incisor rat slices that was used to investigate a wide range of dental tissues infections and repair processes (Sloan and Smith, 1999; Sloan et al., 2000; Ayre et al., 2018). This model was further developed to understand processes involved in bone tissue repair (Sloan et al., 2000; Smith et al., 2010) and to access the biocompatibility and cytotoxicity of drugs (Turner et al., 2002; Waddington et al., 2004). Further details on the *ex vivo* models are provided in Chapter 5.

1.9 Aims and objectives

The aim of this study is to develop a new therapy for oral diseases by utilising a hydrogel as a drug delivery vehicle in combination with an antimicrobial.

Due to the emergence of antimicrobial resistance, the antibacterial and antifungal properties of natural compounds (i.e. essential oils) will be evaluated. Given the *in vivo* application of the novel therapy, the cytotoxicity of the compounds that show the best antimicrobial properties will be investigated. The results obtained by the antimicrobial screening and the cytotoxicity assessment will allow the identification of the best compound to be used in combination with the hydrogel.

Lastly, because of the limits of *in vitro* experiments, an *ex vivo* rodent mandible model to mimic oral candidiasis will be developed. This model will allow the evaluation of the essential oil in an environment that better mimics the *in vivo* conditions.

As mentioned above, the target of the novel therapy is oral diseases and in particular oral candidiasis and periodontal diseases. Oral candidiasis is caused

by *C. albicans*, while periodontal disease is a polymicrobial infection that involves anaerobic bacteria and bone damage. For this reason, the therapy is developed on *C. albicans* and as proof of concept the antimicrobial activity of essential oils is evaluated on a range of periodontal bacteria.

Specific objectives are:

a) To evaluate the antifungal activity of a range of essential oils (12 in total), two terpenes (E-cinnamaldehyde and linalool) and two biocides (chlorhexidine and triclosan) against two *C. albicans* strains in the planktonic and biofilm growth mode
b) To evaluate the antibacterial activity of 5 essential oils, E- cinnamaldehyde and chlorhexidine against two *F. nucleatum* strains, *P. gingivalis* and *P. intermedia,* in the planktonic growth mode
c) To evaluate the cytotoxicity of selected essential oils and chlorhexidine on mouse fibroblasts
d) To evaluate the pro- and anti-inflammatory response of *Melissa officinalis* essential oil on human blood cells
e) To evaluate the antifungal properties of a methylcellulose hydrogel with *Melissa officinalis* essential oil
f) To develop an *ex vivo* rodent mandible model to mimic oral candidiasis

Chapter 2

Antimicrobial properties of essential oils and biocides

2.1 Oral candidiasis

2.1.1 *Candida*

The genus *Candida* comprises over 150 species of 'yeast-like' fungi. *Candida* infections have been reported at most body sites, but they are most frequently encountered on the oral and vaginal mucosa. Although *Candida albicans* is generally isolated from 80% of all oral candidiasis, this organism is also prevalent as a harmless member of the oral microbiota. It is estimated that commensal carriage rate in humans is between 30 and 50% (Singh et al., 2015).

As already mentioned, four main oral diseases named oral candidiasis are associated with *Candida*: pseudomembranous candidiasis, acute atrophic candidiasis, chronic hyperplastic candidiasis, and chronic atrophic candidiasis.

2.1.1.1 Pseudomembranous candidiasis

Pseudomembranous candidiasis is an acute infection characterised by the presence of non-adherent white pseudomembranes on the oral mucosa. The white pseudomembranes, consisting of desquamated epithelial cells, fibrin and fungal hyphae, can be scraped off with a swab to expose the underlying erythematous mucosa. Diagnosis is usually straightforward and can be confirmed microbiologically either by staining a smear from the affected area or by culturing a swab or an oral rinse (Akpan and Morgan, 2002; Williams and Lewis, 2011).

Pseudomembranous candidiasis is mainly found in immunosuppressed patients (e.g. HIV and AIDS) and can be associated with use of steroid inhalers that decrease local immunity, promoting *Candida* growth (Fukushima et al., 2003; Muzyka, 2005; Williams and Lewis, 2011).

2.1.1.2 Acute atrophic candidiasis

Acute atrophic candidiasis is most frequently seen on the dorsum of the tongue and is associated with the prescription of antibiotics. The antibiotic therapy reduces the normal numbers of the oral bacterial community allowing *C. albicans* to overgrow in the mouth (Farah et al., 2000). Acute atrophic candidiasis presents as a red, painful area of the mucosa and may also be seen with patients in conjunction with low serum vitamin B12, low folate and low ferritin concentrations (Akpan and Morgan, 2002). Generally, it is sufficient to interrupt the antibiotic treatment to go back to the healthy level of *Candida* (Williams and Lewis, 2011).

2.1.1.3 Chronic hyperplastic candidiasis

Chronic hyperplastic candidiasis is characterised by hyphal penetration of the oral epithelium (Sitheeque and Samaranayake, 2003) and the presence of an inflammatory cell infiltrate (Williams and Lewis, 2011). Because *Candida* invades the deepest tissues, it cannot be removed simply by scraping. Of particular concern with chronic hyperplastic candidiasis is the potential risk of squamous cell carcinoma (SCC) development at the lesioned site (Krogh et al., 1987; Williams et al., 2001). The exact role of *Candida* in the development of oral cancer is unclear but might be related to the organism's ability to generate carcinogenic nitrosamines from salivary precursor molecules (Williams et al., 2001; Sitheeque and Samaranayake, 2003; Bakri et al., 2010; Ramirez-Garcia et al., 2016) and induce metastasis through a pro-inflammatory response (Ramirez-Garcia et al., 2016).

2.1.1.4 Chronic atrophic candidiasis

Chronic atrophic candidiasis, also known as denture stomatitis, is the most frequently occurring form of oral candidiasis and is present in up to 65% of denture wearers (Williams and Lewis, 2011). This form of oral candidiasis is believed to be promoted through the occurrence of tissue damage caused by frictional irritation of the palatal mucosa by the denture (Williams and Lewis, 2011).

2.1.1.5 *Candida* virulence factors

- Adherence

To colonise, infect and invade the tissues, *Candida albicans* needs first to adhere to the epithelium (Williams and Lewis, 2011). Attachment of *Candida* cells can be mediated by both non-specific and specific factors. Non-specific factors include cell surface hydrophobicity and electrostatic forces (Ramage et al., 2005), whilst specific factors include adhesins (e.g. Als1p, Ala1p, Hwp1p, Int1p, Mnt1p) that bind to complementary receptors present on the host cells or the extracellular matrix proteins (e.g. fibronectin, laminin, collagen, fibrinogen) (Calderone and Fonzi, 2001).

- Morphology

Candida albicans has the ability to grow in different morphological forms including yeast cells, pseudohyphae and true filamentous hyphae. When *C. albicans* attaches to host surfaces, it can switch from a yeast morphology to a filamentous form. The filamentous form can promote adhesion and penetration of the epithelium and increase resistance to phagocytosis by host immune cells. It is

generally believed that the yeast form is predominant in commensal carriage, whilst the hyphal form predominates in tissue penetration, although others have demonstrated that both forms can be associated with commensalism and disease (Calderone and Fonzi, 2001; Soll, 2002; Huang, 2012).

- Phenotypic switching

Candida albicans has the ability to switch spontaneously, reversibly and at high frequencies between different phenotypic forms (Anderson et al., 1990; Gow, 1997; Huang, 2012). Phenotypic switching is characterised by an altered gene expression that causes changes in the antigenicity (Anderson et al., 1990), adhesion (Kennedy et al., 1988; Vargas et al., 1994), sensitivity to neutrophils and oxidants (Kolotila and Diamond, 1990), secretion of proteinases and drug susceptibility (Soll et al., 1989; Vargas et al., 2000; Antony et al., 2009).

- Production of hydrolytic enzymes

Candida albicans secretes hydrolytic enzymes, such as the secreted aspartyl proteinases (SAP) and phospholipases (PL) that destruct the host tissues. Phospholipases are enzymes that hydrolyse phospholipids into fatty acids and contribute to the host cellular lysis and to the exposure of adhesive receptors (Hube and Naglik, 2001; Naglik et al., 2004). Hydrolytic enzymes contribute to host tissue invasion by digesting or destroying cell membranes and by degrading host surface molecules. There is some evidence that hydrolytic enzymes are able to damage cells and molecules of the host immune system, thereby avoiding or resisting antimicrobial activity (Schaller et al., 2005a). Phospholipases (PLs)

contribute to the pathogenicity of *C. albicans* by damaging host-cell membranes, facilitating host-tissue invasion (Borst and Fluit, 2003).

2.2 Periodontal diseases

Periodontal diseases identify an inflammation state of the gingiva and supporting structures of the periodontum (i.e. alveolar bone, periodontal ligament, root cementum) that in the most severe cases lead to tooth loss. Periodontal diseases can be grouped into gingivitis and periodontitis. Gingivitis is an inflammation of the gingiva that results in redness, swelling and bleeding of the tissue (Tonetti et al., 2013; The American Academy of Periodontology, 1999). Since the periodontal ligament and alveolar bone are unaffected, tooth attachment is not compromised. However, gingivitis can turn into periodontitis, a more severe form characterised by the destruction of periodontal ligament and alveolar bone and the migration of the epithelial ligament cells (How et al., 2016). Periodontal diseases also include an aggressive form, named acute periodontitis that mainly affects young people. Other acute forms are classified as necrotising ulcerative gingivitis, which destroys the gingival tissue, and necrotising ulcerative periodontitis in which the bone around the teeth is infected or exposed (Petersen and Ogawa, 2012).

2.2.1 Microbial complexes in the subgingival plaque

As mentioned in Section 1.2.2, the main aetiology of periodontal diseases is the oral microflora and in particular the pathogenic dental plaque associated with it. The “ecological plaque hypothesis” is commonly accepted to describe the development of this pathogenic status (Marsh, 1991). According to this

hypothesis, periodontal diseases are due to a perturbation of the oral environment that causes a shift in the balance of the oral microflora. When the perturbation occurs, pathogenic organisms that in physiological conditions are weakly competitive and present only in a small portion, become prevalent and stronger, leading to periodontal diseases (Marsh, 1991; Marsh, 1994; Marsh and Lewis, 2009).

Socransky et al. (1998) identified the presence of microbial complexes in the subgingival plaque that can be linked to the development of periodontal diseases. Analysing subgingival plaque samples from 185 subjects, the study recognised five different clusters (Figure 2.1). The “red complex” composed by *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* is strongly related to pocket depth. Indeed, its presence increases with pocket depth and bleeding on probing. Similarly, the “orange complex” is found in the deeper pockets and precedes the colonisation of the “red complex”, since bacteria of the “red complex” are rarely found in the absence of the “orange complex”. By contrast, bacteria of the “green”, “yellow” and “purple complex” are poorly associated with the “orange” and “red complex” and are supposed to be host compatible and distinctive of a healthy periodontum status (Socransky et al., 1998).

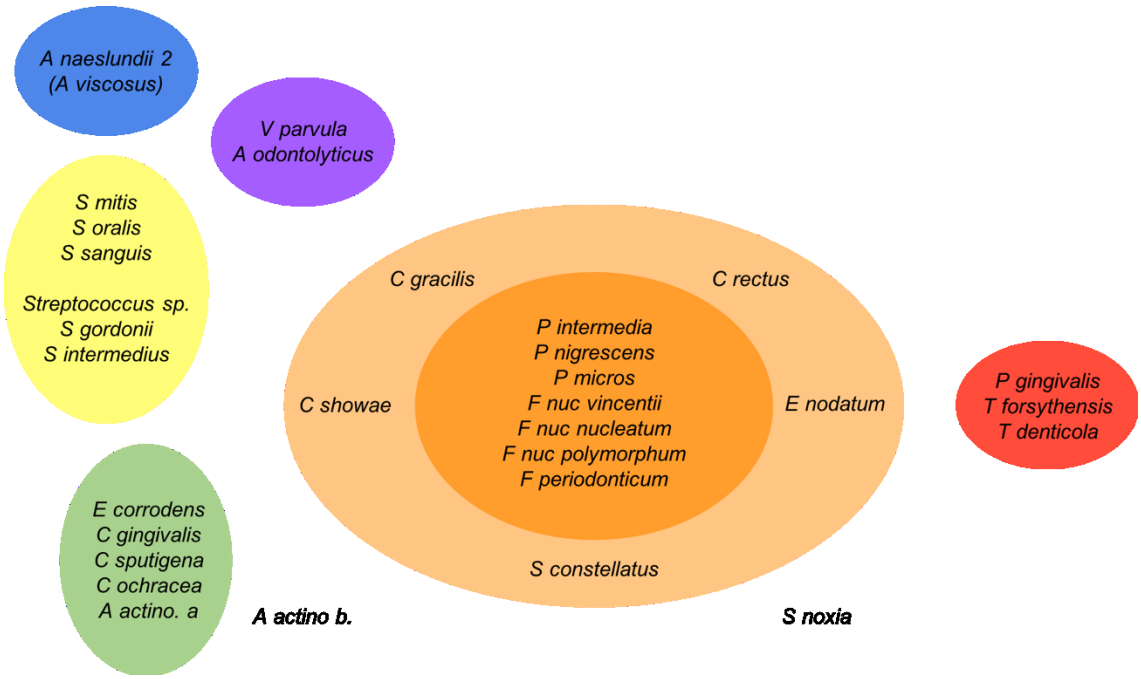


Figure 2.1 - Representation of the relationships of species within microbial complexes and between the microbial complexes. “Red” and “orange complex” are commonly found in the presence of periodontal diseases (Socransky et al., 1998)

2.2.1.1 *Porphyromonas gingivalis*

Porphyromonas gingivalis is a Gram-negative and obligate anaerobic bacterium. It is rod shaped and forms black-pigmented colonies on blood agar plates. *Porphyromonas gingivalis* is one of the main etiological agents implicated in periodontal diseases. How et al. (2016) showed that it was almost non-detectable in subgingival healthy sites or plaque-associated gingivitis, while it was present in 85.7% of subgingival samples collected from individuals suffering from chronic periodontitis. *Porphyromonas gingivalis* is a later coloniser of dental plaque, adhering to the earlier ones. It belongs to the “red complex” and resides mainly in the deepest layers of the periodontal pocket, being able to produce energy

from amino acids in an environment that typically lacks sugar (Bostanci and Belibasakis, 2012; How et al., 2016).

2.2.1.1.1 *Porphyromonas gingivalis* virulence factors

Porphyromonas gingivalis produces a wide range of virulence factors that allow the penetration and as well direct and indirect destruction of the host tissues (Section 1.2.2.1).

The main virulence factors include:

- Capsule

The colonisation of the host environment is due to the ability of bacteria to adhere to a surface and resist to salivary flow. Adhesion is regulated by adhesins that can both be found on the cell wall and on other bacterial structures, such as the capsule and the fimbriae (Marcotte and Lavoie, 1998). In addition, the capsule regulates co-aggregation to other bacteria (Rosen and Sela, 2006; How et al., 2016). Moreover, it can increase bacterial resistance to phagocytosis and antimicrobial peptides, allowing a prolonged presence of bacteria within host sites. For these reasons, the presence and the type of capsule determine the different virulence of *P. gingivalis* strains: capsulated *P. gingivalis* strains are more virulent and cause more invasive infections compared to non-capsulated *P. gingivalis* strains, that are less resistant and quickly phagocytised (Lainel et al., 1997; How et al., 2016).

- Fimbriae

Fimbriae are protrusions of the outer membrane of the bacterial wall. Two fimbriae are present on the *P. gingivalis* membrane encoded by the *fimA* and *mfa1* genes (Amano, 2010). They are involved in the progression of periodontal diseases, being able to interact with host cells, bacteria and molecules present in the extracellular matrix. Moreover, they increase the host response by stimulating the macrophage production of interleukins (Amano, 2010).

- Proteases

Porphyromonas gingivalis proteases are involved in periodontal tissue destruction. Secreted proteases both degrade extracellular proteins (e.g. collagen, immunoglobulin and complement factors) and downregulate the innate immune response (Curtis et al., 2001; Bao et al., 2014).

- Lipopolysaccharide

Lipopolysaccharides are found on the outer bacterial membrane and are composed of a glycan polymer named O-antigen, a core that contains oligosaccharides and a hydrophobic domain named lipid A, which is the virulence factor. The lipopolysaccharide interferes both with the innate immune system, increasing the resistance and the number of bacteria at the site, and with the osteogenic differentiation and mineralisation processes, causing bone disruption (Herath et al., 2011; Kato et al., 2013; How et al., 2016).

- Outer membrane proteins

The outer membrane is an asymmetrical bilayer composed by phospholipids and lipopolysaccharides. Being the external part of the bacteria, it is involved in the

interactions with other bacteria, such as in the formation of oral biofilms, and it acts as a barrier, regulating the movement of molecules through the membrane (Nikaido, 2003; How et al., 2016).

2.2.1.2 *Fusobacterium nucleatum*

Fusobacterium nucleatum is a non-motile Gram-negative bacterium. Despite being anaerobic, it can grow in the presence of up to 6% of oxygen (Bolstad and Jensen, 1996). *Fusubacterium nucleatum* has a crucial role in the development of periodontal diseases, acting as a bridge between early and late colonisers. Early colonisers, mainly the mitis-group of streptococci (e.g. *Streptococcus oralis*, *Streptococcus sanguinis* and *Streptococcus gordonii*) and *Actinomyces* spp., can specifically adhere to the coated tooth surface and co-aggregate with other primary colonisers and *F. nucleatum*. By contrast, secondary colonisers can only co-aggregate with late colonisers and *F. nucleatum.* Therefore, *F. nucleatum* acts as a bridge allowing the co-aggregation between primary and secondary colonisers (Figure 2.2) (Marsh and Lewis, 2009; Seneviratne et al., 2011).

Besides its crucial role in the progression of periodontal diseases, *F. nucleatum* is the periodontal bacterium which is most frequently involved with non-oral infection (e.g. skin ulcers, lung and urinary infections) (Bolstad and Jensen, 1996; Han, 2015).

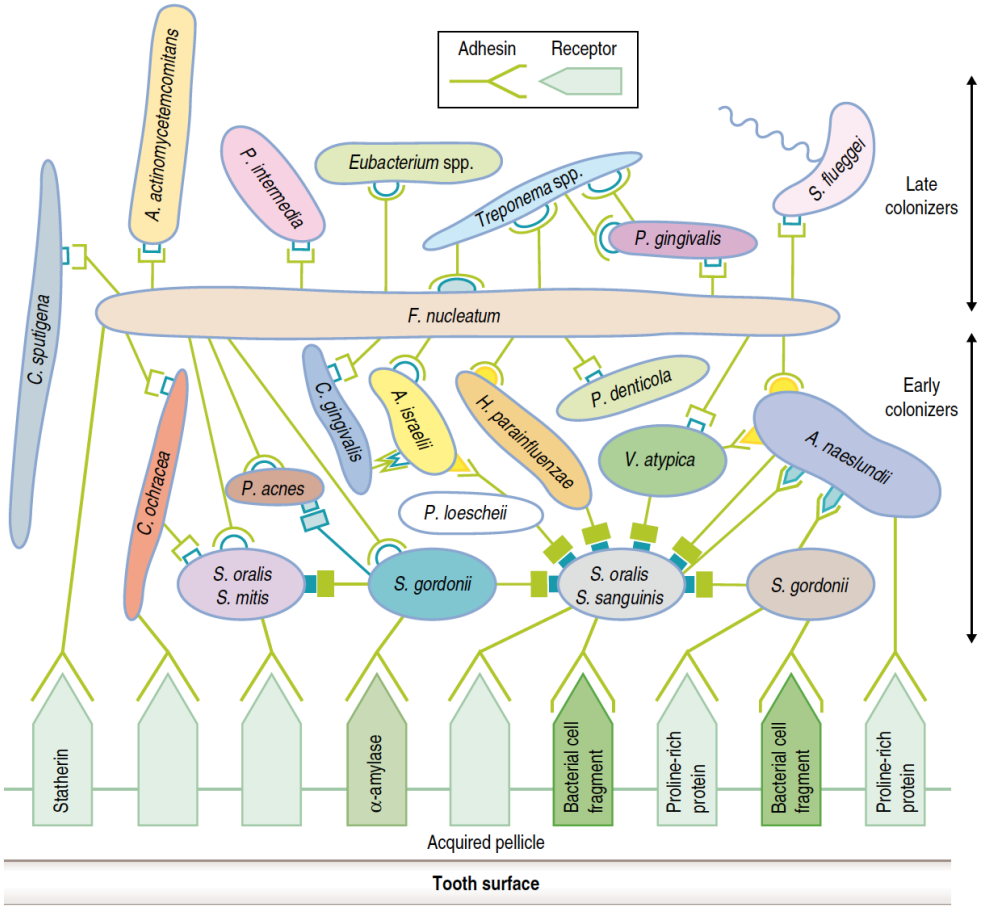


Figure 2.2 - Schematic representation of the patterns of coaggregation/coadhesion in human dental plaque. *Fusobacterium nucleatum* acts as a bridge between early and late colonisers (Marsh and Lewis, 2009)

2.2.1.2.1 *Fusobacterium nucleatum* virulence factors

Virulence factors of *F. nucleatum* are related to its ability to colonise host tissues and to induce a host response. *Fusobacterium nucleatum* can bind to both mammalian cells (e.g. epithelial and endothelial cells, polymorphonuclear leukocytes, monocytes, erythrocytes, fibroblasts) and salivary macromolecules (extracellular matrix proteins and human IgG) (Winkler et al., 1987; Bolstad and Jensen, 1996; Han et al., 2000). A crucial role in the adhesion and subsequent invasion of the host tissues is played by the surface adhesin FadA, which is a surface adhesin unique to oral *F. nucleatum* strains (Han and Wang, 2013). In

addition, *F. nucleatum* can interfere with the host immune system by promoting the secretion of pro-inflammatory molecules (e.g. TNFα and IL-1α) or by suppressing the T-cell response (Bolstad and Jensen, 1996; Han et al., 2000; Han and Wang, 2013).

2.2.1.3 *Prevotella intermedia*

Prevotella intermedia is a black-pigmented Gram-negative anaerobic bacillus, frequently found in the deep pockets of patients that suffer from periodontal diseases (Dahlen, 1993). Similarly, to *P. gingivalis*, *P. intermedia* binds to proteins of the extracellular matrix and co-aggregate with other bacteria.

2.2.1.3.1 *Prevotella intermedia* virulence factors

Prevotella intermedia can both invade host cells, such as gingival fibroblasts, and degrade the extracellular matrix by synthesising proteinases and activating the matrix metalloproteinases (Eley and Cox, 2003; Bao et al., 2008; Alauzet et al., 2010). *Prevotella intermedia* interferes with the host immune system by degrading the complement factor C3 (Potempa et al., 2009). On the other hand, *P. intermedia*'s lipopolysaccharides increase the host inflammatory response by promoting the release of pro-inflammatory mediators (Iki et al., 1997; Tokuda et al., 2001). Moreover, lipopolysaccharides inhibit bone formation by interfering with mineralisation and alkaline phosphatase activity (Alauzet et al., 2010).

2.3 Essential oils: mode of action

Essential oils are natural products produced by aromatic plants and are mainly composed of terpenes and terpenoids (Nazzaro et al., 2013). Antimicrobial, antiseptic, anti-inflammation and anti-oxidant activity of essential oils, alone and in combination with commercial agents, are well known (Bakkali et al., 2008; Santos et al., 2012; Gyawali and Ibrahim, 2014; Morais-braga et al., 2016). However, limited knowledge exists regarding essential oil activity against biofilms and also host cell cytotoxicity. Being lipophilic, essential oils typically integrate into membrane structures causing increased cell permeability, leaching of intracellular components and inactivation of enzymes (Sikkematb and Bontt, 1994; Bakkali et al., 2008).

Concerning the antifungal potential of the selected essential oils, Cardoso et al. (2016) found that basil essential oil and linalool were able to inhibit ergosterol synthesis, which is a sterol that controls the membrane fluidity and integrity. In addition, they observed irregularities in the membrane, presence of vesicles, and cell wall thickening that increase the sensitivity of *Candida* to antimicrobial treatments (Cardoso et al., 2016). Similarly, cinnamon essential oil caused changes in *Candida's* morphology and damaged the wall, the organelles and the cytoplasm (Castro and Lima, 2013; Wang et al., 2018). Singh et al. (2016) observed that when *C. albicans* was treated with citronellal, which is one of the main compounds of geranium and citronellol essential oil, ergosterol levels were reduced and the homeostasis of the *Candida* membrane changed, increasing *Candida*'s sensitivity to treatments. In addition, the production of oxygen reactive species caused oxidative and genotoxic stresses (Singh et al., 2016). E-cinnamaldehyde interfered with the ergosterol synthesis and changed the membrane permeability, causing the leaching of intracellular compounds (e.g.

ATP, K+ and H+ ions) (Ultee and Kets, 1999; Rajput and Karuppayil, 2013; Shreaz et al., 2013; Shreaz et al., 2016). Moreover, E-cinnamaldehyde altered the lipid profile of *C. albicans* wall (Wendakoon and Sakaguchi, 1995; Shreaz et al., 2016). Rajkowska et al. (2016) investigated the mode of action of tea tree and peppermint oil and found that despite different chemical compositions these oils altered the permeability of the *Candida* wall without inhibiting its synthesis. In addition, Samber et al. (2015) observed that peppermint reduced ergosterol levels and inhibited the PM-ATPase causing intracellular acidification and cell death. Furthermore, essential oils can also interact with the mitochondrial membrane leading to cidal effects (Nazzaro et al., 2013). The modes of action of the selected essential oils and two terpenes (E-cinnamaldehyde and Linalool) against *Candida* are summarised in Table 2.1

Table 2.1 - Mode of action of selected essential oils and terpenes against *Candida*

Antimicrobial	Mode of action	Reference
Basil	Ergosterol inhibition Reduction in capsule size Irregularities in the membrane Cell wall thickening Presence of vesicles	Cardoso et al. 2016
Cinnamon	Organelles and cytoplasm destruction Cell wall damage	Castro and Lima 2013 Wang et al. 2018
Citronella	Reduction in ergosterol level Increased production of reactive oxygen species Interference with membrane homeostasis	Singh, Fatima and Hameed 2016
Geranium	Reduction in ergosterol level Increased production of reactive oxygen species Interference with membrane homeostasis	Singh, Fatima and Hameed 2016
Peppermint	Alteration in wall permeability Inhibition of the PM-ATPase Intracellular acidification Reduction in ergosterol level	Rajkowska et al. 2016 Samber et al. 2015
Tea tree oil	Altered wall permeability	Rajkowska et al. 2016
E-cinnamaldehyde	Inhibition of cell wall synthesis Altered regulation of intracellular ATP and ions Alteration in cell morphology Alteration in lipid profile Reduction in ergosterol level	Rajput and Karuppayil 2013 Shreaz et al. 2013, 2016 Ultee and Kets 1999
Linalool	Ergosterol inhibition Reduction in capsule size Irregularities in the membrane Cell wall thickening Presence of vesicles	Cardoso et al. 2016

Currently, knowledge is limited regarding the mode of action of essential oils against periodontal pathogens. Recently, Wang et al. (2018) investigated the effect of cinnamon oil on *P. gingivalis* and observed that bacteria treated with the

essential oil showed a loss of membrane integrity and an enhanced permeability that led to cell death. Overall, the modes of action of essential oils on bacteria are similar to those reported on *C. albicans* and mainly linked to the hydrophobicity of essential oils. Changes in membrane permeability result in leaching of intracellular components. Moreover, the destruction of the cell membrane causes changes in some vital functions, such as the production of ATP, synthesis of macromolecules, secretion of enzymes and nutrient processing. In addition, essential oils can interfere with protein synthesis and pH homeostasis (Faleiro, 2011). Besides changes in the morphology, essential oils can modify the periplasmic spaces and the presence of fimbriae. Lastly, natural compounds can inhibit quorum sensing, a cell-cell communication system based on the production of small molecules (Faleiro, 2011).

2.4 Biocides: mode of action

Chlorhexidine is an antiseptic agent commonly used in hospitals for sanitation and disinfection, and in oral products such as mouthwashes (Cordenonsi et al., 2013). Chlorhexidine shows antimicrobial properties against a wide range of microorganisms, such as Gram-positive and Gram-negative bacteria and fungi. The antimicrobial properties of CHX are mainly due to the leakage of intracellular constituents. CHX interacting with the outer cell layers changes the permeability, without causing lysis or cell death. However, the uptake of CHX by passive diffusion causes attack of the bacterial cytoplasmic or inner membrane or the yeast plasma membrane that results in the leaching of intracellular constituents (Donnell and Russell, 1999). The uptake of CHX is very rapid, within 20 seconds (Fitzgerald et al., 1989; Donnell and Russell, 1999). Interestingly, the leakage of

the internal constituents is only partially a function of the CHX concentrations. Indeed, by increasing the CHX concentration, the release of intracellular compounds increases, but higher concentrations of CHX cause the coagulation of intracellular compounds resulting in a decrease in leakage rate (Hugo and Longworth, 1964; Hugo and Longworth, 1965; Donnell and Russell, 1999).

Triclosan is a biocide commonly found in personal care products (e.g. shampoos, hand soap, deodorants), in medical devices (e.g. surgical sutures and catheters) and in oral formulations (e.g. toothpastes and mouthwashes) (Yueh and Tukey, 2016) . It exhibits bacteriostatic and fungistatic activity at low concentrations (0.1 to 10 μg/ml) and bactericidal and fungicidal activity at higher dosages (World Health Organization, 2006). The uptake of triclosan interferes with lipid synthesis, by inhibiting the NADH-dependent enoyl-acyl carrier protein (ACP) reductase, and RNA and proteins synthesis. In addition, at lethal concentrations triclosan causes K+ leakage (Donnell and Russell, 1999; Ikinci et al., 2002; Russell, 2004; Russell, 2018).

2.5 Synergistic activity

The low rate of discovery of new antibiotics and antifungals and the concomitant increase of resistance led to an interest in investigating the potential of blending antimicrobials. Drug combinations could both delay the emergence of antimicrobial resistance and enhance the antimicrobial properties allowing the use of lower doses of each drug. In particular, the combination of two agents can be synergistic if the effect is much stronger than an additive effect, or indifferent if it is weaker. When the blend shows much lower antimicrobial properties than

those observed by the use of the single drug, the effect is antagonistic. In this case, one of the drugs inhibits the effect of the other (Cuenca-Estrella, 2004; Bollenbach, 2015) (Figure 2.3).

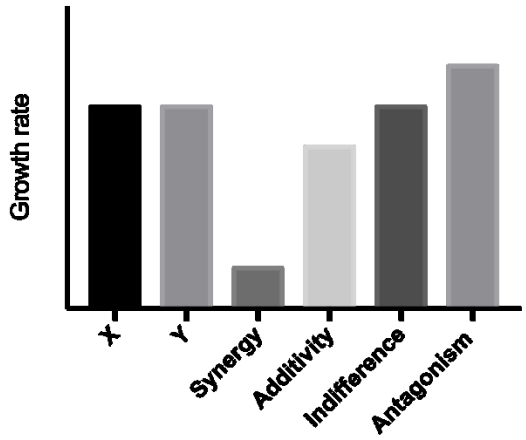


Figure 2.3 - Synergistic, additive, indifferent and antagonistic effect obtained by the combination of drug X and Y

The “checkerboard” method is used to investigate the synergy between two compounds (X and Y). The microorganism is cultured in the presence of 4-5 double serial dilutions below the minimum inhibitory concentration of the compound X combined with 4-5 double serial dilutions below the minimum inhibitory concentration of the compound Y. The fraction inhibitory concentration (FIC) index is the mathematical expression used to represent the interaction between the compounds X and Y. The FIC is expressed as (2.1):

$$FIC = A + B \tag{2.1}$$

with $A = \frac{MIC\ X\ in\ combination\ with\ Y}{MIC\ X\ alone}$ and $B = \frac{MIC\ Y\ \ in\ combination\ with\ X}{MIC\ Y\ alone}$

where X is the first antimicrobial and Y the second antimicrobial.

A FIC index < 0.5 implies synergy, 0.5 < FIC index < 1 implies additivity, 1 < FIC index < 4 implies indifference and a FIC index > 4 implies antagonism (Hsieh et al., 1993).

Several studies evaluated the synergistic activity of essential oils in combination with antifungal agents, revealing that essential oils can be a complementary therapy in the treatment of antifungal infections. Citronellol, geraniol, geranyl acetate, linalool, basil and peppermint combined with fluconazole showed a synergistic effect against *C. albicans*, decreasing the MIC of fluconazole up to 64-fold (Zore et al., 2010; Zore et al., 2011; Samber et al., 2015; Cardoso et al., 2016). Similarly, the combination of myrtle with amphotericin B showed a marked synergism against *C. albicans* (Cannas et al., 2013). In addition, CHX combined with eucalyptus or tea tree oil had a synergistic effect against both *C. albicans* and *S. epidermidis* biofilm (Karpanen et al., 2008; Hendry et al., 2009). Even if a synergistic effect of blends of essential oils was not found, an additive effect such as with clove and cinnamon oil may be present against *C. albicans* (Horváth et al., 2016). Few data on the synergistic effect of essential oils against periodontal pathogens are available. Didry et al. (1994) found an additive effect between some terpenes commonly found in the essential oils (cinnamaldehyde, thymol, carvacrol and eugenol), and Shapiro et al. (1994) observed a synergetic effect between peppermint and tea tree oil against *A. actinomycetemcomita*ns and *P. gingivalis*.

2.6 Aims and objectives

The aim is to develop a novel antimicrobial therapy for oral diseases, focusing on oral candidiasis and periodontal diseases. Due to the emergence of antimicrobial resistance, the antibacterial and antifungal properties of natural compounds (i.e. essential oils) are evaluated. The specific objectives are:

a) To evaluate the antifungal activity of a range of essential oils (12 in total), two terpenes (E-cinnamaldehyde and linalool), and two biocides, namely chlorhexidine and triclosan, against two *C. albicans* strains in the planktonic growth mode
b) To evaluate the antibacterial activity of 5 essential oils, E- cinnamaldehyde and chlorhexidine against a range of anaerobic bacteria involved with periodontal diseases (two *Fusobacterium nucleatum* strains, *Porphyromonas gingivalis* and *Prevotella intermedia*) in the planktonic growth mode
c) To evaluate the antifungal activity of a range of essential oils (12 in total), two terpenes (E-cinnamaldehyde and linalool), and two biocides, namely chlorhexidine and triclosan, against two *C. albicans* strains in the biofilm growth mode
d) To investigate the synergistic effect between the compounds that showed the best antifungal activity

2.7 Materials and methods

2.7.1 Essential oils and biocides preparation

Twelve commercial essential oils (Essential Oils Direct Ltd., Oldham, UK) (Table 2.2), two terpenes (E-cinnamaldehyde and linalool) (Sigma-Aldrich, Gillingham, UK)), two biocides, chlorhexidine digluconate (CHX) (Sigma-Aldrich, Gillingham, UK) and triclosan (Irgasan from Sigma-Aldrich, Gillingham, UK) were evaluated.

Table 2.2 **-** List of commercial essential oils tested

Plant Species	Essential Oil	Origin
Ocimum basilicum	Basil oil	Leaves
Citrus bergamia	Bergamot FCF oil	Peel
Cinnamomum zeylanicum	Cinnamon leaf oil	Leaves
Cymbopogon winterianus	Citronella oil	Aerial parts
Pelargonium graveolens	Geranium oil	Flowering herb
Lavandula angustifolia	Lavender oil	Flowering herb
Melissa officinalis	Melissa oil	Leaves and tops
Myrtus communis	Myrtle oil	Leaves
Mentha piperita	Peppermint oil	Whole plant
Salvia officinalis	Sage oil	Leaves
Mentha spicata	Spearmint oil	Aerial parts
Melaleuca alternifolia	Tea tree oil	Leaves and twigs

The commercial essential oils were tested at a range of concentrations against planktonic growth (0.007% (v/v) to 2% (v/v)) and biofilms (0.125% (v/v) to 8% (v/v)). All agents were prepared in Sabouraud dextrose broth (SDB; Oxoid Ltd, Basingstoke, UK) for *Candida* or fastidious anaerobe broth (FAB, Lab M, Lancashire, UK) for periodontal pathogens, respectively. To enhance dispersion of essential oils in the medium, 1% (v/v) Tween 80 (Sigma-Aldrich, Gillingham, UK) was added. Since the concentrations of essential oils in biofilm studies were

higher, 0.015% (w/v) Agar Bacteriological (LP0011; Oxoid Ltd, Basingstoke, UK) was also added to the culture medium (Mann and Markham, 1998). Serial doubling dilutions of CHX were prepared in water at concentrations between 3.1 $\times$ 10^{-4} % (v/v) and 0.04% (v/v), and from 6.2 × 10^{-4} % (v/v) to 0.08% (v/v) for planktonic and biofilm growth experiments, respectively. A 20% (w/v) stock solution of triclosan was prepared in Dimethyl Sulphoxide (DMSO) (Fisher Scientific, Loughborough, UK). Serial doubling dilutions of the stock solution were prepared in SDB yielding final concentrations from 5.2 × 10^{-6} % (v/v) to 6.7 × 10^{-4} % (v/v) and from 1.7 × 10^{-4} % (v/v) to 5 × 10^{-3}% (v/v) for planktonic and biofilm experiments, respectively.

2.7.2 Microorganisms

Candida albicans NYCY 1363 and *C. albicans* 135BM2/94 were used to test the antifungal properties of essential oils and biocides. *Candida albicans* 135BM2/94 is a clinical strain from the School of Dentistry (Cardiff University), which has been described as a high invader using an *in vitro* tissue model (Malic et al., 2007). Strains were subcultured onto Sabouraud dextrose agar (SDA) (CM0041; Oxoid Ltd, Basingstoke, UK) and grown at 37 °C in an aerobic incubator for 24 hours. A colony of *C. albicans* was inoculated in 20 ml of SDB and incubated aerobically with shaking (200 rpm) overnight at 37 °C. The overnight culture was prepared in SDB to a turbidity equivalent to a 0.5 McFarland Standard (10^5 CFU/ml) and used for further experiments.

Periodontal pathogens, namely, *Fusobacterium nucleatum* KS 515, *Fusobacterium nucleatum* THOWN, *Porphyromonas gingivalis* and *Prevotella intermedia* were used to investigate the antibacterial properties of the above-

mentioned antimicrobials. Strains were subcultured onto fastidious anaerobe agar (FAA, Lab M, Lancashire, UK) supplemented with 10% (v/v) horse blood defibrinated (TCS Biosciences Ltd, Buckingham, UK) and grown at 37 °C in an anaerobic incubator for 24 hours (*Fusobacterium nucleatum)*, 48 hours (*Prevotella intermedia)* or 72 hours (*Porphyromonas gingivalis)*. A colony was inoculated in 10 ml of FAB and incubated anaerobically for 24 - 72 hours at 37 °C. The overnight culture was prepared to a turbidity equivalent to 10^8 CFU/ml and used for further experiments.

2.7.3 Minimum inhibitory concentration (MIC) and minimal lethal concentration (MLC)

The minimum inhibitory concentration (MIC) and the minimal lethal concentration (MLC) were determined using a broth microdilution assay. The method was adapted from that previously reported by Malic et al. (2013). Briefly, 100 µl of antimicrobial and 100 µl of the overnight culture diluted to 1×10^5 CFU/ml (*Candida*) or 1×10^8 CFU/ml (anaerobic bacteria) were added to the wells of 96-well microtitre plates (Thermo Fisher Scientific, Hemel Hempstead, UK). Controls included microorganism suspension cultured in SDB/FAB, with or without 0.5% (v/v) of Tween 80. In addition, when triclosan was tested, SDB containing 1% (v/v) DMSO was used as a control. The plates were covered with the lids supplied by the manufacturer which were sprayed with 3% (v/v) of Triton 100-X (Sigma-Aldrich, Gillingham, UK) in pure ethanol to reduce condensation. The plates inoculated with *Candida* were incubated aerobically at 37 °C with shaking at 130 rpm, for 24 hours. The plates containing anaerobic bacteria were incubated anaerobically at 37 °C with shaking at 130 rpm, for 24 - 72 hours. Growth was estimated by measuring turbidity of each well by spectrophotometric absorbance

at 600 nm (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer), shaking for 3 seconds before the reading. The absorbance readings were standardised against microbial-free controls. The minimal inhibitory concentration 80 (MIC80) was defined as the lowest concentration of the antimicrobial agent that showed at least 80% reduction in absorbance compared to the control. The minimal lethal concentration (MLC) was determined by plating content of selected wells (where no visible growth was evident) onto SDA and incubating for 24 hours at 37 °C. The MLC was defined as the lowest concentration of antimicrobial agent that killed the *Candida* as shown by no colony growth on SDA. All concentrations were tested in quadruplicate and on three separate occasions.

2.7.4 Minimal biofilm eradication concentration (MBEC)

The minimal biofilm eradication concentration (MBEC) method was adapted from Malic et al. (2013). Briefly, a 96-well microtitre plate containing 200 µl of an overnight culture diluted to 1×10^5 CFU/ml was incubated for 48 hours at 37 °C without agitation to allow biofilm formation. Controls included *Candida* suspension cultured in SDB, with or without 1% (v/v) of Tween 80 and 0.015% (w/v) agar bacteriological. When triclosan was tested, SDB containing 8% (v/v) DMSO was also used as a control. After 48 hours, the SDB was removed and the microtitre plate inverted onto tissue paper to remove residual medium. The biofilm was washed three times with 100 µl of PBS. One hundred microlitres (100 µl) of test agent was added to the biofilm and the plate incubated statically for 24 hours at 37 °C. After incubation, test agent was removed, and the biofilm washed twice with 100 µl of PBS. Two hundred microlitres (200 µl) of SDB was added to each well and the biofilm disrupted by repeated pipetting. The three replicates

were then pooled by pipetting into an eppendorf tube which was then centrifuged for 3 minutes at 3000 rpm (Hettich Universal Mikro 12-24, Hettich, Tuttlingen, Germany). The supernatant containing the residual test agent was discarded and the microorganisms re-suspended in fresh SDB and added to three wells of a fresh 96-well plate. The turbidity of the suspension was measured by spectrophotometer absorbance at 600 nm prior to and after incubation for 24 hours at 37 °C with shaking at 130 rpm. The minimal biofilm eradication concentration 80 (MBEC80) was defined as the lowest antimicrobial concentration that prevented at least 80% regrowth of *Candida*. All experiments were conducted on three separate occasions.

2.7.5 Checkerboard method: essential oils and biocides synergy

The checkerboard method was used to investigate the synergistic activity of essential oils and biocides against *Candida*. Table 2.3 summarises the blends of essential oils and biocides screened.

Table 2.3 -The blends of essential oils and biocides tested are highlighted in green

	CHX	Cinnamon	E-cinnamaldehyde	Geranium	Lavender	Peppermint	Spearmint
CHX							
Cinnamon							
E-cinnamaldehyde							
Geranium							
Lavender							
Peppermint							
Spearmint							

Four two-folds dilutions of the MICs were prepared (i.e. MIC/2, MIC/4, MIC/8 and MIC/16). Since the final concentrations in the 96-well plates were diluted 1:4, stock solutions were four times more concentrated. Fifty microlitres (50 µl) of compound X and 50 µl of compound Y were added to a 96-well plate and mixed gently by pipetting up and down. Each well was inoculated with 100 µl of a standardised culture of *C. albicans* at a concentration of 1×10^5 CFU/ml (see Section 2.7.2). The 96-well plate was incubated for 24 hours at 37 °C and the growth was assessed by measuring the turbidity of each well by spectrophotometric absorbance at 600 nm as described in Section 2.7.3. The experiments were carried out in triplicates and the relative growth was measured by averaging three replicate wells. The synergy was evaluated when the combinations of compounds X and Y caused a reduction in growth of at least 80% (i.e. MIC80).

The fractional inhibitory concentration (FIC) index was used to assess the interaction between two compounds (see Section 2.5).

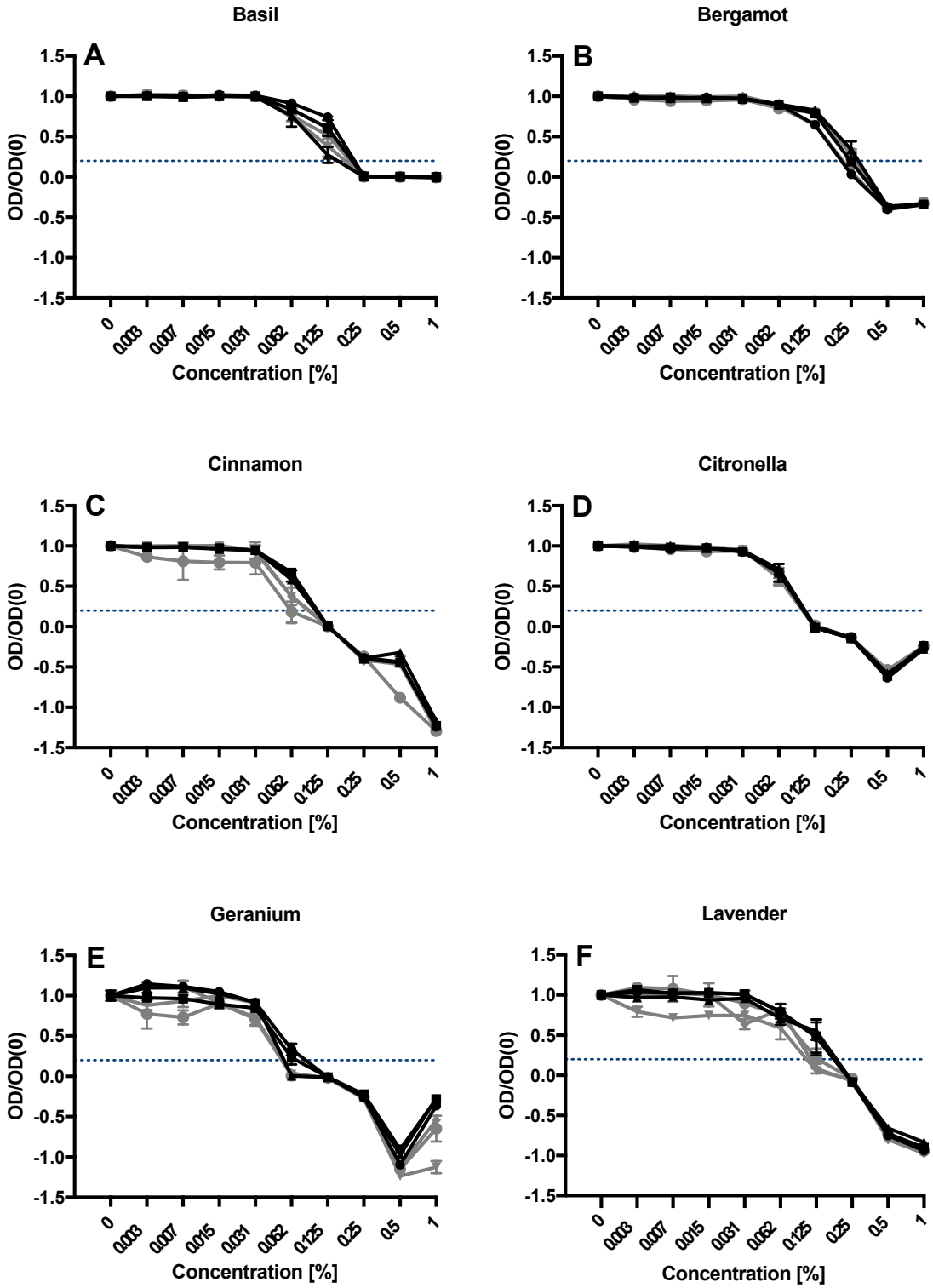
2.7.6 Statistical analysis

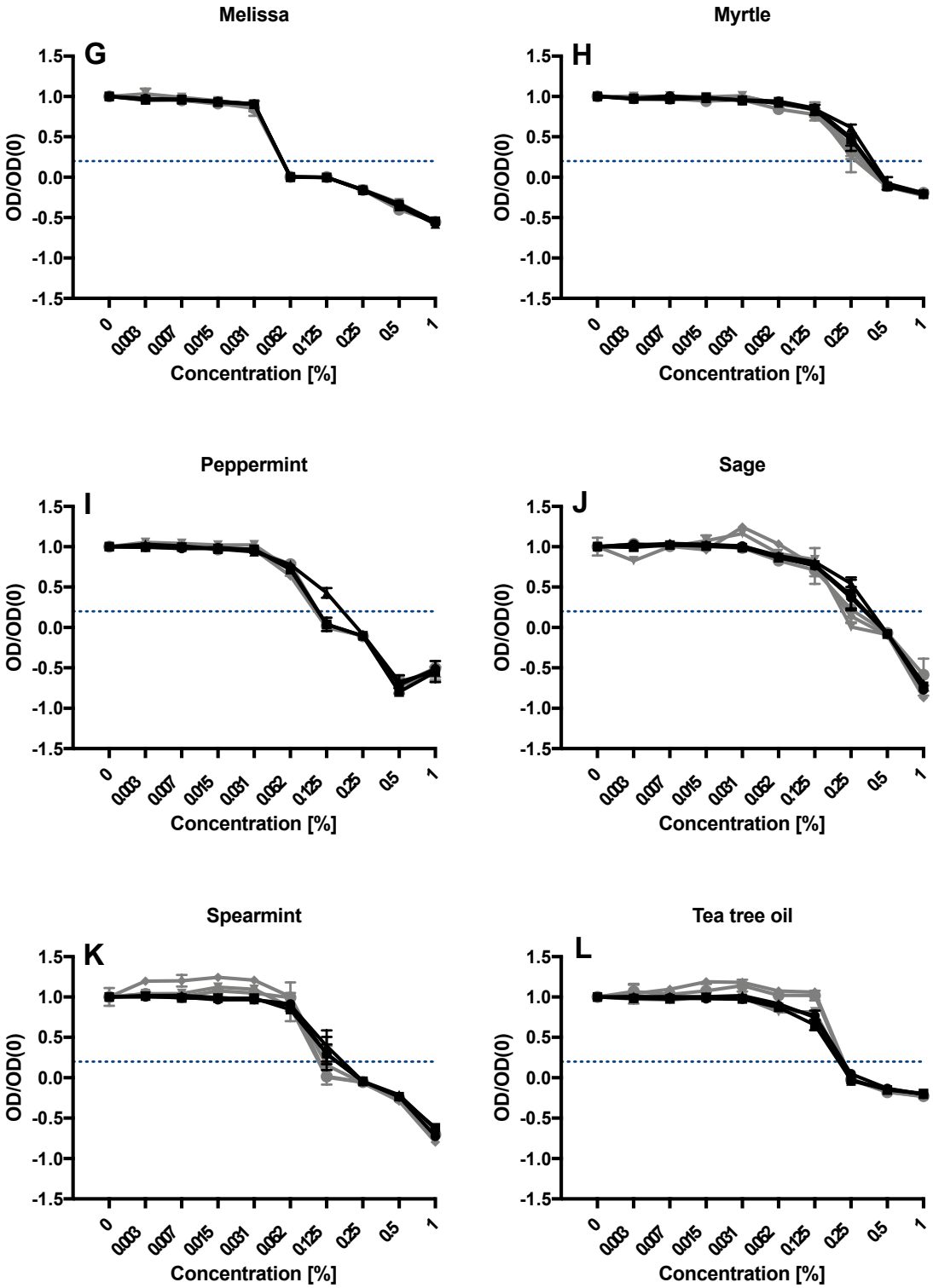
Statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). Data were presented as arithmetic mean ± SD. The difference between treatments was statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. Statistically significant differences were set at $p < 0.05$.

2.8 Results

2.8.1 Minimum inhibitory concentration (MIC) and minimal lethal concentration (MLC) for *Candida albicans*

The antifungal activities of the essential oils and biocides against *C. albicans* in planktonic form was investigated using the microdilution assay. Both *C. albicans* strains were susceptible to all biocides and essential oils tested (Figure 2.4). Indeed, a drop in *C. albicans* growth was observed with increasing concentrations of the compounds. The MIC80 (the concentration that leads to 80% reduction in growth) is represented by the intersection between the blue line (i.e. 80% reduction compared to the control) and the *C. albicans* growth curves.


Basil
A
Bergamot
B
Cinnamon
C
Citronella
D
Geranium
E
Lavender
F
OD/OD(0)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
0
0.003
0.007
0.015
0.031
0.062
0.125
0.25
0.5
1
Concentration [%]


Melissa
G
Myrtle
H
Peppermint
I
Sage
J
Spearmint
K
Tea tree oil
L
OD/OD(0)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
0
0.003
0.007
0.015
0.031
0.062
0.125
0.25
0.5
1
Concentration [%]

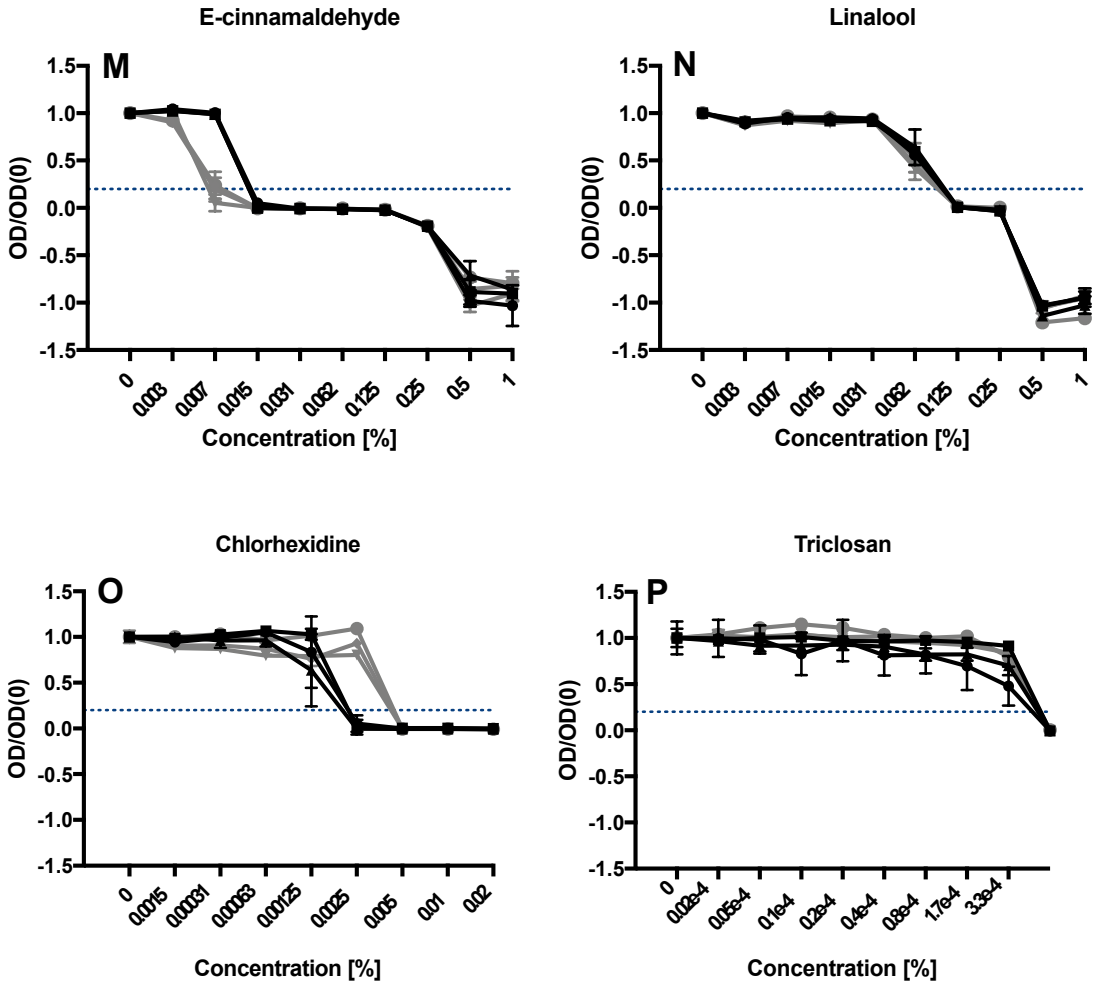


Figure 2.4 - Relative growth of *C. albicans* NYCY 1363 (black) and *C. albicans* 135BM2/94 (grey) in the planktonic growth mode in the presence of basil (A), bergamot (B), cinnamon (C), citronella (D), geranium (E), lavender (F), melissa (G), myrtle (H), peppermint (I), sage (J), spearmint (K), tea tree oil (L), E-cinnamaldehyde (M), linalool (N), chlorhexidine (O) and triclosan (P). Data represent the mean of three independent experiments, each performed in quadruplicate

The minimum inhibitory concentration 80 of the test agents against *C. albicans* NCYC 1363 and *C. albicans* 135BM2/94 are summarised in Table 2.4. The commercial essential oils that inhibited the growth at the lowest concentrations were melissa and geraniol, while myrtle and sage had the lowest fungistatic potential ($p < 0.001$).

Table 2.4 - Minimum inhibitory concentration 80 of essential oils and biocides against *C. albicans* NYCY 1363 and *C. albicans* 135BM2/94 in the planktonic form

Antimicrobial	**Minimum Inhibitory Concentration 80 [% (v/v)] [(g/l)]**	
	***C. albicans* NYCY 1363**	***C. albicans* 135BM2/94**
Basil	0.1 (0.9)	0.1 (0.9)
Bergamot	0.3 (2.6)	0.3 (2.6)
Cinnamon	0.1 (1.0)	0.1 (1.0)
Citronella	0.1 (0.9)	0.1 (0.9)
Geranium	0.07 (0.6)	0.06 (0.5)
Lavender	0.2 (1.8)	0.1 (0.9)
Melissa	0.06 (0.5)	0.06 (0.5)
Myrtle	0.4 (3.5)	0.3 (2.7)
Peppermint	0.1 (0.9)	0.1 (0.9)
Sage	0.4 (3.7)	0.3 (2.7)
Spearmint	0.2 (1.6)	0.1 (1.1)
Tea tree oil	0.2 (1.8)	0.2 (1.8)
E-cinnamaldehyde	0.03 (0.3)	0.01 (0.1)
Linalool	0.1 (0.9)	0.1 (0.9)
CHX	2×10^{-3} (2.1×10^{-2})	5×10^{-3} (5.3×10^{-2})
Triclosan	5.66×10^{-4} (8.4×10^{-3})	5.89×10^{-4} (8.8×10^{-3})

Minimal inhibitory concentration 80 (MIC80) defined as the lowest concentration of the antimicrobial agent that led to 80% reduction in absorbance compared to controls without agent. MIC values are in % (v/v) and in brackets are the equivalent MIC values in (g/l).

Fungicidal activity was also expressed as the lowest concentration of antimicrobial agent that killed the microorganism (minimal lethal concentration) (Table 2.5). All tested compounds, with exception of triclosan, had minimal lethal concentrations against *C. albicans* at concentrations tested. These lethal concentrations were generally higher than the previously established MICs.

Table 2.5 - Minimal lethal concentration of essential oils and biocides against *C. albicans* NYCY 1363 and *C. albicans* 135BM2/94 in the planktonic growth mode

Antimicrobial	Minimal Lethal Concentration [% (v/v)] [(g/l)]	
	***C. albicans* NCYC 1363**	***C. albicans* 135BM2/94**
Basil	0.5 (4.5)	0.5 (4.5)
Bergamot	0.5 (4.4)	0.5 (4.4)
Cinnamon	0.1 (1.0)	0.1 (1.0)
Citronella	0.1 (0.9)	0.1 (2.7)
Geranium	0.1 (0.9)	0.1 (0.9)
Lavender	0.5 (4.4)	0.3 (2.6)
Melissa	0.1 (0.9)	0.1 (0.9)
Myrtle	1 (8.8)	1 (8.8)
Peppermint	0.3 (2.7)	0.1 (0.9)
Sage	1 (9.2)	1 (9.2)
Spearmint	1 (9.2)	1 (9.2)
Tea tree oil	0.5 (4.5)	0.3 (2.7)
E-cinnamaldehyde	0.03 (0.3)	0.03 (0.3)
Linalool	0.3 (2.6)	0.3 (2.6)
CHX	2.5×10^{-3} (2.7×10^{-2})	5×10^{-3} (5.3×10^{-2})
Triclosan	NA	NA

Minimal lethal concentration was defined as the lowest concentration of the antimicrobial agent that killed *C. albicans.* Minimal lethal concentration (MLC) values are in % (v/v) and in brackets are the equivalent MLC values in (g/l). NA = no antimicrobial activity at tested concentrations.

2.8.2 Minimum inhibitory concentration (MIC) for periodontal pathogens

The antibacterial activity of five essential oils, E-cinnamaldehyde, and CHX against periodontal bacteria was investigated using the microdilution assay. *F. nucleatum* KS 515, *F. nucleatum* THOWN*, P. intermedia* and *P. gingivalis* strains were susceptible to all biocides and essential oils tested (Figure 2.5).

The minimum inhibitory concentration 80 of the selected agents against periodontal anaerobic bacteria are shown in Table 2.6.

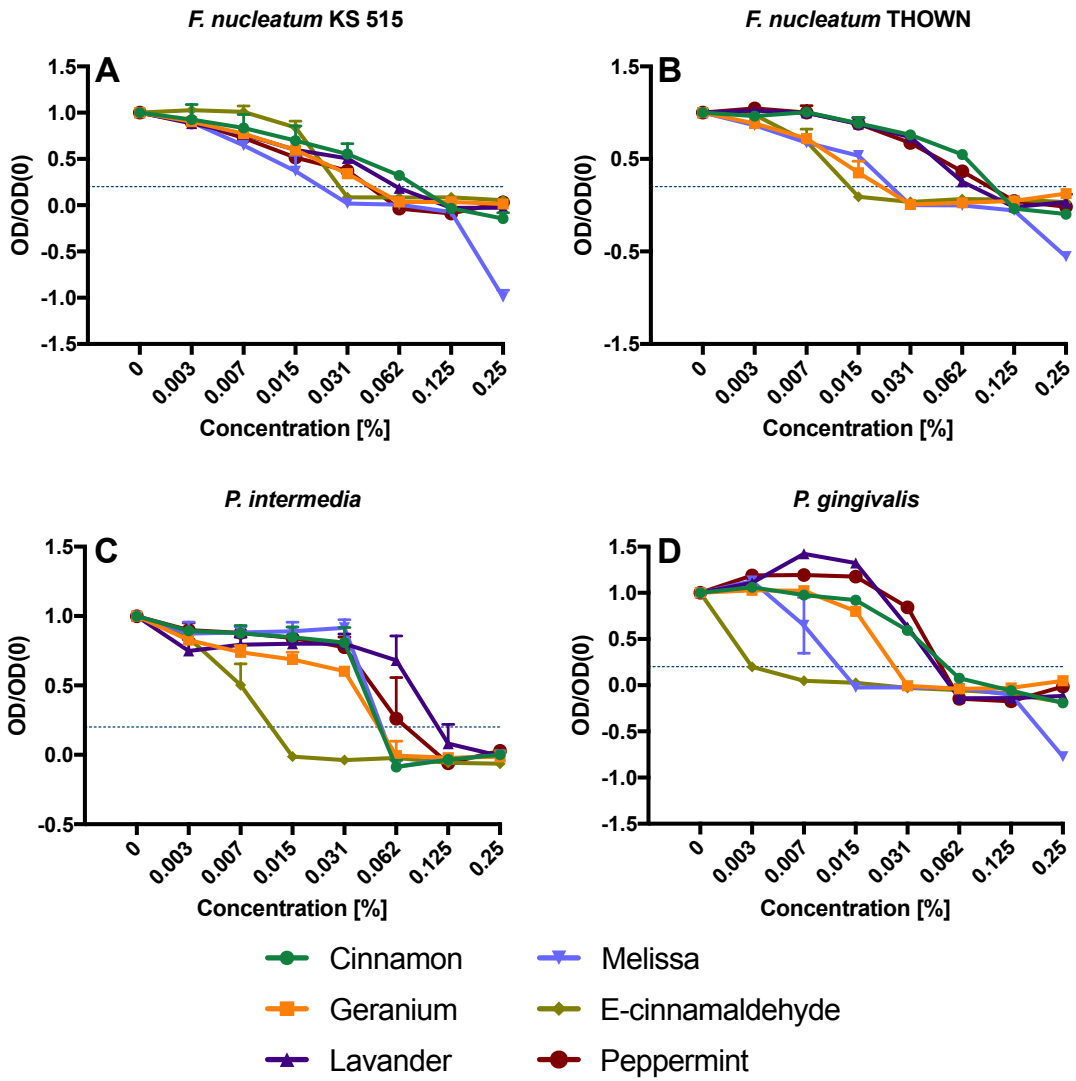


Figure 2.5 - Relative growth of *F. nucleatum* KS 515 (A), *F. nucleatum* THOWN (B), *P. intermedia* (C) and *P. gingivalis* (D) in the planktonic growth mode in the presence of cinnamon (green), geranium (orange), lavender (purple), melissa (lilac), E-cinnamaldehyde (ochre) and peppermint (purple). Data represent the mean of three independent experiments, each performed in quadruplicate

Table 2.6 - Minimum inhibitory concentration 80 of essential oils and biocides against periodontal pathogens in the planktonic form

Antimicrobial	Minimum inhibitory concentration 80 [% (v/v)] [(g/l)]			
	***F. nucleatum* KS 515**	***F. nucleatum* THOWN**	***P. gingivalis***	***P. intermedia***
Cinnamon	0.08 (0.9)	0.1 (1.0)	0.06 (0.5)	0.05 (0.5)
Geranium	0.05 (0.4)	0.05 (0.5)	0.03 (0.2)	0.05 (0.5)
Lavender	0.06 (0.5)	0.1 (0.8)	0.05 (0.4)	0.1 (0.8)
Melissa	0.02 (0.2)	0.03 (0.2)	0.01 (0.1)	0.05 (0.5)
Peppermint	0.04 (0.4)	0.07 (0.6)	0.05 (0.5)	0.08 (0.7)
E-cinnamaldehyde	0.03 (0.3)	0.01 (0.1)	0.004 (0.05)	0.01 (0.1)
CHX	2×10^{-3} (2×10^{-2})	2×10^{-3} (2×10^{-2})	3×10^{-3} (3×10^{-2})	0.5×10^{-3} (0.5×10^{-2})

Minimal inhibitory concentration 80 (MIC80) defined as the lowest concentration of the antimicrobial agent that led to 80% reduction in absorbance compared to controls without agent. MIC values are in % (v/v) and in brackets are the equivalent MIC values in (g/l).

2.8.3 Minimal biofilm eradication concentration (MBEC)

The antifungal activity of biocides and commercial essential oils against *C. albicans* biofilms was expressed as the minimal biofilm eradication concentration (MBEC) (Malic et al., 2013). Most test agents were not active against biofilms at concentrations tested and did not prevent regrowth after removal of the antimicrobial (Figure 2.6). The antimicrobials that exhibited an MBEC against both tested *C. albicans* strains were melissa, geranium, E-cinnamaldehyde and linalool (Table 2.7).

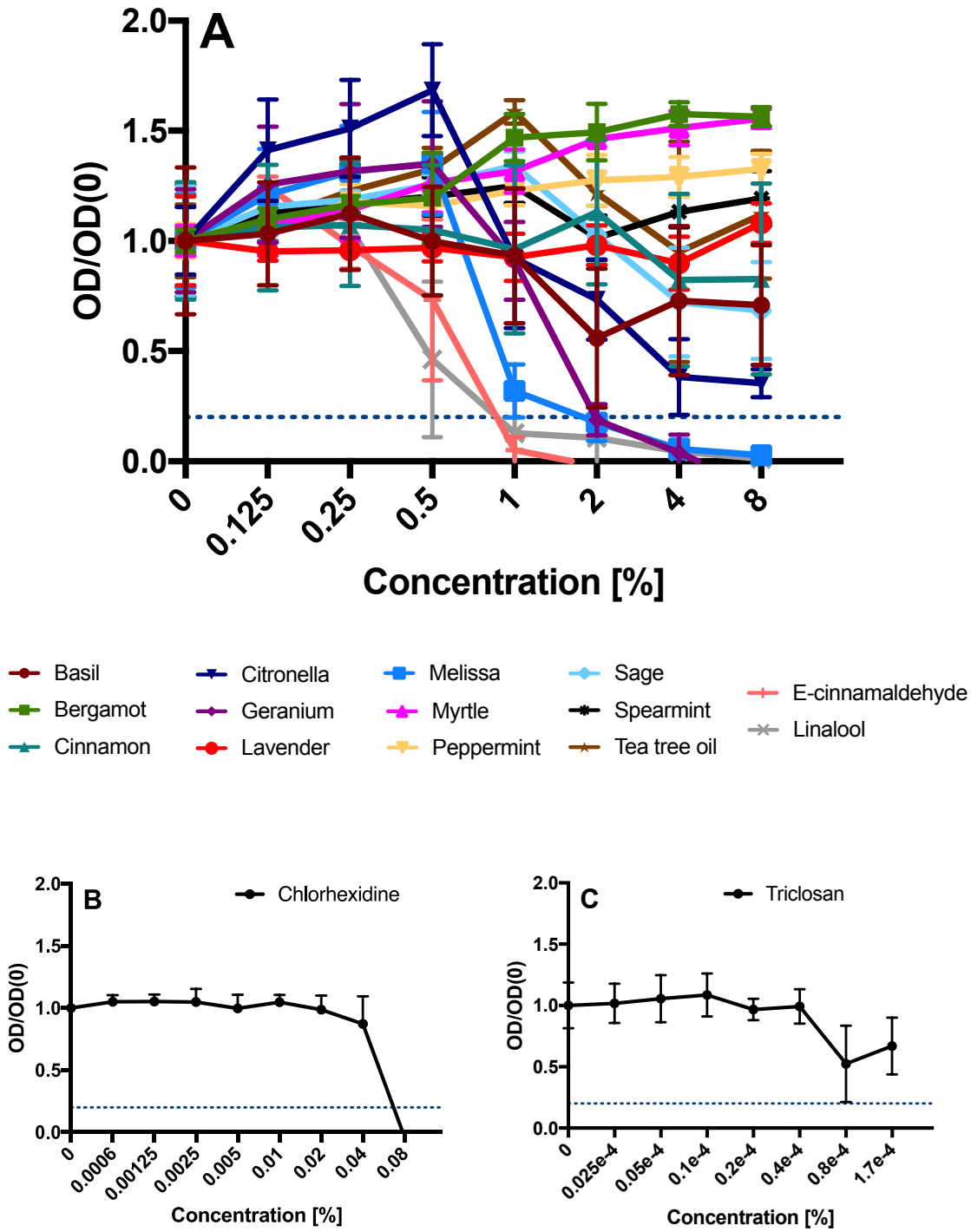


Figure 2.6 - Relative re-growth of *C. albicans* NYCY 1363 after the application on preformed biofilms of 12 essential oils and two terpenes (A), chlorhexidine (B) and triclosan (C). After application of the antimicrobials on pre-formed biofilms for 24 hours, cells were resuspended in a free-antimicrobial medium and re-growth was measured after 24 hours. The minimal biofilm eradication concentration 80 (MBEC80) was defined as the lowest antimicrobial concentration that prevented at least 80% regrowth of *Candida* (blue dotted line). Data represent the mean of three independent experiments, each performed in triplicate

Table 2.7 - Minimal biofilm eradication concentration 80 of essential oils and biocides against *C. albicans* NCYC 1363 and *C. albicans* 135BM2/94

Antimicrobial	Minimal Biofilm Eradication Concentration 80 [% (*v*/*v*)] [(g/l)]	
	***C. albicans* NYCY 1363**	***C. albicans* 135BM2/94**
Basil	NA	NA
Bergamot	NA	NA
Cinnamon	NA	NA
Citronella	NA	NA
Geranium	2.5 (22.3)	2 (17.9)
Lavender	NA	NA
Melissa	1.5 (13.3)	1.5 (13.3)
Myrtle	NA	NA
Peppermint	NA	NA
Sage	NA	NA
Spearmint	NA	NA
Tea tree oil	NA	NA
E-cinnamaldehyde	0.8 (8.4)	0.8 (8.4)
Linalool	1 (8.7)	1.5 (13.1)
CHX	0.07	NA
Triclosan	NA	NA

Minimal biofilm eradication concentration 80 (MBEC80) defined as the lowest antimicrobial concentration that prevented at least 80% regrowth of *Candida*, after the biofilm was treated with antimicrobials for 24 hours. MBEC values are in % (v/v) and in brackets are the equivalent MBEC values in (g/l). NA = no antimicrobial activity at tested concentrations.

2.8.4 Essential oils and biocides synergy

Table 2.8 summarises the FIC index of the blends screened. None of the combinations tested showed a synergistic activity but most of them had an additive effect. Some of the blends had a FIC of 0.6. The lowest concentration of CHX (3.75×10^{-4} % (v/v)) that inhibited *C. albicans* growth was obtained by combining CHX with E-cinnamaldehyde at a concentration of 7.5×10^{-3} % (v/v) and 0.015% (v/v) for *C. albicans* NYCY 1363 and *C. albicans* 135BM2/94, respectively. When cinnamon essential oil was used in combination with 0.0075% (v/v) E-cinnamaldehyde, it was possible to decrease its concentration by 13 times (from 0.105% (v/v) to 7.7×10^{-3} % (v/v)) to obtain the same level of inhibition. A

concentration of geranium essential oil 10 times lower than the MIC (7.5×10^{-4} % (v/v)) effectively inhibited *C. albicans* growth when it was combined with 0.015% (v/v) E-cinnamaldehyde or 0.031% (v/v) cinnamon essential oil. A concentration of peppermint essential oil of 9.3×10^{-3} % (v/v)) was sufficient to inhibit *C. albicans* growth blended with 0.015% (v/v) or 7.5×10^{-3} %(v/v) of E-cinnamaldehyde for *C. albicans* NYCY 1363 and *C. albicans* 135BM2/94, respectively. Moreover, the addition of peppermint essential oil to spearmint essential oil decreased the spearmint essential oil concentration between 13 and 15 times below the MIC value. Lavender essential oil exhibited an additive effect only when it was combined with cinnamon essential oil against *C. albicans* 135BM2/94.

Table 2.8 - Fractional inhibitory concentration (FIC) index

		C. albicans NYCY 1363			*C. albicans* 135BM2/94		
Compound X	**Compound Y**	**FIC**	**MIC_Xc (MIC_X) % (v/v)**	**MIC_Yc (MIC_Y) % (v/v)**	**FIC**	**MIC_Xc (MIC_X) % (v/v)**	**MIC_Yc (MIC_Y) % (v/v)**
CHX	**Cinnamon**	1.34	0.0015 (0.002)	0.062 (0.105)	0.69	0.000375 (0.005)	0.062 (0.082)
CHX	**E-cinnamaldehyde**	0.72	0.000375 (0.002)	0.015 (0.028)	0.61	0.000375 (0.005)	0.0075 (0.0139)
CHX	**Geranium**	N/A	-	-	1.72	0.003 (0.005)	0.062 (0.055)
CHX	**Lavender**	N/A	-	-	N/A	-	-
Peppermint	**CHX**	N/A	-	-	N/A	-	-
Spearmint	**CHX**	N/A	-	-	N/A	-	-
Cinnamon	**E-cinnamaldehyde**	0.61	0.0077 (0.105)	0.0075 (0.028)	1.15	0.0077 (0.082)	0.0075 (0.0139)
Cinnamon	**Geranium**	0.7	0.031 (0.105)	0.0077 (0.071)	0.76	0.031 (0.082)	0.0077 (0.055)
Cinnamon	**Lavender**	N/A	-	-	1.45		
Cinnamon	**Peppermint**	1.22	0.062 (0.105)	0.0375 (0.134)	0.99	0.031 (0.082)	0.0375 (0.108)
Cinnamon	**Spearmint**	0.73	0.062 (0.105)	0.025 (0.167)	0.99	0.062 (0.082)	0.025 (0.115)
E-cinnamaldehyde	**Geranium**	0.65	0.015 (0.028)	0.0077 (0.071)	0.71	0.0075 (0.0139)	0.031 (0.055)
E-cinnamaldehyde	**Lavender**	0.6	0.015 (0.028)	0.0125 (0.190)	1.17	0.015 (0.0139)	0.0125 (0.118)
Peppermint	**E-cinnamaldehyde**	0.61	0.0093 (0.134)	0.015 (0.028)	0.62	0.0093 (0.108)	0.0075 (0.0139)
E-cinnamaldehyde	**Spearmint**	0.61	0.015 (0.028)	0.1 (0.167)	0.64	0.0075 (0.0139)	0.05 (0.115)
Geranium	**Lavender**	N/A	-	-	N/A	-	-
Geranium	**Peppermint**	N/A	-	-	N/A	-	-
Geranium	**Spearmint**	N/A	-	-	1.33		
Lavender	**Spearmint**	N/A	-	-	0.94	0.0125 (0.118)	0.1 (0.115)
Lavender	**Peppermint**	N/A	-	-	N/A	-	-
Peppermint	**Spearmint**	0.72	0.0187 (0.134)	0.1 (0.167)	0.92	0.0093 (0.108)	0.1 (0.115)

Fractional inhibitory concentration (FIC) of the blends of Compound X and Compound Y against *C. albicans* NYCY 1363 and *C. albicans* 135BM2/94. MIC_Xc and MIC_Yc represent the MIC of the compound X and Y in combination, while MIC_X and MIC_Y the MICs of the compound X and Y alone. NA = no antimicrobial activity at tested concentrations.

2.9 Discussion

Essential oils are natural products often extracted from plants and they frequently exhibit antimicrobial, antiseptic, anti-inflammatory and anti-oxidant activities (Bakkali et al., 2008). The primary aim of this research was to evaluate the antifungal activity of 12 commercial essential oils against *C. albicans*. All tested commercial essential oils demonstrated antifungal activity against planktonic *C. albicans*, with MICs ranging from 0.06% (v/v) to 0.4% (v/v) and MLCs from 0.1% (v/v) to 1% (v/v). Comparison of results with those of other studies is problematic given differences in assay techniques (Janssen et al., 1986; Hammer et al., 1999). In addition, the botanical source, climate and environmental conditions, time of harvesting and extraction method can affect both composition and antimicrobial activity of commercial essential oils (Janssen et al., 1986; Carvalhinho et al., 2012; Szweda et al., 2015). The effect of plant origin on antimicrobial properties can be appreciated by comparing the activity of cinnamon oil extracted from *Cinnamomum zeylanicum* leaves and *Cinnamomum aromaticum* leaves. Both types of cinnamon oils are from the evergreen cinnamomum plant but *Cinnamomum aromaticum* extract contains a higher amount of E-cinnamaldehyde, which could explain the higher antifungal activity (MICs 0.0006% (v/v) - 0.0096% (v/v)) (Szweda et al., 2015) compared to the present study using *Cinnamomum zeylanicum* (MIC 0.1% (v/v)) extract. The impact that the amount of E-cinnamaldehyde has on antifungal properties of an essential oil was also evident in this study (MICs of 0.03% (v/v) and 0.01% (v/v)). Geranium and melissa oils exhibited the highest antifungal potential. Both commercial oils contain geraniol and citronellol, which are antifungal (Zore et al., 2010) and likely responsible for the similar antifungal activity of these two oils

(geranium and melissa essential oil) ($p > 0.90$). However, the MIC of melissa oil was lower than that previously reported (Mimica-Dukic et al., 2004; Abdellatif et al., 2014). This present study revealed antifungal effects for bergamot oil (MIC of 0.3% (v/v) and MLC of 0.5% (v/v)) which previously only had limited attention. The MIC of basil oil 0.1% (v/v) (0.9 g/l) was lower than previously reported, namely 0.5% (v/v) (Hammer et al., 1999) and 0.312% (v/v) (Szweda et al., 2015) but comparable to the MIC (1250 µg/ml) found against a fluconazole resistant *C. albicans* strain (Cardoso et al., 2016). The main compound of basil and lavender oils is linalool, which previously has had MICs described ranging from 0.06% (v/v) to 0.12% (v/v) (Marcos-arias et al., 2011). Comparing activity of pure linalool to those of basil and lavender oils, the anticandidal activity of terpene was not significantly higher than that of basil ($p > 0.99$). Tea tree oil had a MIC of 0.2% (v/v) similar to a study recorded by Hammer et al. (1998) against *C. albicans*.

Sage oil exhibited MICs of 0.3% (v/v) (2.7 g/l) and 0.4% (v/v) (3.7 g/l), which were comparable to the MIC of 2.78 g/l reported using a disk diffusion method (Sookto et al., 2013), but lower than the MIC of 1.32 mg/ml measured by broth microdilution assay (Nacsa-farkas et al., 2014). Despite their differences in composition, peppermint and spearmint oils had similar antifungal activities with MICs of 0.1% (v/v) and 0.1% (v/v) - 0.2% (v/v), respectively ($p > 0.07$). However, while the MICs of spearmint oil were similar to those reported by Hammer et al. (1999), the MIC of peppermint oil was higher than that found by Thosar et al. (2013). Myrtle oil had the lowest antifungal potential, even though its MICs were lower than those previously reported by Mahboubi and Bidgoli (2010) (MIC of 0.8 - 1.6% (v/v)). CHX and triclosan, two biocides whose antimicrobial properties are widely recognised, and both commonly added to mouthwashes and toothpastes, were also evaluated in this study. Triclosan exhibited fungistatic activity at

concentrations lower than those used in toothpaste formulations (0.3% (w/v) (Brading et al., 2004)), but did not exhibit fungicidal effects at tested concentrations.

The majority of agents had limited antibiofilm activity. Bacteria in biofilms can be between 10 and 1000 times more tolerant to antibiotics than their planktonic counterparts and similar findings have been reported for *Candida* (Hawser and Douglas, 1995). The mechanisms by which biofilm cells have elevated antimicrobial tolerance are complex and likely multifactorial. These include altered gene expression following surface attachment, reduced growth rates in biofilms, variable nutrient availability that induces changes in phenotype and the presence of extracellular polymeric substances that impedes penetration of agents into the biofilm (Douglas, 2003; Stewart and Franklin, 2008; Singh et al., 2017). Few studies have previously reported activity of commercial essential oils or biocides against *C. albicans* biofilms (de Campos Rasteiro et al., 2014; Almeida et al., 2016). In the present study, melissa oil, geranium oil, E-cinnamaldehyde and linalool had anti-biofilm activity, whilst CHX only had anti-biofilm activity against *C. albicans* NCYC 1363 but not *C. albicans* 135BM2/94. A 3 minutes application of cinnamon (1 mg/ml) and citronella (1 mg/ml) oils has been found to reduce biofilm cell numbers immediately after treatment but this effect was not evident 48 hours post treatment (Almeida et al., 2016). These results concur with the current study, where no antibiofilm activity was noted for cinnamon and citronella oils after 24 hours. A MBEC of tea tree oil of 12.5% (v/v) had previously been reported (de Campos Rasteiro et al., 2014), which is a higher concentration than tested in this study (8% (v/v)); difficulties in forming a stable suspension of the oil-medium using 1% (v/v) Tween 80 were encountered.

To investigate the potential use of the novel oral therapy developed in the current study as treatment for both oral candidiasis and periodontal diseases, 5 essential oils out of the 12 investigated against *Candida* were also evaluated against periodontal pathogens (two *F. nucleatum* strains*, P. gingivalis and P. intermedia*). It is well-known that the growth of anaerobic bacteria takes longer and is more challenging compared to that of aerobic bacteria or fungi. Hence, the oils tested were those that exhibited the highest antifungal potential against *Candida.* Comparing the MICs for *Candida* and periodontal pathogens, it can be observed that periodontal bacteria were less resistant to the treatments, with the concentrations needed to inhibit periodontal bacteria lower than those needed to inhibit *Candida* growth.

Few studies focused on the antibacterial properties of the essential oils tested in the current project. Iauk et al. (2003) investigated the antibacterial potential of melissa and found MICs higher than 0.2% (v/v) against oral anaerobic pathogens. These values were greater than those observed in the current study that showed that concentrations of 0.05% (v/v) were sufficient to inhibit the growth of the 4 strains tested. The MICs of peppermint, cinnamon and lavender were approximately 10 times lower than those reported by Shapiro et al. (1994), Bardaji et al. (2016) and Takarada et al. (2004). To the best of the author's knowledge, the antibacterial properties of geranium essential oil against periodontal bacteria has not been previously evaluated, but this study showed an antibacterial potential at concentrations lower than 0.05% (v/v).

The antimicrobial activity of essential oils against periodontal biofilms was not evaluated. The biofilm formation obtained in 96-well plates was not sufficient to apply the method described in Section 2.7.4. Coating of plates with saliva or more

sophisticated biofilm models, such as the constant depth film fermenter (CDFF), could be applied for this purpose (Walker and Sedlacek, 2007; Mcbain, 2009). In particular, the CDFF was originally developed to model dental plaque biofilms and allows the control of key parameters such as the nutrient source, the temperature, the gas mixture and the substratum (Wilson, 1999).

The potential synergy between essential oils and biocides was evaluated on *C. albicans* with the checkerboard method (Section 2.7.5). The combinations tested were decided according to the composition of the essential oils and their MICs. Firstly, the oils that showed a greater antifungal activity were selected, secondly among these essential oils those with a different main composition were chosen. Indeed, it was supposed to be more probable to find a synergistic effect between compounds with a different composition than with a similar one. The checkerboard method showed that a synergistic activity was not present between the blends of essential oils and biocides. Despite the lack of synergy, there was an additive activity between some of the compounds with FICs ranging between 0.6 and 1.72. In particular, some of the blends had a FIC of 0.6, close enough to the FIC of 0.5 necessary to claim a synergistic effect (Hsieh et al., 1993). This means that it was possible to achieve the same growth inhibition by blending two compounds at lower concentrations than the MICs of the single compounds. These results were in accordance with those reported by Horváth et al. (2016), who found an additive effect by blending essential oils. By contrast, several authors reported a synergistic effect when the essential oils were combined with antifungal agents such as fluconazole and amphotericin B (Zore et al., 2010; Zore et al., 2011; Cannas et al., 2013; Samber et al., 2015; Cardoso et al., 2016). In conclusion, even though a synergistic relationship between the essential oils and

the biocides was not found, the blends decreased the concentrations. Future work should focus on evaluating the FIC index of essential oils in combination with antifungal agents to possibly get a synergistic relationship that would decrease the use of commercial antifungals.

2.10 Conclusions

This study showed that all twelve essential oils, two terpenes and two biocides (triclosan and CHX) had antifungal activity against *C. albicans* in planktonic form. Five compounds (CHX, E-cinnamaldehyde, linalool, geranium and melissa) were also active against biofilms, which are usually a challenge to effectively inhibit. The antifungal potential of these essential oils could be a future therapeutic for topical candidiasis as an option to overcome emerging antifungal drug resistance. In addition, the essential oils tested were also active against bacteria commonly associated with periodontal diseases in planktonic form, showing that they might be used not only against oral candidiasis but also as treatment for periodontal diseases.

Although the checkerboard method did not show a synergistic effect in the blends of essential oils and CHX, an additive effect between the compounds decreased the concentrations needed to inhibit *C. albican*s growth.

Chapter 3

Evaluation of the cytotoxicity and immune response to antimicrobials

3.1 Introduction

Management of oral candidiasis, most frequently caused by *Candida albicans*, is limited due to the relatively low number of antifungal drugs and the emergence of antifungal resistance (Williams and Lewis, 2011). For these reasons, an interest in the antimicrobial properties of essential oils and biocides has developed. In Chapter 2 a range of essential oils and biocides have been screened for antimicrobial properties. However, the evaluation of the host response to a treatment is a crucial step in the development of a new therapy. Both the cytotoxicity and the host inflammatory response need to be assessed.

3.1.1 Evaluation of the cytotoxicity

A range of different methods to evaluate the cytotoxicity is available. The following sections describe some commonly used methods and highlight their advantages and disadvantages.

3.1.1.1 Dye exclusion method

The dye exclusion method (e.g. Trypan blue exclusion method) is used to determine the number of viable cells in a suspension. This method is based on the differing permeability of cells to a dye: live cells are impermeable to the dye, while dead cells are stained. Like this, it is possible to count the number of viable cells. Despite being simple and cost effective, the dye exclusion method has some limitations. Firstly, the counting can be performed manually or automatically. In the former case, it is time-consuming and subject to operator error, while in the latter case expensive instruments are required. In addition, since the viability is assessed by cellular membrane integrity, it is possible to have

false positive or false negative results. Indeed, cells might be able to repair an abnormal membrane and become fully healthy or vice-versa, cells with an integral membrane might have their functions compromised (Posimo et al., 2014; Strober, 2015).

3.1.1.2 Metabolic assays

Metabolic assays evaluate the cytotoxicity by investigating the cellular metabolic activity. Metabolic tests can target viable or dead cells.

The viability assays include:

- Tetrazolium reduction assay

Tetrazolium reduction assays are based on the conversion of a substrate to a colour product. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay was the first developed cell viability assay (Riss et al., 2013). MTT is a soluble salt that in the presence of nicotinamide adenine dinucleotide (NADH) is converted into purple formazan. Dead cells cannot convert the MTT into formazan. Hence, it is possible to correlate the purple colour to the number of metabolically active cells. In particular, the intensity of the colour is a function of the concentration of MTT, the time of incubation, the number of cells and their metabolic activity. The main disadvantages are the incubation time that introduces an additional step and cell handling, and importantly the toxicity of the MTT. In addition, being a metabolic assay, the intensity of the purple does not always correlate linearly with the cell numbers. Indeed, when cells are in the growth phase they are metabolically active but when they reach the stationary phase the metabolism slows down and decreases the conversion into formazan (Riss et al., 2003; Fotakis and Timbrell, 2006; Riss et al., 2013).

- Resazurin reduction assay

Resazurin is a permeable redox indicator that is reduced by viable cells into resorufin. Resorufin is pink coloured and fluoresces red. Therefore, it is possible to correlate the fluorescence to metabolically active cells. The main advantages are the higher sensitivity compared to the MTT assay and the low costs associated with it. However, resazurin is toxic, and cells have to be incubated between 1 to 4 hours. In addition, the fluorescent signal can interfere with the compound tested (Riss et al., 2003; Riss et al., 2013).

- Protease viability marker assay

The viability of cells can be investigated from the activity of the proteases that are specific to viable cells. Therefore, when a fluorogenic protease substrate penetrates into the membrane, a fluorescent signal proportional to the viable cells is generated. The main advantages of this assay are the short incubation time (0.5 to 1 hour) and the absence of cytotoxicity that allows usage in combination with other metabolic tests (Riss et al., 2013).

- Adenosine triphosphate (ATP) assay

Dead cells lose the ability to produce ATP. Therefore, it is possible to evaluate the cell viability by measuring the content of ATP. In this assay, cells are lysed to release the ATP, and ATP reacts with a luciferin substrate by generating light. Therefore, by measuring the luminescence, it is possible to assess the number of viable cells. The main advantages are the high sensitivity, the absence of interference with the compound tested and the short incubation time. The main drawback is the high cost associated with this particular assay (Riss et al., 2013).

Table 3.1 summarises the main characteristics of some commercially available viability assays.

Table 3.1 - Comparison of some commercially available cell viability assays

Metabolic assay	Parameter measured	Detection method	Incubation time	Advantages	Disadvantages
MTT	Tetrazolium	Colorimetric	1 to 4 hours	Easy Cost effective	Toxic Long incubation
AlamarBlue	Resauzurin	Fluorometric Colorimetric	1 to 4 hours	Easy Cost effective More sensitive	Interferences Toxic Long incubation
CellTiter-Fluor	Protease	Fluorometric	0.5 to 1 hour	Non-toxic Short incubation	Expensive
CellTiter-Glo	ATP	Bioluminescent	10 minutes	Most sensitive No artefacts Fastest	Most expensive

Cytotoxicity assays evaluate the number of dead cells or the mechanisms of death. Cytotoxicity assays include:

- Lactate dehydrogenase (LDH) assay

The damaged membranes of dead cells cause the leaching of LDH. In the LDH colorimetric assay, LDH produces NADH that converts iodonitrotetrazolium (INT) into formazan (a red colour). In the LDH fluorometric assay, the NADH converts the resazurin into a fluorescent compound named resorufin. For both assays, the LDH quantified in the medium can be related to the number of dead cells. The main advantages of this assay are the reproducibility and the simplicity (Riss et al., 2003; Fotakis and Timbrell, 2006)

- Caspase assay

Caspase is an enzyme activated during apoptosis. When the enzyme cleaves a fluorogenic substrate, a fluorescent signal is produced and can be correlated to the number of apoptotic cells. The caspase assay can be used in combination with metabolic assays to investigate if the mechanism that causes a decreased metabolic activity is due to necrosis or apoptosis (Riss et al., 2003).

3.1.2 Immune response to *Candida* infection

Once the relationship with *Candida* switches from a commensal to a pathogenic form, the host responds with a series of mechanisms to protect itself from the infection. The host immune response is characterised by two phases: an acute response and a specific response, driven by the innate and adaptive immune system (Deorukhkar, 2017).

The innate immunity is the first line of defence against any invasion of the human body and comprises of a set of physical barriers (e.g. the epithelium and the skin), production of non-specific antimicrobial factors (e.g. histatin, calprotectin and lactoferrin), secretion of cytokines and chemokines that inhibit *Candida* growth and the presence of phagocytic cells (Antachopoulos and Roilides, 2005; Kiyoura and Tamai, 2015).

C. albicans cell wall is composed of an outer layer of mannoproteins, an inner layer of β-1,3 and β-1,6-glucans and an innermost layer of chitin (Qin et al., 2016). Receptors present on the host cell membrane, named pattern recognition receptors (PRRs), are able to recognise microbial patterns, named pathogen-associated molecular patterns (PAMPs), and initiate the immune response. Pattern recognition receptors (PRRs) include Toll-like receptors (TLRs) (e.g.

TLR1, TLR2, TL4, TLR6) and C-type lectin receptors (CLRs) (e.g. dectin-1, dectin-2, dectin-3) that through different pathways produce cytokines and chemokines (Qin et al., 2016) (Figure 3.1).

The recognition of *C. albicans* leads to the recruitment of phagocytic cells. Polymorphonuclear neutrophils (PMNs) are the main phagocytic cell of the innate immune system and are activated by several cytokines (e.g. IL-6, IL-8, IL-17, and TNFα). Polymorphonuclear neutrophils internalise and inactivate *C. albicans* by the production of reactive oxygen species (ROS) and lysosomal enzymes (Qin et al., 2016). In addition, PMNs recruit monocytes that differentiate into macrophages and continue the immune response. Besides phagocytosis, monocytes produce immune-regulatory cytokines, such as IL-10 and IL-12, that are crucial to the development of an adaptive immune response (Xiong et al., 2000).

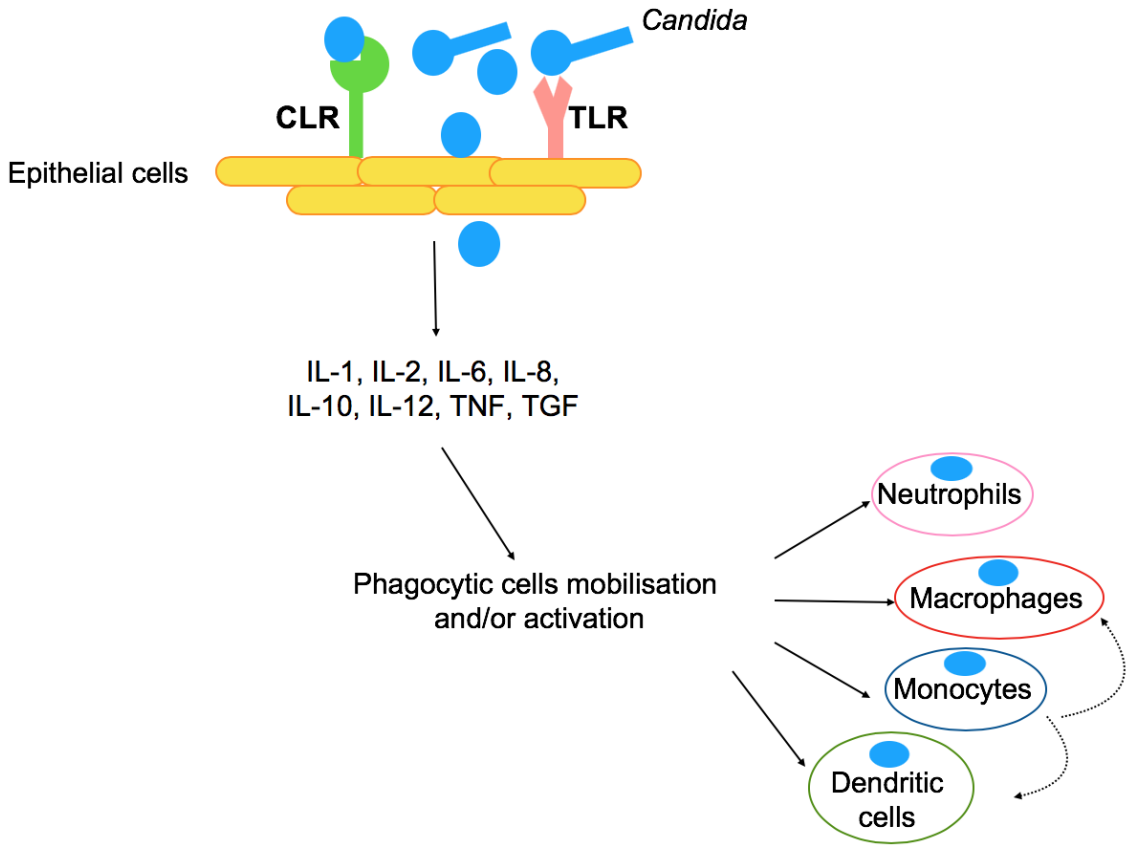


Figure 3.1- Simplified representation of the innate immune response after *Candida* infection. Once *Candida* is recognised by cell receptors, chemokines and cytokines are released and phagocytic cells activated

Dendritic cells are part of the innate immunity and play a crucial role in the activation of the adaptive immune response. Pattern recognition receptors present on the membrane of dendritic cells recognise *C. albicans* and orchestrate phagocytosis (Patin et al., 2018). After phagocytosis, fungal peptide antigens are exposed to the surface of dendritic cells and are recognised by T-cells (Qin et al., 2016). Activated T-cells generate a T-helper response according to the cytokines present in the environment that can be protective (Th1 and Th17), non-protective (Th2) or regulative (Treg) (Richardson and Moyes, 2015) (Figure 3.2). Th1 phenotype is mainly associated with the secretion of interferon IFNγ and IL-12, while Th17 is characterised by the presence of IL-1β, IL-6, IL-23 and TGFβ (Table

3.2). These protective responses lead to macrophage activation, neutrophil migration, generation of cytotoxic CD4+ T-cells, and production of opsonising antibodies (Antachopoulos and Roilides, 2005; Conti et al., 2009; Gozalbo et al., 2014). Whereas, Th2 downregulates the inflammatory response and is characterised by the presence of IL-4 and IL-10, that inhibit the production of interferon IFNγ and IL-12 (Xiong et al., 2000). Treg regulates the inflammatory response by secreting TGFβ, crucial for Th17, or by inhibiting the function of dendritic cell and Th1 cells (Josefowicz et al., 2012; Shachar and Karin, 2013; Rogers et al., 2103).

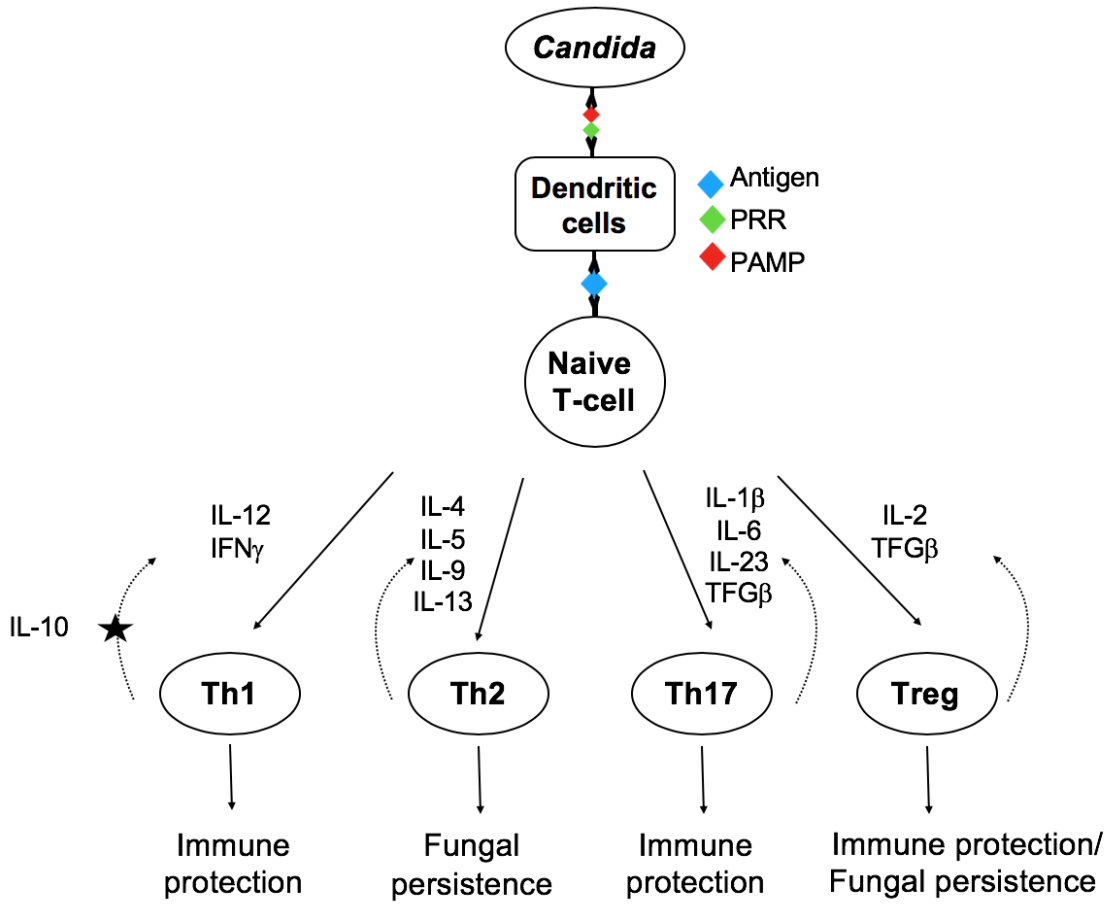


Figure 3.2 - Simplified representation of the adaptive T-cell response to *Candida* infection. Fungal antigens are recognised by T-cells. According to the cytokines present in the environment, T-cells generate an immune response that can protect the host from invasion or promote infection

Table 3.2 summarises the main functions of some cytokines involved in the immune response.

Table 3.2 - Main functions of some cytokines involved in the immune response

	Main functions	References
IL-1β	- Induction of Th17 - Induction of pro-inflammatory cytokines - Regulation of cell proliferation, differentiation, and function of many innate and specific immunocompetent cells	(Akdis et al., 2011; Akdis et al., 2016)
IL-6	- Promotion of Th17 response - Promotion of T-cell proliferation - Promotion of neutrophil migration and leukocyte activation	(Dongari-Bagtzoglou et al., 1999; Akdis et al., 2016)
IL-10	- Affection of dendritic cells - Inhibition of IL-12 and IFNγ - Inhibition of macrophage and monocyte activation - Inhibition of pro-inflammatory cytokines and chemokines	(Shachar and Karin, 2013; Akdis et al., 2016)
IL-12	- Induction of IFNγ and Th1 - Promotion of cytolytic activity of NK cells - Promotion of activity of macrophages	(Akdis et al., 2011; Akdis et al., 2016)
IL-18	- Induction of IFNγ - Promotion of Th1 or Th2 - Recruitment of phagocytes	(Akdis et al., 2016; Qin et al., 2016)
IL-23	- Induction of Th17 - Enhancement of T-cell proliferation - Regulation of antibody production	(Akdis et al., 2016)
TNFα	- Induction of a strong inflammatory response (e.g. migration of neutrophils and leukocytes, production of ROS) - Control of the extent and duration of the inflammatory processes	(Akdis et al., 2016)

3.2 Aims and objectives

A crucial step in the development of a new treatment is the assessment of the host response to that particular treatment and/or intervention. Therefore, the aim of this chapter is to evaluate both the cytotoxicity and inflammatory response to some of the essential oils outlined in Chapter 2.

The specific objectives are:

a) To assess the cytotoxicity on mouse fibroblasts of some essential oils that showed the best antifungal activity against *C. albicans* (i.e. lowest MICs and MBECs), E-cinnamaldehyde and chlorhexidine (as discussed in Chapter 2)
b) To evaluate the immune response using human blood cells with and without *Melissa officinalis* (the best essential oil in terms of antifungal activity and cytotoxicity).
c) To evaluate the immune response using human blood cells infected with *C. albicans* with and without *Melissa officinalis*

3.3 Materials and methods

3.3.1 Half maximal inhibitory concentration on mouse fibroblasts

Mouse fibroblasts NIH 3T3 (Sigma-Aldrich, Gillingham, UK) were cultured in dulbecco modified eagle medium (DMEM) (Sigma-Aldrich, Gillingham, UK) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Paisley, UK), 1% (v/v) penicillin/streptomycin (Sigma-Aldrich, Gillingham, UK) and 1% (v/v) L-glutamine (Sigma-Aldrich, Gillingham, UK).

Serial double dilutions of commercial essential oils, E-cinnamaldehyde and chlorhexidine were prepared in the fibroblast culture medium at final concentrations ranging from 0.007% to 0.25% (v/v) for the commercial essential oils and the terpene, and from 3×10^{-4} % (v/v) to 0.04% (v/v) for chlorhexidine. Fibroblasts were harvested using trypsin EDTA (EDTA 0.25% (w/v), Trypsin 0.53 mM; Thermo Fisher Scientific, Hemel Hempstead, UK) and diluted to a density of 5×10^5 cells/ml. One hundred microlitres (100 µl) of the cell suspension was used to inoculate a 96-well plate (5×10^4 cells per well) which was then incubated at 37 °C and 5% CO_2 for 1.5 hours. After the incubation step, a 100 µl volume of the antimicrobial was added. After 1 and 24 hours, the medium was removed, and the cells were washed twice with 100 µl of PBS. Three hundred µl (300 µl) of DMEM containing 10% (v/v) of AlamarBlue (AlamarBlue Cell Viability Reagent, Invitrogen) were added to each well and the plate was incubated for 1.5 hours at 37 °C and 5% CO_2. The fluorescence was read with a Synergy HT plate reader (BioTek® Instruments, Winooski, VT, USA) with excitation and emission wavelengths of 545 nm and 590 nm, respectively. The half maximal inhibitory concentration (IC50) was defined as the antimicrobial concentration that inhibited 50% cell proliferation compared to the control (i.e. DMEM without antimicrobial agent). Each condition was studied in triplicates and on three separate occasions.

In addition, the morphology of cells in the presence of the essential oils, E-cinnamaldehyde, and chlorhexidine was analysed by microscopy (Leica Live cell imaging 6000, Heidelberg, Germany)

3.3.2 Inflammatory response to *Melissa officinalis* essential oil

3.3.2.1 Preparation of *Melissa officinalis* essential oil, chlorhexidine and zymosan

Double serial dilutions of *Melissa officinalis* essential oil (Essential Oils Direct Ltd., Oldham, UK) from 1.5% (v/v) to 12% (v/v) were prepared in PBS. A 0.5% (v/v) chlorhexidine digluconate (Sigma-Aldrich, Gillingham, UK) solution was prepared in PBS. A stock solution of 100 µg/ml zymosan (Sigma-Aldrich, Gillingham, UK) was prepared in PBS.

3.3.2.2 *Candida albicans*

Candida albicans 135BM2/94, a clinical strain from the School of Dentistry (Cardiff University), was subcultured onto Sabouraud dextrose agar (SDA) (CM0041 Oxoid) and incubated at 37 °C under aerobic conditions overnight. After successful growth on SDA plates, a colony of *C. albicans* was inoculated in 20 ml of SDB and incubated overnight at 37 °C in an aerobic chamber under shaking conditions at 200 rpm. The overnight culture was prepared in SDB culture medium at 2×10^6 CFU/ml and used for further experiments.

3.3.2.3 *Ex vivo* model of whole blood

Blood was collected from 3 healthy volunteers on the day of the experiment. Ethical approval and informed written consent was previously obtained from

healthy volunteers attending Swansea University (UK), College of Medicine. The following experiments were undertaken at Swansea University, adapting a method described by Al-ishaq et al. (2015).

Five hundred microlitres (500 µl) of blood were added to a 24-well plate. Blood was infected with 5 µl of 2 × 10^6 CFU/ml *C. albicans*, equivalent to a final concentration of 10^4 CFU/ml in each well. Following, blood was treated with 5 µl *Melissa officinalis* essential oil at a range of concentrations from 0.015% (v/v) to 0.12% (v/v), or 5 µl CHX, equivalent to a CHX concentration of 0.005% (v/v). The 24-well plates were incubated for 2 and 4 hours at 37 °C under shaking at 200 rpm.

After incubation, whole blood was collected in an Eppendorf tube and centrifuged for 5 minutes at 8000 rpm at 4 °C (Eppendorf 5415R, Eppendorf UK Limited, Stevenage, UK). Following the centrifugation step, serum was collected and stored at -20 °C for further analysis.

Controls included untreated and uninfected blood, treated and uninfected blood, untreated and infected blood and blood cultured in the presence of 5 µl of PBS and/or SDB. Zymosan (1 µg/ml), a polysaccharide prepared from the cell wall of *Saccharomyces cerevisiae*, was used as a positive control for the host immune response to *C. albicans*. Each experiment was run in triplicate.

Controls on healthy donors included serum isolated on the day of the experiment and immediately centrifuged for 10 minutes at 3000 rpm (Eppendorf 5810R, Eppendorf UK Limited, Stevenage, UK) and stored at -20 °C.

3.3.2.4 ELISA

Duoset ELISAs (R&D systems, Abingdon, UK) for human IL-6 and IL-10 were carried out according to the manufacturers' instructions. Healthy volunteer's sera were diluted 1:10 in PBS. Each condition was run in duplicate.

3.3.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). All the data were presented as arithmetic mean ± SEM. The difference between treatments was statistically analysed using the one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. Statistically significant differences were set at $p < 0.05$.

3.4 Results

3.4.1 Half maximal inhibitory concentration

The half maximal inhibitory concentration (IC50) of CHX, cinnamon, E-cinnamaldehyde, geranium and melissa on fibroblast proliferation after a 1 hour and 24 hour exposure was determined (Figure 3.3 and Table 3.3). The highest cytotoxicity occurred with E-cinnamaldehyde, followed by geranium ($p < 0.0001$), which halved proliferation even at the lowest concentration tested. Indeed, a concentration of 0.003% (v/v) E-cinnamaldehyde and 0.01% (v/v) geranium inhibited 50% of cell proliferation (Table 3.3). Melissa was the least cytotoxic commercial essential oil, halving proliferation at 0.03% (v/v) ($p < 0.0001$). A 1 hour exposure of fibroblasts to cinnamon resulted in similar cytotoxicity as melissa, but prolonged exposure led to higher cytotoxicity ($p < 0.0001$). A 1 hour application of CHX was cytotoxic only at the highest concentration tested (IC50 of 0.01% (v/v)) which was higher than the MIC, while a 24 hour exposure at 7×10^{-4} % (v/v) was sufficient to halve fibroblast proliferation.

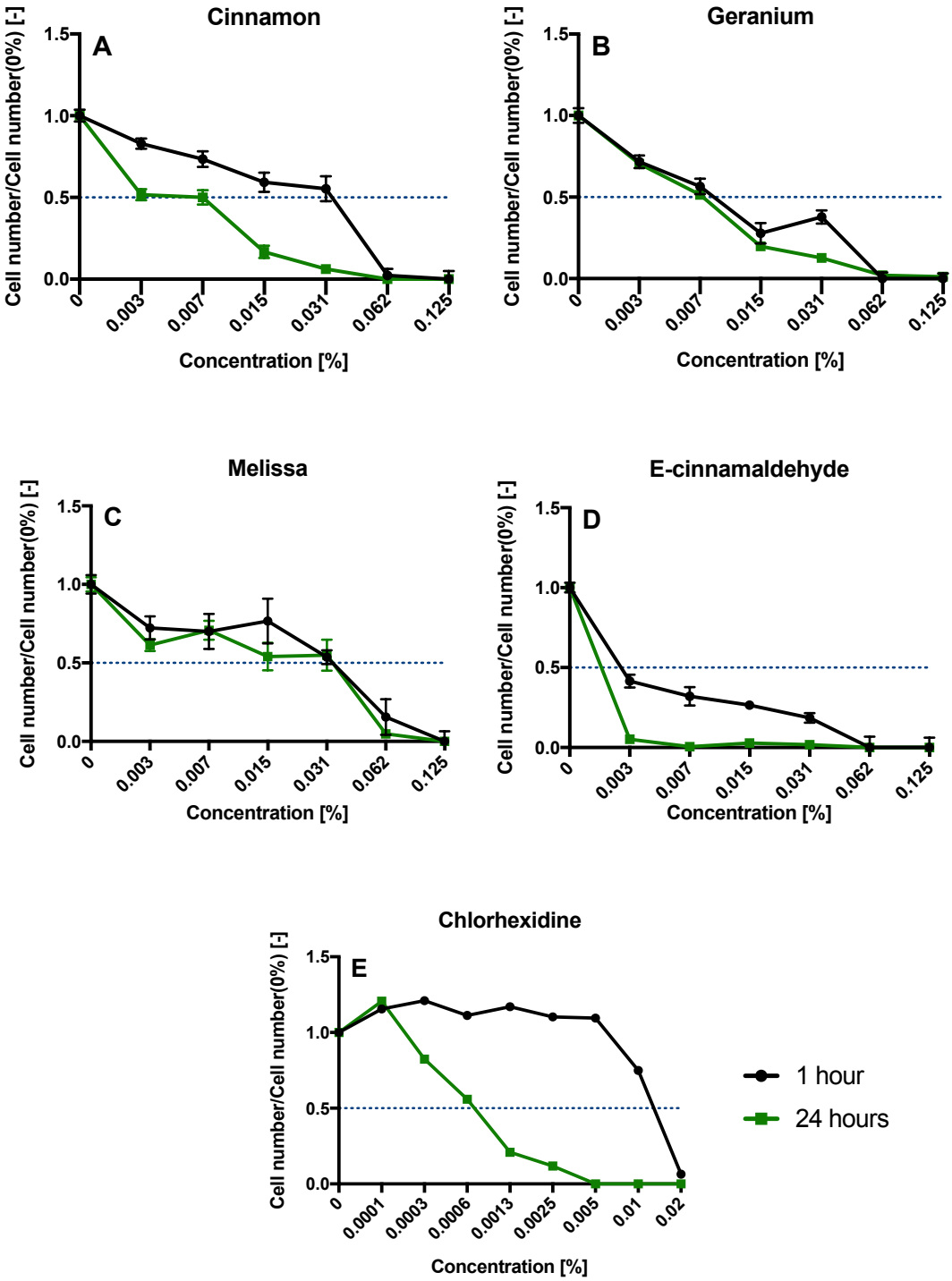


Figure 3.3 - Cytotoxicity of selected antimicrobials against murine fibroblasts. Fibroblast numbers (normalised by the control (0% (v/v) antimicrobial)) after a 1 hour (black) and 24 hour application (green) of cinnamon (A), geranium (B), melissa (C), E-cinnamaldehyde (D) and chlorhexidine (E). Data represent the mean of three independent experiments, each performed in triplicate

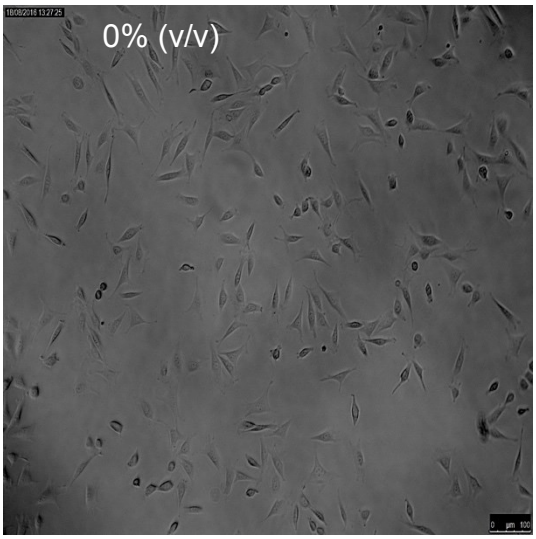
Table 3.3 - Half maximal inhibitory concentration (IC50) against fibroblasts after 1 hour and 24 hour application of the antimicrobial

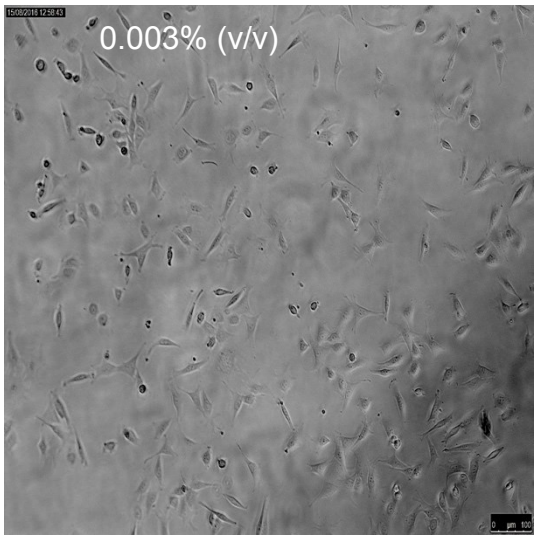
	Half maximal inhibitory concentration [% (v/v)] [g/l]	
Antimicrobial	**1 hour**	**24 hours**
Cinnamon	0.03 (0.36)	0.01 (0.11)
Geranium	0.01 (0.08)	0.01 (0.07)
Melissa	0.03 (0.3)	0.03 (0.3)
E-cinnamaldehyde	0.003 (0.03)	0.002 (0.02)
CHX	0.01 (0.15)	7.32×10^{-4} (0.008)

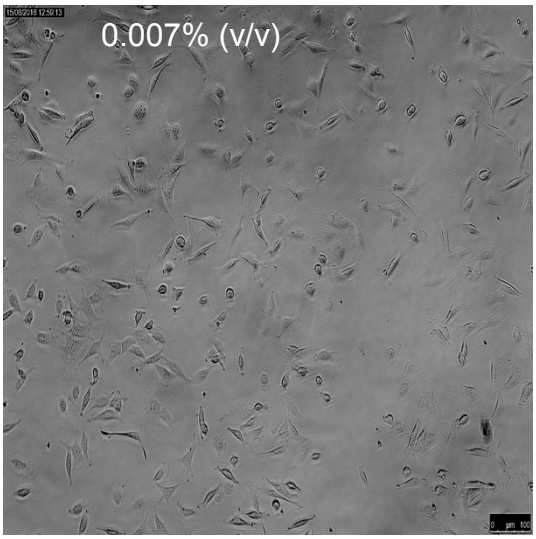
Half maximal inhibitory concentration (IC50) defined as the antimicrobial concentration that inhibits 50% of cell proliferation compared to controls without agent. Values are given in percentages and g/l in brackets.

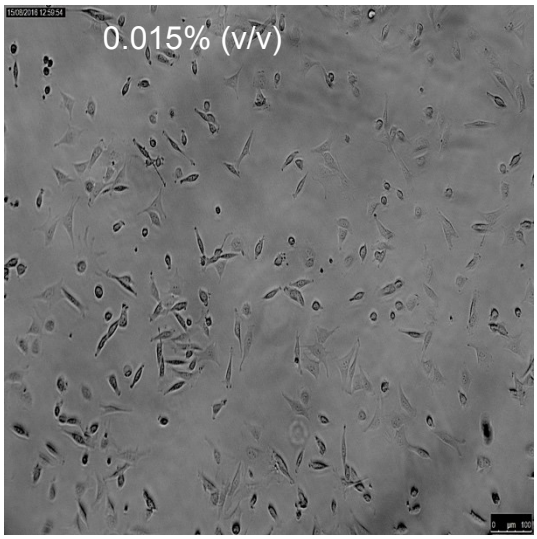
Figure 3.4 shows the morphology of mouse fibroblasts cultured in the presence of essential oils (Figure 3.4-A/C), E-cinnamaldehyde (Figure 3.4-D), and CHX (Figure 3.4-E) for 1 hour. As can be observed, by increasing the concentrations of the essential oils and terpene, the formation of round shaped cells increased. By contrast, cells cultured in the presence of 0.1% (v/v) CHX still showed the spindle shape, which is typical of healthy fibroblasts, as can be observed from the control.

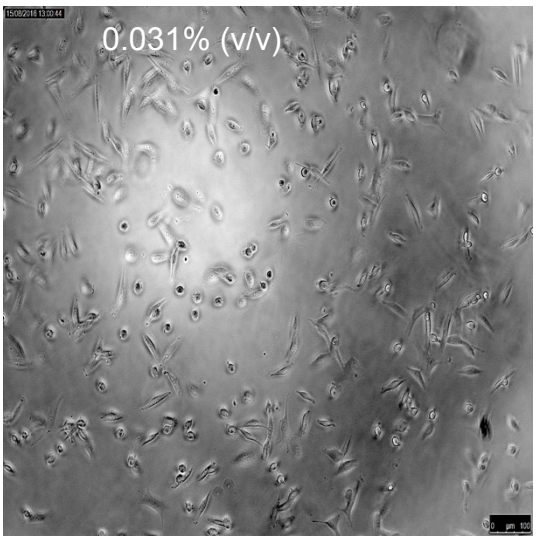
Cinnamon (A)

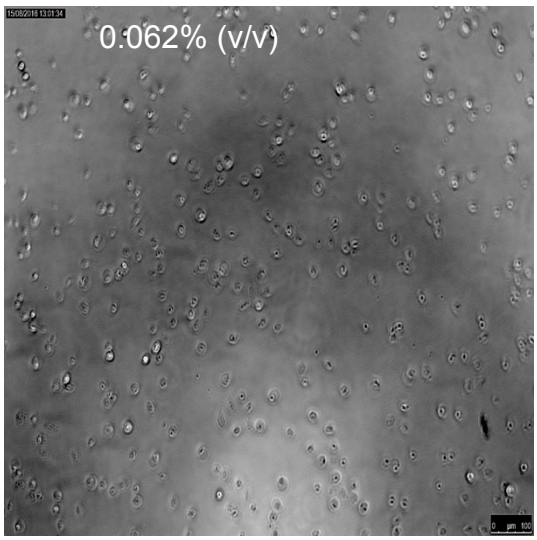




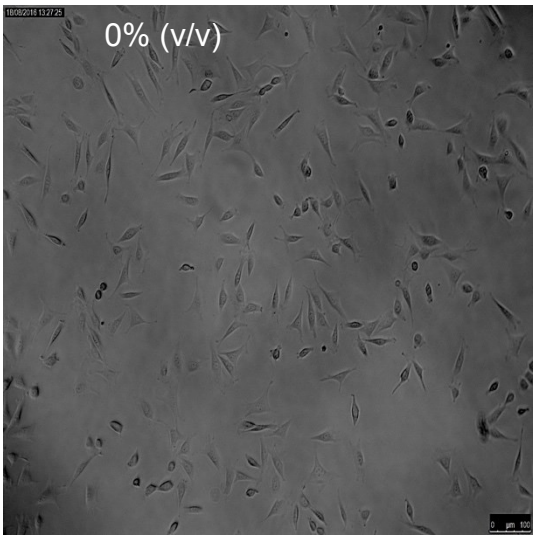


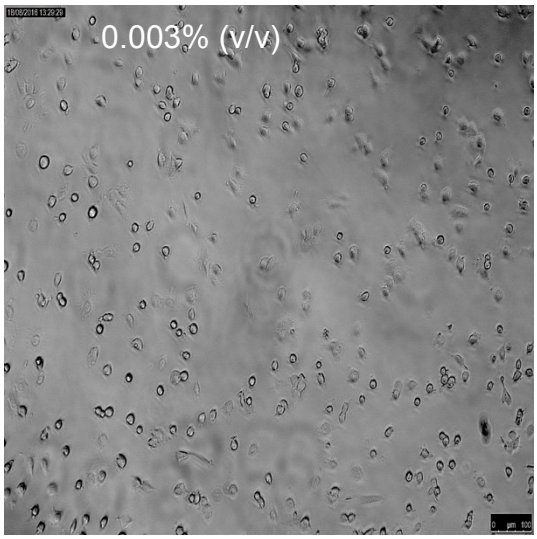


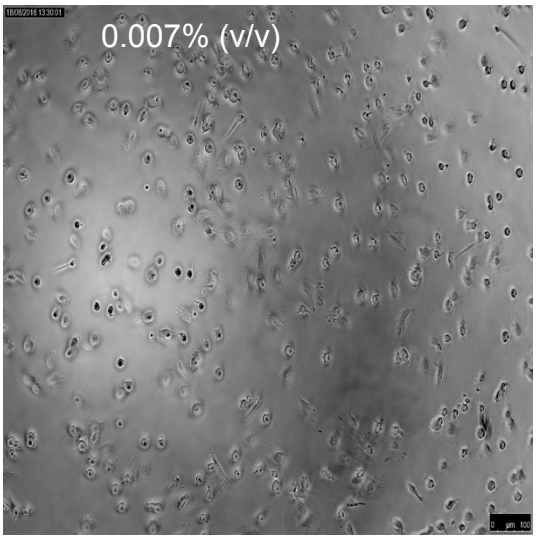


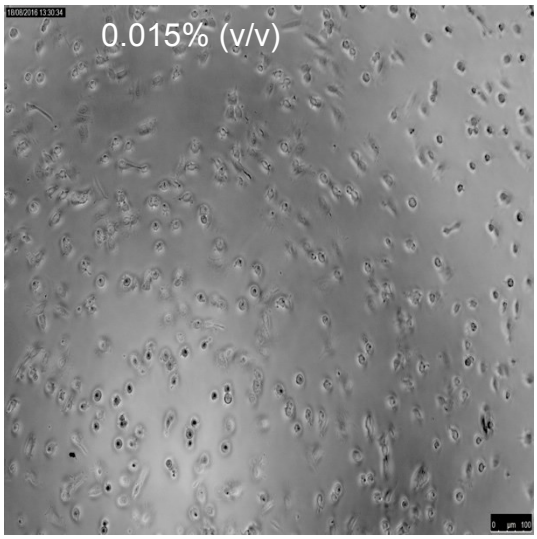


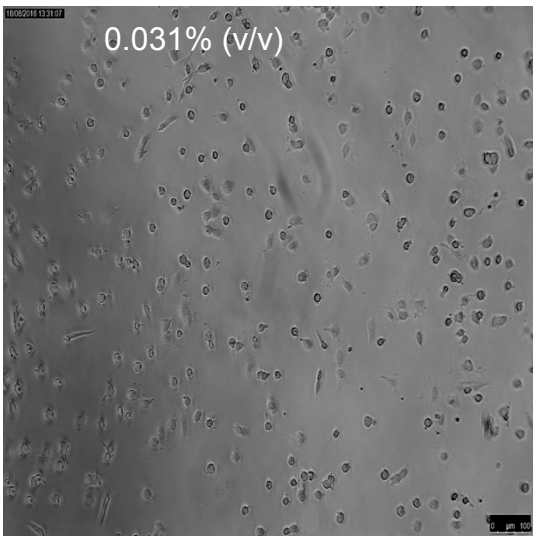
Geranium (B)

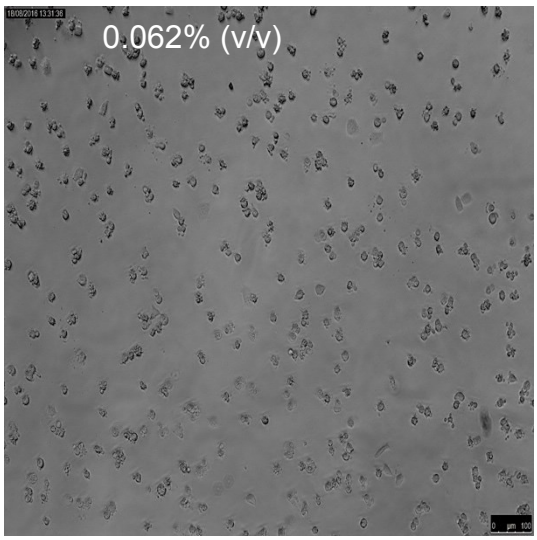




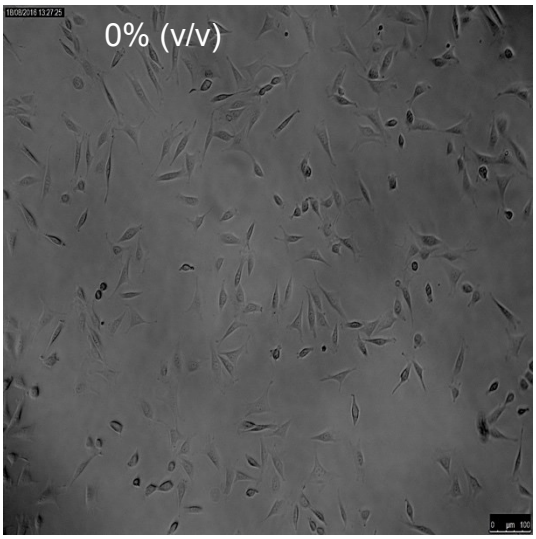


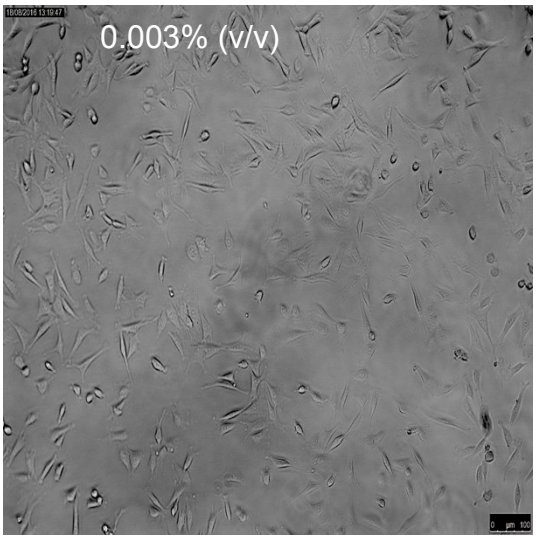


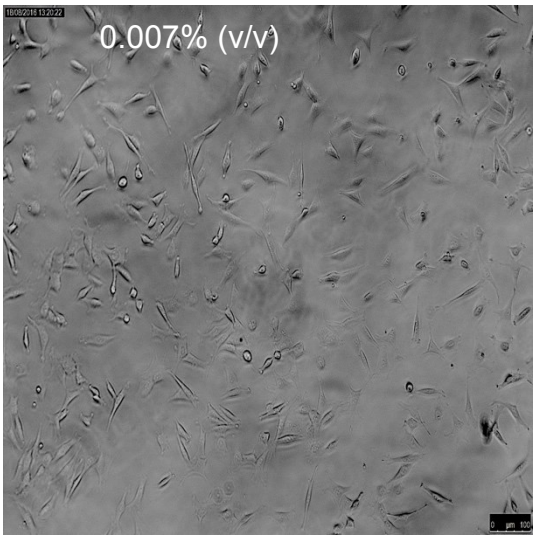


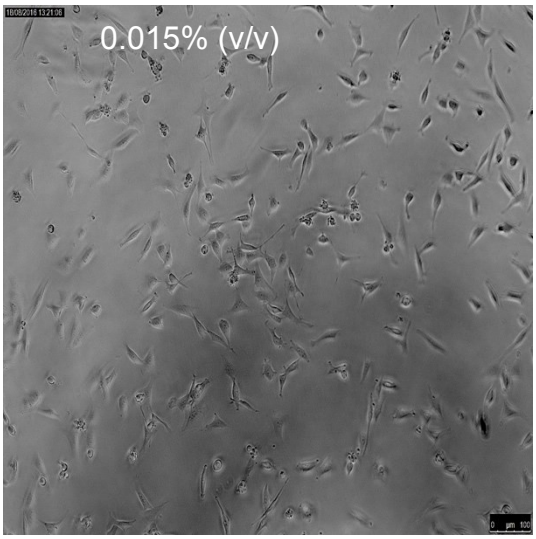


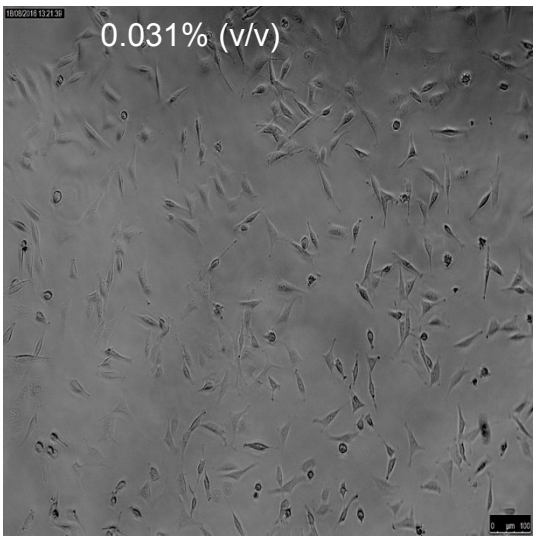
Melissa (C)

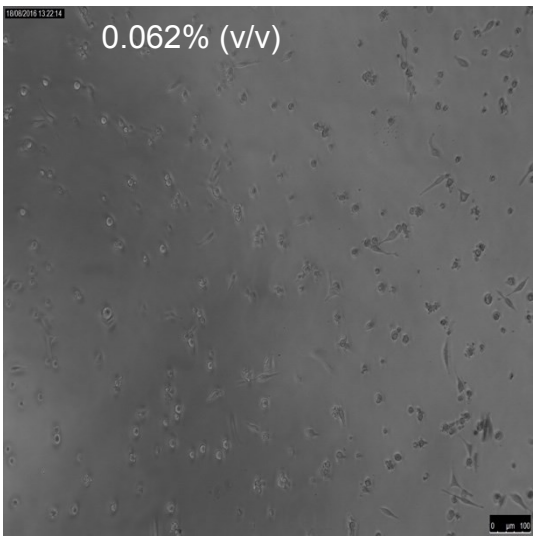




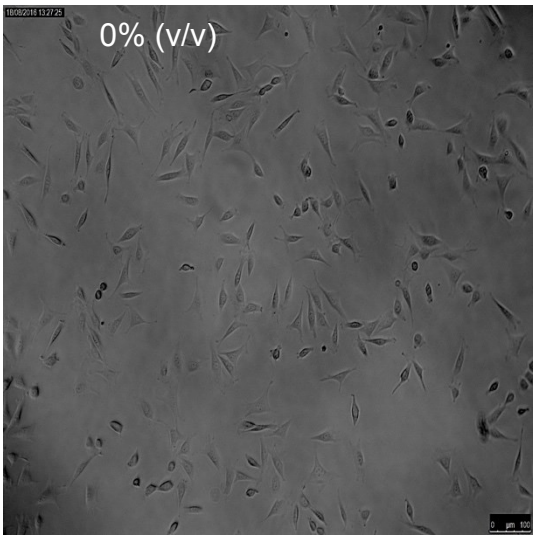


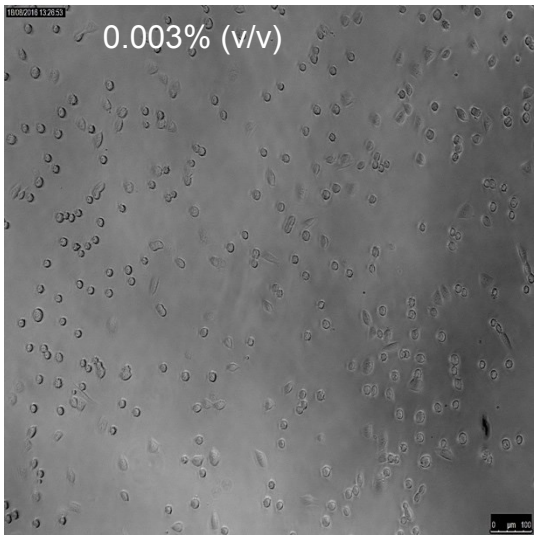


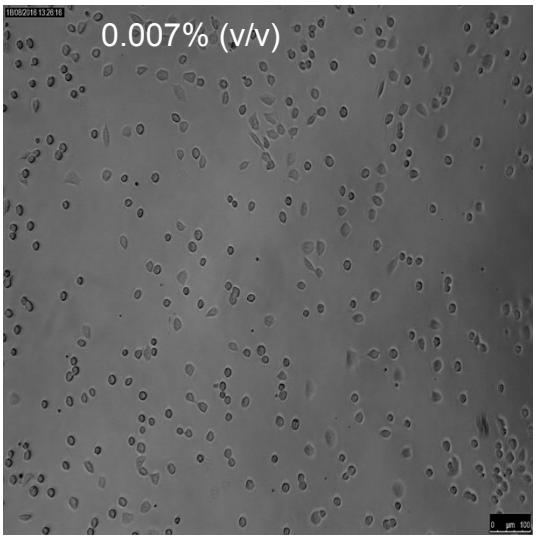


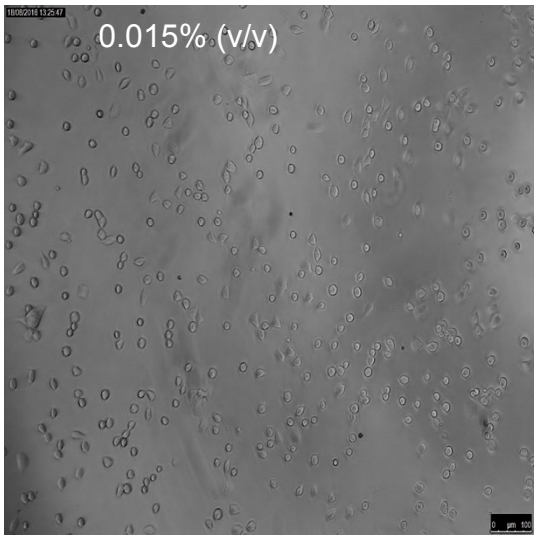


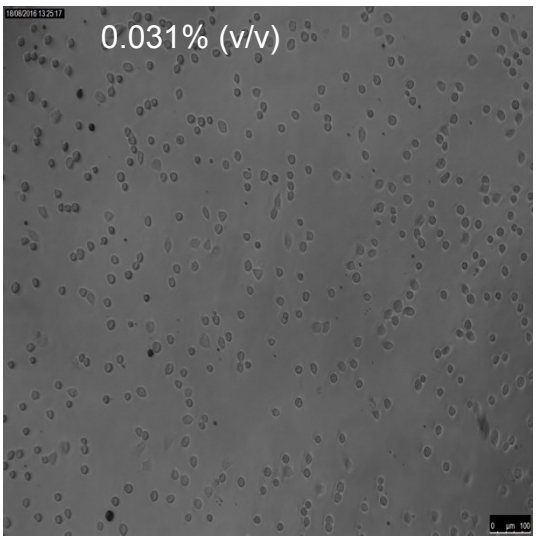
E-cinnamaldehyde (D)

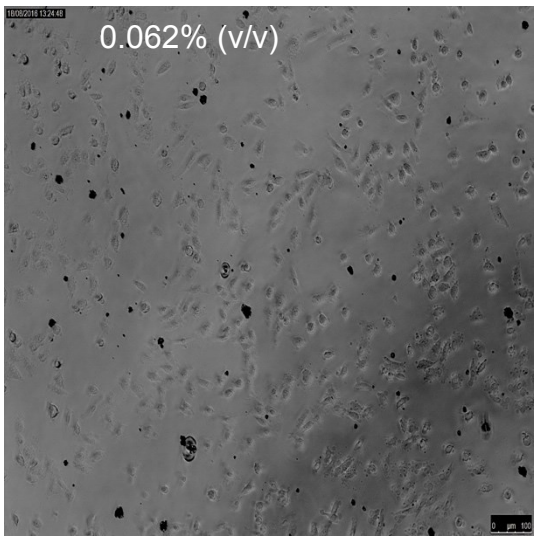




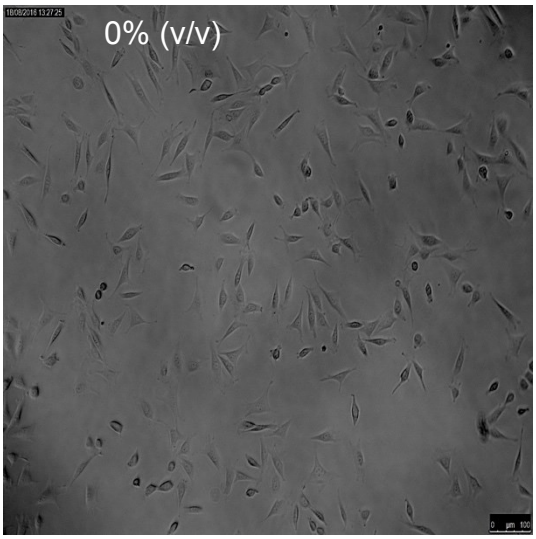


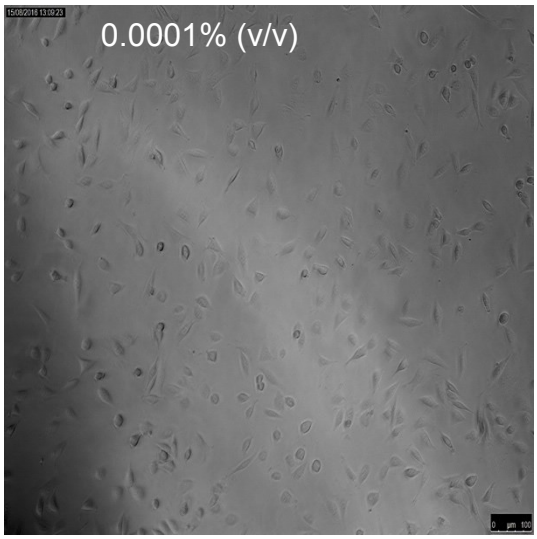


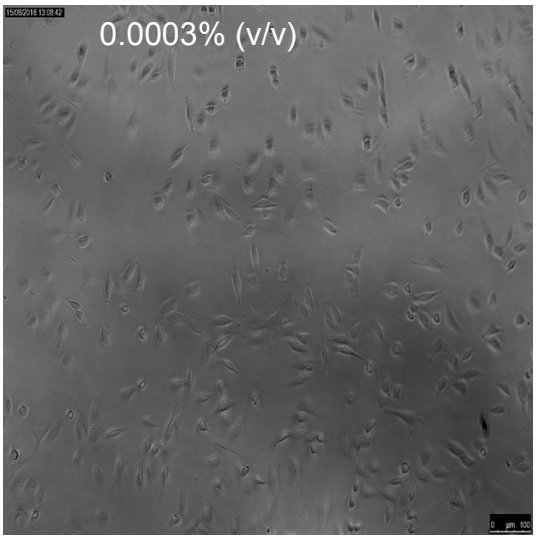


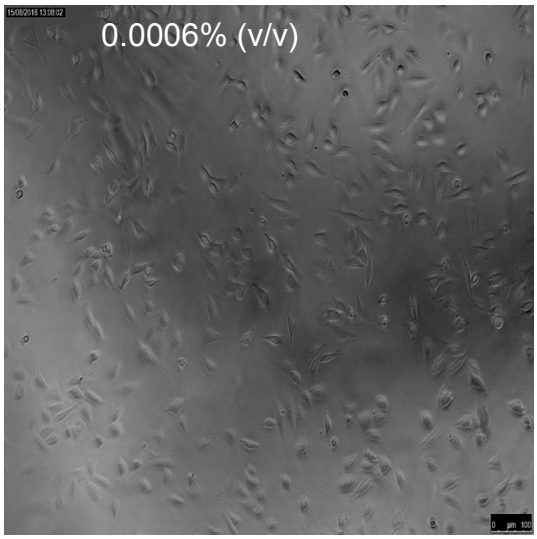


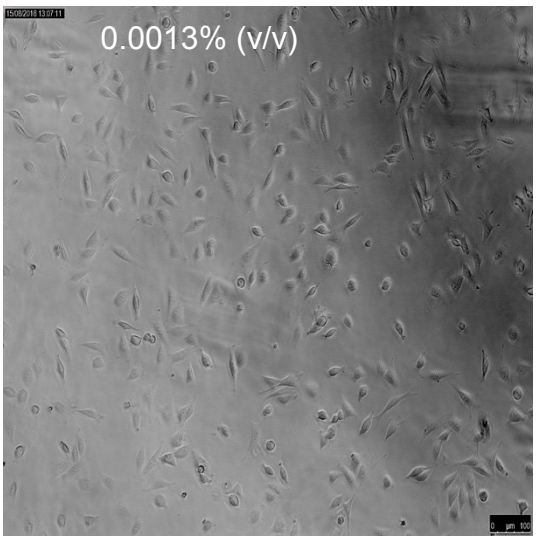
Chlorhexidine (E)

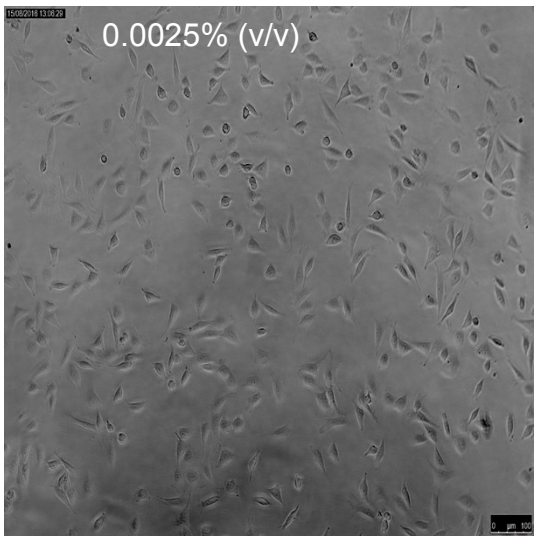












Chlorhexidine (E)

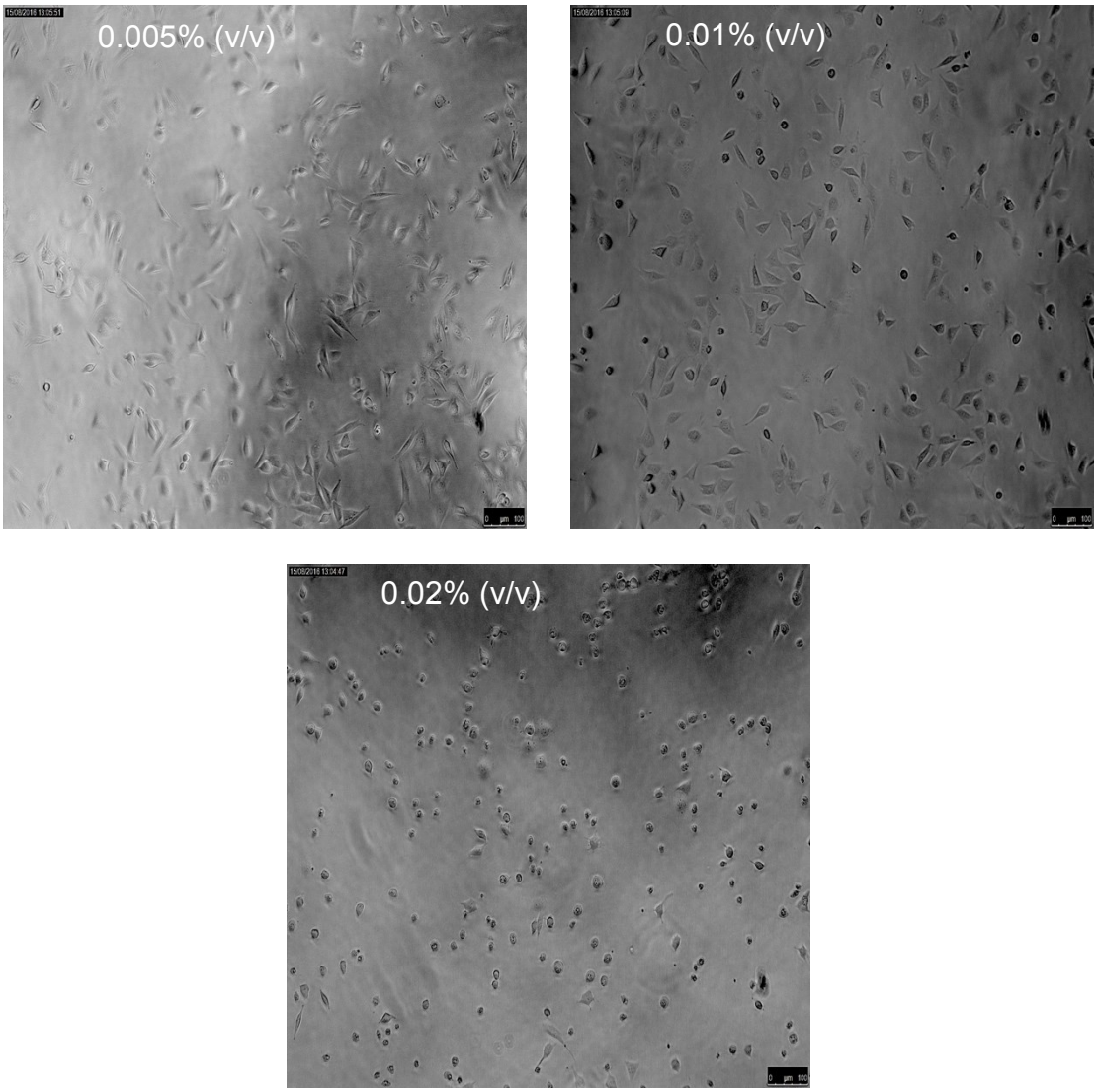


Figure 3.4 - Mouse fibroblasts cultured for 1 hour in the presence of different concentrations of cinnamon (A), geranium (B), melissa (C), E-cinnamaldehyde (D) and chlorhexidine (E). Scale bar = 100 μm

3.4.2 Inflammatory response of whole blood

The inflammatory response to *C. albicans* infection and *Melissa officinalis* essential oil was evaluated on human whole blood. Blood was obtained by three healthy donors and two cytokines were targeted: IL-6, a pro-inflammatory marker, and IL-10, an anti-inflammatory interleukin.

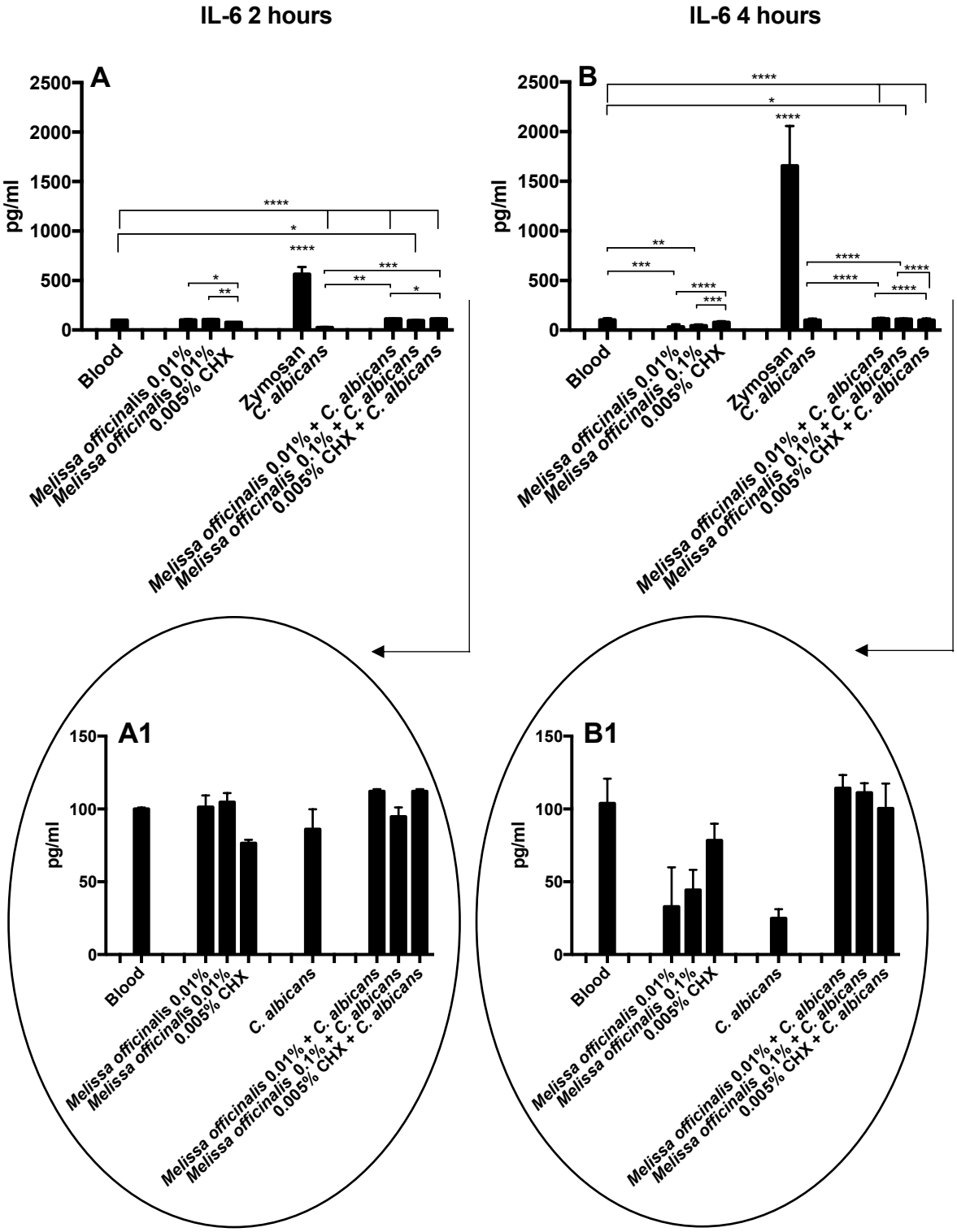
Concerning the production of IL-6, it was observed that 2 out of 3 donors did not show an inflammatory response. Indeed, the production of IL-6 was not detectable with *C. albicans* and/or *Melissa officinalis* but it was only trackable when the whole blood was cultured in the presence of the positive control (zymosan) (Figure 3.5-C/D/E/F).

Concerning the inflammatory response of “Donor 1”, when the blood was infected for 2 and 4 hours with *C. albicans*, there was not a significant pro-inflammatory response (Figure 3.5-A/B). In particular, after 4 hours the inflammatory response decreased and a significant production of IL-10 was observed (Figure 3.6-B). Similarly, a 4 hour addition of *Melissa officinalis* showed an anti-inflammatory response by decreasing the production of IL-6 (Figure 3.5-B).

When the whole blood was both infected with *C. albicans* and treated with the essential oil, a significant pro-inflammatory response was observed compared to the control (Figure 3.5-B). Concerning the effect of CHX, a significantly higher production of IL-6 compared to *Melissa officinalis* was observed after 4 hours, while a significant decrease in IL-6 was observed when CHX was combined with *Candida* (Figure 3.5-B). When CHX was applied for 2 hours, no differences were detected in the production of IL-10 compared to *Melissa officinalis* (Figure 3.6-A).

Concerning the anti-inflammatory response of “Donor 2” and “Donor 3”, IL-10 of “Donor 3” was not detectable. The anti-inflammatory response of “Donor 2” is outlined in Figure 3.6-C/D. Infection with *Candida* caused a significant production

of IL-10 (Fig. 3.6-A/B).



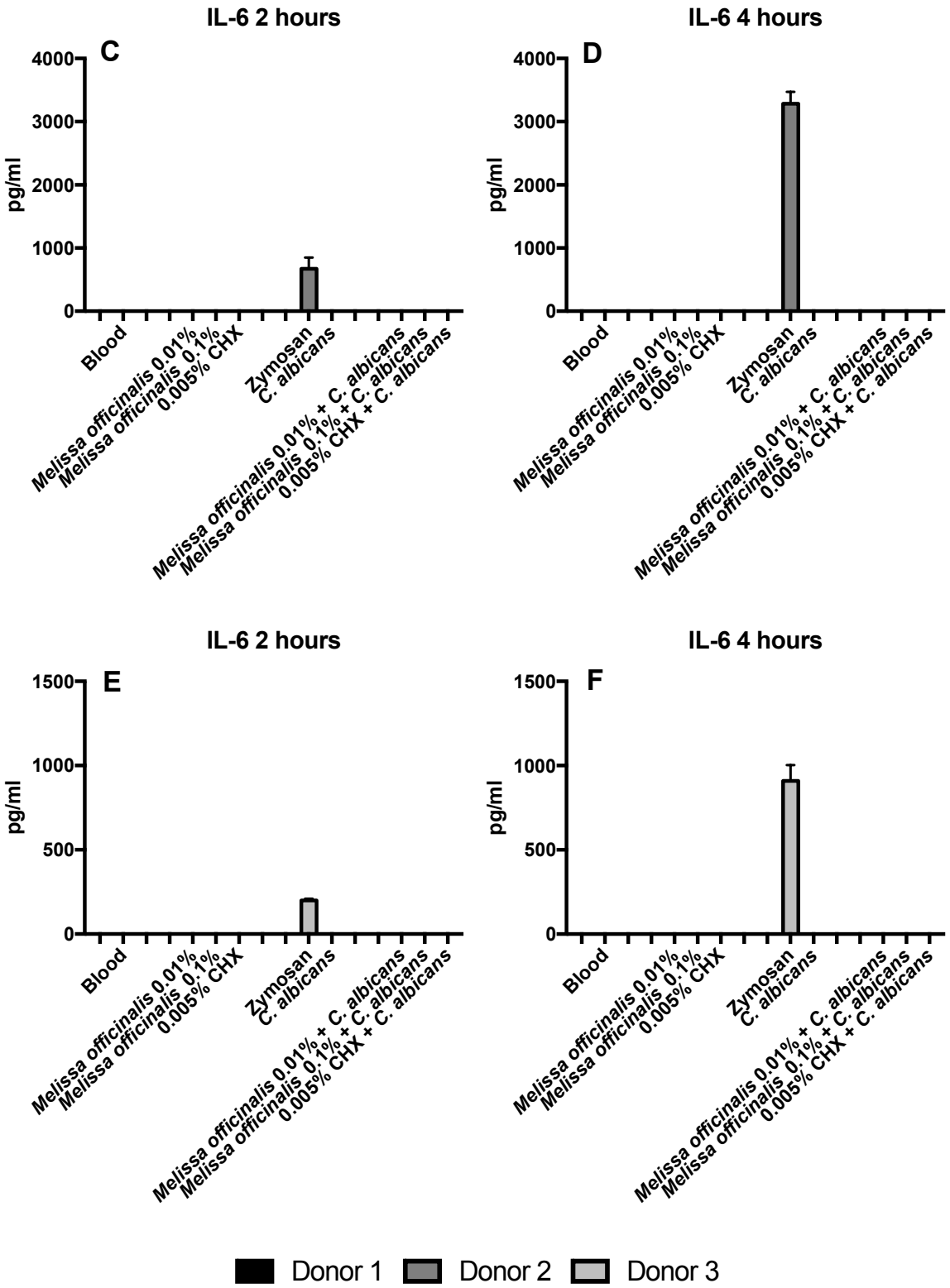


Figure 3.5 – Production of IL-6 from whole blood cultured for 2 and 4 hours in the presence of 0.01% *Melissa officinalis*, 0.1% *Melissa officinalis* and 0.005% CHX with or without *C. albicans*. Control included untreated and uninfected blood and zymosan. A1 and B1) IL-6 from "Donor 1" plotted without Zymosan.

Data represent the mean of three independent experiments, each performed in duplicate. * equivalent to $p < 0.05$, ** equivalent to $p < 0.01$, *** equivalent to $p < 0.001$, **** equivalent to $p < 0.0001$

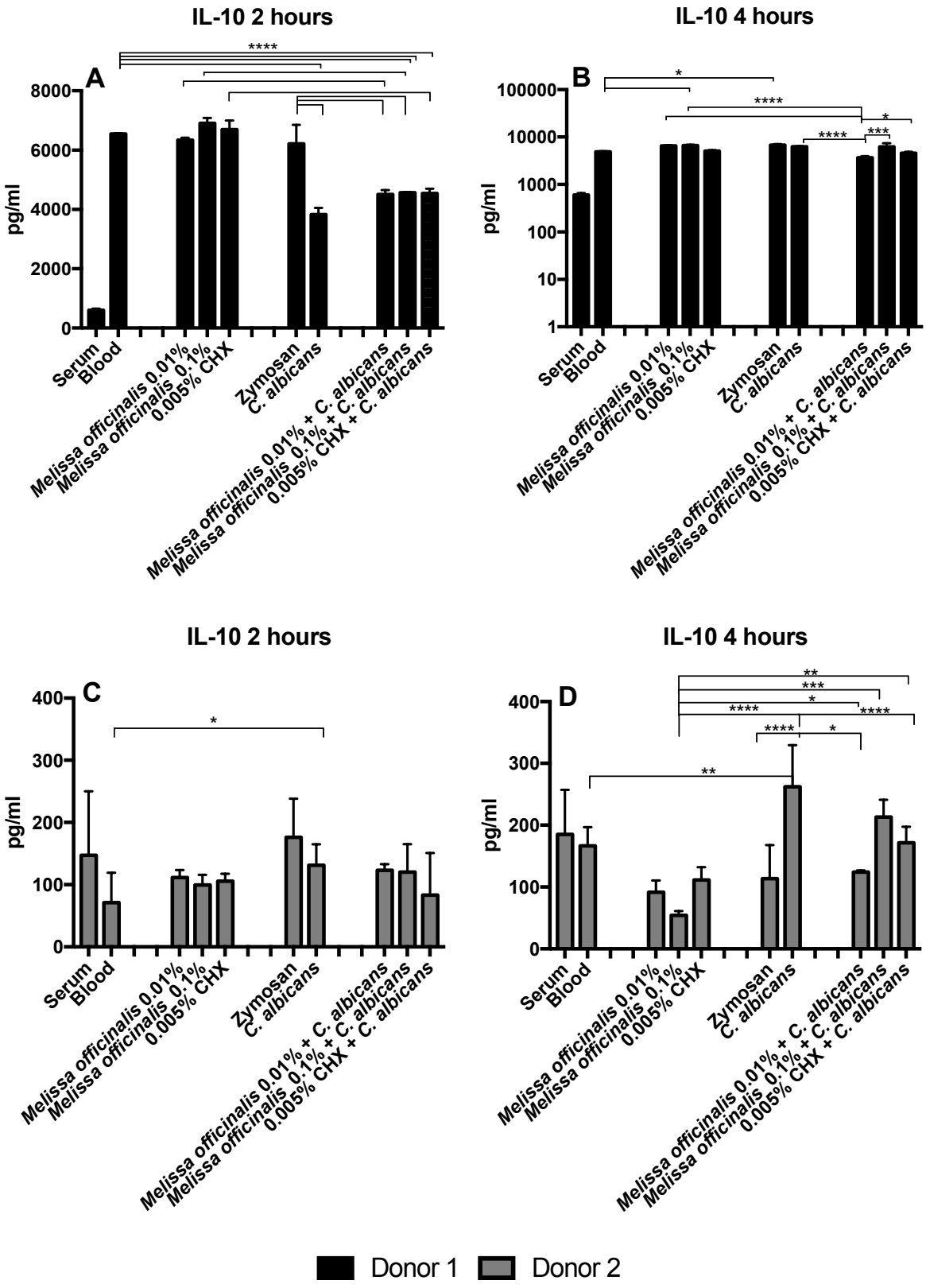


Figure 3.6 - Production of IL-10 from whole blood cultured for 2 and 4 hours in the presence of 0.01% *Melissa officinalis*, 0.1% *Melissa officinalis* and 0.005% CHX with or without *C. albicans*. Control included untreated and uninfected blood, and zymosan.
Data represent the mean of three independent experiments, each performed in duplicate.
* equivalent to p< 0.05, ** equivalent to p< 0.01, *** equivalent to p< 0.001, **** equivalent to p< 0.0001

3.5 Discussion

Essential oils have been used for centuries in traditional medicine. Antimicrobial, anti-aseptic, anti-inflammation and anti-oxidant activity of essential oils is well known (Nazzaro et al., 2013), but limited knowledge exists regarding their cytotoxicity.

The screening of the cytotoxicity of essential oils was performed against mouse fibroblasts. Essential oils that showed the best antifungal activity against *C. albicans* (cinnamon, geranium and melissa) and the best terpene (E-cinnamaldehyde) were selected (see Chapter 2). In addition, chlorhexidine was used as control. Few studies have investigated the cytotoxic effects of these oils. Cytotoxicity of CHX, cinnamon, E-cinnamaldehyde, geranium and melissa oils was a dose- and time-dependent. Overall, the commercial essential oils halved fibroblast proliferation at concentrations lower than their MICs. The IC50 values for E-cinnamaldehyde, geranium and cinnamon oils were actually 10-fold lower than their MIC, while melissa oil had an MIC80 of 0.06% (v/v) and an IC50 of 0.03% (v/v). Although a different assay and cell type was used, the melissa oil results (IC50 0.3 g/L) were in accordance with those of Paul et al. (2013), who did not see a significant change in leukocytes viability after 3 hour treatment with 150 µg/ml melissa oil. Several studies have used E-cinnamaldehyde to inhibit proliferation of cancer cells and reported IC50s ranging from 45.8 to 129.4 mM (Ka et al., 2003), higher concentrations than those obtained in this study with fibroblasts (0.16–0.26 mM). Barros et al. (2016) found that at concentrations lower than those evaluated in this study (5 µg/ml), *Cinnamomum zeylanicum* oil had cytotoxicity towards erythrocytes. A 1 hour exposure of fibroblasts to CHX (0.01% (v/v)) halved cell proliferation compared to controls. However, this

concentration was lower than the MICs (2.5×10^{-3} % (v/v) and 5×10^{-3} % (v/v)) found in the current study. This finding was similar to the cytotoxic effect of CHX previously reported using macrophages and human alveolar bone cells (Cabral and Fernandes, 2007; Li et al., 2012).

In conclusion, the results of the cytotoxic screening combined with the antifungal activity showed that the essential oil with the lowest cytotoxicity and the best antimicrobial properties was melissa essential oil. Therefore, melissa (*Melissa officinalis)* was used for further experiments.

The pro and anti-inflammatory host response to *Melissa officinalis* essential oil was evaluated on human blood cells harvested from three healthy individuals. The whole blood model was developed by Al-ishaq et al. (2015) to investigate the role of polysaccharide intercellular adhesin (PIA) in prosthetic joint infection (PJI). The *ex vivo* model of human whole blood allowed to better mimic the *in vivo* conditions. However, the use of human blood led to a high variability according to the donors and their general health (e.g. maybe one of the donors had a cold). Importantly 2 out of 3 donors did not show a significant pro-inflammatory response, except for the culture of whole blood with zymosan. Zymosan has been widely used to generate an immune response (e.g. stimulation of the production of cytokines and chemokines, and of the mechanisms of phagocytosis) (Underhill, 2003; Nohmi et al., 2015).

Concerning the anti-inflammatory response of “Donor 2”, even when the IL-10 production was detectable, the levels were not within the range of the standard curve, which was prepared according to the manufacturer’s protocol. Consequently, the results were shown but due to the low expression, they are

not discussed in more details. The low values could be due to the volunteer's sera being diluted too much, maybe a 1:3 dilution would be more sensitive and should be considered for future experiments.

"Donor 1" showed both a pro and anti-inflammatory response to the infection and treatment. IL-6 is a pro-inflammatory cytokine that it is commonly associated with *C. albicans* infection and with the Th17 cell-mediated response. Several studies reported a high expression of IL-6 *in vitro, ex vivo,* and *in vivo* in the presence of *Candida* (Steinshamn and Waage, 1992; Dongari-Bagtzoglou et al., 1999; Xiong et al., 2000; Kim et al., 2005). In general, an increased IL-6 production over time was detected, and Mostefaoui et al. (2004) reported no significant difference in comparison with the control after a 2 hour infection. This was in accordance with the kinetic release of IL-6 found in this study. However, after 4 hours a decrease in the IL-6 production was observed. The decrease of IL-6 was concomitant with an IL-10 increase. IL-10 is an anti-inflammatory cytokine that inhibits the production of IL-12 and the formation of Th1 cells, promoting a Th2 response that inhibits the activity of macrophages. IL-10 is one of the mechanisms used by *C. albicans* to evade the immune system (Xiong et al., 2000; Netea et al., 2004; Luo et al., 2013).

The addition of *Melissa officinalis* essential oil to the whole blood did not cause a significant immune response, except at a 4 hour treatment with 0.1% (v/v) that led to an anti-inflammatory response. Bounihi et al. (2013) showed that *Melissa officinalis* had an anti-inflammatory activity by inhibiting or decreasing oedema in rats. To the best of the author's knowledge, no data on the *in vitro* anti-inflammatory activity of *Melissa officinalis* are available. In any case, essential oils that have some of the main compounds in common with *Melissa officinalis* (e.g. citral, geraniol and citronellol) showed inhibition of IL-1 and IL-6 in mouse

macrophages stimulated with LPS, and suppression of the adherence reaction of neutrophils induced by TNFα (Abe et al., 2003; Sforcin et al., 2009).

When *Melissa officinalis* essential oil and *Candida* were added to the whole blood for 4 hours, the capacity of evading the immune system of *Candida* seemed to fail and a pro-inflammatory response was observed.

Chlorhexidine, a biocide commonly found in oral products, was used as a control. It was observed that chlorhexidine did not cause an immune response. Indeed, no significant differences were noticed with the untreated blood. This was in contrast with the anti-inflammatory properties that were reported in other studies, such as the inhibition of the production of IL-6 or the prevention of neutrophil inactivation (Montecucco et al., 2009; Vitt et al., 2017).

3.6 Conclusion

Cytotoxicity screening revealed that the commercial essential oils halved fibroblast proliferation at concentrations lower than those required to inhibit *C. albicans* growth. In particular, *Melissa officinalis* was the essential oil that exhibited the lowest cytotoxicity and best antimicrobial properties. The evaluation of the inflammatory response to *Melissa officinalis* on whole blood highlighted that the essential oil had an anti-inflammatory potential and inhibited the immune system's evasion of *C. albicans*.

Further investigation of the inflammatory response on the *ex vivo* whole blood model should be performed to increase the number of donors and expectantly decrease the variability in the response.

In general, even if these results showed that commercial essential oils were cytotoxic, it should be taken into account that cytotoxicity was conducted in a 2D-culture, which is notably different from *in vivo* conditions. Further investigation on mammalian cells could be performed in 3D-culture or *ex/in vivo* models to better mimic the biological structure of the tissues. In addition, the potential use of the essential oils in synergy with antifungals, as discussed in Chapter 2, would allow decreasing the concentrations needed to kill *C. albicans,* avoiding the cytotoxic effect.

Chapter 4

Development of a methylcellulose hydrogel with *Melissa officinalis* essential oil

4.1 Introduction

4.1.1 Hydrogels

Hydrogels are 3D-networks obtained from natural and/or synthetic polymers that once crosslinked form an insoluble structure (Slaughter et al., 2009). The peculiarity of hydrogels is their ability to absorb and retain a significant amount of water without dissolving. Swollen hydrogels have some properties in common with living tissues (e.g. consistency, low interfacial tension, water content) and are biocompatible (Bhattarai et al., 2010). Therefore, they have been widely used in tissue and regenerative medicine, mainly as drug and/or cell delivery systems and as pre-formed or injectable cell scaffolds (Slaughter et al., 2009).

According to the gelation process, they can be classified into physical and chemical hydrogels. Chemical hydrogels are formed by covalent bonds between the polymeric chains that result in strong and stable gels. Chemical crosslinking can be obtained by crosslinking with aldehydes or enzymes, by free radical polymerisation, by condensation or addition reactions, and by high energy radiation (e.g. gamma rays and electron beams) (Akhtar et al., 2016).

Physical hydrogels are characterised by physical interactions, such as hydrogen bonds, ionic interactions, hydrophobic association and entangled chains (Maitra and Shukla, 2014). Among the different types of hydrogels, it is worth mentioning the stimuli-responsive ones. These hydrogels gellify after the application of an external stimulus (e.g. temperature, pH, electric field and light) (Gulrez et al., 2011). In particular, the thermosensitive gels can crosslink at body temperature, allowing their injection at room temperature and the gelation *in situ*.

4.1.1.1 Methylcellulose hydrogel

Methylcellulose is a cellulose ether obtained by the substitution of the hydroxyls group (-OH) at C2, C3 and/or C6 of anhydro-D-glucose with a methyl group (-CH_3) (Figure 4.1)

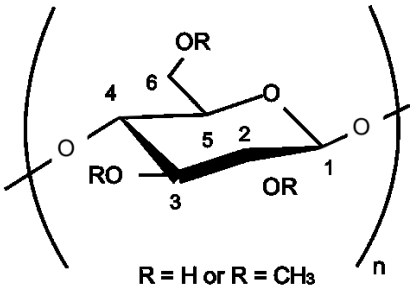


Figure 4.1 - Repetitive unit of methylcellulose

Methylcellulose is water-soluble and forms thermosensitive hydrogels. The gelation process is characterised by two phases: the hydrophobic interactions between the methylated zone form a “clear loose gel” at relatively low temperatures, while further increasing the temperature the phase separation leads to the formation of a “turbid strong gel” (Figure 4.2).

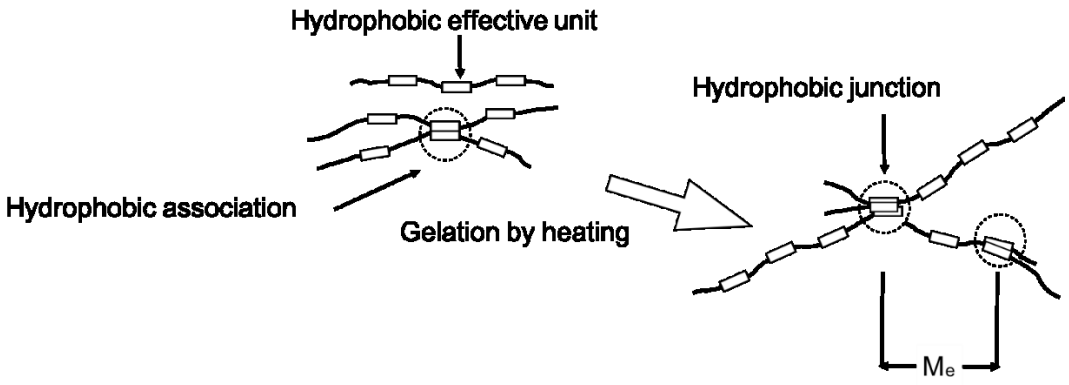


Figure 4.2 - Schematic representation of the gelation process of methylcellulose. At low temperatures, hydrophobic forces develop between the hydrophobic units of methylcellulose. By increasing the temperature, the “turbid strong gel” forms with hydrophobic junctions characterised by hydrophobic association and a constant mean length (M_e) between two junctions. Figure adapted from Li et al. (2001)

The gelation process of methylcellulose can be investigated by evaluating the storage (G') and loss (G'') modulus at different temperatures (see Section 4.1.2.1). At low temperatures, the storage modulus crosses the loss modulus and the gel is formed. However, by further increasing the temperature, the storage modulus reaches a plateau and a stronger gel is obtained (Figure 4.3).

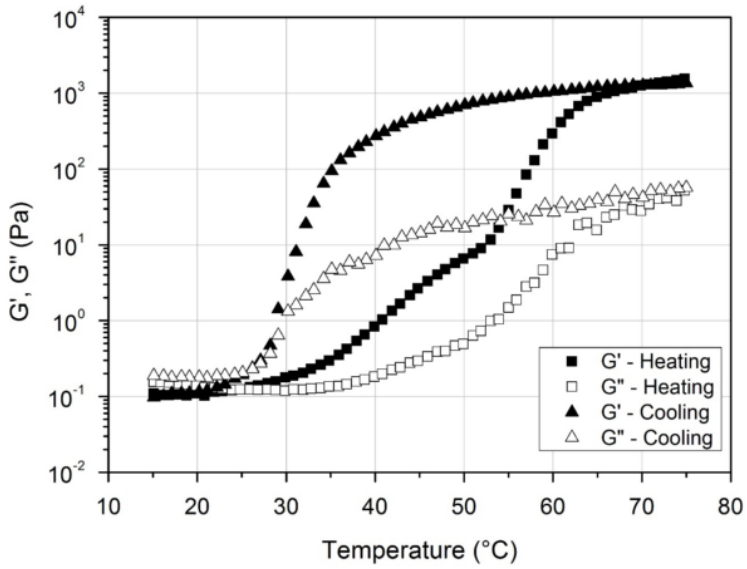


Figure 4.3 - Temperature dependence of dynamic viscoelasticity for methylcellulose (10 g/l) in aqueous solution. Constant frequency of 0.5 Hz, strain 1%, and temperature rate of 0.5°C/min. ■, □ on heating; ▲, △ on cooling. G′, solid symbols, and G″, open symbols (Nasatto et al., 2015a)

In general, the rheological properties of methylcellulose (e.g. gelation time) can be tailored by changing the polymer concentration, the molecular weight and by using additives such as salts, alcohols and surfactants (Nasatto et al., 2015a).

4.1.1.2 Applications and antifungal activity of methylcellulose hydrogels

Methylcellulose is widely used in food packaging. Formulations of methylcellulose films containing essential oils have been developed. Campos et al. (2014) synthesised methylcellulose films containing Ginja cherry extract and evaluated the antimicrobial properties by using a zone inhibition method and time-kill assay,

observing the inhibition of a range of Gram-positive and Gram-negative organisms. Similarly, Ashman et al. (2011) developed a methylcellulose film containing olive leaf extract, while Otoni et al. (2014) investigated the potential of a methylcellulose film loaded with clove bud (*Syzygium aromaticum*) and oregano (*Origanum vulgare*) essential oil.

Concerning the use of methylcellulose as a drug delivery system, methylcellulose is a bioadhesive polymer that shows adhesion to mucosal surfaces (Klouda, 2015). This characteristic prolongs the retention of the drug delivery system at the site (Gafitanu et al., 2017). Formulations of methylcellulose hydrogel containing silver oxide nanoparticles (Kim et al., 2018) and of hydroxypropyl methylcellulose hydrogel loaded with fluconazole (Gafitanu et al., 2017) have been successfully synthesised as treatment for burn wound healing and vaginal fungal infection, respectively.

Kong et al. (2016) developed a hydroxypropyl methylcellulose hydrogel for use against oral candidiasis. A 4% (w/w) hydroxypropyl methylcellulose hydrogel was formulated with 2 mg/ml of Histatin-5, a peptide with antimicrobial properties. The authors tested the antifungal activity of the hydrogel against *C. albicans* standard strain SC5314, observing Histatin-5 release from the hydrogel within 2 hours and a significant decrease in CFU/ml compared to the control (i.e. hydroxypropyl methylcellulose hydrogel without antimicrobial). In addition, the novel formulation was tested *in vivo*. The tongues of mice were infected with *C. albicans* and the hydrogel was applied for 1 hour. Untreated mice developed oral candidiasis with tissue damage and *C. albicans* was recovered from the site. By contrast, most of the mice treated with the hydrogel showed less fungal adherence, hyphal penetration and tissue damage (Kong et al., 2016).

4.1.2 Rheology

Rheology is the study of the deformation and flow of matter (Barnes et al., 1989). Elastic solid materials are materials in which a linear relationship between the shear stress and strain exists. When a constant shear stress is applied, an immediate and constant deformation is observed. Since there is no energy dissipation, once the shear stress is removed the material goes back to its original shape. The elastic behaviour can be described by Hooke's law (4.1):

$$\sigma = E * \varepsilon \quad (4.1)$$

where σ is the shear stress applied, ε the deformation and E the Young's modulus, representative of the material stiffness (Schramm, 1998; Morton and Hearle, 2008; Murata, 2012).

Newtonian fluids are fluids in which a linear relationship between the shear stress and shear rate exists. Once the shear stress is removed, the material does not return to its original shape because all the energy applied is dissipated by friction during the deformation. The behaviour of ideal fluids is described by Newton's law (4.2):

$$\sigma = \eta * \dot{\gamma} \quad (4.2)$$

where σ is the shear stress applied, $\dot{\gamma}$ the shear rate and η the viscosity, representative of the material flow resistance (Schramm, 1998; Morton and Hearle, 2008; Murata, 2012).

In fact, materials do not show a viscous or elastic behaviour but a combination of both, named viscoelasticity. If the deformation is proportional to the shear (i.e.

linear viscoelastic region), the viscoelastic behaviour can be described by combining Hooke's and Newton's law.

The viscoelasticity of a material can be studied by a dynamic rheological analysis: a sinusoidal strain is applied to the material and the resulting sinusoidal stress is measured. Elastic solid materials show a stress and strain in phase, purely viscous materials are characterised by a 90° phase lag, while viscoelastic materials exhibit a phase lag ranging from 0° to 90° (Figure 4.4) (Schramm, 1998; Morton and Hearle, 2008; Murata, 2012).

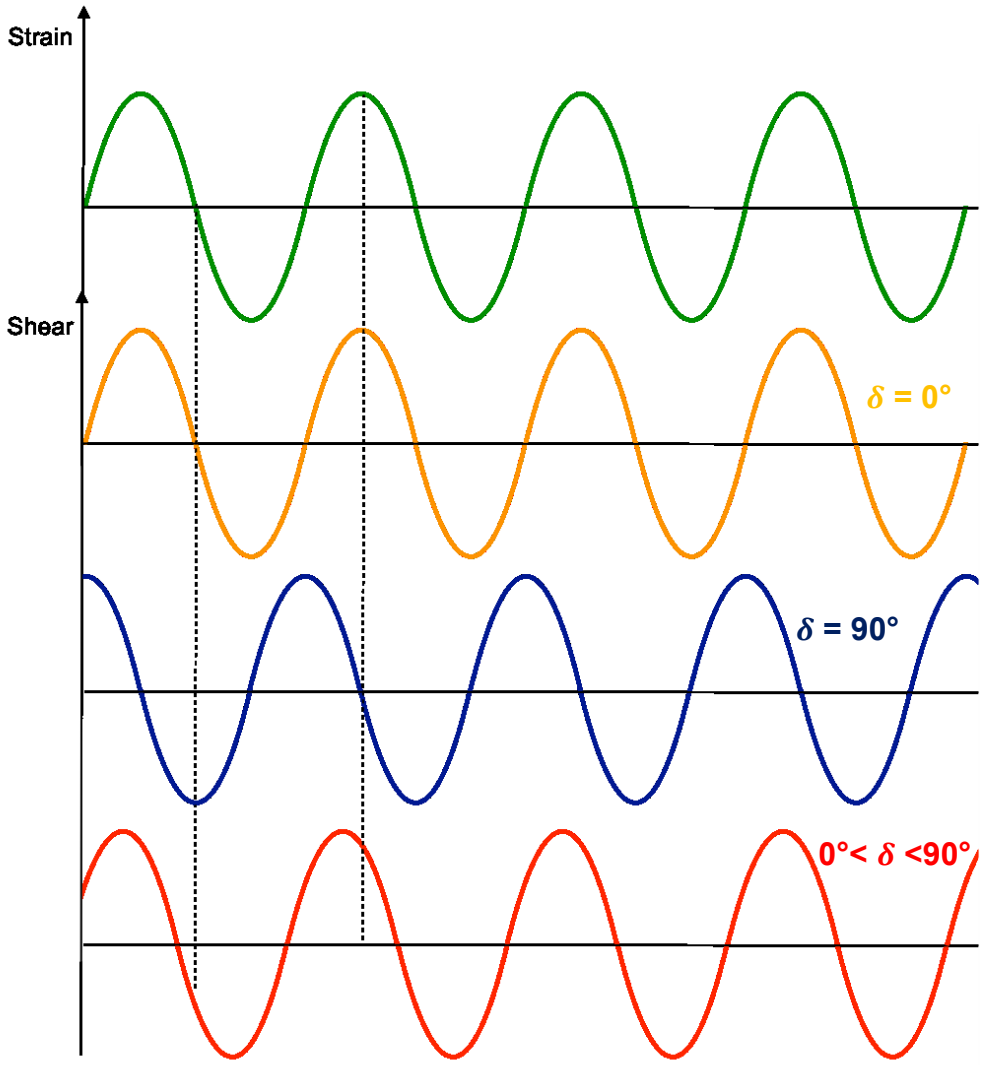


Figure 4.4 - Representative image of a dynamic rheological analysis. A sinusoidal strain (green) is applied to the sample and the resulting shear is measured. Elastic solid materials (orange) show a stress and strain in phase, purely viscous materials (blue) have a 90° phase lag, and viscoelastic materials (red) exhibit a phase lag (δ) ranging from 0° to 90°

Therefore, the viscoelastic materials can be described as a combination of an elastic component (in phase) and a viscous component (out of phase) (4.3).

$$\sigma = \sigma_0 \sin(\omega t + \delta) = \sigma_0 \sin \omega t + \sigma_0 \cos(\omega t) \quad (4.3)$$

where σ is the shear stress, σ_0 is the amplitude, ω is the angular frequency, and δ is the phase lag.

Equation 4.3 can be rewritten as:

$$\sigma = G' \varepsilon_0 \sin \omega t + G'' \varepsilon_0 \cos(\omega t) \quad (4.4)$$

where G' is the storage modulus, linked to the ability of the material to store energy and G'' is the loss modulus representing the energy dissipation. In other words, the storage modulus is the elastic component of the material while the loss modulus is the viscous component.

When $G'' > G'$ the material behaves as a liquid-like material, while when $G' > G''$ it behaves as a solid-like material (e.g. hydrogel) (Schramm, 1998; Morton and Hearle, 2008; Murata, 2012).

4.1.2.1 Rheological analysis

Rheological analysis investigates the rheological properties of a material. The synthesis of a novel hydrogel to be used *in vivo* should involve the investigation of the storage and loss modulus, which influence the drug release, the mechanical properties, the gelation time at body temperature and the injectability (i.e. viscosity). Rheological analysis commonly involves:

- Strain sweep

A sinusoidal strain with increasing amplitudes is applied to the material (Figure 4.5). The response of the material is monitored at constant frequencies and temperatures. This experiment is usually run to identify the linear viscoelastic region (LVR) (Schramm, 1998; Zuidema et al., 2014).

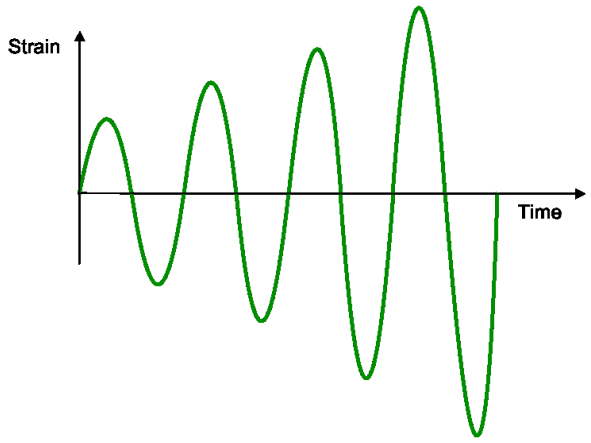


Figure 4.5 - Sinusoidal strain with increasing amplitudes

- Frequency sweep

The response of the material to increasing frequencies of deformation is evaluated at constant temperature and strain amplitude (Figure 4.6) (Schramm, 1998; Zuidema et al., 2014).

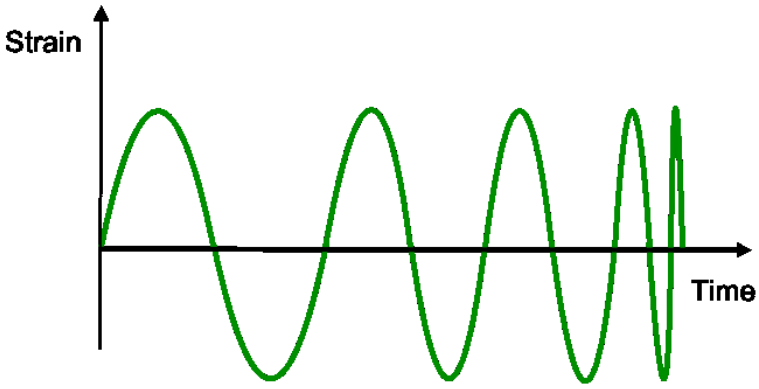


Figure 4.6 - Sinusoidal strain with increasing frequencies

- Temperature sweep

The rheological properties of the material are evaluated at increasing temperatures at a constant strain. This experiment can be run to determine the gelation temperature, that is the temperature at which the storage modulus exceeds the loss modulus (Figure 4.3) (Schramm, 1998; Zuidema et al., 2014).

- Time sweep

The rheological properties of the material are evaluated over time at a constant frequency, amplitude and temperature. This experiment is performed to investigate the gelation time at a certain temperature (e.g. body temperature) (Figure 4.7) (Schramm, 1998; Zuidema et al., 2014).

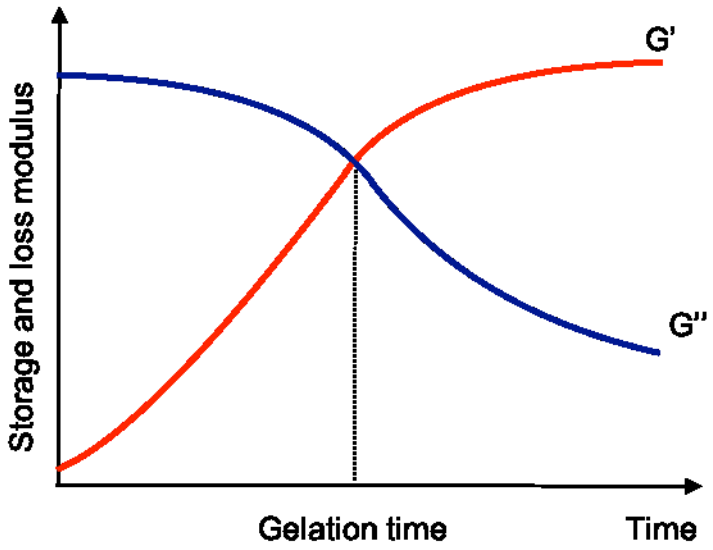


Figure 4.7 - Representative plot of the gelation time. Initially the material is liquid (G'' > G') while during time the storage modulus increases, and the gel is formed (G'> G'')

4.2 Aims and objectives

The aim is to develop a methylcellulose hydrogel with *Melissa officinalis* essential oil as treatment for oral candidiasis. The hydrogel is intended to be used as a vehicle to deliver the antimicrobial at the site of infection or as a denture coating. The specific objectives are:

a) To evaluate the rheological properties and gelation time of 4 methylcellulose hydrogels: i) 10% (w/w) methylcellulose with 1% (v/v) *Melissa officinalis*, ii) 10% (w/w) methylcellulose with 2% (v/v) *Melissa officinalis*, ii) 12% (w/w) methylcellulose with 1% (v/v) *Melissa officinalis*, and iv) 12% (w/w) methylcellulose with 2% (v/v) *Melissa officinalis*
b) To evaluate the release of *Melissa officinalis* essential oil from the methylcellulose hydrogels synthesised
c) To evaluate the antifungal activity in terms of zone of inhibition and time-kill assay of the most promising hydrogels synthesised

4.3 Material and methods

4.3.1 Hydrogel preparation

Methylcellulose was purchased from Sigma-Aldrich (M7140, Sigma-Aldrich, Gillingham, UK). Table 4.1 lists the hydrogel formulations prepared according to the manufacturing protocol, adjusted to allow addition of *Melissa officinalis* essential oil. Briefly, sterile distilled water, with or without 1% or 2% (v/v) *Melissa officinalis* and Tween 80 in equal amounts, was heated to 80 °C for 10 minutes. Ten percent (10% w/w) or 12% (w/w) methylcellulose were gently added into the solvent. Once the powder was fully wet, the temperature was reduced by placing the solution on ice. As the temperature lowered, methylcellulose became water-soluble and a clear viscous solution was formed. The solution was carefully vortexed until the powder was not visible anymore, and then incubated overnight at 4 °C shaking at 200 rpm. Prior to use, the solution was vortexed and equilibrated at room temperature for 1 hour to allow bubble dispersion. The equilibrated solution was transferred to a petri dish and incubated at 37 °C until a stable hydrogel was formed, within 30 minutes. Once a stable hydrogel was formed discs were cut with a sterile cork-borer at the different sized (0.75, 10 or 11.25 mm in diameter) and used for further experiments (Sections 4.3.4, 4.3.5, 4.3.6).

Table 4.1 - List of the methylcellulose hydrogels synthesised

	Methylcellulose % (w/w)	***Melissa officinalis* % (v/v)**
Hydrogel A	10	1
Hydrogel B	10	2
Hydrogel C	12	1
Hydrogel D	12	2

4.3.2 Rheology

The rheological characterisation was performed with a Discovery Hybrid Rheometer DHR-2 (TA Instruments, New Castle, DE) equipped with a 20 mm diameter plate. One hundred and eighty microlitres (180 µl) of the equilibrated solution was placed on the rheometer and the plate was lowered to the gap height used during the experiments (500 µm). To avoid solvent evaporation a Solvent Trap (TA Instruments, New Castle, DE) was used as well. Table 2 summarises the testing parameters set to evaluate the rheological properties of the 4 hydrogels (Table 4.2). The method was adapted from that previously reported by Zuidema et al. (2014). Before analysing the strain and frequency sweep, the solution was kept at 37 °C for 8 minutes to allow the formation of a stable hydrogel. Each sample was used for one experiment and each test was performed in triplicate.

Table 4.2 - Temperatures, gap sizes, equilibrium times, frequencies, percent strains and running times set for all rheological tests

Hydrogel	Parameters	Strain Sweep	Frequency Sweep	Time Sweep
Hydrogel A Hydrogel B Hydrogel C Hydrogel D	Temperature	37 °C	37 °C	37 °C
	Gap size	500 µm	500 µm	500 µm
	Equilibrium time	8 min	8 min	-
	Frequency	1 Hz	0.01 - 100 Hz	1 Hz
	% Strain	0.1% - 100%	1%	1%
	Running time	-	-	10 minutes

4.3.3 *Melissa officinalis* composition

Essential oils are natural products mainly composed of terpenes and terpenoids and other molecules (Nazzaro et al., 2013). Prior to evaluating the release of *Melissa officinalis* from the methylcellulose hydrogels, the composition of the essential oil was analysed by gas chromatography (Agilent Technologies 7890B, Cheadle, UK) coupled to a mass spectrometer (Agilent Technologies 5977B, Cheadle, UK). The gas chromatograph was equipped with a capillary column Agilent J&W HP-5ms (30 m × 0.25 mm, 0.25 μm film thickness) (Agilent Technologies, Cheadle, UK).

The method was adapted from that previously reported by Rajkowska et al. (2016). The initial temperature was set to 50 °C and held for 3 minutes, followed by a linear increase up to 300 °C (40 °C/min) and held for 2 minutes. A 5:1 split ratio with an injection volume of 2 μl was set. Inlet and transfer line temperatures were kept at 275 °C and 300 °C, respectively. Helium, CP grade, was used as a carrier gas at a flow rate of 1 ml/min. Quadrupole temperature was fixed to 150 °C. The mass spectra were obtained by scanning a range of masses from 45 to 550 atomic mass unit (AMU). The ion source temperature was 230 °C and the ionisation was obtained by electron impact at 70 eV. All samples were prepared in dichloromethane (DCM) and the composition of the oil was analysed by comparing the mass spectra with those of the computer library (NIST14 MS Search library).

Once the compounds were identified, abundance was determined using standard curves of each compound. Briefly, analytical standards of citronellol, citronellal, geraniol, and linalool (Sigma-Aldrich, Gillingham, UK) were purchased. Double serial dilutions of each compound from 20 ppm to 2.5 ppm were prepared in DCM and analysed by GC-MS. The correlation between peak areas and

concentrations was obtained and used to evaluate the abundance of each compound.

4.3.4 *Melissa officinalis* release

Ten percent (10% w/w) and 12% (w/w) methylcellulose hydrogels containing 1% and 2% (v/v) *Melissa officinalis* essential oil were prepared as described in Section 4.3.1. Once gellified at 37 °C, 1 g of hydrogel was cut with a 11.25 mm diameter sterile cork-borer and incubated in 20 ml distilled water in a sterile glass universal at 37 °C under shaking conditions at 150 rpm (New Brunswick™ I26, Eppendorf UK Limited, Stevenage, UK). Two millilitres (2 ml) of liquid were collected at 1, 2, 3, 4, 5, 6, 7, 8, 24, 30 and 48 hours and replaced by 2 ml of distilled water, to keep the volume constant throughout the experiment.

To evaluate the *Melissa officinalis* oil release, 1 ml of each liquid sample was added to 5 ml DCM and vortexed briefly before incubation overnight at 4 °C with shaking at 200 rpm to allow dispersion of *Melissa officinalis* oil into DCM. After incubation, the sample was centrifuged at 3000 rpm (SIGMA 3-16 Centrifuge, Sigma Centrifuges, Newtown, UK) for 5 minutes and the DCM, containing the essential oil, was collected in a 1 ml vial and analysed by GC-MS (Section 4.3.3).

Controls included the initial composition of the hydrogels. To extract the oil within the hydrogel, 1 g of hydrogel was placed into 10 ml DCM, vortexed briefly and incubated overnight at 4 °C with shaking at 200 rpm. After incubation, the hydrogel within the DCM was centrifuged at 3000 rpm for 10 minutes and the DCM was collected in a vial and run in the GC-MS. All experiments were completed in triplicate and drug release was expressed as percentage cumulative release and milligrams released.

4.3.5 Disc diffusion method

The antifungal activity of 10% (w/w) methylcellulose hydrogels containing *Melissa officinalis* was evaluated by agar disc diffusion method. The method was adapted from that previously reported by Campos et al. (2014). Briefly, 100 µl of an overnight *C. albicans* 135BM2/94 diluted to 10^7 CFU/ml (for details see Section 2.7.2) was uniformly spread onto SAB agar plates. Ten percent methylcellulose (10% (w/w)) hydrogels with 1 or 2% (v/v) *Melissa officinalis* were prepared as described in Section 4.3.1. Once the solution gellified, 10 mm discs in diameter were cut with a sterile cork-borer, placed onto the agar and incubated overnight at 37 °C. After overnight incubation the diameter of the resulting zone of inhibition was measured. Control included 10% (w/w) methylcellulose hydrogel without essential oil. All tests were performed in duplicate on three separate occasions.

4.3.6 Time-kill assay

One hundred and fifty microlitres (150 µl) of an overnight *C. albicans* 135BM2/94 culture diluted to a turbidity equivalent to 0.5 McFarland Standard (see Section 2.7.2) was added with 150 µl of SDB to a sterile 1.5 ml Eppendorf tube. A disc 0.75 mm in diameter (equivalent to 0.15 g) of 10% (w/w) methylcellulose hydrogels with 1 or 2% (v/v) *Melissa officinalis* essential oil was added to the tube. The cultures were incubated at 37 °C with shaking at 150 rpm. At 2, 4, 6 and 24 hours the suspension containing *C. albicans* 135BM2/94 was collected, and viable cell numbers were counted. Samples were serially diluted in PBS and 50 µl was spread onto SAB agar plates with a Whitley Automated Spiral Plate (WASP, Don Whitley Scientific Limited, Shipley, UK). Agar plates were incubated overnight at 37 °C, and CFUs/ml were counted. Each experiment was performed in duplicate on three separate occasions. Controls included *C. albicans* cultured

in SDB with or without 10% (w/w) methylcellulose hydrogel. The method was adapted from that previously reported by Kong et al. (2016).

4.3.7 Statistical analyses

Statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). Data were presented as arithmetic mean ± SD or mean ± SEM. The difference between treatments was statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. Statistically significant differences were set at $p < 0.05$.

4.4 Results

4.4.1 Rheology

The viscoelastic properties of a hydrogel are independent of the strain and frequency applied up to a certain critical level. Beyond this point, the viscoelastic behaviour is not linear. Hence prior to study the gelation time, it is crucial to identify the test parameters (i.e. frequency and strain) in which the hydrogel responds linearly.

4.4.1.1 Strain sweep

The strain amplitude dependence of the storage and loss modulus was measured stimulating the hydrogel with strains between 0.1% and 100% at a constant frequency of 1 Hz. The storage modulus was constant at strains below 10%, while it increased for strains above 10%, leading to the network destruction and loss of mechanical properties (Figure 4.8). Figure 4.9 compares the storage modulus, representative of the solid behaviour of the material, of the four hydrogels tested. As can be observed, increasing the percentage of methylcellulose led to an increase in the storage modulus, while the presence of *Melissa officinalis* essential oils did not affect the rheological behaviour.

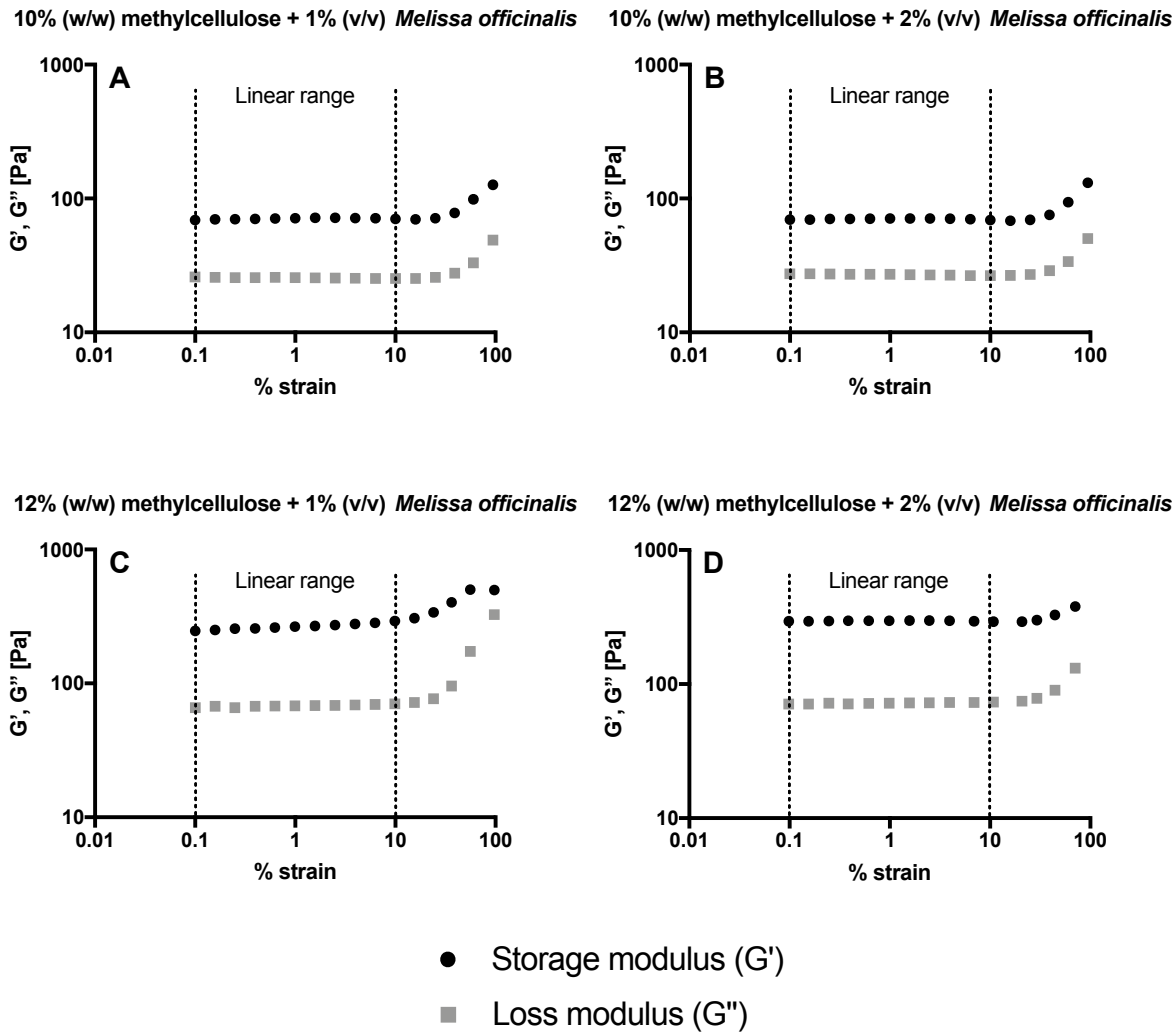


Figure 4.8 - Strain sweep for 10% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (A), 10% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (B), 12% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (C), 12% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (D). The linear viscoelastic limit was determined with respect to the percentage of strain. G' (black circle) and G'' (grey square) were determined at strains ranging from 0.1 to 100%. Data represent the mean of three independent experiments

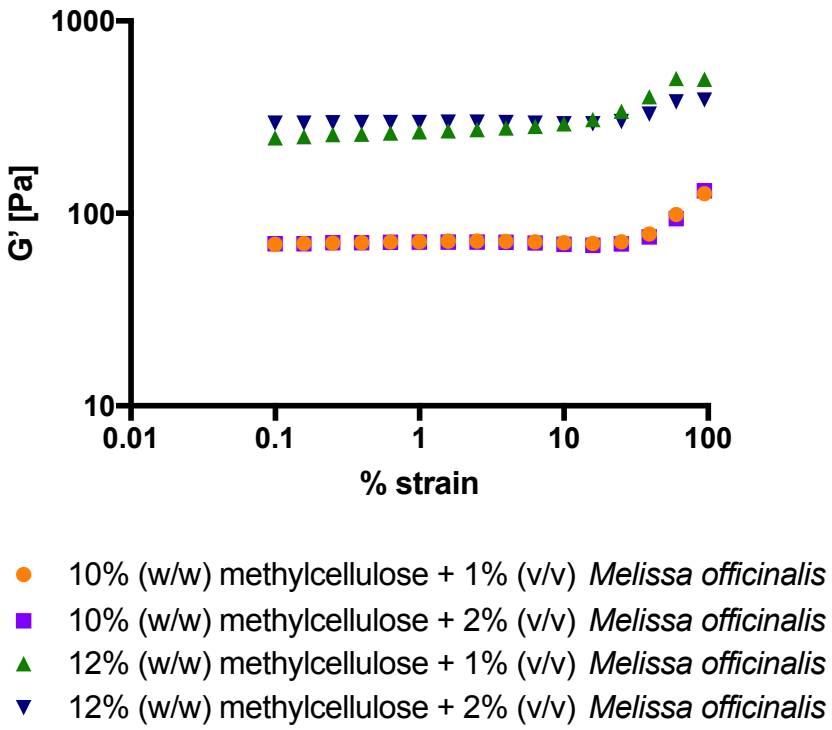


Figure 4.9 - Storage modulus (G') with respect to the strain of 10% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (orange), 10% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (violet), 12% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (green), 12% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (blue). Data represent the mean of three independent experiments

4.4.1.2 Frequency sweep

The frequency amplitude dependence of the storage and loss modulus was measured by stimulating the hydrogel with frequencies ranging from 0.01 Hz to 100 Hz at a constant strain amplitude of 1%. The storage modulus was constant at frequencies lower than 10 Hz, while at frequencies above 10 Hz the loss modulus exceeded the storage modulus (data not presented) leading to the destruction of the hydrogel (Figure 4.10). Twelve percent (12% (w/w)) methylcellulose hydrogel showed higher mechanical properties, while the presence of *Melissa officinalis* essential oils did not affect the rheological behaviour (Figure 4.11).

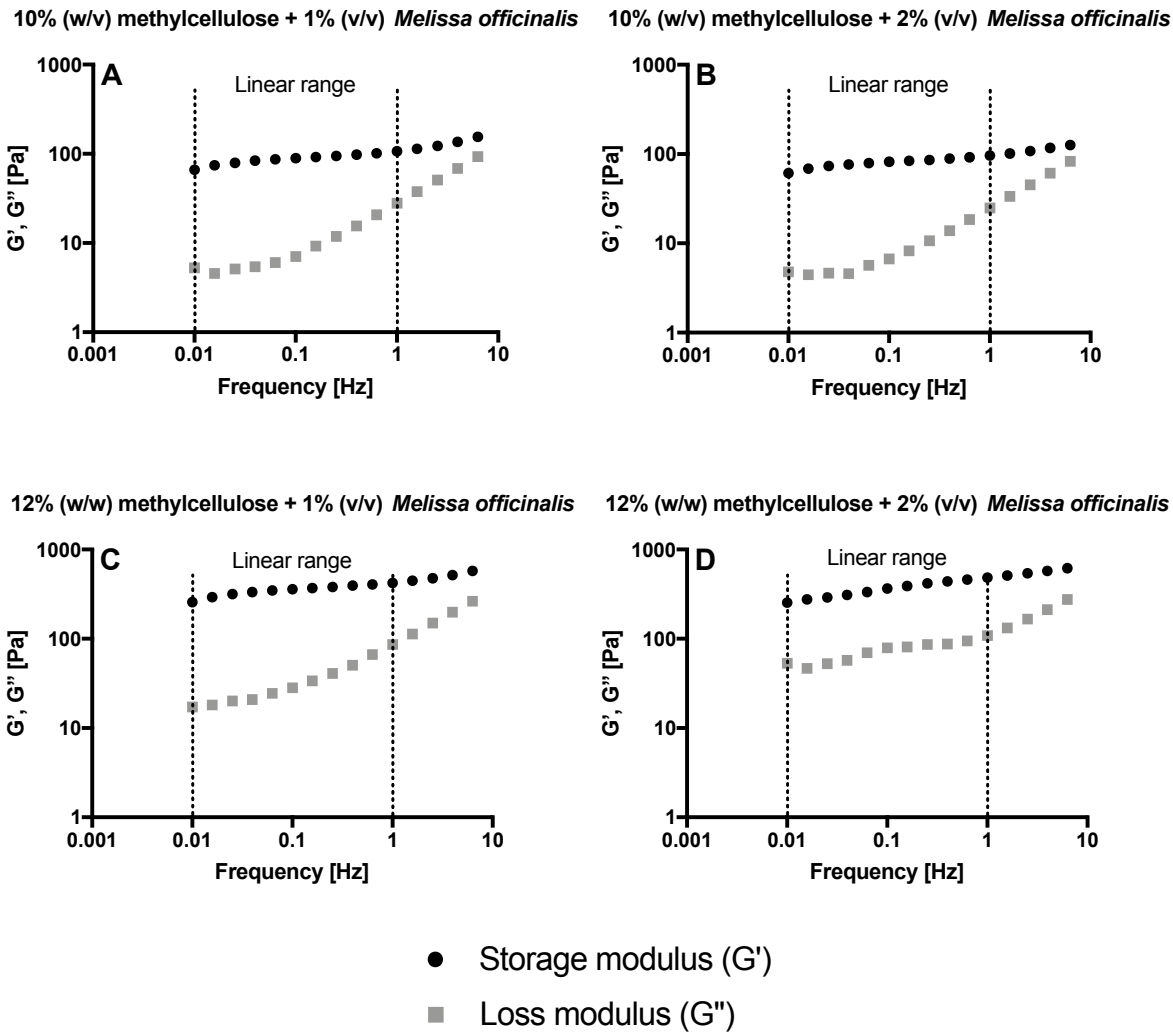


Figure 4.10 - Frequency sweep for 10% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (A), 10% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (B), 12% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (C), 12% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (D). The linear viscoelastic limit was determined with respect to the frequencies. G' (black circle) and G'' (grey square) were determined at angular frequencies ranging from 0.01 Hz to 100 Hz. Data represent the mean of three independent experiments

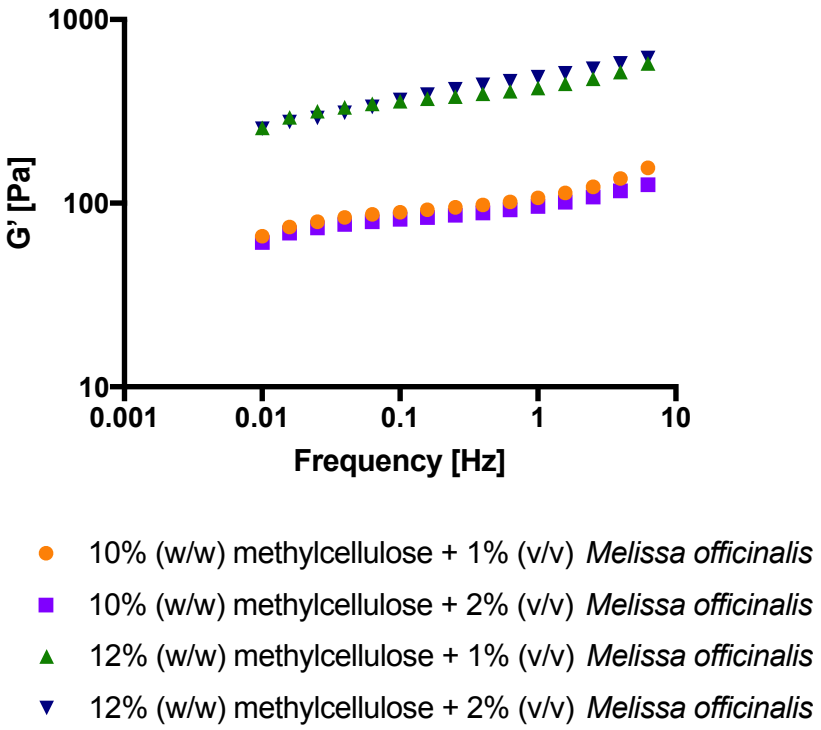


Figure 4.11 - Storage modulus (G') with respect to the angular frequencies of 10% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (orange), 10% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (violet), 12% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (green), 12% (w/w) methylcellulose (w/w) and 2% (v/v) *Melissa officinalis* (blue). Data represent the mean of three independent experiments

4.4.1.3 Time sweep - Gelation time

The gelation time, that is the time needed to form the hydrogel at 37 °C, was studied by applying to the hydrogel a 1% strain at 1 Hz frequency. In this range, the viscoelastic properties of the hydrogel were independent of the strain and frequency applied, as seen in the previous experiments. Figure 4.12 shows the storage and loss modulus as a function of time. Initially, the solution was liquid (the loss modulus exceeded the storage modulus), while over time the storage modulus increased. At a certain point, the storage and loss modulus crosslinked and at this point the hydrogel was formed. Figure 4.13 shows tan(δ), which is the ratio between the loss and storage modulus. When tan(delta) is equal to 1, the

hydrogel is formed. Twelve percent (12% (w/w)) methylcellulose gellified more quickly at 37 °C ($p<0.001$) (Table 4.3).

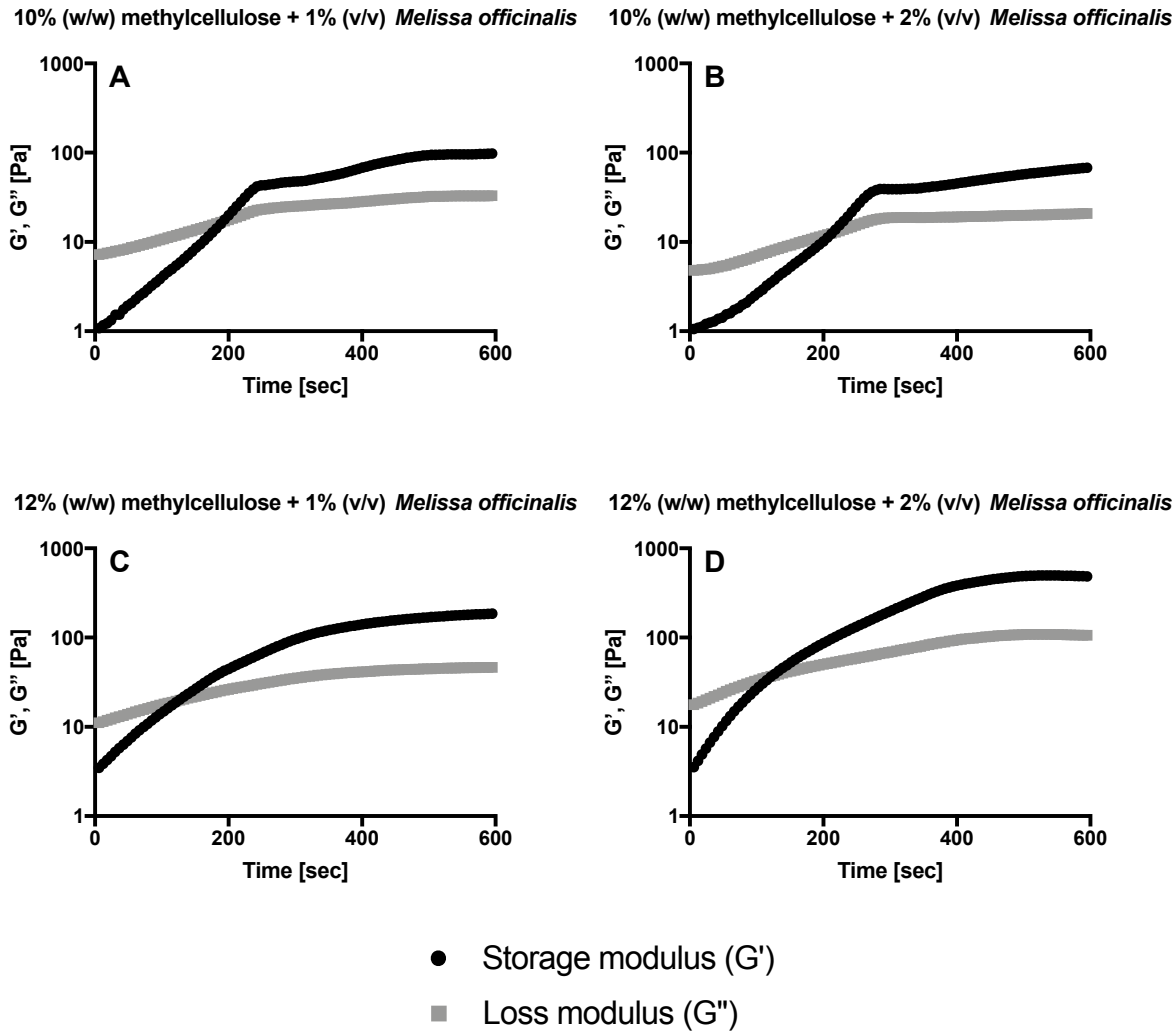
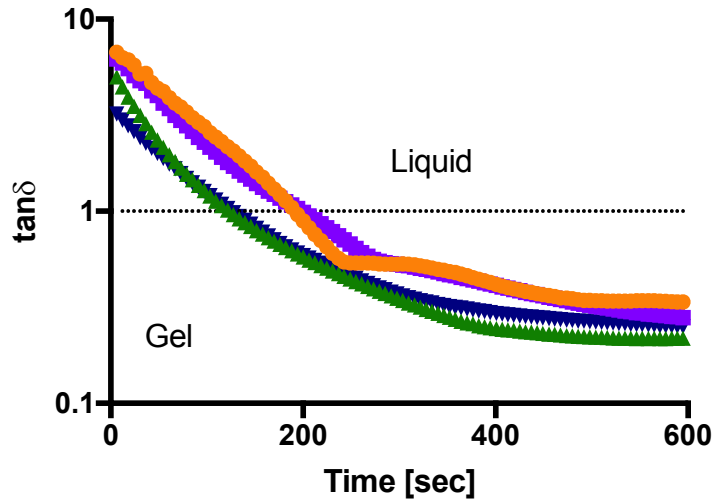


Figure 4.12 - Time sweep for 10% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (A), 10% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (B), 12% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (C), 12% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (D). G' (black) and G'' (grey) were determined at an amplitude strain of 1% and angular frequency of 1 Hz. Data represent the mean of three independent experiments



- 10% (w/w) methylcellulose + 1% (v/v) *Melissa officinalis*
- 10% (w/w) methylcellulose + 2% (v/v) *Melissa officinalis*
- 12% (w/w) methylcellulose + 1% (v/v) Melissa of ficinalis
- 12% (w/w) methylcellulose + 2% (v/v) *Melissa officinalis*

Figure 4.13 - Tanδ at an amplitude strain of 1% and frequency of 1 Hz of 10% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (orange), 10% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (violet), 12% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (green), 12% (w/w) methylcellulose (w/w) and 2% (v/v) *Melissa officinalis* (blue). Data represent the mean of three independent experiments

Table 4.3 - Gelation time at 37 °C of 10% (w/w) methylcellulose with 1 or 2% (v/v) *Melissa officinalis*, and 12% (w/w) methylcellulose with 1 or 2% (v/v) *Melissa officinalis*. *** equivalent to p< 0.001

	Gelation time at 37 °C [sec]
10% (w/w) methylcellulose + 1% (v/v) *Melissa officinalis*	167 ± 14
10% (w/w) methylcellulose + 2% (v/v) *Melissa officinalis*	188 ± 7
12% (w/w) methylcellulose + 1% (v/v) *Melissa officinalis*	117 ± 6 (***)
12% (w/w) methylcellulose + 2% (v/v) *Melissa officinalis*	122 ± 2 (***)

4.4.2 *Melissa officinalis* composition

The *Melissa officinalis* essential oil chromatogram is shown in figure 4.14. Peaks were qualitatively identified by matching the mass spectra with those found in the NIST14 MS Search library. Once the main compounds (i.e. citronellal, citronellol and geraniol) and linalool were identified, quantification was carried out using a standard curve that correlated the peak areas to the concentrations (data not shown).

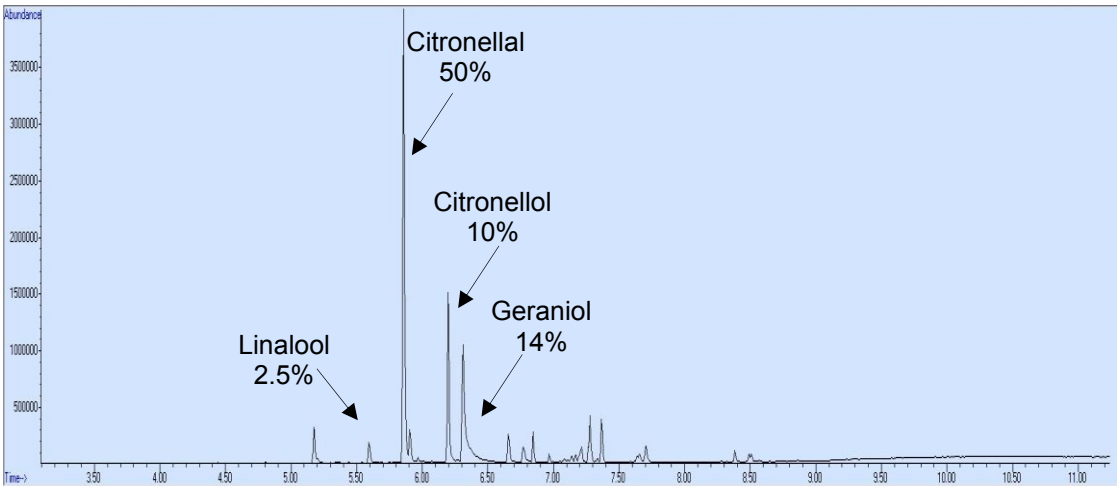
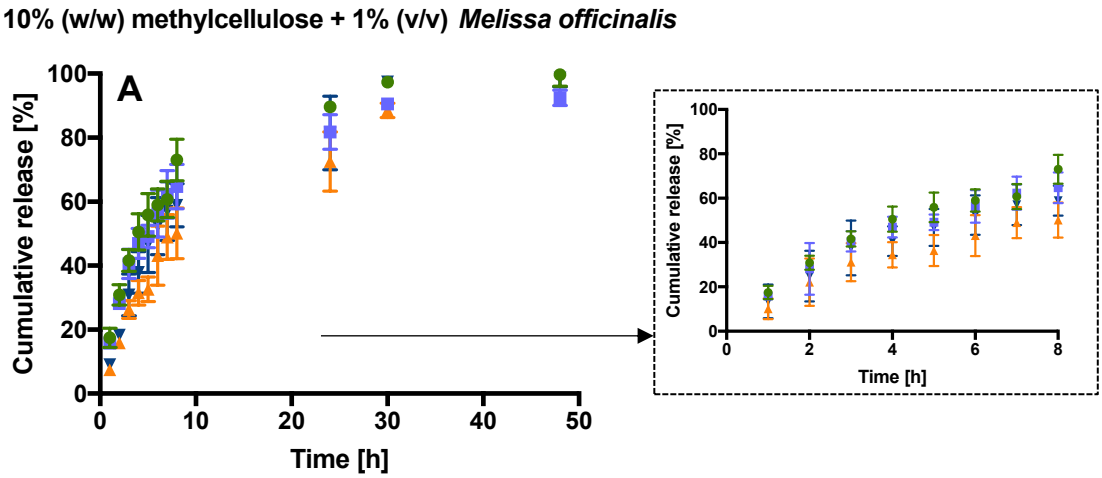


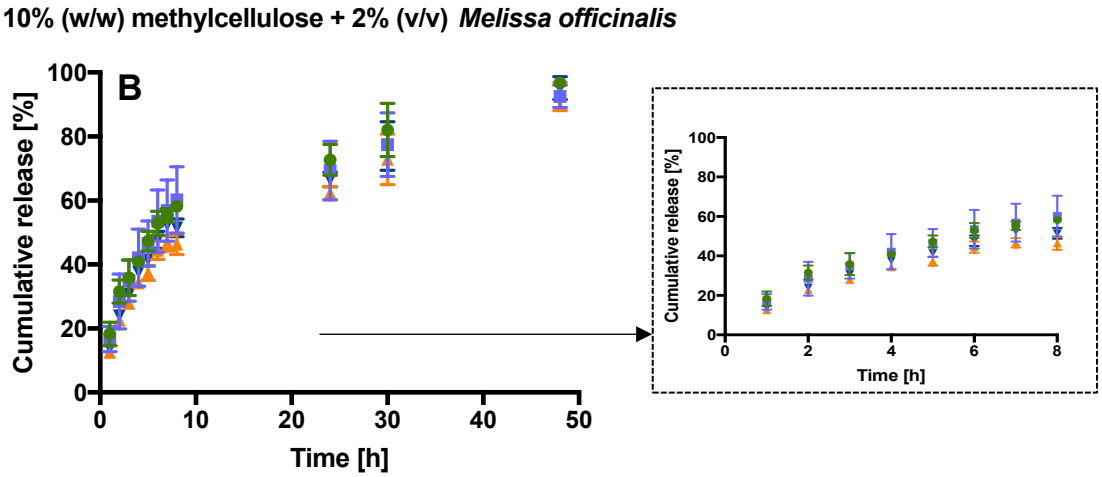
Figure 4.14 - GC-MS chromatogram of *Melissa officinalis* essential oil. The compounds and their abundance are reported as well

4.4.3 *Melissa officinalis* release

Four *Melissa officinalis* compounds were tracked: citronellol, citronellal, geraniol and linalool. Figure 4.15 shows the drug release profile from 10% (w/w) and 12% (w/w) methylcellulose hydrogels with 1 or 2% (v/v) *Melissa officinalis* essential oil over 48 hours. Release was maintained for the duration of the experiment. After 8 hours 50% of the compounds was released, while within 48 hours between 88% and 100% of each compound leached out (Figure 4.15). As expected, by increasing the initial concentration of *Melissa officinalis* into the hydrogel, the

amount of lixiviate increased (Figure 4.16). By contrast, the amount of methylcellulose did not have an impact on the drug release, with the difference in amount released from 10% (w/w) and 12% (w/w) methylcellulose hydrogels not being significant after 48 hours of incubation (Figure 4.16).





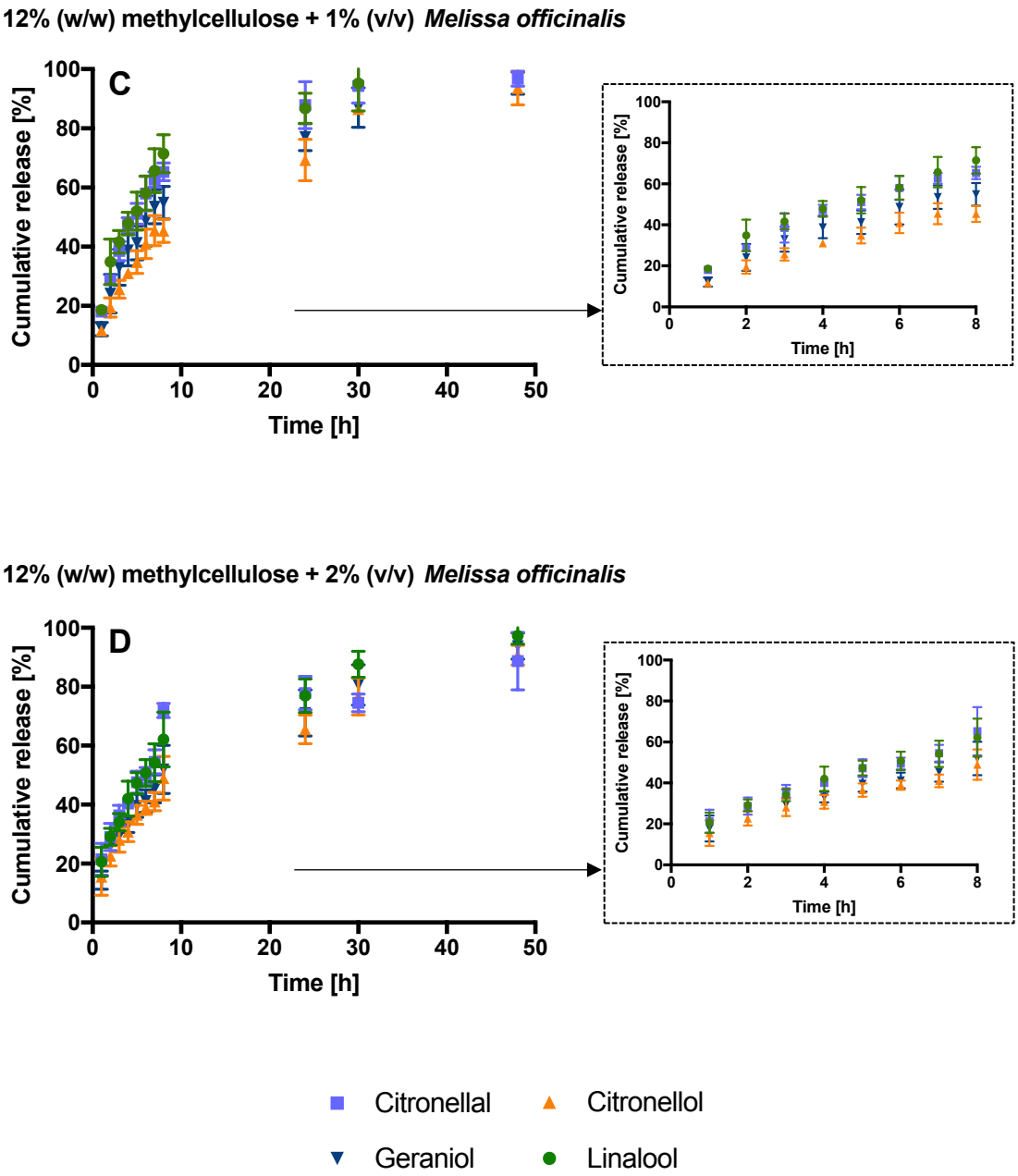
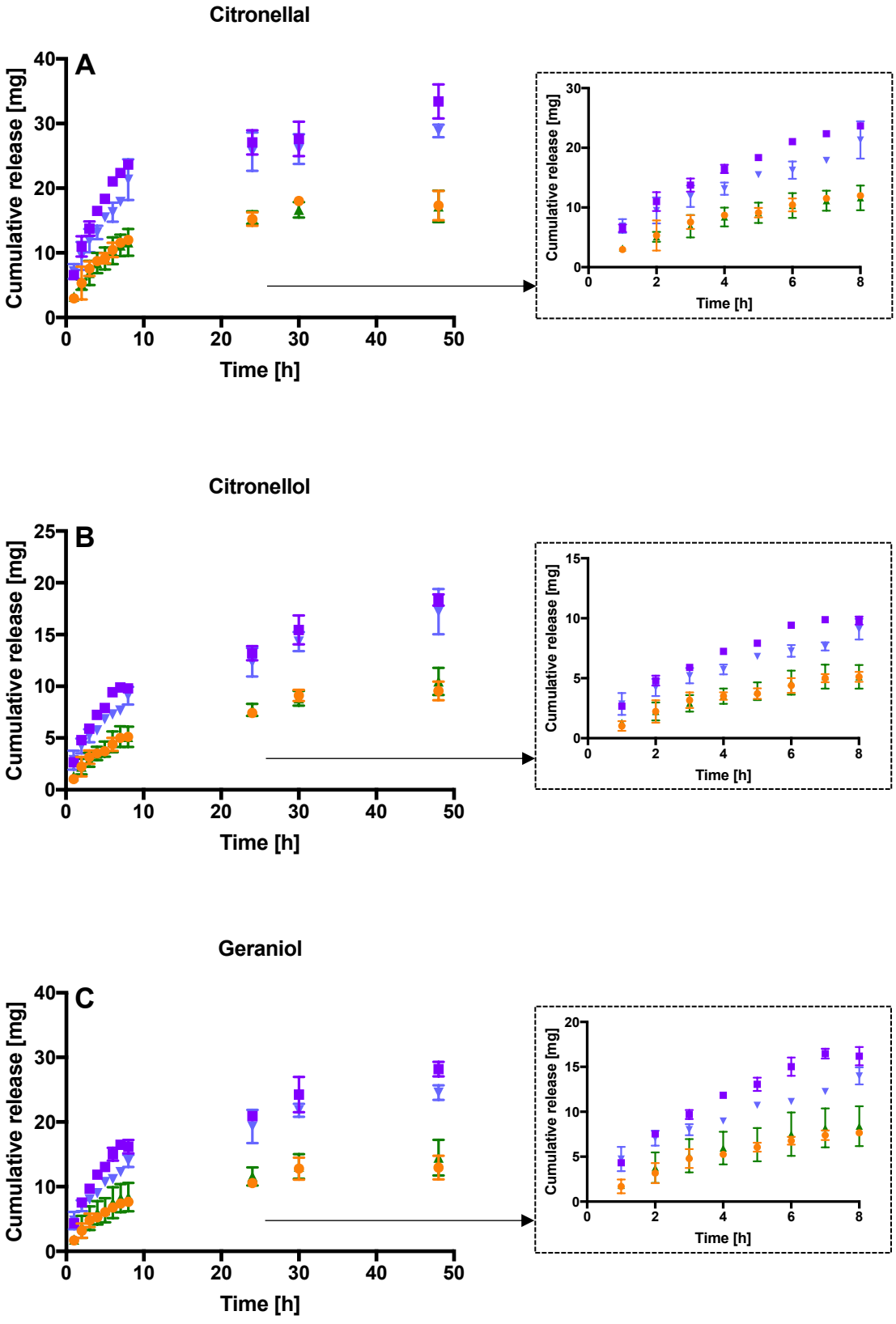


Figure 4.15 - Percentage cumulative release of citronellal (violet), citronellol (orange), geraniol (blue), and linalool (green) 10% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (A), 10% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (B), 12% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (C), 12% (w/w) methylcellulose (w/w) and 2% (v/v) *Melissa officinalis* (D). A zoom of the percentage cumulative release between 0 and 8 hours is also shown in the dashed rectangles. Data represent the mean of three independent experiments



- 10% (w/w) methylcellulose + 1% (v/v) *Melissa officinalis*
- 10% (w/w) methylcellulose + 2% (v/v) *Melissa officinalis*
- 12% (w/w) methylcellulose + 1% (v/v) *Melissa officinalis*
- 12% (w/w) methylcellulose + 2% (v/v) *Melissa officinalis*

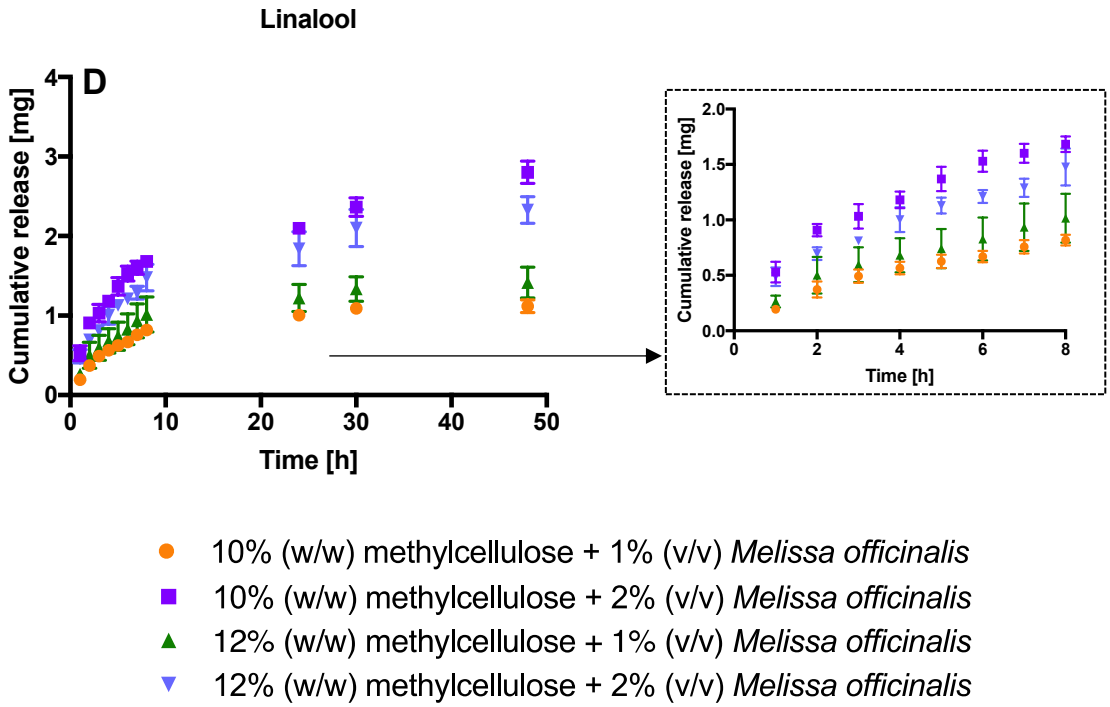


Figure 4.16 - Cumulative release in milligrams of citronellal (A), citronellol (B), geraniol (C) and linalool (D) from 10% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (orange), 10% (w/w) methylcellulose and 2% (v/v) *Melissa officinalis* (violet), 12% (w/w) methylcellulose and 1% (v/v) *Melissa officinalis* (green), 12% (w/w) methylcellulose (w/w) and 2% (v/v) *Melissa officinalis* (blue). A zoom of the cumulative release between 0 and 8 hours is also shown in the dashed rectangles. Data represent the mean of three independent experiments

4.4.4 Disk diffusion method

Firstly, the antifungal activity of the methylcellulose hydrogels was determined by the disc diffusion method. The inhibitory zone was measured, and the diameters are reported in Table 4.4. The 10% (w/w) methylcellulose hydrogels with 1 or 2% (v/v) *Melissa officinalis* essential oil had an antifungal activity, while methylcellulose itself was not able to prevent *C. albicans* growth (Figure 4.17).

Table 4.4 - Inhibitory zone diameters of 10% (w/w) methylcellulose hydrogels with or without 1 or 2% (v/v) *Melissa officinalis.* **** = $p < 0.0001$

	Inhibitory zone diameter (mm)
10% (w/w) methylcellulose	0
10% (w/w) methylcellulose + 1% (v/v) *Melissa officinalis*	10.2 ± 0.4
10% (w/w) methylcellulose + 2% (v/v) *Melissa officinalis*	17.5 ± 2.6 (****)

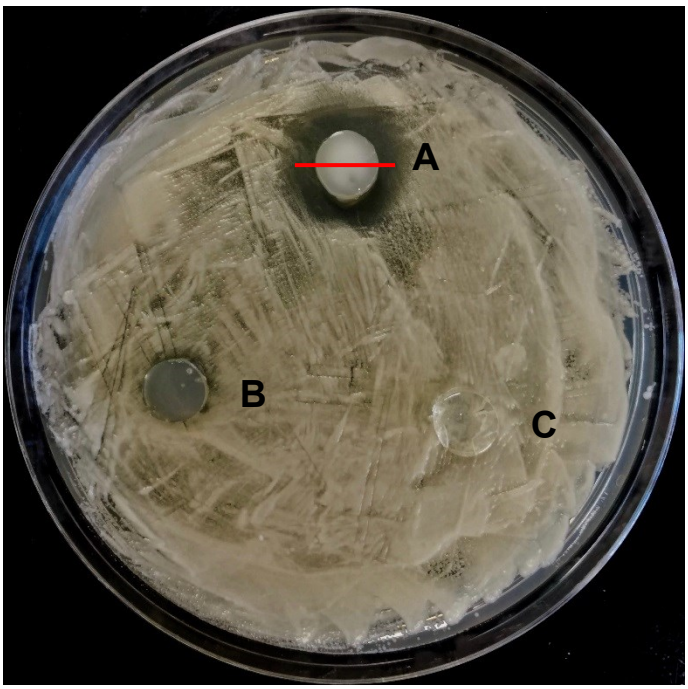


Figure 4.17 - Antimicrobial activity against *C. albicans*. A) 10% (w/w) methylcellulose + 2% (v/v) *Melissa officinalis*, B) 10% (w/w) methylcellulose + 1% (v/v) *Melissa officinalis,* C) 10% (w/w) methylcellulose. Red line – indicates the diameter across the zone of inhibition. Data represent the mean of three independent experiments, each performed in duplicate

4.4.5 Time-kill assay

The antifungal activity was evaluated with a time-kill assay. The amount of *C. albicans* recovered within 2 hours was significantly decreased ($p<0.001$) by 15% and 30% in the presence of 10% (w/w) methylcellulose hydrogel with 1% (v/v) *Melissa officinalis* and 10% (w/w) methylcellulose hydrogel with 2% (v/v) *Melissa officinalis,* respectively (Figure 4.18). After 4 hours of application of 10% (w/w) methylcellulose hydrogels with 2% (v/v) *Melissa officinalis* no viable cells were recovered, while it took 24 hours to completely kill *C. albicans* when it was cultured in the presence of 10% (w/w) methylcellulose hydrogels with 1% (v/v) *Melissa officinalis*. The data presented in figure 4.18 were normalised using the control (i.e. *C. albicans* without hydrogel). It was observed that the presence of 10% (w/w) methylcellulose promoted *C. albicans* growth. This difference was significant until 6 hour incubation ($p < 0.003$), but not after 24 hour incubation ($p > 0.33$)

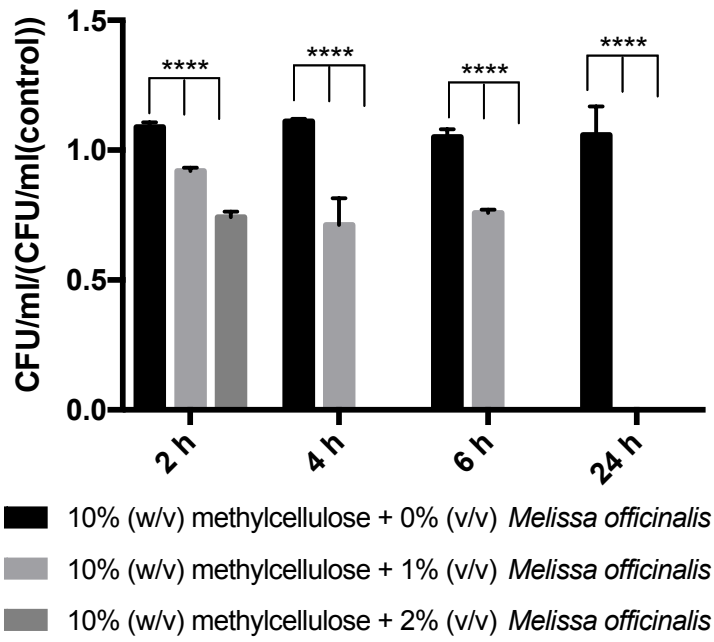


Figure 4.18 - *In vitro* time-kill assay after 2, 4, 6 and 24 hours of exposure of *C. albicans* to 10% (w/w) methylcellulose hydrogels with or without 1 or 2% (v/v) *Melissa officinalis.* CFUs/ml were normalised by the CFUs/ml of the control (*C. albicans* cultured without hydrogel). Data represent the mean of three independent experiments, each performed in duplicate. **** equivalent to $p< 0.0001$

4.5 Discussion

Four hydrogels with different amounts of methylcellulose (10 or 12% (w/w)) and *Melissa officinalis* essential oil (1 or 2% (v/v)) were successfully synthesised. Rheological analysis was carried out to evaluate the gelation time at body temperature. Preliminary experiments were run to identify the amplitude and frequency strain that enabled analysis in the viscoelastic linear region. The essential oil content did not influence the rheological properties, and no significant differences in the loss and storage modulus were observed. By contrast, increasing the amount of methylcellulose led to an increase of the storage modulus. This can be explained by taking into account that a higher amount of methylcellulose (12% (w/w) compared to 10% (w/w)) led to a larger number of crosslinking sites into solution. Therefore, a tighter mesh was formed in the 12% (w/w) methylcellulose hydrogels. For the same reason, it was noticed that 12% (w/w) methylcellulose hydrogels were formed more quickly than 10% (w/w) ones ($p<0.001$).

The synthesised hydrogels are intended to eventually be used as vehicles for drug delivery. Accordingly, it was important that once injected into the mouth, they gellified at 37 °C within an appropriate time. For this reason, the viscoelastic properties of the hydrogel were evaluated only for investigating the gelation time in the viscous linear region. Indeed, the hydrogels were not supposed to be solicited or to resist to a certain deformation such as in load-bearing applications (e.g. cartilage and bone regeneration) (Liu et al., 2017). Therefore, the absolute values of the storage modulus were not further taken into account. Considering the gelation time, both the 10% (w/w) and 12% (w/w) methylcellulose hydrogels were considered suitable for an oral application, being able to gellify in less than three minutes. Consequently, both were examined in terms of drug release.

Before investigating the essential oil release from the hydrogels, the composition of *Melissa officinalis* essential oil was analysed by GC-MS. The main component of the oil was citronellal (50% (v/v)) followed by geraniol (14% (v/v)) and citronellol (10% (v/v)). Linalool (2.5% (v/v)), a terpene with antimicrobial properties (see Section 2.8.1), was also contained in *Melissa officinalis* essential oil. Therefore, it was decided to evaluate the release of the three main compounds as well as of linalool.

Hydrogels are characterised by a 3D-polymeric network that allows liquid and molecules to diffuse. The diffusion of the drug depends on the mesh size: if the mesh size is larger than the drug then molecules are free to migrate through the network, while if the drug size is comparable to the mesh size then the drug is physically entrapped inside the hydrogel (Li and Mooney, 2016). However, the mesh size can change over time allowing the release of the drug. Two main phenomena can lead to changes in the 3D-polymeric network, namely, degradation and swelling. Degradation, a loss of polymer mass, can be mediated by enzyme activity or by hydrolysis. Degradation results in an increase of mesh size that allows drug diffusion. Similarly, swelling due to water absorption, increases the mesh size and allows drug release (Li and Mooney, 2016).

In general, diffusion and drug release are affected by different parameters such as the hydrophilicity/hydrophobicity of the drug, the surface area and geometry of the hydrogel, the type and volume of solvent, the degradation of the hydrogel, the mesh size and the swelling rate (Zarzycki et al., 2010). Different volumes, solvents, ratios of hydrogel to solvent, flasks (e.g. universal, conical flasks), the presence of dialysis membranes and different shaking rate were found to be variable in a review of the literature (Senel et al., 2000; Ji et al., 2010; Kodadova

et al., 2014; Kong et al., 2016; Low et al., 2016). As a result, it was difficult to compare the outcomes obtained within this study with those found by other authors.

The drug release was evaluated over 48 hours and the experiments were carried out by placing 1 g of hydrogel in 20 ml of distilled water. It was observed that the 4 hydrogels showed a similar behaviour in term of percentage cumulative release. After 8 hours 50% of the compounds were released in the solvent, while almost all the oil diffused in water in 2 days. As expected, the milligrams of compounds released were a function of the initial content of oil. Indeed, the amount released from the hydrogel containing 2% (v/v) of *Melissa officinalis* essential oil was significantly greater than that released from the hydrogels synthesised with 1% (v/v) of *Melissa officinalis* essential oil. Interestingly, no significant differences were observed in term of release between the 10 and 12% (w/w) methylcellulose hydrogels. This was in contrast with the observations of Low et al. (2016) and Ikinci et al. (2002) who found that the release of tea tree oil and chlorhexidine from chitosan hydrogels was a function of the crosslinking.

Taking into account that all the hydrogels evaluated gellified at 37 °C and no significant differences were observed in term of percentage cumulative release, it was decided to further evaluate the antifungal activity only of the hydrogels synthesised with 10% (w/w) methylcellulose and 1 or 2% (v/v) *Melissa officinalis* essential oil. This decision was made because it was more complex to prepare 12% (w/w) methylcellulose hydrogels.

Firstly, antifungal activity was screened by the disc diffusion method. It was observed that methylcellulose itself did not inhibit *Candida* growth since a zone

of inhibition was not visible. By contrast, when the methylcellulose hydrogel contained *Melissa officinalis* essential oil a zone of inhibition was observed. In particular, the zone of inhibition was proportional to the amount of *Melissa officinalis* contained in the hydrogel. The hydrogel with 2% (v/v) of *Melissa officinalis* essential oil had higher antifungal activity than that with 1% (v/v) *Melissa officinalis* ($p<0.0001$). These results were in accordance with those stated by Campos et al. (2014) and Ayana and Turhan (2009) who observed a zone of inhibition when methylcellulose hydrogel was loaded with Ginja cherry and olive oil extracts, respectively.

In addition, the antifungal potential of the hydrogels was evaluated with a time-kill assay. It was observed that after 2 hours the presence of 1 or 2% (v/v) *Melissa officinalis* essential oil into the hydrogel significantly decreased the viable cell number compared to the control (10% (w/w) methylcellulose). By comparing the hydrogel with 1 and 2% (v/v) *Melissa officinalis* essential oil, it was noticed that after 4 hours no *C. albicans* was recovered in the presence of 2% (v/v) *Melissa officinalis.* However, it took 24 hours to kill *C. albicans* when it was cultured in the presence of 10% (w/w) methylcellulose + 1% (v/v) *Melissa officinalis* essential oil. Besides evaluating the antifungal potential of the drug released from the hydrogel, the antifungal potential of methylcellulose itself was investigated by comparing the viable cell count with that obtained culturing *C. albicans* in broth without the hydrogel. As already confirmed by the zone inhibition assay, methylcellulose did not show antifungal potential. However, this experiment further highlighted that methylcellulose seemed to promote *C. albicans* growth. The lack of antifungal potential of methylcellulose was also described by Kavanaugh et al. (2014) who used 0.5% methylcellulose to mimic the viscosity of

the mucus environment. They observed that *C. albicans* cultured in the presence of methylcellulose produced hyphal cells.

4.6 Conclusions

Chapter 2 and 3 highlighted that the best essential oil in term of antifungal activity and cytotoxicity was *Melissa officinalis.* The aim of this chapter was to develop a hydrogel to be used as drug delivery system to release *Melissa officinalis* essential oil at the site of infection.

Four hydrogels were successfully synthesised: 10% (w/w) methylcellulose with 1% (v/v) *Melissa officinalis*, 10% (w/w) methylcellulose with 2% (v/v) *Melissa officinalis*, 12% (w/w) methylcellulose with 1% (v/v) *Melissa officinalis* and 12% (w/w) methylcellulose with 2% (v/v) *Melissa officinalis*.

The rheological analysis highlighted that all the hydrogels gellified at body temperature in less than 3 minutes, which was considered to be an appropriate time for a potential gelation in the mouth. *Melissa officinalis* was released over a period of 48 hours meaning that the hydrogels were suitable to be used as drug delivery vehicles. Lastly, the antifungal properties of the hydrogels were confirmed by both the zone of inhibition method and time-kill assay.

Chapter 5

Development of an *ex vivo* mandible rodent model to mimic oral candidiasis

5.1 Introduction

5.1.1 *In vitro*, *in vivo* and *ex vivo* models

A crucial step in the development of a novel antimicrobial treatment is the evaluation of the therapy in a cell model. Because of their simplicity, *in vitro* models are commonly used as a first screening. The main advantage of these models is that they only involve the culture of a single cell type or two cell types in case of co-cultures and 3D-organoid cultures (Sloan et al., 2016). However, their simplicity leads to some important limitations due to the inability to reproduce the *in vivo* spatial cellular organisation and to take into account the cellular interactions that might occur *in vivo*. In addition, the outcome of *in vitro* experiments can be affected by the plastic or glass of the tissue culture plates (Sloan et al., 2016). To overcome these limits, *in vivo* models can be utilised. *In vivo* models are considered as the gold standard for studying the efficacy of a treatment or a regenerative process. Drawbacks associated with *in vivo* models are high costs and ethical issues, as large numbers of animals are required to be used in the experiments. Moreover, flawless data are difficult to obtain because of the intrinsic systemic influences (Sloan & Lynch 2012). Therefore, e*x vivo* models have been recently introduced. These models maintain the natural arrangement of cells and tissues, while removing systemic influences. Furthermore, they limit the costs and the ethical issues as more than one experiment can be carried out on the same animal (Sloan & Lynch 2012).

5.1.2 *In vitro* and e*x vivo* models for oral candidiasis

In vitro and *ex vivo* models have been developed to study oral candidiasis.

Reconstituted human epithelium (RHE) is an *in vitro* commercially available model. SkinEthic Laboratories cultivate epithelial cells on a polycarbonate filter at

the air-liquid interface in a chemically defined medium lacking antibiotics for 5 days. The result is a stratified epithelium, similar to the human one, that expresses markers typical of the natural epithelium (Schaller and Weindl, 2009). This model has been widely used to investigate the pathogenicity of *Candida*, the host immune response to infection and the antifungal potential of antimicrobials (Hernandez and Rupp, 2009). Jayatilake et al. (2006) highlighted the role of SAP and hyphae as virulence factors of *C. albicans*, by observing a decreased tissue invasion in hyphal and SAP mutant. Similarly, Schaller et al. (2005b) reported a diminished host inflammatory response in the presence of SAP inhibitors. Zhao et al. (2005) used the RHE model to show the role of Als2p adhesin in tissue adhesion and invasion. In addition, RHE models allowed evaluating the host inflammatory response. In particular, the production of pro and anti-inflammatory cytokines in the presence of *C. albicans* strains with a different virulence has been investigated (Schaller et al., 2002; Whiley et al., 2012). Reconstituted human epithelium models have also been used to study new antimicrobial treatments, by evaluating the antifungal potential in term of *C. albicans* viability and tissue invasion, and the cytotoxicity of the treatment in term of cellular viability and loss of tissue structure (Bonowitz et al., 2001; Silva et al., 2009; Boros-Majewska et al., 2014).

Ex vivo models used to study *C. albicans* infections have been obtained from pigs and mice. Ohnemus et al. (2008) developed an *ex vivo* porcine oral mucosal infection model. After an infection of 48 hours with *C. albicans*, they observed massive invasion and formation of hyphae. By contrast, when the infection was treated with different doses of Nystatin for 24 hours, only little pseudohyphae formation and tissue invasion were present (Ohnemus et al., 2008). Campos et

al. (2011) evaluated a Nystatin-loaded nanoemulsion formulation on a porcine buccal mucosa reporting a harmless effect of the treatment on cells and the permeation of the drug into the tissue. Peters et al. (2011) used a murine model to evaluate the anticandidal potential of Histatin-5, a peptide contained in saliva. They infected the excised tongues with different concentration of *C. albicans* and they applied the treatment (pure Histain-5 or saliva containing Histain-5).

5.1.3 *Ex vivo* rodent model for oral diseases

Sloan et al. (1998) developed an *ex vivo* culture model of dentine-pulp complex from 28-day-old Wistar rat incisor teeth. The tooth slices were successfully cultured for up to 14 days, a good cell viability was observed, and the tissue organisation was preserved. Since then, tooth slices have been extensively used to investigate dental pulp infection (Ayre et al., 2018), tissue repair processes (Sloan & Smith 1999; Sloan et al. 2000) and to evaluate the biocompatibility and cytotoxicity of dental materials (Murray et al., 2000; Turner et al., 2002; Waddington et al., 2004). Recently, mandibular slice cultures were also established (Smith et al., 2010). Mandible slice cultures were developed to understand processes associated with periodontal diseases and bone tissue repair and to investigate the biocompatibility and cytotoxicity of drugs. Smith et al. (2010) cultured fractured mandible slices to investigate bone repair. The authors maintained a healthy culture for 14 days and observed that the addition of the transforming growth factor beta 1 (TGF-1β) increased the migration and proliferation of osteoblasts and the expression of bone morphogenic proteins. Sloan et al. (2013) developed an *ex vivo* mandible model to study the inflammatory bone destruction. Following *P. gingivalis* LPS administration, an increase in osteoclasts, a decreased viability of ligament fibroblasts and a loss in

the tissue architecture were observed. In addition, increase of monocytes and neutrophils and expression of pro-inflammatory cytokines were evident (Sloan et al., 2013).

5.2 Aims and objectives

In vitro experiments have some limitations mainly due to the impossibility to reproduce the *in vivo* spatial tissue-specific architecture. The *ex vivo* rodent mandible model provides a 3D-environment closer to that of the *in vivo* situation than previously used approaches (e.g. 2D-monolayer cell cultures). The aim of this chapter is to develop an *ex vivo* rodent mandible model to mimic oral candidiasis. The hypothesis of this study is that the *ex vivo* rodent mandible model provides a tissue scaffold and allows the observation of *Candida* growth within this 3D-environment, and the evaluation of *Melissa officinalis* essential oil as a treatment.

To test this hypothesis, the specific objectives are:

a) To monitor the growth of *C. albicans* in a tissue environment through histological examination after 24 and 48 hours of infection
b) To assess the host inflammatory tissue response after infection through real time-polymerase chain reaction (RT-PCR)
c) To investigate the antifungal potential of *Melissa officinalis* essential oil on the *ex vivo* model through histological examination. After infection with *C. albicans*, the mandible is treated with *Melissa officinalis* essential oil and the effect on *C. albicans* viability is determined by viable counts
d) To determine the host inflammatory tissue response after treatment with *Melissa officinalis* by RT-PCR

5.3 Materials and methods

5.3.1 Cell culture medium

Dulbecco's modified eagle medium nutrient mixture F-12 (DMEM/F12) with L-glutamine and phenol red was purchased from Gibco (Paisley, UK). DMEM/F12 was supplemented with 10% (v/v) fetal calf serum (Biosera, East Sussex, UK), 1% (v/v) penicillin/streptomycin (Sigma Aldrich, Dorset, UK), 0.025 mg/ml adenine (Sigma Aldrich, Dorset, UK), 5 µg/ml insulin (Sigma Aldrich, Dorset, UK), 1.36 ng/ml 3,3,5-tri-iodothyronine (Sigma Aldrich, Dorset, UK), 5 µg/ml apo-transferrin (Sigma Aldrich, Dorset, UK), 0.4 µg/ml hydrocortisone (Sigma Aldrich, Dorset, UK), 5 ng/ml epidermal growth factor (Sigma Aldrich, Dorset, UK) and 8.47 ng/ml cholera toxin (Sigma Aldrich, Dorset, UK). The medium composition was adapted from the study of Rheinwald and Green (1975) and Smith et al. (2010).

5.3.2 Microorganisms

Candida albicans 135BM2/94, a clinical strain from the School of Dentistry (Cardiff University), was subcultured onto Sabouraud dextrose agar (SDA) (CM0041 Oxoid) and grown at 37 °C in an aerobic incubator overnight. A colony of *C. albicans* was inoculated in 20 ml of SDB and incubated overnight at 37 °C in an aerobic chamber under shaking conditions at 200 rpm. The overnight culture was prepared in DMEM/F12 culture medium to a turbidity equivalent to 0.5 McFarland Standard (10^5 CFU/ml) and used for further experiments.

5.3.3 Diffusion of *Melissa officinalis* essential oil into agar

Preliminary experiments were carried out to investigate the diffusion of *Melissa officinalis* essential oil from the DMEM/F12 medium to the semi-solid agar that was used to embed the mandible (see Section 5.3.4).

The method was adapted from that previously reported by Smith et al. (2010). Briefly, two percent (2% (w/w)) low melting point agar (agarose type VII; Sigma, Gillingham, UK) was 1:1 mixed with DMEM/F12 culture medium containing *C. albicans* (Section 5.3.2). Three millilitres (3 ml) of the prepared agar/culture medium solution were added to a 24-well plate. Once semi-solid, the agar was transferred to a 0.22 µm-diameter sterile Millipore filter (Millipore, Watford, UK). With the aid of a plastic support, the filter was placed on the surface of DMEM/F12 culture medium (4.5 ml) in a Trowel-type culture in a 6-well plate (Trowell, 1959). The 6 well plate was incubated for 6 hours at 37 °C and 5% CO_2. After the incubation, DMEM/F12 was replaced with DMEM/F12 containing 1% (v/v) *Melissa officinalis* essential oil and 0.5% (v/v) Tween 80 (Sigma-Aldrich, UK), and the plates were incubated for a further 4 hours (10 hours in total), and 24 hours (30 hours in total) at 37 °C and 5% CO_2 (Figure 5.1). Controls included untreated semi-solid agars (i.e. inoculated semi-solid agar cultured without *Melissa officinalis* essential oil for 6, 10 and 30 hours).

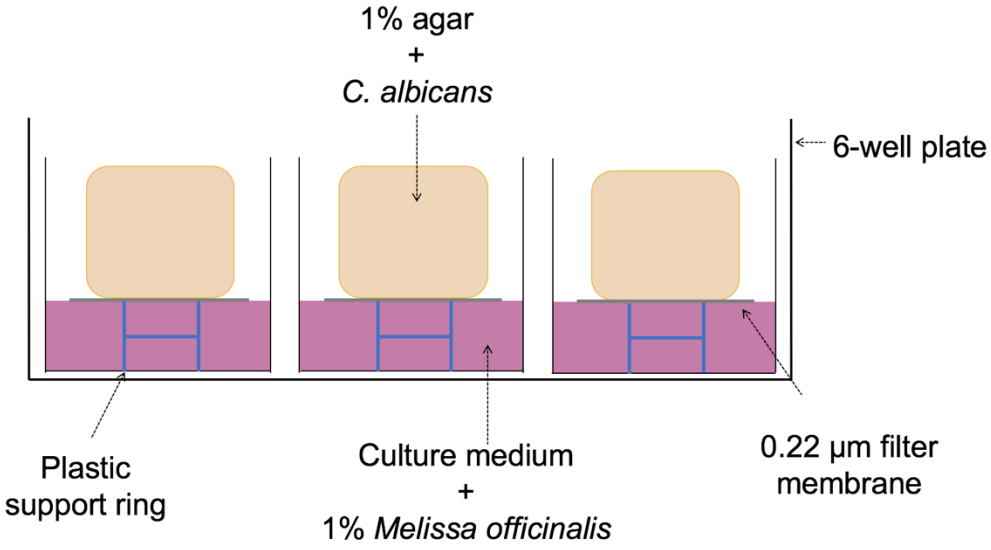


Figure 5.1 - Representation of a 6-well plate containing a plastic support, a 0.22 µl filter, and a 1% (w/w) semi-solidi agar infected with *C. albicans* and treated with 1% (v/v) *Melissa officinalis*

After incubation, 350 µl of semi-solid agar containing *C. albicans* was collected and resuspended in 500 µl of SBD. Samples were serially diluted in PBS and 50 µl were spread onto SAB agar plates with a Whitley Automated Spiral Plate (WASP, Don Whitley Scientific Limited, Shipley, UK). Agar plates were incubated overnight at 37 °C, and the CFUs/ml were counted. Each experiment was performed in duplicates on three separate occasions.

5.3.4 Culture of rodent mandibles

Mandibles were dissected from 28-day-old male Wistar rats, freshly sacrificed by CO_2 asphyxiation. The soft tissues, except for the gingiva, were removed with a scalpel. The condyle, ramus and incisor tooth were cut with a segmented diamond-edged rotary saw cooled with PBS (Figure 5.2).

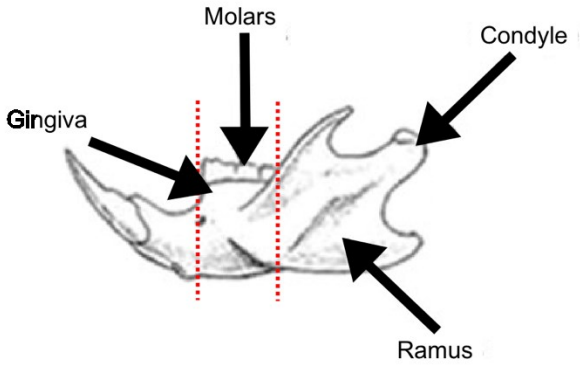


Figure 5.2 - Schematic representation of the dissected mandible preparation. The condyle, ramus, and incisor tooth were cut through the red line using a segmented diamond-edged rotary saw and discarded. Figure adapted from Smith et al. (2010)

The mandibles obtained at the end of the cutting process were collected in DMEM/F12 culture medium and cultured following a protocol slightly adapted from Smith et al. (2010). Mandibles were cultured using the Trowel-type culture (Figure 5.3) (Trowell, 1959). Briefly, 2% (w/w) low melting point agar (agarose type VII; Sigma Gillingham) was prepared in PBS and 1:1 mixed with DMEM/F12. Three millilitres (3 ml) of the solution were pipetted into a 24-well plate and the mandible was added during the solidification process. Once semisolid, the embedded mandibles were transferred to a 0.22 µm-diameter sterile Millipore filter. With the aid of a plastic support, the filter floated on the surface of 4.5 ml DMEM/F12 culture medium in a Trowel-type culture in a 6-well plate. Mandibles were cultured for 24 hours at 37 °C and 5% CO_2 before infection with *C. albicans* and treatment with essential oil.

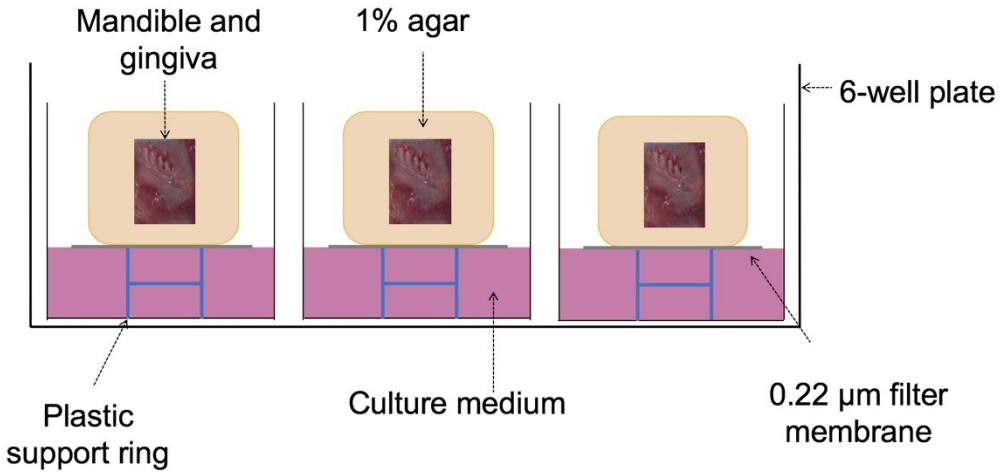


Figure 5.3 - Representation of the Trowel-type culture for the mandible with the attached gingiva

5.3.5 Gingiva infection

After 24 hour incubation (Section 5.3.4), gingiva was infected with *C. albicans* 135BM2/94.

Three infection methods were evaluated by histological analysis (Section 5.3.7):

i. Addition of *C. albicans* into the semi-solid agar by pipetting:

20 µl of an overnight *C. albicans* 135BM2/94 culture diluted to 10^5 CFU/ml (Section 5.3.2) was pipetted into the semi-solid agar containing the mandible, in the proximity of the gingiva (see Section 5.3.4). The Trowel-type culture was incubated for 24 hours at 37 °C and 5% CO_2

ii. Direct contact of the gingiva with *C. albicans* grown onto SAB agar:

100 µl of an overnight *C. albicans* 135BM2/94 culture diluted to 10^5 CFU/ml (Section 5.3.2) was uniformly spread onto SAB agar plates and grown at 37 °C in an aerobic incubator overnight. The gingiva was placed onto the agar plate, in contact with *C. albicans* for 5 minutes. The infected gingiva and mandible were embedded in semi-solid agar as described in Section 5.3.4 and incubated for 24 hours at 37 °C and 5% CO_2

iii. Embedment of the mandible into semi-solid agar containing *C. albicans*: 2% (w/w) low melting point agar (agarose type VII; Sigma Gillingham) was mixed 1:1 with the DMEM/F12 culture medium containing 10^5 CFU/ml of *C. albicans* (Section 5.3.2). Three millilitres (3 ml) of the culture were pipetted into a 24-well plate and the mandible was added during the solidification process. Once semisolid, the embedded mandibles were transferred to a sterile 0.22 µm-diameter Millipore filter (Millipore, Watford, UK) supported by a plastic ring. Each well of the 6-well plate was filled with 4.5 ml DMEM/F12 to the filter level to allow the perfusion of the cell culture medium into the semi-solid agar and into the mandible (Figure 5.4). The mandibles were incubated at 37 °C and 5% CO_2 for 6, 24 and 48 hours. Controls included uninfected mandibles. This was the method chosen and used in the further experiments.

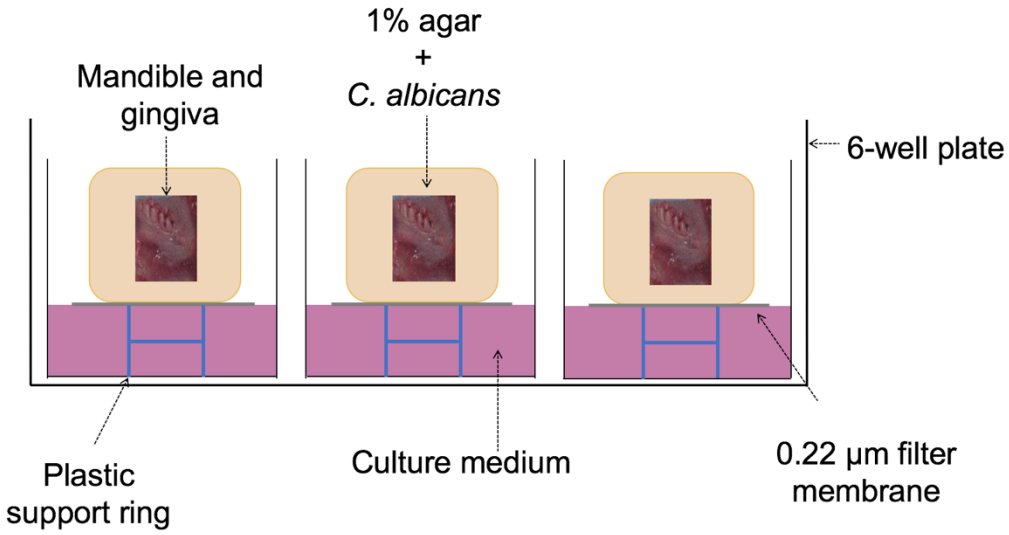


Figure 5.4 - Representation of the Trowel-type culture for the infected gingiva

5.3.6 *Melissa officinalis* essential oil treatment

One percent (1% (v/v)) *Melissa officinalis* essential oil was prepared in DMEM/F12 culture medium. To enhance the dispersion of the essential oil into

the medium, 0.5% (v/v) Tween 80 (Sigma-Aldrich, UK) was added. After infection with *C. albicans* for 6 and 24 hours (Section 5.3.5), the embedded mandibles were treated with 1% (v/v) *Melissa officinalis.* Two methods to treat the mandibles were evaluated by histological analysis.

i. Addition of 1% (v/v) *Melissa officinalis* into the semi-solid agar by pipetting:
   20 µl of 1% (v/v) *Melissa officinalis* were pipetted into the semi-solid agar containing the infected mandible, in the proximity of the gingiva (see Section 5.3.5). Therefore, the Trowel-type culture was incubated for 24 hours at 37 °C and 5% CO_2

ii. Addition of 1% (v/v) *Melissa officinalis* into the DMEM/F12 that diffused into the semi-solid agarose:
   The embedded infected mandibles (Section 5.3.5) were transferred to a 0.22 µm-diameter sterile Millipore filter. With the aid of a plastic support, the filter was placed on the surface of 1% (v/v) *Melissa officinalis* (4.5 ml) in a 6-well plate. The mandibles were incubated for 24 hours at 37 °C and 5% CO_2 (Figure 5.5). Controls included uninfected mandibles treated with 1% (v/v) *Melissa officinalis* essential oil. This method was chosen and used for further experiments.

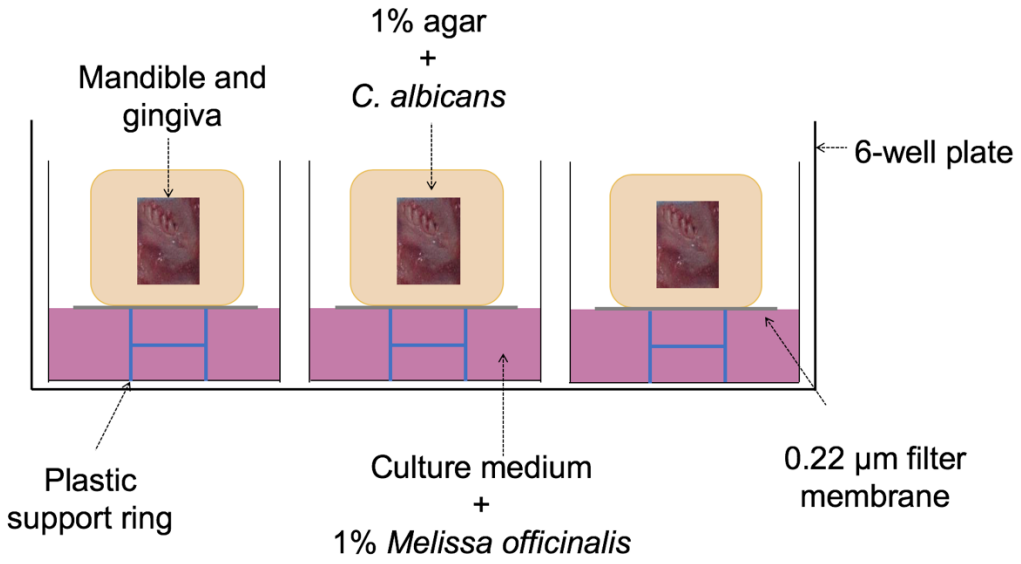


Figure 5.5 - Representation of the application of 1% (v/v) *Melissa officinalis* essential oil to the infected mandibles cultured in a Trowel-type culture

5.3.7 Histological examination

After 6, 24 and 48 hours of culture with or without *C. albicans* and 1% (v/v) *Melissa officinalis*, mandibles were washed briefly in PBS and fixed in 10% (w/v) neutral buffered formalin (Sigma-Aldrich, Gillingham, UK) for 24 hours. Mandibles were demineralised in 15% (w/v) formic acid (Sigma-Aldrich, Gillingham, UK) at room temperature for 72 hours, dehydrated through a series of 70% (v/v), 90% (v/v), and 100% (v/v) graded Ethanol (Sigma-Aldrich, Gillingham, UK), cleared through xylene, and embedded in paraffin wax. Sections (5 µm) were cut on a rotary microtome (Leica Biosystems, Linford Wood, UK), mounted onto super frost microscope slides (Fisher Scientific, Loughborough, UK), and incubated for 1 hour at 60 °C. Lastly, sections were stained with haematoxylin and eosin (H&E, Leica Biosystems, Linford Wood, UK) or periodic acid schiff (PAS, Millipore, Watford, UK) and visualised with the Zeiss AxioImager M1 micrscope (Zeiss, Cambridge, UK). The method was adapted from that previously reported by Ayre et al. (2018).

5.3.8 Colony forming units (CFUs) from *C. albicans*-infected mandibles

Colony forming units (CFUs) of *C. albicans* recovered from infected mandibles were counted adapting a method previously reported by Hayama et al. (2012). After 6, 24 and 48 hours of infection with *C. albicans* and treatment with 1% (v/v) *Melissa officinalis*, mandibles were washed briefly in PBS and homogenised in 10 ml of PBS for 1 minute with a conventional rotor-stator homogeniser (Scilogex, Bedfordshire, UK). The suspension was ultrasonically agitated at 40 Hz for 30 seconds (Branson Ultrasonic Bath 1501, Slough, UK) and spun down for 5 minutes at 2000 rpm (SIGMA 3-16 Centrifuge, Sigma Centrifuges, Newtown, UK). The supernatant was removed. The pellet was suspended in 3 ml of 0.25% (v/v) trypsin EDTA (EDTA 0.25% w/v Tryp 0.53mM) in SDB, and incubated for 15 minutes at 37 °C. After incubation, the suspension was centrifuged (2000 rpm, 4 °C rpm, 5 minutes) two times and the pellet resuspended in 2 ml of PBS. Samples were serially diluted in PBS and 50 µl were spread onto SAB agar plates with a Whitley Automated Spiral Plate (WASP, Don Whitley Scientific Limited, Shipley, UK). Agar plates were incubated overnight at 37 °C, and the CFUs/ml were counted. Each experiment was performed in duplicate on three separate occasions.

All methods described in Sections 5.3.3 - 5.3.8 relating to the preparation and development of the *ex vivo* model were carried out solely by the author in the School Dentistry, Cardiff University.

5.3.9 RT-PCR

5.3.9.1 Primer optimisation

Primers were designed using the NCBI primers design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and purchased from Sigma-Aldrich (Gillingham, UK). The primer sequences and cycling conditions are described in Table 5.1. Primers efficiency was evaluated by running each primer in the presence of 1:5 serial dilutions of cDNA, equivalent to10 ng/µl, 2 ng/µl, 0.4 ng/µl, and 0.08 ng/µl. The efficiency was calculated from the slope of the standard curve.

RT-PCR reactions were run on the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Hemel Hempstead, UK). The amplification was carried out firstly with an activation step at 95 °C (10 minutes), followed by a denaturation step at 95 °C (40 cycles for 15 seconds), and an annealing and elongation step at 60 °C (1 minute). Melt curves were obtained at the end of the cycling process by increasing the temperature by 0.3 °C from 60 to 95 °C (Al-Shanti et al., 2009). A typical reaction mix contained 2 µl of cDNA (equivalent to 20 ng, 4 ng, 0.8 ng, and 0.16 ng), 5 µl of SyberGreen (SensiFAST™ SYBR Lo-ROX Kit, Bioline, London, UK), 0.4 µl of 10 mM forward primer, 0.4 µl of 10 mM reverse primer, and 2.2 µl of DNAse-free water (Promega, Southampton, UK) given a final volume per well of 10 µl. DNAse-free water replaced the primers and served as a negative control. All PCR conditions were run in duplicate.

The most stable reference housekeeping gene, UBC (Ubiquitin C), was chosen according to the NormFinder software (https://moma.dk/normfinder-software) and was used as a reference housekeeping gene for all the experiments.

Table 5.1 - Characteristics of the primers designed. Upper section: reference housekeeping genes, bottom section: cytokines

Gene marker	Primer sequence	Annealing temperature (C°)	Product (bp)	NCBI sequence
β-actin	F: 5'-AGATCAAGATCATTGCTCCTCCT-3'	59.02	174	NM_031144.3
	R: 5'-ACGCAGCTCAGTAACAGTCC-3'	60.04		
B2M	F: 5'-ACTGAATTCACACCCACCGA-3'	59.24	100	NM_012512.2
	R: 5'-ATTACATGTCTCGGTCCCAGG-3'	59.24		
CYC1	F: 5'-GACGATGGTACCCCAGCTAC-3'	59.61	101	NM_001277194.1
	R: 5'-CCCATGCGTTTTCGATGGTC-3'	59.90		
GAPDH	F: 5'-AGTGCCAGCCTCGTCTCATA-3'	60.68	189	NM_017008.4
	R: 5'-TGAACTTGCCGTGGGTAGAG-3'	59.68		
UBC	F: 5'-ACACCAAGAAGGTCAAACAGG-3'	58.35	103	NM_017314.1
	R: 5'-AGACACCTCCCCATCAAACC-3'	59.30		
IL-1β	F: 5'-GCTTCCTTGTGCAAGTGTCT-3'	58.69	160	NM_031512.2
	R: 5'-TCTGGACAGCCCAAGTCAAG-3'	59.60		
IL-6	F: 5'-CTCTCCGCAAGAGACTTCCA-3'	59.11	122	NM_012589.2
	R: 5'-GGTCTGTTGTGGGTGGTATCC-3'	60.34		
IL-10	F: 5'-TGCGACGCTGTCATCGATTT-3'	60.74	186	NM_012854.2
	R: 5'-GTAGATGCCGGGTGGTTCAA-3'	60.04		
IL-18	F: 5'-GATTCGTTGGCTGTTCGGTC-3'	59.07	106	NM_019165.1
	R: 5'-GATTCGTTGGCTGTTCGGTC-3'	59.56		
IL-12	F: 5'-TGGAGCACTCCCCATTCCTA-3'	59.96	106	NM_022611.1
	R: 5'-ACGCACCTTTCTGGTTACACT-3'	59.86		
IL-23	F: 5'-CACACACACCAGTGGGACAA-3'	60.39	140	NM_130410.2
	R: 5'-CCTTTGCAAACAGAACTGGCT-3'	59.59		
TNFα	F: 5'-ACTGAACTTCGGGGTGATCG-3'	59.75	150	NM_012675.3
	R: 5'-TGGTGGTTTGCTACGACGTG-3'	60.88		

5.3.9.2 RNA extraction from the gingiva and RT-PCR

At designated time-points, mandibles were removed from the semi-solid agar and washed briefly in PBS. The gingiva was collected with a scalpel and immediately freeze-dried for 1 hour. RNA was extracted by homogenisation (Scilogex, Bedfordshire, UK) in Trizol reagent (Invitrogen, Loughborough, UK) for 5 minutes, following manufacturer's instruction. RNA was stored at -80 °C and 1 µg of RNA was converted into cDNA with a Promega kit (Southampton, UK) and stored at -20 °C.

RT-PCR reactions were run on the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Hemel Hempstead, UK) as described in Section 5.3.9.1. A typical reaction mix contained 2 µl of cDNA (equivalent to 20 ng), 5 µl of SyberGreen (SensiFAST™ SYBR Lo-ROX Kit, Bioline, London, UK), 0.4 µl of 10 mM forward primer, 0.4 µl of 10 mM reverse primer, and 2.2 µl of DNAse-free water (Promega, Southampton, UK) given a final volume per well of 10 µl. DNAse-free water replaced the primers and served as a negative control.

Ubiquitin C (UBC) served as the reference housekeeping gene in all cases. All RT-PCR conditions were run in duplicate on three separate mandibles. Results were expressed as fold change compared to the control (i.e. uninfected and untreated gingiva cultured for 24 hours).

5.3.10 Statistical analyses

Statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). Data were presented as arithmetic mean ± SD or mean ± SEM. The difference between treatments was statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. Statistically significant differences were set at $p < 0.05$.

5.4 Results

5.4.1 Diffusion of *Melissa officinalis* essential oil into semi-solid agar

Prior to the development of the *ex vivo* model, preliminary experiments were carried out to evaluate the time needed for *Melissa officinalis* to both diffuse into the semi-solid agar and kill *C. albicans.*

After a 6 hour infection, the semi-solid agar was treated with 1% (v/v) *Melissa officinalis* essential oil for 4 and 24 hours, equivalent to a total incubation period of 10 and 30 hours, respectively (Section 5.3.3). Both a treatment for 4 and 24 hours decreased the CFUs/ml recovered from the semi-solid agar compared to the untreated samples (10 and 30 hours infection with *Candida*) ($p<0.0001$) (Figure 5.6). The 4 hour treatment inhibited the growth of *C. albicans* compared to the 10 hour infection, but it was not sufficient to kill the microorganism. Indeed, no significant differences were observed between the sample infected for 6 hours and that infected for 6 hours and treated for 4 hours with the essential oil. By contrast, when the essential oil was applied for 24 hours, the CFUs/ml recovered were significant less than the 6 hours of infection. For this reason, for subsequent experiments *Melissa officinalis* was applied for 24 hours, which inhibited and importantly killed *C. albicans*.

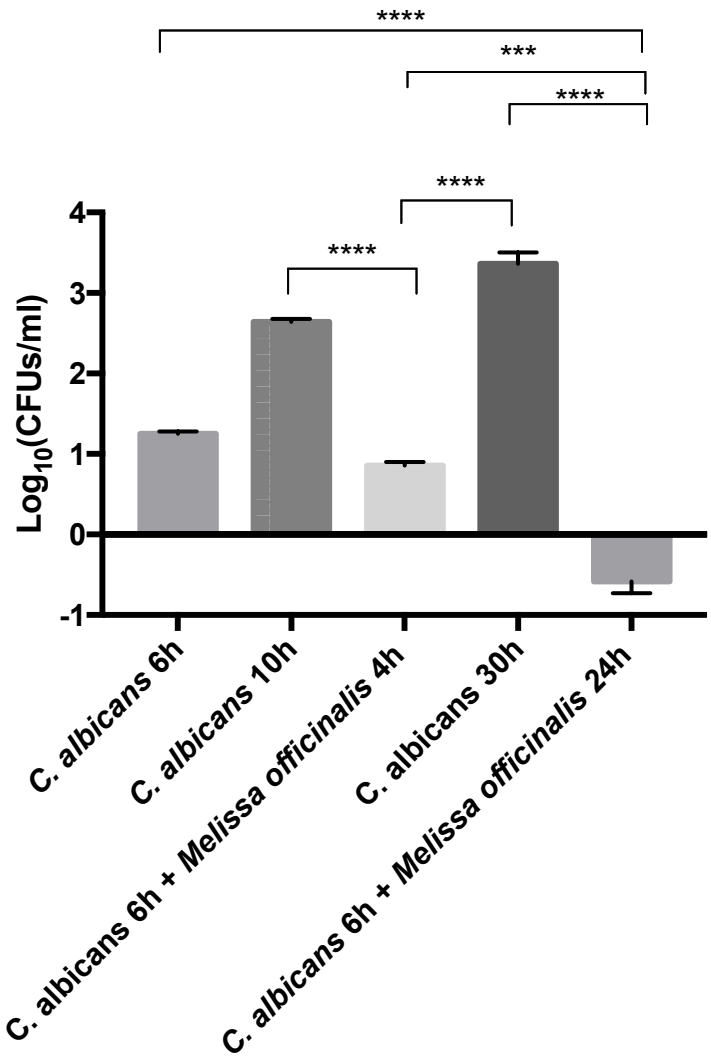


Figure 5.6 – Log_{10} of CFUs/ml recovered from semi-solid agar inoculated *with C. albicans* for 6 hours and treated with 1% (v/v) *Melissa officinalis* essential oil for 4 or 24 hours. Data were normalised by the control (i.e. CFU/ml at 0 hours). Data represent the mean of three independent experiments, each performed in duplicate. *** equivalent to $p< 0.001$, **** equivalent to $p< 0.0001$

5.4.2 Trowel-type culture of the gingiva

The mandibles were cultured with the Trowel-type culture (Figure 5.7). Mandibles were embedded in 1% (w/w) semi-solid agar and nutrients were supplied by diffusion of the cell culture medium through the 0.22 µl Millipore filter into the agar. The uninoculated mandibles appeared clear, while those inoculated were cloudy (Figure 5.7). In accordance with the results in figure 5.6, the 24 hour addition of *Melissa officinalis* inhibited the growth of *C. albicans* (Figure 5.7-D).

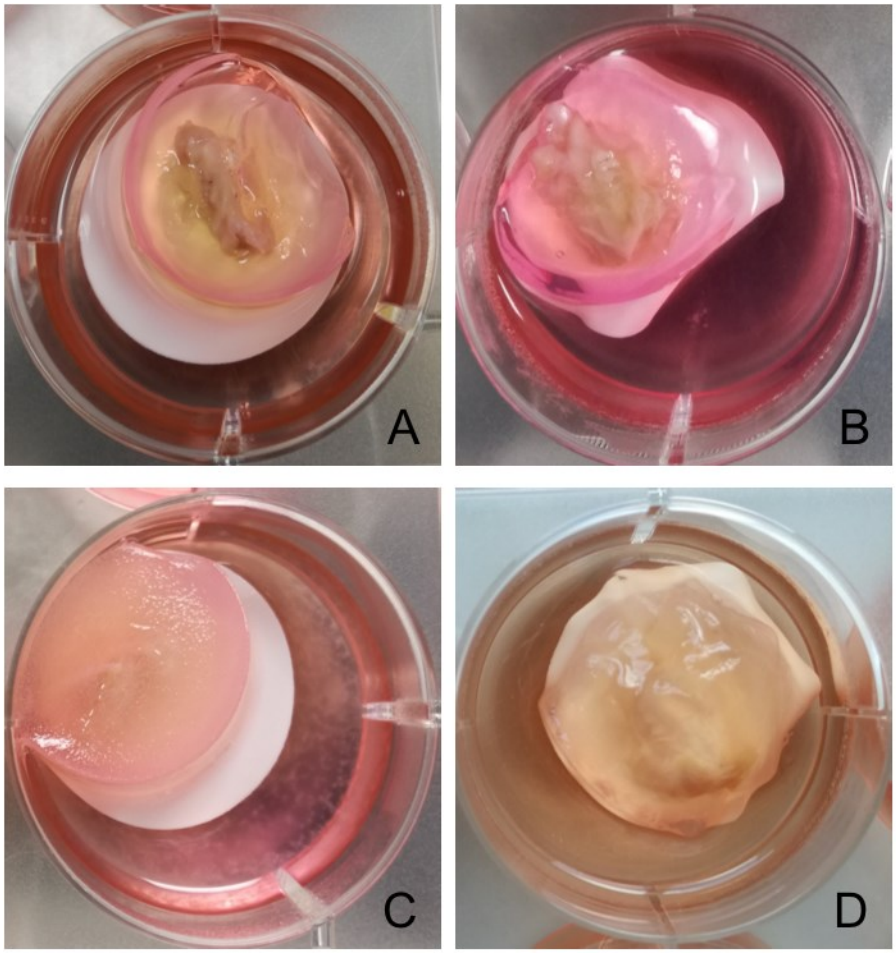


Figure 5.7 – Trowel-type cell culture of A) 24 hour control, B) 24 hour 1% (v/v) *Melissa officinalis*, C) 24 hour *C. albicans*, and D) 6 hour *C. albicans* + 24 hours 1% (v/v) *Melissa officinalis*

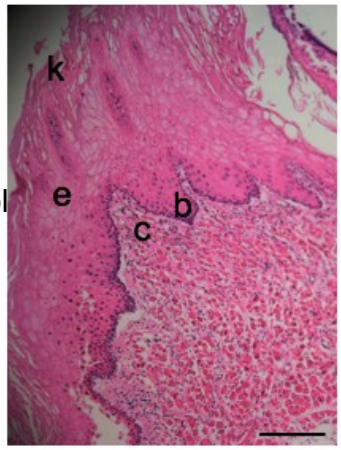
5.4.3 Histological examination

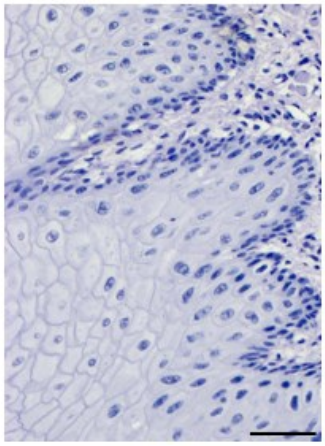
Tissue invasion was monitored by histological examination. Haematoxylin and eosin (H&E) were used to stain cells and PAS to visualise *C. albicans*. Healthy gingiva is characterised by three layers: keratinised layer (k), epithelium layer (e) and the basal layer (b) attached to the connective tissue (c) (Figure 5.8-A).

The healthy structure of the gingiva was maintained in all the conditions tested (Figure 5.8). The gingiva was infected with *C. albicans* for 6, 24 and 48 hours. After 6 hours of infection, *C. albicans* was not visible (Figure 5.8-C). By contrast, when the mandible was infected for 24 hours, *C. albicans* was detectable as

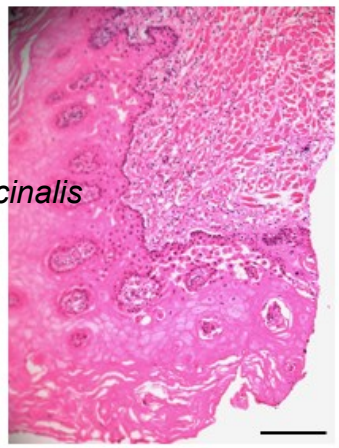
unicellular yeast and hyphal form (Figure 5.8-D). Interestingly, when the infection was prolonged for additional 24 hours (48 hours in total), the number of hyphae and the penetration of *C. albicans* in the deepest layers did not increase, while a uniform layer appeared on the top of the keratinocytes (Figure 5.8-E). After 6 and 24 hour infection, mandibles were treated with 1% (v/v) *Melissa officinalis* essential oil. Figure 5.8-B revealed that the essential oil did not have an impact on the gingival structure. Concerning the antifungal potential of *Melissa officinalis*, it was interesting to note that after 24 hours of infection and addition of *Melissa officinalis*, only few colonies were still detectable (Figure 5.8-F).

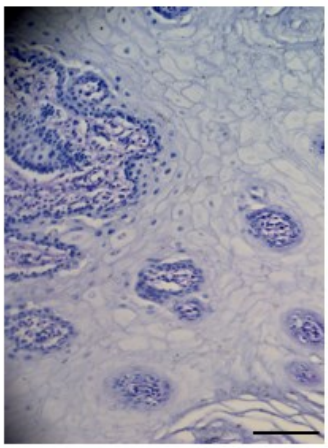
24 hours control
(A)



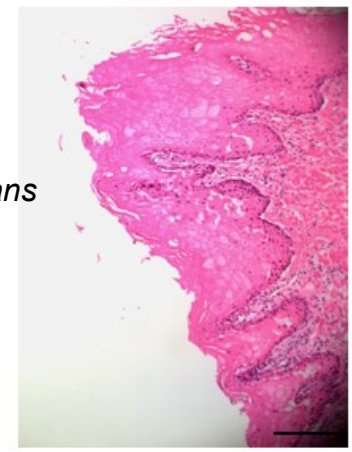


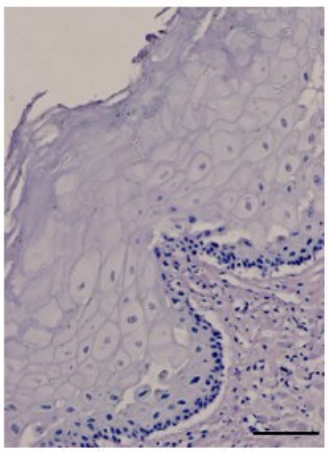
1% (v/v) *Melissa officinalis*
(B)



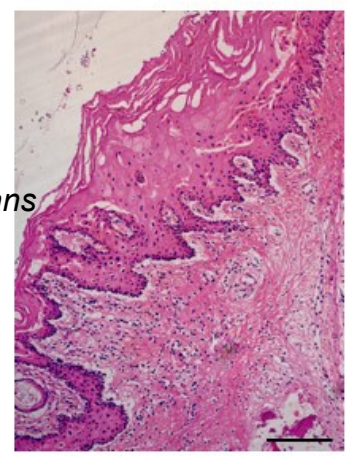


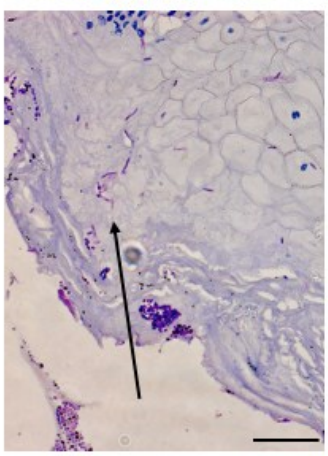
6 hours *C. albicans*
(C)





24 hours *C. albicans*
(D)





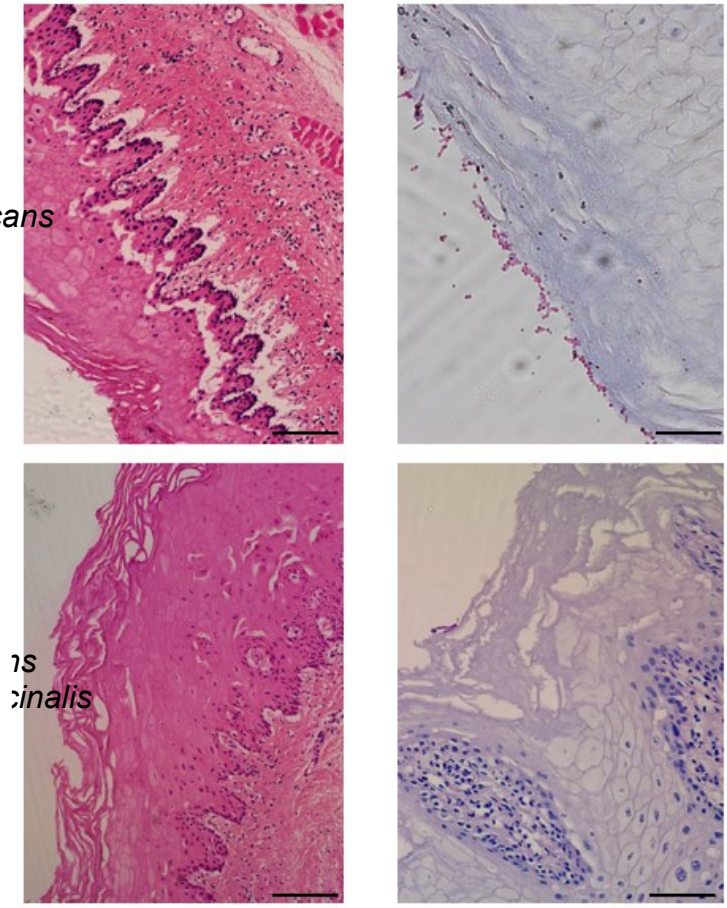


Figure 5.8 - Histological examination of the gingiva stained with H&E (left, scale bar 100 µm) and PAS (right, scale bar 50 µm). A) 24 hour control, B) 24 hour 1% (v/v) *Melissa officinalis*, C) 6 hour *C. albicans*, D) 24 hour *C. albicans*, E) 48 hour *C. albicans*, F) 24 hour *C. albicans* + 1% (v/v) *Melissa officinalis*.
k) indicates the keratinised layer, e) the epithelium layer, b) the basal layer and c) the connective tissue. Arrows point the hyphal form of *C. albicans*

5.4.4 Colony forming units (CFUs)

The antifungal potential of *Melissa officinalis* essential oil was estimated by viable counts of *C. albicans*, which were recovered after *Melissa officinalis* treatment on the infected mandibles. As already observed by histological examination (Section 5.4.3), *Melissa officinalis* prevented *C. albicans* growth. Indeed, the application of *Melissa officinalis* treatment after 6 and 24 hours significantly decreased the CFUs/ml compared to the untreated mandibles ($p<0.01$) (Figure 5.9). In addition, a CFU/ml decrease from the 24 to 48 hour infection was observed, although this difference was not statistically significant.

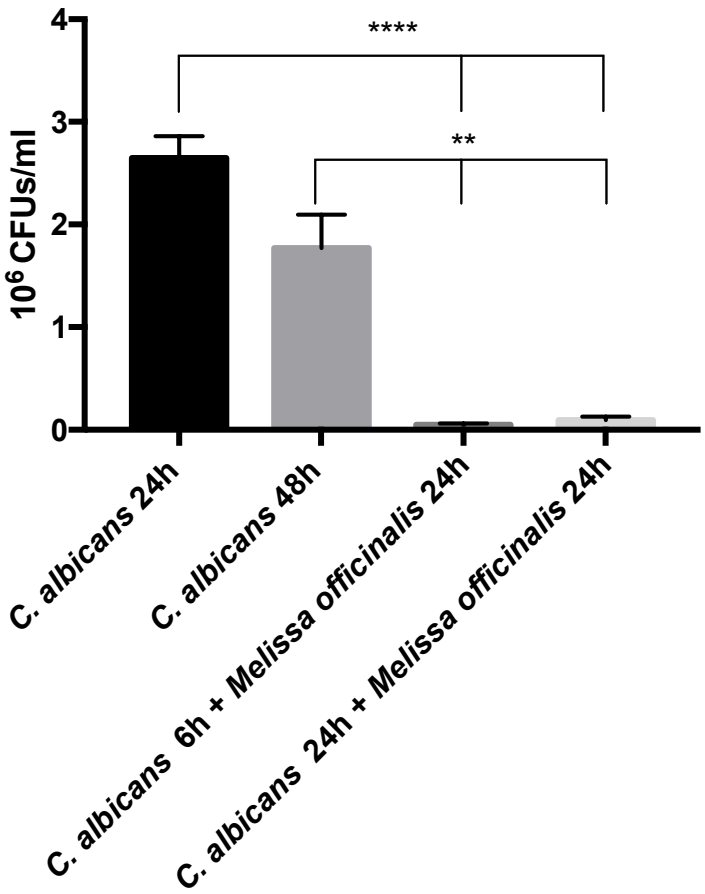


Figure 5.9 - Colony Forming Units (CFUs/ml) recovered from the gingiva after infection with *C. albicans* followed by the application of 1% (v/v) *Melissa officinalis* essential oil. Controls included untreated samples. Data represent the mean of three independent experiments, each performed in duplicate. ** equivalent to $p< 0.01$, **** equivalent to $p< 0.0001$

5.4.5 Primer optimisation

The primers were optimised by running serial dilutions of cDNA as described in Section 5.3.9.1. Figure 5.10 reports the cycle threshold (C_t) values as a function of the content of cDNA, for both the reference housekeeping genes and the gene markers. Table 5.2 shows the efficiency of each primer, calculated from the slopes of the curves reported in Figure 5.10. The best reference housekeeping gene was selected according to the NormFinder software. Ubiquitin C (UBC) was found to be the most stable housekeeping gene and was used as reference gene in the subsequent experiments (Table 5.3).

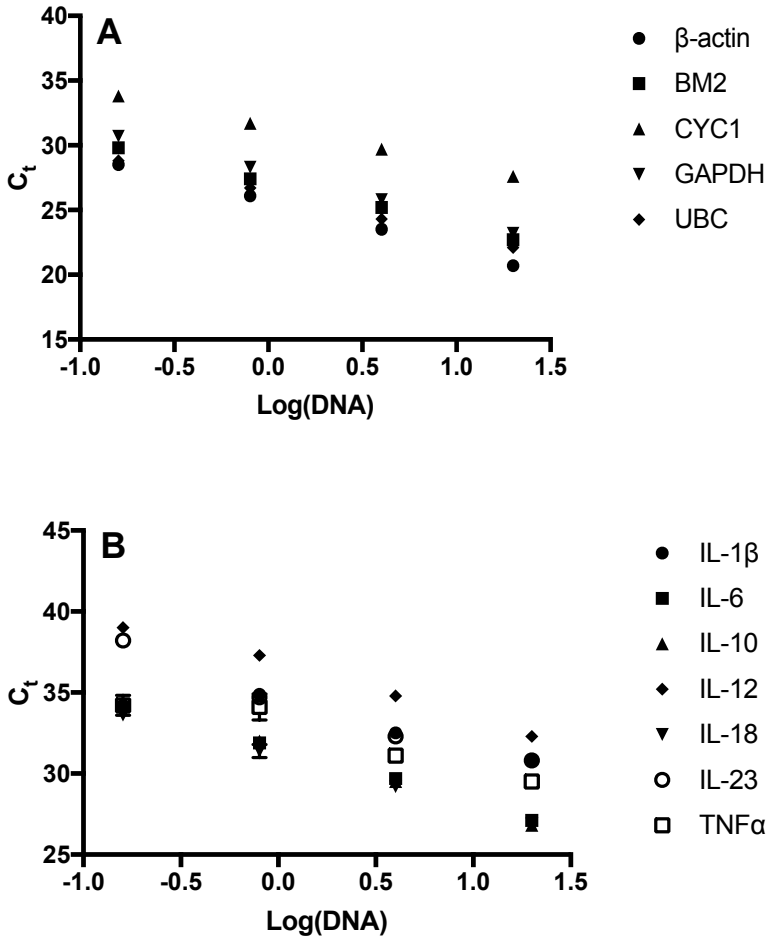


Figure 5.10- Efficiency curves for the reference housekeeping genes (A) and the cytokines (B). Each gene was run in the presence of 1:5 serial dilutions of cDNA

Table 5.2 - Primer efficiency calculated from the slope of the serial dilution curves

Gene marker	Efficiency
β–actin	105%
B2M	99%
CYC1	116%
GAPDH	91%
UBC	103%
IL-1β	106%
IL-6	99%
IL-10	86%
IL-18	104%
IL-12	113%
IL-23	93%
TNFα	102%

Table 5.3 - Stability value for each housekeeping gene calculated with the NormFinder software. The smaller the value, the more stable the gene is

Gene marker	Stability value
β–actin	1.129
B2M	0.779
CYC1	1.076
GAPDH	0.927
UBC	0.422

5.4.6 RT-PCR

Pro and anti-inflammatory responses to *C. albicans* infection and treatment with *Melissa officinalis* essential oil were evaluated by RT-PCR. Four interleukins (IL-1β, IL-6, IL-10 and IL-18) were targeted (

Figure 5.11). No significant differences were observed by culturing the mandibles for 24 or 48 hours. An infection of 6 hours with *C. albicans* did not cause a significant inflammatory response, except for a spike in the IL-10 production, even if this difference was not statistically significant. An infection of 24 hours

caused an acute pro-inflammatory response with a high production of IL-1β, IL-6, IL-18, while the secretion of the anti-inflammatory marker (IL-10) was the lowest. When the mandible was infected for 48 hours, the production of interleukins decreased and only the secretion of IL-1β was significantly higher than the control ($p<0.001$).

Concerning the effect of the essential oil on host cells, except for the high levels of IL-6, no significant differences were observed compared to the untreated samples. The application of *Melissa officinalis* essential oil to the infected samples allowed a significant decrease in the pro-inflammatory response compared to the 24 hours infection with *C. albicans.* However, by comparing this response with that obtained after a 48 hour infection with *C. albicans*, the decrease was significant only in the IL-1β expression.

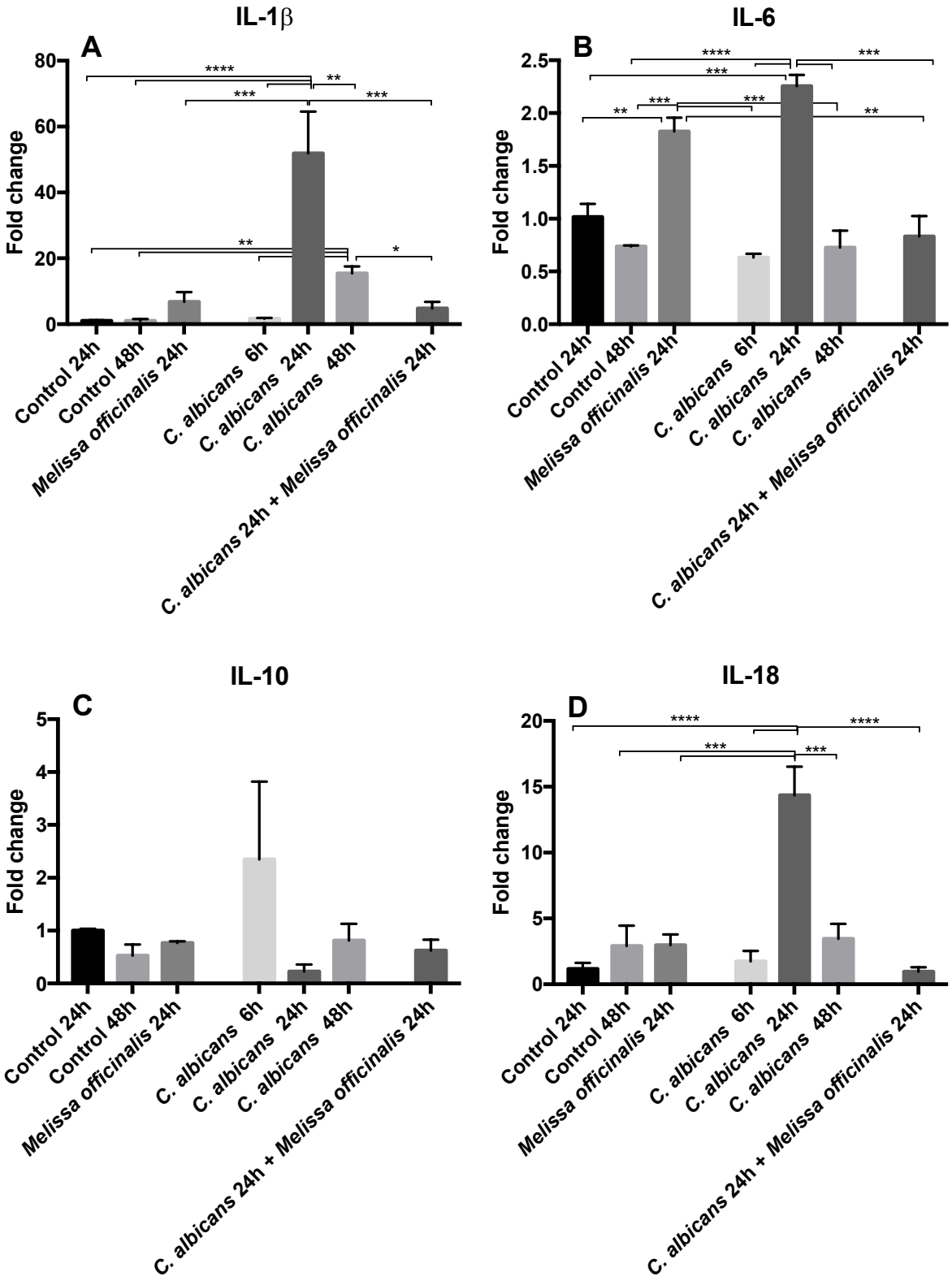


Figure 5.11 - Expression of IL-1β (A), IL-6 (B), IL-10 (C) and IL-18 (D) recovered from the gingiva. Gingiva was infected for 6, 24 and 48 hours with *C. albicans*. After 24 hour infection, 1% (v/v) *Melissa officinalis* essential oil was applied. Controls included untreated and uninfected samples. Data were calculated as fold change compared to the 24 hours culture without *Candida* and *Melissa officinalis.* Data (mean ± SEM) represent the mean of three independent experiments, each performed in duplicate * equivalent to p< 0.05, ** equivalent to p< 0.01, *** equivalent to p<0.001 and **** equivalent to p<0.00

5.5 Discussion

In vitro models are unable to reproduce the *in vivo* spatial cellular organisation and to take into account the cellular interaction that might occur *in vivo* (Sloan et al., 2016). For these reasons, an *ex vivo* model was established with the aim to test the antifungal potential of *Melissa officinalis* essential oil against *C. albicans*. The mandible and the attached gingiva were cultured using the Trowel-type culture (Trowell, 1959), in which the tissue is embedded in a semi-solid agar and cultured at the gas-liquid interface. The Trowel-type culture has been previously reported to be the ideal method for culturing 3D-tissues and has been successfully used to culture mandibles and tooth slices (Sloan et al., 1998; Sloan and Smith, 1999; Waddington et al., 2004; Smith et al., 2010). This is because the more effective nutrient perfusion and oxygen supplementation maintain better tissue morphology and a healthy culture (Smith, 2009).

One percent (1% (v/v)) *Melissa officinalis* essential oil was used because previous experiments showed that this concentration killed *C. albicans* even in a biofilm growth mode (see Section 2.8.3). Two different methods were evaluated for the treatment of the gingiva: addition of the essential oil to the cell culture medium that perfused into the semi-solid agar and direct application of the treatment to the gingiva. It was observed that by pipetting small amount of the treatment (20 µl) into the semi-solid agar, the amount effectively released into the agar was not the same each time the experiment was repeated. This caused a lack of reproducibility, which was also confirmed by histological analysis and showed that the treatment was not always able to kill *C. albicans* (data not shown). For this reason, it was decided to treat the mandible by applying the essential oil in the culture medium.

Since the cell culture medium containing the treatment diffused into the semi-solid agar, the time needed to allow the oil diffusion into the semi-solid agar and to kill *C. albicans* was investigated. It was found that an application of 24 hours of the treatment was necessary to kill *Candida*. Therefore, a 24 hour application was used for all subsequent experiments.

Concerning the infection of the gingiva, three different approaches were evaluated: addition of *C. albicans* into the agar by pipetting, direct contact of the gingiva with *C. albicans* grown onto SAB agar, and embedment of the mandible into semi-solid agar containing *C. albicans*. As mentioned earlier, the pipetting technique was not reproducible, while the direct contact of the mandible with *C. albicans* did not allow the evaluation of the initial inoculum (CFU/ml). Therefore, it was decided to embed the mandibles in previous infected semi-solid agar.

The mandibles were infected for 6 and 24 hours with *C. albicans*, and 1% (v/v) *Melissa officinalis* essential oil was used as a treatment. The histological analysis showed that the structure of the gingiva was maintained over 48 hours and was not altered by the application of *Melissa officinalis* essential oil. It was not possible to quantify the viability of the cells within the gingiva. Therefore, the toxicity of the oil was only investigated qualitatively from histological analysis.

Concerning the inflammatory response, 4 interleukins (IL-1β, IL-6, IL-10 and IL-18) were targeted. Briefly, IL-10 is an anti-inflammatory cytokine that inhibits the production of IL-12 and the formation of Th1 cells, promoting a Th2 response that inhibits the activity of macrophages. The production of IL-10 is one of the virulence factors that *Candida* uses to evade the host immune system (Xiong et

al., 2000; Netea et al., 2004; Luo et al., 2013). IL-1β and IL-6 are pro-inflammatory cytokines involved in the differentiation of T-cells in Th17, in the induction of pro-inflammatory cytokines, in the expression of antimicrobial peptides, and in the migration of neutrophils and the activation of macrophages (Akdis et al., 2011; Richardson and Moyes, 2015; Akdis et al., 2016). IL-18 is a pro-inflammatory cytokine that promotes the recruitment of phagocytic cells and the production of IFNγ, crucial to the development of a Th1 response (Akdis et al., 2016; Qin et al., 2016) . Further details on the immune response are provided in Chapter 3, Section 3.1.2.

The inflammatory response was evaluated using RT-PCR. Significant differences in the inflammatory response to *Melissa officinalis* and the control (i.e. untreated sample) were observed only in the production of IL-6. At the author's knowledge, no data are available on the inflammatory responses generated by the application of *Melissa officinalis* essential oil on the *ex vivo* model. Previous studies reported that geraniol and citronellal, which are the two main compounds of *Melissa officinalis,* had an anti-inflammatory response (de Cássia da Silveira e Sá et al., 2013). In addition, essential oils that have some of the main compounds in common with *Melissa officinalis* (e.g. citral, geraniol and citronellol) inhibited IL-1 and IL-6 in mouse macrophages stimulated with LPS, and suppressed the adherence reaction of neutrophils induced by TNFα (Abe et al., 2003; Sforcin et al., 2009). However, it should be taken into account that different cells or interleukins were targeted in these studies.

The infection of the mandible with *C. albicans* showed that a 6 hour infection was not sufficient to promote adhesion and invasion of *C. albicans.* However, the PCR

analysis highlighted that even if *C. albicans* was not detectable, it caused a host immune response. Indeed, a spike in the IL-10 expression was observed.

A 24 hour infection allowed *C. albicans* to adhere to and invade the gingiva. In particular, the hyphae morphology form, one of the virulence factors of *C. albicans*, was visible. Hyphae are related to greater resistance to phagocytosis compared to the yeast form, as well as a higher invasion ability that causes tissue damage and a down-regulation of pro-inflammatory cytokines (e.g. IFNγ) (Williams and Lewis, 2011; Luo et al., 2013).

When the mandibles were infected for 48 hours, penetration of *Candida* into the gingiva was not evident anymore but a uniform *Candida* layer composed of yeast and hyphae was formed on the surface.

The absence of invasion when the culture was prolonged could be due to the culture medium used. Indeed, mandibles were cultured in DMEM/F12, which is a medium specific for cells. Moreover, *Candida* growth was not supported by the addition of SBD to the cell culture medium. Therefore, it could be possible that the ideal conditions to support the growth and the invasion were not present in the *ex vivo* model, and this was evident in the prolonged culture.

This was also confirmed by determining the CFU/ml after 24 and 48 hours. Even if the difference was not significant, less CFUs/ml were recovered after a 48 hours infection.

Despite this decline in viability and invasive potential, the histological analysis showed tissue damage after 48 hour infection with the detachment of the basal layer from the connective tissue, even if it is possible that the gaps formed during the cutting process. The tissue damage could also be confirmed by the inflammatory responses. Indeed, no significant differences were observed in the

expression of IL-6 and IL-18 between the controls and the infected samples. This could be due to the fact the cytokine-producing cells were killed by *Candida*.

In general, it was observed that during *Candida* infection, there was a similar trend in the expression of the pro-inflammatory cytokines (IL-1, IL-6 and IL-18), opposed to IL-10, characterised by a spike after 24 hour infection. A similar trend for IL-6 was reported by Steinshamn and Waage (1992) that infected granulocytopenic mice with *C. albicans*.

When the mandibles were treated with *Melissa officinalis* essential oil, *C. albicans* was killed as confirmed both by the PAS staining and the CFUs/ml recovered from the mandibles. Taking into account the inflammatory responses to the treatment, no significant differences were observed between treated and untreated samples. However, it was not possible to speculate if this was due to the antifungal potential of the essential oil or to the toxic effect of the essential oil on the cells that inactivated the immune response.

Therefore, further work should focus on the evaluation of the cell viability in the mandible. Truly, only in this way it would be possible to claim an anti-inflammatory and anti-candidal potential of *Melissa officinalis* essential oil.

5.6 Conclusions

An *ex vivo* rodent mandible model was developed. The gingiva was successfully infected, and *Melissa officinalis* essential oil was used as a treatment. The infection and treatment were evaluated by histological examination and by counting the CFUs/ml. Preliminary experiments to investigate the inflammatory responses were carried out by RT-PCR. Results showed a spike in the pro-inflammatory response after an infection of 24 hours. In addition, the application of *Melissa officinalis* essential oil decreased the host response. However, further experiments should be carried out to evaluate the cytotoxicity of the essential oil. Once the optimisation of the model with *C. albicans* is completed, it will be possible to infect the model with periodontal bacteria and evaluate the potential use of *Melissa officinalis* essential oil as a treatment for periodontal diseases.

Chapter 6

General discussion and future work

Candida albicans is a commensal fungus found in 30 - 50% of healthy humans (Singh et al., 2015). However, alterations of the oral environment can lead to the most common human fungal infection, named oral candidiasis. Oral candidiasis comprises four different forms associated with i) the prescription of antibiotics (acute atrophic candidiasis), ii) immunosuppression (pseudomembranous candidiasis), iii) the presence of a denture (denture stomatitis), and iv) the invasion of the oral epithelium (chronic hyperplastic candidiasis) (Williams and Lewis, 2011). Importantly, a possible link between chronic hyperplastic candidiasis and oral cancer has been reported.

Recently, it has also been highlighted that even if oral candidiasis is mainly associated with *C. albicans*, the presence of bacteria at the site plays an important role in the progression of the disease. In particular, higher tissue invasion and production of hyphae were observed when *C. albicans* was co-cultured with *Streptococci* and *Actinomyces* species, meaning that the success of the antifungal therapy can be connected to the concomitant control of the bacteria present at the site (Cavalcanti et al., 2015).

Treatments for oral candidiasis include the use of antifungal agents such as nystatin, amphotericin B, metronidazole and fluconazole. Compared to antibiotics, antifungals on the market are limited and the rise of resistance of *Candida* to these antifungals has been observed. Therefore, an interest in the discovery of new antifungals has developed (Williams and Lewis, 2011; Boros-Majewska et al., 2014).

Essential oils are natural products produced by aromatic plants mainly composed by terpenes and terpenoids. Essential oils interact with the cell membrane

changing its permeability and causing leaching of intracellular components (e.g. radicals, proteins, calcium ions) and the inactivation of enzymatic mechanisms (Bakkali et al., 2008). Moreover, they can penetrate into the cell and interact with the mitochondrial membrane causing cell death (Nazzaro et al., 2013).

In this study, twelve essential oils, two terpenes and two biocides (triclosan and CHX) were evaluated for their antifungal activity against two *C. albicans* strains, a reference strain (*C. albicans* NYCY 1363), and a clinical strain described as a high invader (*Candida albicans* 135BM2/94). The clinical strain was isolated from a patient, who was diagnosed with chronic hyperplastic candidiasis and squamous cell carcinoma (Malic et al., 2007).

This current study confirmed the antifungal potential of essential oils. Indeed, all tested commercial essential oils demonstrated anticandidal activity (MICs from 0.06% (v/v) to 0.4% (v/v)) against planktonic cultures, with a noticeable increase in resistance exhibited by biofilms (MBECs > 1.5% (v/v)).

Since the antimicrobials were applied for 24 hours, future work could investigate the time needed to kill *C. albicans*. In addition, the evaluation of the MBECs could be performed on polymicrobial biofilms or on biofilms in the presence of a conditioning film (e.g. saliva).

Candida albicans, being a eukaryotic organism, shares some features with mammalian cells. Therefore, the development of a treatment that is antifungal but not cytotoxic is crucial. For this reason, the cytotoxicity of the essential oils was evaluated as well. Firstly, a preliminary screening was performed on NIH 3T3 cells, a mouse fibroblast cell line available in-house (Serra et al. 2018). The cytotoxicity of the six compounds active against biofilm growth (cinnamon,

linalool, geranium and melissa, E-cinnamaldehyde, and CHX) was evaluated with an alamarBlue assay. It was observed that the essential oils halved fibroblast proliferation at concentrations lower than their MICs and that melissa was the essential oil that showed the lowest cytotoxicity (MIC80 of 0.06% (v/v) and IC50 of 0.03% (v/v)).

In addition, the cytotoxicity was evaluated on a human cell line to better mimic the *in vivo* response. The cytotoxicity was evaluated on human dental pulp stem cells with a CellTiter-Glo assay. The experiment confirmed that the concentrations needed to kill *C. albicans* were higher than those that inhibited cell proliferation. However, as discussed in Chapter 3, the main disadvantage of this assay is the high cost. For this reason, only one repetition of the experiment was carried out and the data were not shown in this thesis.

It was concluded from analysis of the cytotoxicity that melissa *(Melissa officinalis)* was the essential oil that showed the best compromise between antifungal activity and low cytotoxicity. Hence, *Melissa officinalis* was used in all further experiments.

Even though the screening of the cytotoxicity revealed that the essential oils were generally toxic to cells, some experimental limitations should be taken into account. Indeed, even though the 2D-model was simple and inexpensive, it did not reproduce the *in vivo* spatial organisation. In particular, the treatment was applied to a cellular monolayer. For instance, de Campos Rasteiro et al. (2014) evaluated the potential of 12.5% (v/v) tea tree oil as a treatment for oral candidiasis in a mouse model. They did not report a cytotoxic effect at this

concentration, which was significantly greater than the MIC found in this study and the IC50 of the essential oils screened.

In addition, in the case of oral application, it should be considered that the saliva would dilute the concentration of the essential oil and decrease the contact-time between treatment and cells. This could determine an increase in the resistance of cells to the essential oil. Therefore, it would be ideal to evaluate the effect on cells on a short-time application (e.g. 5 minutes).

Moreover, it should be considered that a synergy between essential oil and antifungals present on the market had been reported (Zore et al., 2010; Zore et al., 2011; Samber et al., 2015; Cardoso et al., 2016). The synergy allows the same antifungal activity by decreasing the doses of both the antifungal and the essential oil, potentially avoiding the cytotoxic effect. Since the aim of the present study was not to use antifungals available on the market, the evaluation of the synergy with essential oils was not screened. However, future work might focus on the investigation of the cytotoxicity of blends essential oil-antifungal on a human cell line.

In general, it is also important to highlight that the essential oils are composed of several terpenes and terpenoids, and not all of them could show an antifungal activity or a cytotoxic effect. Therefore, the evaluation of the MICs and ICs50 of the single compounds would allow the synthesis *ex novo* of an antimicrobial containing the compounds that are antifungal but not those that have an effect on cells. Besides being used as an alternative to commercially available treatments, the best compounds could be used in combination with antimicrobials available to potentiate their action. Therefore, the synergy between the antifungals and the single components of the essential oil could be also evaluated (Abreu et al., 2012).

Besides the cytotoxicity, it is important to evaluate the host response to the treatment. Therefore, the inflammatory response to *Melissa officinalis* in combination with and without *C. albicans* was evaluated. The inflammatory response was investigated on an *ex vivo* whole blood model developed at Swansea University (Al-ishaq et al., 2015). It was observed that *Melissa officinalis* essential oil did not cause a significant inflammatory response, except for a 4 hour addition of 0.1% (v/v) *Melissa officinalis* that caused an anti-inflammatory response. The potential anti-inflammatory activity of essential oils or their main compounds has been previously reported (Abe et al., 2003; Sforcin et al., 2009; Bounihi et al., 2013). In addition, it was observed that the infection of the blood with *C. albicans* in the presence of the essential oil led to a pro-inflammatory response. This could mean that the oil eliminated the capacity of *Candida* to evade the immune system. Indeed, when the blood was infected with *Candida* without the treatment, an anti-inflammatory response was observed.

Despite these observations, it should be considered that only one out of three donors showed an inflammatory response. Therefore, it was not possible to claim a statistical significance of these results. Moreover, the donor that had an inflammatory response showed high levels of IL-10 even in the untreated blood. This could be due to the presence of an infection in the body that was not communicated by the donor when the blood was collected. Future work should be done to include more donors. In addition, the haemolysis caused by the application of the essential oil should be analysed to verify that the cells producing interleukins are still alive.

Once the antimicrobial properties of a new compound have been investigated, a vehicle to deliver the compound at the site of infection has to be synthesised.

Hydrogels are 3D-networks obtained from natural and/or synthetic polymers that have been widely used as drug delivery systems (Slaughter et al., 2009). The thermosensitive hydrogels can crosslink at body temperature, allowing their injection at room temperature and the gelation *in situ*.

Among the different types of hydrogels, it was decided to use a methylcellulose polymer. The decision was established on the low cost of the powder, and on the simple preparation that did not involve the use of a possible cytotoxic crosslinker. In the present study, a methylcellulose hydrogel containing 1 and 2% (v/v) *Melissa officinalis* was successfully developed. In particular, the drug release was maintained for 48 hours, allowing the increase of the retention time of the essential oil at the site of infection. In addition, the synthesised hydrogel had an antifungal activity as shown by the time-kill assay and the zone inhibition method. Future work on the hydrogel should be undertaken to evaluate its potential application as a treatment for oral candidiasis.

Methylcellulose hydrogels have been reported to have mucoadhesive properties (Klouda, 2015). Therefore, it could be injected directly at the infection site. Moreover, in case of denture stomatitis, the hydrogel might be used to coat the denture. In the latter case, further experiments to evaluate the interactions of the hydrogel with *Candida* need to be conducted. In particular, retention assays will allow understanding if the presence of the hydrogel containing the essential oil acts as an additional surface to which *Candida* can adhere, or inhibits the attachment of *Candida* to the denture acrylic (Taylor et al., 1998).

In addition, the efficacy of the hydrogel against biofilms needs to be evaluated. Finally, even if methylcellulose is a biocompatible polymer, it would be interesting to evaluate the cytotoxicity of the hydrogel in combination with the essential oil. Indeed, even if the hydrogel contained 1 or 2% (v/v) *Melissa officinalis* essential

oil, greater than the IC50, the current study has determined that the oil was released over 48 hours. Consequently, the cells would not be exposed to the high concentrations added into the hydrogel but only to the fraction released, that might not exceed the toxic threshold.

As previously mentioned, *in vitro* models have some limitations such as the impossibility to reproduce the *in vivo* spatial cellular organisation (Sloan et al., 2016). For this reason, the final part of the present study was dedicated to the development of an *ex vivo* mandible rodent model. The *ex vivo* rodent mandible model provided a tissue scaffold that allowed observation of *Candida* invasion and adhesion and evaluation of the antifungal potential of *Melissa officinalis*. In particular, it was possible to infect the gingiva with *Candida* and observe the formation of hyphae and penetration over 48 hours. In addition, the application of the essential oil successfully decreased the CFUs/ml recovered compared to the untreated samples. Preliminary RT-PCR experiments highlighted that the presence of *Candida* for 24 hours caused a pro-inflammatory response, while the application of the essential oil decreased the host response.

The main limitation of this model was the impracticality of quantifying cell viability. It was impossible to speculate if the decreased host response in the presence of the treatment was due to the effect of the essential oil on *Candida* or to its cytotoxicity.

In particular, the use of cytotoxicity assays on the model is challenging. Since the mandible was cultured in the Trowel-type culture, it was embedded into the semi-solid agar. This meant that the LDH was not released into the cell culture medium. Similarly, the use of the MTT or alamarBlue assay was not applicable because of the variability of the cell number in each mandible, depending on the rat and the

cutting process. Basically, it would not allow comparison of the colour intensity of the assay between the mandibles (regarding colour intensity, more details in Chapter 3). Therefore, imaging methods such as the counting of the nuclei could be utilised.

Considering that the aim of the project was to develop a novel therapy for oral diseases, the experiments were designed to assess the antimicrobial properties of a range of compounds, to test the cytotoxicity of these compounds, to synthesise and evaluate a delivery vehicle and to develop an *ex vivo* model.

In the first instance, it was decided to target oral candidiasis, which is mainly caused by *Candida*. However, in the future, the hydrogel and the *ex vivo* model could be used for other clinical applications. In particular, these technologies and methodologies could be transferred to other oral diseases, such as periodontal diseases. Periodontal diseases are a polymicrobial infection that involves anaerobic bacteria and bone damage, and are of particular concern, being a risk factor for cardiovascular diseases, the leading cause of death worldwide (Tonetti et al., 2013).

Indeed, once the activity of the essential oil is investigated *in vitro* against periodontal pathogens, as shown in Chapter 1, the hydrogel containing the essential oil can be synthesised as described in Chapter 4 and applied to periodontal bacteria. In addition, once the *ex vivo* model is optimised in term of infection and treatment procedures, measurement of the viability, and extraction of the RNA, it will be possible to adapt the methodology to an infection with periodontal pathogens.

In conclusion, this study attempted to develop a novel antimicrobial therapy for oral diseases by utilising a hydrogel in combination with an essential oil.

The main findings of this study were:

- Essential oils showed antifungal activity against planktonic *C. albicans,* and six of the compounds tested were also active against biofilms
- Cytotoxicity screening revealed that the essential oils halved fibroblast proliferation at concentrations lower than those required to inhibit *C. albicans* growth
- *Melissa officinalis* was the essential oil that exhibited the best antifungal activity and lowest cytotoxicity, and had an anti-inflammatory potential
- The methylcellulose hydrogel containing *Melissa officinalis* was successfully synthesised. The hydrogel gellified at body temperature in less than three minutes and the release of *Melissa officinalis* was maintained over a period of 48 hours. The antifungal potential of the hydrogel with *Melissa officinalis* was confirmed by both zone of inhibition method and time-kill assay
- The *ex vivo* rodent mandible model to better mimic the *in vivo* situation was developed. The gingiva was successfully infected with *C. albicans* and the treatment with *Melissa officinalis* decreased the CFU/ml recovered from the tissue. RT-PCR showed that the application of the essential oil decreased the host inflammatory response

References

Abdellatif, F., Boudjella, H., Zitouni, A. and Hassani, A. (2014) 'Chemical composition and antimicrobial activity of the essential oil from leaves of algerian'. *Experimental and Clinical Sciences Journal,* 13(1) pp. 772-781.

Abe, S., Maruyama, N., Hayama, K., Ishibashi, H., Inoue, S., Oshima, H. and Yamaguchi, H. (2003) 'Suppression of tumor necrosis factor-alpha-induced neutrophil adherence responses by essential oils'. *Mediators of Inflammation,* 12(6) pp. 323-328.

Abreu, A. C., Mcbain, A. J. and Simões, M. (2012) 'Plants as sources of new antimicrobials and resistance-modifying agents'. *Natural Product Reports,* 29(9) pp. 1007-1021.

Ahmed, T. a. E., Dare, E. V. and Hincke, M. (2008) 'Fibrin: a versatile scaffold for tissue engineering applications'. *Tissue Engineering Part B: Reviews,* 14(2) pp. 199-215.

Akdis, M., Aab, A., Altunbulakli, C., Azkur, K., Costa, R. A., Crameri, R., Duan, S., Eiwegger, T., Eljaszewicz, A., Ferstl, R., Frei, R., Garbani, M., Globinska, A., Hess, L., Huitema, C., Kubo, T., Komlosi, Z., Konieczna, P., Kovacs, N., Kucuksezer, U. C., Meyer, N., Morita, H., Olzhausen, J., O'mahony, L., Pezer, M., Prati, M., Rebane, A., Rhyner, C., Rinaldi, A., Sokolowska, M., Stanic, B., Sugita, K., Treis, A., Van De Veen, W., Wanke, K., Wawrzyniak, M., Wawrzyniak, P., Wirz, O. F., Zakzuk, J. S. and Akdis, C. A. (2016) 'Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: receptors, functions, and roles in diseases'. *Journal of Allergy and Clinical Immunology,* 138(4) pp. 984-1010.

Akdis, M., Burgler, S., Crameri, R., Eiwegger, T., Fujita, H., Gomez, E., Klunker, S., Meyer, N., O'mahony, L., Palomares, O., Rhyner, C., Quaked, N., Schaffartzik, A., Van De Veen, W., Zeller, S., Zimmermann, M. and Akdis, C. A. (2011) 'Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases'. *Journal of Allergy and Clinical Immunology,* 127(3) pp. 701-721.

Akhtar, M. F., Hanif, M. and Ranjha, N. M. (2016) 'Methods of synthesis of hydrogels…A review'. *Saudi Pharmaceutical Journal,* 24(5) pp. 554-559.

Akpan, A. and Morgan, R. (2002) 'Oral candidiasis'. *Postgraduate Medical Journal,* 78(922) pp. 455-459.

Al-Ishaq, R., Armstrong, J., Gregory, M., Hara, M. O., Phiri, K., Harris, L. G., Rohde, H., Siemssen, N., Frommelt, L., Mack, D. and Wilkinson, T. S. (2015) 'Effects of polysaccharide intercellular adhesin (PIA) in an *ex vivo* model of whole blood killing and in prosthetic joint infection (PJI): a role for C5a'. *International Journal of Medical Microbiology* 305(8) pp. 948-956.

Al-Shanti, N., Saini, A. and Stewart, C. E. (2009) 'Two-step versus one-step RNA-to-CT 2-step and one-step RNA-to-CT 1-Step: validity, sensitivity, and efficiency'. *Journal of Biomolecular Techniques,* 20(3) pp. 172-179.

Alauzet, C., Marchandin, H. and Lozniewski, A. (2010) 'New insights into *Prevotella* diversity and medical microbiology'. *Future Microbiology,* 5(11) pp. 1695-1718.

Aljehani, Y. A. (2014) 'Risk factors of periodontal disease: review of the literature'. *International Journal of Dentistry,* 2014, May, pp. 182513.

Almeida, D. D., Paula, J. F. D., Almeida, D. D., Williams, D. W., Hebling, J. and Cavalcanti, Y. W. (2016) 'Efficacy of citronella and cinnamon essential

oils on *Candida albicans* biofilms'. *Acta Odontologica Scandinavica,* 74(5) pp. 393-398.
Amano, A. (2010) 'Bacterial adhesins to host components in periodontitis'. *Periodontology 2000,* 52(1) pp. 12-37.
Amorim, C. V. G., Aun, C. E. and Mayer, M. P. A. (2004) 'Susceptibility of some oral microorganisms to chlorhexidine and paramonochlorophenol'. *Brazilian Oral Research,* 18(3) pp. 242-246.
Anderson, J., Mihalik, R. and Soll, D. R. (1990) 'Ultrastructure and antigenicity of the unique cell wall pimple of the *Candida* opaque phenotype'. *Journal of Bacteriology,* 172(1) pp. 224-235.
Anderson, M. (2002) 'Risk assessment and epidemiology of dental caries: review of the literature'. *Pediatric Dentistry Journal,* 24(5) pp. 377-385.
Antachopoulos, C. and Roilides, E. (2005) 'Cytokines and fungal infections'. *British Journal of Haematology,* 129(5) pp. 583-596.
Antony, G., Saralaya, V., Gopalkrishna Bhat, K., Shalini Shenoy, M. and Shivananda, P. G. (2009) 'Effect of phenotypic switching on expression of virulence factors by *Candida albicans* causing candidiasis in diabetic patients'. *Revista Iberoamericana de Micologia,* 26(3) pp. 202-205.
Arens, U. (1999) 'Oral health: diet and other factors'. *Nutrition Bulletin,* 24(1) pp. 41-44.
Ashman, R. B., Vijayan, D. and Wells, C. A. (2011) 'IL-12 and related cytokines: function and regulatory implications in *Candida albicans* infection'. *Clinical and Developmental Immunology,* 2011, September, pp. 1-9.
Aurer, A. and Plančak, D. (2004) 'Antimicrobial treatment of periodontal diseases'. *Acta Stomatologica Croatica,* 38(1) pp. 67-72.
Ayana, B. B. and Turhan, K. N. (2009) 'Use of antimicrobial methylcellulose films to control *Staphylococcus aureus* during storage of Kasar cheese and science'. *Packaging Technology and Science,* 22(8) pp. 461-469.
Ayre, W. N., Melling, G., Cuveillier, C., Natarajan, M., Roberts, J. L., Marsh, L. L., Lynch, C. D., Maillard, J. Y., Denyer, S. P. and Sloan, A. J. (2018) '*Enterococcus faecalis* demonstrates pathogenicity through increased attachment in an *ex vivo* polymicrobial pulpal infection'. *Infection and Immunity,* 86(5) pp. e00871-17.
Babpour, E., Angaji, S. A. and Angaji, S. M. (2009) 'Antimicrobial effects of four medicinal plants on dental plaque'. *Journal of Medicinal Plants Research,* 3(3) pp. 132-137.
Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M. (2008) 'Biological effects of essential oils - A review'. *Food and Chemical Toxicology,* 46(2) pp. 446-475.
Bakri, M. M., Hussaini, H. M., Holmes, A., Cannon, R. D. and Rich, A. M. (2010) 'Revisiting the association between candidal infection and carcinoma, particularly oral squamous cell carcinoma'. *Journal of Oral Microbiology,* 2, December, pp. 1-6.
Banas, J. A. (2017) 'Virulence properties of *Streptococcus mutans*'. *Frontiers in Bioscience,* 1(9) pp. 1267-1277.
Bao, G. J., Kari, K., Tervahartiala, T., Sorsa, T. and Meurman, J. H. (2008) 'Proteolytic activities of oral bacteria on ProMMP-9 and the effect of synthetic proteinase inhibitors'. *The Open Dentistry Journal,* 2, July, pp. 96-102.

Bao, K., Belibasakis, G. N., Thurnheer, T., Aduse-Opoku, J. and Curtis, M. A. (2014) 'Role of *Porphyromonas gingivalis* gingipains in multi-species biofilm formation'. *BCM Microbiology,* 14(258) pp. 1-8.

Bardaji, D. K. R., Reis, E. B., Medeiros, T. C. T., Lucarini, R., Crotti, A. E. M. and Martins, C. H. G. (2016) 'Antibacterial activity of commercially available plant-derived essential oils against oral pathogenic bacteria'. *Natural Product Research,* 30(10) pp. 1178-1181.

Barkvoll, P. and Attramadal, A. (1989) 'Effect of nystatin and chlorhexidine digluconate on *Candida albicans*'. *Oral Surgery, Oral Medicine, Oral Pathology,* 67(3) pp. 279-281.

Barnes, H. A., Hutton, J. F. and Walters, K. (1989) *An introduction to rheology,* London and New York.

Barros, F. J., Costa, R. J. O., Cesário, F. R. a. S., Rodrigues, L. B., De Menezes, I. R. A., Da Costa, J. G. M., Coutinho, H. D. M. and Galvao, H. B. F. (2016) 'Activity of essential oils of *Piper aduncum* anf and *Cinnamomum zeylanicum* by evaluating osmotic and morphologic fragility of erythrocytes.'. *European Journal of Integrative Medicine,* 8(4) pp. 505-512.

Bertolini, M. M., Portela, M. B., Curvelo, J. A., Soares, R. M., Lourenço, E. J. and Telles, D. M. (2014) 'Resins-based denture soft lining materials modified by chlorhexidine salt incorporation: an *in vitro* analysis of antifungal activity, drug release and hardness'. *Dental Materials* 30(8) pp. 793-798.

Bhattarai, N., Gunn, J. and Zhang, M. (2010) 'Chitosan-based hydrogels for controlled, localized drug delivery '. *Advanced Drug Delivery Reviews,* 62(1) pp. 83-99.

Bollenbach, T. (2015) 'Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution'. *Current Opinion in Microbiology,* 27, June, pp. 1-9.

Bolstad, A. I. and Jensen, H. B. (1996) 'Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*'. *Clinical Microbiology Reviews,* 9(1) pp. 55-71.

Bonowitz, A., Schaller, M., Laude, J., Reimer, K. and Korting, H. C. (2001) 'Comparative therapeutic and toxic effects of different povidone iodine (PVP-I) formulations in a model of oral candidosis based on *in vitro* reconstituted epithelium'. *Journal of Drug Targeting,* 9(1) pp. 75-83.

Boros-Majewska, J., Salewska, N., Borowski, E., Milewski, S., Malic, S., Wei, X. Q., Hayes, A. J., Wilson, M. J. and Williams, D. W. (2014) 'Novel Nystatin A1 derivatives exhibiting low host cell toxicity and antifungal activity in an *in vitro* model of oral candidosis'. *Medical Microbiology and Immunology,* 203(5) pp. 341-355.

Borst, A. and Fluit, A. C. (2003) 'High levels of hydrolytic enzymes secreted by *Candida albicans* isolates involved in respiratory infections'. *Journal of Medical Microbiology,* 52(11) pp. 971-974.

Bostanci, N. and Belibasakis, G. N. (2012) '*Porphyromonas gingivali*s: an invasive and evasive opportunistic oral pathogen'. *FEMS Microbiology Letters,* 333(1) pp. 1-9.

Bounihi, A., Hajjaj, G., Alnamer, R., Cherrah, Y. and Zellou, A. (2013) '*In vivo* potential anti-inflammatory activity of *Melissa officinalis* L. essential oil'. *Advances in Pharmacological Sciences,* 2013, November, pp. 1-7.

Brading, M. G., Cromwell, V. J., Green, A. K., Debrabander, S. and Beasley, T. (2004) 'The role of triclosan in dentifrice formulations, with particular reference to a new 0.3 % Triclosan calcium carbonate-based system'. *International Dental Journal,* 54(4) pp. 291-298.

Bryant, S. J. and Anseth, K. S. (2001) 'The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels'. *Biomaterials,* 22(6) pp. 619-626.

Budzyńska, A., Sadowska, B., Więckowska-Szakiel, M. and Różalska, B. (2014) 'Enzymatic profile, adhesive and invasive properties of *Candida albicans* under the influence of selected plant essential oils '. *Acta Biochimica Polonica,* 61(1) pp. 115-121.

Burt, B. (2005) 'Position paper: epidemiology of periodontal diseases'. *Journal of periodontology,* 76(8) pp. 1406-1419.

Cabral, C. T. and Fernandes, M. H. (2007) '*In vitro* comparison of chlorhexidine and povidone – iodine on the long-term proliferation and functional activity of human alveolar bone cells'. *Clinical Oral Investigations,* 11(2) pp. 155-164.

Calderone, R. A. and Fonzi, W. A. (2001) 'Virulence factors of *Candida albicans*'. *Trends in Microbiology,* 9(7) pp. 327-335.

Campos, D., Piccirillo, C., Pullar, R. C., Castro, M. L. and Pintado, M. M. E. (2014) 'Characterization and antimicrobial properties of food packaging methylcellulose films containing stem extract of Ginja cherry'. *Journal of the Science of Food and Agriculture,* 24(10) pp. 2097-2103.

Campos, F. F., Capmany, A. C. C., Delgado, G. R., Serrano, O. L. and Naveros, B. C. (2011) 'Development and characterization of a novel nystatin-loaded nanoemulsion for the buccal treatment of candidosis: ultrastructural effects and release studies'. *International Journal of Drug Development and Research,* 101(10) pp. 3739-3752.

Cannas, S., Molicotti, P., Ruggeri, M., Cubeddu, M., Sanguinetti, M. and Zanetti, S. (2013) 'Antimycotic activity of *Myrtus communis* L. towards *Candida spp.* from clinical isolates'. *The Journal of Infection in Developing Countries,* 7(3) pp. 295-298.

Cardoso, N. N. R., Alviano, C. S., Blank, A. F., Romanos, M. T. V., Fonseca, B. B., Rozental, S., Rodrigues, I. A. and Alviano, D. S. (2016) 'Synergism effect of the essential oil from *Ocimum basilicum* var. Maria Bonita and its major components with fluconazole and its influence on ergosterol biosynthesis'. *Evidence-Based Complementary and Alternative Medicine,* 2016, (May), pp. 5647182.

Carvalhinho, S., Margarida, A. and Sampaio, A. (2012) 'Susceptibilities of *Candida albicans* mouth isolates to antifungal agents, essentials oils and mouth rinses'. *Mycopathologia,* 174(1) pp. 69-76.

Castro, R. D. D. and Lima, E. O. (2013) 'Anti-*Candida* activity and chemical composition of *Cinnamomum zeylanicum* blume essential oil'. *Brazilian Archives of Biology and Technology,* 56(5) pp. 749-755.

Cavalcanti, Y. W., Morse, D. J., Da Silva, W. J., Del-Bel-Cury, A. A., Wei, X., Wilson, M., Milward, P., Lewis, M., Bradshaw, D. and Williams, D. W. (2015) 'Virulence and pathogenicity of *Candida albicans* is enhanced in biofilms containing oral bacteria'. *Biofouling,* 31(1) pp. 27-38.

Chao, S. C., Young, D. G. and Oberg, C. J. (2000) 'Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses'. *Journal of Essential Oil Research,* 12(5) pp. 639-649.

Claxton, L., Taylor, M. and Kay, E. (2016) 'Oral health promotion: the economic benefits to the NHS of increased use of sugarfree gum in the UK'. *British Dental Journal,* 220(3) pp. 121-127.

Conti, H. R., Shen, F., Nayyar, N., Stocum, E., Sun, J. N., Lindemann, M. J., Ho, A. W., Hai, J. H., Yu, J. J., Jung, J. W., Filler, S. G., Masso-Welch, P., Edgerton, M. and Gaffen, S. L. (2009) 'Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis'. *The Journal of Experimental Medicine,* 206(2) pp. 299-311.

Cordenonsi, P., Filippi, C., Vendruscolo, T., Albertina, V., Rigon, C., Flores, C., Baggiotto, J., Christ, R. and Santos, V. (2013) 'Chlorhexidine activity against bacterial biofilms'. *American Journal of Infection Control,* 41(12) pp. 119-122.

Cortés, M. E., Bonilla, J. C. and Sinisterra, R. D. (2011) 'Biofilm formation, control and novel strategies for eradication'. *Science against microbial pathogens: communicating current research and technological advances,* 2 pp. 896-905.

Costa, E., Silva, S., Tavaria, F. and Pintado, M. (2014) 'Antimicrobial and antibiofilm activity of chitosan on the oral pathogen *Candida albicans*'. *Pathogens,* 3(4) pp. 908-919.

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. (1999) 'Bacterial biofilms: a common cause of persistent infections'. *Science,* 284(5418) pp. 1318-1322.

Cuenca-Estrella, M. (2004) 'Combinations of antifungal agents in therapy – what value are they?'. *Journal of Antimicrobial Chemotherapy,* 54, September, pp. 854-869.

Cullinan, M. P., Bird, P. S., Heng, N. C. K., West, M. J. and Seymour, G. J. (2014) 'No evidence of triclosan-resistant bacteria following long-term use of triclosan-containing toothpaste'. *Journal of Periodontal Research,* 49(2) pp. 220-5.

Cullinan, M. P., Westerman, B., Hamlet, S. M., Palmer, J. E., Faddy, M. J. and Seymour, G. J. (2003) 'The effect of a triclosan-containing dentifrice on the progression of periodontal disease in an adult population'. *Journal of Clinical Periodontology,* 30(5) pp. 414-9.

Curtis, M. A., Aduse-Opoku, J. and Rangarajan, M. (2001) 'Cysteine proteases of *Porphyromonas gingivalis*'. *Critical Reviews in Oral Biology & Medicine,* 12(3) pp. 192-216.

Dahlen, G. (1993) 'Role of suspected periodontopathogens in microbiological monitoring of periodontitis'. *Advances in Dental Research,* 7(2) pp. 163-174.

Dann, A. B. and Hontela, A. (2011) 'Triclosan: environmental exposure, toxicity and mechanisms of action'. *Journal of Applied Toxicology,* 31(4) pp. 285-311.

Davies, D. (2003) 'Understanding biofilm resistance to antibacterial agents'. *Nature Reviews Drug Discovery,* 2(2) pp. 114-122.

De Campos Rasteiro, V. M., Da Costa, A. C., Araújo, C. F., De Barros, P. P., Rossoni, R. D., Anbinder, A. L., Jorge, A. O. and Junqueira, J. C. E. (2014) 'Essential oil of *Melaleuca alternifolia* for the treatment of oral candidiasis induced in an immunosuppressed mouse model'. *BMC Complementary and Alternative Medicine,* 14, December, pp. 489-499.

De Cássia Da Silveira E Sá, R., Andrade, L. N. and De Sousa, D. P. (2013) 'A review on anti-inflammatory activity of monoterpenes'. *Molecules,* 18(1) pp. 1227-1254.
Deas, D. E., Moritz, A. J., Sagun, R. S., Gruwell, S. F. and Powell, C. A. (2016) 'Scaling and root planing vs. conservative surgery in the treatment of chronic periodontitis'. *Periodontology 2000,* 71(1) pp. 128-139.
Deorukhkar, S. C. (2017) 'Immunity to *Candida* infection: an overview'. *Journal of Immunology,* 5(1) pp. 1-4.
Dhillon, G. S., Kaur, S., Pulicharla, R., Brar, S. K., Cledón, M., Verma, M. and Surampalli, R. Y. (2015) 'Triclosan: current status, occurrence, environmental risks and bioaccumulation potential'. *International Journal of Environmental Research and Public Health,* 12(5) pp. 5657-5684.
Didry, N., Dubreuil, L. and Pinkas, M. (1994) 'Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria'. *Pharmaceutica Acta Helvetiae,* 69(1) pp. 25-28.
Diniz, I. M. A., Chen, C., Xu, X., Ansari, S., Zadeh, H. H., Marques, M. M., Shi, S. and Moshaverinia, A. (2015) 'Pluronic F-127 hydrogel as a promising scaffold for encapsulation of dental-derived mesenchymal stem cells'. *Journal of Materials Science: Materials in Medicine,* 26(3) pp. 1-10.
Dongari-Bagtzoglou, A., Wen, K. and Lamster, I. B. (1999) '*Candida albicans* triggers interleukin-6 and interleukin-8 responses by oral fibroblasts *in vitro*'. *Oral Microbiology and Immunology,* 14(6) pp. 364-370.
Donnell, G. M. C. and Russell, A. D. (1999) 'Antiseptics and disinfectants: activity, action, and resistance'. *Clinical Microbiology Reviews,* 12(1) pp. 147-179.
Douglas, L. J. (2003) '*Candida* biofilms and their role in infection'. *Trends in Microbiology,* 11(1) pp. 30-36.
Drisko, C. H. (2001) 'Nonsurgical periodontal therapy'. *Periodontology 2000,* 25(1) pp. 77-88.
Dunne, W. M. (2002) 'Bacterial adhesion: seen any good biofilms lately?'. *Clinical Microbiology Reviews,* 15(2) pp. 155-166.
Eley, B. M. and Cox, S. W. (2003) 'Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defenses and tissues and detection in gingival crevice'. *Periodontology 2000,* 31, January, pp. 105-124.
Elisseeff, J., Mcintosh, W., Anseth, K. S., Riley, S., Ragan, P. and Langer, R. (2000) 'Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks'. *Journal of Biomedical Materials Research,* 51(2) pp. 164-171.
Ellepola, A. N. B. and Samaranayake, L. P. (2001) 'Adjunctive use of chlorhexidine in oral candidoses'. *Oral Diseases,* 7(1) pp. 11-17.
Faleiro, M. L. (2011) *'*The mode of antibacterial action of essential oils*'. In:* Mendez-Vilas, A. (ed.) *Science against microbial pathogens: communicating current research and technological advances.* Formatex Research Center.
Fani, M. M., Kohanteb, J. and Araghizadeh, A. (2014) 'Inhibitory activity of *Myrtus communis* oil on some clinically isolated oral pathogens'. *Medical Principles and Practice,* 23(4) pp. 363-368.
Farah, C. S., Ashman, R. B. and Challacombe, S. J. (2000) 'Oral Candidosis'. *Clinics in Dermatology,* 18(5) pp. 553-562.

Felix Lanao, R. P., Jonker, A. M., Wolke, J. G., Jansen, J. A., Van Hest, J. C. and Leeuwenburgh, S. C. (2013) 'Physicochemical properties and applications of poly(lactic-co-glycolic acid) for use in bone regeneration'. *Tissue Engineering Part B Reviews* 19(4) pp. 380-90.

Ferhout, H., Bohatier, J., Guillot, J. and Chalchat, C. (1999) 'Antifungal activity of selected essential oils, cinnamaldehyde and carvacrol against malassezia furfur and *Candida albicans*'. *Journal of Essential Oil Research,* 11(1) pp. 119-129.

Filoche, S. K., Soma, K. and Sissons, C. H. (2005) 'Antimicrobial effects of essential oils in combination with chlorhexidine digluconate'. *Oral Microbiology and Immunology,* 20(4) pp. 221-225.

Fine, D. H., Furgang, D., Markowitz, K., Sreenivasan, P. K., Klimpel, K. and De Vizio, W. (2006) 'The antimicrobial effect of a triclosan/copolymer dentifrice on oral microorganisms in vivo'. *Journal of the American Dental Association* 137(10) pp. 1406-1413.

Finney, M., Walker, J. T., Marsh, P. D. and Brading, M. G. (2003) 'Antimicrobial effects of a novel Triclosan/zinc citrate dentifrice against mixed culture oral biofilms'. *International dental journal,* 53(6) pp. 371-378.

Fitzgerald, K. A., Davies, A. and Russell, A. D. (1989) 'Uptake of 4C-Chlorhexidine diacetate to *Escherichia coli* and *Pseudomonas aeruginosa* and its release by azolectin'. *FEMS Microbiology Letters,* 60(3) pp. 327-332.

Flemming, H.-C. and Wingender, J. (2010) 'The biofilm matrix'. *Nature Reviews Microbiology,* 8(9) pp. 623-633.

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A. and Kjelleberg, S. (2016) 'Biofilms: an emergent form of bacterial life'. *Nature Reviews Microbiology,* 14(9) pp. 563-575.

Fosse, T., Madinier, I., Hitzig, C. and Y., C. (1999) 'Prevalence of b-lactamase-producing strains among 149 anaerobic Gram-negative rods isolated from periodontal pockets'. *Oral Microbiology and Immunology,* 14(6) pp. 352-357.

Fotakis, G. and Timbrell, J. A. (2006) '*In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride'. *Toxicology Letters,* 160(2) pp. 171-177.

Fukushima, C., Matsuse, H., Tomari, S., Obase, Y., Miyazaki, Y., Shimoda, T. and Kohno, S. (2003) 'Oral candidiasis associated with inhaled corticosteroid use: comparison of fluticasone and beclomethasone'. *Annals of Allergy, Asthma and Immunology,* 90(6) pp. 646-651.

Gaffar, A., Scherl, D., Afflitto, J. and Coleman, E. J. (1995) 'The effect of triclosan on mediators of gingival inflammation'. *Journal of Clinical Periodontology,* 22(6) pp. 480-485.

Gafitanu, C. A., Filip, D., Cernatescu, C., Rusu, D., Tuchilus, C. G., Macocinschi, D. and Zaltariov, M.-F. (2017) 'Design, preparation and evaluation of HPMC-based PAA or SA freeze-dried scaffolds for vaginal delivery of fluconazole'. *Pharmaceutical Research,* 34(10) pp. 2185-2196.

Genco, R. J. and Borgnakke, W. S. (2013) 'Risk factors for periodontal disease'. *Periodontology 2000,* 62(1) pp. 59-94.

Gholizadeh, P., Pormohammad, A., Eslami, H., Shokouhi, B., Fakhrzadeh, V. and Kafil, H. S. (2017) 'Oral pathogenesis of *Aggregatibacter*

actinomycetemcomitans'. *Microbial Pathogenesis,* 113, December, pp. 303-311.
Giordani, R., Regli, P., Kaloustian, J., Mikaïl, C., Abou, L. and Portugal, H. (2004) 'Antifungal effect of various essential oils against *Candida albicans*. Potentiation of antifungal action of amphotericin B by essential oil from *Thymus vulgaris*'. *Phytotherapy Research,* 18(12) pp. 990-995.
Gleiznys, A., Zdanavičienė, E. and Žilinskas, J. (2015) '*Candida albicans* importance to denture wearers. A literature review'. *Stomatologija,* 17(2) pp. 54-66.
Glowacki, J. and Mizuno, S. (2008) 'Collagen scaffolds for tissue engineering'. *Biopolymers,* 89(5) pp. 338-344.
Gow, N. A. (1997) 'Germ tube growth of *Candida albicans*'. *Current Topics in Medical Mycology,* 8(1-2) pp. 43-55.
Gozalbo, D., Maneu, V. and Gil, M. L. (2014) 'Role of IFN-gamma in immune responses to *Candida albicans* infections'. *Frontiers in Bioscience,* 19, June, pp. 1279-1290.
Greenstein, G., Berman, C. and Jaffin, R. (1986) 'Chlorhexidine. An adjunct to periodontal therapy'. *The Journal of periodontology,* 57(6) pp. 370-377.
Gromadzkim, S. 2017. *Periodontal disease, pyorrhea, gum disease, periodontitis, bad breath, halitosis* [Online]. Available: http://www.fullhealthsecrets.com/diseases/periodontal-disease-pyorrhea-gum-disease-periodontitis-bad-breath-halitosis/ [Accessed].
Gulrez, S. K. H., Al-Assaf, S. and Phillips, G. O. (2011) *'*Hydrogels: methods of preparation, characterisation and applications*'. In:* Carpi, A. (ed.) *Progress in molecular and environmental bioengineering.* IntechOpen.
Gulsen, D. and Chauhan, A. (2005) 'Dispersion of microemulsion drops in HEMA hydrogel: a potential ophthalmic drug delivery vehicle'. *International Journal of Pharmaceutics,* 292(1-2) pp. 95-117.
Gupta, P., Sarkar, S., Das, B., Bhattacharjee, S. and Tribedi, P. (2016) 'Biofilm, pathogenesis and prevention—a journey to break the wall: a review'. *Archives of Microbiology,* 198(1) pp. 1-15.
Gursoy, U. K., Gursoy, M., Gursoy, O. V., Cakmakci, L., Könönen, E. and Uitto, V. J. (2009) 'Anti-biofilm properties of *Satureja hortensis* L. essential oil against periodontal pathogens'. *Anaerobe,* 15(4) pp. 164-167.
Gyawali, R. and Ibrahim, S. A. (2014) 'Natural products as antimicrobial agents'. *Food Control,* 46, December, pp. 412-429.
Hajishengallis, G. (2015) 'Periodontitis: from microbial immune subversion to systemic inflammation'. *Nature Reviews Immunology,* 15(1) pp. 30-44.
Hammer, K. A., Carson, C. F. and Riley, T. V. (1998) '*In vitro* activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against *Candida* spp.'. *Journal of Antimicrobial Chemotherapy,* 42(5) pp. 591-595.
Hammer, K. A., Carson, C. F. and Riley, T. V. (1999) 'Antimicrobial activity of essential oils and other plant extracts'. *Journal of Applied Microbiology,* 86(6) pp. 985-990.
Han, Y. W. (2015) '*Fusobacterium nucleatum*: a commensal-turned pathogen'. *Current Opinion in Microbiology,* 23, February, pp. 141-147.
Han, Y. W., Shi, W., Huang, G. T., Haake, S. K., Park, N.-H., Kuramitsu, H. and Genco, R. J. (2000) 'Interactions between periodontal bacteria and human oral epithelial cells: *Fusobacterium nucleatum* adheres to and invades epithelial cells.'. *Infection and Immunity,* 68(6) pp. 3140-3146.

Han, Y. W. and Wang, X. (2013) 'Mobile microbiome: oral bacteria in extra-oral infections and inflammation'. *Journal of Dental Research,* 92(6) pp. 485-491.
Harris, R., Nicoll, A. D., Adair, P. M. and Pine, C. M. (2004) 'Risk factors for dental caries in young children: a systematic review of the literature'. *Community Dental Health Journal,* 21(1) pp. 71-85.
Harrison, J. J., Turner, R. J. and Ceri, H. (2005) 'Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa*'. *Environmental Microbiology,* 7(7) pp. 981-994.
Hasan, A. and Palmer, R. M. (2014) 'A clinical guide to periodontology: pathology of periodontal disease'. *British Dental Journal,* 216(8) pp. 457-61.
Hawser, S. P. and Douglas, L. J. (1995) 'Resistance of *Candida albicans* biofilms to antifungal agents *in vitro*'. *Antimicrobial Agents and Chemotherapy,* 39(9) pp. 2128-2131.
Haynes, K. (2001) 'Virulence in *Candida* species'. *Trends in Microbiology,* 9(12) pp. 591-596.
Hedayati, A., Khosropanah, H., Bazargani, A. and Abed, M. (2013) 'Assessing the antimicrobial effect of the essential oil of *Myrtus communis* on the clinical isolates of P*orphyromonas gingivalis*: an *in vitro* study'. *Jundishapur Journal of Natural Pharmaceutical Products,* 8(4) pp. 165-168.
Hendry, E. R., Worthington, T., Conway, B. R. and Lambert, P. A. (2009) 'Antimicrobial efficacy of eucalyptus oil and 1,8-cineole alone and in combination with chlorhexidine digluconate against microorganisms grown in planktonic and biofilm cultures'. *Journal of Antimicrobial Chemotherapy,* 64(6) pp. 1219-1225.
Herath, T. D. K., Wang, Y., Seneviratne, C. J., Darveau, R. P., Wang, C. Y. and Jin, L. J. (2011) 'Heterogeneous LPS of *Porphyromonas gingivalis* differentially modulate the innate immune response of human gingiva'. *BMC Proceedings,* 5(1) pp. P86.
Hernandez, R. and Rupp, S. (2009) 'Human epithelial model systems for the study of *Candida* infections *in vitro*: part II. Histologic methods for studying fungal invasion.'. *In:* Rupp, S. & Sohn, K. (eds.) *Methods in molecular biology.* Humana Press.
Hesse, E., Hefferan, T. E., Tarara, J. E., Haasper, C., Meller, R., Krettek, C., Lu, L. and Yaszemski, M. J. (2011) 'Collagen type I hydrogel allows migration, proliferation and osteogenic differentiation of rat bone marrow stromal cells'. *Journal of Biomedical Materials Research,* 94(2) pp. 442-449.
Hienz, S. A., Paliwal, S. and Ivanovski, S. (2015) 'Mechanisms of bone resorption in periodontitis'. *Journal of Immunology Research,* 2015, May, pp. 1-10.
Higgins, J., Pinjon, E., Oltean, H. N., White, T. C., Kelly, S. L., Martel, C. M., Sullivan, D. J., Coleman, D. C. and Moran, G. P. (2012) 'Triclosan antagonizes fluconazole activity against *Candida albicans*'. *Journal of Dental Research,* 91(1) pp. 65-70.
Horváth, G., Jenei, J. T., Vágvölgyi, C., Böszörményi, A. and Krisch, J. (2016) 'Effects of essential oil combinations on pathogenic yeasts and moulds '. *Acta Biologica Hungarica,* 67(2) pp. 205-214.

How, K. Y., Song, K. P., Chan, K. G. and Caldwell, C. C. (2016) '*Porphyromonas gingivalis* : an overview of periodontopathic pathogen below the gum line'. *Frontiers in Microbiology,* 7(53) pp. 1-14.
Hsieh, M. H., Yu, C. M., Yu, V. L. and Chow, J. W. (1993) 'Synergy assessed by checkerboard. A critical analysis'. *Diagnostic Microbiology and Infectious Disease,* 16(4) pp. 343-349.
Hsu, C.-C., Lai, W.-L., Chuang, K.-C., Lee, M.-H. and Tsai, Y.-C. (2013) 'The inhibitory activity of linalool against the filamentous growth and biofilm formation in *Candida albicans*'. *Medical Mycology,* 51(5) pp. 473-482.
Huang, G. (2012) 'Regulation of phenotypic transitions in the fungal pathogen *Candida albicans*'. *Virulence,* 3(3) pp. 251-261.
Hube, B. and Naglik, J. (2001) '*Candida albicans* proteinases: resolving the mystery of a gene family'. *Microbiology,* 147(8) pp. 1997-2005.
Hugo, W. B. and Longworth, A. R. (1964) 'Some aspects of the mode of action of chlorhexidine'. *Journal of Pharmacy and Pharmacology,* 16, October, pp. 655-662.
Hugo, W. B. and Longworth, A. R. (1965) 'Cytological aspects of the mode of action of chlorhexidine diacetate'. *Journal of Pharmacy and Pharmacology,* 17(1) pp. 28-32.
Iauk, L., Lo Bue, A. M., Milazzo, I., Rapisarda, A. and Blandino, G. (2003) 'Antibacterial activity of medicinal plant extracts against periodontopathic bacteria'. *Phytotherapy Research,* 17(6) pp. 599-604.
Iki, K., Kawahara, K., Sawamura, S., Arakaki, R., Sakuta, T., Sugiyama, A., Tamura, H., Sueda, T., Hamada, S. and Takada, H. (1997) 'A novel component different from endotoxin extracted from *Prevotella intermedia* ATCC 25611 activates lymphoid cells from C3H/HeJ mice and gingival fibroblasts from humans'. *Infection and Immunity,* 65(11) pp. 4531-4538.
Ikinci, G., Şenel, S., Akincibay, H., Kaş, S., Erciş, S., Wilson, C. G. and Hincal, A. A. (2002) 'Effect of chitosan on a periodontal pathogen *Porphyromonas gingivalis*'. *International Journal of Pharmaceutics,* 235(1-2) pp. 121-127.
Janssen, A. M., Scheffer, J. C. and Baerheim Svendsen, A. (1986) 'Antimicrobial activity of essential oils: a 1976-1986 literature review. Aspects of the test methods'. *Planta Medica,* 53(3) pp. 395-398.
Jayatilake, J. a. M. S., Samaranayake, Y. H., Cheung, L. K. and Samaranayake, L. P. (2006) 'Quantitative evaluation of tissue invasion by wild type, hyphal and SAP *mutants* of *Candida albicans*, and non-albicans *Candida* species in reconstituted human oral epithelium'. *Journal of Oral Pathology and Medicine,* 35(8) pp. 484-491.
Ji, Q. X., Zhao, Q. S., Deng, J. and Lü, R. (2010) 'A novel injectable chlorhexidine thermosensitive hydrogel for periodontal application: preparation, antibacterial activity and toxicity evaluation'. *Journal of Materials Science: Materials in Medicine,* 21(8) pp. 2435-2442.
Josefowicz, S. Z., Lu, L.-F. and Rudensky, A. Y. (2012) 'Regulatory T cells: mechanisms of differentiation and function'. *Annual Review of Immunology,* 30, July, pp. 531-564.
Ka, H., Park, H.-J., Jung, H.-J., Choi, J.-W., Cho, K.-S., Ha, J. and Lee, K.-T. (2003) 'Cinnamaldehyde induces apoptosis by ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells'. *Cancer Letters,* 196(2) pp. 143-152.

Kanafani, Z. A. and Perfect, J. R. (2008) 'Resistance to antifungal agents: mechanisms and clinical impact'. *Clinical Infectious Diseases,* 46(1) pp. 120-128.

Karpanen, T. J., Worthington, T., Hendry, E. R., Conway, B. R. and Lambert, P. A. (2008) 'Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*'. *Journal of Antimicrobial Chemotherapy,* 62(5) pp. 1031-1036.

Kato, H., Taguchi, Y., Tominaga, K. and Umeda, M. (2013) '*Porphyromonas gingivalis* LPS inhibits osteoblastic differentiation and promotes pro-inflammatory cytokine production in human periodontal ligament stem cells'. *Archives of Oral Biology,* 59(2) pp. 167-175.

Kavanaugh, N. L., Zhang, A. Q., Nobile, C. J., Johnson, A. D. and Ribbeck, K. (2014) 'Mucins suppress virulence traits of *Candida albicans*'. *mBio,* 5(6) pp. 1-8.

Kennedy, M. J., Rogers, A. L., Hanselmen, L. R., Soll, D. R. and Yancey, R. J. (1988) 'Variation in adhesion and cell surface hydrophobicity in *Candida albicans* white and opaque phenotypes'. *Mycopathologia,* 102(3) pp. 149-156.

Khan, A., Ahmad, A., Akhtar, F., Yousuf, S., Xess, I., Khan, L. A. and Manzoor, N. (2010) '*Ocimum sanctum* essential oil and its active principles exert their antifungal activity by disrupting ergosterol biosynthesis and membrane integrity'. *Research in Microbiologoy,* 161(10) pp. 816-823.

Khor, E. and Lim, L. Y. (2003) 'Implantable applications of chitin and chitosan'. *Biomaterials,* 24(13) pp. 2339-2349.

Kim, H., Jeong, H., Han, S., Beack, S., Hwang, B. W., Shin, M., Oh, S. S. and Hahn, S. K. (2017) 'Hyaluronate and its derivatives for customized biomedical applications'. *Biomaterials,* 123, April, pp. 155-171.

Kim, H. S., Choi, E. H., Khan, J., Roilides, E., Francesconi, A., Kasai, M., Sein, T., Schaufele, R. L., Sakurai, K., Son, C. G., Greer, B. T., Chanock, S., Lyman, C. A. and Walsh, T. J. (2005) 'Expression of genes encoding innate host defense molecules in normal human monocytes in response to *Candida albicans*'. *Infection and Immunity,* 73(6) pp. 3714-3724.

Kim, J. K., Kim, H. J., Chung, J. Y., Lee, J. H., Young, S. B. and Kim, Y. H. (2014) 'Natural and synthetic biomaterials for controlled drug delivery'. *Archives of Pharmacal Research,* 37(1) pp. 60-68.

Kim, M. H., Park, H., Nam, H. C., Park, S. R., Jung, J. Y. and Park, W. H. (2018) 'Injectable methylcellulose hydrogel containing silver oxide nanoparticles for burn wound healing'. *Carbohydrate Polymers,* 181, February, pp. 579-586.

Kiyoura, Y. and Tamai, R. (2015) 'Innate immunity to *Candida albicans*'. *Japanese Dental Science Review,* 51(3) pp. 59-64.

Klouda, L. (2015) 'Thermoresponsive hydrogels in biomedical applications: a seven-year update'. *European Journal of Pharmaceutics and Biopharmaceutics,* 97(B) pp. 338-349.

Kodadova, A., Vitkova, Z., Herdova, P., Oremusova, J., Kodadova, A. and Mikus, P. (2014) 'Formulation of sage essential oil (*Salvia officinalis*, L.) monoterpenes into chitosan hydrogels and permeation study with GC-MS analysis'. *Drug Development and Industrial Pharmacy* 41(7) pp. 1080-1088.

Kolambkar, Y. M., Dupont, K. M., Boerckel, J. D., Huebsch, N., Mooney, D. J., Hutmacher, D. W. and Guldberg, R. E. (2012) 'An alginate-based hybrid system for growth factor delivery in the functional repair of large bone defects'. *Biomaterials,* 32(1) pp. 65-74.

Kolotila, M. P. and Diamond, R. D. (1990) 'Effects of neutrophils and *in vitro* oxidants on survival and phenotypic switching of *Candida albicans* WO-1'. *Infection and Immunity,* 58(5) pp. 1174-1179.

Kong, E. F., Tsui, C., Boyce, H., Ibrahim, A., Hoag, S. W., Karlsson, A. J., Meiller, T. F. and Jabra-Rizk, M. A. (2016) 'Development and *in vivo* evaluation of a novel Histatin-5 bioadhesive hydrogel formulation against oral candidiasis'. *Antimicrobial Agents and Chemotherapy,* 60(2) pp. 881-889.

Kontturi, L. S., Järvinen, E., Muhonen, V., Collin, E. C., Pandit, A. S., Kiviranta, I., Yliperttula, M. and Urtti, A. (2014) 'An injectable, *in situ* forming type II collagen/hyaluronic acid hydrogel vehicle for chondrocyte delivery in cartilage tissue engineering'. *Drug Delivery and Translational Research,* 4(2) pp. 149-158.

Kraivaphan, P., Amornchat, C. and Maneepitsamai, Y. (2013) 'Bactericidal effects of three mint essential oils on *Porphyromonas gingivalis* in planktonic and biofilm cells'. *Research Journal of Medicinal Plants,* 7(2) pp. 100-106.

Krogh, P., Hald, B. and Holmstrup, P. (1987) 'Possible mycological etiology of oral mucosal cancer: catalytic potential of infecting *Candida albicans* and other yeasts in production of N-nitrosobenzylmethylamine.'. *Carcinogenesis,* 8(10) pp. 1543-1548.

Krol, D. M. (2003) 'Dental caries, oral health, and pediatricians'. *Current Problems in Pediatric and Adolescent Health Care,* 33(8) pp. 253-270.

Krzysciak, W., Jurczak, A., Koscielniak, D., Bystrowska, B. and Skalniak, A. (2014) 'The virulence of *Streptococcus mutans* and the ability to form biofilms'. *European Journal of Clinical Microbiology & Infectious Diseases,* 33(4) pp. 499-515.

Labriola, A., Needleman, I. and Moles, D. R. (2005) 'Systematic review of the effect of smoking on nonsurgical periodontal therapy'. *Periodontology 2000,* 37(1) pp. 124-137.

Lainel, M. L., Appelmelk, B. J. and Winkelhoffl, A. J. V. (1997) 'Prevalence and distribution of six capsular serotypes of *Porphyromonas gingivalis* in periodontitis patients'. *Journal of Dental Research,* 76(12) pp. 1840-1844.

Lana, M. A., Ribeiro-Sobrinho, A. P., Stehling, R., Garcia, G. D., Silva, B. K., Hamdan, J. S., Carvalho, M. A., Nicoli, J. R. and Farias, L. M. (2001) 'Microorganisms isolated from root canals presenting necrotic pulp and their drug susceptibility *in vitro*'. *Oral Microbiology and Immunology,* 16(2) pp. 100-105.

Langer, R. and Vacanti, J. P. (1993) 'Tissue Engineering'. *Science,* 260(5110) pp. 920-926.

Laurenti, M. and Abdallah, M.-N. (2015) 'Natural and synthetic hydrogels for periodontal tissue regeneration'. *International Dental Journal of Student Research,* 3(2) pp. 49-51.

Law, D., Moore, C. B., Wardle, H. M., Ganguli, L. A., Keaney, M. G. L. and Denning, D. W. (1994) 'High prevalence of antifungal resistance in

Candida spp. from patients with AIDS'. *Journal of Antimicrobial Chemotherapy,* 34(5) pp. 659-668.
Lawrence, M. and Jiang, Y. (2017) 'Porosity, pore size distribution, micro-structure'. *Bio-aggregates Based Building Materials* Springer.
Lee, K. Y. and Mooney, D. J. (2001) 'Hydrogels for tissue engineering'. *Chemical Reviews* 101(7) pp. 1869-1879.
Lee, K. Y. and Mooney, D. J. (2012) 'Alginate: properties and biomedical applications'. *Progress in Polymer Science* 37(1) pp. 106-126.
Li, J. and Mooney, D. J. (2016) 'Designing hydrogels for controlled drug delivery'. *Nature Reviews Materials,* 1(12) pp. 1-18.
Li, L., Thangamathesvaran, P. M., Yue, C. Y., Tam, K. C., Hu, X. and Lam, Y. C. (2001) 'Gel network structure of methylcellulose in water'. *Langmuir,* 17(26) pp. 8062-8068.
Li, Y.-C., Kuan, Y.-H., Lee, S.-S., Huang, F.-M. and Chang, Y.-C. (2012) 'Cytotoxicity and genotoxicity of chlorhexidine on macrophages *in vitro*'. *Environmental Toxicology,* 29(4) pp. 452-458.
Liljemark, W. F. and Bloomquist, C. (1996) 'Human oral microbial ecology and dental caries and periodontal diseases'. *Critical Reviews in Oral Biology & Medicine,* 7(2) pp. 180-198.
Linnes, M., Ratner, B. D. and Giachelli, C. M. (2007) 'A fibrinogen-based precision microporous scaffold for tissue engineering'. *Biomaterials,* 28(35) pp. 5298-5306.
Listl, S., Galloway, J., Mossey, P. A. and Marcenes, W. (2015) 'Global economic impact of dental diseases'. *Journal of Dental Research,* 94(10) pp. 1355-1361.
Liu, M., Zeng, X., Ma, C., Yi, H., Ali, Z., Mou, X., Li, S., Deng, Y. and He, N. (2017) 'Injectable hydrogels for cartilage and bone tissue engineering'. *Bone Research,* 5, May, pp. 17014.
Lobo, M. S. and Costa, P. (2001) 'Modeling and comparison of dissolution profiles ''. *European Journal of Pharmaceutical Sciences* 13(2) pp. 123-133.
Loesche, W. J. (1976) 'Chemotherapy of dental plaque infections'. *Oral Science Reviews* 9pp. 65-107.
Longitudinal, S., Structural, M. and Models, W. (2015) 'HHS Public Access'. 2 pp. 147-185.
Low, W. L., Kenward, M. a. K., Cairul, M., Mohd, I. and Martin, C. (2016) 'Ionically crosslinked chitosan hydrogels for the controlled release of antimicrobial essential oils and metal ions for wound management applications'. *Medicines (Basel)* 1(3) pp. E8.
Luo, S., Skerka, C., Kurzai, O. and Zipfel, P. F. (2013) 'Complement and innate immune evasion strategies of the human pathogenic fungus *Candida albicans*'. *Molecular Immunology,* 56(3) pp. 161-169.
Macià, M. D., Rojo-Molinero, E. and Oliver, A. (2014) 'Antimicrobial susceptibility testing in biofilm-growing bacteria'. *Clinical Microbiology and Infection,* 20(10) pp. 981-990.
Madinier, I. M., Fosse, T. B. and Hitzig, C. (1999) 'Resistance profile survey of 50 periodontal strains of *Actinobacillus actinomycetemcomitans*'. *Journal of Periodontology,* 70(8) pp. 888-892.
Mahboubi, M. and Bidgoli, F. G. (2010) '*In vitro* synergistic efficacy of combination of amphotericin B with *Myrtus communis* essential oil

against clinical isolates of *Candida albicans*'. *Phytomedicine,* 17(10) pp. 771-774.
Maitra, J. and Shukla, V. K. (2014) 'Cross-linking in hydrogels - A review'. *American Journal of Polymer Science,* 4(2) pp. 25-31.
Maity, P., Moin, A., Gowda, D. V. and Osmani, R. a. M. (2016) 'Ophthalmic drug delivery by contact lenses'. *Journal of Chemical and Pharmaceutical Research,* 8(2) pp. 644-651.
Malic, S., Emanuel, C., Lewis, M. A. and Williams, D. W. (2013) 'Antimicrobial activity of novel mouthrinses against planktonic cells and biofilms of pathogenic microorganisms'. *Microbiology Discovery,* 1(11) pp. 1-5.
Malic, S., Hill, K. E., Ralphs, J. R., Hayes, A., Thomas, D. W., Potts, A. J. and Williams, D. W. (2007) 'Characterization of *Candida albicans* infection of an in vitro oral epithelial model using confocal laser scanning microscopy'. *Oral Microbiology and Immunology,* 22(3) pp. 188-194.
Mann, C. M. and Markham, J. L. (1998) 'A new method for determining the minimum inhibitory concentration of essential oils'. *Journal of Applied Microbiology,* 84(4) pp. 538-544.
Marcos-Arias, C., Eraso, E., Madariaga, L. and Quindós, G. (2011) '*In vitro* activities of natural products against oral *Candida* isolates from denture wearers'. *BMC Complementary and Alternative Medicine* 11, (November), pp. 119.
Marcotte, H. and Lavoie, M. C. (1998) 'Oral microbial ecology and the role of salivary immunoglobulin A'. *Microbiology and molecular biology reviews* 62(1) pp. 71-109.
Marsh, P. D. (1991) 'Sugar, fluoride, pH and microbial homeostasis in dental plaque'. *Proceedings of the Finnish Dental Society,* 87(4) pp. 515-525.
Marsh, P. D. (1994) 'Microbial ecology of dental plaque and its significance in health and disease'. *Advances in Dental Research,* 8(2) pp. 263-271.
Marsh, P. D. and Lewis, M. V. (2009) *Oral Microbiology* 5th Edition. Churchill Livingstone Elsevier.
Masiá, C. M. and Gutiérrez, R. F. (2002) 'Antifungal drug resistance to azoles and polyenes'. *The Lancet Infectious Diseases,* 2(9) pp. 550-563.
Matesanz, P., Herrera, D., Echeverría, A., O'connor, A., González, I. and Sanz, M. (2013) 'A randomized clinical trial on the clinical and microbiological efficacy of a xanthan gel with chlorhexidine for subgingival use'. *Clinical Oral Investigations,* 17(1) pp. 55-66.
Mathur, V. P. and Dhillon, J. K. (2018) 'Dental caries: a disease which needs attention'. *Indian Journal of Pediatrics,* 85(3) pp. 202-206.
Mayer, F. L., Wilson, D. and Hube, B. (2013) '*Candida albicans* pathogenicity mechanisms'. *Virulence,* 4(2) pp. 119-128.
Mcbain, A. J. (2009) 'Chapter 4 - *In vitro* biofilm models: an overview'. *Advances in Applied Microbiology,* 69 pp. 99-132.
Meyvis, T. K. L., Smedt, S. C. D. and Demeester, J. (2000) 'Influence of the degradation mechanism of hydrogels on their elastic and swelling properties during degradation'. *Macromolecular,* 33(13) pp. 4717-4725.
Mimica-Dukic, N., Bozin, B., Sokovic, M. and Simin, N. (2004) 'Antimicrobial and antioxidant activities of *Melissa officinalis* L. (*Lamiaceae*) essential oil'. *Journal of Agricultural and Food Chemistry,* 52(9) pp. 2485-2489.
Montecucco, F., Bertolotto, M., Ottonello, L., Pende, A., Dapino, P., Quercioli, A., Mach, F. and Dallegri, F. (2009) 'Chlorhexidine prevents

hypochlorous acid-induced inactivation of α1-antitrypsin'. *Clinical and Experimental Pharmacology and Physiology,* 36(11) pp. 72-77.
Morais-Braga, M. F. B., Sales, D. L., Carneiro, J. N. P., Machado, J. T., Thassya, A., Santos, L., Freitas, M. a. D., Matos, D., Tintino, S. R., Souza, D. S. L., Menezes, I. R. A., Ribeiro, J., Costa, J. G. M. and Coutinho, H. D. M. (2016) '*Psidium guajava* L. and *Psidium brownianum* Mart *ex* DC.: chemical composition and anti–*candida* effect in association with fluconazole'. *Microbial Pathogenesis,* 95, June, pp. 200-207.
Morton, W. E. and Hearle, J. W. S. (2008) *'*Rheology*'. In:* Morton, W. E. (ed.) *Physical properties of textile fibres.* Woodhead Publishing.
Moshaverinia, A., Chen, C., Akiyama, K., Ansari, S., Xu, X., Chee, W. W., Schricker, S. R. and Shi, S. (2012) 'Alginate hydrogel as a promising scaffold for dental-derived stem cells: an *in vitro* study'. *Journal of Materials Science: Materials in Medicine,* 23(12) pp. 3041-3051.
Mostefaoui, Y., Bart, C., Frenette, M. and Rouabhia, M. (2004) '*Candida albicans* and *Streptococcus salivarius* modulate IL-6, IL-8, and TNF-alpha expression and secretion by engineered human oral mucosa cells'. *Cellular Microbiology,* 6(11) pp. 1085-1096.
Moynihan, P. and Petersen, P. E. (2004) 'Diet, nutrition and the prevention of dental diseases'. *Public Health Nutrition,* 7(1A) pp. 201-226.
Murata, H. (2012) *'*Rheology - Theory and application to biomaterials*'. In:* Gomes, A. D. S. (ed.) *Polymerization.* IntechOpen.
Murray, P. E., Lumley, P. J., Ross, H. F. and Smith, A. J. (2000) 'Tooth slice organ culture for cytotoxicity assessment of dental materials'. *Biomaterials,* 21(16) pp. 1711-1721.
Muzyka, B. C. (2005) 'Oral fungal infections'. *Dental Clinics of North America,* 49(1) pp. 49-65.
Naahidi, S., Jafari, M., Logan, M., Wang, Y., Yuan, Y., Bae, H., Dixon, B. and Chen, P. (2017) 'Biocompatibility of hydrogel-based scaffolds for tissue engineering applications'. *Biotechnology Advances,* 35(5) pp. 530-544.
Nacsa-Farkas, E., Kerekes, E., Kerekes, E. B., Krisch, J., Roxana, P., Vlad, D. C., Ivan, P. and Vágvölgyi, C. (2014) 'Antifungal effect of selected European herbs against *Candida albicans* and emerging pathogenic non-albicans *Candida* species'. *Acta Biologica Szegediensis,* 58(1) pp. 61-64.
Naglik, J., Albrecht, A., Bader, O. and Hube, B. (2004) '*Candida albicans* proteinases and host/pathogen interactions'. *Celluar Microbiology,* 6(10) pp. 915-26.
Narayanan, G., Vernekar, V. N., Kuyinu, E. L. and Laurencin, C. T. (2016) 'Poly(lactic acid)-based biomaterials for orthopaedic regenerative engineering'. *Adv Drug Deliv Rev,* 107, December, pp. 247-276.
Nasatto, P. L., Pignon, F., Silveira, J. L. M., Duarte, M. E. R., Noseda, M. D. and Rinaudo, M. (2015a) 'Influence of molar mass and concentration on the thermogelation of methylcelluloses'. *International Journal of Polymer Analysis and Characterization,* 20(2) pp. 110-118.
Nasatto, P. L., Pignon, F., Silveira, J. L. M., Duarte, M. E. R., Noseda, M. D. and Rinaudo, M. (2015b) 'Methylcellulose, a cellulose derivative with original physical properties and extended applications'. *Polymers,* 7(5) pp. 777-803.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R. and De Feo, V. (2013) 'Effect of essential oils on pathogenic bacteria'. *Pharmaceuticals,* 6(12) pp. 1451-1474.
Netea, M. G., Sutmuller, R., Hermann, C., Van Der Graaf, C. a. A., Van Der Meer, J. W. M., Van Krieken, J. H., Hartung, T., Adema, G. and Kullberg, B. J. (2004) 'Toll-like receptor 2 suppresses immunity against *Candida albicans* through Induction of IL-10 and regulatory T cells'. *The Journal of Immunology,* 172(6) pp. 3712-3718.
Ng, K. W., Torzilli, P. A., Warren, R. F. and Maher, S. A. (2014) 'Characterization of a macroporous polyvinyl alcohol scaffold for the repair of focal articular cartilage defects'. *Journal of Tissue Engineering and Regenerative Medicine,* 8(2) pp. 164-168.
Nhs England. 2014. *Improving dental care – a call to action* [Online]. Available: http://www.england.nhs.uk/wp-content/uploads/2014/02/imp-dent-care.pdf [Accessed].
Nikaido, H. (2003) 'Molecular basis of bacterial outer membrane permeability revisited'. *Microbiology and molecular biology reviews* 67(4) pp. 593-656.
Nohmi, K., Tokuhara, D., Tachibana, D., Saito, M., Sakashita, Y., Nakano, A., Terada, H., Katayama, H., Koyama, M. and Shintaku, H. (2015) 'Zymosan induces immune responses comparable with those of adults in monocytes, dendritic cells, and monocyte-derived dendritic cells from cord blood'. *Journal of Pediatrics,* 167(1) pp. 155-162.
O'brien, F. J. (2011) 'Biomaterials & scaffolds for tissue engineering'. *Materials Today,* 14(3) pp. 88-95.
Ocheng, F., Bwanga, F., Joloba, M., Softrata, A., Azeem, M., Pütsep, K., Obua, C. and Gustafsson, A. (2015) 'Essential oils from Ugandan aromatic medicinal plants: chemical composition and growth inhibitory effects on oral pathogens'. *Evidence-Based Complementary and Alternative Medicine,* 2015, May, pp. 1-10.
Ohnemus, U., Willers, C., Bubenheim, M., Horstkotte, M. A., Houdek, P., Fischer, F., Schmage, P., Moll, I. and Brandner, J. M. (2008) 'An *ex-vivo* oral mucosa infection model for the evaluation of the topical activity of antifungal agents'. *Mycoses,* 51(1) pp. 21-29.
Oka, M., Ushio, K., Kumar, P., Ikeuchi, K., Hyon, S. H., Nakamura, T. and Fujita, H. (2000) 'Development of artificial articular cartilage'. *Proceedings of the Institution of Mechanical Engineers Part H,* 214(1) pp. 59-68.
Oldfield, E. and Feng, X. (2014) 'Resistance-resistant antibiotics'. *Trends in Pharmacological Sciences,* 35(12) pp. 664-674.
Olsen, I. (2015) 'Biofilm-specific antibiotic tolerance and resistance'. *European Journal of Clinical Microbiology & Infectious Diseases,* 34(5) pp. 877-886.
Oosterwaal, P. J. M., Mikx, F. H. M., Van Den Brink, M. E. and Renggli, H. H. (1989) 'Bactericidal concentrations of chlorhexidine-digluconate, amine fluoride gel and stannous fluoride gel for subgingival bacteria tested in serum at short contact times'. *Journal of Periodontal Research,* 24(4) pp. 155-160.
Otoni, C. G., Pontes, S. F. O., Medeiros, E. a. A. and Soares, N. F. (2014) 'Edible films from methylcellulose and nanoemulsions of clove bud (*Syzygium aromaticum*) and oregano (*Origanum vulgare*) essential oils

as shelf life extenders for sliced bread'. *Journal of Agricultural and Food Chemistry,* 62(22) pp. 5214-5219.
Pal, K., Banthia, A. K. and Majumdar, D. K. (2017) 'Polymeric hydrogels: characterization and biomedical applications'. *Designed Monomers and Polymers* 12(3) pp. 197-220.
Paranagama, P. A., Wimalasena, S., Jayatilake, G. S., Jayawardena, A. L., Senanayake, U. M. and Mubarak, A. M. (2002) 'A comparison of essential oil constituents of bark, leaf, root and fruit of cinnamon (*Cinnamomum zeylanicum* Blum) grown in Sri Lanka'. *Journal of the National Science Foundation of Sri Lanka,* 29(3-4) pp. 147-153.
Park, K. M., Lee, S. Y., Joung, Y. K., Na, J. S., Lee, M. C. and Park, K. D. (2009) 'Thermosensitive chitosan – Pluronic hydrogel as an injectable cell delivery carrier for cartilage regeneration'. *Acta Biomaterialia,* 5(6) pp. 1956-1965.
Park, S. N., Lim, Y. K., Freire, M. O., Cho, E., Jin, D. and Kook, J. K. (2012) 'Antimicrobial effect of linalool and α-terpineol against periodontopathic and cariogenic bacteria'. *Anaerobe,* 18(3) pp. 369-372.
Patel, R. 2012. The state of oral health in Europe. Better Oral Health European Platform.
Patin, E. C., Thompson, A. and Orr, S. J. (2018) 'Pattern recognition receptors in fungal immunity'. *Seminars in Cell and Developmental Biology,* S1084-9521(17) pp. 30541-30544.
Paul, J., Adeniran, A., Augusti, A., Vargas, C., Olalekan, O., Hassan, W., Ibrahim, M., Pansera, E. and Francine, D. (2013) 'Antioxidant activity, genotoxicity and cytotoxicity evaluation of lemon balm (*Melissa officinalis* L.) ethanolic extract: its potential role in neuroprotection'. *Industrial Crops & Products,* 51, November, pp. 26-34.
Pazyar, N., Yaghoobi, R., Bagherani, N. and Kazerouni, A. (2013) 'A review of applications of tea tree oil in dermatology'. *International Journal of Dermatology,* 52(7) pp. 784-790.
Peña, A., Sánchez, N. S. and Calahorra, M. (2013) 'Effects of chitosan on *Candida albicans*: conditions for its antifungal activity'. *BioMed Research International,* 2013, May, pp. 1-15.
Peters, B. M., Zhu, J., Jr, P. L. F., Scheper, M. A., Hackett, W., Shaye, S. E. and Jabra-Rizk, M. A. (2011) 'Protection of the oral mucosa by salivary histatin-5 against *Candida albicans* in an *ex vivo* murine model of oral infection'. *FEMS Yeast Research* 10(5) pp. 597-604.
Petersen, P. E. (2003) 'The World Oral Health Report 2003: continuous improvement of oral health in the 21st century--the approach of the WHO Global Oral Health Programme'. *Community dentistry and oral epidemiology,* 31 (1) pp. 3-23.
Petersen, P. E. and Ogawa, H. (2012) 'The global burden of periodontal disease: towards integration with chronic disease prevention and control'. *Periodontology 2000,* 60(1) pp. 15-39.
Pfaller, M. A., Diekema, D. J. and Sheehan, D. J. (2006) 'Interpretive breakpoints for fluconazole and *Candida* revisited: a blueprint for the future of antifungal susceptibility testing'. *Clinical Microbiology Reviews,* 19(2) pp. 435-447.
Pietruska, M., Paniczko, A., Waszkiel, D., Pietruski, J. and Bernaczyk, A. (2006) 'Efficacy of local treatment with chlorhexidine gluconate drugs on the

clinical status of periodontium in chronic periodontitis patients'. *Advances in medical sciences,* 51(1) pp. 162-165.
Plessas, A. (2014) 'Nonsurgical periodontal treatment: review of the evidence'. *Oral health and dental management,* 13(1) pp. 71-80.
Posimo, J. M., Unnithan, A. S., Gleixner, A. M., Choi, H. J., Jiang, Y., Pulugulla, S. H. and Leak, R. K. (2014) 'Viability assays for cells in culture'. *Journal of Visualized Experiments,* 83(83) pp. 1-14.
Potempa, M., Potempa, J., Kantyka, T., Nguyen, K.-A., Wawrzonek, K., Manandhar, S. P., Popadiak, K., K., R., Eick, S. and Blom, A. M. (2009) 'Interpain A, a cysteine proteinase from *Prevotella intermedia*, inhibits complement by degrading complement factor C3'. *PLOS Pathogens,* 5(2) pp. e1000316.
Prashar, A., Locke, I. C. and Evans, C. S. (2004) 'Cytotoxicity of lavender oil and its major components to human skin cells'. *Cell Proliferation,* 37(3) pp. 221-229.
Qin, Y., Zhang, L., Xu, Z., Zhang, J., Jiang, Y.-Y., Cao, Y. and Yan, T. (2016) 'Innate immune cell response upon *Candida albicans* infection'. *Virulence,* 7(5) pp. 512-526.
Quale, J. M., Landman, D., Zaman, M. M., Burney, S. and Sathe, S. S. (1996) '*In vitro* activity of *Cinnamomum zeylanicum* against azole resistant and sensitive *Candida* species and a pilot study of cinnamon for oral candidiasis'. *The American Journal of Chinese Medicine,* 24(2) pp. 103-109.
Rajkowska, K., Nowak, A., Kunicka-Styczy, A. and Siadura, A. (2016) 'Biological effects of various chemically characterized essential oils: investigation of the mode of action against *Candida albicans* and HeLa cells'. *RSC Advances* 6, (October), pp. 97199-97207.
Rajput, S. B. and Karuppayil, S. M. (2013) 'Small molecules inhibit growth, viability and ergosterol biosynthesis in *Candida albicans*'. *Springer Plus,* 2(26) pp. 1-6.
Ramage, G., Saville, S. P., Thomas, D. P. and López-Ribot, J. L. (2005) '*Candida* biofilms: an update'. *Eukaryotic Cell,* 4(4) pp. 633-638.
Ramirez-Garcia, A., Rementeria, A., Aguirre-Urizar, J. M., Moragues, M. D., Antoran, A., Pellon, A., Abad-Diaz-De-Cerio, A. and Hernando, F. L. (2016) '*Candida albicans* and cancer: Can this yeast induce cancer development or progression?'. *Critical Reviews in Microbiology,* 42(2) pp. 181-193.
Ramseier, C. A., Rasperini, G., Batia, S. and Giannobile, W. V. (2012) 'Advanced reconstructive technologies for periodontal tissue repair'. *Periodontology 2000,* 59(1) pp. 185-202.
Ranjha, N. M. and Qureshi, U. F. (2014) 'Preparation and characterization of crosslinked acrylic acid/hydroxypropyl methyl cellulose hydrogels for drug delivery'. *Internation Journal of Pharmacy and Pharmaceuticals Sciences,* 6(4) pp. 400-410.
Reichmann, P., Ko, A., Lin, J., Alcaide, F., Tenover, F. C., Mcdougal, L., Swidsinski, S. and Hakenbeck, R. (1997) 'A global gene pool for high-level cephalosporin resistance in commensal *Streptococcus* species and *Streptococcus pneumoniae*'. *The Journal of Infectious Diseases,* 176(4) pp. 1001-1012.

Rheinwald, J. G. and Green, H. (1975) 'Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells'. *Cell,* 6(3) pp. 331-343.
Rho, K. S., Jeong, L., Lee, G., Seo, B. M., Park, Y. J., Hong, S. D., Roh, S., Cho, J. J., Park, W. H. and Min, B. M. (2006) 'Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing'. *Biomaterials,* 27(8) pp. 1452-1461.
Ribeiro Rosa, E. A. (2015) *Oral candidosis: physiopathology, decision making, and therapeutics,* Springer.
Richardson, J. P. and Moyes, D. L. (2015) 'Adaptive immune responses to *Candida albicans* infection'. *Virulence,* 6(4) pp. 327-337.
Riss, T., O'brien, M. and Moravec, R. (2003) 'Choosing the right cell-based assay for your research'. *Cell Notes,* 6 pp. 6-12.
Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J. and Minor, L. (2013) *'*Cell viability assays*'. In:* Sittampalam Gs, C. N., Brimacombe K, Et Al. (ed.) *Assay Guidance Manual [Internet].* Bethesda (MD):: Eli Lilly & Company and the National Center for Advancing Translational Sciences; .
Roberts, M. E. and Stewart, P. S. (2005) 'Modelling protection from antimicrobial agents in biofilms through the formation of persister cells'. *Microbiology,* 151(1) pp. 75-80.
Roche, Y. and Yoshimori, R. N. (1997) '*In-vitro* activity of spiramycin and metronidazole alone or in combination against clinical isolates from odontogenic abscesses.'. *Journal of Antimicrobial Chemotherapy,* 40(3) pp. 353-357.
Rogers, H., Wei, X. Q., Lewis, M. a. O., Patel, V., Rees, J. S., Walker, R. V., Maggio, B., Gupta, A. and Williams, D. W. (2103) 'Immune response and candidal colonisation in denture associated stomatitis'. *Journal of Clinical and Cellular Immunology-,* 4(6) pp. 178-184.
Romano, L., Battaglia, F., Masucci, L., Sanguinetti, M., Posteraro, B., Plotti, G., Zanetti, S. and Fadda, G. (2005) '*In vitro* activity of bergamot natural essence and furocoumarin-free and distilled extracts, and their associations with boric acid, against clinical yeast isolates'. *Journal of Antimicrobial Chemotherapy,* 55(1) pp. 110-114.
Rosen, G. and Sela, M. N. (2006) 'Coaggregation of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* PK1594 is mediated by capsular polysaccharide and lipopolysaccharide'. *FEMS Microbiology Letters,* 256(2) pp. 304-310.
Rosling, B., Dahlén, G., Volpe, A., Furuichi, Y., Ramberg, P. and Lindhe, J. (1997) 'Effect of triclosan on the subgingival microbiota of periodontitis-susceptible subjects'. *Journal of clinical periodontology,* 24(12) pp. 881-7.
Russell, A. D. (2004) 'Whither triclosan?'. *Journal of Antimicrobial Chemotherapy,* 53(5) pp. 693-695.
Russell, A. D. (2018) 'Similarities and differences in the responses of microorganisms to biocides'. *Journal of Antimicrobial Chemotherapy,* 52(5) pp. 750-763.
Salerno, C., Pascale, M., Contaldo, M., Esposito, V., Busciolano, M., Milillo, L., Guida, A., Petruzzi, M. and Serpico, R. (2011a) '*Candida*-associated denture stomatitis'. *Medicina Oral, Patología Oral y Cirugía Bucal,* 16(2) pp. 139-143.

Salerno, C., Pascale, M., Contaldo, M., Esposito, V., Busciolano, M., Milillo, L., Guida, A., Petruzzi, M. and Serpico, R. (2011b) '*Candida*-associated denture stomatitis'. *Medicina Oral Patologia Oral y Cirugia Bucal,* 16(2) pp. 139-143.

Salim, N., Moore, C., Silikas, N., Satterthwaite, J. D. and Rautemaa, R. (2012) 'Fungicidal amounts of antifungals are released from impregnated denture lining material for up to 28 days'. *Journal of Dentistry,* 40(6) pp. 506-512.

Samber, N., Khan, A., Varma, A. and Manzoor, N. (2015) 'Synergistic anti-candidal activity and mode of action of *Mentha piperita* essential oil and its major components'. *Pharmaceutical Biology,* 53(10) pp. 1496-1504.

Sandasi, M., Leonard, C. M., Van Vuuren, S. F. and Viljoen, A. M. (2011) 'Peppermint (*Mentha piperita*) inhibits microbial biofilms *in vitro*'. *South African Journal of Botany,* 77(1) pp. 80-85.

Sanglard, D. and Odds, F. C. (2002) 'Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences'. *The Lancet Infectious Diseases,* 2(2) pp. 73-85.

Sanjeeta, N. (2014) 'Oral changes in diabetes – a review'. *IOSR Journal of Dental and Medical Sciences,* 13(1) pp. 36-39.

Santana, I. L., Gonçalves, L. M., De Vasconcellos, A. A., Da Silva, W. J., Cury, J. A. and Del Bel Cury, A. A. (2013) 'Dietary carbohydrates modulate *Candida albicans* biofilm development on the denture surface'. *PLOS One,* 8(5) pp. e64645.

Santoro, M., Shah, S. R., Walker, J. L. and Mikos, A. G. (2016) 'Poly(lactic acid) nanofibrous scaffolds for tissue engineering'. *Advanced Drug Delivery Reviews,* 107, December, pp. 206-212.

Santos, K. A., Matias, E. F., Souza, C. E., Tintino, S. R., Braga, M. F., Guedes, G. M., Nogueira, L. F., Morais, E. C., Costa, J. G., Menezes, I. R. and Coutinho, H. D. (2012) 'Anti-*Candida* activity of *Mentha arvensis* and *Turnera ulmifolia*'. *Journal of Medicinal Food,* 15(3) pp. 322-324.

Sardi, J. C., Scorzoni, L., Bernardi, T., Fusco-Almeida, A. M. and Mendes Giannini, M. J. (2013) '*Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options'. *Journal of Medical Microbiology,* 62(1) pp. 10-24.

Saunders, K. A., Greenman, J. and Mckenzie, C. (2000) 'Ecological effects of triclosan and triclosan monophosphate on defined mixed cultures of oral species grown in continuous culture'. *The Journal of antimicrobial chemotherapy,* 45(4) pp. 447-452.

Sbordone, R. L. and Bortolaia, C. (2003) 'Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease'. *Clinical Oral Investigations,* 7(4) pp. 181-188.

Schaller, M., Borelli, C., Korting, H. C. and Hube, B. (2005a) 'Hydrolytic enzymes as virulence factors of *Candida albicans*'. *Mycoses,* 48(6) pp. 365-377.

Schaller, M., Korting, H. C., Borelli, C., Hamm, G. and Hube, B. (2005b) '*Candida albicans*-secreted aspartic proteinases modify the epithelial cytokine response in an *in vitro* model of vaginal candidiasis'. *Infection and immunity,* 73(5) pp. 2758 - 2765.

Schaller, M., Mailhammer, R., Grassl, G., Sander, C. A., Hube, B. and Korting, H. C. (2002) 'Infection of human oral epithelia with *Candida* species

induces cytokine expression correlated to the degree of virulence'. *Journal of Investigative Dermatology,* 118(4) pp. 652-657.
Schaller, M. and Weindl, G. (2009) 'Models of oral and vaginal candidiasis based on *in vitro* reconstituted human epithelia for the study of host-pathogen interactions*'. In:* Rupp, S. & Sohn, K. (eds.) *Host-pathogen interactions.* Humana Press.
Schiott, C., Loe, H., Jensen, B., Kilian, M., Davies, R. M. and Glavind, K. (1970) 'The effect of chlohexidine mouthrinses on the human oral flora'. *Journal of Periodontal Research,* 5(2) pp. 84-89.
Schramm, G. (1998) *A practical approach to rheology and rheometry,* 2nd. Germany: Haake.
Selwitz, R. H., Ismail, A. I. and Pitts, N. B. (2007) 'Dental caries'. *The Lancet,* 369(9555) pp. 51-59.
Senel, S., Ikinci, G., Kas, S., Yousefi-Rad, A., Sargon, M. F. and Hincal, A. A. (2000) 'Chitosan films and hydrogels of chlorhexidine gluconate for oral mucosal delivery'. *International Journal of Pharmaceutics,* 193(200) pp. 197-203.
Seneviratne, C. J., Zhang, C. F. and Samaranayake, L. P. (2011) 'Dental plaque biofilm in oral health and disease'. *The Chinese Journal of Dental Research,* 14(2) pp. 87-94.
Sforcin, J. M., Amaral, J. T., Fernandes, A. J., Sousa, J. P. and Bastos, J. K. (2009) 'Lemongrass effects on IL-1β and IL-6 production by macrophages'. *Natural Product Research,* 23(12) pp. 1151-1159.
Shachar, I. and Karin, N. (2013) 'The dual roles of inflammatory cytokines and chemokines in the regulation of autoimmune diseases and their clinical implications'. *Journal of Leukocyte Biology,* 93(1) pp. 51-61.
Shapiro, S., Meier, A. and Guggenheim, B. (1994) 'The antimicrobial activity of essential oils and essential oil components towards oral bacteria'. *Oral Microbiology and Immunology,* 9(4) pp. 202-208.
Shreaz, S., Bhatia, R., Khan, N., Muralidhar, S., Manzoor, N. and Khan, L. A. (2013) 'Influences of cinnamic aldehydes on H + extrusion activity and ultrastructure of *Candida*'. *Journal of Medical Microbiology,* 62(2) pp. 232-240.
Shreaz, S., Wani, W. A., Behbehani, J. M., Raja, V., Karched, M., Ali, I., Siddiqi, W. A. and Ting, L. (2016) 'Cinnamaldehyde and its derivatives, a novel class of antifungal agents'. *Fitoterapia,* 112, (July), pp. 116-131.
Sikkematb, J. and Bontt, J. a. M. D. (1994) 'Interactions of cyclic hydrocarbons with biological membranes '. *The Journal of Biological Chemistry,* 269(11) pp. 8022-8028.
Silva, B. R. D., Freitas, V. a. a. D., Nascimento-Neto, L. G., Carneiro, V. A., Arruda, F. V. S., Aguiar, A. S. W. D., Cavada, B. S. and Teixeira, E. H. (2012) 'Antimicrobial peptide control of pathogenic microorganisms of the oral cavity: a review of the literature'. *Peptides,* 36(2) pp. 315-321.
Silva, S., Henriques, M., Oliveira, R., Azeredo, J., Malic, S., Hooper, S. J. and Williams, D. W. (2009) 'Characterization of *Candida* parapsilosis infection of an *in vitro* reconstituted human oral epithelium'. *European Journal of Oral Sciences,* 117(6) pp. 669-675.
Sindhura Reddy, N., Sowmya, S., Bumgardner, J. D., Chennazhi, K. P., Biswas, R. and Jayakumar, R. (2014) 'Tetracycline nanoparticles loaded calcium sulfate composite beads for periodontal management'. *Biochimica et Biophysica Acta - General Subjects,* 1840(6) pp. 2080-2090.

Singh, S., Fatima, Z. and Hameed, S. (2015) 'Predisposing factors endorsing *Candida* infections'. *Le Infezioni in Medicina,* 23(3) pp. 211-223.
Singh, S., Fatima, Z. and Hameed, S. (2016) 'Citronellal-induced disruption of membrane homeostasis in *Candida albicans* and attenuation of its virulence attributes'. *Revista Da Sociedade Brasileira De Medicina Tropical* 49(4) pp. 465-472.
Singh, S., Singh, S. K., Chowdhury, I. and Singh, R. (2017) 'Understanding the mechanism of bacterial biofilms resistance to antimicrobial agents'. *The Open Microbiology Journal,* 11(1) pp. 53-62.
Sitheeque, M. a. M. and Samaranayake, L. P. (2003) 'Chronic hyperplastic candidosis/candidiasis (candidal leukoplakia)'. *Critical Reviews in Oral Biology & Medicine,* 14(4) pp. 253-267.
Slaughter, B. V., Khurshid, S. S., Fisher, O. Z., Khademhosseini, A. and Peppas, N. A. (2009) 'Hydrogels in regenerative medicine'. *Advanced Materials,* 21(32-33) pp. 3307-3329.
Sloan, A. J., Colombo, J., Roberts, J., Waddington, R. J. and Ayre, W. N. (2016) 'Tissue culture models and approaches for dental tissue regeneration: current strategies'. *In:* Waddington, R. J. & Sloan, A. J. (eds.) *Tissue engineering and regeneration in dentistry.* Wiley Blackwell.
Sloan, A. J. and Lynch, C. D. (2012) 'Dental tissue repair: novel models for tissue regeneration strategies'. *The open dentistry journal,* 6, December, pp. 214-219.
Sloan, A. J., Rutherford, R. B. and Smith, A. J. (2000) 'Stimulation of the rat dentine-pulp complex by bone morphogenetic protein-7 *in vitro*'. *Archives of Oral Biology* 45(2) pp. 173-177.
Sloan, A. J., Shelton, R. M., Hann, A. C., Moxham, B. J. and Smith, A. J. (1998) 'An *in vitro* approach for the study of dentinogenesis by organ culture of the dentine-pulp complex from rat incisor teeth'. *Archives of Oral Biology,* 43(6) pp. 421-430.
Sloan, A. J. and Smith, A. J. (1999) 'Stimulation of the dentine-pulp complex of rat incisor teeth by transforming growth factor-b isoforms 1-3 *in vitro*'. *Archives of Oral Biology* 44(2) pp. 149-156.
Sloan, A. J., Taylor, S. Y., Smith, E. L., Roberts, J. L., Chen, L., Wei, X. Q. and Waddington, R. J. (2013) 'A novel ex vivo culture model for inflammatory bone destruction'. *Journal of Dental Research,* 92(8) pp. 728-34.
Smith, E. L. 2009. *Development and characterisation of an ex vivo model system for bone repair.* Cardiff University.
Smith, E. L., Locke, M., Waddington, R. J. and Sloan, A. J. (2010) 'An *ex vivo* rodent mandible culture model for bone repair'. *Tissue engineering. Part C, Methods,* 16(6) pp. 1287-1296.
Sochalska, M. and Potempa, J. (2017) 'Manipulation of neutrophils by *Porphyromonas gingivalis* in the development of periodontitis'. *Frontiers in Cellular and Infection Microbiology,* 7, May, pp. 197.
Socransky, S. S., Haffajee, S. S., Cugini, A. D., Smith, C. and Jr, K. (1998) 'Microbial complexes in subgingival plaque'. *Journal of Clinical Periodontology,* 25(2) pp. 134-144.
Soll, D. R. (2002) '*Candida* commensalism and virulence: the evolution of phenotypic plasticity.'. *Acta Tropica,* 81(2) pp. 101-110.
Soll, D. R., Galask, R., Isley, S., Rao, T. V., Stone, D., Hicks, J., Schmid, J., Mac, K. and Hanna, C. (1989) 'Switching of *Candida albicans* during

successive episodes of recurrent vaginitis.'. *Journal of Clinical Microbiology,* 27(4) pp. 681-690.
Solmaz, G. and Korachi, M. (2013) 'Inhibition and disruption properties of chlorhexidine gluconate on single and multispecies oral biofilms'. *Jundishapur Journal of Microbiology,* 6(1) pp. 61-66.
Sookto, T., Srithavaj, T., Thaweboon, S., Thaweboon, B. and Shrestha, B. (2013) '*In vitro* effects of *Salvia officinalis* L. essential oil on *Candida albicans*'. *Asian Pacific Journal of Tropical Biomedicine,* 3(5) pp. 376-380.
Soukoulis, S. and Hirsch, R. (2004) 'The effects of a tea tree oil-containing gel on plaque and chronic gingivitis'. *Australian dental journal,* 49(2) pp. 78-83.
Soysa, N. S., Samaranayake, L. P. and Ellepola, A. N. B. (2008) 'Diabetes mellitus as a contributory factor in oral candidosis'. *Diabetic Medicine,* 23(5) pp. 455-459.
Steinshamn, S. and Waage, A. (1992) 'Tumor necrosis factor and Interleukin-6 in *Candida albicans* infection in normal and granulocytopenic mice'. *Infection and Immunity,* 60(10) pp. 4003-4008.
Stewart, P. S. and Franklin, M. J. (2008) 'Physiological heterogeneity in biofilms'. *Nature Reviews Microbiology,* 6(3) pp. 199-210.
Stoodley, P. and Dirckx P. (2003). 'Biofilm formation in 3 steps'. http://www.biofilm.montana.edu/resources/images/multicellularextracellular/biofilm–formation–3–steps.html. Montana State University, Bozeman, Center for Biofilm Engineering.
Strober, W. (2015) 'Trypan blue exclusion test of cell viability'. *Current Protocols in Immunology,* 21(1) pp. 3-5.
Sulieman, R. T. (2009) 'The antibacterial effect of *Myrtus communis* as root canal irrigant: a comparative study'. *Dentistry Journal* 9(2) pp. 289-296.
Sun, X., Xu, C., Wu, G., Ye, Q. and Wang, C. (2017) 'Poly(lactic-co-glycolic acid): applications and future prospects for periodontal tissue regeneration'. *Polymers,* 9(6) pp. 189.
Sweeney, L. C., Dave, J., Chambers, P. A. and Heritage, J. (2004) 'Antibiotic resistance in general dental practice-a cause for concern?'. *The Journal of antimicrobial chemotherapy,* 53(4) pp. 567-576.
Szweda, P., Gucwa, K., Kurzyk, E., Romanowska, E., Dzierżanowska-Fangrat, K., Zielińska Jurek, A., Kuś, P. M. and Milewski, S. (2015) 'Essential oils, silver nanoparticles and propolis as alternative agents against fluconazole resistant *Candida albicans*, *Candida glabrata* and *Candida krusei* clinical isolates'. *Indian Journal of Microbiology,* 55(2) pp. 175-183.
Taguchi, Y., Hasumi, Y., Abe, S. and Nishiyama, Y. (2013) 'The effect of cinnamaldehyde on the growth and the morphology of *Candida albicans*'. *Medical Molecular Morphology* 46(1) pp. 8-13.
Takarada, K., Kimizuka, R., Takahashi, N., Honma, K., Okuda, K. and Kato, T. (2004) 'A comparison of the antibacterial efficacies of essential oils against oral pathogens'. *Oral microbiology and immunology,* 19(1) pp. 61-64.
Tan, W. H. and Takeuchi, S. (2007) 'Monodisperse alginate hydrogel microbeads for cell encapsulation'. *Advanced Materials,* 19(18) pp. 2696-2701.

Tanzi, M. C. (2010) *Fondamenti di bioingegneria chimica. Non solo biomateriali,* Bologna.
Taylor, R., Maryan, C. and Verran, J. (1998) 'Retention of oral microorganisms on cobalt-chromium alloy and dental acrylic resin with different surface finishes'. *Journal of Prosthetic Dentistry,* 80(5) pp. 592-597.
The American Academy of Periodontology 1999. Informational paper: the pathogenesis of periodontal diseases.
Theilade, E. (1986) 'The non-specific theory in microbial etiology of inflammatory periodontal diseases'. *Journal of Clinical Periodontology,* 13(10) pp. 905-911.
Thirumala, S., Gimble, J. M. and Devireddy, R. V. (2013) 'Methylcellulose based thermally reversible hydrogel system for tissue engineering applications'. *Cells,* 2(3) pp. 460-475.
Thosar, N., Basak, S., Bahadure, R. N. and Rajurkar, M. (2013) 'Antimicrobial efficacy of five essential oils against oral pathogens: an *in vitro* study'. *European Journal of Dentistry,* 7(1) pp. 71-77.
Tokuda, M., Sakuta, T., Fushuku, A. and Torii, M. (2001) 'Regulation of interleukin-6 expression in human dental pulp cell cultures stimulated with *Prevotella intermedia* lipopolysaccharide'. *Journal of Endodontics,* 27(4) pp. 273-277.
Tonetti, M. S., Van Dyke, T. E. and working group 1 of the joint EFP/AAP workshop. (2013) 'Periodontitis and atherosclerotic cardiovascular disease: consensus report of the Joint EFP/AAPWorkshop on periodontitis and systemic diseases'. *Journal of Periodontology* 84(4) pp. 24-29.
Trindade, L. A., Oliveira, J. D. A., Castro, R. D. D. and Lima, O. D. (2015) 'Inhibition of adherence of *C . albicans* to dental implants and cover screws by *Cymbopogon nardus* essential oil and citronellal'. *Clinical Oral Investigations* 19(9) pp. 2223-2231.
Tripodo, G., Trapani, A., Torre, M. L., Giammona, G., Trapani, G. and Mandracchia, D. (2015) 'Hyaluronic acid and its derivatives in drug delivery and imaging: recent advances and challenges'. *European Journal of Pharmaceutics and Biopharmaceutics,* 97(B) pp. 400-416.
Trowell, O. A. (1959) 'The culture of mature organs in a synthetic medium'. *Experimental Cell Research,* 16(1) pp. 118-147.
Tu, Y.-Y., Yang, C.-Y., Chen, R.-S. and Chen, M.-H. (2015) 'Effects of chlorhexidine on stem cells from exfoliated deciduous teeth'. *Journal of the Formosan Medical Association,* 114(1) pp. 17-22.
Turner, D. J., Hoyle, S. L., Snewin, V. A., Gares, M.-P., Brown, I. N. and Young, D. B. (2002) 'An *ex vivo* culture model for screening drug activity against *in vivo* phenotypes of *Mycobacterium tuberculosis*'. *Microbiology,* 148(10) pp. 2929-2936.
Udina, I. G. and Gulenko, O. V. (2018) 'Molecular-genetic mechanisms of caries development'. *Russian Journal of Genetics,* 54(4) pp. 415-422.
Ultee, A. and Kets, E. P. W. (1999) 'Mechanisms of action of carvacrol on the food-borne pathogen bacillus cereus'. *Applied and Environmental Microbiology,* 65(10) pp. 4606-4610.
Underhill, D. M. (2003) 'Macrophage recognition of zymosan particles'. *Journal of Endotoxin Research,* 9(3) pp. 176-180.
Unlu, M., Ergene, E., Unlu, G. V., Zeytinoglu, H. S. and Vural, N. (2010) 'Composition, antimicrobial activity and *in vitro* cytotoxicity of essential oil

from *Cinnamomum zeylanicum* Blume (Lauraceae)'. *Food and Chemical Toxicology,* 48(11) pp. 3274-3280.
Vargas, K., Messer, S. A., Pfaller, M., Lockhart, S. R., Stapleton, J. T., Hellstein, J. and Soll, D. R. (2000) 'Elevated phenotypic switching and drug resistance of *Candida albicans* from human immunodeficiency virus-positive individuals prior to first thrush episode'. *Journal of Clinical Microbiology,* 38(10) pp. 3595-3607.
Vargas, K., Wertz, P. W., Drake, D., Morrow, B. and Soll, D. R. (1994) 'Differences in adhesion of *Candida albicans* 3153A cells exhibiting switch phenotypes to buccal epithelium and stratum corneum'. *Infection and Immunity,* 62(4) pp. 1328-1335.
Vasconcellos, A. a. D., Vasconcellos, A. a. D., Chagas, R. B. and Gonçalves, L. M. (2014) '*Candida*-associated denture stomatitis: clinical relevant aspects'. *Clinical Microbiology,* 3(4) pp. 9-11.
Verma, D., Garg, P. K. and Dubey, A. K. (2018) 'Insights into the human oral microbiome'. *Archives of microbiology,* 200(4) pp. 525-540.
Vitt, A., Slizen, V., Boström, E. A., Yucel-Lindberg, T., Kats, A., Sugars, R. V., Gustafsson, A. and Buhlin, K. (2017) 'Effects of polyhexamethylene guanidine phosphate on human gingival fibroblasts'. *Acta Odontologica Scandinavica,* 75(7) pp. 524-529.
Waddington, R. J., Moseley, R., Smith, A. J., Sloan, A. J. and Embery, G. (2004) 'Fluoride-induced changes to proteoglycan structure synthesised within the dentine-pulp complex *in vitro*'. *Biochimica et Biophysica Acta - General Subjects,* 1689(2) pp. 142-151.
Walker, C. and Sedlacek, M. J. (2007) 'An *in vitro* biofilm model of subgingival plaque'. *Oral Microbiology and Immunology,* 22(3) pp. 152-161.
Wallet, M. A., Calderon, N. L., Alonso, T. R., Choe, C. S., Catalfamo, D. L., Lalane, C. J., Neiva, K. G., Panagakos, F. and Wallet, S. M. (2012) 'Triclosan alters antimicrobial and inflammatory responses of epithelial cells'. *Oral Diseases,* 19(3) pp. 286-302.
Wang, C., Cen, L., Yin, S., Liu, Q., Liu, W., Cao, Y. and Cui, L. (2010) 'A small diameter elastic blood vessel wall prepared under pulsatile conditions from polyglycolic acid mesh and smooth muscle cells differentiated from adipose-derived stem cells'. *Biomaterials,* 31(4) pp. 621-30.
Wang, H. L. and Greenwell, H. (2001) 'Surgical periodontal therapy'. *Periodontology 2000,* 25, January, pp. 89-99.
Wang, Y., Zhang, Y., Shi, Y.-Q., Pan, X.-H., Lu, Y.-H. and Cao, P. (2018) 'Antibacterial effects of cinnamon (*Cinnamomum zeylanicum*) bark essential oil on *Porphyromonas gingivalis*'. *Microbial Pthogenesis,* 116, March, pp. 26-32.
Watt, R. G. and Petersen, P. E. (2012) 'Periodontal health through public health--the case for oral health promotion'. *Periodontology 2000,* 60(1) pp. 147-55.
Webb, B. C. (1998) '*Candida*-associated denture stomatitis. Aetiology and management: a review. Part 2. Oral diseases caused by *candida* species'. *Australian Dental Journal* 43(3) pp. 160-166.
Wendakoon, C. N. and Sakaguchi, M. (1995) 'Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices'. *Journal of Food Protection* 58(3) pp. 280-283.
Whiley, R. A., Cruchley, A. T., Gore, C. and Hagi-Pavli, E. (2012) '*Candida albicans* strain-dependent modulation of pro-inflammatory cytokine

release by *in vitro* oral and vaginal mucosal models'. *Cytokine,* 57(1) pp. 89-97.
Williams, D. and Lewis, M. (2011) 'Pathogenesis and treatment of oral candidosis'. *Journal of Oral Microbiology,* 1(30) pp. 1-11.
Williams, D. W., Bartie, K. L., Potts, A. J., Wilson, M. J., Fardy, M. J. and Lewis, M. A. (2001) 'Strain persistence of invasive *Candida albicans* in chronic hyperplastic candidosis that underwent malignant change'. *Gerodontology,* 18(2) pp. 73-78.
Wilson, M. (1999) 'Use of the constant depth film fermenter in studies of biofilms of oral bacteria'. *Methods in Enzymology,* 310 pp. 264-279.
Winkler, J. R., John, S. R., Kramer, R. H., Hoover, C. I. and Murray, P. A. (1987) 'Attachment of oral bacteria to a basement-membrane-like matrix and to purified matrix proteins'. *Infection and Immunity,* 55(11) pp. 2721-2726.
World Health Organization 2006. WHO guidelines on hand hygiene in health care (advanced draft): global safety challenge 2005-2006: clean care is safer care. Geneva.
Xiong, J., Kang, K., Liu, L., Yoshida, Y., Cooper, K. D. and Ghannoum, M. A. (2000) '*Candida albicans* and *Candida krusei* differentially induce human blood mononuclear cell interleukin-12 and gamma interferon production'. *Infection and Immunity,* 68(5) pp. 2464-2469.
Yuan, T., Zhang, L., Li, K., Fan, H., Fan, Y., Liang, J. and Zhang, X. (2014) 'Collagen hydrogel as an immunomodulatory scaffold in cartilage tissue engineering'. *Journal of Biomedical Materials Research - Part B Applied Biomaterials,* 102(2) pp. 337-344.
Yueh, M.-F. and Tukey, R. H. (2016) 'Triclosan: a widespread environmental toxicant with many biological effects'. *Annual Review of Pharmacology and Toxicology* 6(56) pp. 251-272.
Zarzycki, R., Modrzejewska, Z., Nawrotek, K. and Lek, U. (2010) 'Drug release from hydrogel matrices'. *Ecological Chemistry and Engineering S,* 17(2) pp. 117-136.
Zhao, X., Oh, S. H., Yeater, K. M. and Hoyer, L. L. (2005) 'Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within Als family'. *Microbiology,* 151(Pt 5) pp. 1619-1630.
Zore, G. B., Thakre, A. D., Jadhav, S., Karuppayil, S. M. and Candida, A. (2011) 'Phytomedicine Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle'. *European Journal of Integrative Medicine,* 18(13) pp. 1181-1190.
Zore, G. B., Thakre, A. D., Rathod, V. and Karuppayil, S. M. (2010) 'Evaluation of anti-*Candida* potential of geranium oil constituents against clinical isolates of *Candida albicans* differentially sensitive to fluconazole: inhibition of growth, dimorphism and sensitization'. *Mycoses,* 54(4) pp. 99-109.
Zuidema, J. M., Rivet, C. J., Gilbert, R. J. and Morrison, F. A. (2014) 'A protocol for rheological characterization of hydrogels for tissue engineering strategies'. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials,* 102(5) pp. 1063-1073.

Appendix 1

pathogens

MDPI

Article

Antifungal Activity of Commercial Essential Oils and Biocides against *Candida Albicans*

Elisa Serra [1], Araida Hidalgo-Bastida [1], Joanna Verran [2], David Williams [3] and Sladjana Malic [1,*]

[1] School of Healthcare Science, Manchester Metropolitan University, Manchester M1 5GD, UK; elisa.serra@stu.mmu.ac.uk (E.S.); A.Hidalgo@mmu.ac.uk (A.H.-B.)
[2] School of Research, Enterprise and Innovation, Manchester Metropolitan University, Manchester M1 5GD, UK; J.verran@mmu.ac.uk
[3] School of Dentistry, Cardiff University, Cardiff CF14 4XY, UK; WilliamsDD@cardiff.ac.uk
* Correspondence: S.Malic@mmu.ac.uk

Received: 12 December 2017; Accepted: 23 January 2018; Published: date

Abstract: Management of oral candidosis, most frequently caused by *Candida albicans*, is limited due to the relatively low number of antifungal drugs and the emergence of antifungal tolerance. In this study, the antifungal activity of a range of commercial essential oils, two terpenes, chlorhexidine and triclosan was evaluated against *C. albicans* in planktonic and biofilm form. In addition, cytotoxicity of the most promising compounds was assessed using murine fibroblasts and expressed as half maximal inhibitory concentrations (IC50). Antifungal activity was determined using a broth microdilution assay. The minimum inhibitory concentration (MIC) was established against planktonic cells cultured in a range of concentrations of the test agents. The minimal biofilm eradication concentration (MBEC) was determined by measuring re-growth of cells after pre-formed biofilm was treated for 24 h with the test agents. All tested commercial essential oils demonstrated anticandidal activity (MICs from 0.06% (*v*/*v*) to 0.4% (*v*/*v*)) against planktonic cultures, with a noticeable increase in resistance exhibited by biofilms (MBECs > 1.5% (*v*/*v*)). The IC50s of the commercial essential oils were lower than the MICs, while a one hour application of chlorhexidine was not cytotoxic at concentrations lower than the MIC. In conclusion, the tested commercial essential oils exhibit potential as therapeutic agents against *C. albicans*, although host cell cytotoxicity is a consideration when developing these new treatments.

Keywords: *Candida albicans*; oral candidosis; commercial essential oils; biocides; antifungal activity; minimum inhibitory concentration; minimal biofilm eradication concentration; cytotoxicity

1. Introduction

Candida are commensal fungal microorganisms that can colonise the oral cavity, where they are mainly found on the posterior part of the tongue and the oral mucosa. Changes in the oral environment that lead to increased *Candida* growth can instigate oral candidosis [1]. The rising number of immunocompromised and immunodeficient patients has resulted in an increased incidence of fungal infections. To highlight this, *Candida*-related infections affect 65% of HIV positive individuals and over 80% of AIDS patients [2–4]. The higher life expectancy of the general population has also led to a rise in denture wearing, with a concomitant increase in *Candida*-associated stomatitis [5–7]. Even though more than 17 *Candida* species can cause human infection, oral candidosis are mainly caused by *C. albicans* [8]. In the mouth, *Candida* typically

grows as biofilms, which are three-dimensional structures attached to surfaces including human tissue or abiotic substrates (e.g. a denture). Biofilm cells are embedded in a self-produced extracellular polymeric matrix and importantly often exhibit an elevated tolerance to antimicrobial agents and host defences [5].

Current therapies for oral candidosis include use of topical or systemic antifungal agents, such as polyenes and azoles. Polyenes (e.g. nystatin and amphotericin B) are fungicidal through binding to ergosterol in the fungal cell membrane and inducing cell membrane damage. Azoles, such as fluconazole and miconazole, are fungistatic by inhibiting the enzyme lanosterol demethylase, involved in ergosterol biosynthesis [9]. Importantly, the range of available antifungals are limited compared to antibiotics [9] and coupled with the rise of *Candida* resistance, especially within biofilms, this has led to an interest in the discovery of new antifungal compounds [10].

Essential oils are natural products produced by aromatic plants and are mainly composed by terpenes and terpenoids [11]. Being lipophilic, these oils typically integrate into membrane structures causing increased cell permeability, leaching of intracellular components and inactivation of enzymes [12,13]. Essential oils can act against *Candida* by inhibiting ergosterol synthesis [14–18], altering cell wall morphology [15,17–19], inhibiting enzymes involved in cell wall synthesis [18,20], changing cell membrane permeability [21,22] and producing oxygen reactive species [23]. Furthermore, essential oils can also interact with the mitochondrial membrane leading to cidal effects [11]. Antimicrobial, anti-aseptic, anti-inflammation and anti-oxidant activity of essential oils, alone and in combination with commercial agents is well known [13,24–26]. However, limited knowledge exists regarding essential oil activity against biofilms and also host cell cytotoxicity.

The aim of this study was therefore to investigate the antifungal potential of twelve commercial essential oils and two terpenes (E-cinnamaldehyde and linalool) against *C. albicans* planktonic and biofilm growth. The cytotoxicity of the most active commercial essential oils was established against mouse fibroblasts. Antifungal activity of commercial essential oils was compared to chlorhexidine (CHX) and triclosan. These two biocides have previously shown antimicrobial properties against a wide range of oral pathogens and are frequent components in mouthwashes and toothpastes [27,28].

2. Results

2.1. Minimum Inhibitory Concentration (MIC) 80 and Minimal Lethal Concentration

The minimum inhibitory concentration (MIC) 80 of the test agents against *C. albicans* NCYC 1363 and *C. albicans* 135BM2/94 are shown in Table 1. The commercial essential oils that inhibited the growth at the lowest concentrations were melissa and geraniol, while myrtle and sage had the lowest fungistatic potential ($p < 0.001$).

Fungicidal activity was also expressed as the lowest concentration of antimicrobial agent that killed the microorganism (minimal lethal concentration) (Table 2). All tested compounds, with exception of triclosan, had minimal lethal concentrations against *C. albicans* at tested concentrations. However, these lethal concentrations were generally higher than the previously established MICs.

Table 1. Minimum inhibitory concentration 80 of commercial essential oils and biocides against *C. albicans* NYCY 1363 and *C. albicans* 135BM2/94 in the planktonic form.

Antimicrobial	Minimum Inhibitory Concentration 80 [% (*v*/*v*)] [(g/L)]	
	***C. albicans* NYCY 1363**	***C. albicans* 135BM2/94**
Basil	0.1 (0.9)	0.1 (0.9)
Bergamot	0.3 (2.6)	0.3 (2.6)
Cinnamon	0.1 (1.0)	0.1 (1.0)
Citronella	0.1 (0.9)	0.1 (0.9)

Geranium	0.07 (0.6)	0.06 (0.5)
Lavender	0.2 (1.8)	0.1 (0.9)
Melissa	0.06 (0.5)	0.06 (0.5)
Myrtle	0.4 (3.5)	0.3 (2.7)
Peppermint	0.1 (0.9)	0.1 (0.9)
Sage	0.4 (3.7)	0.3 (2.7)
Spearmint	0.2 (1.6)	0.1 (1.1)
Tea tree oil	0.2 (1.8)	0.2 (1.8)
E-cinnamaldehyde	0.03 (0.3)	0.01 (0.1)
Linalool	0.1 (0.9)	0.1 (0.9)
CHX	2×10^{-3} (2.1×10^{-2})	5×10^{-3} (5.3×10^{-2})
Triclosan	5.66×10^{-4} (8.4×10^{-3})	5.89×10^{-4} (8.8×10^{-3})

Minimal inhibitory concentration 80 (MIC80) defined as the lowest concentration of the antimicrobial agent that led to 80% reduction in absorbance compared to controls without agent. MIC values are in % (*v*/*v*) and in brackets are the equivalent MIC values in (g/L).

Table 2. Minimal lethal concentration of commercial essential oils and biocides against *C. albicans* NYCY 1363 and *C. albicans* 135BM2/94 in the planktonic growth mode.

Antimicrobial	**Minimal Lethal Concentration [% (*v*/*v*)] [(g/L)]**	
	***C. albicans* NCYC 1363**	***C. albicans* 135BM2/94**
Basil	0.5 (4.5)	0.5 (4.5)
Bergamot	0.5 (4.4)	0.5 (4.4)
Cinnamon	0.1 (1.0)	0.1 (1.0)
Citronella	0.1 (0.9)	0.1 (2.7)
Geranium	0.1 (0.9)	0.1 (0.9)
Lavender	0.5 (4.4)	0.3 (2.6)
Melissa	0.1 (0.9)	0.1 (0.9)
Myrtle	1 (8.8)	1 (8.8)
Peppermint	0.3 (2.7)	0.1 (0.9)
Sage	1 (9.2)	1 (9.2)
Spearmint	1 (9.2)	1 (9.2)
Tea tree oil	0.5 (4.5)	0.3 (2.7)
E-cinnamaldehyde	0.03 (0.3)	0.03 (0.3)
Linalool	0.3 (2.6)	0.3 (2.6)
CHX	2.5×10^{-3} (2.7×10^{-2})	5×10^{-3} (5.3×10^{-2})
Triclosan	NA	NA

Minimal lethal concentration was defined as the lowest concentration of the antimicrobial agent that killed *C. albicans*. MLC values are in % (*v*/*v*) and in brackets are the equivalent MLC values in (g/L). NA = no antimicrobial activity at tested concentrations.

2.2. Minimal Biofilm Eradication Concentration 80

The antifungal activity of biocides and commercial essential oils against *C. albicans* biofilms was expressed as the minimal biofilm eradication concentration (MBEC) [29]. Most test agents were not active against biofilms at tested concentrations and did not prevent regrowth after removal of the antimicrobial (Table 3). The antimicrobials that exhibited an MBEC against both tested *C. albicans* strains were melissa geranium, E-cinnamaldehyde and linalool (Table 3).

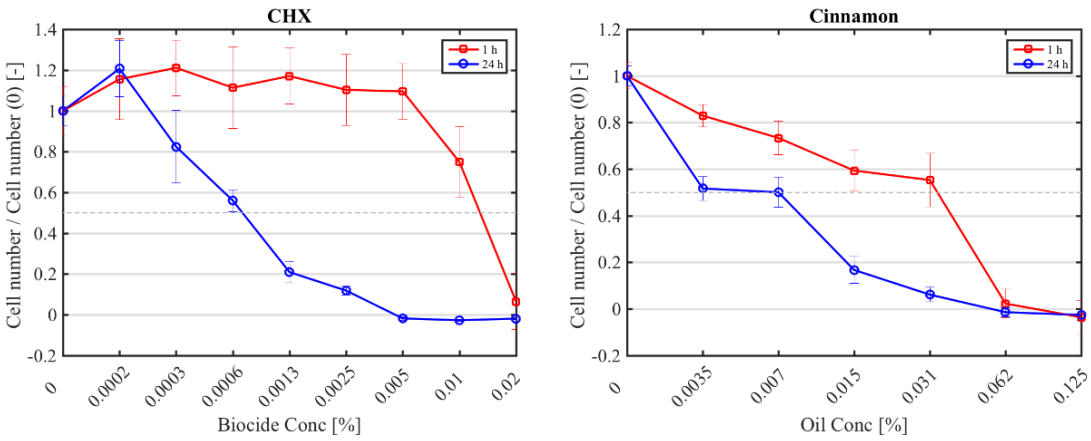
Table 3. Minimal biofilm eradication concentration 80 of commercial essential oils and biocides against *C. albicans* NCYC 1363 and *C. albicans* 135BM2/94.

Antimicrobial	Minimal Biofilm Eradication Concentration 80 [% (*v*/*v*)] [(g/L)]	
	***C. albicans* NYCY 1363**	***C. albicans* 135BM2/94**
Basil	NA	NA
Bergamot	NA	NA
Cinnamon	NA	NA
Citronella	NA	NA
Geranium	2.5 (22.3)	2 (17.9)
Lavender	NA	NA
Melissa	1.5 (13.3)	1.5 (13.3)
Myrtle	NA	NA
Peppermint	NA	NA
Sage	NA	NA
Spearmint	NA	NA
Tea tree oil	NA	NA
E-cinnamaldehyde	0.8 (8.4)	0.8 (8.4)
Linalool	1 (8.7)	1.5 (13.1)
CHX	0.07	NA
Triclosan	$>5 \times 10^{-3}$ (7.45×10^{-2})	$>5 \times 10^{-3}$ (7.45×10^{-2})

Minimal biofilm eradication concentration 80 (MBEC80) defined as the lowest antimicrobial concentration that prevented at least 80% regrowth of *Candida*, after the biofilm was treated with antimicrobials for 24 h. MBEC values are in % (*v*/*v*) and in brackets are the equivalent MBEC values in (g/L). NA = no antimicrobial activity at tested concentrations.

2.3. Half Maximal Inhibitory Concentration (IC50) against Fibroblasts

The half maximal inhibitory concentration (IC50) CHX, cinnamon, E-cinnamaldehyde, geranium and melissa on fibroblast proliferation after a 1 h and 24 h exposure was determined (Figure 1; Table 4). The highest cytotoxicity occurred with E-cinnamaldehyde, followed by geranium ($p < 0.0001$), which halved proliferation even at the lowest concentration tested. Indeed, a concentration of 0.003% (*v*/*v*) E-cinnamaldehyde and 0.01% (*v*/*v*) geranium inhibited 50% of cell proliferation (Table 4). Melissa was the least cytotoxic commercial essential oil, halving proliferation at 0.03% (*v*/*v*) ($p < 0.0001$). A 1 h exposure of fibroblasts to cinnamon resulted in similar cytotoxicity as melissa but prolonged exposure led to higher cytotoxicity ($p < 0.0001$). A 1 h application of CHX was cytotoxic only at the highest concentration tested (IC50 of 0.01% (*v*/*v*)) which was higher than the MIC, while a 24 h exposure at 7×10^{-4}% (*v*/*v*) was sufficient to halve fibroblast proliferation.



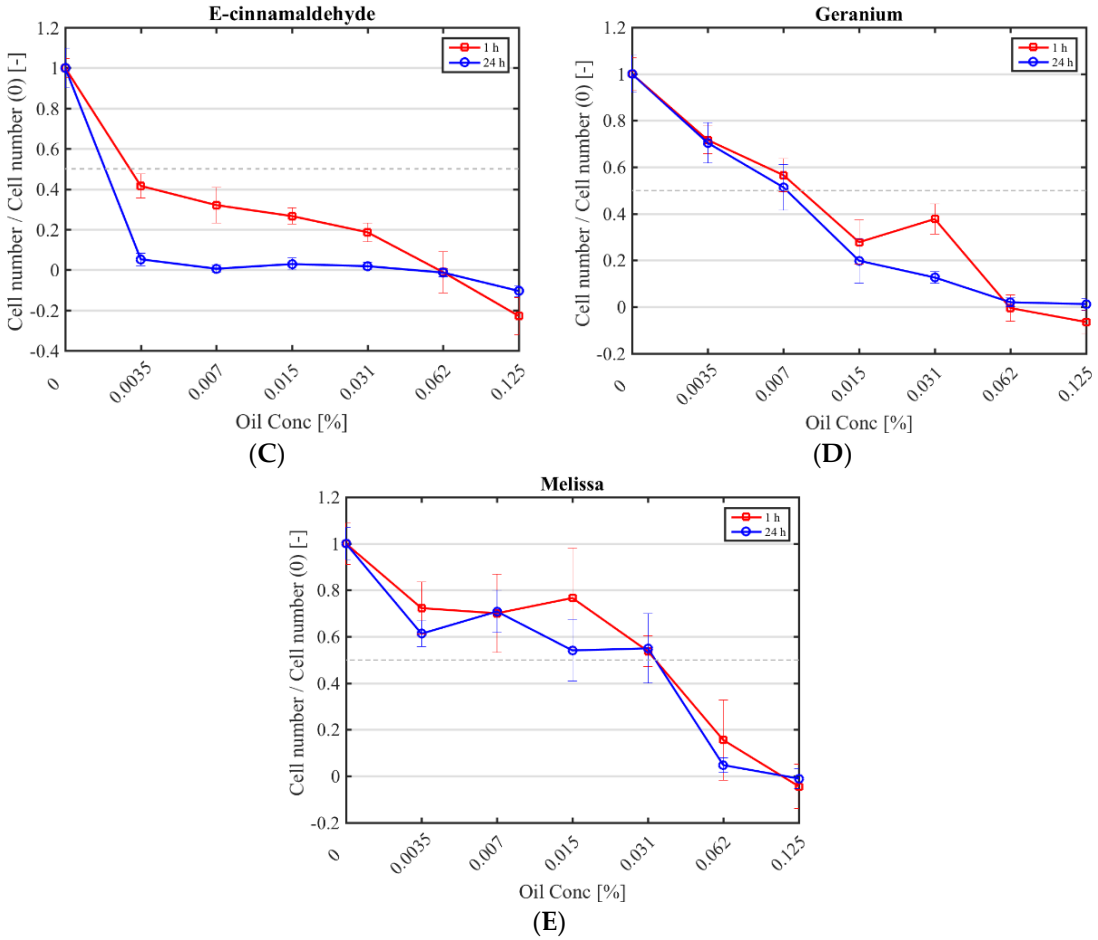


Figure 1. Cytotoxicity of selected antimicrobials against murine fibroblasts. Fibroblast numbers (normalised by the control (0% (*v*/*v*) antimicrobial) after a 1 h (red square) and 24 h application (blue circle) of CHX (**A**); cinnamon (**B**); E-cinnamaldehyde (**C**); geranium (**D**) and melissa (**E**).

Table 4. Half maximal inhibitory concentration (IC50) against fibroblasts after 1 h and 24 h application of the antimicrobial.

Antimicrobial	Half Maximal Inhibitory Concentration [% (*v*/*v*)] [(g/L)]	
	1 h	24 h
Cinnamon	0.03 (0.36)	0.01 (0.11)
Geranium	0.01 (0.08)	0.01 (0.07)
Melissa	0.03 (0.3)	0.03 (0.3)
E-cinnamaldehyde	0.003 (0.03)	0.002 (0.02)
CHX	0.01 (0.15)	7.32×10^{-4} (0.008)

Half maximal inhibitory concentration (IC50) defined as the antimicrobial concentration that inhibits the 50% of cell proliferation compared to controls without agent. IC50 values are in % (*v*/*v*) and in brackets are the equivalent IC50 values in (g/L).

3. Discussion

Essential oils are natural products often extracted from plants and they frequently exhibit antimicrobial, anti-aseptic, anti-inflammatory and anti-oxidant activities. The primary aim of this research was to evaluate the antifungal activity of 12 commercial essential oils against *C. albicans*. All tested commercial essential oils demonstrated antifungal activity against planktonic *C. albicans*, with MICs ranging from 0.06% (*v*/*v*) to 0.4% (*v*/*v*) and MLCs from 0.1% (*v*/*v*) to 1% (*v*/*v*). Comparison of results with those of other studies is problematic given differences in assay techniques [30,31]. In addition, the botanical source, climate and environmental conditions, time

of harvesting and extraction method can affect both composition and antimicrobial activity of commercial essential oils [31–33].

The effect of plant origin on antimicrobial properties can be appreciated by comparing the activity of cinnamon oil extracted from *Cinnamomum zeylanicum* leaves and *Cinnamomum aromaticum* leaves. Both types of cinnamon oils are from the evergreen cinnamomum plant but *Cinnamomum aromaticum* extract contains a higher amount of E-cinnamaldehyde, which could explain the higher antifungal activity (MICs 0.0006% (*v*/*v*)–0.0096% (*v*/*v*)) [32] compared to the present study using *Cinnamomum zeylanicum* (MIC 0.1% (*v*/*v*)) extract. The impact that the amount of E-cinnamaldehyde has on antifungal properties of an essential oil was also evident in this study (MICs of 0.03% (*v*/*v*) and 0.01% (*v*/*v*)). Geranium and melissa oils exhibited highest antifungal potential. Both commercial oils contain geraniol and citronellol, which are antifungal [34] and likely responsible for the similar antifungal activity of these oils ($p > 0.90$). However, the MIC of melissa oil was lower than that previously reported [35,36]. This present study revealed antifungal effects for bergamot oil (MIC of 0.3% (*v*/*v*) and MLC of 0.5% (*v*/*v*)) which has previously only had limited attention. The MIC of basil oil 0.1% (*v*/*v*) (0.9 g/L) was lower than previously reported, namely 0.5% (*v*/*v*) [30] and 0.312% (*v*/*v*) [32] but comparable to the MIC (1250 μg/mL) found against a fluconazole resistant *C. albicans* strain [15]. The main compound of basil and lavender oils is linalool, which previously has had MICs ranging from 0.06% (*v*/*v*) to 0.12% (*v*/*v*) [37]. Comparing activity of pure linalool to those of basil and lavender oils, the anticandidal activity of terpene was not significantly higher than that of basil ($p > 0.99$). Tea tree oil had an MIC of 0.2% (*v*/*v*) and this was similar to that recorded by Hammer et al. against *C. albicans* [38]. Sage oil exhibited MICs of 0.3% (*v*/*v*) (2.7 g/L) and 0.4% (*v*/*v*) (3.7 g/L), which were comparable to the MIC of 2.78 g/L reported using a disk diffusion method [39] but lower than the MIC of 1.32 mg/mL measured by broth microdilution assay [40]. Despite their differences in composition, peppermint and spearmint oils had similar antifungal activities with MICs of 0.1% (*v*/*v*) and 0.1% (*v*/*v*)–0.2% (*v*/*v*), respectively ($p > 0.07$). However, while the MICs of spearmint oil were similar to those reported by Hammer et al. [30], the MIC of peppermint oil was higher than that found by Those et al. [41]. Myrtle oil had the lowest antifungal potential, even though its MICs were lower than those previously reported by Mahboubi et al. (MIC of 0.8–1.6% (*v*/*v*)) [42]. CHX and triclosan, two biocides whose antimicrobial properties are widely recognised and both commonly added to mouthwashes and toothpastes, were also evaluated in this study. Triclosan exhibited fungistatic activity only at concentrations higher than those used in toothpaste formulations (0.3% (*w*/*v*) [43]) but did not exhibit fungicidal effects at tested concentrations.

The majority of agents had limited antibiofilm activity. Bacteria in biofilms can be between 10 and 1000 times more tolerant to antibiotics than their planktonic counterparts and similar findings have been reported for *Candida* [44]. The mechanisms by which biofilm cells have elevated antimicrobial tolerance are complex and likely multifactorial. These include altered gene expression following surface attachment, reduced growth rates in biofilms, variable nutrient availability that induces changes in phenotype and the presence of extracellular polymeric substances that impedes penetration of agents into the biofilm [45]. Few studies have previously reported activity of commercial essential oils or biocides against *C. albicans* biofilms [46,47]. In the present study, from melissa oil, geranium oil, E-cinnamaldehyde and linalool all had anti-biofilm activity, whilst CHX only had anti-biofilm activity against *C. albicans* NCYC 1363. A 3 min application of cinnamon (1 mg/mL) and citronella (1 mg/mL) oils has been found to reduce biofilm cell numbers immediately after treatment but this effect was not evident 48 h post treatment [46]. These results concur with the current study, where no antibiofilm activity was noted for cinnamon and citronella oils after 24 h. An MBEC of tea tree oil of 12.5% (*v*/*v*) had previously been reported [47], which is a higher concentration (8% (*v*/*v*)) than tested in this study, as difficulties were encountered in forming a stable suspension of the oil-medium using 1% (*v*/*v*) Tween 80.

Few studies have investigated the cytotoxic effects of these oils. Cytotoxicity of CHX, cinnamon, E-cinnamaldehyde, geranium and melissa oils had a dose- and time-dependent

cytotoxicity. Overall, the commercial essential oils halved fibroblast proliferation at concentrations lower than their MICs. The IC50 values for E-cinnamaldehyde, geranium and cinnamon oils were actually 10-fold lower than their MIC 80, while melissa oil had an MIC 80 of 0.06% (*v*/*v*) and an IC50 of 0.03% (*v*/*v*). Although a different assay and cell type was used, the melissa oil results (IC50 0.3 g/L) were in accordance with those of Paul et al. [48] who did not see a significant change in leukocytes viability after 3 h treatment with 150 µg/mL melissa oil. Several studies have used E-cinnamaldehyde to inhibit proliferation of cancer cells and reported IC50s ranging from 45.8 to 129.4 mM [49], higher than those obtained in this study with fibroblasts (0.16–0.26 mM). Barros et al. found that at concentrations lower than those evaluated in this study (5 µg/mL), *Cinnamomum zeylanicum* oil had cytoxicity towards erythrocytes [50]. A 1 h exposure of fibroblasts to CHX (0.01% (*v*/*v*)) halved cell proliferation compared to controls. However, this concentration was lower than the MICs (2.5×10^{-3}% (*v*/*v*) and 5×10^{-3}% (*v*/*v*)) found in the current study. This finding was similar to the cytotoxic effect of CHX previously reported using macrophages [51] and human alveolar bone cells [52]. Even if these results showed that commercial essential oils were cytotoxic, it should be taken into account that cytotoxicity was conducted in 2D culture, which is notably different from in *vivo* conditions. Further investigation on mammalian cells could be performed in 3D culture or *ex/in vivo* models to better mimic the biological structure of the tissues.

4. Materials and Methods

4.1. Essential Oils and Biocides Preparation

Twelve commercial essential oils (Essential Oils Direct Ltd., Oldham, UK) (Table 5), two terpenes (E-cinnamaldehyde and linalool (Sigma-Aldrich, Gillingham, UK)), chlorhexidine digluconate (CHX) (Sigma-Aldrich, Gillingham, UK) and triclosan (Irgasan from Sigma-Aldrich, Gillingham, UK) were evaluated.

Table 5. List of commercial essential oils tested.

Plant Species	Essential Oil	Origin
Ocimum basilicum	Basil oil	Leaves
Citrus bergamia	Bergamot FCF oil	Peel
Cinnamomum zeylanicum	Cinnamon leaf oil	Leaves
Cymbopogon winterianus	Citronella oil	Aerial parts
Pelargonium graveolens	Geranium oil	Flowering herb
Lavandula angustifolia	Lavender oil	Flowering herb
Melissa officinalis	Melissa oil	Leaves and tops
Myrtus communis	Myrtle oil	Leaves
Mentha piperita	Peppermint oil	Whole plant
Salvia officinalis	Sage oil	Leaves
Mentha spicata	Spearmint oil	Aerial parts
Melaleuca alternifolia	Tea tree oil	Leaves and twigs

The commercial essential oils were tested at a range of concentrations against planktonic growth (2% (*v*/*v*) to 0.007% (*v*/*v*) and biofilms (8% (*v*/*v*) to 0.125% (*v*/*v*)). All agents were prepared in Sabouraud Dextrose Broth (SDB; Oxoid Ltd, Basingstoke, UK). To enhance dispersion of essential oils in the medium, 1% (*v*/*v*) Tween 80 (Sigma-Aldrich, Gillingham, UK) was added. In the case of biofilm studies, 0.015% (*w*/*v*) Agar Bacteriological (LP0011; Oxoid Ltd, Basingstoke, UK) was added to SDB [53]. CHX was used in SDB at concentrations between 0.04% (*v*/*v*) to 3.1×10^{-4}% (*v*/*v*) and from 0.08% (*v*/*v*) to 6.2×10^{-4}% (*v*/*v*) for planktonic and biofilm growth experiments, respectively. A 20% (*w*/*v*) stock solution of triclosan was prepared in Dimethyl Sulfoxide (DMSO) (Fisher Scientific, Loughborough, UK). Serial doubling dilutions of the stock solution were prepared in SDB yielding final concentrations from 5.2×10^{-6}% (*v*/*v*) to 6.7×10^{-4}%

(*v*/*v*) and from 1.7 × 10^{-4}% (*v*/*v*) to 5 × 10^{-3} (*v*/*v*) for planktonic and biofilm experiments, respectively.

4.2. Microorganisms

Candida albicans NYCY 1363 and *C. albicans* 135BM2/94 were used to assess antifungal activity of commercial essential oils and biocides. *Candida albicans* 135BM2/94 is a clinical strain from the School of Dentistry (Cardiff University, Cardiff, UK), which has been described as a high invader of tissues [54]. Strains were subcultured onto Sabouraud Dextrose Agar (SDA) (CM0041; Oxoid Ltd, Basingstoke, UK) and grown at 37 °C in an aerobic incubator for 24 h. A colony of *C. albicans* was inoculated in 20 mL of SDB and incubated aerobically with shaking (150 rev/min) overnight at 37 °C. The overnight culture was prepared in SDB to a turbidity equivalent to a 0.5 McFarland Standard and used for further experiments.

4.3. Minimum Inhibitory Concentration and Minimal Lethal Concentration

The minimum inhibitory concentration (MIC) and the minimal lethal concentration (MLC) were determined using a broth microdilution assay. The method was adapted from that previously reported by Malic et al. [29]. Briefly, 100 µL of antimicrobial and 100 µL of overnight culture diluted to 1 × 10^5 CFU/mL were added to the wells of 96-well microtitre plates (Thermo Fisher Scientific, Hemel Hempstead, UK). Controls included *Candida* suspension cultured in SDB, with or without 0.5% (*v*/*v*) of Tween 80. In addition, when triclosan was tested, SDB containing 1% (*v*/*v*) DMSO was used as control. The plates were covered with the lids supplied by the manufacturer and sprayed with 3% (*v*/*v*) of Triton 100-X (Sigma-Aldrich, Gillingham, UK) in pure ethanol to reduce condensation. The plates were incubated aerobically at 37 °C with shaking at 110 rpm, for 24 h. Growth was estimated by measuring turbidity of each well by spectrophotometric absorbance at 620 nm (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer), shaking 3 s before the reading. The absorbance readings were standardised against microbial-free controls. The minimal inhibitory concentration 80 (MIC 80) was defined as the lowest concentration of the antimicrobial agent that showed at least 80% reduction in absorbance compared to the control. The MLC was determined by plating selected well contents (where no visible growth was evident) on to SDA and incubating for 24 h at 37 °C. The MLC was defined as the lowest concentration of antimicrobial agent that killed the *Candida* as shown by no colony growth on SDA. All concentrations were tested in quadruplicate and on three separate occasions.

4.4. Minimal Biofilm Eradication Concentration 80

The minimal biofilm eradication concentration (MBEC) method was adapted from Malic et al. (2013) [29]. Briefly, a 96-well microtitre plate containing 200 µL of an overnight culture diluted at 1 × 10^5 CFU/mL was incubated for 48 h at 37 °C without agitation to allow biofilm formation. Controls included *Candida* suspension cultured in SDB, with or without 1% (*v*/*v*) of Tween 80 and 0.015% (*w*/*v*) Agar Bacteriological. When triclosan was tested, SDB containing 8% (*v*/*v*) DMSO was also used as control. After 48 h, the SDB was removed and the microtitre plate inverted onto tissue paper to remove residual medium. The biofilm was washed three times with 100 µL of PBS. One hundred µl of test agent was added to the biofilm and the plate incubated statically for 24 h at 37 °C. After incubation, test agent was removed and the biofilm washed twice with 100 µL of PBS. Two hundred µL of SDB was added to each well and the biofilm disrupted by repeated pipetting. The three replicates were then pipetted into a microcentrifuge tube which was then centrifuged for 3 min at 3000 rev/min (Hettich Universal Mikro 12-24, Hettich, Tuttlingen, Germany). The supernatant containing residual test agent was discarded and the microorganisms resuspended in fresh SDB and three wells of a 96-well plate were inoculated with the suspension. The turbidity of the suspension was measured by spectrophotometer absorbance at 620 nm prior to and after incubation for 24 h at 37 °C with shaking at 110 rev/min. The minimal biofilm

eradication concentration 80 (MBEC80) was defined as the lowest antimicrobial concentration that prevented at least 80% regrowth of *Candida*. All experiments were conducted on three separate occasions.

4.5. Half Maximal Inhibitory Concentration

Mouse fibroblasts (NIH 3T3; Sigma-Aldrich, Gillingham, UK) were cultured in Dulbecco Modified Eagle Medium (DMEM) (Sigma-Aldrich, Gillingham, UK) supplemented with 10% (*v*/*v*) foetal bovine serum (FBS) (Life Technologies, Paisley, UK), 1% (*v*/*v*) penicillin/streptomycin (Sigma-Aldrich, Gillingham, UK) and 1% (*v*/*v*) L-glutamine (Sigma-Aldrich, Gillingham, UK). Serial doubling dilutions of commercial essential oils and biocides were prepared in the fibroblast culture medium at final concentrations ranging from 0.25% to 0.007% (*v*/*v*) for the commercial essential oils and from 0.04% to 3×10^{-4}% (*v*/*v*) for chlorhexidine. Fibroblasts were harvested using trypsin EDTA (EDTA 0.25% (*w*/*v*), Trypsin 0.53 mM, Thermo Fisher Scientific, Hemel Hempstead, UK) and diluted to a density of 5×10^5 cells/mL. One-hundred µl of the cell suspension was used to inoculate a 96-well plate (5×10^4 cells per well) which was then incubated at 37 °C and 5% CO_2 for 1.5 h. A 100-µL volume of the antimicrobial was then added. After 1 and 24 h, the medium was removed and the cells washed twice with 100 µL of PBS. Three hundred µL of DMEM containing 10% (*v*/*v*) of alamarBlue (AlamarBlue Cell Viability Reagent, Invitrogen, Paisley, UK) was added to each well and the plate incubated for 1.5 h. Fluorescence was read with a Synergy HT plate reader (BioTek® Instruments, Winooski, VT, USA) with excitation and emission wavelengths of 545 nm and 590 nm, respectively. The half maximal inhibitory concentration (IC50) was defined as the antimicrobial concentration that inhibited 50% cell proliferation compared to the control (i.e. DMEM without antimicrobial agent). Each condition was studied in triplicate and on three separate occasions.

4.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 7.0. Data were presented as arithmetic mean ± SD. The difference between treatments was statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. Statistically significant differences were set at $p < 0.05$.

5. Conclusions

This study showed that all the twelve commercial essential oils, two terpenes and triclosan and CHX had antifungal activity against planktonic *C. albicans*. Six of these compounds (CHX, cinnamon, E-cinnamaldehyde, linalool, geranium and melissa) were also active against *C. albicans* biofilms, which are usually challenging to effectively inhibit. Cytotoxicity screening revealed that the commercial essential oils halved fibroblast proliferation at concentrations lower than those required to inhibit *C. albicans* growth. Further investigation on the effect of these agents against mammalian cells is however warranted before any in *vivo* application. The antifungal potential of these essential oils could be a future therapeutic for topical candidosis as an option to overcome emerging antifungal drug resistance.

Author Contributions: Elisa Serra performed all the experiments, analysed the data and wrote a draft of the paper; Araida Hidalgo-Bastida designed the experiments related to cell culture (cytotoxicity work), reviewed and revised the draft manuscript; Sladjana Malic, Joanna Verran and David Williams drafted the work, designed the experiments related to the microbiology work, oversaw the laboratory work, reviewed and revised the draft manuscript; All authors approve the final manuscript for submission.

References

1. Muzyka, B.C. Oral fungal infections. *Dent. Clin. N. Am.* **2005**, *49*, 49–65.
2. Palmer, G.D.; Robinson, P.G.; Challacombe, S.J.; Birnbaum, W.; Croser, D.; Erridge, P.L.; Hodgson, T.; Lewis, D.; McLaren, A.; Zakrzewska, J.M. Aetiological factors for oral manifestations of HIV. *Oral Dis.*

1996, *2*, 193–197.
3. McCarthy, G.M.; Mackie, I.D.; Koval, J.; Sandhu, H.S.; Daley, T.D. Factors associated with increased frequency of HIV-related oral candidiasis. *J. Oral Pathol. Med.* **1991**, *20*, 332–336.
4. Farah, C.S.; Ashman, R.B.; Challacombe, S.J. Oral candidosis. *Clin. Dermatol*. **2000**, *18*, 553–562.
5. Gleiznys, A.; Zdanavičienė, E.; Žilinskas, J. Candida albicans importance to denture wearers. A literature review. *Stomatologija* **2015**, *17*, 54–66.
6. Yasui, M.; Ryu, M.; Sakurai, K.; Ishihara, K. Colonisation of the oral cavity by periodontopathic bacteria in complete denture wearers. *Gerodontology* **2012**, *29*, 494–502.
7. Douglass, C.W.; Shih, A.; Ostry, L. Will there be a need for complete dentures in the United States in 2020? *J. Prosthet. Dent*. **2010**, *87*, 5–8.
8. Sardi, J.C.O.; Scorzoni, L.; Bernardi, T.; Fusco-Almeida, A.M.; Mendes Giannini, M.J. Candida species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* **2016**, *62*, 10–24.
9. Williams, D.; Lewis, M. Pathogenesis and treatment of oral candidosis. *J. Oral Microbiol.* **2011**, *1*, 1–11.
10. Boros-Majewska, J.; Salewska, N.; Borowski, E.; Milewski, S.; Malic, S.; Wei, X.Q.; Hayes, A.J.; Wilson, M.J.; Williams, D.W. Novel Nystatin A1 derivatives exhibiting low host cell toxicity and antifungal activity in an in vitro model of oral candidosis. *Med. Microbiol. Immunol*. **2014**, *203*, 341–355.
11. Nazzaro, F.; Fratianni, F.; De Martino, L.; Coppola, R.; De Feo, V. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* **2013**, *6*, 1451–1474.
12. Sikkematb, J.; Bontt, J.A.M. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **1994**, *269*, 8022–8028.
13. Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. Biological effects of essential oils—A review. *Food Chem. Toxicol.* **2008**, *46*, 446–475.
14. Adil, M.; Singh, K.; Verma, P.K.; Khan, A.U. Eugenol-induced suppression of biofilm-forming genes in Streptococcus mutans: an approach to inhibit biofilms. *J. Glob. Antimicrob. Resist.* **2014**, *2*, 286–292.
15. Cardoso, N.N.R.; Alviano, C.S.; Blank, A.F.; Romanos, M.T.V; Fonseca, B.B.; Rozental, S.; Rodrigues, I.A.; Alviano, D.S. Synergism effect of the essential oil from Ocimum basilicum var. Maria bonita and its major components with fluconazole and its influence on ergosterol biosynthesis. *Evid. Based Complement Altern. Med.* **2016**, *2016*, 5647182, doi:10.1155/2016/5647182.
16. Rajput, S.B.; Karuppayil, S.M. Small molecules inhibit growth, viability and ergosterol biosynthesis in Candida albicans. *Springerplus* **2013**, *2*, 1–6.
17. Samber, N.; Khan, A.; Varma, A.; Manzoor, N. Synergistic anti-candidal activity and mode of action of Mentha piperita essential oil and its major components. *Pharm. Biol*. **2015**, *53*, 1496–1504.
18. Shreaz, S.; Wani, W.A.; Behbehani, J.M.; Raja, V.; Karched, M.; Ali, I.; Siddiqi, W.A.; Ting, L. Cinnamaldehyde and its derivatives, a novel class of antifungal agents. *Fitoterapia* **2016**, *112*, 116–131.
19. de Castro, R.D.; Lima, E.O. Anti-Candida activity and chemical composition of Cinnamomum zeylanicum blume essential oil. *Braz. Arch. Biol. Technol.* **2013**, *56*, 749–755.
20. Bang, K.; Lee, D.; Park, H.; Rhee, Y. Inhibition of fungal cell wall synthesizing enzymes by trans-cinnamaldehyde. *Biosci. Biotechnol. Biochem*. **2000**, *64*, 1061–1063.
21. Cox, S.D.; Mann, C.M.; Markham, J.L.; Bell, H.C.; Gustafson, J.E.; Warmington, J.R.; Wyllie, S.G. The mode of antimicrobial action of the essential oil of Melaleuca alternifolia (Tea tree oil). *J. Appl. Microbiol.* **2000**, *88*, 170–175.
22. Rajkowska, K.; Nowak, A.; Kunicka-styczy, A.; Siadura, A. Biological effects of various chemically characterized essential oils: investigation of the mode of action against Candida albicans and HeLa cells. *RSC Adv.* **2016**, *6*, 97199–97207.
23. Singh, S.; Fatima, Z.; Hameed, S. Citronellal-induced disruption of membrane homeostasis in Candida albicans and attenuation of its virulence attributes. *Rev. Soc. Bras. Med. Trop.* **2016**, *49*, 465–472.
24. Gyawali, R.; Ibrahim, S.A. Natural products as antimicrobial agents. *Food Control* **2014**, *46*, 412–429.
25. Morais-Braga, M.F.B.; Sales, D.L.; Carneiro, J.N.P.; Machado, J.T.; Thassya, A.; Santos, L.; de Freitas, M.A.; Matos, D.; Tintino, S.R.; Souza, D.S.L.; et al. Psidium guajava L. and Psidium brownianum Mart ex DC.: chemical composition and anti—Candida effect in association with fluconazole. *Microb. Pathog.* **2016**, *95*, 200–207.
26. Santos, K.K.; Matias, E.F.; Souza, C.E.; Tintino, S.R.; Braga, M.F.; Guedes, G.M.; Nogueira, L.F.; Morais, E.C.; Costa, J.G.; Menezes, I.R.; et al. Anti-Candida activity of Mentha arvensis and Turnera ulmifolia.

J. Med. Food **2012**, *15*, 322–324.
27. Greenstein, G.; Berman, C.; Jaffin, R. Chlorhexidine. An adjunct to periodontal therapy. *J. Periodontol*. **1986**, *57*, 370–377.
28. Cullinan, M.P.; Westerman, B.; Hamlet, S.M.; Palmer, J.E.; Faddy, M.J.; Seymour, G.J. The effect of a triclosan-containing dentifrice on the progression of periodontal disease in an adult population. *J. Clin. Periodontol.* **2003**, *30*, 414–419.
29. Malic, S.; Emanuel, C.; Lewis, M.A.; Williams, D.W. Antimicrobial activity of novel mouthrinses against planktonic cells and biofilms of pathogenic microorganisms. *Microbiol. Discov.* **2013**, *1*, 11, doi:10.7243/2052-6180-1-11.
30. Hammer, K.A.; Carson, C.F.; Riley, T.V. Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* **1999**, *86*, 985–990.
31. Janssen, A.M.; Scheffer, J.C.; Svendsen, A.B. Antimicrobial activity of essential oils: a 1976–1986 literature review. Aspects of the test methods. *Planta Med.* **1986**, *53*, 395–398.
32. Szweda, P.; Gucwa, K.; Kurzyk, E.; Romanowska, E. Essential oils, silver nanoparticles and propolis as alternative agents against fluconazole resistant Candida albicans, Candida glabrata and Candida krusei clinical isolates. *Indian J. Microbiol*. **2015**, *55*, 175–183.
33. Carvalhinho, S.; Margarida, A.; Sampaio, A. Susceptibilities of Candida albicans mouth isolates to antifungal agents, essentials oils and mouth rinses. *Mycopathologia* **2012**, *174*, 69–76.
34. Zore, G.B.; Thakre, A.D.; Rathod, V.; Karuppayil, S.M. Evaluation of anti-Candida potential of geranium oil constituents against clinical isolates of Candida albicans differentially sensitive to fluconazole: Inhibition of growth, dimorphism and sensitization. *Mycoses* **2010**, *54*, 99–109.
35. Mimica-Dukic, N.; Bozin, B.; Sokovic, M.; Simin, N. Antimicrobial and antioxidant activities of Melissa officinalis L. (Lamiaceae) essential oil. *J. Agric. Food Chem*. **2004**, *52*, 2485–2489.
36. Abdellatif, F.; Boudjella, H.; Zitouni, A.; Hassani, A. Chemical composition and antimicrobial activity of the essential oil from leaves of algerian. *Exp. Clin. Sci. J.* **2014**, *13*, 772–781.
37. Marcos-Arias, C.; Eraso, E.; Madariaga, L.; Quindós, G. In vitro activities of natural products against oral Candida isolates from denture wearers. *BMC Complement Altern. Med*. **2011**, *11*, 119, doi:10.1186/1472-6882-11-119.
38. Hammer, K.A.; Carson, C.F.; Riley, T.V. In vitro activity of essential oils, in particular Melaleuca alternifolia (tea tree) oil and tea tree oil products, against Candida spp. *J. Antimicrob. Chemother.* **1998**, *42*, 591–595.
39. Sookto, T.; Srithavaj, T.; Thaweboon, S.; Thaweboon, B.; Shrestha, B. In vitro effects of Salvia officinalis L. essential oil on Candida albicans. *Asian Pac. J. Trop. Biomed*. **2013**, *3*, 376–380.
40. Nacsa-Farkas, E.; Kerekes, E.; Kerekes, E.B.; Krisch, J.; Roxana, P.; Vlad, D.C.; Ivan, P.; Vágvölgyi, C. Antifungal effect of selected European herbs against Candida albicans and emerging pathogenic non-albicans Candida species. *Acta Biol. Szeged*. **2014**, *58*, 61–64.
41. Thosar, N.; Basak, S.; Bahadure, R.N.; Rajurkar, M. Antimicrobial efficacy of five essential oils against oral pathogens: An in vitro study. *Eur. J. Dent*. **2013**, *7*, doi:10.4103/1305-7456.119078.
42. Mahboubi, M.; Bidgoli, F.G. Phytomedicine In vitro synergistic efficacy of combination of amphotericin B with Myrtus communis essential oil against clinical isolates of Candida albicans. *Phytomedicine* **2010**, *17*, 771–774.
43. Brading, M.G.; Cromwell, V.J.; Green, A.K.; Debrabander, S.; Beasley, T. The role of Triclosan in dentifrice formulations, with particular reference to a new 0.3% Triclosan calcium carbonate-based system. *Int. Dent. J*. **2004**, *54*, 291–298.
44. Hawser, S.P.; Douglas, L.J. Resistance of Candida albicans biofilms to antifungal agents in vitro. *Antimicrob. Agents Chemother.* **1995**, *39*, 2128–2131.
45. Douglas, L.J. Candida biofilms and their role in infection. *Trends Microbiol*. **2003**, *11*, 30–36.
46. de Almeida, Lde.F.D; Paula, J.F.; Almeida, R.V.; Williams, D.W.; Hebling, J.; Cavalcanti, Y.W. Efficacy of citronella and cinnamon essential oils on Candida albicans biofilms. *Acta Odontol. Scand*. **2016**, *74*, 393–398.
47. de Campos Rasteiro, V.M.; da Costa, A.C.; Araújo, C.F.; de Barros, P.P.; Rossoni, R.D.; Anbinder, A.L.; Jorge, A.O.; Junqueira, J.C. Essential oil of Melaleuca alternifolia for the treatment of oral candidiasis induced in an immunosuppressed mouse model. *BMC Complement. Altern. Med.* **2014**, *14*, 489–499.
48. Paul, J.; Adeniran, A.; Augusti, A.; Vargas, C.; Olalekan, O.; Hassan, W.; Ibrahim, M.; Pansera, E.;

Francine, D. Antioxidant activity, genotoxicity and cytotoxicity evaluation of lemon balm (Melissa officinalis L.) ethanolic extract: Its potential role in neuroprotection. *Ind. Crop. Prod.* **2013**, *51*, 26–34.
49. Ka, H.; Park, H.; Jung, H.; Choi, J.; Cho, K.; Ha, J.; Lee, K. Cinnamaldehyde induces apoptosis by ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells. *Cancer Lett.* **2003**, *196*, 143–152.
50. Barros, F.J.; Costa, R.J.O.; Cesário, F.R.A.S.; Rodrigues, L.B.; de Menezes, I.R.A.; da Costa, J.G.M.; Coutinho, H.D.M.; Galvao, H.B.F. Activity of essential oils of Piper aduncum anf and Cinnamomum zeylanicum by evaluating osmotic and morphologic fragility of erythrocytes. *Eur. J. Integr. Med*. **2016**, *8*, 505–512.
51. Li, Y.C.; Kuan, Y.H.; Lee, S.S.; Huang, F.M. Cytotoxicity and genotoxicity of chlorhexidine on macrophages in vitro. *Environ. Toxicol.* **2014**, *29*, 452–458.
52. Cabral, C.T.; Fernandes, M.H. In vitro comparison of chlorhexidine and povidone-iodine on the long-term proliferation and functional activity of human alveolar bone cells. *Clin. Oral Investig*. **2007**, *11*, 155–164.
53. Mann, C.M.; Markham, J.L. A new method for determining the minimum inhibitory concentration of essential oils. *Appl. Microbiol*. **1998**, *84*, 538–544.
54. Malic, S.; Hill, K.E.; Ralphs, J.R.; Hayes, A.; Thomas, D.W.; Potts, A.J.; Williams, D.W. Characterization of Candida albicans infection of an in vitro oral epithelial model using confocal laser s canning microscopy. *Microbiol. Immunol*. **2007**, *22*, 188–194.