An investigation of the prebiotic properties of Australian honeys

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ABSTRACT

Non-digestible carbohydrates that promote specific, favourable changes in the composition and functionality of the gut microbiota, and thus contribute to improving health and well-being, are referred to as prebiotics. These favourable changes might be due to a relative increase in the numbers of potentially beneficial bacteria, and/or an increase in the metabolic activity of gut microbiota to produce more beneficial substances, such as short chain fatty acids (SCFA). An established method for quantifying the prebiotic effect of a carbohydrate utilises the Prebiotic Index (PI), which is the ratio of the changes in the populations of the potentially beneficial and potentially harmful bacteria.

Honey contains non-digestible oligosaccharides and there is some evidence that certain honeys could induce beneficial changes in the gut, however there is limited information on Australian floral varieties of honey.

The aim of this work was to conduct an in-depth investigation of the prebiotic properties of Australian honeys from a variety of floral sources. Three broad approaches were used. Initially, the influences of the monosaccharides in high fructose content Australian honeys on the composition and metabolic activity of gut microbes were tested in microcosms established with human intestinal microbiota.

Secondly, the impact of the monosaccharides and non-digestible components (oligosaccharides) found naturally in honey on the growth and metabolic function of the gut microbiota was tested. The non-digestible components were obtained by simulating gastrointestinal conditions using a digestion process that mimicked the upper regions of the gastrointestinal tract. Both whole and digested honeys were assayed in microcosms established using human intestinal microbiota to simulate the lower regions of the gastrointestinal tract. Standard culture-based techniques were used to determine the impact of the honey monosaccharides and oligosaccharides on the numbers of the major bacterial groups of the gut. Molecular profiling of the microcosm microbiota enriched with four of the honeys supported the data from the culture-based methodology. The PI values of the honeys were determined, and the effect of the honeys on SCFA production by the gut microbiota was measured.

Finally, the effect of the *in vitro* fermentation of honey by the gut microbiota on the growth of three enteropathogens and a probiotic strain was determined as an assessment of the resilience of the microcosm to an introduced species.

It was shown that the saccharides present in honey affected the bacterial composition of the gut microbiota. While much of the compositional changes can be attributed to the complex sugars that remain after digestion (oligosaccharides), the simple sugars (monosaccharides) in honey also contributed to modulation of the gut microbiota. The presence of the monosaccharides in honey significantly affected the changes in the microbial composition. Positive changes in the potentially beneficial lactobacilli populations were observed when high fructose content honeys were used, and these effects were attributed to the fructose components of the honeys.

The second part of this study focused on the oligosaccharide components of the honey. This was the first study to investigate the impact of simulated digestion of Australian honeys on their prebiotic activity. The oligosaccharide components of all of the tested varieties of Australian honeys exerted favourable effects on the gut ecosystem by promoting the growth of the beneficial bacteria at levels similar to commercial prebiotic, inulin. The honeys suppressed the potentially harmful populations of the gut, and the growth of clostridia specifically was significantly impaired in the presence of the honeys. All honeys had positive PI values, and they also enhanced the production of SCFA, especially the butyric acid, but both of these parameters varied considerably depending on the honey type.

Finally, the fermentation of honey by human gut microbiota resulted in the production of compounds with inhibitory activity against three common enteropathogens, namely *Salmonella typhimurium*, *Clostridium difficile* and *Escherichia coli*, and at levels that matched the commercial prebiotic control, inulin. Most of the honeys tested were more effective at inhibiting the growth of *C. difficile* than the prebiotic control, and inclusion of inulin in the microcosms had little added inhibitory effect on *C. difficile* growth, suggesting that the honeys allowed enhanced production of the inhibitory substances. These compounds did not inhibit the growth of a commercial beneficial probiotic strain, *Lactobacillus fermentum*.

In summary, the results from this investigation demonstrate that Australian honeys have considerable prebiotic capacity which was comparable to or better than inulin. The prebiotic properties of the honeys were associated with the fructose and the oligosaccharides of the honeys which promoted the beneficial bacteria, inhibited the potential pathogens and elevated butyric acid levels.

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Abstract

Non-digestible carbohydrates that promote specific, favourable changes in the composition and functionality of the gut microbiota, and thus contribute to improving health and well-being, are referred to as prebiotics. These favourable changes might be due to a relative increase in the numbers of potentially beneficial bacteria, and/or an increase in the metabolic activity of gut microbiota to produce more beneficial substances, such as short chain fatty acids (SCFA). An established method for quantifying the prebiotic effect of a carbohydrate utilises the Prebiotic Index (PI), which is the ratio of the changes in the populations of the potentially beneficial and potentially harmful bacteria.

Honey contains non-digestible oligosaccharides and there is some evidence that certain honeys could induce beneficial changes in the gut, however there is limited information on Australian floral varieties of honey.

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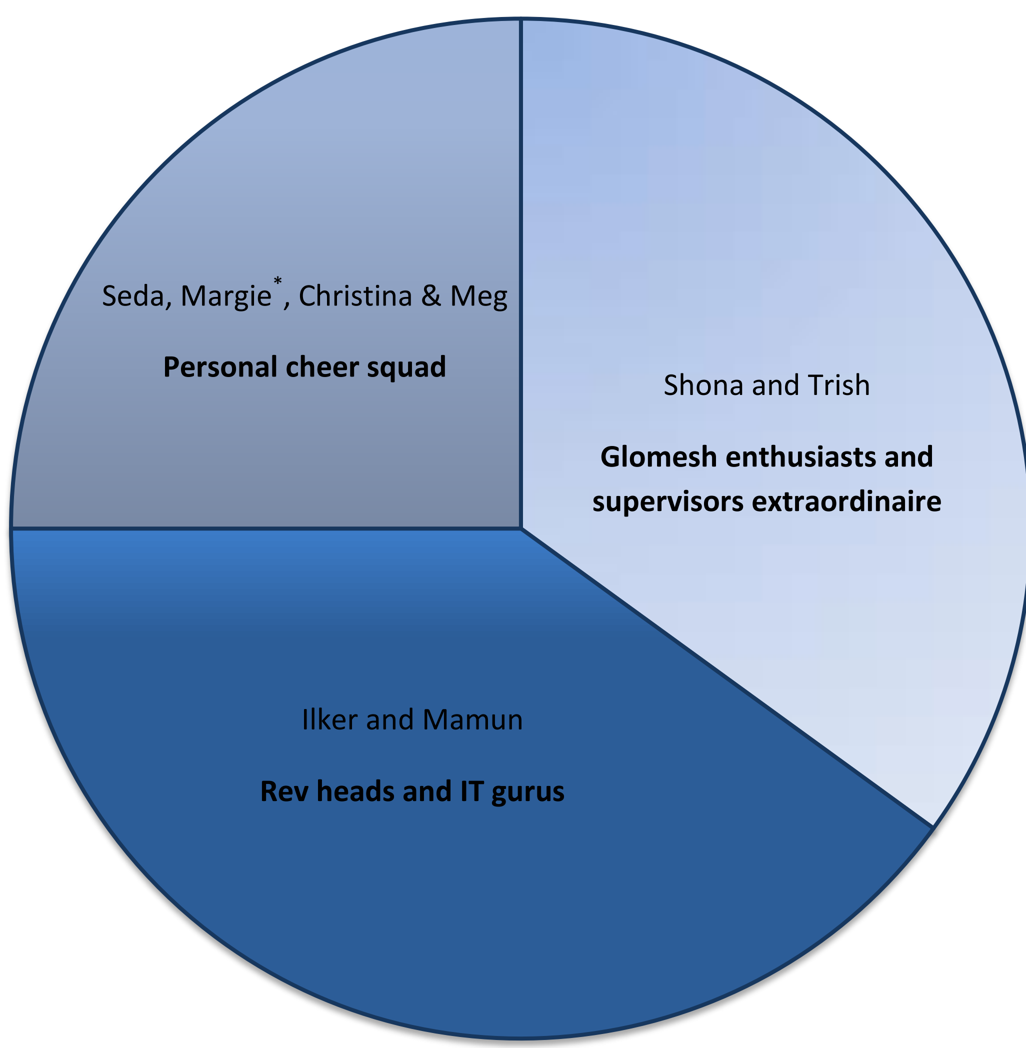


* Without whom a pie chart would never have made it into this thesis.

Table of Contents

Originality statement i
Abstract ii
Acknowledgements iv
Table of Contents vi
List of Tables xi
List of Figures xii
List of Abbreviations xv
1 CHAPTER ONE Introduction 1
1.1. Honey 1
1.1.1. Honey throughout the ages 2
1.1.2. The variable therapeutic properties of honey 3
1.1.3. Honey in gastroenterology 4
1.1.4. Honey in the human diet 5
1.2. Gut microbiology 5
1.2.1. Microbes in the gut 5
1.2.2. The implications of gut microbiota in disease and the immune response 8
1.2.3. Contributions of molecular techniques to understanding the composition and physiology of the gut 9
1.2.4. The impact of host diet on the gut ecosystem 10
1.3. Prebiotics 11
1.3.1. What are prebiotics? 11
1.3.2. Health benefits associated with prebiotics 12
1.3.3. Types of prebiotics and their mechanism of action 13
1.3.4. Determining the prebiotic effect of carbohydrates 16
1.3.5. The Prebiotic Index 16
1.4. Honey and its potential as a prebiotic 16
1.5. Aims of this study 18

2 CHAPTER TWO Impact on the gut microbiota of honey with a high fructose content 19
2.1 Introduction 19
2.1.1 The sugars in honey 19
2.1.2 The fate of simple sugars of honey in the gut 19
2.1.3 Sources of fructose and the issues associated with fructose consumption 20
2.1.4 The intestinal microbiota 22
2.1.5 Aim 23
2.2 Materials and methods 23
2.2.1 Experimental approach 23
2.2.2 Honeys and control carbohydrates 24
2.2.3 Quantification of fructose and glucose 24
2.2.4 Honey digests 24
2.2.5 *In vitro* intestinal microcosms 25
2.2.6 Enumeration of major viable bacterial groups 26
2.2.7 Short chain fatty acid analysis 26
2.2.8 Statistical analysis 27
2.3 Results 27
2.3.1 Monosaccharide constituents of honey 27
2.3.2 The influence of honey saccharides on the growth of potentially beneficial groups 28
2.3.3 The effect of saccharides on the growth of enteric bacteria in the microcosms 32
2.3.4 Clostridial growth in microcosms 32
2.3.5 Summary of overall changes in bacterial profiles 35
2.3.6 Correlation of overall changes in bacterial groups 38
2.3.7 Short chain fatty acid profiles in microcosms 39
2.4 Discussion 41
2.4.1 Significance of fructose 41
2.4.2 Enteric bacterial growth in microcosms 43
2.4.3 Inhibition of potentially harmful bacteria by honey 43

2.4.4 Complex interrelationships in the gut 44

2.4.5 Substrate digestion in *in vitro* gut models 44

2.5 Conclusion 45

3 CHAPTER THREE Impact of the oligosaccharides in Australian honeys on the constituents of the gut microbiota and the prebiotic properties of the honey 47

3.1 Introduction 47

3.1.1 Determining the prebiotic effect of carbohydrates 47

3.1.2 Significance of Australian honey as a prebiotic 48

3.1.3 Aims 49

3.2 Materials and methods 49

3.2.1 Experimental approach 49

3.2.2 Honeys and other carbohydrates 50

3.2.3 Honey digests 52

3.2.4 Intestinal microcosms 52

3.2.5 Enumeration of major viable bacterial groups 52

3.2.6 Short chain fatty acid analysis 52

3.2.7 Measurement of Prebiotic Index (PI) 52

3.2.8 Statistical analysis 53

3.2.9 Molecular fingerprinting 53

3.3 Results 55

3.3.1 Prebiotic Index of digested and whole honey 55

3.3.2 In-depth analysis of the PI values of digested Australian honeys 59

3.3.3 Investigation of the relationship between PI and oligosaccharide concentration in Australian honeys 64

3.3.4 Effect of honey on growth of major bacterial groups 66

3.3.5 Molecular profiling of microbiota from selected honey-enriched microcosms 79

3.3.6 SCFA production in microcosms 81

3.4 Discussion 90

3.4.1 The impact of *in vitro* digestion on the prebiotic assessment of honeys 90

3.4.2 The beneficial effects of digested honeys on gut microbiota 91

3.4.3 Relationship between PIs and oligosaccharide concentrations in honey ... 101

3.4.4 Limitations of PI 102

3.5 Conclusion 104

4 CHAPTER FOUR Impact of the honey-enriched intestinal microcosm on introduced bacterial species 106

4.1 Introduction 106

4.1.1 Bacterial pathogens of the gut 106

4.1.2 Inhibitory effects of commensal gut microbiota on enteropathogens 107

4.1.3 Effect of honey on enteropathogens 109

4.1.4 Aim 110

4.2 Methods 110

4.2.1 Growth of *Salmonella typhimurium* in intestinal microcosms 110

4.2.2 *S. typhimurium* growth curve 110

4.2.3 Measurement of *S. typhimurium* growth in honey-enriched intestinal microcosms 110

4.2.4 Inhibitory effects of honey-enriched microcosm supernatants on intestinal bacterial cultures 111

4.3 Results 113

4.3.1 Growth of *S. typhimurium* in honey-enriched microcosms 113

4.3.2 Growth of intestinal bacterial cultures in microcosm supernatants 118

4.4 Discussion 140

4.4.1 Impact of honey enrichment in microcosms on *S. typhimurium* 141

4.4.2 Effects of honey on introduced intestinal bacterial cultures 142

4.5 Conclusion 146

5 CHAPTER FIVE General discussion 147

5.1 Summary and comparison of the prebiotic properties of Australian floral honeys 147

5.1.1 Assessment of prebiotic potential of honeys *in vitro* 150

5.1.2 Proposed revised prebiotic score 151

5.2 Additional potential benefits of honey consumption on gut health 153
5.3 Future work and human clinical study 154
5.4 Conclusions 154
References 156
Appendices 173
Appendix 1 173
Appendix 2 175
Appendix 3 178
Appendix 4 180

List of Tables

Table 1.1 | Composition, source and properties of common prebiotics 14
Table 2.1 | Honeys used in study 24
Table 2.2 | Selective media for enumeration of the major bacterial groups 26
Table 2.3 | Amount of fructose and glucose as monosaccharides in the honeys 27
Table 2.4 | Correlation of changes in the counts of the major bacterial groups in microcosms 38
Table 2.5 | Enumeration of short chain fatty acids in microcosms using whole and digested substrates 40
Table 3.1 | Honey samples used in *in vitro* microcosm studies 50
Table 3.2 | Donors of faecal microbiota for the *in vitro* microcosm studies 52
Table 3.3 | PCR protocol 54
Table 3.4 | Correlation between maltose + oligosaccharide concentration and PI of digested honeys determined by microcosms established using various microbiota ... 66
Table 3.5 | Enumeration of butyric acid in microcosms 83
Table 3.6 | Enumeration of propanoic acid in microcosms 86
Table 3.7 | Enumeration of acetic acid in microcosms 88
Table 4.1 | Bacterial strains used in inhibition assays 112
Table 5.1 | Summary and comparison of the effects of Australian floral honeys on human gut microbiota 149
Table 5.2 | Revised prebiotic score of Australian floral varieties of honey 152

List of Figures

Figure 1.1 | Mesophilic rock painting of honey collection from a wild nest 2
Figure 1.2 | Factors affecting gut microbial ecology and effects on host health 8
Figure 2.1 | Composition and effects of human gut bacteria 23
Figure 2.2 | Growth of bifidobacteria in microcosms using whole and digested carbohydrate sources 30
Figure 2.3 | Growth of lactobacilli in microcosms using whole and digested carbohydrate sources 31
Figure 2.4 | Growth of enteric bacteria in microcosms with added whole honey 33
Figure 2.5 | Growth of clostridia in microcosms using whole and digested carbohydrate sources 34
Figure 2.6 | Overview of total change in bacterial counts in microcosms established with adult microbiota 36
Figure 2.7 | Overview of total change in bacterial counts in microcosms established with infant microbiota 37
Figure 3.1 | Prebiotic index of previously tested honeys (with and without digestion) 56
Figure 3.2 | Prebiotic index of 22 Australian honeys (with and without digestion) 58
Figure 3.3 | Prebiotic indices of jarrah honeys 60
Figure 3.4 | Prebiotic indices of red stringybark honeys 61
Figure 3.5 | Prebiotic indices of spotted gum honeys 62
Figure 3.6 | Prebiotic indices of yellow box honeys 63
Figure 3.7 | Prebiotic indices of canola honeys 64
Figure 3.8 | PI and oligosaccharide concentrations of digested honeys 65
Figure 3.9 | Impact of digested honeys on bifidobacteria numbers in microcosms established using microbiota from five donors 68
Figure 3.10 | Impact of digested honeys on lactobacilli numbers in microcosms established using microbiota from five donors 70
Figure 3.11 | Impact of digested honeys on bacteroides numbers in microcosms established using microbiota from five donors 72
Figure 3.12 | Impact of digested honeys on clostridia numbers in microcosms established using microbiota from five donors 74
Figure 3.13 | Impact of digested honeys on enteric bacteria numbers in microcosms established using microbiota from five donors 76
Figure 3.14 | Impact of digested honeys on enterococci numbers in microcosms established using microbiota from five donors 78
Figure 3.15 | TRFLP profiles generated using restriction enzyme *Rsa*I for microbiota from intestinal microcosms enriched with honeys 80

Figure 3.16 | TRFLP profiles determined using restriction enzyme *Msp*I of microbiota from microcosms enriched with honeys 81
Figure 4.1 | *S. typhimurium* growth in microcosms established with jarrah honeys . 114
Figure 4.2 | *S. typhimurium* growth in microcosms established with red stringybark honeys 115
Figure 4.3 | *S. typhimurium* growth in microcosms established with spotted gum honeys 116
Figure 4.4 | *S. typhimurium* growth in microcosms established with yellow box honeys 117
Figure 4.5 | *S. typhimurium* growth in microcosms established with canola and manuka honeys 118
Figure 4.6 | Growth of *S. typhimurium* in supernatants from jarrah honey microcosms 121
Figure 4.7 | Growth of *C. difficile* in supernatants from jarrah honey microcosms 122
Figure 4.8 | Growth of *E. coli* in supernatants from jarrah honey microcosms........ 123
Figure 4.9 | Growth of *L. fermentum* in supernatants from jarrah honey microcosms 124
Figure 4.10 | Growth of *S. typhimurium* in supernatants from red stringybark honey microcosms 125
Figure 4.11 | Growth of *C. difficile* in supernatants from red stringybark honey microcosms 126
Figure 4.12 | Growth of *E. coli* in supernatants from red stringybark honey microcosms 127
Figure 4.13 | Growth of *L. fermentum* in supernatants from red stringybark honey microcosms 128
Figure 4.14 | Growth of *S. typhimurium* in supernatants from spotted gum honey microcosms 129
Figure 4.15 | Growth of *C. difficile* in supernatants from spotted gum honey microcosms 130
Figure 4.16 | Growth of *E. coli* in supernatants from spotted gum honey microcosms 131
Figure 4.17 | Growth of *L. fermentum* in supernatants from spotted gum honey microcosms 132
Figure 4.18 | Growth of *S. typhimurium* in supernatants from yellow box honey microcosms 133
Figure 4.19 | Growth of *C. difficile* in supernatants from yellow box honey microcosms 134
Figure 4.20 | Growth of *E. coli* in supernatants from yellow box honey microcosms 135

Figure 4.21 | Growth of *L. fermentum* in supernatants from yellow box honey microcosms 136
Figure 4.22 | Growth of *S. typhimurium* in supernatants from canola and manuka honey microcosms 137
Figure 4.23 | Growth of *C. difficile* in supernatants from canola and manuka honey microcosms 138
Figure 4.24 | Growth of *E. coli* in supernatants from canola or manuka honey microcosms 139
Figure 4.25 | Growth of *L. fermentum* in supernatants from canola or manuka honey microcosms 140

List of Abbreviations

BMI	Body mass index
bp	Base pairs
CFU	Colony forming units
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
FOS	Fructo-oligosaccharides
GC-MS	Gas chromatography-mass spectrometry
GI	Glycaemic index
GIT	Gastrointestinal tract
GOS	Galacto-oligosaccharides
HBA	Horse blood agar
HFCS	High fructose corn syrup
HREA	Human Research Ethics Advisory
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IMO	Isomalto-oligosaccharides
MGO	Methylglyoxal
MPE	Measure of prebiotic effect
MRS	de Man Rogosa Sharpe
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Prebiotic Index
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
SCFA	Short chain fatty acid(s)
TNF	Tumor necrosis factor
TOS	Transgalacto-oligosaccharides
TRFLP	Terminal restriction fragment length polymorphism
TSA	Tryptone soya agar
TSB	Tryptone soya broth
WCA	Wilkens-Chalgren Anaerobe
XOS	Xylo-oligosaccharides

1 CHAPTER ONE
Introduction

The gut microbiota plays an important role in human health and it is recognised that a healthy balance of gut microbiota is essential for host health and well-being, and disturbances to this balance can result in the development and progression of numerous diseases. Consequently, there is increasing interest in the manipulation of the gut microbiota by dietary means to a more remedial or beneficial balance. The use of live beneficial bacterial supplements (probiotics) or non-digestible foods that can selectively stimulate the growth of beneficial indigenous gut microbes (known as prebiotics) are the main approaches used.

1.1. HONEY

Honey is a complex carbohydrate that is produced by honey bees, and the honey most commonly collected by humans is made by the European honey bee, *Apis mellifera*. Honey is usually produced from the nectar of flowering plants, but honeydew honey is sometimes produced by honey bees collecting sap that is exuded from other insects such as aphids (Crane, 1999).

Honey is a super-saturated sugar solution, containing 15-21 % water (Molan, 1992). Fructose is the predominant sugar, with concentrations ranging from 36-50 %, followed by glucose making up 28-36 % of its final composition. The exact composition of honey is highly variable and dependant on the floral source as well as external contributions from seasonal, environmental, processing and storage factors. Honey contains at least 181 substances, mainly carbohydrates, but also phytochemicals (plant-derived substances), minerals, proteins, amino acids, enzymes and vitamins (Alvarez-Suarez *et al.*, 2010).

Although much of the carbohydrate component is made up of sugars i.e. monosaccharides, that are in the immediately digestible form in the small intestine, there are di-, tri- and oligosaccharides that are present in smaller quantities (Bogdanov *et al.*, 2004). The many oligosaccharides and low-weight polysaccharides in honey are likely to resist degradation by host enzymes, and therefore could be used by the colonic microbiota as a nutrient source. So, honey has potential for use as a natural prebiotic and as described in the following sections it has been exploited therapeutically and medicinally for thousands of years.

1.1.1. Honey throughout the ages

Honey has been important to humans for thousands of years. It was highly regarded in many early civilisations for its nutritional and therapeutic properties (Ransome, 1937; Crane, 1980; Crane, 1999). The relationship between bees and humans dates back to as early as the Stone Age, and there are numerous examples of rock art depicting people collecting honey, for example, Figure 1.1 (Crane, 1986; Crane, 1999).

The first known written reference to honey, a Sumerian tablet writing dated to 2100-2000 BC, mentions the use of honey as a drug and an ointment (Crane, 1980). A range of therapeutic uses of honey are noted in the Qur'an, the Bible, the Torah and other ancient sacred texts (Beck, 1938; Zumla and Lulat, 1989). These texts promoted its use for the treatment of ailments including eye conditions, gastrointestinal upsets, burns, ulcers and other skin lesions (Zumla and Lulat, 1989; Crane, 1999). Honey was an integral part of the medical practices of numerous cultures throughout Africa, Asia, the Arab world and Europe for thousands of years (Crane, 1999). However, it was largely displaced from use in modern medicinal practices with the introduction of antibiotics in the mid-1940s (White, 1966; Molan, 1992).

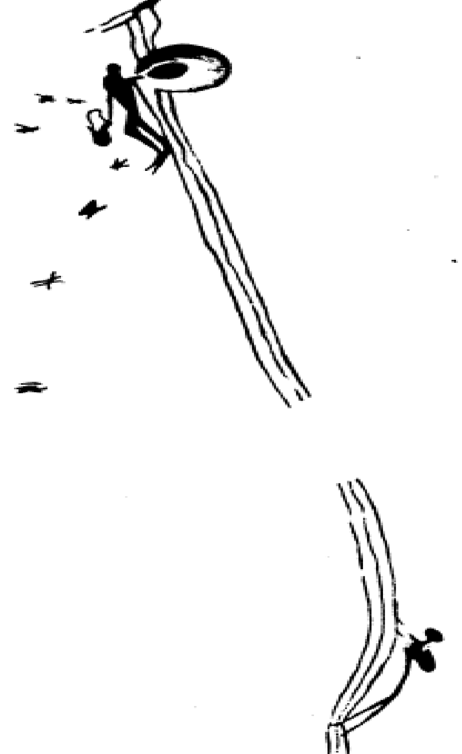


Figure 1.1 | Mesophilic rock painting of honey collection from a wild nest
La Arana shelter, Valencia, Spain (Crane, 1999).

In addition to its therapeutic benefits, honey was held in high regard for its nutritional properties as it was the only available natural sweetener (Bogdanov *et al.*, 2008). It was an important source of carbohydrate, with significant contributions to the diets of many ancient civilisations including the Egyptians, Greeks, Romans and Chinese as well as

indigenous Australian Aboriginal tribes and the Guayaki Indians of Paraguay (Allsop and Brand Miller, 1996).

However, crusaders first encountered the sugar cane in the 11th century and by the 1550s, industrial sugar began to replace honey in the diet. By the early 1700s, the supply of industrial sugar boomed, and it became more affordable so honey was no longer the standard sweetener (Alvarez-Suarez *et al.*, 2010).

1.1.2. The variable therapeutic properties of honey

Honey provides numerous nutritional and therapeutic benefits including antimicrobial, antioxidant, anti-inflammatory and wound healing activities. Of these, the most extensively studied through *in vitro* and *in vivo* experiments and human clinical trials has been the antimicrobial activity of honey (reviewed in (Molan, 1992; Molan, 1995; Bogdanov, 1997; Molan, 1998; Molan, 2002; Bogdanov *et al.*, 2008; Alvarez-Suarez *et al.*, 2010)). Without doubt, the continued medicinal use of honey as a therapeutic agent can be attributed to its broad-spectrum antimicrobial properties, which have proven effective against many pathogenic organisms, including multi-drug resistant strains. The antimicrobial activity of honey is multi-factorial and is derived from osmolarity, acidity, production of hydrogen peroxide, and the presence of non-peroxide factors (Molan, 1992; Molan, 1992). There have been no documented cases of microbial resistance to the inhibitory effects of honey, nor could resistance to honey be induced (Blair *et al.*, 2009; Cooper *et al.*, 2010; Maddocks and Jenkins, 2013).

However, not all honeys are the same. Many ancient cultures appreciated that different floral sources gave rise to honeys with different therapeutic properties. For example, Aristotle (384-322 B.C.) noted that pale honeys were best used as a salve for sore eyes and wounds (Molan, 1999). Although often viewed today as a generic product with little regard to its floral source, the floral species-specific organoleptic characteristics create great diversity in honey. For instance, a survey of 345 New Zealand honeys revealed considerable variation in the antibacterial activity, with approximately a third of the honeys showing activity near or below the level of detection and others with extremely potent inhibitory effects equivalent to 58 % phenol, a standard reference antiseptic (Allen *et al.*, 1991). In addition, the source of antimicrobial activity differed according to honey type. In some honeys, the activity was due predominantly to the production of hydrogen peroxide, whereas in other honeys the activity was attributed to the non-peroxide components in the honey.

In a more recent study, the antibacterial activities of 477 Australian honeys were assayed, and these also varied considerably (Irish *et al.*, 2011). The study showed that the

predominant reason for antibacterial activity (either hydrogen peroxide or non-peroxide) and levels of activity were dependant on the floral sources of the honeys, and also that the storage conditions (particularly temperature) affected the activity.

The antioxidant effect of honey is largely attributed to its phenolic compounds, which when ingested can provide protection in the blood stream and within cells (Schramm *et al.*, 2003; Cooper *et al.*, 2009). As with the antimicrobial activity, the antioxidant capacity of honeys is highly variable and dependant on floral source. Generally, darker honeys have shown higher levels of antioxidant activity than their lighter counterparts (Estevinho *et al.*, 2008).

The anti-inflammatory, antimicrobial and wound healing properties of some honeys have been utilised extensively in the treatment of surgical and traumatic wounds, burns and ulcers (Molan, 1999; Molan, 2001; Molan, 2001; Molan, 2002). The efficacy of honey as a wound dressing is again highly dependent on the type of honey used, which is influenced by the floral source, local environment as well as processing and storage conditions.

In summary, all honeys can provide nutritional and therapeutic benefits, but not all honeys will exert the same health benefits, and honeys from different locations and different floral sources will have different properties.

1.1.3. Honey in gastroenterology

The use of honey to treat gastrointestinal conditions has been documented throughout history. For example, Roman physicians prescribed different types of honey as a cure for both diarrhoea and constipation (ca. 25AD), and Islamic holy scripts dating back to the 8th century show the prophet Muhammad recommending the use of honey for diarrhoea (Crane, 1999; Bogdanov *et al.*, 2008). The use of honey in the prevention and treatment of peptic ulcers, gastritis and gastroenteritis have been reported in various books and publications from Eastern Europe and from Arab countries (Crane, 1980).

Studies have shown that the ingestion of honey shortens the duration of bacterial diarrhoea in children (Haffejee and Moosa, 1985). Honey is also as effective as glucose in ensuring the recovery of patients with viral gastroenteritis (Salem, 1981; Haffejee and Moosa, 1985). Honey exhibits a potent *in vitro* inhibition of *Helicobacter pylori*, the causative agent of peptic ulcers and gastritis (Al Somal *et al.*, 1994; McGovern *et al.*, 1999; Osato *et al.*, 1999). Other studies suggest that honey has a protective effect on the stomach (Al-Swayeh and Ali, 1998). Honey can also have a laxative effect, and the consumption of relatively large amounts of honey (50 to 100 g) can be a mild laxative in some individuals, due to insufficient absorption of the fructose in honey (Bogdanov *et al.*, 2008).

1.1.4. Honey in the human diet

The importance of honey in the diets of human foragers has been documented throughout history and it has been suggested that honey (and residual bee larvae in wild honeys) may have been an important source of energy, fat, and protein (reviewed in (Crittenden, 2011)). Routine consumption of honey has also been identified as an important part of human evolution by providing a considerable amount of energy, supplementing meat and plant foods. It has been suggested that honey, an energy dense food that is easy to digest, may have played an important role (along with meat and tubers) in shifting the diet of early humans. This idea is supported by the appearance of Oldowan tools that may have been used for honey collecting, along with the evolution of larger hominin brains (requiring regular consumption energy rich foods) and the reduction of molar size, suggestive that hominins were consuming foods that required less mechanical breakdown (Crittenden, 2011).

A number of recent infant nutrition studies showed the benefits of a diet supplemented with honey (reviewed in (Bogdanov *et al.*, 2008)). For example, infants whose diets included honey were of healthier weight and showed improved blood formation than those on a diet without honey. Another study showed that honey was better tolerated by babies than sucrose and these infants showed weight gain, less susceptibility to illness, increase of haemoglobin, better skin colour and fewer digestion problems (Bogdanov *et al.*, 2008).

The consumption of honey in overweight and obese adult human subjects showed reduced cardiovascular risk factors, reduced BMI and no increase in body weight when compared to the sucrose control (Yaghoobi *et al.*, 2008).

1.2. GUT MICROBIOLOGY

1.2.1. Microbes in the gut

The human gut is a complex microbial ecosystem; members of the human intestinal microbiota are made up of several hundred species representing nine bacterial and one archaeal division. It has been estimated that approximately 400-500 species of bacteria reside in the colon alone, with numbers as high as 10^{12} bacteria per gram of faeces (Kurokawa *et al.*, 2007).

Colonisation of the gastrointestinal tract (GIT) occurs at birth. As the infant passes through the birth canal it is exposed to the mother’s commensal bacteria, which results in the initial inoculation of the otherwise sterile gut. Bacteria from the surrounding environment can also contribute to the primary bacterial community that establishes in

the gut (Gibson *et al.*, 2004). The infant gut microbiota, particularly when the infant is breast-fed, is dominated by populations of bifidobacteria. In contrast, there are considerable differences in the composition of the adult human gut microbiota from one individual to another and the adult gut is comprised of a range of anaerobic bacteria with members of the bacteroides genus accounting for the highest numbers (Gibson, 1999; Manning and Gibson, 2004).

Intestinal bacteria are able to produce a range of compounds that have both positive and negative effects on gut physiology as well as other systemic influences. An example of this is the production of short-chain fatty acids (SCFA) from the metabolism of complex carbohydrates, which can then be further metabolised locally or systemically to provide energy generation for the host (Gibson and Roberfroid, 1995; Manning and Gibson, 2004). The physiology and structure of the gut can affect the activity of these bacteria. Changes in substrate availability, redox potential, pH, oxygen tension and distribution in the colon are all factors that can influence fluctuations in bacterial metabolism and there is a high degree of heterogeneity in the gut ecosystem. Microorganisms in the proximal colon have a plentiful supply of nutrients and grow at a faster rate causing a decrease in pH as a result of SCFA production. In the distal colon, where substrate availability is lower, bacteria grow more slowly and the pH approaches neutral (Gibson and Roberfroid, 1995).

Substrates for fermentation in the human colon primarily come from dietary carbohydrates that have not been digested in the upper GIT (discussed in Section 1.3). In many cases, the metabolic end products excreted by one species can serve as a growth substrate for another. For example, bacteroides are capable of degrading polysaccharides to shorter chain oligosaccharides that other species utilise (Gibson and Roberfroid, 1995; Maathuis *et al.*, 2009).

Intestinal bacteria can be broadly categorised into species that are benign, those that exert harmful effects or those that have beneficial effects on the host. It is generally accepted that bifidobacteria and lactobacilli have health-promoting effects due to their ability to inhibit the growth of harmful bacteria, stimulate immune functions, improve digestion and absorption of essential nutrients and synthesise vitamins. In contrast, bacteroides and clostridia are implicated in the more pathogenic effects including diarrhoea, infections, liver damage, carcinogen production and intestinal putrefaction (Gibson, 1999; Gibson *et al.*, 2004).

There has been particular interest in promoting the beneficial (bifidobacteria and lactobacilli) populations in the gut. The health promoting effects of bifidobacteria have

been studied extensively (reviewed in (Gibson and Roberfroid, 1995)) and these bacteria are known to aid host health by:

- producing SCFA, which lower pH and exert an antibacterial effect;
- excreting additional metabolic end products (e.g. bacteriocins), which have direct broad-spectrum inhibitory effects;
- producing B-group vitamins and digestive enzymes;
- restoring normal intestinal flora following antibiotic therapy; and
- promoting immunological attack against malignant cells.

Although the gut microbiota plays a fundamentally important role in health and disease, this ecosystem remains incompletely characterised and its diversity poorly defined (Eckburg *et al.*, 2005; Thomas and Ockhuizen, 2012; Kovatcheva-Datchary and Arora, 2013). Due to their major role in host health, there is ongoing interest in the manipulation of the composition of gut microbiota toward a more remedial community (Gibson and Roberfroid, 1995; Scott *et al.*, 2011; Rauch and Lynch, 2012). An upset of the gut microbial community can lead to unfavourable symptoms of acute gastroenteritis as well as the possibility of more chronic disorders such as inflammatory bowel disease (IBD) and colonic cancer (Gibson and Fuller, 2000; Gentschew and Ferguson, 2012). Gut microbial ecology can be affected by a variety of factors including diet, medication, stress, age and general living conditions (Gibson and Fuller, 2000; Sommer and Backhed, 2013), as shown in Figure 1.2.

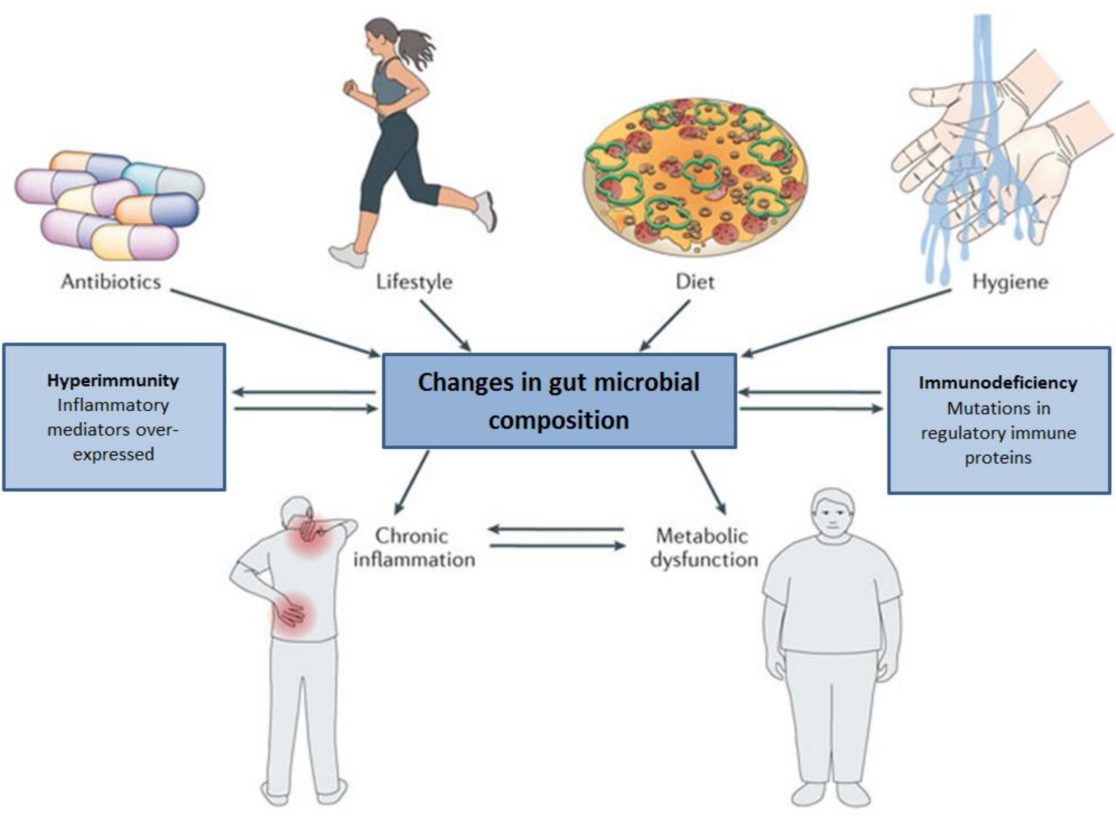


Figure 1.2 | Factors affecting gut microbial ecology and effects on host health

Gut microbial composition is affected by a variety of environmental factors including medication, diet and lifestyle. The genetic disposition of the host also contributes: hyperimmunity which occurs when inflammatory mediators are over-expressed; or immunodeficiency due to mutations in regulatory immune proteins. Alterations in the intestinal microbiota composition influence the levels of immune mediators, inducing chronic inflammation and metabolic dysfunction (modified from (Sommer and Backhed, 2013)).

1.2.2. The implications of gut microbiota in disease and the immune response

Due to the increased interest in the impact of gut microbiota on human health, the contributions of the gut microbes to disease, especially within the GIT, have been widely studied (Almansa *et al.*, 2012; Clarke *et al.*, 2012; Ceapa *et al.*, 2013). Nutrient absorption, secretion of certain electrolytes and water as well as the storage and excretion of waste materials are the major biological functions of the intestine (Gibson and Roberfroid, 1995). The commensal bacteria of the gut possess metabolic capabilities that are lacking in the human host, and contribute to host nutrition by improving the efficacy of energy harvested from food and by synthesising essential vitamins. An imbalance of the intestinal microbiota can predispose individuals to a variety of disease states, ranging from inflammatory bowel disease to allergy and obesity (Kurokawa *et al.*, 2007; Ha, 2011).

Altered patterns in the gut microbiota have been associated with allergies and other immune related diseases, inflammatory bowel diseases, as well as metabolic and degenerative diseases (Ha, 2011; Ceapa *et al.*, 2013).

Commensal bacteria of the gut also provide a barrier to infection by pathogenic organisms by impairing the ability of new organisms to gain access to attachment sites. In particular, *Lactobacillus acidophilus* inhibits receptors for a number of virulent gut pathogens (Gibson and Roberfroid, 1995). Bifidobacteria produce antimicrobial substances, inhibiting cell entry and killing intracellular *Salmonella typhimurium* (Eckburg *et al.*, 2005). Studies also show that *Lactobacillus* species may aid digestion of lactose in lactose-intolerant individuals, reduce constipation and infantile diarrhoea, help resist infections caused by enteric pathogens, prevent traveller's diarrhoea and help relieve irritable bowel syndrome (Gibson *et al.*, 2004; Clarke *et al.*, 2012).

However, increased levels of certain bacteria in the intestine have been implicated as causative agents in both colonic and systemic disorders. Intestinal pathologies range from antibiotic-associated colitis and inflammatory bowel disease to colorectal cancer and necrotising enterocolitis (Gibson *et al.*, 2004; Manichanh *et al.*, 2006). Systemically, gut microbiota imbalances can cause gut-origin septicaemia, pancreatitis and multiple organ failure (Gibson and Roberfroid, 1995; Gibson *et al.*, 2004).

Other studies suggest that the gut microbiota, in particular high numbers of Firmicutes and Bacteroidetes, may make a significant contribution to the pathophysiology of obesity (Ley *et al.*, 2005; Ley *et al.*, 2006).

Understanding the intestinal microbiota profile in relation to health and disease could help in the diagnosis of health risks. Beneficial manipulation of the intestinal microbiota allows targeted nutritional approaches that can reduce the severity of disease or improve health outcomes (Ha, 2011; Ceapa *et al.*, 2013).

However, although the association of specific commensal bacterial species in health and disease is recognised, it is not always clear whether this is a causative relationship or an effect (Thomas and Ockhuizen, 2012; Ceapa *et al.*, 2013).

1.2.3. Contributions of molecular techniques to understanding the composition and physiology of the gut

Current understanding of the composition and functions of the human intestinal microbiota is still somewhat limited due to the highly complex mixed microbial communities that are present.

Until relatively recently, almost all our knowledge and understanding of the gut microbiota has been obtained by culture-based techniques. That is, isolating and culturing organisms from faecal or intestinal material. Culture-based approaches are still widely used as part of many standard testing procedures, particularly in studies on the human intestinal ecosystem, as they are cost-effective and reproducible (Tannock, 1999; Chassard *et al.*, 2008; O'Toole and Cooney, 2008; Sekirov *et al.*, 2010). However, cultivation of microbes as a way to characterise entire microbial communities has significant limitations. It is well recognised that most microbes cannot be cultured using standard culture techniques. Despite the shortcomings, culture-based techniques are useful in obtaining an understanding of the diversity and role of the intestinal environment (Tannock, 1999; O'Toole and Cooney, 2008).

There has been a shift to using culture-independent molecular approaches to characterise the diverse ecosystem in the gut, and elucidate its role in health and disease (Backhed *et al.*, 2005). Molecular methods have some distinct advantages over culture-based technologies as they can encompass full microbial diversity through broad-range sequencing of 16S rRNA genes (Ley *et al.*, 2006). Despite the high diversity of bacterial species, they share common genomic features, which allow large-scale comparative metagenomic analyses when studying communities (Kurokawa *et al.*, 2007).

The most comprehensive enumerations of microbial diversity within the mammalian gut have come from sequencing 16S rRNA genes, obtained directly from DNA extracted from gut mucosal biopsies or faeces, and using PCR primers to target to broad phylogenetic groups (Ley *et al.*, 2006).

1.2.4. The impact of host diet on the gut ecosystem

The role of the host diet in the development of microbial composition has been studied using molecular approaches. The impact of diet is seen as early as infancy where the composition and diversity of the microbiota of breast-fed and formula-fed infants was found to differ significantly (Schwartz *et al.*, 2012).

Molecular technique based studies have helped elucidate how certain members of the gut microbiota contribute to the maintenance and functional capability of the human intestine. A 16S rDNA sequencing based study comparing the impact of diet on the gut microbiota of children from Europe and a rural African village showed that the African microbiome was enriched with Actinobacteria and Bacteroidetes with a depletion of Firmicutes (De Filippo *et al.*, 2010). The African microbiome contained an abundance of *Xylanibacter* and *Prevotella*, leading the authors to hypothesise that the microbiota of the African individuals co-evolved with their diet, and that the members of these genera could

improve the ability to extract calories from indigestible plant polysaccharides. In another sequencing study, the microbiota of malnourished children from Bangladesh were characterised by lower abundance of Bacteroidetes and a dominance of Proteobacteria, and showed a lower overall diversity of gut microbiota when compared to their healthy counterparts (Monira *et al.*, 2011).

The importance of bacterial functions related to carbohydrate metabolism in the colon has been established by a number of metagenomic studies (Gill *et al.*, 2006; Kurokawa *et al.*, 2007). These studies showed that polysaccharides and peptides that are indigestible by the host are major drivers of the colonic microbial composition (Ottman *et al.*, 2012) and that diet has a crucial influence on intestinal microbial composition and activity.

1.3. PREBIOTICS

1.3.1. What are prebiotics?

Prebiotics are classified as functional foods, that is foods that provide health benefits beyond basic nutrition (Gibson and Roberfroid, 1995; Gibson *et al.*, 2004). The premise behind prebiotics is to stimulate potentially beneficial bacteria that are already residing in the gut, rather than to introduce exogenous species (as is the case with probiotics - live microbial food supplements) (Manning and Gibson, 2004).

Prebiotics are carbohydrates that are neither hydrolysed nor absorbed in the upper part of the GIT, so they reach the colon intact and are available to be used as a selective substrate by the commensal bacteria (Gibson and Roberfroid, 1995). Prebiotics must specifically stimulate the growth of beneficial rather than harmful bacteria, ultimately leading to a change in the colonic microbial populations in favour of a healthier composition (Gibson and Roberfroid, 1995; Gibson and Fuller, 2000; Rastall and Gibson, 2002; Gibson *et al.*, 2004).

The preferred target organisms for prebiotics are the beneficial indigenous gut flora, bifidobacteria and lactobacilli (Gibson and Fuller, 2000). Ideally, the most efficient prebiotics may also reduce or suppress the numbers and activities of potentially pathogenic bacteria such as toxin-producing clostridia, proteolytic bacteroides and toxigenic *Escherichia coli* (Manning and Gibson, 2004).

In order to be classified as a prebiotic, a substance must satisfy a set of criteria which include the ability of the carbohydrate to (Gibson *et al.*, 2004):

i) resist host digestion, gastric acidity, hydrolysis by mammalian enzymes and absorption;

ii) undergo fermentation by intestinal microbiota; and

iii) selectively stimulate intestinal microbiota, favouring those capable of exerting beneficial effects to the host.

1.3.2. Health benefits associated with prebiotics

Due to the selective stimulation of the beneficial gut populations, the ingestion of prebiotics has health promoting effects including immunostimulation, improved digestion and absorption, vitamin synthesis, inhibition of the growth of potential pathogens, cholesterol reduction and lowering of gas distension (Grizard and Barthomeuf, 1999; Manning and Gibson, 2004; Roberfroid *et al.*, 2010; Gibson *et al.*, 2010; Chauhan and Chorawala, 2012; Dewulf *et al.*, 2013). The use of prebiotics can also exert adverse effects on enteropathogens, capable of causing acute gastroenteritis and food poisoning, as lactobacilli and bifidobacteria secrete natural antimicrobials that have a broad spectrum of activity (Manning and Gibson, 2004). In addition, the administration of prebiotics can result in improved mineral (especially calcium) absorption (Manning and Gibson, 2004; Raschka and Daniel, 2005; Lobo *et al.*, 2009). While the small intestine is primarily the site of absorption, significant amounts of minerals are absorbed through the length of the gut. The SCFA end-products that reduce luminal colonic pH are likely to increase calcium solubility. They enter the colon in protonated form, dissociating the proton in the intracellular environment in exchange for calcium ions before being absorbed into the body for host energy use (Manning and Gibson, 2004). Prebiotics can also modulate lipid metabolism, most likely via fermentation products.

It has been suggested that prebiotics are protective against the development of colon cancer, which is the second most prevalent cancer in humans (Manning and Gibson, 2004). Several bacterial species commonly found in the colon produce carcinogens and tumour promoters from the metabolism of various food components. Diet-mediated intervention to protect against colon cancer due to the slow, progressive nature of the disease is aided by the fact that the colonic microbes can be influenced through diet (Manning and Gibson, 2004). The mechanisms proposed for the protective properties of prebiotics are:

i) Production of protective metabolites

The production of butyric acid by indigenous colonic bacteria stimulates apoptosis in colonic cancer cell lines and is the preferred energy source for healthy coloncytes. Some prebiotics are capable of increasing the level of butyric acid formed in the gut. However, the beneficial bifidobacteria and lactobacilli do not produce butyric acid, rather it is produced as a metabolic end-product of clostridia and Eubacteria. So, the

development of prebiotics that stimulate benign Eubacteria but not toxic clostridia is desirable

ii) Manipulating colonic fermentation away from protein and lipid metabolism

Protein and lipid fermentation in the gut leads to the production of potentially carcinogenic or pre-carcinogenic end products. Prebiotics shift the metabolism in the gut to a saccharolytic (carbohydrate) one, which results in the production of beneficial or benign end products. In the presence of prebiotics, the potentially pathogenic species of bacteroides and clostridia shift away from a proteolytic fermentation to a saccharolytic one (Manning and Gibson, 2004).

1.3.3. Types of prebiotics and their mechanism of action

Non-digestible oligosaccharides in general and fructo-oligosaccharides (FOS) in particular are prebiotics. FOS are short- and medium-length chains of β-D-fructans in which the fructosyl units are bound by a β (2→1) osidic linkage (Gibson and Roberfroid, 1995). Depending on chain length relative to the number of osyl units and the degree of polymerisation (DP), FOS can be categorised as either being oligofructose (DP < 9, average 4.8) or inulin (DP up to 60, average 12). FOS are selectively fermented by most strains of bifidobacteria, allowing their proliferation at the expense of bacteroides, clostridia or coliforms (Gibson *et al.*, 2004). In addition to inulin and oligofructose, there are a number of carbohydrates that have potential prebiotic status, as reviewed by Gibson *et al.* (Gibson *et al.*, 2004; Gibson *et al.*, 2010). These are summarised in the Table 1.1 below.

Table 1.1 | Composition, source and properties of common prebiotics

Carbohydrate	Composition	Source	Criteria*			Considerations
			A	B	C	
Inulin	β (2-1) fructans	Extracted from chicory root	✓	✓	✓	Extensive testing *in vitro* and *in vivo;* studies performed using pure, mixed and faecal cultures; human studies show non-digestibility and ability to stimulate beneficial bifidobacteria and lactobacilli
Fructo-oligosaccharide (FOS) (aka oligofructose)	β (2-1) fructans	Transfructosylation from sucrose, or hydrolysis of chicory inulin	✓	✓	✓	As above
Galacto-oligosaccharides (GOS) and Transgalacto-oligosaccharides (TOS)	Mixture of oligo-galactose (85%) with glucose and lactose	Produced from lactose by enzymic (β-galactosidase) transglycosylation	✓	✓	✓	*In vivo* and human studies show significant increases in bifidobacteria and lactobacilli at the expense of potentially pathogenic enterics and bacteroides; inconclusive non-digestibility data
Lactulose	Galactosyl β (1-4) fructose	Isomerisation of lactose, used as laxative	✓	✓	✓	Human studies show significant increases in bifidobacteria with some studies showing marked decreases in *Clostridium perfringens*, streptococci and bacteroides; lack of non-digestibility data; selective stimulation of bacteria inconsistent
Isomalto-oligosaccharides (IMO)	α (1-4) glucose and branched α (1-6) glucose	Starch hyrolysed by combined action of α-amylase and pullulanase, resultant sugar converted to IMO by α-glucosidase	?	✓	?	Inconsistencies in digestibility; enters colon in variable amounts; metabolised by bifidobacteria but also potentially pathogenic bacteroides, *Enterococcus faecalis* and clostridia; selective stimulation unknown

Carbohydrate	Composition	Source	Criteria* A	B	C	Considerations
Lactosucrose	Mixture of lactose, glucose and fructose	Enzymic inversion of lactose and sucrose by β-fructofuranosidase	?	✓	✓	No data to support non-digestibility; allows stimulation of bifidobacteria and suppression of clostridia
Xylo- oligosaccharides (XOS)	β (1-4)-linked xylose	Enzymic hydrolysis of xylan from maize cobs	?	✓	?	Recognised as indigestible dietary fibre; metabolised by bifidobacteria and lactobacilli but also *C. difficile* and *E. coli*; tested in pure culture studies only; *in vitro* studies do not show selective stimulation; rat and human studies show significant increases in bifidobacteria and organic acid production
Soyabean oligosaccharides	α-galactosyl sucrose derivatives (raffinose and stachyose)	Extracted from soya bean whey	✓	✓	?	Lack of conclusive selective stimulation; human volunteer trials show significant increases in bifidobacteria and *L. acidophilus*
Gluco- oligosaccharides	α (1-2) linkages	Sucrose synthesised by dextran sucrase in the presence of maltose; produced via fermentation using *Leuconostoc mesenteroides*	?	✓	?	Non-digestible in animal models; utilised by bifidobacteria and lactobacilli in pure culture studies

Carbohydrates identified in bold text are classified as commercially available prebiotics. The remaining carbohydrates show prebiotic potential, but are not yet accepted as prebiotics due to lack of data from human studies.

***Criteria for classification: (A) non-digestibility including resistance to gastric acid, hydrolysis by enzymes and gastrointestinal absorption; (B) fermentation by intestinal microbiota; and (C) selective stimulation of growth and/or activity of intestinal bacteria.**

1.3.4. Determining the prebiotic effect of carbohydrates

Many *in vitro* and *in vivo* approaches have been used to measure the efficacy of both probiotics and prebiotics. The survivability of live organisms poses great challenges for the administration of probiotics, while changing the composition of the microbiota by selectively increasing the number or metabolism of the beneficial populations shows significant promise.

For testing prebiotic efficacy *in vitro*, researchers commonly use fermenter or continuous culture systems. In simple fermenter models, the substrate is added at a known concentration to a vessel with either a faecal inoculum (preferred) or defined, pure cultures (may be single species or a mixture). The suspension is incubated anaerobically and sampled at regular intervals to determine the changes in bacterial population (Gibson and Fuller, 2000).

The ultimate test of prebiotic efficacy would come from *in vivo* tests, particularly well-controlled human studies. Animals (rats or mice) have been used to determine the effect of substrates on the faecal microbiota, and this provides significant insights into effects such as gas production, weight changes and toxicology. However, there are differences between animal and human faecal microbiota (Gibson and Fuller, 2000). While sterile animals supplemented with human faecal microbiota give a better representation of the human gut populations, these experiments are limited still by the differences in the physiology and anatomy of the animal and human gut (Gibson and Fuller, 2000; Rastall and Maitin, 2002).

1.3.5. The Prebiotic Index

Palframan *et al.* introduced the concept of a Prebiotic Index (PI) as a means of quantifying prebiotic efficacy and providing a way for comparing the activities of differing products (Palframan *et al.*, 2003). The PI provides a quantitative score, and it works on the assumption that an increase in the populations of bifidobacteria and lactobacilli are positive, whereas increases in the bacteroides and clostridia populations are negative, when compared to their starting levels (Palframan *et al.*, 2003).

1.4. HONEY AND ITS POTENTIAL AS A PREBIOTIC

There have been a small number of studies investigating potential prebiotic effects of honey using a number of different approaches (Shamala *et al.*, 2000; Chick *et al.*, 2001; Kajiwara *et al.*, 2002; Sanz *et al.*, 2004; Shin and Ustunol, 2005; Haddadin *et al.*, 2007; Ustunol, 2007; Jan Mei *et al.*, 2010). The oligosaccharide constituents of honey have been

shown to have prebiotic effects, similar to that of fructooligosaccharides (Bogdanov *et al.*, 2008). However, these studies have been limited and often the honeys were poorly characterised with unknown floral source and chemical composition.

In one *in vitro* study of clover honey from USA, a strain-specific growth promoting effect on bifidobacteria was observed, similar to that of commercial oligosaccharides (Ustunol, 2007). Another study on five human intestinal bifidobacteria cultures showed a growth-promoting effect similar to that of FOS, GOS and inulin (Kajiwara *et al.*, 2002). Other researchers showed that the growth of four probiotic strains was improved, and production of lactic acid by *B. bifidum* was significantly enhanced in dry milk supplemented with honey compared to other sweeteners (Chick *et al.*, 2001). Three Jordanian honeys had a positive influence on the growth and metabolism (SCFA production) of two beneficial bacterial strains of human intestinal origin, when compared to the other sugar controls (Haddadin *et al.*, 2007). Three different honeys (sourwood, alfalfa and sage) also promoted the growth and activity of five bifidobacteria strains isolated from the human intestine (Shin and Ustunol, 2005).

The beneficial effects observed in the above *in vitro* studies were attributed mainly to the oligosaccharide components of the honey. One important thing to note when testing the prebiotic potential of honey is that different honeys contain source-specific oligosaccharides (Sanz *et al.*, 2004). A study of New Zealand native honeys showed that their composition included isomaltose and melezitose (Weston *et al.*, 2000), while others reported the presence of raffinose in Italian honey (Oddo and Piazza, 1995). Further, some of the stimulatory compounds in honey could be absorbed during transit through the upper GIT of the host as whole honey is composed largely of monosaccharides, which would not be expected to reach the large intestine *in vivo* (Sanz *et al.*, 2005; Haddadin *et al.*, 2007).

Sanz *et al.* (2005) quantified the *in vitro* effect of honey oligosaccharides on the growth of faecal bacteria using the PI calculation. Their results showed that the positive impact of the honey oligosaccharides on the microbiota was similar to that of the commercial prebiotic, FOS. Similarly, the growth of *Bifidobacterium longum* was enhanced in the presence of two wild and one commercial honey from Malaysia, all of which had been pre-treated to remove the simple sugars (Jan Mei *et al.*, 2010).

There is very little data available on the prebiotic capabilities of Australian honeys. Australia is home to a vast range of unique flora that consequently gives rise to a number of unique honeys that are very different from those of the northern hemisphere in terms of composition, therapeutic potential, taste and sugar content. A study of 18 Australian

honeys by Conway *et al.* (2010) showed that the honeys promoted the growth of beneficial bacteria such as lactobacilli and bifidobacteria in pure culture experiments, as well as in microcosms established with human gut microbiota. In addition, the oligosaccharide components of the honeys enhanced the growth of the beneficial populations in the microcosms significantly, often at the expense of the less desirable ones, warranting further investigation.

1.5. AIMS OF THIS STUDY

Very little is known about the prebiotic activity of Australian honey. One previous study has shown that the saccharides in honey were capable of promoting the growth of potentially beneficial bacteria in pure culture studies, and to a very limited extent in mixed culture studies representative of the human gut. A comprehensive study of Australian honeys of known saccharide composition using a number of approaches would be invaluable in determining and quantifying their prebiotic potential.

The overall aim of the research presented in this thesis was to investigate the prebiotic properties of Australian honeys from various floral varieties.

In detail, the aims were to investigate:

i) the influence of the monosaccharides of high fructose honey on the composition and activity of the gut microbiota
ii) the influence of the oligosaccharides of Australian honeys on the gut microbiota and the prebiotic potential of the honey
iii) the impact of honey on the inhibitory effect that gut microbiota can have on introduced and/or invading species

2 CHAPTER TWO
Impact on the gut microbiota of honey with a high fructose content

2.1 INTRODUCTION

2.1.1 The sugars in honey

Honey is a natural sugar solution made up predominantly of the monosaccharides, fructose (36 – 50 %) and glucose (28 – 36 %) (Molan, 1992), that are readily absorbed in the small intestine. Sucrose and maltose (disaccharides) are also found in honey at high levels, (Da Costa Leite *et al.*, 2000; Sanz *et al.*, 2004; Tewari and Irudayaraj, 2004; Shin and Ustunol, 2005), and other di-, tri-, and oligosaccharides are present in smaller quantities (Bogdanov *et al.*, 2004). The complex mixture of saccharides in honey varies widely from one type to another and is influenced mainly by floral source as well as geographic, climatic and processing conditions (Luchese, 2012).

2.1.2 The fate of simple sugars of honey in the gut

Fructose and glucose have the same molecular formula, but differ in their molecular structure (Erejuwa and Sulaiman, 2012). The digestion, absorption and metabolism of these sugars also differs (Bray *et al.*, 2004). As they are monosaccharides, fructose and glucose do not require hydrolysis by the gastrointestinal tract enzymes and are readily absorbed (Shepherd and Gibson, 2006). When disaccharides such as sucrose enter the intestine, they are not directly absorbed, but cleaved by enzymes. For sucrose, the enzyme sucrase hydrolyses the sugar into the monosaccharides glucose and fructose (Shepherd and Gibson, 2006; Erejuwa and Sulaiman, 2012).

Glucose is absorbed in the brush border membrane of the small intestinal epithelium by a sodium/glucose-galactose co-transporter, SGLT1 (Hopkins *et al.*, 1998; Ferder *et al.*, 2010; Erejuwa and Sulaiman, 2012). In contrast, fructose is absorbed further down in the duodenum and jejunum by the fructose-specific GLUT5 transporter and a facultative, non-sodium dependent process (Hopkins *et al.*, 1998; Erejuwa and Sulaiman, 2012). After absorption, glucose and fructose are either transported to the liver (where fructose can be converted to glucose), or pass into general circulation (Erejuwa and Sulaiman, 2012).

Fructose absorption appears to be limited relative to glucose, as some individuals have a low fructose tolerance and absorption capacity and consequently can develop symptoms

of diarrhoea and flatulence after fructose consumption (Ravich *et al.*, 1983). The failure to completely absorb fructose in the small intestine is known as fructose malabsorption and this results in fructose reaching the colon (Shepherd and Gibson, 2006). Colonic bacteria rapidly ferment fructose to gases (hydrogen, carbon dioxide and methane) and short chain fatty acids (Hopkins *et al.*, 1998; Shepherd and Gibson, 2006). Thus, if sufficient amounts of fructose reach the colon, luminal distention can occur. This potentially leads to bloating, flatulence, abdominal discomfort and a laxative effect (reviewed in (Shepherd and Gibson, 2006 and Tappy and Lê, 2010)).

Studies have shown that in the presence of glucose, intestinal absorption of fructose is markedly improved. For example, in its monosaccharide form, fructose malabsorption was noted in some human subjects subsequent to fructose challenge studies at various concentrations (10 g to 50 g) (Rumessen and Gudmand-Høyer, 1986). The absorption capacity of fructose given as sucrose was markedly improved by the same subjects. In addition, the absorption capacity of fructose was greatly enhanced by the addition of glucose (monosaccharide) to the mixture in a dose dependent manner, with the greatest effect being seen for equivalent amounts of fructose and glucose. Although the mechanisms behind this are not completely understood, it has been proposed that when fructose and glucose are administered in equimolar amounts, absorption of the monosaccharides may occur by the disaccharidase-related transport system as if they were the product of the enzymatic hydrolysis of sucrose (Riby *et al.*, 1993).

As such, it is possible that the fructose in honey can also be better absorbed compared to fructose alone, as it exists with an almost equal amount of glucose and also because the disaccharidase-related transport system is already in effect due to the presence of sucrose in honey. Furthermore, since fructose can reach the large intestine as a monosaccharide, this could have positive benefits (if not in excess) as it could have a positive effect on the gut microbiota.

2.1.3 Sources of fructose and the issues associated with fructose consumption

Fructose is found in the diet in three main forms: as a pure monosaccharide (present in fruits and honey), as the disaccharide, sucrose (hydrolysed by sucrase to its fructose and glucose constituents), and as oligosaccharides in the polymers known as inulin, fructan and fructo-oligosaccharide that are present in some vegetables and wheat (Shepherd and Gibson, 2006; Gibson *et al.*, 2007). The 1960s saw the development of corn-derived sweeteners such as high fructose corn syrup (HFCS), most commonly available as HFCS-55 containing 55 % fructose and 45 % glucose (Tappy and Lê, 2010). The high sweetening power, long shelf-life, low cost and favourable organoleptic properties of HFCS

contributed to a significant increase in its consumption, particularly in the United States (White, 2008; Tappy and Lê, 2010). The increased usage of HFCS meant that dietary fructose consumption increased considerably, and is now part of the Western diet at levels usually in excess of the daily recommended intake.

Hepatic metabolism of fructose favours lipogenesis and unlike glucose, fructose does not stimulate insulin secretion or enhance leptin production, which are key signals in the regulation of food intake (Bray *et al.*, 2004; Ferder *et al.*, 2010). This suggests that dietary fructose may contribute to increased energy intake and weight gain, particularly if glucose levels are low. An increase in fructose consumption has been identified as a contributing factor to the increased prevalence of obesity observed over the past few decades (Bray *et al.*, 2004; Tappy and Lê, 2010; Stanhope, 2012). Fructose consumption, particularly in excess, has also been associated with other deleterious effects such as hypertension and reduced insulin sensitivity (Ferder *et al.*, 2010). When HFCS is used as a sweetener in soft drinks and baked or processed foods, fructose consumption approaches levels at which malabsorption can be observed in otherwise healthy adults (Gibson *et al.*, 2007). Although the HFCS also contains glucose (in almost equimolar amounts to fructose) which could improve the absorption of fructose, it is possible that the sheer quantity of HFCS is the contributing factor to the detrimental effects seen, and that the benefits when combined with glucose can only occur provided the levels of consumption of HFCS is reasonable.

Fructose, in its monosaccharide form or as part of the disaccharide sucrose, has also generated interest from its potential roles in the pathogenesis of non-alcoholic fatty liver disease, the pathogenesis of dental caries and symptom generation in patients with irritable bowel syndrome (IBS) (reviewed in (Gaby, 2005)). In addition, observational studies support the possibility of a link between increased fructose consumption (particularly as part of HFCS in carbonated drinks) and the obesity epidemic (Ludwig *et al.*, 2001; Schulze *et al.*, 2004).

This has led to the classification of fructose as a potentially problematic food, and diets that restrict consumption of fructose and FODMAPs (fermentable oligosaccharides, disaccharides, monosaccharides and polyols) have been prescribed for patients suffering with IBS and obesity (Shepherd and Gibson, 2006). In these diets, naturally occurring sources of fructose (such as that in honey or fruit), are commonly labelled as unfavourable foods because their fructose content can outweigh the glucose.

However, a recent study demonstrated that adding selected sugar (resulting in a low fructose diet) or a diet with natural fruit supplements (moderate fructose diet) resulted in weight loss of obese patients (Madero *et al.*, 2011). In addition, the study revealed that

the moderate natural fructose diet was superior to the low-fructose diet suggesting that natural fructose might be differently absorbed in the body compared with fructose from added sugars.

Many of the adverse effects of fructose are associated with its excess consumption in the monosaccharide or disaccharide (as part of sucrose) forms. Unfortunately, this distinction is not always clear in the literature, and as a result, fructose in its oligosaccharide (polymer) form is also mistakenly associated with the deleterious effects of high fructose consumption. In contrast to the adverse effects of some forms of fructose, the beneficial effects of fructose in its oligosaccharide (polymerised) form (e.g. as fructo-oligosaccharide) on human health have been demonstrated in numerous studies, as polymerised fructose is recognised for its prebiotic properties (Gibson and Wang, 1994; Alles *et al.*, 1996; Grizard and Barthomeuf, 1999; Kaplan and Hutkins, 2000; Rycroft *et al.*, 2001).

Consequently, it is not possible to extrapolate from studies using the monosaccharide fructose to conclude how the other fructose saccharides (whether as di-, tri-, or oligosaccharides) in honey will impact on the body.

2.1.4 The intestinal microbiota

Bacteria in the gut can be broadly categorised into groups that can exert harmful effects and those that can have health promoting influences on the host (Gibson and Roberfroid, 1995; Gibson *et al.*, 2004). The composition and effects of the predominant human gut bacteria are addressed in Section 1.2.1, and summarised in Figure 2.1.

Some researchers have shown positive effects of honey on gut microbes (Shamala *et al.*, 2000; Chick *et al.*, 2001; Kajiwara *et al.*, 2002; Sanz *et al.*, 2004; Sanz *et al.*, 2005; Shin and Ustunol, 2005; Haddadin *et al.*, 2007; Ustunol, 2007; Jan Mei *et al.*, 2010). Similar positive findings were reported by our group for some Australian honeys (Conway *et al.*, 2010). In this study it was shown that banksia honey, reported by the supplier to have a high fructose content, and the fructose monosaccharide control promoted growth of lactobacilli (potentially beneficial) and suppressed coliforms (potentially harmful) when studying the impact on the entire gut microbiota. From this initial observation, it could be postulated that honeys high in fructose may not be detrimental to health and that fructose might exert benefits on the gut microbiota.

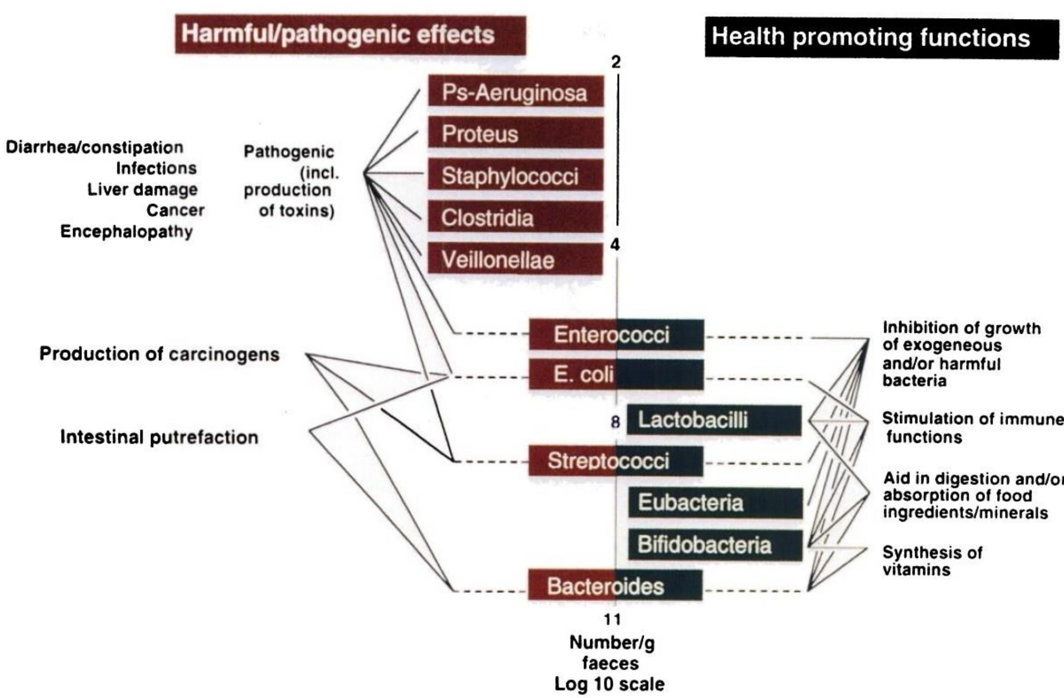


Figure 2.1 | Composition and effects of human gut bacteria
Approximate numbers of faecal bacterial groups and associated harmful or beneficial influences on human health (source (Gibson and Roberfroid, 1995)).

2.1.5 Aim

The previous studies exploring the prebiotic potential of Australian honeys have shown that the banksia honey, reported to be high in fructose, had potential health benefits as levels of lactobacilli were elevated. This observation warranted further investigation in view of the negative reports about fructose and the possible beneficial implications of the preliminary studies.

The aim of the work presented in this chapter was to investigate the influence of the sugars in Australian floral varieties of honey with high fructose content, on the composition and activity of the gut microbiota. While it is anticipated that the oligosaccharides in the honey could contribute to beneficial effects based on previous findings in our laboratory, the high fructose content of honeys needs to be addressed.

2.2 MATERIALS AND METHODS

2.2.1 Experimental approach

Three honeys, two of Australian floral variety and one commercially available medical honey (Medihoney^{TM}), were sourced for this study. The influence of honey sugars on the

gut microbiota was investigated using *in vitro* microcosms seeded with human faecal samples as representative of the colonic microbiota. The microcosms were prepared using the method reported by Rang *et al.* (1996). Faecal samples were obtained from both an adult and an infant donor. The honeys were tested in their undigested (whole) and digested states to determine the influence of the simple sugars. The effects of honey sugars were determined by monitoring changes in the bacterial population and short chain fatty acid production.

2.2.2 Honeys and control carbohydrates

Honeys used in this study are detailed in Table 2.1. The honeys were chosen based on information about their sugar compositions - yellow box was identified as relatively high in glucose, while the banksia was relatively high in fructose. The manuka honey (Medihoney™) was chosen as it has been studied extensively for its other therapeutic properties, namely its antimicrobial activity. Control sugars used in the study were the monosaccharides fructose and glucose as well as the oligosaccharide inulin, all obtained from Sigma-Aldrich. A negative control with no added carbohydrate (i.e. medium only) was also included.

Table 2.1 | Honeys used in study

Reference number	Floral variety	Scientific name	Source/Distributor
Honey i	Yellow box	*Eucalyptus* spp.	NSW beekeeper
Honey ii	Banksia	*Banksia* spp.	NSW beekeeper
Honey iii*	Manuka	*Leptospermum scoparium*	Comvita Ltd.

*** Commercial name: Medihoney™ Antibacterial Medical Honey**

2.2.3 Quantification of fructose and glucose

Fructose and glucose monosaccharides in the honey samples were quantified using the Fructose Assay Kit and the Glucose (HK) Assay Kit, respectively (obtained from Sigma-Aldrich). The kits were used according to the manufacturer's instructions. Briefly, the concentration of each monosaccharide was determined by enzymatic reaction and spectrophotometric absorbance reading. Each honey was tested in triplicate.

2.2.4 Honey digests

In order to simulate passage through the upper regions of the digestive tract, digested samples of the honeys were prepared by pre-treatment with digestive enzymes followed by dialysis to simulate absorption. Control monosaccharides fructose and glucose as well as the oligosaccharide inulin were also treated in the same manner as the honey. The

enzymatic digestion methodology was a slightly modified version of those reported by Beer *et al*. (Beer *et al.*, 1997) and Kedia *et al.* (Kedia *et al.*, 2008).

Honey or sugar samples were diluted (5 % w/v) in phosphate buffered saline (pH 6.9; 10mM) prior to the addition of α-amylase (Sigma-Aldrich; final concentration 1.25 mg/ml) and incubation for 30 min at 37 °C. After pH adjustment to 2.0 using HCl, pepsin was added (Sigma-Aldrich; final concentration 3.8 mg/ml) and the mixture incubated for 60 min at 37 °C. Pancreatin and bile salt solution (Sigma-Aldrich; final concentration 0.75 mg/ml) were added after neutralisation of the pH to 6.9 followed by incubation at 37 °C for three hours. Following dialysis (cut-off 1000 Da, Spectrum Labs) against sodium phosphate buffer (pH 6.9) the dialysate was filter sterilised. The resultant material is referred to as “digested honey”.

Glucose and fructose assay kits (Sigma-Aldrich) were used according to the manufacturer’s instructions for enzymatic determination of glucose and fructose in the substrates to confirm that the digestion had successfully removed these simple sugars.

2.2.5 *In vitro* intestinal microcosms

Microcosms were established *in vitro* using human faecal samples from healthy human volunteers to allow examination of the effect of honeys and sugars on the complex intestinal microbial population, using the method described by Conway *et al*. (Conway *et al.*, 2010). Approval for the study was obtained from the Human Research Ethics Advisory (HREA) panel, University of New South Wales (HREA-11041). Consent was obtained before taking part in the study.

Two healthy female donors provided samples, one adult (aged 45-60) and one infant (12 months; partially breast fed). Freshly voided faecal samples were collected and transferred to sterile specimen jars and stored at -80 °C.

The microcosms were established using Wilkens-Chalgren Anaerobe (WCA) broth (Oxoid) containing faecal suspension (final concentration 10 %) and either whole or digested honeys or control sugars (final concentration 1 %). For the negative control, additional WCA was added instead of the honey. Samples were collected at 0 and 48 hours for enumeration of the major bacterial groups, short chain fatty acid analysis (SCFA) and pH measurement.

The experiments were performed in triplicate and on four separate occasions.

2.2.6 Enumeration of major viable bacterial groups

The major culturable bacterial groups were enumerated. Samples from the microcosms were serially diluted in WCA broth, plated using the micro-drop technique (10 µl drop) in triplicate on selective media and incubated as outlined in the Table 2.2. Counts were expressed as log_{10} CFU (colony forming units) per ml.

All dehydrated media were obtained from Oxoid and prepared according to the manufacturer's instructions. All antibiotics and reagents were obtained from Sigma-Aldrich and stored as appropriate.

Table 2.2 | Selective media for enumeration of the major bacterial groups

Bacterial group	Media	Additions/Treatments	Incubation	Code
Total anaerobes	Wilkins-Chalgren anaerobe agar	None	Anaerobic 37 °C, 48 hrs	WCA
Bacteroides	Wilkins-Chalgren anaerobe agar	Kanamycin (0.1 g/L) Vancomycin (7.5 mg/L)	Anaerobic 37 °C, 48 hrs	WCA-KV
Clostridia	Columbia horse blood agar	Aliquot diluted in equal volume of 100 % ethanol, incubated for 1 hour at room temperature prior to serial dilutions	Anaerobic 37 °C, 48 hrs	HBA
Bifidobacteria	Reinforced clostridial agar	Aniline blue (0.3 g/L) Dicloxacillin (2 mg/L)	Anaerobic 37 °C, 48 hrs	RCA-AD
Lactobacilli	Rogosa agar	None	Anaerobic 37 °C, 48 hrs	ROG
Gram-negative enterics*	MacConkey no. 3 agar	None	Aerobic 37 °C, 24 hrs	MAC
Enterococci	M17 agar	pH adjusted to 9.6 using 3 M NaOH	Aerobic 37 °C, 24 hrs	M17

***Includes *Salmonella* spp., *Shigella* spp. and *Escherichia coli*.**

2.2.7 Short chain fatty acid analysis

The amounts of SCFA produced in the microcosms with the added substrates of honey, digested honey and control sugars were determined by gas chromatography-mass spectrometry (GC-MS) using an internal standard method.

Aliquots (5 ml in triplicate) from the intestinal microcosms were centrifuged at 10,000 x g for 15 min and the supernatant was decanted and filter sterilised using a 0.22 µm syringe driven filter (Merck Millipore).

The internal standard, 2-ethylbutyric acid (Sigma-Aldrich), was added to give a final concentration of 3mM in each of the supernatants prior to ether extraction of the SCFA for analysis, as previously described (Weaver *et al.*, 1989). A standard solution of volatile SCFA (containing 10 mM each of: acetic, propanoic, isobutyric, butyric, isovaleric, pentanoic, isocaproic, hexanoic and heptanoic acid; Supelco) was used to generate a standard curve with concentration range 0.1 – 10 mM. Samples were analysed by GC-MS (Focus DSQ II, Thermo Scientific) fitted with an HP-FFAP column (J&W 50m, 0.2 mm, 0.332 µm; Agilent Technologies) using 2 µl of the ether extracts for chromatographic injection.
Concentrations of SCFA were determined using Xcalibur software (Thermo Scientific) by calculating the relative area of the peaks in the chromatographs.

2.2.8 Statistical analysis

Statistical analysis of the results was performed with R software (version 3.0.2) for Windows. The Kolmogorov-Smirnov test was used to confirm normal distribution. Analysis of variance was tested by one-way ANOVA, and then followed by Tukey's (HSD) test to identify significance between groups. P-values <0.05 were considered significant.

2.3 Results

2.3.1 Monosaccharide constituents of honey

The quantification of fructose and glucose levels in the honeys confirmed that the yellow box honey was higher in glucose relative to banksia (33.2 % compared to 28.1 %), and that banksia was higher in fructose (50.9 % compared to 40.9 %; Table 2.3). Fructose and glucose concentrations for the manuka honey were relatively low (38.4 % and 29.0 %, respectively), and were in accordance to the values obtained from Comvita Ltd. (37.9% and 29.7 %, respectively) (Darcy, 2013).

Table 2.3 | Amount of fructose and glucose as monosaccharides in the honeys
Concentrations are expressed as the mean percentage of total saccharides (w/w) ± SD from three separate trials.

Honey	Fructose (% in sample)	Glucose (% in sample)
Honey i (Yellow box)	40.9 ± 2.2	33.2 ± 1.9
Honey ii (Banksia)	50.9 ± 1.4	28.1 ± 1.4
Honey iii (Manuka)	38.4 ± 0.2	29.0 ± 0.2

2.3.2 The influence of honey saccharides on the growth of potentially beneficial groups

Bifidobacteria and lactobacilli numbers increased in the microcosms for each of the three honeys and the sugar controls.

The increase of these bacterial groups when the adult and infant microbiota were used was statistically significant for all honeys and control sugars ($p<0.01$ in all cases). The difference in the initial and final counts was also significant in the negative control (medium only, $p<0.05$), however there was always a smaller increase in numbers compared to the other substrates tested (see Figures 2.2 A and B, and 2.3 A and B).

Bifidobacteria counts

When the microcosms were established using the adult microbiota, the following was observed for bifidobacteria:

- the increase in numbers of bifidobacteria using all honeys and control sugars were significantly greater than that of the negative control ($p<0.01$ in all cases, Figure 2.2 A)
- counts were most elevated when yellow box and banksia honeys were used as substrates
- counts from the use of these two honeys were not statistically different to each other, and were as effective as the oligosaccharide control, inulin ($p>0.05$)

When the microcosms were established using infant microbiota, the following was observed for bifidobacteria:

- manuka honey was as effective as the oligosaccharide control, inulin, and both resulted in significantly higher counts of bifidobacteria than noted in the negative control ($p<0.01$ for both, Figure 2.2 B)
- there was a trend of elevated counts of bifidobacteria when either the yellow box honey, banksia honey or the monosaccharides were used when compared to the negative control, however these were not statistically significant.

The digested honeys and were also tested to investigate the influence of the simple sugars of honey on bacterial numbers (Figures 2.2 C and D), and the following was observed:

- honey digests produced similar results to the oligosaccharide control, inulin
- digests of the monosaccharide controls (fructose and glucose) yielded bifidobacteria levels similar to the negative control

- microcosms established with manuka honey and adult microbiota showed a marked increase in bifidobacteria numbers in its digested form compared to its whole form (Figure 2.2 A and C)
- all whole (undigested) honeys were as effective as the saccharide controls in elevating counts of lactobacilli in microcosms established with either microbiota.

Lactobacilli counts

The following changes in lactobacilli counts were observed in the microcosms established using adult microbiota:

- yellow box and banksia honeys in their whole states, as well as the fructose control, allowed a significant increase in number of lactobacilli from the initial values (Figure 2.3 A)
- the largest increase in lactobacilli numbers was observed when the control saccharide, fructose, was used (Figure 2.3), and this was significantly higher than the negative control ($p<0.01$) and the glucose control ($p<0.05$).

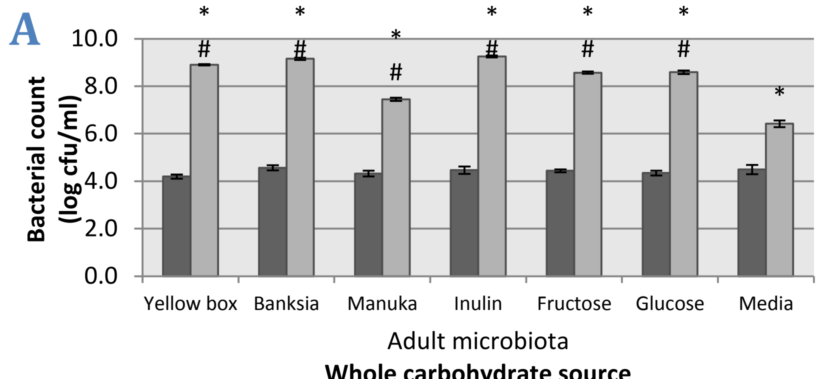
Microcosms established using infant microbiota showed the following:

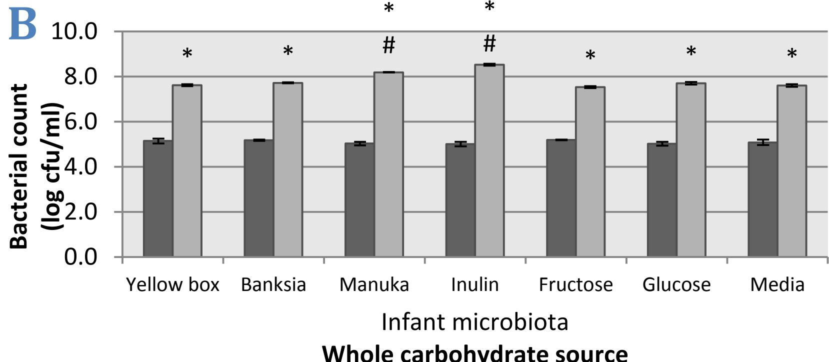
- lactobacilli numbers in microcosms using whole honey, or the saccharide controls, were significantly higher than the negative control (Figure 2.3 B).

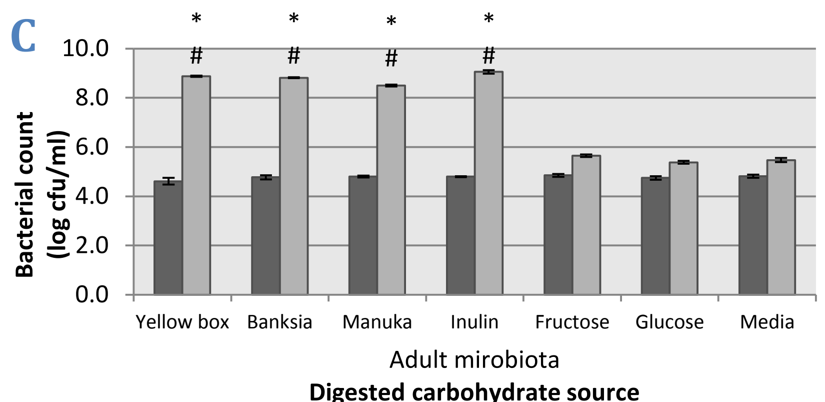
Of the honeys, banksia (with the highest fructose concentration) produced the highest lactobacilli levels, followed by yellow box and then manuka. Banksia was not significantly more effective than yellow box ($p=1.000$), however both were significantly more effective at stimulating lactobacilli growth than manuka ($p<0.05$). Banksia and yellow box honey were comparable to the fructose control ($p=0.126$ and 0.164, respectively), and all three were significantly more effective than the commercially available oligosaccharide (inulin) at boosting lactobacilli numbers ($p<0.05$).

The use of digested honeys in the microcosms showed the following:

- all honeys and the inulin control allowed significantly higher numbers of lactobacilli compared to the negative control (and the digested fructose and glucose controls which resembled the negative control)
- there were no significant differences in the change in lactobacilli counts when yellow box, banksia, manuka or inulin were used ($p>0.05$ in all cases).







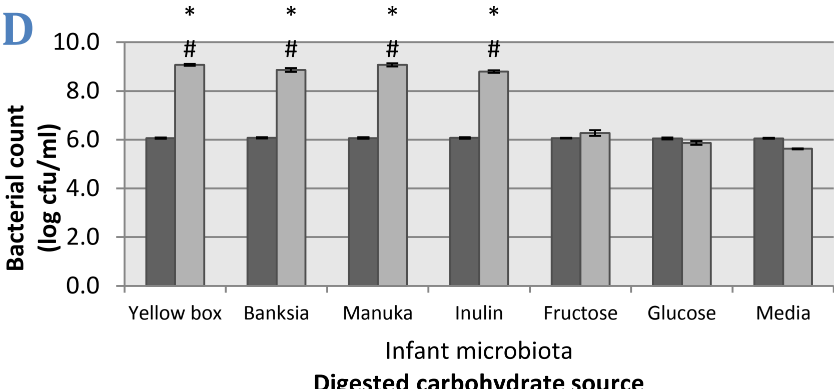


Figure 2.2 | Growth of bifidobacteria in microcosms using whole and digested carbohydrate sources

Initial and final counts (dark and light grey bars, respectively) are expressed as mean log cfu/ml ± SD from four separate experiments. Symbols indicate statistically significant difference ($p<0.05$) from: (*) initial to final values and (#) negative control media.

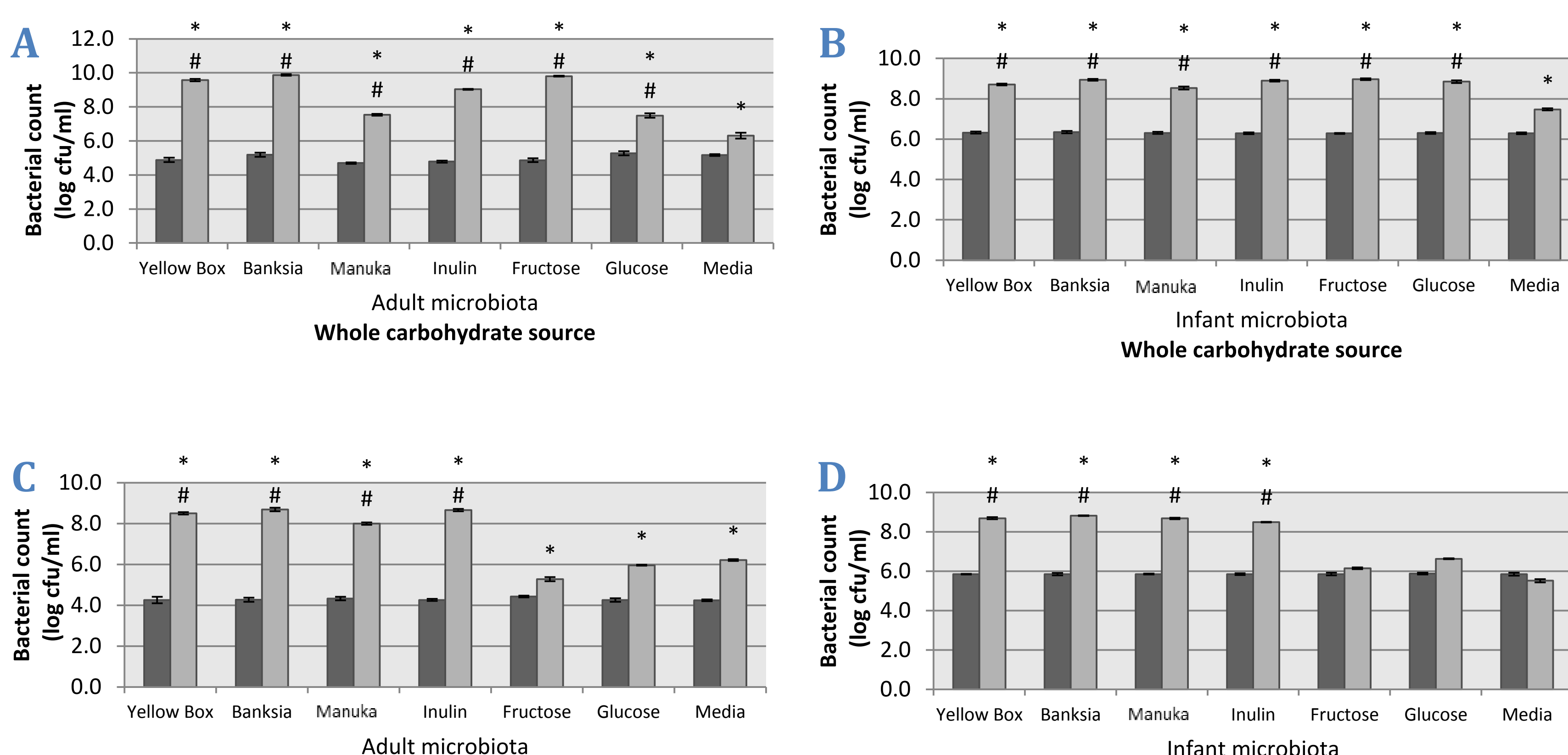


Figure 2.3 | Growth of lactobacilli in microcosms using whole and digested carbohydrate sources

Initial and final counts (dark and light grey bars, respectively) are expressed as mean log cfu/ml ± SD from four separate experiments. Symbols indicate statistically significant difference (p<0.05) from: (*) initial to final values and (#) negative control media.

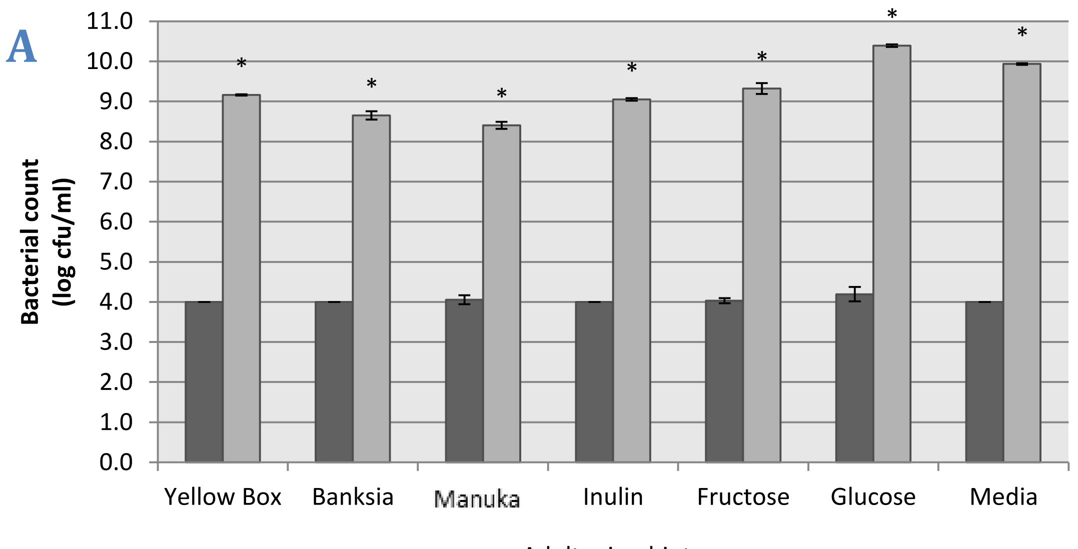
2.3.3 The effect of saccharides on the growth of enteric bacteria in the microcosms

The yellow box honey (highest glucose concentration) supported the growth of enterics at significantly higher levels than the banksia and manuka honey samples in the microcosms established using the adult microbiota ($p<0.05$) (Figures 2.4 A and B). The increases in the enteric levels in the microcosms using any of the three honeys or the fructose control were significantly lower ($p<0.05$) than that observed in the glucose and negative control. The highest increase in enteric numbers in the microcosms established using either the adult or infant microbiota was observed when the glucose control was used, followed by the negative control. These were not significantly different to each other in either the adult or the infant microcosm ($p=0.105$ and 0.999, respectively). Enteric levels were more elevated using the control monosaccharides compared to the honeys or the oligosaccharide control, inulin.

2.3.4 Clostridial growth in microcosms

Clostridia numbers did not increase in the microcosms with added honeys (Figure 2.5). When manuka honey (undigested) was used in the microcosms established with adult microbiota, the numbers of clostridia were significantly lower than the initial values ($p<0.05$, Figure 2.5 A). Clostridial counts were significantly lower than the negative control in the microcosms established using undigested honeys and either the adult or infant microbiota (Figure 2.5 A and B).

When the digested honey samples were used in microcosms with adult microbiota, the counts of clostridia in the presence of yellow box were significantly lower than the negative control (Figure 2.5 C).



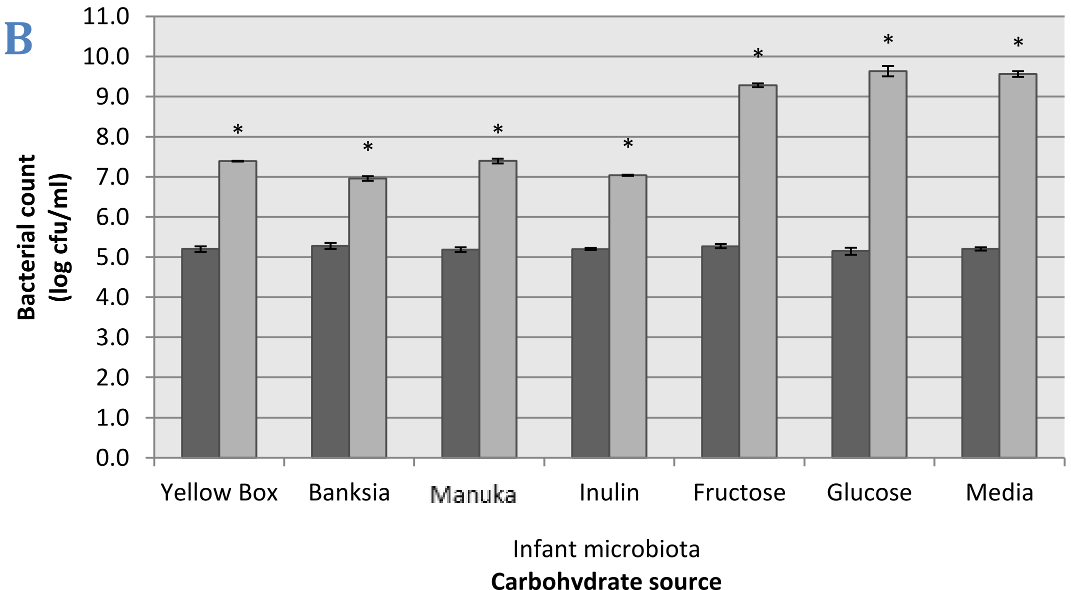


Figure 2.4 | Growth of enteric bacteria in microcosms with added whole honey
Initial and final counts (dark and light grey bars, respectively) are expressed as mean log cfu/ml ± SD from four separate experiments.
Symbols indicate statistically significant difference ($p<0.05$) from (*) initial to final values. There were no significant differences in the final counts of enteric bacteria relative to the negative control.

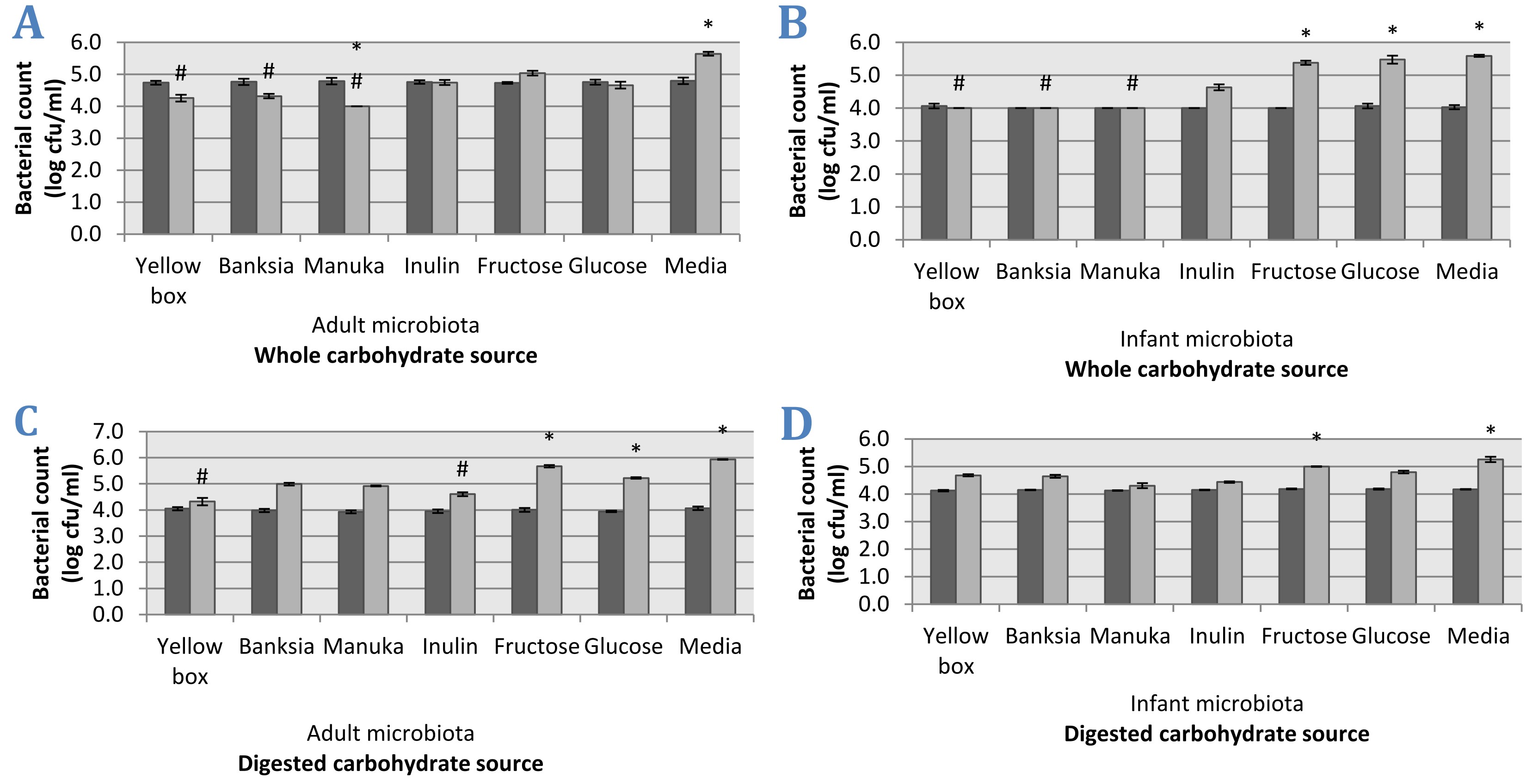


Figure 2.5 | Growth of clostridia in microcosms using whole and digested carbohydrate sources

Initial and final counts (dark and light grey bars, respectively) are expressed as mean log cfu/ml ± SD from four separate experiments. Symbols indicate statistically significant difference ($p<0.05$) from: (*) initial to final values and (#) negative control media.

2.3.5 Summary of overall changes in bacterial profiles

The changes in the numbers of the various bacterial groups for the adult microbiota with either the undigested whole honey and control saccharides (Figure 2.6 A) or with the digested substrates (Figure 2.6 B) show that overall the patterns were similar for the honeys and oligosaccharide (inulin) control. Overall, the digested monosaccharide controls (fructose and glucose) were consistent with the negative control. The microcosms using the infant microbiota had similar total changes as presented in Figure 2.7 A and B.

The following patterns were observed:

- changes in bacteroides and bifidobacteria counts were greater for whole honeys compared to digested honeys
- change in clostridial numbers were lower using whole honeys
- whole honeys resulted in similar counts of lactobacilli to those obtained when digested honey was used. This was also true for enterococci
- the change in enteric bacteria was higher using whole honeys when the adult microbiota were used, and higher with digested honeys when infant microbiota were used
- yellow box and banksia honey in the microcosms resulted in higher numbers of bifidobacteria and lactobacilli relative to the manuka honey. The yellow box and banksia honey were also noted to have relatively higher levels of fructose than the manuka sample.

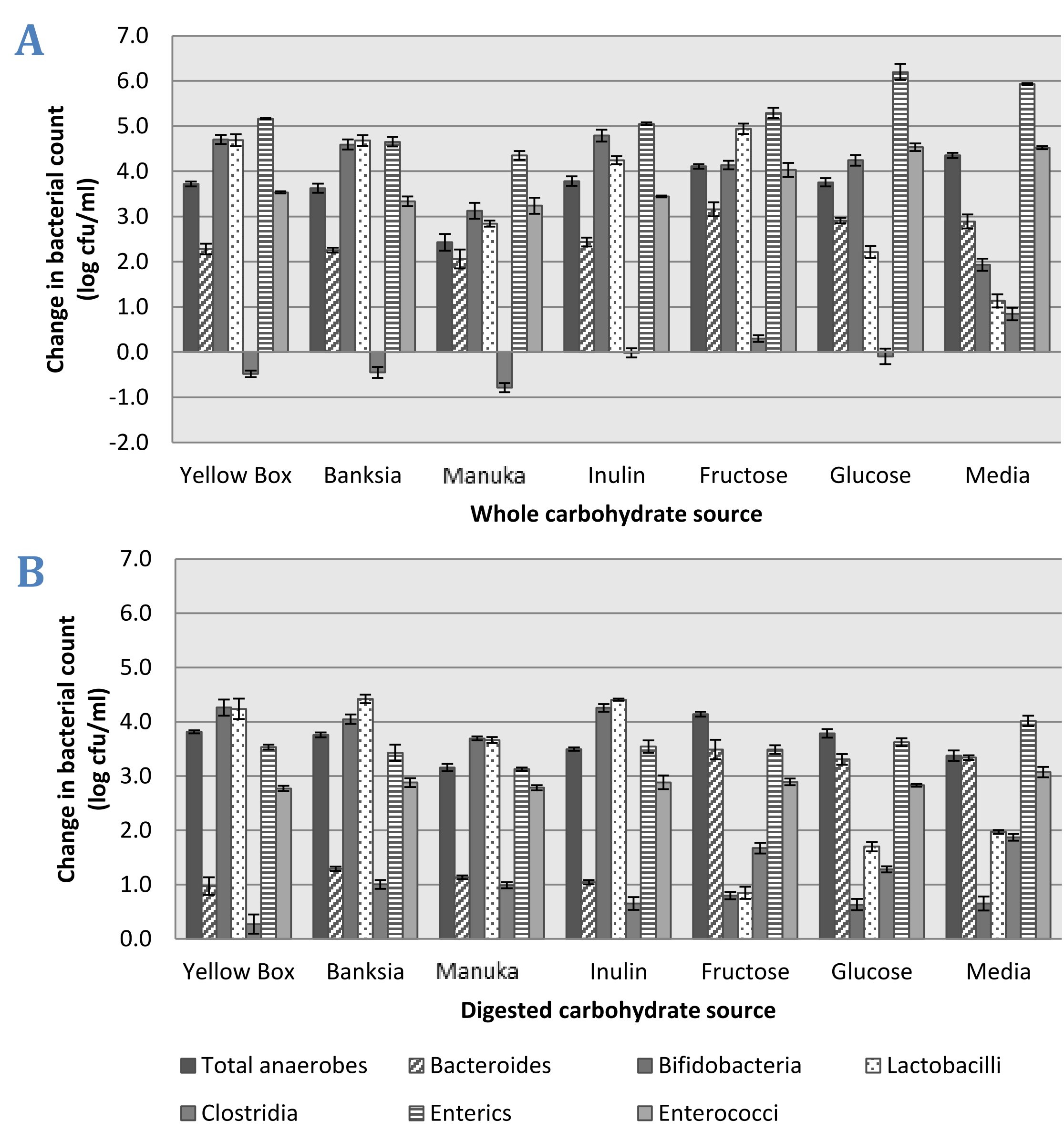


Figure 2.6 | Overview of total change in bacterial counts in microcosms established with adult microbiota

Difference between initial and final log counts expressed as change. Mean results from four separate trials (cfu/ml ± SD).

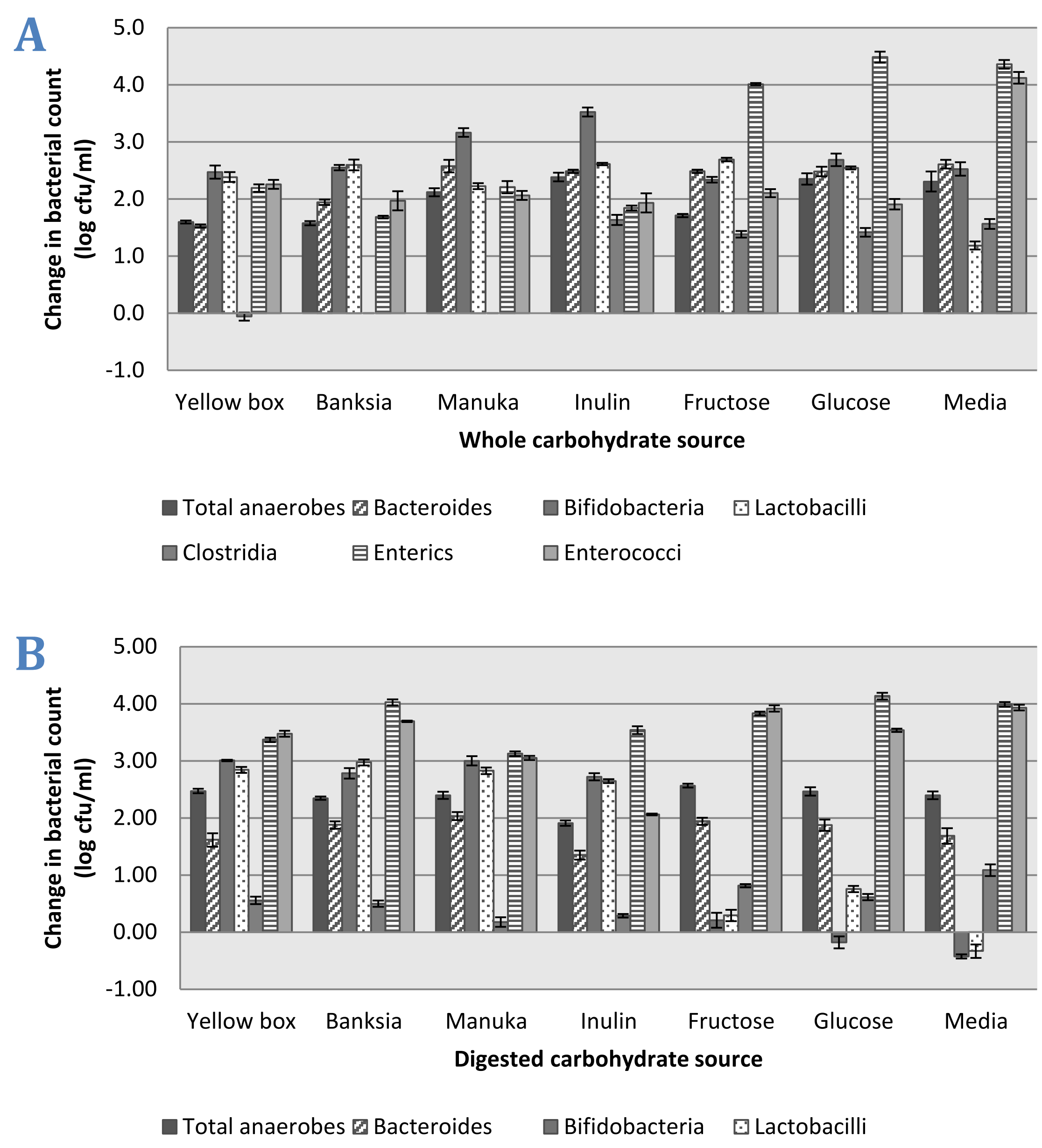


Figure 2.7 | Overview of total change in bacterial counts in microcosms established with infant microbiota

Difference between initial and final log counts expressed as change. Mean results from four separate trials (cfu/ml ± SD).

2.3.6 Correlation of overall changes in bacterial groups

Some correlations were found between bacterial groups in the different microcosms (Table 2.4) and they are expressed as the correlation coefficient (R) whereby a value of ±1.0 represents a linear relationship (perfect correlation). A positive (+) value indicates that as the numbers of one bacterial group increases, so do the other. A negative (-) value indicates that the counts of one bacterial group increases as the other decreases.

A strong positive correlation was found between bifidobacteria and lactobacilli in the adult microcosms, using whole and digested substrates, and in the infant microcosms using digested substrates. In the adult microcosms, a positive correlation was also observed between bacteroides and clostridia, regardless of whether the substrate was digested. In the adult microcosm using whole substrates, a strong positive correlation was found between enterococci and enterics (R = 0.95). As numbers of bacteroides increased in the adult microcosm, the levels of bifidobacteria and lactobacilli decreased when using digested substrates (R values -0.99 and -0.96, respectively). A strong negative correlation was also observed between lactobacilli and enterococci in the infant microcosm using whole substrates (R = -0.94).

Table 2.4 | Correlation of changes in the counts of the major bacterial groups in microcosms

Correlation coefficient (R) calculated using Microsoft Excel. R values were determined by comparing the change in counts of each bacterial group against one another.

Microbiota	Bacterial group correlations in microcosms using whole honeys	R value	Bacterial group correlations in microcosms using digested honeys	R value
Adult	Enterococci - Enterics	0.95	Bacteroides - Bifidobacteria	-0.99
	Bacteroides - Enterococci	0.85	Bacteroides - Lactobacilli	-0.96
	Bifidobacteria - Lactobacilli	0.81	Bifidobacteria - Lactobacilli	0.96
	Bacteroides - Clostridia	0.81	Bacteroides - Clostridia	0.87
			Bifidobacteria - Clostridia	-0.87
			Lactobacilli - Clostridia	-0.83
Infant	Lactobacilli - Enterococci	-0.94	Bifidobacteria - Lactobacilli	0.98
			Lactobacilli - Clostridia	-0.86
			Bifidobacteria - Clostridia	-0.81

2.3.7 Short chain fatty acid profiles in microcosms

Nine SCFA profiles were determined in the microcosms. The results of three SCFAs of most clinical importance, namely acetic acid, propanoic acid and butyric acid, are summarised in Table 2.5. The remaining profiles can be seen Appendix 1.

Below is a summary of the SCFA findings:

- butyric acid levels were significantly higher than the negative control and the oligosaccharide control in the presence of digested banksia honey
- when digested banksia honey was used in the adult microcosms, the concentration of butyric acid was 10 times greater than the negative control, and three times that of the oligosaccharide control, inulin
- when digested banksia honey was used in the infant microcosms, the amount of butyric acid was over 20 times greater than the negative control, and over four times greater than that of the oligosaccharide control, inulin
- butyric acid levels in the microcosms using digested yellow box and manuka honey were similar to those in the negative control
- when whole honeys were used, butyric acid levels in the microcosms were similar to the negative control, and significantly lower than in the saccharide controls in most cases
- acetic acid and propanoic acid amounts in the microcosms using whole honeys were comparable to those in the negative control, as well as the oligosaccharide (inulin) control. This was also true for the digested honey studies, with the exception of the manuka-enriched microcosms which had significantly lower levels of acetic acid compared to the inulin control ($p=0.01$).

Table 2.5 | Enumeration of short chain fatty acids in microcosms using whole and digested substrates

Mean SCFA concentrations ± SD from three separate trials. Values in italics represent the starting concentration of SCFA, and values in bold show a significant increase from the initial level.

		Acetic acid (mM)				Propanoic acid (mM)				Butyric acid (mM)			
Donor	**Substrate**	**Whole**		**Digested**		**Whole**		**Digested**		**Whole**		**Digested**	
Adult	*Initial*	*3.13*	*±0.17*	*3.13*	*±0.17*	*5.74*	*±0.29*	*5.74*	*±0.29*	*5.20*	*±0.02*	*5.20*	*±0.02*
Adult	Yellow box	3.13	±0.10	2.94	±0.10	4.62	±0.50	4.43	±0.50	**2.70**	±0.07	3.78	±0.07
Adult	Banksia	3.32	±0.10	2.61	±0.11	4.81	±0.50	**3.64**	±0.33	**2.89**	±0.07	**38.10***	±0.26
Adult	Manuka	3.51	±0.10	1.98	±0.14	5.00	±0.50	**3.33**	±0.02	**3.08**	±0.07	3.68	±0.02
Adult	Inulin	3.70	±0.10	**5.33***	±0.11	5.19	±0.50	4.67	±0.69	**13.27***	±0.07	**11.93***	±0.07
Adult	Fructose	4.68*	±0.11	2.18	±0.11	5.92*	±0.19	**3.42**	±0.19	6.44*	±0.14	3.94	±0.14
Adult	Glucose	4.90*	±0.02	2.10	±0.02	6.18*	±0.06	**3.38**	±0.06	6.69*	±0.09	3.89	±0.09
Adult	Media	2.74	±0.26	2.85	±0.26	**3.58**	±0.46	**3.67**	±0.31	3.71	±0.06	3.72	±0.06
Infant	*Initial*	*1.31*	*±0.09*	*1.31*	*±0.09*	*3.72*	*±0.09*	*3.72*	*±0.09*	*3.57*	*±0.19*	*3.57*	*±0.19*
Infant	Yellow box	1.73	±0.39	2.49	±0.11	3.91	±0.09	3.50	±0.06	**2.01**	±0.04	4.04	±0.06
Infant	Banksia	2.14	±0.78	2.86	±0.06	4.10	±0.09	4.77	±0.13	2.20	±0.04	**86.07***	±4.65
Infant	Manuka	2.56	±1.17	2.26	±0.05	4.29	±0.09	3.43	±0.06	2.39	±0.04	4.27	±0.27
Infant	Inulin	2.98	±1.56	2.68	±0.45	4.48	±0.09	4.06	±0.12	**12.58***	±0.04	**19.41***	±0.63
Infant	Fructose	**8.62***	±0.54	3.12	±0.02	**6.61***	±0.10	3.81	±0.10	**6.58***	±0.09	3.78	±0.09
Infant	Glucose	**4.91***	±0.12	3.53	±0.12	**5.17***	±0.10	3.79	±0.10	4.51	±0.08	3.13	±0.08
Infant	Media	2.47	±0.03	2.57	±0.03	3.49	±0.07	3.78	±0.11	3.84	±0.06	3.86	±0.13

* **Indicates statistically significant difference ($p < 0.05$) from the negative control.**

2.4 DISCUSSION

The work presented in this chapter was an investigation of the effect of the saccharides in honey on the composition and activity of gut bacteria *in vitro* and in particular, the high fructose content honey since preliminary studies in our laboratory suggested potential positive benefits. The results show that the sugars in honey, both simple and complex, stimulate the growth of different bacterial groups and that the levels of fructose correlated with the promotion of potentially beneficial bacteria. This suggests a rethink of the current stance on the impact of some of the less complex carbohydrates, particularly fructose, on human health.

2.4.1 Significance of fructose

The findings presented in this chapter showed that the saccharides in honey promote the growth of beneficial bacterial populations, namely the bifidobacteria and lactobacilli (Figure 2.2 and 2.3). There was a positive impact on the growth of bifidobacteria and lactobacilli when fructose was administered in the microcosms, in the form of honey or as the pure monosaccharide control. The effect of the fructose in honeys and as the monosaccharide control was more pronounced on the lactobacilli populations. For example, the levels of lactobacilli were markedly increased when banksia honey was used in the microcosms compared to the other two honeys tested, and this was attributed to the higher fructose concentration in the honey. In addition, the yellow box honey allowed greater increases in the numbers of lactobacilli relative to the manuka honey, and this was consistent with the fructose content of the honeys as the yellow box had a higher fructose concentration relative to the manuka sample. The counts of lactobacilli were also elevated in microcosms seeded with the pure fructose control, compared to the other monosaccharide control, glucose. The work in this chapter supports the findings of a previous study done in our lab, showing that lactobacilli counts increase in intestinal microcosms with added fructose, either as the monosaccharide or in honey (Conway *et al.*, 2010). The study by Conway *et al.* used the same yellow box and banksia honeys as were used in this chapter, and showed that the growth of lactobacilli was consistent with the findings here which correlated with the levels of fructose measured in the honeys. That is, that the banksia honey alone showed distinct increases in the counts of lactobacilli and this honey had a higher content of fructose. Significant increases in the growth of lactobacilli were also noted when the fructose monosaccharide control was used.

There was a growth promoting effect of the high fructose honeys on bifidobacteria as the yellow box and banksia honeys allowed for increased growth of this group relative to the manuka honey, which had a lower fructose content. This data supports previous findings,

that fructose is capable of elevating the beneficial bacterial populations of the gut, particularly bifidobacteria (Hopkins *et al.*, 1998).

Digestion of the honeys showed that although the honeys still elevated lactobacilli and bifidobacteria numbers considerably, in most cases it was at lower levels than when the honey was used in its whole form, suggestive that the simple sugars contributed to the higher lactobacilli counts. That the honeys elevated both the lactobacilli and bifidobacteria when used in their digested form is suggestive of the beneficial effects of the complex sugars (which may include fructo-oligosaccharides) present in honey, which functioned at least as well as the commercial oligosaccharide control, inulin. This suggests that the sugars in honey, both simple and complex, have the potential to confer beneficial effects on the gut microbiota.

In addition to the oligosaccharides in honey, previous studies suggest that the large quantities of monosaccharides may also enhance the growth of intestinal microbes (Chick *et al.*, 2001; Sanz *et al.*, 2005; Popa and Ustunol, 2011). Specifically, the effects of honey on the growth of probiotic strains *Lactobacillus acidophilus*, *Lactobacillus delbrukeii* and *Bifidobacterium bifidum* were comparable to that of fructose and sucrose (Chick *et al.*, 2001).

It could be argued that the beneficial effects of fructose on the intestinal bacteria would not be applicable to the *in vivo* situation, as fructose is expected to be absorbed in the small intestine before reaching the colon. However, the inability of the body to completely absorb fructose, as well as the availability of fructose in its polymeric forms (fructans and fructo-oligosaccharides), contribute to the fact that fructose can reach the lower gut where it can be used by the intestinal bacteria as has been previously suggested (Hopkins *et al.*, 1998).

Although there have been a number of deleterious effects associated with increased dietary fructose, these have mostly been identified when fructose is consumed in its monosaccharide or disaccharide forms (HFCS and as sucrose), and especially in excess quantities. Fruit, fruit products and honey provide the largest source of naturally occurring fructose in the US diet (72.5 %) (Sievenpiper, 2012). The intake of these natural forms of fructose is relatively low, accounting for only 15 % of total fructose intake in the Western diet (Marriott *et al.*, 2009; Tappy and Lê, 2010). Naturally occurring fructose from fruit at a daily intake of approximately 60 g per day showed decreased body weight and no adverse effects on lipids, blood pressure or insulin resistance when compared with a calorie equivalent, low fructose control diet in overweight humans (Madero *et al.*, 2011).

Consequently, the impact of fructose saccharides (mono-, di-, tri-, or oligosaccharides) in honey cannot be likened to those identified in studies using the monosaccharide fructose.

2.4.2 Enteric bacterial growth in microcosms

The results presented in this chapter suggest that the growth of enteric bacteria in the microcosms conformed to the levels of glucose in the honey. The highest counts of enteric bacteria were observed when yellow box honey (higher glucose content of 33.2 %, relative to the other two honey samples) was used as a substrate in the microcosms. The use of banksia and manuka honeys in the microcosms resulted in similar growth of the enteric bacteria, and this was in agreement with the glucose content of these two honeys which were very similar (28.1 and 29.0 %, respectively). Enteric bacteria also responded more favourably to the glucose control when compared to the fructose control. The growth of enterics in the presence of honey relative to the glucose control showed that the enteric levels were consistently lower when using honey as a substrate. This could mean that the enteric bacteria were susceptible to the antibacterial properties of the honeys (which would be removed once the honeys were digested) or compounds produced by the lactobacilli which could grow using the other components in the honey. Another explanation is that the enterics were not able to use the simple sugars in honey when they were administered with complex sugars and the other constituents in honey. Alternatively, it could be that the other bacterial groups were using the honey constituents more effectively than the enteric bacteria. The results confirm those in the previous study performed in our laboratory that showed that the yellow box honey stimulated the growth of coliforms at levels comparable to the glucose control (Conway *et al.*, 2010), linking the levels of glucose in the honey with growth of the coliforms.

2.4.3 Inhibition of potentially harmful bacteria by honey

The growth of the potentially harmful bacterial group, clostridia, was highly susceptible to compositional changes in the microbiota as well as the choice of substrate in the microcosms. The results using whole honeys showed an inhibitory effect on clostridia, with counts declining or remaining unchanged. It should be noted, however, that the method used to isolate and enumerate the clostridial group (ethanol treatment) kills the vegetative cells; therefore the counts are representative of the spores which may explain the lower numbers. In addition, there were differences in the levels of clostridia in the microcosms established using the adult or infant microbiota and these could be explained by the bacterial compositional variations in adults and infants.

Species of clostridia (implicated in wound infections) have previously been identified as being susceptible to the antibacterial effects of honey (Efem and Iwara, 1992; Hammond and Donkor, 2013). Therefore, the inhibitory effects observed here may also be attributed

to the antibacterial properties of the honeys, which is supported by the data obtained using the digested samples. Digestion inactivates and removes the antibacterial component of honey (by heat and dialysis), and once removed, increases in the clostridial levels could be expected and were observed.

2.4.4 Complex interrelationships in the gut

The correlation studies (Table 2.4) highlighted possible relationships between bacterial groups in the microcosms, suggestive that different groups of bacteria were affected by the change in numbers of other groups. In most cases, increases in the potentially beneficial bifidobacteria and lactobacilli were observed together. When only the complex sugars in honey were available (i.e. in the digested studies), the increase in numbers of the beneficial bacteria often corresponded with decreases in the potentially harmful clostridia and bacteroides numbers. This decrease could be due to the production of inhibitory substances, which the beneficial bacteria are known to secrete (Gibson and Roberfroid, 1995; Gibson, 1999; Manning and Gibson, 2004), or because the beneficial bacteria were able to use the complex sugars as a substrate more readily than the potentially harmful groups as has been previously proposed (Gibson *et al.*, 2004; Roberfroid *et al.*, 2010).

The complexity of the gut composition was further illustrated when the SCFA profiles of the microcosms were considered. An increase in butyric acid production in the microcosms (Table 2.5) often corresponded with increased numbers of bacteroides, enterococci or enterics (Figure 2.6 and 2.7) and it is possible that these bacteria were the main producers of butyric acid. The compositional changes, substrate availability and pH (i.e. production of SCFA which reduce pH) are likely to have influenced the fluctuations in the metabolism of bacteria as has been previously suggested (Gibson and Roberfroid, 1995).

2.4.5 Substrate digestion in *in vitro* gut models

A comparison of the overall changes in the counts of the major bacterial groups (Figure 2.6 and 2.7) revealed that in some cases, the effects of honey on the gut microbiota were grossly over or underestimated when the honeys were used in their whole state. Despite this, there were similar patterns in the overall changes observed when using the honeys in their digested and whole forms, suggesting that the numbers of bacteria were mainly influenced by the non-digestible components of the honeys.

In addition, enumeration of butyric acid in the microcosms showed that production of this acid was significantly increased when digested banksia honey was used, relative to its undigested counterpart. It is possible that the non-digestible components of the honey were used selectively by bacterial groups capable of butyric acid production. Increased

butyric acid production was not noted when the two other honey samples were used in the microcosms, and this could be due to the compositional variations of the honeys. Another possible explanation is that the balance between the production and consumption of butyric acid in the microcosms varied, as butyric acid and other SCFAs produced in the human gut are quickly absorbed or utilised (Cummings, 1981; Hamer *et al.*, 2008; Gibson *et al.*, 2010).

Digestion of the substrates used in studies that modulate gut bacteria by dietary means is an important consideration. Previous studies of the effects of oats, fibres and oligosaccharides found in other carbohydrates (e.g. maize) on the gut microbiota employ a digestive step to simulate passage of these carbohydrates in the human body (Hayakawa *et al.*, 1990; Beer *et al.*, 1997; Mandalari *et al.*, 2007; Kedia *et al.*, 2008; Maathuis *et al.*, 2009; Hur *et al.*, 2011). Prior studies of the beneficial effects of honey on the gut microbiota have used natural honey without digestion or pre-treatment to isolate the oligosaccharides (Kajiwara *et al.*, 2002; Shin and Ustunol, 2005; Haddadin *et al.*, 2007; Ustunol, 2007; Jan Mei *et al.*, 2010). This study is the first to compare the effect of some honeys in their whole and digested forms on the gut microbiota. The literature on the beneficial effects of honey oligosacchardies (i.e. digested honey) on the gut microbiota is limited to a handful of studies (Sanz *et al.*, 2005; Conway *et al.*, 2010), therefore further work is warranted in order to elucidate the expected *in vivo* benefits of honey consumption on the human gut microbiota.

2.5 CONCLUSION

The work presented in this chapter showed that the saccharides present in honey can affect the bacterial composition of the gut. While much of the compositional changes can be attributed to the complex sugars that remain after digestion, the simple sugars in honey can also contribute to modulation of the gut microbiota. Positive changes in lactobacilli levels were observed when high fructose honeys were used in this study, and these effects were attributed to the fructose components of the honeys. It is possible that the beneficial effects could occur *in vivo* with consumption of excess quantities of these saccharides or if they are incompletely absorbed in the body. Many studies exploring the potential benefits of honey use undigested, whole honey. As was observed, the presence of the monosaccharides in honey may grossly affect the changes in the gut microbes.

Finally, the detrimental effects associated with dietary fructose need to be revisited, particularly when discussing the inclusion of naturally occurring fructose (such as in honey) in the diet. The damaging effects of fructose consumption are not associated with low or moderate levels, but with high levels of fructose, which are often in excess of normal recommended caloric consumption. Therefore, they should not be extended to

the consumption of honey. Despite its relatively high fructose content, honey could provide health beneficial effects due to its numerous bioactive constituents and through the promotion of the potentially beneficial gut microbiota by the fructose.

3 CHAPTER THREE
Impact of the oligosaccharides in Australian honeys on the constituents of the gut microbiota and the prebiotic properties of the honey

3.1 INTRODUCTION

3.1.1 Determining the prebiotic effect of carbohydrates

Prebiotics are non-digestible carbohydrates, such as fructo-oligosaccharides (FOS), that are capable of selectively stimulating the growth and/or metabolic activity of beneficial indigenous gut microbiota. Prebiotic testing *in vitro* commonly involves the use of fermenter models, or microcosms, established using defined pure cultures or a faecal inoculum. Pure culture studies are useful in comparative evaluations of metabolism and provide a valuable approach to mechanistic studies, however, they do not take into account the microbial competition that is present in the human GIT (Shin and Ustunol, 2005). For this reason, studies involving mixed (faecal) cultures exhibit a closer representation of the interactions that occur in the human gut. In addition, to more closely mimic the activities of the upper GIT on the carbohydrate, the samples may also be given an enzymatic and acidic pre-treatment, simulating passage through the body.

Much of our knowledge of the prebiotic effect of carbohydrates on the gut microbiota has been obtained by culture-based techniques, that is, by quantitating changes in the composition of the gut microbiota in the fermenter systems. However, it is recognised that many microbes cannot be cultured using standard culture techniques and culture-based data need to be supported by culture-independent molecular methods such as molecular enumeration (e.g. quantitative PCR or fluorescent *in situ* hybridisation), molecular fingerprinting (e.g. terminal restriction fragment length polymorphism) or metagenomic analyses.

For a carbohydrate to be considered as a prebiotic it should have particular characteristics, and one of these is non-digestibility, which includes resistance to gastric acidity, hydrolysis by enzymes and gastrointestinal absorption. In addition, the carbohydrate must be fermentable by intestinal microbes, and allow for selective stimulation of their growth and/or metabolic activity (Gibson and Roberfroid, 1995).

An effective prebiotic is generally accepted as one that:

- stimulates the growth of potentially beneficial bacteria, and/or
- inhibits or suppresses the growth of potentially harmful bacteria, and/or

- allows for increased metabolic production of short chain fatty acids (SCFA).

The benefits of promoting the growth of the potentially beneficial bacteria, bifidobacteria and lactobacilli, are linked to the saccharolytic metabolism of these organisms. This leads to enhanced levels of SCFA, particularly lactic and acetic acid, thereby reducing the colonic pH (Gibson, 1999) and this has been associated with protection against potential pathogens, reduction of diarrhoeal disease, improved digestion and absorption, and immunostimulation (Chauhan and Chorawala, 2012; Gentschew and Ferguson, 2012; Ceapa *et al.*, 2013; Kovatcheva-Datchary and Arora, 2013). In contrast, promoting the growth of the potentially harmful bacteria (clostridia and bacteroides) can have serious negative impacts as these groups are involved in intestinal diseases and can produce carcinogens (Gibson, 1999; Gibson *et al.*, 2004). However, some bacteria labelled as potentially harmful, such as bacteroides, also have a range of health promoting functions including synthesis of vitamins, stimulation of immune functions, inhibition of enteropathogens and stimulation of SCFA production (Gibson and Roberfroid, 1995). This is particularly important to note as bacteroides constitutes the highest number of bacteria in the adult human gut (Gibson and Roberfroid, 1995).

The Prebiotic Index (PI) is commonly used as a means of quantifying the prebiotic effect of a carbohydrate (Palframan *et al.*, 2003). The PI is calculated as a ratio of potentially beneficial (bifidobacteria and lactobacilli) and potentially harmful (bacteroides and clostridia) bacteria while taking into account the overall changes.

3.1.2 Significance of Australian honey as a prebiotic

It has been shown that honey has prebiotic potential because it can support the growth of bifidobacteria and lactobacilli (Chick *et al.*, 2001; Kajiwara *et al.*, 2002; Shin and Ustunol, 2005; Haddadin *et al.*, 2007; Ustunol, 2007; Jan Mei *et al.*, 2010; Popa and Ustunol, 2011). However, many of these studies were performed using pure bacterial cultures only, and used honey in its whole (undigested) state, without removing the simple sugars that are unlikely to survive digestion *in vivo*. Furthermore, many of the honeys were not well-characterised and it is accepted that there is a large variation in the composition and properties of different honeys, depending on the type of flowers visited by the bees.

For honey to exhibit prebiotic activity, at least some components need to survive digestion in order to reach the colon to be used as a substrate for the intestinal microbiota. As oligosaccharide components are most likely to survive this digestion, these are of interest when investigating the prebiotic potential of honey.

As Australia is home to a vast range of unique melliferous flora (that is, plants with flowers that produce nectar that bees can turn into honey), this gives rise to many unique honeys.

Although some work has been conducted to investigate the antimicrobial properties of Australian honeys (Irish, 2004), very little is known about the prebiotic activity of these honeys. One previous study investigating the prebiotic components of 18 honeys of different floral variety showed that these honeys supported the growth of lactobacilli and bifidobacteria in pure culture studies. Additionally, honey oligosaccharides from two of these honeys promoted potentially beneficial bacteria in pure culture studies, and stimulated their growth in faecal microcosms (Conway *et al.*, 2010). It was therefore of interest to extend these studies to the oligosaccharides in well-characterised Australian honeys.

3.1.3 Aims

Studies of the effects of Australian honeys on the gut microbiota are very limited, but preliminary studies have shown that components in Australian honeys have prebiotic activity. An in-depth study of the impact of Australian honeys on the overall changes in the microbial composition and activity of the gut is warranted. As the composition of honey is known to vary depending on floral source, and the microbial composition of the gut is known to vary among individuals, investigating the effects of a range of Australian floral varieties of honey on microbiota from multiple individuals would be of value.

The aim of this study was to investigate the prebiotic properties of well-characterised Australian honeys by examining the oligosaccharides under simulated gastrointestinal conditions using a digestion process for the upper regions of the tract, and human intestinal microbiota for the large intestine in order to understand the prebiotic activities of components in the honeys. The approach taken included:

i) exposing the honeys to simulated upper tract conditions using enzymes and dialysis
ii) measuring the impact of the honeys on the numbers of the major bacterial groups of the gut in microcosms using the standard culture-based technique supported by molecular profiling of selected samples
iii) determining the Prebiotic Index (PI) of the honeys, and
iv) measuring the effect of honeys on short chain fatty acid (SCFA) production by gut microbes.

3.2 MATERIALS AND METHODS

3.2.1 Experimental approach

The prebiotic properties of 25 Australian honeys were investigated using *in vitro* human intestinal microcosms by quantifying the changes in the major bacterial groups in the

microcosm, determining the Prebiotic Index (PI) and monitoring metabolic activity (via SCFA production). The compositional changes in the gut microbiota were also investigated using molecular profiling of selected samples.

3.2.2 Honeys and other carbohydrates

Honeys studied in this chapter are detailed in Table 3.1. Honey i, ii and iii were those used in Chapter 2. Honeys 1 to 22 were provided by the Rural Industries Research and Development Corporation (RIRDC) as part of a collaborative study (*Value-adding to honey* (Dawes and Dall, 2014)). The reference numbering system was used to identify the honeys throughout this research, rather than by referring to their floral type or source, in order to reduce any potential bias. Once the analyses were completed, the honeys were identified for discussion of the outcomes.

Table 3.1 | Honey samples used in *in vitro* microcosm studies

Sample ID number	Reference number	Common name	Scientific name	Source
-	Honey i	Yellow box	*Eucalyptus* spp.	NSW beekeeper
-	Honey ii	Banksia	*Banksia* spp.	NSW beekeeper
-	Honey iii*	Manuka	*Leptospermum scoparium*	Comvita Ltd.
7843WES	Honey 1	Jarrah	*Eucalyptus marginata*	RIRDC
7863WES	Honey 2	Jarrah	*Eucalyptus marginata*	RIRDC
8012WES	Honey 3	Jarrah	*Eucalyptus marginata*	RIRDC
8105WES	Honey 4	Jarrah	*Eucalyptus marginata*	RIRDC
8113WES	Honey 5	Jarrah	*Eucalyptus marginata*	RIRDC
7264DEN	Honey 6	Red stringybark	*Eucalyptus macrorhyncha*	RIRDC
7369HOL	Honey 7	Red stringybark	*Eucalyptus macrorhyncha*	RIRDC
7460EMM	Honey 8	Red stringybark	*Eucalyptus macrorhyncha*	RIRDC
7515BBN	Honey 9	Red stringybark	*Eucalyptus macrorhyncha*	RIRDC

Sample ID number	Reference number	Common name	Scientific name	Source
7526BOM	Honey 10	Red stringybark	*Eucalyptus macrorhyncha*	RIRDC
3747RUT	Honey 11	Spotted gum	*Corymbia maculata*^	RIRDC
3854DEN	Honey 12	Spotted gum	*Corymbia maculata*^	RIRDC
3883SNO	Honey 13	Spotted gum	*Corymbia maculata*^	RIRDC
4442BOM	Honey 14	Spotted gum	*Corymbia maculata*^	RIRDC
5485BOM	Honey 15	Spotted gum	*Corymbia maculata*^	RIRDC
5735SPI	Honey 16	Yellow box	*Eucalyptus melliodora*	RIRDC
7130SMI	Honey 17	Yellow box	*Eucalyptus melliodora*	RIRDC
7141WRI	Honey 18	Yellow box	*Eucalyptus melliodora*	RIRDC
7427RUT	Honey 19	Yellow box	*Eucalyptus melliodora*	RIRDC
7626DEN	Honey 20	Yellow box	*Eucalyptus melliodora*	RIRDC
8168KLI	Honey 21	Canola	*Brassica* spp.	RIRDC
8193SNO	Honey 22	Canola/stringybark	*Brassica/Eucalyptus* spp. blend	RIRDC

***Commercial name - Medihoney™**

^ Formerly referred to as *Eucalyptus maculata*

The control carbohydrates used in the study were fructose, glucose and inulin, all obtained from Sigma-Aldrich, and the fructo-oligosaccharide referred to as Raftilose P95 (FOS) was obtained from Orafti. The inulin and FOS served as the positive controls for oligosaccharides; and glucose and fructose as controls for the monosaccharides.

Negative controls with no added carbohydrate (i.e. medium only) were also included.

3.2.3 Honey digests

Honeys were digested to remove simple sugars by pre-treatment with digestive enzymes, followed by dialysis, as detailed in Section 2.2.4. The resultant dialysate referred to subsequently as honey digests or digested honey contained the honey oligosaccharides.

3.2.4 Intestinal microcosms

Microcosms were established *in vitro* using human microbiota as outlined in Section 2.2.5. Faecal samples were provided by five donors, D1 to D5 (Table 3.2). Approval for the study was obtained from the Human Research Ethics Advisory (HREA) panel, University of New South Wales (approval number HREA-11041).

Table 3.2 | Donors of faecal microbiota for the *in vitro* microcosm studies

Donor	Gender	Age group
D1	Female	45-60
D2	Female	Infant, partially breast fed
D3	Male	18-30
D4	Female	18-30
D5	Male	45-60

Microcosms were prepared with the honeys or control sugars as added substrate (using both D1 and D2 microbiota in separate microcosms), and the digested fractions thereof using D1 to D5 microbiota separately. A microcosm with no added substrate (i.e. 10 % inoculum in medium only) was used as the negative control.

The microcosms were incubated and aliquots taken as previously described (Section 2.2.5). The experiments were performed on at least three separate occasions, and were analysed in triplicate on any given day.

3.2.5 Enumeration of major viable bacterial groups

Aliquots from the microcosms were used to enumerate the major culturable bacterial groups, using the micro-drop technique on selective media, as detailed in Section 2.2.6. Counts are expressed as $\log_{10}$ CFU per ml.

3.2.6 Short chain fatty acid analysis

The SCFA produced in the microcosms were extracted in ether and then quantified using GC-MS, as detailed in Section 2.2.7.

3.2.7 Measurement of Prebiotic Index (PI)

The PI was calculated according to the method developed by Palframan *et al.* (2003).

The following equation was used to determine the PI:

PI = (Bif/Total) – (Bac/Total) + (Lac/Total) – (Clos/Total)

where

Bif = final number of bifidobacteria / initial number of bifidobacteria

Bac = final number of bacteroides / initial number of bacteroides

Lac = final number of lactobacilli / initial number of lactobacilli

Clos = final number of clostridia / initial number of clostridia

Total = final number of total anaerobes / initial number of total anaerobes

3.2.8 Statistical analysis

Statistical analysis of the results was performed with R software (version 3.0.2) for Windows. The Kolmogorov-Smirnov test was used to confirm normal distribution. Analysis of variance was tested by one-way ANOVA, and then followed by Tukey's (HSD) test to identify significance between groups. P-values <0.05 were deemed significant.

3.2.9 Molecular fingerprinting

3.2.9.1 DNA extraction

Aliquots from the microcosms were centrifuged and the pellet stored at -80 °C. DNA was extracted from the pellet (thawed on ice) using the Isolate Fecal DNA Kit (Bioline), as per the manufacturer's instructions. Briefly, lysis buffer was added to the pellet and then processed in a bead beater (TissueLyser II, Qiagen). Following centrifugation, the filtrate was mixed with DNA binding buffer, centrifuged and washed with pre wash buffer. After a final wash with DNA wash buffer, the DNA was eluted in a final volume of 50 µl buffer. DNA was quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

3.2.9.2 PCR conditions

The standard mix used for a 25 µl PCR was composed of EconoTaq™ PLUS Green 2X Master Mix (Lucigen), fluoro-labelled forward primer 27F 6-FAM (IDT), reverse primer 519 R (IDT), extracted DNA, and made up to volume with sterile molecular grade water (5 Prime Inc).

A Mastercycler™ (Eppendorf) was used with the following settings.

Table 3.3 | PCR protocol

	Temperature	Time	
Initialisation	94 °C	3 min	
Denaturation	94 °C	30 sec	Repeated for 25 cycles
Annealing	56 °C	30 sec	
Extension	72 °C	30 sec	
Final extension	72 °C	5 min	
Hold	4 °C		

The quality of the PCR product was visualised using standard agarose gel electrophoresis, containing GelRed (Biotium). The gel was imaged using a Gel Doc 2000 System (Bio Rad).

PCR products were purified using DNA Clean and Concentrator™ -5 kits (Zymo Research) according to the manufacturer's instructions. Briefly, binding buffer was added to the PCR product, and centrifuged in a silica column. DNA was washed twice and eluted into 20 µl sterile molecular grade water.

DNA was quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

3.2.9.3 Restriction enzyme digest

Purified PCR products (100 – 200 ng concentration) were digested with restriction enzymes *Msp*I and *Rsa*I separately (New England Biolabs) for terminal restriction fragment length polymorphism (TRFLP) analysis. The sample and restriction enzyme mix was incubated in the thermocycler (Eppendorf) for 3 hours at 37 °C. The restriction enzymes were then deactivated by heating at 80 °C (*Msp*I) or 65 °C (*Rsa*I), for 20 min.

The resultant DNA was purified using Zymo DNA Clean and Concentrator™ kits as above and eluted in a total volume of 1 – 3 µl, containing 5 – 10 ng DNA.

3.2.9.4 TRFLP analysis

Sample analysis was conducted by the Ramaciotti Centre for Gene Function Analysis (Biological Sciences Building, University of New South Wales). Fragments were analysed on the ABI 3730 Capillary Sequencer (Applied Biosystems) using the GeneScan™ LIZ 600 size standard.

The electropherograms were analysed in Peak Scanner (Version 1.0, Applied Biosystems), from base pair ranges 20 to 600 (covering the 36 labelled fragments in the LIZ 600 size standard, represented by the orange peaks in the program). The software allowed visual confirmation of the 6-FAM labelled sample peaks (blue) and the electropherogram data

was exported and edited in Excel (Microsoft) to allow compatibility with the T-REX software package.

All data was then analysed through T-REX (Culman *et al.*, 2009) allowing representation of true peaks after filtering out background fluorescence from the electropherograms. Any peaks that occurred in the negative control were assumed to be residual noise and removed from all samples.

3.3 RESULTS

3.3.1 Prebiotic Index of digested and whole honey

Changes in the counts of key bacterial groups in the *in vitro* microcosms were used to quantify the prebiotic effect of the honeys. Both undigested (whole) and digested honey samples were tested and the results expressed as Prebiotic Index (PI).

The PI values of the honeys assayed in Chapter 2 were determined for both whole and digested honey (i.e. honey oligosaccharides) using the adult (D1) and infant (D2) microbiota, and the results are shown in Figure 3.1. These honeys were used to develop the PI methods used in this chapter, before testing the larger set of 22 honeys.

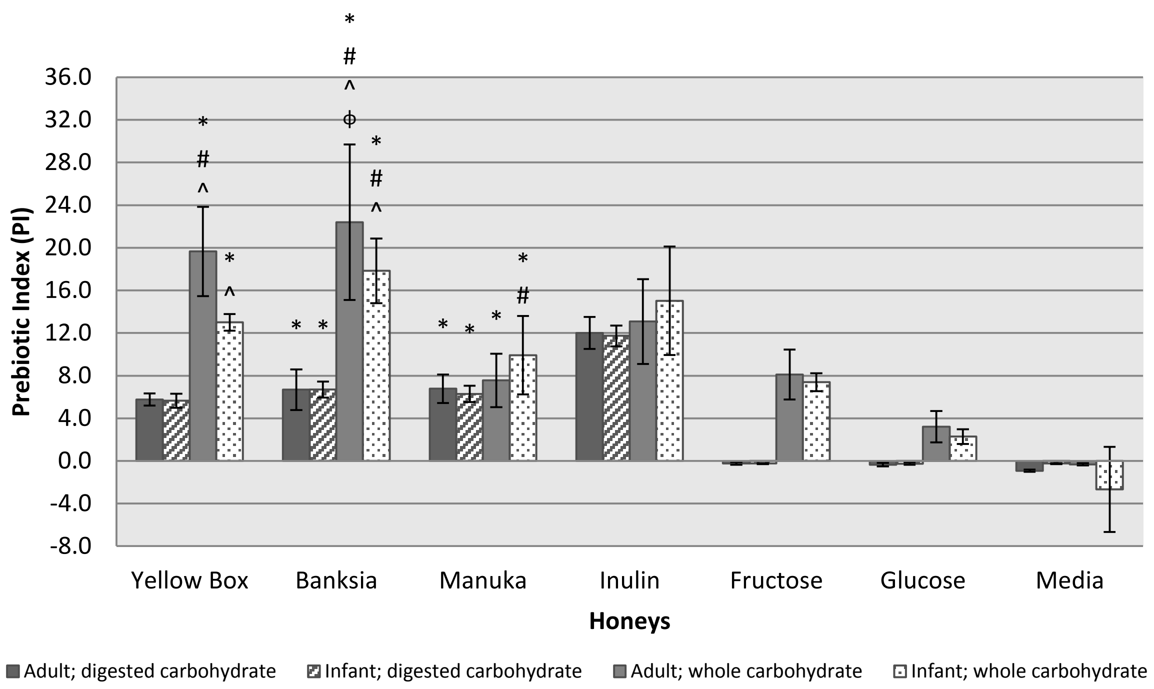


Figure 3.1 | Prebiotic index of previously tested honeys (with and without digestion)

PIs based on changes in key bacterial groups during fermentation in microcosms, established using adult or infant microbiota and digested or undigested (whole) honeys. Results expressed as mean PI ± SD from four separate trials. Symbols indicate statistically significant difference ($p<0.05$): (*) relative to negative control, (#) relative to fructose control, (^) relative to glucose control, and (ɸ) relative to inulin control. In addition to inulin, monosaccharides (fructose and glucose) were used as positive controls for the assays using whole honey due to the presence of these sugars in undigested samples.

When used in their undigested state, all three honeys exhibited significantly higher PI than the negative control when the adult microbiota were used in the microcosms ($p<0.01$ for all).

In their whole (undigested) forms, the PI value of banksia was similar to that of yellow box honey, and both were significantly higher than the PI of manuka honey ($p<0.01$) when the adult microbiota were used. The PI values of yellow box and banksia honeys were significantly higher than for the fructose and glucose controls ($p<0.01$), whereas the PI for the manuka sample was comparable to both monosaccharides ($p>0.05$). The PI of banksia was significantly higher than the inulin control ($p=0.00$).

In their digested states, banksia and manuka honey showed significantly higher PI values relative to the negative control ($p=0.02$ for both), and the PI values of all three honeys were similar to the inulin control ($p>0.05$). The use of undigested yellow box and banksia honeys in the microcosms resulted in significantly higher PI values relative to their

digested counterparts ($p<0.05$ for all). In contrast, the PI of manuka honey was similar in both cases.

The patterns in PI using the infant microbiota were largely supportive of those observed when the adult microbiota was used.

The PI values of 22 honeys in their digested (oligosaccharides) and undigested are shown in Figure 3.2. Many honey samples in their digested and undigested states had significantly higher PI values than the negative control. The PI of undigested (whole) honey was similar to that of the digested honey, with some exceptions.

In the microcosms established using adult microbiota:

- red stringybark honey 10 and yellow box honey 20 had higher PI values in their whole state compared to the digested counterparts ($p<0.05$)
- in their digested form, jarrah honeys 4 and 5 had higher PI values compared to their whole form ($p<0.01$).

In the microcosms established using infant microbiota:

- jarrah honey 3, spotted gum honey 11, and yellow box honeys 18 and 20 had significantly higher PI values in their whole states ($p<0.05$) relative to their digested state
- jarrah honeys 2 and 4 had significantly higher PI values in their digested state compared to when they were used whole ($p<0.01$).

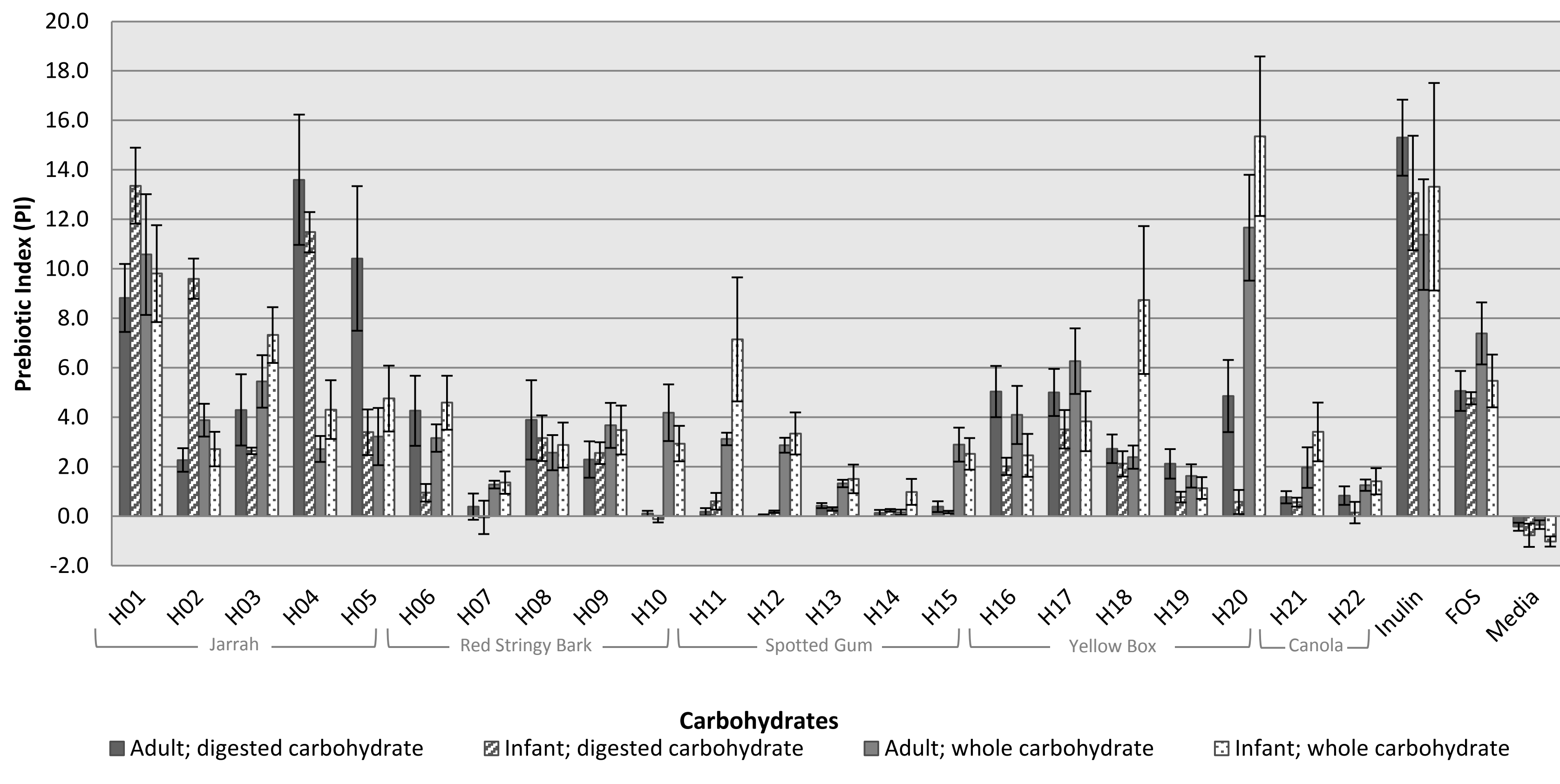


Figure 3.2 | Prebiotic index of 22 Australian honeys (with and without digestion)

PIs based on changes in key bacterial groups during fermentation in microcosms, established using adult (D1) or infant (D2) microbiota and digested (oligosaccharides) or whole honeys. Results expressed as mean PI ± SD from four separate trials. Symbols indicate statistically significant difference (p<0.05): (*) relative to the negative control (medium only), and (^) of digested honey relative to whole honey counterparts. Inulin and FOS included as prebiotic (positive) controls.

3.3.2 In-depth analysis of the PI values of digested Australian honeys

As the use of digested honey samples more closely reflects what happens *in vivo*, further experiments were performed using digested samples which contained the oligosaccharides. The 22 RIRDC sourced honeys were examined further in microcosms established using microbiota from five different donors to investigate the prebiotic activity of the honeys in gut ecosystems of varying microbial composition. Some differences were observed in the PI of various honeys depending on the microbiota used. However, there were no significant differences in the PI values of the positive controls (inulin and FOS), or in the negative control when the microcosms were established using the different microbiota from the different donors.

The PI results were analysed by honey type, and are shown in Figures 3.3 to 3.7.

Some general trends were observed. For example, there was a consensus that the jarrah honeys had the highest PI values relative to the other honey types, followed by the yellow box and red stringybark samples. Conversely, the spotted gum and canola honeys typically had the lowest PI values of the honey types tested.

3.3.2.1 PIs of digested jarrah honey samples

Below is a summary of the PIs of the five jarrah honeys (Figure 3.3) from microcosms established using D1 microbiota:

- PIs of honeys 1, 4, and 5 were significantly higher than the negative control ($p<0.01$)
- PIs of honeys 4 and 5 were similar to the inulin control ($p>0.05$), and honeys 1-3 significantly lower relative to inulin ($p<0.05$)
- PI of honey 4 was significantly higher than the FOS control ($p=0.00$), and honeys 1, 2, 3, and 5 similar to FOS ($p>0.05$)
- PIs of honeys 1, 4, and 5 (PI 8.8, 13.6, and 10.4, respectively) were higher than honey 2 (PI 2.3, $p=0.00$) and honey 3 (PI 4.3, $p=0.01$).

The PI from the use of D2 microbiota largely resembled the D1 studies, with the exception of honey 2, which gave a significantly higher PI.

When microcosms were established using D3, D4 and D5 microbiota all five honey samples had significantly higher PI than the negative control ($p<0.05$), similar PI to the inulin control ($p>0.05$), and similar or better PI than the FOS control.

In conclusion, all of the jarrah samples resulted in a positive PI, and this was at least as good as the prebiotic controls, inulin and FOS, in most cases. In addition, jarrah samples 1 and 4 showed consistently higher PI than the other jarrah samples, with PIs ranging from 8.8 to 19.1 for honey 1, and 11.5 to 19.9 for honey 4.

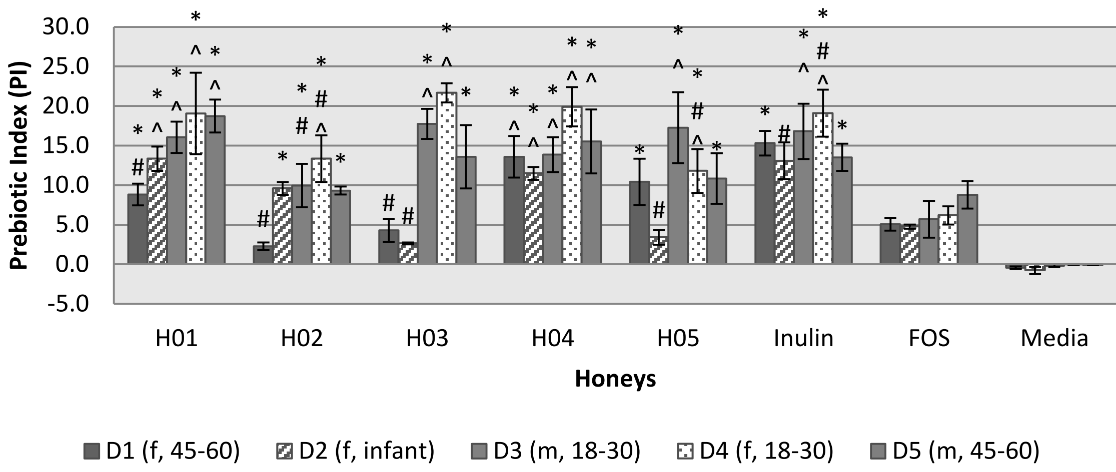


Figure 3.3 | Prebiotic indices of jarrah honeys

PIs determined from microcosms established using digested honeys (H) and microbiota from different donors D1 to D5, consisting of three females (f) and two males (m) of different ages. Results expressed as mean PI ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (*) relative to the negative control media, (#) relative to the positive control inulin, and (^) relative to the positive control FOS.

3.3.2.2 PIs of digested red stringybark honey samples

The PIs of the five red stringybark honeys (Figure 3.4) from microcosms established using D1 microbiota are summarised below:

- PIs of all red stringybark samples were positive, but not significantly higher than the negative control ($p>0.05$)
- PIs of all five red stringybark honeys were significantly lower than the inulin control ($p<0.01$)
- PIs of all five red stringybark honeys were similar to the FOS control ($p>0.05$).

PIs of honeys from the microcosms established using the other microbiota resembled those of the D1 studies, with some small exceptions. For example, when D4 or D5 microbiota were used, the PI of red stringybark honeys 8 and 9 (PI between 5.7 and 7.8) were significantly higher than the negative control ($p<0.05$), and the PI of honey 10 (PI 6.7) was also significantly higher relative to the negative control when D4 microbiota were used ($p<0.05$).

Of the red stringybark samples, honeys 8 and 9 had the highest PIs ranging from 2.3 to 7.8, and honey 7 had the lowest PI (ranging from 0.1 to 2.2).

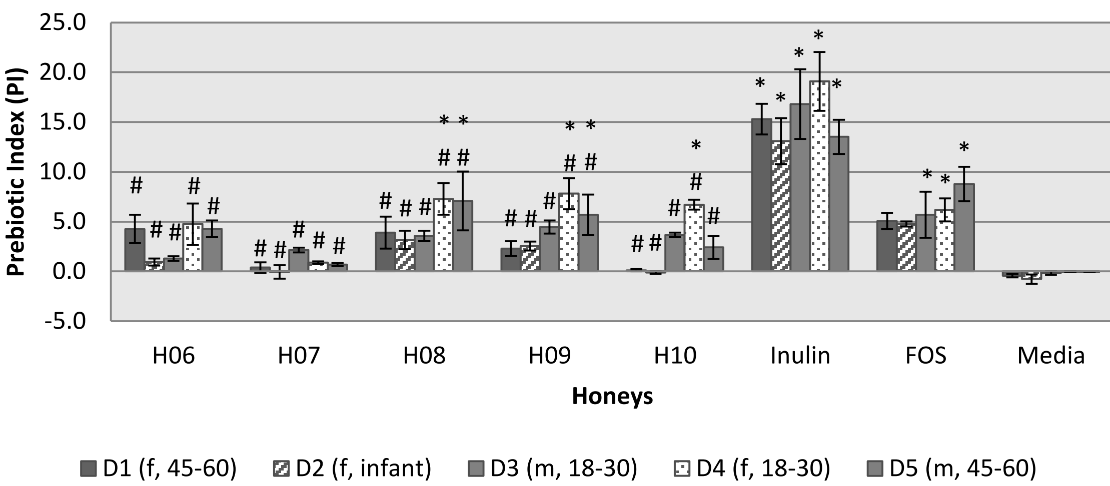


Figure 3.4 | Prebiotic indices of red stringybark honeys

PIs determined from microcosms established using digested honeys (H) and microbiota from different donors D1 to D5, consisting of three females (f) and two males (m) of different ages. Results expressed as mean PI ± SD from three separate trials. Symbols indicate statistically significant difference (p<0.05): (*) relative to the negative control media, (#) relative to the positive control inulin, and (^) relative to the positive control FOS.

3.3.2.3 PIs of digested spotted gum honey samples

A summary of the PI values of five spotted gum honeys (Figure 3.5) from microcosms established using D1 microbiota can be found below:

- PIs of all spotted gum samples were positive, but not significantly higher than the PI of the negative control (p>0.05)
- PIs of all spotted gum samples were significantly lower than the inulin control (p<0.01)
- PIs of all spotted gum samples were similar to the FOS control (p>0.05).

The spotted gum PIs for the other donor microbiota reflected those of the D1 studies, with a few variations. The PI of honey 11 (PI 7.7) was significantly higher than the negative control and the FOS control when D3 microbiota was used (p<0.05).

Overall, spotted gum honey 11 had the highest PI (ranging from 0.2 to 7.7) and honey 13 had the lowest PI (between 0.1 and 0.7), relative to the other spotted gum samples.

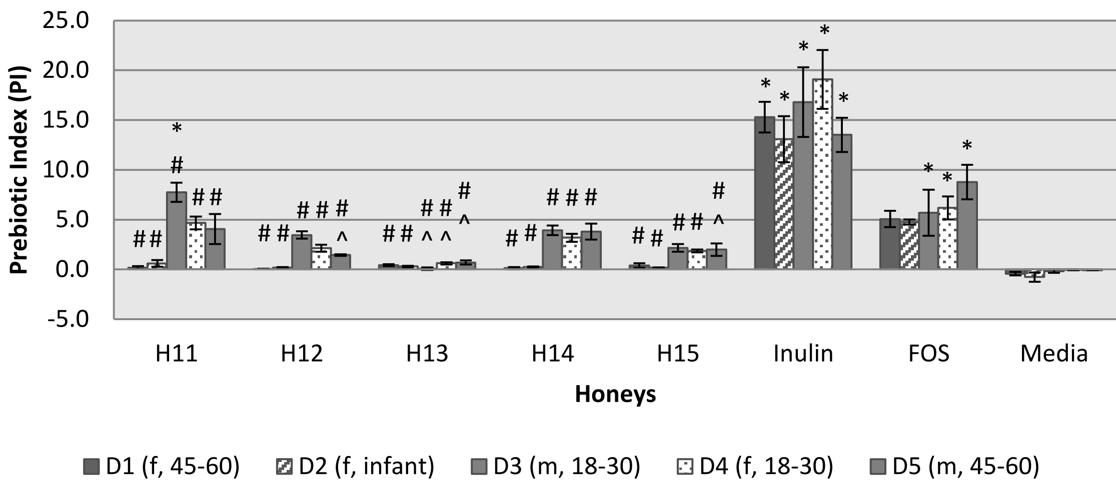


Figure 3.5 | Prebiotic indices of spotted gum honeys

PIs determined from microcosms established using digested honeys (H) and microbiota from different donors D1 to D5, consisting of three females (f) and two males (m) of different ages. Results expressed as mean PI ± SD from three separate trials. Symbols indicate statistically significant difference (p<0.05): (*) relative to the negative control media, (#) relative to the positive control inulin, and (^) relative to the positive control FOS.

3.3.2.4 PIs of digested yellow box honey samples

The PIs of yellow box honeys (Figure 3.6) from microcosms established using D1 microbiota are summarised below:

- PIs of all yellow box honeys were positive, but not significantly higher than the negative control (p>0.05)
- PIs of all yellow box honeys were significantly lower than the inulin control (p<0.01)
- PIs of all yellow box honeys were similar to the FOS control (p>0.05).

The trends seen in the yellow box PIs from the microcosms established using the D2 microbiota were mostly similar to the D1 studies. However, there were some differences noted when the D3, D4 and D5 microbiota were used. Yellow box samples 17, 18 and 19 (PI between 5.9 and 7.9) had significantly higher PI values relative to the negative control when D3 and D5 microbiota were used. Yellow box honey 16 had a significantly higher PI value of 7.3 relative to the negative control when D4 microbiota was used.

Yellow box honey 17 had the highest PI (ranging from 3.6 to 6.3), and honey 20 had the lowest PI (between 0.6 and 4.9) relative to the other yellow box samples.

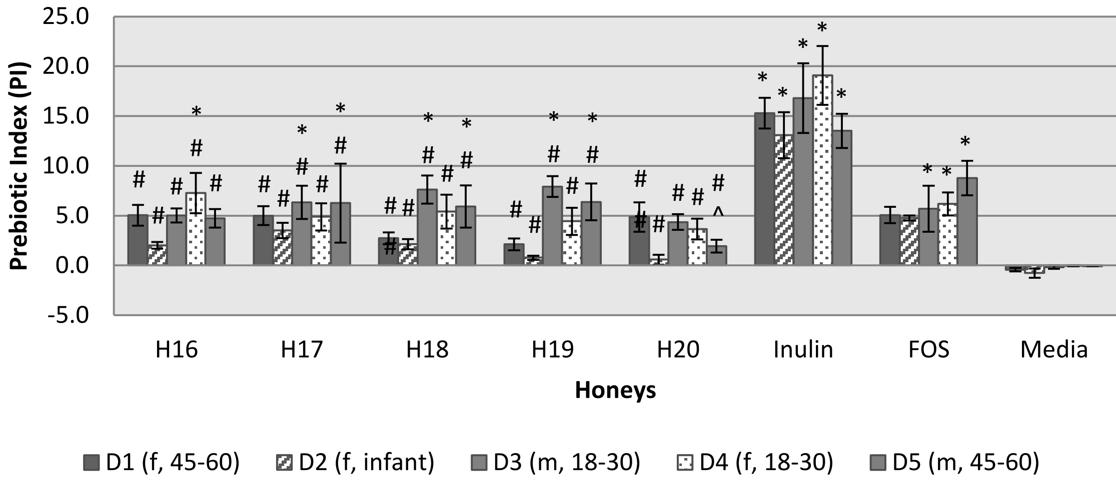


Figure 3.6 | Prebiotic indices of yellow box honeys

PIs determined from microcosms established using digested honeys (H) and microbiota from different donors D1 to D5, consisting of three females (f) and two males (m) of different ages. Results expressed as mean PI ± SD from three separate trials. Symbols indicate statistically significant difference (p<0.05): (*) relative to the negative control media, (#) relative to the positive control inulin, and (^) relative to the positive control FOS.

3.3.2.5 PIs of digested canola honey samples

Below is the summary of the PI values of the two canola honeys (Figure 3.7) from microcosms established using the D1 microbiota:

- PIs of both canola samples were positive, but not significantly higher than the negative control (p>0.05)
- PIs of both canola samples were significantly lower than the inulin control (p<0.05)
- PIs of both canola samples were comparable to the FOS control (p>0.05).

The canola honey PIs from microcosms established using the other microbiota were similar to the D1 studies, with two exceptions. Canola honey 21 had a significantly lower PI relative to FOS when D4 microbiota were used, and both canola honeys had significantly lower PIs compared to the FOS control when D5 microbiota were used (p<0.01).

There were no significant differences in the PIs of the canola honeys, although it was noted that honey 22 had slightly higher PI (ranging from 0.1 to 3.5) relative to honey 21 (PI 0.6 to 2.6).

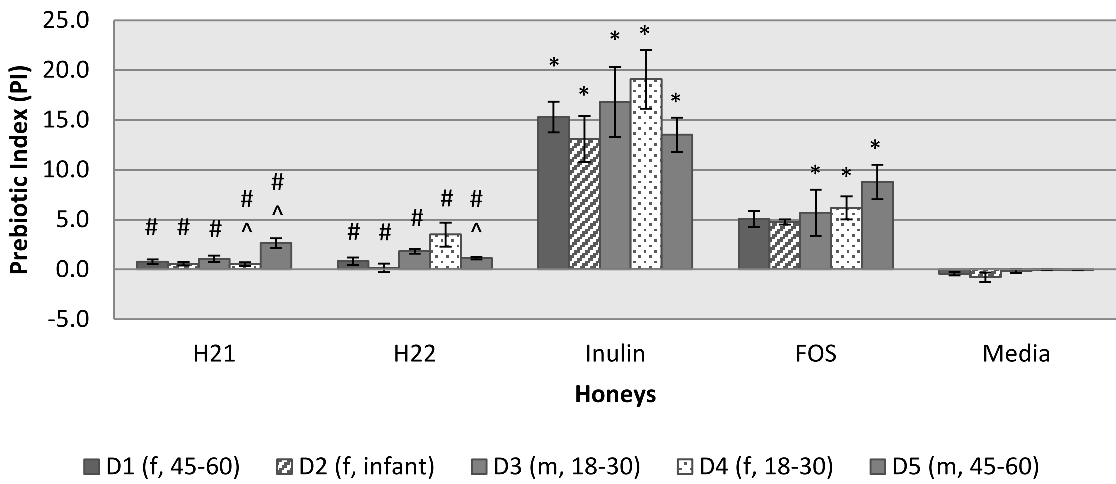


Figure 3.7 | Prebiotic indices of canola honeys

PIs determined from microcosms established using digested honeys (H) and microbiota from different donors D1 to D5, consisting of three females (f) and two males (m) of different ages. Results expressed as mean PI ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (*) relative to the negative control media, (#) relative to the positive control inulin, and (^) relative to the positive control FOS.

3.3.3 Investigation of the relationship between PI and oligosaccharide concentration in Australian honeys

As the PI obtained from the digested samples was due to the non-digestible components of honey, it was of interest to compare PI values with the honey oligosaccharide (i.e. non-digestible sugar) concentrations in the whole honey (Figure 3.8). The oligosaccharide concentrations were determined externally (Appendix 2), and made available as the combined percentage of maltose and oligosaccharide in each honey sample.

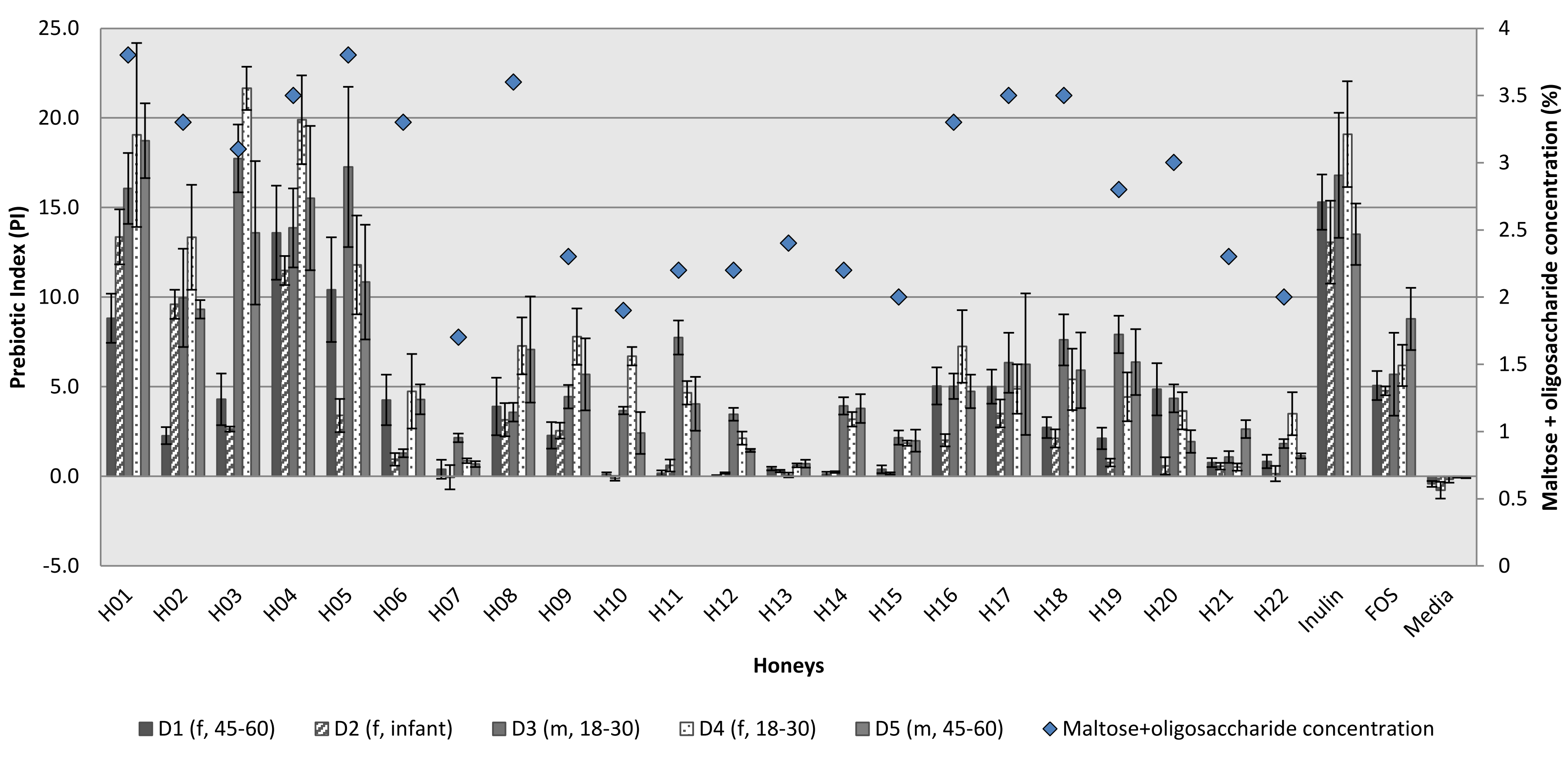


Figure 3.8 | PI and oligosaccharide concentrations of digested honeys

PIs determined from microcosms established using digested honeys (H) and microbiota from donors D1 to D5 of different gender (m,f) and age groups represented by the bar graph on primary axis (left-hand side). Results expressed as mean PI ± SD from three separate trials. Maltose + oligosaccharide concentration (% in each whole honey sample) displayed as blue scatter plot on secondary (right-hand side) axis.

The comparison of digested honey PI and the corresponding maltose + oligosaccharide concentrations of whole honey suggested that there was a positive correlation, since honey with higher maltose + oligosaccharide concentrations generally had higher values for the PI. However, the observed relationship was not conclusive, with a number of outliers, for example, honey 13 which had a very low PI and relatively high concentration of maltose + oligosaccharide concentration. Correlation and regression tests were performed by comparing the PIs of each of the honeys to the maltose + oligosaccharide concentrations determined in the honey samples (Table 3.4 and Appendix 3). The analyses were performed on the PI values obtained from microcosms established using all of the microbiota sources (D1-D5). The results were suggestive of a weak exponential relationship between PI and maltose + oligosaccharide concentration, as the correlation coefficient (R) values in some cases were just below 0.8, which is generally accepted as showing correlation (perfect correlation represented by R=1.00).

Table 3.4 | Correlation between maltose + oligosaccharide concentration and PI of digested honeys determined by microcosms established using various microbiota

Microbiota used to establish microcosm	Correlation coefficient (R)
D1 (female, 45-60)	0.78
D2 (female, infant)	0.64
D3 (male, 18-30)	0.62
D4 (female, 18-30)	0.61
D5 (male, 45-60)	0.73

3.3.4 Effect of honey on growth of major bacterial groups

The key culturable bacterial groups in the microcosms, using different donor microbiota (D1-D5) and digested honeys, were enumerated to determine the effect of the honeys on the growth of these bacteria. The change in bacterial numbers from the initial to final counts in the microcosms was determined.

3.3.4.1 Effect of honey on growth of bifidobacteria

The change in bifidobacteria numbers (Figure 3.9) in microcosms established using D1 microbiota are summarised below:

- all 22 digested honeys allowed a significant increase in bifidobacteria counts relative to the negative control ($p<0.05$). This trend was also seen in the microcosms established using the other microbiota, with some exceptions. Specifically, three spotted gum samples (H13-H15) in the D3 microcosms, one yellow box (H18) and both canola (H21 and H22) in the D4 microcosms, and one sample each of the red stringybark (H7), spotted gum (H13), yellow box (H17) and canola (H21) honeys in the

D5 microcosms showed an elevation of bifidobacteria at levels similar to the negative control

- some varieties of honey promoted the growth of bifidobacteria at levels comparable to the inulin control. These included some jarrah samples (H1, H2, and H5), some yellow box samples (H17 and H19), and the canola honeys (H21 and H22). The bifidobacteria counts in the microcosms established using D2 microbiota were similar to the D1 studies. In contrast, the bifidobacteria in the microcosms established using D3, D4 and D5 microbiota were enhanced at levels similar to the inulin control for most of the honeys tested.
- some digested honey samples promoted the growth of bifidobacteria at levels similar to the FOS control, floral varieties jarrah (H1, H2 and H5), yellow box (H16, H17 and H20) and canola (H21 and H22). These patterns were largely reflected in the studies using D2 microbiota, but differed in the microcosms established using D3, D4 and D5, where the majority of the honey samples were as effective at increasing the bifidobacteria levels as the FOS control.

Collectively, the jarrah honeys (H1-H5) were found to promote the most positive change in bifidobacteria numbers relative to the other honey types.

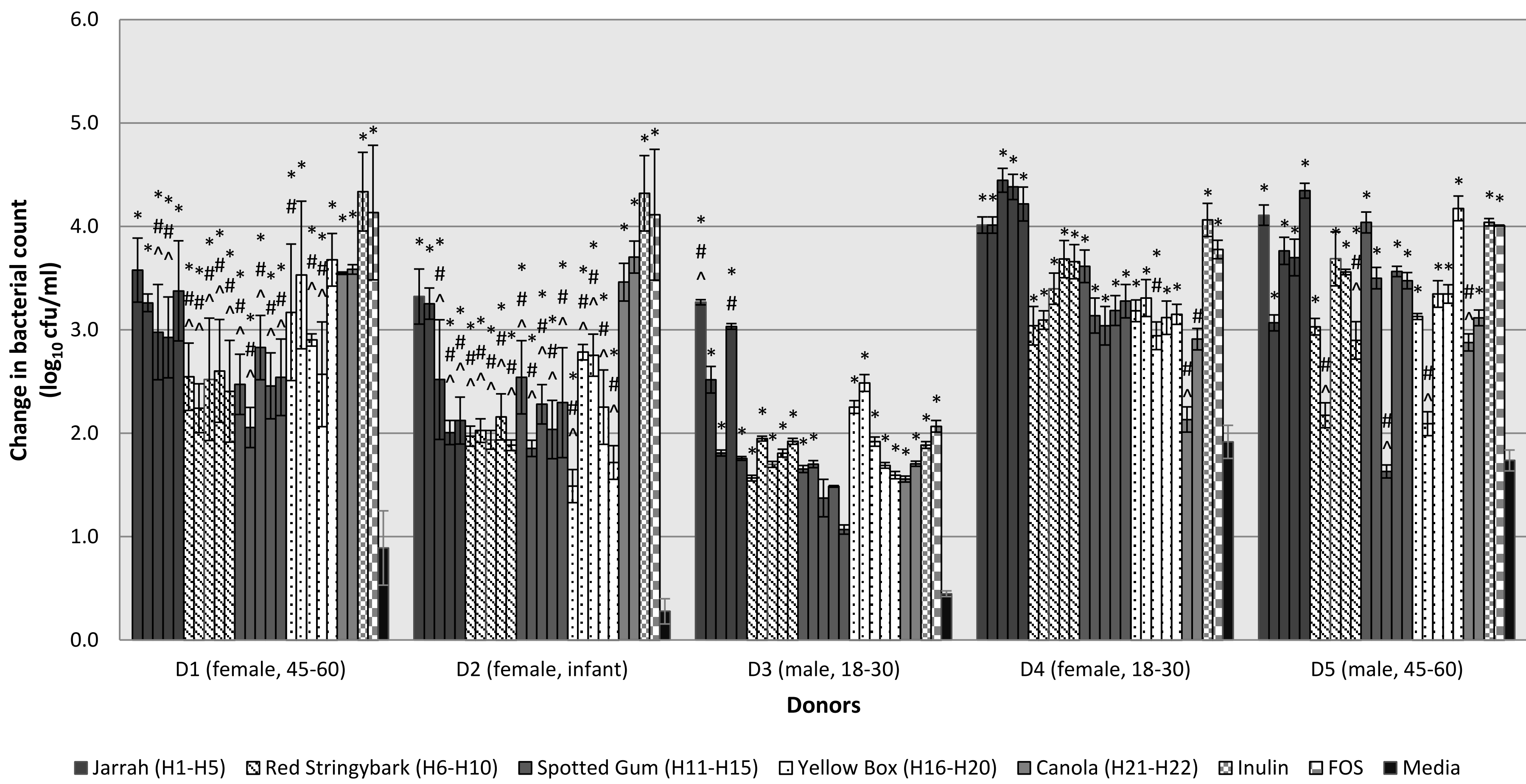


Figure 3.9 | Impact of digested honeys on bifidobacteria numbers in microcosms established using microbiota from five donors

Mean change in bacterial counts (from initial to final) in microcosms established using microbiota from all donors. Results expressed as mean change $\log_{10}$ cfu/ml ± SD from three separate trials. Symbols indicate a statistically significant difference ($p<0.05$) relative to: (*) negative control media, and positive controls: (#) inulin, and (^) FOS.

3.3.4.2 Effect of honey on growth of lactobacilli

Increases in lactobacilli counts in the presence of digested honeys (Figure 3.10) in microcosms established using D1 microbiota are summarised below:

- lactobacilli numbers significantly increased in the presence of all honeys relative to the negative control, except for jarrah honey 3 and spotted gum 11. These results were largely reflective of the trends when other microbiota were used in the microcosms as most of the honeys significantly promoted lactobacilli growth
- levels of lactobacilli were significantly lower than inulin in the presence of some jarrah (H2-H5), all red stringybark (H6-H10) and all spotted gum (H11-H15) that were used in the microcosms. Similarly, in the microcosms established using D2 microbiota, and some of these spotted gum as well as some yellow box (H16-H18) honeys, lactobacilli counts were lower than the inulin control. In contrast, changes in lactobacilli numbers were comparable to the inulin control for the majority of the honey samples when the microcosms were established with microbiota from D3, D4 or D5.
- increases in lactobacilli numbers using most of the digested honey samples were similar to the FOS positive control, although the change in lactobacilli counts using some jarrah (H2-H5), red stringybark (H6-H10) and spotted gum (H11, H12 and H15) was significantly lower. Similar trends were observed when the microcosms were established using D2 microbiota. Contrastingly, almost all honey samples allowed lactobacilli to grow at levels comparable to the FOS control in microcosms established with D3, D4 or D5 microbiota.

Overall, the change in numbers of lactobacilli in the microcosms was most obvious when jarrah and yellow box honeys were used.

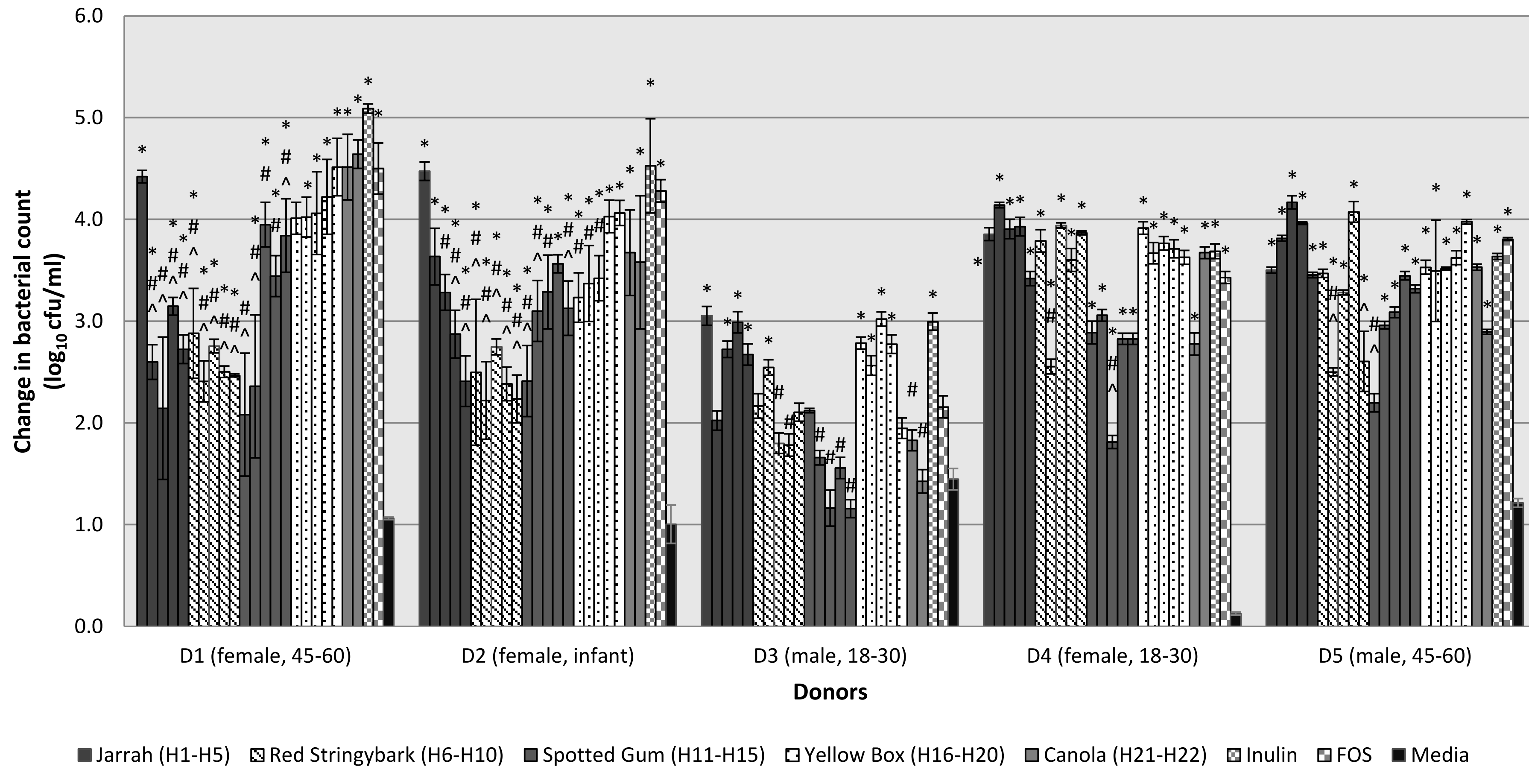


Figure 3.10 | Impact of digested honeys on lactobacilli numbers in microcosms established using microbiota from five donors

Mean change in bacterial counts (from initial to final) in microcosms established using microbiota from all donors. Results expressed as mean change log_{10} cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$) relative to: (*) negative control media, and positive controls: (#) inulin, and (^) FOS.

3.3.4.3 Effect of honey on growth of bacteroides

The levels of bacteroides (Figure 3.11) in the microcosms established using D1 microbiota are summarised below:

- bacteroides counts were significantly lower in the presence of any of the 22 honeys relative to the negative control, with the exception of one canola sample (H22). Although the microcosms established using D2 microbiota showed similar patterns in bacteroides numbers, there were differences when D3, D4 and D5 microbiota were used as the levels of bacteroides were comparable to the negative control
- the change in bacteroides counts was significantly lower than both the inulin and FOS controls for most of the honeys tested. This was also supported in the microcosms established using D2 microbiota, but not in those using D3, D4 or D5 which showed that bacteroides numbers were similar in the presence of the honeys relative to either of the positive controls.

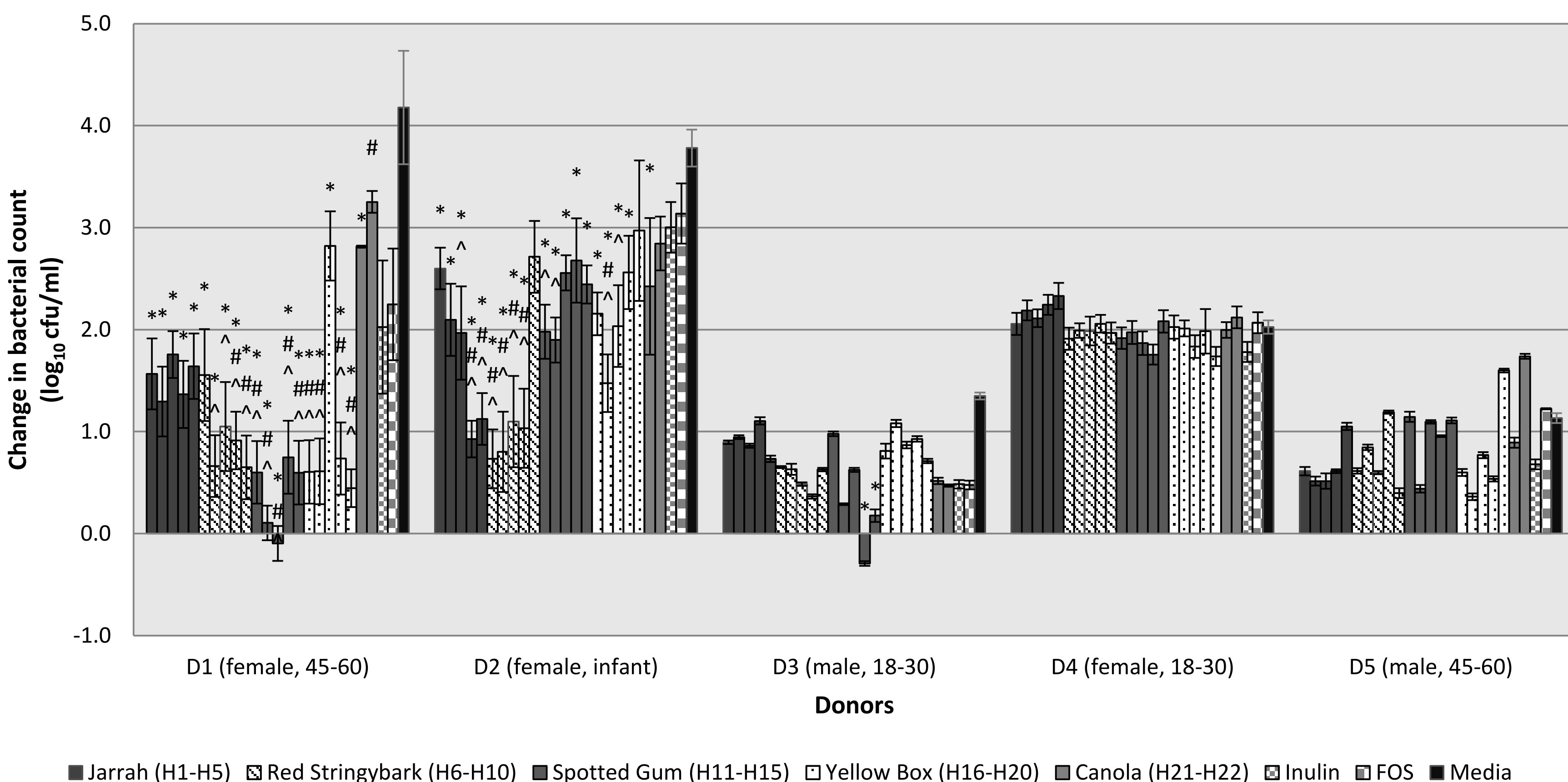


Figure 3.11 | Impact of digested honeys on bacteroides numbers in microcosms established using microbiota from five donors

Mean change in bacterial counts (from initial to final) in microcosms established using microbiota from all donors. Results expressed as mean change log_{10} cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$) relative to: (*) negative control media, and positive controls: (#) inulin, and (^) FOS.

3.3.4.4 Effect of honey on growth of clostridia

The change in number of clostridia (Figure 3.12) in the microcosms established using D1 microbiota are summarised below:

- numbers of clostridia were significantly lower in the presence of most of the honey samples compared to the negative control. This pattern was also noted in the microcosms established with the other microbiota, with the exception of D3 in which the clostridial levels in the presence of honey were similar to the negative control
- the counts of clostridia were significantly lower in the presence of all jarrah, red stringybark and spotted gum honeys, and two of the yellow box samples compared to inulin. This trend was not reflected in the microcosms using the D2-D5 microbiota. In most cases, the effect of the honeys on the growth of clostridia was similar to the inulin control. Similar patterns were also noted when the honeys were compared to the FOS control.

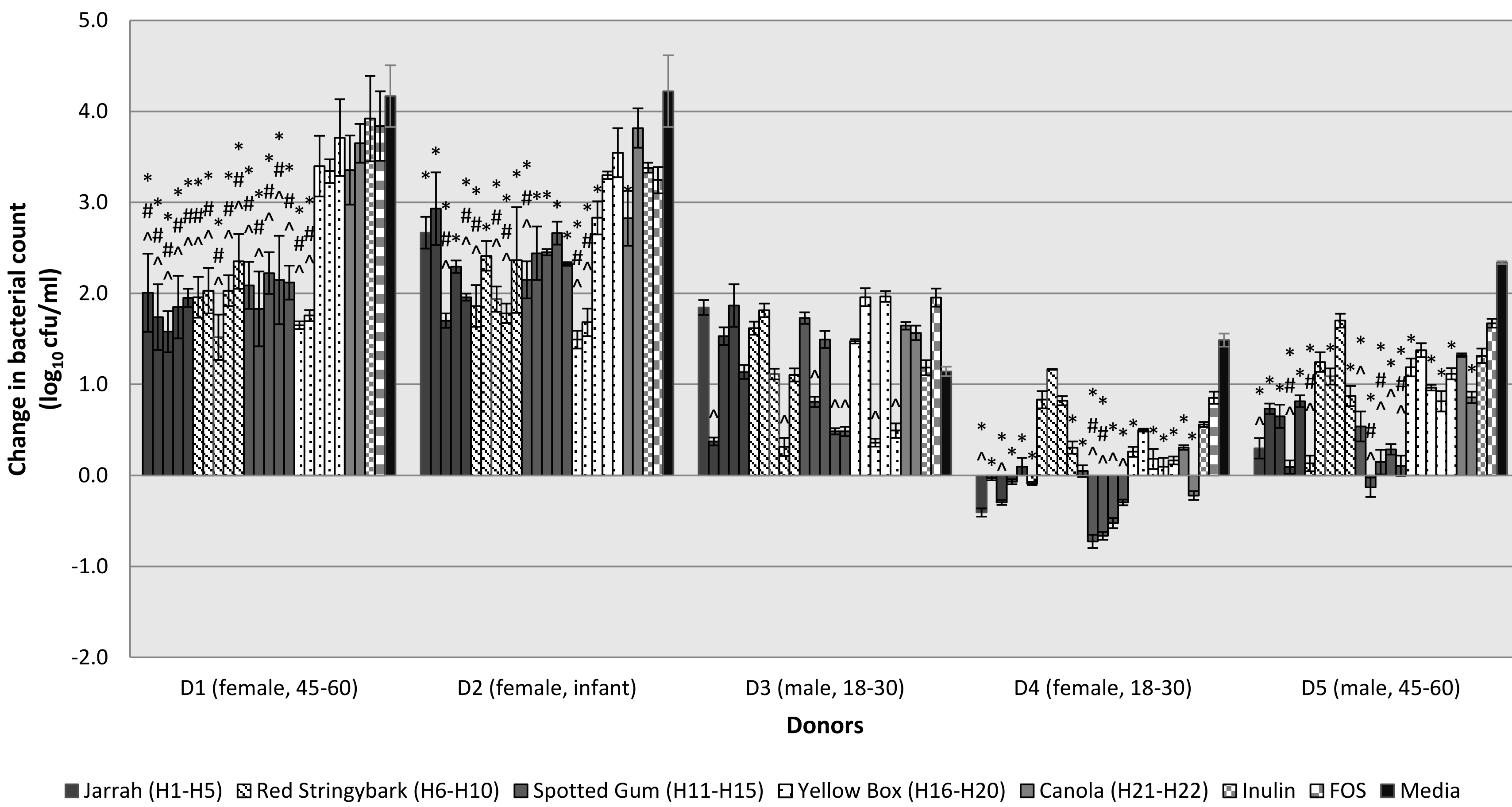


Figure 3.12 | Impact of digested honeys on clostridia numbers in microcosms established using microbiota from five donors

Mean change in bacterial counts (from initial to final) in microcosms established using microbiota from all donors. Results expressed as mean change $\log_{10}$ cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$) relative to: (*) negative control media, and positive controls: (#) inulin, and (^) FOS.

3.3.4.5 Effect of honey on growth of enteric bacteria

The change in the number of enteric bacteria (Figure 3.13) in the microcosms established using D1 microbiota are summarised below:

- counts of enteric bacteria were significantly lower in the presence of the honeys relative to the negative control. These results were confirmed in the microcosms established using D2 and D3 microbiota, but some differences were noted when D4 and D5 microbiota were used. For example, the enteric counts were similar to the negative control when most of the honeys were used in microcosms established with D4, and similar for half of the honeys in microcosms established using D5
- all honeys allowed the enteric bacteria to grow to similar levels as the inulin control. This trend was also true when the microcosms were established using D3, D4 and D5 microbiota, but not when D2 microbiota were used
- numbers of enteric bacteria were similar in the presence of the honeys relative to the FOS control. This was also true when the microcosms were established with the microbiota from the other donors, except for a small number of honey samples.

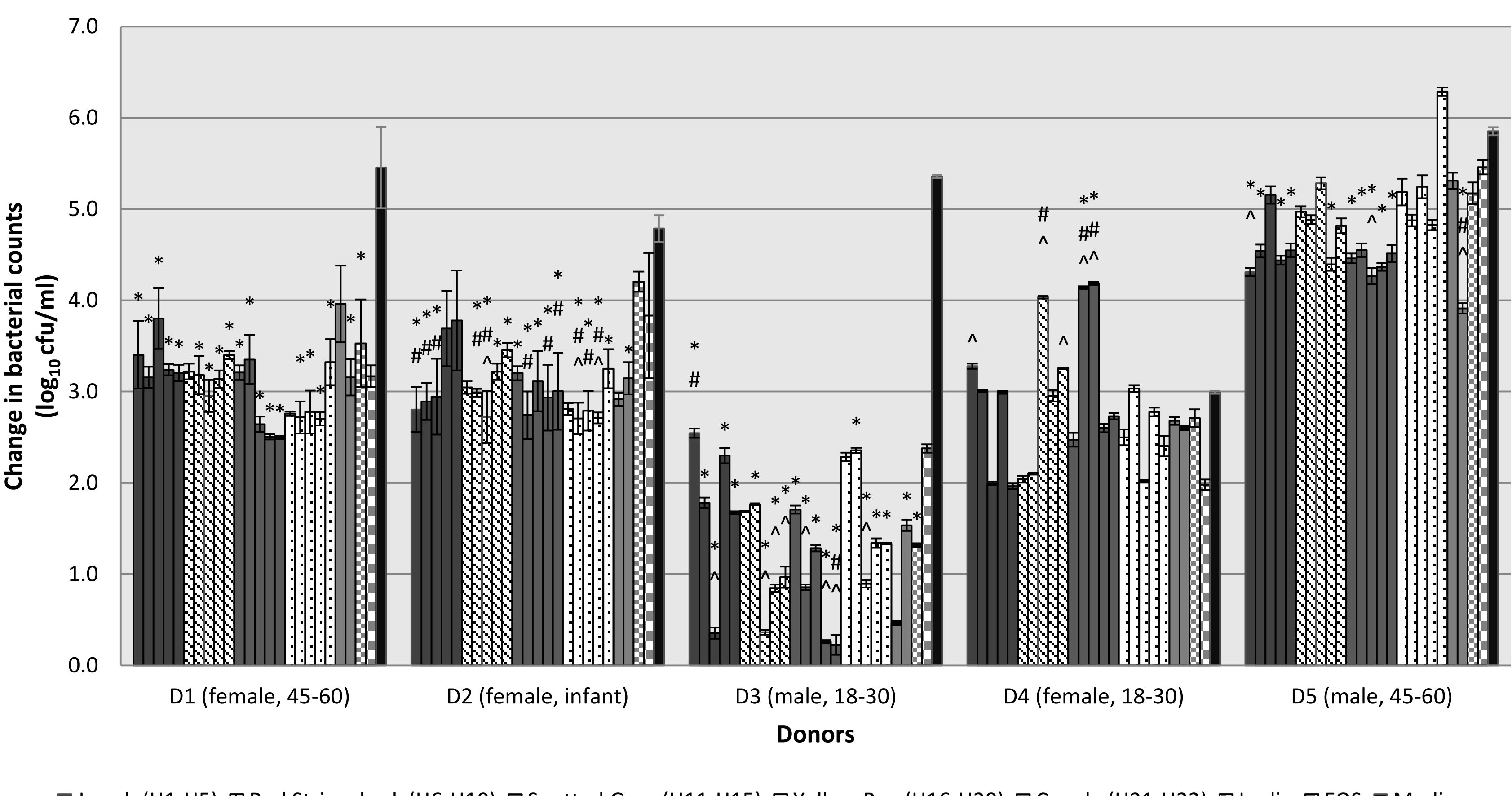


Figure 3.13 | Impact of digested honeys on enteric bacteria numbers in microcosms established using microbiota from five donors

Mean change in bacterial counts (from initial to final) in microcosms established using microbiota from all donors. Results expressed as mean change log_{10} cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$) relative to: (*) negative control media, and positive controls: (#) inulin, and (^) FOS.

3.3.4.6 Effect of honey on growth of enterococci

Enterococci levels (Figure 3.14) in the microcosms established using D1 microbiota are summarised below:

- counts were significantly lower in the presence of all 22 honeys compared to the negative control. This pattern was supported in the microcosms established with D2 and D3 microbiota, but in those established with D4 and D5, the levels of enterococci were similar to the negative control for a majority of the honey samples
- significantly smaller increases in enterococci levels were observed in the presence of most of the honey samples compared to the inulin and FOS controls. This was also true for the microcosms established using D2 and D3 microbiota, however, when D4 and D5 were used, the counts of enterococci were mostly similar to the positive controls.

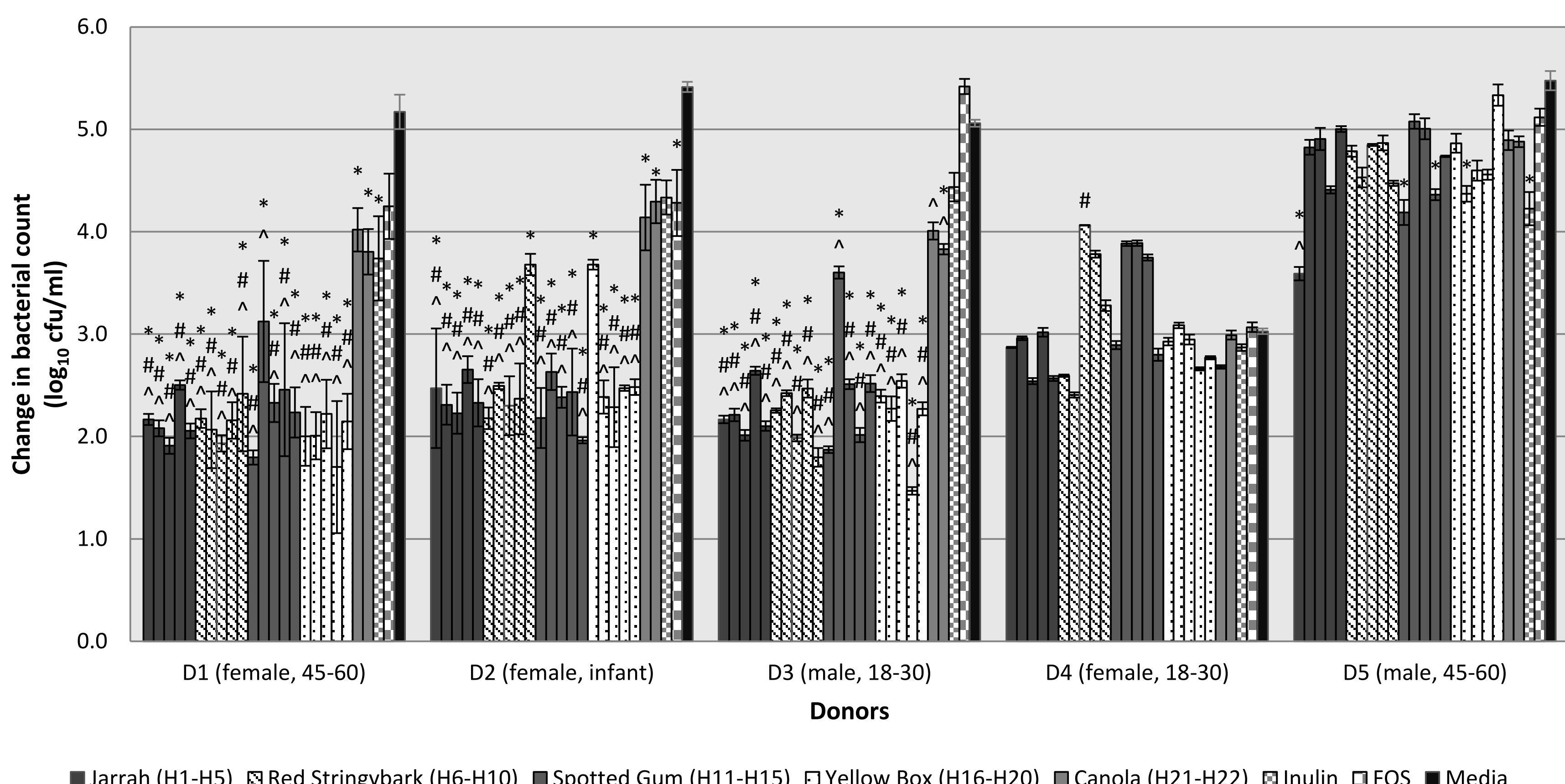


Figure 3.14 | Impact of digested honeys on enterococci numbers in microcosms established using microbiota from five donors

Mean change in bacterial counts (from initial to final) in microcosms established using microbiota from all donors. Results expressed as mean change $\log_{10}$ cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$) relative to: (*) negative control media, and positive controls: (#) inulin, and (^) FOS.

3.3.5 Molecular profiling of microbiota from selected honey-enriched microcosms

The microcosm samples selected for molecular fingerprinting were chosen based on the PI values as well as the butyric acid production so that the parameters of low and high butyrate production and low and high PI values were covered. Microbiota in microcosms established using digested honeys 4, 7, 13 and 17 were studied using TRFLP to determine their molecular microbial profile. In addition, the inulin positive control and the no added honey (negative) control were included. Examples of the TRFLP profiles of the gut microbiota in honey-enriched, inulin-enriched and no honey added microcosms are shown in Figures 3.15 and 3.16 for the two different restriction enzymes used. These results were chosen to highlight the differences in the microbial diversity and/or abundance in the honey-enriched and no honey added microcosms. It is commonly accepted that each peak in the electropherogram represents one species, and the height (fluorescence intensity) of the peak corresponds to the relative abundance of the species. Although there were donor specific variations in response to the different honey types, the results presented are representative of the key observations, i.e. that the honeys markedly changed either the diversity or abundance of the microbiota compared to the negative control, whereas the variation in microbial fingerprints were fewer when the honeys and inulin control were compared.

The TRFLP profiles (using restriction enzyme *Rsa*I) of the D4 gut microbiota in microcosms established using different honeys (honeys 4, 7 and 17), inulin or no honey added are shown in Figure 3.15. Compared to the negative (no honey added) control, the profiles using any of the honeys or inulin in the microcosms were markedly different, as the negative control showed six peaks between 420 and 480 bp, and one peak at 200 bp that did not appear in the other profiles. The profiles using honeys 7 and 17 were more similar to the inulin control than honey 4, due to a small number of similar peaks between 20 and 120 bp that were not recognised in the honey 4 profile.

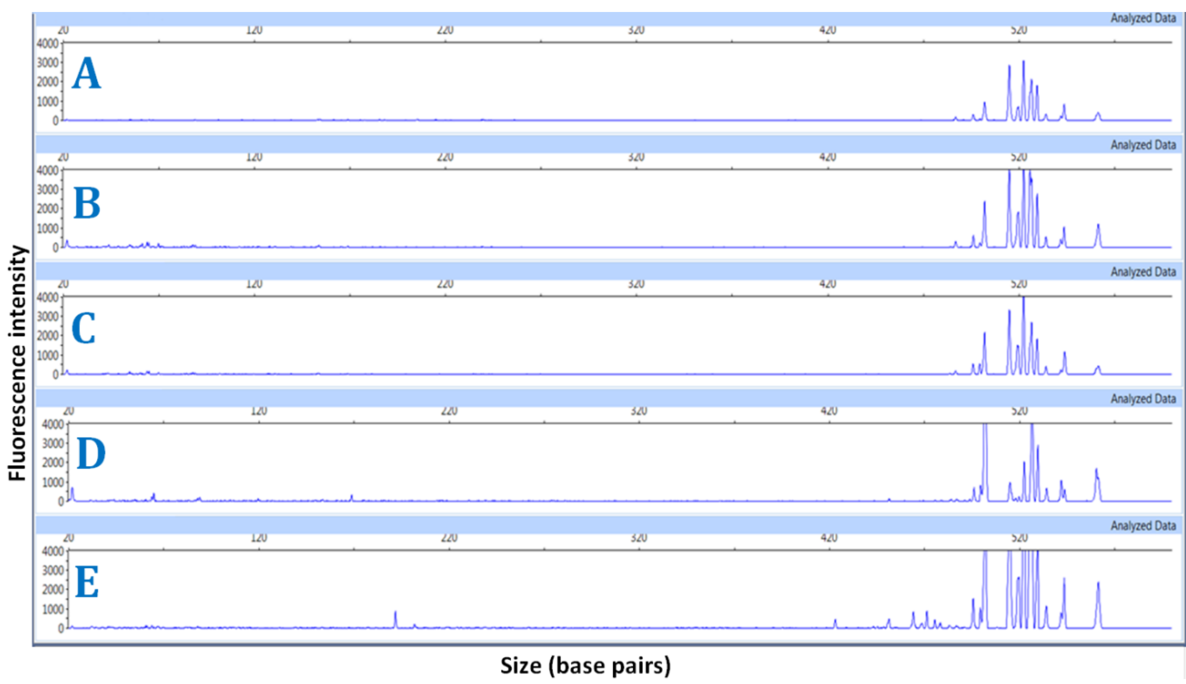


Figure 3.15 | TRFLP profiles generated using restriction enzyme *Rsa*I for microbiota from intestinal microcosms enriched with honeys

Microcosms established using: microbiota from D4 and either (A) honey 4, (B) honey 7, (C) honey 17, (D) inulin or (E) medium only (negative control)

The TRFLP profiles (using restriction enzyme *Msp*I) of the D5 gut microbiota in microcosms established with jarrah 4 or no honey added (negative control) are shown in Figure 3.16. The fingerprint of the microbiota grown in the honey-enriched microcosms was found to be similar in diversity (based on number and position of peaks) to the negative control. However, there was variation in the abundance of the microbiota (based on intensity, y-axis). For example, in the profiles of the microbiota of the honey-enriched microcosms, the intensity of the peaks between 90-99 bp, and at 180, 284 and 562 bp were markedly higher relative to the negative control, whereas the intensity of the peak at 295 bp was higher in the negative control.

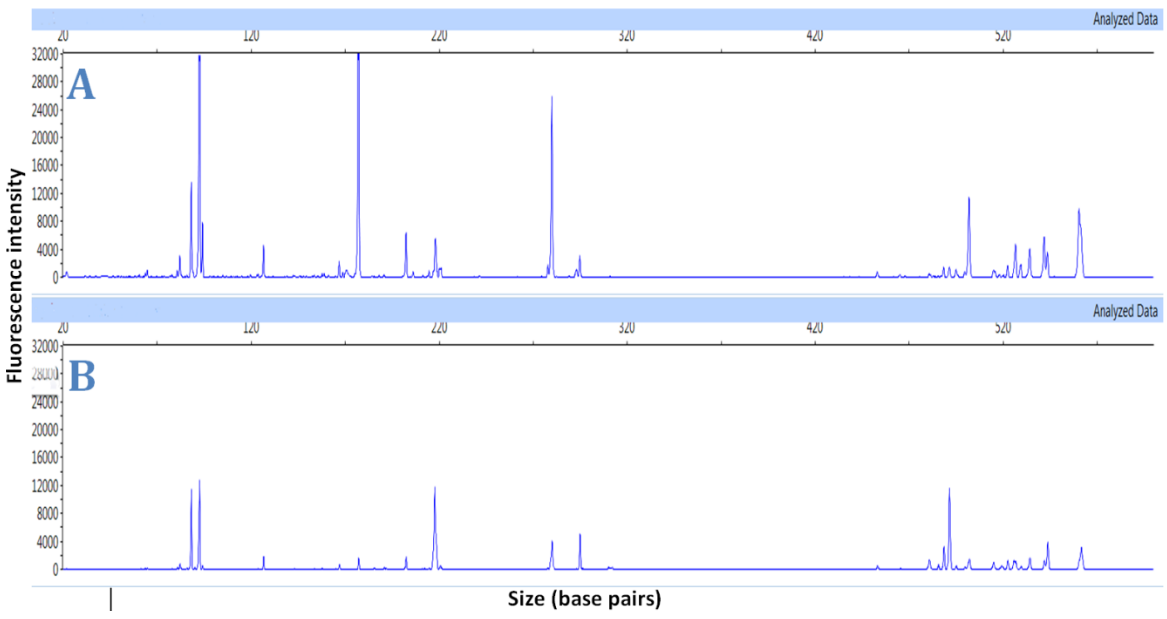


Figure 3.16 | TRFLP profiles determined using restriction enzyme *Msp*I of microbiota from microcosms enriched with honeys

Microcosms were established using microbiota from D5 and (A) jarrah honey 4, and (B) medium only (negative control)

3.3.6 SCFA production in microcosms

The SCFA produced by the gut microbiota in microcosms were enumerated before the addition of digested honey and after the anaerobic incubation with the digested honey. Separate microcosms were established for each honey using microbiota from each donor (D1 to D5), and were analysed for levels of butyric acid (Table 3.5), propanoic acid (Table 3.6), and acetic acid (Table 3.7). Of the three SCFA, butyric acid levels were most markedly increased from the initial levels in the microcosms, followed by substantial increases in acetic acid levels. The increases in propanoic acid levels were relatively low compared to the other two fatty acids.

Microcosms established using D1 microbiota showed:

- that the inclusion of honey allowed for higher production of butyric acid relative to the negative control when the jarrah honeys, and one spotted gum (honey 13) sample were used. These results were also representative of the butyric acid production in microcosms established using D2 microbiota. In contrast, the presence of the honeys in the microcosms established with the other microbiota (D3-D5) showed marked variations, as all honey samples allowed significantly higher production of butyric acid relative to the negative control, with one exception (red stringybark honey 7 when tested with the D3 microbiota). The

microcosms established using D4 and D5 microbiota showed the largest increases in production of butyric acid, of up to 46-times the initial levels detected.

- relative to the inulin control, butyric acid production was much lower with most of the honeys tested, namely all red stringybark, most spotted gum (H11, H12, H14 and H15), all yellow box and all canola samples. The microcosms established with D2 showed similar butyric acid production to the D1 studies in the presence of the honeys. Production of butyric acid in the microcosms set up using D3, D4 and D5 microbiota was enhanced at levels comparable to inulin in the presence of almost all varieties of honey tested, with especially high levels detected in the D4 and D5 microcosms
- relative to the FOS control, some digested jarrah honeys (H1, H2, H3 and H5) allowed for significantly higher levels of butyric acid production, whereas some red stringybark (H6-H9), spotted gum 15 and yellow box 16 showed significantly lower butyric acid levels. In the microcosms established using D2 microbiota, butyric acid levels in the presence of honey were similar to FOS, with the exception of the jarrah samples which were significantly higher. Butyric acid levels in the microcosms established using the other donor microbiota (D3-D5) were significantly enhanced relative to the FOS control for the majority of the honey samples.

Table 3.5 | Enumeration of butyric acid in microcosms

Mean SCFA concentrations ± SD from three separate trials. Values in italics represent the starting concentration of SCFA, and values in bold show a significant increase from the initial level.

		Butyric acid (μM)									
		D1 (female, 45-60)		D2 (female, infant)		D3 (male, 18-30)		D4 (female, 18-30)		D5 (male, 45-60)	
Honey type	Substrate										
	Initial	*0.50*	*±0.07*	*0.32*	*±0.04*	*0.84*	*±0.09*	*0.93*	*±0.09*	*1.07*	*±0.06*
Jarrah	H1	**14.20**[*^]	±1.73	**9.40**[*^]	±0.53	**16.74**[*#^]	±1.75	**22.87**[*#^]	±2.22	**34.25**[*#^]	±1.92
	H2	**12.84**[*^]	±1.22	**6.78**[*]	±0.28	**21.19**[*#^]	±2.21	**46.84**[*#^]	±4.54	**33.06**[*#^]	±1.86
	H3	**15.35**[*^]	±0.96	**8.60**[*^]	±0.12	**18.73**[*#^]	±1.95	**31.87**[*#^]	±3.09	**31.56**[*#^]	±1.77
	H4	**8.71**[*]	±0.79	**4.81**[*]	±0.25	**16.96**[*#^]	±1.77	**27.85**[*#^]	±2.70	**35.40**[*#^]	±1.99
	H5	**10.63**[*^]	±0.60	3.82[#]	±0.20	**15.22**[*#^]	±1.59	**32.13**[*#^]	±3.12	**36.96**[*#^]	±2.08
Red stringybark	H6	1.23[#^]	±0.27	0.64[#]	±0.06	**10.22**[*]	±1.07	**29.93**[*#^]	±2.90	**37.62**[*#^]	±2.11
	H7	0.87[#^]	±0.21	0.61[#]	±0.04	4.51[#]	±0.47	**13.98**[*]	±1.36	**18.75**[*]	±1.05
	H8	1.82[#^]	±0.19	0.82[#]	±0.03	**12.08**[*]	±1.26	**31.04**[*#^]	±3.01	**49.88**[*#^]	±2.80
	H9	1.10[#^]	±0.12	0.68[#]	±0.03	**11.16**[*]	±1.16	**27.85**[*#^]	±2.70	**27.40**[*#^]	±1.54
	H10	2.13[#]	±0.20	0.69[#]	±0.03	**13.50**[*^]	±1.41	**27.27**[*#^]	±2.65	**40.22**[*#^]	±2.26
Spotted gum	H11	2.36[#]	±0.14	1.73[#]	±0.13	**15.61**[*#^]	±1.63	**26.60**[*#^]	±2.58	**53.65**[*#^]	±3.02
	H12	4.10[#]	±0.19	1.07[#]	±0.03	**16.18**[*#^]	±1.69	**28.35**[*#^]	±2.75	**39.49**[*#^]	±2.22
	H13	**7.55**[*]	±0.58	**4.73**[*]	±0.17	**18.90**[*#^]	±1.97	**32.70**[*#^]	±3.17	**44.79**[*#^]	±2.52
	H14	2.74[#]	±0.34	1.43[#]	±0.04	**15.92**[*#^]	±1.66	**20.62**[*#^]	±2.00	**38.47**[*#^]	±2.16
	H15	1.64[#^]	±0.11	1.43[#]	±0.04	**14.71**[*^]	±1.53	**32.36**[*#^]	±3.14	**41.20**[*#^]	±2.32
Yellow box	H16	1.89[#^]	±0.09	1.97[#]	±0.04	**11.21**[*]	±1.17	**28.45**[*#^]	±2.76	**38.47**[*#^]	±2.16
	H17	2.39[#]	±0.14	2.64[#]	±0.22	**9.84**[*]	±1.03	**20.45**[*^]	±1.98	**28.56**[*#^]	±1.60
	H18	3.77[#]	±0.09	3.39[#]	±0.31	**12.98**[*^]	±1.35	**33.97**[*#^]	±3.30	**36.90**[*#^]	±2.07
	H19	2.60[#]	±0.15	3.35[#]	±0.34	**12.38**[*^]	±1.29	**27.97**[*#^]	±2.71	**33.86**[*#^]	±1.90
	H20	3.38[#]	±0.17	3.37[#]	±0.32	**11.45**[*]	±1.19	**29.36**[*#^]	±2.85	**29.31**[*#^]	±1.65

		Butyric acid (µM)									
		D1		**D2**		**D3**		**D4**		**D5**	
Honey type	**Substrate**	**(female, 45-60)**		**(female, infant)**		**(male, 18-30)**		**(female, 18-30)**		**(male, 45-60)**	
Canola	H21	2.16$^{\#}$	±0.09	1.18$^{\#}$	±0.08	**10.59***	±1.10	**28.93**$^{*\#\wedge}$	±2.81	**26.28**$^{*\wedge}$	±1.48
	H22	2.08$^{\#}$	±0.22	2.27$^{\#}$	±0.08	**9.82***	±1.02	**25.72**$^{*\#\wedge}$	±2.50	**27.51**$^{*\wedge}$	±1.55
Positive control	Inulin	**10.35**$^{*\wedge}$	±0.82	**7.94***	±0.32	**11.22**$^{*\wedge}$	±1.17	**16.62***	±1.61	**16.31***	±0.92
	FOS	**5.97**$^{*\#}$	±0.35	3.66	±0.32	**8.27**$^{*\#}$	±0.86	**13.62***	±1.32	**15.44***	±0.87
Negative control	Media	0.65	±0.08	0.59	±0.14	2.47	±0.26	3.08	±0.30	4.19	±0.24

Symbols indicate statistically significant difference ($p < 0.05$) relative to the: (*) negative control, (#) positive control inulin and (^) positive control FOS.

The propanoic acid levels in the microcosms established using D1 microbiota in the presence of honey were mostly unchanged ($p>0.05$) from the negative control, with the exception of a small number of samples (Table 3.6). Similar trends were observed in the microcosms established using the other microbiota sources. Honeys that gave rise to significantly higher propanoic acid levels in any of the microcosms were jarrah samples 2, 3 and 5, red stringybark sample 8 and yellow box sample 20.

It was noted that the positive prebiotic controls showed levels of propanoic acid production similar to the negative control in the microcosms. There was one exception to this trend, namely in the microcosm established using the D1 microbiota and the inulin control, the positive control allowed significantly higher production of butyric acid relative to the negative control.

The acetic acid concentrations in the microcosms increased significantly in the microcosms established using the D1 microbiota in the presence of most of the honeys relative to the negative control (Table 3.7), and these trends were also observed in the microcosms established using D2 and D3 microbiota. In the microcosms established using D4 and D5 microbiota, all honeys allowed for significantly higher production of acetic acid relative to the negative control.

The production of acetic acid in the presence of honeys was similar to the positive inulin control in microcosms established using four of the five microbiota sources (D1-D4). In the microcosms established with D5 microbiota, the majority of the honey samples allowed for significantly higher production of acetic acid relative to inulin ($p<0.05$). These trends were also generally reflective of those when the data were compared to the FOS positive control.

Table 3.6 | Enumeration of propanoic acid in microcosms

Mean SCFA concentrations ± SD from three separate trials. Values in italics represent the starting concentration of SCFA, and values in bold show a significant increase from the initial level.

		Propanoic acid (µM)									
Honey type	**Substrate**	**D1 (female, 45-60)**		**D2 (female, infant)**		**D3 (male, 18-30)**		**D4 (female, 18-30)**		**D5 (male, 45-60)**	
	Initial	*0.29*	*±0.02*	*0.25*	*±0.01*	*0.22*	*±0.00*	*0.19*	*±0.01*	*0.35*	*±0.01*
Jarrah	H1	2.96	±0.17	2.60	±0.15	3.26	±0.06	2.74	±0.07	3.20	±0.06
	H2	3.25	±0.18	1.30	±0.07	3.25	±0.06	**5.04***	±0.14	3.32	±0.06
	H3	**4.34***	±0.24	1.32	±0.07	3.88	±0.07	3.48	±0.09	4.23	±0.08
	H4	3.48	±0.20	1.32	±0.07	3.48	±0.06	3.16	±0.09	3.66	±0.07
	H5	**4.28***	±0.24	1.31	±0.07	3.30	±0.06	3.64	±0.10	3.90	±0.07
Red stringybark	H6	0.85	±0.05	0.74	±0.04	2.23	±0.04	3.01	±0.08	4.09	±0.08
	H7	0.82	±0.05	0.33	±0.02	2.59	±0.05	3.51	±0.10	3.12	±0.06
	H8	1.93	±0.11	0.84	±0.05	2.37	±0.04	3.64	±0.10	**4.89***	±0.09
	H9	0.95	±0.05	0.53	±0.03	2.60	±0.05	3.53	±0.10	2.62	±0.05
	H10	0.83	±0.05	0.31	±0.02	2.79	±0.05	3.02	±0.08	3.89	±0.07
Spotted gum	H11	0.84	±0.05	0.31	±0.02	1.86	±0.03	2.84	±0.08	3.77	±0.07
	H12	1.38	±0.08	0.36	±0.02	1.58	±0.03	3.22	±0.09	3.50	±0.06
	H13	1.84	±0.10	1.01	±0.06	1.66	±0.03	3.00	±0.08	3.16	±0.06
	H14	1.49	±0.08	0.51	±0.03	1.40	±0.03	2.46	±0.07	3.11	±0.06
	H15	0.85	±0.05	0.84	±0.05	1.03	±0.02	3.22	±0.09	4.23	±0.08
Yellow box	H16	1.66	±0.09	1.49	±0.08	2.21	±0.04	3.37	±0.09	3.53	±0.07
	H17	1.24	±0.07	0.97	±0.05	2.03	±0.04	2.66	±0.07	3.43	±0.06
	H18	1.21	±0.07	0.96	±0.05	2.03	±0.04	3.18	±0.09	3.36	±0.06
	H19	1.51	±0.08	0.61	±0.03	2.15	±0.04	2.84	±0.08	3.20	±0.06
	H20	0.96	±0.05	0.83	±0.05	2.00	±0.04	**4.39***	±0.12	3.33	±0.06

Honey type	Substrate	Propanoic acid (µM) D1 (female, 45-60)		D2 (female, infant)		D3 (male, 18-30)		D4 (female, 18-30)		D5 (male, 45-60)	
Canola	H21	0.96	±0.05	0.72	±0.04	2.01	±0.04	4.13	±0.11	2.63	±0.05
	H22	0.95	±0.05	0.77	±0.04	2.03	±0.04	3.58	±0.10	2.61	±0.05
Positive control	Inulin	**4.48** *	±0.25	3.86	±0.22	3.03	±0.06	3.86	±0.11	2.91	±0.05
	FOS	2.86	±0.16	1.71	±0.10	2.90	±0.05	3.54	±0.10	2.35	±0.04
Negative control	Media	0.32	±0.02	0.55	±0.03	0.37	±0.01	0.23	±0.01	0.56	±0.01

Symbols indicate statistically significant difference (p <0.05) relative to the: (*) negative control, (#) positive control inulin and (^) positive control FOS.

Table 3.7 | Enumeration of acetic acid in microcosms

Mean SCFA concentrations ± SD from three separate trials. Values in italics represent the starting concentration of SCFA, and values in bold show a significant increase from the initial level.

		Acetic acid (µM)									
Honey type	**Substrate**	**D1 (female, 45-60)**		**D2 (female, infant)**		**D3 (male, 18-30)**		**D4 (female, 18-30)**		**D5 (male, 45-60)**	
	Initial	*0.21*	*±0.01*	*0.15*	*±0.01*	*0.57*	*±0.02*	*0.77*	*±0.03*	*0.67*	*±0.03*
Jarrah	H1	**8.29***	±0.33	**7.35***	±0.29	**4.68^**	±0.19	**8.73***	±0.35	**9.19***	±0.36
	H2	2.33#^	±0.09	2.04^	±0.08	**5.37*^**	±0.21	**10.93***	±0.43	**8.31***	±0.33
	H3	**4.59#^**	±0.17	3.84^	±0.15	**6.14*^**	±0.24	**10.19***	±0.40	**8.62***	±0.34
	H4	3.31#^	±0.13	2.52^	±0.10	**5.32*^**	±0.21	**8.95***	±0.35	**8.97***	±0.36
	H5	**4.38#^**	±0.17	3.46^	±0.14	4.37^	±0.17	**10.30***	±0.41	**11.64*#^**	±0.46
Red stringybark	H6	**9.44***	±0.37	**8.13***	±0.32	**8.16***	±0.32	**10.37***	±0.41	**9.79*#**	±0.39
	H7	**9.41***	±0.37	**8.49***	±0.34	**8.19***	±0.32	**11.56***	±0.46	**12.80*#^**	±0.51
	H8	**10.48***	±0.42	**9.50***	±0.38	**8.73***	±0.35	**10.22***	±0.40	**13.95*#^**	±0.55
	H9	**7.32*^**	±0.29	**6.41***	±0.25	**8.53***	±0.34	**10.21***	±0.40	**7.37***	±0.29
	H10	**8.40***	±0.33	**7.40***	±0.29	**8.16***	±0.32	**10.92***	±0.43	**11.12*#^**	±0.44
Spotted gum	H11	**7.70***	±0.31	**7.38***	±0.29	**8.02***	±0.32	**9.89***	±0.39	**13.82*#^**	±0.55
	H12	**7.39***	±0.29	**6.46***	±0.26	**8.18***	±0.32	**10.65***	±0.42	**10.90*#^**	±0.43
	H13	3.31^	±0.13	**6.73***	±0.27	**6.12*^**	±0.24	**9.08***	±0.36	**11.30*#^**	±0.45
	H14	1.29^	±0.05	0.98#^	±0.04	**4.66^**	±0.18	**6.54*^**	±0.26	**11.48*#^**	±0.45
	H15	**8.44***	±0.33	**8.04***	±0.32	**8.16***	±0.32	**9.31***	±0.37	**12.80*#^**	±0.51
Yellow box	H16	**10.05***	±0.40	**7.41***	±0.29	**8.19***	±0.32	**7.87*^**	±0.31	**11.00*#^**	±0.44
	H17	**8.91***	±0.35	**7.46***	±0.30	**8.28***	±0.33	**10.53***	±0.42	**9.46***	±0.37
	H18	**13.36***	±0.53	**8.21***	±0.33	**8.30***	±0.33	**8.50***	±0.34	**11.25*#^**	±0.45
	H19	**12.24***	±0.48	**5.21***	±0.21	**8.16***	±0.32	**8.06*^**	±0.32	**8.12***	±0.32
	H20	**10.96***	±0.43	**9.01***	±0.36	**10.16***	±0.40	**10.55***	±0.42	**9.17***	±0.36

Honey type	Substrate	Acetic acid (µM) D1 (female, 45-60)		D2 (female, infant)		D3 (male, 18-30)		D4 (female, 18-30)		D5 (male, 45-60)	
Canola	H21	**9.84***	±0.39	**5.62***	±0.22	**8.32***	±0.33	**9.12***	±0.36	**7.59***	±0.30
	H22	**5.63*^**	±0.22	3.87^	±0.15	**6.34*^**	±0.25	**8.57***	±0.34	**7.41***	±0.29
Positive control	Inulin	**9.56***	±0.38	**5.80***	±0.23	**7.17*^**	±0.28	**7.95***	±0.31	**5.66***	±0.22
	FOS	**11.32***	±0.45	**8.11***	±0.32	**10.92***	±0.43	**12.39***	±0.49	**6.89***	±0.27
Negative control	Media	0.83	±0.03	0.75	±0.03	0.74	±0.03	1.04	±0.04	1.01	±0.04

Symbols indicate statistically significant difference ($p < 0.05$) relative to the: (*) negative control, (#) positive control inulin and (^) positive control FOS.

3.4 DISCUSSION

The work presented in this chapter shows that the Australian honeys that were tested have prebiotic properties based on their ability to positively influence the gut microbiota. All honeys tested support the growth of the potentially beneficial lactic acid bacteria (bifidobacteria and lactobacilli) at levels comparable to the commercial prebiotics, inulin and FOS. Previous work has shown that honey can beneficially stimulate the gut microbiota at levels similar to commercial prebiotic oligosaccharides such as FOS, GOS and inulin (Shamala *et al.*, 2000; Chick *et al.*, 2001; Kajiwara *et al.*, 2002). However, these studies use honey in its whole, undigested form which is not expected to reflect the *in vivo* situation. In addition, often the honey used was viewed as a generic product, with little regard to its floral source and composition, which are known to influence the properties of the honey. In this study, a large number of well-characterised Australian honeys of known floral source and sugar composition were tested for their effects on the growth and metabolic function of human gut microbiota from a number of donors using three techniques, namely cultural enumeration, detection of SCFA production and molecular fingerprinting. The honeys were tested in their whole and digested forms, and the results suggested that the non-digestible components of the digested honey (such as the oligosaccharides) are most likely to be driving the prebiotic activity of the honeys, as the effects were evident even after the digested honey samples were used (Figures 3.9 and 3.10).

3.4.1 The impact of *in vitro* digestion on the prebiotic assessment of honeys

The current PI studies showed distinct differences between the values obtained using undigested (whole) honey compared to digested honey (Figures 3.1 and 3.2). In general, the whole honey samples resulted in higher PI values than their digested counterparts. This could lead to certain samples being misjudged as having good (or better) prebiotic activity than they could be anticipated to have if tested *in vivo*. Since the non-digestible components are of interest as prebiotic candidates, measuring the PI of whole honey could be misleading, as demonstrated in Figures 3.1 and 3.2, for example.

However, it is interesting to note that in some cases, for example jarrah honeys 2 and 4, the honeys in their digested form had higher PI values than their undigested counterparts. This could be attributed to the removal of the antimicrobial components in the honeys in the digestive pre-treatment steps, and it has been shown previously that some jarrah honeys have significant antibacterial activity (Irish *et al.*, 2011).

In the previous chapter, results based on the bacterial group counts and SCFA production in microcosms also indicated that the use of whole rather than digested honeys generally overestimated the beneficial effects of honey on the gut microbiota. Although the studies using whole honey samples can provide important mechanistic data and can be especially helpful in identifying the contributions of the components in honey that are incompletely digested or malabsorbed (e.g. fructose), the digested honey studies more closely replicate the *in vivo* situation.

In previous studies likening the prebiotic activity of honey to that of commercial prebiotic oligosaccharides such as FOS, GOS and inulin (Shamala *et al.*, 2000; Chick *et al.*, 2001; Kajiwara *et al.*, 2002), it is likely that the prebiotic effects can too be attributed to the oligosaccharides present in the honeys tested; however without the digestive pre-treatment step they do not negate the contribution of the simple sugars and other digestible components of honey. Sanz *et al.* (2005), using an *in vitro* fermentation system, studied the effect of honey oligosaccharides on the growth of faecal bacteria (Sanz *et al.*, 2005). These researchers identified that by using whole honey, the high amounts of glucose and fructose present (which would normally be metabolised in the GIT) could contribute to the growth of bacteria using *in vitro* systems, as was also seen in the work presented in this thesis.

Although it is impossible to mimic the *in vivo* situation due to the inherent complexity of the digestive process, *in vitro* digestion models that employ pre-treatments with digestive enzymes, bile salts and consecutive incubations to simulate the GIT can provide a more realistic approach to the assessment of functional foods, particularly for prebiotic activity. Human digestion models contribute substantially to understanding the fate of a carbohydrate following ingestion, and studies of the prebiotic activity of oats, brans and other non-digestible carbohydrates have utilised digestive pre-treatment steps to more closely replicate the *in vivo* situation (Beer *et al.*, 1997; Kedia *et al.*, 2008; Hur *et al.*, 2011). Similar methods were adopted here for the assessment of the prebiotic activity of honey oligosaccharides.

3.4.2 The beneficial effects of digested honeys on gut microbiota

The results from this chapter show that Australian floral varieties of honey have a number of beneficial effects on the human gut microbiota. As these effects were observed after the digestive pre-treatment of the honeys, it shows that the non-digestible components of the honeys, such as the oligosaccharides, were responsible.

The digested honeys produced favourable changes in the human gut microbiota in the following ways:

i) prebiotic activity was comparable to commercial prebiotics, as measured by PI
ii) enhanced growth of potentially beneficial bacteria
iii) inhibition of potentially harmful bacteria
iv) increased production of SCFA, particularly the clinically important butyric acid.

The beneficial effects (discussed in detail below) are comparable to commercially available oligosaccharides, inulin and FOS. The results obtained here are of note, especially when the oligosaccharide concentrations of the honeys are taken into consideration (Figure 3.8); they make up only 2 to 4 % of the final sugar composition of the honey samples. Since the oligosaccharide concentrations in the microcosms established with honey were 25 to 50 times less than the positive controls, it is possible that the prebiotic activity of the honey oligosaccharides would surpass those of the positive controls if used at the same concentrations. In future studies, it would be of interest to establish the microcosms with the oligosaccharide concentrations of the positive control similar to those in the honeys as this would allow a more accurate comparison of the prebiotic effects.

3.4.2.1 Prebiotic activity of digested honey determined by PI

The PI value is based on a ratio of potentially beneficial to potentially harmful bacteria, with increases in the numbers of potentially beneficial bacteria scoring positively, and increases in the less desirable bacteria scoring negatively (Palframan *et al.*, 2003). A positive PI value implies that the beneficial bacteria outnumbered the potentially harmful ones, and can be used as a means of identifying carbohydrates with prebiotic potential. The cultural enumeration work performed in this chapter demonstrated that the numbers of lactobacilli and bifidobacteria in the microcosms exceeded those of clostridia and bacteroides when honey was supplied as a substrate. Therefore, it is not surprising that the PI values for all of the Australian floral varieties of honey tested here were positive, indicating that they could be effective prebiotics (Figures 3.4 to 3.7).

As lactobacilli numbers had the largest increases (Figure 3.10), this group was identified as driving the PI values. However, there were considerable differences in the PI values of the different honey samples, even those from within the same honey type, and this could be explained by the compositional variations of the individual honey samples. For example, the PI of the jarrah honeys varied considerably from one sample to another (Figure 3.3), with honey 1 and 4 exhibiting much higher PIs than honey 2 and 3 when the D1 microbiota were used in the microcosms.

It has been previously documented that the composition of honey greatly impacts on its therapeutic potential, and has been discussed extensively in studies of the antimicrobial activity of honey (Molan, 1985; Molan, 1992; Molan, 2000; Molan, 2002). The concentration of oligosaccharides (measured as maltose + oligosaccharide) in the honey samples ranged between 2 and 4% of the total sugars. The change in oligosaccharide concentration for the various honeys could explain the differences in the PIs, however the influence of other non-digestible constituents in the honey samples on the PI could also have an impact.

In addition, the PI of individual honey samples varied depending on the microbiota used in the microcosms. For example, the PI of yellow box honey 19 was relatively high when the microcosms were established using D3, D4 and D5 microbiota, and much lower when the microbiota from D1 and D2 were used (Figure 3.6). It is possible that the variations in the honey PIs determined from different sources of microbiota could be explained by the compositional differences of the microbiota, that is, the numbers and species of bacteria differ considerably from donor to donor. However, as there were no significant differences in the positive (prebiotic) and negative (no honey added) microcosm control PI values for the different donors, it is possible that the complexity of the honeys reveals differences not detected when pure inulin or FOS were used. If the PI of honeys can be expected to vary depending on the microbiota used to establish the microcosms, various other measures of the prebiotic potential of honey should be considered in addition to PI to provide a more robust representation of their expected health benefits on the gut microbiota *in vivo*.

3.4.2.2 Enhanced growth of beneficial bacteria

All of the Australian floral varieties of honey tested in this chapter showed significant increases in the numbers of potentially beneficial bifidobacteria and lactobacilli in the microcosms (Figure 3.9 and 3.10). The bifidobacteria numbers in the microcosms were increased by up to 4.5 log_{10} from their starting values, and these were approximately 2 log_{10} greater than the changes observed in the negative control. Similarly, the lactobacilli counts increased by up to 5 log_{10} from the initial values, and these were 2-3 log_{10} greater than the negative control.

Although the different honey samples did enhance these bacteria at different levels, it was clear that the digested honeys were capable of selectively stimulating the growth of the potentially beneficial gut populations. There were several differences noted in the overall change of these bacteria related to the microbiota used, for example the microcosms established with D3 microbiota showed smaller overall change in lactobacilli and bifidobacteria numbers compared to the other donors (Figure 3.9 and 3.10). The smaller

changes could be attributed to the fewer starting numbers of these bacteria, as the initial lactobacilli and bifidobacteria numbers in the D3 microbiota were at least one log_{10} less than the other donors (data not shown). The variations in the increases of bacterial counts depending on the specific microbiota used also suggest that the growth promoting effects of the various honeys on bifidobacteria and lactobacilli may be strain specific. A recent study of the growth of five lactobacillus strains using inulin, FOS, lactulose, raftilose and honeys showed that individual strains responded more favourably to particular carbohydrate (Nagpal and Kaur, 2011). A strain specific effect was also identified in a previous *in vitro* study testing the prebiotic effects of clover honey (from USA) on commercial strains of bifidobacteria (Ustunol, 2007).

Despite these variations, the Australian honey samples tested here showed promotion of the growth of lactobacilli and bifidobacteria in the microcosms at levels that matched those achieved by the positive prebiotic controls. These results add to previous studies that have shown the bifidogenic effects of honey (Chick *et al.*, 2001; Kajiwara *et al.*, 2002; Shin and Ustunol, 2005; Ustunol, 2007), and those that have shown the ability of honey to support and promote the growth of lactobacilli (Shamala *et al.*, 2000; Chick *et al.*, 2001; Nagpal and Kaur, 2011). For example, Kajiwara *et al.* (2002) showed that honey had a growth promoting effect similar to that of FOS, GOS and inulin on five human intestinal bifidobacteria cultures (Kajiwara *et al.*, 2002). Another study using pure cultures of *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus delbrukeii* sub-species *bulgaricus* or *Bifidobacterium bifidum* compared bacterial growth in non-fat dry milk supplemented with 5% (w/w) honey, fructose or sucrose (Chick *et al.*, 2001). Results showed that honey supported the growth of all four organisms similar to that of other sweeteners. Another report compared the effect of three honeys (sourwood, alfalfa and sage) on the growth of five strains of bifidobacteria of human intestinal origin (Shin and Ustunol, 2005). Again, all honeys promoted the growth and activity of the bifidobacteria tested, in some cases at the expense of less desirable *Clostridium perfringens* and *Enterococcus aerofaciens* (Shin and Ustunol, 2005).

Following digestion, the Australian floral varieties of honey studied here enhanced the levels of the beneficial bacterial groups, indicating that the prebiotic activity observed was mainly due the non-digestible components, including oligosaccharides. As the oligosaccharide concentrations in the honeys tested were similar to one another (i.e. between 2 to 4 %) and because they were determined as a combined oligosaccharide and maltose concentration, it was difficult to extrapolate the influence of oligosaccharide concentration on the prebiotic activities. The beneficial activity of the honey oligosaccharides supports what has been previously suggested by Sanz *et al.* (2005), who showed that honey oligosaccharides, separated from the monosaccharides by three

different methods, significantly increased the counts of bifidobacteria and lactobacilli (Sanz *et al.*, 2005). Additionally, the prebiotic activity of three Malaysian honeys from Perak (two wild and one commercial sample) was tested after the removal of the simple sugars by adsorption onto activated charcoal. All three honeys were shown to support the growth of *Bifidobacterium longum*. The prebiotic effect observed was attributed to the fructo-oligosaccharides that were identified in the honeys (Jan Mei *et al.*, 2010). In a study by Shin & Ustunol (2005), the increase in bifidobacteria numbers conformed to the oligosaccharide content of the honeys, whereby the sourwood honey (10.9 % oligosaccharide concentration) was the most effective at boosting bifidobacteria levels, followed by alfalfa (5.5 % oligosaccharide) and then sage (3.8 % oligosaccharide), however no significant differences were identified between the honeys.

The results in this chapter show that lactobacilli were largely more responsive to the growth enhancing effects of the honeys tested here. Interestingly, studies using inulin, FOS or other commercial prebiotics have generally shown a more bifidogenic effect rather than an elevation of the lactobacilli (reviewed in (Gibson, 1999 and Gibson *et al.*, 2004)).

The improved growth of bifidobacteria and lactobacilli in the presence of various honeys could have industrial applications, for example honey could be used as a sweetener (and preservative) for food products containing probiotic cultures (such as yoghurts).

3.4.2.3 Inhibition of potentially harmful bacteria

The inclusion of honey in the microcosms had a considerable inhibitory effect overall on the potentially harmful bacterial groups, clostridia and bacteroides and if the microbiota of all donors was considered collectively, the changes in these bacterial group numbers were significantly lower than the other bacterial groups counted (in the presence of honey).

Reduced numbers of clostridia in the presence of honey were also observed in Chapter 2. It should be noted that the treatment used for enumerating clostridia (ethanol treatment) was selective for spores. The lower numbers of clostridia could be a result of the treatment killing the vegetative cells. The inhibitory effect of honey on clostridia was not donor specific, although there were differences in the level of inhibition depending on the microbiota used to set up the microcosms (Figure 3.12). The difference in bacterial composition in the donor samples would account for the variation in the inhibitory effect observed. The change in clostridial numbers in microcosms established using microbiota from D3, D4 and D5 donors was substantially lower than those in the microcosms established using D1 and D2 (Figure 3.12). This may have been due to the variation in the

initial numbers of clostridia in the microbiota samples, as these donors had much higher levels of clostridia D1 and D2 (data not shown).

A suppressive effect was also seen on the bacteroides counts overall, as well as the enterics in most cases, with the exception being the studies using D5 microbiota. In microcosms established using D3, D4 and D5 microbiota, the change in bacteroides numbers in the presence of honey was not significantly different to the negative control (Figure 3.11) and this was in contrast to the results seen when the microbiota of the other two (D1 and D2) donors were used. As with the clostridial results, this may have been due to the compositional variation of the microbiota samples, as these donors had higher initial levels of bacteroides than D1 and D2 (data not shown).

In the whole honey studies, the inhibitory effects of the various honeys on the potentially pathogenic bacterial groups could be partially attributed to the antimicrobial properties of honey including osmolarity, acidity, production of hydrogen peroxide and presence of non-peroxide factors, as previously identified (Molan, 1992; Molan, 1992). Honey has been previously shown to inhibit gut pathogens (Shamala *et al.*, 2002; Badawy *et al.*, 2004; Alnaqdy *et al.*, 2005; Wilkinson and Cavanagh, 2005; Lin *et al.*, 2011; Hammond and Donkor, 2013), and has been used extensively throughout history to prevent and treat peptic ulcers, diarrhoea and bacterial gastroenteritis due to its antimicrobial properties (Crane, 1980; Haffejee and Moosa, 1985; Bogdanov *et al.*, 2008). However, it is unlikely that the antimicrobial properties of honey would remain active once the honey samples were subjected to the digestive pre-treatment steps.

One explanation for the lower numbers of clostridia and bacteroides could be that these bacteria could not use the digested honey components as well as the other groups in the microcosms. It is possible that the potentially harmful groups were only able to grow well after cross-feeding, i.e. unable to use the oligosaccharides and other non-digestible components unless they had been degraded by other bacterial groups as suggested by (Gibson and Roberfroid, 1995). Additionally, the metabolic end products excreted by one species in the gut can serve as a growth substrate for others (Gibson and Roberfroid, 1995; Maathuis *et al.*, 2009) and it is possible that the metabolic end products of certain species in the presence of honey changed to those that were not able to be used by the potentially harmful bacteria.

It is also likely that the suppressive effect observed in the digested honey studies is due to the increases in the aforementioned beneficial bacterial groups, lactobacilli and bifidobacteria. These bacterial groups are known for their ability to inhibit the growth of harmful bacteria by competition and the production of antimicrobial substances (Gibson

and Roberfroid, 1995; Eckburg *et al.*, 2005). Increased numbers of lactobacilli and/or bifidobacteria have been associated with resistance to infections by enteric pathogens (Gibson *et al.*, 2004; Manning and Gibson, 2004; Clarke *et al.*, 2012).The decreases in numbers of clostridia, bacteroides and enterics (Figures 3.11 to 3.13) generally correlated to the relative increases of lactobacilli and/or bifidobacteria. This supports what has been observed previously when faecal bacteria were incubated with FOS or inulin, as both selectively stimulated the growth of bifidobacteria while maintaining *E. coli* or clostridia levels low (Wang and Gibson, 1993; Reddy, 1999).

The bacterial enumeration results (Figures 3.9 to 3.14) presented here suggest that the lactobacilli and bifidobacteria in the microcosms can readily use the non-digestible components of the honey samples, as their growth was significantly enhanced relative to the potentially harmful clostridia and bacteroides. It is probable that the digested honey allowed the lactobacilli and bifidobacteria to produce enhanced levels of SCFA as well as other inhibitory substances that aided in suppressing the growth of the other groups. The suppression of less desirable bacterial groups by lactobacilli and bifidobacteria has been shown in studies that used commercially available prebiotics (Gibson and Roberfroid, 1995; Gibson, 1999; Gibson *et al.*, 2004). It has been shown that the dietary administration of FOS or inulin increased bifidobacteria counts at the expense of enteropathogens (bacteroides, clostridia, fusobacteria and/or Gram-positive cocci) (Gibson *et al.*, 1995). Another study using three honeys of different floral source (sourwood, alfalfa and sage) also found that the honeys inhibited the growth of some less desirable bacteria including *Clostridium perfringens* and *Eubacterium aerofaciens* (Shin and Ustunol, 2005). These researchers also performed co-culturing experiments (that is, the previously mentioned species together with *Bifidobacterium* spp.), and results showed that *C. perfringens* and *E. aerofaciens* were inhibited in the presence of honey and further inhibited in the presence of *Bifidobacterium* spp. as the growth of *Bifidobacterium* spp., and the lactic and acetic acid production were enhanced by the honeys.

3.4.2.4 Molecular profiling to support culture-based findings

The molecular fingerprint of the gut microbiota of some donors in honey-enriched, inulin-enriched and no honey added microcosms were determined by TRFLP (Figures 3.15 and 3.16). There were differences noted in the molecular fingerprints of D4 microbiota in honey- or inulin-enriched microcosms compared to the no honey added microcosm (negative) control. The cultural enumeration studies noted that the negative control showed significantly lower levels of lactobacilli and bifidobacteria, and significantly higher levels of clostridia (Section 3.3.4) and this could account for the additional peaks in between the 420 – 480 bp range. In addition, there were some differences noted in the profiles of the microbiota grown in red stringybark 7, yellow box 17 or the inulin-enriched

microcosms, compared to the jarrah 4 enriched ones. From the enumeration studies, it was found that in the microcosms established with jarrah 4, the final numbers of clostridia were significantly lower, and bifidobacteria and bacteroides significantly higher relative to the red stringybark 7, yellow box 17 and inulin studies (which were similar) and this could account for the differences in microbial fingerprint.

The TRFLP profiles of the D5 gut microbiota from microcosms enriched with jarrah 4 honey or from microcosms with no added honey showed that although the diversity (number of peaks) was comparable, the relative abundance (peak height) of the species changed noticeably (Figure 3.15). In studies that have used similar parameters (i.e. primers, restriction enzymes and human faecal samples), peaks between 90-99 were identified as *Bacteroides* spp.; at 212-213 bp, 220-225 bp, and 285 -295 bp were identified as *Clostridium* spp.; and at 562 bp were identified as *Lactobacillus* spp. (Wang *et al.*, 2004; Sjöberg *et al.*, 2013). If these identifications are applied to the TRFLP profiles seen in this thesis, it can be concluded that the profiles of the microbiota in the jarrah honey 4 enriched microcosms showed higher abundance of *Bacteroides* spp., and *Lactobacillus* spp., whereas the clostridial species were varied as some peak intensities were higher in the honey-enriched microcosms and some higher in the no honey added control microcosms. The cultural enumeration data shows that the lactobacilli numbers in the honey-enriched microcosms were significantly higher than the negative control (no honey added), and that the final clostridia numbers between the two microcosms were comparable, and this is supported by the TRFLP findings. However, the bacteroides counts were substantially higher in the no honey added microcosms which is not reflected in the TRFLP profile. It could be argued that there are a number of unidentified peaks on the electropherogram, which could explain the disagreement in the bacteroides data. As a next step, the peaks (i.e. bacterial species) will be identified via a clone library to determine whether changes in the molecular profile correspond to those observed in the cultural enumeration studies. In future work, the use of genus- or species-specific primers would offer substantial benefits in identifying the changes in the microbial composition of the gut, as it is known that some groups (e.g. lactobacilli) have been under-represented using universal primers (Li *et al.*, 2007). While not all microcosms were examined using the molecular profiling, the profiles presented in Figures 3.15 and 3.16 are consistent with the findings using the culture-based analyses. It is acknowledged that the cultural enumeration methodology fails to detect species and that the molecular profile gives a more total picture of the microbiota, however for the purpose of this study comparing a large number of parameters and including both negative and positive controls, the cultural enumeration of the major groups yielded valuable information about the prebiotic activities of the whole and digested honeys.

3.4.2.5 Increased production of SCFA

The fermentation of certain oligo- and polysaccharides in the large intestine produces millimolar amounts of SCFA, including butyric acid shown to be clinically important (Cummings, 1981; German, 1999; Topping and Clifton, 2001; Roberfroid *et al.*, 2010). In the work presented in this chapter, the non-digestible components of the honeys also showed a favourable effect on butyric, acetic and propanoic acid production by human gut microbiota as increases observed were similar to that achieved by the positive controls which were commercial prebiotics (Table 3.5 to 3.7). Previous studies have shown increases in the production of lactic acid and SCFA by *Bifidobacterium* spp. and *Lactobacillus acidophilis* in the presence of honey (Chick *et al.*, 2001; Haddadin *et al.*, 2007; Riazi and Zia, 2008). Additionally, the effect of the honey sample on these microbes as measured by the production of lactic and acetic acid, was comparable to that of FOS, galactooligosaccharide or inulin (Kajiwara *et al.*, 2002).

Although increases in both bifidobacteria and lactobacilli numbers were observed (Figure 3.9 and 3.10) in the microcosms, there did not seem to be a clear correlation between increases in these groups and the levels of SCFA produced, nor was there a relationship with SCFA increases and changes in numbers of the other key bacterial groups. There did not seem to be a correlation between the concentration of oligosaccharides in the honey samples and the production of any of the SCFA tested. Although a relationship was not identified in previous studies, in the work presented by Shin *et al.* (2005), the lactic and acetic acid production was generally in accordance with the oligosaccharide levels in the honeys, that is, the sourwood honey (10.9 % oligosaccharide) allowed for highest production of the acids, whereas production was lower with the use of the alfalfa honey (5.5 %) and then sage honey (3.8 %) (Shin and Ustunol, 2005). It should be noted, however, that the difference in the acid production was not significantly different amongst the honeys, nor were there any effects of the honeys on bacterial growth by these workers.

It is likely that the increase in SCFA levels in the microcosms (Table 3.5 to 3.7) was due to a combination of oligosaccharide concentration in the honeys, and selective fermentation of these oligosaccharides which resulted in changes of the overall bacterial composition. Additionally, the SCFA may have been produced by species in the microcosms that were not culturable (therefore not enumerated), or the SCFA were being used in the microcosms in the cases that showed lower production.

The production of SCFA was influenced by honey type, which suggests that there may be different oligosaccharides in the honey samples and/or additional components derived from the floral source of the honey. SCFA production was also affected by bacterial

species, as there were obvious differences in the levels of SCFA depending on which of the microbiota were used to set up the microcosm. Although lactobacilli and bifidobacteria are involved in the production of acetic acid in the colon, the production of propanoic acid and butyric acid are attributed mainly to clostridia and bacteroides as suggested by Gibson (Gibson, 1999). Interestingly, despite the high levels of butyric acid in the microcosms, the honeys generally had a suppressive effect on the potentially harmful clostridia and bacteroides. It is possible that the butyric acid was produced by species or genera that were not able to be enumerated due to the limitations of cultural enumeration methods, or that the butyric acid was produced mainly by the other bacterial groups in this case. Approaches to future studies could involve monitoring the carbohydrate use of each of the bacterial groups, culture-independent enumeration techniques of the bacterial groups, and sampling for SCFA more frequently to determine if the increase in SCFA levels corresponded to increases in particular bacterial groups or species at any given time.

Although differences were noted between the donors, the inclusion of digested honey in the microcosms had a positive effect on SCFA production, and this effect was particularly impressive when the multitude-fold increases in butyric acid production were observed. For example, the butyric acid levels increased by up to ten-fold in the presence of most honey samples relative to the negative control in the microcosms established D3 and D5 microbiota, and by up to fifteen-fold in the microcosms established using the D4 microbiota (Table 3.5). All three SCFA tested here have important health promoting benefits to the host and account for the majority of those produced in the gut. Butyric acid in particular has been recognised as the preferred energy source for colonic epithelial cells and plays a key role in the maintenance of colonic homeostasis (German, 1999; Hamer *et al.*, 2008). Butyric acid exerts potent effects on a variety of mucosal functions such as inhibition of inflammation and carcinogenesis, and thus has been identified as having an valuable role in the prevention of colorectal cancer as previously suggested (Cummings, 1981; Topping and Clifton, 2001; Wollowski *et al.*, 2001; Hamer *et al.*, 2008; Roberfroid *et al.*, 2010). Dietary fibre correlated with increased butyric acid concentration in the colon and decreased colonic cell proliferation in an animal model (Boffa *et al.*, 1992). This suggests that the production of butyric acid by fermentation of dietary fibre may be an important mediator of the protective effects against human colon cancer that epidemiological studies have linked with fibre as previously proposed (German, 1999). Because of the important role of butyric acid, and the relatively low consumption of fermentable dietary fibre in the modern diet, there has been much interest in adding fibre sources to foods that rely on slow bacterial fermentation as these have been found to increase colonic butyric acid concentrations (German, 1999; Gibson, 1999). The large increases in butyric acid production in the presence of the honeys tested here are

especially valuable when these factors are considered, and honey could be used extensively as a functional food ingredient by providing a source of fermentable oligosaccharides. Most microorganisms are known to prefer carbohydrate (saccharolytic) fermentation which has been recognised as taking place predominantly in the proximal colon (Hamer *et al.*, 2008). In the distal colon where fermentable carbohydrates are depleted, there is a shift to proteolytic (protein) fermentation which can result in the production of carcinogens. As a result, the distal part of the colon is the predominant location of several gastrointestinal disorders, and it has been postulated that the production of toxic metabolites and a lower availability of SCFA are involved in the pathogeneses of these diseases (Hamer *et al.*, 2008; Sekirov *et al.*, 2010).

Increased production of SCFA has been observed with FOS, inulin, germinated barley foodstuff, guar gum, oat bran, corn starch and isomalt (Hamer *et al.*, 2008). The results obtained in this chapter show that honey is at least as effective at boosting SCFA levels as both commercial prebiotics, inulin and FOS. The ability of human gut microbiota to selectively ferment the honey oligosaccharides from a range of Australian floral varieties of honey to produce large quantities of SCFA shows great promise for the use of honey for intestinal health benefits.

3.4.3 Relationship between PIs and oligosaccharide concentrations in honey

A relationship was noted between the PIs of the honeys tested and their corresponding oligosaccharide + maltose concentrations, however this relationship was weak as there were a number of outliers. Only one previous study has determined PI values of honey oligosaccharides, and the results from this study suggested that PI value may have been influenced by the concentration of oligosaccharides in the samples (Sanz *et al.*, 2005). These researchers extracted the honey oligosaccharides using three techniques (activated charcoal, yeast treatment and nanofiltration), and each fraction had a slightly different oligosaccharide content. Of the fractions, the PI value was the highest for the charcoal fraction which contained the highest oligosaccharide content. The FOS control (pure oligosaccharide) showed the highest PI of the study. However, it should be noted that the oligosaccharide concentrations following the various extraction techniques were not significantly different to each other, nor were the corresponding PI values to each other. Therefore, no conclusions can be made regarding a correlation between PI and the oligosaccharide content of the honeys.

There are several factors to consider before a conclusive relationship between PI and oligosaccharide content can be assumed. In the present study, the oligosaccharide content of the honeys was determined in combination with the maltose concentrations

(Appendix 2). In order to identify a correlation between PI and oligosaccharide concentration, the oligosaccharide components must be quantified without maltose (which would not be expected survive the digestive pre-treatment steps and therefore not contribute to the calculation of the PI from the *in vitro* microcosms).

Additionally, the concentrations of oligosaccharide + maltose in the honey samples were within a very small range of 2 – 4%, whereas the resulting PI values were far more varied. While oligosaccharide content may be useful as an initial assessment of prebiotic potential, the results indicate that the PI values of the various honeys were influenced by more than just the oligosaccharides. It would be of interest to test a range of honeys with varying oligosaccharide concentrations to better understand if they were the main determinants of the PI.

3.4.4 Limitations of PI

The Prebiotic Index calculation was used to deliver a quantitative means of comparing the prebiotic activity of different honeys in an *in vitro* gut model. Although PI can provide an initial assessment of the expected beneficial effects of honey on gut microbiota, it does not fully reflect prebiotic capability as it only considers the change in numbers of key bacterial groups. The results presented in this chapter revealed that all of the honeys tested had significant growth promoting effects on the potentially beneficial lactobacilli and bifidobacteria (Figure 3.9 and 3.10). Often, this was at the expense of the potentially harmful clostridia and bacteroides, resulting in a high PI value relative to the negative control (Figures 3.3 to 3.7). The benefits of the various honeys on the gut microbiota extend beyond what was depicted by the PI. For example, if the PI alone was used to assess the prebiotic capabilities of the honeys tested, it could be assumed that generally the jarrah honeys showed most potential as a prebiotic while the spotted gum honeys were least effective. However, when the effects of the honeys on the growth of the bacterial groups not included in the PI equation are considered (i.e. enteric bacteria and enterococci), along with the effects on SCFA production (e.g. increased butyric acid production), different conclusions may be drawn. This was particularly evident when SCFA (especially butyric acid) levels were analysed as those honeys that performed poorly based on PIs showed promise as SCFA boosting honeys, such as the spotted gum honeys - honey 13 in particular (Table 3.5).

The PI calculation is based on changes of bacteria in real numbers (rather than $\log_{10}$ values) which are normalised by expressing the values in relation to their starting levels, and then as a proportion of total bacterial counts (Palframan *et al.*, 2003). One limiting factor of the PI calculation is obtaining the count for total bacteria, particularly when cultural enumeration techniques are used. Although selective and non-selective culture

based approaches have been the standard techniques used to quantitate faecal bacterial populations, it is known that not all bacteria can be cultivated, resulting in a misrepresentation of bacterial population sizes and microbial diversity (O'Sullivan, 1999; Gracias and McKillip, 2004; Macfarlane and Macfarlane, 2004; Sekirov *et al.*, 2010). Additionally, methods of sampling, storage and cultivation technique (for example, media used) have been identified as leading to differences in total bacterial counts (Bonten *et al.*, 1997). This in turn can affect PI values which can limit the ability to quantitatively compare results across studies as the original authors of the PI calculation had intended. The use of molecular enumeration techniques, such quantitative PCR and fluorescent *in situ* hybridisation (FISH) can be used to provide more sensitive enumeration.

Another limitation of the PI calculation is that only four genera are considered, and these are given equal weight in the equation, as also acknowledged by the authors (Palframan *et al.*, 2003). These authors suggested that the equation could be weighted to take into account the numerically more dominant species, or expanded to include other bacterial groups. Inclusion of other bacterial groups that were studied in this chapter, namely the enterics and enterococci, would alter the PI values substantially. However, the difficulties of how to categorise the bacteria must be considered. Favourable bacterial groups are usually characterised by a beneficial metabolism to the host, and inarguably, lactobacilli and bifidobacteria are known to exert beneficial effects (reviewed in (Gibson and Roberfroid, 1995; Roberfroid *et al.*, 2010)). On the other hand, there is less consensus with the classification of intermediate genera, including enterococci, bacteroides, clostridia, streptococci and eubacteria, as they can be classified as potentially beneficial to health or potentially harmful, depending on the species, as previously noted (Roberfroid *et al.*, 2010). The current PI equation stipulates that bacteroides and clostridia are potentially harmful, as bacteria that are associated with toxin formation, pathogenicity and carcinogen production (usually as a result of proteolytic fermentation in the gut) generally belong to species within these groups. As such, it is reasonable to expect that the inclusion of other intermediate groups in the current PI calculation would also be treated as potentially harmful, skewing the final PI score towards a negative one. However, it should be noted that the potentially health damaging effects are only likely to occur if the species responsible become dominant, therefore the health contributing effects of these genera should not be overlooked. This is particularly important when bacteroides are considered, as these bacteria are numerically high, identified as representing up to 30 % of the total microbiota (Gibson, 1999). Additionally, it is known that some of the potentially harmful bacteria are capable of growing on the metabolic end products of other species (Macfarlane and Gibson, 1994; Macfarlane and Macfarlane,

2004), which could lead to a favourable shift in gut fermentation (i.e. from proteolytic to saccharolytic fermentation).

An alternative measure of prebiotic effect was proposed by Vulevic *et al.* (2004), namely the Measure of Prebiotic Effect (MPE). The MPE was determined by three separate equations that measured bacterial population changes, fermentation end products (SCFA), and substrate assimilation (Vulevic *et al.*, 2004). The change in bacterial populations was determined by using a 'modified' PI approach that, in addition to the previous four genera, also included eubacteria (classified as having health promoting effects), *E. coli* and sulfate-reducing bacteria (classified as having potentially harmful effects). However, the issue with categorising bacteria as having health promoting or health damaging effects remains an important consideration. SCFA production is a valuable inclusion in the prebiotic assessment of a carbohydrate, and the MPE incorporates the proportion of lactate relative to overall SCFA (acetic acid, butyric acid, propanoic acid and lactate) levels. It could be argued that each of these SCFA has substantial contributions to host well-being, and consideration of these SCFA individually may better elucidate the prebiotic activity of a carbohydrate.

The PI scores calculated by Palframan *et al.* (2003) were supportive of the qualitative conclusions drawn from a number of previous studies. However, in its current form, the PI score alone is not necessarily fully indicative of the prebiotic potential of a carbohydrate. This can be seen in the work presented here, as the PI does not always accurately reflect the full spectrum of beneficial effects (e.g. marked increases in the potentially beneficial populations, or in the production of butyric acid) of the various honeys on the gut microbiota composition and metabolic function.

The MPE does provide a more thorough analysis of prebiotic potential, but also faces some limitation with respect to categorising of bacterial groups and SCFA analysis. A further developed approach for quantitative analysis of prebiotic potential is warranted, and should include changes in beneficial bacterial populations and metabolic activity of gut microbes. Inulin, FOS and GOS are widely available commercial prebiotics, and a score based on prebiotic effects of carbohydrates (for example, PI, changes in bacterial populations, SCFA production) relative to these commercial prebiotics may be an alternative method of comparison between studies.

3.5 CONCLUSION

This study was the first in-depth assessment of the prebiotic properties of a large number of Australian honeys, of known floral source and sugar composition, using a number of approaches (microcosms, detection of SCFA, molecular fingerprinting) and human gut

microbiota from a number of donors. It is also the first to investigate the impact of digestion of carbohydrates on their prebiotic activity.

The work presented in this chapter demonstrates that Australian floral varieties of honey, and the non-digestible components in these honeys in particular, can exert beneficial effects on human gut microbes. All of the honeys tested were found to positively impact the gut microbiota in microcosms by

- promoting the growth of the beneficial bacteria, particularly lactobacilli, at levels similar to commercial prebiotics,
- suppressing the growth of the potentially harmful groups, such as clostridia, at levels that matched or surpassed the commercial prebiotics, and
- enhancing production of SCFA, particularly the clinically important butyric acid.

In many cases, the increased growth of the potentially beneficial bacteria corresponded to decreased numbers of the potentially harmful groups, resulting in a positive PI. The PI values of the honeys were varied, as was the level of SCFA production in the presence of different honey samples, which may have been caused by the presence of different types and quantities of oligosaccharides, or other non-digestible components in the honeys.

The results presented here show that all honeys tested had beneficial effects on gut microbiota, and that these benefits were varied. Depending on the desired beneficial effect, different honeys could be used to confer specific health benefits. For example a honey that had an especially enhancing effect on lactobacilli or bifidobacteria could be incorporated into probiotic containing foods, or a honey particularly effective in inhibiting clostridia could be used as part of a management of clostridia-related diarrhoea. Overall, this study was a comprehensive analysis of the prebiotic properties of Australian honeys and demonstrates various beneficial effects of honey on the gut microbiota. These benefits are likely to be effective *in vivo*, as the prebiotic assessment of the honeys was done using digested samples and mixed populations of human gut microbiota, factors that were often overlooked in previous studies. It is clear from the work presented in this chapter that Australian floral varieties of honey possess non-digestible components with prebiotic characteristics, warranting further investigation of the oligosaccharides in the honeys and also assessment *in vivo*.

4 CHAPTER FOUR

Impact of the honey-enriched intestinal microcosm on introduced bacterial species

4.1 INTRODUCTION

4.1.1 Bacterial pathogens of the gut

The most commonly identified bacteria in colonic diseases are enterotoxigenic strains of *Escherichia coli*, as well as species belonging to the genera *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, and *Aeromonas* (Gibson and Macfarlane, 1994).

A healthy balance of intestinal microbiota is essential for host health and well-being, however the indigenous gut microbiota contain certain bacteria that have the potential to become pathogenic, including species of *Bacteroides* and *Clostridium* (Gibson, 1999). An overgrowth of certain bacterial populations can result in a variety of detrimental conditions (Sekirov *et al.*, 2010). For example, the use of antibiotics can disrupt the ecological balance of the gut, leading to an overgrowth of potentially pathogenic bacteria such as the toxigenic *Clostridium difficile*, which is now implicated as the principal causative agent of pseudomembranous colitis (Gibson and Macfarlane, 1994; Guarner and Malagelada, 2003). Antibiotic-induced alterations in the intestinal microbiota were also shown to predispose the host to a higher risk of non-typhoidal *Salmonella* infection (Gradel *et al.*, 2008), demonstrating that both opportunists and pathogens are able to benefit from disturbances in the ecological balance of the gut.

The commensal bacteria have been implicated in the pathogenesis of human diseases such as inflammatory bowel disease (IBD) and colon cancer, particularly in genetically susceptible individuals (O'Hara and Shanahan, 2006). Studies have shown that IBD does not develop under germ-free conditions, suggesting a relation between IBD and normal colonic microbiota (reviewed in (Yan and Polk, 2004)). Germ-free animal models have also been used to show increased susceptibility to infection by certain bacteria, viruses and parasites in the absence of a conventionally colonised gut (reviewed in (Round and Mazmanian, 2009)). For example, germ-free animals showed decreased immune resistance to infection and increased mortality when exposed to the enteric pathogen *Shigella flexneri* (Sprinz *et al.*, 1961). In addition, germ-free animals were found to have reduced digestive enzyme activity, vascularity, cytokine production and muscle wall thickness (Shanahan, 2002).

The involvement of intestinal microbiota in the initiation and development of colon cancer has been suggested, through the production of metabolites that function as carcinogens

or their precursors (reviewed in (Heavey and Rowland, 2004)). Species of bacteroides and clostridia have been implicated in the incidence and growth rate of colonic tumours induced in animals (reviewed in (Guarner and Malagelada, 2003)). A study comparing human faecal microbiota of populations with high and low risks of colon cancer identified that the presence of *Bacteroides vulgatus* and *Bacteroides stercoris* were associated with increased risk of colon cancer whereas low risk was associated with the presence of *Lactobacillus* species and *Eubacterium aerofaciens* (Moore and Moore, 1995).

In addition, dysfunction of the gut mucosal barrier can lead to translocation of many enteric bacteria to extra-intestinal sites, such as the lymph nodes, liver and spleen. Dissemination of enteric bacteria throughout the body can lead to sepsis, shock, multisystem organ failure, or even death of the host (Guarner and Malagelada, 2003).

4.1.2 Inhibitory effects of commensal gut microbiota on enteropathogens

Under normal homeostatic conditions the intestinal microbiota are crucial in preventing colonisation by pathogens (Gorbach *et al.*, 1988; Gibson, 1999). For infection to occur the enteric pathogen needs to colonise the host, and to do so they need to be able to compete with a stable, dense and established bacterial population, and many also need to penetrate the mucus overlying the epithelial wall that acts as a barrier to infection (Round and Mazmanian, 2009; Sekirov *et al.*, 2010).

Reduced microbial diversity, i.e. the number and types of species, in the gut has been linked to compromised health (Ismail *et al.*, 2012; Cozen *et al.*, 2013; Storro *et al.*, 2013). Studies of germ-free mice showed that restoration with conventional intestinal microbiota allowed restoration of the mucosal immune system (Umesaki *et al.*, 1995), highlighting the influence of the commensal bacteria on intestinal physiology. Microbiota transplantation studies in animals have also shown that the transmission of an imbalanced gut microbiota to their healthy counterparts is sufficient to induce disease, suggesting a causative relationship (Turnbaugh *et al.*, 2008).

There are several proposed mechanisms by which commensal bacteria might inhibit enteric pathogens; these have been discussed by Gibson and Macfarlane ((Gibson and Macfarlane, 1994)) and are summarised below:

- *Competition for nutrients.* Although the gut contents are a plentiful source of nutrients, the resident gut microbes are adapted to rapidly consume all resources, depriving intruders of essential nutrients for growth. In addition, studies of germ-free mice colonised with *Bacteroides thetaiotaomicron* revealed a symbiotic relationship between the host and the bacterium, whereby production of an

essential nutrient by the host was determined by the bacterium actively indicating how much was needed (Hooper *et al.*, 1999). It is possible that other commensal bacteria also have a symbiotic relationship with the host, preventing over-production of nutrients that could otherwise favour intrusion by pathogenic microbial competitors.

- *Direct antagonism*. Fermentation by the gut microbiota leads to the production of metabolites including bacteriocins and short chain fatty acids (SCFA) (or volatile acids) which can exert inhibitory effects on invading microbes. The metabolic end products excreted by the intestinal microorganisms, particularly the acids, contribute to lowering the pH of the gut to levels below those at which pathogens are able to grow competitively (Gibson *et al.*, 1997). Fatty acids have been found to have a particularly suppressive effect on *E. coli* and *Shigella* species (Gibson and Wang, 1994). In addition, many species of lactobacilli and bifidobacteria can excrete natural antibiotics with broad spectrum activity, and bifidobacterial species have been shown to exert inhibitory effects on both Gram-positive and -negative pathogens including *Salmonella* spp., *Campylobacter* spp. and *E. coli* (Gibson and Wang, 1994).

- *Competition for adhesion sites*. Colonisation of the host receptors is an essential component of pathogenicity in the gut for some bacteria, e.g. *E. coli*, and non-pathogenic species (part of the normal gut microbiota) can prevent adhesion by their pathogenic counterparts by competitive inhibition of receptors.

- *Stimulation of immune responses.* Commensal bacteria (particularly lactobacilli) profoundly influence the development of the gut mucosal immune system (Weinstein and Cebra, 1991), and it is likely that the composition of the colonising microbiota influences individual variations in immunity (O'Hara and Shanahan, 2006).

Gram-positive anaerobic faecal bacteria (lactobacilli and bifidobacteria) had a greater inhibitory effect on the growth of enteric pathogens than Gram-negative isolates *in vitro* (Gomes *et al.*, 2006), a contributing factor to the growing interest in boosting the populations of these Gram-positive populations through the use of probiotic supplements as well as prebiotics.

Re-population of the gut microbiota with selective commensal (lactobacilli or bifidobacteria) can have applications in the treatment and/or prevention of diarrhoea, IBD and other intestinal disorders. The indigenous lactic acid bacteria of the human

gastrointestinal tract have been implicated in improved colonisation resistance. For example, increased numbers of bifidobacteria in breast-fed infants contributed towards improved exclusion of pathogens compared to those infants who were formula fed (reviewed in (Gibson *et al.*, 1997)).

4.1.3 Effect of honey on enteropathogens

Honey has inhibitory and bactericidal activity against many enteropathogenic organisms, including:

- *Salmonella* spp., such as *S. typhimurium*, *S. missisippi* and *S. enteritidis* (including multi-drug resistant strains) (Molan, 2001; Adebolu, 2005; Willix *et al.*, 1992; Badawy *et al.*, 2004; Lin *et al.*, 2011)
- *Shigella* spp., including *S. dysenteriae, S. flexneri* and *S. sonnei* (Molan, 2001; Adebolu, 2005; Al-Waili, 2004; Lin *et al.*, 2011)
- Enteropathogenic *E. coli*, including *E. coli* 0157:H7 (including multi-drug resistant strains) (Molan, 2001; Adebolu, 2005; Willix *et al.*, 1992; Al-Waili *et al.*, 2005; Al-Waili, 2004; Badawy *et al.*, 2004)
- *Enterobacter cloacae, Enterobacter aerogenes* (Al-Waili, 2004; Lin *et al.*, 2011)
- *Yersinia enterocolitica* (Lin *et al.*, 2011)

Apart from the direct antimicrobial activity of honey, it has been shown to prevent attachment of *Salmonella* spp. to mucosal epithelial cells, thus acting as a preventative in the establishment of infection (Alnaqdy *et al.*, 2005).

The use of honey in an oral rehydration solution for infants and children with gastroenteritis caused by *Salmonella*, *Shigella* and *E. coli* was effective in shortening the duration of diarrhoea (Haffejee and Moosa, 1985), and the authors attributed this effect to the antibacterial activity of the honey. This study also showed that administration of honey via a parenteral route to control *E. coli* 0157:H7 and *S. typhimurium* infection in mice was effective. The authors proposed that this effect could be attributed to the antibacterial activity of the honey and honey stimulating proliferation of lymphocytes and phagocytes that are involved in activating the immune response, as previously suggested (Molan and Russell, 1988; Tonks *et al.*, 2001). A more recent study also demonstrated that the addition of honey to the oral rehydration solution to treat gastroenteritis in infants and children reduced the frequency of bacterial and non-bacterial diarrhoea (Abdulrhman *et al.*, 2010).

4.1.4 Aim

The aim of the work presented in this chapter was to investigate the effect of *in vitro* fermentation of honey by human gut bacteria on the growth of introduced and/or invading species, using two main approaches:

i) monitoring the growth of an enteropathogen after seeding into microcosms established using human gut microbiota in the presence of honey
ii) determining whether substances produced by human gut microbiota during fermentation in *in vitro* honey-enriched microcosms were inhibitory to enteropathogens or a probiotic strain.

4.2 METHODS

4.2.1 Growth of *Salmonella typhimurium* in intestinal microcosms

The growth of the gut pathogen *Salmonella enterica* subsp. *enterica* serovar Typhimurium in the presence of faecal bacteria was explored using honey-enriched intestinal microcosms (set up as described in Section 2.2.5).

4.2.2 *S. typhimurium* growth curve

S. typhimurium (UNSW 078 300) was obtained from the School of Biotechnology and Biological Sciences culture collection at the University of New South Wales, Australia. This strain was resistant to streptomycin at a concentration of 2 mg/ml.

The culture was grown aerobically in Tryptone Soya Broth (TSB) (Oxoid) at 37 °C for 24 hours. The culture was centrifuged (3000 g for 10 min), supernatant discarded and the pellet resuspended in phosphate buffered saline (PBS). After another round of centrifuging, the resultant pellet was resuspended in 1 ml PBS and this was used to make solutions of different optical density (OD) values between 0.1 and 0.9 at 595 nm. The solutions at each of the ODs were serially diluted in peptone water (Oxoid), plated using the micro-drop plate technique (10 µl drop) on Tryptone Soya Agar (TSA)(Oxoid) and incubated aerobically at 37 °C for 24 hours. A standard curve was generated using the bacterial counts versus OD values.

4.2.3 Measurement of *S. typhimurium* growth in honey-enriched intestinal microcosms

Separate intestinal microcosms using faecal samples from donors D1, D2 and D3 (see Table 3.2) were prepared as per Section 2.2.5 using digested honey. Honeys 1 -22 and honey iii (manuka) (see Table 3.1) were used in this assay, along with the positive

prebiotic control, inulin. A microcosm containing no honey/sugar (i.e. microbiota in medium only) was set up as a negative control.

All microcosms were seeded with *S. typhimurium* (approximately 5 x 10^3 cells determined spectrophotometrically at 595 nm) and incubated anaerobically at 37 °C for 48 hours.

Plate counts for *S. typhimurium* were performed at 0 hour and 48 hours on TSA plates supplemented with 2 mg/ml streptomycin (Sigma) and incubated aerobically at 37 °C for 24 hours.

During the microcosm studies, it was noted that the pH of the microcosms dropped from pH 7.0 to between pH 5.5 – 6.5 during the 48 hour incubation. It was established that the pH drop occurred in the first 6 – 8 hours of incubation and that the pH stabilised to between pH 5.5 – 6.5 after 8 – 10 hours. In order to account for inhibition of *S. typhimurium* due to changes in pH of microcosms alone, a set of controls containing *S. typhimurium* in growth media were set up with pH levels adjusted to cover a range between pH 4.5 to 6.5 (in increments of 0.5, using 1M HCl) after 6 – 8 hours of incubation. The pH controls were incubated and plate counts performed as above.

Plate counts were performed in triplicate, and the assay was conducted on three separate occasions.

4.2.4 Inhibitory effects of honey-enriched microcosm supernatants on intestinal bacterial cultures

The effect of inhibitory substances produced by faecal bacteria during fermentation in the *in vitro* honey-enriched microcosms on three gut pathogens was explored. In order to determine whether the fermentation products of faecal bacteria had an adverse effect on the growth of probiotic bacteria, one probiotic strain was also included in these assays.

The bacterial strains used are summarised in Table 4.1. Growth curves were generated for each of the bacterial strains as per Section 4.2.2, using the appropriate growth media and conditions as outlined in Table 4.1.

Table 4.1 | Bacterial strains used in inhibition assays

Bacterial strains were all obtained from the School of Biotechnology & Biological Sciences, University of New South Wales culture collection. All growth media were obtained from Oxoid.

Strain	Reference code	Effect on gut health	Growth media	Growth conditions
Salmonella typhimurium	078 300	Gastroenteritis	Tryptone soya broth (TSB) Tryptone soya agar (TSA)	Aerobic 37 °C, 24 hrs
Escherichia coli	027 500	Gastroenteritis	Tryptone soya broth (TSB) Tryptone soya agar (TSA)	Aerobic 37 °C, 24 hrs
Clostridium difficile	523 900	Antibiotic-associated diarrhoea	Thioglycollate broth Columbia horse blood agar (HBA)	Anaerobic 37 °C, 48 hrs
Lactobacillus fermentum PC1	511 400	Probiotic	Mann Rogosa Sharpe (MRS) agar MRS broth	Anaerobic 37 °C, 48 hrs

Aliquots from microcosms established using faecal material from donors D1, D2 and D3 (Section 3.2.4) were centrifuged at 10,000 x g for 15 min. The supernatant was removed and filter sterilised using a 0.22 µm syringe driven filter (Merck Millipore).

Twenty-four hour bacterial cultures were diluted 1/100 in the appropriate liquid growth media (as in Table 4.1) prepared at double strength. Aliquots of the cultures were added to the microcosm supernatants in a 1:1 ratio (total volume 200 µl) in a 96-well microtitre plate (Nunc).

The positive growth control was prepared by adding WCA broth to the 1 % bacterial culture wells in a 1:1 ratio. The negative controls were prepared with 100 µl of WCA broth mixed in a 1:1 ratio with either TSB, thioglycollate broth or MRS broth (double strength, uninoculated).

In order to ascertain the effect of pH on the inhibition of the strains, the test wells (containing 1 % bacterial strain in double strength growth media) had pH-adjusted WCA broth added at various pH, ranging from pH 4.5 to pH 7, in increments of 0.5.

The microtitre plates were incubated at 37 °C for 24 hours; aerobically for the *S. typhimurium* and *E. coli* assays and anaerobically for the *C. difficile* and *L. fermentum* assays. The growth of the strains was determined by recording the OD at 595 nm on a microplate reader (BioRad) at 0, 4, 8, 12 and 24 hours and the growth curve of each culture used to estimate viable cells as colony forming units (CFU). Each sample was prepared in triplicate in the microtitre plates, and the assay was repeated twice more on completely independent occasions.

4.3 RESULTS

4.3.1 Growth of *S. typhimurium* in honey-enriched microcosms

The indigenous gut microbiota are known to inhibit the growth of other microbes that invade the gut by a range of mechanisms such as nutrient competition and by the production of inhibitory compounds during fermentation.

S. typhimurium was seeded into and later enumerated from honey-enriched microcosms to determine the effect of the different honeys on the growth of this pathogen in the presence of indigenous gut microbes. The results were analysed by honey type and are shown in Figure 4.1 to 4.5. Controls with various pH levels (ranging from pH 4.5 to 6.5 and adjusted after 6 hours of incubation) were included in the assays to investigate the effect that decreased pH in the microcosms had on the numbers of *S. typhimurium*. The pH controls always had the highest counts of *S. typhimurium* (with an increase of approximately 2.5 log from the initial seeded value) and the counts were comparable at the different pH values ($p=1.00$).

The microcosms were established using human microbiota from three donors (D1, D2 and D3) and results were consistent across the three donors. The *S. typhimurium* counts were significantly lower than the pH controls for all honeys tested ($p<0.05$ in all cases).

The inclusion of jarrah honeys (H1 – H5) had an overall inhibitory effect on *S. typhimurium* growth in the microcosms established using the D1 microbiota, as there were lower counts of the bacterium when compared to the negative control, medium only (Figure 4.1). The numbers were significantly lower when honeys 1, 4 and 5 were used ($p<0.01$). The jarrah honeys had a similar effect on *S. typhimurium* growth to the prebiotic control (inulin) in the microcosms ($p>0.05$). Of the jarrah honeys, honey 1 and 4 were most effective at reducing the numbers of *S. typhimurium* in the microcosms, as the counts showed an increase of only 0.46 and 0.85 log, respectively, from the initial seeded value (5×10^3 cfu/ml). The other three jarrah honeys showed a 1-log increase of *S. typhimurium* counts from the initial counts.

Some similar trends were seen in the microcosms established with the other microbiota. In the microcosms established with D2 microbiota, lower numbers of *S. typhimurium* were observed in the honey microcosms compared to the negative control, media. This was significant in all jarrah honeys ($p<0.05$) except honey 4. The effects of the jarrah honeys on *S. typhimurium* numbers were similar to inulin, except in the case of honey 4 when the counts were significantly higher than this prebiotic control ($p<0.05$). The *S. typhimurium* was more sensitive to inhibition in the microcosms using D2 relative to D1 microbiota.

When honeys 3 and 5 were used, there was a decrease in bacterial numbers from the initial value seeded into the microcosms by 0.78 and 0.61 log, respectively. Honey 4 showed a 1-log increase from the initial counts, while the increases in numbers using honeys 1 and 2 were less than 1-log. The D3 microbiota established microcosms showed that the use of jarrah honeys significantly reduced the numbers of *S. typhimurium* ($p<0.01$) when compared to the negative control (media), and the counts were similar to those when inulin was used. Furthermore, the counts of *S. typhimurium* in these microcosms were notably lower than the initial values that were added by up to 0.93 log, making the *S. typhimurium* most susceptible to the inhibitory effects taking place in the microcosms established with the D3 microbiota.

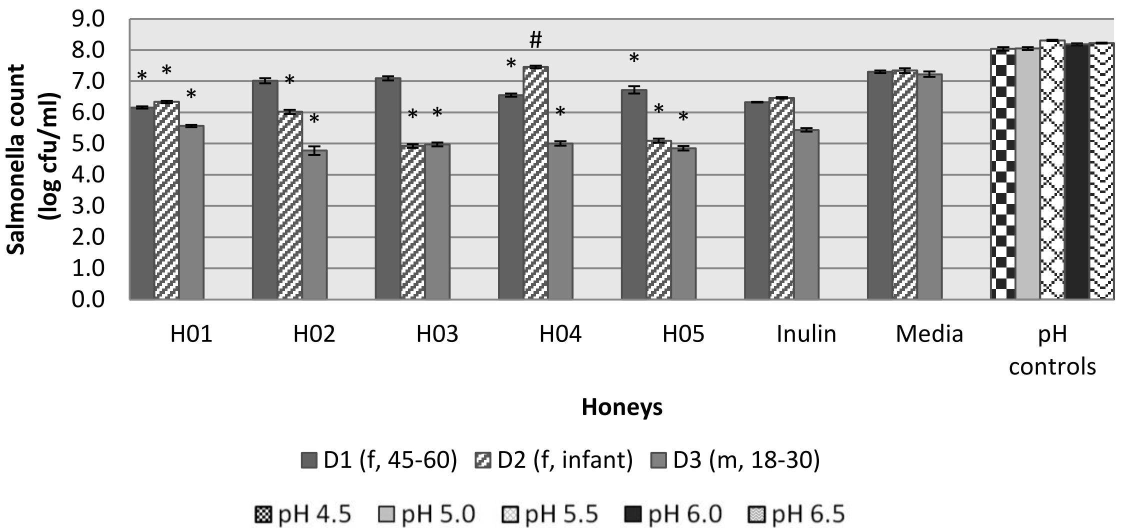


Figure 4.1 | *S. typhimurium* growth in microcosms established with jarrah honeys

Growth of *S. typhimurium* in microcosms established using microbiota from donors D1 to D3 of different gender (m, f) and age, and digested jarrah honeys. Results expressed as log cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (*) from the negative control media and (#) from the prebiotic positive control inulin.

The red stringybark honeys (H6-H10) used in the microcosms established with D1 microbiota showed lower numbers of *S. typhimurium* when compared to the negative control (media), and this was significant when honey 6 was used ($p<0.05$) (Figure 4.2). The growth of the bacterium in the presence of all five red stringybark samples was similar to the prebiotic control, inulin ($p>0.05$). Of these honey samples, honey 6 was most effective at preventing the growth of *S. typhimurium* as the counts increased by only 0.36 log, compared to an average 1-log increase when the other four honeys were used.

The counts from microcosms using D2 and D3 microbiota generally supported what was seen for the D1 studies, however there were some differences. The counts of *S.*

typhimurium were lower when the honeys were used in the microcosms compared to the negative control, however this was significant in honeys 8, 9, and 10 ($p<0.01$) which was different to what was observed in the D1 studies. The counts of *S. typhimurium* in these microcosms were lower than the levels that were originally seeded by an average of 0.6 log. All five red stringybark honeys allowed for similar growth levels of *S. typhimurium* when compared to the prebiotic control, inulin ($p>0.05$) as was shown in the studies using D1 microbiota.

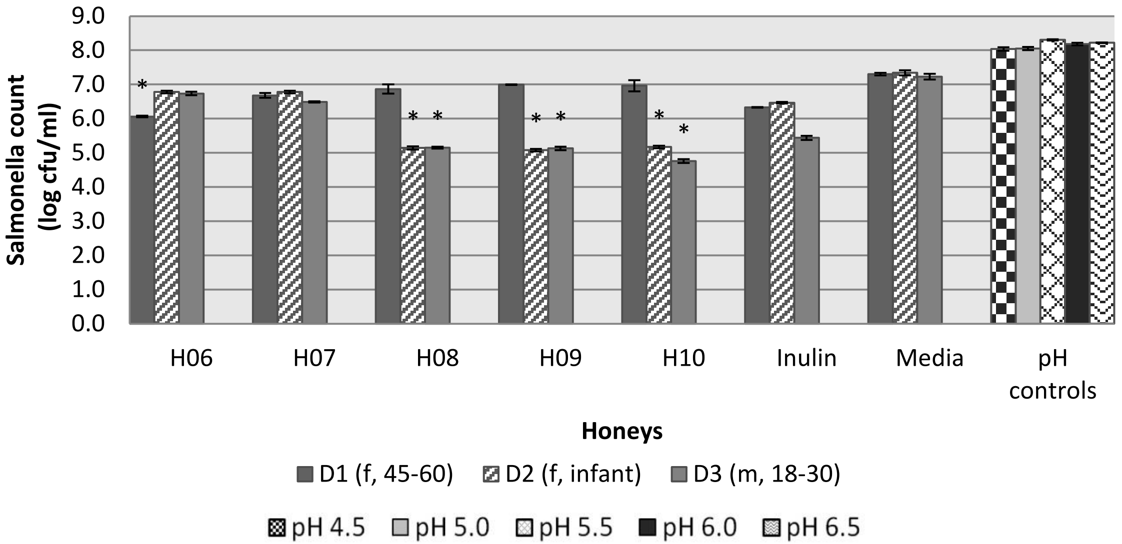


Figure 4.2 | *S. typhimurium* growth in microcosms established with red stringybark honeys

Growth of *S. typhimurium* in microcosms established using microbiota from donors D1 to D3 of different gender (m, f) and age, and digested red stringybark honeys. Results expressed as log cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (*) from the negative control media and (#) from the prebiotic control inulin.

The addition of spotted gum honeys to the microcosms established with D1 microbiota resulted in lower *S. typhimurium* numbers compared to the negative control (media), and this was significant when honeys 11 and 15 were used ($p<0.05$) (Figure 4.3). All spotted gum honeys had a similar effect to inulin on the growth of the bacterium ($p>0.05$), except for honey 14 which allowed for a significantly higher number of *S. typhimurium* ($p<0.05$). Honey 15 was the most effective of the spotted gum samples at inhibiting the growth of *S. typhimurium* in the microcosms with an increase of 0.14 log from the initial value, followed by honey 11 which showed an increase of 0.5 log. The remaining three spotted gum honeys allowed growth of over 1-log from the starting levels of *S. typhimurium*.

The growth of *S. typhimurium* in the D2 and D3 microcosms showed some differences from the D1 studies. All spotted gum honeys showed lowered numbers of *S. typhimurium*

compared to the negative control, with honeys 12, 14 and 15 resulting in significantly lower counts (p<0.01). Honey 15 was again the most effective at suppressing the growth of *S. typhimurium* and the final counts were lower than the initial seeded values in the microcosms established with D2 or D3 microbiota. Honeys 12 and 14 had a less than 1-log increase in *S. typhimurium* numbers, while honeys 11 and 15 showed a larger difference from the initial values. The results when honey 11 was used in the D2 and D3 studies were in contrast to the D1 results, as there was a 1.2 to 1.5-log increase in *S. typhimurium* numbers. Similar to the results seen in D1 microbiota studies, *S. typhimurium* counts showed no significant difference when the spotted gum honeys were compared to the prebiotic control, inulin, except when honey 13 was used in combination with D3 microbiota.

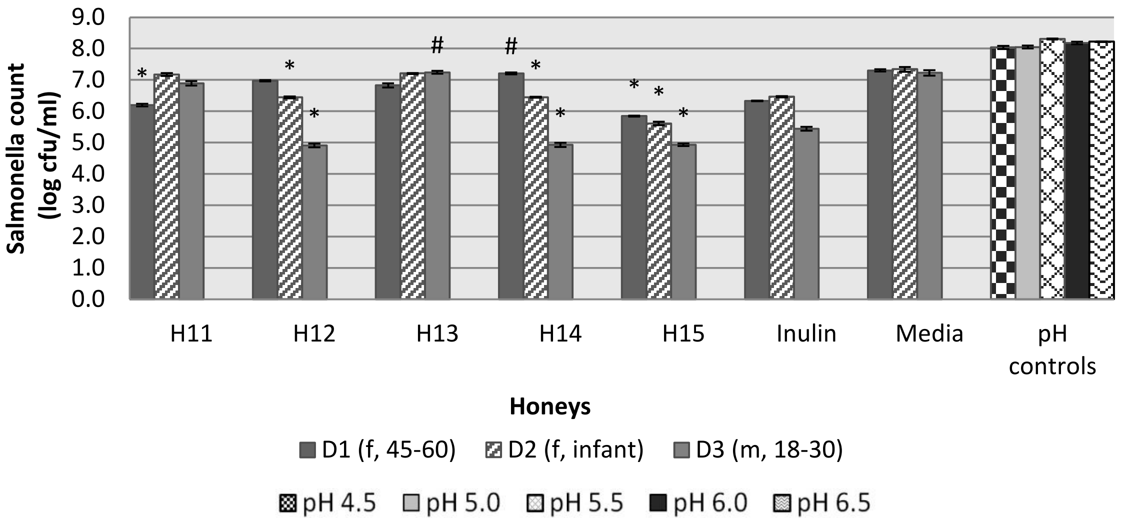


Figure 4.3 | *S. typhimurium* growth in microcosms established with spotted gum honeys
Growth of *S. typhimurium* in microcosms established using microbiota from donors D1 to D3 of different gender (m, f) and age, and digested spotted gum honeys. Results expressed as log cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference (p<0.05): (*) from the negative control media and (#) from the prebiotic control inulin.

Four (honeys 16 to 19) of the five yellow box samples showed significant (p<0.01) suppression of *S. typhimurium* in the microcosms established with D1 microbiota (Figure 4.4), with counts increasing from the initial values by less than 1-log. All yellow box honeys behaved similarly to the inulin control (p>0.05).

Results from the microcosms established D2 and D3 microbiota showed several differences to those seen above. Honeys 18 to 20 significantly inhibited *S. typhimurium* compared to the media control in studies using D2 microbiota. The effect of the honeys on *S. typhimurium* growth was similar to the inulin control, except when honey 17 was used

in the microcosm as this resulted in a significantly higher count of the bacterium (p<0.05). When D3 microbiota was used, *S. typhimurium* counts were significantly lower in all of the yellow box honey microcosms compared to the negative control (p<0.01), and similar to the inulin control (p>0.05).

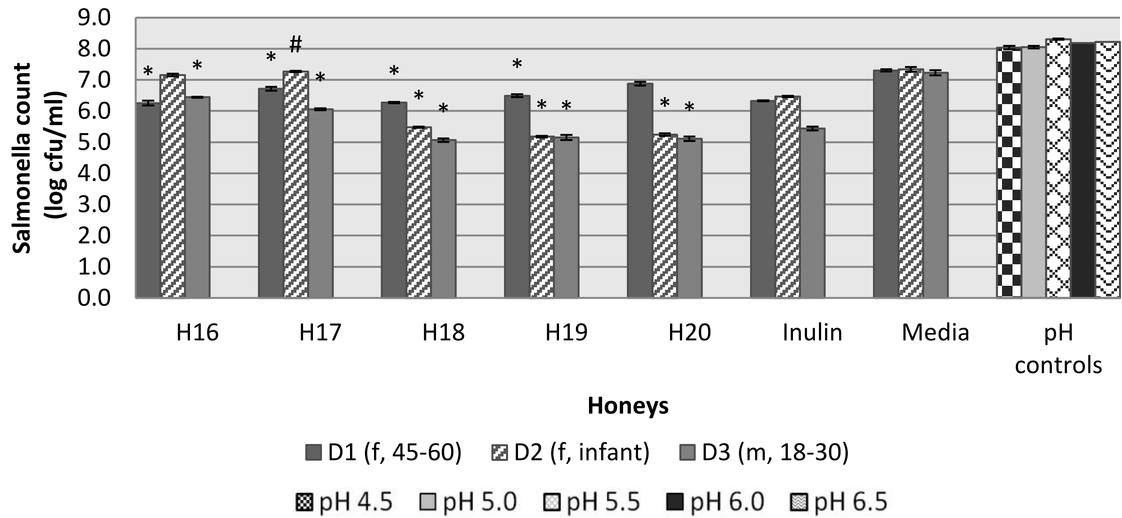


Figure 4.4 | *S. typhimurium* growth in microcosms established with yellow box honeys
Growth of *S. typhimurium* in microcosms established using microbiota from donors D1 to D3 of different gender (m, f) and age, and digested yellow box honeys. Results expressed as log cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference (p<0.05): (*) from the negative control media and (#) from the prebiotic control inulin.

When canola honey (honey 21 and 22) was used in the D1 microbiota established microcosms, the numbers of *S. typhimurium* were comparable to both the media control and the inulin control (Figure 4.5). The manuka honey sample allowed for significantly lower (p<0.01) *S. typhimurium* counts than the negative control with an increase of just 0.07 log from the initial values. This honey also behaved like the inulin control (p>0.05).

The experiments using the D2 and D3 microbiota gave different results to those using D1 microbiota. The presence of either of the canola honey samples or the manuka sample in the microcosms resulted in significantly lower counts of *S. typhimurium* compared to the negative control (p<0.01). The results were comparable to those obtained when inulin was used in the microcosms.

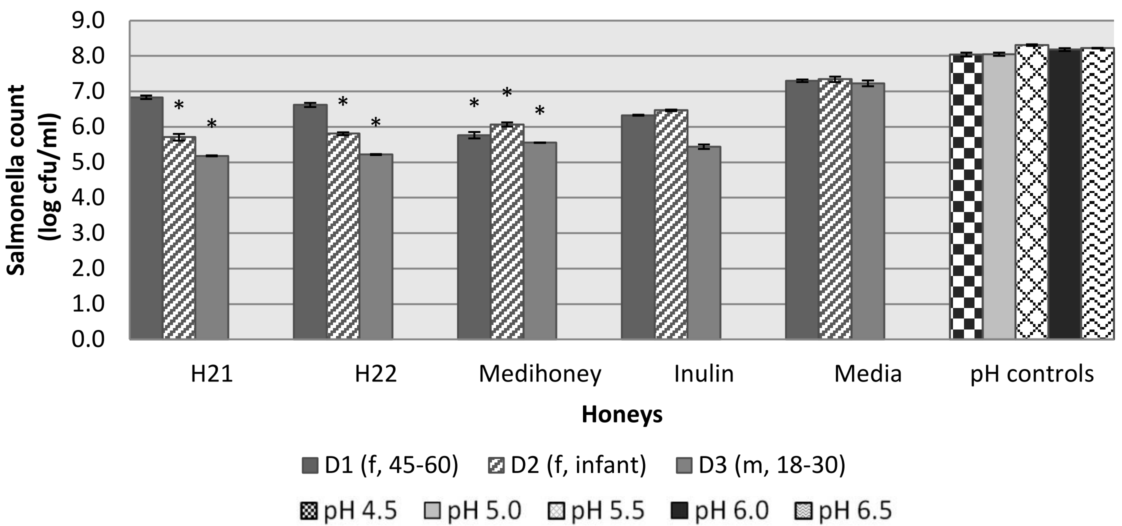


Figure 4.5 | *S. typhimurium* growth in microcosms established with canola and manuka honeys

Growth of *S. typhimurium* in microcosms established using microbiota from donors D1 to D3 of different gender (m, f) and age, and digested canola or manuka honeys. Results expressed as log cfu/ml ± SD from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (*) from the negative control media and (#) from the prebiotic control inulin.

The results indicated that the growth of *S. typhimurium* was highly dependent not only on the type of honey used in the microcosm, but also the microbiota source. The difference between initial and final counts of *S. typhimurium* did not exceed 1-log in approximately half (n=11) of the honey samples used in the microcosms established with D1 microbiota. Sixteen of the 23 honeys tested in the microcosms using D2 microbiota had below 1-log increase in numbers of this bacterium. Inhibition of *S. typhimurium* was most effective in the microcosms established with D3 microbiota, with 20 of the 23 honeys tested showing less than 1-log increase in numbers of the gut pathogen.

4.3.2 Growth of intestinal bacterial cultures in microcosm supernatants

The effect of the inhibitory substances produced by gut microbes in the presence of honey was studied by growing three known gut pathogens in the supernatants from the microcosms. A probiotic strain (*L. fermentum*) was also grown in the supernatants to determine whether the inhibitory substances had an adverse effect on the growth of potentially beneficial bacteria. The use of the filtered supernatants eliminated the contribution of competition with the faecal microbiota on the growth of the test microorganisms.

Growth of each test organism was determined spectrophotometrically over a 24-hour period, and the results were analysed by honey type.

Control wells containing the test organisms incubated in media of various pH levels (ranging from 4.5 to 7.0 in increments of 0.5) were included to determine the effect of pH on their growth (Appendix 4). Adjusting the pH of the growth media to pH 4.5, 5.0 and 5.5 at the start of the assay showed significant inhibition in the growth of all three enteropathogens (*S. typhimurium*, *C. difficile*, and *E. coli*) as well as the probiotic *L. fermentum* strain when compared to the positive growth control ($p<0.01$). Additionally, the growth of all bacteria at these lower pH levels was significantly reduced when compared to their growth in media at pH 6.0, 6.5 and 7.0 ($p<0.01$). The results indicate that low pH affects the growth of the test microbes adversely, and this should be considered in the analyses of the supernatant assays as the final pH of most microcosms was between 5.5 and 6.5.

Positive growth controls (i.e. test organism in growth media only) were included in each of the assays. In addition, a negative supernatant control (i.e. the supernatant from microcosms with no added carbohydrate) and a prebiotic supernatant control (i.e. the supernatant from microcosms established using inulin) were also included for comparison to the growth control. Results from the control assays showed the following:

- the growth of *S. typhimurium* was significantly reduced in the presence of supernatants from the microcosm controls (no added carbohydrate, and inulin-enriched) when compared to the positive growth control, and growth in the supernatants of the inulin-enriched microcosm was significantly lower than the negative supernatant control
- the growth of *C. difficile* was significantly reduced in the presence of the supernatants from the microcosm controls (no added carbohydrate, and inulin-enriched) when compared to the positive control, and growth in the supernatants of the inulin-enriched and negative supernatant control was comparable
- the growth of *E. coli* was significantly reduced in the presence of supernatants from the microcosm controls (no added carbohydrate, and inulin-enriched), and growth in the supernatants of the inulin-enriched microcosm was significantly lower than the negative supernatant control
- the growth of *L. fermentum* in the presence of supernatants from the microcosm controls (no added carbohydrate, and inulin-enriched) were similar to each other, and also to the positive growth control

4.3.2.1 Growth of microbes in supernatants from jarrah honey microcosms

The supernatant from microcosms established with D1 microbiota and jarrah honey samples (H1 to H5) had a significant ($p<0.05$) inhibitory effect on *S. typhimurium* compared to the positive (+) growth control at the 24-hour time interval (Figure 4.6). The

inhibitory effect could be attributed to the presence of honey in the microcosms as the negative supernatant control (no added honey) had significantly higher counts of *S. typhimurium* ($p<0.05$) than four of the five honey samples (honeys 1 to 4). The numbers of *S. typhimurium* in the microcosm supernatant using Honey 5 was not significantly lower than the negative supernatant control ($p=0.26$). The effects of the jarrah honey microcosm supernatants on the growth of *S. typhimurium* were comparable to the prebiotic (inulin) control ($p>0.05$). There were no significant differences in *S. typhimurium* numbers amongst the different jarrah samples. The various time intervals did not highlight any differences between the samples, and generally the numbers of *S. typhimurium* were lowest in the first four hours, approximately equal at the 4- and 8-hour intervals, and highest at 24 hours.

The results when supernatants from the microcosms established with other donor material were generally supportive of the above results. When D2 and D3 microbiota were used in the microcosms, the resulting supernatant had a significantly negative effect on *S. typhimurium* growth compared to the positive growth control ($p<0.05$). The supernatant from D3 microbiota established microcosms allowed for significantly lower *S. typhimurium* numbers when most of the jarrah samples (honeys 1, 2, 3 and 5) were used relative to the negative supernatant control (no added honey) ($p<0.05$), as was seen above. However, when D2 were used, the effect of the supernatant from these microcosms on the numbers of *S. typhimurium* was similar to the negative supernatant (no added honey) control ($p>0.05$). The results obtained when D1 and D3 were used in the microcosms more closely resembled each other than when D2 microbiota were used.

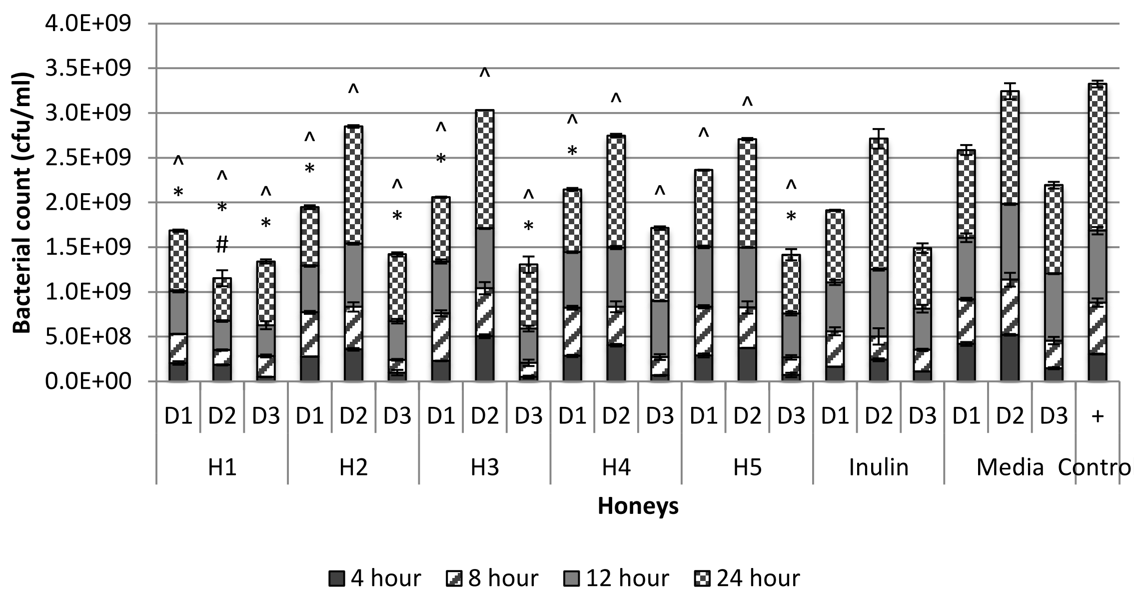


Figure 4.6 | Growth of *S. typhimurium* in supernatants from jarrah honey microcosms

Growth of *S. typhimurium* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested jarrah honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

C. difficile growth was adversely affected in the presence of supernatants from the microcosms established using jarrah honey and D1 microbiota (Figure 4.7). When the supernatants were used, the numbers of *C. difficile* were significantly lower than the positive growth control (p<0.01) at the 24-hour interval. When supernatants from the honey microcosms were used, significantly lower numbers of *C. difficile* were observed when compared to the supernatant from the inulin-enriched microcosm (p<0.05). The *C. difficile* numbers were also significantly lower (p<0.05) compared to the negative supernatant (no added honey) control in most cases (all honeys except for honey 1). Growth of *C. difficile* was very low in the 4-hour and 8-hour intervals when grown in the presence of the supernatant from the honey microcosms, and increased at 12- and 24 hours. These results were unlike those seen in the positive growth control, as well as the inulin-enriched microcosm supernatant, where *C. difficile* showed some growth in the first four hours, relatively higher growth at the 8- and 12-hour intervals and highest growth at 24 hours.

When the supernatants from the microcosms established with D2 and D3 were used, the results were varying. When D3 microbiota was used in the microcosms, the results were in

accordance with D1 studies. However, when D2 microbiota was used to set up the microcosms, some differences were seen. The supernatants from the honey microcosms all inhibited the growth of *C. difficile* significantly compared to the positive growth control (p<0.05), however, the results were similar to those observed when the supernatants from the inulin-enriched microcosms and the negative supernatant control microcosm. The *C. difficile* numbers were most affected in the assays using the supernatant from microcosms established with D3 microbiota.

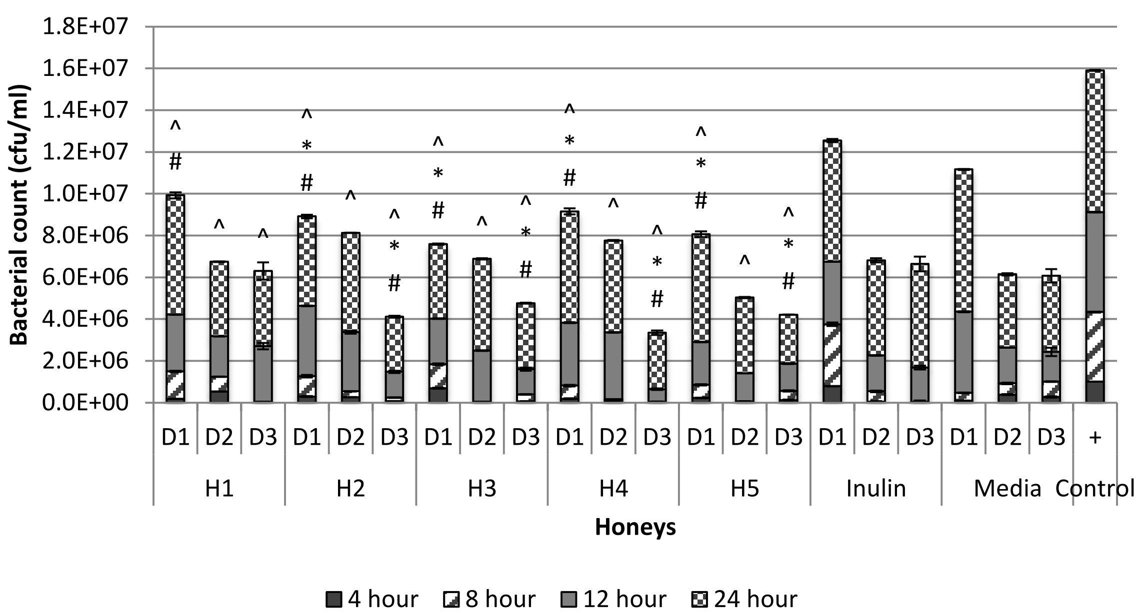


Figure 4.7 | Growth of *C. difficile* in supernatants from jarrah honey microcosms

Growth of *C. difficile* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested jarrah honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The growth of *E. coli* was significantly reduced in the presence of supernatants from the microcosms established using jarrah honey and D1 microbiota (Figure 4.8). In the presence of the supernatants, the counts of *E. coli* were significantly lower than the positive growth control at 24 hours (p<0.05). The supernatants from the honey microcosms were also found to be significantly more inhibitory on *E. coli* growth the negative supernatant control in most cases (p<0.05). The effect of the honey-enriched microcosm supernatants on the numbers of *E. coli* were similar to those of the supernatant from inulin-enriched microcosm supernatants (p>0.05).

There were no notable differences when microbiota from the other two donors were used in the assays.

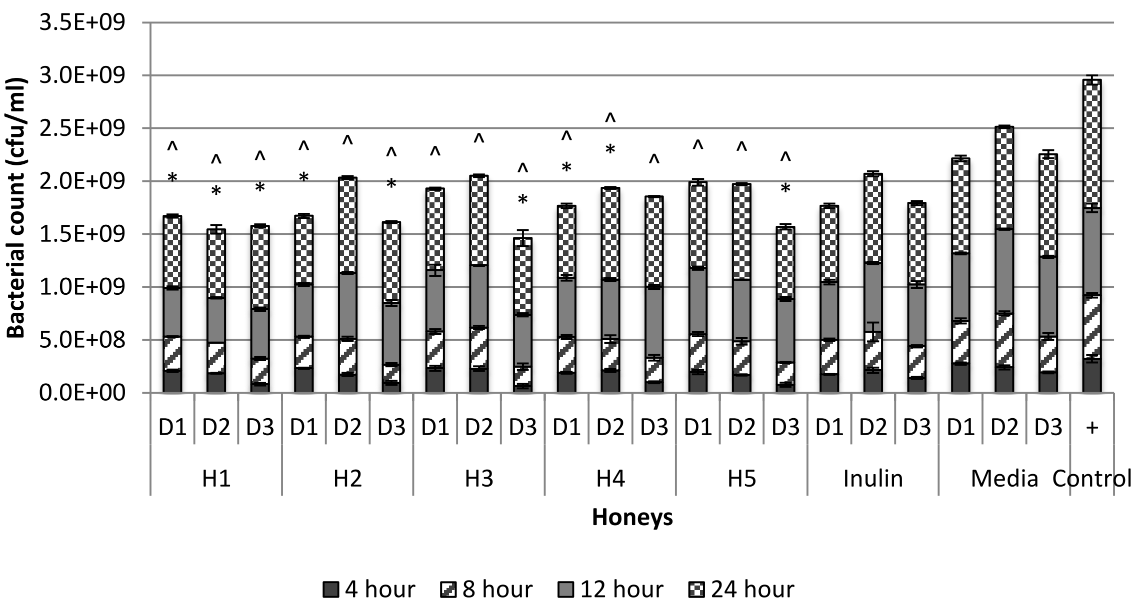


Figure 4.8 | Growth of *E. coli* in supernatants from jarrah honey microcosms

Growth of *E. coli* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested jarrah honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The effect of the supernatants from microcosms using jarrah honeys and the D1 microbiota on the growth of a probiotic strain, *L. fermentum*, was also studied (Figure 4.9). In the presence of the supernatants from the honey-enriched microcosms, *L. fermentum* counts were significantly lower than the positive growth control in all but the honey 3 assays. They were also lower than the counts in the presence of the inulin-enriched microcosm supernatants, as well as the negative supernatant control.

However, these results were not supported when the microbiota D2 and D3 were used. *L. fermentum* growth in these assays was comparable to the positive growth control, and also similar to the growth when the supernatants from inulin-enriched or no honey microcosms were used.

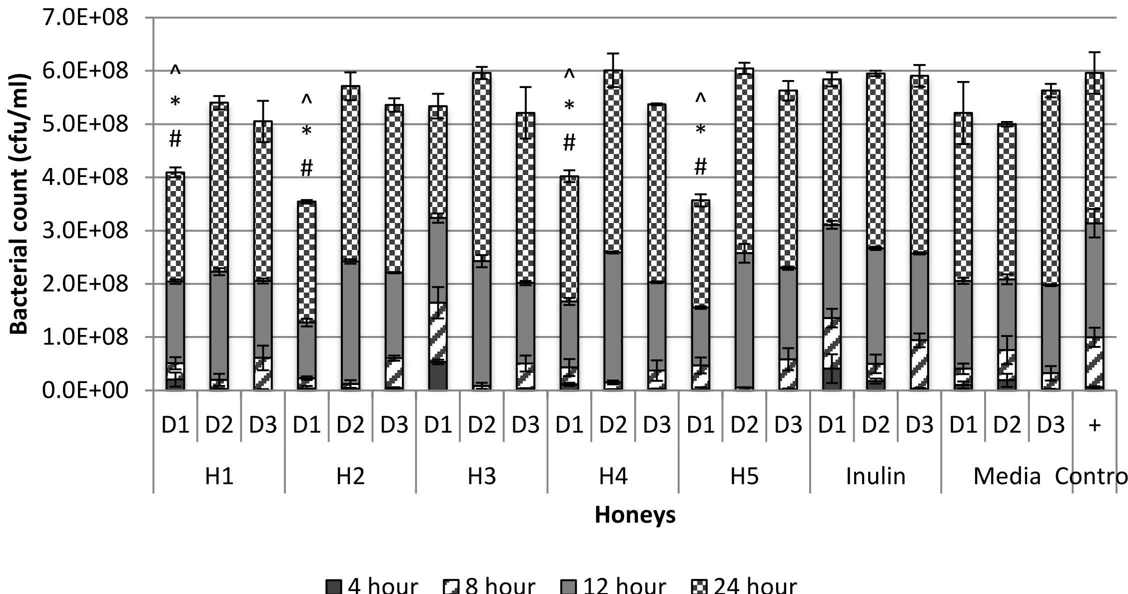


Figure 4.9 | Growth of *L. fermentum* in supernatants from jarrah honey microcosms

Growth of *L. fermentum* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested jarrah honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

Overall, the inclusion of jarrah honeys in the microcosms resulted in the production of inhibitory substances by the faecal microbiota (present in the supernatants) that affected the growth of the three bacterial pathogens adversely, but still allowed the growth of the probiotic strain in most cases.

4.3.2.2 Growth of microbes in supernatants from red stringybark honey microcosms

The growth of *S. typhimurium* was significantly lower in the presence of supernatants from the microcosms established using red stringybark honey (honeys 6 to 10) and the D1 microbiota (Figure 4.10). In the presence of the supernatants from the honey-enriched microcosms, the counts of *E. coli* were significantly lower than the positive growth control at 24 hours (p<0.05). No significant differences in *E.* coli counts were found when the organism was grown in the supernatants of the honey-enriched microcosms relative to those that were inulin-enriched or with no honey added (p>0.05).

When microbiota from the other two donors were used in the microcosms, the results of *S. typhimurium* growth in the presence of these microcosm supernatants were largely supportive of the trends above. However, when D3 microbiota were used, the

supernatant from the microcosms established using honey showed significant inhibition of *S. typhimurium* (p<0.05) compared to the no honey microcosm supernatant. The results arising from the use of D3 microbiota showed different patterns in *S. typhimurium* growth with very low to low growth observed at the 4- and 8-hour time intervals, respectively, compared to the assays performed using the other donor microbiota.

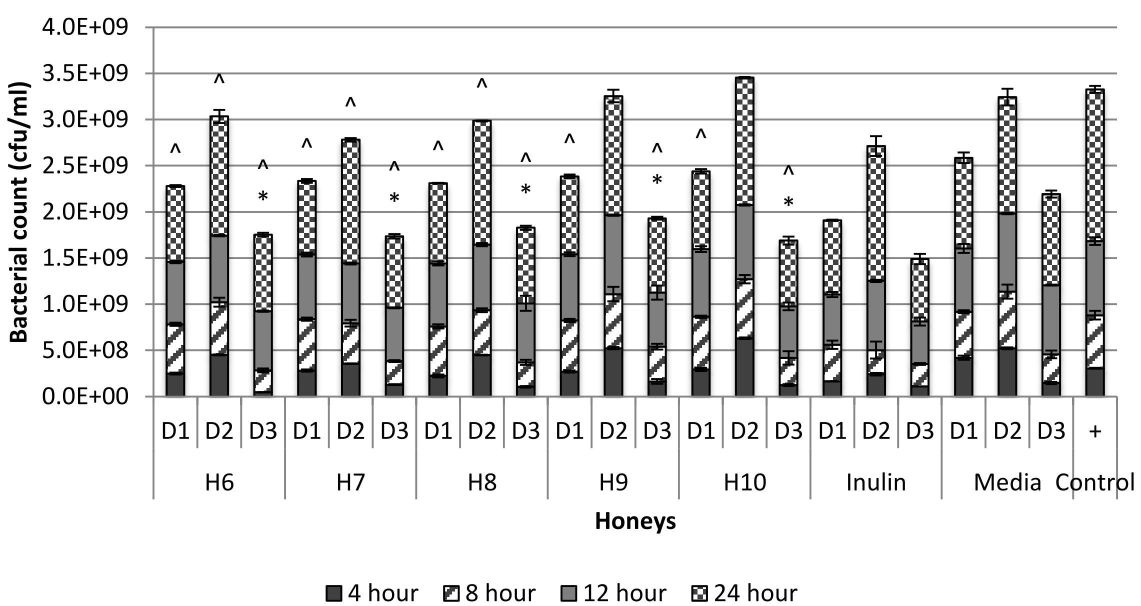


Figure 4.10 | Growth of *S. typhimurium* in supernatants from red stringybark honey microcosms

Growth of *S. typhimurium* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested red stringybark honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

C. difficile growth was significantly affected in the presence of the supernatants from the microcosms established using red stringybark honeys and D1 microbiota compared to the positive growth control (Figure 4.11). Additionally, the inclusion of honey in the microcosm resulted in supernatants that were significantly inhibitory towards *C. difficile* compared to the inulin-enriched microcosm control as well as the no honey microcosm control (p<0.05). There was very little growth of *C. difficile* in the first eight hours of the assay, with much of the observed growth occurring between the 12 to 24 hour intervals.

The data when D2 and D3 microbiota were used with the honeys also showed that the addition of honey to the microcosms produced a supernatant that had a significant inhibitory effect on the growth of *C. difficile* relative to the positive growth control.

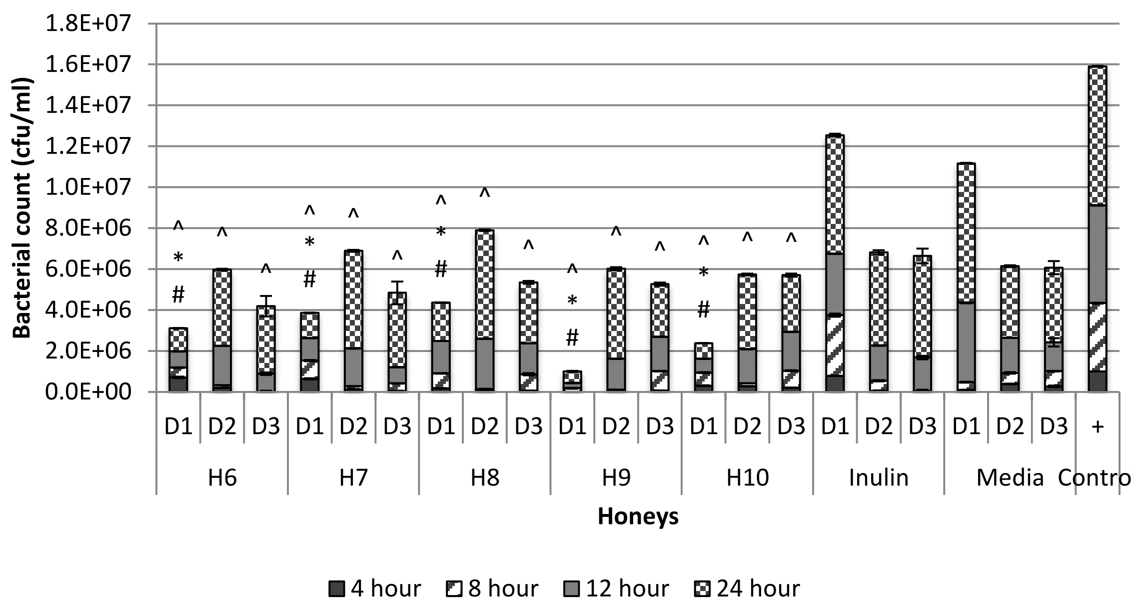


Figure 4.11 | Growth of *C. difficile* in supernatants from red stringybark honey microcosms

Growth of *C. difficile* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested red stringybark honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

When red stringybark honeys were added to the microcosms established with D1 microbiota, all of the resulting supernatants had a significant inhibitory effect on the growth of *E. coli* at 24 hours relative to the positive growth control ($p<0.05$) (Figure 4.12). The counts of *E. coli* in the presence of the honey-enriched microcosm supernatants were mostly similar to those observed in the presence of the supernatants from the inulin-enriched and no honey microcosms ($p>0.05$).

The data obtained when D2 and D3 microbiota were used in the microcosms largely resembled what was seen above.

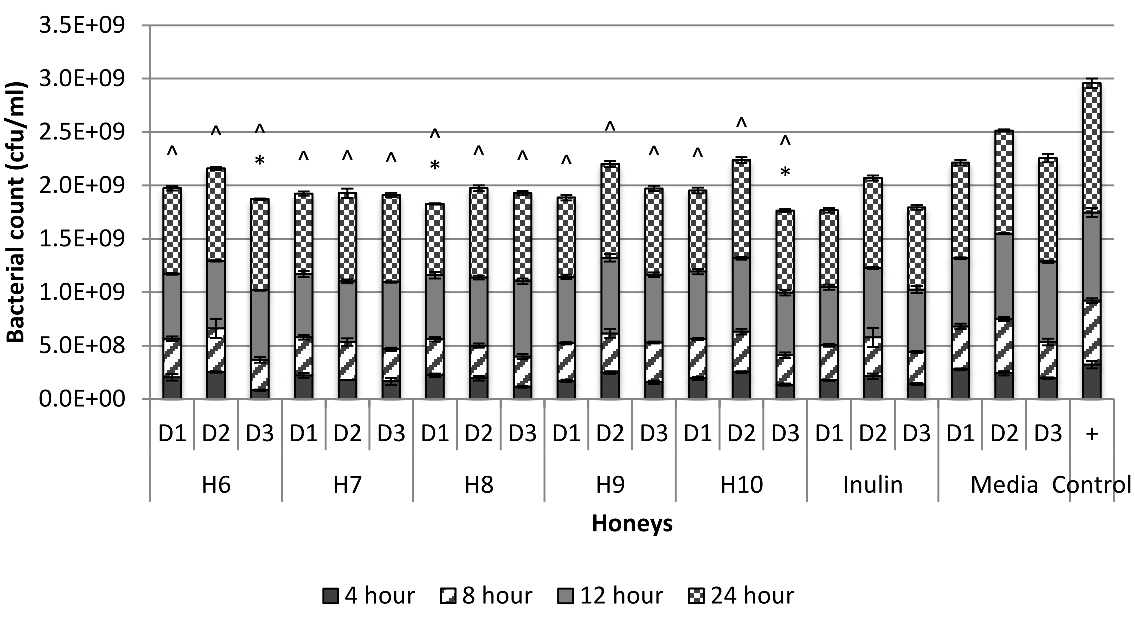


Figure 4.12 | Growth of *E. coli* in supernatants from red stringybark honey microcosms

Growth of *E. coli* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested red stringybark honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The growth of *L. fermentum* was largely unaffected in the presence of the supernatants from microcosms established using red stringybark honey D1 microbiota compared to the positive growth control (Figure 4.13). The honey-enriched microcosm supernatants did not affect the growth of *L. fermentum* any differently to the no honey microcosm supernatant. Growth of *L. fermentum* was negatively affected compared to the inulin-enriched microcosm supernatant when honeys 7 or 9 were used (p=0.04), however, these honeys did not result in significant differences in *L. fermentum* levels when microbiota from the other donors were used in the assays. *L. fermentum* growth was most prominent after eight hours in almost all of the assays.

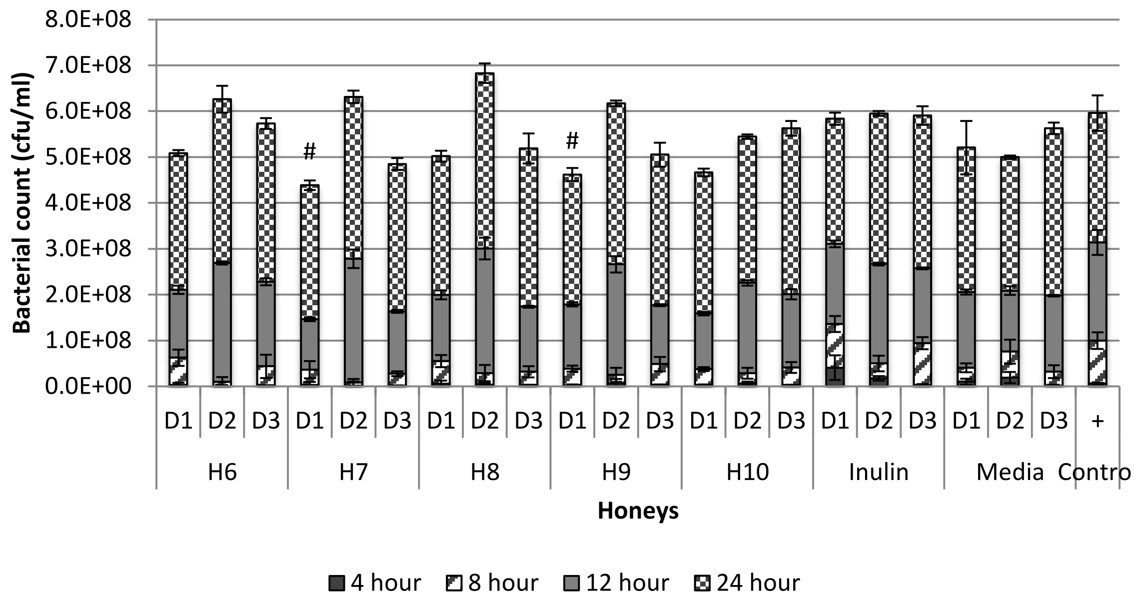


Figure 4.13 | Growth of *L. fermentum* in supernatants from red stringybark honey microcosms

Growth of *L. fermentum* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested red stringybark honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

As with the jarrah samples, the inclusion of red stringybark honeys in the microcosms produced supernatants with inhibitory effects on all three gut pathogens, without adverse effects on the growth of the probiotic strain.

4.3.2.3 Growth of microbes in supernatants from spotted gum honey microcosms

The inclusion of spotted gum honeys (H11-15) in microcosms established with D1 microbiota resulted in supernatants that had a significantly inhibitory effect on *S. typhimurium* growth at 24 hours compared to the positive growth control (Figure 4.14). The *S. typhimurium* counts were significantly lower in the presence of the honey-enriched microcosm supernatants compared to the supernatants from the no honey microcosms ($p<0.05$), and similar to those from microcosms established using inulin ($p>0.05$).

The results from the assays using microbiota from D2 and D3 donors in the microcosms were largely supportive of the above trends, except when counts of *S. typhimurium* from the honey-enriched microcosm supernatants were compared to the inulin-enriched

microcosm control. In these assays, most of the supernatant samples from the honey D2 established microcosms showed significantly more inhibition of *S. typhimurium* compared to the inulin-enriched ones (p<0.05).

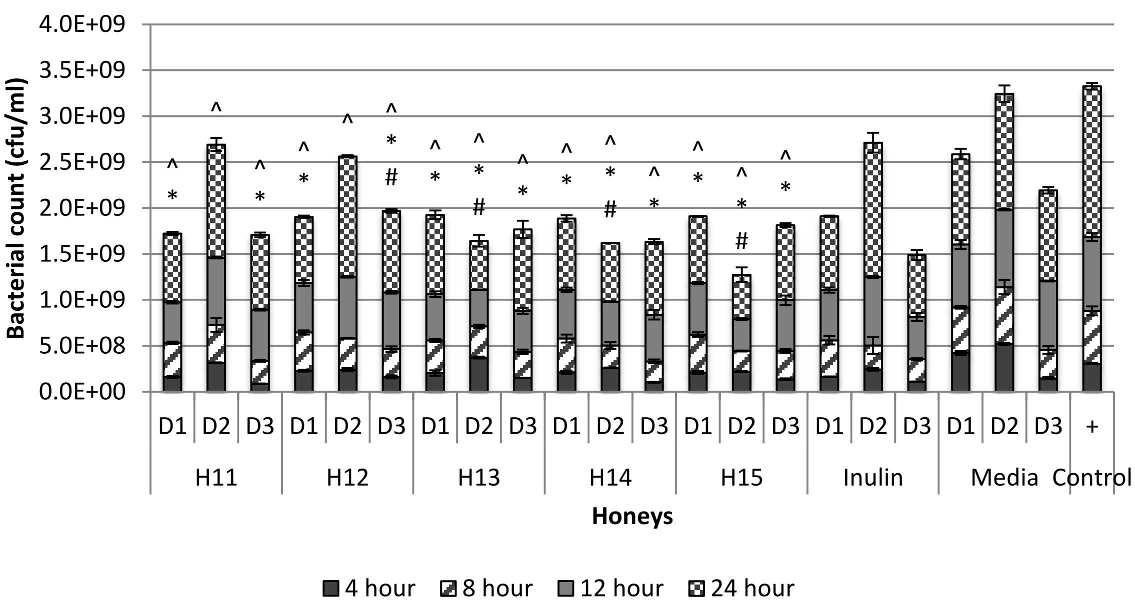


Figure 4.14 | Growth of *S. typhimurium* in supernatants from spotted gum honey microcosms

Growth of *S. typhimurium* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested spotted gum honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The numbers of *C. difficile* when grown in supernatants from microcosms established using spotted gum honey (honeys 11 to 15) and D1 microbiota were significantly lower than the positive growth control (p<0.01), and also significantly lower than when grown in the inulin-enriched or no honey microcosm supernatant (Figure 4.15). The one exception was honey 14 which yielded results that were comparable to both the inulin-enriched and no honey microcosm controls. The growth in the presence of the different honey-enriched microcosm supernatants varied, with some exhibiting very low levels of *C. difficile* at all time intervals (honeys 11 and 15), some showing most growth at the 12- and 24-hour time points (honeys 12 and 13) and one with consistently increasing growth after four hours (honey 14).

The data obtained when microcosms were established using D2 or D3 microbiota also showed significant inhibition of *C. difficile* compared to the positive growth control.

Although *C. difficile* numbers were generally lower in the presence of supernatants from the honey-enriched microcosm supernatants compared to those established with no honey or inulin, the efficacy of the honeys varied depending on the microbiota source. Additionally, there were differences in the growth of *C. difficile* at the various time intervals when different honeys and sources of microbiota were used in the assays.

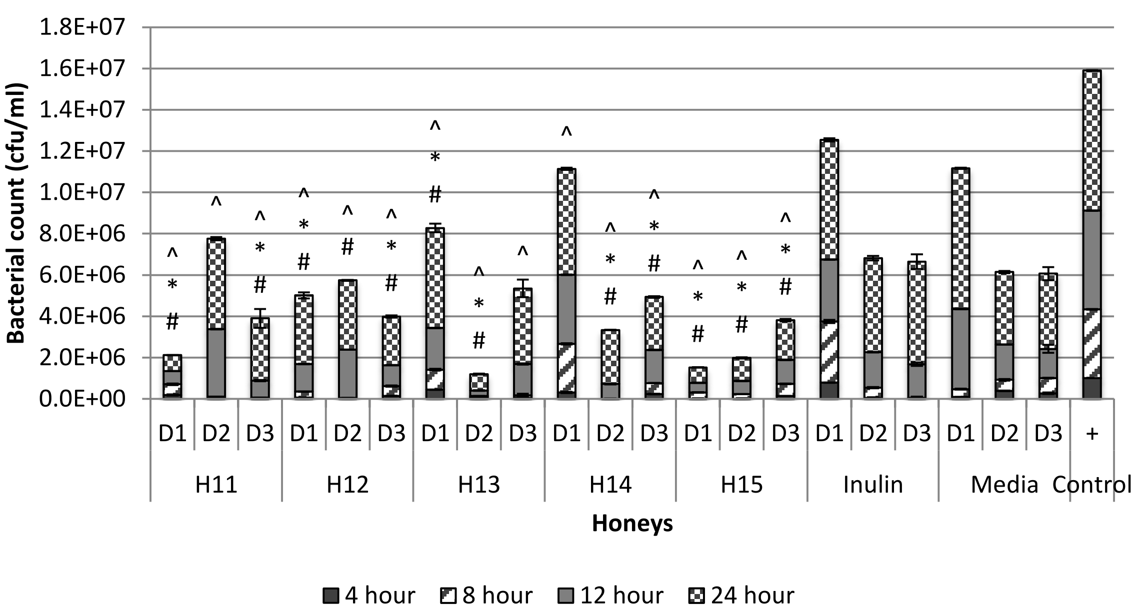


Figure 4.15 | Growth of *C. difficile* in supernatants from spotted gum honey microcosms
Growth of *C. difficile* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested spotted gum honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The inclusion of spotted gum honey in the microcosms established with D1 also had a suppressive effect on *E. coli* relative to the positive growth control (Figure 4.16). The *E. coli* counts in the honey microcosm assays were comparable to those obtained in the inulin-enriched microcosms ($p>0.05$).

There were a few differences noted when the microcosms were established with D2 microbiota, with honeys 14 and 15 allowing significantly lower numbers of *E. coli* when compared to the no honey and inulin-enriched microcosm controls. The use of D3 microbiota also showed slightly different trends in resultant *E. coli* counts, as counts were comparable to both the inulin-enriched and no honey microcosm controls.

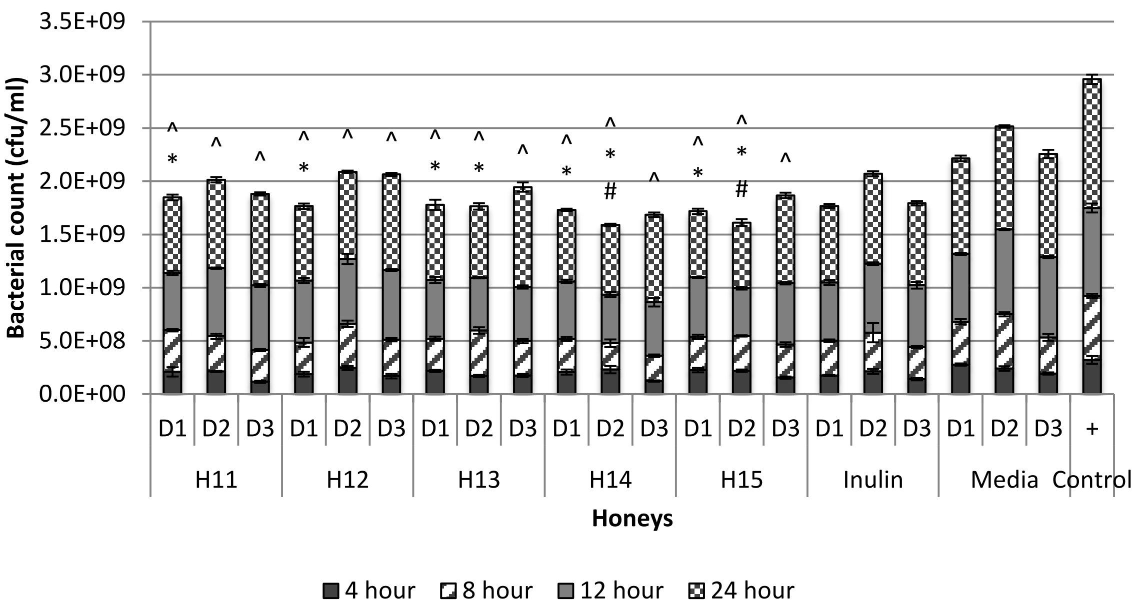


Figure 4.16 | Growth of *E. coli* in supernatants from spotted gum honey microcosms

Growth of *E. coli* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested spotted gum honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The overall growth of the probiotic strain, *L. fermentum*, was largely unaffected in the presence of the supernatants from microcosms established using spotted gum samples and D1 microbiota (Figure 4.17). However, when honeys 12 and 13 were used in the microcosms it was noted that *L. fermentum* numbers were significantly lower than those compared to the growth control and also the inulin-enriched and no honey microcosm controls (p<0.05).

This observation was donor specific, as *L. fermentum* counts were not affected when microbiota from the other two donors were used in the assays.

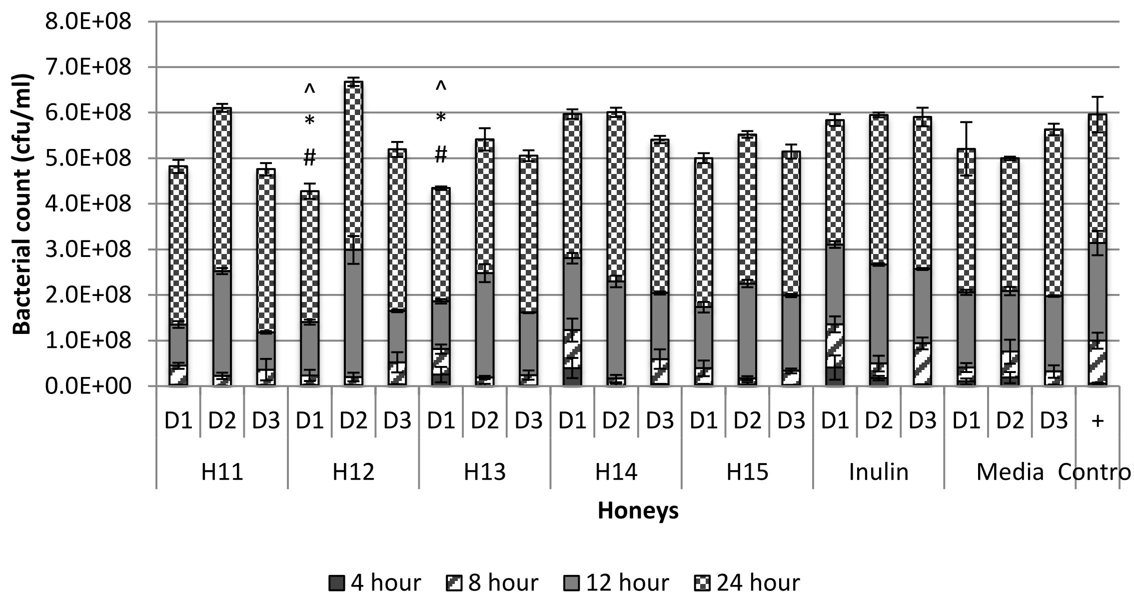


Figure 4.17 | Growth of *L. fermentum* in supernatants from spotted gum honey microcosms

Growth of *L. fermentum* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested spotted gum honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

Overall, the results from the spotted gum honeys largely supported those seen when the jarrah and red stringybark samples were used in the microcosms.

4.3.2.4 Growth of microbes in supernatants from yellow box honey microcosms

Microcosms established using yellow box honey (honeys 16 to 20) and D1 microbiota gave rise to supernatants with significantly inhibitory effects on the growth of *S. typhimurium* (p<0.05) compared to the positive growth control (Figure 4.18). Additionally, the inhibitory effect could be attributed to the presence of the honeys in the microcosms, as the counts of *S. typhimurium* in the supernatant of the microcosm with no honey added were significantly higher (p<0.05). The presence of honeys in the microcosms had a similar effect to the inulin-enriched microcosm supernatants on the growth of *S. typhimurium* in most cases, however honeys 17 and 18 did show significantly lower counts of the enteropathogen (p<0.01).

When microbiota from the other donors were used in the microcosms, the trends mainly resembled those seen from the D1 studies.

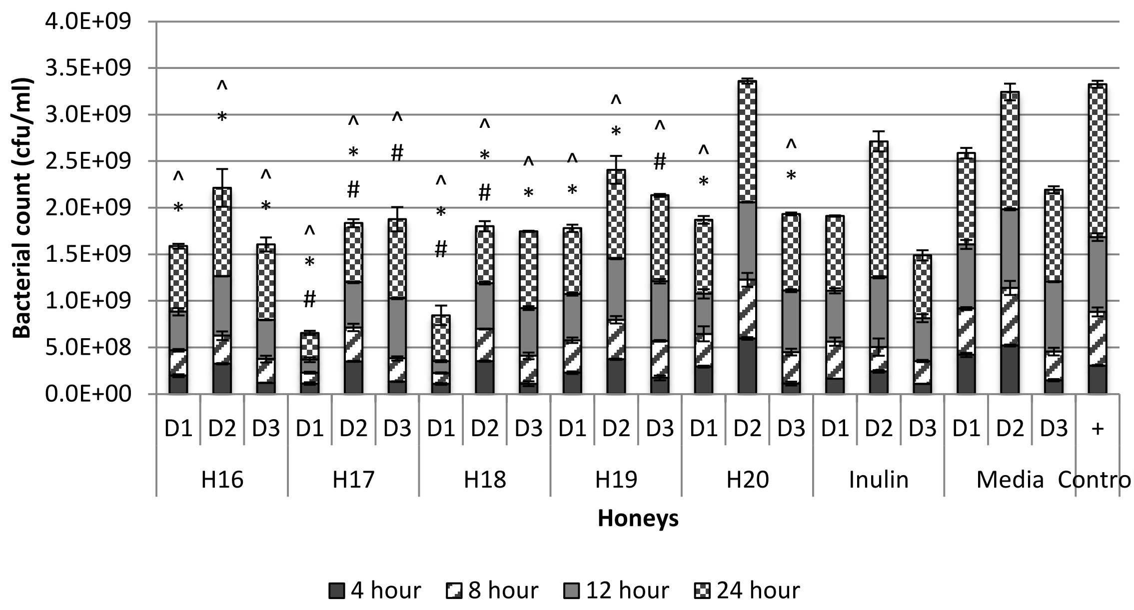


Figure 4.18 | Growth of *S. typhimurium* in supernatants from yellow box honey microcosms

Growth of *S. typhimurium* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested yellow box honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

As was seen with the previous honeys, the growth of *C. difficile* was also very notably affected when yellow box honey was included in the microcosms (Figure 4.19). All yellow box samples generated microcosm supernatants using D1 microbiota that significantly inhibited *C. difficile* growth ($p<0.01$ when compared to the positive control). In addition, the inclusion of yellow box honeys in the microcosms had a significantly more suppressive effect on *C. difficile* growth compared to the inulin-enriched and no honey microcosm controls ($p<0.05$).

The results from experiments set up using the other donor microbiota were largely supportive of the D1 studies.

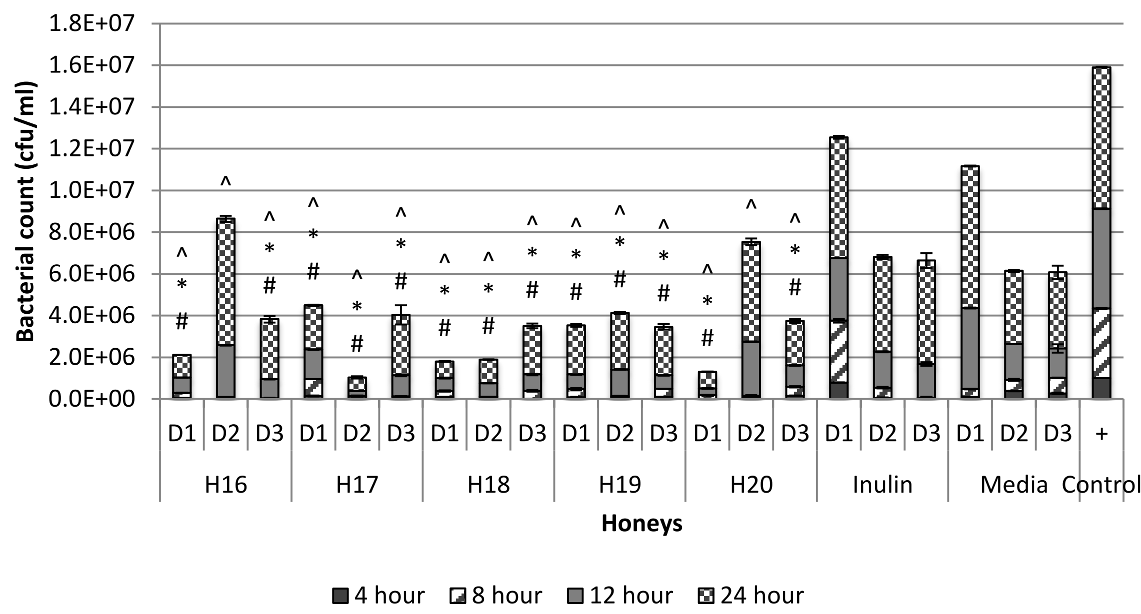


Figure 4.19 | Growth of *C. difficile* in supernatants from yellow box honey microcosms

Growth of *C. difficile* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested yellow box honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The supernatants from microcosms established using yellow box honey D1 microbiota showed reduced counts of *E. coli* ($p<0.05$) compared to the positive growth control (Figure 4.20). The bacterial counts were similar to those obtained from the inulin-enriched and no honey microcosm controls ($p>0.05$).

E. coli counts when grown in the supernatant from microcosms established using D3 microbiota mirrored those results. When D2 microbiota were used some differences were noted, namely assays using honeys 17 and 18 showed significantly lower *E. coli* counts when compared to the no honey microcosm control ($p=0.04$).

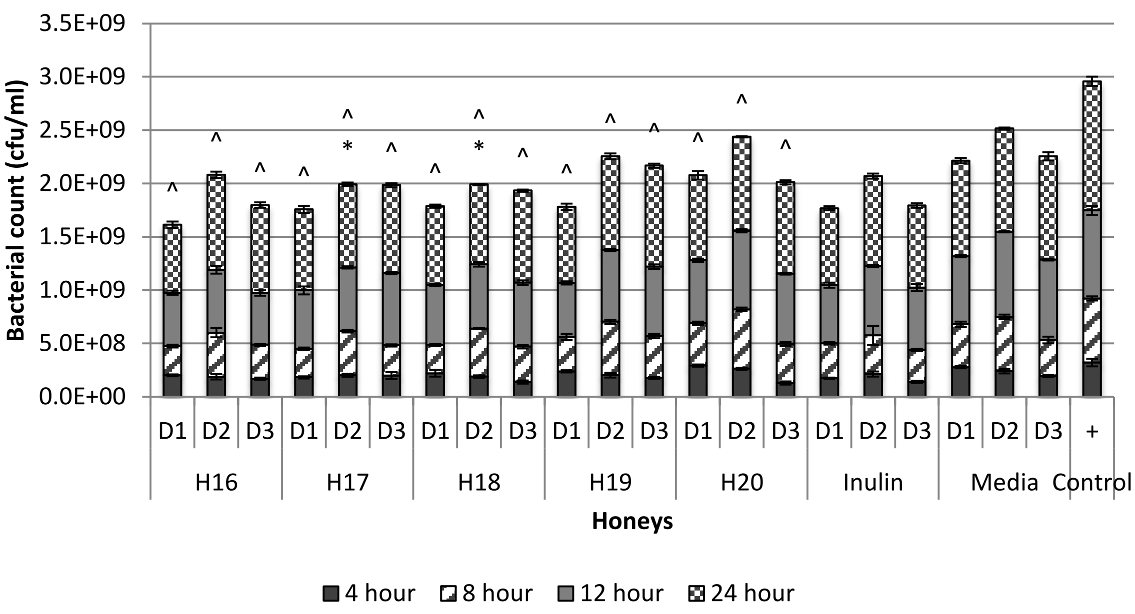


Figure 4.20 | Growth of *E. coli* in supernatants from yellow box honey microcosms

Growth of *E. coli* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested yellow box honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

L. fermentum growth was unaffected in the presence of the supernatants from yellow box enriched microcosms compared to the positive growth control ($p>0.05$), regardless of which yellow box sample or microbiota source was used in the microcosms. (Figure 4.21).

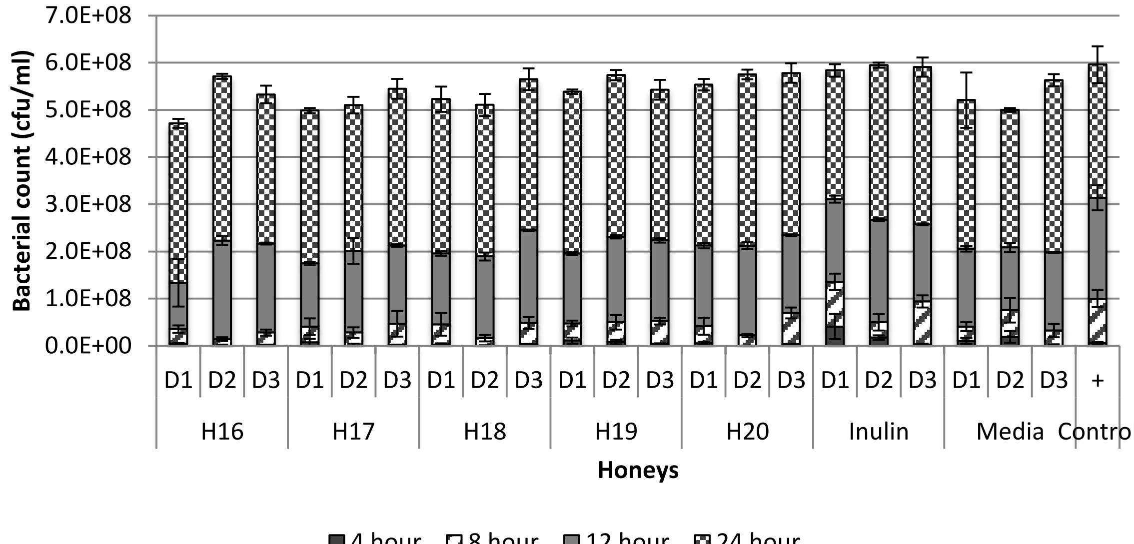


Figure 4.21 | Growth of *L. fermentum* in supernatants from yellow box honey microcosms

Growth of *L. fermentum* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested yellow box honey. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference (p<0.05): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The results obtained from the yellow box honey studies confirm those of the other honeys presented in this chapter, that is, the honeys mostly inhibit the pathogenic strains while the growth of the probiotic strain was unaffected.

4.3.2.5 Growth of microbes in supernatants from canola and manuka honey microcosms

Microcosms established with D1 microbiota and canola samples (honeys 21 and H22) gave rise to supernatants that significantly reduced the growth of *S. typhimurium* (Figure 4.22). The *S. typhimurium* counts were also significantly lower than the positive growth control when manuka honey was used in the microcosms (p<0.05). The inhibitory effect of the supernatants derived from microcosms established using these three honeys were similar to those when the microcosms were set up using inulin (p=1.00). Furthermore, the inhibitory effect of the honey-enriched microcosm supernatants was significantly higher than that of the no honey (negative) microcosm control (p<0.05), therefore the inhibitory effect could be attributed to the presence of the honeys in the microcosms.

The results from the D3 studies mostly confirmed those of the D1 studies, but the supernatants from canola enriched microcosms using D2 microbiota were not inhibitory.

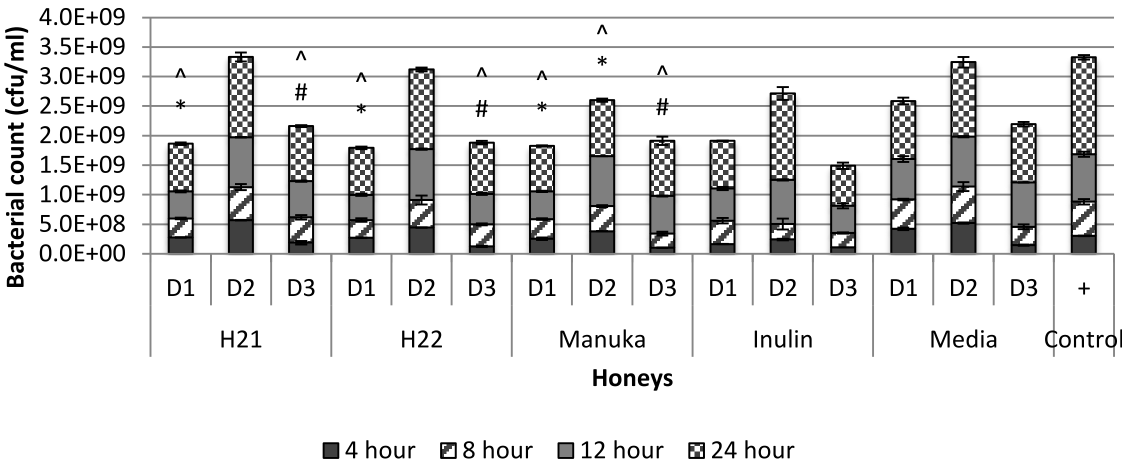


Figure 4.22 | Growth of *S. typhimurium* in supernatants from canola and manuka honey microcosms

Growth of *S. typhimurium* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested canola and manuka honeys. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The supernatants from D1 microbiota established microcosms and canola or manuka honey were able to inhibit *C. difficile* growth, with counts significantly lower than the positive growth control ($p<0.01$), and significantly lower than the inulin-enriched and no honey microcosm controls ($p<0.05$) (Figure 4.23). The growth of *C. difficile* was low at all time intervals, with much of the growth occurring between 12 and 24-hours.

When D3 microbiota were used in the microcosms, the results resembled those from the D1 studies. However, there were some differences when D2 microbiota were used, whereby the inhibitory effect of the honeys was no different to the inulin-enriched and no honey microcosm controls.

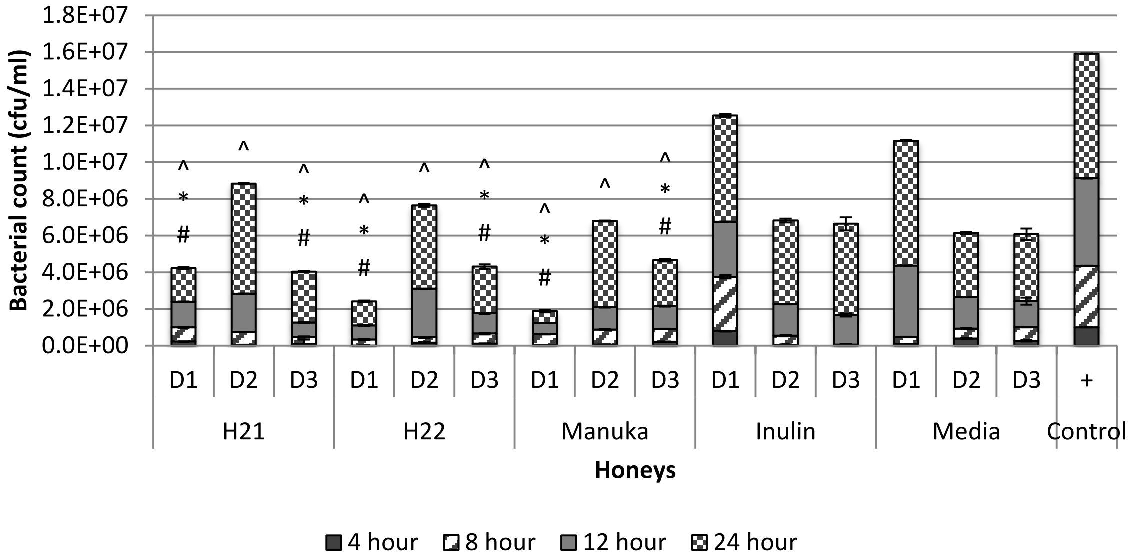


Figure 4.23 | Growth of *C. difficile* in supernatants from canola and manuka honey microcosms

Growth of *C. difficile* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested canola or manuka honeys. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

E. coli numbers were significantly lower than the positive growth control when incubated in the presence of supernatants from the D1 established microcosms with either canola sample or the manuka sample (Figure 4.24). The counts from the honey assays were largely equivalent to those of the inulin-enriched and no honey controls ($p>0.05$).

Results using the other donor microbiota also supported these findings.

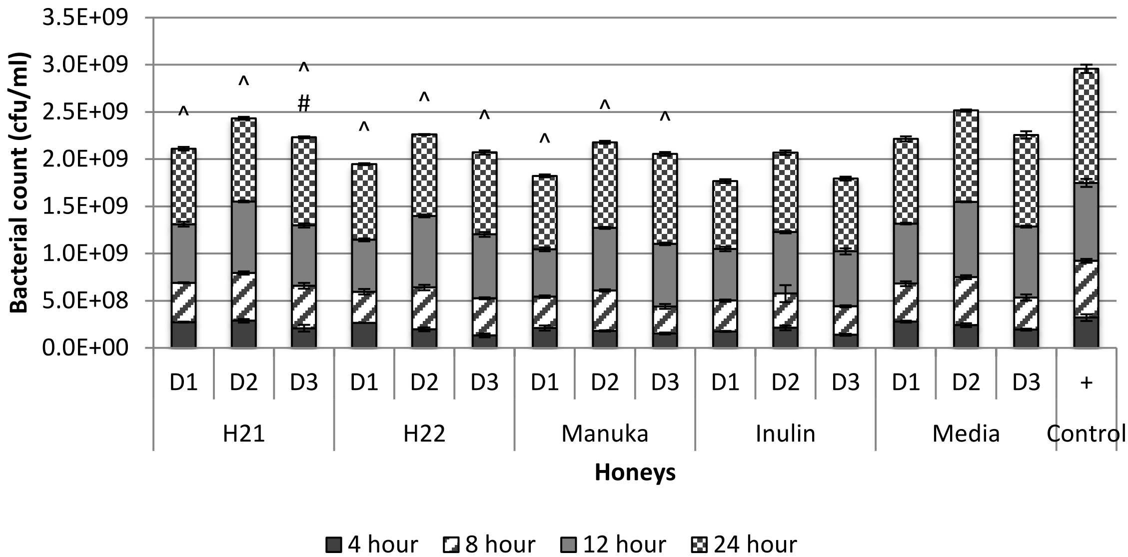


Figure 4.24 | Growth of *E. coli* in supernatants from canola or manuka honey microcosms

Growth of *E. coli* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested canola or manuka honeys. Results expressed as cfu/ml ± SD at different time intervals, from three separate trials. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

The growth of *L. fermentum* in the supernatants of microcosms set up using D1 microbiota and the canola honey or manuka honey samples was comparable to the positive growth control (Figure 4.25). The growth was also similar to that in the presence of supernatant from the no honey microcosm control, however *L. fermentum* counts were lower when the canola honeys were used in the microcosms compared to the inulin-enriched microcosm control ($p<0.05$).

The use of D2 microbiota showed some changes in *L. fermentum* growth in the corresponding supernatant. The counts of the probiotic strain were comparable to the positive growth control, and also similar to the no honey and the inulin-enriched microcosm control ($p>0.05$). The use of D3 microbiota in the microcosms had a different effect to what was seen in D1 and D2 studies. Counts of *L. fermentum* in the presence of the honey-enriched microcosm supernatants were similar to the positive growth control, however the use of canola honeys showed a reduction in the counts when compared to the inulin-enriched and no honey microcosm controls ($p<0.05$).

When all results were considered, the supernatants from the microcosms established with canola and manuka honeys were inhibitory against the enteropathogens compared to the positive growth control, while still allowing elevated counts of *L. fermentum*.

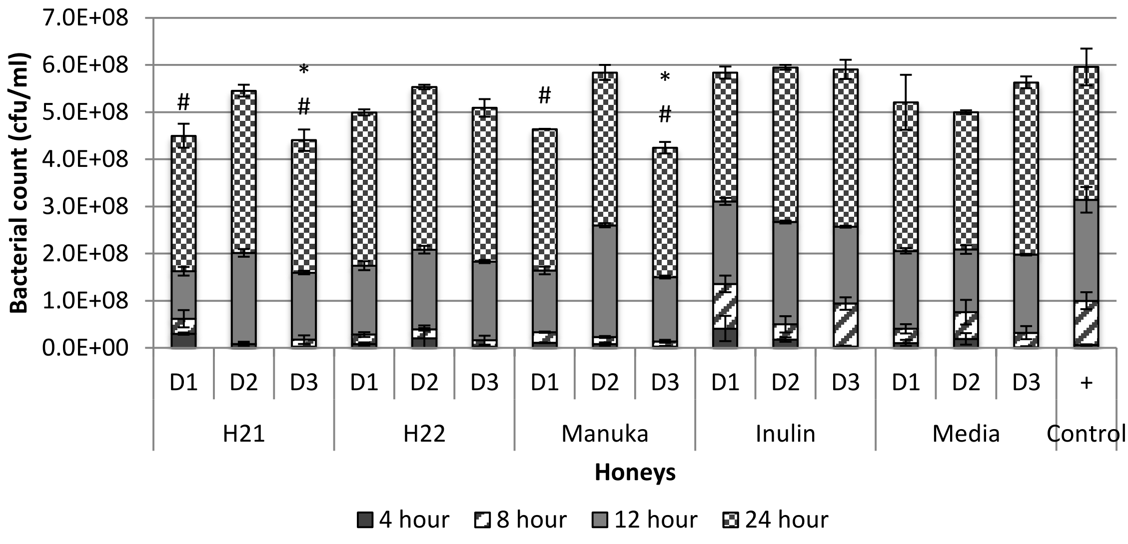


Figure 4.25 | Growth of *L. fermentum* in supernatants from canola or manuka honey microcosms

Growth of *L. fermentum* in supernatants of microcosms established using microbiota from donors D1 to D3 and digested canola or manuka honey. Symbols indicate statistically significant difference ($p<0.05$): (^) from the positive growth control (test organism in growth media), (*) from the negative supernatant control (supernatant from microcosms with no added honey) and (#) from the prebiotic inulin control (supernatant from inulin-enriched microcosms).

4.4 Discussion

The work presented in this chapter was an investigation into the effect of Australian honeys on the growth of enteropathogens in *in vitro* microcosms established with human gut microbiota. The results showed that inclusion of honey in the microcosms had adverse effects on the growth of enteropathogens *S. typhimurium* and *E. coli,* which are commonly implicated in gastroenteritis, as well as *C. difficile* which is associated with diarrhoea following antibiotic therapy. The inhibitory effects can be mainly attributed to the beneficial changes observed in the gut microbiota in the presence of honey. In this study, it is likely that the gut microbiota suppressed the growth of the enteropathogens by competing for nutrients (i.e. when *S. typhimurium* was seeded into microcosms) and also by direct antagonism through the production of inhibitory compounds (i.e. supernatant assays). This supports the findings from previous chapters, that honey has an overall positive effect on the composition and function of the human gut microbiota *in vitro*.

4.4.1 Impact of honey enrichment in microcosms on *S. typhimurium*

When *S. typhimurium* was seeded into microcosms established with human gut microbiota, an inhibitory effect on its growth was observed. Results from the pH controls, adjusted after 6 hours of incubation to mimic the pH drop in the microcosms (ranging between pH 4.5 to 6.5) showed no inhibitory effect on the growth of *S. typhimurium*. This suggests that the inhibitory effects in the microcosms were not due to changes in pH. The negative control (microcosm established with human gut microbiota and no added honey) confirmed that the microbiota were responsible for the inhibition of *S. typhimurium* as these counts were lower than the pH controls. Furthermore, inhibition was more pronounced in the microcosms containing any of the honey samples, indicating that the presence of honey resulted in further inhibition of the enteropathogen. A similar effect was seen in the inulin-enriched (prebiotic control) microcosms, suggesting that it may be the oligosaccharides in honey that offer the gut microbiota an advantage over the enteropathogen. It is likely that the inhibition of *S. typhimurium* was caused by competition for nutrients, i.e. the gut microbiota were able to use the components in the digested honeys as a growth substrate more readily than *S. typhimurium*. It is also probable that the gut microbiota inhibited the growth of *S. typhimurium* by direct antagonism; the drop in pH in the microcosms suggests that the microbiota produced SCFA which are known to offer benefits to the commensal bacteria while inhibiting intruders (Gibson and Wang, 1994). The inhibition of *S. typhimurium* may also be attributed to the production of additional antimicrobial substances by the gut microbiota, as the numbers of lactobacilli and bifidobacteria increased markedly in the microcosms in the presence of honey (see Chapter 3) and these bacteria are known to produce such substances (Gibson and Wang, 1994).

All honey samples tested showed inhibition of *S. typhimurium*, however, the level of inhibition varied depending on the honey sample and microbiota source used. When D1 microbiota were used in the microcosms, inhibition of *S. typhimurium* was more prominent in the presence of honeys (e.g. honeys 1 and 6) that gave rise to higher numbers of lactobacilli and bifidobacteria (based on bacterial counts in microcosms from Chapter 3). In cases where the D3 microbiota were used, honeys that were more inhibitory of *S. typhimurium* (honeys 2-5, 7-10, 12, 14-15, 18-20) were those that had previously shown lower numbers of enterics and clostridia in the microcosms (see Chapter 3). There were no obvious patterns observed when D2 microbiota were used in the assays, although the increased inhibition of *S. typhimurium* in the presence of some jarrah samples (honeys 3 and 5) and some yellow box samples (honeys 18-20) corresponded to larger changes in the clostridia numbers according to enumeration of clostridia in the

previous chapter. The different trends observed between the microbiota sources can be attributed to variations in the composition of the microbiota, for example the infant (D2) microbiota had the highest number of lactobacilli and bifidobacteria whereas the adult (D1 and D3) microbiota had higher counts of enterics, as has been previously documented (Gibson, 1999; Manning and Gibson, 2004; Ceapa *et al.*, 2013). When the data from all microbiota sources were considered together, there were no patterns to suggest that one honey type was superior to another. A possible explanation for this is that the oligosaccharide concentrations of the honeys (measured as a combined oligosaccharide + maltose concentration, as per Appendix 2) varied by only 2.1 % across the samples, despite their fructose, glucose and sucrose concentrations showing higher variation of 10.1, 12.8 and 6.2 %, respectively. Most honeys showed inhibition of *S. typhimurium* at levels comparable to the prebiotic control, inulin, suggesting that the bacterial isolate was not able to effectively use oligosaccharides (in the control or those present in honey) as a growth substrate. That the honeys performed as well as the inulin control is remarkable considering the oligosaccharide (and maltose combined) concentrations in the honeys ranged from 1.7 to 3.8 % compared to inulin, made up solely (100 %) of oligosaccharide.

One of the benefits of using oligosaccharides (prebiotics) as a nutrient source for the commensal microbiota is that they favour saccharolytic fermentation, rather than the potentially harmful proteolytic fermentation, by selectively promoting the growth of lactobacilli and bifidobacteria in the gut (reviewed in (Ceapa *et al.*, 2013)). As these beneficial gut populations are readily capable of using oligosaccharides, they can outnumber potential pathogens by competing for nutrients as was observed in these studies. Furthermore, saccharolytic fermentation leads to enhanced levels of fatty acids that reduce colonic pH and the production of other inhibitory compounds that have been linked to improved protection against potential pathogens (Gibson and Roberfroid, 1995), as well as a myriad of other intestinal health benefits including improved digestion and absorption and immunostimulation (Fioramonti *et al.*, 2003; Veereman, 2007). The pH drop in the microcosms observed here can be attributed to the increased levels of SCFA production by the gut microbiota as shown in previous chapters. It is likely that the production of these SCFA also aided in the inhibition of *S. typhimurium* in the microcosms. Therefore, the inhibition of *S. typhimurium* was likely the result of a combination of the competition for nutrients and the production of inhibitory substances by the commensal bacteria (discussed further below).

4.4.2 Effects of honey on introduced intestinal bacterial cultures

The commensal gut microbiota, particularly lactobacilli and bifidobacteria, are known to produce inhibitory substances effective against a range of enteropathogens. In order to

determine whether the addition of honey could enhance the inhibitory activity of the commensal bacteria, the enteropathogens *S. typhimurium*, *C. difficile* and *E. coli* were grown in the supernatants of the microcosms. The results showed that the supernatants impeded the growth of the enteropathogens, thereby suggesting that the commensal bacteria produced antimicrobial products in the presence of honey.

When the different honeys were considered, all honey enriched microcosms contained substances in the supernatant that were inhibitory to the *S. typhimurium* isolate. There were some cases where different honeys resulted in supernatants with varying levels of inhibition, and this could be due to the honey type and also dependent on the microbiota source. The honeys did have varying levels of oligosaccharides, and this coupled with the differences in microbiota composition could lead to the production of different inhibitory substances, or different amounts of the substances being produced. Typically, the experiments set up using the adult (D1 and D3) microbiota more closely resembled one another, whereas those using the infant (D2) microbiota showed some differences. For example, the *S. typhimurium* counts in supernatants from microcosms established using the infant microbiota were higher when jarrah, red stringybark, some yellow box, canola, and manuka honeys were used, compared to when the adult microbiota were used. This could be because the adult microbiota were composed of higher numbers of aerobic enterics compared to the infant microbiota and this had an effect on the types of inhibitory substances produced. The adult gut microbiota profile is more complex compared to that of infants, i.e. no longer dominated by *Bifidobacterium* spp., higher in aerobic enteric bacteria and higher in diversity of other anaerobic bacteria (Ceapa *et al.*, 2013). Faecal bifidobacterial counts in humans older than 55 years old are known to show a marked decrease in comparison to younger adults (Mitsuoka, 1990; Kleessen *et al.*, 1997). It has been suggested that reduced pathogen resistance may be linked to decreased numbers of bifidobacteria (especially in the elderly) and the production of natural resistance factors, i.e. that the natural gut microbiota is compromised because of the reduced numbers of bifidobacterial (Gibson, 1999). As such, prebiotic based strategies, aimed at restoring the bifidobacterial populations, may prove effective for selected populations such as the elderly.

The results from this thesis showed that a number of digested honeys have positive effects on the beneficial populations of the gut, regardless of the starting numbers of these populations present in the donor. Therefore, honey oligosaccharides (and the other non-digestible components in honey) can serve as an important tool in rebalancing the gut microbiota, similar to commercial prebiotics. The potential for prebiotic treatments has been documented in a number of studies (discussed in (Gibson *et al.*, 2004)). For example, a more recent study has shown that inulin-type fructans induced selective changes in the

composition of the gut microbiota in obese women, leading to changes in key metabolites associated with obesity and diabetes (Dewulf *et al.*, 2013).

An obvious drop in pH was observed in the microcosms, suggesting that honey allowed for enhanced production of SCFA (as shown in the previous chapter), which are known to have suppressive effects on the growth of potentially pathogenic bacteria. In order to elucidate the effect of decreased pH on the growth of the enteropathogens, pH controls were established where the test organisms were incubated in media of various pH ranging from 4.5 to 7.0. The growth of the enteropathogens was significantly hindered in the more acidic controls (pH 4.5, 5.0 and 5.5), but not at the higher pH values tested (pH 6.0, 6.5 and 7.0). This finding shows that low pH has a negative effect on the growth of the enteropathogens. However, it is probable that pH is not the only factor responsible for inhibition of the enteropathogens in the assays, as the pH drop in the microcosms occurs gradually (after 6 – 8 hours of incubation) whereas the controls were adjusted to the lower pH from the beginning of the assays. This is further confirmed when the results from the *S. typhimurium* seeded microcosm assays are considered, in which the pH controls showed no significant inhibition of the test organism when pH was adjusted after 6 hours of incubation. It is therefore reasonable to assume that the inhibitory effects against enteropathogens are multi-factorial. Interestingly, the inhibitory substances in the supernatants of the microcosms did not have an effect on the growth of a commercial probiotic strain, *L. fermentum*. This indicates that the SCFAs and other inhibitory compounds may be produced by similar lactic acid bacteria (lactobacillus and bifidobacteria) and have specific inhibitory activity against Gram-negative organisms, which are often identified as potentially pathogenic in the gut.

From the previous chapter, it is known that the jarrah honeys allowed for greater production of butyric and propanoic acid, whereas the acetic acid levels were comparable with the other honeys. Despite these differences, the pH of the microcosms were consistently between 5.5 – 6.0 most likely because of the large quantities of lactic acid produced by the lactobacilli. There was no indication that the higher volumes of SCFA produced in the microcosms resulted in lower counts of the enteropathogens, implying that even the lower quantities produced allowed adequate inhibition of the tested enteropathogens. It is known that the anaerobic breakdown of substrates, including undigested polysaccharides or oligosaccharides, resistant starch and fibre, enhances the formation of lactic acid bacteria and the production of SCFA as fermentation products (Wollowski *et al.*, 2001). These effects have been associated with increased protection from pathogens ((Gibson *et al.*, 1995; Gibson, 1999)), and the results from this chapter suggest that digested honey can similarly promote these beneficial effects in the gut.

The inclusion of any of the honey samples in the microcosms generally produced inhibitory substances that were at least as effective as those produced in the presence of the prebiotic control, inulin. Inhibition of *S. typhimurium* and *E. coli* was significantly better in the presence of honey or inulin compared to when the microcosms were established with medium alone. This indicates that the microbiota in the microcosms were capable of using the digested components of the honeys to produce antimicrobial substances in the supernatant. The growth of *C. difficile* did not resemble that of the other two enteropathogens; *C. difficile* was especially sensitive to the inhibitory substances and there were greater variations in the counts depending on the honey type and microbiota source used to establish the microcosms. Most of the honeys tested were more effective at inhibiting the growth of *C. difficile* than the prebiotic control, inulin. It was noted that inclusion of inulin in the microcosms had little added inhibitory effect on *C. difficile* growth, as the counts were similar to those obtained when the bacterium was grown in supernatants from the medium-only microcosms. These data support that the honeys allowed enhanced production of the inhibitory substances. Numerous other studies have documented the production of inhibitory substances by the commensal bacteria on potentially harmful bacteria (Ramare *et al.*, 1993; Dabard *et al.*, 2001; Destoumieux-Garzon *et al.*, 2002; Riley and Wertz, 2002; Lievin-Le Moal and Servin, 2006). For example, compounds secreted by *Lactobacillus* were shown to decrease colonisation by pathogenic *E. coli in vivo* (Medellin-Pena and Griffiths, 2009). Furthermore, lactobacilli produce lactic acid which not only directly inhibits the growth of many bacteria, but also affects the permeability of the outer membrane of Gram-negative bacteria (Alakomi *et al.*, 2000).

The sensitivity of clostridia to the effects of honey in the microcosms was also noted in the previous chapter. Therefore, the application of honey for intestinal diseases, especially those in which clostridia are implicated, could provide health benefits. In addition, since *L. fermentum* was not susceptible to the inhibitory substances produced in the microcosms, honey could be administered with commercial probiotic strains (i.e. a synbiotic – combination of probiotic and prebiotic) to further improve intestinal health. Synbiotics have previously been shown to protect mice against *Salmonella typhimurium* infection more effectively than when the probiotic or prebiotic were administered alone (Asahara *et al.*, 2001). Based on the results presented in this thesis, the oligosaccharides in digested honey can function effectively as prebiotics and are an ideal candidate for use in combination with probiotics. This approach offers a potentially greater positive effect by stimulating the beneficial populations of the gut and promoting production of inhibitory substances against pathogens, while still supporting the growth of the commercial probiotics strains.

The combination of a prebiotic with a probiotic has been shown to protect mice against *Salmonella typhimurium* infection more effectively than when the probiotic or prebiotic were administered alone (Asahara *et al.*, 2001). Based on the results presented in this thesis, honey oligosaccharides can function effectively as prebiotics and are an ideal candidate for use in combination with probiotics. This approach offers a potentially greater positive effect by stimulating the beneficial populations of the gut and promoting production of inhibitory substances against pathogens, while still supporting the growth of the commercial probiotics strains.

4.5 CONCLUSION

Australian honeys were shown to have an inhibitory effect on several enteropathogens by inducing favourable compositional and functional changes in human gut microbiota *in vitro*. In the presence of honey, inhibition of the growth of bacterial isolates implicated in diseases of the gut occurred through the production of inhibitory substances and most probably by competition for nutrients. Furthermore, the inhibitory substances that were produced by the intestinal microbiota in the presence of honey did not inhibit the growth of a commercial probiotic strain, further supporting the use of honey as an agent to promote intestinal health. The results presented in this chapter further support those in the previous chapters by showing that the Australian honey samples tested have an overall positive effect on human gut microbiota *in vitro*.

5 CHAPTER FIVE
General discussion

5.1 SUMMARY AND COMPARISON OF THE PREBIOTIC PROPERTIES OF AUSTRALIAN FLORAL HONEYS

The gut microbiota plays a critical role in the health and well-being of humans. As a result, there is much interest in the manipulation of the gut microbiota to a more favourable composition by dietary means. In particular, non-digestible carbohydrates – known as prebiotics – have been used to promote specific, favourable changes in the composition and functionality of the gut microbiota. Honey contains non-digestible oligosaccharides and there is evidence that some honeys could induce beneficial changes in the gut.

The work presented in this thesis was an in-depth investigation of the prebiotic properties of Australian floral honeys using *in vitro* microcosms.

In Chapter 2, the impact of the saccharides of high fructose Australian honeys on the microbial composition and metabolic activity of the gut was explored. In this chapter, the honeys were tested with and without digestion to investigate the contribution of both the monosaccharides and oligosaccharides on the observed effects.

The findings in Chapter 2 showed that the oligosaccharides in the tested honeys enhanced the growth and/or metabolic activity of potentially beneficial bacteria in intestinal microcosms. Chapter 3 focused specifically on digested honeys, i.e. the oligosaccharide components within the honeys, and their ability to induce changes in the gut. In this chapter, the effect of 25 well-characterised Australian honeys were investigated using a number of approaches including culture-based techniques supported by molecular profiling, measuring the effect of the honeys on SCFA production by the gut microbes, and quantifying the prebiotic effect of the honeys by calculating their Prebiotic Index (PI) values.

Chapter 4 detailed an investigation of the impact of honey on the production of inhibitory compounds by the gut microbiota. The effect of *in vitro* fermentation of honey, by human gut microbiota, on the growth of introduced bacterial species (three enteropathogens and one probiotic strain) was explored.

A summary of the findings from the numerous approaches and techniques that were used to investigate the prebiotic properties of Australian honeys is shown in Table 5.1, and this

allows for a comparison of the activity of the different honeys, as well as the different techniques used throughout this project.

Table 5.1 | Summary and comparison of the effects of Australian floral honeys on human gut microbiota

Honey type	Sample	Lac[a]	Bif[b]	Clo[c]	Ent[d]	PI[e]	Butyric acid[f]	ST inhibition[g]	Effect of microcosm supernatants			
									ST[h]	CD[i]	EC[j]	LF[k]
Yellow box	i	+++	+++	++	++	++	-	NA	NA	NA	NA	NA
Banksia	ii	+++	+++	+	+	++	+++	NA	NA	NA	NA	NA
Manuka	iii	+++	+++	+++	+++	++	-	+++	+++	+++	++	++
Jarrah	H1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
	H2	++	+++	+++	+++	+++	+++	++	+++	+++	+++	++
	H3	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
	H4	+++	+++	+++	++	+++	+++	++	+++	+++	++	++
	H5	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
Red stringybark	H6	++	+	++	+++	+	+++	++	+++	+++	++	+++
	H7	++	+	++	++	+	+++	++	+++	+++	++	+++
	H8	++	++	+++	+++	++	+++	+++	++	+++	+++	+++
	H9	++	++	++	+++	++	+++	+++	+++	+++	++	+++
	H10	++	++	++	+++	+	+++	+++	+++	+++	++	+++
Spotted gum	H11	+	++	+++	+++	++	+++	++	+++	+++	++	+++
	H12	+	+	+++	+++	+	+++	+++	+++	+++	++	+++
	H13	++	+	+++	+++	-	+++	++	+++	+++	+++	++
	H14	++	+	+++	+++	+	+++	++	+++	+++	+++	+++
	H15	++	+	+++	+++	+	+++	+++	+++	+++	+++	+++
Yellow box	H16	+++	++	+++	+++	++	+++	++	+++	+++	++	+++
	H17	+++	++	+++	++	++	+++	++	+++	+++	++	+++
	H18	+++	++	+++	+++	++	+++	+++	+++	+++	++	+++
	H19	+++	++	+++	+++	++	+++	+++	+++	+++	++	+++
	H20	++	++	+++	++	+	+++	+++	+++	+++	++	+++
Canola	H21	++	++	++	++	+	+++	+++	++	+++	++	++
	H22	++	++	+++	+++	+	+++	+++	++	+++	++	+++

Table legend:

Scoring system ranges from (-) no difference to relevant negative control to (+++) honey at least as effective as prebiotic control(s)

NA- not tested

Blue shades represent level of beneficial effect: light-, mid- and dark-blue corresponding to good, very good and excellent, respectively

Lac[a] - Increase in lactobacilli counts in microcosms

Bif[b] - Increase in bifidobacteria counts in microcosms

Clo[c] - Decrease in clostrida counts in microcosms

Ent[d] - Decrease in enteric bacteria counts in microcosms

PI[e] -Prebiotic Index values calculated from bacterial counts in microcosms

Butyric acid[f] - Increase in butyric acid levels in microcosms

ST inhibition[g] - Inhibition of *S. typhimurium* seeded into microcosms

ST[h] - Inhibitory effect of microcosm supernatant on growth of *S. typhimurium*

CD[i] - Inhibitory effect of microcosm supernatant on growth of *C. difficile*

EC[j] - Inhibitory effect of microcosm supernatant on growth of *E. coli*

LF[k] - Growth of *L. fermentum* in microcosm supernatant

5.1.1 Assessment of prebiotic potential of honeys *in vitro*

As summarised in Table 5.1 above, all varieties of the Australian floral honeys tested in this project confer benefits to the gut microbiota, either by enhancing the numbers of the beneficial bacteria, reducing numbers of the potentially harmful bacteria, increasing production of butyric acid, increasing protection against enteropathogens, or by supporting the growth of a commercial probiotic. The extent of the beneficial impact varied according to the honey type. Different conclusions of the prebiotic potential of each honey can be drawn, depending on the technique used for prebiotic assessment, and this is discussed further below.

The Prebiotic Index (PI) is a quantitative comparative assessment of the prebiotic capabilities of carbohydrates (Palframan *et al.*, 2003). Based on the PI in Table 5.1, it can be inferred that of the honeys tested, the jarrah honeys (all five samples) are the most promising candidates for prebiotic use because their PIs are equivalent to those of the commercial prebiotic, inulin.

However, if the PI values alone are used to determine prebiotic potential, there are a number of important factors contributing to the beneficial effect that honey has on the gut microbiota that would be overlooked. In addition, while there is a consensus that bifidobacteria and lactobacilli are health promoting populations in the gut, there is less agreement on the other bacterial groups used in the prebiotic calculation, as they can be considered as both potentially harmful and potentially beneficial. This is particularly notable when bacteroides are considered, as they are the most numerically dominant group in the gut and therefore changes in the counts of bacteroides can skew the PI

calculation significantly. Furthermore, the PI values of the honeys varied depending on the microbiota source used. Consequently, there is a need for a revised prebiotic scoring system that takes into account the numerous techniques for measuring prebiotic potential.

5.1.2 Proposed revised prebiotic score

The summary in Table 5.1 shows that certain honeys offer a greater beneficial effect than others, relative to both negative and commercial prebiotic (inulin) controls. Those honeys that showed no difference when compared to the negative control were scored with the negative symbol (-), and those that were more beneficial than the negative control were scored with plus symbols ranging from (+) to (+++), (and lightest to darkest blue), with (+++) signifying that the effects were most similar to inulin.

When the individual techniques for measuring beneficial effects of the honeys on gut microbiota are considered, the ranking results differ from when PI alone is used. For example, if the growth of lactobacilli is the only technique used to deduce prebiotic potential, the yellow box honeys are more effective than the jarrah samples, and if only butyric acid production is taken into account, then all honeys performed equally as well as each other and the commercial prebiotic. Therefore, including more factors in the prebiotic assessment of the honeys (and other non-digestible carbohydrates) would provide a broader understanding of the expected health benefits. Comparison to a commercial prebiotic can offer a means to standardise the prebiotic score.

Although this is a qualitative approach, it can be converted to a simple quantitative tool by assigning numerical values to the symbols, with (-) equivalent to 0, (+) equivalent to 1, and so on. The resulting scores can then be tallied and expressed as a ratio of the total possible score, dependant on how many of the techniques are performed. For example, the prebiotic scores of some honeys would be calculated as below:

- banksia honey (honey ii) scored 13 out of a possible 18 (six techniques used, with possible highest score of three per test), resulting in a final prebiotic score of 0.72
- jarrah honey (honey 1) scored 31 out of a possible 33 (eleven techniques used), resulting in a final prebiotic score of 0.94.

The ratio could be a useful means of comparing the prebiotic capabilities of different carbohydrates, similar to the manner in which PI has been used in the past. Using the techniques summarised in Table 5.1 and the calculation above, the tested honeys were assigned a prebiotic score and this is shown below in Table 5.2.

Table 5.2 | Revised prebiotic score of Australian floral varieties of honey

Revised prebiotic score calculated based on the numerous effects of the honeys relative to a negative (no honey added) and positive (commercial prebiotic) controls. A score of 1.0 denotes that the test sample was at least as effective as the commercial prebiotic and a score of 0.0 denotes that the test sample was no more effective than the negative control.

Honey type	Sample	Prebiotic score
Yellow box	i	**0.67**
Banksia	ii	**0.72**
Manuka	iii	**0.82**
Jarrah	H1	**0.94**
	H2	**0.91**
	H3	**0.91**
	H4	**0.88**
	H5	**0.91**
Red stringybark	H6	**0.76**
	H7	**0.73**
	H8	**0.88**
	H9	**0.85**
	H10	**0.82**
Spotted gum	H11	**0.82**
	H12	**0.79**
	H13	**0.76**
	H14	**0.82**
	H15	**0.85**
Yellow box	H16	**0.88**
	H17	**0.85**
	H18	**0.91**
	H19	**0.91**
	H20	**0.82**
Canola	H21	**0.73**
	H22	**0.82**

Based on these data, it can be concluded that of the honeys tested, jarrah honeys 1, 2, 3, 5 and yellow box honeys 18 and 19 show the highest prebiotic potential. Overall, the scores ranged from 0.67 to 0.94 and most of the honeys (18 samples out of 25) scored 0.80 and above, including some that had given rise to low PI values, such as red stringybark 10, spotted gum 14 and 15, yellow box 20, and canola 22.

This approach allows a more comprehensive understanding of the prebiotic capabilities of carbohydrates.

In addition, modifications could be made to the calculations to measure the effectiveness of a given carbohydrate. For example, four plus symbols (++++) could be used to identify those carbohydrates that behave significantly better than a prebiotic control, resulting in a final score above 1.0.

5.2 Additional potential benefits of honey consumption on gut health

The results from many *in vitro* studies suggest that the consumption of honey will have a prebiotic effect on the host by stimulating the growth of the beneficial populations in the gut (Shamala *et al.*, 2000; Chick *et al.*, 2001; Kajiwara *et al.*, 2002; Shin and Ustunol, 2005; Haddadin *et al.*, 2007; Ustunol, 2007; Jan Mei *et al.*, 2010). A previous study has also shown that potentially probiotic *Lactobacillus* species had a preference for honey over other carbohydrates, inulin and gum acacia (Dhewa and Goyal, 2009). The beneficial effects of honey on the gut microbiota can be attributed mainly to the oligosaccharide components that are likely to survive digestion following consumption, as indicated by Sanz *et al* (Sanz *et al.*, 2004), Conway *et al* (Conway *et al.*, 2010) and the work presented in this thesis. Furthermore, it has been proposed that the oligosaccharides in honey could prevent colonisation of pathogens in the gastrointestinal tract (such as *Helicobacter pylori*) by attaching to the bacterial cell walls and preventing adhesion to human tissues (Al Somal *et al.*, 1994).

Since numerous intestinal disturbances have been linked to changes in the gut microbiota, antibiotic therapies are commonly used in the clinical management of these diseases. However, some diseases, such as inflammatory bowel disease, are becoming increasingly difficult to treat as the use of antibiotics can induce antibiotic resistant strains causing further impairment (Guarner and Malagelada, 2003). For this reason, there has been an interest in using probiotics, or prebiotics that selectively promote beneficial populations, for health benefits. In this thesis, the honeys tested have shown prebiotic capabilities through the promotion of the beneficial populations, while reducing the numbers of the less desirable ones, such as clostridia which are commonly implicated in antibiotic-associated diarrhoea. In addition, the work presented in this chapter shows that the gut microbiota is able to use the honey to inhibit enteropathogens and that the honey supported growth of a commercial probiotic strain. Therefore, there is potential for honey to play a valuable role in reducing the negative impact of some antibiotics. However, it should be noted that there are variations in the composition of honeys, mainly influenced

by plant source, and this does affect their activity as has been previously identified (Molan, 1992).

5.3 FUTURE WORK AND HUMAN CLINICAL STUDY

Although there are numerous studies showing the effectiveness of some honeys as prebiotic agents that are capable of selectively promoting the gut microbiota, more research needs to be done in the field. The effectiveness of honeys from a wider range of floral sources, Australian and internationally sourced, should be further investigated. In addition, it would be of use to study honeys with varying levels of oligosaccharides in order to investigate the link between oligosaccharide concentration and PI, growth or suppression of certain bacteria, and production of SCFA or other beneficial compounds.

Also, although culture-dependent techniques offer useful insight into the composition and function of the gut microbiota, molecular techniques are being increasingly used to understand the complex microbial population of the gut. The research presented here used molecular techniques to investigate the prebiotic properties of some honeys, and although only four honeys were studied that data strongly suggests that more work in this area should be further explored.

Controlled clinical trials would be invaluable in measuring the prebiotic potential of honey, and based on the promising *in vitro* results presented in this thesis, a double-blinded human clinical study has been conducted (reporting on this was outside the scope of this thesis).

The clinical study was modelled on one performed by Wallace *et al*. (2010), which showed no impact on the gut microbiota when subjects consumed manuka honey. Four different honeys were chosen to cover the parameters of low and high butyric acid production, and low and high PI values as found in the work presented here. The effect of consuming 20g of a honey daily for 4 weeks was determined using the methods described in this thesis, and the results were supportive of the *in vitro* findings. That is, selected Australian honeys offer significant prebiotic benefits when consumed by human subjects (manuscript in preparation).

5.4 CONCLUSIONS

This study is the first comprehensive assessment of the prebiotic properties of a large number of Australian honeys, in both their whole and digested forms, on various microbiota, using a number of different approaches. The results from this investigation indicate that Australian honeys have considerable potential for use as prebiotics.

The saccharides present in honey, both simple and complex, can affect the bacterial composition of the gut favourably as demonstrated by analysis of the microbial profiles and calculation of the Prebiotic Index. Although the compositional changes can be attributed mainly to the oligosaccharide components of the honeys, the monosaccharides can also contribute to the modulation of the gut microbiota. Enhanced levels of lactobacilli were observed in the presence of high fructose content honeys, and these effects were attributed to the fructose components.

In addition, the non-digestible components (oligosaccharides) of the honeys contributed to the beneficial effects on the composition and also the metabolic activity of the gut microbiota, as reflected in the elevated levels of the SCFA (particularly butyric acid). All honeys tested promoted the growth of the potentially beneficial populations of the gut and inhibited intestinal pathogens. These benefits were demonstrable at levels that matched or surpassed commercial prebiotics, even though the oligosaccharide concentration of the honeys was markedly less than the controls of commercial prebiotics. The concentration of the inulin (1 %) was the same as the concentration of the honey (1 %) of which less than 5 % was oligosaccharides. Interestingly the honeys suppressed the pathogen *C. difficile* but the inulin did not.

It was concluded that the tested Australian honeys had considerable prebiotic capacity which was comparable to or better than inulin. The prebiotic properties of the honeys were associated with the fructose and the oligosaccharides of the honeys which promoted the beneficial bacteria, inhibited the potential pathogens and elevated butyric acid levels.

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Appendices

Appendix 1

Analysis of SCFA produced by gut microbiota determined by GC-MS.

		Isobutyric acid (mM)		Pentanoic acid (mM)		Isovaleric acid (mM)	
Donor	**Substrate**	**Digested**	**Whole**	**Digested**	**Whole**	**Digested**	**Whole**
D1	Initial	*3.15 ±0.06*	*3.15 ±0.06*	*5.98 ±0.03*	*5.98 ±0.03*	*3.79 ±0.02*	*3.79 ±0.02*
D1	Honey i	**3.38** ±0.06	**3.57** ±0.06	3.17 ±0.05	**6.17** ±0.03	3.55 ±0.06	**3.98** ±0.02
D1	Honey ii	3.03 ±0.04	**3.76** ±0.06	3.16 ±0.06	**6.36** ±0.03	3.49 ±0.02	**4.17** ±0.02
D1	Honey iii	2.89 ±0.09	**3.95** ±0.06	3.28 ±0.16	**6.55** ±0.03	3.36 ±0.05	**4.36** ±0.02
D1	Inulin	**3.85** ±0.1	**4.14** ±0.06	3.19 ±0.03	**6.74** ±0.03	**6.41** ±0.27	**4.55** ±0.02
D1	Fructose	2.92 ±0.06	**5.42** ±0.06	3.28 ±0.16	5.78 ±0.16	3.47 ±0.06	**5.97** ±0.06
D1	Glucose	2.76 ±0.06	**5.56** ±0.06	3.16 ±0.05	5.96 ±0.05	3.19 ±0.02	**5.99** ±0.02
D1	Media	2.83 ±0.04	2.78 ±0.06	3.32 ±0.03	3.17 ±0.04	3.28 ±0.02	3.19 ±0.07
D2	Initial	*2.68 ±0.03*	*2.68 ±0.03*	*3.23 ±0.09*	*3.23 ±0.09*	*2.90 ±0.03*	*2.90 ±0.03*
D2	Honey i	**2.98** ±0.04	**2.87** ±0.03	3.12 ±0.01	**3.42** ±0.09	**3.89** ±0.03	**3.25** ±0.32
D2	Honey ii	**18.92** ±0.04	**3.06** ±0.03	**5.03** ±0.05	**5.22** ±0.05	**3.52** ±0.03	**3.87** ±0.27
D2	Honey iii	**2.90** ±0.03	**3.25** ±0.03	3.13 ±0.02	**3.32** ±0.02	**3.79** ±0.05	**4.15** ±0.25
D2	Inulin	**4.51** ±0.09	**3.44** ±0.03	**3.30** ±0.07	**3.49** ±0.07	**13.07** ±0.92	**13.43** ±0.69
D2	Fructose	**3.25** ±0.05	**6.05** ±0.05	**3.25** ±0.14	**6.05** ±0.14	**4.35** ±0.31	**7.15** ±0.31
D2	Glucose	**2.79** ±0.02	**4.17** ±0.02	3.18 ±0.06	**4.56** ±0.06	**3.47** ±0.16	**4.85** ±0.16
D2	Media	**2.80** ±0.09	2.64 ±0.03	3.19 ±0.06	**3.59** ±0.03	**3.31** ±0.21	**3.16** ±0.06

		Isocaproic acid (mM)		Hexanoic acid (mM)		Heptanoic acid (mM)	
Donor	**Substrate**	**Digested**	**Whole**	**Digested**	**Whole**	**Digested**	**Whole**
D1	Initial	*5.88 ±0.1*	*5.88 ±0.1*	*6.22 ±0.09*	*6.22 ±0.09*	*6.36 ±0.2*	*6.36 ±0.2*
D1	Honey i	2.95 ±0.06	**6.07** ±0.1	2.88 ±0.22	**6.41** ±0.09	2.98 ±0.04	**6.55** ±0.2
D1	Honey ii	3.07 ±0.05	**6.26** ±0.1	2.70 ±0.08	**6.60** ±0.09	2.47 ±0.06	**6.74** ±0.2
D1	Honey iii	2.89 ±0.09	**6.45** ±0.1	2.85 ±0.08	**6.79** ±0.09	2.69 ±0.17	**6.93** ±0.2
D1	Inulin	4.72 ±0.27	**6.64** ±0.1	2.73 ±0.07	**6.98** ±0.09	2.63 ±0.12	**7.12** ±0.2
D1	Fructose	3.05 ±0.06	5.55 ±0.06	2.91 ±0.11	5.41 ±0.11	2.50 ±0.01	5.00 ±0.01
D1	Glucose	2.94 ±0.08	5.74 ±0.08	4.97 ±0.16	**7.77** ±0.16	**7.99** ±0.03	**10.79** ±0.03
D1	Media	2.96 ±0.07	2.91 ±0.01	5.01 ±0.09	4.31 ±0.53	2.54 ±0.03	2.51 ±0.01
D2	Initial	*2.55 ±0.1*	*2.55 ±0.1*	*9.47 ±0.03*	*9.47 ±0.03*	*2.56 ±0.08*	*2.56 ±0.08*
D2	Honey i	**2.68** ±0.02	**2.74** ±0.1	2.46 ±0.06	**9.66** ±0.03	2.50 ±0.05	**2.75** ±0.08
D2	Honey ii	**16.84** ±0.31	**17.03** ±0.31	**69.00** ±5.33	**69.19** ±5.33	2.52 ±0.01	**2.71** ±0.01
D2	Honey iii	**2.78** ±0.15	**2.97** ±0.15	**27.50** ±1.64	**27.69** ±1.64	2.33 ±0.15	2.52 ±0.15
D2	Inulin	**2.74** ±0.07	**2.93** ±0.07	2.62 ±0.06	2.81 ±0.06	**9.21** ±0.01	**9.40** ±0.01
D2	Fructose	**2.78** ±0.03	**5.58** ±0.03	2.55 ±0.01	5.35 ±0.01	2.52 ±0.02	**5.32** ±0.02
D2	Glucose	**2.72** ±0.04	**4.10** ±0.04	2.65 ±0.04	4.03 ±0.04	2.56 ±0.03	**3.94** ±0.03
D2	Media	2.50 ±0.11	2.39 ±0.13	2.62 ±0.1	2.56 ±0.05	**2.57** ±0.06	2.32 ±0.1

APPENDIX 2

External analysis of sugars in honey.

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Bulleen ◆ Victoria ◆ 3105
Australia

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Info@ChemicalAnalysis.com.au

Analytical Report

Analysis of Sugars in Honey

Client:	Pestat Pty Ltd
Client Contact:	Dr. David Dall
Client Address:	LPO Box 5055, University of Canberra, Bruce, ACT, 2617
Date:	29th October, 2010
Job No:	C12081
Client Reference:	Analysis Request Form dated 14th October, 2010.
Job Coordinator:	Dr Iain Cook
Samples Received:	15th October, 2010
Request:	Analysis of sugars (glucose, fructose, sucrose, maltose, and total oligosaccharides).

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Samples:

CA Sample ID	Customer Sample ID
C12081/1	7843WES Honey Sample 01; jarrah 1
C12081/2	7863WES Honey Sample 02; jarrah 2
C12081/3	8012WES Honey Sample 03; jarrah 3
C12081/4	8105WES Honey Sample 04; jarrah 4
C12081/5	8113WES Honey Sample 05; jarrah 5
C12081/6	7264DEN Honey Sample 06; red stringbark 1
C12081/7	7369HOL Honey Sample 07; red stringbark 2
C12081/8	7460EMM Honey Sample 08; red stringbark 3
C12081/9	7515BBN Honey Sample 09; red stringbark 4
C12081/10	7526BOM Honey Sample 10; red stringbark 5
C12081/11	3747RUT Honey Sample 11; spotted gum 1
C12081/12	3854DEN Honey Sample 12; spotted gum 2
C12081/13	3883SNO Honey Sample 13; spotted gum 3
C12081/14	4442BOM Honey Sample 14; spotted gum 4
C12081/15	5485BOM Honey Sample 15; spotted gum 5
C12081/16	5735SPI Honey Sample 16; yellow box 1
C12081/17	7130SMI Honey Sample 17; yellow box 2
C12081/18	7141WRI Honey Sample 18; yellow box 3
C12081/19	7427RUT Honey Sample 19; yellow box 4
C12081/20	7626DEN Honey Sample 20; yellow box 5
C12081/21	8168KLI Honey Sample 21; canola 1
C12081/22	8193SNO Honey Sample 22; C/SB 2

TGA 208741

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Page 1 of 3

C12081 Ver: 01

Experimental:

Samples were prepared in Milli-Q water (deionized Type I) at a concentration of approximately 10 000 mg/L. Samples were analysed using HPLC with ELSD detection. Calibration curves were generated for each sugar in the ranges of 2000 to 5000 mg/L (fructose), 1500 to 3000 mg/L (glucose), and 250 to1000 mg/L (sucrose and maltose). Total oligosaccharides are reported as maltose + total oligosaccharides due to overlapping peaks.

Results:

Table 1. Sugar content results for samples C12081/1 to C12081/22

Sample	w/w% [1]				
	Fructose	**Glucose**	**Sucrose**	**Maltose + Total Oligosaccharides[2]**	**Total Saccharides**
C12081/1	40.7	22.9	7.3	3.8	74.8
C12081/2	39.6	27.9	4.5	3.3	75.3
C12081/3	42.1	23.6	6.8	3.1	75.6
C12081/4	41.0	23.0	7.4	3.5	74.9
C12081/5	35.3	20.5	5.4	3.8	65.0
C12081/6	42.7	21.2	6.2	3.3	73.5
C12081/7	38.3	28.9	5.6	1.7	74.4
C12081/8	41.2	27.1	7.1	3.6	79.0
C12081/9	42.6	24.7	4.5	2.3	74.0
C12081/10	43.5	24.5	5.2	1.9	75.2
C12081/11	43.1	25.6	3.4	2.2	74.3
C12081/12	45.4	24.4	3.0	2.2	75.0
C12081/13	43.8	25.6	3.4	2.4	75.2
C12081/14	42.7	27.5	2.7	2.2	75.1
C12081/15	43.2	27.9	2.7	2.0	75.9
C12081/16	38.2	26.8	3.8	3.3	72.2
C12081/17	43.2	24.2	5.2	3.5	76.1
C12081/18	42.5	24.2	5.4	3.5	75.6
C12081/19	42.0	24.2	6.3	2.8	75.3
C12081/20	43.4	23.8	5.4	3.0	75.6
C12081/21	38.8	33.3	1.2	2.3	75.6
C12081/22	42.1	26.1	4.4	2.0	74.7

[1] Results are an average of duplicate sample preparations.
[2] Results calculated using maltose calibration curve.

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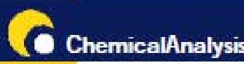

	Name	Position	Signature	Date
Prepared by:	Carrie Brisbane	Analyst		___/_____/______
Approved by:	Dr Iain Cook	Principal Scientist		___/_____/______

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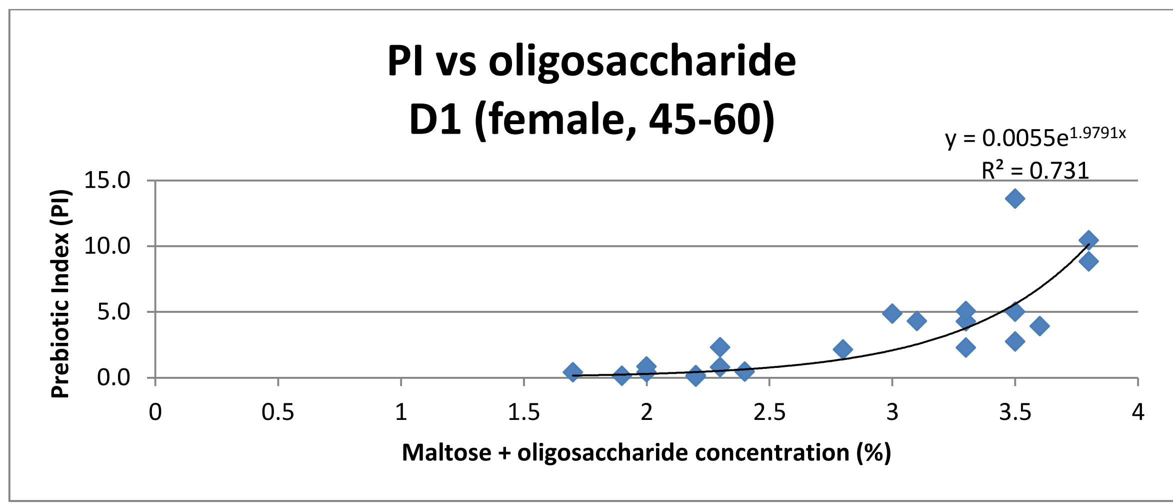
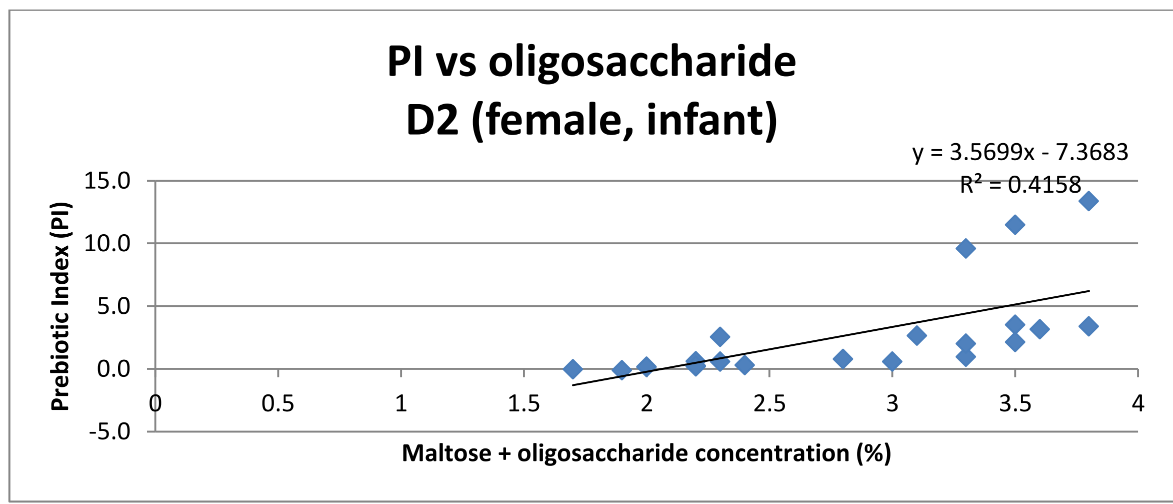
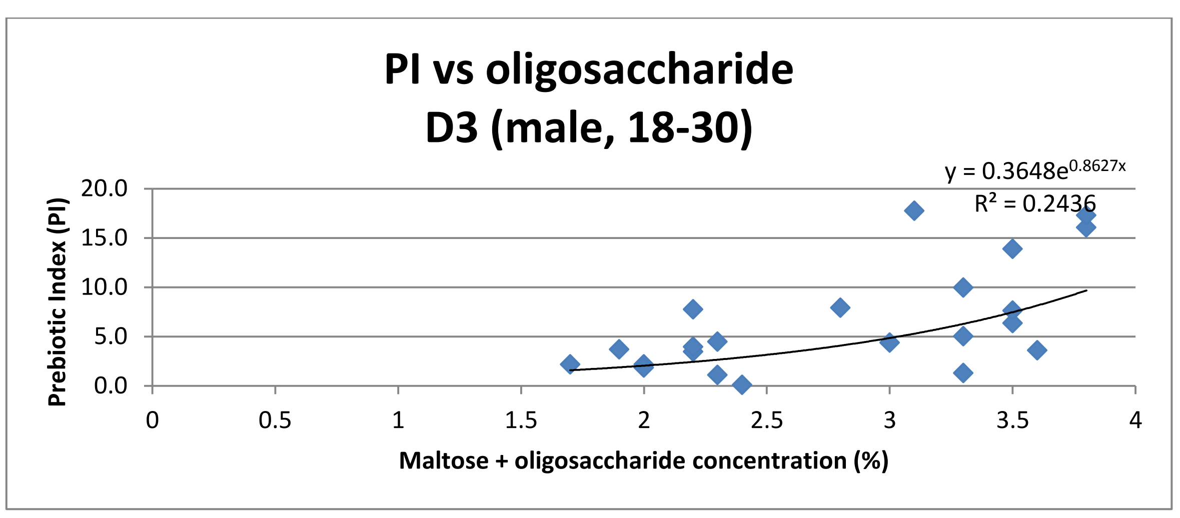
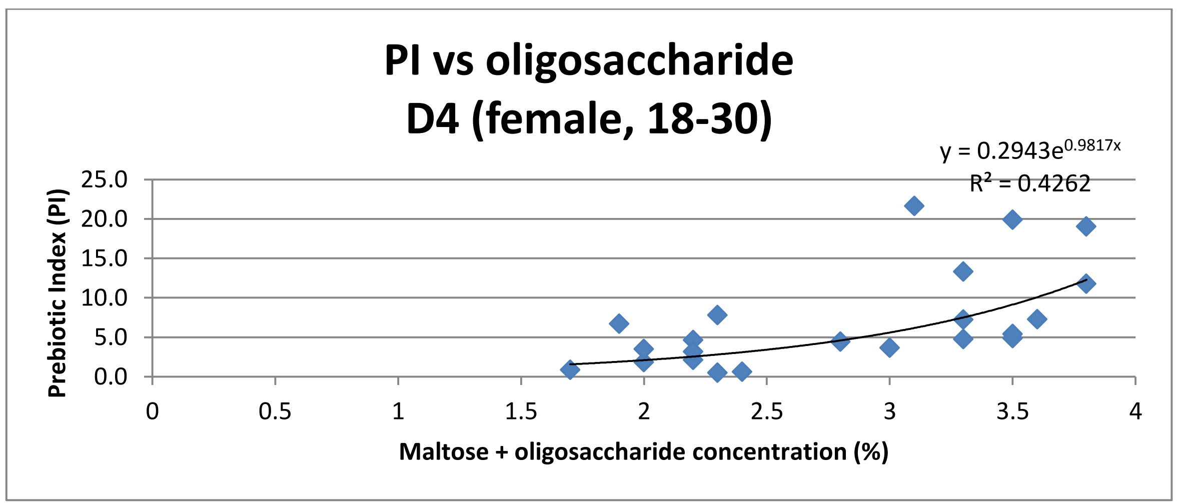
APPENDIX 3

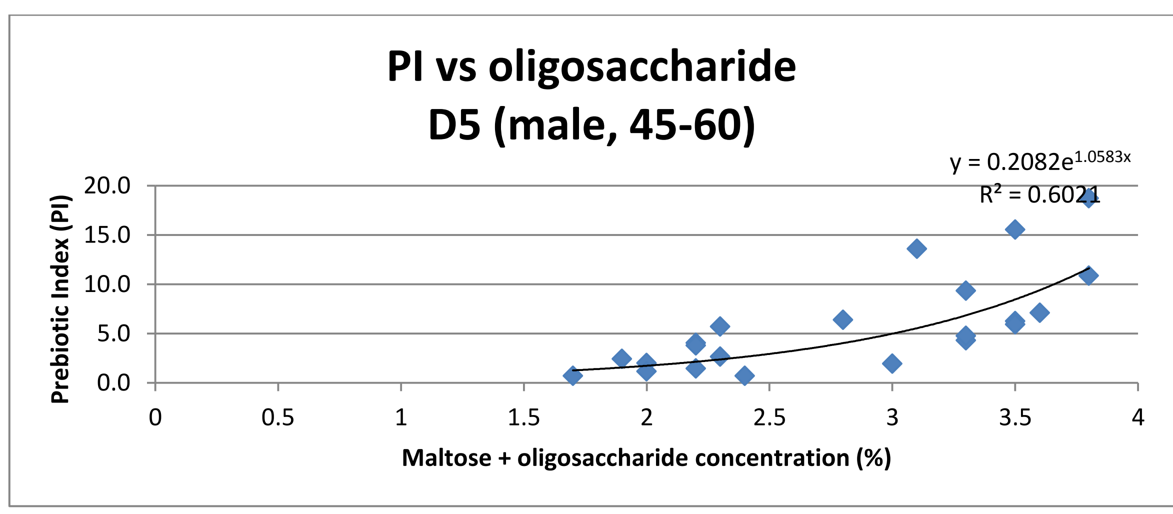
Regression analysis graphs of Prebiotic Indices (PI) of digested honeys versus corresponding maltose + oligosaccharide concentrations (%) in whole honeys.





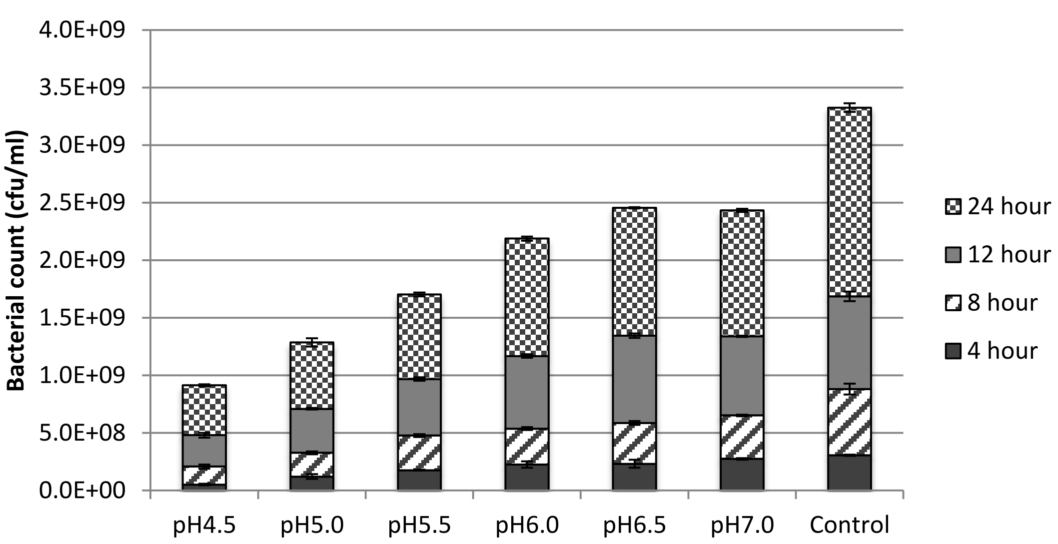

PI vs oligosaccharide
D3 (male, 18-30)
y = 0.3648e^0.8627x
R² = 0.2436
Prebiotic Index (PI)
20.0
15.0
10.0
5.0
0.0
0
0.5
1
1.5
2
2.5
3
3.5
4
Maltose + oligosaccharide concentration (%)

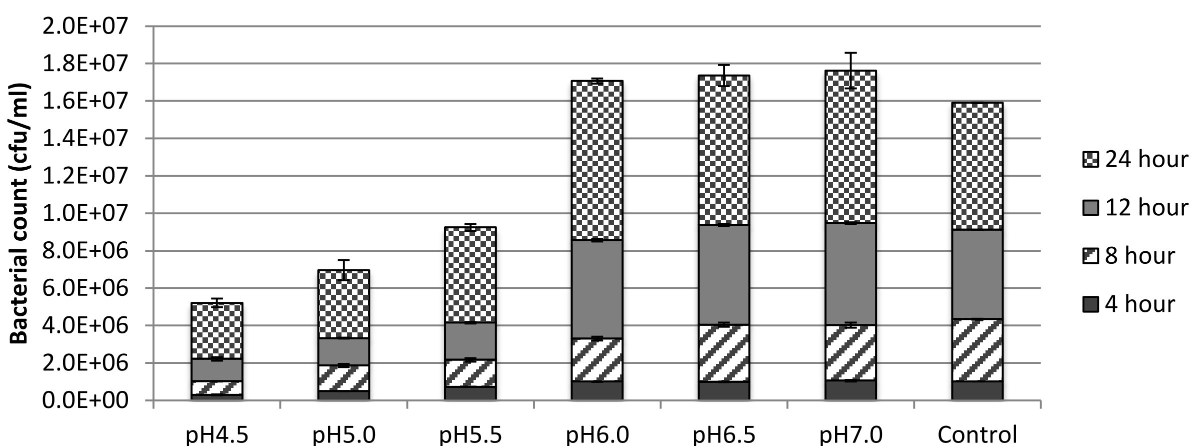

PI vs oligosaccharide
D4 (female, 18-30)
y = 0.2943e^0.9817x
R² = 0.4262
Prebiotic Index (PI)
25.0
20.0
15.0
10.0
5.0
0.0
0
0.5
1
1.5
2
2.5
3
3.5
4
Maltose + oligosaccharide concentration (%)

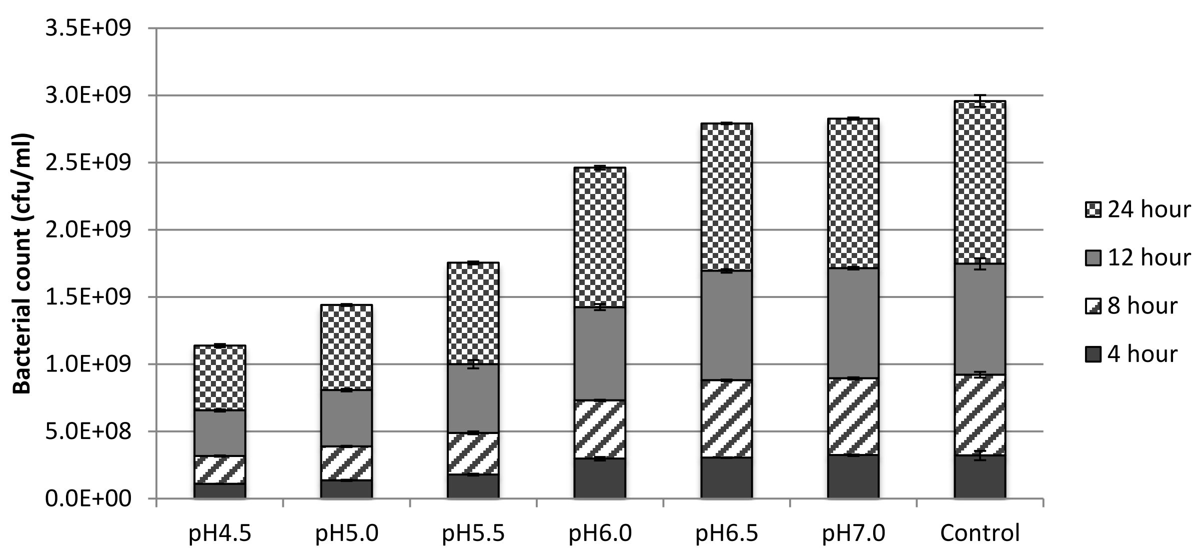

PI vs oligosaccharide
D5 (male, 45-60)
y = 0.2082e^1.0583x
R² = 0.6021
Prebiotic Index (PI)
20.0
15.0
10.0
5.0
0.0
0
0.5
1
1.5
2
2.5
3
3.5
4
Maltose + oligosaccharide concentration (%)

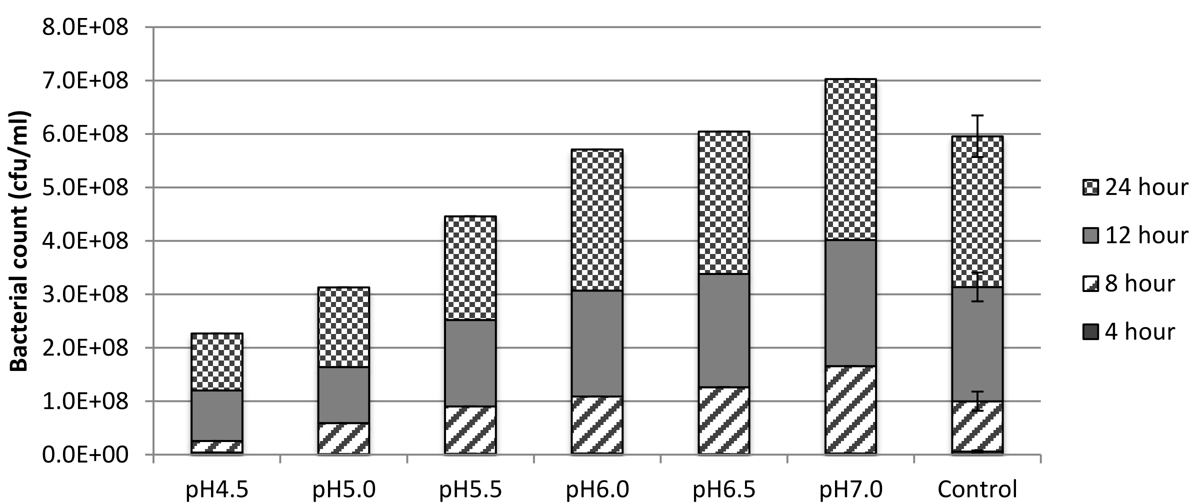
APPENDIX 4

Control wells containing the test organisms were incubated in media of various pH levels to determine the effect of pH on their growth. The pH of these assays was adjusted at the beginning of the experiment.






pH controls - *Escherichia coli*
Bacterial count (cfu/ml)
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
pH4.5
pH5.0
pH5.5
pH6.0
pH6.5
pH7.0
Control
24 hour
12 hour
8 hour
4 hour


pH controls - *Lactobacillus fermentum*
Bacterial count (cfu/ml)
8.0E+08
7.0E+08
6.0E+08
5.0E+08
4.0E+08
3.0E+08
2.0E+08
1.0E+08
0.0E+00
pH4.5
pH5.0
pH5.5
pH6.0
pH6.5
pH7.0
Control
24 hour
12 hour
8 hour
4 hour