

**Identification and characterization of bioactivity of simulated
gastrointestinal digested indigenous southern African honey
samples**

by

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Abstract

Development of disease in the gastrointestinal tract is predominantly due to oxidative damage, infection and inflammation which if left unresolved lead to conditions such as inflammatory bowel disease. Functional foods can prevent/reduce the effects of oxidative damage, infection and inflammation. Honey is a well described functional food due to its several bioactivities. However, a functional food needs to be bioavailable and some bioactive molecules found in honey such as methylglyoxal (MGO) and polyphenols have been reported to have reduced activity following digestion. Therefore, the aim of this study was to determine the effects of digestion on the antioxidant, antibacterial and anti-inflammatory activities of southern Africa honey samples.

Manuka (control) and three honey samples from the southern African region, Agricultural Eucalyptus (AE), south eastern Mozambique (SEMh), Western Cape, Fynbos (WC) were subjected to simulated gastroduodenal digestion and the bioactivities of the undigested (UD), gastric digested (GD) and gastroduodenal (GDD) fractions were determined.

Total polyphenol content, Trolox equivalent antioxidant capacity and oxygen radical absorbance capacity were determined. Cellular antioxidant activity (CAA) using the human colon adenocarcinoma (Caco-2) cells and the dichlorofluorescein diacetate assay was also determined. A polyphenol mixture (PP) and a synthetic honey (sugars and polyphenols) were also digested and the antioxidant properties determined. Antibacterial effects against Gram-negative *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) and Gram-positive *Bacillus subtilis* (*B. subtilis*) and *Staphylococcus aureus* (*S. aureus*) bacteria were then evaluated using the microbroth dilution, minimum inhibitory concentration (MIC) assay. The contribution of sugars, MGO, H₂O₂, polyphenols and cationic peptides to antibacterial activity was also evaluated. Cytotoxicity against the mouse fibroblast (L929) cells, inhibition of nitric oxide (NO) formation, inhibition of *E. coli* induced NO levels in L929 cells and inhibition of LPS induced activation of human platelets was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide assay, sodium nitroprusside assay and scanning electron microscopy, respectively.

All honeys had antioxidant activity similar to Manuka honey. With GD the antioxidant properties of all honey fractions was unchanged. Following GDD, antioxidant activity was either unchanged or increased. In contrast, for CAA a strong pro-oxidant effect was observed. Using PP mixtures, findings were that this effect was not due to pH dependent polyphenol degradation associated with an increase in hydrogen peroxide (H₂O₂) levels, but possibly due

to the presence of β -Carotene, which in the presence of O_2 forms the carotenoid peroxy radical.

All honeys had MIC's between 25 – 30% (v/v) against all tested bacteria, with the exception of MANc 6.25% (v/v) against *S. aureus* and AE 40% (v/v) against *B. subtilis*. With GD, the antibacterial activity, was unchanged. With GDD the antibacterial activity of MANc was retained. The activity of AE, SEMh and WC was unchanged against *P. aeruginosa* and *S. aureus* but was reduced against *E. coli* and *B. subtilis*. The MIC of MGO was 0.8 - 1.2 mM against all bacteria while the MIC for H_2O_2 was 9 mM for all bacteria except *B. subtilis* (90 mM). Honey sugars did not achieve MIC. Therefore, the activity of AE, SEMh and WC was concluded not to be due to MGO or H_2O_2 as higher levels than what is present in honey were needed to achieve MIC, but due to sugars in combination with an unidentified component.

At honey concentrations that did not lead to cytotoxicity (<3% v/v), NO formation was reduced by 50%, 62%, 63% and 50% for MAN UMF10+, AE, SEMh and WC, respectively. *E. coli* induced NO formation in L929 cells and platelet activation was inhibited to varying degrees, with AE and SEMh honeys being the most effective.

In conclusion, at physiologically relevant levels, the beneficial effects of honey in a gastric environment was the reduction of oxidative damage and inflammation while in a gastroduodenal environment the predominant effect was anti-inflammatory.

Keywords: Antibacterial, anti-inflammatory, antioxidant, bioactive, digestion, functional food, gastroduodenal, Manuka honey, oxidative damage, polyphenol.

Publications in the field and conferences attended

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Declaration

I, June Serem hereby declare that this research dissertation is my own work, has not been presented for any degree of another University and has been checked for plagiarism;

Signed:

A handwritten signature in black ink, appearing to be 'June Serem', written in a cursive style.

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List of abbreviations and chemical formulae

%	percentage
°C	degrees Celsius
µm	micro meter
µL	micro litre
µmol TE/g	micromolar Trolox equivalent/gram
µmol TE/mL	micromolar Trolox equivalent/millilitre
µM	micro molar
<u>A</u>	
a_w	low water activity
A	antagonistic
A-549	human lung adenocarcinoma cell line
AA	arachidonic acid
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt
ABTS ^{•+}	ABTS radical
ACE	angiotensin I converting enzyme
AD	after death
AE	agricultural Eucalyptus
AGE.	advanced glycation end products
AlCl ₃	aluminium chloride
AMP	antimicrobial peptide
ANOVA	analysis of variance
ATCC	American Type Culture Collection
AUC	area under curve
<u>B</u>	
BC	before Christ
BCA	bicinchoninic acid
<i>B. cereus</i>	<i>Bacillus cereus</i>
BD-1	bee defensin 1
BSA	bovine serum albumin
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
<u>C</u>	
CA	caffeic acid
C-A	co- additive
CAA	cellular antioxidant activity
Caco-2	human colon adenocarcinoma cell line
CAPE	caffeic acid phenethyl ester
CAT	catechin
CD	Crohn's disease

CD14	human gene – cluster of differentiation 14
CECT	Spanish Type Culture Collection
CFU	colony forming unit
cm ²	centimeter squared
Co	cobalt
CO ₂	carbon dioxide
COX	cyclooxygenase
CuSO ₄ ·5H ₂ O	copper sulphate pentahydrate
<u>D</u>	
DCFH-DA	dichlorofluorescein diacetate
ddH ₂ O	double distilled water
dddH ₂ O	double distilled deionised water
DLD-1	human colon carcinoma cell line
DMEM	Dulbecco's Modified Essential Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
<u>E</u>	
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
EGCG	epigallocatechin
ELISA	enzyme linked immunosorbent assay
Em	Emission wavelength
EPFKISIHLL	amino acid sequence of jellein 3
EtOH	ethanol
EU	endotoxin units
Ex ₄₈₅	excitation wavelength
<u>F</u>	
F-C	Folin-Ciocalteu's
FCS	foetal calf serum
Fe	iron
FeSO ₄ ·(NH ₄) ₂ SO ₄ ·6H ₂ O	ammonium ferrous sulphate
FL	fluorescence
FOX	ferrous ion oxidation xylenol orange
Fru	fructose
<u>G</u>	
g	gram
G100	growth control (100% growth)
GA	gallic acid
GAE/g	gallic acid equivalent/gram
GAE/mL	gallic acid equivalent/milliliter

GD	gastric digested
GDD	gastroduodenal digested
GIT	gastrointestinal tract
Glc	glucose
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione- S-transferases
H	
h	hours
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
H ₂ SO ₄	sulphuric acid
HCl	hydrochloric acid
HEAE	honey extracted in ethyl acetate
HeLa	human cervical cancer cell line
HepG2	human hepatoma cell line
HME	honey extracted in methanol
HORAC	hydroxyl radical averting capacity
HO•	hydroxyl radical
<i>H. pylori</i>	<i>Helicobacter pylori</i>
HUVECs	human umbilical vein endothelial cells
I	
IBD	inflammatory bowel disease
IL-1	Interleukin 1 (Pro-inflammatory)
IL-1 β	Interleukin 1 Beta (Pro-inflammatory)
IL-6	Interleukin 6 (Pro-inflammatory)
IL-8	Interleukin 8 (Pro-inflammatory)
IL-10	Interleukin 10 (Anti-inflammatory)
iNOS	inducible nitric oxide
K	
kDa	kilo dalton
K ₂ S ₂ O ₈	potassium peroxodisulfate
L	
L	litre
L929	mouse fibroblast cell line
LAL	limulus ameobocyte lysate
LB	Luria Bertani
LBP	LPS-binding protein

LC-MS	liquid chromatography-mass spectrometry
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
LOX	lipoxygenase
LPS	lipopolysaccharide
M	
mM	millimolar
mL	millimeter
mg/kg	milligram/kilogram
mg/g	milligram/gram
M	metal
MAL	maltose
MANc	Manuka control honey
MAN UMF10+	Manuka UMF 10+ honey
MAPK	mitogen activated protein kinase
MCF-7	human breast cancer cell line
MD-2	myeloid differentiation factor 2 (MD-2)
MDA-MB-231	human breast cancer cell line
MGO	methylglyoxal
MIC	minimum concentration of a compound at which bacteria is inhibited/killed
Min	minutes
MRJP	major royal jelly protein
MRP	Maillard reaction product
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide) assay
MUC-2	mucin
N	
nm	nanometer
Na ₂ CO ₃	sodium carbonate anhydrous
Na ₂ CO ₃ ·H ₂ O	sodium carbonate monohydrate
NaCl	sodium chloride
NaH ₂ PO ₄	sodium phosphate
NaHCO ₃	sodium bicarbonate
Na ₂ HPO ₄ ·2H ₂ O	sodium phosphate dibasic dihydrate
NaOH	sodium hydroxide
NED	N-(1-naphthyl)ethylenediamine
NF- κβ	nuclear transcription factor kappa beta
NO	nitric oxide
NO ₂ ⁻	nitrite

NO ₃ ⁻	nitrate
NOS	nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
<u>O</u>	
O ₂	oxygen
O ₂ ^{•-}	superoxide anion
OD	optical density
ORAC	oxygen radical absorbance capacity
<u>P</u>	
p	probability value
ppm	parts per million
pH	acidity or basicity of an aqueous solution
PAL	phenylalanine ammonia-lyase
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	photo diode array
PFKISHL	amino acid sequence of jellein 1
PGE ₂	prostaglandin E ₂
PGI ₂	prostacyclin
PMN	polymorphonuclear cells
PP	polyphenol mix
PRR	pattern recognition receptors
<u>Q</u>	
QUE	quercetin
qPCR	quantitative polymerase chain reaction
<u>R</u>	
rpm	revolutions per minute
R ²	coefficient of determination
RAW 264.7	mouse macrophage cell line
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RS	Revamil source
<u>S</u>	
S	synergism
SA	sugar analogue
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SEM	standard error of mean

SEMh	south eastern Mozambique
<i>S. epidermidis</i>	<i>Staphylococcus epidermidis</i>
SH	synthetic honey
SHIME	simulated human intestinal microbial ecosystem
SIRS	systemic inflammatory response syndrome
<i>S. mutans</i>	<i>Streptococcus mutans</i>
SNP	sodium nitroprusside
SOD	superoxide dismutase
SPS	polyanetholesulfonic acid sodium salt
<i>S. pyogenes</i>	<i>Streptococcus pyogenes</i>
<i>S. sonnei</i>	<i>Shigella sonnei</i>
STAT	signal transducer and activator transcription
SUC	sucrose
<u>I</u>	
T0	time at 0 hours
T24	time at 24 hours
TEAC	Trolox equivalent antioxidant capacity
TIM	gastrointestinal model
TLR	toll-like receptor
TNF- α	tumour necrosis factor alpha
TPC	total polyphenol content
TPFKISIH	amino acid sequence of jellein 4
TPFKISIHL	amino acid sequence of jellein 2
T-TEST	students T-TEST
<u>U</u>	
UC	ulcerative colitis
UD	undigested
UMF	unique Manuka Factor
UPLC	ultraperformance liquid chromatography
<u>V</u>	
v/v	volume to volume
vitamin C	ascorbic acid
vitamin E	α -tocopherol
VRE	vancomycin resistant <i>Enterococcus</i>
<u>W</u>	
w/v	
WB	whole blood
WC	Western Cape, Fynbos
WS-1	human fibroblast cell line
<u>X</u>	

xg

Z

Zn

centrifugal force

zinc

Chapter 1: Introduction

The holy Qur'an *'Thy Lord has inspired the Bees, to build their hives in hills, on trees and in man's habitations, From within their bodies comes a drink of varying colours, wherein is healing for mankind, Verily in this is a Sign, for those who give thought'*.

St Ambrose *'The fruit of the Bees is desired of all, and is equally sweet to kings and beggars and it is not only pleasing but profitable and healthful, it sweetens their mouths, cures their wounds and conveys remedies to inward ulcers'*.

Honey is a sweet natural substance and its efficacy for the treatment of ailments has been recorded since earliest of times. In 5000 BC the Egyptians used honey for wound management and the Greeks, Chinese and Romans used honey as an antiseptic for sores and ulcers (Kassim *et al.*, 2012). Sumeria 2100 – 2000 BC writings identified honey as a drug or ointment and Aristotle 384 – 322 BC recorded that pale honey was useful as an eye and wound ointment (Mandal, Mandal 2011). Celsus, 25 AD recommended the use of honey as a therapy against diarrhoea (Bogdanov *et al.*, 2008) while Galen 190 AD recommended the use of oxymel (a mixture of vinegar and honey) to cure epilepsy (Eadie 2004). Not only medical but also holy texts identify honey as a remedy where both the Bible and the Holy Hadith recommend the use of honey as a treatment for a variety of ailments (Bogdanov *et al.*, 2008).

Although some of the suggested remedies are not proven such as honey as an antiepileptic agent (Eadie 2004), from scientific studies it can be seen that honey promotes the healing of wounds and ulcers as well as stomach ailments such as diarrhoea due its antibacterial (Weston, *et al.*, 1999, Molan 2001, Brady *et al.*, 2004, Wilkinson, Cavanagh 2005, Kwakman, Zaat 2012, Anthimidou, Mossialos 2013, Zainol *et al.*, 2013) and its anti-inflammatory activity (Kassim *et al.*, 2010, Alzubier, Okechukwu 2011, Bashkaran *et al.*, 2011, Ahmad *et al.*, 2012, Kassim *et al.*, 2012, Aziz *et al.*, 2014, Almasaudi *et al.*, 2017). This has led to the field of 'Apitherapy' where diseases/conditions are treated with bee related products, with honey being the most common product.

Literature states that honey of different floral and geographical origins (Martos *et al.*, 1997, Beretta *et al.*, 2005, Blasa *et al.*, 2006, Fiorani *et al.*, 2006, Bertoncej *et al.*, 2007, Joseph *et al.*, 2007, Estevinho *et al.*, 2008, Rasmussen *et al.*, 2008, Al *et al.*, 2009, Kaškonienė *et al.*, 2009, Makawi *et al.*, 2009, Pyrzyńska, Biesaga 2009, Truchado *et al.*, 2009, Alvarez-Suarez *et al.*, 2010a, Alvarez-Suarez *et al.*, 2010b, Saxena *et al.*, 2010) have antioxidant, anti-inflammatory, anti-tumour, anti-fungal and antibacterial properties. Therefore as a

multifunctional therapeutic product these honeys not only reduce infection, but also reduce the levels of reactive oxygen species (ROS), inflammation and promote wound healing.

Several honey types such as New Zealand Manuka honey and Revamil source (RS) honey have been developed to clinically treat wounds. This therapeutic application is based on the antibacterial activity of these honeys which is due to the specific presence of methylglyoxal (MGO) in Manuka honey and hydrogen peroxide (H₂O₂) and antimicrobial peptides such as bee defensin-1 (BD-1) in RS honey (Weston *et al.*, 1999, Molan 2001, Brady *et al.*, 2004, Wilkinson, Cavanagh 2005, Kwakman, Zaat 2012, Anthimidou, Mossialos 2013, Zainol *et al.*, 2013).

Like New Zealand, the southern African region is has unique floral biodiversity and biospheres. In addition two sub-species of honey bees are found in these region. These are *Apis mellifera capensis* and *Apis mellifera scutella* more commonly known as the Cape and the African bee, respectively. The Cape bee is found in the Fynbos region of the Western Cape while the African bee is found north of this region. The antioxidant (Serem, Bester 2012) and the antibacterial activity (Basson, Grobler 2008, Manyi-Loh *et al.*, 2010) of a selection of honey from the southern African region has been determined. Little is known if the unique floral source and bee species from this region will translate into honey with unique therapeutic properties as has been found for Manuka honey.

Based on human and animal based studies, honey protects against bacterial, *Helicobacter pylori* (*H. pylori*) (Kim 2005, Manyi-Loh *et al.*, 2010) and drug, indomethacin, alcohol, ammonia and aspirin (Bogdanov *et al.*, 2008) induced gastritis. From these studies, honey is identified as a functional food product that can prevent or reduce infection and inflammation of the gastric mucosa. Information on the effect of gastroduodenal digestion on bioactivity is largely unknown especially related to the development of colon cancer and prevention or treatment of inflammatory bowel disease (IBD).

The ability of flavonoids to reduce ROS, reactive nitrogen species (RNS) levels, inflammation and improve epithelium barrier function (Veza *et al.*, 2016), makes honey, if activity is retained following digestion, an attractive functional food for the prevention and alleviation of inflammation associated with IBD. In addition, honey contains sugars such as fructose (Fru) that has a prebiotic effect (Bogdanov *et al.*, 2008).

For honey to have beneficial effects on the mucosa of the gastrointestinal tract (GIT), bioactivity should not be altered following digestion. Only a single study could be found investigating the

effects of digestion on the antioxidant activity of honey O'Sullivan *et al.*, (2013) while to our knowledge no studies could be found on the effects of digestion on the antibacterial and anti-inflammatory activity of honey. Especially related to the antibacterial activity of honey, Daglia *et al.*, (2013) reported that MGO binds digestive enzymes, antimicrobial peptides can undergo proteolytic degradation, and flavonoids that have anti-inflammatory activity can undergo neutral pH degradation (Bermúdez-Soto *et al.*, 2007).

The contribution that this thesis aims to make is to address this gap in knowledge that although claims are made that honey has beneficial effects, with digestion polyphenols present in honey can degrade adversely affecting bioavailability. Therefore the effects of GIT digestion on the antioxidant, antibacterial and anti-inflammatory activity of honey was undertaken. To achieve this and to identify if honey from the southern African region has bioactivity comparable to Manuka honey (a well-known medical grade honey) the bioactivity of undigested and digested southern African honey was compared to Manuka honey. Possible modes of action related to observed antioxidant, antibacterial and anti-inflammatory effects were explored.

Chapter 2: Literature review

2.1 Honey as a functional food

Functional foods are nutritional foods or products that in addition to having nutritional function also contain molecules that can prevent disease. For example the Mediterranean diet, which is rich in fruit, vegetables, olive oil as well as nuts and seeds, has shown to reduce the risk of diseases of lifestyle and this has been shown to be due to high levels of bioactive molecules. Characteristics that may qualify a food as a functional food include the presence of dietary fibres, vitamins, minerals, antioxidants, oligosaccharides, essential fatty acids, lactic acid bacteria cultures and lignins. Some well-known functional foods include those that contain carotenoids (most fruit and vegetables), dietary fibre (wheat bran, oats, and barley), fatty acids (salmon and fish oils), phenolic compounds (fruits, vegetables and tea) and those containing prebiotics/probiotics and bioactive peptides (yoghurt and other dairy products) (Shahidi 2004).

Nutraceuticals are defined as products isolated from food and generally sold in medicinal form (Romano *et al.*, 2012) and have been shown to or believed to contribute to mental and physical well-being as well as preventing or reducing the risk factors of chronic diseases of lifestyle. Scientific endeavour focuses on the identification of foods with functional properties that can be developed into new nutraceuticals. Alternatively these functional foods are a source of phytochemicals with unique structure and biochemical properties, which can act as lead compounds for the development of therapeutic drugs.

Honey which has been identified as a functional food and a nutraceutical product is derived from the nectar of plants and its composition is influenced by plant species, climate, environmental conditions and contribution of the beekeeper. Bee products such as royal jelly, propolis and honeys with high levels of specific bioactive molecules can be classified as nutraceutical products. Honey consists of 70% saccharides, mainly Fru and glucose (Glc) (Finola *et al.*, 2007), 10% water, organic acids, mineral salts [the most abundant being potassium (40 – 3500 mg/100g)] (Bogdanov *et al.*, 2008), vitamins (such as vitamin B6, thiamine, niacin riboflavin, pantothenic acid and β -Carotene) (da Silva *et al.*, 2016) proteins and polyphenols.

Honey has free amino acids with proline being the predominant amino acid (Ouchemoukh *et al.*, 2007). The enzymes found in honey are invertase (α -glucosidase/saccharase), amylase, glucose oxidase and in lower quantities catalase and acid phosphatase. Invertase, which makes up 50% of the enzymes, converts the sucrose in honey to its monosaccharide components (Glc and Fru). Amylase produced by the salivary glands of the bee, hydrolyses

plant starch and is a minor 2% component of honey. Glucose oxidase present at the same quantities as amylase forms H_2O_2 (a potent antimicrobial) by oxidizing Glc to gluconic acid and H_2O_2 (Babacan, Rand 2005, Kunieda *et al.*, 2006, Aurongzeb, Azim 2011). In contrast, catalase found in honey is an enzymatic antioxidant which converts H_2O_2 to H_2O (Vertuani *et al.*, 2004). Both enzymes ensure a dynamic equilibrium in honey between H_2O_2 formation and enzymatic degradation of H_2O_2 . Peptides contributing to the antimicrobial activity include BD-1 (Aurongzeb, Azim 2011) found in honey and royalsins present in both royal jelly and honey (Kwakman, Zaat 2012).

2.2 Medicinal properties of honey

Honey has been used in ethno medicine since early times for the treatment of burns, GIT disorders, asthma, infected wounds and skin ulcers and these beneficial effects are due to the antioxidant, antimicrobial, anti-inflammatory, anti-allergic, antioxidant, vascular and cytotoxic anti-tumour properties of honey (Beretta *et al.*, 2005, Blasa *et al.*, 2007, Ouchemoukh *et al.*, 2007).

The major components that contribute to the antioxidant activity of honey are the polyphenols and include the flavonoids (chrysin, pinocembrin, quercetin, kaempferol, luteolin, and myricetin) and phenolic acids (caffeic, ferulic, benzoic and gallic acid) (Estevinho *et al.*, 2008, Kaškonienė *et al.*, 2009, Pyrzynska, Biesaga 2009, Truchado *et al.*, 2009). Non-nutrient antioxidants are formed as products of the Maillard reaction when sugars condense with free amino acids, leading to the formation of a variety of brown pigments, the Maillard reaction pigments (MRPs) (Turkmen *et al.*, 2006). In food, MGO is also formed via the Maillard reaction, which involves the degradation of glycated proteins and monosaccharides. The antibacterial activity of Manuka honey is due to the high levels of MGO found in the nectar of the *Leptospermum scoparium* flower (Mavric *et al.*, 2008).

2.3. Antioxidant molecules and cellular antioxidant pathways

Antioxidants are defined as molecules, enzymes and peptides that prevent oxygen consumption or that are readily oxidized. In a cellular system, the definition can be summarized as compounds that prevent oxidative reactions by scavenging ROS before cellular and tissue damage can occur (Lobo *et al.*, 2010). Using these definitions an antioxidant can be characterized as a substance that is a hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor and/or a metal chelating agent.

The activity of antioxidants can be divided into three lines of defence (Table 2.1). The first line of defence (primary defence), mostly constitutes enzymatic antioxidants which suppress the formation of free radicals. The second line of defence (secondary defence), consists mostly of non-enzymatic antioxidants which suppress chain initiation or break chain propagation. The final and third line of defence (tertiary defence), consists mostly of proteolytic enzymes and proteinases in the cytosol and mitochondria, which are the repair systems (Lobo *et al.*, 2010). The tripeptide, glutathione (GSH) (Table 2.1) is involved in both primary and secondary defence. GSH consists of cysteine, glycine and glutamic acid. The cysteine residue, which contains a thiol group, is the reducing agent and can be reversibly oxidized and reduced. GSH plays an important role in reducing free radicals and becomes oxidized GSSG and reduced back to GSH. The GSH:GSSG ratio is an indicator of oxidative stress in mammalian cells where under normal, conditions GSH is the predominant species. Associated with the GSH system are the antioxidant enzymes: GSH reductase (GR), GSH peroxidase (GPx) and GSH-S-transferases (GST), each performing specific functions. GR ensures correct disulfide bond formation in many proteins and participates in the metabolism of xenobiotics. GPx oxidises GSH and catalyses the conversion of H₂O₂/organic peroxide to ethanol (EtOH) or water. Finally, GST is involved in the cellular defence against xenobiotics and carcinogens (Rahman 2007).

Other antioxidant enzymes of the primary defence system are superoxide dismutase (SOD) which breaks down the superoxide anion and catalase, which breaks down H₂O₂ (Lobo *et al.*, 2010). Catalase is an enzyme that forms part of the primary defences (with SOD and the glutathione systems), by quenching oxidants such as H₂O₂ (Table 2.1). Found in the peroxisomes of cells, catalase catalyses the conversion of two molecules of H₂O₂ to two molecules of H₂O and one of O₂. The decomposition of H₂O₂ to H₂O and O₂ is important, as in the presence of transition metals e.g. iron, the formation of the hydroxyl (HO•), a more reactive radical than H₂O₂, is formed (Michiels *et al.*, 1994). Absence of these enzymes results in the accumulation of radicals, which lead to diseases associated with ROS and these include GIT inflammatory disorders, cancers as well as neurodegenerative diseases such as Alzheimer's disease (Rahman 2007). High levels of these enzymes are found in the cell membrane of erythrocytes as well as the endothelium of the cardiovascular system for protection against oxidative damage (Rahman 2007).

Table 2.1: Summary of antioxidants and defence mechanisms that prevent oxidative stress (Noguchi *et al.*, 2000, Simon *et al.*, 2000).

<u>PRIMARY DEFENCE</u>	<u>SECONDARY DEFENCE</u>	<u>TERTIARY DEFENCE</u>
<p>(Preventative antioxidants) Suppress formation of free radicals</p> <p>1.1. Glutathione (GSH) systems: Function as detoxification metabolism and break down H₂O₂ and organic hydroperoxides</p> <p>e.g. (GSH, GSH reductase, GSH peroxidase, GSH S-transferases)</p> <p>1.2. Superoxide dismutase (SOD): Catalyze breakdown of superoxide anion $2O_2^{\bullet -} \longrightarrow H_2O_2 + O_2$ e.g. (Mn-SOD – mitochondria & peroxisomes Fe-SOD – peroxisomes Cu,Zn-SOD – cytosol & peroxisomes)</p> <p>1.3. Catalase: Breaks down H₂O₂</p> $2H_2O_2 \longrightarrow 2H_2O + O_2$	<p>(Scavenging antioxidants) Suppress chain initiation/break chain propagation</p> <p>2.1. Ascorbic acid (vitamin C, (Vit C)): Reducing agent, therefore can neutralize ROS e.g. H₂O₂ generated</p> <p>Hydrophilic dietary compounds maintained in reduced form by GSH</p> $Vit\ C\ (red) + H_2O_2 \xrightarrow[\text{isomerase}]{\text{Protein disulfide}} Vit\ C\ (ox) + H_2O + O_2$ <p>2.2. Tocopherol (vitamin E): Lipophilic compound that prevents lipid peroxidation $\alpha\text{-tocopherol (red)} + LOOH\ (lipid\ radicals) \longrightarrow L + H_2O + \alpha\text{-tocopherol (ox)}$</p> $\alpha\text{-tocopherol (ox)} \xrightarrow{\text{Ascorbate/thiol/ubiquinol}} \alpha\text{-tocopherol (red)}$ <p>2.3. GSH: Reversibly oxidized and reduced. Maintained in reduced form by GSH reductase</p> $GSH + oxidant \xleftarrow{\text{GSH reductase}} GSH\ (red) + oxidant\ (red)$	<p>(Repair/<i>de novo</i> antioxidants) Repair antioxidant systems</p> <p>3.1. Enzymes: Proteolytic enzymes</p> <p>3.2. DNA repair systems: Glycosylases and nucleases</p>

Cu,Zn-SOD – copper, zinc superoxide dismutase
 DNA – deoxyribonucleic acid
 Fe-SOD – iron superoxide dismutase
 Mn-SOD – manganese superoxide dismutase
 ox – oxidation
 red – reduction

Secondary defence is made up of scavenging non-enzymatic essential vitamin antioxidants such as vitamin C (ascorbic acid) and vitamin E (α -tocopherol). Vitamin C is a hydrophilic, reducing agent and therefore can neutralize ROS such as the hydroxyl radical generated from H_2O_2 (Table 2.1). Vitamin E is lipophilic, highly bioavailable and is a reducing agent that reduces lipid radicals, becoming oxidized forming a tocopheryl/tocopheroxyl radical, which is then reduced by vitamin C to its reduced form (Table 2.1) (Buettner 1993, Lobo *et al.*, 2010).

Tertiary defence is made up of repair antioxidants, such as proteolytic, glycosylase and nuclease enzymes, which repair damage to proteins and DNA caused by free radicals (Lobo *et al.*, 2010). These enzymes play an important role as unquenched ROS could cause the carbonylation of proteins as well as amino acid modifications. These modifications include methionine sulfoxide reduction and the formation of protein carbonyls, which can alter signal transduction mechanisms, affect enzyme activity, heat stability, and susceptibility to proteolysis which in turn lead to disease as well as accelerated aging (Lobo *et al.*, 2010).

A further mode of defence, not listed is an endogenous source of phytochemicals, such as the Mediterranean diet which has high levels of bioactive phytochemicals (Visioli *et al.*, 2004). These phytochemicals include vitamins and polyphenols that prevent the formation of free radicals, scavenge free radicals produced by oxidative stress and in some instances repair the effects of oxidative stress (Table 2.1) (Noguchi, *et al.*, 2000, Simon *et al.*, 2000).

2.3.1 Polyphenols as antioxidants

Dietary non-enzymatic antioxidants besides vitamin C and E include polyphenols comprising of flavonoids and phenolic acids. Polyphenols have been shown to be effective ROS scavengers and have greater antioxidant properties *in vitro* than tocopherols and ascorbate. Polyphenols can effectively scavenge $O_2^{\bullet-}$, H_2O_2 and HO^{\bullet} . Their antioxidant activity arises from their high reactivity as hydrogen electron donors, their ability to stabilize and delocalize the unpaired electron (chain breaking function) as well as their ability to chelate transition metal ions (termination of the Fenton reaction) (Vertuani *et al.*, 2004).

Polyphenols are products of secondary metabolism that occurs in plants (Pyrzynska, Biesaga 2009). Primary metabolism results in the formation of carbohydrates and protein metabolites that are important cellular constituents. After the formation of metabolites from primary metabolism, secondary metabolism begins with phenylalanine (produced in the shikimate pathway) which forms when simple carbohydrates are first converted to erythrose-4-phosphate then to phosphoenolpyruvate through the pentose phosphate and glycolysis pathways, respectively (Dixon, Paiva 1995, Ewané *et al.*, 2012). Phenylalanine links primary metabolism

(shikimate pathway) to secondary (phenylpropanoid) metabolism. The classes of phenylpropanoids that arise from phenylalanine are summarised below (Figure 2.1).

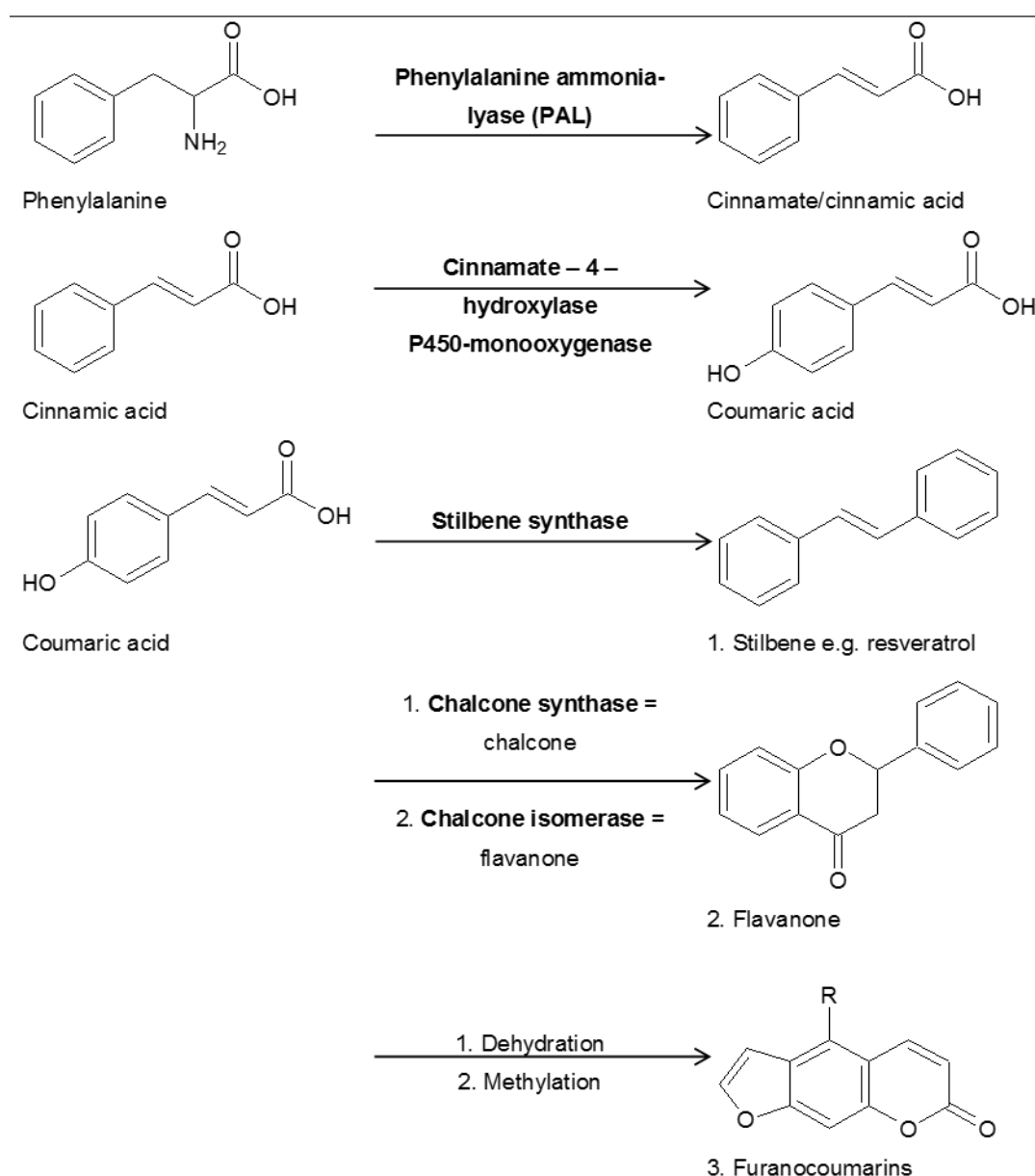


Figure 2.1: Secondary metabolism in plants that results in stilbenes, flavanones and furanocoumarins formation (Dixon, Paiva 1995, Daniel *et al.*, 1999).

Of the phenylpropanoids, flavonoids are the most widely occurring phenolic phytochemical found in plant-based foods. These arise from chalcones (a product of phenylalanine, Figure 2.1) and results in flavonoid classes including, flavanones, flavones, isoflavonoids, flavonols, dihydroflavonols and anthocyanidins (Figure 2.2) (Koes *et al.*, 1994). Flavonoid classes are made up of three benzene rings, two aromatic and one heterocyclic, with the aromatic rings labelled A and B and the heterocyclic ring labelled C (Figure 2.2). Classification is according to the oxidation of the C ring (Rice-Evans 2001). In their natural dietary form most of these

flavonoids are glycosylated except the catechins. Flavonoids that have been reported to have high antioxidant activity include catechin, taxifolin, luteolin and quercetin. This is attributed to their hydroxylation patterns, and the presence of a double bond between C2 and C3 (Figure 2.2) (Vertuani *et al.*, 2004, Lago *et al.*, 2014).

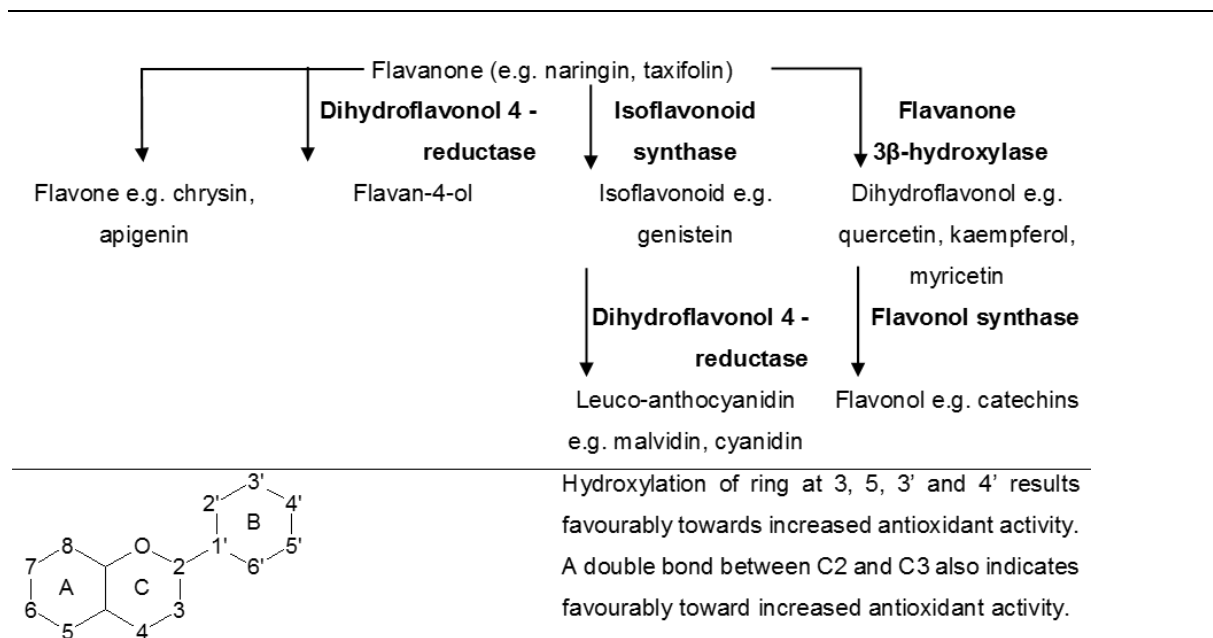
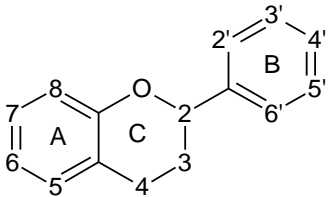
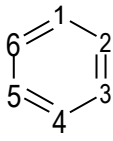
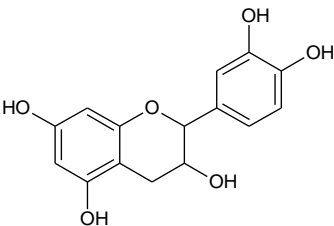
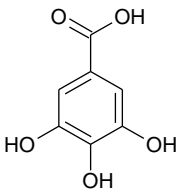
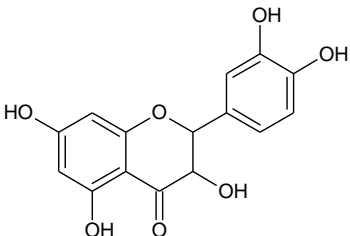
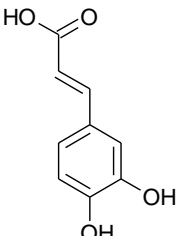
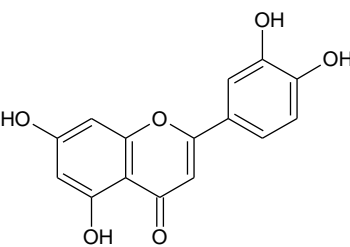
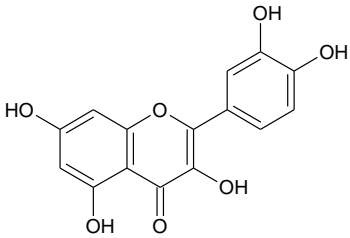


Figure 2.2: Formation of flavonoids from chalcone a product of the shikimate pathway (Koes *et al.*, 1994).

Of the flavonoids studied, catechin, taxifolin, luteolin and quercetin (in decreasing order of activity) have been found to have high antioxidant activity (Wolfe, Liu 2008). Although the basic structure of these flavonoids is different, their hydroxylation pattern of 3, 5, 7, 3', 4' – OH is similar (Table 2.2) and this is associated with high antioxidant capacity (Vertuani *et al.*, 2004).

Table 2.2: Structure and hydroxylation patterns of selected flavonoids and phenolic acids (Sroka, Cisowski 2003, Wolfe, Liu 2008).

Flavonoids	Phenolic acids
	
<p>Catechin</p> 	<p>Gallic acid</p> 
<p>Taxifolin</p> 	<p>Caffeic acid</p> 
<p>Luteolin</p> 	
<p>Quercetin</p> 	

Another major group of phenylpropanoids are the phenolic acids, which are derived from the shikimate pathway from phenylalanine via cinnamic acid, the cinnamic acid derivatives, e.g. caffeic acid (Table 2.2) and the benzoic acid derivatives (Figure 2.3). Phenolic acids similar to flavonoids have structural features that contribute to antioxidant activity (Mandal *et al.*, 2010, Ewané *et al.*, 2012,).

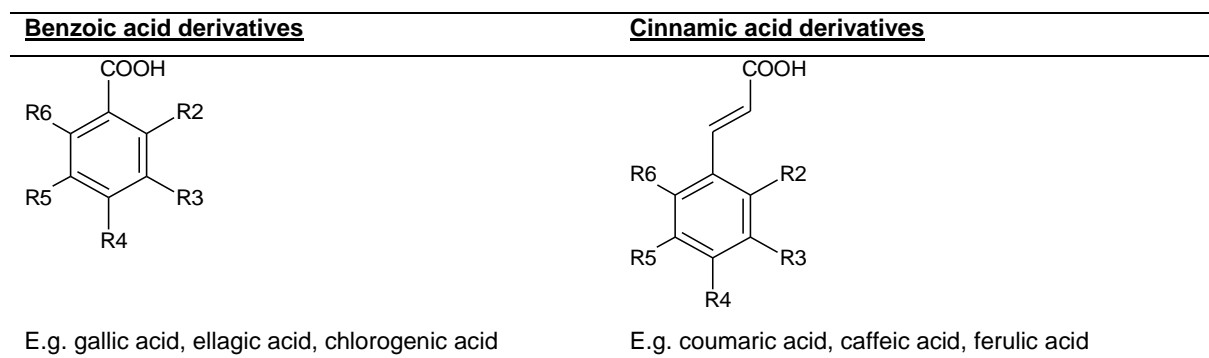


Figure 2.3: Phenolic acid backbone and associated phenolic acid derivatives.

The factors that determine increased antioxidant activity of phenolic acids, as reported by Rice-Evans *et al.*, (1996), are not only the number and position of hydroxyl groups (Sroka, Cisowski 2003) but also the presence of a carboxylate. Specifically the ortho (o-) (position 1,2) and para (p-) (position 1,4) hydroxyl configurations determines antioxidant activity. Gallic acid, which is both carboxylated and has 3 hydroxyl groups was reported to have the highest activity, closely followed by pyrogallol, which though not carboxylated also has 3 hydroxyl groups, when compared to 3-hydrobenzoic and 2-hydrobenzoic acid which though carboxylated have each only one hydroxyl group. Compared to caffeic acid, gallic acid had stronger activity, and this was due to the presence of an extra hydroxyl group and possibly due to the fact that caffeic acid is acetylated on its first carbon compared to gallic acid, which is carboxylated.

2.3.2 Antioxidant properties of honey

The polyphenol composition of honey is directly linked to the botanical source of the nectar. The antioxidant activity of honey is due to the presence of non-enzymatic (polyphenols) and enzymatic (catalase) antioxidants. Catalase in honey originates from the plant from where the honey bee collects its nectar. In honey, the main function of catalase is to regulate the enzyme glucose oxidase which converts glucose to gluconic acid and H_2O_2 in the presence of H_2O (Jeffrey, Echazarreta 1996). Catalase breaks down the formed H_2O_2 to H_2O and O_2 . Catalase in honey does not contribute significantly to its antioxidant activity compared to the non-enzymatic antioxidants. Furthermore heat processing and/or digestion can destroy the activity of catalase and glucose oxidase (Bucekova *et al.*, 2018).

The antioxidant content and activity of honey from many regions of the world have been determined and this includes honey from Algeria (Ouchemoukh *et al.*, 2007), Burkina Faso (Beretta *et al.*, 2005, Meda *et al.*, 2005), Sudan (Makawi *et al.*, 2009), Tunisia (Martos *et al.*, 1997), Cameroon (Joseph *et al.*, 2007), Morocco (Malika *et al.*, 2005), Italy (Beretta *et al.*, 2005, Blasa *et al.*, 2006, Fiorani *et al.*, 2006), Romania (Al *et al.*, 2009), Slovenia (Bertoncelj

et al., 2007), India (Saxena *et al.*, 2010), Cuba (Alvarez-Suarez *et al.*, 2010a, Alvarez-Suarez *et al.*, 2010b) and USA (Rasmussen *et al.*, 2008). The structures of the most commonly identified in honey (Table 2.3) and specifically for Manuka *Leptospermum* honeys, the polyphenol combination has been identified to be: Myricetin, tricetin, quercetin, luteolin, kaempferol, kaempferol 8-methyl ether, pinocembrin, chrysin, gallic acid, ellagic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid and syringic acid (Pyrzynska, Biesaga 2009).

Table 2.3: Summary of polyphenols found in some honeys from different regions of the world.

<u>Sources</u>	<u>Flavonoid</u>	<u>Phenolic acids</u>	<u>Reference/s</u>
<u>Europe</u>			
North Portugal	Naringenin, kaempferol, apigenin, pinocembrin, chrysin	Protocatechuic acid, p-hydroxybenzoic acid, caffeic acid, chlorogenic acid, vanillic acid, p-coumaric acid, benzoic acid, ellagic acid cinnamic acid	Estevinho <i>et al.</i> , 2008
Italy (Raw)	Luteolin, quercetin, 8-methoxykaempferol, apigenin, fisetin, kaempferol, isorhamnetin, acacetin, tamarixetin, chrysin, galangin		Fiorani <i>et al.</i> , 2006
<u>Australasia</u>			
Australia/ New Zealand	Myricetin, tricetin, quercetin, luteolin, quercetin-3-methyl ether, quercetin-3,3' – dimethyl ether, kaempferol, kaempferol 8-methyl ether, pinocembrin, pinobanksin, chrysin, genkwanin	Gallic acid, ellagic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, syringic acid	Pyrzynska, Biesaga 2009
<u>Northern America</u>			
North America	Quercetin, pinocembrin, kaempferol, chrysin, galangin	p-Hydrobenzoic acid, vanillic acid, p-coumaric acid, cis, trans-absicic acid, cinnamic acid	Rasmussen <i>et al.</i> , 2008
Cuba	Phloroglucinol, myricetin, quercetin-diglycoside, quercetin-o-rhamnoside, quercetin, kaempferol-7-rhamnoside, kaempferol, isorhamnetin, 8-methoxykaempferol	Vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid	Alvarez-Suarez <i>et al.</i> , 2010a
<u>Africa</u>			
Sudan	Quercetin, hesperitin, kaempferol, apigenin, isorhamnetin		Makawi <i>et al.</i> , 2009
Tunisia	Pinobankisin, hesperetin, quercetin, luteolin, 3-methylquercetin, 8-methoxykaempferol, quercetin 3,7-dimethyl ether, galangin, apigenin, isorhamnetin, pinocembrin, 3,5,7-trihydroxyflavanone, hesperetin, kaempferol, chrysin, galangin 3-methyl ether, myricetin 3,7,4',5'-methylether, pinocembrin 7-methyl ether	Ellagic acid, gallic acid, phenylethyl caffeate caffeic acid	Martos <i>et al.</i> , 1997, Pyrzynska, Biesaga 2009

From a previous study, the antioxidant activity of honey from the southern African region was determined. This study included wild and agricultural honey from the Cape, Gauteng and south eastern Mozambique region. These honeys were found to have high antioxidant activity and protected cells against peroxy oxidative damage (Serem, Bester 2012).

2.4 Antimicrobial activity of honey

Microbes are implicated in many infectious diseases the most common of these being bacterial infection. The first antibiotics prior to 1942 were either specific for Gram-positive bacteria (penicillin, gramicidin, and actinomycin) or Gram-negative bacteria (streptomycin). There were several limitations to the use of these antibiotics e.g. penicillin was highly selective, high concentrations of actinomycin were required to treat infections and streptomycin, though water soluble with limited toxicity, was not very effective against Gram-positive and negative bacteria. Streptomycin was the first antibiotic discovered that had activity against both Gram-negative and positive bacteria (Schatz *et al.*, 2005).

Conventional antibiotics (Figure 2.4), inhibit deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis (Sang, Blecha 2008). However, bacteria develop resistance by inactivating antibiotics via enzyme reactions, transporting antibiotics out via efflux pumps and mutating protein targets so that the antibiotics cannot bind. In addition, bacteria can produce excessive amounts of the antibiotic target such as protein or DNA, which results in the ratio of target to antibiotic being so large that the antibiotic is ineffective.

Honey from different areas have been shown to have *in vitro* antimicrobial activity against both Gram-positive and negative bacteria and these include *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis* and *H. pylori*. Honey with antibacterial activity include medical grade RS honey (Kwakman *et al.*, 2010), Manuka honey (Henriques *et al.*, 2010, Henriques *et al.*, 2011, Kwakman *et al.*, 2011), honey derived from Australian flora (Irish *et al.*, 2011), and South Africa honey (Manyi-Loh *et al.*, 2010). Furthermore, Medihoney from Australia used as a wound healing product, has been shown to inhibit resistant bacteria such as strains of *methicillin resistant S. aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) (George, Cutting 2007).

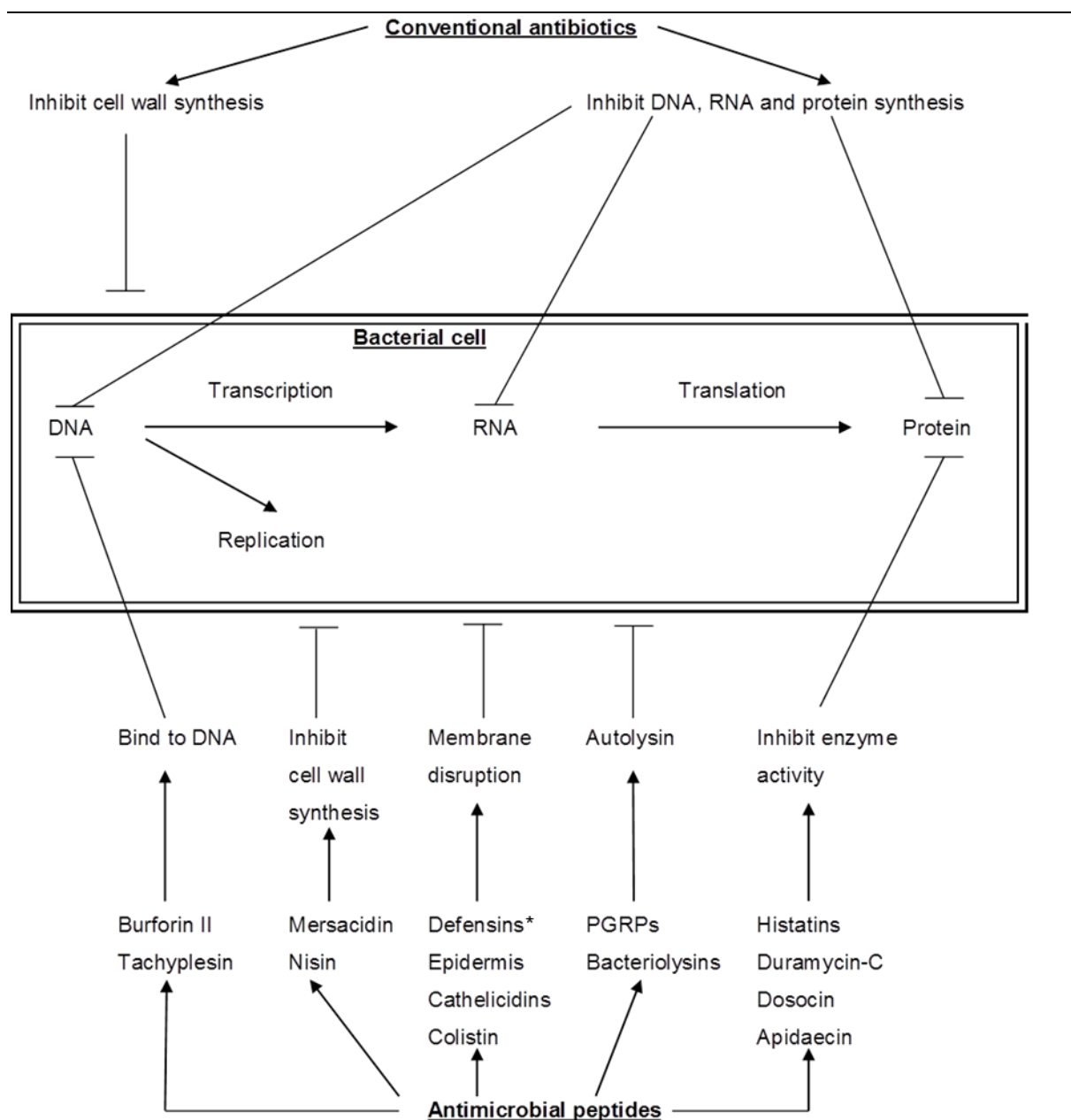


Figure 2.4: Summary of mechanism of action intracellularly of antibiotics vs. antimicrobial peptides, adopted from (Sang, Blecha 2008). *Includes bee defensin 1.

Honey is a supersaturated sugar solution, and its high osmolarity causes strong interaction between water and sugar molecules, that leaves very little water to support the growth of microorganisms. The pH of honey is low (3.2 – 4.5) and as such is a hostile environment for most bacteria. The presence of H₂O₂ in several types of honey inhibits the growth of most bacteria. Flavonoids and phenolic acids such as catechin, apigenin, myricetin, caffeic acid and ferulic acid present in honey have been shown also to have antibacterial activity (Wahdan 1998, Emsen 2007, Henriques *et al.*, 2010, Irish *et al.*, 2011).

The antibacterial activity of specific honey types such as Manuka honey is due to the presence of high MGO levels (Henriques *et al.*, 2010) while the AMP, BD-1 and the presence of H₂O₂ contributes to the antibacterial activity of RS honey (Kwakman *et al.*, 2010, Sherlock *et al.*, 2010).

2.4.1 Hydrogen peroxide, methylglyoxal and antimicrobial peptides

H₂O₂ in honey is formed from the oxidation of glucose by the enzyme glucose oxidase when honey is ripening. The antibacterial activity of H₂O₂ is due to its oxidising nature causing oxidative damage to the bacterial cell wall, proteins, enzymes and DNA resulting in bacteria not responding to proliferative signals (Brudzynski *et al.*, 2011). Brudzynski *et al.*, (2011) reported that the antibacterial activity of Buckwheat honey correlated with the H₂O₂ content of this honey and with dilution formation of H₂O₂ is favoured.

MGO is a precursor of advanced glycation end products (AGE) and is found mostly in sugar-rich and intense heated products or in hyperglycaemic conditions (Degen *et al.*, 2013). Under physiological conditions, MGO is formed as the result of the degradation of triose phosphates (an intermediate of the glycolysis pathway), the catabolism of ketone bodies and amino acids such as threonine (Figure 2.5). In food, MGO is formed via the Maillard reaction, which involves the degradation of glycated proteins and monosaccharides. MGO was first identified in coffee (1.6 – 7.0 mg/L), and has also been found in wine (0.1 – 2.9 mg/L), yoghurt (0.6 – 1.3 mg/kg), soy sauce (8.7 mg/kg) and in Manuka honey (40 – 760 mg/kg) (Degen *et al.*, 2013). In Manuka honey the presence of high levels MGO is due the high levels of MGO in the nectar of *Leptospermum scoparium* (Mavric *et al.*, 2008).

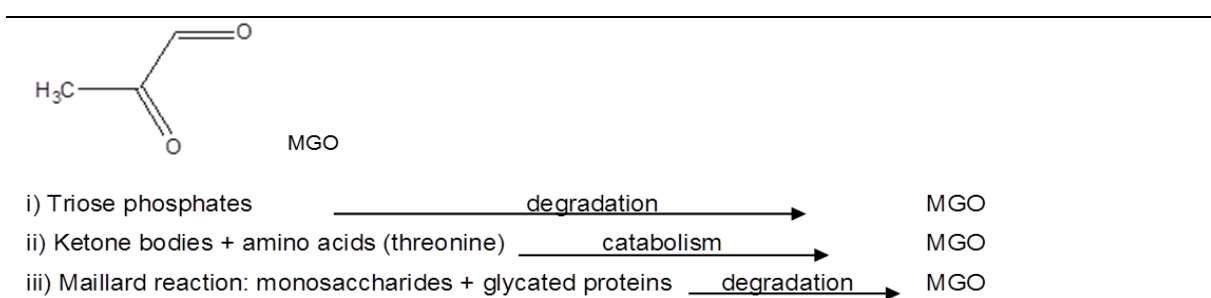


Figure 2.5: Chemical structure and the biochemical processes involved in the formation of methylglyoxal (MGO).

MGO effectively kills bacteria (Mavric *et al.*, 2008, Fidaleo *et al.*, 2010, Kilty *et al.*, 2011, Mukherjee *et al.*, 2011), however high levels of MGO *in vitro* induces apoptosis (Lee *et al.*, 2009a). *In vivo*, exposure of rats to 60 mg MGO per kg body weight, intraperitoneally or

subcutaneously, presented with biochemical and molecular abnormalities, characteristic of diabetes type 2 (Degen *et al.*, 2013).

AMPs are peptides that can kill microbes usually via a different mode of action than conventional antibiotics where AMPs act through membrane targeting and pore-forming mechanisms, making the development of resistance more difficult due to the non-specificity of killing when compared to traditional antibiotics (Figure 2.4), (Sang, Blecha 2008). Several AMPs have additional intracellular targets and these include inhibition of cell wall synthesis, inhibition of enzyme activity and DNA degradation (Figure 2.4).

AMPs can be cationic or anionic, consist of 2 – 100 amino acid residues and have activity due to charge and hydrophobicity. Cationic peptides have 2 – 9 positive charges because of the high content of basic amino acids (lysine, arginine and histidine). The AMP, melittin is found in honeybee venom (Sang, Blecha 2008). AMPs are also produced in honeybees after infection with *E. coli* and these are hemonoptecin, apidaecin, abaecin peptides and BD-1. Of these, only BD-1, also known as royalisin, has also been identified in royal jelly and honey. BD-1 is identified in RS but not in Manuka honey (Kwakman, Zaat 2012).

Antibacterial glycoproteins in honey include major royal jelly proteins 1 precursor (MRJP1) which can agglutinate bacteria, induce phenotypic changes and increase bacterial permeability leading to cell lysis. MRJP1 consists of four antimicrobial peptides, known as jelleins Brudzynski *et al.*, (2015). Jelleins I – IV are not cytolytic while I – III have antimicrobial activity against Gram-positive and negative bacterial as well as yeast (Fontana *et al.*, 2004).

Synergism between the physiochemical characteristics (osmolarity, acidity and low water activity) and molecules with antibacterial activity (MGO, H₂O₂, BD-1) contributes to the antimicrobial activity of honey. The molecules in southern African honey contributing to the antibacterial activity is unknown.

2.5 Anti-inflammatory activity of honey

Inflammation is a reaction of tissues/cells to foreign hostile agents that results in the oxidation of biomolecules (Wang *et al.*, 1999). This process starts with the inducers of inflammation, followed by mediators, which propagate the inflammatory process, and ends with effectors, which regulate the end products of inflammation. Regulation of end products of inflammation mostly involves the microcirculation and includes the movement of serum proteins and leukocytes (neutrophils, eosinophils and macrophages) from blood to the extra-vascular tissue

(Table 2.4) (Santangelo *et al.*, 2007). Once in tissue leukocytes specifically neutrophils, release their toxic contents i.e. ROS, RNS and proteinases to act against predominately microbial inducers e.g. lipopolysaccharide (LPS) and host tissue. This is then followed by resolution, where the mediator lipoxin inhibits recruitment of neutrophils and instead promotes the recruitment of monocytes that remove dead cells and initiates tissue remodelling marking the end of the acute phase of inflammation (Medzhitov 2008). Inflammation without resolution then recruits macrophages and T cells to act against inducers, and failing this chronic inflammation that contributes to the development of cancer, atherosclerosis and arthritis occurs (Wang *et al.*, 1999, Medzhitov 2008).

A major pathway of inflammation, the arachidonic acid (AA) dependent pathway, involves the lipid mediators (mediator 4, Table 2.4). These mediators are derived from phospholipids specifically cytosolic phospholipase A₂ which is activated by intracellular Ca²⁺ to generate AA and lysophosphatidic acid. These acids are then converted to prostaglandins and thromboxanes by cyclooxygenase (COX) and leukotrienes and lipoxins by lipoxygenase (LOX), (Figure 2.6) (Geronikaki, Gavalas 2006). The prostaglandins and thromboxanes then propagate inflammation by inducing fever and affecting vasculature, whereas leukotrienes promote inflammation and lipoxins promote resolution of inflammation (Figure 2.6, Table 2.4) (Geronikaki, Gavalas 2006, Santangelo *et al.*, 2007, Medzhitov 2008).

Table 2.4: Inducers, mediators and effectors of inflammation (Santangelo *et al.*, 2007, Medzhitov 2008).

Inducers		
Exogenous		Endogenous
Microbial	Non-microbial	
Pathogen associated molecular patterns Virulence factors	Allergens/irritants Foreign bodies Toxic compounds	Cell or tissue derived Plasma or extracellular matrix derived
Mediators		Effectors
1. Vasoactive amines e.g. histamine, serotonin		Mast cells & platelets – affects vasculature (dilation/constriction)
2. Vasoactive peptides e.g. substance P, kinins, fibrino peptide A & B, fibrin degradation products (from Hageman factor, thrombin, plasmin)		Vasodilatation & increased vascular permeability Act directly, or indirectly by causing release of histamine
3. Anaphylatoxins e.g. C3a, C4a, C5a		Promote granulocyte & monocyte recruitment and induces mast cell degranulation, affecting vasculature
4. Lipid mediator's e.g. Eicosanoids: prostaglandins + thromboxanes, Eicosanoids: leukotrienes + lipoxins Platelet activating factor		PGE ₂ /PGI ₂ = vasodilatation; PGE ₂ = hyperalgesic /potent inducer of fever Inhibit inflammation, promote resolution of inflammation and tissue repair Recruit leukocytes, vasodilatation/vasoconstriction
5. Inflammatory cytokines e.g. TNF- α , IL-1, IL-6		Mostly mast cells and macrophages – activate endothelium & leukocytes; induce acute-phase response
6. Chemokines		Many cell types – leukocyte extravasation, chemotaxis
7. Proteolytic enzymes e.g. Elastin, cathepsins, matrix metalloproteinases		Degrade extracellular matrix & basement proteins; host defence, tissue remodelling, leukocyte migration
Substance P – neuropeptide/neurotransmitter Peptide A/B – neuropeptides C3a/C4a/C5a – complement anaphylatoxins (cationic peptides) TNF- α – tumour necrosis factor alpha IL-1/IL-6 – interleukin 1/interleukin 6 PGE ₂ /PGI ₂ – prostaglandin E ₂ /prostaglandin I ₂ (prostacyclin)		

Other pathways occur independent of AA are the AA – independent pathways, and these include the following mediators, free radicals and enzymes such as nitric oxide synthase (NOS) with inducible (iNOS) being associated with inflammation. Nuclear transcription factor kappa beta (NF- κ B) is the second kind of mediator found in the cytoplasm which is activated by free radicals and inflammatory signals, which moves to the nucleus where gene transcription occurs. This leads to cellular events such as apoptosis, proliferation, invasion and inflammation associated with cancer, atherosclerosis, myocardial infarction, diabetes and inflammatory disorders such as asthma and IBD (Yoon, Baek 2005). A focus of anti-inflammatory research is the identification of molecules that can be used to inhibit key role players in the inflammatory process such as iNOS, LOX and NF- κ B.

Inflammation is commonly treated with non-steroidal anti-inflammatory drugs (NSAIDs) which have different mechanisms. These include drugs that strongly inhibit COX-1 (aspirin, ibuprofen, diclofenac, indomethacin, naproxen, piroxicam), weak inhibitors of COX-2 (celecoxib, etodolac, meloxicam, nimesulide), strong inhibitors of COX-2 (rofecoxib) and weak inhibitors of both COX-1 and COX-2 (5 - amino salicylic acid, sodium salicylate, nabumetone, sulfasalazine) (Rao, Knaus 2008). However, NSAIDs have negative side effects especially to the GIT as COX-1 plays an important role in protecting the stomach lining, therefore, if inhibited, it may cause side effects ranging from gastric discomfort to ulceration. In addition, NSAIDs have been shown to be ineffective when used for the treatment of chronic inflammatory conditions such as arthritis, IBD, vasculitis and lupus. For this reason, the need for novel anti-inflammatory molecules with fewer side effects is increasing (Yoon, Baek 2005, Kupeli *et al.*, 2007, Medzhitov 2008).

Ayurvedic and Chinese medicine and other traditional systems use natural sources and plants to treat inflammatory conditions. Functional foods can also contribute by either preventing or reducing inflammation, specifically by promoting or inhibiting particular enzymes and/or free radicals in the inflammatory pathways. For example, foods with compounds that inhibit COX, LOX 5 (leukotrienes) and ROS/RNS can reduce inflammation, whereas those with compounds that activate neutrophils and lipoxins (LOX 15) can also effectively reduce inflammation (Figure 2.6).

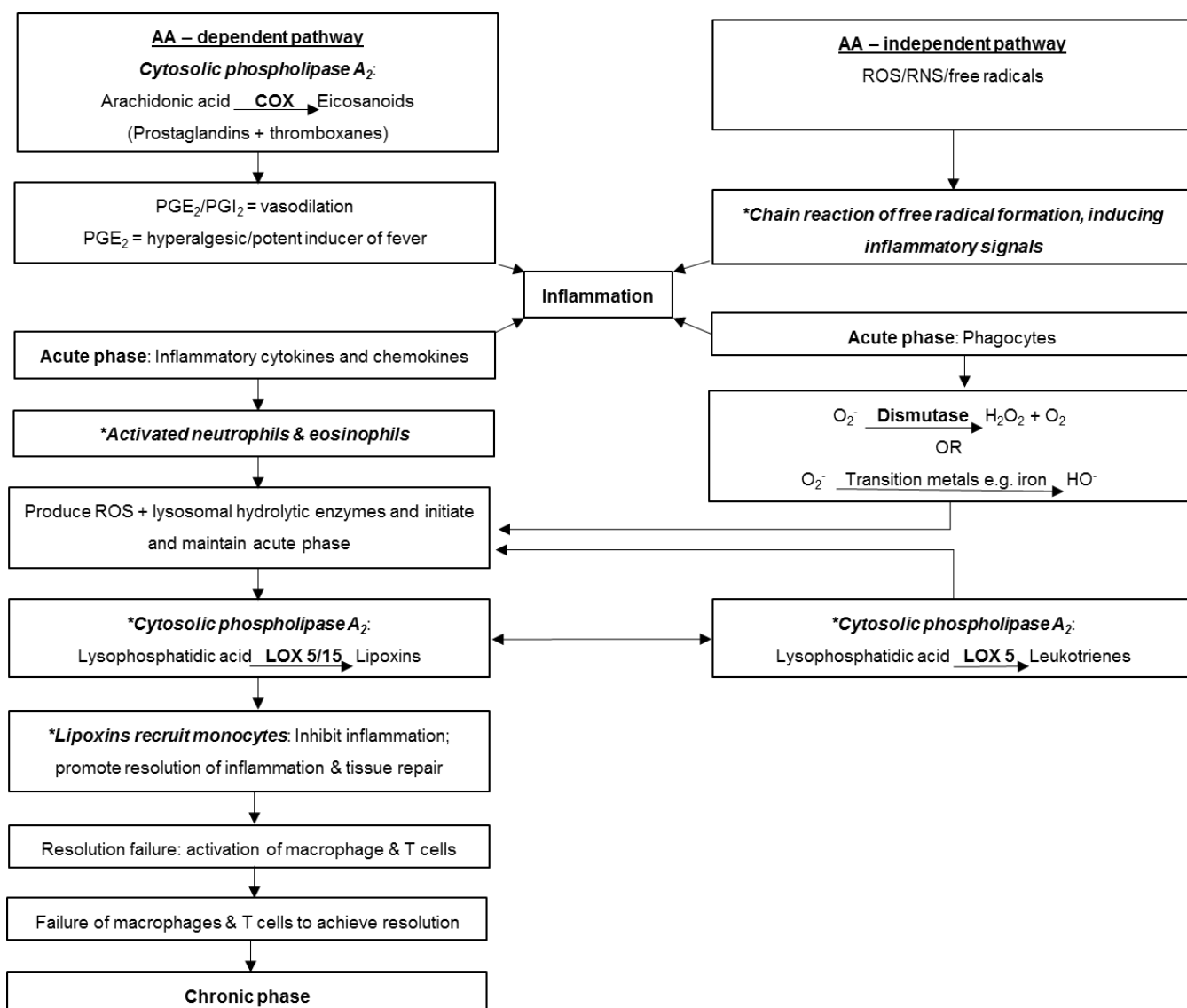


Figure 2.6: Inflammation pathways induced via lipid mediators and free radicals (Santangelo *et al.*, 2007, Medzhitov 2008). * indicate points at which inflammation could be propagated/inhibited.

AA – arachidonic acid, COX – cyclooxygenase, ROS – reactive oxygen species, RNS – reactive nitrogen species, PGE – prostaglandin, O_2^- – superoxide anion, O_2 – oxygen, H_2O_2 – hydrogen peroxide, OH^- – hydroxyl radical, LOX – lipoxygenase.

Several honey types have been shown to have anti-inflammatory activity, the most well-known of which is Manuka honey. Wang, Huang (2013), have shown that apigenin, a common flavonoid also present in honey, inhibited NF- κ B activation, scavenged free radicals and stimulated mucin (MUC-2) secretion in cultured gastric adenocarcinoma cells. Inhibition of NF- κ B reduces inflammation and the presence of free radicals, while MUC-2 secretion improves the secreted mucus barrier, which is important in inflammatory disorders such as gastritis and Crohn’s disease. Malaysian honey, which contains ellagic acid, caffeic acid, chrysin and quercetin, has been shown to down regulate NF- κ B which results in reduced synthesis of iNOS causing NO inhibition in a dose-dependent manner without affecting the viability of RAW 264.7 cells (Kassim *et al.*, 2010). Caffeic acid phenethyl ester (CAPE) a natural product secreted by

honeybees, and also present in honey also inhibits NF- κ B, reducing inflammation (Yoon, Baek 2005). Most studies conclude that anti-inflammatory activity of honey is due to the presence of phenolic compounds (Safi *et al.*, 2016, Almasaudi *et al.*, 2017), and this is confirmed by testing the activity of specific flavonoids. Catechin and epigallocatechin (EGCG) was found to inhibit COX-2, quercetin inhibited LOX 12, while kaempferol, quercetin and myricetin inhibited LOX 5. All of these flavonoids, except EGCG are found in honey (Table 2.3) (Yoon, Baek 2005).

2.6 Gastrointestinal digestion

In the GIT, digestion can result in a loss of activity due to the degradation of molecules or alternatively a gain of activity can occur due to the release or formation of bioactive molecules from the food matrix. Factors that contribute to these effects are pH, ionic strength and enzyme activity (Hur *et al.*, 2011).

The various stages of digestion can be grouped into oral, gastric and the gastro-duodenal phase, with each phase involving different enzymes and pH conditions. Studies have been done to simulate these processes to observe the effect of digestion on various foods (Neilson *et al.*, 2007, Wootton-Beard *et al.*, 2011, Stalmach *et al.*, 2012). These studies can be grouped into *in vivo*, human and animal studies or *in vitro*, laboratory based studies. Due to ethical considerations and complexities involved in human clinical studies, research focuses on laboratory based models and these include *ex vivo*, *in vitro* and *in silico* methods.

2.6.1 *In vivo* models

Human studies involve the analysis of blood, plasma, urine and fecal matter following the digestion of a specific drug, molecule, food or food mixtures. Data generated provides information on the end result of digestion rather than the effect of the different stages of digestion (Borgstrom *et al.*, 1957). Although also an indirect method, the use of ileostomy does provide some information on the processes involved in GIT digestion (Englyst, Cummings 1985). The most commonly used method that incorporates all compartments/phases of digestion is the use of intubation. This procedure involves intubating a patient, followed by feeding the patient a meal with a reference marker, then sampling the digests at the various levels of the GIT via the tube (Schnabel 2001). The Miller-Abbott tube, allows direct investigation on effect of enzymes and/or gastric juices on particular types of food as well as the effect of specific physiological conditions such as fasting (Karr *et al.*, 1935, Borgstrom *et al.*, 1957). These pioneering studies provided important basic information related to the biochemical and physiological functioning of the GIT. However, these studies are time consuming and expensive and the use of such an invasive method in human subjects has led

to several ethical issues. This has led to the increasing popularity of animal models or *in vitro* methods.

Animal studies are less expensive methods to study digestion, but still require ethical consent. Similar to human studies, indirect or direct methods can be used. Animals also provide an important source of GIT tissue an example of which is the *ex vivo*, inverted gut sac model. In this model, sections of the rat intestine, is inverted, with the mucosa on the outside. One end is closed and is suspended in a solution containing the drug or food of interest. The rate of absorption can be studied using different sections of the small or large intestine. Limitations of this model are that the tissue viability is short, costly due to the number of rats required and the mucosa is not a true representation of the human GIT (Williamson, Clifford 2010).

2.6.2 *In vitro* models

In vitro studies are aimed to mimic/simulate human GIT digestion as accurately and rapidly as possible. These models differ in complexity and are designed to take into account the phase, rate and the temperature of digestion as well as type of food being digested. The most commonly studied phases of digestion are the gastric and gastroduodenal phase. The oral phase is not as widely researched, but recent research involves the inclusion of this phase of digestion. In addition to the phase of digestion, it is also important to consider the digestive enzymes that are used. These enzymes are usually obtained from omnivorous animals such as rats, pigs or even from human volunteers and include amylase, pepsin, pancreatin, trypsin, chymotrypsin, peptidase and lipase. Bile salts and mucin are often also included. The enzymes that are used depend on the kind of food being digested, and are lipase for lipids, proteases for protein and amylase for starch digestion. In addition, enzyme concentrations should be representative of physiological concentrations. In the simulated *in vitro* processes, these enzymes at physiological concentrations are added sequentially to simulate normal GIT digestion (Hur *et al.*, 2011). Other important factors that are also controlled is the pH of each phase, transition time as well as temperature.

Besides the presence of enzymes, the microbial environment of the GIT plays an important role in digestion. These functions include metabolic fermentation of non-digestible dietary residue, production and absorption of vitamin K, trophic functions, which maintain homeostasis of the GIT immune system and protective functions which offer a barrier against pathogens (Guarner, Malagelada 2003). Several *in vitro* models only focus on the enzymatic component of digestion and not the contribution of the microbial environment. Simulated human intestinal microbial ecosystem (SHIME) is an example of a model that simulates the small and large intestine digestion taking into account, both enzyme and bacterial interactions and consists of

several chambers or reactors. The reactors are pH controlled, contain different volumes of sample and enzymes and are run for different intervals. The reactors representing the duodenum and jejunum, ileum and caecum and the ascending colon which are inoculated with a particular concentration of bacteria. Due to the controlled experimental environment, the effects of altered pH, bacteria composition and volume can be investigated (Molly *et al.*, 1993). The TNO human gastrointestinal tract simulator (TIM) is another example of this model, which is made up TIM-1 representing stomach, duodenum, jejunum, and ileum digestion and TIM-2 representing the large intestine. In each compartment, the effects of temperature, pH, peristaltic movement and rate of water absorption can be determined. The type and concentration of fermentation products that also serve as substrates can be monitored as these molecules move from one compartment to another (Minekus *et al.*, 1999). These models vary with construction, enzymes used, number of phases used and the time for each phase of digestion and are often automated. Although more representative of actual GIT digestion, these models are time consuming and due to increased complexity have potential for increased error. Consensus is that initial studies should involve the simple basic models with further research making use of more complex models such as the SHIME model. Further research should also include the effect of liver and kidney metabolism and conjugation.

2.7 Digestion of honey associated compounds

This section of the literature review will specifically focus on the effect of digestion on the bioactive components MGO, H₂O₂, proteins and polyphenols that are present in honey.

2.7.1 Gastrointestinal digestion of methylglyoxal

With digestion, MGO is more stable under gastric digestion (pH 2) and less stable under gastroduodenal conditions (pH 7). The loss in MGO after digestion has been identified to be due to interaction of the MGO with proteins, such as the digestive enzymes in the GIT (Degen *et al.*, 2013).

2.7.2 Gastrointestinal digestion of polyphenols

Polyphenols, have been shown to have excellent biological activities *in vitro*, however these effects *in vivo* are disappointing and this is attributed to the poor bioavailability of polyphenols in the small intestine (Dryden *et al.*, 2006). The poor bioavailability is a major criticism of antioxidant studies however, polyphenols do have beneficial effects and these include direct protection of the GIT mucosal membrane against oxidative damage generated by the micro flora of the GIT (Rice-Evans *et al.*, 1996, Bourne, Rice-Evans 1998). Honey contains flavonoids and phenolic acids with reported antioxidant, antimicrobial and anti-inflammatory activity (Noguchi *et al.*, 2000). Several of the polyphenol compounds found in honey (Table 2.2), such

as quercetin, epicatechin and caffeic acid (Spencer *et al.*, 2004) have been shown to be bioavailable however, those that are not available may have prebiotic and/or beneficial effects on the mucosa of the GIT. In addition polyphenols have been found to modulate cellular activity due to especially flavonoids targeting several enzymes and/or receptors in the GIT (Pérez-Cano *et al.*, 2014).

At birth, the GIT is a sterile environment in which a stable microbial population is established (Ewaschuk, Dieleman 2006). A delicate balance between intestinal bacteria and bacteria from the external often-pathogenic environment has to be maintained. A change in this balance can cause alteration of the mucosal homeostasis resulting in chronic inflammation in the GIT. Factors that help maintain this balance include diet, antibiotics, genetics and hygiene. Diet is increasingly being identified as an important factor since the identification of probiotics and prebiotics. Prebiotics by definition are non-digestible food ingredients such as dietary carbohydrate that stimulate the growth and metabolism of endogenous enteric bacteria that improve host health (Fooks *et al.*, 1999, Ewaschuk, Dieleman 2006). Polyphenols have a prebiotic effect, as when they are ingested, a majority are seen as xenobiotics hence are not absorbed in the small intestine. They then concentrate in the colon, wherein they can be broken down by gut microbiota and be reabsorbed or inhibit the growth of pathogenic bacteria while increasing the growth of commensal bacteria. These two pathways then result in beneficial health outcomes such as anticancer effects (Cardano *et al.*, 2013). Probiotics are defined as living organisms in food/dietary supplements, which improve health of host beyond their inherent basic nutrition (Fooks *et al.*, 1999, Ewaschuk, Dieleman 2006). Undigested polyphenols that are transported to the colon would then be classed as prebiotics. The effects of prebiotics and probiotics have been shown in the improvement of GIT disorders and ailments through various mechanisms (Figure 2.7). Decreased prebiotics, probiotics and synbiotics (a combination of dietary prebiotics and probiotics (Ewaschuk, Dieleman 2006)) increases predisposition to IBD (Ewaschuk, Dieleman 2006). For example, a decrease in prebiotics and probiotics can lead to Ulcerative Colitis (UC) whereas a decrease in probiotics and synbiotics can lead to inflamed ileal Crohn's Disease (CD). Consequently, functional foods with probiotics and symbiotic effects can effectively reduce inflammation associated with IBD.

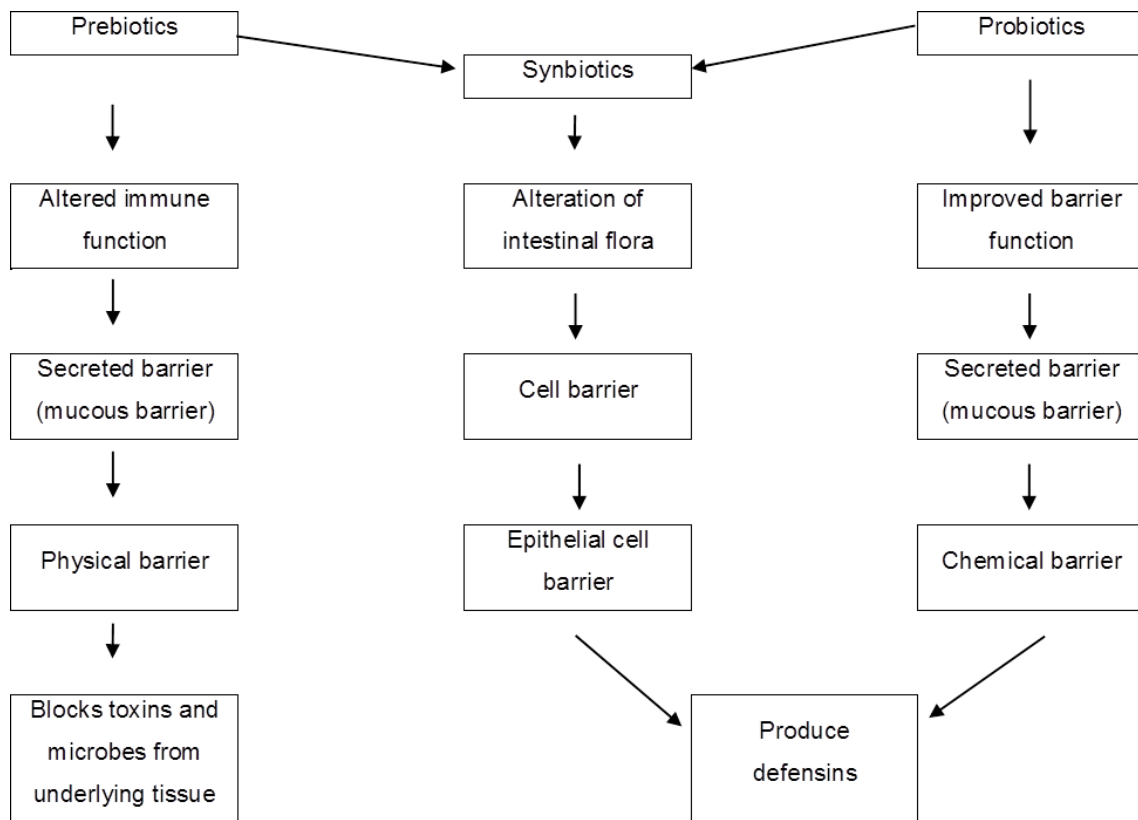


Figure 2.7: The effects of prebiotics, probiotics and synbiotics on the GIT (Ewaschuk, Dieleman 2006)

2.7.3 Gastrointestinal digestion of proteins

Protein digestion begins in the stomach and involves breaking down of proteins into large peptides, tripeptides and dipeptides by pepsin, followed by the proteolytic activity of predominately, trypsin and chymotrypsin in the duodenum. The final stage of protein digestion that consists of two parts occurs in the enterocytes that line the villi of the small intestine. At the brush border, polypeptides are converted into tripeptides, dipeptides and amino acids by aminopolypeptidase and dipeptidases. These products move into the enterocytes, where peptidase converts them to amino acids, which are transported via the blood to the liver for the biosynthesis of new protein (Hall 2006).

Bioactive proteins can be divided into those that are naturally present and those that arise from digestion of intact dietary protein sources (Kitts, Weiler 2003). Bioactive peptides, which contain between 2 – 20 amino acid residues, can then be defined as specific breakdown products of proteins that have a beneficial effect on cellular and tissue function. Proteins in food can be hydrolysed into biologically active peptides via three routes, enzymatic hydrolysis with digestive enzymes, microbial activity of fermented foods and action of enzymes derived from proteolytic microorganisms (Korhonen, Pihlanto 2006). Common sources of bioactive peptides are milk, eggs, fish and cereals. Casein, a protein found in milk, when digested with

trypsin, yields casein angiotensin I converting enzyme (ACE) inhibitor protein with ACE-inhibitory activity, and when digested with chymosin/rennin yields casecidin, which has antimicrobial activity (Kitts, Weiler 2003). Another example is ovalbumin, a protein found in egg, which when digested yields ovokinin, a peptide that has antihypertensive activity (Kitts, Weiler 2003). Other digestive enzymes that form bioactive peptides include alcalase, chymotrypsin, pancreatin, pepsin and thermolysin (Korhonen, Pihlanto 2006). It is unknown whether the digestion of honey associated proteins results in the formation of bioactive peptides or causes a loss of BD-1 and jellein activity.

2.8 Beneficial effects of honey in the gastrointestinal tract

As a functional food, honey has several nutritional benefits such as increasing the level of vitamin C and β -Carotene in blood plasma. Honey has also been shown to be a gastro protective agent against microbes, the most common being *H. pylori* (Kim 2005, Manyi-Loh *et al.*, 2010). This bacterium produces ammonia which creates an environment that promotes the survival of this microbe, before it infiltrates the gastric epithelium. Ammonia in the GIT causes a chain reaction, leading to cell death and vacuolation of gastric cells resulting in inflammation and associated gastroenteritis (Suerbaum, Josenhans 1999, Kim 2005), gastric carcinomas and peptic ulcers (Kim 2005). Prevention of these ailments is two-fold: firstly to reduce the effects of ammonia and then to allow the healing and subsequent restoration of the structural integrity and function of the gastric mucosal barrier. Manyi-Loh *et al.*, (2010) investigated the ability of extracts of three South African honeys to inhibit *H. pylori*. All honey samples had activity at honey concentrations greater than 10% with the lowest activity at 75% (v/v). In rat studies, honey reduced ulcers caused by indomethacin, alcohol, ammonia and aspirin (Bogdanov *et al.*, 2008) and this was due to the ROS scavenging activity and anti-inflammatory activity of honey.

IBD is an inflammatory disorder of the intestines of the GIT and includes CD and UC. In CD inflammation can affect any part of the GIT although inflammation is commonly located in the small bowel and colon while in UC inflammation is restricted to the large bowel and rectum (Veza *et al.*, 2016). As the cause of IBD is not completely known, treatment mostly focuses on the eradication of inflammation and maintaining the non-inflamed state in the intestine. To achieve this therapies include the use of aminosalicylates and immunosuppressant's, however, long term use could lead to chronic side effects (Veza *et al.*, 2016).

In the GIT functional foods and nutraceutical products are attractive treatment options for the prevention, treatment and maintenance of a GIT free of inflammation.

Polyphenols specifically the flavonoids have been shown to have anti-inflammatory effects *in vitro* and *in vivo* (Pérez-Cano *et al.*, 2014, Gil-Cardoso *et al.*, 2016, Vezza *et al.*, 2016), therefore flavonoid rich vegetables, fruits and other food derived products such as tea and honey (Table 2.3) will have anti-inflammatory beneficial effects in the GIT if the structure and function is retained following digestion. Honey is a rich source of flavonoids (Table 2.3) and in addition honey is a prebiotic due to the presence of Fru that cannot be digested and can alter human gut flora beneficially by enhancing the growth of bifidobacteria and lactobacilli (Bogdanov *et al.*, 2008). Little is known about how honey can protect against inflammatory disorders of the GIT such as IBD, however studies have shown that polyphenols have ameliorating effects on peptic ulcers (Farzaei *et al.*, 2015) and can improve colonic microflora (Parkar *et al.*, 2008) indicating the potential of honey as a source of flavanoids and prebiotics and therefore as a functional food that can prevent and reduce inflammation in the GIT.

2.9 Background to this study

The antioxidant, antibacterial and anti-inflammatory activity of honey and its main constituent molecules is well described, however as a functional food, honey is consumed and little is known about the effects of digestion on bioactivity although *in vivo* studies do indicate beneficial effects. Many studies, determine for example the polyphenol profile of a honey or identify the component of honey responsible for antibacterial activity. These molecules are then evaluated for bioactivity. Due to the complexity of honey, the variable effects of digestion on the constituent bioactive molecules and the possibility of synergy between molecules, it is important to evaluate honey as a functional food to determine the effects of digestion on bioactivity prior to the identification of specific molecules contributing to these effects.

The southern African region has a unique floral biodiversity where honey is produced by the African honeybee, *Apis mellifera scutellata* and in the Western Cape honeybee, *Apis mellifera capensis*. From a previous study in our laboratory honey from this region and produced by these bee subspecies has been identified to have high antioxidant activity and to protect cells against oxidative damage (Serem, Bester 2012). The antibacterial and anti-inflammatory activity of these honeys is unknown. Furthermore, as a functional food the effects of digestion on bioactivity is also unknown.

2.10 Aim and objectives

The aim of this study was to determine if following simulated *in vitro* GIT digestion the antioxidant, antibacterial and anti-inflammatory activity of a selection of southern African and

Manuka honey is retained, thereby identifying honey as an important functional food that can reduce infection and/or improve the cellular features of inflammatory disorders of the GIT.

The specific objectives related to antioxidant activity were to:

1. Determine the effect of simulated *in vitro* GIT digestion on the total polyphenol content (TPC) and antioxidant activity using the oxygen radical absorbent capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) of the undigested (UD), gastric (GD) and gastroduodenal (GDD) digested honey samples.
2. Further elucidate, using polyphenol mixtures and a synthetic honey the effects of digestion on polyphenol stability.
3. Determine the cellular antioxidant activity (CAA) using the dichlorofluorescein diacetate (DCFH-DA) assay with Caco-2 cells of UD and digested honey samples.
4. Further elucidate, using polyphenol mixtures and a synthetic honey the effects of digestion on CAA.

The specific objectives related to antibacterial activity were to:

1. Determine the minimum inhibitory concentration (MIC) of Manuka (control) and three southern Africa honeys against Gram-negative (*E. coli* and *P. aeruginosa*) and positive (*B. subtilis* and *S. aureus*) bacteria.
2. Determine the MIC of MGO and H₂O₂ and the contribution of MGO, H₂O₂, sugars and antimicrobial peptides found in honey to the measured antibacterial activity.
3. Evaluate using a simulated model of GIT digestion, the effects of digestion on the antibacterial activity of honey.

The specific objectives related to anti-inflammatory activity were to:

1. Determine the flavonoid content of UD, GD and GDD.
2. Determine the ability of UD, GD and GDD to scavenge nitric oxide (NO).
3. Evaluate the cytotoxicity of UD, GD and GDD in the L929 cell line.
4. Determine the ability of UD, GD and GDD to inhibit *E. coli* induced NO formation in the L929 cell line.
5. To determine the ability of UD and GDD honey to prevent LPS induced platelet activation.

Chapter 3: Elucidating the effect of gastrointestinal digestion on the antioxidant properties of honey

Abstract

Honey is a functional food with antioxidant activity that can scavenge (ROS) that contributes to the development of cancer, inflammatory disorders as well as cardiovascular and neurological diseases. Polyphenols are stable at a low pH but undergo degradation at neutral pH, which may negatively impact on activity especially following duodenal digestion. The aim of this study was to determine using a simulated digestion model, the effect of gastrointestinal (GIT) digestion on the antioxidant properties of honey.

Three southern African honeys: (Agricultural Eucalyptus (AE), south eastern Mozambique (SEMh), Western Cape, Fynbos (WC) and a control honey Manuka (MANc)) were subjected to simulated gastroduodenal digestion. The total polyphenol content (TPC) and antioxidant activity, (Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbency capacity (ORAC) assays) of the undigested (UD), gastric (GD) and gastroduodenal (GDD) fractions was determined. Using human colon adenocarcinoma (Caco-2) cells the cellular antioxidant activity (CAA) was evaluated with the dichlorofluorescein diacetate (DCFH-DA) assay. To better understand the effects of digestion on polyphenols, polyphenol (PP) mixtures, and a synthetic honey (SH) consisting of sugars and polyphenols was also subjected to simulated GIT digestion and the antioxidant properties were determined. The effect of digestion on specific polyphenols in the mixtures was determined with liquid chromatography-mass spectrometry (LC-MS) analysis.

With GD the antioxidant properties and CAA was unchanged compared to undigested honey implying that honey can protect the stomach mucosa against oxidative damage. With GDD an increase in the TPC of MANc and AE and the antioxidant activity of MANc and SEMh (ORAC assay) was observed. The TPC and antioxidant activity of the other honey samples were unchanged. In contrast, the CAA of the GDD was lost, and was the greatest for WC, AE, MANc > SEMh. This loss was associated with an increase in H₂O₂ levels with MANc > AE, SEMh, WC. CAA findings following gastroduodenal digestion were different to measured antioxidant properties. Using polyphenol mixtures, findings of previous studies that at neutral pH, polyphenol degradation associated with an increase in H₂O₂ levels was confirmed. However, with SH, polyphenol degradation did not occur. It was then postulated that loss of CAA was due to H₂O₂ decomposition at neutral pH causes the formation of carotenoid peroxy radicals that act as pro-oxidants.

In conclusion, the antioxidant activity of honey with digestion is retained, loss of CAA associated with a strong pro-oxidant effect is not associated with polyphenol degradation but is possibly due to the formation of carotenoid peroxy radicals.

3.1 Introduction

Foods that have physiological beneficial effects beyond inherent nutrition of such as food are known as functional foods. Honey is a plant derived functional food that contains sugars, MGO, H₂O₂, proteins and enzymes as well as phenolic compounds, such as flavonoids which include, quercetin (QUE), kaempferol, luteolin, galangin, myricetin, chrysin (Blasa *et al.*, 2007) and catechin (CAT) (Ahmed, Othman 2013). Phenolic acids also present in honey include, caffeic (CA), gallic (GA), coumaric ferulic, ellagic and chlorogenic acid (Blasa *et al.*, 2007). The bioavailability of polyphenols is limited, however, the ability of these polyphenols to act as prebiotics or probiotics and to reduce the levels of ROS can have beneficial effects in preventing and improving the symptoms associated with diseases associated with the GIT such as cancer, gastric ulcers and IBD including UC and CD (Yoon, Baek 2005, Gómez-Caravaca *et al.*, 2006).

GIT has variable effects on the bioactivity of polyphenols and with digestion the antioxidant activity and the structure of polyphenols contributing to activity of honey may become altered as has been described for tea (Neilson *et al.*, 2007) as well as grape (Stalmach *et al.*, 2012) and vegetable juice (Wootton-Beard *et al.*, 2011). GIT associated pH changes and enzymatic activity can alter antioxidant activity. Low pH as such as found in the stomach can increase polyphenol extraction while in contrast the higher, neutral pH environment of the duodenum can cause oxidation of the polyphenols leading to a loss of antioxidant activity (Bermúdez-Soto *et al.*, 2007, O'Sullivan *et al.*, 2013). Digestive proteolytic activity can cause the degradation of proteins/peptides such as catalase and BD-1 found in honey or may result in the formation of bioactive peptides with antioxidant activity (Rice-Evans *et al.*, 1996, Bourne, Rice-Evans 1999, Sarmadi, Ismail 2010).

Several honey types including southern African honey contains H₂O₂, which in the presence of metals (ferrous iron) can via the Fenton reaction form hydroxyl radicals (Jung *et al.*, 2009). In addition, honey contains high levels of Glc and Fru and the cytotoxicity of these monosaccharides is due to the formation of reactive carbonyl metabolites or carbonyl oxidation products. This occurs when non-enzymatic fragmentation of sugar glycolaldehydes undergoes autoxidation with the formation of superoxide radicals which react with proteins and nucleic acids to form carbonylated adducts. D-glucose undergoes H₂O₂ oxidation with the formation of several smaller end products, such as formic, acetic and glycolic acid which are cytotoxic (Moreno *et al.*, 2012). Toxic AGE-2 forms following the binding of D-glyceraldehyde to proteins. AGE-3 is formed from glycolaldehyde. In addition dicarbonyls, glyoxal and MGO also present in honey form AGE-5 and AGE-4, respectively (Yang *et al.*, 2011).

In a study by Lee *et al.*, (2009b) it was shown that Fru alone was not cytotoxic towards hepatocytes, but in the presence of non-toxic levels of H₂O₂ became toxic. Synergic toxicity was found between H₂O₂, glyoxal and D-glyceraldehyde and this effect was Fe(II) dependent. HPLC analysis by Manini *et al.*, (2006) showed that following H₂O₂/Fe(II) oxidation of Fru or glycolaldehyde, glyoxal was the major product that formed. Yang *et al.*, (2011) reported that hydroxypyruvate in the presence of H₂O₂ and Cu(II) and Fe(II) as catalysts, was found to be the most toxic metabolite. This reaction resulted in the formation of ROS, glycolaldehyde and glyoxal (Figure 3.1).

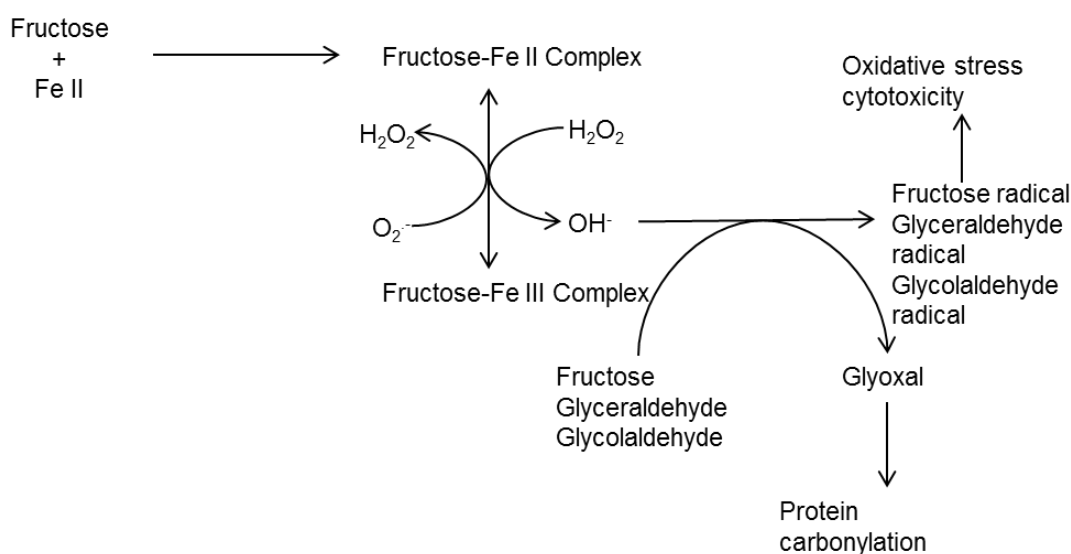


Figure 3.1: Proposed schematic for protein carbonylation and cytotoxicity of Fru metabolites and oxidative products (Dong *et al.*, 2010).

Therefore digestion can have variable effects, such as increased extraction or degradation, a probiotic effect or the formation of toxic Fru metabolites and oxidative products. In this chapter, the effects of GIT digestion on the antioxidant properties of three southern African honey samples compared to Manuka honey was determined. In addition, the observed effects were further studied, using polyphenols and polyphenol (PP) mixtures, as well as a synthetic honey (SH).

The specific objectives were to:

1. Determine the effect of simulated *in vitro* GIT digestion on TPC and antioxidant activity using the ORAC and TEAC assays of the undigested and digested honey samples.
2. Further elucidate, using a PP mix and SH the effects of digestion on polyphenol stability.
3. Determine the CAA using the DCFH-DA assay with Caco-2 cells of undigested and digested honey samples.

4. Further elucidate, using a PP mix and SH the effects of digestion on CAA.

3.2 Materials

3.2.1 Honey samples

Three honey samples identified to have high antioxidant activity from a previous study (Serem, Bester 2012), namely, agricultural Eucalyptus (AE) south eastern Mozambique (SEMh), Western Cape, Fynbos (WC). A commercially available Manuka (MANc) honey was included as a control, due to its published beneficial effects.

3.2.2 Reagents, equipment & disposable plastic ware

Sodium phosphate dibasic dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), sodium phosphate (NaH_2PO_4), sodium chloride (NaCl), dimethyl sulphoxide (DMSO), xylol orange, ammonium ferrous sulphate ($\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), Glc, Fru, maltose (MAL) and sucrose (SUC) were of analytical quality and were obtained from Merck Chemicals, Modderfontein South Africa (SA). Hydrogen peroxide (H_2O_2), Folin-Ciocalteu's (F-C) reagent, sodium carbonate anhydrous (Na_2CO_3), GA, CA, CAT, QUE, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), ABTS (2,2'-Azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt), dichlorofluorescein diacetate (DCFH-DA), sorbitol, potassium peroxodisulfate ($\text{K}_2\text{S}_2\text{O}_8$), Trolox, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and fluorescein sodium salt were obtained from the Sigma-Aldrich Company, Atlasville, SA. Dulbecco's Modified Essential Medium (DMEM), foetal calf serum (FCS), antibiotic solution (streptomycin, penicillin and fungizone) were obtained from Highveld Biological Company, Johannesburg, SA. Trypsin was obtained from Life Technologies Laboratories and was supplied by Gibco BRL products, Johannesburg, SA. Sartorius cellulose acetate membrane filters 0.22 μm were obtained from National Separations, Johannesburg, SA.

Equipment used included: Lambda LS50B spectrophotometer from Perkin Elmer, Boston, MA, USA supplied by Separations Scientific, Honeydew, SA, a BioTek plate reader purchased from Analytical and Diagnostic Products (ADP) Johannesburg, SA was used. A Hermle Z300 centrifuge, 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 labtechnologies, Offenburg, Germany and a water bath from EcoBath Labotec, Cape Town, SA.

Disposable plastic ware included, 96 well plates, 50 mL, 15 mL tubes and pipette tips (10, 25, 100, 200, and 1000 μL) which were obtained from Greiner Bio-one also supplied by LASEC.

3.2.3 Laboratory facilities

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

3.3 Methods

3.3.1 Sample preparation

Honey samples were diluted to 90% (v/v), with ddH₂O to aid with solubility. From this stock solution further dilutions were prepared as required for each assay.

Polyphenol solutions of 1 mg/mL were prepared and these were CA, GA, CAT and QUE. These concentrations were selected based on previous studies where the total polyphenol content of honey from southern Africa was 0.68 – 1.67 mg GAE/mL with a mean of 0.99 mg GAE/mL (Serem, Bester 2012). For the preparations of these solutions, 1 mg/mL of each polyphenol was dissolved in DMSO and solutions of CA, GA, CAT and QUE were prepared. PP mixtures (each polyphenol at a concentration of 1 mg/mL) were also made at double (CA+GA, CA+CAT, CA+QUE, GA+CAT, GA+QUE, CAT+QUE), triple (CA+GA+CAT, CA+GA+QUE, GA+CAT+QUE, CA+CAT+QUE) and quadruple (CA+GA+CAT+QUE) combinations.

A sugar analogue solution containing Fru, Glc, MAL and SUC as found in honey was prepared. The final sugar content was 40% Fru, 30% Glc, 10% MAL and 2% SUC (Beretta *et al.*, 2005, Bertoncelj *et al.*, 2007, Kwakman *et al.*, 2011). For the SH; CA, GA, CAT, QUE at 1 mg/mL each was added to the sugar analogue solution. All honey, PP mixtures and SH were diluted further in buffer or medium depending on assay.

3.3.2 Phosphate buffered saline, Dulbecco's Modified Essential Medium and trypsin

Phosphate buffered saline (PBS) (final concentration 0.1 M) was prepared by mixing three solutions: a) 405 mL of Na₂HPO₄ (12.78 g in 450 mL ddH₂O (0.2 M)), b) 95 mL of NaH₂PO₄·H₂O (2.76 g in 100 mL ddH₂O (0.2 M)) and c) 8.77 g of NaCl, and made up to 1 litre with ddH₂O. pH was then adjusted to 7.4 and stored at room temperature. DMEM was prepared as per manufacturer's instructions, 13.55 g DMEM in 1 litre of dddH₂O, with NaHCO₃ (3.7 g/L). This mixture was adjusted to pH 7.4, sterile filtered and to this 10% foetal calf serum (FCS) and 1% antibiotic solution (streptomycin, penicillin and fungizone) was added. DMEM was stored at 4°C until needed and replaced on a monthly basis. Trypsin was made as a 5% solution (w/v) in 0.1 M PBS and sterile filtered. Trypsin was kept at -4°C until needed.

3.3.3 Simulated *in vitro* gastrointestinal digestion

A simple and efficient *in vitro* digestion method was needed to simulate what happens during digestion. Therefore a modified method of Daglia *et al.*, (2013) was used to simulate the *in vitro* GIT model. This model consisted of subjecting samples (both honey, PP mixtures and SH) to gastric followed by gastroduodenal digestion. For gastric digestion the pH of the samples was lowered to 2 using 1M HCl before the addition of pepsin (20 mg/mL, 5 μ L for every mL of sample) followed by the incubation of the samples in a water bath (37°C for 30 min). Gastroduodenal digestion then followed, which involved increasing the pH of the sample to 7 using 1M NaHCO₃, before adding the pancreatic enzymes (4 mg/mL, 5 μ L for every mL of sample) followed by incubating the samples in a water bath (37°C for 60 min.). Enzyme activity was stopped by heating samples at 95°C for 5 min. Control pH 2 and 7 samples were subjected to digestion without the addition of enzymes. Samples obtained from digestion were: U, GD and GDD.

3.3.4 Total polyphenolic content assay

The Folin-Ciocalteu (F-C) assay is based on an oxidation-reduction reaction during which the phenolate ion is oxidized under alkaline conditions while reducing the phosphotungstic-phospho-molybdic complex in the reagent to a blue coloured solution. The F-C assay was used to determine TPC according to Amin *et al.*, (2006) which was modified for a 96 well format.

F-C reagent was diluted 15x in ddH₂O. Gallic acid (0 - 0.03 mg/mL) was used to prepare the standard curve. To 10 μ L volume of a 10% (v/v) honey solution and 10 μ L of the polyphenol, PP mixtures and SH a 50 μ L volume of F-C was added, followed by a 50 μ L volume of a 7.5% w/v) NaCO₃ solution. The samples were mixed well and the absorbance measured at 630 nm. TPC was expressed as mg gallic acid equivalents (GAE)/g of honey (converted to per gram by incorporating the wet weight of honey) and GAE/mL for the polyphenols, PP mixtures and SH.

3.3.5 Trolox equivalent antioxidant capacity assay

The TEAC assay was done according to Awika *et al.*, (2003) The ABTS^{••} was freshly generated by adding 3 mM of K₂S₂O₈ solution to 8 mM ABTS and the mixture was left to react in the dark for at least 12 hours at room temperature. The working solution was prepared by diluting ABTS stock solution with 0.1 M phosphate buffer, pH 7.4. Trolox was used as a standard, final concentration 0 – 3.3 μ M. A 290 μ L volume of the working solution was added to 10 μ L of a 10% (v/v) honey solution (UD, GD and GDD) as well as the polyphenol solutions, PP mixtures and SH. The reaction mixtures were left to stand at room temperature and the

absorbance was measured at 734 nm after 30 min for the samples and 15 min for the standards, using the Lambda EZ150 spectrophotometer. To eliminate the possible effects of interference each sample served as its own control i.e. all components, no ABTS added. The results were expressed as $\mu\text{mol TE/g}$ of sample for honey (converted to per gram by incorporating the wet weight of honey) and $\mu\text{mol TE/mL}$ for all polyphenol containing solutions.

3.3.6 Oxygen radical absorbance capacity assay

Antioxidant activity was measured with the ORAC assay based on a modified method of Ou *et al.*, (2002b). A concentration of 0.88 mM of fluorescein stock solution was made up in PBS. To prepare the working solution fluorescein stock solution was diluted 100,000x, yielding a fluorescein concentration of 8.82 nM. A volume of 165 μL of working solution fluorescein was added to 10 μL sample followed by 25 μL AAPH (240.74 mM), yielding final concentrations of fluorescein (7.26 nM) and AAPH (30.13 mM). A 10 μL volume of a 10% v/v honey solutions (UD, GD and GDD) as well a polyphenol solutions, mixtures and SH with a final polyphenol concentration of 0.4 mg/mL were added to the wells of a 96 well plate followed by 165 μL fluorescein and 25 μL AAPH. The solutions were mixed well and fluorescence measured at 37°C, every 2 min for 2 hours at an excitation wavelength of 485 nm (Ex_{485}) and an emission wavelength of 520 nm (Em_{520}) using a fluorescence plate reader (FLUOstar Omega, BMG Labtechnologies Offenburg, Germany). The final ORAC values of the samples were calculated by using the net area under the decay curves (AUC) and were expressed as $\mu\text{mol TE/g}$ for honey (converted to per gram by incorporating the wet weight of honey) or $\mu\text{mol TE/mL}$ for all polyphenol containing solutions.

3.3.7 Maintenance of the Caco-2 cell line

Caco-2 cells were maintained in DMEM supplemented with 10% foetal calf serum (FCS) and a 1% antibiotic (penicillin, streptomycin and amphotericin B) solution. Vials were thawed rapidly in warm 37°C water. The cells were suspended in 5 mL supplemented medium and collected by centrifugation (200xg for 2 min). The supernatant was removed and the cells were re-suspended in fresh medium and plated in a 25 cm² culture flask. The flask was then incubated at 37°C at 5% CO₂. Once cells were confluent, medium was removed from the flask and the cells were passaged with a 1 mL 5% trypsin solution (w/v prepared in PBS). When the cells had detached 5 mL fresh medium was added to flask and cells were collected via centrifugation at 200xg for 2 min. The medium was then removed and cells were re-suspended in 5 mL medium. The cell concentration was then determined using the trypan blue exclusion method, by counting a 10 μL aliquot of cells using a haemocytometer and the required the concentration of cells plated was according to the specified protocol.

3.3.8 Cellular protective effects

A modified method of Blasa *et al.*, (2011) was used. Caco-2 cells were plated at a volume of 100 μL at a concentration of 2×10^4 cells/mL in a 96 well plate and were cultured for a further 24 hours at 37°C at 5% CO_2 . To determine the cellular protective effects a 50 μL volume of a 75 μM dichlorofluorescein diacetate (DCFH-DA) solution was added to each well (final concentration 25 μM) and after an incubation period of 1 hour at 37°C , the medium was carefully removed and the cells were washed once with 300 μL PBS. A 50 μL volume of sample (honey final concentration 5% (v/v) UD, GD and GDD) and the polyphenol solutions, polyphenol mixtures and SH was then added followed by 50 μL of AAPH solution (final concentration 7.5 mM). Change in fluorescence was measured every 2 min for 60 min Ex_{485} and Em_{520} using a fluorescence plate reader (BMG Labtechnologies Offenburg, Germany). The gradient of change in fluorescence was measured and data was expressed relative to the effect of AAPH, 100% damage.

3.3.9 Ferrous ion oxidation xylenol orange assay

H_2O_2 formation as the result of NaHCO_3 mediated polyphenol degradation in cell culture medium occurs (Halliwell *et al.*, 2000, Odiatou *et al.*, 2013). The H_2O_2 content of UD, GD and GDD was determined using a slightly modified version of FOX assay (Banerjee *et al.*, 2003). The FOX reagent was prepared as follows: A concentration of 100 μM of xylenol orange was mixed with 100 mM sorbitol to a final volume of 50 mL. A volume of 65 μL H_2SO_4 and a concentration of 250 μM ammonium ferrous sulphate were added to act as a catalyst. A 150 μL volume of the FOX reagent was added to 10 μL of a 10% v/v honey solutions (UD, GD and GDD), the polyphenol solutions, PP mixtures and SH as well as the H_2O_2 standard (standard curve, final concentration range 0 – 0.0375 μM) in a 96 well plate. All samples were then incubated at room temperature in the dark for 30 min, after which the absorbance was measured at 570 nm. Data was expressed as mM H_2O_2 for honey samples and μM H_2O_2 for polyphenols.

3.3.10 Synergistic, antagonistic and additive effects

Using the definitions of Becker *et al.*, (2004) synergistic, antagonistic and additive effects were determined by comparing expected with observed values. If there was a several fold increase in activity or a formed compound compared to expected levels then the reaction is synergistic (S). If there was a reduction in activity or formed compound compared to expected levels then the effect is antagonistic effect (A). Lastly, if the expected was the same as the observed levels of activity or formed compound the effects was a co-additive (C-A) effect.

3.3.11 Liquid chromatography-mass spectrometry analysis

LC–MS analyses was performed on a Waters (Milford, MA, USA) Synapt G2 quadrupole time of flight mass spectrometer coupled to a Waters Acquity ultraperformance liquid chromatograph (UPLC) fitted with an Acquity photo diode array (PDA) detector. Separation was achieved on a Waters acquity UPLC ® HSS T3 (2.1×150 mm, 1.8 µm particle size) with 0.1% formic acid as mobile phase A and acetonitrile + 0.1% formic acid as mobile phase B (Long *et al.*, 2012). The gradient was started with 100% using 0.1% (v/v) formic acid (solvent A) and was kept at 100% for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, 44% solvent B over 4 min and finally to 100% solvent B over 5 min. The column was subjected to 100% solvent B for an additional 2 min. The column was then re-equilibrated over 1 min to yield a total run time of 15 min. A flow rate of 0.4 mL min⁻¹ was applied (Albrecht *et al.*, 2012). Data was then acquired in MSE (mode selecting element) mode, consisting of a scan using low collision energy and a scan using a collision energy ramp from 25 to 60 V. Data was scanned using a scan rate of 0.2 s over the range m/z 100–1000 (Stander *et al.*, 2013).

Samples used were digested and undigested GA, CA, CAT, QUE, GA+CA+CAT+QUE, PP Mix and SH at a polyphenol final concentration of 100 µg/mL. Subsequent dilutions were then performed to make a range of calibration standards used to calibrate the instrument response to the compounds of interest.

3.3.12 Statistical analysis

All experiments were done at least in triplicate (except LC-MS) and represented as mean ± SEM. Analysis was done using the students T-TEST and one way ANOVA followed by the Tukey post-hoc test with significant differences determined at $p < 0.05$.

3.4 Results and discussion

In vitro digestion models have been found to positively correlate with animal or human studies (Bouayed *et al.*, 2011) and also have the advantage of being cheaper, faster, having fewer ethical issues as well as being able to provide important information prior to the implementation of animal models.

To determine the effect of simulated *in vitro* GIT digestion on the antioxidant properties of honey, four honey samples were subjected to gastric and gastroduodenal digestion. Three of these honey samples were from the southern African region and have been shown to have high antioxidant content and activity as well as the ability to protect cells *in vitro* against oxidative damage (Serem, Bester 2012). MANc honey sample was included as a control. Only

the data of the UD and the GDD are presented. All data related to the antioxidant properties of the GD are presented in the Appendices 1 – 6.

3.4.1 With gastroduodenal digestion the antioxidant properties of honey is unaltered

The TPC of UD honey samples ranged from 0.85 – 1.31 mg GAE/g. Levels were similar as reported by Serem, Bester (2012) and within range reported for Burkinabe and Algerian honeys (Beretta *et al.*, 2005, Ouchemoukh *et al.*, 2007). The TPC of SEMh and WC was significantly greater than MANc honey.

Following GD digestion compared to UD the only significant increase in TPC was observed for AE, (Appendix 1). For GDD compared to UD a statistically significant increase in TPC (Table 3.1) of 18% and 24%, respectively for MANc and AE was measured. For GD digests the significantly highest honeys again were SEMh and WC (Appendix 1), while following GDD differences for SEMh and WC were not significant. Compared to MANc, following digestion the TPC of SEMh was significantly greater than MANc honey. O'Sullivan *et al.*, (2013) investigated the effect of GDD on the polyphenol content of four honey samples, Manuka (UMF 5), Irish honey and economy brands (Tesco & Lidl) and found no significant differences in TPC between UD and GDD samples.

For GD digests an increase in TPC may be due to increased extraction of polyphenols at low pH (Bermúdez-Soto *et al.*, 2007). Bermúdez-Soto *et al.*, (2007) identified that in general the polyphenol content of orange juice, pomegranate juice, cocoa, soy bread, raspberry, onions and apples, were either stable/increased with GD but were less stable after GDD. Interestingly, the finding of the study by O'Sullivan *et al.*, (2013) and of this study is that following digestion the TPC of honey is either increased or unchanged.

Antioxidant activity determined with the TEAC assay was the highest for SEMh and activity was significantly greater than MANc honey. With GD (Appendix 1) and GDD (Table 3.1), antioxidant activity was unchanged. With GDD, antioxidant activity was unchanged for MANc, AE and WC and was reduced for SEMh, although activity was still greater than MANc.

Likewise with the ORAC assay, the antioxidant activity of the honey samples were the highest for SEMh>WC>AE>MANc. Following GD (Appendix 1) antioxidant activity was increased for MANc and WC, whereas for GDD antioxidant activity using the ORAC assay was significantly increased for MANc, and SEMh (Table 3.1). The correlations between TPC vs. TEAC and TPC vs. ORAC were 0.76 and 0.75, respectively. A similar high degree of correlation was also

found in a previous study which evaluated the antioxidant properties of honey from the southern African region (Serem, Bester 2012).

Table 3.1: The TPC, TEAC and ORAC activity of UD and GDD honey.

TPC (mg GAE/g)				
	MANc	AE	SEMh	WC
UD	0.85 ± 0.065	0.95 ± 0.05	1.31 ± 0.025 [#]	1.27 ± 0.03 [#]
GDD	1.00 ± 0.037 [*]	1.18 ± 0.07 [*]	1.39 ± 0.12 [#]	1.16 ± 0.11
TEAC assay (µmol TE/g)				
	MANc	AE	SEMh	WC
UD	10.48 ± 0.25	15.45 ± 1.29	22.16 ± 2.10 [#]	17.84 ± 0.99
GDD	9.15 ± 1.20	14.31 ± 0.83	18.16 ± 1.56 [#]	15.43 ± 0.80
ORAC assay (µmol TE/g)				
	MANc	AE	SEMh	WC
UD	87.58 ± 10.88	94.13 ± 4.40	124.43 ± 8.15	114.82 ± 8.14
GDD	131.77 ± 12.36 [*]	117.53 ± 12.48	139.76 ± 11.07 [*]	122.78 ± 15.78

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, UD – undigested, GDD – gastroduodenal digested, TPC – total polyphenol content, TEAC – Trolox equivalent antioxidant capacity, ORAC – oxygen radical absorbance capacity, GAE – gallic acid equivalents, TE – Trolox equivalent.

Data is expressed as an average of at least 3 experiments ± SEM

*Indicates significant differences at p< 0.05, UD compared to GDD, using either one way ANOVA or T-TEST.

[#]Indicates honeys with significantly higher values compared to MANc

Literature states that polyphenols are unstable at pH 7 and undergo degradation as has been described for individual polyphenols (Krook, Hagerman, 2012), tea associated polyphenols (Neilson *et al.*, 2007), grape (Stalmach *et al.*, 2012) and vegetable juices (Wootton-Beard *et al.*, 2011). In contrast, with GDD, there was no decrease in TPC or measured antioxidant activity.

Honey contains a complex mixture of polyphenols (Table 2.3) and factors that could contribute to honey polyphenols being resistant to pH dependent degradation are polyphenol complexity and possibly associated synergistic protective effects (Wahdan 1998, Beretta *et al.*, 2005, Bertoncelj *et al.*, 2007, Kwakman *et al.*, 2011). Due to the differences in the type and the concentrations of polyphenols found in different types of honey such as used in this study, these effects were further investigated using polyphenol solutions and PP mixtures as well as a SH.

3.4.2. With gastroduodenal digestion the antioxidant properties of polyphenols and polyphenol mixtures are reduced

Bicarbonate ions are an important component of the buffering system in cell culture media and the addition of polyphenol compounds to cell culture media has resulted in increased toxicity against several cell lines. Further research attributed this toxicity to the formation of H_2O_2 by polyphenols in the presence of bicarbonate ions (Halliwell *et al.*, 2000, Odiatou *et al.*, 2013). Examples of such studies have been done on, oleuropein and hydroxytyrosol, (Odiatou *et al.*, 2013), (-)-epigallocatechin (EGC), EGCG, CAT and QUE (Long *et al.*, 2010). The authors also reported that in DMEM hydroxytyrosol, delphinidin chloride and rosmarinic acid undergo rapid oxidation to form H_2O_2 . In contrast apigenin, curcumin, hesperetin, naringenin, resveratrol and tyrosol do not undergo oxidation. The authors identified that the presence of adjacent phenol –OH groups are required for the formation of H_2O_2 . For hydroxytyrosol Odiatou *et al.*, (2013), describes that the reaction occurs in three steps: reaction of hydroxides and phenolic hydrogen to form phenoxide anions; followed by the phenoxide anion reacting with oxygen to form phenoxyl radicals and finally the phenoxyl radical forms H_2O_2 and o-benzoquinone. Likewise in the GDD phase of digestion, bicarbonate ions are also present and it can be assumed that at the neutral pH associated with duodenal digestion polyphenols will degrade. However, in honey, polyphenols appear to be resistant to degradation (Table 3.1).

To elucidate the factors that prevent polyphenol degradation, the effect of digestion on the stability of polyphenols alone and as part of mixtures was evaluated. The TPC and antioxidant activity with the TEAC and ORAC assays was determined.

After GDD of CA, GA, CAT and QUE, a statistically significant loss of TPC was found for GA and CAT while the TPC for CA was unchanged (Table 3.2). CA appears to be resistant to pH dependent degradation. The slight increased TPC observed for QUE may be related to the increased solubility of this flavonoid. For all polyphenol combinations there was a statistically significant loss of TPC. The highest fold loss for the double combinations was 4.14 for CAT+QUE and the least was 1.42 for CA+GA. The triple combinations lost TPC content in the range of 1.52 – 2.65 fold, while in the quadruple combination GA+CA+CAT+QUE, the fold loss of TPC was 2.17. To summarise, with GDD the TPC content of polyphenols alone, except for CA or as part of a polyphenol mixture, was reduced.

Table 3.2: TPC (mg GAE/mL) of UD and GDD polyphenols and PP mixtures.

<u>Single polyphenols</u>						
	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>		
UD	0.04 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.02 ± 0.004		
GDD	0.05 ± 0.01	0.04 ± 0.003*	0.08 ± 0.01*	0.04 ± 0.01		
<u>Double combinations</u>						
	<u>CA +GA</u>	<u>GA+CAT</u>	<u>GA+QUE</u>	<u>CA+CAT</u>	<u>CA+QUE</u>	<u>CAT+QUE</u>
UD	0.17 ± 0.02	0.22 ± 0.02	0.18 ± 0.02	0.26 ± 0.03	0.18 ± 0.04	0.29 ± 0.02
GDD	0.12 ± 0.01*	0.11 ± 0.01*	0.12 ± 0.02*	0.11 ± 0.01*	0.12 ± 0.01*	0.07 ± 0.01*
<u>Triple combinations</u>						
	<u>GA+CA+CAT</u>	<u>GA+CA+QUE</u>	<u>GA+CAT+QUE</u>	<u>CA+CAT+QUE</u>		
UD	0.38 ± 0.04	0.39 ± 0.04	0.37 ± 0.05	0.34 ± 0.05		
GDD	0.25 ± 0.01*	0.23 ± 0.01*	0.14 ± 0.002*	0.16 ± 0.01*		
<u>Quadruple combinations</u>						
	<u>GA+CA+CAT+QUE</u>					
UD	0.50 ± 0.03					
GDD	0.23 ± 0.01*					

TPC – total polyphenol content, GAE – gallic acid equivalent, CA – caffeic acid, GA – gallic acid, CAT – catechin, QUE – quercetin, UD – undigested, GDD – gastroduodenal digested.

Each polyphenol in solution was at a concentration of 0.1 mg/mL.

Data is expressed as an average of at least 3 experiments ± SEM.

*Indicates significant differences at $p < 0.05$, GDD compared to UD, using one way ANOVA or a T-TEST.

Antioxidant activity measured with the TEAC assay revealed the same trend as observed for TPC. For the single polyphenols there was a significant loss in antioxidant activity with GDD for GA, CAT and QUE while activity for CA was unchanged (Table 3.3). The greatest loss antioxidant activity of 10.25 fold was found for GA. For the double polyphenol combinations, there was a significant loss in antioxidant activity of 1.44 – 6.17 fold with the greatest loss for CAT+QUE. Evaluation of the triple polyphenol combinations revealed a 2.09 – 4.36 fold loss of antioxidant activity, with the greatest decrease for the combination of GA+CAT+QUE. For the combination GA+CA+CAT+QUE loss of antioxidant activity was 3.41 fold (Table 3.3).

Table 3.3: Antioxidant activity: TEAC assay ($\mu\text{mol TE/mL}$) of UD and GDD polyphenols and PP mixtures.

<u>Single</u>						
	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>		
UD	0.11 \pm 0.03	0.41 \pm 0.03	0.29 \pm 0.03	0.25 \pm 0.02		
GDD	0.07 \pm 0.02	0.04 \pm 0.01*	0.13 \pm 0.02*	0.14 \pm 0.03*		
<u>Double combinations</u>						
	<u>CA+GA</u>	<u>GA+CAT</u>	<u>GA+QUE</u>	<u>CA+CAT</u>	<u>CA+QUE</u>	<u>CAT+QUE</u>
UD	0.41 \pm 0.03	0.54 \pm 0.04	0.33 \pm 0.02	0.59 \pm 0.01	0.27 \pm 0.03	0.74 \pm 0.04
GDD	0.19 \pm 0.02*	0.18 \pm 0.02*	0.23 \pm 0.02*	0.21 \pm 0.02*	0.16 \pm 0.02*	0.12 \pm 0.02*
<u>Triple combinations</u>						
	<u>GA+CA+CAT</u>	<u>GA+CA+QUE</u>	<u>GA+CAT+QUE</u>	<u>CA+CAT+QUE</u>		
UD	0.86 \pm 0.04	0.67 \pm 0.01	0.96 \pm 0.04	0.63 \pm 0.02		
GDD	0.37 \pm 0.05*	0.32 \pm 0.03*	0.22 \pm 0.03*	0.23 \pm 0.03*		
<u>Quadruple combinations</u>						
	<u>GA+CA+CAT+QUE</u>					
UD	0.99 \pm 0.08					
GDD	0.29 \pm 0.03*					

TEAC – Trolox equivalent antioxidant capacity, TE – Trolox equivalent, CA – caffeic acid, GA – gallic acid, CAT – catechin, QUE – quercetin UD – undigested, GDD – gastroduodenal digested.

Each polyphenol in solution was at a concentration of 0.1 mg/mL.

Data is expressed as an average of at least 3 experiments \pm SEM.

*Indicates significant differences at $p < 0.05$, GDD compared to UD, using one way ANOVA or a T-TEST.

Evaluation of the antioxidant of CA, GA, CAT and QUE with the ORAC assay showed that the antioxidant activity of CA following GDD was 1.40 fold increased while that of GA, CAT and QUE was reduced by 3.85, 1.38 and 2.72, fold respectively (Table 3.4). For the combination GA+CA antioxidant activity following GDD was increased by 1.38 fold, unchanged for GA+QUE and reduced by a range 1.43 – 3.08 for all other double combinations. Measured antioxidant activity was reduced with a range of 1.06 – 1.82 fold for all triple polyphenol combinations and was 1.31 fold reduced for GA+CA+CAT+QUE.

Table 3.4: Antioxidant activity: ORAC assay ($\mu\text{mol TE/mL}$) of UD and GDD polyphenols and PP mixtures.

<u>Single</u>						
	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>		
UD	0.38 \pm 0.10	0.35 \pm 0.07	0.66 \pm 0.05	0.68 \pm 0.15		
GDD	0.53 \pm 0.09*	0.13 \pm 0.07*	0.48 \pm 0.07*	0.25 \pm 0.05*		
<u>Double combinations</u>						
	<u>GA+CA</u>	<u>GA+CAT</u>	<u>GA+QUE</u>	<u>CA+CAT</u>	<u>CA+QUE</u>	<u>CAT+QUE</u>
UD	0.47 \pm 0.05	1.04 \pm 0.02	0.97 \pm 0.05	1.60 \pm 0.05	1.44 \pm 0.07	1.70 \pm 0.01
GDD	0.65 \pm 0.04*	0.58 \pm 0.05*	0.93 \pm 0.04	0.52 \pm 0.03*	1.01 \pm 0.05*	0.73 \pm 0.02*
<u>Triple combinations</u>						
	<u>GA+CA+CAT</u>	<u>GA+CA+QUE</u>	<u>GA+CAT+QUE</u>	<u>CA+CAT+QUE</u>		
UD	1.77 \pm 0.13	1.75 \pm 0.14	1.50 \pm 0.12	1.53 \pm 0.17		
GDD	1.66 \pm 0.13*	0.96 \pm 0.09*	0.97 \pm 0.07*	1.06 \pm 0.10*		
<u>Quadruple combinations</u>						
	<u>GA+CA+CAT+QUE</u>					
UD	1.59 \pm 0.11					
GDD	1.21 \pm 0.14*					

ORAC – oxygen radical absorbance capacity, TE – Trolox equivalent, CA – caffeic acid, GA – gallic acid, CAT – catechin, QUE – quercetin, UD – undigested, GDD – gastroduodenal digested.

Each polyphenol in solution was at a concentration of 0.1 mg/mL.

Data is expressed as an average of at least 3 experiments \pm SEM.

*Indicates significant differences at $p < 0.05$, GDD compared to UD, using one way ANOVA or a T-TEST.

Differences between the levels of reduction in the antioxidant activity measured with the TEAC assay vs. the ORAC assay may be based on the differences in the principles of each assay, where the TEAC assay as an end point assay does not take into account differences in reaction rates. Also no correlation between TEAC values and the number of electrons donated has been reported (Huang *et al.*, 2014). GSH, ascorbic acid, α -tocopherol and uric acid have similar TEAC values of 1.28, 1.05, 0.97 and 1.01, respectively. However, GSH has only one electron to donate while ascorbic acid, α -tocopherol and uric acid can donate two electrons. CA and ferulic acid that are structurally similar have reported TEAC values of 2 and 1.90, respectively, however QUE and kaempferol that are also structurally similar have TEAC values of 3 and 1, respectively. These differences may be related to differences in the rate of reactions, which accounts for the 15 min vs. 30 min required for the Trolox standard and honey samples, respectively (Section 3.3.5). The ORAC assay on the other hand is hydrogen atom transfer based and time-based quantification takes into account differences in the rate of reactions and therefore is more precise and accurate (Huang *et al.*, 2014).

It can be concluded that polyphenols in mixtures such as found in honey are susceptible to GDD degradation, however some polyphenols such as CA seem to be more resistant to degradation at neutral pH associated with GDD and when combined with other polyphenols

may reduce the degree of degradation. The findings presented (Table 3.2, 3.3 and 3.4) confirms that with digestion polyphenols degrade in the same manner that polyphenols degrade in cell culture media containing NaHCO_3 .

However, the degree or extent of degradation may be reduced or less than expected due to synergistic effects when phenolic compounds transfer electrons to each other causing regeneration and a reduction in free radicals (Palafox-Carlos *et al.*, 2012). Alternatively occurring at a neutral pH, preventing degradation. Becker *et al.*, (2004) defined synergism in antioxidant evaluation protocols as 'A number of compounds when present together have a more pronounced effect than that which would be derived from a simple additive concept'. The same effect as the sum of the individual components is known as an additive or co-antioxidant effect. Antagonism occurs when an antioxidant reduces the antioxidant activity of another antioxidant. Heo *et al.*, (2007), evaluated the activity of double and triple combinations of polyphenols (chlorogenic acid, CAT, cyanidin 3-rutinoside and quercetin 3-rutinoside) using the TEAC assay. Findings of this study were that only an additive or co-additive effects were observed with no synergism or antagonism. Palafox-Carlos *et al.*, (2012) studied the effects of various phenolic acids (chlorogenic acid, GA, protocatechuic acid and vanillic acid) as double, triple and quadruple mixtures using the DPPH assay. Individually GA had the highest antioxidant activity. Additive/co-antioxidant effects were observed for protocatechuic acid + vanillic acid and chlorogenic acid + vanillic acid. Antagonistic effects were observed only for GA + vanillic acid and protocatechuic acid + chlorogenic acid + vanillic acid.

A similar strategy can be used to determine if as part of mixtures such as found in honey, polyphenol degradation at neutral pH is reduced due to synergistic interactions i.e. if degradation of a polyphenol is inhibited by the presence of another polyphenol. For TPC synergistic effects were observed for GA+QUE, CA+CAT, CA+QUE and CAT+QUE, all triple and the quadruple combinations (Table 3.5). This implies that with increased complexity of polyphenol mixtures such as found in honey, polyphenol degradation can be to some degree inhibited.

The TEAC assay interactions were mostly co-antioxidant effects with 3 combinations showing antagonistic effects (GA+CAT, GA+QUE and CA+QUE) and 2 combinations showing synergistic effects (CA+CAT and CAT+QUE) (Table 3.5). For the ORAC assay, combinations CA+CAT, CA+QUE, CAT+QUE, only GA+CA+CAT were synergistic. For all assays synergistic effects were observed for CA+CAT and CAT+QUE (Table 3.5). Findings are that polyphenol degradation in complex mixtures is a function of type and concentration of other polyphenols that can either protect or promote polyphenol degradation. .

Table 3.5: Antioxidant interactions between combined polyphenols.

<u>Single</u>	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>		
Observed – TPC	0.04 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.02 ± 0.004		
Observed – TEAC	0.11 ± 0.03	0.41 ± 0.03	0.29 ± 0.03	0.25 ± 0.02		
Observed – ORAC	0.38 ± 0.10	0.35 ± 0.07	0.66 ± 0.05	0.68 ± 0.15		
<u>Double combinations</u>	<u>GA+CA</u>	<u>GA+CAT</u>	<u>GA+QUE</u>	<u>CA+CAT</u>	<u>CA+QUE</u>	<u>CAT+QUE</u>
Expected – TPC	0.15	0.21	0.13	0.14	0.08	0.12
Observed – TPC	0.17 ± 0.02 (C-A)	0.22 ± 0.02 (C-A)	0.18 ± 0.02* (S)	0.26 ± 0.03* (S)	0.18 ± 0.04* (S)	0.29 ± 0.02* (S)
Expected – TEAC	0.52	0.70	0.66	0.40	0.36	0.54
Observed – TEAC	0.41 ± 0.03 (C-A)	0.54 ± 0.04* (A)	0.33 ± 0.02* (A)	0.59 ± 0.01* (S)	0.27 ± 0.03* (A)	0.74 ± 0.04* (S)
Expected – ORAC	0.73	1.01	1.03	1.04	1.06	1.34
Observed – ORAC	0.47 ± 0.05 (C-A)	1.04 ± 0.02 (C-A)	0.97 ± 0.05 (C-A)	1.60 ± 0.05* (S)	1.44 ± 0.07* (S)	1.70 ± 0.01* (S)
<u>Triple combinations</u>	<u>GA+CA+CAT</u>	<u>GA+CA+QUE</u>	<u>GA+CAT+QUE</u>	<u>CA+CAT+QUE</u>		
Expected – TPC	0.25	0.17	0.23	0.16		
Observed – TPC	0.38 ± 0.04* (S)	0.39 ± 0.04* (S)	0.37 ± 0.05* (S)	0.34 ± 0.05* (S)		
Expected – TEAC	0.81	0.77	0.95	0.65		
Observed – TEAC	0.86 ± 0.04 (C-A)	0.67 ± 0.01 (C-A)	0.96 ± 0.04 (C-A)	0.63 ± 0.02 (C-A)		
Expected – ORAC	1.39	1.41	1.69	1.72		
Observed – ORAC	1.77 ± 0.13* (S)	1.75 ± 0.14 (C-A)	1.50 ± 0.12 (C-A)	1.53 ± 0.17 (C-A)		
<u>Quadruple combinations</u>	<u>GA+CA+CAT+QUE</u>					
Expected – TPC	0.27					
Observed – TPC	0.50 ± 0.03* (S)					
Expected – TEAC	1.06					
Observed – TEAC	0.99 ± 0.08 (C-A)					
Expected – ORAC	2.07					
Observed – ORAC	1.59 ± 0.11 (C-A)					

TPC – total polyphenol content, TEAC – Trolox equivalent antioxidant capacity, ORAC – oxygen radical absorbance capacity, CA – caffeic acid, GA – gallic acid, CAT – catechin, QUE – quercetin, S = synergistic effects, C-A = co-additive effects and A = antagonistic effects.

Each polyphenol in solution was at a concentration of 0.1 mg/mL. Data is expressed as an average of at least 3 experiments ± SEM. *Indicates significant differences between observed and expected values at $p < 0.05$, using the T-TEST.

To establish if H₂O₂ formation did occur as a result of polyphenol degradation under gastroduodenal conditions as has been described for cell culture media (Long *et al.*, 2010) the H₂O₂ levels in GDD of CA, GA, CAT and QUE alone and in combination were determined. The H₂O₂ content of the UD polyphenols and polyphenol mixtures ranged from 0.02 – 0.27 μM, with the highest being the CA+GA+CAT+QUE combination (Table 3.6). H₂O₂ remains relatively unchanged with GD (Appendix, Table A6) with levels of 0.05 – 0.13 μM. With GDD a significant increase in H₂O₂ levels was measured for all polyphenols and polyphenol mixtures, Table 3.6. With digestion, the highest H₂O₂ level for individual polyphenols was found for GA which was 17 fold greater than in the UD sample. For the polyphenol combinations the greatest increase in H₂O₂ formation was observed for the combination of GA+CAT, with a 77 fold increase in H₂O₂ levels (Table 3.6).

Table 3.6: H₂O₂ levels (μM) in UD and GDD polyphenols and PP mixtures.

Single						
	CA	GA	CAT	QUE		
UD	0.10 ± 0.01	0.07 ± 0.03	0.08 ± 0.04	0.08 ± 0.02		
GDD	0.87 ± 0.08*	1.22 ± 0.12*	1.08 ± 0.13*	0.44 ± 0.07*		
Fold	9	17	14	6		
Double combinations						
	GA+CA	GA+CAT	GA+QUE	CA+CAT	CA+QUE	CAT+QUE
UD	0.06 ± 0.04	0.02 ± 0.06	0.19 ± 0.03	0.09 ± 0.01	0.09 ± 0.02	0.11 ± 0.05
GDD	1.46 ± 0.19*	1.54 ± 0.22*	1.10 ± 0.12*	0.81 ± 0.14*	1.05 ± 0.14*	1.20 ± 0.18*
Fold	24	77	6	9	12	11
Triple combinations						
	GA+CA+CAT	GA+CA+QUE	GA+CAT+QUE	CA+CAT+QUE		
UD	0.10 ± 0.02	0.12 ± 0.02	0.14 ± 0.03	0.08 ± 0.04		
GDD	1.38 ± 0.15*	1.01 ± 0.05*	1.30 ± 0.14*	1.29 ± 0.12*		
Fold	14	8	9	16		
Quadruple combinations						
	GA+CA+CAT+QUE					
UD	0.27 ± 0.02					
GDD	1.41 ± 0.13*					
Fold	5					

CA – caffeic acid, GA – gallic acid, CAT – catechin, QUE – quercetin, PP – polyphenol mixture, H₂O₂ – hydrogen peroxide, UD – undigested, GDD – gastroduodenal digested.

Each polyphenol was assayed at a concentration of 0.1 mg/mL.

Data is expressed as an average of at least 3 experiments ± SEM.

*Indicates significant differences at p< 0.05, GDD compared to UD, using one way ANOVA or a T-TEST.

All polyphenols and polyphenol samples were prepared in 1% DMSO/PBS and at this pH some degradation may have already occurred and this could account for the levels of H₂O₂ measured

in the UD samples. Hanham *et al.*, (1983) identified that CA undergoes auto-oxidation and in a phosphate-buffered saline/dextrose solution and concentration of H₂O₂ in a freshly prepared CA solution the molar ratio increased from 1:0.003 to 1:0.0324, an increase in H₂O₂ levels from 3 µM to 32.4 µM. Similarly in all UD polyphenol solutions some auto-oxidation was observed. Following GDD the H₂O₂ levels measured in all polyphenols and PP mixtures was increased. Using the same assay as in the present study, Schuck *et al.*, (2013) reported that in cell culture media 100 µM GA forms 108 ± 3.6 µM H₂O₂ (molar ratio 1:1.08) after 4 hours. In the present study with GDD for 1 hour, 0.1 mg/mL (588 µM) GA, final concentration 36.75 µM forms 1.22 ± 0.12 µM H₂O₂ a molar ratio of 1:0.03, much lower to that reported by Schuck *et al.*, (2013). Long *et al.*, (2000) reported that after 1 hour in DMEM media, 100 µM QUE formed 25.4 ± 6.2 µM and CAT formed 6.1 ± 2.2 µM H₂O₂ with a molar ratio of 1:0.25 and 1:0.061, respectively, which is similar to that found in the present study. Likewise with GDD the amount of H₂O₂ that formed was increased for CA, GA, CAT and QUE (Table 3.6).

Kurin *et al.*, (2012) reported that resveratrol, QUE, ethyl gallate and CAT found in red wine inhibited the proliferation of vascular smooth cells thereby reducing the development of atherosclerosis. Strong synergism was found between QUE and resveratrol and moderate synergism between QUE and CAT. These synergistic effects in the inhibition of cellular proliferation may be due to increased H₂O₂ formation or polyphenol targeting of proliferation.

Possible synergistic effects were evaluated to determine whether in complex mixtures the synergistic interaction between polyphenols (Table 3.7) reduces the levels of H₂O₂ that forms (antagonistic H₂O₂ formation) or alternatively if antagonistic effects increases the expected levels of H₂O₂ (synergistic H₂O₂ formation). For all combinations the polyphenols acted synergistically to cause antagonistic formation of H₂O₂, where the observed levels of H₂O₂ were less than expected, which confirms that synergistic interactions occur in complex mixtures where polyphenols inhibit polyphenol degradation and reduce H₂O₂ formation.

Table 3.7: Observed and expected H₂O₂ levels (µM) in GDD polyphenols and PP mixtures.

Single						
	CA	GA	CAT	QUE		
Observed	0.87 ± 0.08	1.22 ± 0.12	1.08 ± 0.13	0.44 ± 0.07		
Double combinations						
	GA+CA	GA+CAT	GA+QUE	CA+CAT	CA+QUE	CAT+QUE
Expected	2.09	2.30	1.66	1.95	1.31	1.52
Observed	1.46 ± 0.19	1.54 ± 0.22	1.10 ± 0.12	0.81 ± 0.14	1.05 ± 0.14	1.20 ± 0.18
Effect	A	A	A	A	A	A
Triple combinations						
	GA+CA+CAT	GA+CA+QUE	GA+CAT+QUE	CA+CAT+QUE		
Expected	3.17	2.53	2.74	2.39		
Observed	1.38 ± 0.15	1.01 ± 0.05	1.30 ± 0.14	1.29 ± 0.12		
Effect	A	A	A	A		
Quadruple combinations						
	GA+CA+CAT+QUE					
Expected	3.61					
Observed	1.41 ± 0.13					
Effect	A					

CA – caffeic acid, GA – gallic acid, CAT – catechin, QUE – quercetin, PP – polyphenol mixture, A – antagonistic effects, nd – not determined, GDD – gastroduodenal digested, H₂O₂ – hydrogen peroxide.

Data is expressed as an average of at least 3 experiments ± SEM.

3.4.3. Sugar in honey, protects polyphenols against pH dependent degradation

For all honeys evaluated no degradation was observed as generally TPC and antioxidant activity was either unchanged or slightly increased. Under the conditions used in this study, it was found that polyphenols do undergo degradation, which is associated with increased H₂O₂ formation. Therefore other molecules besides polyphenols may be protecting the polyphenols found in honey against degradation. As sugars are the major component of honey the possibility of these sugars protecting polyphenols against oxidative damage was further evaluated.

GDD resulted in a decrease in the TPC and antioxidant activity of individual polyphenols and polyphenol combinations. The effect of GDD on the polyphenol content and antioxidant activity of PP mix (CA+GA+CAT+QUE) and SH (sugars as found in honey containing PP mix) was evaluated (Table 3.8). The measured TPC of the undigested SH was less than PP mix alone. This implies that sugars, inhibit polyphenol determination with the F-C assay while no such inhibition was observed for antioxidant activity measured with the TEAC and ORAC assays.

With gastroduodenal digestion of the PP mix, there was a statistically significant fold decrease of 2.17, 3.41 and 1.31 fold in TPC and antioxidant activity measured with the TEAC and ORAC

assays, respectively. For SH there was no significant change in TPC and antioxidant activity measured with the ORAC assay (Table 3.8). In contrast with the TEAC assay measured antioxidant activity was reduced, however this was less than that observed for the PP mix (3.41 vs 1.74). This implies that sugars found in honey may prevent the pH dependent degradation of polyphenols.

Table 3.8: Antioxidant content and activity of UD and GDD, PP mix and SH.

	TPC (mg GAE/mL)		TEAC (μmol TE/g)		ORAC (μmol TE/g)	
	PP mix	SH	PP mix	SH	PP mix	SH
UD	0.50 ± 0.03	0.19 ± 0.01 [#]	0.99 ± 0.08	1.20 ± 0.06	1.59 ± 0.13	1.45 ± 0.10
GDD	0.23 ± 0.01*	0.18 ± 0.02 [#]	0.29 ± 0.03*	0.69 ± 0.10* [#]	1.21 ± 0.15*	1.42 ± 0.11
Fold	2.17	1.06	3.41	1.74	1.31	1.02

TPC – total polyphenol content, TEAC – Trolox equivalent antioxidant capacity, ORAC – oxygen radical absorbance capacity, GAE – gallic acid equivalent, TE – Trolox equivalent, CA – caffeic acid, GA – gallic acid, CAT – catechin, QUE – quercetin, PP – polyphenol mixture, SH – synthetic honey, UD – undigested, GDD – gastroduodenal digested.

For PP mix and SH, final polyphenol concentration 0.4mg/mL.

Data is expressed as an average of at least 3 experiments ± SEM.

*Indicates significant differences at p< 0.05, GDD compared to UD, using one way ANOVA or a T-TEST.

[#]Indicates significant differences at p< 0.05, PP mix compared to PP mix + sugar.

Bold indicates fold loss that is statistically significant.

To confirm whether the presence of sugars protect against polyphenol degradation, the amount of each polyphenol in individual PP samples, all PP mix and SH was determined by LC-MS analysis. In the PP mix with GDD only CA was resistant to the effects of digestion where 84.7% CA remained while levels of GA, CAT and QUE were reduced. GDD digestion of the SH showed that the presence of sugars protected polyphenols CA, GA and CAT against degradation with 93.89%, 77.51% and 94.33% of CA, GA and CAT remaining after GDD (Table 3.9).

In summary, the polyphenol content and antioxidant activity of four honey samples was either increased or unchanged after GD and GDD. This is in contrast with findings of other studies where GDD reduces the antioxidant properties of juices or fruit extracts (Cilla *et al.*, 2009, Tagliacruzchi *et al.*, 2010). This part of the study confirmed that at neutral pH and in the presence of NaHCO₃, polyphenols degrade with the formation of H₂O₂. The degree of degradation was related to the complexity of the polyphenol mixture. The stability of polyphenols in honey was due to the protective effect of the sugars found in honey and possibly to some degree synergism between polyphenols.

Table 3.9: LC-MS analysis of percentage polyphenol content of UD and GDD in PP mix and SH.

<u>Individual PP</u>	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>
<u>UD</u>	24.7	53.9	38.2	1.1
<u>GDD</u>	0.2	nd	0.5	nd
% Remaining	0.04%	0%	0.13%	0%
<u>PP mix</u>	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>
<u>UD</u>	32.7	57.8	42.3	nd
<u>GDD</u>	24.7	nd	0.7	nd
% Remaining	84.7%	0%	1.65%	0%
<u>SH</u>	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>
<u>UD</u>	32.7	57.8	42.3	nd
<u>GDD</u>	30.7	44.8	39.3	nd
% Remaining	93.89%	77.51%	94.33%	nd

CA – caffeic acid, GA – gallic acid, CAT – catechin, QUE – quercetin, PP – polyphenol mixture, SH – synthetic honey, UD – undigested, GDD – gastroduodenal digested.

3.4.4. With gastroduodenal digestion, hydrogen peroxide formation causes a loss of cellular antioxidant activity

The antioxidant activity of the honey samples evaluated in this study are unaltered and therefore to further confirm these beneficial properties of honey it was necessary to determine if these effects also occur in a biologically relevant environment. Standard antioxidant assays only measure changes in the antioxidant activity usually associated with the presence of polyphenols. Honey is a complex mixture of many types of molecules which have been shown to be cytotoxic and these include Glc, Fru (Figure 3.1) and MGO.

Antioxidant assays such as the TEAC and ORAC assays provide no information on cellular aspects of antioxidant activity which includes the uptake, distribution as well as possible cellular toxicity. In the present study, for the determination of CAA, the Caco-2 cells, a colon adenocarcinoma cell line was used. This is a GIT cell line and therefore is physiologically relevant when investigating antioxidant effects during the digestion process. In addition the CAA is similar to the ORAC assay as in both assays AAPH is used to generate physiologically relevant peroxy radicals while the CAA provides more information of effects in a physiological environment.

In the CAA assay, AAPH induces 100% oxidative damage and in the presence of individual polyphenols and complex mixtures such as honey and plant extracts containing polyphenols the percentage oxidative damage is reduced. Undigested honey samples, MANc, AE, SEMh and WC reduce the % oxidative damage by 61.64%, 71.36%, 58.80% and 69.76%, respectively. AE had the highest CAA levels while the lowest was measured for SEM (Table 3.10). With gastric digestion, CAA was unchanged for MANc and AE and was increased for SEMh and WC (Appendix 5) indicating that at a low pH, polyphenols are stable and a low pH favours increased

polyphenol extraction. This implies that with gastric digestion honey can protect the mucosa of the stomach against oxidative damage. In addition, the polyphenol content of the honey samples is sufficient to counteract Fru mediated formation of oxidative molecules (Figure 3.1).

With GDD, CAA was lost and a pro-oxidant effect was observed (Table 3.10). For MANc, AE and WC the measured CAA was greater than the 100% oxidative damage measured for AAPH alone. The fold increase compared to UD was 3.24, 5.02, 1.80 and 5.47 for MANc, AE, SEMh and WC, respectively with WC, AE, MANc > SEMh. No correlation was found between CAA and assays used to determine polyphenol content and antioxidant activity. In contrast, to the UD and GD samples with GDD, the CAA of honey is lost and a strong toxic, pro-oxidant effect is observed.

Table 3.10: DCFH DA assay (% oxidative damage) of UD and GDD honey.

	<u>MANc</u>	<u>AE</u>	<u>SEMh</u>	<u>WC</u>
UD	38.36 ± 10.10	28.64 ± 4.11	41.20 ± 3.46	30.24 ± 5.65
GDD	124.08 ± 23.70*#	143.79 ± 25.75*#	74.10 ± 10.03*	165.41 ± 33.05*#
Fold increase	3.24 (223.45%↑)	5.02 (402.03%↑)	1.80 (79.86%↑)	5.47 (447.10%↑)

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, DCFH-DA – dichlorofluorescein diacetate, UD – undigested, GDD – gastroduodenal digested.

Data is expressed as an average of at least 3 experiments ± SEM.

*Indicates significant differences at p< 0.05, GDD compared to UD, using one way ANOVA or a T-TEST.

#Indicates highest values amongst honeys.

Few studies where honey has undergone digestion then subjected to CAA assays have been done, making comparison of these results to other studies difficult. O’Sullivan *et al.*, (2013) digested honeys and measured cellular viability of exposed Caco-2 cells. Results showed that cytotoxicity was observed at 2.5 – 7.5 mg/mL for undigested honey and 1 – 3 mg/mL for digested honeys, which implies that with digestion there is an increase in cytotoxicity.

Many honeys contain H₂O₂ and the presence of H₂O₂ has been identified as one of the molecules responsible for the antibacterial activity of honey. Honey has inherent H₂O₂ content formed by the oxidation of Glc by the enzyme glucose oxidase when honey is ripening (Irish *et al.*, 2011). Honey types with high inherent H₂O₂ content are Buckwheat honeys from Canada with a H₂O₂ content of 0.248 ± 0.02 – 2.68 ± 0.04 mM (Brudzynski *et al.*, 2011). Increased cytotoxicity may be related to increased H₂O₂ levels due to dilution effects or some degree of polyphenol degradation and therefore H₂O₂ levels in the GDD were measured.

The H₂O₂ content of MANc, AE, SEMh and WC honey was 2.17, 0.4, 1.57 and 0.91 mM, respectively (Table 3.11). With GD, H₂O₂ levels remained relatively unchanged (Appendix 5), but

following GDD there was a significant increase in the formation of H₂O₂ which was statistically increased from that measured in the UD except for SEMh. Levels increased by 1.87, 7.85, 1.48 and 2.91 fold for MANc, AE, SEMh and WC, respectively with a range of 2.32 ± 0.22 – 4.06 ± 0.25 Mm (Table 3.11).

Table 3.11: H₂O₂ (mM) of UD and GDD honey.

	MANc	AE	SEMh	WC
UD	2.17 ± 0.12 [#]	0.4 ± 0.14	1.57 ± 0.38 [#]	0.91 ± 0.23
GDD	4.06 ± 0.25 ^{*#}	3.14 ± 0.01 [*]	2.32 ± 0.22	2.65 ± 0.25 [*]
Fold increase	1.87	7.85	1.48	2.91

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, H₂O₂ – hydrogen peroxide, UD – undigested, GDD – gastroduodenal digested.

Data is expressed as an average of at least 3 experiments ± SEM.

*Indicates significant differences at p< 0.05, GDD compared to UD, using one way ANOVA or a T-TEST.

[#]Indicates highest value amongst honeys.

Perna *et al.*, (2012) reported that the most abundant metals in Italian honey are Fe and Zn with levels of 15.04 ± 10.12 and 11.85 ± 11.24 ppm, respectively. The Fe and Zn content of Manuka honey was 16.34 ± 0.85 and 4.57 ± 0.15 mg/kg, respectively (Kek *et al.*, 2016). These metals are sufficient to catalyse the formation of hydroxyl radicals from H₂O₂. Therefore in honey the concentration of metal ions, such as Fe can catalyse the formation of hydroxyl radicals and should be determined.

Using a PP mixture and a SH, the effect of polyphenol degradation and the effect of increased levels of H₂O₂ on CAA was determined. The PP mix after GDD still effectively protected Caco-2 cells against oxidative damage. Following GDD, although increased due to polyphenol degradation, sufficient amounts of non-degraded polyphenols e.g. CA are able to scavenged generated radicals. A similar pattern emerged for the SH, although no change in the % CAA was observed due to sugars protecting polyphenols from degradation (Table 3.12).

Table 3.12: Percentage oxidative damage and H₂O₂ (μM) levels for UD and GDD polyphenols.

	% Cellular antioxidant activity		H ₂ O ₂ (μM)	
	PP mix	SH	PP mix	SH
UD	13.99 ± 4.59	15.88 ± 6.27	0.27±0.02	0.44±0.08
GDD	19.29 ± 11.33	13.05 ± 7.14	1.41±0.13*	1.88±0.15*
Percentage change	37.53% ↑	-	5↑	4.3↑

H₂O₂ – hydrogen peroxide, PP – polyphenol mixture, SH – synthetic honey, UD – undigested, GDD – gastroduodenal digested.

Each polyphenol was assayed at a concentration of 0.1 mg/mL.

Data is expressed as an average of at least 3 experiments ± SEM.

*Indicates significant differences at p< 0.05, GDD compared to UD, using one way ANOVA or a T-TEST.

With GDD a significant increase in H₂O₂ levels was measured for all polyphenols and polyphenol mixtures (Table 3.6) although this did reduce %CAA for the polyphenol mix, levels were not above 100% as observed for MANc, SEMh and WC (Table 3.12).

No probable reason for the observed strong pro-oxidant effect could be found. However, Girard-Lalancette *et al.*, (2009) reported that carrot and broccoli juice with high antioxidant activity measured with the ORAC assay caused a pro-oxidant effect increasing measured %CAA to 204 ± 3% and 179 ± 4%, respectively. After boiling (30 min at 100°C) this effect was reduced and both vegetable juices were still found to be strongly antioxidant. This pro-oxidant effect was also further confirmed in the human fibroblasts (WS-1), lung adenocarcinoma (A549) and the human colon carcinoma (DLD-1) cell lines. α-Carotene was found to be the responsible for this effect and synergistic interactions between α- and β- Carotene enhanced this pro-oxidant effect.

The findings of the present study are that polyphenols in honey are stable and do not degrade with GIT digestion. In contrast, % oxidative damage is unexpectedly high only following GDD which may be due to changes in O₂ levels where β-Carotene becomes pro-oxidant. Future research should focus on quantifying β-Carotene levels in southern African honey and then evaluating using a model system the interaction of β-Carotene with H₂O₂ at neutral pH.

3.5 Conclusion

In summary, polyphenols in UD and GD can directly scavenge ROS, however with GDD a more complex effect emerges. At a neutral pH and the presence NaHCO₃, polyphenols degrade, however in honey with digestion, sugars protect against degradation and antioxidant activity is comparable to UD and GD samples. Evaluation of CAA post-digestion shows a strong pro-oxidant effect which is not due to increased H₂O₂ levels, but may be due to β-Carotene peroxy radical formation as a result of H₂O₂ degradation and O₂ formation at neutral pH.

Chapter 4: Antibacterial activity of honey samples and associated antibacterial components: sugars, hydrogen peroxide, methylglyoxal and polyphenols

Abstract

The antibacterial activity of honey, is type specific where methylglyoxal (MGO) is the major antibacterial molecule in Manuka, H₂O₂ and bee defensin-1 (BD-1) in Rewarewa, and H₂O₂ in buckwheat honey. Components contributing to the antibacterial activity of southern African honey and whether following gastrointestinal (GIT) digestion, antibacterial activity is retained, is unknown. The aim of this study was to determine the antibacterial activity of southern African honey and then tentatively identify the contributing components and lastly to determine if activity is retained following digestion.

The antibacterial activity of three southern African honeys, Agricultural Eucalyptus (AE), south eastern Mozambique (SEMh), Western Cape, Fynbos (WC) and control honeys; commercial Manuka (MANc) and medical grade Manuka (MAN, UMF10+) was determined. The effects of simulated *in vitro* GIT digestion on activity was also determined. Using the microbroth dilution assay, the minimum inhibitory concentration (MIC) (v/v) against Gram-negative, *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) and Gram-positive, *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*) bacteria of undigested (UD), gastric (GD) and the gastroduodenal digested (GDD) samples was determined. The contribution of sugars, MGO, H₂O₂, polyphenols and BD-1 to activity was also evaluated.

Against *E. coli*, the MIC for MANc, AE and WC was 30% and for SEMh was 25%. For *P. aeruginosa* all honeys had an MIC of 25%. The MIC for MGO was 1.0 and 1.2 mM for *E. coli* and *P. aeruginosa* respectively with an MIC of 9 mM for H₂O₂ for both bacteria. For *B. subtilis*, the MIC for MAN and WC was 25%, AE was 40% and for SEMh was 30%. For *S. aureus*, the MIC for MANc was 6.25% and was 25% for all other honeys. The MIC for MGO was 0.8 and 1.2 mM and for H₂O₂ was 90 and 9 mM for *B. subtilis* and *S. aureus* respectively. Honey sugars had a bacteriostatic effect. MGO and H₂O₂ content in southern African honeys was too low to contribute significantly to activity. With GD, antibacterial activity was unaltered. In contrast, with GDD a loss of activity was seen for AE, SEMh and WC against *E. coli* and *B. subtilis* but not in MANc. No significant loss was seen for all honeys against *S. aureus* or *P. aeruginosa*. Bacteria type selectivity, proteolytic degradation with digestion (*in silico* determination) and limited polyanetholsulphonate binding indicates that BD-1 is probably not the source of antibacterial activity.

Due to bacteria selectivity, identified following digestion, a small molecule with specific bacteria targeting should be considered as antibacterial component of the honeys. Retention of

antibacterial activity following digestion although selective following GDD identifies southern African honey as a functional food.

4.1 Introduction

Honey has been used extensively as a therapeutic agent specifically due to its antibacterial properties (George, Cutting 2007, Henriques *et al.*, 2010, Kwakman *et al.*, 2010, Manyi-Loh *et al.*, 2010, Al-Waili *et al.*, 2011, Kwakman *et al.*, 2011). Manuka's popularity in wound care is due to its antibacterial activity against a range of Gram-positive and negative bacteria (Tan *et al.*, 2009, Sherlock *et al.*, 2010, Kwakman *et al.*, 2011, Kwakman, Zaat 2012, Anthimidou, Mossialos 2013, Al-Nahari *et al.*, 2015). Manuka is similar to most honeys in terms of polyphenol content, sugars and pH, but also contains MGO, (Alvarez-Suarez *et al.*, 2014) which has been identified as the major molecule contributing to the antibacterial activity of Manuka honey. Leptosperin which is a glycoside of methyl syringate found exclusively in Manuka honey has been identified as an additional antibacterial ingredient (Kato *et al.*, 2014). The MGO levels found in Manuka honey is due to the high levels of dihydroxyacetone in the nectar of the *Leptospermum scoparium* flower which is converted to MGO (Mavric *et al.*, 2008). Based on the MGO content of Manuka honey, this honey is classified according to its unique Manuka factor (UMF). The higher the value the higher the MGO content and consequently antibacterial activity. Studies have shown that MGO inhibits, *E. coli* and *S. aureus* (Mavric *et al.*, 2008), *P. aeruginosa* (Mukherjee *et al.*, 2011), *P. aeruginosa*, *S. aureus* and MRSA (Kilty *et al.*, 2011) as well as *S. aureus* and *Staphylococcus epidermidis* (*S. epidermidis*) (Fidaleo *et al.*, 2010).

RS honey, which is produced under controlled conditions in greenhouses has been developed as a wound healing product. The antibacterial activity of this honey is attributed to the presence of BD-1, also known as royalsin, and H₂O₂. This honey has shown to have antibacterial activity against *B. subtilis*, MRSA, *E. coli* and *P. aeruginosa* (Kwakman *et al.*, 2011). The antibacterial activity of Buckwheat honey is due to the presence of H₂O₂ which effectively kills *E. coli* and *B. subtilis* (Brudzynski *et al.*, 2011). H₂O₂ in honey is produced by the enzyme glucose oxidase as honey ripens (Wahdan 1998), and has well described antimicrobial activity.

Antimicrobial peptides, defensins produced by honey bees are melittin, hemenoptecin, apidaecin and BD-1. Melittin is found in bee venom and hemenoptecin, apidaecin and abaecin are formed in response to *E. coli* infection (Kwakman, Zaat 2012). Only BD-1, a cationic peptide that contributes to the antibacterial activity of RS honey is found in honey. Synergism between BD-1 and H₂O₂ contributes to the antibacterial activity of RS honey especially against *B. subtilis*.

Sugars make up 70% of honey, with the major sugars being Fru and Glc and the minor sugars being MAL and SUC (Wahdan 1998, Beretta *et al.*, 2005, Bertoneclj *et al.*, 2007, Sherlock *et al.*, 2010, Kwakman *et al.*, 2011). These sugars contribute to the antimicrobial activity of honey mainly due to their osmotic action and their ability to debride wounds through the action of drawing out water, consequently drawing out foreign substances from wounds. Studies on the effectiveness of sugar to inhibit and/or kill bacteria (Wahdan 1998, Sherlock *et al.*, 2010) as well as the effect of sugar combined with H₂O₂ to aid wound healing (Tanner *et al.*, 1988) has been investigated.

Polyphenols also present in honey, have reported antibacterial activity and identified mechanisms of action includes cytoplasmic membrane damage, inhibition of energy metabolism, nucleic acid synthesis as well as cell membrane and wall synthesis (Cushnie, Lamb 2011). Examples of phenolic compounds with activity are flavan-3-ol/flavonols with activity against *E. coli*, *S. aureus*, *Bacillus cereus* (*B. cereus*), and *H. pylori* amongst others, and phenolic acids against *S. aureus*, *Listeria monocytogenes* (*L. monocytogenes*), *E. coli* and *P. aeruginosa* (Daglia 2012). Scottish honey effectively killed *Acinetobacter calcoaceticus* (*A. calcoaceticus*), *E. coli*, *P. aeruginosa* and *S. aureus* and the antibacterial activity of this honey was attributed to the presence of a novel, fatty diacid glycoside (Fyfe *et al.*, 2017).

Although, honey has well described antibacterial activity and several factors that contribute to the antibacterial activity of specific honey types has been identified, the effect of digestion on these components and associated antibacterial activity is unknown. Retention of antibacterial activity can contribute to the health of the GIT, by preventing for example gastric *H. pylori* (Manyi-Loh *et al.*, 2010) infections and *E. coli* induced diarrhoea.

The aims of the research presented in this study is to determine compared to Manuka honey the antibacterial activity of a three honey samples representative of honey found in the southern African region. Then to tentatively identify the major components responsible for antibacterial activity in these honeys. Lastly, to determine, whether as a functional food the antibacterial activity of these honeys is retained following GD and GDD.

The specific objectives were to:

1. Determine the minimum inhibitory concentration (MIC) of Manuka (control) and three southern African honeys against Gram-negative (*E. coli* and *P. aeruginosa*) and positive (*B. subtilis* and *S. aureus*) bacteria.
2. Determine the MIC of MGO and H₂O₂ and the contribution of MGO, H₂O₂ and the sugars found in honey to the measured antibacterial activity.

3. Evaluate using a simulated model of GIT digestion, the effects of digestion on the antibacterial activity of honey.
4. Determine if AMPs such as BD-1 contribute to the antibacterial activity of southern African honey.

4.2 Materials

4.2.1 Samples and bacterial strains

Honey, polyphenol and sugar samples used were the same as used in chapter 3 and included MANc while Manuka UMF10+ (MAN UMF10+) was used as a control for the determination of the effect of digestion on antibacterial activity. Bacteria strains used were *E. coli* (700928), *P. aeruginosa* (10145), *B. subtilis* (13933) from the ATCC and *S. aureus* (U3300) kindly donated by the University of KwaZulu Natal.

4.2.2 Reagents, equipment & disposable plastic ware

Reagents were similar to those used in Chapter 3 as well as $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$, Na_2 tartrate dibasic, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, polyanetholesulfonic acid sodium salt (SPS), MGO (40% solution) and melittin were obtained from Sigma-Aldrich Company, Atlasville, SA. Tryptone and yeast extract were obtained from Merck Chemicals, Modderfontein SA. Bovine serum albumin (BSA) was obtained from BioRad, Johannesburg, South Africa. Less than 5 kDa cut-off membrane filters were obtained from Corning, The Scientific Group, SA.

4.2.3 Laboratory facilities

All research was conducted in the research facilities of the Department of Anatomy, Faculty of Health Sciences and the Department of Biochemistry, Genetics and Microbiology, Faculty of Natural and Agricultural Sciences, University of Pretoria.

4.3 Methods

4.3.1 Sample preparation

Honey, sugar, sugar analogue, polyphenols, PP mix and SH were prepared as described in Chapter 3.

4.3.2 Luria Bertani broth

For bacterial growth, Luria Bertani (LB) broth which is composed of 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in 1 litre of ddH₂O, pH 7.5 was used.

4.3.3 Simulated *in vitro* GIT digestion

Simulated *in vitro* GIT digestion was done as described in Chapter 3.

4.3.4 Antibacterial activity assay

The antibacterial activity of the different samples was determined using the microbroth dilution assay as described by Sherlock *et al.*, (2010), with a few modifications. Bacteria were cultured overnight in LB broth (prepared then diluted 100 times in LB broth and proliferated until an optical density at 600 nm (OD₆₀₀) of 0.4 – 0.7 was obtained. Bacteria were then diluted with LB broth to a starting OD₆₀₀ of 0.02. Fifty µL of bacteria were added to 50 µL of sample at a range of determined concentrations in sterile 96 well microtiter plates. Sterile controls contained broth only whereas growth controls contained bacteria and broth. Plates were incubated in the dark at 37°C for 24 h with shaking at 150 rpm. Data was analysed according to Sherlock *et al.*, (2010). Optical density was determined at 600 nm before (T0) and after (T24) incubation. The OD at 24 h was subtracted from the OD at 0 h. The growth control was assigned 100% growth (G100). Percent inhibition of growth was then determined using the formula. Percent inhibition = $100 - ((T24 - T0) / G100) * 100$. The minimum concentration of test sample that completely inhibited growth (100%) was defined as the MIC.

4.3.4.1 Inhibitory activity of honeys, sugar analogue and synthetic honey

For honey and sugar analogue (40% Fru, 30% Glc, 10% MAL and 2% SUC) (Beretta *et al.*, 2005, Bertoncej *et al.*, 2007, Kwakman *et al.*, 2011) a two-fold serial dilutions were prepared at final concentrations ranging from 6.25 to 40% (v/v).

4.3.4.2 Inhibitory activity of methylglyoxal and hydrogen peroxide

MGO was tested at final concentrations of 0 – 2.2 mM and H₂O₂ at a final H₂O₂ concentrations of 0.0009 to 900 mM.

4.3.4.3 Inhibitory activity of polyphenols

The effects of the PP mix and SH prepared as described in section 3.3.1 was determined at a 10x dilution.

4.3.5 Inhibitory activity of peptides

Proteins and peptides such as BD-1 and other small molecules such as MGO and H₂O₂ can contribute to the antibacterial activity of honey. A strategy similar to that described by Kwakman *et al.*, (2010) was used. Selective filtration can be used to provide an indication of the size of the molecule/s involved. A reduction in expected antibacterial activity indicates that a >5kDa molecule is responsible for antibacterial activity and if activity is unchanged

molecules <5 kDa are responsible for activity. Cationic antimicrobial peptides such as BD-1 will bind anionic SPS with a subsequent loss of antibacterial activity.

A 10 (v/v) UD, MAN UMF10+ and WC honey solution was filtered using a <5 kDa cut-off membrane filter, then SPS was added to the filtrate, final concentration 0.05% w/v to neutralise cationic peptides. The antibacterial activity of this sample was compared to a filtered 10% (v/v) honey sample not treated with SPS. Cationic AMP, melittin was used as a control.

4.3.6 Peptide concentration determination

The peptide content of <5 kDa filtered honey samples was determined using the bicinchoninic acid (BCA) assay according to Smith *et al.*, (1985). BCA solution was made by mixing 50 parts of solution A (1% BCA, 2% Na₂CO₃·H₂O, 0.13% Na₂ tartrate dibasic, 0.4% NaOH and 0.95% NaHCO₃, adjusted to pH 11.25) to 1 part of solution B (4% CuSO₄·5H₂O). A volume of 10 µL (100x diluted) filtered honey samples were incubated with 200 µL of the BCA solution. A BSA standard curve with a final concentration of 0 to 0.05 mg/mL was used to determine the protein content of the honey samples.

4.3.7 Statistical analysis

All experiments were done at least in triplicate and represented as mean ± SEM. Analysis was done using the students T-TEST and one way ANOVA followed by the Tukey post-hoc test with significant differences determined at $p \leq 0.05$.

4.4 Results and discussion

4.4.1 Physiochemical characteristics of honey samples

Honey has specific physiochemical characteristics that make it a good antibacterial agent. These characteristics were compared to MANc honey. The pH, moisture, and the reducing sugar content was similar for all honeys. Therefore for all honey samples the contribution of pH and osmotic effects to the antibacterial activity of these honeys would be similar. Protein, phenolic acid and especially the flavonoid content of southern African honey were statistically greater than MANc honey (Table 4.1).

Table 4.1: Physicochemical characteristics of honey samples.

	MAN	AE	SEMh	WC
pH	4.10 (UMF 5+)#	4.53*	4.93*	4.24*
Moisture content (%)	11.59 (UMF 5+)#	11.45*	20.73*	14.45*
Reducing sugars (g/100g)	58.61 ± 0.42 (UMF 5+)#	61.88 ± 2.72	60.30 ± 16.53	52.99 ± 7.57
Proteins (g/100 g)	0.50 ± 0.02 (UMF 5+)#	0.93 ± 0.04*	1.06 ± 0.05*	0.96 ± 0.03*
Peptide content <5kDa (mg/mL)	0.92 ± 0.01 (UMF 10+)C4	0.69 ± 0.02C4	0.73 ± 0.02C4	0.84 ± 0.01C4
TPC (mg GAE/100 g)	42.96 (UMF 5+)#	97.14 ± 4.98*	122.39 ± 2.73*	112.50 ± 0.70*
TFC (mg CE/100 g)	9.76 (UMF 5+)#	30.16 ± 1.70*	33.00 ± 1.70*	38.75 ± 2.60*
MGO (g/100g)	0.0038 – 0.0761 (UMF 5+ - 25 +)†	ND^	ND^	ND^
H₂O₂ (mM)	0.72 – 1.04 (UMF 20+ - 25+)§/2.17±0.12 (commercial)C3	0.4 ± 0.14C3	1.57 ± 0.38C3	0.91 ± 0.23C3

MAN – Manuka honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, TPC – total polyphenol content, TFC – total flavonoid content, MGO – methylglyoxal, H₂O₂ – hydrogen peroxide.

Data obtained from: (Serem, Bester 2012)*, (Moniruzzaman *et al.*, 2013)# (Mavric *et al.*, 2008)†, (Brudzynski *et al.*, 2011)§,(Chapter 3)C3, (Chapter 4)C4 and ND, not determined for these specific samples, ^ 0.092 – 0.1216 mg/100g for southern Africa honey (Rabie 2016).

4.4.2 Honey has antibacterial activity against Gram-negative and positive bacteria

The antibacterial activity of honey samples MANc, AE, SEMh and WC was determined against two Gram-negative bacteria. Against, *E. coli* the antibacterial activity of SEMh was greater than MANc, AE and AE (Figure 4.1, Table 4.2). The antibacterial activity of MANc, AE, SEMh and WC against *P. aeruginosa* was similar (Figure 4.2, Table 4.2).

Table 4.2: Antibacterial activity of honey, MGO and H₂O₂ against Gram-negative bacteria.

Bacteria	MIC (%)*				MIC (mM)[mg/kg]	
	MANc	AE	SEMh	WC	MGO	H₂O₂
<i>E. coli</i>	30	30	25	30	1.0 [72.06]	9.0
<i>P. aeruginosa</i>	25	25	25	25	1.2 [86.47]	9.0

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, MGO – methylglyoxal, H₂O₂ – hydrogen peroxide, MIC – minimum inhibitory concentration.

* Honey tested between final concentrations of 6.25 – 40 % (v/v). MIC reported as minimum concentration of test sample that completely inhibited growth (100%).

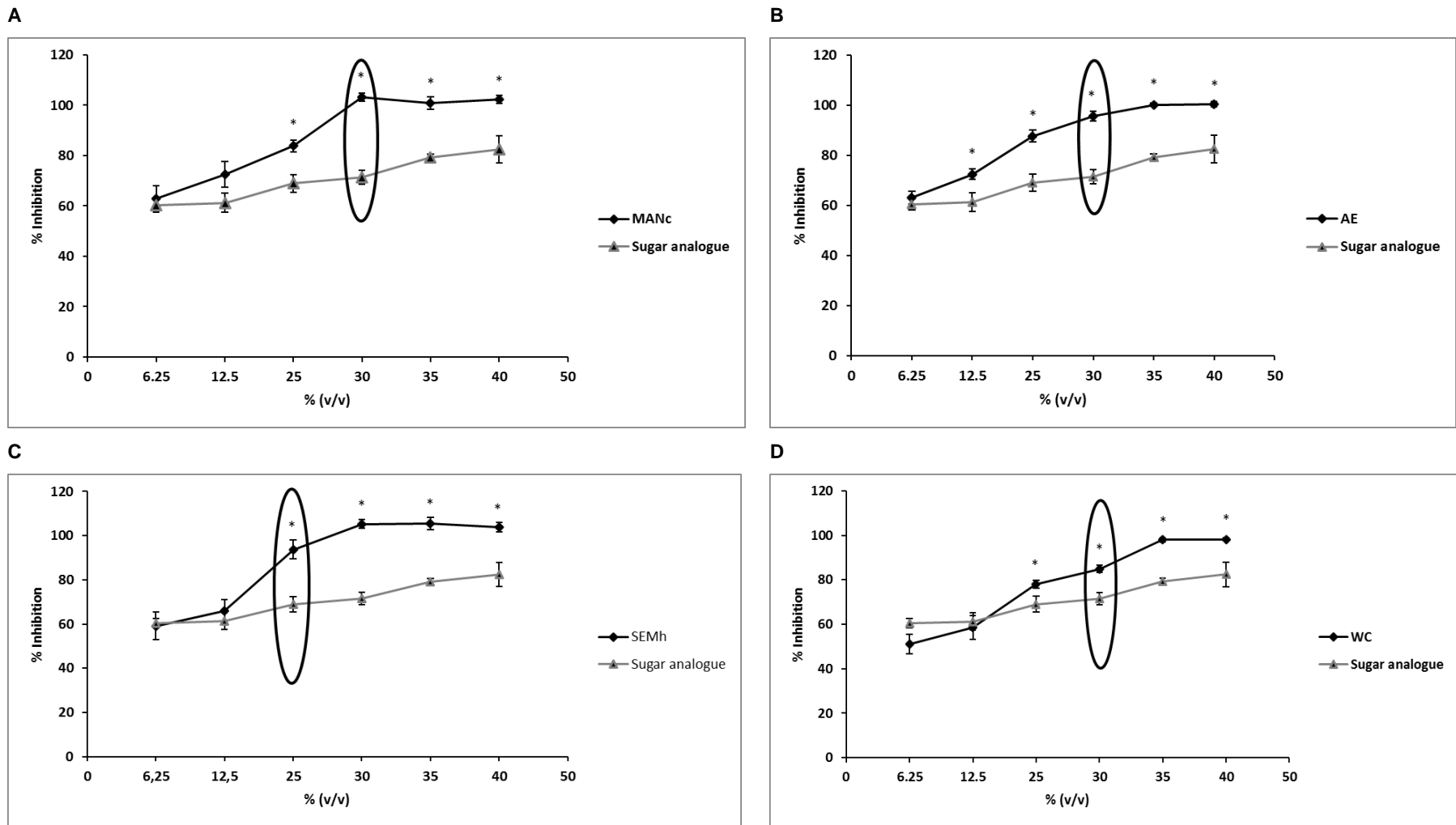


Figure 4.1: Antibacterial activity of A) MAN, B) AE, C) SEMh and D) WC honey samples compared to a sugar analogue against *E. coli* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean±SEM. * represents significant differences between concentration points of sugar analogue vs. honey sample. Circled area Indicates MIC of honey. Differences between sugar analogue and honey indicates additional components contributing to antibacterial activity at the MIC of each honey.

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey.

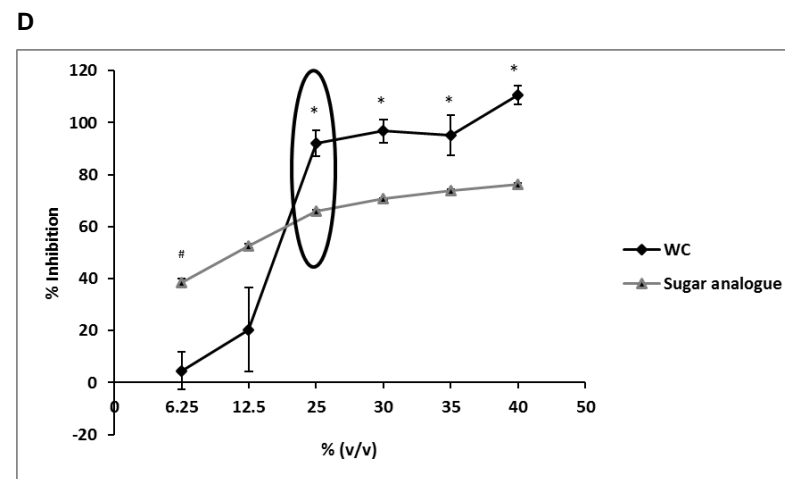
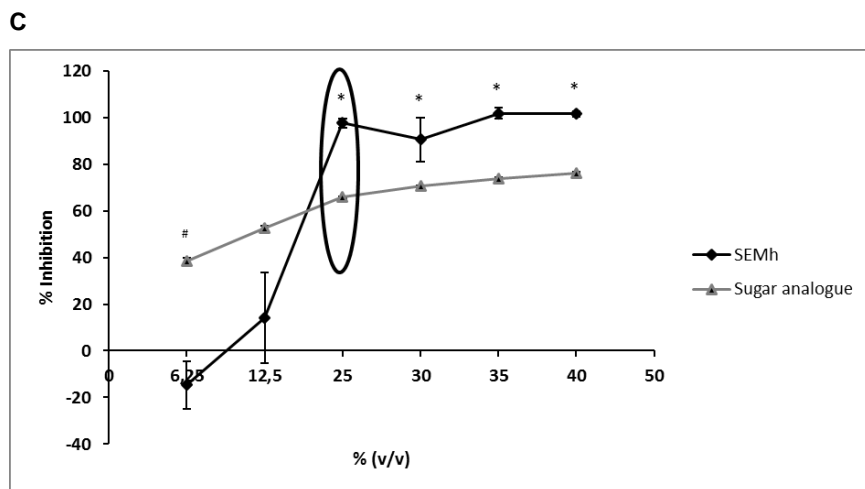
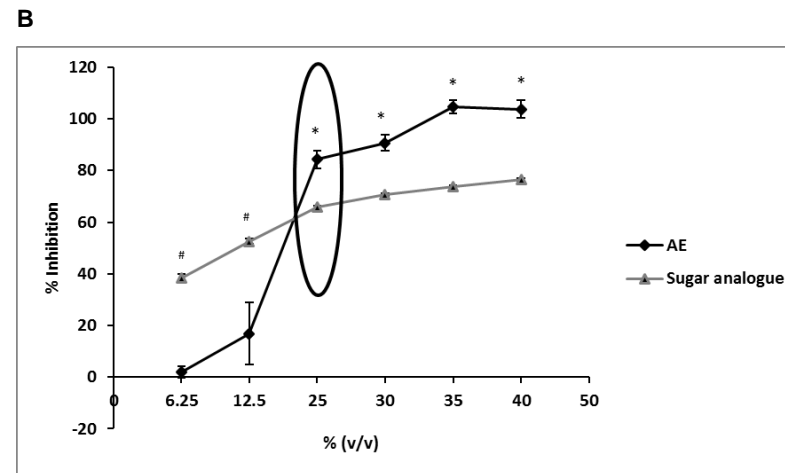
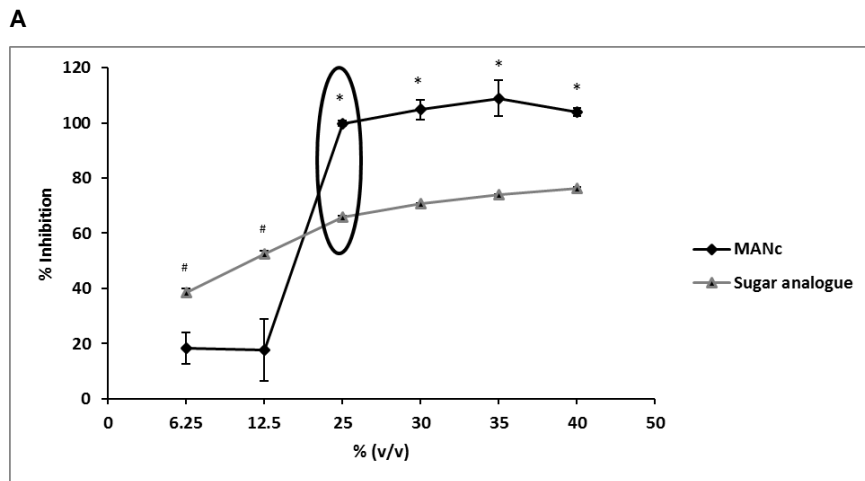


Figure 4.2: Antibacterial activity of A) MAN, B) AE, C) SEMh and D) WC honey samples compared to a sugar analogue against *P. aeruginosa* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean \pm SEM. * represents significant differences between concentration points of sugar analogue vs. honey sample. Circled area Indicates MIC of honey. Differences between sugar analogue and honey indicates additional components contributing to antibacterial activity at the MIC of each honey.

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey.

Tan *et al.*, (2009) investigated the inhibitory effects of Manuka honey compared to that of Tualang honey (*Koompassi excelsa*). MAN UMF10+ had an MIC₉₅ of 17.50% against *P. aeruginosa* and a lower MIC₉₅ of 20.00% against *E. coli*. Tualang honey in comparison to Manuka had similar MIC₉₅ against *P. aeruginosa* but a higher MIC₉₅ of 22.50% against *E. coli*. Zainol *et al.*, (2013) evaluated the antibacterial activity of five selected Malaysian honeys. Three honeys, Acacia, Gelam and Tualang more effectively inhibited the growth of *P. aeruginosa* than *E. coli*. Likewise in the present study, MANc, AE and WC also better inhibited *P. aeruginosa* than *E. coli*.

Honey samples were also tested for antibacterial activity against two Gram-positive bacteria, *B. subtilis* and *S. aureus*. The % MIC of honeys MANc, AE, SEMh and WC was determined (Figure 4.3/4.4, Table 4.3). Against *B. subtilis*, the antibacterial activity of WC was similar to MANc while the inhibitory effect was lower for AE and SEMh. Using a commercial Manuka sample, Basson, Grobler (2008) showed that the MIC was 25% against *E. coli* and *S. aureus*. Likewise, South African Bluegum (similar type to AE) and Pincushion honey had an MIC of 25%. Fynbos (similar type to WC) had an MIC of 50% against *E. coli*. Against *S. aureus* the MIC was 25% for all three honeys (Bluegum, Pincushion and Fynbos). Similar to the present study it was shown that the MIC for WC was 25% for *S. aureus* whereas WC more effectively inhibited *E. coli* than reported in the study of Basson, Grobler (2008) with a MIC of 30%.

Table 4.3: Antibacterial activity of honey, MGO and H₂O₂ against Gram-positive bacteria.

Bacteria	MIC (%)*				MIC (mM)[mg/kg]	
	MANc	AE	SEMh	WC	MGO	H ₂ O ₂
<i>B. subtilis</i>	25	40	30	25	0.8 [57.64]	90
<i>S. aureus</i>	6.25	25	25	25	1.2 [86.47]	9

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, MGO – methylglyoxal, H₂O₂ – hydrogen peroxide, MIC – minimum inhibitory concentration.

* Honey tested between final concentrations of 6.25 – 40 % (v/v). MIC reported as minimum concentration of test completely inhibited growth (100%).

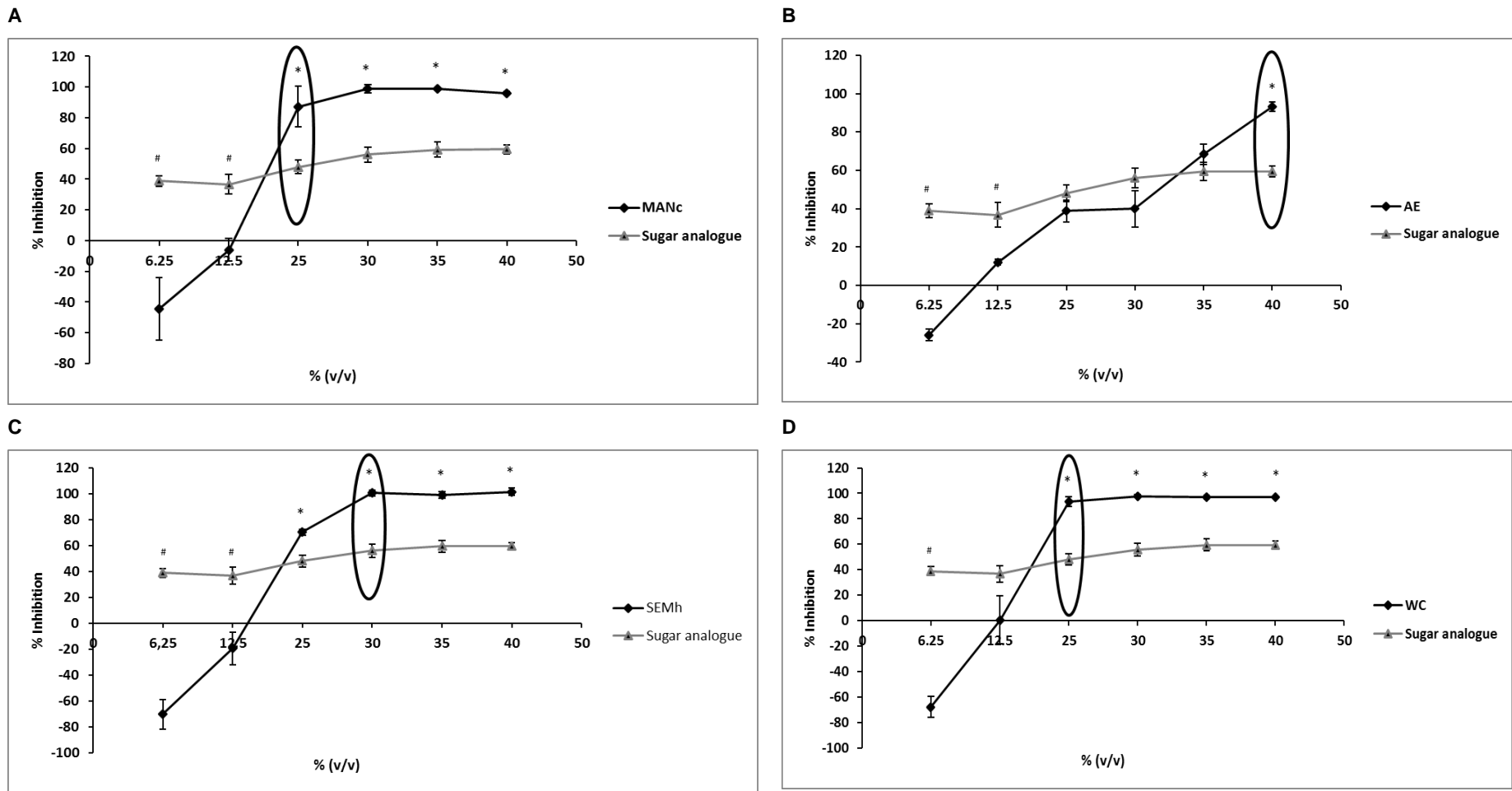


Figure 4.3: Antibacterial activity of A) MAN, B) AE, C) SEMh and D) WC honey samples compared to a sugar analogue against *B. subtilis* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean±SEM. * represents significant differences between concentration points of sugar analogue vs. honey sample. Circled area Indicates MIC of honey. Differences between sugar analogue and honey indicates additional components contributing to antibacterial activity at the MIC of each honey.

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey.

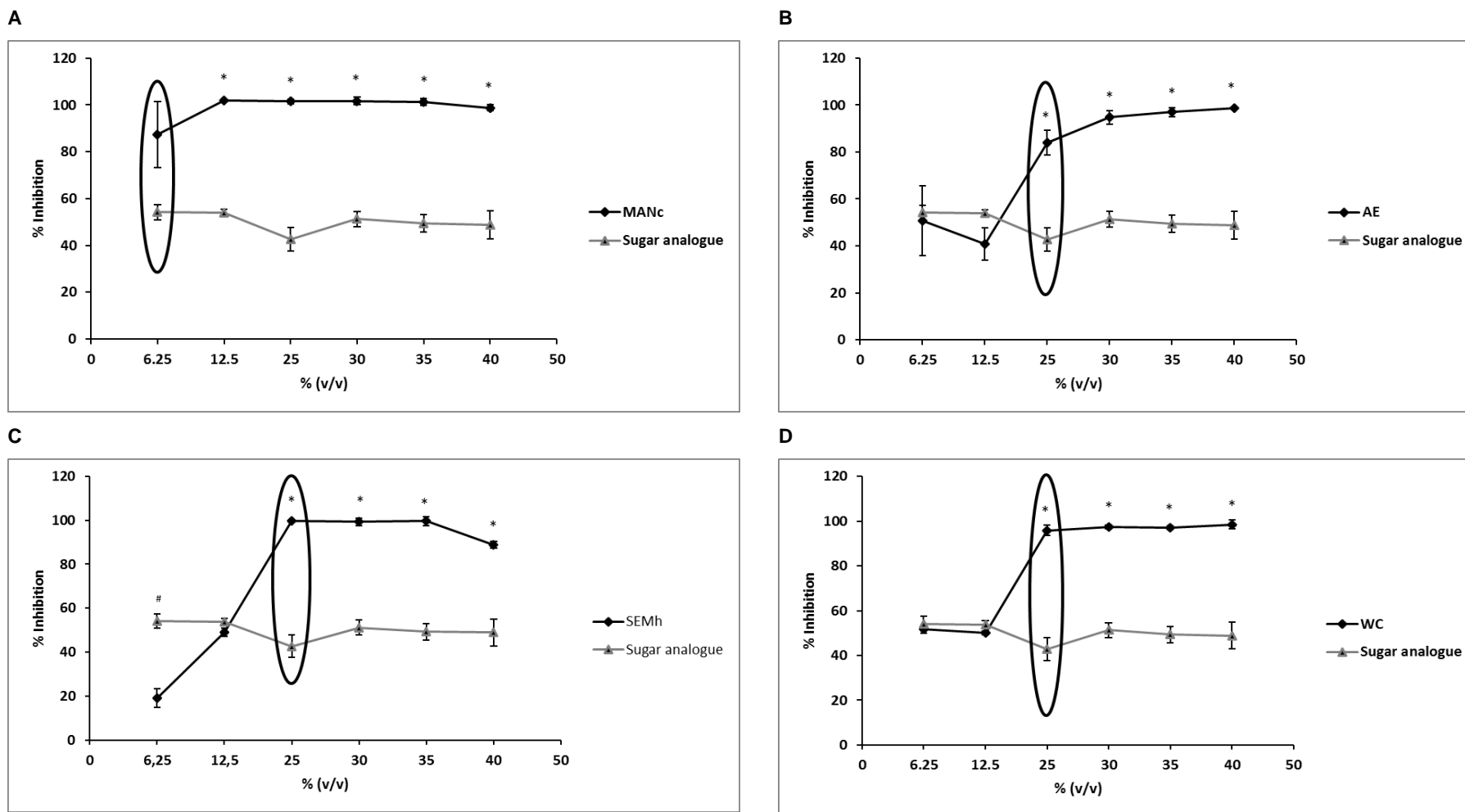


Figure 4.4: Antibacterial activity of A) MAN, B) AE, C) SEMh and D) WC honey samples compared to a sugar analogue against *S. aureus* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean±SEM. * represents significant differences between concentration points of sugar analogue vs. honey sample. Circled area indicates MIC of honey. Differences between sugar analogue and honey indicates additional components contributing to antibacterial activity at the MIC of each honey.

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey.

Brudzynski *et al.*, (2011) evaluated the antibacterial activity of Canadian honeys compared to Manuka UMF 20+ and UMF 25+. The authors reported the MIC against *E. coli* to be 25% for both Manuka UMF 20+ and UMF 25+. Against *B. subtilis* the MIC was reported to be 6.25% also for both Manuka UMF 20+ and UMF 25+, indicating bacterial strain specific activity. Brady *et al.*, (2004) evaluated the antibacterial activity of 179 non-Manuka New Zealand honeys and reported that the MIC against *E. coli* was between 11.2 – 23.4% and for *S. aureus* using the well diffusion assay (reported as % phenol equivalent) was 5.0 – 27.9%. The antibacterial activity of Manuka honey against *E. coli* and *S. aureus* was 6.3% and 28.4%, respectively, although its UMF was not specified.

Gallardo-Chacón *et al.*, (2008) evaluated the antibacterial activity of 31 honeys against different strains of *E. coli* and MRSA. All honey samples and artificial honey inhibited the growth of *E. coli* at an MIC of 25%. It was concluded that the inhibition of *E. coli* was due to the inhibitory effects of sugars. In contrast, the inhibition of MRSA was not due to the presence of high sugar but due to the presence of H₂O₂ in 12 samples and proteinaceous compounds in two samples. Proteins responsible for antibacterial activity were not identified.

From the above graphs (Figure 4.1, 4.2, 4.3 and 4.4) and from the literature provided, it can be concluded that activity against bacteria is dependant largely on strain, as can be seen with AE honey that has an activity between 25 – 30% against all bacteria but 40% against *B. subtilis* and MANc honey that also has activity between 25 – 30% against all bacteria but 6.25% against *S. aureus*. In addition at honey concentrations that cause MIC, the sugar analogues had lower activity. Therefore indicating that sugar contributed to activity but other compounds in honey synergistically with sugar caused complete inhibition of bacteria.

4.4.3 Contribution of pH, sugars, methylglyoxal and hydrogen peroxide to the antibacterial activity of honey

Besides the physiochemical properties such as pH and high sugar content, the presence of bioactive constituent molecules such as MGO, H₂O₂, polyphenols and AMPs also contributes to the antibacterial activity of honey.

4.4.3.1 Effect of pH and digestion on antibacterial activity

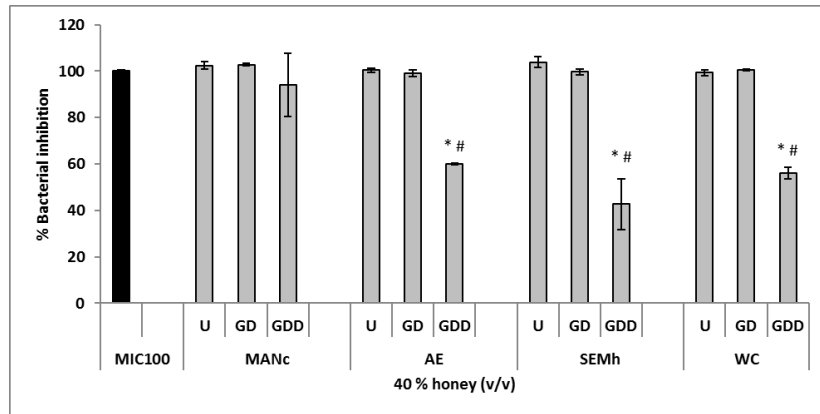
The pH of the honeys evaluated in this study was 4.10 – 4.93 with MANc honey having the lowest pH and SEMh the highest pH. A study by Kwakman *et al.*, (2010) where honey was titrated from its usual low pH to pH 7, showed that honey loses its antibacterial activity to a level that matched that of a sugar solution where > 40% honey is required to achieve inhibition.

Al-Waili (2004), evaluated the antimicrobial activity of honeys titrated to an acidic (4.1 – 4.4), neutral (7.2 – 7.4) and alkaline (9.0 – 9.2) pH against various bacteria isolated from human specimens. The change in MIC against *E. coli* from natural to acidic and then from neutral to alkaline pH, was 30% and 30% and then 50% and 60%, respectively. For *P. aeruginosa* this was 30% and 20% and then 50% and 80% and for *S. aureus* was 60% and 30% and then 60% and 50%. Therefore, if pH is a determining factor, following GDD, antibacterial activity against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* would be reduced. If pH alone contributed to loss of antibacterial activity, a similar loss of activity would be observed for all bacteria as was described by Al-Waili (2004). Likewise, recently Salonen *et al.* (2017) reported that pH did not contribute significantly to the antibacterial activity of Nordic unifloral honey.

Simulated GIT digestion on the antibacterial activity of MANc, AE, SEMh and WC honey was determined. Not only does simulated digestion provide information on the effect of digestion on the properties of honey as a functional food, but can also be used to determine if proteins or peptides contribute to the antibacterial of honey. To achieve this, a 40% solution was used. GD did not alter the antibacterial activity of MANc, AE, SEMh and WC against Gram-negative and positive bacteria (Figure 4.5). Following GDD the antibacterial activity of MANc against all bacteria was unaltered. In contrast for AE, SEMh and WC approximately 50% of activity against *E. coli* and *B. subtilis* was lost while antibacterial activity was unchanged against *P. aeruginosa* and *S. aureus*, except for sample SEMh (Figure 4.5). A 40 – 50% loss of activity indicates that the bioactive component responsible for activity is possibly a protein or peptide that is being broken down via digestion.

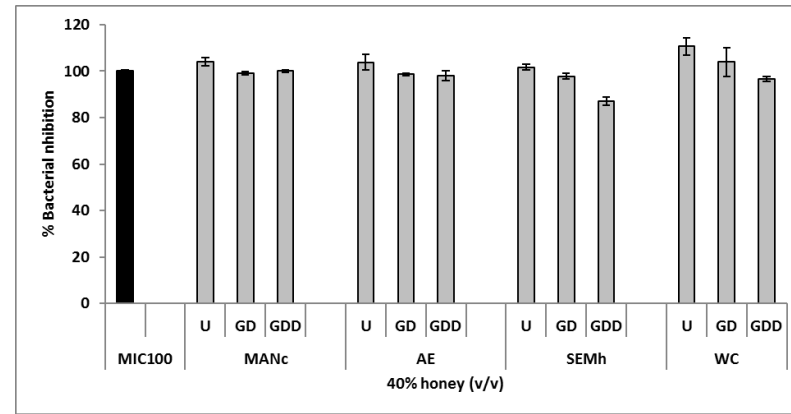
In Manuka honey, antibacterial activity is due to the presence of MGO. Daglia *et al.*, (2013) reported that with simulated GIT digestion of a standard solution containing 0.83 mM MGO there was a loss of 52.5% and 79.3% in MGO levels following GD and GDD respectively. For Manuka honey with a MGO content of 637.4 ± 11.8 mg/kg the loss of MGO content was 19.2% and 52.1% for each respective stage of digestion. For honey with a MGO content of 33.9 mg/kg there was a loss of activity of 59.8% and 87.8% following GD and GDD, respectively. If MGO contributes to the antibacterial activity, it would be expected that with digestion that there would be a significant loss of antibacterial activity following digestion. No loss of activity was observed which implies that other molecules such as the glycoside of methyl syringate is responsible for antibacterial activity of MANc.

E. coli



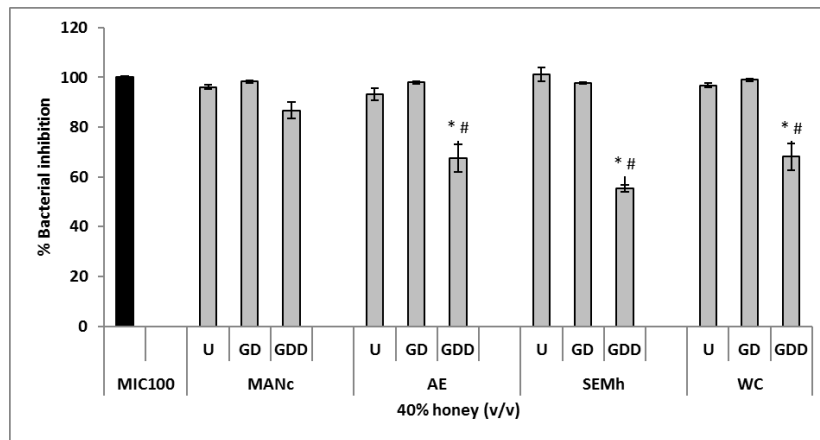
Gram-negative

P. aeruginosa



Gram-positive

B. subtilis



S. aureus

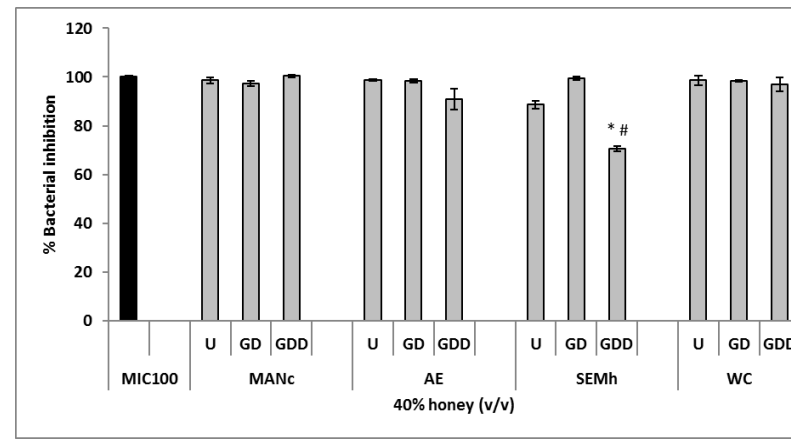


Figure 4.5: Effect of simulated *in vitro* gastroduodenal digestion of MANc, AE, SEMh and WC honey samples on antibacterial activity against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*B. subtilis* and *S. aureus*). Data is an average of at least 3 experiments and reported as % bacteria inhibition \pm SEM. * indicates significant difference compared to MIC100, # indicates significant difference of UD compared to GDD.

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey.

4.4.3.2 Inhibitory activity of sugars

Ripened honey consists of 80% sugars, majority of these being Fru, Glc, maltose and sucrose. The concentration of Fru and Glc are approximately 40 and 30%, respectively. The minor sugars, maltose and sucrose are found at approximate concentrations of 10% and 2%, respectively (Wahdan 1998, Beretta *et al.*, 2005, Bertoncej *et al.*, 2007, Kwakman *et al.*, 2011). Sugar has documented activity against bacterial species and has been shown to aid wound healing by creating an environment with low water activity (a_w) which inhibits or stresses bacterial growth (Chirife *et al.*, 1983).

The antibacterial effects of a sugar analogue containing these four sugars was determined. For both Gram-negative and positive bacteria no MIC could be calculated for the sugar analogue. However, against *E. coli* and *P. aeruginosa* over the concentration range evaluated the sugar analogue shows a linear increase in inhibition with a line equation (correlation) of $y = 0.6732x + 53.94$ ($R^2 = 0.945$) and $y = 1.096x + 35.77$ ($R^2=0.955$), respectively (Appendix 11) which implies that the sugar analogue better inhibited *P. aeruginosa* than *E. coli*.

For Gram-positive bacteria, against *B. subtilis* the sugar analogue shows a linear increase in inhibition with a line equation (correlation) of $y = 0.748x + 31.14$ ($R^2 = 0.925$) (Appendix 11). However, against *S. aureus* no linear inhibition is seen with an equation of $y = -0.167x + 54.17$ ($R^2= 0.272$) (Appendix 11). From the gradients, sugar in honey contributes to the inhibition of *E. coli*, *P. aeruginosa* and *B. subtilis* and *S. aureus*, although against *S. aureus* this activity is not dose-dependent.

The difference between the antibacterial activity of MANc, AE, SEMh, WC and the sugar analogues at the MIC of each honey (Figures 4.1 – 4.4) indicates that other factors other than the sugars contributes to the antibacterial activity of the honey samples. The large difference between, the sugar analogue and the honey samples for *S. aureus* indicates that activity is specific and non-sugar components are contributing to activity and is similar to that observed by Gallardo-Chacón *et al.*, (2008) against MRSA.

In general Gram-negative bacteria were more sensitive than Gram-positive bacteria to the effects of sugars. Likewise Kwakman *et al.*, (2010) reported that Gram-positive *B. subtilis*, vancomycin resistant *Enterococcus faecium* and MRSA was less sensitive to a sugar analogue compared to Gram-negative *E. coli* and ciprofloxacin resistant *P. aeruginosa* bacteria. In a further study, Kwakman *et al.*, (2011) confirmed that Gram-positive *B. subtilis* and MRSA were less sensitive to a sugar analogue compared to Gram-negative *E. coli* and *P. aeruginosa*.

Although many studies only reported on the MIC values of different types of honey, evaluation of the curve shape or form obtained from testing a concentration range of honey provides some important information on the effect of different honey types. For both *P. aeruginosa* and *B. subtilis* at low concentrations a majority of the honeys promoted the growth of bacteria greater than the sugar analogue. Shamala *et al.*, (2000) reported that a 1% honey solution stimulated the growth of *Lactobacillus acidophilus* and *Lactobacteria plantarum* by 10 – 100 fold compared to a 1% SUC and a 1% Glc:lactose (1:1) solution. Stimulation of the growth of these bacteria has a beneficial effect as these bacteria decrease toxic products and establish a balanced microflora population (Shamala *et al.*, 2000). This is important as often with digestion samples are diluted and especially for patients with IBD which is associated with a disrupted GIT microbiota (Sartor 2004, 2008), honey may have beneficial effects.

4.4.3.3 Inhibitory activity of methylglyoxal and hydrogen peroxide

MGO and H₂O₂ have been identified as the major antibacterial components of honey. The aim of this part of the study is to determine if the levels of MGO and H₂O₂ in MAN, AE, SEMh and WC are sufficient to inhibit the growth of bacteria.

4.4.3.3.1 Inhibitory activity of methylglyoxal

Alvarez-Suarez *et al.*, (2014) reported that Manuka's antibacterial activity is due to MGO, and leptosperin (Kato *et al.*, 2014). Rabie *et al.*, (2016), based on the morphological effects of MGO confirmed that these changes in was due to MGO in Manuka honey. Findings were that the morphology of *E. coli* and *B. subtilis* was unaltered following exposure to 0.5 mM (36.03 mg/kg) MGO. At 1 mM MGO bacteria had fewer fimbriae and the flagella were less or absent while exposure to 2 mM MGO resulted in a loss of fimbriae and flagella and bacteria were rounded with shrinkage and loss of membrane integrity. The lack of fimbriae and the flagella limits bacteria adherence and motility while shrinkage and loss of membrane integrity indicates cell death.

The MIC of MGO against *E. coli* and *P. aeruginosa* was 1.0 mM and 1.2 Mm, respectively (Table 4.2) and for *B. subtilis* and *S. aureus* was 1.2 mM and 0.8 Mm, respectively (Table 4.3). Likewise Fidaleo *et al.*, (2010) reported that MGO inhibited *E. coli* and *S. aureus* at 1.1 mM while Mavric *et al.*, (2008) reported that 1.05 mM MGO inhibited *S. aureus*.

Mavric *et al.*, (2008) found that MGO levels in Manuka UMF honey vary from 38.4 ± 5.0 to 761 ± 25 mg/kg (0.701 ± 0.091 to 13.90 ± 0.46 mM) for UMF5 to UMF25 honey. Levels in commercial honeys ranged from n.d. – 5.7 mg/kg with an average of 3.1 mg/kg which is equivalent to 0.058 mM MGO. The MIC of MANc honey is 25% against two of the bacteria

tested (Table 4.2, 4.3), therefore this honey contains 0.0145 mM MGO, which is not sufficient to inhibit/kill any of the bacteria used in the present study. Synergistic effects between the sugars and MGO may increase the ability of MGO to kill *E. coli* or alternatively other components can contribute to this activity. Rabie (2016) reported that the MGO content of southern Africa honey (0.92 – 1.216 mg/kg) was not sufficient to kill bacteria.

4.4.3.3.2 Inhibitory activity of hydrogen peroxide

Most honeys contain H₂O₂, and its concentration increases with dilution when glucose is oxidised by glucose oxidase to gluconic and H₂O₂. H₂O₂ is found in varying concentrations in different honeys and this may account for differences in antimicrobial activity (Tan *et al.*, 2009). H₂O₂ is a potent antibacterial at low concentrations, hence its use as a disinfecting solution. At concentrations found in disinfecting solutions 0.8 – 8 M, H₂O₂ inhibits *Staphylococcus*, *Streptococcus*, *Pseudomonas* species and *Bacillus* spores (Brudzynski *et al.*, 2011). At concentrations as low as 1.25 or 2.5 mM, H₂O₂ can cause bacterial inhibition (Brudzynski *et al.*, 2012). The MIC of H₂O₂ determined in the present study using the microbroth dilution assay against *E. coli* and *P. aeruginosa* was 9 mM for both bacteria (Table 4.2) and against Gram-positive bacteria *B. subtilis* and *S. aureus*, the MIC was 90 and 9 mM, respectively (Table 4.3). Brudzynski *et al.*, (2011), reported that 1.25 mM H₂O₂ is sufficient to inhibit *E. coli* by 90% whereas double the concentration to achieve 90% inhibition against *B. subtilis* was needed. This is similar to results obtained in this study, although ten-fold higher H₂O₂ concentrations are required to inhibit *B. subtilis*. The H₂O₂ content of AE, SEMh and WC is 0.4 ± 0.14, 1.57 ± 0.38, 0.91 ± 0.23 mM, respectively (Table 4.1) which may be sufficient to inhibit growth at the respective MIC values but not sufficient to kill bacteria.

In a study by Tanner *et al.*, (1988), sugar was mixed with H₂O₂ (0.15% w/v, 4.11 mM) and this mixture showed efficacy in healing chronic wounds that had previously not responded to conventional treatment. H₂O₂ is reported to be the major antibacterial component of buckwheat and clover honeys. Brudzynski *et al.*, (2011) reported MIC's against *E. coli* and *B. subtilis* of 6.25%, 12.5%, 25% and 50% of buckwheat honey samples with H₂O₂ contents of 2.68 ± 0.04, 2.12 ± 0.22, 0.74 ± 0.01 and 0.25 ± 0.02 mM, respectively. SEMh honey with an MIC of 25% and 30% against *E. coli* and *B. subtilis* respectively, has a H₂O₂ content of 1.57 ± 0.38 mM similar to that of blueberry and buckwheat honey with a H₂O₂ content of 0.52 ± 0.11 and 0.744 ± 0.01 mM, respectively. Therefore for MANc, SEMh and WC, H₂O₂ levels are sufficient to inhibit bacterial growth (Table 3.11). Furthermore an increase in H₂O₂ levels following digestion to 4.06 ± 0.25, 3.14 ± 0.01, 2.32 ± 0.22 and 2.65 ± 0.25 mM for MANc, AE, SEMh and WC, respectively (Table 3.11) indicates that the H₂O₂ content of these honeys are sufficient to kill bacteria.

4.4.3.4 Inhibitory activity of polyphenols

Both MGO and H₂O₂ have been identified as the major antibacterial components present in honey (Brudzynski 2006, Mavric *et al.*, 2008, Brudzynski *et al.*, 2011, Kilty *et al.*, 2011, Brudzynski *et al.*, 2012). Other studies have identified that polyphenols also inhibit bacteria (Daglia 2012). In this study the contribution of these components to the antibacterial activity of honey was determined. Lu *et al.*, (2013) reported that MGO and H₂O₂ production did not account for all the antibacterial activity in Manuka, Kanuka and clover honey and recently it was found that leptosperin, a novel glycoside of methyl syringate also contributed to the antibacterial activity of Manuka honey (Kato *et al.*, 2014).

The polyphenol content of southern African honey is 0.99 mg GAE/g (Serem, Bester 2012). The TPC of MANc, AE, SEMh and WC is 1.17, 1.30, 1.78 and 1.69 mg GAE/mL, respectively. The effect of a 10% polyphenol mix (CA+GA+CAT+QUE) and a SH containing the same concentrations of polyphenols was evaluated. The PP mix inhibited bacteria growth of *E. coli*, *P. aeruginosa* and *B. subtilis* but not *S. aureus* (Table 4.4). Inhibition of *S. aureus* was increased from not detectable levels to 69.40 ± 7.03% against *S. aureus* indicating synergistic bacteria killing effects between sugars and polyphenols. The MIC of MANc against *S. aureus* is 6.25% and at that concentration sugars had a minimal effect on the growth of *S. aureus* (Figure 4.4) further supporting the possible synergism between polyphenols and sugars. Likewise but to a lesser extent, a synergistic antibacterial effect was observed between polyphenols and sugars for *E. coli*.

Table 4.4: Inhibitory effects (%) of a PP mixture and a SH against Gram-negative and positive bacteria.

	PP Mix	SH
<u>Gram-negative</u>		
<i>E. coli</i>	16.90 ± 5.77	34.83 ± 2.84*
<i>P. aeruginosa</i>	20.20 ± 5.64	24.50 ± 4.82
<u>Gram-positive</u>		
<i>B. subtilis</i>	34.98 ± 2.34	34.20 ± 5.69
<i>S. aureus</i>	nd	69.40 ± 7.03*

Polyphenol mixture (PP) containing 0.1 mg/mL of gallic acid, caffeic acid, catechin and quercetin, final concentrations (0.59 mM gallic acid, 0.55 mM caffeic acid, 0.34 mM catechin and 0.33 mM quercetin). Synthetic honey (SH) containing the polyphenol mixture plus sugars (Fructose – 40%, Glucose 30%, Maltose 10% and sucrose 2%).

Data is an average of at least 3 experiments reported as % inhibition ± SEM. * indicates significant differences between the PP mix and SH.

Inhibition of bacterial growth by polyphenols depends on bacterial strain. A study by Cueva *et al.*, (2010), showed that CAT had no effect on *S. aureus* (Merck culture collection EP167) and *P. aeruginosa* (Merck culture collection PA01) over a concentration range of 0.625 – 1 mg/mL.

Also against non-pathogenic *E. coli* (ATCC 25922) and pathogenic with virulence factor deleted *E. coli* (CECT 5947), CAT had no effect. CAT did show a dose inhibitory response against *E. coli* (Merck culture collection IpxC/tolC) genetically modified to increase outer membrane permeability to approximately 40%. The authors concluded that inhibition of bacteria by phenolic compounds is strain dependent and that Gram-positive bacteria appeared more susceptible to inhibition by phenolic compounds compared to Gram-negative bacteria. Orhan *et al.*, (2010) evaluated the antibacterial effects of several flavonoids against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*. Generally Gram-positive bacteria were more sensitive to the effects of flavonoids than Gram-negative bacteria. Likewise in the present study, *S. aureus* was more sensitive to the effects of a SH containing besides phenolic acids also flavonoids, CAT and QUE.

The MIC of GA was reported by Borges *et al.*, (2013) to be 1.5 mg/mL (8.82 mM) for *E. coli* and 0.5 mg/mL (2.93 mM) for *P. aeruginosa*. Wu *et al.*, (2013) evaluated the structure-activity relationship of flavonoids using *E. coli* as a model bacteria. The lowest MIC₅₀ of 25.00, 35.76 and 36.72 ug/mL (0.087 mM, 0.118 mM and 0.144 mM) were reported for kaempferol, QUE and chrysin, respectively. Using liposomes these authors reported that flavonoids mediated bacteria killing by reducing membrane fluidity. Initial investigations in our laboratory, has identified kaempferol as a flavonoid present in southern African honey. The concentrations of polyphenols found in honey in this study (approx. 1 mg/mL) are sufficient to effectively inhibit the bacterial growth (Borges *et al.*, 2013). In addition honey can also contain unique polyphenols and derivatives that may have unique bioactivity such as leptosperin in Manuka honey and fatty diacid glycoside found in Scottish honey.

Although the antibacterial activity of plant extracts rich in polyphenols have been widely investigated (Rice-Evans *et al.*, 1996, Rice-Evans *et al.*, 1997, Rice-Evans 2001, Pérez-Vicente *et al.*, 2002, Riso *et al.*, 2005), the contribution of polyphenols to the antibacterial activity of honey is neglected. The reason for this is that it difficult to knock out the activity of polyphenols as can be done for H₂O₂ with catalase, MGO with GSH, antimicrobial peptides with SPS and protein or peptides with enzymatic digestion (Kwakman *et al.*, 2010, Kwakman *et al.*, 2011, Kwakman, Zaat 2012).

Studies on the synergism between honey and other molecules or mixtures such as antibiotics and extracts have been undertaken. Briozzo *et al.*, (1989) investigated the antibacterial activity of a mixture of polyphenols, 63% (w/w) sugars and clove oil 0.4% (v/v) which contains 90 – 95% of the polyphenol eugenol. This mixture had a germicidal effect against *S. aureus*, *P. aeruginosa*, *E. coli*, *Proteus vulgaris* and *C. albicans*. Within 2 – 7 min bacterial growth was

reduced from 10^7 to 10 CFU/mL. The antibacterial activity of the sugar solution was low compared to clove oil, however, clove oil was insoluble in water and very soluble in the sugar solution. Therefore the benefit of solubility and additional antimicrobial activity identifies the importance of synergism in complex mixtures such as honey. Likewise a beneficial, antibacterial effect was observed by Jayaraman *et al.*, (2010) for a mixture of polyphenols and antibiotics. Against *P. aeruginosa* a synergistic effect was observed between polyphenols (protocatechuic acid (MIC = 2 mg/mL), GA (MIC = 2 mg/mL), ellagic acid (MIC = 4 mg/mL), myricetin (MIC = 0.5 mg/mL)) and sulfamethoxazole (MIC = 0.128 mg/mL). For example, individually 0.5 mg/mL GA and 0.032 mg/mL sulfamethoxazole (concentrations that are $\frac{1}{4}$ of what was needed to achieve MIC) each showed no inhibition of bacteria, however when combined an MIC was achieved. A similar effect was observed for 0.5 mg/mL GA and 0.008 mg/mL tetracycline. Observed synergism between polyphenols and antibiotics was attributed to polyphenols making bacteria more susceptible to various drugs by for example, disrupting membrane fluidity (Wu *et al.*, 2013). The benefits of this effect is that the toxicity of antibiotics are reduced. Likewise synergism between polyphenols can also contribute to increased antibacterial activity. In the present study synergism has been observed for polyphenols in combination with honey sugars especially against *E. coli* and *S. aureus* indicating that as with H_2O_2 the presence of sugars enhance the antibacterial properties of polyphenols. It is important to identify and quantify the polyphenols found in AE, SEMh and WC as there may be specific synergistic interactions between polyphenols and/or as described for Manuka honey (Kato *et al.*, 2014) unique phenolic molecules with antibacterial activity.

4.4.3.5 The contribution of antimicrobial peptides

Kwakman *et al.*, (2010) showed that RS honey that contains BD-1 and H_2O_2 rapidly kills *B. subtilis* after 2 h with no activity against *E. coli* or MRSA (Kwakman *et al.*, 2011). Recombinant BD-1 was found to reduce that viability of *S. aureus* and *P. aeruginosa* biofilms (Sojka *et al.*, 2016).

Defensins are cationic peptides and will bind negatively polyanionic SPS thereby inhibiting the activity of the defensin, thereby reducing antibacterial activity. This strategy was used by Kwakman *et al.*, (2010) to identify the BD-1 as the major antibacterial component of RS honey. Using WC honey, which has similar antibacterial profiles as AE and SEMh (MIC of 25 – 30%) against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis* except for AE honey against *B. subtilis* (MIC 40%), the effects of defensin inactivation was determined. Filtration of MAN UMF10+, AE, SEMh and WC revealed that each fraction contained peptides with a molecular mass of <5 kDa and these were 0.92 ± 0.01 , 0.69 ± 0.02 , 0.73 ± 0.02 and 0.84 ± 0.01 mg/mL for MAN

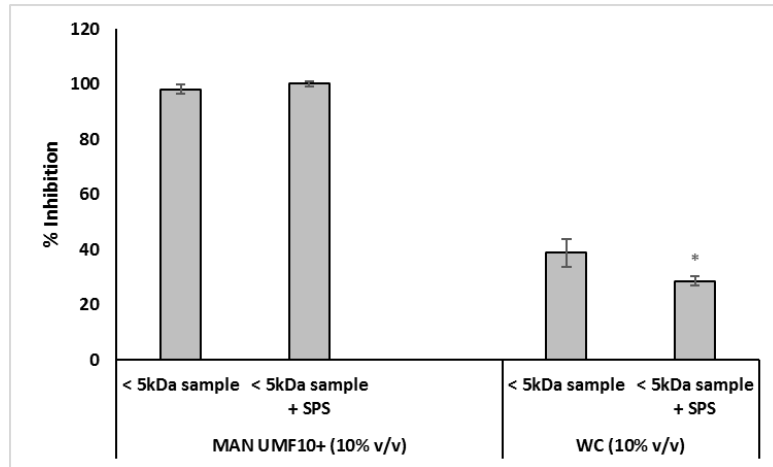
UMF10+, AE, SEMh and WC, respectively (Table 4.1). This fraction will also contain all other small molecules found in honey including MGO, H₂O₂, sugars and polyphenols.

Addition of SPS will result in the capture of any cationic peptides thereby reducing the antibacterial activity of the fraction. As a control the cationic antimicrobial peptide, melittin, derived from bee venom was used (Kwakman, Zaat 2012). SPS was found to effectively reduce the antimicrobial activity of melittin. The observed effect was a reduction of antibacterial activity of a 50 µM (0.142 g/mL) melittin solution from 100% to 50.96%, 51.64%, 84.30% and 47.44% for *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*, respectively, where *S. aureus* was the most sensitive and *B. subtilis* the least sensitive to the effects of melittin.

WC honey was used as a representative sample of southern Africa honey and the antibacterial activity of a 10% solution of the < 5 kDa was 38.85%, 35.08%, 58.97% and 88.49% against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*, respectively. With the addition of SPS to bind cationic antimicrobial peptides only activity against *E. coli* was reduced by 10.28% (Figure 4.6). Kwakman *et al.*, (2010) reported that for Revamil honey, the addition of SPS resulted in a loss of antibacterial activity from 6-8 to 1 Log CFU/mL for all bacteria evaluated. The small loss of activity against *E. coli* indicates that in BD-1 or any other cationic peptides are not responsible for the antibacterial activity of WC honey.

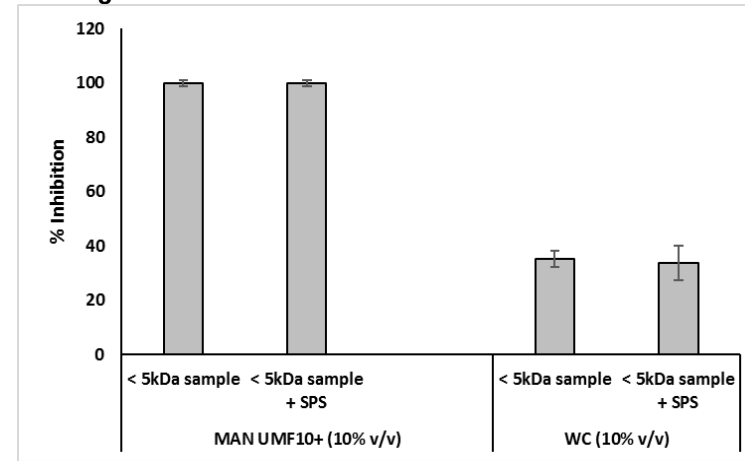
Gallardo-Chacón *et al.*, (2008) evaluated the effect of proteinase K and α-chymotrypsin digestion on the antibacterial activity of several monofloral and multifloral honeys. Enzymatic digestion altered the antibacterial activity of two honeys and inhibition was either partially or total following proteinase K or α-chymotrypsin digestion. Only antibacterial activity against MRSA was affected. In the present study following GDD only the activity of SEMh against *S. aureus* was affected. Following digestion none of the honeys evaluated by Gallardo-Chacón *et al.*, (2008) showed reduced activity against *E. coli*, *S. sonnei*, *S. mutans* and *E. faecalis*. The authors identified three inhibitory agents and these were a high sugar concentration, H₂O₂ formation, and the presence of proteinaceous antimicrobial compounds. The authors did not identify the proteinaceous antimicrobial compounds contributing to antibacterial activity.

E. coli

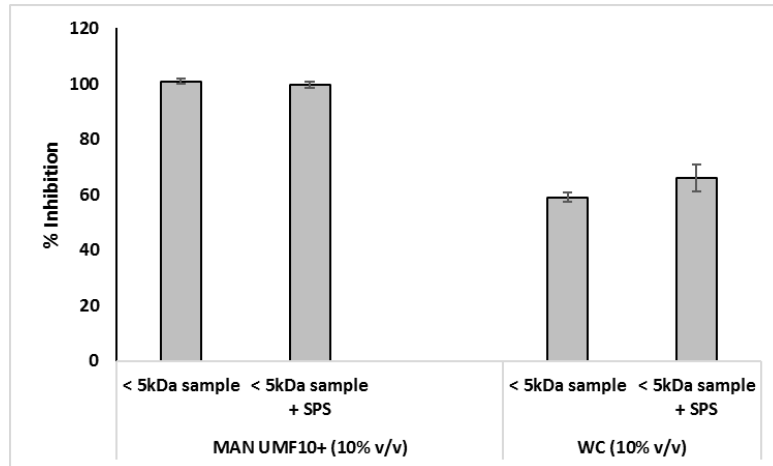


Gram-negative

P. aeruginosa



B. subtilis



Gram-positive

S. aureus

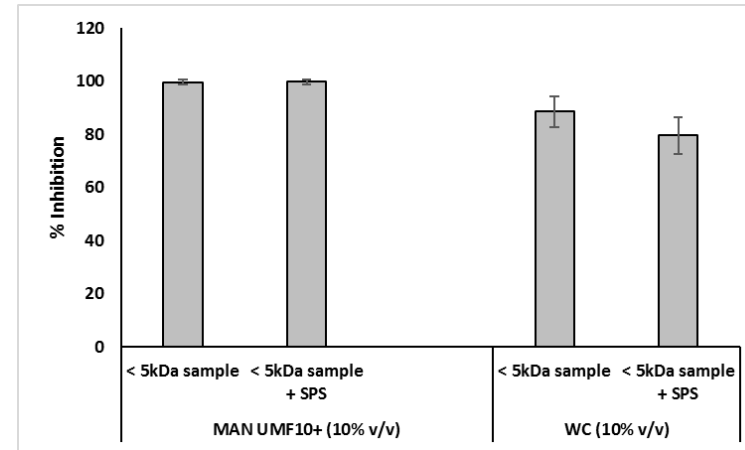


Figure 4.6: The antibacterial activity of a <5kDa filtered (MAN UMF10+ and WC) honey samples with or without SPS treatment on the antibacterial activity of on Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*B. subtilis* and *S. aureus*). Data is an average of at least 3 experiments reported as % bacterial inhibition \pm SEM. *indicates significant difference of untreated sample compared to SPS treated sample.

MAN UMF10+ – Manuka UMF10+ honey, WC – Western Cape, Fynbos honey, SPS – polyanetholesulfonic acid sodium salt.

The MIC of H₂O₂ is 9 mM against both *E. coli* and *P. aeruginosa* (Table 4.2). If H₂O₂ was the major contributing molecule to the antibacterial activity of southern African honey, it would be expected that the extent of change in activity following GDD would be the same. However, findings were that with digestion the antibacterial activity was reduced for *E. coli* but not *P. aeruginosa*, which implies that although H₂O₂ may contribute to the antibacterial activity of these honeys other molecules are involved.

Protein and/or peptides are susceptible to proteolytic degradation with digestion and will result in loss of activity. Using ExPASy peptideCutter it was found that within the sequence of BD-1 there are 22 cleavage sites which includes pepsin digestion sites. This implies that with gastric digestion the antibacterial activity of BD-1 would be lost. For all honeys no change in antibacterial activity was observed following gastric digestion. SPS, binds small cationic peptides and if these peptides contribute to antibacterial activity, binding will result in a loss of activity. Using WC as a representative honey sample only a small change in activity against *E. coli* was observed which implies that antibacterial profile of southern Africa honeys is different than that observed by Kwakman *et al.*, (2011) for Manuka and RS honey and Sojka *et al.*, (2016) for honeydew honey.

Brudzynski *et al.*, (2015) reported the presence of antimicrobial peptides, jelleins derived from royal jelly protein 1 (MRJP1) in honey. MRJP1 is the precursor of three antimicrobial peptides jelleins 1, 2 and 4. These authors found that the high mannose structure of MRJP1 may be responsible for the lectin-type activity of MRJP1 while the jellein-like sequences are responsible for the cell wall effects. Jelleins are composed of 8-9 amino acids, are amidated at the C terminal and have a net charge of +2. Jellein 1 (PFKISIH) and jellein 2 (TPFKISIH) is active against Gram-positive and negative bacteria and these include *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa*. Jellein 3 (EPFKISIH) has less broad activity while jellein 4 (TPFKISIH) is inactive (Romanelli *et al.*, 2011). These authors identified that the amino acid sequence contained in jellein 1 is necessary for antimicrobial activity.

Using ExPASy peptide cutter and the sequence of jellein 1, it was found that there are three cleavage sites at amino acids 1, 7 and 8 and with pepsin digestion a core of FKISIH. Romanelli *et al.*, (2011) do not provide information of the specific sequence that is required for activity, however a smaller derived sequence of 5 amino acids may still has antibacterial activity and this may account for the antibacterial activity observed following gastric digestion. Further digestion will create a smaller sequence of 4 amino acids (ISIH) with a single charge, which in turn may either have no activity or may have selective activity as has been reported for

several antimicrobial peptides and this may account for the differences seen following digestion.

In the previous chapter, it was postulated that the low levels of H₂O₂ and metals may result in the non-enzymatic fragmentation of Glc and Fru resulting in the formation of fragmentation products which cause cellular protein carbonylation (Figure 3.1). Likewise bacterial protein can undergo carbonylation with a subsequent loss of bacteria viability. If this is the mode of action, following GDD, there would be increased antibacterial activity rather than a loss of activity due to increased H₂O₂ levels that form (Table 3.11). With GDD, the pH is adjusted to 7.00, which has been reported to reduce the antibacterial activity of honey (Al-Waili 2004) and this may be due to the decomposition of H₂O₂ forming H₂O and O₂ at pH 7.

In the present study it was found that with digestion there was a decrease in the antibacterial activity against *E. coli* and not *P. aeruginosa*, indicating *P. aeruginosa* is a more sensitive Gram-negative bacteria to antibacterial components of honey. Similar MIC values for MGO and H₂O₂ as well as a similar response to SH (Table 4.4) indicates that these factors are not responsible for the observed differences. Although it is usually accepted that both bacteria are Gram-negative and therefore will react similarly there are major differences between these bacteria. The outer membrane permeability of *P. aeruginosa* is 12 – 100 fold lower than *E. coli*. Therefore *E. coli* is more sensitive to antibiotics such as ceftazidime and cefotaxime than *P. aeruginosa* with MIC₅₀ values of 0.125 vs 24/16 ug/mL, respectively (Hancock, 1998). The relative membrane permeability of *E. coli* is 100% and that of *P. aeruginosa* is 1- 8% and this is due to the number and type of porins present in the cell membrane. However, the same study also noted despite the higher permeability of *E. coli* some antibiotics needed higher concentrations to inhibit *E. coli* compared to *P. aeruginosa* (cephaloridine, piperacillin, tobramycin and nalidixic acid), indicating a different mechanism of action other than membrane permeability (Hancock, 1998).

A similar difference in activity was also observed between H₂O₂, BD-1, MGO and additional cationic and non-cationic compounds where killing of *B. subtilis* was rapid (after 2 h) while the killing of MRSA was slow (after 24 h) (Kwakman *et al.*, 2011) which identifies that other bacteria dependent factors play a role in bacterial killing.

Other cationic and non-cationic compounds were speculated to further contribute to the antibacterial activity of Manuka honey. Recently in Fyfe *et al.*, (2017) identified that a fatty diacid glycoside contributes to the antibacterial activity of Scottish honeys. Likewise in the present study indications are that MGO and BD-1 do not contribute to the antibacterial activity

of southern Africa honey. However sugars, H₂O₂ in combination with other novel antimicrobial molecules, possibly a polyphenol may act synergistically showing selective killing of bacteria following GIT digestion.

4.4.3.6 Effect of digestion on the health benefits of honey – antibacterial activity

Simulated digestion does not alter the antibacterial activity of MAN against all bacteria evaluated. With GD, all honeys maintained MIC, therefore honey from the southern African region can be hypothesised to have the ability to protect the mucosa of the stomach against infections such as *H. pylori*, which if not eliminated can lead to inflammation, peptic ulcers and gastric adenocarcinomas. With GDD there is a loss of activity, although some activity is retained. At low concentrations honey may have a prebiotic effect causing growth of beneficial gut microbiota while killing pathogenic microbiota (Section 4.4.3.2) and at higher concentrations an antibacterial effect although less than that found following GD. Therefore in the small intestine, MAN, AE, SEMh and WC and protect against gastroenteritis caused by overgrowth of bacteria which often results in diarrhoea, constipation and abdominal discomfort. These symptoms if not treated could develop into IBD whose main characteristic is altered microbiota in the gut (Zhong *et al.*, 2017).

4.5 Conclusion

Evaluated southern African honey had an MIC between 25 – 40% (v/v) and MANc an MIC between 6.25 – 30% (v/v). Sugars found in honey had bacteriostatic effects. The levels of MGO are low, however, H₂O₂ levels may be sufficient to contribute to the antibacterial activity of southern African honey. Polyphenols were found to act synergistically with sugars especially against *E. coli* and *S. aureus*. Pepsin digestion associated with the gastric phase had no effect antibacterial activity. However following, trypsin and chymotrypsin digestion antibacterial activity of AE, SEMh and WC was lost against *E. coli* and *B. subtilis* but not *P. aeruginosa* or *S. aureus*. This effect was probably not due to the presence of BD-1 and therefore the presence of additional non-cationic peptides with selective antibacterial activity should be considered. The recent identification of a novel antibacterial, the glycoside of methyl syringate in Manuka (Kato *et al.*, 2014) and a fatty diacid glycoside in Scottish honey (Fyfe *et al.*, 2017) indicates that future research should also focus on the identification of similar compounds in southern African honey with selective antibacterial activity that may act synergistically with sugars and H₂O₂ (gastroduodenal phase) to kill bacteria.

Chapter 5: Anti-inflammatory activity of undigested and *in vitro* digested honey samples and polyphenols

Abstract

Honey has well described antioxidant, antibacterial and anti-inflammatory activity and this has resulted in the use of honey as a wound healing product. Animal based studies of inflammation have shown that honey reduces inflammation in the gastrointestinal tract (GIT). However, little is known regarding the effect of the different phases of GIT digestion on the anti-inflammatory properties of honey related to the reduction of inflammation associated with *Helicobacter pylori* (*H. pylori*) infection and inflammatory bowel disorders such as irritable bowel disease (IBD) which includes Ulcerative Colitis (UC) and Crohn's disease (CD).

Three southern African honey samples Agricultural Eucalyptus (AE), South Eastern Mozambique (SEMh), Western Cape, Fynbos (WC) and a honey control, Manuka (MAN UMF10+) were subjected to simulated *in vitro* GIT digestion. Each fraction, undigested (UD), gastric (GD) and gastroduodenal digested (GDD) were investigated for nitric oxide (NO) scavenging activity using the sodium nitroprusside (SNP) assay and ability to reduce NO levels following the stimulation of L929 (mouse fibroblast) cells with *E. coli*. Lastly the ability of the UD and GDD digests to reduce lipopolysaccharide (LPS 011:B4) induced activation of platelets in whole blood was determined using scanning electron microscopy.

Cytotoxicity against L929 cells was only observed at honey final concentrations $\geq 3\%$. At low concentrations all honey samples reduced NO formation, while at concentrations $>25\%$ NO production was increased for MAN UMF10+ and WC. UD, GD and GDD samples at 10% inhibited NO formation. At 1% honey samples were not cytotoxic and also inhibited *E. coli* induced NO formation in L929 cells. LPS induced increased human platelet pseudopodia formation, spreading and aggregation associated with platelet activation. One percent solutions of UD and GDD did not cause platelet activation. UD and GDD fractions inhibited LPS induced platelet activation to varying degrees and the predominant morphological effect was the inhibition of pseudopodia formation. AE and SEMh compared to WC and MAN UMF10+ were the most effective honey samples in reducing platelet activation.

In conclusion honey has chemical and cellular anti-inflammatory activity which is maintained post-digestion. Not only does digested honey reduce NO formation but also reduces LPS induced platelet activation which shows potential therapeutic effects for patients with inflammatory conditions of the GIT.

5.1 Introduction

In the GIT, communication/cross talk between host cells and microbes is essential for the maintenance of homeostasis between cells and bacteria. Toll-like receptors on the intestinal epithelial cells are part of pattern recognition receptors that recognize certain bacterial molecules such as LPS initiating an immune response. Therefore, inhibition of this receptor activity can lead to the development of chronic inflammation (Pérez-Cano *et al.*, 2014). A typical example of such dysregulation is associated with IBD which includes UC and CD. Both are disorders of the GIT and there is increasing evidence that in both diseases inflammation and platelet activation contribute to the pathogenesis of disease (Voudoukis *et al.*, 2014).

UC is characterised by diffuse mucosal inflammation, which produces inflammatory mediators and consequently chronic inflammation leads to mucosal ulceration (Xavier, Podolsky 2007). Characteristic of UC is the depletion of goblet cells and lower mucin formation, which makes the mucosa vulnerable to inflammatory injury. In addition, aggregation of macrophages and consequent release of inflammatory mediators such as NO further potentiates inflammation (Xavier, Podolsky 2007). Characteristic of CD is the presence of epithelioid granulomas. Around the nodules are infiltration of lymphoid and plasma cells. In both diseases, the interaction between the hosts immune response and commensal bacteria play a pivotal role in the initiation and disease pathology (Xavier, Podolsky 2007). Enteric bacteria such as *E. coli* can adhere and infect the epithelium. Altered mucosal structure and permeability results in increased blood levels of LPS which promotes platelet activation and aggregation (Voudoukis *et al.*, 2014). Xavier, Podolsky (2007), identified that both inflammation and coagulation play an important role in the pathogenesis and the clinical manifestations of IBD.

Choghakhori *et al.*, (2017) reported that in IBD patients TNF α , IL-17, malondialdehyde and cytokine levels were increased while the IL-10 and total antioxidant capacity was reduced. Several other studies have identified that excessive ROS, RNS, lipid peroxidation and protein carbonylation reduced GSH levels and reduced functioning of cellular antioxidant pathways (Veza *et al.*, 2016). Several flavonoids, in animal models of colitis, reduce ROS, lipid peroxidation and improve the antioxidant status of the GIT mucosa (Veza *et al.*, 2016). Honey is a rich source of flavonoids which has beneficial antioxidant and anti-inflammatory effects (Table 5.1). Beneficial effects include scavenging activity, improving the cellular redox homeostasis and reducing the levels of inflammatory markers as well as NO formed by inducible nitric synthase (iNOS) which is expressed by inflammatory cells in response to bacterial products and pro-inflammatory cytokines. Excessive NO production interacts with

superoxide anions forming peroxy nitrates which then induces mucosal cellular and tissue damage (Veza *et al.*, 2016).

Table 5.1: Anti-inflammatory effects of honey.

Honey	Model	Effect	Reference
Manuka	Trinitrobenzene sulphonic acid (TNBS)-induced colitis.	↑catalase, superoxide dismutase (SOD), reduced glutathione	(Prakash <i>et al.</i> , 2008)
	<i>H. pylori</i> infections.	↓IL-8	(Keenan <i>et al.</i> , 2012)
	LPS stimulated THP 1 (monocytic leukemic) cells.	↓TNF-α	(Chen <i>et al.</i> , 2016)
	Acetic-induced gastric ulcers in rats.	↓TNF-α, IL-6, IL-1β, ↑GPx, SOD, IL-10	(Almasaudi <i>et al.</i> , 2017)
Gelam	Stimulated RAW 264.7 cells TNF-α-induced cytotoxicity of L929 cells.	↓NO, ↓TNF-α	(Kassim <i>et al.</i> , 2010)
	Endotoxemia.	↓NO, ↑heme oxygenase-1	(Kassim <i>et al.</i> , 2012)
	Carrageenan-induced paw edema.	↓Paw-size, ↓NO, ↓TNF-α, IL- and ↓PGE2 (possibly via inhibition of COX-2 and iNOS)	(Hussein <i>et al.</i> , 2012)
	Periodontitis induced Sprague Dawley rats.	↓IL-1β	(Aziz <i>et al.</i> , 2014)
	Glucose-induced oxidative stress in pancreatic hamster cells (HIT-T15).	↓ROS induced phosphor-JNK, IL-1β, phosphorylated IKK-β, IL-6, TNF-α, all related with inflammatory pathways	(Safi <i>et al.</i> , 2016)
Tualang	Alkali injury in rabbit's eyes.	↓Polymorphonuclear leucocytes (PMNs)	(Bashkaran <i>et al.</i> , 2011)
	UVB exposed murine keratinocytes (PAM212).	↓IL-1β, IL-6, TNF-α in	(Ahmad <i>et al.</i> , 2012)
Yemen Sidr	Carageenan-induced and histamine-induced paw odema and yeast-induced hyperpyrexia.	Inhibits inflammation	(Alzubier, Okechukwu 2011)
Taiwan	Human colon carcinoma (WiDr) treated with honey sample.	↓IL-8	(Liu <i>et al.</i> , 2013)
Buckwheat	Stimulated human PMNs.	↓ROS	(Van den Berg <i>et al.</i> , 2008)

LPS – lipopolysaccharide, IL – interleukin, TNF-α – tumour necrosis factor alpha, GPx – glutathione peroxide, NO – nitric oxide, PGE – prostaglandin, COX – cyclooxygenase, iNOS – nitric oxide synthase, ROS – reactive oxygen species.

Under normal physiological conditions, endothelial NOS (eNOS) produces low levels of NO that regulates colon blood flow, prevents platelet activation and produces ROS which has an antibacterial function. Platelets derived NO also prevents the aggregation of other platelets (Barale *et al.*, 2017). However, excessive iNOS activity and NO formation by macrophages and other inflammatory cells will have a deleterious effect on cell and tissue structure as well as function.

Platelet activation is mediated via the Toll-like receptor 4 (TLR4)/MyD88 and cGAMP-dependent protein pathway (Zhang *et al.*, 2009). Effects can be the direct activation of TLR by acting on the receptor and associated adaptor proteins or via signal transduction where the

flavonoids interfere with upstream and downstream kinases as well as transcription factors involved in the activation of inflammatory and immune responses. Flavonoids that effectively down regulate TLR (TLR2 and/or TLR4) include engeletin, astilbin, QUE, EGCG and baicalin and these effects have been evaluated in macrophages, endothelial keratinocytes and neuron cell cultures but not platelets (Pérez-Cano *et al.*, 2014). In addition, several flavonoids have been shown to suppress the NF- κ B signal pathway, inhibition of the kinases or the modulation of signal transduction events upstream of the relevant mitogen activated protein kinase (MAPK) pathways as well as the Janus Kinases signal transducers and activators of transcription (JAK-STAT) signalling pathway (Veza *et al.*, 2016).

Treatment of IBD, is multifactorial and involves anti-inflammatory and immunomodulatory therapy and modulation of the intestinal microbiome as well as symptomatic treatment. Traditional anti-inflammatory therapy such as the use of 5-aminosalicylates, corticosteroids, thiopurines and methotrexate have several side effects. Newer therapy options are antibodies to TNF, adhesion molecules, IL-12/23 and JAK kinase inhibitors that target inflammatory pathways (Bernstein 2015). For the prevention and treatment of IBD, multifunctional nutraceutical foods rich in flavonoids are exciting options to traditional therapies. This is due to the multi-functionality of flavonoids which would reduce ROS and NO levels, restore bacteria-host cell cross talk and prevent platelet activation by promoting the re-establishment of the integrity of the mucosa (Wang, Huang 2013). Furthermore, flavonoids can further regulate the microbiota – host relationship, by creating a prebiotic and probiotic environment as well as reducing the effect of LPS via TLRs as described above (Pérez-Cano *et al.*, 2014). Several *in vivo* studies have also identified the ability of flavonoids to inhibit TLR. Studies on rats orally fed with cocoa rich in procyanidins resulted in various modulation of TLR 2, 4, 7 and 9. In a clinical trial in which healthy subjects were on a high fat, high carbohydrate diet in addition to orange juice (rich in flavanones), TLR2 and TLR4 were found to be down-regulated. (Pérez-Cano *et al.*, 2014).

The aim of the research presented in this study is to determine if Manuka and southern African honey samples can scavenge NO, inhibit *E. coli* induced NO production and inhibit LPS induced platelet activation. Then to determine following digestion if these beneficial effects are retained.

The specific objectives were to:

1. Determine the ability of UD, GD and GDD to scavenge NO.
2. Evaluate the cytotoxicity of UD, GD and GDD in the L929 cell line.

3. Determine the ability of UD, GD and GDD to inhibit *E. coli* induced NO formation in the L929 cell line.
4. Lastly to determine the ability of UD and GDD honey to prevent LPS induced platelet activation.

5.2 Materials

5.2.1 Samples and cell lines

Honey digests were prepared as described in Chapter 3 and 4. The L929 murine fibroblast cell line used was from CELLONEX and was purchased from Separations, Johannesburg, SA. The L929 cells used in these experiments were between passage 55 – 65.

5.2.2 Reagents, equipment & disposable plastic ware

Reagents were similar to those used in Chapter 3, and also included sodium nitroprusside (SNP), sodium nitrite (NaNO_2), sodium hydroxide (NaOH), aluminium chloride (AlCl_3), N-(1-naphthyl)ethylenediamine (NED), sulphanilamide, phosphoric acid (H_3PO_4) purchased from Sigma Aldrich, SA and *E. coli* strain 25922 from the ATCC, used for LPS production. The LAL kit was obtained from Thermo Scientific, IL USA.

5.2.3 Laboratory facilities

All research was conducted in the research facilities of the Department of Anatomy, Faculty of Health Sciences and the Unit for Microscopy and Microanalysis at the University of Pretoria.

5.3 Methods

5.3.1 Samples

All honey samples were the same as used in Chapters 3 and 4.

5.3.2 Buffers and cell culture media and solutions

PBS and cell culture reagents were prepared as described in Chapter 3.

5.3.3 Simulated *in vitro* gastrointestinal digestion

Simulated *in vitro* GIT digestion of the honey samples was the same as described in Chapter 3.

5.3.4 Total flavonoid content assay

Flavonoids have been reported to play a role in reducing inflammation, therefore the TFC was determined using the aluminium chloride based on the formation of a red aluminium complex where flavonoid acts as a bidental ligand, forming complexes with the C-4 keto group and either the C-3 or C-5 OH group of flavones and flavonols (Amaral *et al.*, 2009). TFC was measured using a modified method of Amaral *et al.*, (2009). Catechin (0 - 0.21 mg/mL) was used to prepare a standard curve. A 10 μ L of a 10% (v/v) honey solution was added to each well of a 96 well microplate. To each well, 30 μ L of a 2.5% sodium nitrite, followed by a 20 μ L of a 2.5% aluminium chloride and then 100 μ L of a 2% sodium hydroxide were then added. The mixture was mixed well and the absorbance was read at 450 nm using a BioTek plate reader. For each sample a blank consisting of 10 μ L of a 10% (v/v) honey solution with 150 μ L PBS was run to correct for any colour interference. TFC of each honey sample was expressed as mg catechin equivalents (CE)/g of honey (converted to per gram by incorporating the wet weight of honey).

5.3.5 Chemical nitric oxide scavenging activity

SNP at physiological pH spontaneously releases NO, which then competes with O₂ to form (nitrites, NO₂⁻), or (nitrates, NO₃⁻). Compounds that scavenge NO reduces the levels of nitrite. Nitrite levels can be quantified with the Griess reagent. For the measurement of NO scavenging capacity a 5 mM SNP was prepared and left in the light for 1 hour, to trigger NO production. After which a volume of 80 μ L 5 mM SNP was added to 20 μ L sample, mixed well and incubated in the dark for 1 h. After incubation 100 μ L of Griess reagent (1:1 of 0.2% solution w/v of N-(1-naphthyl)ethylenediamine (NED) and a 2% solution w/v of sulphanilamide in 5% phosphoric acid) was added to the mixture and then the absorbance was read at 570 nm. To quantify data a standard curve of 0 – 0.05 mM NaNO₂ was prepared. Honey and polyphenol samples were tested for residual NO or nitrite levels. Results were then expressed as % NO produced. For undigested honey the concentrations that effectively scavenged NO was identified, at this concentration the effect of digestion was determined.

5.3.6 Maintenance of the L929 cells

L929 mouse fibroblast cells were used as they are a standard toxicology cell line. These cells were maintained in 2x diluted Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FCS and a 1% antibiotic solution (concentrations similar to Chapter 3). Vials were thawed rapidly in warm water at 37°C. The cells were suspended in 5 mL RPMI/FCS and collected by centrifugation. The supernatant was removed and the cells were re-suspended in fresh RPMI/FCS and plated in a 25 cm² culture flask. The flask was then incubated at 37°C

at 5% CO₂. Once the cells were confluent, the medium was removed from the flask and the cells were passaged by addition of 5% trypsin and incubated for 2 – 3 min at. To the flask containing the detached cells, 5 mL fresh RPMI/FCS added and the cells were collected via centrifugation at 200xg for 2 min. The medium was then removed and cells were re-suspended in 5 mL RPMI/FCS. The cell concentration was determined by counting a 10 µL aliquot of cells using a haemocytometer and the trypan blue exclusion method.

5.3.7 Cytotoxicity using L929 cells

A volume of 90 µL L929 cells was plated in a 96-well plate at a concentration of 4X10⁴/mL and incubated at 37°C/5% CO₂ overnight to attach. Using a concentration series (volume of 10 µL) of undigested samples honey for 24 hours, the honey concentrations that did not lead to a loss of L929 viability was determined. At an identified honey concentration that did not lead to a loss of cell viability (<10%) the effect of digestion was determined.

5.3.7.1 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide) assay

The MTT assay is a cytotoxicity assay that was originally reported to measure cell viability through mitochondrial function. Yellow soluble MTT dye is reduced by the enzyme succinate dehydrogenase in metabolically active mitochondria to a purple insoluble formazan product. Therefore, the activity of mitochondria could be correlated to cellular viability (Mosmann 1983, Chiba *et al.*, 1998). However, it has been recently reported that the MTT dye is not reduced in the mitochondria, due to its strong oxidative ability but in the endoplasmic reticulum/cytoplasm where it is reduced by nicotinamide enzymes resulting in formazan products being released in lipid droplets (Stockert *et al.*, 2012). However, this assay can still be used as an indicator of cellular viability. A 10 µL volume of the MTT solution (1 mg/mL in 0.1 M PBS) was added to the wells of a 96-well plate containing L929 cells and was then incubated for 3 hours at 37°C. After 3 hours, the dye and medium was removed and the plate was left to dry. The purple insoluble formazan product was then solubilised using 25% DMSO in EtOH and read at 570 nm. Results were reported as percentage viability, compared to 100% control not exposed to honey.

5.3.8 Nitric oxide production in the L929 cells

LPS is found in the cell wall of Gram-negative bacteria and was used to stimulate the L929 cells to produce NO, using fixed *E. coli*.

5.3.8.1 Preparation of fixed *E. coli*

From the original frozen stock, same as used in Chapter 4, Section 4.3.4, *E. coli* was streaked and left overnight at 37°C. Thereafter 2 – 3 colonies were then picked and grown in 10 mL of LB broth overnight at 37°C, to ensure that the bacteria reached the stationary phase of growth. The bacteria were then fixed with formaldehyde (final concentration 2%) for 1 h at room temperature before being collected by centrifugation. The pelleted bacteria were washed twice with sterile PBS. Bacteria were then re-suspended to the original 10 mL volume, sonicated for 1 h and aliquots were stored at -80°C until required.

5.3.8.2 Limulus amoebocyte lysate assay

To determine the endotoxin units (EU) present in *E. coli* and LPS, the limulus amoebocyte lysate (LAL) assay was used. This involved creating a standard 0 – 1 EU/mL, from LPS (stock solution, 35 EU/mL). A volume of 20 µL of each concentration of standard or 20 µL of sample was incubated at 37°C to equilibrate the temperature. A volume of 10 µL LAL reagent (diluted according to manufacturer's instructions) was then added and further incubated for 10 min at 37°C. Thereafter a volume of 20 µL, 2 mM chromogenic substrate was added, then mixed before a further 10 min incubation at 37°C. Ten microliters of 25% acetic acid was finally added to stop the reaction. The mixture was then read at 405 nm and EU/mL was calculated from the standard.

5.3.8.3 Nitric oxide production in L929 using fixed *E. coli*

A volume of 80 µL L929 cells were plated at a concentration of 1.25×10^6 cells/mL in RPMI/FCS in a 96-well plate. To this 10 µL of honey sample (final concentration, 1% solution), UD, GD or GDD and 10 µL of fixed *E. coli* was added, yielding a final cell concentration of 1×10^6 cells/mL. After 24 h incubation at 37°C, 5% CO₂, 50 µL of supernatant was collected to which 50 µL Griess reagent (prepared as described in section 5.3.4) was added and then the absorbance was read at 570 nm. To the remaining 50 µL in the 96-well plate containing L929 cells, 5 µL MTT (as described in section 5.3.6.1) was added to determine cell viability.

5.3.9 Effect on platelet activation on whole blood using lipopolysaccharide

Whole blood (WB) was collected from healthy, consenting donors (Research Ethics Committee, University of Pretoria, ethics number: 476/2014). Using a sterile needle whole blood was collected into citrate tubes. For these experiments the negative control consisted of 80 µL whole blood (WB) and 20 µL PBS, while the positive control was 80 µL WB, 10 µL LPS and 10 µL PBS.

Honeys have been reported to contain residual amounts of LPS and therefore to determine whether the honey samples activate platelets and/or are cytotoxic, 10 μ L sample and 10 μ L PBS was added to 80 μ L WB. The effect of UD and GDD on LPS induced platelet activation was determined by adding 10 μ L honey sample and 10 μ L LPS to 80 μ L. The final volume of all samples was 100 μ L and the final concentration of LPS (011:B4) was 50 ng/mL. The samples were incubated for 30 min at room temperature.

After incubation 10 μ L of the samples were smeared on a cover slip and left to dry for 5 min. Coverslips were then washed in PBS before being fixed in a solution of 2.5% formaldehyde and 2.5% glutaraldehyde in a 0.075 M phosphate buffer (pH 7.4) (NaP buffer) for 30 min. After fixation, the samples were then rinsed 3 times for 10 minutes with NaP buffer. The samples were placed in a secondary fixative of 1% osmium tetroxide for 30 minutes before the samples were washed again 3 times with NaP buffer as described above. The samples were then dehydrated using increasing concentrations of ethanol for 10 min each (30%, 50%, 70% and 90%). Samples were further dehydrated 3x in 100% EtOH for 10 min each. The samples were dried using hexamethyldisilazane (HMDS) for 1 h and were then mounted with carbon tape on aluminium stubs before being coated with carbon. The samples were then viewed with a Zeiss Ultra plus FEG scanning electron microscope. Images were taken at 1kV. The extent of platelet activation was evaluated by using the following scoring system where (-) represents no activation (samples are similar to control), (+) activation properties present at few/low quantities, (++) activation properties present at moderate quantities and (+++) activation properties present at extensive quantities.

5.3.10 Statistical analysis

All quantitative experiments were done at least in triplicate and represented as mean \pm SEM. Analysis was done using the students T-TEST and one way ANOVA followed by the Tukey post-hoc test with significant differences determined at $p \leq 0.05$.

5.4 Results and Discussion

5.4.1 With gastroduodenal digestion flavonoid content of honey is unaltered

Honeys had a TFC range between 0.17 – 0.29 mg CE/g with SEMh having the highest content (Table 5.2). With GDD there was no loss in activity, similar to antioxidant activity measured with the TPC, TEAC and ORAC assays (Table 3.1).

Table 5.2: TFC of UD and GDD honeys.

	TFC (mg CE/g)			
	MANc	AE	SEMh	WC
UD	0.20 ± 0.02	0.19 ± 0.02	0.29 ± 0.04	0.17 ± 0.06
GDD	0.29 ± 0.04	0.27 ± 0.03	0.40 ± 0.06	0.18 ± 0.03

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, TFC – total flavonoid content, UD – undigested, GDD – gastroduodenal digested.

Data is expressed as an average of at least 3 experiments ± SEM.

There was no significant differences between UD and GDD, or amongst honeys, $p < 0.05$ using one way ANOVA.

5.4.2 Honey has scavenging effects against nitric oxide production

The honey samples contained no NO or nitrite content, indicating that without the presence of an NO-inducer these honeys did not have the ability to produce NO (Appendix 14). At the lowest concentration of 6.25% (v/v) all honey samples inhibited NO formation by 60 – 70% (v/v) (Figure 5.1). At higher concentrations of 12.5 – 40% (v/v) of AE and SEMh, no further inhibition of NO formation was observed. In contrast, to an expected increase in NO scavenging there was a loss of NO scavenging and this was observed for MAN UMF10+ (line equation = $2.728x + 4.9535$ and $R^2=0.9229$) and WC (line equation = $1.0172x + 22.714$ and $R^2=0.943$). AE and SEMh honeys, both have anti-inflammatory activity related to NO scavenging ability. In contrast, MAN UMF 10+ and WC reduce NO production at lower concentrations but NO formation is increased at higher concentrations.

Mukohda *et al.*, (2010) reported that MGO enhanced SNP induced relaxation in rat aorta via a ROS/RNS-independent pathway. Although the authors concluded that this was not due to NO formation by SNP, the combination of SNP and MGO could increase NO production. With MAN UMF10+ and WC honey NO production increases at high concentrations which according to Majtan (2014), honey can either stimulate or inhibit inflammation depending on the phase of inflammation. In the acute phase of inflammation, honey activates monocytes/macrophages, neutrophils and keratinocytes, which then produce cytokines, ROS and matrix metalloproteinase, respectively while in the chronic phase of inflammation the above inducers are inhibited and consequently the effectors of inflammation are inhibited. Therefore MAN UMF10+ and WC could possibly be anti-inflammatory at low concentrations and pro-inflammatory at higher concentrations.

Honey samples were then digested and assayed at a concentration of 10% (v/v) as this was the concentration all the UD honey samples reduced NO levels. No significant changes in NO scavenging activity were found with GD digestion (Appendix 12). Following GDD, all honey samples except MAN UMF10+ maintained their NO scavenging activity when compared to

UD samples (Table 5.3). MAN UMF10+ with GDD showed a decrease in NO scavenging activity (Table 5.3). The NO scavenging of all the GDD honey samples was similar.

Table 5.3: Effect of 10% (v/v) honey digests on NO production.

	MAN UMF10+	AE	SEMh	WC
UD	50.28 ± 1.01	37.74 ± 3.38	36.78 ± 1.30	49.71 ± 2.62
GDD	34.20 ± 1.18*	36.34 ± 2.10	37.78 ± 2.48	37.07 ± 1.90

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, UD – undigested, GDD – gastroduodenal digested.

%NO production of UD and GDD compared to SNP alone no honey added. All data is an average of at least three experiments ± SEM. * indicates significant differences between UD and GDD samples using one way ANOVA, p<0.05.

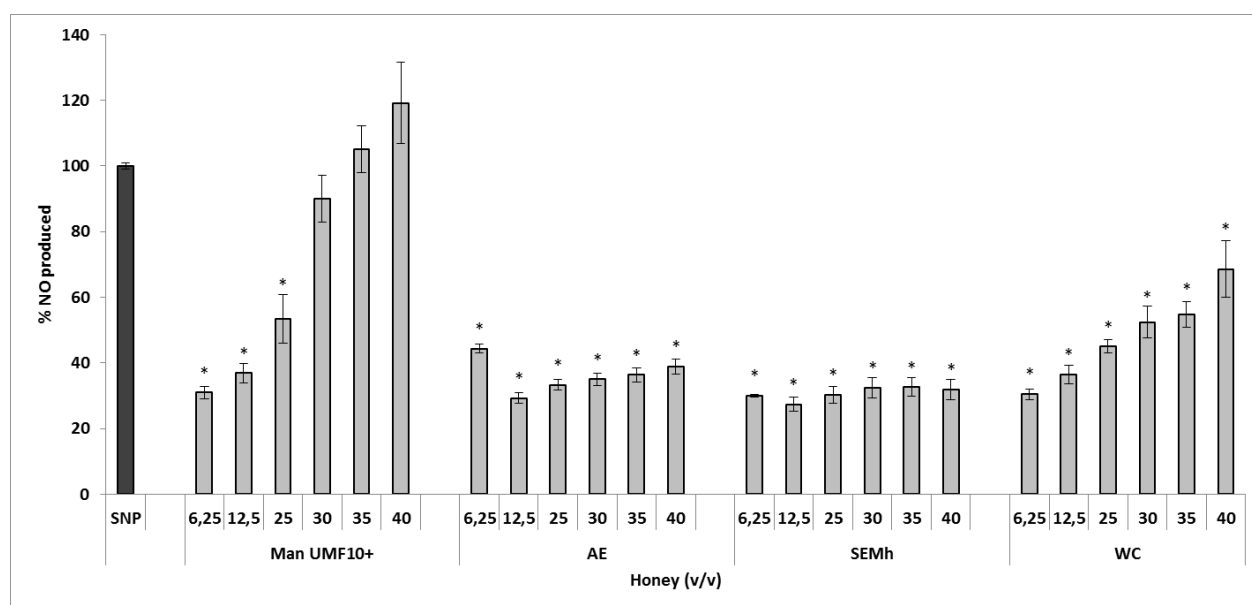


Figure 5.1: % NO production of 6.25 - 40% honey solutions in the presence of SNP, compared to SNP alone (100% NO produced). Data is expressed as an average of at least 3 experiments ± SEM. * Indicates significant decrease in NO levels compared to control (SNP in the absence of honey) p< 0.05, using one way ANOVA.

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, NO – nitric oxide.

5.4.3 Honey shows cytotoxicity against L929 cells at high concentrations

To determine if the UD and GDD digested honey samples inhibit NO production in the L929 cell line, the cytotoxicity of the UD and the GDD honey samples was determined. The cytotoxic effect of 24 hours exposure to 0.63 – 4% v/v on the L929 cell line is presented (Table 5.2). With increasing concentrations, all honey samples caused a decrease in cell viability. Compared to the control (no honey added), loss of cell viability was significant for 3-4% for MAN UMF10+ and AE as well as 3.5% and 4% for SEMh and WC.

Fauzi *et al.*, (2011), observed that for Tualang honey between 1 – 10% (v/v) apoptosis was induced in breast (MCF-7 and MDA-MB-231) and cervical cancer (HeLa) cell lines in a dose-

dependent manner, while having no effect on non-cancerous breast (MCF-10A) cell line. Exposure of MCF7 cell to MAN UMF10+ for 24 hours resulted in a decrease in cell viability, where at 4% only 30% of cell viability was retained (Portokalakis *et al.*, 2016). Likewise, in the present study, L929 cell viability was reduced to 50%. The L929 cell line is a mouse fibroblast cell whereas the RAW 264.7 cell line is a mouse macrophage cell line. Toxicity to both these cell lines (RAW 264.7 cells, Appendix 15) at higher honey concentrations of > 4% may imply potential anti-cancer activity and this effect, especially of southern African honey samples, should be investigated further.

At a final honey concentration of 1% (v/v) no cytotoxicity was observed for all UD samples. The effect of digestion on cell viability was then determined. GD showed the same trend as GDD samples showing no significant loss of cell viability and no significant differences when compared to UD (Appendix 13). No change in cell viability was observed following GDD compared to UD (Table 5.4).

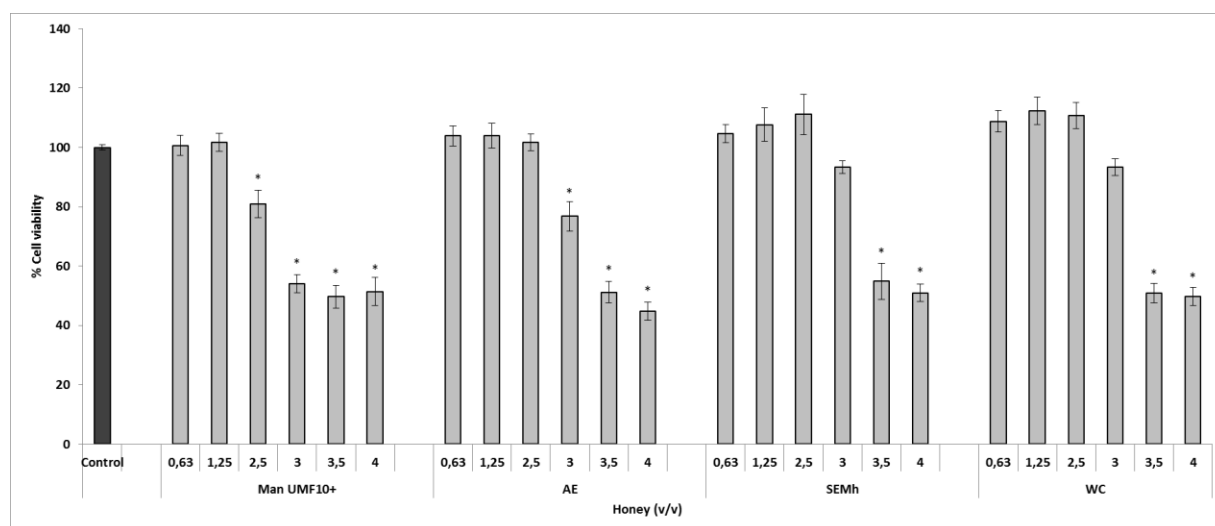


Figure 5.2: Cytotoxicity of 0.63 – 4% v/v undigested honey samples exposed to the L929 cell line for 24 h evaluated using the MTT assay. Data is an average of at least 3 experiments represented as mean \pm SEM. * indicates significant decreases compared to control, using one way ANOVA, $P < 0.05$.

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey.

Table 5.4: Effect of 1% (v/v) honey digests on L929 cell viability.

	MAN UMF10+	AE	SEMh	WC
UD	93.30 \pm 2.21	100.14 \pm 6.70	95.41 \pm 4.88	94.27 \pm 2.26
GDD	96.28 \pm 2.40	99.45 \pm 7.35	92.34 \pm 3.40	98.13 \pm 4.45

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, UD – undigested, GDD – gastroduodenal digested.

% Cell viability of UD and GDD honey samples. All data is an average of at least three experiments \pm SEM. * indicates significant differences between UD and GDD samples using one way ANOVA, $p < 0.05$.

5.4.4 Honey inhibits *E. coli* induced nitric oxide production in L929 cells

The ability of 1% (v/v) honey samples and digests to inhibit LPS induced NO formation in the L929 cell line was then determined (Table 5.4). In this study, the ability of several different forms of LPS to induce NO production in the L929 cell line was evaluated. Pure LPS (0111:B4) and LPS (055:B5) did not induce NO formation. Using fixed, dead *E. coli*, significant increase in NO levels was achieved. This lack of NO production by LPS may be due to several factors such as the source or the form of LPS, LPS aggregation and the levels of LPS binding protein in heat inactivated FCS. Katz *et al.*, (1996) described the potent signalling of human leukocytes by *E. coli* and this was the result of direct interaction between the whole bacteria and host cells. This is either due to direct cell-cell interactions or the delivery of high levels of LPS concentrated on the bacterial cell wall. The authors reported that a single *E. coli* bacterium contains $\pm 2 \times 10^6$ LPS molecules. In the present study endotoxin units were determined using the LAL assay and it was found that the LPS (011:B4) and LPS (055:B5) had 2.1 and 2.4EU/mL, respectively whereas the *E. coli* used had 2.3 EU/mL. Therefore, 1×10^6 L929 cells were exposed to 2.3 EU, which caused a 164 μ M increase in NO levels.

The ability of UD and GDD honey samples to reduce *E. coli* induced NO levels was then determined. All UD honey samples reduced cellular NO levels to a range of 42.23 – 73.01%. With GDD, this activity was maintained within a range of 47.74 – 74.64% (Table 5.5). Differences between the UD and GDD honey samples as well as differences between samples at the different stages of digestion was not significant. No studies could be found evaluating the effect of digestion on the anti-inflammatory properties of honey.

Table 5.5: Effect of 1% (v/v) honey digests on the cellular NO scavenging activity in L929 cells.

	MAN UMF10+	AE	SEMh	WC
UD	73.01 \pm 11.70	42.23 \pm 6.34	65.60 \pm 16.03	63.56 \pm 10.78
GDD	68.39 \pm 11.83	52.39 \pm 9.40	74.64 \pm 16.75	47.74 \pm 14.84

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, UD – undigested, GDD – gastroduodenal digested, NO – nitric oxide, L929 – mouse fibroblast cells.

% NO reduction in UD and GDD honey samples. All data is an average of at least three experiments \pm SEM. * indicates significant differences between UD and GDD samples using one way ANOVA, $p < 0.05$.

5.4.5 Lipopolysaccharide shows increased platelet activation in whole blood

LPS is an endotoxin found in the walls of Gram-negative bacteria that causes endotoxemia in both humans and animals. Endotoxemia is characterised by increased cytokines such as NO, which result in acute or chronic inflammatory related pathologies (Kassim *et al.*, 2012). Once LPS is released systemically, it binds to CD14 causing an inflammatory response which could lead to sepsis, an uncontrolled inflammatory response (Murch *et al.*, 2007). *E. coli* is the most

common Gram-negative pathogen in these infections and interacts directly with platelets via LPS. The important structural components of LPS responsible for this interaction are the O-antigen and the inner core regions of LPS (Lenehan *et al.*, 2016).

LPS has been shown to induce NO production in RAW 264.7 macrophages (Woo *et al.*, 2005, Kassim *et al.*, 2010, Majtan 2014, Malan *et al.*, 2016,). Mouse bone marrow derived macrophages in the presence of LPS also produce NO, however co-cultures of macrophages with platelets produce lower levels of NO, TNF- α and IL-6 than macrophages exposed only to LPS (Ando *et al.*, 2016). The suppression of NO production by platelets found by Ando *et al.*, (2016) was in part due to factors secreted by platelets whereas the suppression of NO by macrophages was due to the modulation of the NF- κ B signalling pathway. Another study by Barale *et al.*, (2017) showed that glucagon-like peptide and related peptides also increases NO levels therefore reducing platelet activation. It can thus be concluded that reduced NO levels in blood would cause platelet activation, whereas increased NO levels in blood would retard this process. However, with systemic infections this normal physiological process which is tightly regulated is disrupted and an uncontrolled inflammatory effect occurs.

Cytotoxicity, of drugs, molecules and complex mixtures including honey involves the use of cell line based assays where several functional parameters are measured such as cell viability and lactate dehydrogenase (LDH) leakage and/or markers of apoptosis (Chiba *et al.*, 1998). To investigate possible toxic effects on the cell membrane, the haemolysis assay is often used (Ximenes *et al.*, 2010). Characteristic features of toxicity are the formation of echinocytes and spherocytes as well as increased release of the cytoplasmic content which includes haemoglobin which can be quantified using spectrophotometric methods.

Micrographs of WB (negative control) and WB exposed to LPS (positive control) are shown (Figure 5.3). In the negative control, most red blood cells have a typical discoid shape. Exposure to LPS, honey alone and combinations of honey and LPS showed the presence of a few echinocytes and spherocytes (Figures 5.4, 5.5, 5.6 and 5.7). This implies that LPS alone and in combination with UD and GDD honey did not result in red blood cell haemolysis and this confirms the results obtained with L929 cells.

In the negative controls (not exposed to honey and/or LPS) (Figures 5.3A, B) there are minimal changes in platelet morphology (orange arrows). Few pseudopodia (blue arrows) are present which may be due to contact activation that occurs during sample preparation. In the positive control (WB with LPS) (Figure 5.3C), there is an increased number of activated platelets and

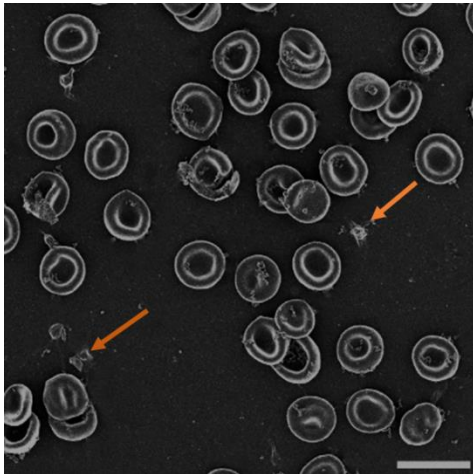
associated formation of long slender pseudopodia (blue arrows, Figures 5.3E, F). Platelet aggregation (yellow arrow, Figure 5.3D) and spreading is present (green arrow, Figure 5.3D, F).

Using washed human platelets, Zhang *et al.*, (2009) determined if LPS directly activates platelets. LPS alone did not activate the washed platelets but in the presence of platelet agonist's collagen and thrombin, LPS-induced activation occurred. In the presence of platelet enriched plasma, collagen induced platelet activation occurred. LPS from different sources was also evaluated and findings were that 0127:B28 > 0111:B4 > 055:B5 potentiated collagen induced platelet activation. In the present study WB is used which contains plasma, erythrocytes, leukocytes as well as platelets. Successful platelet activation associated with pseudopodia formation and platelet spreading was observed and this is due to the presence of the required plasma components as well as the cellular component of blood such as the leukocytes that contribute to LPS-mediated platelet activation (Zhang *et al.*, 2009).

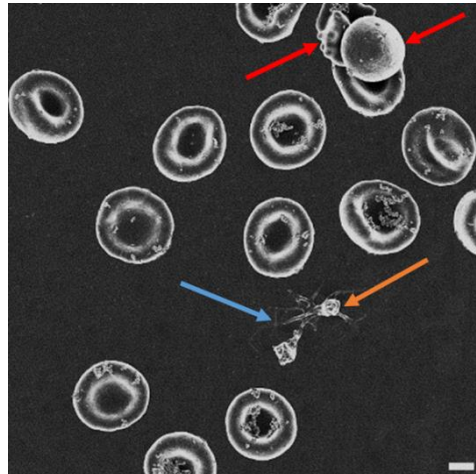
LPS activation of platelets occurs when LPS binds LPS-binding protein (LBP) which is recognised by CD14. The LPS-LBP-CD14 complex is then transferred to TLR4 which recognises LPS with the help of myeloid differentiation factor 2 (MD-2). Intracellular activation then occurs with the help of multiple adaptor proteins such as myeloid differentiation factor 88 (MyD88) (Zhang *et al.*, 2009, Ando *et al.*, 2016). Therefore, modulation of platelet activation occurs not through the ability of LPS directly stimulating NO in blood, which would inhibit platelet activation and aggregation but predominantly indirectly via the TLR4/MyD88 and cGMP-protein kinase dependent pathways (Zhang *et al.*, 2009, Ando *et al.*, 2016).

NEGATIVE CONTROLS (whole blood only)

A

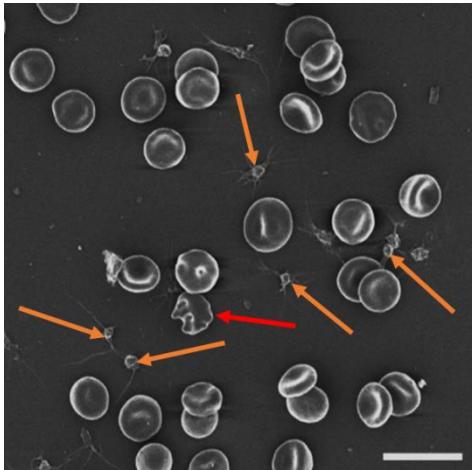


B

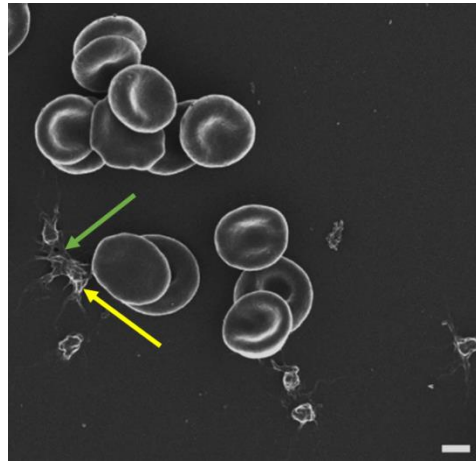


POSITIVE CONTROLS (whole blood + LPS)

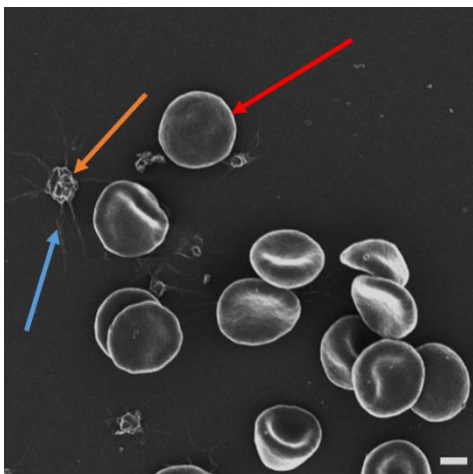
C



D



E



F

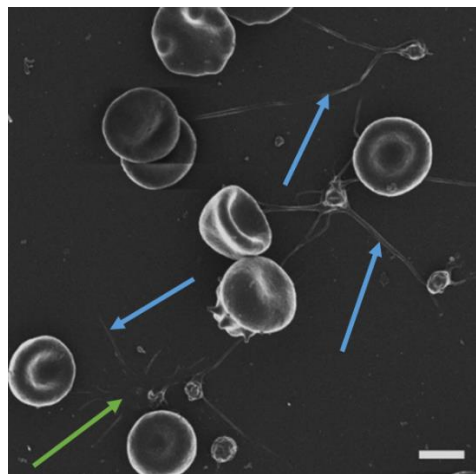


Figure 5.3. Representative scanning electron micrographs of whole blood alone and whole blood exposed to lipopolysaccharide (LPS). Figure A and B indicate the negative control, with minimal structural alterations. Figures C and D shows an increase in platelet activation in the positive control. Figures E and F shows an increase in platelet activation in the positive control. Orange arrows - body of the platelets; blue arrows - pseudopodia; green arrows - platelet spreading; yellow arrows - aggregation, red arrows - abnormal red blood cell morphology (Scale bars: A and C: 10 μ m; B and D: 2 μ m)

5.4.6 Honey shows protection against morphological changes associated with LPS-induced platelet activation

Honey contains a mixture of components in its food matrix, most of which are beneficial, some of which are detrimental. Honey contains approximately $5.6 \times 10^{-7} - 6.9 \times 10^{-6}$ $\mu\text{g/mL}$ LPS (Tonks *et al.*, 2003), therefore the possible activation effects of honey in the absence of LPS was determined. Other cytotoxic components of honey include MGO, H_2O_2 and in some honeys such as honey from Turkey, derived from *Rhododendron* or containing grayanotoxins sources also known as mad honey (Gunduz *et al.*, 2008) may also cause cellular damage and the activation of platelets.

The effect of honey samples on their own and honey samples in the presences of LPS on the morphology of platelets (Figures 5.4 – 5.11, Table 5.6). In the negative control not exposed to LPS and/or honey some platelets had few pseudopodia and membrane ruffling. Exposure to LPS caused to formation of distinctive long thin pseudopodia, some platelet spreading and aggregation (Figure 5.3).

UD honey samples alone with no LPS added, MAN UMF10+, and SEMh caused some pseudopodia formation and membrane ruffling (Figure 5.4A and B, 5.8A and B) similar to the negative control (Figure 5.3A, B). Pseudopodia formation without membrane ruffling was observed for AE and WC (Figure 5.6A and B, Figure 5.10A and B). Effects were MAN UMF10+ > SEMh > WC, AE with AE and WC causing no changes to platelet morphology.

Exposure of WB to UD honey and LPS in combination, reduced the effect of LPS alone (Figure 5.3). Comparisons between the undigested AE honey samples not and exposed to LPS revealed no differences in morphology. For SEMh there is a loss of membrane ruffling, for MAN UMF10+ honey increased platelet spreading was observed and for WC increased ruffling and spreading of platelets were also observed, although all observed effects were less, especially related to pseudopodia formation when compared to WB exposed only to LPS (Figure 5.3F). Effects were UMF10+ MAN, WC > AE, SEMh with AE and SEMh having no effect. In summary, all UD honey samples were not cytotoxic and caused minor morphological changes associated with platelet activation. Furthermore, UD honey reduced LPS induced platelet activation.

WB was also exposed to the GDD samples with and without LPS. MAN UMF10+ in the absence of LPS caused the formation of pseudopodia, membrane ruffling, spreading and platelet aggregation (Figure 5.5A, B) although to a lesser extent than what was observed for LPS alone. For AE, pseudopodia formation, membrane ruffling and platelet spreading was

seen (Figure 5.7A, B) and for WC only pseudopodia formation and spreading (Figure 5.11A, B) while for SEMh only induced pseudopodia formation (Figure 5.9A, B) was observed. For the GDD evaluated (no LPS added) MAN UMF10+ > AE > WC > SEMh with SEMh having the lowest effect.

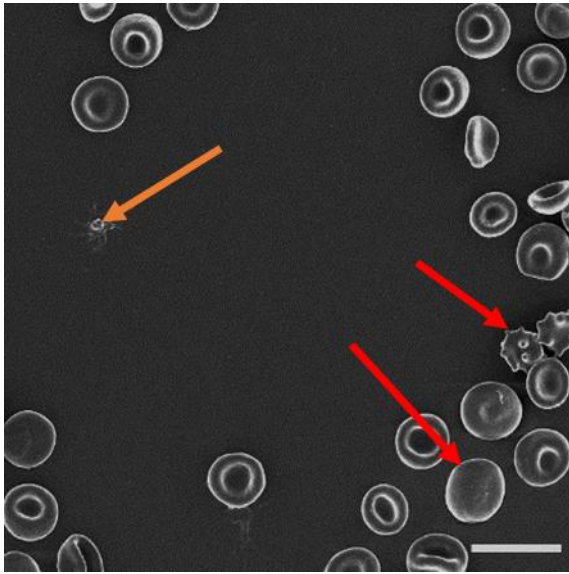
Together with LPS only minor changes to platelet morphology was observed (Figure 5.5C and D, 5.7C and D, 5.9C and D, 5.11C and D). Only for WC in the presence of LPS an increase in platelet spreading was observed (Figure 5.11C and D) although not to the same extent as LPS alone. In summary, all honey GDD protected platelets against LPS-induced changes in morphology and the effect was WC > MAN UMF10+ > SEMh > AE with AE protecting the most effective in preventing LPS induced platelet activation. In this study the observed inhibition of platelet activation can be due to the direct scavenging of NO via the cGMP-dependent protein kinase pathway or the inhibition of the TLR4/MyD88 pathway as has been described for several flavonoids (Pérez-Cano *et al.*, 2014).

Inhibition of NO production will lower NO levels causing platelet activation while inhibition of the TLR4/MyD88 pathway will inhibit platelet activation. The findings of this study, is that UD and GDD honey inhibits LPS mediated activation of platelets, which suggests that the TLR4/MyD88 pathway is the predominant pathway involved in LPS mediated platelet activation. Little is known if flavonoids directly bind LPS, as this would be an additional mode of action.

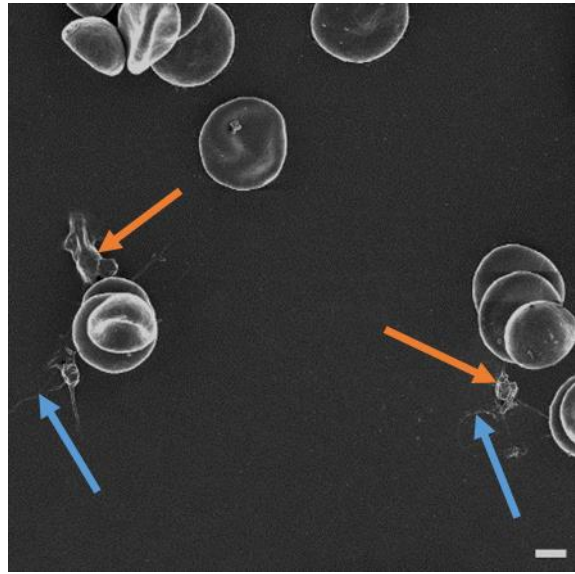
In summary UD and GDD honeys, inhibit NO formation associated with inflammation both in a chemical and cellular environment, as well as possibly the platelet TLR4/MyD88 pathway that following exposure to LPS causes platelet activation. Consequently, honey has anti-inflammatory effects and will therefore have beneficial effects in inflammatory conditions such as those seen in patients with IBD.

UD – MAN UMF10+ (-LPS)

A

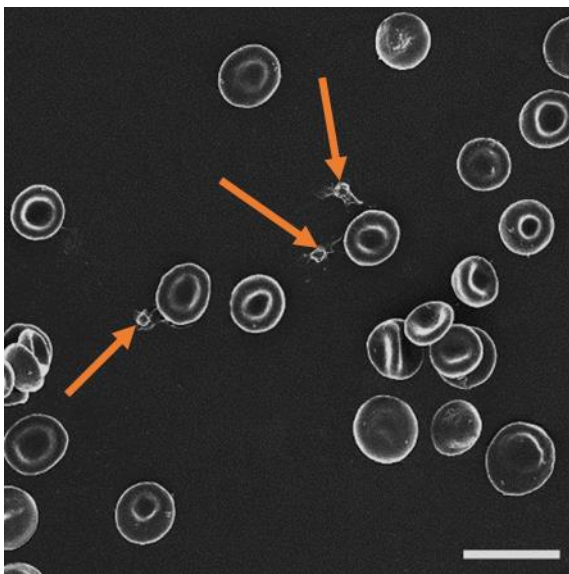


B



UD – MAN UMF 10+ (+LPS)

C



D

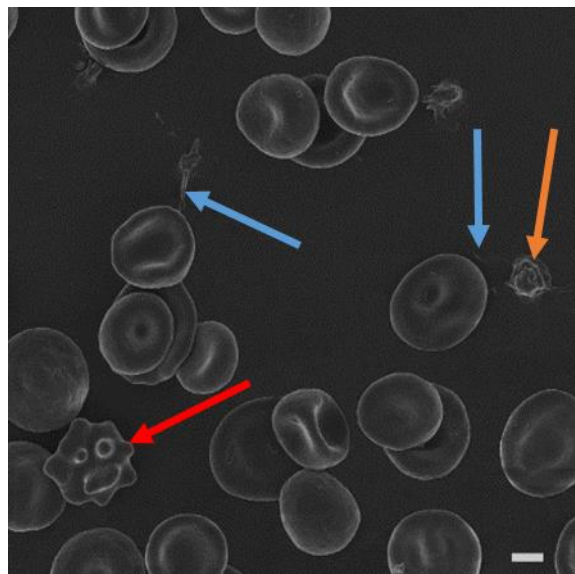
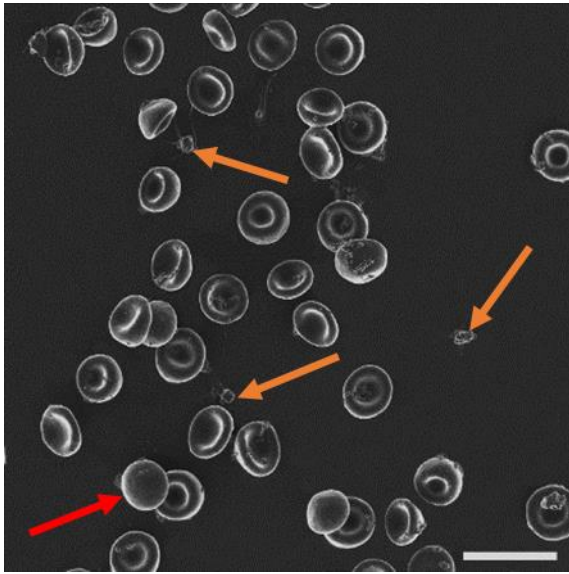


Figure 5.4: Scanning electron micrographs of whole blood exposed to 1% (final concentration) of UD honey samples (A-D) without (A & B) or with (C & D) LPS. Orange arrows: platelet body, blue arrows: pseudopodia, green arrows: platelet spreading, red arrows: abnormal red blood cell morphology. (Scale bars: A and C: 10 μ m; B and D: 2 μ m).

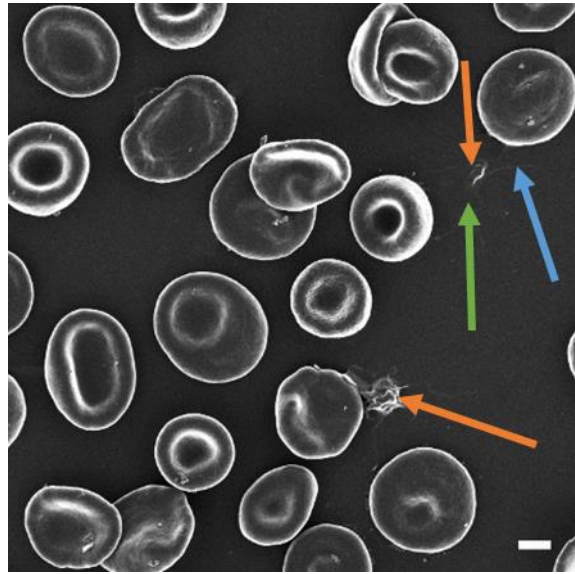
MAN UMF10+ – Manuka UMF10+ honey, LPS – lipopolysaccharide, UD – undigested.

GDD – MAN UMF10+ (-LPS)

A

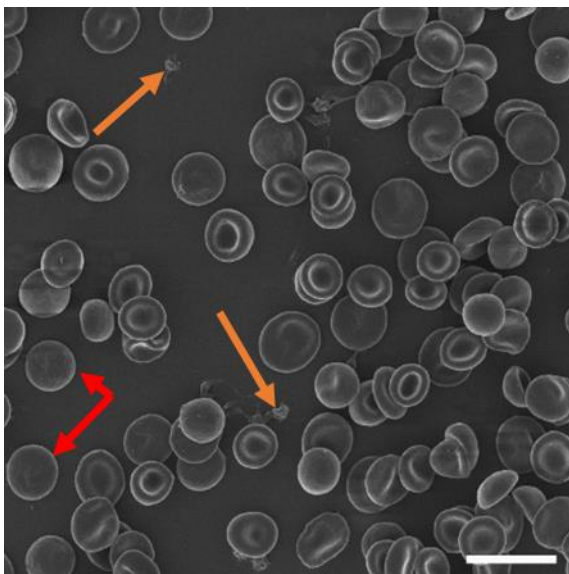


B



GDD – MAN UMF10+ (+LPS)

C



D

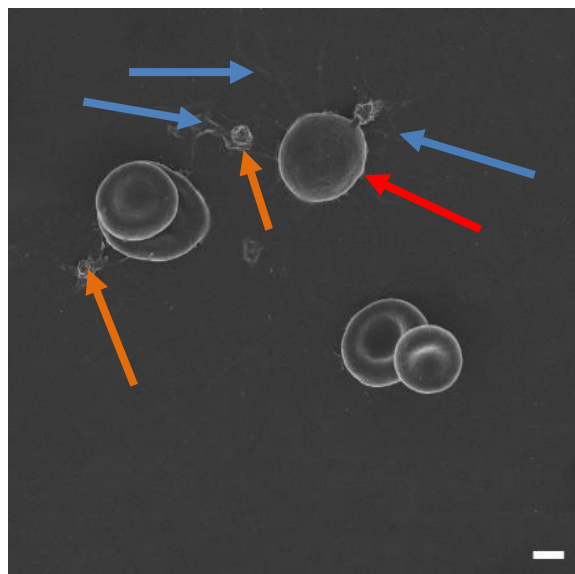
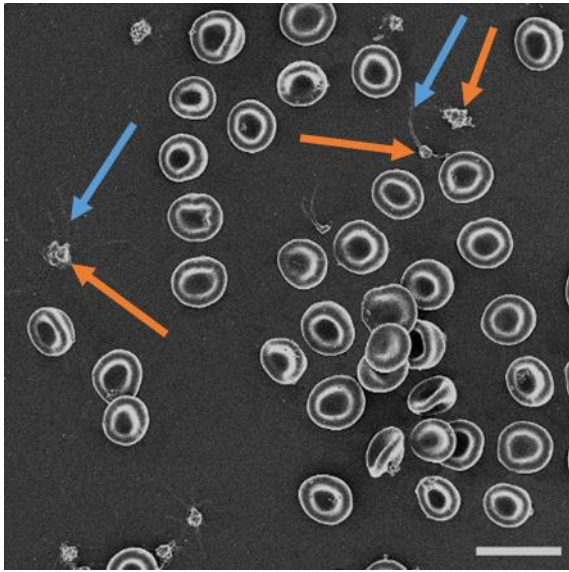


Figure 5.5: Scanning electron micrographs of whole blood exposed to 1% (final concentration) of GDD honey samples (A-D) without (A & B) or with (C & D) LPS. Orange arrows: platelet body, blue arrows: pseudopodia, green arrows: platelet spreading, red arrows: abnormal red blood cell morphology. (Scale bars: A and C: 10 μ m; B and D: 2 μ m).

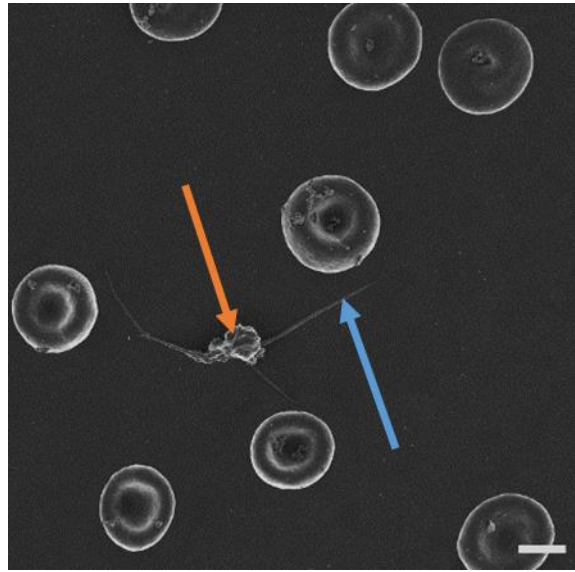
MAN UMF10+ – Manuka UMF10+ honey, LPS – lipopolysaccharide, GDD – gastroduodenal digested.

UD – AE (-LPS)

A

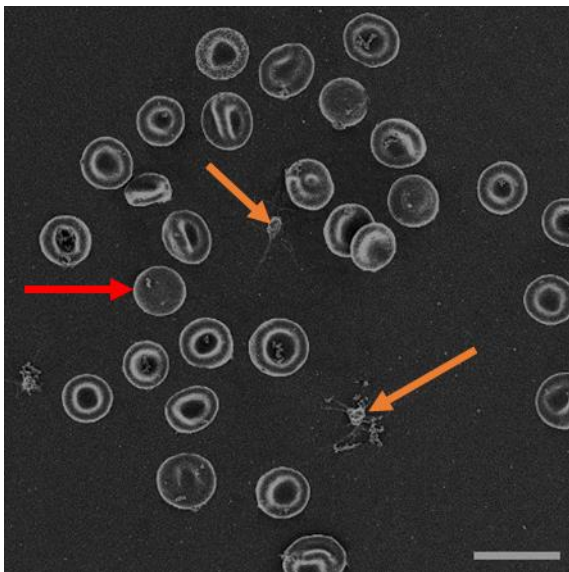


B



UD – AE (+LPS)

C



D

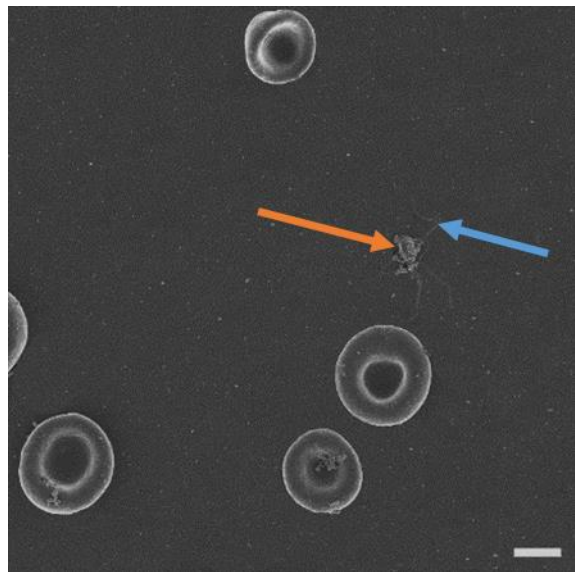
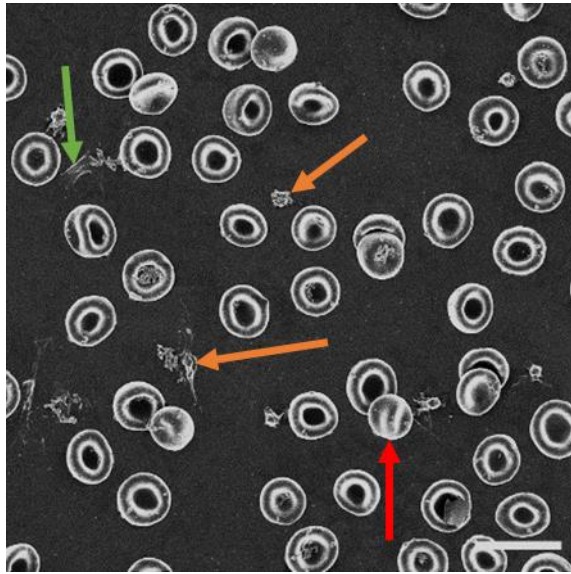


Figure 5.6: Scanning electron micrographs of whole blood exposed to 1% (final concentration) of UD honey samples (A-D) without (A & B) or with (C & D) LPS. Orange arrows: platelet body, blue arrows: pseudopodia, green arrows: platelet spreading, red arrows: abnormal red blood cell morphology. (Scale bars: A and C: 10 μ m; B and D: 2 μ m).

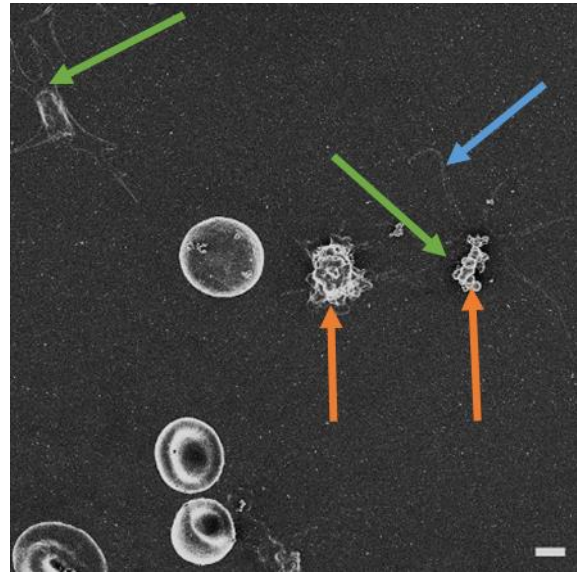
AE – Agricultural Eucalyptus honey, LPS – lipopolysaccharide, UD – undigested.

GDD – AE (-LPS)

A

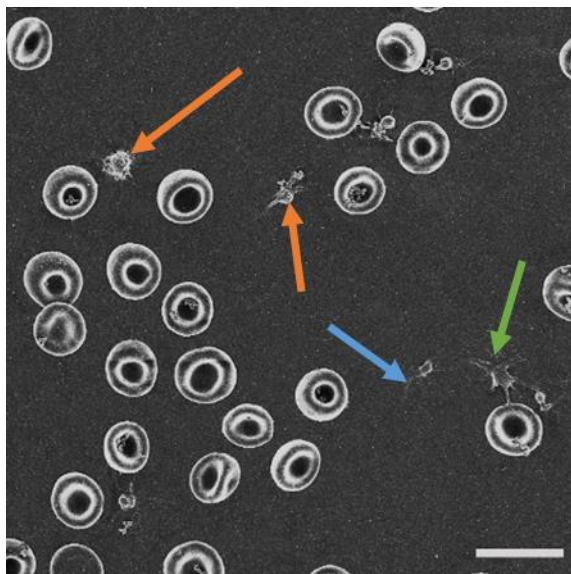


B



GDD – AE (+LPS)

C



D

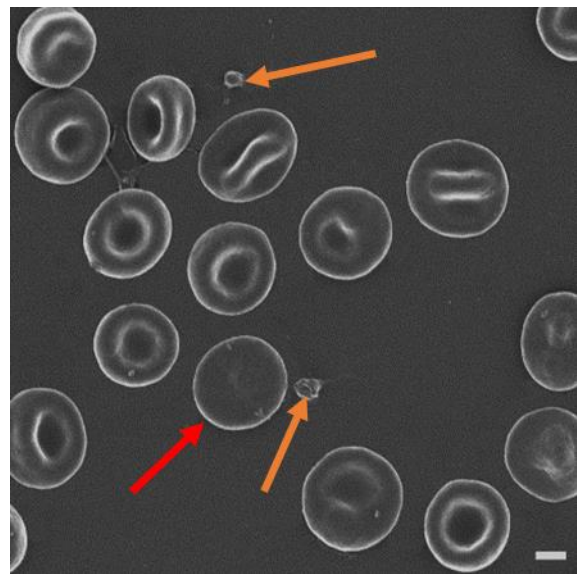
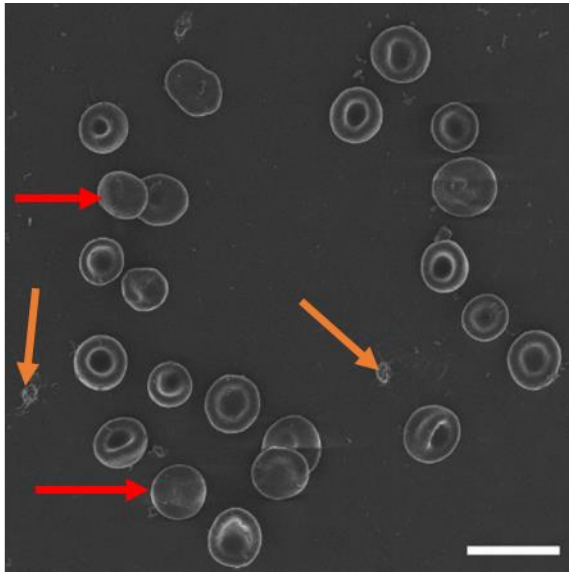


Figure 5.7: Scanning electron micrographs of whole blood exposed to 1% (final concentration) of GDD honey samples (A-D) without (A & B) or with (C & D) LPS. Orange arrows: platelet body, blue arrows: pseudopodia, green arrows: platelet spreading, red arrows: abnormal red blood cell morphology. (Scale bars: A and C: 10 μ m; B and D: 2 μ m).

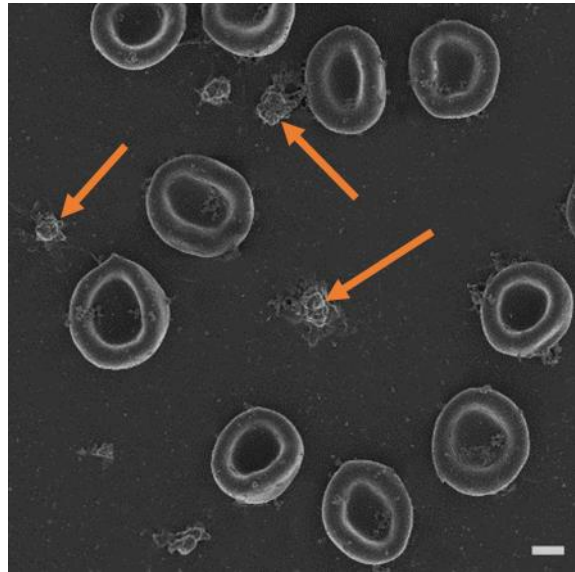
AE – Agricultural Eucalyptus honey, LPS – lipopolysaccharide, GDD – gastroduodenal digested.

UD – SEMh (-LPS)

A

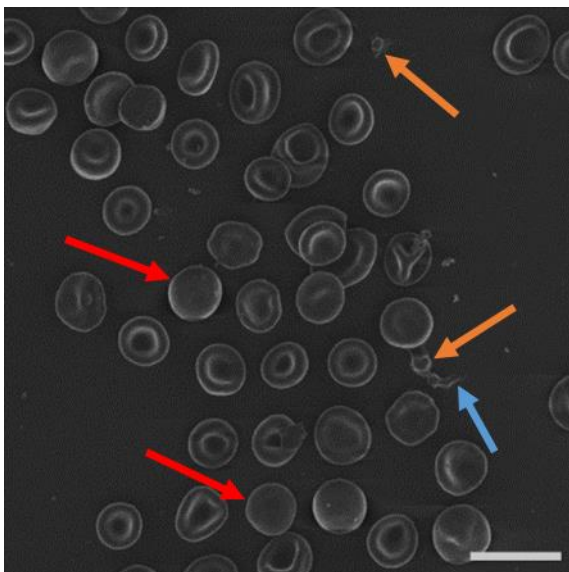


B



UD – SEMh (+LPS)

C



D

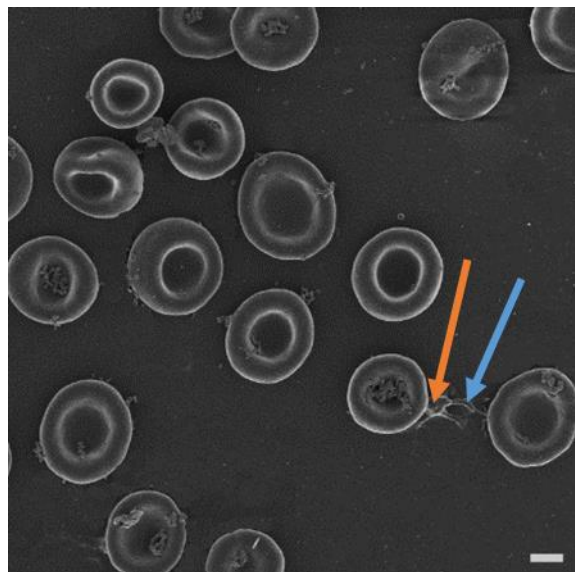
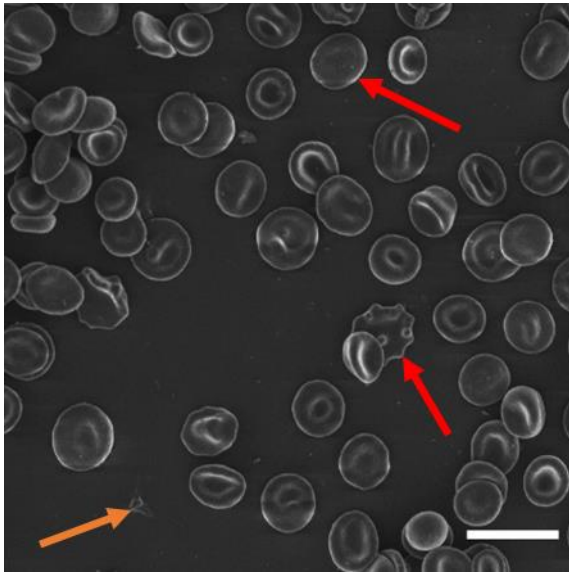


Figure 5.8: Scanning electron micrographs of whole blood exposed to 1% (final concentration) of UD honey samples (A-D) without (A & B) or with (C & D) LPS. Orange arrows: platelet body, blue arrows: pseudopodia, green arrows: platelet spreading, red arrows: abnormal red blood cell morphology. (Scale bars: A and C: 10 μ m; B and D: 2 μ m).

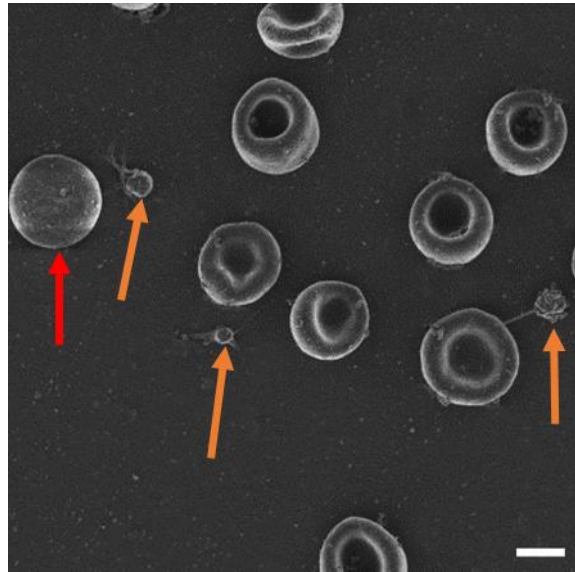
SEMh – south eastern Mozambique honey, LPS – lipopolysaccharide, UD – undigested.

GDD – SEMh (-LPS)

A

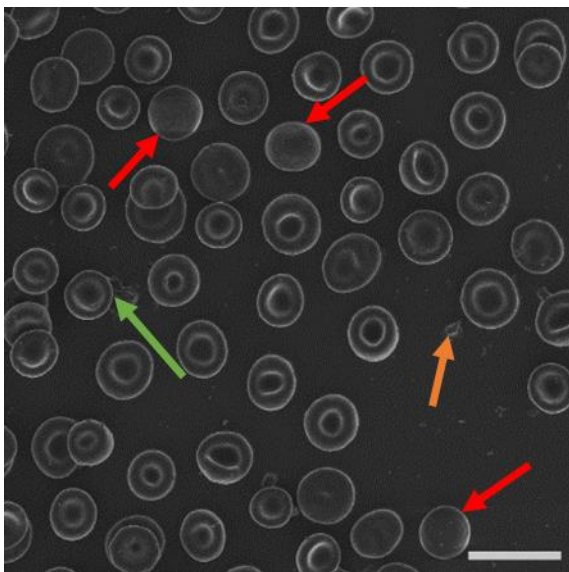


B



GDD – SEMh (+LPS)

C



D

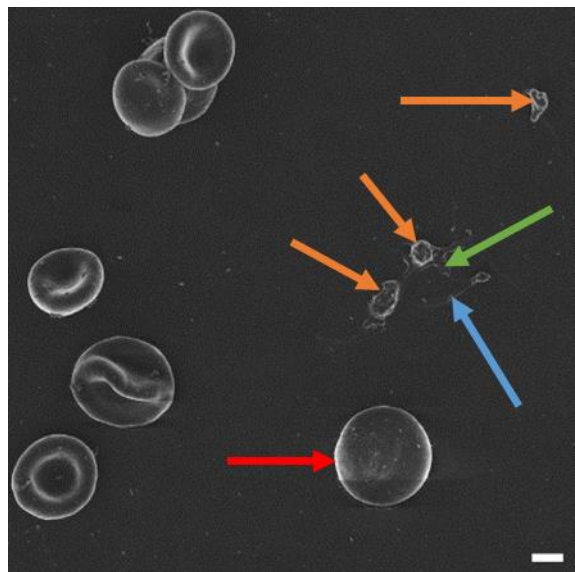
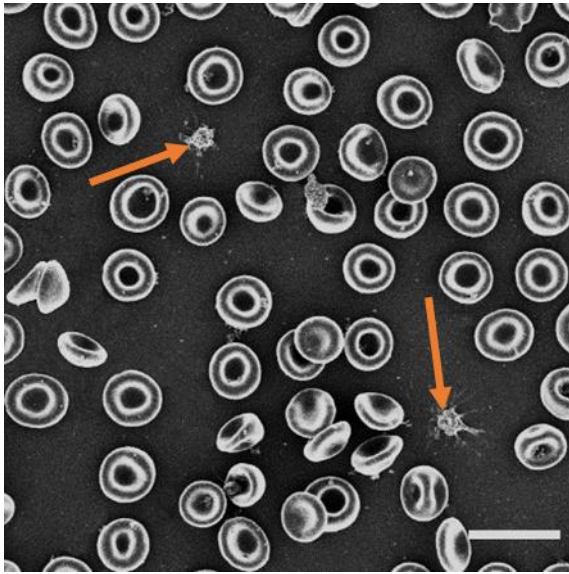


Figure 5.9: Scanning electron micrographs of whole blood exposed to 1% (final concentration) of GDD honey samples (A-D) without (A & B) or with (C & D) LPS. Orange arrows: platelet body, blue arrows: pseudopodia, green arrows: platelet spreading, red arrows: abnormal red blood cell morphology. (Scale bars: A and C: 10 μ m; B and D: 2 μ m).

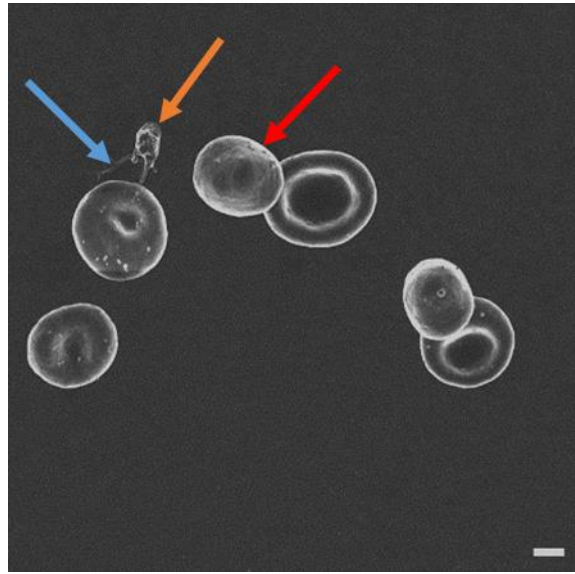
SEMh – south eastern Mozambique honey, LPS – lipopolysaccharide, GDD – gastroduodenal digested.

UD – WC (-LPS)

A

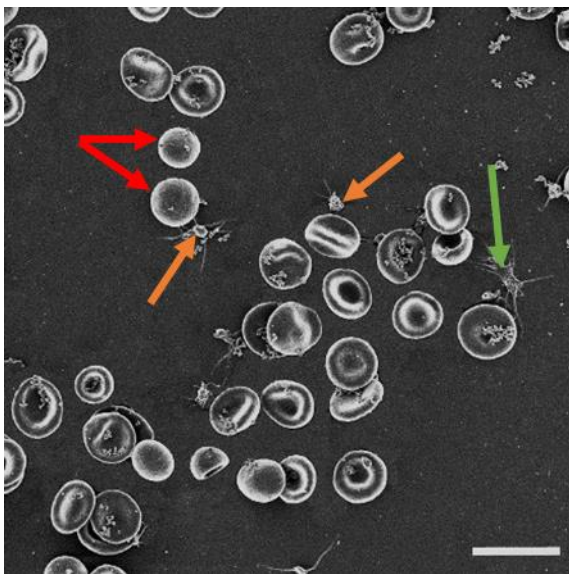


B



UD – WC (+LPS)

C



D

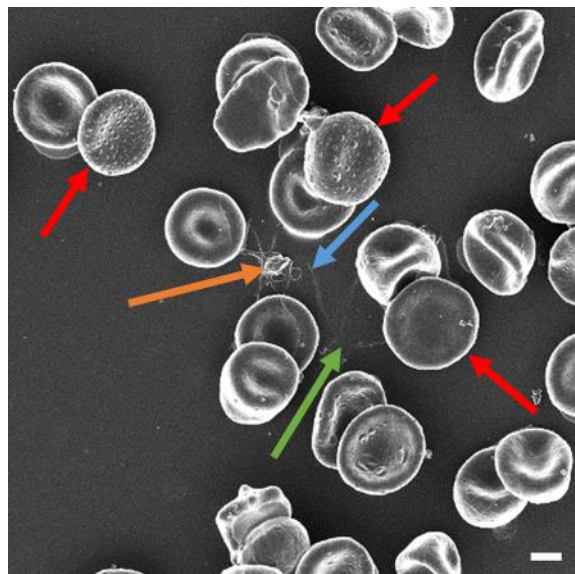
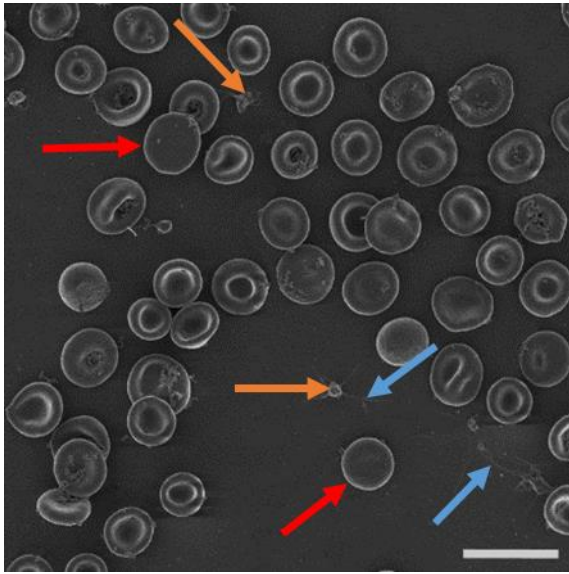


Figure 5.10: Scanning electron micrographs of whole blood exposed to 1% (final concentration) of UD honey samples (A-D) without (A & B) or with (C & D) LPS. Orange arrows: platelet body, blue arrows: pseudopodia, green arrows: platelet spreading, red arrows: abnormal red blood cell morphology. (Scale bars: A and C: 10 μ m; B and D: 2 μ m).

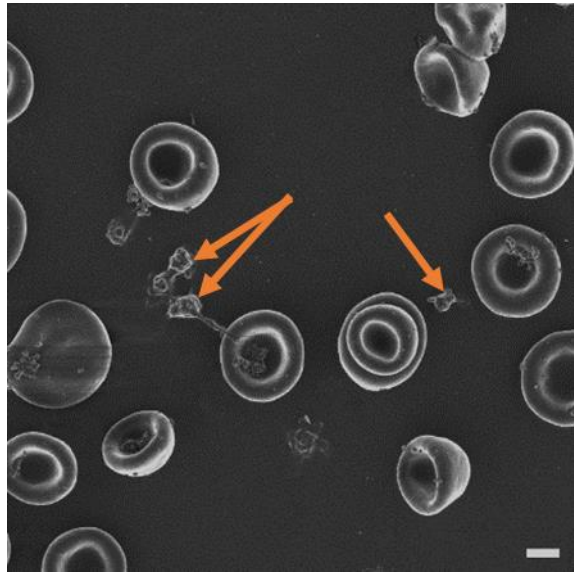
WC – Western Cape, Fynbos honey, LPS – lipopolysaccharide, UD – undigested.

GDD – WC (-LPS)

A

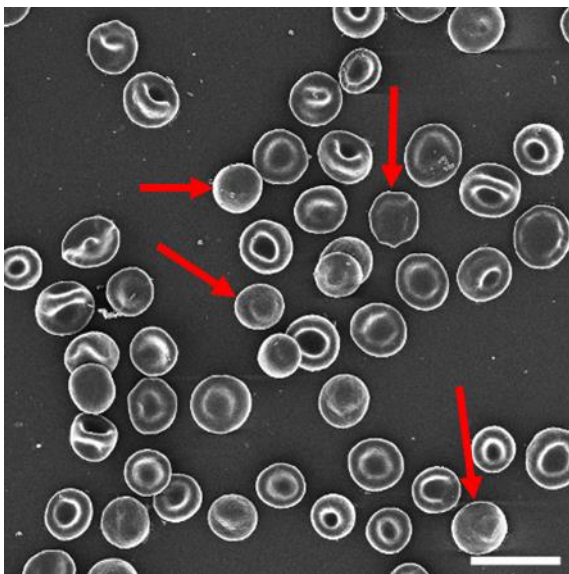


B



GDD – WC (+LPS)

C



D

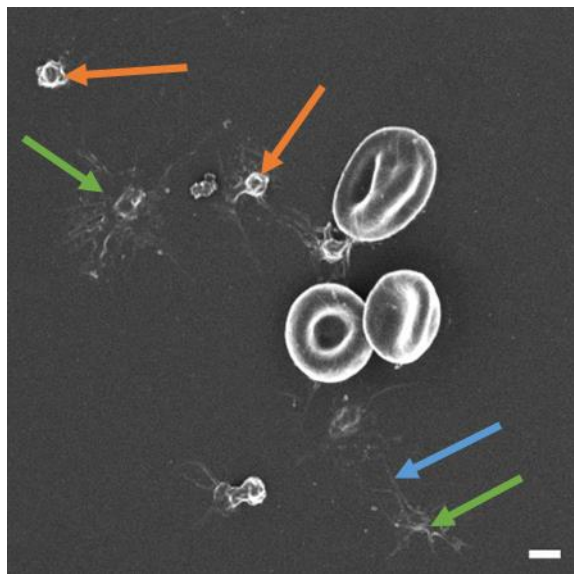


Figure 5.11: Scanning electron micrographs of whole blood exposed to 1% (final concentration) of GDD honey samples (A-D) without (A & B) or with (C & D) LPS. Orange arrows: platelet body, blue arrows: pseudopodia, green arrows: platelet spreading, red arrows: abnormal red blood cell morphology. (Scale bars: A and C: 10 μ m; B and D: 2 μ m).

WC – Western Cape, Fynbos honey, LPS – lipopolysaccharide, GDD – gastroduodenal digested.

Table 5.6: Summary of the effect of UD and GDD honey on platelet morphology.

	<u>Platelets</u>				<u>Red blood cells*</u>
	<u>Pseudopodia</u>	<u>Ruffling</u>	<u>Spread/flattened</u>	<u>Aggregation</u>	
CONTROLS					
<u>Negative (-LPS)</u>	+	+	-	-	All normal
<u>Positive (+LPS)</u>	+++	-	+	+	N* + Few primary echinocytes
MAN UMF10+					
<u>UD</u>	+	+	-	+	N + Few primary echinocytes and spherocytes
<u>UD (+LPS)</u>	+	+	+	+	N + Few primary echinocytes and spherocytes
<u>GDD</u>	+	+	+	+	N + Few spherocytes
<u>GDD (+LPS)</u>	+	+	+	-	N + Few spherocytes
AE					
<u>UD</u>	+	-	-	-	All normal
<u>UD (+LPS)</u>	+	-	-	-	N + Few primary echinocytes
<u>GDD</u>	+	+	+	-	N + Few spherocytes
<u>GDD (+LPS)</u>	+	-	-	-	N + Few spherocytes
SEMh					
<u>UD</u>	+	+	-	-	N + Few spherocytes
<u>UD (+LPS)</u>	+	-	-	-	N + Few spherocytes
<u>GDD</u>	+	-	-	-	N + Few primary echinocytes and spherocytes
<u>GDD (+LPS)</u>	+	+	-	-	N + Few primary echinocytes and spherocytes
WC					
<u>UD</u>	+	-	-	-	N + Few spherocytes
<u>UD (+LPS)</u>	+	+	+	-	N + Few spherocytes
<u>GDD</u>	+	+	-	-	N + Few spherocytes
<u>GDD (+LPS)</u>	+	+	++	-	N + Few spherocytes

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, UD – undigested, GDD – gastroduodenal digested, LPS – lipopolysaccharide, N – normal.

*In all controls and samples a majority of the red blood cells had normal (N) morphology the presence of echinocytes and spherocytes refer to a minority of the red blood cells.

- not present
- + present (few)
- ++ present (moderate)
- +++ present (extensive)

Chapter 6: Concluding discussion

Honey has been used as a food since the earliest of times. Scientific endeavour has confirmed the efficacy of honey as a functional food as it has physiological effects beyond nutrition. Functional foods can prevent and help reduce the effects of oxidative damage, infection and inflammation and would have beneficial effects thereby preventing the development of cancer and GIT associated inflammatory disorders. Honey is a well described functional food due to the presence of vitamins, amino acids and sugars as well as bioactive polyphenols and other molecules such as methylglyoxal (MGO), hydrogen peroxide (H₂O₂) and antimicrobial peptides (AMPs) (Blasa *et al.*, 2007).

The physiological effects of honey include antioxidant effects, due to the polyphenols present in honey which can scavenge ROS and nitric oxide (NO), thereby indirectly also reducing RNS levels (Bogdanov *et al.*, 2008, da Silva *et al.*, 2016). Honey has antimicrobial effects with a wide range of activity against Gram-positive and negative bacteria including *H. pylori*. Antibacterial activity is due to the presence of MGO a major component of Manuka honey, H₂O₂ and antimicrobial peptides such as bee defensin 1 (BD-1), and H₂O₂. In addition, several polyphenols including the flavonoids, in honey have well described antibacterial activity (Tan *et al.*, 2009, Sherlock *et al.*, 2010, Kwakman *et al.*, 2011, Kwakman, Zaat 2012, Anthimidou, Mossialos 2013, Al-Nahari *et al.*, 2015). Honey is effective as an antimicrobial to the point of some honeys such as Medihoney or Manuka being labelled as medical grade honey in the wound healing industry (Mavric *et al.*, 2008). In addition to antibacterial effects, flavonoids found in honey have several additional anti-inflammatory effects and these include inhibition of cyclooxygenase-2 (COX-2), reduction of pro-inflammatory mediator production, inhibition of the signal transducer and activator transcription (STAT), mitogen activated protein kinase (MAPK) and nuclear factor kappa beta (NF-κβ) pathways, leukocyte migration, T cell activity, and improvement of epithelial barrier function as well as antimicrobial effects and gut microbiota modulation (Veza *et al.*, 2016).

A lot of studies have been done on honey, however, very few involve African honeys, specifically honeys from the southern African region. The southern African region has unique floral species which could lend different properties to the honeys from this region compared to other honeys. In addition the bioavailability of these honeys from the southern African region is unknown.

Therefore, the aim of this study was to address the gap in knowledge on southern African honey, by determining the effects of digestion and to identify the components of honey that following digestion contribute to the antioxidant, antibacterial and anti-inflammatory activity.

To achieve this three honey samples from the southern African region (Agricultural Eucalyptus (AE), south eastern Mozambique (SEMh), (Western Cape, Fynbos (WC) and Manuka (MAN) (control) were subjected to simulated gastroduodenal digestion and the antioxidant, antibacterial and anti-inflammatory activity of the undigested (UD), gastric digested (GD) and gastroduodenal (GDD) fractions was determined. Then, the factors contributing to the observed activity were tentatively identified.

For the determination of the antioxidant properties, the total polyphenol content (TPC) and the antioxidant activity was determined with the Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbency capacity (ORAC) assays. Using human colon adenocarcinoma (Caco-2) cells the cellular antioxidant activity (CAA) was evaluated with the dichlorofluorescein diacetate (DCFH-DA) assay. To better understand the effects of digestion on polyphenols, a polyphenol (PP) mixture and a synthetic honey (SH) consisting of sugars and polyphenols was also subjected to simulated GIT digestion and the antioxidant properties were determined. The effect of digestion on specific polyphenols in the mixtures was determined with liquid chromatography-mass spectrometry (LC-MS) analysis.

GD did not cause any significant changes in the antioxidant properties of the honey samples. CAA was also unchanged for all honey types. This implies that honey protects the stomach mucosa against oxidative damage. GDD digestion caused a significant increase in the TPC of MANc and AE and the antioxidant activity of MANc and SEMh (ORAC assay) while the TPC and antioxidant activity (TEAC and ORAC assays) of other honey samples were unchanged. In contrast, the CAA of the GDD was lost and was the greatest for WC, AE, MANc > SEMh associated with an increase in H₂O₂ levels with the highest being for MANc > AE, SEMh, WC.

In this study, findings related to TPC and measured antioxidant activity and CAA were contradictory. Using polyphenol mixtures, findings of previous studies that at neutral pH, polyphenol degradation associated with an increase in H₂O₂ levels was confirmed. However, using a SH, polyphenol degradation did not occur. The observed pro-oxidant effect was hypothesised to be due to the presence of β -Carotene in honey which in an acidic environment acts as an antioxidant scavenger for both AAPH generated peroxy radicals and hydroxyl radicals (Woods *et al.*, 1999, El-Agamey *et al.*, 2004). Therefore it is hypothesised that with GDD which occurs at a neutral pH, H₂O₂ will decompose to form O₂ and together with β -Carotene will form the carotenoid peroxy radical which would have a strong pro-oxidant effect

The antibacterial effects against Gram-negative (*E. coli* and *P. aeruginosa*) and positive (*B. subtilis* and *S. aureus*), bacteria were then evaluated using the microbroth dilution assay and the minimum inhibitory concentration (MIC) was calculated. The contribution of sugars, MGO

and H₂O₂, polyphenols to measured activity as well as the possible contribution of peptides and specifically cationic peptides such as BD-1 was also determined. The antibacterial activity of each honey against all bacteria was reassessed following GD and GDD.

Against *E. coli*, the MIC for MANc, AE and WC was 30% (v/v) and for SEMh was 25% (v/v). For *P. aeruginosa* all honeys had an MIC of 25% (v/v). The MIC for MGO was 1.0 and 1.2 mM for *E. coli* and *P. aeruginosa* respectively while the MIC of 9 mM for H₂O₂ was the same for both bacteria. Honey sugars had a bacteriostatic effect. For Gram-positive *B. subtilis*, the MIC for MANc and WC was 25% (v/v), AE was 40% (v/v) and for SEMh was 30% (v/v). For *S. aureus*, the MIC for MANc was 6.25% (v/v) and was 25% for all other honeys. The MIC for MGO was 0.8 and 1.2 mM while that of H₂O₂ was 90 and 9 mM for *B. subtilis* and *S. aureus* respectively. The sugars in honey also had a bacteriostatic effect. Using a PP mixture and a SH containing polyphenols, it was found that polyphenols had an antibacterial effect which was enhanced when combined with sugars against *E. coli* and *S. aureus*. The presence of cationic antimicrobial peptides was determined in the <5kDa fraction. No SPS binding occurred and antibacterial activity of this fraction was unaltered indicating that cationic peptides such as BD-1 do not contribute significantly to the antibacterial activity of southern African honey. It was concluded that the sugars had a bacteriostatic effect, H₂O₂ (depending on concentration) and polyphenols (depending on type, structure, stability and concentration) may act synergistically to kill bacteria.

With GD, the antibacterial activity of all honeys against Gram-positive and negative bacteria was unchanged. Likewise the antibacterial activity of MANc was unchanged following GDD. With digestion the antibacterial activity of AE, SEMh and WC was unchanged against *P. aeruginosa* and *S. aureus* (although some reduction in activity was observed for SEMh) but was reduced against *E. coli* and *B. subtilis*.

As MANc retained activity against all bacteria, this implies that MGO does not contribute to the antibacterial activity of southern African honey. BD-1 has been shown to kill *P. aeruginosa* and *S. aureus* (Sojka *et al.*, 2016). However, *in silico* evaluation has identified that BD-1 activity is lost following GIT digestion. Furthermore the <5kDa fraction of WC has limited binding to polyanetholsulphonate, which indicates that BD-1 or other cationic peptides are not the bioactive component. H₂O₂ and polyphenols that have been shown not to degrade in the presence of sugars at neutral pH and consequently non-degraded polyphenols as found in undigested honey may contribute to the antibacterial activity of honey following GDD. Due to bacteria selectivity, a small molecule such as a flavonoid with specific membrane or protein targeting should be considered. Examples of probable candidate molecules are leptosperin

found in Manuka honey (Kato *et al.*, 2014) or a fatty diacid glycoside (Fyfe *et al.*, 2017) found in Scottish honey.

Further studies focused on the ability of the digests to reduce levels of NO, associated with inflammation. NO scavenging activity was determined with the chemical, SNP assay and the cellular, *E. coli* induced L929 model. Lastly the ability of the UD and GDD digests to reduce lipopolysaccharide (LPS 011:B4) induced activation of platelets in human whole blood was evaluated.

Cytotoxicity against the L929 cell line was only observed at honey concentrations >3%. At low concentrations all honey samples reduced NO formation, while at concentrations >25% NO production was increased for MAN UMF10+ and WC. UD, GD and GDD samples at 10% inhibited NO formation and at 1% was not cytotoxic and inhibited *E. coli* induced NO formation in L929 cells. LPS induces increased human platelet pseudopodia formation, spreading and aggregation associated with platelet activation. One percent solutions of UD and GDD did not cause platelet activation. UD and GDD fractions inhibited LPS induced platelet activation to varying degrees and the predominant morphological effect was the inhibition of pseudopodia formation. AE and SEMh compared to WC and MAN UMF10+ were the most effective in reducing platelet activation.

In conclusion, following digestion, in the stomach, the antioxidant, antibacterial and anti-inflammatory activity of all honey was retained. Related to antioxidant, antibacterial activity against Gram-negative bacteria and anti-inflammatory activity the bioactivity of southern African honey was similar to MANc. Following GDD for southern African honey variable effects were observed, CAA was partially lost and a specific strain-dependent partial loss of antibacterial activity was observed. However, cellular NO scavenging activity was retained and following and LPS mediated platelet activation associated with systemic exposure was inhibited. Therefore it can be concluded that honey from the southern African region is a functional food which is bioactive and bioavailable and has predominantly an antioxidant and anti-inflammatory beneficial effect in the stomach and an anti-inflammatory effect in rest of the GIT.

Limitations and recommendations

The first limitation of this study was that the type and concentrations of specific polyphenols in each honey type was not identified. If this was known, a more accurate evaluation of the effects of digestion on specific polyphenols could be determined as well as the stability of these polyphenols at neutral pH. This is especially important as in the present study southern African honeys have been identified to have beneficial effects. Using established LC-MS

protocols and bioactivity guided LC-MS these polyphenols can be identified and the possible presence of novel glycosides as found in Manuka and Scottish honey can be determined.

A novel finding was that the GDD fractions induced a strong pro-oxidant effect and it was postulated that this was due to carotenoid peroxy radical formation due to increased O₂ levels as a result of H₂O₂ degradation. To further investigate these effects it will be necessary to determine the β-Carotene levels in southern African honey. Using a SH containing β-Carotene and H₂O₂ at levels found in honey, whether the observed pro-oxidant effect is as a result of carotenoid peroxy radical formation following GDD can be established.

Honey from the southern African region were found to have antibacterial activity against Gram-negative and Gram-positive bacteria. MGO levels were too low to contribute to measured antibacterial activity. Although H₂O₂ levels are low, possible synergism with AMPs such as BD-1 may contribute to activity as has been described for RS honey. With digestion, selective loss of antibacterial activity, against *E. coli* and *B. subtilis*, digestive degradation (*in silico* analysis) and limited SPS binding, indicates that BD-1 does not contribute to the antibacterial activity of southern African honey. However this can and should be confirmed with a recently developed enzyme-linked immunosorbent assay (ELISA) method described by Valachová *et al.*, (2016). Digestion of honey with proteinase K and subsequent retesting will also confirm that antibacterial activity is not due to the presence of an antimicrobial peptide. As discussed in Chapter 4, jelleins identified with matrix-assisted laser desorption ionisation-time of flight (MALDI TOF) and electrospray quadrupole time of flight mass spectrometry (ESI-Q-TOF-MS/MS) analysis may also contribute to the antibacterial activity of these honeys. As the sequence of these peptides are known, these peptides and digested derivatives can be synthesised and the antibacterial activity and selectivity can be evaluated.

Honey sugars were found to have a bacteriostatic effect and together with H₂O₂ and polyphenols may act synergistically to inhibit bacteria. However, selective loss of activity with digestion indicates that a molecule with selective antibacterial activity contributes to the antimicrobial activity of these honeys. This should be further investigated using honey from a specific region such as honey from the Western Cape where honey is produced by the same bee subspecies and the floral origin is unique and well defined. Collecting samples within the same region of the Western Cape at a specific time of year will provide an accurate determination and confirmation of the major compounds in this honey contributing to antioxidant, antibacterial and anti-inflammatory activity.

Southern African and Manuka honey scavenged *E. coli* induced NO formation by L929 cells. Whether the observed effect is due to direct NO scavenging or the inhibition of iNOS is

unknown. The role of polyphenols, and specifically the effect of those polyphenols identified in southern African honey should be investigated. This can be achieved with Western blotting and/or real time PCR (qPCR) of iNOS RNA levels.

GDD honey inhibited LPS induced activation of human platelets. Due to the complexity of honey it is unknown, which components are inhibiting platelet activation. Although it has been shown that flavonoids inhibit TLR4, this must be confirmed by firstly identifying the flavonoids present in these honeys and then evaluating each identified flavonoid for activity. Furthermore, the ability of these flavonoids to regulate the TLR4/MyD88 pathway should be investigated using Western blotting and/or qPCR.

Future research should focus of the development of honey as a nutraceutical product for the improvement of symptoms associated with inflammation of the GIT. Key to this process is the identification of the bioactive molecules in honey and the use of animal models of CD or IBD. This would further prove the anti-inflammatory efficacy of southern African honey and more strongly link the data in this study to the actual human inflammatory diseases.

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Appendices

Appendix 1: Table A1

Table A1: The TPC and antioxidant activity of the GD honey.

<u>TPC (mg GAE/100g)</u>				
	<u>MANc</u>	<u>AE</u>	<u>SEMh</u>	<u>WC</u>
GD	116.57 ± 3.35*	108.03 ± 6.29	156.47 ± 9.35	149.41 ± 3.15
<u>TEAC assay (µmol TE/g)</u>				
	<u>MANc</u>	<u>AE</u>	<u>SEMh</u>	<u>WC</u>
GD	9.30 ± 1.29	15.34 ± 1.02	20.27 ± 2.26	19.52 ± 2.51
<u>ORAC assay (µmol TE/g)</u>				
	<u>MANc</u>	<u>AE</u>	<u>SEMh</u>	<u>WC</u>
GD	119.10 ± 17.60*	80.82 ± 10.87	133.35 ± 8.56	143.69 ± 16.74*

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, TPC – total polyphenol content, TEAC – Trolox equivalent antioxidant capacity, ORAC – oxygen radical absorbance capacity, GAE – gallic acid equivalent, TE – Trolox equivalent, UD – undigested, GD – gastric digest.

Data is expressed as an average of at least 3 experiments ± SEM. *Indicates significant differences at p< 0.05, GD compared to UD, using either one way ANOVA or T-TEST.

Appendix 2: Table A2

Table A2: TPC (mg GAE/mL) of undigested and digested polyphenols and PP mixtures.

<u>Single polyphenols</u>						
	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>		
GD	0.12 ± 0.01*	0.11 ± 0.01	0.10 ± 0.01	0.02 ± 0.003		
<u>Double combinations</u>						
	<u>CA+GA</u>	<u>GA+CAT</u>	<u>GA+QUE</u>	<u>CA+CAT</u>	<u>CA+QUE</u>	<u>CAT+QUE</u>
GD	0.15 ± 0.01	0.19 ± 0.02	0.21 ± 0.03	0.22 ± 0.02	0.16 ± 0.03	0.19 ± 0.02*
<u>Triple combinations</u>						
	<u>GA+CA+CAT</u>	<u>GA+CA+QUE</u>	<u>GA+CAT+QUE</u>	<u>CA+CAT+QUE</u>		
GD	0.39 ± 0.02	0.29 ± 0.04	0.32 ± 0.03	0.35 ± 0.04		
<u>Quadruple combinations</u>						
	<u>GA+CA+CAT+QUE</u>					
GD	0.43 ± 0.03					

GA – gallic acid, CA – caffeic acid, CAT – catechin, QUE – quercetin, PP – polyphenol mixture, TPC – total polyphenolic content, GAE – gallic acid equivalent, UD – undigested, GD – gastric digest.

Each polyphenol was assayed at a concentration of 0.1 mg/mL. Data is expressed as an average of at least 3 experiments ± SEM. *Indicates significant differences at p< 0.05, GDD compared to UD, using one way ANOVA or T-TEST.

Appendix 3: Table A3

Table A3: Antioxidant activity: TEAC assay ($\mu\text{mol TE/mL}$) of digested polyphenols/PP mixtures.

<u>Single</u>						
	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>		
GD	0.08 \pm 0.02	0.33 \pm 0.02*	0.21 \pm 0.02*	0.08 \pm 0.03*		
<u>Double combinations</u>						
	<u>CA+GA</u>	<u>GA+CAT</u>	<u>GA+QUE</u>	<u>CA+CAT</u>	<u>CA+QUE</u>	<u>CAT+QUE</u>
GD	0.37 \pm 0.03*	0.43 \pm 0.01*	0.27 \pm 0.03*	0.38 \pm 0.004*	0.19 \pm 0.01*	0.62 \pm 0.01*
<u>Triple combinations</u>						
	<u>GA+CA+CAT</u>	<u>GA+CA+QUE</u>	<u>GA+CAT+QUE</u>	<u>CA+CAT+QUE</u>		
GD	0.62 \pm 0.05*	0.43 \pm 0.01*	0.67 \pm 0.03*	0.59 \pm 0.02		
<u>Quadruple combinations</u>						
	<u>GA+CA+CAT+QUE</u>					
GD	0.67 \pm 0.08*					

GA – gallic acid, CA – caffeic acid, CAT – catechin, QUE – quercetin, PP – polyphenol mixture, TEAC – Trolox equivalent antioxidant capacity, TE – Trolox equivalent, UD – undigested, GD – gastric digest.

Each polyphenol was assayed at a concentration of 0.1 mg/mL. Data is expressed as an average of at least 3 experiments \pm SEM. *Indicates significant differences at $p < 0.05$, GDD compared to UD, using one way ANOVA or T-TEST.

Appendix 4: Table A4

Table A4: Antioxidant activity: ORAC assay ($\mu\text{mol TE/mL}$) of UD and GD polyphenols/PP mixtures.

<u>Single</u>						
	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>		
GD	0.64 \pm 0.08*	0.27 \pm 0.05*	0.64 \pm 0.06	0		
<u>Double combinations</u>						
	<u>GA+CA</u>	<u>GA+CAT</u>	<u>GA+QUE</u>	<u>CA+CAT</u>	<u>CA+QUE</u>	<u>CAT+QUE</u>
GD	0.35 \pm 0.03*	0.66 \pm 0.05*	0.77 \pm 0.03*	0.89 \pm 0.12*	0.72 \pm 0.08*	1.38 \pm 0.11*
<u>Triple combinations</u>						
	<u>GA+CA+CAT</u>	<u>GA+CA+QUE</u>	<u>GA+CAT+QUE</u>	<u>CA+CAT+QUE</u>		
GD	1.17 \pm 0.09*	0.97 \pm 0.11*	0.80 \pm 0.09*	0.75 \pm 0.04*		
<u>Quadruple combinations</u>						
	<u>GA+CA+CAT+QUE</u>					
GD	1.17 \pm 0.07*					

GA – gallic acid, CA – caffeic acid, CAT – catechin, QUE – quercetin, PP – polyphenol mixture, ORAC – oxygen radical absorbance capacity, TE – Trolox equivalent, UD – undigested, GD – gastric digest.

Each polyphenol was assayed at a concentration of 0.1 mg/mL. Data is expressed as an average of at least 3 experiments \pm SEM. *Indicates significant differences at $p < 0.05$, GDD compared to UD, using one way ANOVA or T-TEST.

Appendix 5: Table A5

Table A5: DCFH DA assay (% oxidative damage) and H₂O₂ (mM) levels of UD and GD honey.

	<u>MANc</u>	<u>AE</u>	<u>SEMh</u>	<u>WC</u>
GD (% oxidative damage)	34.21 ± 10.15	31.63 ± 3.34	19.00 ± 2.21*	16.39 ± 1.59*
GD (H₂O₂ levels)	1.42±0.33	0.37±0.18	1.15±0.22	0.63±0.26

MANc – Manuka commercial honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, DCFH-DA – dichlorofluorescein diacetate, H₂O₂ – hydrogen peroxide, UD – undigested, GD – gastric digested.

Data is expressed as an average of at least 3 experiments ± SEM. *Indicates significant differences at p< 0.05, GD compared to UD, using either one way ANOVA or T-TEST.

Appendix 6: Table A6

Table A6: H₂O₂ (µM) levels in GD polyphenols and PP mixtures.

<u>Single</u>						
	<u>CA</u>	<u>GA</u>	<u>CAT</u>	<u>QUE</u>		
GD	0.12±0.02	0.09±0.03	0.10±0.04	0.08±0.04		
<u>Double combinations</u>						
	<u>GA+CA</u>	<u>GA+CAT</u>	<u>GA+QUE</u>	<u>CA+CAT</u>	<u>CA+QUE</u>	<u>CAT+QUE</u>
GD	0.08±0.03	0.08±0.04	0.12±0.08	0.08±0.04	0.07±0.03	0.07±0.04
<u>Triple combinations</u>						
	<u>GA+CA+CAT</u>	<u>GA+CA+QUE</u>	<u>GA+CAT+QUE</u>	<u>CA+CAT+QUE</u>		
GD	0.10±0.02	0.07±0.04	0.052±0.02	0.13±0.02		
<u>Quadruple combinations</u>						
	<u>GA+CA+CAT+QUE</u>					
GD	0.11±0.04					

GA – gallic acid, CA – caffeic acid, CAT – catechin, QUE – quercetin, PP – polyphenol mixture, H₂O₂ – hydrogen peroxide UD – undigested, GD – gastric digest.

Each polyphenol was assayed at a concentration of 0.1 mg/mL.

Data is expressed as an average of at least 3 experiments ± SEM.

*Indicates significant differences at p< 0.05, GDD compared to UD, using one way ANOVA or a T-TEST.

Appendix 7: Figure A1

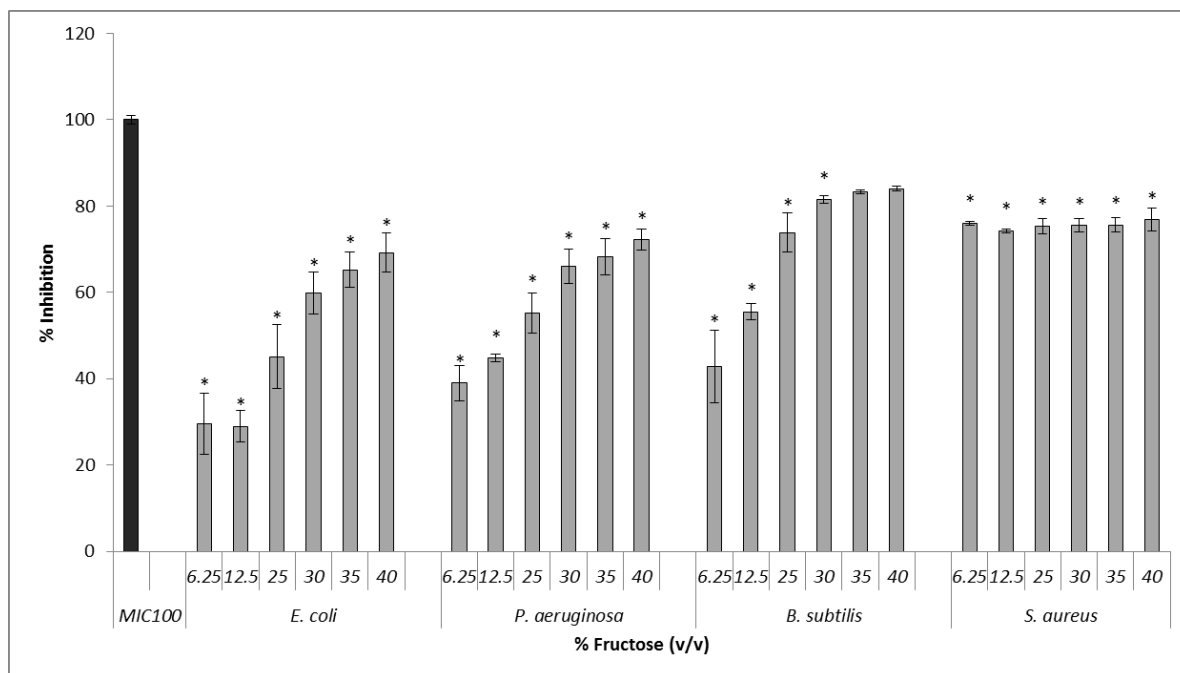


Figure A1: Antibacterial activity of fructose against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean±SEM. * represents significant differences between 100% MIC compared to various concentrations of fructose using one way ANOVA, p<0.05.

Appendix 8: Figure A2

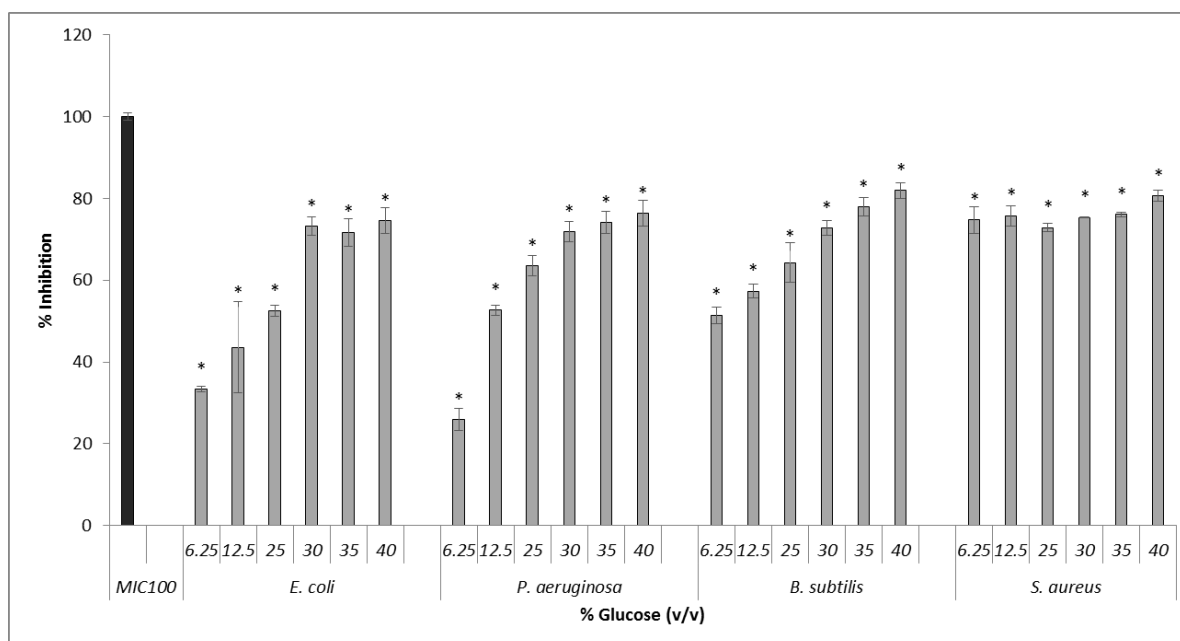


Figure A2: Antibacterial activity of glucose against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean±SEM. * represents significant differences between 100% MIC compared to various concentrations of glucose using one way ANOVA, p<0.05.

Appendix 9: Figure A3

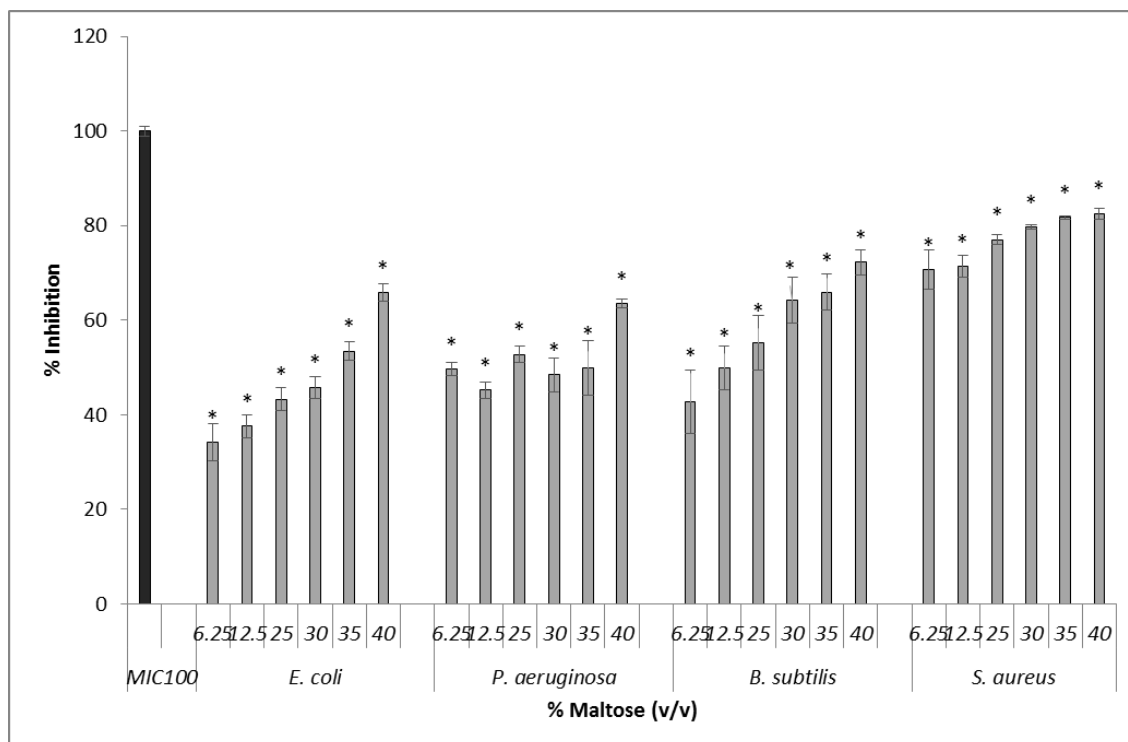


Figure A3: Antibacterial activity of maltose against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean±SEM. * represents significant differences between 100% MIC compared to various concentrations of maltose using one way ANOVA, p<0.05.

Appendix 10: Figure A4

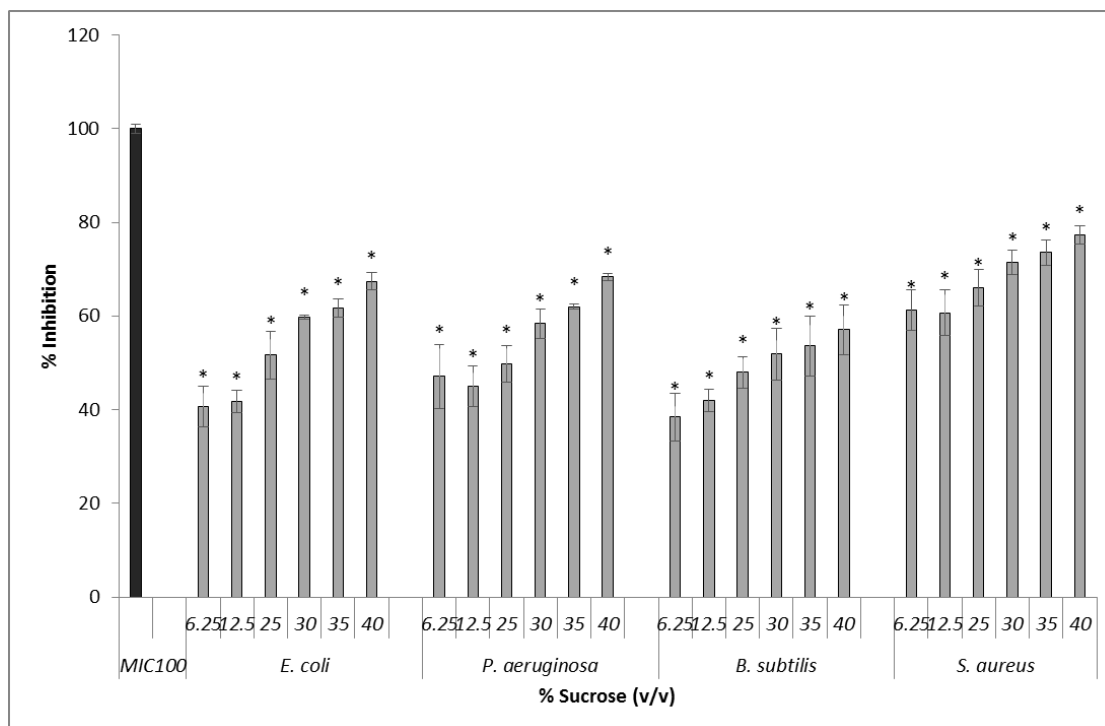


Figure A4: Antibacterial activity of sucrose against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean±SEM. * represents significant differences between 100% MIC compared to various concentrations of sucrose using one way ANOVA, p<0.05.

Appendix 11: Figure A5

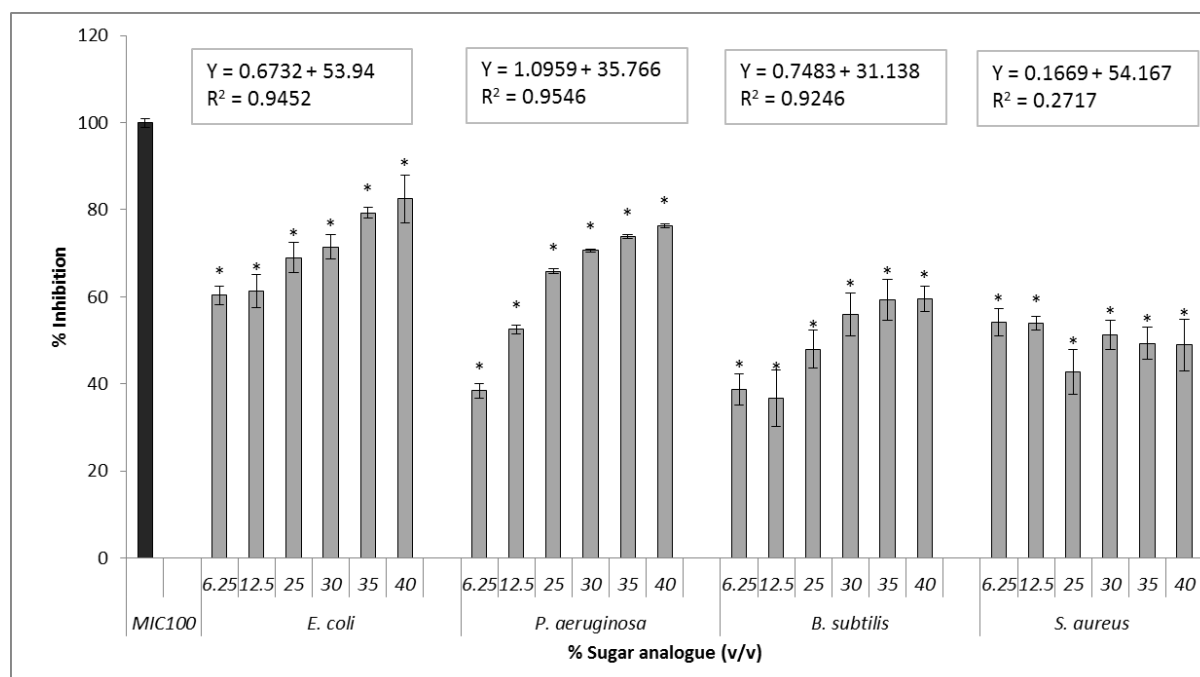


Figure A5: Antibacterial activity of a sugar analogue against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* determined with the microbroth dilution assay. Data was an average of at least 3 experiments reported as mean \pm SEM. * represents significant differences between 100% MIC compared to various concentrations of the sugar analogue using one way ANOVA, $p < 0.05$.

Appendix 12: Table A7

Table A7: Percentage NO production of a 10% (v/v) GD honey.

	MAN UMF10+	AE	SEMh	WC
GD	55.28 \pm 1.22	39.90 \pm 3.92	40.68 \pm 3.06	54.77 \pm 4.44

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, UD – undigested, GD – gastric digested.

Percentage NO scavenging activity of undigested vs. digested honey samples. All data is an average of at least three experiments \pm SEM. * indicates significant differences between UD and GD samples using one way ANOVA, $p < 0.05$.

Appendix 13: Table A8

Table A8: Percentage cell viability of a 10% (v/v) GD honey.

	MAN UMF10+	AE	SEMh	WC
GD	94.18 \pm 1.09	97.88 \pm 5.96	95.58 \pm 5.25	95.19 \pm 3.67

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, UD – undigested, GD – gastric digested.

Percentage cell viability of undigested and gastroduodenal digested honey samples. All data is an average of at least three experiments \pm SEM. * indicates significant differences between UD and GD samples using one way ANOVA, $p < 0.05$.

Appendix 14: Figure A6

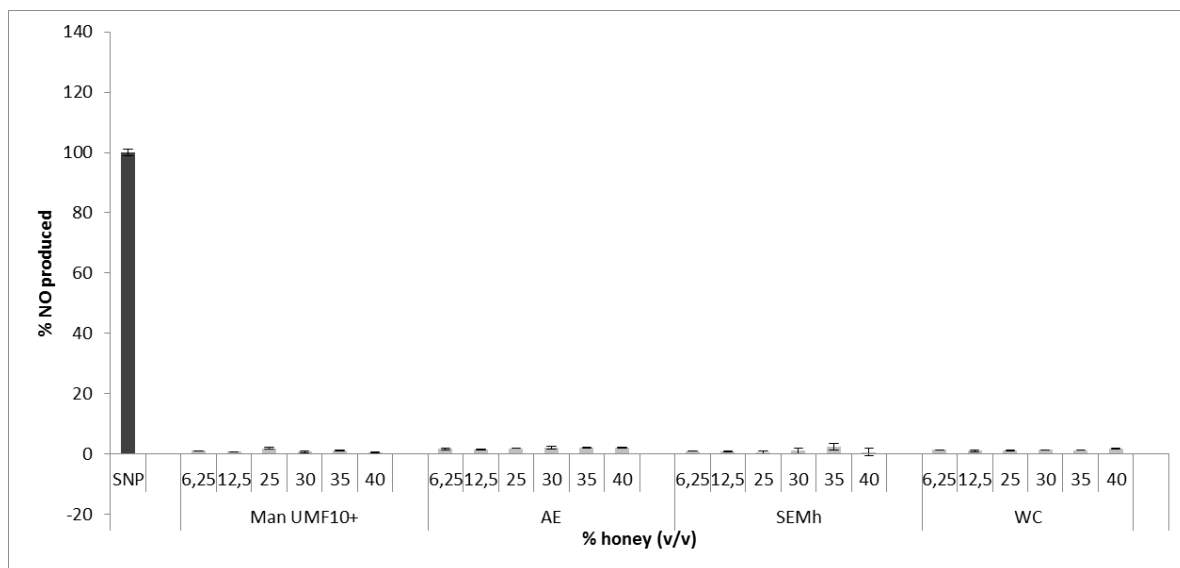


Figure A6: Percentage residual NO production of undigested polyphenol samples (without the presence of SNP). Data is expressed as an average of at least 3 experiments \pm SEM. All samples were significantly different compared to control (SNP) $p < 0.05$, using one way ANOVA.

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey, UD – undigested, GD – gastric digested, NO – nitric oxide, SNP – sodium nitroprusside.

Appendix 15: Figure A7

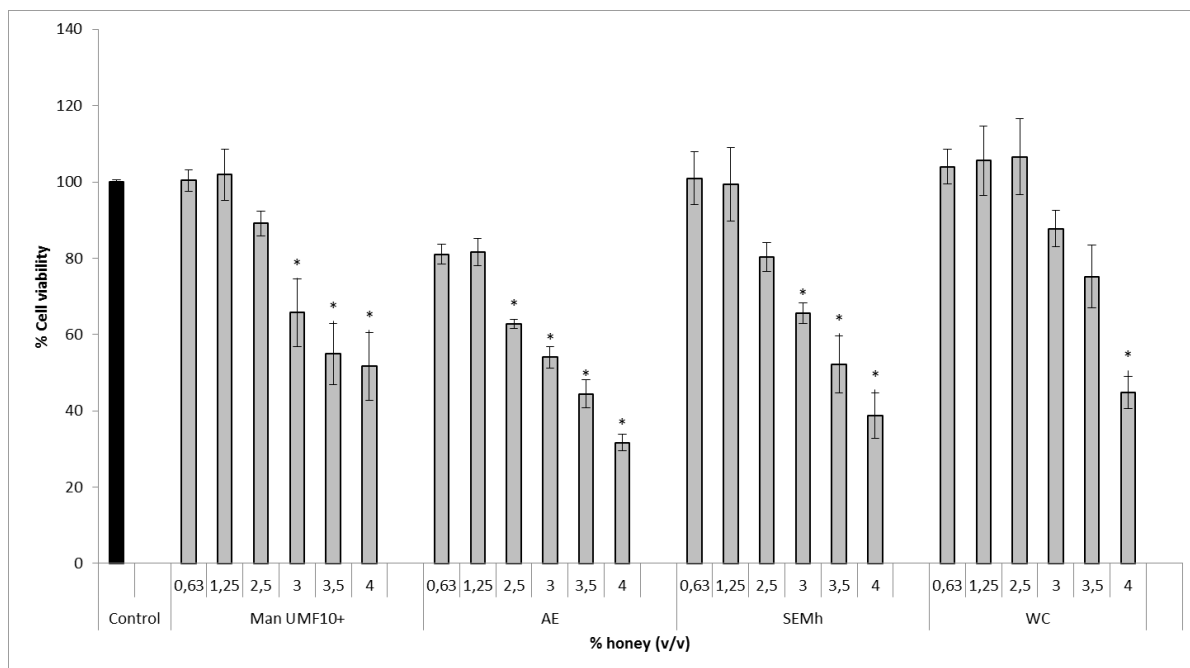


Figure A7: Cytotoxicity of undigested honey samples, 0.63 – 4% v/v on the RAW 264.7 cell line. Data is an average of at least 3 experiments represented as mean \pm SEM. * indicates significant decreases compared to control, using one way ANOVA, $P < 0.05$.

MAN UMF10+ – Manuka UMF10+ honey, AE – Agricultural Eucalyptus honey, SEMh – south eastern Mozambique honey, WC – Western Cape, Fynbos honey.

Appendix 16: Ethics clearance letters

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 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

27/11/2014

Approval Certificate New Application

Ethics Reference No.: 476/2014

Title: Bioactivity of simulated gastrointestinal digested indigenous Southern African honey samples

Dear Miss June Serem

The **New Application** as supported by documents specified in your cover letter for your research received on the 24/10/2014, was approved by the Faculty of Health Sciences Research Ethics Committee on the 26/11/2014.

Please note the following about your ethics approval:

- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (**476/2014**) 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.
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 2004 (Department of Health).

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✉ Private Bag X323, Arcadia, 0007 - 31 Bophelo Road, HW Snyman South Building, Level 2, Room 2.33, Gezina, Pretoria



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Faculty of Health Sciences Research Ethics Committee

29/03/2018

Miss June Serem
Department of Anatomy
University of Pretoria

Dear Miss June Serem

RE.: 476/2014 ~ Letter dated 9 March 2018

Protocol Number	476/2014
Protocol Title	Bioactivity of simulated gastrointestinal digested indigenous Southern African honey samples
Principal Investigator	Miss June Serem, Tel: Email: Dept: Anatomy, University of Pretoria

We hereby acknowledge receipt of the following document:

- Extension of study until the end of December 2018

which has been approved at 28 March 2018 meeting.

With regards

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

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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.



UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences Research Ethics Committee

30/05/2018

**Approval Certificate
Amendment**

(to be read in conjunction with the main approval certificate)

Ethics Reference No.: 476/2014

Title: New title: Identification and characterization of bioactivity of simulated gastrointestinal digested indigenous southern African honey samples. Old title: Bioavailability of simulated gastrointestinal digested indigenous southern African honey samples.

Dear Miss June Cheptoo JC Serem

The **Amendment** as described in your documents specified in your cover letter dated 20/04/2018 received on 20/04/2018 was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 30/05/2018.

Please note the following about your ethics amendment:

- Please remember to use your protocol number (476/2014) 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.
- The Amendment will be ratified at the meeting of 30/05/2018.

Ethics amendment 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); MPharmD; 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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**** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, Tswelopele Building, Level 4-60**