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The bioactivity of Fynbos honey compared with Manuka honey in a salt- and enzyme-based synthetic saliva

by

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Supervisor: Prof MJ Bester

Co-supervisor: Dr JC Serem

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Abstract

Candida albicans (C. albicans) is a common fungus that can cause oral candidiasis; especially in immune-compromised patients. Honey has previously shown anti-fungal, antioxidant and anti-inflammatory activities which may have beneficial effects in the oral cavity. The bioactivity of Fynbos honeys, a Manuka honey and oral digests was determined.

In this laboratory-based study, four Fynbos (FB) honey samples and a Manuka honey (MAN UMF10+) were incubated with a synthetic saliva for 2 min to simulate oral digestion. For each honey, three samples were generated; undigested, oral digest control and oral digest.

The ability of these samples to inhibit the growth of *C. albicans* (Strain 90028 (ATCC® 90028™)) was determined. Concentration ranges of 5 - 9% and 25 - 45% did not inhibit the growth of planktonic cells. Solutions of 7 - 9% FB1 and 45% FB2 and FB3, 40 and 45% FB4 as well as 30 - 35% MAN UMF 10+ honey inhibited the growth of *C. albicans* biofilms. With oral digestion, inhibition was unaltered. Although exposure did not alter viability, scanning electron microscopy analysis of cells in *C. albicans* biofilms exposed to 5% FB and MAN UMF10+ honey for 24 h revealed morphological changes with an increase in round/oval yeast cells instead of pseudohyphae or true hyphae associated with biofilms.

The ability to reduce the oxidative damage often associated with candidiasis firstly, the total polyphenolic content and then the antioxidant activity with the Trolox equivalent antioxidant capacity and oxygen radical absorbance capacity assays was determined. Nitric oxide (NO) scavenging activity was determined with the sodium nitroprusside assay. The ability of each honey to protect bovine serum albumin (BSA) and low density lipoprotein (LDL) against MGO and reactive oxygen species (ROS)-mediated oxidative damage was also determined. All honeys contained comparable amounts of polyphenols, scavenged ROS and reduced NO levels indicating antioxidant related bioactivity. Each honey protected BSA, but not LDL against oxidative damage. Oral digestion caused minor changes in the measured parameters.

Further studies were undertaken in cellular models. Honey solutions of 1.25 - 5% did not alter the cell number and viability of L929 (murine fibroblasts) and Caco-2 (human colon adenocarcinoma) cells following 24 h exposure, determined with the crystal violet and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide assays, respectively. The cellular antioxidant activity (CAA) of 1.25% honey in both cell lines was determined using the dichlorofluorescein diacetate assay. All honey samples had CAA activity that was not affected with oral digestion.

In the murine macrophages (RAW 264.7), the pro- and anti-inflammatory effects of each honey and oral digests was determined. Honeys, FB1, FB3 and MAN UMF10+ at a concentration of 1.25% were pro-inflammatory while in contrast, FB2 and FB4 were anti-inflammatory. All honeys and oral digests were to various degrees able to inhibit lipopolysaccharide-mediated NO formation in this cell line.

In conclusion, FB and MAN UMF10+ honeys reduced the growth of biofilms and at 5% caused changes in *C. albicans* morphology. The antioxidant properties of all honeys were retained following oral digestion. In cellular models, the honeys and oral digests showed no cytotoxicity, had cellular antioxidant activity and either a pro- or anti-inflammatory activity at the concentrations evaluated. Therefore, in this laboratory-based study, FB honeys had beneficial effects, that may translate into therapeutic benefits.

Keywords: Fynbos, Manuka, honey, *Candida albicans*, oral digestion, chemical antioxidant activity, cellular antioxidant activity, anti-inflammatory activity, RAW 264.7, anti-fungal activity.

Declaration

I, Anwani Wendy Nekhumbe hereby declare that this research thesis is my own work. It is being submitted for the degree, Master of Science in Anatomy with specialisation in Human Cell Biology at the University of Pretoria, South Africa. This thesis has not been presented by me for any degree at this or any other University;

Signed: ……

Date: ……

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Dedicated to my loving grandmother, Patience (Pheziwe), my guardian angel.

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%	percentage
˚C	degrees centigrade
±	plus or minus
µg/mL	micrograms per millilitre
µL	microliters
µM	micromolar
µmol	micromole
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azo-bis(3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
ARV	antiretroviral
BD-1	bee defensin-1
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CA	caffeic acid
$CaCl_2$	calcium chloride
Caco-2	human colon adenocarcinoma cell line
CAUTI	catheter-associated urinary tract infections
CFU/mL	colony forming unit/millilitre
CLABSI	central line-associated bloodstream infections
cm^2	centimetres square
CO_2	carbon dioxide
COX	cyclooxygenase
CV	crystal violet
DCFH-DA	dichlorofluorescein diacetate
$dddH_2O$	deionised double distilled water
ddH_2O	double distilled water
DM	diabetes mellitus
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulphoxide
DMT2	diabetes mellitus-type 2
DNA	deoxyribonucleic acid
ECACC	European collection of cell cultures
eNOS	endothelial nitric oxide synthase
FB	Fynbos
FB1 – FB4	Fynbos honey 1 to 4
FBS	foetal bovine serum

F-C reagent	Folin-Ciocalteu reagent
FCS	foetal calf serum
g	grams
GA	gallic acid
GI	glycaemic index
g/L	gram per litre
h	hour/hours
H_2O	water
H_2O_2	hydrogen peroxide
HAI	hospital-acquired infection
HIV	human immunodeficiency virus
HMDS	hexamethyldisilazane
ICU	intensive care unit
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IL	interleukin
iNOS	inducible nitric oxide synthase
K_2HPO_4	dipotassium phosphate
$K_2S_2O_8$	potassium peroxodisulfate
K_2SO_4	potassium sulphate
KCl	potassium chloride
KSCN	potassium thiocyanate
L929	mouse fibroblast cells
LPS	lipopolysaccharide
LOOH	lipid hydroperoxide
M	molar
MAN	Manuka
MBC	minimum bactericidal concentration
MDA	malondialdehyde
mg	milligrams
mg/mL	milligram per millilitre
$MgCl_2$	magnesium chloride
MGO	methylglyoxal
MGCN	MGO-conjugated chitosan nanoparticles
MIC	minimum inhibitory concentration
min	minutes
mL	millilitre
mm^3	millimetre cubed
MRSA	methicillin resistant *Staphylococcus aureus*

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	sodium carbonate anhydrous
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaF	sodium fluoride
$NaHCO_3$	sodium hydrogen carbonate
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NED	N-1-napthylethylenediamine dihydrochloride
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NH_4Cl	ammonium chloride
nm	nanometres
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen
OH^-	hydroxide ion
OI	opportunistic infection
ORAC	oxygen radical absorbance capacity
OsO_4	osmium tetraoxide
PBS	phosphate buffered saline
pH	logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution
P-value	probability value
PRRs	pattern recognition receptors
PAMPs	pathogen associated molecular patterns
RAW 264.7	mouse murine macrophage cell line
ROS	reactive oxygen species
rpm	revolutions per minute
SAP	secreted aspartyl proteinases
sec	seconds
SEM	standard error of mean
SNP	sodium nitroprusside
SSI	surgical site infections
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TLR4	Toll-like receptor 4
TNF-α	tumour necrosis factor - alpha
TPC	total polyphenol content
UMF	unique Manuka factor

VAP	ventilator-associated pneumonia
v/v	volume to volume
w/v	weight to volume
x	times

Chapter 1 : Introduction

Candida albicans (*C. albicans*) is found naturally in the human microbiome, colonising the gastro-intestinal tract, oral cavity, skin and reproductive tract (Nobile and Johnson, 2015). Infections by *C. albicans* such as oral candidiasis affect the young and the old alike as well as those who are immune or medically compromised. For example, 7 million people out of the South African population of 56.5 million are infected with HIV/AIDS, an important predisposing factor for the increased fungal infections (Schwartz *et al.*, 2019). Consequently, there is a need for anti-fungal therapies that are effective and readily available.

The most common oral fungal infections are due to *C. albicans* which is a dimorphic or pleomorphic yeast that can survive in different environmental extremes by switching between the two different morphologies (Blignaut, 2017). The most common morphology is the round/oval yeast cells known as blastoconidia, which can switch to the elongated (pseudohyphae or true hyphae) yeast cells that are associated with candidiasis (Blignaut, 2017) - a superficial or life-threatening infection (Yan *et al.*, 2019). In candidiasis, the biofilms formed by hyphal yeast cells are impenetrable, and resistant to anti-fungal treatment. In the oral cavity, candidiasis is associated with white lesions most prevalent on the tongue, a burning oral sensation, easy bleeding on affected sites and changes in taste perception (Millsop and Fazel, 2016).

Candidiasis is associated with inflammation and free radicals are important mediators in this process. Nitric oxide (NO) regulates diverse cellular functions, however NO can react with reactive oxygen species (ROS) forming highly reactive nitrogen species (RNS) (Romero-Puertas and Sandalio, 2016). Both RNS and ROS cause increased inflammation and further protein, lipid and nucleic acid oxidation which leads to cellular dysfunction and cell death. Effective scavenging by either enzymatic and non-enzymatic antioxidants, can reduce NO and ROS levels (Romero-Puertas and Sandalio, 2016), thereby reducing the associated inflammation.

The classes of anti-fungal drugs used to treat fungal infections are the azoles, polyenes, echinocandins, allylamines and fluorinated pyrimidine analogues. Unfortunately, *C. albicans* biofilms are generally resistant to treatment by these drugs (Kabir *et al.*, 2012). Also, *C. albicans* biofilms are reported to become resistant to drugs such as amphotericin B, fluconazole and caspofungin, when used incorrectly for the treatment of oral candidiasis, (Kabir *et al.*, 2012). Therefore, there is a need to find novel anti-fungal treatments for oral candidiasis. Therapies that reduce the growth of *C. albicans* while reducing associated

inflammation would be of benefit to patients, promoting rapid recovery without the need for systemic treatment.

Honey is an old, traditional medicine and extensive research has led to the development of medical-grade honey such as Revamil source honey (Kwakman *et al.*, 2011), chestnut and thyme honeys (Halstead *et al.*, 2016) that are used for the treatment of wounds, burns, colds, ulcers and digestion issues (Niaz *et al.*, 2017; Hermanns *et al.*, 2019).

Medical-grade Manuka (MAN) honey is sourced from *Leptospermum scoparium* which is native to New Zealand and Australia (Carter *et al.*, 2016). It has antibacterial activity which is due to high levels of methylglyoxal (MGO), hydrogen peroxide (H_2O_2), bee defensin-1 (BD-1) and phenolic compounds such as caffeic acid (CA), gallic acid (GA), quercetin and chrysin (Alvarez-Suarez *et al.*, 2014). In laboratory-based studies, MAN honey has been shown to inhibit and eradicate the growth of free-floating planktonic and biofilms of many bacterial species such as *Streptococcus* and *Staphylococcus* species, *Escherichia coli (E. coli), Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Carter *et al.*, 2016). In addition, the high viscosity of honey generates a protective barrier over wounds which limits the growth of microorganisms (Alvarez-Suarez *et al.*, 2014).

Honey also promotes the secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor (TNF-α) which mediate an immune response to heal wounds (Tonks *et al.*, 2007). Used orally in a mouthwash or consumed, MAN honey has been shown to reduce infection against microbes that cause plaque and gingivitis (English *et al.*, 2004), inflammation and prevent oxidative damage to the oral mucosa (Alvarez-Suarez *et al.*, 2014). The polyphenols with radical scavenging activity, reduce the levels of ROS and RNS, but also can modulate inflammatory pathways (Hussain *et al.*, 2016; Yahfoufi *et al.*, 2018).

Unfortunately, in South Africa, MAN honey is imported and expensive. Local honey such as Fynbos (FB) honey, produced by the Cape honeybee which populates the Fynbos biome in the Western Cape province of South Africa, is readily available. FB honey has an antibacterial activity against Gram-positive and -negative bacteria (Magoshi, 2017; Serem, 2018). The anti-fungal activity of FB honey against *C. albicans* has been evaluated in several studies (Theunissen *et al.*, 2001; Basson and Grobler, 2008; Khan *et al.*, 2014), although low inhibition was reported. In contrast, FB honey has antioxidant (Serem and Bester, 2012; Magoshi, 2017), and anti-inflammatory properties (Hadagali and Chua, 2014; Magoshi, 2017; Serem, 2018) and therefore, may still be beneficial in reducing inflammation associated with candidiasis.

With digestion, the antimicrobial, antioxidant and anti-inflammatory activities of honey change. This is due to MGO binding digestive enzymes (Daglia *et al.*, 2013), H_2O_2 degradation (Majtan *et al.*, 2014b), proteolysis of BD-1 (Kwakman and Zaat, 2012) and the pro-oxidant effects or the oxidation of polyphenols at neutral pH (O'Sullivan *et al.*, 2013). It is not possible to exactly duplicate human saliva, as it is a complex mixture of numerous constituents which change according to the time of the day (Gal *et al.*, 2001). Knowing that the oral cavity has a neutral pH environment, with high levels of salts containing the enzymes α-amylase and lipase, numerous digestion and odontology studies have formulated synthetic saliva with varying formulae for different *in vitro* studies. For instance, human saliva contains organic components like glycoproteins which are responsible for its natural viscosity, such components are necessary for biological studies, however, some components of human saliva are left out of formulae when the substance has no detectable effect (Gal *et al.*, 2001). Little is known regarding the effect of human saliva on the anti-fungal, antioxidant and anti-inflammatory effects of honey.

Therefore, the aim of this laboratory-based study was to investigate the anti-fungal, antioxidant and anti-inflammatory activities of undigested and oral digested FB honeys compared with MAN UMF10+ honey in order to determine if the afore-mentioned activities of each honey are altered following a simulated oral digestion model.

Chapter 2 : Literature review

2.1 Introduction

Hospital-acquired infections (HAIs) are infections that occur in patients during the process of care in a hospital or other healthcare facility that was not present at the time of admission (Nejad *et al.*, 2011). These are opportunistic infections that often affect patients that are immune-compromised, infected with the human immunodeficiency virus (HIV), have cancer, diabetes mellitus (DM) and those undergoing surgical procedures (de Arruda Caceres *et al.*, 2015). The most common fungal opportunistic infection in humans is by the *Candida* species, of which *C. albicans* is the most common (Mayer *et al.*, 2013).

C. albicans biofilms have become resistant to anti-fungal drugs such as the azoles, itraconazole and fluconazole, although still sensitive to amphotericin B and ketoconazole (Zaidi *et al.*, 2016). In the oral cavity, topical nystatin is used in the treatment of oral candidiasis (Park *et al.*, 2017). Reported resistance to the azole class of drugs, especially with prolonged use (Zhang *et al.*, 2020) emphasises the need for new and novel therapies.

2.2 Hospital-acquired infections

The World Health Organization (WHO), defines a HAI as an infection that occurs in a patient while they are in the process of care in a hospital or other healthcare facility that was not present at the time of admission (Nejad *et al.*, 2011). HAIs are caused by multi-resistant pathogens that cause illnesses, disability, prolonged hospital stays and may even result in death (Nejad *et al.*, 2011). The types of HAIs and a short description of each is presented in Table 2.1 (Khan *et al.*, 2017).

Table 2.1: Types of hospital-acquired infections.

Infection	Description
Central line-associated bloodstream infections (CLABSI)	• Deadly HAI • Catheters used to provide fluid and medication with prolonged use causing bloodstream infections
Catheter-associated urinary tract infections (CAUTI)	• Caused by natural microflora of patient • Catheters act as conduits for entry of bacteria
Surgical site infections (SSI)	• Commonly caused by *Staphylococcus aureus* (*S. aureus*) • Caused by natural microflora of patient
Ventilator-associated pneumonia (VAP)	• Occurs in patients on mechanically-assisted ventilators

HAIs are commonly caused by microorganisms such as bacteria, viruses and fungi (Khan *et al.*, 2017), the different types of microorganisms associated with HAIs and the types of infections that occur are summarised in Table 2.2 (Khan *et al.*, 2017).

Table 2.2: Pathogens associated with infection.

Microorganism	Types of infections
Bacteria	• *Acinetobacter* – infections occurring in the intensive care unit (ICU) • *Bacteroides fragilis* – causes infections when combined with others • *Clostridium difficile* – inflammation of colon • Methicillin resistant *Staphylococcus aureus* (MRSA) – sepsis and pneumonia
Virus	• Hepatitis B and C • Influenza • Human immunodeficiency virus (HIV) • Rotavirus • Herpes simplex virus
Fungi	Causes infections in immune-compromised individuals • *Aspergillus spp.* – via environmental contamination • *Cryptococcus neoformans* – via contaminated air • *C. albicans* – from patient's microflora

The most vulnerable patient populations are immune-compromised individuals (Mehta *et al.*, 2014). Additional factors that increase susceptibility to infection are age (younger children and the elderly) and patients with chronic co-morbidities (asthma, DM, cancer, arthritis, etc.).

South Africa is a developing country and according to Statistics South Africa (2017a), in 2015 more than half the population of South Africa was living under the poverty line (as defined in the South African Multidimensional Poverty Index by Statistics South Africa), with the poverty headcount increasing from 53.2% as reported in 2011, to 55.5% in 2017 (Statistics South Africa, 2017b). As a result of poverty, only 17 out of 100 individuals have medical aid (Statistics South Africa, 2017b) and thus many individuals are dependent on public healthcare, resulting in an increased burden on the system (Coovadia *et al.*, 2009). Consequently, HAIs are often underestimated due to the lack of surveillance in healthcare facilities (Lowman, 2016). The true burden of HAIs is unknown, but it is known that it affects the public healthcare system more than it does the private healthcare system (Lowman, 2016).

2.2.1 Oral C. albicans infections

C. albicans can cause life-threatening, as well as superficial infections such as vaginal or oral candidiasis (Mayer *et al.*, 2013). In healthy individuals, *C. albicans* usually remains benign, but in immunodeficient individuals, the infections often occur in the oral cavity where oral candidiasis affects the oropharynx and the oesophagus (Mayer *et al.*, 2013).

Statistics South Africa (2020) has reported that a total of 7.8 million individuals in South Africa are living with HIV, accounting for 13% of the South African population. AIDS is the advanced clinical manifestation of HIV where the lymphocyte count ($CD4^+$) of the patient is 200/mm^3 and

below (de Arruda Caceres *et al.*, 2015). People living with HIV are highly susceptible to opportunistic infections (OI) because of immunosuppression, however with antiretroviral (ARV) therapy, the prevalence of these infections has decreased and are no longer the major cause of morbidity and mortality (de Arruda Caceres *et al.*, 2015). HIV patients are still infected by OIs for the following reasons: individuals are unaware of their HIV status and only start ARV treatment when an OI is identified, lack of access to ARVs, as well as poor response to ARV treatment due to lack of adherence and drug toxicity (de Arruda Caceres *et al.*, 2015). The most common OI that affects people living with HIV is pneumocystosis which leads to severe pneumonia and oral-oesophageal candidiasis (de Arruda Caceres *et al.*, 2015).

In addition, the incidence of DM and especially DM-type 2 (DMT2) in South Africa is increasing. This is due to urbanisation which has influenced diet, resulted in lifestyle changes, leading to an increase in body weight and co-morbidities (Pheiffer *et al.*, 2018). These patients are highly susceptible to *C. albicans* infections. The prevalence of oral candidiasis in patients with DM type 1 and type 2 is 84% and 68%, respectively, compared to non-diabetic patients at 27% (Rodrigues *et al.*, 2019). The pathophysiology in DM patients is due to hyperglycaemia, high glucose levels in saliva, low salivary pH and poor oral hygiene (Rodrigues *et al.*, 2019).

Opportunistic infections like *C. albicans* also affect the young with a developing immune system and the elderly who are immune-compromised often due to underlying diseases and conditions (Flevari *et al.*, 2013). *C. albicans* affects young patients with dental caries (Xiao *et a*l., 2018) and is also the consequence of excessive antibiotic and steroid use (Jain *et al*., 2010). In elderly patients, *C. albicans* occurs due to ageing physiological changes, concomitant drug use and oropharyngeal colonisation by *C. albicans*. Colonisation of dental prosthesis and urinary tract infections usually occur after broad spectrum antibiotic use (Flevari *et al.*, 2013).

2.2.2 The oral cavity as a site of infection

In healthy individuals, *C. albicans* can remain benign as the stratified squamous epithelium in the oral mucosa serves as a protective barrier against infections and helps maintain a healthy oral mucosa. The keratinisation of this epithelium limits colonisation by *C. albicans* (Jain *et al.*, 2010).

Saliva also contributes to the protection of the oral mucosa. Whole saliva is a hypotonic and neutral to alkaline solution (pH 6.5 - 7.6, average pH 6.7) (Baliga *et al.*, 2013; Pedersen and Belstrom, 2019). The inorganic components of saliva are the ions sodium, chloride, phosphate, potassium, magnesium, calcium, bicarbonate and trace elements. Bicarbonate,

phosphate and proteins provide the salivary buffer system which is important to maintain a healthy microbial presence. The bicarbonate concentration and pH increase when the rate of flow of the saliva increases. Calcium and phosphate in saliva maintain the saturation of hydroxyapatite which builds tooth enamel (Pedersen and Belstrom, 2019). The main proteins found in saliva are Immunoglobulin A (IgA), lysozyme, lactoferrin, α-amylase and mucosal glycoproteins (Minekus *et al.*, 2014).

The α-amylase enzyme in saliva breaks down starches into maltoses where it cleaves the α-1-4 glycosidic bonds (Butterworth *et al.*, 2011). Secretion of salivary α-amylase is stimulated by autonomic nerve stimulation mainly by the serous acinar cells of the parotid gland. Although honey has a high glucose and fructose content, the content of complex carbohydrates is low (Swallow and Low, 1990) and therefore the effect of α- amylase will be minimal.

Other enzymes, although not major components, are proteolytic and lipolytic which break down proteins and fats, respectively. This enzymatic activity gives saliva the ability to interact with different types of macromolecules found in food and these enzymes also release or modify molecules which give different foods different tastes (Neyraud *et al.*, 2012).

The oral cavity possesses diverse microflora, bacterial and fungal. Microorganisms are able to bind to and colonise surfaces like the enamel because of a film produced by saliva which has a high content of proline-rich proteins known as enamel pellicle. The enamel pellicle is important for the maintenance of dental health, it lubricates and maintains mineral homeostasis on tooth surfaces (Pedersen and Belstrom, 2019). The oral cavity is covered by salivary pellicle which contains mucins, cystatins and α-amylase. Saliva supplies these microorganisms with nutrients due to the proteolytic activity in the oral cavity that breaks down starches, lipids and proteins from food (Pedersen and Belstrom, 2019).

Saliva also contains antimicrobial peptides and proteins with antimicrobial properties and these are lysozyme, lactoferrin, mucins, statherin, histatins and immunoglobulins (Ig) (Pedersen and Belstrom, 2019; Vila *et al.*, 2019).

Lysozyme is an antimicrobial protein present in the salivary and enamel pellicle, it is produced by the major and minor salivary glands as well as by leukocytes. Its mechanism of action is hydrolysing 1,4-glycosidic bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine in the polysaccharide layer of the cell wall of Gram-positive bacteria, and also promotes the clearance of microorganisms from the oral cavity (Pedersen and Belstrom, 2019). Lactoferrin is an antimicrobial glycoprotein that binds iron, thereby depriving microorganisms of iron

essential for their growth. Lactoferrin originates from neutrophil granulocytes and it increases infection and inflammatory conditions. Mucins are glycosylated proteins important for maintaining lubrication of the teeth and the oral mucosa. Statherin is a phosphoprotein which binds hydroxyapatite contributing to the formation of the enamel pellicle while the histatins are cationic peptides, that also help to form the enamel pellicle (Pedersen and Belstrom, 2019). The Igs present in saliva are IgA and IgG, these help to bind antigens in the oral cavity (Pedersen and Belstrom, 2019).

The antimicrobial peptides present in saliva are the first line of defence against foreign microbes, helping to maintain a healthy equilibrium in the oral cavity (Vila *et al.*, 2019). In immunocompromised patients such as those with HIV infection who have oral candidiasis, the health of the oral cavity is compromised.

2.2.3 Candida albicans

Candida are a common fungus of which *C. albicans* is found naturally as part of the human microbiome (Mayer *et al.*, 2013). It can be found in the oral cavity or in the genitourinary tract of about 70% of humans (Kabir *et al.*, 2012).

Infections by *C. albicans* range from superficial mucosal infections to life-threatening systemic infections. Infection can be divided into four stages, initial colonisation followed by superficial, then deep-seated, then systemic infections (Jacobsen *et al.*, 2014). The most common and initial stage of infection is superficial infection where infection occurs because of an imbalance in the microbiome or because of the host's compromised immune system. In systemic infections, the fungus is disseminated through the bloodstream which results in the life-threatening infection of internal organs (Jacobsen *et al.*, 2014).

C. albicans is a pleomorphic fungus that exists as a round/oval yeast, pseudohyphae or true hyphae (Mayer *et al.*, 2013). *C. albicans* exists either as planktonic, unattached, floating cells or as complex biofilms that grow on biotic and abiotic surfaces. Abiotic surfaces such as dental enamel and implanted medical devices are the major sites where biofilms can form (Kabir *et al.*, 2012).

Biofilms can be found as a community of different cell species or cell types of the same species (Silva *et al.*, 2017). *C. albicans* biofilms consist of a high concentration of different cell types; that include round, budding yeast-form cells, oval pseudohyphal cells and elongated hyphal cells which are all surrounded by a protective extracellular matrix (ECM) which protects the cells (Nobile and Johnson, 2015). This trait makes these biofilms resistant to anti-fungal agents.

Infection by *Candida albicans* is initiated by a single fungal yeast cell that adheres to a substrate forming a foundation layer of basal yeast cells. Then cell proliferation takes place across the foundation layer and the cells start to form elongations which become filamentous hyphal forms. In the maturation step, these hyphal forms start to form biofilms, and then with biofilm maturation, an ECM accumulates providing structural support. Colonisation takes place in the dispersal step where non-adherent yeast cells are released into the surroundings to colonise other surfaces (Nobile and Johnson, 2015; Tsui *et al.*, 2016). The stages of *C. albicans* biofilm formation are summarised in Figure 2.1.

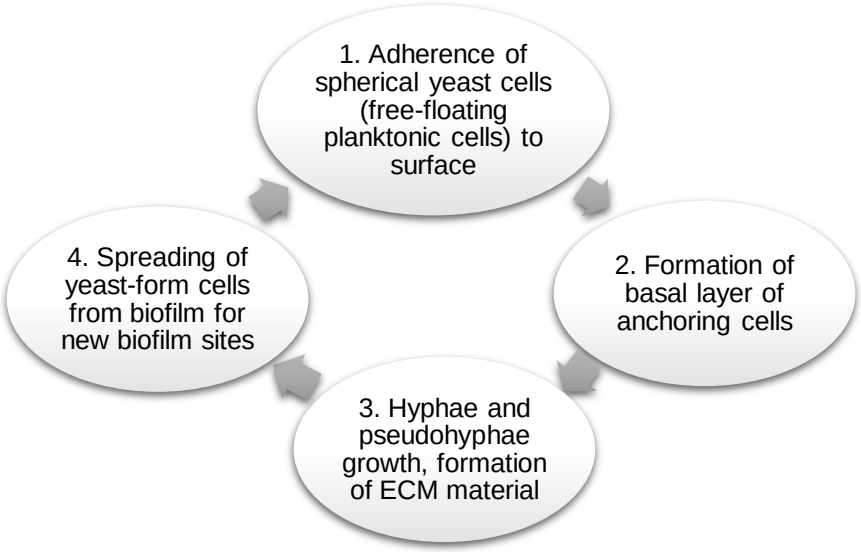

Figure 2.1: The formation of *C. albicans* biofilms from free-floating planktonic cells.

The ECM of *C. albicans* helps protect the cells from the host's defence system and anti-fungal drugs by preventing anti-fungal drug diffusion and penetration (Silva *et al.*, 2017). *C. albicans* biofilms are extremely drug-resistant to many anti-fungal drugs and originally this was thought to be caused by the ECM that surrounds and protects the cells, but there are conflicting views. Some researchers found that damaged biofilms still show a high resistance to drugs (Kabir *et al.*, 2012), but more recent studies have shown that the chemical composition of the ECM plays a major role in the resistance of *Candida* species (Silva *et al.*, 2017).

2.3. Control and treatment of *C. albicans* infections

Anti-fungal drugs that kill the planktonic form of *C. albicans* are polyenes (amphotericin B and nystatin in combination with cyclosporine A), azoles (fluconazole and voriconazole) and echinocandins (caspofungin). The most common drugs used for the treatment of oral infections are from the azole class of drugs namely, fluconazole and ketoconazole as well as nystatin.

The membrane of fungi is partly made up of ergosterol, where it is involved in the fluidity of the membrane, the regulation, distribution and arrangement of integral membrane proteins as well as control of the fungal cell cycle (Alcazar-Fuoli and Mellado, 2013). Anti-fungal targets include ergosterol and 1,3-β-D-glucan synthesis (Alcazar-Fuoli and Mellado, 2013).

Polyenes, allylamines and azoles are three classes of drugs that target ergosterol (Alcazar-Fuoli and Mellado, 2013). Azoles inhibit ergosterol synthesis by targeting the enzyme, lanosterol 14α-demethylase which results in the toxic accumulation of sterols which kills fungal cells. The enzyme, 1,3-β-D-glucan synthase is associated with the fungal cell wall and is required for the synthesis of 1,3-β-D-glucan polymers which are major components of the fungal cell wall (Walker *et al.*, 2011). Inhibition of this enzyme by the echinocandin class of anti-fungal drugs, namely caspofungin, anidulafungin, and micafungin, compromises the integrity of the cell wall by decreasing the β-D-glucans present in the cell wall (Lima *et al.*, 2019). The effects of polyenes include membrane permeabilization and pore formation which destroys the proton gradient leading to the outflow of cell contents (Silva *et al.*, 2017) and cell death.

Unfortunately, *Candida* species are becoming resistant to the available anti-fungal drugs and because of the adverse effects of high dosages of anti-fungal drugs in immune-compromised patients, the development of new anti-fungal therapies is becoming more essential (Kabir *et al.*, 2012). With oral candidiasis there are no specific therapies that can help prevent or inhibit biofilm formation on dentures, although topical use of anti-fungals and antimicrobials as well as physical cleaning of the dentures can prevent infection and biofilm formation (Williams and Lewis, 2011).

2.3.1 Combating C. albicans by oxidative and nitrosative stress

The formation of ROS that are produced by phagocytes protects against infection. *In vivo*, pathogenic cells such as neutrophils kill *C. albicans* by producing ROS. In a similar manner, *C. albicans, in vivo* are also vulnerable to RNS (Hwang *et al.*, 2002). To ensure survival, *C. albicans* has antioxidant defence systems that help the fungus resist the host immune response (Hwang *et al.*, 2002). Therefore *in vivo*, Hwang *et al.* (2002) suggests that exogenous antioxidant systems would bolster the virulence of *C. albicans* whereas ROS formation would attenuate the virulence of *C. albicans*. Drugs that kill *C. albicans* by the formation of reactive species are miconazole, echinocandins and liposomal formulations of amphotericin B (Delattin *et al.*, 2013).

However, an excess of ROS and RNS is associated with increased inflammation and this compromises the integrity of the mucosa and can contribute to the development of systemic infections. Scavenging of these oxidative species would reduce inflammation, but may promote the growth of *C. albicans*. This highlights the fine balance between the immune system and *C. albicans*, where disruption favours colonisation and the formation of biofilms. Therefore, therapies that directly kill *C. albicans* while reducing inflammation would be beneficial.

2.3.2 Honey as a therapy against C. albicans

Honey is widely used as a therapy, due to several properties including antioxidant, anti-inflammatory and antimicrobial activity (Table 2.3) (Samarghandian *et al.*, 2017). Honey has been used as a treatment for wounds, diarrhoea, eye diseases, coughs and sore throat, sunburn and the prevention of infections and scars (Bellik and Boukraa, 2014). In addition, honey has been identified as a promising anti-fungal for the treatment of *C. albicans* (Bellik and Boukraa, 2014), however the levels of sugar and antioxidants are of concern as these could potentially increase the virulence of *C. albicans*.

Table 2.3: Therapeutic bioactivity of honey.

Effect	Mechanism
Antioxidant	• Contains phenolic compounds ○ Predicted by honey colour: darker honey, greater antioxidant activity • Protection against reactive oxygen species
Antimicrobial	• Properties such as low osmotic pressure, low pH and the presence of hydrogen peroxide inhibits the growth of yeast and bacteria
Anti-inflammatory	• Due to the phenolic and flavonoid compounds • Suppress pro-inflammatory activities • Increases leukocytes and antibody production
Wound healing	• Promotes leukocytes to release cytokines • Activates immune responses
Diabetes mellitus	• Honey used in conjunction with anti-diabetic therapy • Reduces glycaemic index • Reduces blood glucose levels
Anti-cancer	• Interferes with cell signalling pathways • Induces apoptosis
Treatment of diarrhoea	• Oral rehydration therapy • Used in infants and children in hospitals with gastro-enteritis to decrease diarrhoea

2.4 Antimicrobial, antioxidant and anti-inflammatory properties of honey

Several components in honey have been identified to contribute to the antimicrobial activity of honey and these are sugars, H_2O_2, polyphenols, MGO and antimicrobial peptides (AMP) (Serem, 2018; Albaridi, 2019).

The sugars present in honey are fructose (38.5%), glucose (31%), maltose (7%), trisaccharides (4%) and sucrose (1.5%) (Albaridi, 2019) and the presence of these sugars creates a high osmotic concentration. The water activity (a_w) of honey is low and therefore does not promote the growth of microorganisms. The high sugar and low water content of honey results in osmotic pressure where microbial cells become dehydrated and this causes cell death. However, this effect is reduced as honey is diluted (Albaridi, 2019). The different virulence factors of *C. albicans* are affected by glucose such as the ability to form biofilms (Van Ende *et al.*, 2019). Glucose functions as a morphogen in *C. albicans* and it activates the transition of yeast cell to hyphal cell (Brown *et al.*, 2006; Van Ende *et al.*, 2019). *C. albicans* has over 20 hexose transporters for the transport of glucose, and therefore a sugar sensing and signalling mechanism in *C. albicans* is important as it is essential for the virulence of *C. albicans*.

Horizontal gene transfer 4 (Hgt4) is a sensor of sugar in *C. albicans*, sensing glucose, mannose and fructose when these sugar levels are low. Hgt4 is required for fermentation and the absence of this sensor may indicate the inability of *C. albicans* to colonise the host, especially in the gut, making it a potential drug target (Brown *et al.*, 2006). High levels of glucose inhibit morphogenesis, while the formation of hyphae is induced at sugar levels lower than 0.25%. In the presence of high sugar levels, *Candida* cells form more dispersed biofilms as a survival mechanism (Van Ende *et al.*, 2019). In stress conditions, stress resistant genes are expressed in *C. albicans* and with increased glucose levels, oxidative and osmotic stress genes are expressed (Van Ende *et al.*, 2019), protecting *C. albicans* against oxidative stress.

H_2O_2 is also a main component responsible for the antimicrobial activity of honey (Brudzynski *et al.*, 2011; Albaridi, 2019) and is formed by glucose oxidase that oxidises glucose to produce gluconic acid and H_2O_2. Activity of this enzyme increases as honey is diluted and the pH level increases, with maximum formation of H_2O_2 when honey is diluted to between 30 and 50% (Albaridi, 2019). H_2O_2 causes oxidative stress in microbials such as damage to membrane layers, proteins enzymes and DNA. Activity is influenced by the many compounds found in honey, that either enhance or diminish the oxidative effects of H_2O_2 (Brudzynski *et al.*, 2011).

Honey contains variable amounts of MGO, which is typically high in MAN honey. The Unique Manuka Factor (UMF) rating of MAN honey indicates the total phenol content as well as the MGO content i.e. the higher the UMF rating, the higher the total phenol and MGO content in the MAN honey (Johnston *et al*., 2018); with MAN UMF10+ containing 263 mg/kg MGO (approximately 1 mM). MAN honey is considered the golden standard when investigating the antimicrobial effects of honey.

Studies have shown that MGO effectively inhibits the growth of Gram-negative and -positive bacteria, with reported bacteriostatic and bactericidal effects (Rabie *et al.*, 2016). MGO acts as an antimicrobial by altering the structure of bacterial fimbriae and flagella and as well by inducing damage to their cell membranes (Rabie *et al.*, 2016; Nolan *et al.*, 2019). MGO in MAN honey can react with macromolecules such as DNA, RNA and proteins, forming radicals such as advanced glycation end products (AGEs) which means that it can potentially cause cell damage in mammals (Carter *et al.*, 2016). Khan *et al.* (2020) assessed the activity of free MGO and MGO-conjugated chitosan nanoparticles (MGCN) against *C. albicans* that is fluconazole-resistant in mice. The percentage survival rate of the mice exposed to 10 mg/kg MGO and MGCN was 50% and 90%, respectively. MGO and MGCN (doses 1, 5 and 10 mg/kg) further inhibited the *in vivo* replication of *C. albicans* and eliminated fluconazole-resistant systemic candidiasis. No renal and hepatic toxicity was observed, indicating lack of

toxicity. *In vitro*, the susceptibility of *C. albicans* to fluconazole, MGO and MGCN was further investigated. In the control group, extensive hyphal formation was observed while at a concentration of 1 and 2 µg/mL fluconazole, hyphal formation was also not inhibited. In contrast, at the same concentrations both MGO and MGCN significantly inhibited hyphal formation in *C. albicans*.

AMPs have broad spectrum activity protecting the host against infections by both Gram-positive and -negative bacteria, viruses, fungi and unicellular protozoa (Mahlapuu *et al.*, 2016). A common mechanisms of action is the disruption of the membrane of bacteria by disintegrating the lipid bilayer and being pro- and anti-inflammatory mediators (Mahlapuu *et al.*, 2016). Other mechanisms of action are the inhibition of DNA replication and protein synthesis (Bahar and Ren, 2013). The AMPs that have been identified in the honeybee are apidaecins, abaecin, hymenoptaecin and BD-1 and BD-2 (Danihlı´k *et al.*, 2016).

Apidaecins and abacin are proline-rich peptides whose levels are up-regulated in response to bacterial infection. Apidaecins and hymenoptaecins have shown a higher efficacy against Gram-negative bacteria, with abaecin having less activity. Apidaecin permeates the bacterial lipid bilayer of *E. coli* and induces an irreversible permeate/transporter uptake and a dose-dependent inhibition of protein synthesis (Danihlı´k *et al.*, 2016).

Hymenoptaecin is a glycine-rich peptide, while BD-1 and -2 are cysteine-rich peptides. BD-1 has been identified as an important antimicrobial component of honey, and BD-2 has been expressed in the honeybee in response to lipopolysaccharide injections. BD-1 is effective against both Gram-positive and -negative bacteria (Danihlı´k *et al.*, 2016).

Polyphenols found in honey include catechin, chrysin, quercetin, chlorogenic acid, coumaric acid, GA and *p*-coumaric acid (Cianciosi *et al.*, 2018). Polyphenols can promote the antimicrobial activity by directly producing H_2O_2 and by triggering the Fenton reaction where Fe (III) is reduced to Fe (II) creating ROS such as hydroxyl radicals (Bucekova *et al.*, 2018) This pro-oxidant reaction occurs under neutral to basic pH conditions, and inhibits the growth of microbes (Bucekova *et al.*, 2018).

In addition, polyphenols present in honey have antioxidant and anti-inflammatory properties and these properties have been widely investigated (Serem and Bester, 2012; Vallianou *et al.*, 2014; Magoshi, 2017; Miguel *et al.*, 2017; Cianciosi *et al.*, 2018; Serem, 2018). However, polyphenols are unstable at a neutral to slightly alkaline pH as found in the gastro-intestinal

tract (GIT) and this affects the properties of polyphenols (Honda *et al.*, 2019). Stability in such environments is dependent on polyphenol type and structure (Friedman and Jurgens, 2000), and pH sensitive polyphenols include the phenolic acids; CA, chlorogenic, and GA. Although many honey types have been identified with health benefits, little is known regarding the effect of neutral and alkaline environments such as those found in the oral cavity on bioactivity.

This study will focus on honey from the Fynbos region of South Africa, and the known antimicrobial, antioxidant and anti-inflammatory properties of Fynbos (FB) honey will be discussed in greater detail.

2.4.1 Honey from the Fynbos region

Fynbos is found in the Western Cape Province (WP) of South Africa (SA). This is an endemic ecosystem which consists of unique vegetation; this region is known as the Fynbos ecoregion (du Preez, 2014). Fynbos means fine bush and it is derived from the Dutch word fijn-bosch. This region is located on the coast of the WP, extending from Table Mountain in Cape Town to Port Elizabeth in the Eastern Cape Province (du Preez, 2014). The Fynbos biome is strictly made up of three different vegetation types; Fynbos, Renosterveld and Strandveld (Rebelo *et al.*, 2006). These vegetation types occur during winter- and summer-rainfall seasons and consist mainly of evergreen shrubs with small leaves which have adapted to veld fires (Rebelo *et al.*, 2006). Well known plants which have medicinal properties in the Fynbos ecoregion are Buchu which has antiseptic properties, *Protea repens* known as suikerkan or suikerbos in Afrikaans which is used to remedy chest disorders as well as *Aspalanthus linearis* known as red bush tea (rooibos tee in Afrikaans) (du Preez, 2014). This Fynbos ecoregion is populated by the Cape honeybee (Apis *millifera capensis*) which produces Fynbos honey (du Preez, 2014). This honeybee is adapted to survive the cold and wet winters of the WP (Rebelo *et al.*, 2006). The known history of Fynbos honey dates back to the mid-17th century when Jan van Riebeeck instructed his men to collect honey from the local Khoisan people who obtained honey from the Fynbos region (du Preez, 2014). As it is today, Fynbos honey was used for the treatment of skin problems and wound repair (du Preez, 2014).

FB honey does not have high broad-spectrum antimicrobial activity (Basson and Grobler, 2008). Serem (2018) found that FB honey at a concentration of between 25 and 40% inhibited the growth of Gram-positive and -negative bacteria. In a study by (Khan *et al.*, 2014), the antimicrobial properties of 42 South African honeys, which included honeys sourced from *Erica* species (Fynbos), *Eucalyptus* species and *Acacia* species was determined. The study identified that the antimicrobial efficacy of South African honeys were more sensitive to

bacterial species than yeast species. Only one FB honey sample displayed broad-spectrum antimicrobial activity. This can be corroborated in a different study by Theunissen *et al.* (2001), where concentrations of FB honey higher than 5% caused a partial inhibition of *C. albicans* planktonic cells. No further detailed studies have been undertaken to investigate the anti-fungal effects against *C. albicans,* especially the effects against biofilms.

Magoshi (2017) evaluated the antioxidant and anti-inflammatory activity of FB honey which were compared with those of MAN UMF15+ honey. The study evaluated the effect of gastric and gastro-duodenal digestion on the bioactivity of FB honey. The antioxidant activity of FB honey was maintained after digestion, meanwhile for MAN honey, the antioxidant activity increased with gastric digestion and was maintained or decreased with gastro-duodenal digestion. MAN honey had a higher antioxidant activity than FB honey. The chemical and cellular NO scavenging activity of the FB honeys was determined. The FB honeys were able to scavenge between 35.67 – 59.73% of the NO produced by sodium nitroprusside. The pro- and anti-inflammatory activity was identified in the honeys in the RAW 264.7 cell line. Honey alone had a pro-inflammatory effect and induced NO, while following lipopolysaccharide (LPS) stimulation of RAW 264.7, FB honey was able to reduce increased NO levels indicating a disruption of the inflammatory pathways. The NO scavenging activity of MAN honey was higher than that of FB honey.

Magoshi (2017) found that following gastro-duodenal digestion, antioxidant and anti-inflammatory activity was retained indicating that the polyphenols in honey were stable. Likewise, both the pro- and anti-inflammatory effects were retained after gastric and gastro-duodenal digestion. A limitation of this study, was that the oral phase of digestion was not included.

To develop FB honey for the treatment of oral candidiasis, it is important to determine whether in the physiological environment of the oral cavity, activity is retained. Variability in the composition of saliva and low volumes makes the use of saliva collected from donors difficult. Furthermore, the complexity of saliva makes it very hard to reproduce for research purposes. An ideal synthetic saliva must closely mimic human saliva and should be able to lubricate and protect the oral mucosa from microorganisms (Silva *et al.*, 2017). Commonly used synthetic saliva is made of natural mucins which can be provided by pig gastric mucosa and this type of synthetic saliva has shown lubricating properties comparable to human saliva (Petrou and Crouzier, 2018). Other ingredients that are contained in synthetic saliva are electrolytes such as fluoride, calcium and phosphate required for remineralisation (Sejdini *et al.*, 2018) as well as α-amylase that is involved in the digestion of starches. The first level of testing for the

evaluation of antimicrobial and anti-inflammatory activity, as used in the present study is the use of a synthetic saliva, that is at the correct pH, ionic strength and contains α-amylase.

2.5 Aims and objectives

Therefore, the aim of this study was to determine if the anti-fungal, antioxidant, and anti-inflammatory activities of several FB honeys are comparable to a medical-grade MAN UMF10+ honey. Then to determine if following oral digestion, the bioactivity of each honey type is altered.

The aims of each research chapter were:

Chapter 3 - To determine if the anti-fungal activity against *C. albicans,* of several FB honeys are comparable to MAN UMF10+ honey. Then to determine if following oral digestion, the bioactivity of each honey type is altered.

Chapter 4 - To determine if the antioxidant properties of several FB honeys are comparable to MAN UMF10+ honey. Then to determine if following oral digestion, the bioactivity of each honey type is altered.

Chapter 5 - To evaluate the cellular toxicity, antioxidant and anti-inflammatory activities of several FB honeys compared to MAN UMF10+ honey. Then to determine if following oral digestion, the bioactivity of each honey type is altered.

Chapter 3 : Anti-fungal activity of undigested and oral digested honey

3.1 Introduction

Oral candidiasis is an opportunistic mucosal infection in the oral cavity caused by *C. albicans* (Pankhurst, 2009). Infection is common in individuals that are immune-compromised, including young children and the aged, as well as patients with DM. Symptoms of oral candidiasis are inflammation, lesions on the tongue and cheek, and dysphagia due to the lesions which reach the oropharynx (Coronado-Castellote and Jimenez-Soriano, 2013).

Honey is a known ancient therapy that has good antimicrobial activity (Irish *et al.*, 2011). This is due to the presence of bee-derived glucose oxidase that produces H_2O_2 from glucose and oxygen (Irish *et al.*, 2011), BD-1 and MGO. In MAN honey, MGO is the main antimicrobial component (Johnston *et al.*, 2018). All honey contains a mixture of polyphenols such as quercetin, naringenin and catechin (Marin *et al.*, 2015) that have antimicrobial properties (Marin *et al.*, 2015; Samarghandian *et al.*, 2017). Additive or synergistic interactions between H_2O_2 and/or MGO and the polyphenols may contribute to antimicrobial effects (Alvarez-Suarez *et al.*, 2014; Albaridi, 2019). The sugars in honey make up 80% of its constituents, and play a role in the antimicrobial activity of honey, by inducing osmotic stress due to low moisture content (Kwakman and Zaat, 2012).

Suhana *et al.* (2015) compared the anti-fungal activity of Malaysian honeys to MAN honey. The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of MAN honey was 22% and 25% (v/v), respectively, compared with 25% for an artificial sugar indicating that MAN honey did not inhibit planktonic *C. albicans,* but did have fungicidal activity. All Malaysian honeys evaluated had no activity against *C. albicans*. Anand *et al.* (2019) compared an *Agastache* honey with New Zealand MAN honey in *C. albicans* skin infections, and found that MAN honey had fungistatic activity at a 40% concentration.

A 31% solution of Portuguese lavender honey reduced the growth rate of *C. albicans* by 10% (Estevinho *et al.*, 2011). Moussa *et al.* (2012) reported no inhibition with the radial diffusion assay, while in contrast, the MIC_{50} against planktonic *C. albicans* was 70.09 – 79.27% (v/v). Irish *et al.* (2006) determined the MIC of several honeys, and reported a MIC of 39.9 ± 1.7% for Comvita@ WoundCare 18+ with a lower MIC of 18.5 ± 2.1% for Jarred honey compared with 42.6% for an artificial honey. These studies indicate that honey may have some anti-fungal activity against planktonic *C. albicans*, although findings are not conclusive and vary depending on the honey type.

Ansari *et al.* (2013) reported that Jujube honey inhibited biofilm formation and disrupted established biofilms at a concentration of 40%. Other studies on the biofilm inhibition of honey are limited.

Aim

The aim of the research presented in this chapter was to determine if the anti-fungal activity against *C. albicans,* of several FB honeys are comparable to MAN UMF10+ honey, and further determine the possible anti-fungal activity of MGO and an artificial honey solution.

Objectives

1. To determine the anti-fungal activity of FB honey, MAN UMF10+ honey, MGO and an artificial honey solution.
2. To determine the effect of oral digestion on the anti-fungal properties of each honey, MGO and an artificial honey solution.

Changes in the following parameters were evaluated;

a) The growth of *C. albicans* planktonic cells and biofilms.
b) The effects of MGO and an artificial honey solution, on the growth of *C. albicans* planktonic cells and biofilms.
c) Lastly, the effect on *C. albicans* morphology with scanning electron microscopy.

3.2 Materials

3.2.1: Reagents

Potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), monopotassium phosphate (KH_2PO_4), ammonium chloride (NH_4Cl), sodium carbonate (Na_2CO_3), α-amylase from porcine pancreas, MGO, fructose, glucose, maltose, sucrose, Yeast Peptone Dextrose (YPD) agar, Rosewell Park Memorial Institute-1640 (RPMI-1640) medium and morpholinopropanesulfonic acid (MOPS) were obtained from Sigma Aldrich Company, Johannesburg, SA. While, anhydrous glucose was from Merck (Davidson Rd. Wadeville, Gauteng, SA).

Additional reagents used in this part of the study were CellTiter-Blue® Reagent, poly-L-lysine solution, glutaraldehyde and hexamethyldisilazane (HMDS) obtained from Sigma Aldrich Company, Johannesburg, SA.

3.2.2 C. albicans

The American Type Culture Collection (ATCC) supplied the *C. albicans* strain 90028 (ATCC® 90028™).

3.2.3 Honey samples

The honey samples used in this study were FB1 (Highveld Honey Farms, SA), FB2 (Karoo Gold Pure Honey, SA), FB3 (local health store, SA), FB4 (Wolvefontein Farms, Gouritsmond, SA) and MAN UMF10+ (Tahi Manuka Honey UMF10+) was obtained from a local health store in Pretoria, SA. The criteria for selecting the honey samples was random. All the honey samples were from different FB regions and to obtain a range of bioactivity.

3.2.4 Solutions

The synthetic saliva that was used in this study for oral digestion was a modified formula adapted from Minekus *et al.* (2014). A 50 mL synthetic saliva stock solutions were prepared and consisted of 1.86 g KCl, 3.4 g KH_2PO_4, 4.2 g $NaHCO_3$, 0.714 g $MgCl_2$, 2.65 g Na_2CO_3, 1.33 g NH_4Cl and 1.66 g $CaCl_2$ each diluted in ddH_2O. A 1.25X synthetic saliva salt solution was prepared by mixing together 15.1 mL KCl, 3.7 mL KH_2PO_4, 6.8 mL $NaHCO_3$, 0.5 mL $MgCl_2$, 0.06 mL Na_2CO_3, 0.06 mL NH_4Cl diluted to 473.78 mL ddH_2O which was adjusted to pH 7 with NaOH and HCl. $CaCl_2$ and 1500 U/mL α-amylase prepared in the synthetic salt solution was added to a final concentration of 0.75 mM and 75 U/mL, respectively. The control was prepared as described above except that the α-amylase was replaced with the synthetic saliva solution.

Phosphate buffered saline (PBS) (final concentration 0.1 M) was prepared by mixing three solutions: 405 mL of Na_2HPO_4 (12.78 g in 450 mL ddH_2O (0.2 M)), 95 mL of $NaH_2PO_4 \cdot H_2O$ (2.76 g in 100 mL ddH_2O (0.2 M)) and 8.77 g of NaCl and made up to 1 L with ddH_2O. The pH was then adjusted to 7.4 and stored at room temperature.

3.2.5 Equipment

The equipment used was a VWR spectrophotometer from Radnor, PA, USA, a SpectraMax Paradigm Multi-Mode microplate reader from Molecular devices, Separations Johannesburg, SA and a Zeiss Ultra Plus FEG-SEM, Zeiss Crossbeam 540 FEG-SEM.

3.2.6. Laboratory facilities

Research was conducted in the research facilities of the Biotherapeutics Division of Biochemistry in the Department of Biochemistry, Genetics and Microbiology of the Faculty of Natural and Agricultural Sciences, and the Laboratory for Microscopy and Microanalysis at the University of Pretoria.

3.3 Methods

3.3.1 Honey solutions and simulated oral digestion

For this study three samples were generated. These were;

Undigested (UD): Honey was diluted in ddH_2O to respective concentrations. A 50% (v/v) solution of each honey was prepared. This solution was further diluted to prepare 25%, 30%, 35%, 40% and 45% (v/v) solutions. Each solution was then further diluted 5X to prepare low concentration solutions of 5, 6, 7, 8 and 9% (v/v).

Oral digest control (no α-amylase added) (ODC): For the preparation of ODC, a 1:1 ratio of the diluted honey to the control solution (Section 3.2.4) was prepared.

Oral digest (α-amylase added) (OD): For the preparation of OD, a 1:1 honey ratio of the diluted honey to the synthetic saliva solution (Section 3.2.4) was prepared.

Samples were incubated for 2 min at 37ºC to simulate normal oral digestion or the use of honey as a mouthwash. To deactivate the α-amylase activity, the synthetic saliva mixtures were snap frozen in liquid nitrogen for 30 s.

A 40 mM MGO working solution was prepared from a 6.5 M MGO solution in ddH_2O and was subjected to oral digestion as described above. The undigested and oral digested MGO was diluted to generate a low and high concentration series of 0.5 – 2 mM and 5 – 20 mM, respectively.

An artificial honey solution, representing the sugar-based analogue of honey consisted of 40% fructose, 30% glucose, 10% maltose and 2% sucrose, was prepared and was subjected to oral digestion and was the further diluted to 1% and 50% (v/v) solutions.

3.3.2 Candida albicans cultures

C. albicans stock solutions were stored at - 80ºC in 30% glycerol (v/v). Overnight cultures were grown in YPD agar (1% yeast extract, 2% peptone, 2% glucose, and 1.5% agar). *C. albicans* was grown in RPMI-1640 medium and was buffered to a pH of 7 using MOPS,

containing L-glutamine, phenol red and no bicarbonate. The media was supplemented with glucose to a final concentration of 20 g/L glucose (2% w/v) to yield RPMI-1640 containing 2% glucose (RPMI/Glc).

3.3.2.1 Candida albicans planktonic cell preparation

The minimum inhibitory concentration assay was performed according to the method of the European Committee on Antimicrobial Susceptibility Testing (Rodriguez-Tudela *et al.*, 2008) definitive document, with minor modifications (Rodriguez-Tudela *et al.*, 2008). Five representative colonies from a 24 h culture on YPD agar medium, were suspended in 5 mL of water. The inoculum was vortexed for 15 s, and a volume of 200 µL was used to measure the optical density at 530 nm (OD_{530}). The cell density of the inoculum was adjusted to obtain an OD_{530} value within the range 0.12 to 0.15, which correlates to a yeast suspension of 1-5 x 10^6 colony forming units per mL (CFU/mL). An OD_{530} value above the range was adjusted, by diluting the inoculum to a final volume of 1 mL, using the equations below:

$$\frac{\boldsymbol{OD_{530}}}{\mathbf{0.15}} = \boldsymbol{dilution\ factor}$$

$$\frac{\mathbf{1000}\ \boldsymbol{\mu L}}{\boldsymbol{dilution\ factor}} = \boldsymbol{\mu L\ inoculum}$$

The remaining volume was made up with water. A final 10X dilution was performed to yield a final yeast suspension of 1-5 x 10^5 CFU/mL in a volume of 5 mL. Upon preparing the inoculum, working solutions of undigested and oral digested FB and MAN UMF10+ honeys were prepared as described in section 3.3.1.

A volume of 50 µL of the different honey concentrations was transferred to a 96-well plate in duplicate. To each well, 50 µL of the final yeast suspension was added, to yield a final volume of 100 µL. The growth control included 50 µL of cells and 50 µL of RPMI/Glc. The blank included 50 µL of ddH_2O and 50 µL of RPMI/Glc. The 96-well plate was then left to incubate for 24 h at 37°C. Following incubation, the absorbance was read at 530 nm and the established absorbance was used to determine the inhibition (%) of the honey samples against *C. albicans*, using the following equation (Hsieh *et al.*, 1993; Rodriguez-Tudela *et al.*, 2008):

$$\left(\frac{\boldsymbol{average\ absorbance\ of\ wells - average\ absorbance\ of\ blank}}{\boldsymbol{average\ absorbance\ of\ growth\ control - average\ absorbance\ of\ blank}}\right) \times \mathbf{100}$$

3.3.2.2 Candida albicans biofilm preparation

Five colonies were inoculated in 20 mL YPD broth and the cells were grown for 18 hours at 30°C and 150 rpm. The cells were aliquoted in 1 mL volumes into 1.5 mL Eppendorf tubes and centrifuged for 10 min at 2000×g. The supernatant was discarded. The cells were then washed with 1000 µL RPMI-1640 medium and centrifuged for 2 min at top speed (14100×g). The supernatant was removed and the cells were resuspend in 1000 µL RPMI-1640 by vortexing. The optical density (OD_{620}) was measured and diluted to an OD_{620} that corresponds to a cell density of 1×10^6 CFU/ml. A 100 µL volume of the cell suspension was pipetted into the wells of a 96-well plate and was incubated for 1 h and 30 min at 37ºC to allow attachment. The media was then removed from the wells and the plates were thoroughly washed with 1X PBS to remove non-adherent cells. To evaluate the effects of the low sample concentrations, including oral digests, 90 µL of RPMI-1640 was added into each well, followed by 10 µL of honey, MGO or artificial honey sample. For the higher concentrations, 50 µL of RPMI-1640 was added into the wells followed by 50 µL of honey, MGO or artificial honey sample. This was incubated for another 24 h at 37ºC and sealed with parafilm. After incubation, the inhibition assay was performed. The growth control was the wells containing 100 µL of RPMI-1640 (no sample added) and the blank contained only 100 µL of ddH_2O.

3.3.2.3 CellTiter-Blue assay

Principle

CellTiter-Blue is a fluorometric method that is used to estimate the number of viable biofilm cells cultured in multi-well plates. Blue resazurin is the active compound in CellTiter-Blue which forms pink resorufin in the presence of viable cells (O'Brien *et al.*, 2000).

Method

The honey sample was removed from each well and the plate was gently washed with 1X PBS. A volume of 100 µL of 10% CellTiter-Blue solution was added into each well and then the plate was wrapped in foil to avoid photodegradation and incubated for 1 h at 37°C. The amount of resorufin formed was quantified fluorescently at an Ex of 535 nm and an Em of 590 nm with a SpectraMax Paradigm Multi-Mode microplate reader.

3.4 Scanning electron microscopy

Poly-L-lysine-coated glass cover slips

Principle

Poly-L-lysine is polycationic adhesive that is used to secure individual anionic cells and particles in order to ease processing and viewing under the scanning electron microscope (Taylor, 2008).

Method

To grow *C. albicans* and enable the evaluation of morphology with scanning electron microscopy, poly-L-lysine coated coverslips were prepared. The glass cover slips were washed five times with a 5% (w/v) alkaline NaOH solution prepared in a 30% (v/v) solution of ethanol. The coverslips were then left in 100% ethanol overnight and dried completely. Each cover slip was coated with 1 mL poly-L-lysine solution and incubated at room temperature for 2 h. The poly-L-lysine was removed by washing the cover slips ten times with sterile water and were then dried for a minimum of three days before use.

Ultrastructure evaluation

Principle

The scanning electron microscope uses solid pieces of the specimen rather than the ultrathin sections of tissue. This allows the three-dimensional view of the surface of the cells and subcellular structures of the specimen. A small piece of the specimen is dried and coated with carbon, or another type of heavy metal. The specimen is scanned by an electron beam coming from the electron gun and low-energy secondary electrons are produced by the interaction of the incident electrons (primary electrons) with atoms in the surface layer of the specimen. The low-energy secondary electrons are used to create a high-resolution, three-dimensional image of the specimen (Jensen, 2012) such as *C. albicans*.

Method

Cells were grown on the glass cover slips coated with poly-L-lysine. The *C. albicans* cells on coverslips were then exposed to honey samples as described in section 3.3.1. After exposure the cells were then fixed in 2.5% GA/FA fixative (1 mL GA, 1 mL FA, 3 mL ddH_2O, 5 mL buffer) for 30 min. The cells were then washed with buffer 3X (0.075 M phosphate buffer pH 7.4) with 15 min in between washes. Cells on coverslips were then dehydrated in 30%, 50%, 70%, 90% ethanol with 15 min intervals for each. Cells were then finally dehydrated in 100% ethanol 3X for 15 min each. HMDS (100%) was then added for 1 h, then removed. After which, 1 - 2 drops of HMDS were added on the glass cover slips and dried overnight. The samples were then

mounted on aluminium stubs and coated with carbon. Samples were viewed with a Zeiss Ultra Plus FEG-SEM.

3.5 Statistical analysis

All experiments were done at least three times in triplicate yielding 9 data points, unless otherwise stated, and presented as mean ± SEM. Statistical analysis was first done by determining normality of data using D'Agostino Pearson test and confirmed using the Shapiro-Wilks test. Depending on normality of data, significant differences were determined using one-way ANOVA followed by the Tukey post-hoc test for data with a normal data set or the Kruskal-Wallis test for data with a skewed data set. Significant differences were determined at $p \leq 0.05$.

3.6 Results

3.6.1 Inhibition of Candida albicans planktonic cell growth

The anti-fungal activity of undigested and oral digested FB and MAN UMF10+ honey was tested against *C. albicans* planktonic cells using a low (5 - 9%) and a high (25 - 45%) concentration range as presented in Figures 3.1 and 3.2, respectively.

For UD, the low honey concentration range promoted the growth of *C. albicans* planktonic cells when compared to 100% for the growth control, with a statically significant increase in growth in the range of 408.42 - 428.06%. Using a 5% honey solution, the effect of oral digestion was then determined (Figure 3.1B). Also for 5% UD, ODC and OD, the growth of *C. albicans* planktonic cells was increased compared with the growth control. Differences between FB honeys and MAN UMF10+ were not significant. Significant differences were observed between the UD and OD, as well as the ODC and OD of FB4. Significant differences were also observed between the UD and ODC of MAN UMF10+. For the other honeys, with oral digestion, differences between undigested, ODC and OD were not observed.

For the higher concentrations, UD caused significant growth of planktonic cells. For 25 – 45% there was a dose response with a negative dosage response, or decrease in growth for FB2 with $y = -16x + 995.2$, $R^2 = 0.945$, and FB3 with $y = -68.301x + 546.9$, $R^2 = 0.948$, but not for FB1, FB4 and MAN UMF10+ (Figure 3.2A). The effects of oral digestion on the ability of a 25% solution honey to inhibit the growth of *C. albicans* planktonic cells was then determined (Figure 3.2B). Growth was increased compared with the growth control with no differences between UD, ODC and OD of all honey samples.

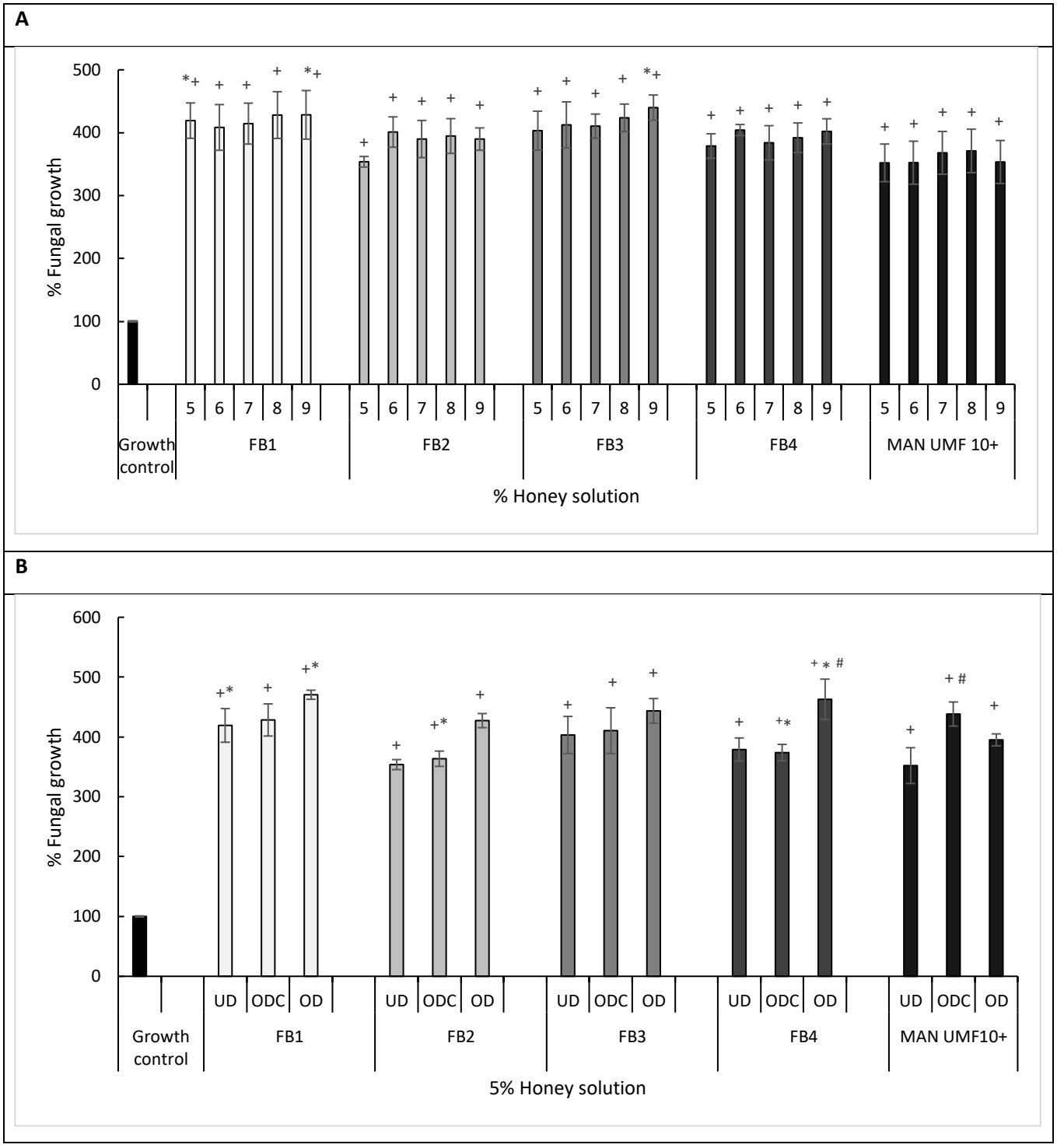

Figure 3.1: Inhibition of planktonic *C. albicans* growth by Fynbos (FB) and Manuka (MAN UMF10+) honeys. Figure A: inhibition of growth by 5 - 9% undigested (UD) honey solutions and Figure B: Inhibition of growth by 5% solutions of UD, oral digest control (ODC) and oral digest (OD) honey samples. + Indicates significant differences between the growth control and the honey samples, * indicates significant difference of the FB honey samples compared with MAN UMF10 (at the same concentrations) and # indicates differences between UD and ODC and OD of a specific honey, $p<0.05$.

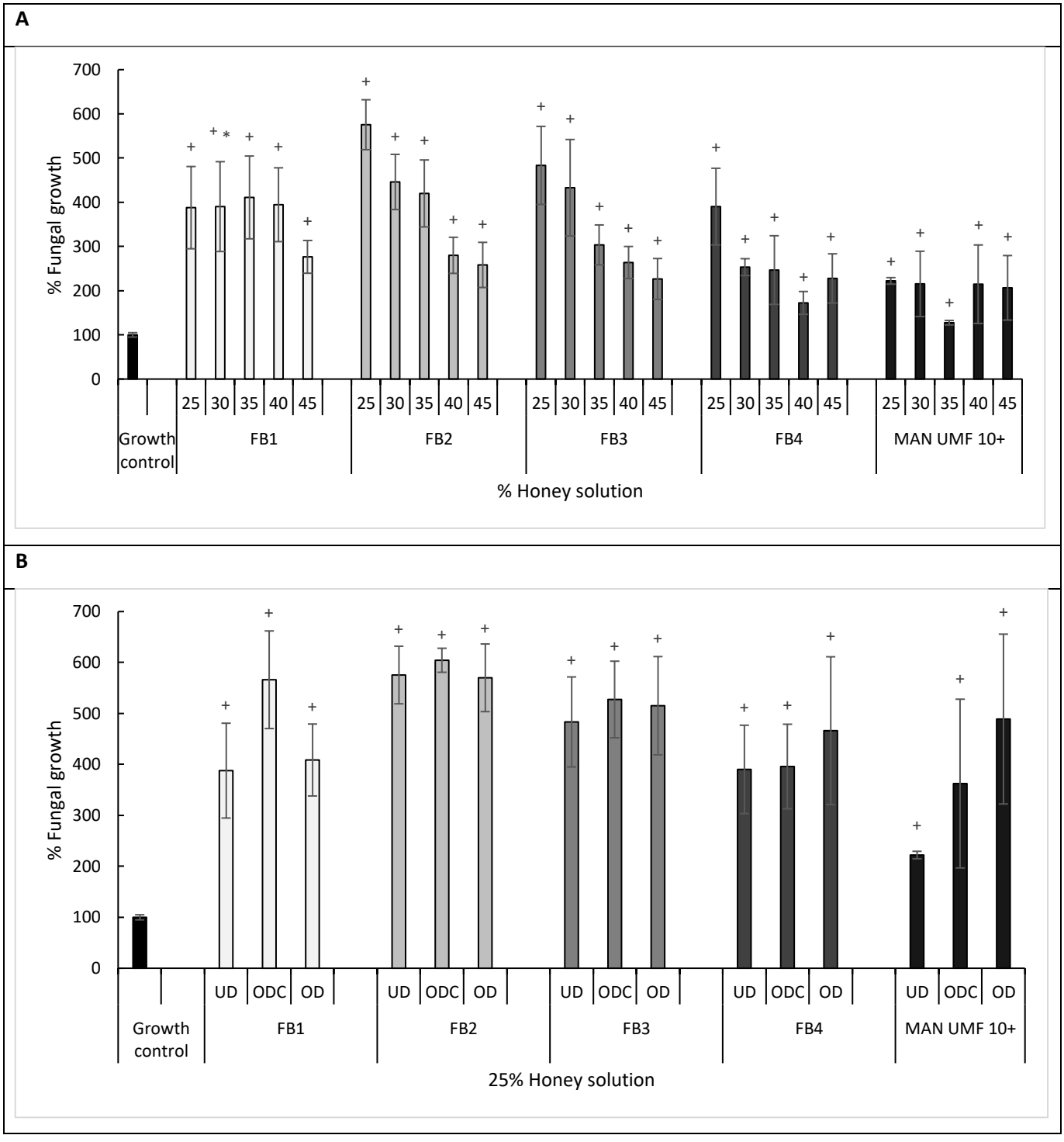

Figure 3.2: Inhibition of planktonic *C. albicans* growth by Fynbos (FB) and Manuka (MAN UMF10+) honeys. Figure A: inhibition of growth by 25 - 45% undigested (UD) honey solutions and Figure B: Inhibition of growth by 25% solutions of UD, oral digest control (ODC) and oral digest (OD) honey samples. * indicates significant difference of the FB honey samples compared with MAN UMF10+ and $^{+}$ indicates significant difference of growth control compared to honey samples, $p<0.05$

3.6.2 Inhibition of biofilm Candida albicans growth

The anti-fungal activity of FB and MAN UMF10+ honeys against *C. albicans* biofilms was then evaluated. In Figures 3.3 and 3.4 the effects of low and high honey dilutions on the growth of *C. albicans* biofilms is presented, respectively.

In Figure 3.3A dilutions of 7 - 9% FB1 and 9% FB3 showed a significant reduction on the % growth of *C. albicans* biofilms and this effect was significantly less than that observed for MAN UMF10+. No statistical differences were observed between before and after oral digestion (Figure 3.3B).

In Figure 3.4A, the higher concentrations (25 – 45%) compared with the growth control, 45% FB2, FB3 and FB4 reduced the growth of the *C. albicans* biofilms to 63.69%, 54.88% and 41.75%, respectively. In contrast, 30-45% MAN UMF10+ caused a significant reduction in biofilm growth with inhibition of 15.66% at 45%. Compared to MAN UMF10+, undigested FB1 and FB2 (30 and 45%), FB3 (40 and 45%) and FB4 (35 - 45%) honey samples were significantly different, where MAN UMF10+ was able to better inhibit the growth of *C. albicans* biofilms. No significant differences were observed between undigested and orally digested honey samples (Figure 3.4B). For the orally digested honey samples, FB1 and FB3 OD honey samples showed a significantly better biofilm inhibition when compared to MAN UMF10+, $p<0.05$.

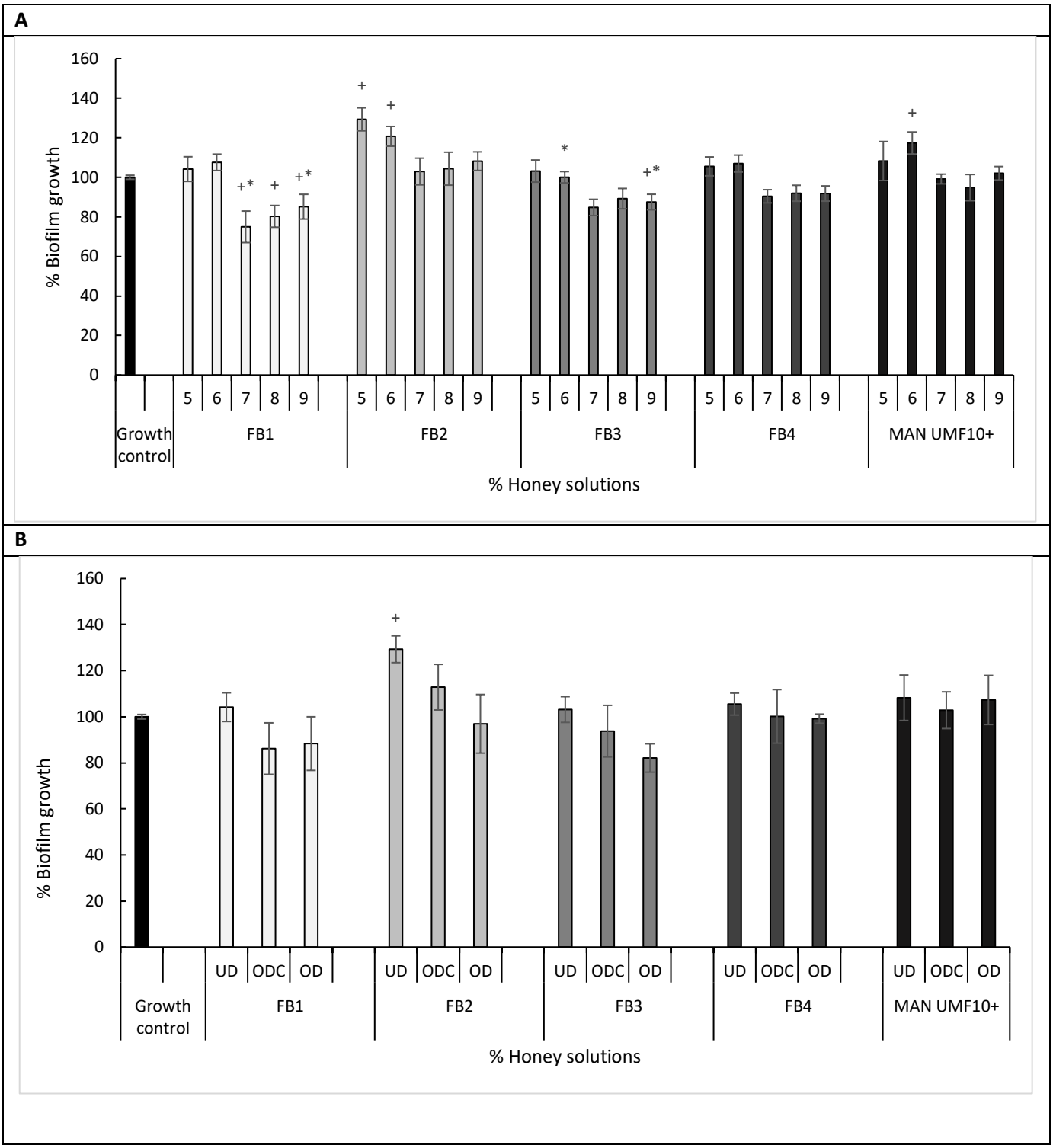

Figure 3.3: Inhibition of *C. albicans* biofilm growth by Fynbos (FB) and Manuka (MAN UMF10+) honeys. Figure A: inhibition of growth by 5 - 9% undigested (UD) honey solutions and Figure B: Inhibition of growth by 5% solutions of UD, oral digest control (ODC) and oral digest (OD) honey samples. + Indicates significant differences between the growth control and the honey samples, * indicates significant difference of the FB honey samples compared with MAN UMF10 (at the same concentrations) and # indicates differences between UD and ODC and OD of a specific honey, $p<0.05$.

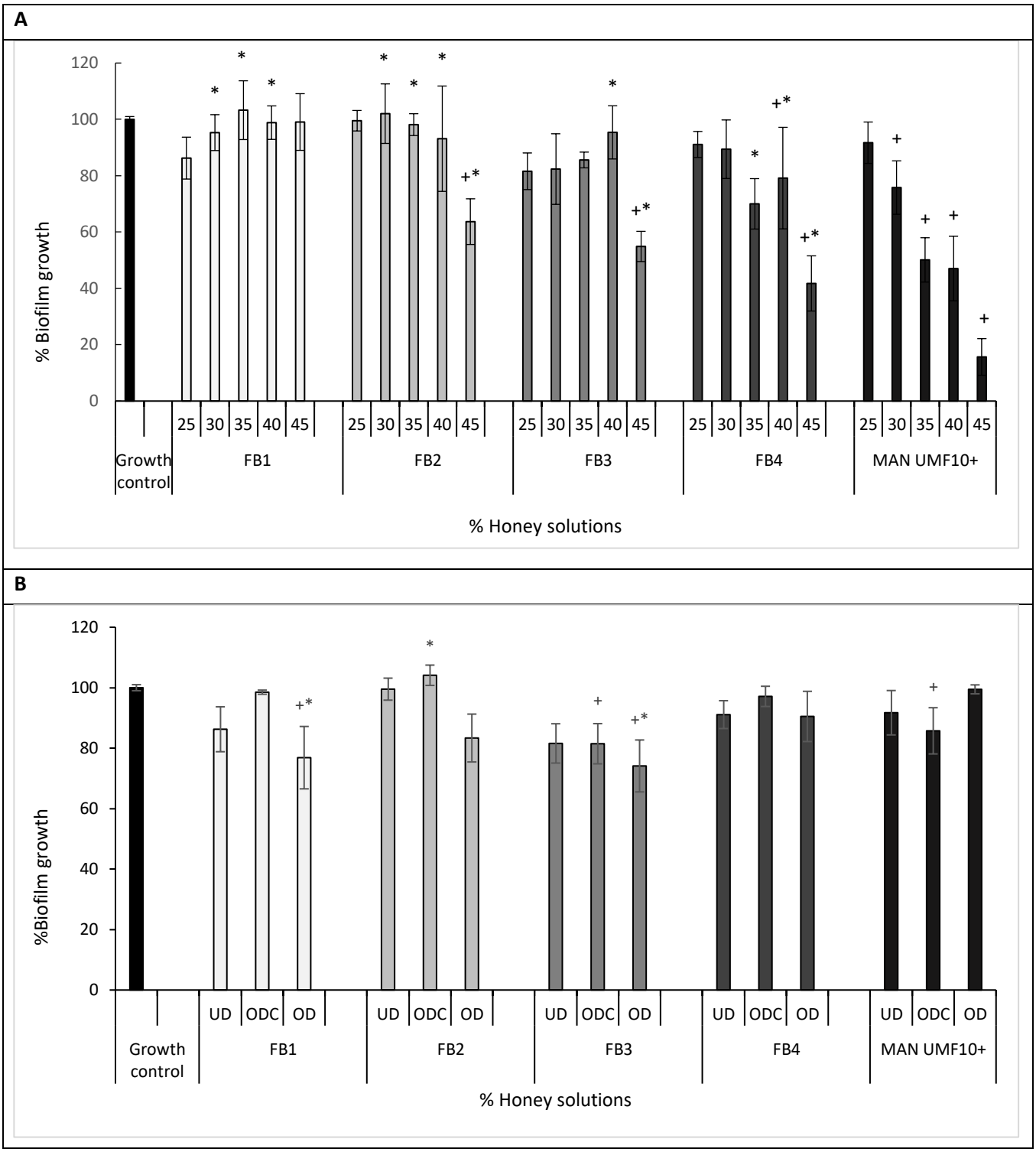

Figure 3.4: Inhibition of *C. albicans* biofilm growth by Fynbos (FB) and Manuka (MAN UMF10+) honeys. Figure A: inhibition of growth by 25 - 45% undigested (UD) honey solutions and Figure B: Inhibition of growth by 25% solutions of UD, oral digest control (ODC) and oral digest (OD) honey samples. $^{+}$ Indicates significant difference between the growth control compared with the honey samples, * indicates significant difference of the FB honey samples compared with MAN UMF10+, and no significant difference observed between UD, ODC and OD honey samples, $p<0.05$.

3.6.3 Inhibition of C. albicans planktonic cell growth by methylglyoxal and an artificial honey solution

As sugars and MGO contribute to the anti-fungal effects of honey, the effects of MGO and an artificial honey solution were determined individually (Figure 3.5 and 3.6). Low MGO concentrations of 0.5 - 2.0 mM did not inhibit the growth of planktonic *C. albicans*. Following oral digestion for 1 mM, 1.5 mM and 2 mM MGO the growth of planktonic *C. albicans* compared with the control was promoted. Differences between UD and OD, were significant, $p<0.05$ at a concentration of 5 mM.

In contrast, higher concentrations of 5 – 20 mM, significantly inhibited growth compared with the control. A dosage dependent inhibition of growth was observed for the undigested ($y = -65.495x + 405.15$, $R^2 = 0.895$) and oral digested MGO ($y = -21.529x + 126.25$, $R^2 = 0.951$) although this was only significant compared with the growth control for 15 and 20 mM undigested MGO, and 5 mM digested MGO.

The 50% artificial honey inhibited the growth of planktonic *C. albicans,* but this effect was reduced following oral digestion and was not statistically different from the growth control and the UD artificial honey solution.

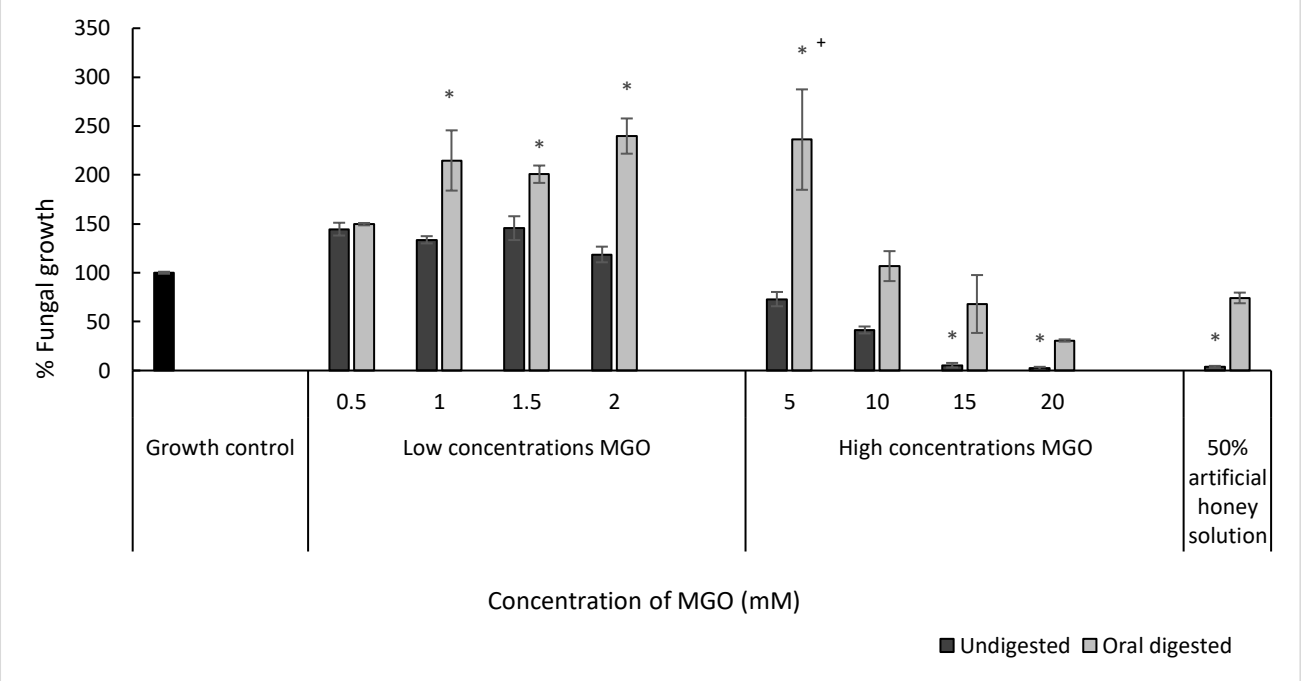

Figure 3.5: Inhibition of the growth of planktonic *C. albicans* by of undigested (U), oral digested (OD), methylglyoxal (MGO) at low (0.5 – 2 mM) and high concentrations (5 - 20 mM) as well as a 50% artificial honey solution. $^{+}$ indicates significant differences between the growth control and MGO or the artificial honey solution (undigested and digested) and $^{\#}$ indicates differences between UD and OD MGO and the artificial honey solution, $p<0.05$.

3.6.4 Inhibition of C. albicans biofilm growth by methylglyoxal and an artificial honey solution

The effects of MGO, a 1% and 50% artificial honey solution on the growth of *C. albicans* biofilms were also determined. Low concentrations, 0.5 – 2 mM MGO (Figure 3.6) showed very little to no inhibition to the growth of *C. albicans* biofilms. The orally digested MGO samples showed better inhibition, especially for the concentrations 1 and 1.5 mM. At these concentrations, no significant differences were found between undigested and orally digested MGO, as well as when compared to the growth control.

Higher concentrations, 5 – 20 mM were able to inhibit the growth of *C. albicans* biofilms and the differences between the UD and OD of these MGO concentrations was significant, $p<0.05$. The growth of *C. albicans* biofilms in the presence of a 1% and 50% artificial honey solution was not inhibited, when compared with the growth control (Figure 3.6). Likewise, with oral digestion biofilm growth was not significantly altered.

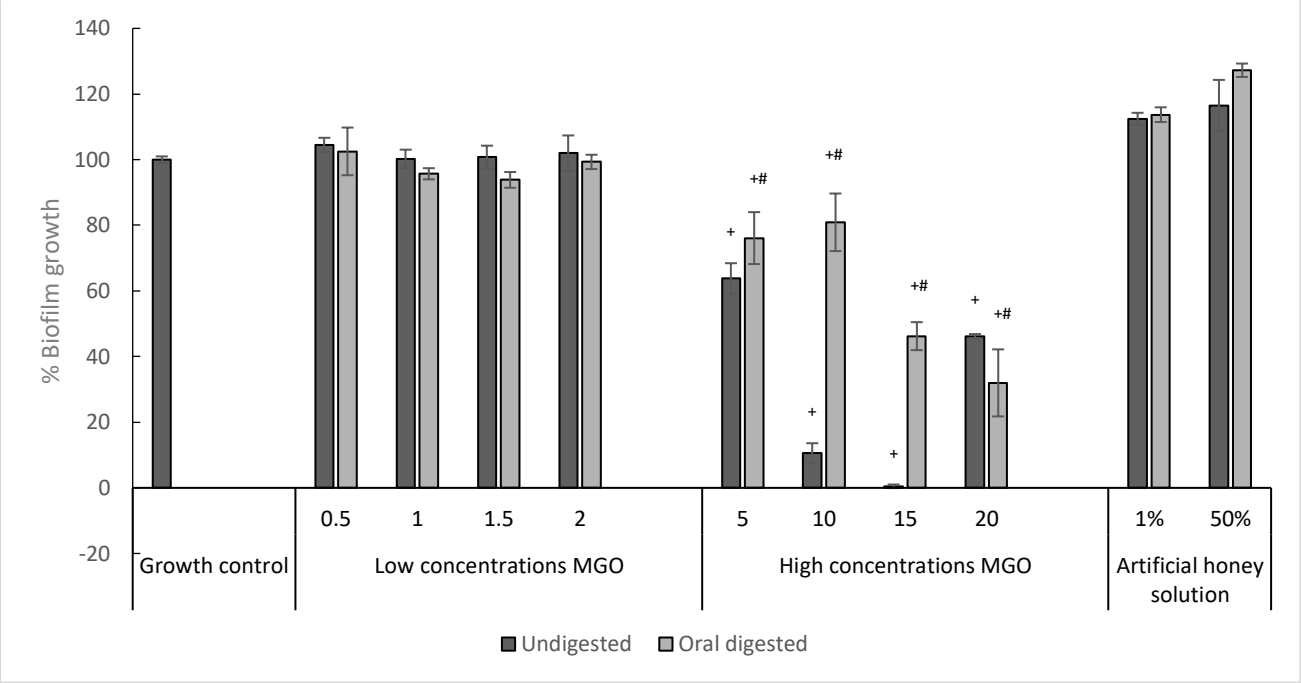

Figure 3.6: Inhibition of *C. albicans* biofilm growth by undigested (U) and oral digested (OD) methylglyoxal (MGO) at low (0.5 – 2 mM) and high concentrations (5 - 20 mM) as well as a 1% and 50% artificial honey solution. $^{+}$ indicates significant differences between the growth control and MGO or the artificial honey solution (undigested and digested) and $^{\#}$ indicates differences between UD and OD MGO and the artificial honey solution, $p<0.05$.

3.6.5 The effect of low concentrations of honey on the morphology of Candida albicans biofilms

The morphogenic effects of 5% undigested and oral digested FB and MAN UMF10+ honey on *C. albicans* cells in biofilms was evaluated. Changes in morphology was evaluated at three different magnifications and the micrographs are a representative of the average appearance.

In Figure 3.7, the growth control cells indicate the normal morphology of *C. albicans* cells in biofilms that develop from the growth and proliferation of free-floating planktonic cells. With sample processing for scanning electron microscopy, the ECM is lost and only the cellular components of the biofilm are retained. The growth control shows a dense network of true hyphae (UD), indicated with red arrows. With OD, a dense network of true hyphae is also present, although not as dense as the UD. Pseudohyphal cells usually have a thicker width in the middle than at the ends (ellipsoidal) unlike hyphal cells which are more uniform in shape and have true septa with no constrictions (Thompson *et al.*, 2011).

For both undigested and oral digested FB honeys and the MAN UMF10+ honey, the typical biofilm structure is absent but rather clumps and individual round/oval yeast cells are distributed over the surface indicating the presence of planktonic cells rather than biofilms. There was no observable difference between UD and OD samples.

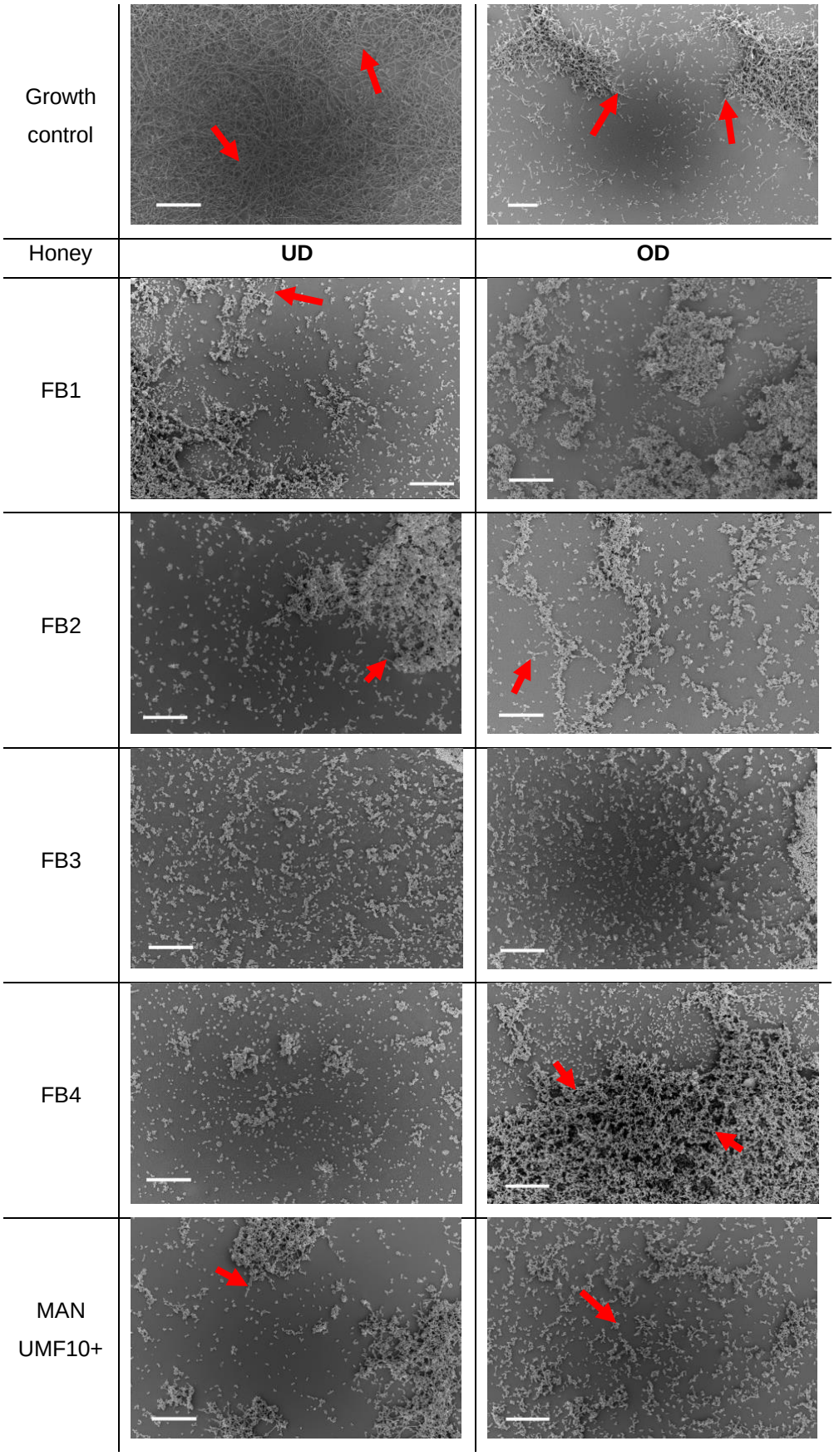

Figure 3.7: Scanning electron micrographs of *Candida albicans* biofilms. Samples are the growth control, and 5% undigested (UD) and oral digested (OD) Fynbos (FB) and Manuka (MAN UMF10+) honey. Red arrows indicate hyphae. Scale bars: 100 µm for 150X magnification.

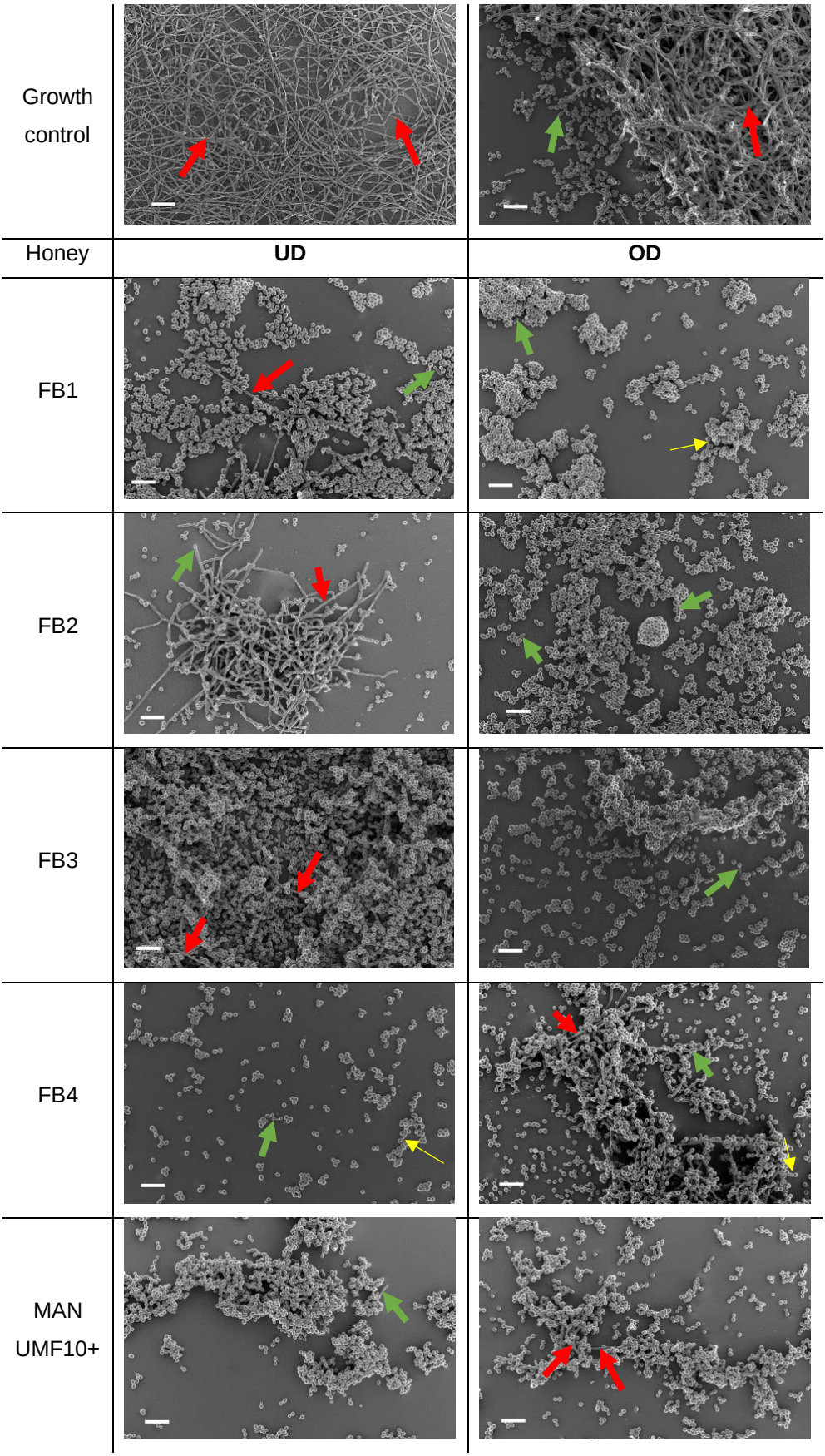

Figure 3.8: Scanning electron micrographs of *Candida albicans* biofilms. Samples are the growth control, and 5% undigested (UD) and oral digested (OD) Fynbos (FB) and Manuka (MAN UMF10+) honey. Red arrows indicate hyphae, green arrows indicate pseudohyphae and yellow arrows indicate hyphal septa. Scale bars: 20 µm for 400X magnification.

In Figure 3.9, at a magnification of 2000X, the growth control images show the intrinsic network of hyphae (red arrows) with many pseudohyphae (green arrows) and a few round yeast (y) cells. A number of shrunken cells or cells with indentations are also present (white arrows).

With the cells exposed to FB and MAN UMF10+ honeys, there was an increased prevalence of round/oval yeast cells than pseudohyphae as well as a few true hyphae, which supports the findings in Figure 3.3, that biofilm growth and related biofilm formation is inhibited. The orange arrows indicate truncated bud scars where the fragile hyphal septa (yellow arrows) were present. At this high magnification (Figure 3.10), exposure to OD honey, causes some cells to have splitting of the cell wall (purple arrow).

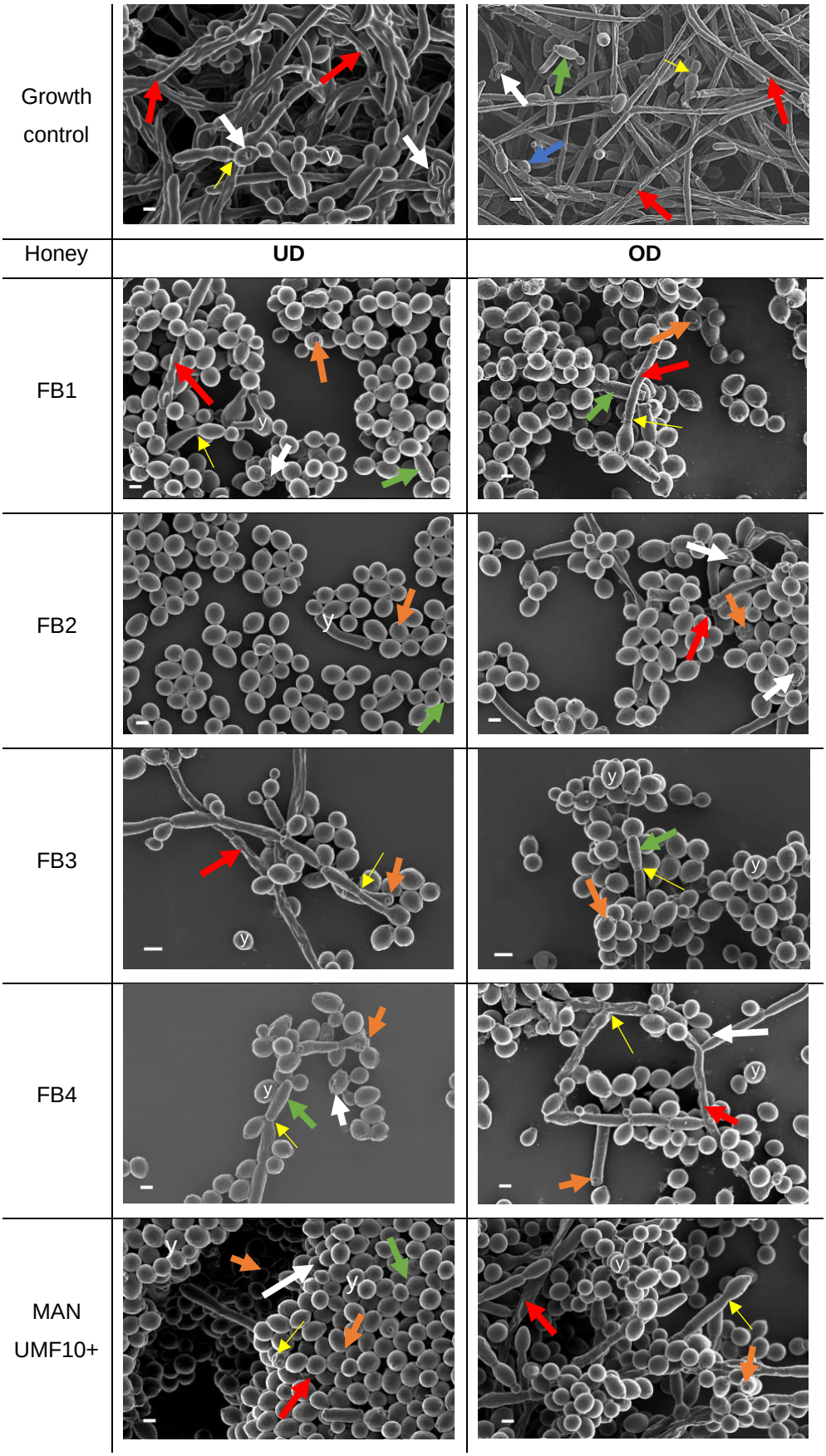

Figure 3.9: Scanning electron micrographs of *Candida albicans* biofilms. Samples are the growth control, and 5% undigested (UD) and oral digested (OD) Fynbos (FB) and Manuka (MAN UMF10+) honey. Red arrows indicate hyphae, green arrows indicate pseudohyphae, yellow arrows indicate hyphal septa, white arrows indicate shrunken cells and uneven cells, orange arrows indicate truncated bud scars, blue arrow indicates splitting of the cell wall and (y) indicates round/oval yeast cells. Scale bars: 2 µm for 2000X magnification.

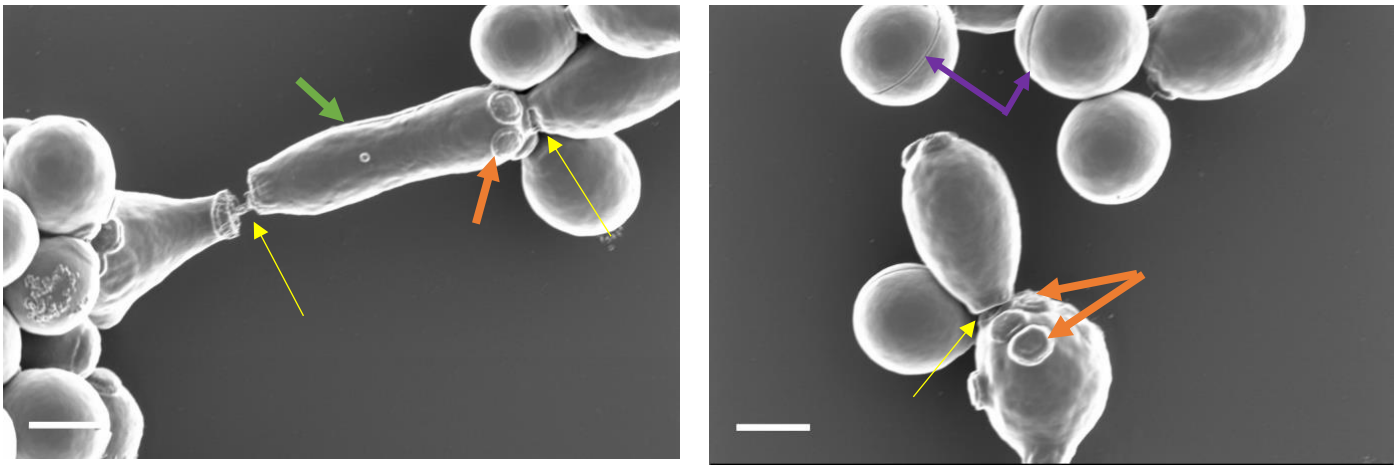

Figure 3.10: Scanning electron micrographs showing examples of pseudohyphae. *Candida albicans* biofilms exposed to 5% oral digested (OD) Fynbos 1 (FB1) honey. Yellow arrows indicate hyphal septa, orange arrows indicate truncated bud scars, green arrows indicate pseudohyphae and purple arrows indicate a splitting of the surface of the cells. Scale bars: 2 µm for 6000X magnification.

3.7 Discussion

The effects of undigested honey on the growth of planktonic and *C. albicans* biofilms were determined. At both low and high concentrations, the growth of planktonic *C. albicans* cells were not inhibited. In contrast, the higher concentrations of the honey samples inhibited the growth of the *C. albicans* biofilms, with the lower concentrations showing less inhibition. This is indicative of a concentration dependent inhibition of the growth of *C. albicans* biofilms.

Anand *et al.* (2019) evaluated the effects of Manuka UMF22+ honey on the growth of planktonic *C. albicans* cells. Using the microbroth dilution assay, 40% Manuka UMF22+ honey inhibited the growth of *C. albicans* and a clinical isolate of *C. albicans* by 50%. In contrast, super Manuka honey MGO-400, did not inhibit the growth of planktonic *C. albicans* or a clinical isolate. Medihoney, Comvita® Wound Care 18+, inhibited growth with a MIC of 39.9 ± 1.7% (Irish *et al.*, 2006). In the present study, at 45%, the highest concentration evaluated no inhibition was observed.

Lavender honey inhibited the growth of *C. albicans* by 31 ± 0.05% (Estevinho *et al.*, 2011) and Jarrah honey by 18.5 ± 2.7% (Irish *et al.*, 2006). Hau-Yama *et al.* (2019) reported a dose dependent inhibitory effect of honey from the stingless bee *Melipona beecheii* against planktonic *C. albicans* with a MIC of 35%. In the study of Anand *et al.* (2019), the effects of Agastache, Tea tree, Jelly bush and Jarrah honey were determined and the MIC values for all honeys was 40%. Against a clinical isolate, the MIC was 40% for Agastache and Tea tree honey but absent for Jelly bush and Jarrah honey. For southern African honeys, Theunissen *et al.* (2001), reported 29.4% inhibition by a 25% solution of Wasbessie honey, with little or no inhibition at this concentration

for Fynbos and Blue gum honey. Likewise, in the present study no inhibition of the growth even at 45% was observed.

Anand *et al.* (2019) attributed the anti-fungal activity to the increase in H_2O_2 levels in honey following dilution of 155 µM measured for Tea tree, and Jarrah honey each. MAN honey, in contrast, has low H_2O_2 levels and even although this honey contains MGO, poor inhibition of *C. albicans* and a clinical isolate was obtained.

Few studies have investigated the effect of honey on biofilm growth. Ansari *et al.* (2013) investigated the effect of Jujube honey on the growth of *C. albicans* and biofilm formation. Findings were that low concentrations of <10% promoted biofilm growth while higher concentrations inhibited biofilm formation with 100% inhibition at a 40% concentration. Low MAN UMF10+ and FB honey concentrations of 4 - 9% did not inhibit the growth of *C. albicans* biofilms. At higher concentration of 45% growth was reduced to 63.7%, 54.9% and 41.75% for FB2, FB3 and FB4 honeys, respectively. At the same concentration biofilm growth was reduced to 15.7% for MAN UMF10+. This indicates that MAN UMF10+ and FB honey do not inhibit the growth of *C. albicans* planktonic cells, but does inhibit the growth of biofilms.

During the glycation reaction of amino acids with MGO, superoxide anions increase, generating two types of free radicals, cross-linked radical cations and MGO radical anions. These effects can cause damage to nucleic acids and proteins in the eukaryotic cell, inhibiting cell division (Johnston *et al.*, 2018; Kwak *et al.*, 2018). High concentrations, 5 - 20 mM MGO inhibited the growth and *C. albicans* biofilm formation, while little to no effect was observed at lower concentrations and this may be due to the alleviation of oxidative stress by glutathione and glyoxalase systems that are well-established in *C. albicans* (Hasim *et al.*, 2014). Inhibition of both forms of *C. albicans* indicates a non-specific mode of oxidative mediated inhibition as described above and not the selective inhibition of biofilms as observed in the present study.

As described in section 2.4, the presence of sugar in honey can create a high osmotic concentration leaving little water available for survival. This can explain how the 50% artificial honey solution inhibited the growth of *C. albicans* planktonic cells. Man *et al.* (2017) investigated the role of glucose and fructose, as important nutritional factors that influence the growth rate of *C. albicans* in diabetics as oral candidiasis is common in patients with poor glycaemic control. The results of this study showed that the growth of planktonic *C. albicans* was dependent on the concentration of glucose while fructose inhibited growth. The artificial honey solution, used in the present study included 40% fructose, 30% glucose, 10% maltose and 2% sucrose. Therefore the presence of fructose may inhibit growth. In contrast, following digestion this inhibitory effect is lost.

Sugars in honey can also act as an energy source promoting biofilm growth and play an important role in the virulence of *C. albicans*, where *C. albicans* biofilms disperse more yeast cells as a survival mechanism in high sugar states, thus causing an increased accumulation of biofilm colony sites. However, in this study, artificial honey at high concentrations did not inhibit biofilm growth although with scanning electron microscopy, an increase in the number of round yeast cells were observed in the *C. albicans* biofilms.

Magoshi (2017) reported that the H_2O_2 content of FB honey was 0.8 – 1.78 mmol/L (n= 6) and for MAN UMF15+ the H_2O_2 content was 0.97 mmol/L. Brudzynski *et al.* (2012) reported that killing was due to hydroxyl ion formation from H_2O_2 and treatment of the honey samples reduced the bacterial killing of H206 honey but not MAN honey. Although H_2O_2 was identified as the source of activity, the retention of activity after catalase treatment indicates the involvement of other molecules such as MGO or possibly polyphenols. In the present study, low dilutions of MGO did not have an effect on *C. albicans* planktonic cells and biofilms, further indicating the possible involvement of other molecules.

Honey contains polyphenols including phenolic acids and flavonoids. Polyphenols identified in MAN honey are the phenolic acids including CA, isoferulic acid, *p*-coumaric acid, GA, 4-hydroxybenzoic acid and syringic acid (Alvarez-Suarez *et al.*, 2014). Of these polyphenols, GA inhibited the growth of *C. albicans* biofilms with a MIC of between 1.18 – 73.5 mmol/L (Teodoro *et al.*, 2015). The mechanism of action of phenolic acids against the growth of *C. albicans* have been identified as apoptotic, such as damaging the cell membrane of the fungus, and increasing ROS (Teodoro *et al.*, 2015), further supporting radical-mediated killing.

Using scanning electron microscopy the effects of 5% FB and MAN UMF10+ honeys on the morphology of the cells in a *C. albicans* biofilm compared to the control was determined. Planktonic cells are round/oval yeast cells, which then develop into pseudohyphae then into true hyphal cells which are the major cell type present in biofilms and are associated with a dense ECM. For continued biofilm growth, single cell dispersal occurs from biofilms (Bester *et al.*, 2013). In pseudohyphae, septae form, and the daughter cell remains attached to the mother cell (Sudbery *et al.*, 2004). Nuclear division and septum ring formation takes place around the mother cell's neck in yeast cells and pseudohyphae. In true hyphae, this takes place within the germ tube during the first division (Thompson *et al.*, 2011). In the control biofilms, the presence of true hyphae is observed indicating that biofilms after 24 h growth have been established. In the present study, after 24 h, to a 5% MAN UMF10+ and FB honey solution the predominant cell type in the biofilm was cells with a planktonic-like morphology. Changes in cell morphology, would not necessary result in a change in cell viability measured with the CellTiter-Blue assay.

A study by Hau-Yama *et al.* (2019) reported that the exposure of *C. albicans* biofilms to 20% (v/v) and above concentrations of honey from the stingless bee *Melipona beecheii* inhibited biofilm formation. Altered ultrastructure and morphology of *C. albicans* cells included the formation of blebs such as those seen for cells undergoing apoptosis. Apoptosis is a programmed cell death that occurs as a homeostatic mechanism that maintains cell population in tissues, and it is a part of normal development and ageing (Elmore, 2007). This process is characterised by cell shrinkage, pyknosis (DNA fragmentation, chromatin condensation and cytoplasm compacting), cell blebbing of the plasma membrane, detaching of cells and cell fragmentation forming apoptotic bodies (Elmore, 2007). In addition, increased surface roughness was observed as found in Figure 3.10. In this study, the control cells morphology was normal with round/oval shaped cells with polarisation and bud formation. However, with exposure to honey, this polarisation was disrupted and disruption of the cell wall as well as cytoplasm shrinkage was observed.

The Ansari *et al.* (2013) study, found that in control biofilms cells, a smooth cell wall coated with exopolysaccharide material was observed. The growth of biofilms and established biofilms were disrupted following exposure to 40% Jujube honey for 24 h. Evaluation with scanning electron microscopy, revealed that exposure resulted in a lack of exopolysaccharide material and the formation of small pores, vesicle formation due to lytic activity and shrinkage due to plasmolysis. Atomic force microscopy also revealed a reduction in the thickness of the biofilms. Likewise, in the present study, using FB1 (Figure 3.10) as an example, the surface of the pseudohyphae was rough, splitting of the cell surface and cell shrinkage was observed. The fixation method used in the present study, resulted in the loss of the ECM, the effect on this component of the biofilm could not be evaluated. This study reveals that *C. albicans* cell viability was not altered and cellular changes associated with apoptosis had occurred.

Using a simulated model, the effects of oral digestion on the anti-fungal activity of MAN UMF10+ and FB honey were determined. With oral digestion, no changes in the selective inhibition of the growth of *C. albicans* biofilms were observed. Only at 5 - 20 mM MGO differences in the inhibition of the growth of planktonic *C. albicans* was observed. This effect may be related to the ability of MGO to bind proteolytic digestive enzymes. Daglia *et al.* (2013) investigated the influence of *in vitro* simulated gastro-duodenal digestion on the MGO concentration of MAN honey. MGO reacts with the digestive enzymes by carbonylating their amino acids. These structural changes did not affect the activity of the enzymes, and thus not the digestion process. However, this binding effect may reduce the amount of free MGO that can have an inhibitory effect (Daglia *et al.*, 2013).

In the presence of oral digestive salts and enzymes, the inhibitory effect of 50% sugars against planktonic *C. albicans* cells was lost while the artificial honey solutions had no effect on biofilm growth.

3.8 Conclusion

In this study the low concentrations of 7 - 9% FB1 honey, and high concentrations of 45% FB2 and FB3, 40 - 45% of FB4 and 30 -35% MAN UMF 10+ inhibited the growth of *C. albicans* biofilms but not planktonic cells. With oral digestion, the inhibitory effect of 5% MAN UMF10+ and several FB honeys was unaltered. Scanning electron microscopy analysis of FB and MAN UMF10+ revealed changes to the morphology of cells found in *C. albicans* biofilms, with the majority of the cells having a round/oval morphology typical of planktonic *C. albicans* cells, indicating a lack of differentiation into typical cell morphology associated with biofilms. Some ultrastructural changes associated with apoptosis were observed.

Chapter 4 : Antioxidant content and scavenging activities of undigested and oral digested Fynbos honey.

4.1 Introduction

Antioxidants are natural or synthetic substances that prevent and delay the auto-oxidation of free radicals (Brewer, 2011). This is due to either the quenching or the scavenging of ROS by chelation of metal ions, inhibiting the auto-oxidative chain reaction or decreasing the concentration of localised oxygen (Brewer, 2011). Non-nutrient antioxidants are phenolic acids, flavonoids, carotenoids and tocopherols (Brewer, 2011) while nutritive antioxidants have an important biochemical function, but can also act as antioxidants and these include vitamins E and C (Sindhi *et al.*, 2013).

Plants are particularly rich in antioxidants as they are constantly under oxidative stress by free radicals, ROS and pro-oxidants such as heat and light or radiation (Brewer, 2011). Consequently, plants and plant products such as honey are a rich source of natural dietary antioxidants (Khalil *et al.*, 2010). Antioxidants in honey include phenolic acids, flavonoids, enzymes, organic acids, ascorbic acid, amino acids and proteins as well as Maillard reaction products (Khalil *et al.*, 2010).

Honey has well-described antioxidant activity (Alvarez-Suarez *et al.*, 2014; Bellik and Boukraa, 2014; Samarghandian *et al.*, 2017), however, the stability of polyphenols are variable with gastro-intestinal digestion and this may compromise activity (Seraglio *et al.*, 2017). Likewise, the neutral pH in the oral cavity can also compromise the stability of flavonoids such as GA and CA (Friedman and Jurgens, 2000).

Antioxidant activity is determined using two different reaction types, the electron transfer (ET) and the hydrogen atom transfer (HAT) reactions. ET-based assays measure the capacity of an antioxidant to reduce oxidants, while most HAT-based assays act by having an antioxidant and substrate compete for a peroxyl radical and consequently, are considered to be physiologically relevant. Physiologically, peroxyl radicals can bind with NO forming highly reactive peroxynitrite radicals. These radicals are •NO-derived oxidants and are associated with hypertension, cancer and inflammation (Radi, 2018). Reduction of the levels of peroxynitrite radicals can be either due to peroxyl radical scavenging and/or NO scavenging activities.

The next level of testing is to evaluate the ability of antioxidants to protect cellular macromolecules including proteins and lipids against oxidative damage as damage to these structures can lead to cellular dysfunction and cell death. *In vitro,* these specific effects can be determined by measuring the ability to protect bovine serum albumin (BSA) against MGO-induced glycation and low density lipoprotein (LDL) against Cu^{2+}-mediated oxidation. In subsequent experiments, and later in this study, the *in vitro* cellular effects can be evaluated, providing information on the total cellular effects. Infection by candidiasis is associated with increased ROS production which may damage

the oral cavity. Even though a reduction in ROS may increase the virulence of *Candida* infections it would be interesting to observe if honey in the oral cavity environment may have additional benefits by reducing ROS and NO levels as well as the ability to protect cellular macromolecules against damage.

Aim

Therefore, the aim of the research presented in this chapter was to determine the antioxidant properties of several FB honeys comparable to MAN UMF10+ honey, pre and post oral digestion, and to relate this to anti-fungal activity seen in the previous chapter.

Objectives

The objectives were:

1. To compare the antioxidant properties of undigested FB honey with MAN UMF10+ honey.
2. Then for each honey, determine the effect of oral digestion on the antioxidant properties.

Changes in the following antioxidant parameters were evaluated;

a) The total polyphenol content (TPC) evaluated with Folin-Ciocalteu (F-C) assay.
b) The antioxidant activity evaluated with the Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbent capacity (ORAC) assay representing ET and HAT reactions, respectively.
c) The NO scavenging activity evaluated with the sodium nitroprusside (SNP) scavenging assay.
d) The protection of protein against MGO glycation and LDL against Cu^{2+}-mediated peroxidation.

4.2 Materials

4.2.1 Reagents, disposable plastic ware and equipment

The reagents used were gallic acid (GA), Trolox (6-hydroxy-2,5,7,8–tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu (F-C) reagent, sodium carbonate anhydrous (Na_2CO_3), potassium peroxodisulfate ($K_2S_2O_8$), (2,2'-azo-bis(3-ethylbenzothiazoline-6-sulfuric acid), diamonium salt (ABTS), fluorescein sodium salt (FL), lipopolysaccharide (LPS), sodium nitroprusside (SNP), sodium nitrite ($NaNO_2$), sulphanilamide (SA), N-1-naphthylethylenediamine dihydrochloride (NED), phosphoric acid solution (H_3PO_4), bovine serum albumin (BSA), sodium hydroxide (NaOH), methylglyoxal (MGO), low density lipoprotein (LDL), copper sulphate ($CuSO_4$), Hepes buffer, malondialdehyde (MDA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA) and thiobarbituric acid (TBA). All reagents were obtained from Sigma-Aldrich, Johannesburg, SA.

The disposable plastic ware used were 96-well plates, 25 cm^2 tissue culture flasks, 50 mL and 15 mL centrifuge tubes and pipette tips (10, 25, 100, 200, and 1000 µL) from Greiner Bio-one were supplied by LASEC. Sartorius cellulose acetate membrane filters 0.22 µm were obtained from Sartorius, SA.

Equipment used in this part of the study were a Zeiss Axio cam Erc5s, Olympus IX71 light microscope was supplied by Wirsam Scientific & Precision Equipment, Cape Town, SA. A Lambda LS5OB spectrophotometer from Perkin Elmer, Boston, MA, USA supplied by Separations Scientific, Honeydew, SA, a BioTek plate reader from Winooski, Vermont, USA was purchased from Analytical and Diagnostic Products (ADP) Johannesburg, SA. A Crison GLP 21 pH Meter and Eppendorf pipettes from Eppendorf AG Hamburg, Germany were all supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, SA. A FLUOstar OPTIMA plate reader from BMG lab technologies, Offenburg, Germany and a water bath from EcoBath Labotec, Cape Town, SA.

4.2.2 Laboratory facilities

All research was conducted in the research facilities of the Department of Anatomy of the Faculty of Health Sciences, University of Pretoria.

4.3 Methods

4.3.1 Sample preparation

4.3.1.1 Oral digestion of honey samples

The same oral digestion method as described in section 3.3.1 was used.

4.3.2 Buffers and solutions

ORAC PBS was prepared by mixing 0.5495 g of Na_2HPO_4 base in ddH_2O with 0.8445 g of $NaH_2PO_4 \bullet H_2O$, all mixed in 1 L of ddH_2O. This mixture was then pH adjusted to 7 using NaOH and HCL to increase the pH and reduce the pH, respectively.

For the determination of TPC, the Folin-Ciocalteu (F-C) reagent was prepared by diluting the F-C reagent 15X in ddH_2O, and a 7.5% w/v $NaCO_3$ in ddH_2O was also prepared. A GA concentration series of 0 to 0.2 mg/mL in ddH_2O was prepared.

For the TEAC assay, a stock solution of ABTS was prepared by mixing 3 mM $K_2S_2O_8$ and 8 mM ABTS. This was done by dissolving 4.05 mg $K_2S_2O_8$ in 5 mL, 0.1 M PBS and 21.9 mg ABTS in 5 mL 0.1 M PBS. Both solutions were mixed together and the mixture was incubated in the dark for 12 h before use. From this stock solution, a working solution was prepared by diluting the stock

solution 30X in 0.1 M PBS. A Trolox standard series with a concentration range of between 0 and 1 mM in in ddH_2O was prepared.

For the ORAC assay the following solutions were prepared. A 0.139 nM fluorescein stock solution was prepared by dissolving 3.32 mg of fluorescein in 10 mL ORAC PBS to prepare the F_0 solution. Furthermore, a F_1 solution was made by diluting 10 µL of the F_0 solution in 10 mL ORAC PBS. The working solution was made by diluting 100 µL F_1 solution in 10 mL ORAC PBS.

4.3.3 Total polyphenolic content assay

Principle

The total polyphenolic content of a compound can be quantified using the F-C method. In this method, a redox reaction takes place where the mixture of phosphomolybdate and phosphotungstate forms a complex that reacts with polyphenolic antioxidants. Under alkaline conditions polyphenols are oxidized and the phosphomolybdate-phosphotungstate complex is reduced to a blue-coloured solution (Huang *et al.*, 2005), where the intensity correlated with the polyphenolic content with GA as standard.

Method

The undigested and oral digested honey samples were diluted to yield 12.5, 25 and 50% solutions. A 10 µL volume of each, was added in triplicate to the wells of a 96-well plate followed by 50 µL of the 15X diluted F-C solution. Then, a 50 µL of 7.5% Na_2CO_3 was added to a final volume of 110 µL. A GA standard curve with a final concentration range of 0 - 0.04 mg/mL in ddH_2O or synthetic saliva. All data was expressed as milligram GA equivalents per 100 g (mg GAE/100g).

4.3.4 Trolox equivalent antioxidant capacity assay

Principle

The TEAC assay is an electron transfer based assay where $ABTS^{2-}$ is oxidised to $ABTS^{+\bullet}$, a green-coloured radical, by $K_2S_2O_8$. In this assay, the ability of a polyphenol to scavenge the $ABTS^{+\bullet}$ radicals is measured which causes a loss of colour (Zulueta *et al.*, 2009).

Method

From a 1 mM Trolox stock solution, a concentration series of 0 - 0.033 mM was prepared. The undigested and oral digested honeys were diluted to yield final concentrations of 1.25, 2.5 and 5%. In a 96-well plate, 10 µL of the Trolox standards and the undigested and oral digested honey samples, were added into the wells of a 96 well plate. To the wells, 290 µL TEAC reagent working solution was added, and the mixtures incubated at room temperature for 30 min before the

absorbance was read at 734 nm. Activity was expressed as micromolar Trolox equivalence per 100 grams (µmolTE/100 g).

4.3.5 Oxygen radical absorbance capacity assay

Principle

The ORAC assay investigates the ability of antioxidants to prevent the quenching of fluorescence (Huang *et al.*, 2005). In this assay, the *in situ* peroxyl-free radicals generated by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) are able to react with a fluorescent probe. These free radicals generated in the presence of oxygen oxidise the fluorescent probe thereby depleting fluorescence. In the presence of an antioxidant, the fluorescence depletion is inhibited as the antioxidant donates the hydrogen atoms to stabilise the radical leaving the fluorescent probe intact (Lü *et al.*, 2010; Uribarri *et al.*, 2010; Siddiqui *et al.*, 2016).

Method

A concentration series of 0 - 0.05 mM from a 1 mM Trolox in ddH_2O was prepared. In a 96-well plate, to 165 µL of fluorescein working solution, a 10 µL solution of the Trolox standards and 1.25% (v/v) honey samples (undigested and oral digested) were added. Then 25 µL of a 65 mg/mL AAPH solution was added (final concentration of 8.13 mg/mL). The plate was gently mixed and the fluorescence was measured every minute for 2 h at an excitation wavelength (Ex) of 485 nm and an emission wavelength (Em) of 520 nm. Using the standard curve, the final ORAC values were calculated and were expressed as micromolar Trolox equivalents (µmolTE/100 g).

4.3.6 Sodium nitroprusside (SNP) assay

Principle

Nitric oxide is a molecule which mediates many physiological processes such as vasodilation, inflammation and immunity (Tuteja *et al.*, 2004). This involvement in physiological process can be used to quantify the presence of inflammation in cells. Sodium nitroprusside in aqueous solution at physiological pH generates NO (Jagetia and Baliga, 2004; Fraisse *et al.*, 2018) which interacts with oxygen (O_2) to produce NO_2^- which can be measured using the Griess reagent. Molecules that scavenge NO are in competition with O_2 which results in the reduced production of NO_2^- (Marcocci *et al.*, 1994). The Griess reaction is a method used to analyse NO_2^- where NO_2^- reacts under acidic conditions with sulfanilic acid to form a diazonium cation which couples to the aromatic amine 1-naphthylamine to produce a red-violet colour soluble azo dye (Tsikas, 2007).

Method

A 5 mM SNP solution was prepared in PBS and was left at room temperature for 1 h. To a 50 µL of 12.5, 25 and 50% (v/v) honey solutions, 50 µL of 5 mM SNP was added. Controls consisted of 50 µL 5mM SNP solution with 50 µL PBS (positive control), and 100 µL PBS (negative control).

After 1 h incubation at room temperature, NO levels were quantified with 50 µL Griess reagent. Griess reagent consisted of 1% (w/v) SA solution in a 2.5% H_3PO_4 solution was added to the honey solutions. After an incubation period of 10 min, 50 µL of a 0.1% NED in a 2.5% H_3PO_4 was added. The samples were mixed well and the absorbance read at 570 nm. The data was expressed as the percentage NO (%NO) scavenging activity relative to the control, no honey added.

4.3.7 Anti-glycation assay

Principle

Glycation is the spontaneous, non-enzymatic reaction that occurs between sugars and free amino groups of proteins, lipids or nucleic acids (Uribarri *et al.*, 2010; Siddiqui *et al.*, 2016) to form AGEs. AGEs can promote oxidative stress and inflammation by binding to cell surface receptors and forming cross-links with proteins which alters the structure and function of the proteins (Uribarri *et al.*, 2010). Stable N-carboxymethyl-lysine (CML) and the highly reactive MGO are well-known examples of AGEs (Uribarri *et al.*, 2010). The anti-glycation assay measures the ability of molecules, including antioxidants to inhibit AGE formation. In this assay, MGO was used as a reducing agent and BSA as the reference target protein.

Several models for measuring anti-glycation effects involves the use of glucose, fructose and MGO as glycating agents (Putta and Kilari, 2015). Honey contains 30% and 40%, glucose and fructose, respectively, while the amount of MGO is variable. Furthermore, honey also contains polyphenols that have anti-glycation activity, therefore, depending on concentration there will be a dynamic equilibrium between glycation and anti-glycation effects. Thus, the ability of honey to induce AGE formation was determined before anti-glycation activity was evaluated.

Method

The anti-glycation assay was adapted from (Siddiqui *et al.*, 2016) and as MAN honey contains MGO, the ability of this MGO to induced AGE compared with FB honey was determined. Then, the ability of both undigested and oral digested honeys to inhibit AGE formation was also determined.

To determine the ability of honey to inhibit AGE formation, to the wells of opaque 96-well plates (with clear bottoms) 50 µL of each 12.5% honey sample (undigested and oral digested) was added, followed by 50 µL of a 40 mg/mL BSA solution. All mixtures were made to a final volume of 200 µL with NaP buffer (0.1 M, pH 7). Controls consisted of undigested or oral digested honey with buffer only, final volume 200 µL. The positive control contained 50 µL BSA, 50 µL of a 56 mM MGO solution while the negative control contained only BSA, with both mixtures made to a final volume of 200 µL with NaP. The plate was then incubated at 37˚C and 5% CO_2 for 7 days.

After incubation, fluorescence was measured at an Ex of 330 nm and Em of 420 nm, respectively. All data was expressed as percentage inhibition or induction of AGE by MGO.

To evaluate the ability of honey to induce AGE formation, 50 µL of each honey sample and 150 µL of NaP were pipetted into a 96-well plate. The positive control contained BSA, MGO and NaP and the negative control was made up of BSA and NaP buffer only. Samples were then incubated at 37˚C and 5% CO_2 for 7 days and fluorescence was measured at an Ex of 330 nm and an Em of 420 nm. Data was expressed as %AGE formation, with the positive control forming 100% and the negative control 0% AGE.

4.3.8 Lipid peroxidation assay

Principle

Lipid peroxidation is the process in which oxidants attack lipids that have carbon-carbon double bonds such as poly-unsaturated fatty acids. Lipids are attacked by oxygen insertion resulting in the formation of lipid peroxyl radicals and hydroperoxides (LOOH). Another product formed by lipid peroxidation is MDA and it is used as a biomarker for lipid peroxidation (Hodges *et al.*, 1999; Ayala *et al.*, 2014).

The lipid peroxidation assay, measures the ability of functional foods to prevent the oxidation of LDL. In this assay, Cu^{2+} acts as a catalyst with the formation of LOOH. Honey contains metals such as zinc, nickel and chromium (Aghamirlou *et al.*, 2015) and these can catalyse the Fenton reaction. Honey also contains polyphenols, and especially the flavonoids such as catechin and chrysin which can chelate metals (Kumar and Pandey, 2013). In addition, polyphenols can inhibit the effects of LOOH formed as a result of this reaction. The ability of honey to induce LDL peroxidation due to the metal content of honey was determined and then the ability of honey to reduce Cu^{2+}-mediated LDL peroxidation was evaluated

Method

The ability of the honey samples and oral digests to inhibit peroxidation was evaluated by combining 10 µL of the honey samples, 100 µL of 100 µg protein/mL LDL and 10 µL of 55 µM $CuSO_4$. After mixing the solution, they were incubated for 16 h at 37°C.

The ability of the honey samples and oral digests to induce lipid peroxidation was determined and the same solution was prepared as described above, but $CuSO_4$ was excluded. Likewise, these solutions were incubated for 16 h at 37°C.

The positive control was 100 µL of 100 µg protein/mL LDL, 10 µL of 55 µM $CuSO_4$ and 10 µL of 5 mM Hepes buffer; and the negative control was 100 µL of LDL and 20 µL of 5 mM Hepes buffer. Following the 16 h incubation, to each of the Eppendorf tubes, 10 µL of 1 mM BHT was added to

stop the lipid peroxidation reaction. Following this, 60 µL of a 5 mM Hepes buffer was added, followed by 50 µL of a 20% (v/v) TCA solution prepared in Hepes buffer; followed by 75 µL of a 1% (w/v) TBA solution prepared in 0.3% NaOH. The tubes were then incubated in the 60°C water bath for 2 h.

A 20 µM MDA solution was used to prepare a standard curve of 0, 0.5, 1, 2.5, 5, 10 and 20 µM in Hepes buffer. The MDA standard was also incubated for 2 h with only 50 µL TCA and 75 µL TBA.

After incubation, the Eppendorf tubes were centrifuged at 1409 *x*g for 5 min and then 75 µL of the supernatant was transferred to 96-well plates and the fluorescence was measured at an Ex of 544 nm and an Em of 590 nm. All data was expressed relative to the positive control, 100% and the negative control, 0% lipid peroxidation.

4.4 Statistical analysis

All experiments were done at least three times in triplicate yielding 9 data points, unless otherwise stated, and presented as mean ± SEM. Statistical analysis undertaken as described in Section 3.5.

4.5 Results

4.5.1 Total polyphenolic content assay

Firstly, the TPC of undigested and oral digested FB and MAN UMF10+ was determined. The TPC for the undigested honey FB samples were significantly ($p<0.05$) less than MAN UMF10+. Of the FB honeys, FB3 and FB4 had the highest TPC (Table 4.1). With oral digestion, the TPC of FB1 and FB2 was significantly less than that measured for MAN UMF10+, while the TPC content of FB3 and FB4 was similar to MAN UMF10+. With oral digestion, TPC was increased following oral digestion of FB1, FB2 and FB3 for both ODC and OD, which indicates that in the salt solution associated with oral digestion, there is increased extraction of polyphenols. In contrast for FB4, there was a significant increase in TPC with OD only. With oral digestion for all FB honeys there was a 1.09 – 1.16 fold increase in TPC.

Table 4.1: The total polyphenolic content and fold change of undigested and oral digested honeys.

	Polyphenol content (mgGAE/100g)				
	FB1	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
UD	65.63 ± 0.18*	40.94 ± 0.17*	70.08 ± 0.23*	72.76 ± 0.28*	94.14 ± 0.29
ODC	70.27 ± 0.41*#	43.05 ± 0.34*#	75.60 ± 0.59*#	73.83 ± 0.13*	87.55 ± 0.05#
OD	71.25 ± 0.31*#	47.33 ± 0.27*#	81.29 ± 0.49#	81.46 ± 0.07#	89.85 ± 0.37#
Fold change					
UD vs. OD	**1.09**	**1.16**	**1.16**	**1.12**	**0.95**

* Indicates significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD) and # indicates differences between UD, ODC and OD for each honey, $p<0.05$.

4.5.2 Trolox equivalent antioxidant capacity assay

The antioxidant activities of oral digested and undigested honeys were determined with the TEAC assay and the fold change in activity is presented in Table 4.2. The range of antioxidant activity for UD FB honey was 366.46 ± 5.00 - 671.93 ± 5.29 µmolTE/100g. The UD honeys FB1, FB2 and FB4 showed a significantly lower antioxidant capacity when compared to MAN UMF10+. Following oral digestion, the antioxidant activity of only FB2 was again less than MAN UMF10+. Oral digestion did not alter the antioxidant activity of all the honey samples. All honey samples were a 1.22 to 1.44 fold higher, indicating increased extraction of molecules with antioxidant activity.

Table 4.2: The Trolox equivalent antioxidant capacity and fold change of undigested and oral digested honey.

	Antioxidant activity (TEAC assay, µmolTE/100g)				
	FB1	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
UD	585.84 ± 5.33*	366.46 ± 5.00*	699.47 ± 5.74	671.93 ± 5.29*	824.09 ± 8.11
ODC	722.97 ± 10.27	424.87 ± 10.13*	812.66 ± 9.85	769.55 ± 9.42	972.82 ± 8.32
OD	758.06 ± 11.11	526.15 ± 8.48*	901.37 ± 7.79	821.63 ± 8.21	1052.86 ± 10.06
Fold change					
UD vs. OD	**1.29**	**1.44**	**1.29**	**1.22**	**1.28**

* Indicates significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD). No significant differences seen between UD, ODC and OD for each honey, $p<0.05$.

4.5.3 Oxygen radical absorbance capacity assay

The ORAC assay is considered physiologically relevant as antioxidants directly protect fluorescein against AAPH-induced oxidative damage. The antioxidant activity and fold change of undigested and oral digested honeys (FB and MAN UMF10+) is presented in Table 4.3. The range for antioxidant activity range for the FB honeys 3581.22 ± 2.92 µmolTE/100g - 4765.11 ± 0.87

µmolTE/100g and was similar to the activity of MAN UMF10+ which was 4640.39 ± 0.15 µmolTE/100g. With oral digestion, the range of antioxidant activity was 2003.57 ± 11.12 - 4416.99 ± 10.36 µmolTE/100g and the antioxidant activity of FB2 was significantly less than MAN UMF10+ honey. With oral digestion, the antioxidant activity measured with the ORAC assay was reduced for all the honey samples. The fold change was 0.56 – 0.94 for all honeys with the greatest loss of activity evaluated for FB2 although the change in antioxidant activity was not statistically significant.

Table 4.3: The oxygen radical absorbance capacity and fold change of undigested and oral digested honey.

	Antioxidant activity (ORAC assay, µmolTE/100g)				
	FB1	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
UD	4548.15 ± 1.71	3581.22 ± 2.92	4454.52 ± 0.05	4765.11 ± 0.87	4640.39 ± 0.15
ODC	2176.04 ± 6.37	2003.57 ± 11.12*	4088.39 ± 2.17	4416.99 ± 10.36	4138.08 ± 2.38
OD	3529.48 ± 4.74	2017.16 ± 1.67*	4398.99 ± 4.95	4219.08 ± 7.30	4373.92 ± 8.67
Fold change					
UD vs. OD	**0.78**	**0.56**	**0.99**	**0.89**	**0.94**

* Indicates significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD). No significant differences between UD, ODC and OD for each honey, $p<0.05$.

4.5.4 Chemical NO scavenging activity

Before determining the NO scavenging activity of each honey and oral digests, the residual nitrite levels in each honey sample and oral digests was determined and levels were expressed as a percentage of the control, SNP forming 2.78 mM NO (100%). The nitrite levels of FB honey and MAN UMF10+ were 0.42 - 1.75% which were significantly less than the control, therefore all honey samples did not contain or induce NO formation from SNP (Table 4.4). No significant differences were observed when comparing undigested and oral digested FB honeys to MAN UMF10+ honey. Furthermore, oral digestion did not induce a significant change to NO levels with 0.68 – 2.71 fold change in NO levels.

Table 4.4: The residual % NO levels and fold change of undigested and oral digested honey.

	FB1	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
UD	0.80 ± 0.23	0.42 ± 0.07	1.75 ± 0.88	0.72 ± 0.06	1.00 ± 0.13
ODC	1.05 ± 0.10	0.68 ± 0.12	0.94 ± 0.02	0.90 ± 0.04	0.64 ± 0.10
OD	1.36 ± 0.10	1.14 ± 0.13	1.20 ± 0.18	1.07 ± 0.12	1.52 ± 0.27
Fold change					
UD vs. OD	**1.7**	**2.71**	**0.68**	**1.48**	**1.52**

No significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD) and no significant differences between UD, ODC and OD for each honey, $p<0.05$.

The ability of the honey samples to scavenge NO generated from SNP was then determined (Table 4.5). Low percentage values indicate effective NO scavenging for all honey samples with the highest scavenging activity for UD MAN UMF10+. The NO scavenging activity of FB4 was similar to MAN UMF10+, while the NO scavenging activity of FB1, FB2 and FB3 was significantly less. Following oral digestion, the NO scavenging activity of FB4 was similar to MAN UMF10+. Oral digestion did not cause a significant change in NO scavenging activity for all honey samples with a range of fold change of 0.92 – 1.08.

Table 4.5: The % NO scavenging activity and fold change of undigested and oral digested honey.

	FB1	FB2	FB3	FB4	MAN UMF10+
UD	69.11 ± 2.54*	70.24 ± 2.87*	65.67 ± 2.65*	48.53 ± 1.40	47.24 ± 1.59
ODC	63.50 ± 3.27*	71.39 ± 3.85*	64.83 ± 7.55	54.14 ± 7.86	47.29 ± 8.45
OD	75.23 ± 9.94*	70.22 ± 4.84*	60.15 ± 7.61	49.87 ± 1.51	49.34 ± 2.16
Fold change					
U vs. OD	**1.08**	**0.99**	**0.92**	**1.03**	**1.04**

* Indicates significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD) and no differences between UD, ODC and OD for each honey were observed, $p<0.05$.

Using chemical-based assays, all honey samples scavenged ROS and NO, both effects preventing the formation of peroxynitrite radicals. Generally, with oral digestion, some changes in activity were observed, however sufficient scavenging activity was retained.

4.5.5 Advanced glycation end-products (AGEs)

The percentage glycation of BSA by MGO (56 mM) was considered as the positive control (100%). The percentage glycation of BSA by honey (no MGO added) was determined (Table 4.6). The levels of AGE formation for UD, ODC and OD honey were significantly lower than the BSA + MGO control, 100% AGEs. MAN UMF10+ contains 263 mg/kg MGO (Girma *et al.*, 2019) and the levels in a 1.25% solution (3.29 mg/kg) was not sufficient to induce AGE formation. During the 7 days incubation, BSA can undergo spontaneous structural changes which were prevented by the honey and consequently the %AGE values which are negative.

BSA and MGO induce 100% glycation. UD, ODC and OD honey significantly reduced the glycation of BSA where percentage values indicate prevention of BSA glycation. Compared with MAN UMF10+, UD FB2, FB3 and FB4 have better anti-glycation properties. No differences were found following oral digestion for FB honey compared with MAN UMF10+. For each honey, oral digestion, did not cause significant changes in activity.

Table 4.6: The percent (%) formation of advanced glycation end-products induced by honey alone and in combination with MGO of undigested and oral digested honey samples.

Control (BSA alone):	**0**				
Control (BSA + MGO):	**100**				
			Undigested		
	FB1	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
Honey + BSA	-9.06 ± 0.15*	-13.54 ± 0.36*	-8.44 ± 0.33*	-10.11 ± 0.13*	-2.39 ± 0.41
Honey + BSA + MGO	30.62 ± 2.16	21.17 ± 2.02*	22.64 ± 3.11*	25.89 ± 2.94*	32.54 ± 3.85
			Oral digest control		
Honey + BSA	-8.92 ± 0.39*	-13.90 ± 0.13*	-6.75 ± 0.78*	-9.34 ± 0.97*	-2.03 ± 0.74
Honey + BSA + MGO	28.80 ± 4.35	29.77 ± 4.67	33.06 ± 6.20	30.60 ±. 4.51	34.22 ± 4.22
			Oral digest		
Honey + BSA	-7.83 ± 0.62	-10.17 ± 1.46	-7.15 ± 1.33	-8.51 ± 1.25	-1.72 ± 0.97
Honey + BSA + MGO	23.42 ± 2.79	27.10 ± 8.84	30.32 ± 8.46	31.81 ± 5.86	31.55 ± 2.54
Fold change					
Honey + BSA + MGO	**0.76**	**1.28**	**1.34**	**1.23**	**0.97**

* Indicates significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD), no differences between UD, ODC and OD for each honey and no significant differences observed between the control and the honey samples, $p<0.05$.

4.5.6 Lipid peroxidation assay

Finally, the effects on LDL peroxidation were determined. The ability of each honey and oral digest to induce lipid peroxidation was determined prior to the evaluation of the ability of each honey and oral digest to prevent LDL peroxidation (Table 4.7). The undigested and oral digested honey caused very little to no LDL peroxidation, with only 0.27 ± 0.34 - 4.31 ± 2.93% activity compared with the control (LDL and Cu^{2+}, 100% lipid peroxidation). No significant differences were found between the FB honey and MAN UMF10+ honey. Also, for each honey, with oral digestion, no changes were observed.

Honey, UD, ODC and OD combined with LDL in the presence of $CuSO_4$, showed little to no protection against lipid peroxidation with a range of 94.71 -100.73% compared with the control, 100% for UD honey. Following oral digestion the range was 86.06 – 100.12% with lack of a significant difference compared with the control.

Table 4.7: Percentage LDL peroxidation induced and inhibited of undigested and oral digested honey samples.

Control LDL alone	**0**				
Control LDL +Cu^{2+}	**100**				
			Undigested		
	FB1	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
Honey + LDL only	1.28 ± 0.43	1.79 ± 1.30	4.31 ± 2.93	1.82 ± 0.87	1.39 ± 0.71
Honey+ LDL + Cu^{2+}	98.09 ± 2.36	99.72 ± 1.88	100.73 ± 3.76	96.60 ± 4.31	94.71 ± 4.97
			Oral digest control		
Honey + LDL only	0.32 ± 0.34	1.56 ± 1.15	0.95 ± 0.60	0.88 ± 0.35	0.76 ± 0.29
Honey+ LDL + Cu^{2+}	91.42 ± 6.86	95.38 ± 8.55*	94.00 ± 7.91	83.75 ± 5.38	82.93 ± 7.05
			Oral digest		
Honey + LDL only	1.12 ± 0.24	0.27 ± 0.71	0.92 ± 0.41	0.52 ± 0.59	0.65 ± 0.20
Honey+ LDL + Cu^{2+}	99.40 ± 3.34	100.12 ± 3.05	96.57 ± 2.84	86.06 ± 1.77	92.78 ± 2.15

* indicates significant difference of FB (Fynbos) compared to MAN UMF10+ (Manuka) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD), no significant differences observed UD, ODC and OD for each honey and no significant differences seen between the control and the honey samples, $p<0.05$.

4.6 Discussion

Monofloral honeys are produced from one specific type of flower (Gül and Pehlivan, 2018). Examples are MAN honey derived from *Leptospermum scoparium* and FB honey, from the *Ericaceae* family of flowers. Monofloral honeys can differ from other honeys in moisture content, conductivity and colour (Vanhanen *et al.*, 2011).

The range of TPC for the FB honey used in the present study was 40.94 ± 0.17 - 81.46 ± 0.07 mg GAE/100g and were similar to the range of 68.85 - 167.96 mg GAE/100g for FB honey reported by Magoshi (2017). The TPC of honeys from other sources was between 110.39 - 196.50 mg GAE/100g for Malaysian, Tualang, Gelam and Acacia honey (Chua *et al.*, 2013) which were higher than that found for FB honey in the present study. Attanzio *et al.* (2016) determined the TPC of monofloral honeys made by the black honeybee *Apis mellifera ssp. Sicula*, an African subspecies found in Sicily, Italy. The TPC of 30 samples from different floral sources were then compared to MAN honey. The reported TPC was 16.5 ± 0.8 - 133.3 ± 4.8 mg GAE/100g, levels comparable to that found in FB honey. Honey samples with the highest TPC were the Dill, Eucalyptus, Honeydew citrus and Ferula honeys and the TPC was greater than 100 mg GAE/100g, and greater than the TPC of MAN UMF10+ honey (50.3 ± 1.9 mg GAE/100g).

O'Sullivan *et al.* (2013) showed that after digestion, polyphenols are subject to degradation and this affected the antioxidant content and activity of honey. Changpraykaewa and Petchlert (2015) evaluated the antioxidant content with the TPC assay of three fruit juices (pineapple, pomegranate and roselle) pre-digestion, and following oral digestion, gastric and intestinal digestion. These

researchers reported an increase in antioxidant activity following oral digestion. Magoshi (2017) conducted a study to evaluate the effect of *in vitro* simulated gastro-duodenal digestion on the antioxidant activity of FB honeys compared with MAN UMF15+ honey. The TPC of Fynbos honey was 44% less than the MAN UMF15+ honey. Following gastric digestion, the TPC was increased while after gastro-duodenal digestion, TPC was reduced. The reduction in TPC was attributed to the instability of polyphenols in neutral/alkaline conditions associated with duodenal digestion. The oral activity is also a neutral pH environment however exposure time may be critical, but may not have an effect as exposure in the oral cavity is 2 min and in the duodenum, 1 h, where a decrease in TPC and associated antioxidant activity may be time dependent.

Polyphenols are generally accepted to contribute to antioxidant activity, however a study by Brudzynski *et al.* (2011) identified that other high molecular weight melanoidins were the main components responsible for radical scavenging capacity in Canadian honeys, which were unheated or heat-treated. In addition, other compounds such as ascorbic acid, enzymes like catalase and glucose oxidase and carotenoid-like substances can also contribute to antioxidant activity (Gorjanović *et al.*, 2012). This highlights that other molecules found in honey may further contribute to antioxidant activity and highlights the importance of several antioxidant assays with different mechanisms of action. Therefore, in the present study, antioxidant activity was determined with the TEAC and ORAC assays.

The ability of the honey samples and oral digests to prevent electron transfer in the TEAC assay was determined. Magoshi (2017) and Serem and Bester (2012) reported similar TEAC values of 434 - 1228 µmol TE/100g, and 536 - 1128 µmol TE/100g, respectively for FB honey. In the present study, levels were 366.46 ± 5.00 - 699.47 ± 5.74 µmol TE/100g for UD and 424.87 ± 10.13 - 901.37 ± 7.79 µmol TE/100g for oral digested FB honey. For MAN UMF10+, levels were 824.09 ± 8.11 for UD and 1052.86 ± 10.06 µmol TE/100g oral digested honey. Following oral digestion, antioxidant activity was slightly increased for FB and MAN UMF10+ honeys, although not statistically significant.

Yusof *et al.* (2018) determined the Iron (III) reducing antioxidant capacity for MAN honey with different UMF values; UMF5+, UMF10+, UMF15+ and UMF18+ and the TEAC values for these honeys were reported as 1455 µmol TE/100g, 1722 µmol TE/100g, 1753 µmol TE/100g and 1900 µmol TE/100g, respectively. These values are comparable to the MAN UMF10+ honey used in the present study, the range of which was between 824.09 ± 8.11 and 1052.86 ± 10.06 µmol TE/100g for both undigested and oral digested samples.

The evaluation of antioxidant activity with the TEAC assay was followed by determining the ability of the honeys and oral digests to protect fluorescein against the effects of the physiologically relevant AAPH radical. The ORAC values were between 3581.22 ± 2.92 - 4765.11 ± 0.87

µmolTE/100g for UD FB honeys, and 2003.57 ± 11.12 - 4398.99 ± 4.95 µmolTE/100g for the ODC and OD FB honeys. For MAN UMF10+, the ORAC values were 4640.39 ± 0.15 µmolTE/100g (UD). 4138.08 ± 2.38 µmolTE/100g (ODC), 4373.92 ± 8.67 µmolTE/100g (OD). Although antioxidant activity was reduced following oral digestion, these differences were not significant when compared with the undigested honey samples.

A study by Gheldof *et al.* (2002) measured the *in vitro* antioxidant activity of 14 honey samples using the ORAC assay, the ORAC value for the honey with the highest antioxidant activity was 975 µmolTE/100g for the Buckwheat honey. Compared to the present study, the ORAC values for the FB honeys (3581.22 ± 2.92 – 4765.11 ± 0.87 µmolTE/100g) and MAN UMF10+ honey (4640.36 ± 0.15 µmolTE/100g) were much higher than the Buckwheat honey. Magoshi (2017) determined antioxidant activity using the ORAC assay and determined ORAC values between 1987 and 6289 µmolTE/100g for the FB honeys and 4671 µmolTE/100g for MAN UMF15+ honey while Serem and Bester (2012) determined ORAC values of between 371 and 4926 µmolTE/100g for honeys from the southern African region which included several FB samples. These studies support the results found in the present study, regarding antioxidant activity of the FB and MAN UMF10+ honeys measured with the ORAC assays.

Magoshi (2017) reported similar chemical NO scavenging activity for FB honey. With gastric digest and gastro-duodenal digestion, NO scavenging activity was unchanged and was between 35.67 - 59.73%, 37.19 - 63.02%, and 27.83 - 63.36% for undigested, gastric and gastro-duodenal digested FB honey respectively. A study by Serem (2018) showed that at concentrations as low as 6.25% honey samples, including Fynbos honey, were able to inhibit the production of NO by as much as 60 - 70% while at concentrations higher than 12.5%, no further scavenging but rather a loss of scavenging activity was observed. As observed in the study of Magoshi (2017) following gastric and gastroduodenal digestion of FB honey, no significant changes in NO scavenging activity was observed. In contrast for MAN UMF10+ a decrease in NO scavenging activity, was observed.

MGO can be bound by phenolic compounds thereby reducing MGO levels (Lo *et al.*, 2011). The MGO content of MAN UMF10+ honey is 747 ± 40 mg/kg (Legnanga, 2017). The phenolic acids found in MAN UMF10+ are CA, 4-hydroxybenzoic acid, p-coumaric acid, GA, isoferulic acid and syringin acid, with only CA and GA having the ability to trap MGO (Legnanga, 2017). ROS formation is also associated with glycation effects and therefore the ROS scavenging activity of the polyphenols found in honey also contributes in reducing glycation (Lo *et al.*, 2011; Cianciosi *et al.*, 2018), and consequently, the ability of the honey samples to inhibit glycation was determined. Also, in patients with untreated diabetes, systemic levels of MGO are increased and this contributes to poor wound healing (Berlanga *et al.*, 2005; Sams-Dodd and Sams-Dodd, 2018).

Therefore, the ability of polyphenols to scavenge ROS and reduce MGO levels in the oral mucosa would promote the health of the oral cavity by reducing glycation and ROS formation. However, systemic high glucose levels associated with untreated diabetes as well as high glucose levels as found in honey can promote the growth of *C. albicans* (Brown *et al.*, 2006; Van Ende *et al.*, 2019), as is shown in Chapter 3.

The MGO levels in MAN honey can be as high as 150 mg/kg (Majtan, 2011). MGO has antibacterial and anti-fungal activity, however high levels can cause the glycation of the proteins associated with the squamous stratified epithelium of the oral cavity. This can lead to cellular damage and eventually death. Therefore, in the oral cavity the beneficial antioxidants effects must outweigh the effects and consequences of protein glycation.

Honey alone (Table 4.6) did not cause BSA glycation, but seems to be able to protect BSA against changes that occur during the incubation at 37ºC for 7 days. The ability to reduce the effects of added MGO was determined. For UD MAN UMF10+ honey, the percentage decrease in AGE formation was from 100% to 32.54 ± 3.85%. For the UD FB honey the percentage decrease in AGE formation was from 100% to a range 21.17 ± 2.02% - 30.62 ± 2.16 % for FB honey. When compared with MAN UMF10+, FB2, FB3 and FB4 showed a significantly higher inhibition of AGE formation.

Piwowar *et al.* (2019) studied the anti-glycoxidative ability of selected phenolic compounds. The percentage inhibition of AGE formation was: 82.87 ± 4.14 % for 2 µM and 81.65 ± 4.08% for 20 µM CA, 79.53 ± 3.98% for 2 µM and 90.99 ± 4.55% for 20 µM ferulic acid, 81.82 ± 4.09 % for 2 µM and 87.00 ± 4.35% for 20 µM chlorogenic acid as well as 71.52 ± 3.58% for 2 µM and 80.71 ± 4.04% for 20 µM quercetin. These phenolic compounds are found in honey indicating that polyphenols in honey potentially inhibit AGE formation.

Besides direct binding of MGO causing the reduction in ROS, MGO can also bind proteolytic digestive enzymes. Daglia *et al.* (2013) investigated the influence of *in vitro* simulated gastro-duodenal digestion on the MGO concentration of MAN honey. MGO carbonylates the amino acids of proteolytic enzymes. These structural changes did not affect the activity of the enzymes, and thus not the digestion process. Likewise, MGO can bind amylase, thereby reducing the effective concentrations of MGO, however the lack of a significant difference between ODC and OD indicates that this is not a major effect although the effect on enzymatic activity is unknown. Therefore, after oral digestion the anti-glycation activity of all honeys is retained.

Lipids together with proteins are important constituents of the plasma membrane of epithelial cells. Oxidative stress can cause lipid peroxidation of polyunsaturated fatty acids which, in turn, leads to the formation of lipoperoxyl radical (LOO•) that can cause cellular dysfunction and cell death. UD MAN UMF10+ and FB honey did not significantly cause LDL oxidation or reduce

peroxidation. The reduction of peroxidation is mediated either by metal chelation by flavonoids or the scavenging of LOOH. These results indicate that the flavonoids present in these honey samples and oral digests have poor metal chelation ability and do not effectively scavenge LOOH. In addition, this can also indicate that the majority of the polyphenols found in these honeys are phenolic acids.

Therefore, the predominant effect on macromolecules is the prevention of protein glycation by MGO. In addition, the poor glycation of BSA by the honey samples, with no additional added MGO confirms that in low dilutions of honey, that glycation does not significantly contribute to the inhibition of *C. albicans* growth.

The antioxidant activity of the honey samples evaluated in this study was retained post oral digestion. The honey samples were also able to scavenge NO, which plays a major role in forming RNS and this can be attributed to the presence of polyphenols.

4.7 Conclusion

Both MAN UMF10+ and FB honey have antioxidant activity due to the presence of polyphenols. With oral digestion, antioxidant content and activity is retained or increased for FB honeys and MAN UMF10+ when evaluated with the TPC and TEAC assays, respectively. The ORAC assay showed a non-significant reduction in activity. All honeys and oral digests scavenged NO, indicating the ability to prevent RNS formation. All honeys, undigested and oral digested do not cause protein glycation and effectively prevent AGE formation. Lipid peroxidation was not induced or prevented by undigested and oral digested MAN UMF10+ and FB honeys. All honeys had antioxidant activity and protected protein such as albumin against MGO-mediated oxidative damage indicating a beneficial effect in oral health.

Chapter 5 : Toxicity, cellular antioxidant and anti-inflammatory effects of undigested and oral digested honey.

5.1 Introduction

Cellular models, are valuable options that provide some information on the cellular effects of absorption, distribution, metabolism, excretion and toxicity. These models also provide further evidence for the findings related to antioxidant and anti-inflammatory effects.

Although, honey including MAN UMF10+ and FB honey used in this study have been shown to have antioxidant and anti-glycation activity, the effects in a cellular environment are more complex. In cells and tissue, oxidative stress is a loss of balance between oxidative and anti-oxidative systems which results in the over-production of free radicals and ROS (Rani *et al.*, 2016). Cells contain enzymatic and non-enzymatic antioxidants which protect them against ROS (Nimse and Pal, 2015). Cellular antioxidant activity (CAA), measures the sum of these effects and addresses the limitations associated with antioxidant assays. The CAA of six FB honeys (Magoshi, 2017), thirteen honey samples from the regions of Southern African (Serem and Bester, 2012) was determined. All honeys were able to protect several cell lines against oxidative stress. As such, these honeys can be developed as wound healing products, however systemically, the effects of salts, proteolytic activity and pH can alter CAA and so for such applications these honeys must be further investigated.

Inflammation is an immune response against foreign pathogens (Mittal *et al.*, 2014) and with activation, an acute inflammatory response is initiated by immune cells to the site of infection (Mittal *et al.*, 2014). In this innate immune response, neutrophils attach to endothelial cells and migrate towards the site of infection to phagocytose invading pathogens and secrete pro-inflammatory and vasoactive mediators which include histamine, cyclo-oxygenase-generated prostaglandins, cytokines and kinins as well as ROS that acts as a mediator of inflammation (Mittal *et al.*, 2014). Honey-derived polyphenols reduce ROS levels as well as ROS-mediated inflammatory responses. Honey is also reported to suppress the pro-inflammatory activities of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Samarghandian *et al.*, 2017). Inhibition of iNOS, reduces NO levels, thereby inhibiting NO-mediated effects. In addition, the reduction in NO levels prevents the formation of peroxynitrite radicals, and associated oxidative stress. Several FB honeys have been reported to scavenge NO by between 35.67% and 59.73% (Magoshi, 2017)

Although the lack of toxicity as well as the beneficial cellular antioxidant and anti-inflammatory effects of honey has been described, little is known regarding the effects of oral digestion on toxicity and the above-mentioned activities.

Aim

Therefore, the aim of the research presented in this chapter was to evaluate the cellular toxicity, antioxidant and anti-inflammatory activities of several FB honeys compared with MAN UMF10+ honey. Then to determine the effects of oral digestion on the bioactivity of each honey type.

Objectives

The objectives were:

1. To evaluate the cellular toxicity, antioxidant and anti-inflammatory activities of several FB honeys compared with MAN UMF10+ honey.
2. Then to determine the effects of oral digestion on the cellular toxicity, antioxidant and anti-inflammatory activities of each honey type.

Changes in the following parameters were evaluated;

a) Cell number and viability of the murine fibroblasts (L929) and human colon adenocarcinoma (Caco-2) cell lines, evaluated with the crystal violet (CV) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assays, respectively.
b) Cellular antioxidant activity with the dichlorofluorescein diacetate (DCFH-DA) assay in the L929 and Caco-2 cell lines.
c) Cellular anti-inflammatory activity, related to NO scavenging in LPS-stimulated murine fibroblasts (RAW 264.7) cells.

5.2 Materials

5.2.1 Reagents, equipment and disposable plastic ware

Dulbecco's modified essential medium (DMEM), foetal calf serum (FCS), trypan blue, ethylenediaminetetraacetate acid (EDTA) and antibiotic solution (streptomycin, penicillin and fungizone) were obtained from Sigma Aldrich Company, Johannesburg, SA. Trypsin was obtained from Life Technologies Laboratories and supplied by Gibco BRL products, Johannesburg, SA. Water was double distilled (ddH_2O) and de-ionised ($dddH_2O$) with a continental water system and medium, enzyme solutions and buffers were sterilised by filtration through a Millex 0.2 µm filter. Glassware was sterilized at 121˚C for 30 min in a Prestige Medical Autoclave (series 100).
All reagents used for oral digestion were the same as used in Chapter 3. For the evaluation of cytotoxicity and cellular antioxidant activity, Crystal Violet (CV) and dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma Aldrich Company, Johannesburg, SA. Acetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), formic acid, dimethyl sulphoxide (DMSO) were obtained from Merck, SA. All disposable plastic ware and equipment that was used was the same as indicated in chapter 3.

5.2.2 Honey samples

The same honey samples collected and evaluated in Chapter 3 were used. Honeys were diluted 10X to yield 1.25, 2.5 and 5% honey solutions.

5.2.3 Cell lines

The human colon adenocarcinoma cell line (Caco-2) was kindly donated by the Department of Food Sciences, University of Pretoria, and was originally from the American Tissue Culture Collection (ATCC) at passage number 28 and was used from passage number 34 – 38 in the following experiments. The mouse fibroblast cell line (L929) was bought at passage number 40 from CELLONEX, Separations, Johannesburg, SA and was used from passage number 42 - 49. The macrophage cell line RAW 264.7 was used from passage number 7 - 10 in the following experiments, and was obtained from CELLONEX, Separations, SA.

5.3 Methods

Sample preparation and oral digestion was the same as described in section 3.3.1.

5.3.1 Cell culture media and trypsin

DMEM was prepared as per manufacturer's instructions, 13.55 g DMEM was dissolved in 1 L of dddH_2O, with $NaHCO_3$ (3.7 g/L). The pH was adjusted to pH 7.4, and the medium was sterile filtered before 10% foetal calf serum (FCS) and 1% antibiotic solution (streptomycin, penicillin and fungizone) was added. This DMEM/FCS was stored at 4˚C until needed and replaced on a monthly basis. A 0.25% solution (w/v) trypsin solution in 0.1 M PBS and a 0.02% EDTA solution in 0.1 M PBS were prepared and sterile filtered, before being stored at -4˚C until needed.

5.3.2 CV and MTT working solutions

A CV solution was prepared by dissolving 0.1 g CV powder in 0.75% formic acid, the pH was adjusted to pH 3.5 with NaOH or HCl and the solution was made to a final volume of 100 mL. A 1 mg/mL solution of MTT was prepared by dissolving 1 mg MTT in 1 mL of PBS.

5.3.3 Maintenance of cell lines

L929: Vials of frozen L929 cells were thawed rapidly at 37˚C before suspended in 5 mL DMEM/FCS and then collected by centrifugation (626 *x*g for 2 min). The supernatant was removed, and the cells re-suspended in fresh DMEM/FCS and were then plated in a 25 cm^2 culture flask and cultured at 37˚C at 5% CO_2 for 3 days. Once cells were confluent, the medium was removed and the cells were dispersed with 1 mL of the trypsin/EDTA solution (w/v). The addition of 9 mL fresh DMEM/FCS medium deactivated trypsin activity. The cells were then

collected by centrifugation, resuspended in 1 mL DMEM/FCS, then the cell concentration was determined by counting the number of viable cells in a 10 µL aliquot with the trypan blue exclusion assay using a haemocytometer. Cultures were re-established or diluted to a concentration of $4x10^4$ viable cells/mL for various experiments.

Caco-2: The cells were maintained in DMEM supplemented with 20% foetal calf serum (FCS), a 1% antibiotic (penicillin, streptomycin and amphotericin B) and a 1% non-essential amino acids (NEAA) solution (DMEM/FCS). Vials were thawed rapidly at 37˚C. The cells were suspended in 5 mL DMEM/FCS and collected by centrifugation (626 *x*g for 2 min). The supernatant was removed, and the cells were re-suspended in fresh DMEM/FCS and plated in a 25 cm^2 culture flask and were cultured at 37˚C at 5% CO_2. Once cells were confluent after 3 days, the medium was removed, then 5 mL of 0.25% trypsin/EDTA was added. To the detached cells, 5 mL fresh DMEM/FCS and the cells were then collected by centrifugation at 626 *x*g for 2 min. The medium was then removed, and the number of viable cells were determined as described for the L929 cells. The cells were then diluted to either establish further cultures or $4X10^4$ cells/mL for the subsequent experiments.

RAW 264.7: The cells were grown in a flask until confluent in DMEM/FCS. Once confluent, the cells were cultured for a further 24 h in serum-free medium. The medium was then removed and the cells were scraped and diluted to 1.25 x 10^6 cells/mL for plating.

5.3.4 Cytotoxicity in the L929 and Caco-2 cell lines

A volume of 90 µL containing $4X10^4$ cells/mL L929 or Caco-2 cells added to the wells of a 96-well plate and were then incubated at 37˚C and 5% CO_2 overnight. The established cultures of L929 cells were exposed to a 10 µL of the 1.25, 2.5 and 5% (v/v), honey solutions for 24 h. Cell viability and number was determined with the MTT and the CV assays, respectively.

5.3.5 Crystal Violet (CV) assay

Principle

CV (Figure 5.1) is a positively charged dye and binds to negatively charged proteins and nucleic acids staining cells purple. Qualitatively, CV staining is used to evaluate cellular structure, while subsequent extraction of the dye and measurement of colour provides quantitative data.

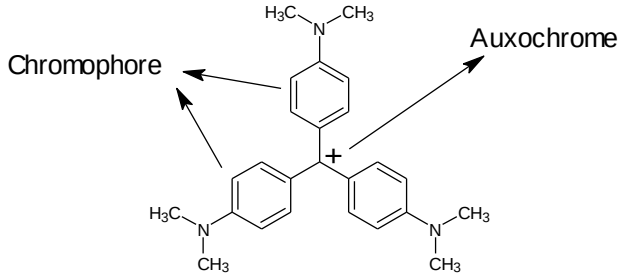

Figure 5.1: Chemical structure of crystal violet after deprotonation in water.

Method

The L929 and Caco-2 cells were exposed to honey sample as described in sections 5.3.4. A 10 µL of a 20% formaldehyde solution (final concentration of 2%) was added to each well, and then after an incubation period of 30 min, the fixative was removed and the plates were left to dry. The attached cells were stained with 100 µL of a 0.1% CV solution for 30 min at room temperature. Excess CV was then removed, the plates were then washed with ddH_2O, and were left to dry. Images of the attached cells were taken with an Olympus IX71 light microscope. For quantification, the bound dye was solubilised with 100 µL of 10% acetic acid and absorbance was measured at 630 nm with a Lambda LS5OB spectrophotometer. Results were reported as % cell number relative to control, where only 10 µL PBS added.

5.3.6 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay

Principle

The MTT assay is a reduction assay, which measures cell viability and the quantity of formazan formed is assumed to be directly proportional to the number of viable cells. Metabolically active cells convert MTT into a purple coloured formazan product (Figure 5.2) while dead cells lack the ability to convert MTT into formazan. Thus, the colour change serves as a convenient marker of viable cells. Generally, it is accepted that in the mitochondria, MTT is reduced, however recent studies have reported that MTT reduction also occurs in the cytoplasm (Śliwka *et al.*, 2016).

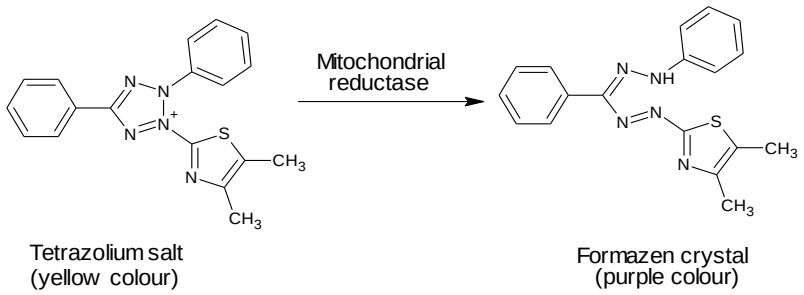

Figure 5.2: Conversion of tetrazolium salt to formazan crystal.

Method

The L929 and Caco-2 cells were exposed to honey sample as described in sections 5.3.4. A 10 µL of the MTT solution was added to each well and the cell culture plates were incubated for a further 3 h period at 37˚C. The medium was then removed, and the plates blotted dry. The MTT formazan crystals were solubilised with 100 µL of 25% DMSO in ethanol with shaking for 10 min. The absorbance was then measured at 570 nm. Results were reported as % cell viability relative to the control, where cells were exposed to PBS only.

5.3.7 Dichlorofluorescein diacetate (DCFH-DA) assay

Principle

ROS are free radical molecules with unpaired electrons, making them highly reactive (Campbell and Farrell, 2012). Antioxidants are able to donate electrons to these molecules in order to stabilise them. The DCFH-DA assay measures the amount of ROS produced in the presence of a radical. DCFH-DA (Figure 5.3) is a cell-permeable probe and it is deacetylated by esterases to DCFH and consequently cannot cross the cell membrane. In the presence of ROS, DCFH is oxidised into the fluorescent product DCF (Huang *et al.*, 2005).

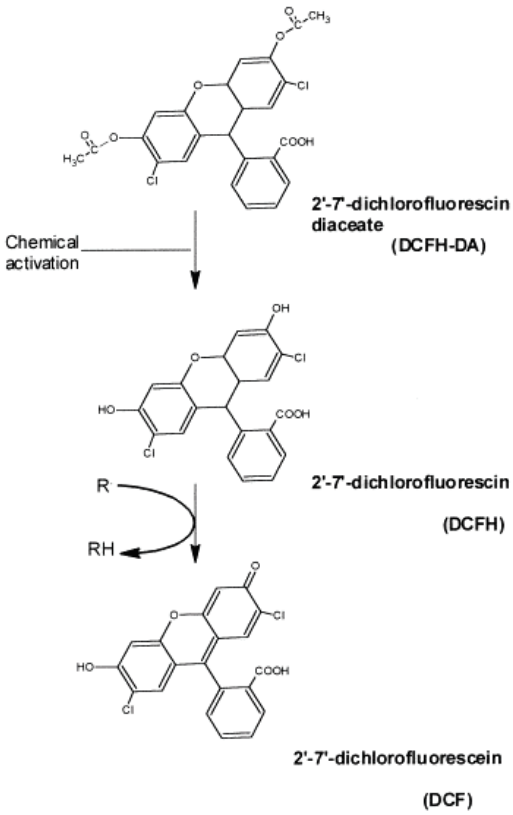

Figure 5.3: Mechanism of DCFH-DA de-esterification to DCFH, which is oxidised to fluorescent DCF by reactive oxygen species. Adapted from Bourre *et al.* (2002).

Method

In a 96-well plate, L929 cells were plated at a concentration of 4 x 10^4 cells/mL at 100 µL and then were incubated for 24 h at 37°C. After this incubation period, 50 µL of the DCFH-DA working solution (75 µM) was added to each well and the plate incubated at 37°C for a further 1 h. The medium containing DCFH-DA was then removed and the plate was gently washed with PBS. A volume of 30 µL of 1.25% honey samples, was added into the respective wells, followed by 30 µL of AAPH, at a starting concentration of 0.1 mg/mL, which yielded a final concentration of 0.05 mg/mL. The negative control consisted of 60 µL PBS, and a positive control consisted of 30 µL of PBS and 30 µL of AAPH. Vehicle controls included 60 µL of the OD solution (to show that the OD solution on its own did not produce ROS) and 30 µL OD solution plus 30 µL of AAPH (to show that the OD solution in the presence of AAPH, did not scavenge ROS). The change in fluorescence was then measured immediately every 2 min over a 0 - 60 min period at an Ex of 485 nm and an Em of 520 nm. The gradient of the change in fluorescence was calculated and the data expressed as % damage, with AAPH alone causing 100% damage.

5.3.8 RAW 264.7/Lipopolysaccharide cellular anti- inflammatory assay

Principle

The RAW 264.7 cell line is a murine-derived macrophage cell line which is typically used to test the immune response of substances (Merly and Smith, 2017). LPS, a bacterial endotoxin stimulates the production of NO by RAW 264.7 cells and eventually undergo phagocytosis (Taciak *et al.*, 2018). The cell line can be used to determine if molecules in complex mixtures such as honey can inhibit or induce NO formation having either an anti- or a pro-inflammatory effect.

Method

The anti-inflammatory effect of the honey samples was determined in RAW 264.7 cells by stimulating them with LPS before the addition of the honey samples. To determine if the honey samples had a pro-inflammatory effect, RAW 264.7 cells were not stimulated (no LPS added) and only the honey samples were added. For both experiments NO levels and cell viability were determined with the Griess reaction and MTT assay, respectively.

In this study, 80 µL of $1.25X10^6$ cells/mL RAW 264.7 cells were exposed to a 10 µL of 1.25% honey sample, without or after stimulation with 10 µL of 1 µg/mL LPS, this yielded a final cell concentration of $1X10^6$ cells/mL and 100 ng/ml LPS. The cell cultures were then incubated for 24 h at 37˚C, 5% CO_2. After incubation, a volume of 50 µL of the supernatant was collected and 50 µL of the Griess reagent as described in Section 4.3.7 was added. For the NO quantification, controls were background controls (medium only), cells stimulated with LPS, cells alone, without LPS and cells with honey only.

The viability of the cells in the remaining 50 µL of medium was determined with the MTT assay as described in section 5.3.6. Results for NO production and cell viability were reported as % of the respective controls.

5.4 Statistical analysis

All experiments were done at least three times in triplicate yielding 9 data points, unless otherwise stated, and presented as mean ± SEM. Statistical analysis undertaken as described in Section 3.5.

5.5 Results

5.5.1 Cytotoxicity

Both the L929 and Caco-2 cells were exposed to 1.25%, 2.5% and 5% UD, ODC and OD honey for 24 h before the cell number and viability was determined with the MTT and CV assays,

respectively. Activity of UD, ODC and OD honey was compared with MAN UMF10+ honey. Also the differences between UD, ODC and OD for each honey types were compared.

5.5.1.1 Effects of oral digestion on cell number (CV assay)

In Table 5.1, the effects on several dilution of each honey on cell number was determined. Compared to the control, all honeys showed no significant decrease ($p \leq 0.05$) in cell number. When comparing the undigested and oral digested FB honey samples to MAN UMF10+, there were no significant differences in cell number. Likewise, no change in cell number was found following the oral digestion of each honey. The fold change in cell number following oral digestion varied from 0.92 – 0.99, 0.91 – 1.09 and 0.96 – 1.09 for the honey solutions 1.25%, 2.5% and 5%, respectively.

Table 5.1: Percentage L929 cell number and fold change following exposure to undigested (UD), oral digest control (ODC) and oral digests (OD) honey samples.

Control: 100	**FB1**	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
1.25% UD	82.98 ± 0.72	84.39 ± 0.86	82.19 ± 0.73	84.18 ± 0.55	82.95 ± 0.19
1.25% ODC	79.19 ± 7.19	83.98 ± 4.90	83.59 ± 7.19	79.64 ± 7.51	81.76 ± 8.18
1.25% OD	82.51 ± 3.46	82.08 ± 2.84	78.65 ± 5.73	77.61 ± 6.44	76.52 ± 6.74
Fold change, UD vs. OD	**0.99**	**0.97**	**0.96**	**0.92**	**0.99**
2.5% UD	85.09 ± 1.36	81.90 ± 1.98	82.99 ± 0.91	81.29 ± 1.58	86.31 ± 3.59
2.5% ODC	76.06 ± 3.86	75.98 ± 4.56	73.61 ± 3.37	76.93 ± 4.49	75.79 ± 4.40
2.5% OD	77.68 ± 3.92	83.17 ± 5.34	80.36 ± 6.35	77.94 ± 6.12	76.11 ± 7.34
Fold change, UD vs. OD	**0.91**	**1.09**	**1.09**	**1.01**	**1.00**
5% UD	90.65 ± 7.72	91.44 ± 8.83	88.96 ± 7.83	89.93 ± 10.67	89.01 ± 8.46
5% ODC	76.45 ± 4.91	79.07 ± 3.77	78.97 ± 6.36	78.97 ± 5.26	81.62 ± 5.96
5% OD	90.87 ± 12.28	94.08 ± 13.56	87.24 ± 14.37	87.72 ± 15.94	85.62 ± 13.94
Fold change, UD vs. OD	**1.00**	**1.03**	**0.98**	**1.09**	**0.96**

No significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD), no significant differences between UD, ODC and OD for each honey and no significant differences between the control and honey samples, $p<0.05$.

In cell culture, L929 fibroblasts have an elongated, bipolar (green arrows) and a flattened multipolar (orange arrows) shape (Thibeault *et al.*, 2008; Fernandes *et al.*, 2016), as shown in Figure 5.4.

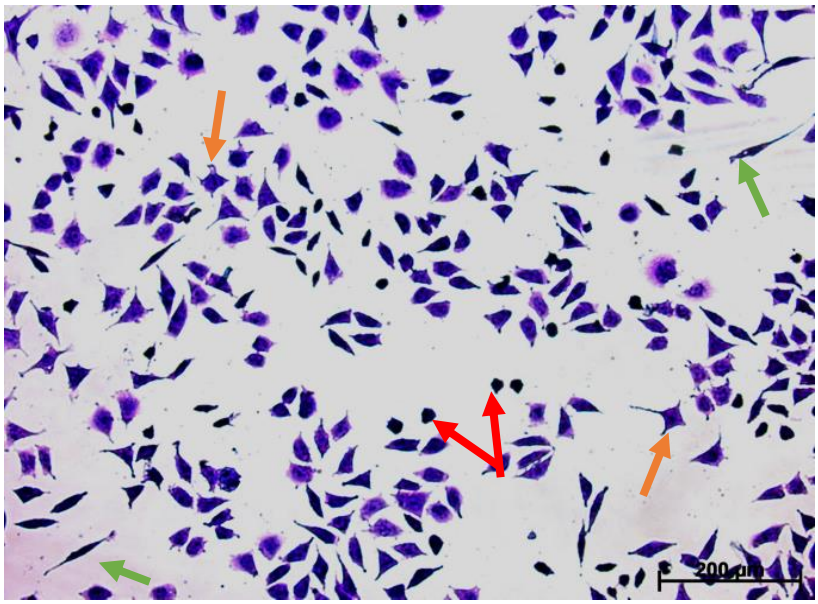

Figure 5.4: Control L929 fibroblast cells showing elongated bipolar fibroblasts in green arrows and a multipolar shape shown in the orange arrows. Red arrows show round cells detaching from the surface. Scale bar: 200 µm.

Due to the lack of significance differences between exposure to 1.25, 2.5 and 5% honey, only the effects of 5% undigested, ODC and OD honey on the morphology of L929 cells were evaluated. Exposure resulted in the L929 cells maintaining their characteristic bipolar and multipolar shapes. A few cells appearing to be detaching from the surface can be seen as round cells (red arrows) which indicates that a few of the cells died but differences compared with the control are not significant (Table 5.1).

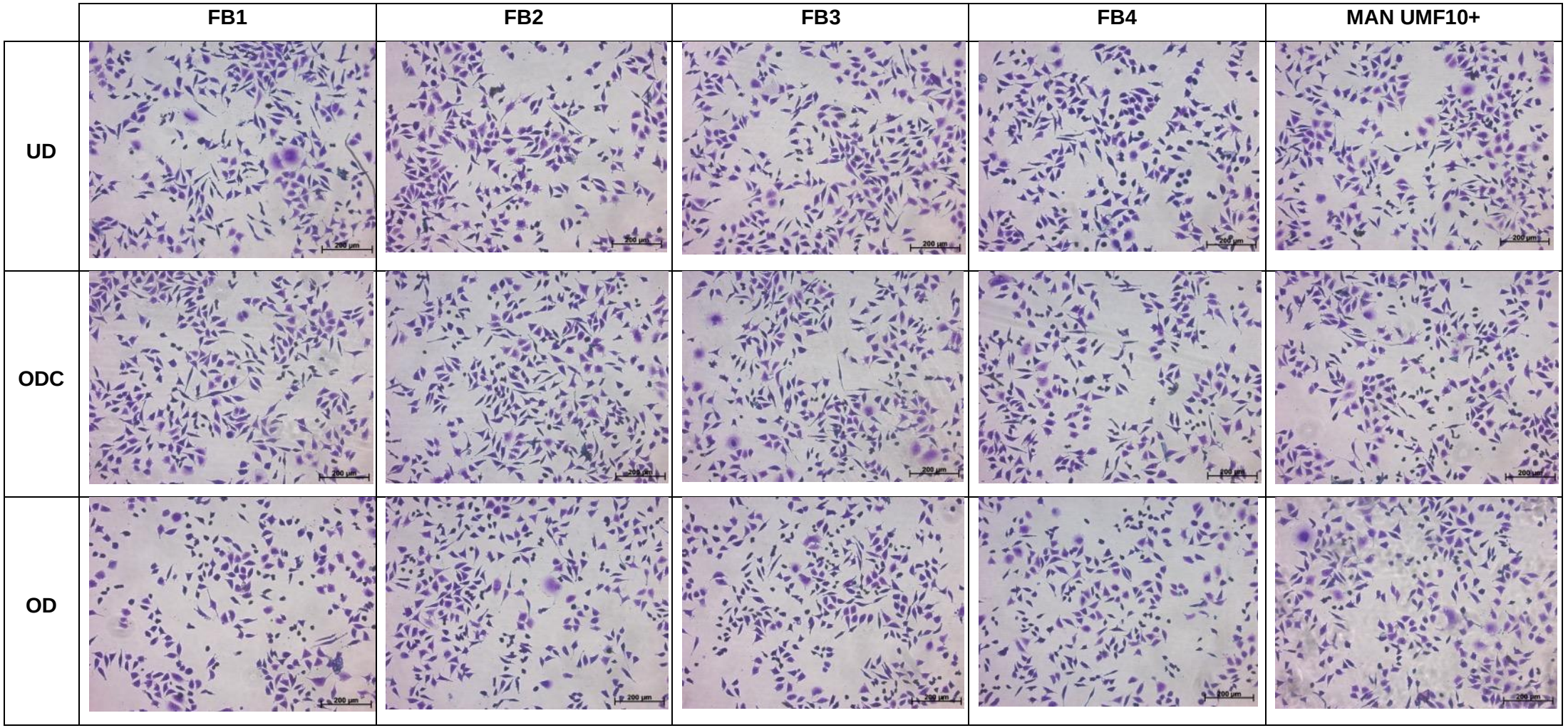

Figure 5.5: L929 fibroblast cells exposed to 5% undigested (UD), oral digest control (ODC) and oral digest (OD), Fynbos (FB) honeys and Manuka (MAN UMF10+) honey. Scale bars: 200 µm.

In cell culture, epithelial cells grow and have a typical squamous and cuboidal shape. Epithelial cells form a monolayer, with the formation of tight, adhering and gap junctions between cells. Even when plated at low concentrations, islands of cells with these typical interactions form as shown in Figure 5.6. In Table 5.2, the % cell number of the Caco-2 cell line after exposure to UD, ODC and OD honey at different concentrations is presented compared to the control, where no honey was added. No change in cell number was observed at all evaluated concentrations when undigested and oral digested FB honey was compared with MAN UMF10+. For each honey, there was no differences between UD, ODC and OD (Table 5.2) indicating that oral digestion did not increase the toxicity of honey. The fold change in the Caco-2 cell number was 0.92 -1.18, 0.99 – 1.14 and 1.04 – 1.21 for the 1.25%, 2.5% and 5% honey solutions, respectively.

Table 5.2: Percentage Caco-2 cell number and fold change following exposure to undigested (UD), oral digest control (ODC) and oral digests (OD) honey samples.

Control: 100	FB1	FB2	FB3	FB4	MAN UMF10+
1.25% UD	82.82 ± 4.33	91.75 ± 3.79	89.51 ± 4.44	99.44 ± 4.58	110.99 ± 8.71
1.25% ODC	95.92 ± 5.07	99.22 ± 3.66	99.99 ± 6.72	99.10 ± 3.52	96.08 ± 3.15
1.25% OD	95.90 ± 8.36	108.50 ± 6.10	101.89 ± 2.80	91.46 ± 10.13	109.53 ± 3.80
Fold change, UD vs. OD	**1.16**	**1.18**	**1.14**	**0.92**	**0.99**
2.5% UD	90.01 ± 9.04	87.14 ± 4.85	84.06 ± 2.28	92.55 ± 1.92	90.15 ± 1.98
2.5% ODC	92.72 ± 1.84	98.43 ± 4.46	101.40 ± 7.01	92.18 ± 3.18	94.81 ± 5.30
2.5% OD	102.92 ± 0.76	114.21 ± 3.65	100.25 ± 3.34	98.22 ± 3.99	106.21 ± 3.50
Fold change, UD vs. OD	**1.14**	**1.16**	**0.99**	**1.07**	**1.12**
5% UD	91.4 ± 5.74	89.18 ± 6.57	90.02 ± 3.83	84.87 ± 4.39	84.38 ± 5.07
5% ODC	88.64 ± 6.44	91.87 ± 3.69	87.89 ± 2.24	94.58 ± 0.81	96.80 ± 8.06
5% OD	103.27 ± 0.35	104.61 ± 5.96	93.78 ± 2.40	100.39 ± 7.22	102.33 ± 6.35
Fold change, UD vs. OD	**1.13**	**1.17**	**1.04**	**1.18**	**1.21**

No significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD), no significant differences between UD, ODC and OD for each honey and no significant differences between the control and honey samples, $p<0.05$.

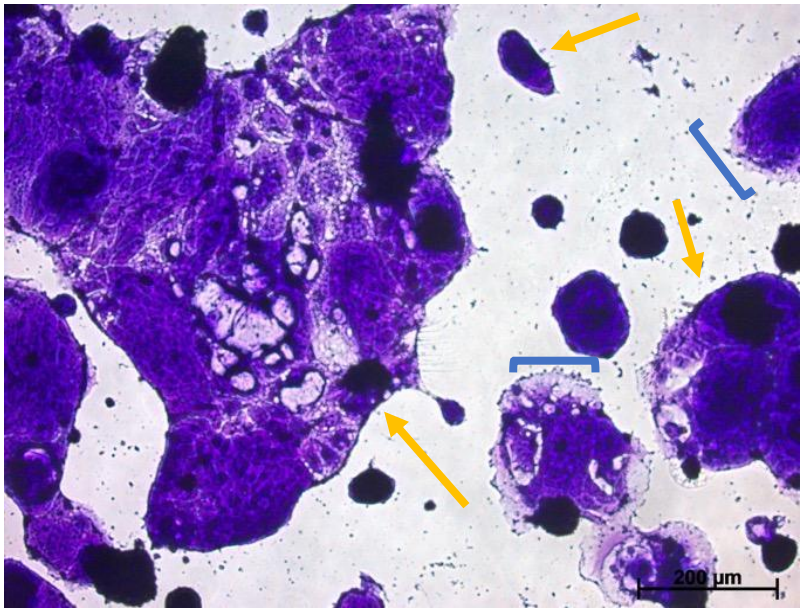

Figure 5.6: Control Caco-2 cells showing islands of cells (yellow arrows) and ruffling indicated by blue brackets. Scale bar: 200 µm.

In figure 5.7, the morphology of the exposed Caco-2 cells exposed to 5% undigested, ODC and OD were compared with the control (Figure 5.6). Following exposure, the morphology of the Caco-2 cells was similar to the control, confirming the lack of toxicity.

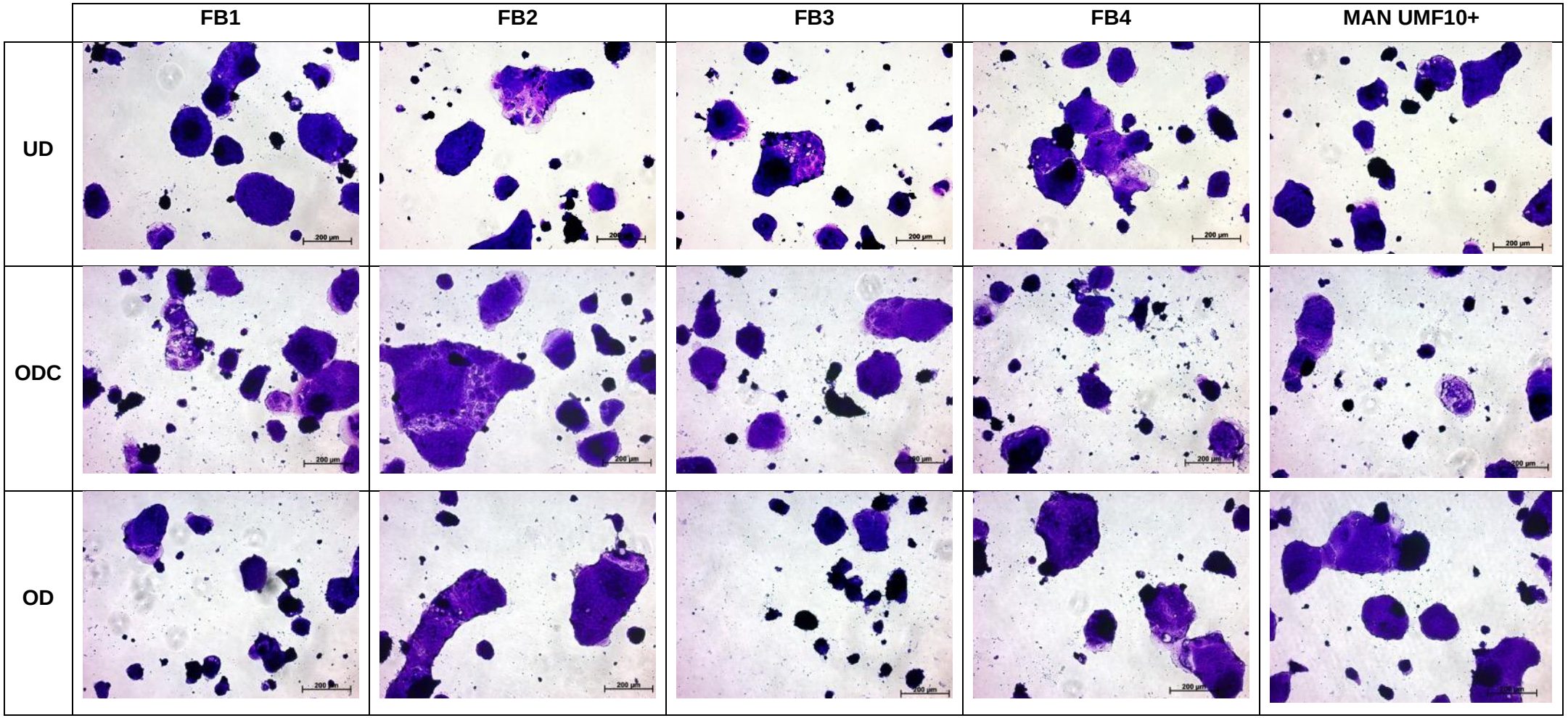

Figure 5.7: Caco-2 cells exposed to 5% undigested (UD), oral digest control (ODC) and oral digest (OD) Fynbos (FB) and Manuka (MAN UMF10+) honeys. Scale bars: 200 µm.

5.5.2 Effect of undigested, oral digested control and digested honey of cell viability (MTT assay)

The effects of different concentrations of the UD, ODC and OD following 24 h exposure on the viability of L929 cells were determined (Table 5.3). No change in cell viability was observed for the UD, ODC and OD, FB honey samples compared with MAN UMF10+. The fold change between undigested and OD was 1.02 – 1.22, 1.00 – 1.10 and 1.04 – 1.15 for the 1.25%, 2.5% and 5% honeys, respectively. Statistical analysis revealed no significant changes, $p \leq 0.05$ in viability.

Table 5.3: Percentage L929 cell viability and fold change following exposure to undigested (UD), oral digest control (ODC) and oral digests (OD) honey samples.

Control: 100	FB1	FB2	FB3	FB4	MAN UMF10+
1.25% UD	80.87 ± 6.79	83.20 ± 7.09	77.99 ± 8.78	82.96 ± 6.38	82.63 ± 8.32
1.25% ODC	74.71 ± 7.25	80.42 ± 5.93	78.28 ± 8.62	80.79 ± 7.28	83.33 ± 7.46
1.25% OD	98.67 ± 14.05	86.67 ± 6.00	86.25 ± 6.05	84.76 ± 7.35	88.68 ± 7.26
Fold change, UD vs. OD	**1.22**	**1.04**	**1.11**	**1.02**	**1.07**
2.5% UD	81.10 ± 14.44	87.26 ± 10.22	80.98 ± 10.57	83.01 ± 11.53	79.76 ± 8.05
2.5% ODC	75.89 ± 8.21	82.79 ± 7.50	80.88 ± 9.21	84.30 ± 5.66	84.29 ± 5.94
2.5% OD	87.11 ± 8.55	82.71 ± 6.83	86.95 ± 7.26	93.11 ± 7.44	90.53 ± 8.50
Fold change, UD vs. OD	**1.07**	**1.00**	**1.08**	**1.10**	**1.07**
5% UD	79.10 ± 12.11	84.67 ± 10.37	83.76 ± 10.14	90.64 ± 8.83	81.68 ± 9.19
5% ODC	74.67 ± 7.47	85.52 ± 7.21	82.23 ± 8.67	91.26 ± 5.50	83.79 ± 3.60
5% OD	90.29 ± 2.60	92.19 ± 6.26	87.39 ± 7.036	99.49 ± 5.19	93.69 ± 5.46
Fold change, UD vs. OD	**1.14**	**1.09**	**1.04**	**1.10**	**1.15**

No significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD), no significant differences between UD, ODC and OD for each honey and no significant differences between the control and honey samples, $p<0.05$.

As in the L929 cell line, the effects of different concentrations of the UD, ODC and OD following 24 h exposure on the viability of Caco-2 cells was determined (Table 5.4). There was also no significant differences between the UD, ODC and OD, FB honeys and MAN UMF10+ honey. In Table 5.4, the effects of oral digestion on activity was determined. The fold change between UD and OD was 0.83 – 1.01, 0.91 – 1.00 and 0.82 – 0.98 for the 1.25%, 2.5% and 5% honey solutions, respectively. Statistical analysis revealed no significant changes, $p \leq 0.05$ in viability following oral digestion.

Table 5.4: Percentage Caco-2 cell viability and fold change following exposure to undigested (UD), oral digest control (ODC) and oral digests (OD) honey samples

Control: 100	FB1	FB2	FB3	FB4	MAN UMF10+
1.25% UD	104.92 ± 14.99	95.09 ± 9.11	94.75 ± 9.15	98.60 ± 14.45	108.61 ± 12.23
1.25% ODC	97.64 ± 12.10	92.44 ± 9.00	99.82 ± 15.01	116.78 ± 14.49	110.23 ± 9.72
1.25% OD	86.65 ± 3.34	98.3 ± 9.54	87.42 ± 4.39	88.97 ± 1.91	97.89 ± 6.77
Fold change, UD vs. OD	**0.83**	**1.01**	**0.92**	**0.90**	**0.90**
2.5% UD	96.17 ± 12.96	91.18 ± 8.41	94.34 ± 5.67	101.50 ± 7.35	101.18 ± 8.93
2.5% ODC	90.79 ± 6.51	103.30 ± 8.65	95.78 ± 6.33	97.19 ± 4.23	106.43 ± 11.82
2.5% OD	88.82 ± 15.09	86.41 ± 7.78	94.98 ±17.22	91.51 ± 16.11	91.81 ± 13.62
Fold change, UD vs. OD	**0.92**	**0.95**	**0.99**	**1.00**	**0.91**
5% UD	91.44 ± 6.72	83.62 ± 5.92	85.58 ± 4.42	90.43 ± 7.18	93.65 ± 7.91
5% ODC	87.64 ± 7.19	89.92 ± 4.58	82.97 ± 5.67	89.83 ± 9.41	89.92 ± 8.79
5% OD	75.03 ± 11.03	81.85 ± 13.70	80.79 ± 16.09	78.39 ± 13.63	79.46 ± 17.74
Fold change, UD vs. OD	**0.82**	**0.98**	**0.94**	**0.87**	**0.85**

No significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD), no significant differences between UD, ODC and OD for each honey and no significant differences between the control and honey samples, $p<0.05$.

5.5.3 Oxidative effect and cellular antioxidant activity (DCFH-DA assay)

The DCFH-DA assay was used in this study firstly to determine the possible oxidative effects of the undigested and oral digested honey samples, and then the ability of these samples to quench the oxidative effects of the radical AAPH.

The percentage oxidative damage caused by AAPH alone was 100%. In the L929 cell line, compared with the effects of AAPH the effects of each honey sample alone, with no added AAPH was minimal and differences between FB honeys and MAN UMF10+ honey were not significant (Table 5.5) for the UD, ODC and OD honey. All honey samples inhibited the effects of AAPH from 100% to a range of 7.49 ± 3.77% - 17.39 ± 6.84% for the UD honey with FB4 having the best CAA. The oxidative effects of AAPH were quenched to 4.66 ± 1.07 - 16.59 ± 4.74 and 4.45 ± 0.22 - 13.61 ± 1.28 for the ODC and OD honey, respectively with MAN UMF10+ honey having the best CAA following oral digestion, although differences were not statistically significant, $p \leq 0.05$.

Table 5.5: Percentage oxidative damage in L929 cells induced by undigested (UD), oral digest control (ODC) and oral digests (OD) 1.25% (v/v) honey samples with (A) no AAPH and (B) AAPH added.

No AAPH added – Oxidative effect					
Control: 100	**FB1**	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
1.25% UD	-0.84 ± 0.24^{+}	-2.05 ± 0.33^{+}	-1.30 ± 0.17^{+}	-2.07 ± 0.26^{+}	-2.04 ± 0.22^{+}
1.25% ODC	-0.20 ± 0.97^{+}	-1.90 ± 0.38^{+}	-0.64 ± 0.68^{+}	-1.87 ± 0.58^{+}	-2.06 ± 0.49^{+}
1.25% OD	0.91 ± 1.65^{+}	-1.35 ± 0.58^{+}	-0.22 ± 0.56^{+}	-1.56 ± 0.38^{+}	1.11 ± 0.54^{+}
Fold change, UD vs. OD	**-1.08**	**0.65**	**0.17**	**0.75**	**0.54**
AAPH added - Antioxidant effect					
1.25% UD	15.00 ± 5.37^{+}	17.39 ± 6.84^{+}	14.36 ± 6.61^{+}	7.49 ± 3.77^{+}	8.56 ± 4.37^{+}
1.25% ODC	16.59 ±4.74^{+}	13.79 ± 4.49^{+}	14.74 ± 3.75^{+}	6.06 ± 1.35^{+}	4.66 ± 1.07^{+}
1.25% OD	13.17 ± 1.18^{+}	11.24 ±0.14^{+}	13.61 ± 1.28^{+}	10.59 ± 1.08^{+}	4.45 ±0.22^{+}
Fold change, UD vs. OD	**0.88**	**0.65**	**0.95**	**1.41**	**0.52**

$^{+}$ Indicates significant differences between the control and honey samples, no significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) and no significant differences between UD, ODC and OD for each honey, $p<0.05$.

The effects on CAA were then determined in the Caco-2 cell line. Compared with the effects of AAPH, 100%, the effects of each honey sample alone, in the Caco-2 cells were minimal and differences between FB honeys and MAN UMF10+ honey were not significant (Table 5.6) for UD, ODC, control and OD honey. All honey samples inhibited the effects of AAPH from 100% to a range of 9.93 ± 4.56% - 20.51 ± 7.75% for the UD honey with FB4 honey having the best CAA. The oxidative effects of AAPH were quenched to between 6.61 ± 1.99 % and 20.14 ± 6.87% for the ODC and OD honey, with MAN UMF10+ and FB4 having the best CAA, respectively although, differences were not significant, $p \leq 0.05$.

In both the L929 and Caco-2 cell lines, all honey samples and their oral digests effectively inhibited the oxidative effect of AAPH.

Table 5.6: Percentage oxidative damage in Caco-2 cells induced by undigested (UD), oral digest control (ODC) and oral digests (OD) 1.25% (v/v) honey samples with (A) no AAPH and (B) AAPH added.

No AAPH added - Oxidative effect					
Control: 100	**FB1**	**FB2**	**FB3**	**FB4**	**MAN UMF10+**
1.25% U	-0.69 ± 0.17^{+}	-1.10 ± 0.35^{+}	-0.53 ± 0.48^{+}	-1.08 ± 0.42^{+}	-1.27 ± 0.54^{+}
1.25% ODC	-0.54 ± 0.25^{+}	-0.98 ± 0.67^{+}	-0.67 ± 0.56^{+}	-1.19 ± 0.72^{+}	1.33 ± 0.71^{+}
1.25% OD	0.06 ± 0.78^{+}	-1.38 ± 0.89^{+}	-0.34 ± 0.53^{+}	-1.17 ± 0.07^{+}	1.17 ± 0.14
Fold change, U vs. OD	**-1.36**	**0.93**	**0.55**	**1.08**	**-0.64**
AAPH added - Antioxidant effect					
1.25% U	20.51 ± 7.75^{+}	19.54 ± 7.58^{+}	17.61 ± 7.05^{+}	9.93 ± 4.56^{+}	7.73 ±3.14^{+}
1.25% ODC	14.34 ± 5.41^{+}	14.53 ± 6.24^{+}	13.37 ± 4.83^{+}	6.61 ± 1.99^{+}	7.94 ± 2.80^{+}
1.25% OD	20.14 ± 6.87^{*+}	17.29 ± 7.44^{+}	16.32 ± 6.83^{+}	10.11 ± 3.58^{+}	6.27 ± 1.53^{+}
Fold change, U vs. OD	**1.01**	**0.97**	**1.02**	**1.13**	**0.81**

* Indicates significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+). $^{+}$ Indicates significant differences between the control and honey samples, and no significant differences between UD, ODC and OD for each honey, $p<0.05$.

5.5.4 Cellular pro-inflammatory and anti-inflammatory activity

The RAW 264.7 cell line was used to determine the anti- and pro-inflammatory activities of undigested and oral digested FB and MAN UMF10+ honeys. Table 5.7 indicates these activities at a concentration of 1.25%. The honey samples, no LPS added, showed slight pro-inflammatory activity. The undigested honeys FB1, FB3 and MAN UMF10+ compared with the control (100%) induced 36.62 ± 13.13%, 37.28 ± 9.82 and 17.15 ± 16.28% NO production, respectively with FB3 showing the most pro-inflammatory effect. FB2 honey did not induce NO, with FB4 also showing a significantly low pro-inflammatory effect at 5.23 ± 1.87 %. With oral digestion, the trend remained the same. Differences compared with the control were significant with $p<0.05$.

The ability to reduce NO levels induced by LPS in the RAW 264.7 cell line was then determined. LPS induced the formation of 4.17 mM NO. NO levels were significantly reduced for all UD honey ($p<0.05$) compared with the control (100%). With ODC honeys, all honeys except FB4 significantly reduced NO produced by LPS, whereas for OD honeys only FB1 honey significantly reduced NO produced by LPS. This indicates that with oral digestion some protective effect against LPS-induced NO formation was lost.

Table 5.7: Percentage NO production of 1.25% undigested Fynbos and Manuka (MAN UMF10+) honey (pro-inflammatory activity) and honey + LPS (anti-inflammatory activity) samples in RAW 264.7 cells

Control (LPS): 100					
			Undigested		
	FB1	**FB2**	**FB3**	**FB4**	**MAN (UMF10+)**
Honey	36.62 ± 13.13^{+}	-0.45 ± 4.46^{+}	37.28 ± 9.82^{+}	5.23 ± 1.87^{+}	17.15 ± 16.28^{+}
Honey + LPS	73.89 ± 5.31^{+}	80.71 ± 1.07^{+}	64.11 ± 6.06^{+}	80.49 ± 3.88^{+}	79.35 ± 4.97^{+}
			Oral digest control		
	FB1	**FB2**	**FB3**	**FB4**	**MAN (UMF10+)**
Honey	46.23 ± 11.85^{+}	-4.04 ± 4.14*$^{+}$	39.77 ± 9.95^{+}	5.96 ± 1.13^{+}	33.01 ± 10.57^{+}
Honey + LPS	69.93 ± 4.33^{+}	77.29 ± 2.53^{+}	66.15 ± 4.94^{+}	91.11 ± 6.12	72.58 ± 5.61^{+}
			Oral digest		
	FB1	**FB2**	**FB3**	**FB4**	**MAN (UMF10+)**
Honey	45.69 ± 12.31^{+}	-1.30 ± 3.44*$^{+}$	44.67 ± 12.74^{+}	14.87 ± 7.42^{+}	39.93 ± 10.39^{+}
Honey + LPS	72.97 ± 3.96^{+}	87.68 ± 9.43	75.09 ± 8.24	91.60 ± 14.53	90.03 ± 10.31

* Indicates significant difference of Fynbos (FB) compared with Manuka (MAN UMF10+) honey for undigested (UD), oral digested, control (ODC) and oral digested (OD), $^{+}$ Indicates significant differences between the control and honey samples and no significant differences between UD, ODC and OD for each honey, $p<0.05$.

5.6 Discussion

Cellular *in vitro* models provide more information on the possible effectiveness of molecules in reducing oxidative damage and inflammation. The advantage of these models is that they are a representative of a specific cell type and additional effects such as cellular metabolism and excretion are also taken into account. The first level of testing is the determination of cellular toxicity.

The oral cavity mucosa consists of stratified squamous epithelium and the deeper lamina propria (Groeger and Meyle, 2019). The mouse fibroblast, L929 cell line is a subclone of the L-parental strain derived from subcutaneous and adipose tissue of a C_3H mouse, and following subsequent cloning, the strain NCTC clone 929, strain L, known as the L929 cell line (Fedoroff and Cook, 1959) was established. This cell line is widely used to evaluate toxicity. The human colon adenocarcinoma (Caco-2) cell line is an epithelial cell line (Rousset, 1986) is widely used as a predictor of the oral absorption of drugs in humans due to the permeation characteristics of drugs across Caco-2 cell monolayers (Yang *et al.*, 2017). Although both cell types are not associated with the oral mucosa they are representative of the cells found in the oral mucosa.

Honey is a complex mixture of sugars, polyphenols, enzymes, catalase, carotenoids, amino acids and other proteins such as BD-1 (Khalil *et al.*, 2010) which may induce cellular toxicity. Effects on cell number and viability was determined with the CV and MTT assays, respectively. The CV provided more consistent results with lower standard error of mean (SEM) values. In

contrast, the results with the MTT assay were more variable as the MTT assay measures the metabolic activity of the cells, which can be influenced by the components of honey such as the sugars that can modify cell metabolism and reactions (Śliwka *et al.*, 2016). In addition, MTT tetrazolium salt may be reduced within the cytoplasm, on the surface of the cell, lysosomal and endosomal membranes or in the extracellular environment; and not necessarily a reflection of mitochondrial activity (Śliwka *et al.*, 2016). However, in the present study, both the undigested and oral digested MAN UMF10+ and FB honeys did not cause a significant decrease in cell number or viability and the observed morphology was similar to the controls. Therefore, at concentrations that reduce the growth of *C. albicans*, this honey is not cytotoxic.

In a study by Porcza *et al.* (2016), the anti-proliferative activity in Caco-2 cells of a 10% sugar solution (4% fructose and 3% glucose), a MAN (UMF15+) honey and a raw, unprocessed honey after 24 h exposure was determined. A reduction in cell viability measured using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay, revealed inhibition, by both the sugar and honey solutions, although inhibition by the honey solutions was greater than the sugar solution. This effect was attributed to fructose and the polyphenols present in honey.

A study by Al-Jadi *et al.* (2014) investigated the effect of the Malaysian honey, Gelam honey, and its major components on the proliferation of cultured rat fibroblast (3Y1) cells. After 24 h exposure at concentrations of 0.195 – 195 mg/mL (with 1 mL equivalent to 1.3 g (Serem and Bester, 2012)), which is equivalent to a 0.15 - 15% honey solution did not inhibit the growth of 3Y1 fibroblasts evaluated with the MTT assay. The equivalent sugar solutions and H_2O_2 solution promoted cellular growth while the phenolic extracts inhibited growth. In a complex mixture such as honey, the observed effect is the sum of all effects and in the present study, there was a lack of inhibition at the concentrations evaluated.

Chai and Chopra (2013) compared the *in vitro* anti-cancer activity of three types of MAN honeys, Active 5+, Manuka 6^+ and Manuka 15+, as well as Australian Pure, Gale's commercial honey, Pure Raw Bee Flower and Sun honey. After 24 h exposure, cell viability was determined with the MTT assay. A 10 % solution caused a decrease in viability from 100% for the control to 37.7%, 38.0%, 47.7%, 45.3% 45.7% and 33.2%, respectively.

Portokalakis *et al.* (2016) also investigated the anticancer effects of MAN honey with UMF ratings 5+, 10+, 15+ and 18+. The MAN honeys showed a dose-dependent loss of MCF-7 cell viability after 24 h evaluated with the MTT assay. Concentrations of 4.7% (w/v) for UMF5+ honey to 2.2% (w/v) for UMF18+ caused 50% inhibition indicating that MAN honey is cytotoxic to MCF-7 breast cancer cells *in vitro*.

In contrast, in the L929 and Caco-2 cell lines the effect of 24 h exposure to 1.25%, 2.5% and 5% FB and MAN UMF10+ honey and oral digests resulted in no significant loss of activity at all concentrations evaluated.

Oxidative stress due to ROS causes cellular damage by oxidation of cell membranes and proteins (Pizzino *et al.*, 2017,) and DNA damage leading to apoptosis which leads to cell death and inflammation (Alarifi *et al.*, 2016). To counteract these effects, cells contain enzymatic and non-enzymatic antioxidants molecules, but high cellular levels of ROS can overwhelm the antioxidant capacity of cells (He *et al.*, 2017).

In honey, the presence of MGO and/or H_2O_2 can induce cellular oxidative damage and therefore the cellular oxidative effects of the undigested and oral digested MAN UMF10+ and FB honeys was determined. In contrast, antioxidants such as polyphenols also present in honey can react with oxidising agents such as free radicals preventing damage to biomolecules (Campbell and Farrell, 2012). The type and concentration of polyphenols (Alvarez-Suarez *et al.*, 2014) of honey is determined by the floral source and external factors such as the environment (Khalil *et al.*, 2010) and consequently honey from different seasons, geographical regions and from different bee types may have different CAA.

The honey samples did not cause ROS but did protect L929 cells against oxidative damage with a reduction in oxidative damage from 100% to a range of 7.49 ± 3.77% - 17.39 ± 6.84 % for the undigested honey and in Caco-2 cells from 100% to a range of 9.93 ± 4.56 % - 20.51 ± 7.75%. In both cell lines, FB4 honey had the best anti-oxidative effect. This confirms the findings of Magoshi (2017) where MAN UMF15+ and FB4 honeys were the most effective in protecting Caco-2 cells against oxidative damage.

In Chapter 4, the MAN UMF10+ and FB honey and oral digests contained polyphenols and had antioxidant activity and the findings confirm that this activity translates into beneficial cellular effects, although MGO and H_2O_2 have potential oxidative effects. Likewise, in a study by Serem and Bester (2012) three FB honeys were found to also protect the Caco-2 and the SC-1 mouse fibroblast cell lines against oxidative damage, but did not determine the effects of digestion. The greatest protection was observed for FB4 and MAN UMF10+ compared to the other FB honey types, indicating the antioxidant properties of some FB honey is comparable to MAN UMF10+ honey. Magoshi (2017) also investigated the protection against oxidative damage of undigested and digested FB honeys in the Caco-2 and SC-1 cell lines; after gastric digestion there was no change to oxidative damage but after gastro-duodenal digestion oxidative damage was increased. This is potentially related to exposure time, where exposure in the oral cavity was limited to 2 min, while in the gastro-duodenal phase of digestion exposure was longer.

NO is a well-known mediator of acute and chronic inflammation as it is generated during immune and inflammatory responses (Coleman, 2001). It can be a toxic agent towards infectious organisms, such as *C. albicans* and is an important component of the innate immune system. NO has both pro-inflammatory and anti-inflammatory properties (Coleman, 2001). NO is synthesised from L-arginine and oxygen by nitric oxide synthase (NOS) enzymes making use of electrons donated by nicotinamide adenine dinucleotide phosphate (NADPH) through the intermediate N-hydroxy-L-arginine converting L-arginine to NO and L-citrulline. There are three types of NOS enzymes and these are neuronal NOS (nNOS or NOS 1), endothelial NOS (eNOS or NOS 3) and the inducible form of NOS (iNOS or NOS 2) (Coleman, 2001). iNOS is induced during inflammation and results in increased levels of NO. iNOS is expressed by macrophages in inflamed tissue and the cytokine responsible for its expression is Interferon gamma (IFN-ɣ) (Korhonen *et al.*, 2005).

Excessive NO production can have detrimental effects which includes the formation of peroxynitrite radicals in an environment with increased ROS. The pro-inflammatory activity found in the honeys may be advantageous as it is ideal for acute wound healing (Majtan *et al.*, 2014a). In chronic inflammation, honey can have an anti-inflammatory effect and can inhibit the release of pro-inflammatory cytokines and other factors such as ROS (Majtan *et al.*, 2014a; Magoshi, 2017). This can be advantageous in oral candidiasis.

Mouse macrophages produce NO in response to bacterial LPS (Korhonen *et al.*, 2005). The NO then regulates signalling cascades, vascular responses, cytokine production and apoptosis, if necessary (Korhonen *et al.*, 2005). Tonks *et al.* (2007), reported that in human monocytic cells, MAN honey stimulates the production of pro-inflammatory cytokines IL-6, IL-1β and TNF-α. The mechanism of activation was mediated by the pattern recognition receptor Toll-like receptor 4 (TLR4) which is responsible for activating the innate immune system (Vaure and Liu, 2014).

The ability of the honey samples to induce NO was determined and was compared to the effects of LPS that induced 4.17 mM NO. Both the honeys and oral digests in the absence of LPS caused an increase in NO levels. Compared to the control, normalised to 100%, NO levels for undigested honey was increased to 36.62%, 37.28% and 17.15% for FB1, FB3 and MAN UMF10+, respectively. After oral digestion levels were retained and were 45.69%, 44.67% and 39.93% for FB1, FB3 and MAN UMF10+, respectively. In contrast, levels for FB2 and FB4 were significantly lower.

The question arises that if honey induces NO production, why is this NO not being scavenged by polyphenols? Honey is a complex mixture of different types and concentrations of polyphenols, where depending on type and concentration, either a pro-inflammatory or anti-

inflammatory effect is observed. In RAW 264.7 macrophages, polyphenols were found to affect the activity of cyclooxygenase, lipoxygenase and NOS, and their inhibition moderates the production of important mediators in inflammation (Yahfoufi *et al.*, 2018). Poor scavenging effects and increased stimulation would result in increased NO levels as observed for FB1, FB3 and MAN UMF10+. Other naturally-derived compounds such as polyphenols found in olives, wine and turmeric counteract oxidative stress and enhance the generation of NO and thereby, improving endothelial function (Forte *et al.*, 2016).

LPS-mediated stimulation of RAW 264.7 cells increases NO levels. The consequence of direct scavenging would reduce these levels as observed for FB2 and FB4, where both honeys did not cause an increase in NO levels. Polyphenols can also bind LPS and this can reduce the levels of free LPS able to stimulate NO production. Although honey alone can have a stimulatory effect, the binding of added LPS may reduce NO levels.

Magoshi (2017) investigated the production of NO of honeys using the RAW 264.7 cell model, induced to produce NO with LPS and IFN-γ. The undigested honeys, no LPS or IFN-γ added induced an average NO level of 23.57% which is comparable to the findings of the present study. Gastro-duodenal digestion resulted in an increase in NO production of an average of 91.91%, which was similar to that produced by cells and LPS/IFN-γ.

Honey can contain low levels of LPS derived from bee-associated bacteria or that have been acquired during processing or storage. Gram-negative species such as *Enterobacter*, *Escherichia coli*, *Klebsiella* and *Pseudomonas* are commonly found in honey, withstanding the concentrated sugar and acidic honey environment (Olaitan *et al.*, 2007). LPS derived from Gram-negative bacteria can stimulate NO production. With digestion, proteolytic activity can result in the release of smaller membrane associated proteins such as LPS that can stimulate NO formation.

Few studies have investigated the effects of honey on NO formation, and most studies have focused on NO scavenging effects. The effects of Malaysian honey extracts on the production of NO in RAW 264.7 cells that were induced with LPS and IFN-γ have been investigated by Kassim *et al.* (2010). The ethyl acetate extract (EAE) with high levels of ellagic acid, myricetin and ferulic acid induced and inhibited NO in a dosage dependent manner. The authors identified that there was an increase in NO production and a decrease in cell viability after stimulation with LPS/IFN-γ in the RAW 264.7 macrophages. This then induces iNOS which increases cellular NO concentrations. NO mediates toxicity by generating reactive nitrogen derivatives such as peroxynitrite which acts upon cellular targets such as DNA and proteins. From their study, the authors could conclude that honey can inhibit NO production and, in turn,

the formation of peroxynitrite. This activity has also been observed with phenolic acids such as GA, CA, ferulic acid and quercetin.

Compared to the undigested and oral digest control honeys, there was no change in produced NO levels while only a significant increased scavenging was found for FB2. This difference to the study of Magoshi (2017) is due to only α-amylase being present in the oral digests whereas for gastro-duodenal digestion, enzymes such as pepsin and pancreatin digest proteins with the subsequent release of LPS.

A pro-inflammatory effect was observed for the honeys FB1, FB3 and MAN UMF10+ without the presence of LPS. In wound healing, pro-inflammatory effects (coagulation, inflammation, cell proliferation and tissue remodelling) (Tonks *et al.*, 2007) are observed and aid in the wound healing process. However, if this state is prolonged, neutrophils accumulate at the site of the wound and activate cytokines which then cause a chronic state of inflammation (Alvarez-Suarez *et al.*, 2014) especially if associated with infection.

In the presence of LPS, the honeys had significant anti-inflammatory activity showing that some of these honeys can also act as anti-inflammatory agents and can promote the wound healing process which is initiated by inflammation, acting as a good alternative therapy to regular anti-inflammatory agents that completely slow down the process of wound healing (Hadagali and Chua, 2014).

The association of *C. albicans* with inflammation is that with an increase in the fungi load there is a subsequent increased pro-inflammatory state, the purpose of which is to eradicate infection (Sarazin *et al.*, 2010). A study by Sarazin *et al.* (2010) evaluated pro- and anti-inflammatory cytokine secretion by macrophages in relation to levels of *C. albicans*. High yeast concentrations, reduced the pro-inflammatory state (reduced TNF-α production) and stimulated an anti-inflammatory state (increased IL-10 production) which may favour the growth of *C. albicans*. However, other studies have shown that the inhibition of cyclo-oxygenase isoenzymes with aspirin is effective in reducing the germination of hyphae in *C. albicans* (Rusu *et al.*, 2015).

Whether a pro-inflammatory or anti-inflammatory state in the oral cavity promotes or prevents *C. albicans* infections is unknown and may be related to whether *C. albicans* in the oral cavity are planktonic or grow as biofilms. Further investigations need to be undertaken with *C. albicans* (planktonic and biofilms) and macrophages as co-cultures to elucidate the mechanism of action.

5.7 Conclusion

The undigested and oral digested FB and MAN UMF10+ honeys did not alter the cell number and viability as well as the morphology of L929 and Caco-2 cells following exposure for 24 h. Both the honeys and oral digests inhibited AAPH-mediated oxidative damage in the Caco-2 cell line. Honeys and oral digests of FB1, FB3 and MAN UMF10+ induced NO levels in the RAW 264.7 cell line indicating a pro-inflammatory effect, however also had an anti-inflammatory effect in the presence of LPS. In contrast, FB2 did not induce NO formation and FB4 induced negligible NO, also inhibiting LPS-induced NO levels indicating an anti-inflammatory effect. The mechanisms involved needs to be elucidated. At the concentrations that inhibit *C. albicans* biofilm formation, MAN UMF10+ and FB honeys and oral digests have additional benefits which are a reduction in ROS levels and modulation of NO levels which may contribute to tissue recovery.

Chapter 6 : General discussion

Honey is a repeat essential on the grocery lists of many South African households as reports on its beneficial bioactivities are on the rise. Honey has been used for centuries as a therapeutic product and increasing research has identified its health benefits. The most extensively researched honey is Manuka (MAN) honey, indigenous to New Zealand and Australia. This has led to the development of certified medical-grade MAN honey that is used for the treatment of wounds based on its antibacterial, anti-oxidative and anti-inflammatory properties. MAN honey is expensive to import into South Africa, therefore there is a need to identify honeys from South Africa with health benefits.

To achieve this, a number of investigations have been done on the bioactivity of different types of honey from South Africa and this includes the anti-fungal activity of three South African honeys (Theunissen *et al.*, 2001), the antimicrobial activity of Fynbos (FB) honey (Basson and Grobler, 2008), the antioxidant properties of southern African honeys (Serem and Bester, 2012), the wound healing and anti-fungal activities of South African honeys (Khan *et al.*, 2014), the antioxidant and anti-inflammatory activities of simulated gastric digested and gastro-duodenal digested FB honey (Magoshi, 2017) and lastly the effect of simulated digestion on the anti-bacterial, antioxidant and anti-inflammatory activities of southern Africa honeys (Serem, 2018).

In an endeavour to identify if FB honey can reduce oral *Candida albicans* (*C. albicans)* infections and reduce associated reactive oxygen species (ROS) formation and inflammation, the present study was undertaken. In the oral cavity, honey is exposed to salts, neutral pH and enzymes such as α-amylase which can affect anti-fungal, antioxidant and anti-inflammatory activities. This type of study is important due to the development of *C. albicans* infections in patients with immune-compromised systems such as those with HIV/AIDS, receiving chemotherapy, and patients with type 2 diabetes mellitus (DM-T2) and the young with immature immune systems. In addition, the increasing prevalence of drug resistance makes finding alternative therapies essential.

The Cape Honeybee produces FB honey in the Fynbos biome of the Western Cape province of South Africa. This unique biome has produced many well-known plants such as those used to make Buchu and Rooibos products. This makes it a highly sought area for the use of its plants as most have medicinal properties. Four FB honeys from different local honey suppliers were used in this study and they were compared to a medical-grade MAN UMF10+ honey. In the laboratory, each sample was subjected to simulated oral digestion, using synthetic saliva. For each honey three samples were generated and these were, undigested (UD), oral digest

control (ODC) and oral digest (OD). The effect of low (5 - 9%) and high (25 - 45%) concentrations on the growth of planktonic cells and biofilms was determined.

In planktonic cells, both UD FB and MAN UMF10+, at low and high concentrations induced the growth of *C. albicans*. Evaluation of the effect of oral digestion showed that the UD, ODC and OD samples also stimulated the growth of planktonic *C. albicans*. This effect has not previously been reported and may be due to the presence of sugars that supports the growth of planktonic cells or may be related to the method used for quantification. For the inhibition of growth the absorbance is measured at 530 nm. Honey is known to absorb at this region of the absorbance spectrum and therefore the findings of this study must be confirmed by using alternative quantification methods such as the CellTiter-Blue assay.

In contrast, low concentrations (7 – 9%) UD FB and MAN UMF10+ inhibited the growth of *C. albicans* biofilms by between 68.00 and 86.46%, and high concentrations between 30 and 45% also showed inhibition. Oral digestion did not alter this inhibitory effect.

MGO, is a major component of MAN honey responsible for antibacterial activity. A concentration of 5 – 20 mM MGO inhibited the growth of planktonic and *C. albicans* biofilms. MAN UMF 10+ contains approximately 1 mM MGO, consequently the MGO content is not sufficient to inhibit growth, where the highest MAN UMF 10+ concentration evaluated contains approximately 0.45 mM MGO. The sugars found in honey were able to inhibit the planktonic form of *C. albicans*, but not biofilms.

Although there was no change in cell viability, honey may alter the biofilm developmental process. Therefore, the ultrastructural morphology of *C. albicans* cells in biofilms following 24 h exposure to MAN UMF10+ and FB honey was evaluated with scanning electron microscopy. Changes in cell morphology were observed where instead of the presence of true hyphae, the majority of cells had an oval/round yeast cell shape typical of planktonic cells. Some of these cells also showed signs of apoptosis, with the surface of the cells being uneven with disruption of the cell membrane. For future studies, quantitative apoptotic assays such as the caspase 3/7 assay can be used to confirm morphological observations using flow cytometry and western blotting.

Other studies have also reported the inhibition of biofilms where 25 - 50% honey solutions inhibited the growth of biofilms of *C. albicans* (Ansari *et al.*, 2013; Hau-Yama *et al.*, 2019; Fernandes *et al.*, 2020). Inhibition of biofilm growth but not the growth of planktonic cells indicates possible specific transition targeting for example by polyphenols rather than a generalised effect.

A limitation in this part of the study is that the specific molecules involved were not identified. As honey is a complex mixture, it would be of value to firstly evaluate, in more detail, the effects of a sugar solution (artificial honey), a protein and polyphenol extract as described by Al-Jadi *et al.* (2014). If activity is associated with the polyphenol fraction, the polyphenols in honey can be extracted using liquid-liquid extraction (LLE) or solid phase extraction (SPE), and further characterised by using high-performance liquid chromatography (HPLC), ultraviolet detection and mass spectrometry (MS) (Istasse *et al.*, 2016). Then the specific identified polyphenols can be tested to determine the effect at relevant concentrations on *C. albicans* biofilms.

C. albicans (ATCC® 90028™) is a model organism, and does not represent clinical isolates. Further studies of relevant clinical isolates would be beneficial, especially *C. albicans* isolates from HIV patients as candidiasis and associated biofilm formation in the oral cavity is common. In a time based study, the effects on biofilm formation and development can be determined with scanning electron microscopy. Simultaneously, the effects on the regulators of this process can be identified. In the present study changes in the morphology of the cells in biofilms was evaluated. No information could be obtained on the structural features of the extracellular matrix (ECM) following exposure due to the fixation method used. Using cryopreservation methodologies, the effects on ECM formation and structure can be evaluated. Furthermore, biofilm eradication studies can also be undertaken to determine if the honey samples are able to eradicate fully-developed *C. albicans* biofilms.

The polyphenol content and associated antioxidant activity of UD, ODC and OD FB and MAN UMF10+ honey samples were determined. The antioxidant activity was determined using the Trolox equivalent antioxidant capacity (TEAC) and the oxygen radical absorbance capacity (ORAC) assays. Using the sodium nitroprusside (SNP) assay, the ability of the honeys and oral digests to scavenge nitric oxide (NO) was also determined. The ability of these honeys and oral digests to protect proteins and lipids against oxidative damage was determined with the anti-glycation and lipid peroxidation assays.

The total polyphenolic content (TPC) was the highest for MAN UMF10+ and for the FB honey levels were FB4 > FB3 > FB1 > FB2. After oral digestion, TPC levels were increased for FB honey but were reduced for MAN UMF10+. With the TEAC assay, undigested honeys had lower activity compared to MAN UMF10+, however, there was an increase in antioxidant activity post digestion to match MAN UMF10+, except for FB2 that consistently showed a lower antioxidant activity when compared with MAN UMF10+. Antioxidant activity with the ORAC assay was similar in FB honeys compared to MAN UMF10+, however, after oral digestion a decrease in antioxidant activity was observed for FB2 when compared to MAN UMF10+. The ability of each honey and oral digests to scavenge NO was determined with the

SNP assay. All honeys and oral digests scavenged NO, thereby having the ability to reduce RNS by either scavenging ROS and/or NO.

The undigested and oral digested FB and MAN UMF10+ honey did not induce, but inhibited AGE formation. Inhibition of AGE formation was between 65.78 - 78.83% for the FB honeys. Similarly, the honeys and oral digests did not induce lipid peroxidation or protect LDL from oxidation.

Alkaline conditions such as that associated with digestion can cause the degradation of polyphenols (Friedman and Jurgens, 2000). The oral cavity is a neutral/alkaline environment yet, under the oral digestion conditions no change in activity was observed indicating beneficial effects in the oral cavity. The degradation of polyphenols in an alkaline environment is a function of time and the type of polyphenols, where polyphenols such as caffeic acid (CA), chlorogenic, and gallic acid (GA) readily undergo oxidation (Friedman and Jurgens, 2000). The short digestion times may limit oxidation and/or the polyphenols found in MAN UMF10+ and FB honey are resistant to degradation. This again highlights the need to identify the polyphenols present in FB honey and oral digests. Other macromolecules such as DNA are also susceptible to oxidative damage which can lead to mutations, increasing the risk for cancer. Increased cancers associated with the oral cavity are squamous cell carcinomas, salivary gland carcinomas and mucosal melanomas (Montero and Patel, 2015). Therefore, initial evaluation of a protective effect can be undertaken to determine if these honeys and oral digests can protect plasmid DNA against AAPH-induced oxidative damage as described by Serem and Bester (2012).

Subsequently, these effects were determined in cellular models, which provide a more accurate indication. To be of therapeutic value, it was necessary to show that at the concentrations used to inhibit *C. albicans* biofilms, the honeys and oral digests did not adversely affect the growth of cells representative of cell types found in the oral cavity. Therefore, the effect of MAN UMF 10+ and FB honey on the cell number and viability of the mouse fibroblast (L929) and the human colon adenocarcinoma (Caco-2) cell lines was determined using the crystal violet (CV) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assays. At all concentrations evaluated, the honeys and the oral digests did not cause a change in cell number, viability or cell morphology. Additional methods such as electron microscopy can further be used to confirm the lack of cytotoxicity at these and higher concentrations.

The dichlorofluorescein diacetate (DCFH-DA) assay was used to determine whether the honeys and oral digests induced oxidative damage and/or protected L929 and Caco-2 cells against oxidative damage. With cellular antioxidant activity (CAA), minimal oxidative damage

was observed for all the honeys and oral digests, both protecting L929 and Caco-2 cells against the oxidative effects of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH). Honeys that showed the best effects were FB4 and MAN UMF10+, which are the darker honeys. Increased antioxidant activity has been previously reported for dark honeys (Alvarez-Suarez *et al.*, 2014). FB4 and MAN UMF10+ honeys were able to quench the effects of the radical by between 90% and 95%, respectively. For the OD samples, the % oxidative damage decreased when compared to the undigested honey samples.

The cellular pro-inflammatory and anti-inflammatory activities of FB and MAN UMF10+ were determined using the murine macrophage RAW 264.7/LPS cellular inflammation assay. The honey samples showed a pro-inflammatory activity especially for FB1 and FB3. However, FB2 honey did not induce NO production, while the effect of FB4 was only 5.23% compared with the control (RAW 264.7 cells exposed to lipopolysaccharide (LPS)), with levels normalised to 100%. Honeys that were exposed to LPS showed potential anti-inflammatory activity in the following order FB3 > FB1 > MAN UMF10+ > FB2 > FB4. The same effect was observed with the ODC honeys, while the OD honeys induced high NO levels with limited NO scavenging.

A limitation for the cytotoxicity and CAA assays was that the L929 and Caco-2 cell lines that were used are not derived from the oral cavity. Better cell lines that can be used are human tongue carcinoma cell lines CAL27 and CAL33 or primary cultures of oral epithelial and fibroblast cells.

The observed pro- and anti-inflammatory effects highlight the importance of identifying the molecules present in honey, then using *in vitro* assays and cell lines, the effects on other pro-inflammatory mediators such as interleukin (IL)-6 and IL-8 can be determined. Furthermore, using techniques such as western blotting, the effects on the expression of protein and enzymes of the NO pathway can be determined. Polyphenols can bind LPS, reducing LPS levels and the ability of LPS to induce NO. This mechanism can be evaluated by LPS binding assay using polymyxin B as described by Delehanty *et al.* (2007) for polyphenolic metabolites plant proanthocyanidins from cranberries, tea and grapes.

This anti-biofilm activity of UMF10+ and FB honey can be enhanced by synergism where honey in combination with known anti-fungal antibiotics or other natural anti-fungal products can enhance activity and can prevent the attachment of *C. albicans* to cell surfaces, prevent replication and biofilm formation and also eradicate established biofilms. Synergistic studies have been undertaken by Serpa *et al.* (2012) and have shown a loss in viability of *Candida* strains including *C. albicans* at a MIC_{50} of the flavonoid baicalein in combination with fluconazole evaluated with the microdilution assay and the checkerboard microdilution assay.

In conclusion, some FB honeys have comparable antioxidant and anti-inflammatory activities to MAN UMF10+, FB honey was able to inhibit the growth of biofilms and the same effect was observed with MAN UMF10+. With MAN UMF10+ being a medical-grade honey, and having observed similar results to FB honey, further studies should be done to ascertain that FB honey can be developed as a medical-grade honey. This study has determined that FB honey has the potential to become a therapeutic product that can be used in conjunction with other anti-fungal therapies, specifically for infections by *C. albicans* such as oral candidiasis. The next step in this process is to evaluate the effect of epithelial and fibroblast co-cultures infected with *C. albicans*. This will determine whether at concentrations that prevent biofilm growth, cellular antioxidant effects are observed. In addition, human isolated leukocytes can be used to evaluate the pro- or anti-inflammatory effects at these concentrations.

FB honey has shown potential to inhibit the growth of *C. albicans* biofilms while at the same time protecting cells of the oral cavity against oxidative damage while modulating inflammatory effects.

Chapter 7 : References

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Chapter 8 : Appendix

8.1 Ethics clearance

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

16/08/2018

Approval Certificate
New Application

Ethics Reference No: 431/2018

Title: The bioactivity of Fynbos honey compared with Manuka honey in a salt- and enzyme-based synthetic saliva.

Dear Anwani Wendy Nekhumbe

The **New Application** as supported by documents specified in your cover letter dated 18/07/2018 for your research received on the 18/07/2018, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 15/08/2018.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**431/2018**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

11 September 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 431/2018
Title: The bioactivity of Fynbos honey compared with Manuka honey in a salt- and enzyme-based synthetic saliva.

Dear Ms AW Nekhumbe

The **Annual Renewal** as supported by documents received between 2019-08-05 and 2019-09-11 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-09-11.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-09-11.
- Please remember to use your protocol number (431/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

18 September 2020

Approval Certificate
Annual Renewal

Ethics Reference No.: 431/2018
Title: The bioactivity of Fynbos honey compared with Manuka honey in a salt- and enzyme-based synthetic saliva.

Dear Ms AW Nekhumbe

The **Annual Renewal** as supported by documents received between 2020-08-28 and 2020-09-09 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2020-09-09 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2021-09-18.
- Please remember to use your protocol number (431/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
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