Antimicrobial Activity of Functional Food Ingredients Focusing on Manuka Honey Action against *Escherichia coli.*

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Douglas Ian Rosendale

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Abstract

The goal of this research was to identify functional food ingredients/ingredient combinations able to manage the growth of intestinal microorganisms, and to elucidate the mechanisms of action of the ingredient(s).

By developing a high-throughput *in vitro* microbial growth assay, a variety of pre-selected ingredients were screened against a panel of bacteria. Manuka honey UMF$^{(TM)}$ 20+ and BroccoSprouts$^{(R)}$ were identified as the most effective at managing microbial growth, alone and in combination. Manuka honey was particularly effective at increasing probiotic growth and decreasing pathogen growth. Testing of these two ingredients progressed to an animal feeding trial. Here, contrary to the *in vitro* results, it was found that no significant *in vivo* effects were observed.

All honeys are known to be antimicrobial by virtue of bee-derived hydrogen peroxide, honey sugar-derived osmotic effects, and the contribution of low pH and the other bioactive compounds present, hence their historical usage as an antiseptic wound dressing. The *in vitro* antimicrobial effect of manuka honey has currently been the subject of much investigation, primarily focusing on the Unique Manuka Factor (UMF), recently identified as methylglyoxal, a known antimicrobial agent. This work has taken the novel approach of examining the effects of all of the manuka honey antimicrobial constituents together against *Escherichia coli*, in order to fully establish the contribution of these factors to the observed *in vitro* antimicrobial effects.

For the first time, it has been demonstrated that the *in vitro* antimicrobial activity of manuka honey is primarily due to a combination of osmotically active sugars and methylglyoxal, both in a dose-dependent manner, in a complex relationship with pH, aeration and other factors. Interestingly, the manuka honey was revealed to prevent the antimicrobial action of peroxide, and that whilst methylglyoxal prevented *E. coli* growth at the highest honey doses tested, at low concentrations the osmotically active sugars were the dominant growth-limiting factors.

Contrary to the literature, it was discovered that methylglyoxal does not kill *E. coli*, but merely extended the lag phase of the organism. In conjunction with the lack of antimicrobial activity *in vivo*, this is a landmark discovery in the field of manuka honey research, as it implies that the value of manuka honey lies more towards wound dressing applications and gastric health than as a dietary supplement for intestinal health.

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µm	micrometer
mm	millimitre
m	metre
µL	microlitre
mL	millilitre
L	litre
µM	micromolar
mM	millimolar
M	molar
APCs	antigen presenting cells
sIgA	secretory immunoglobulin A
℃	degrees Celcius
16S rRNA	16 S subunit of ribosomal polymerase gene
6-MPT	6-methylpterin
ANOVA	analysis of variance
APC	antigen presenting cells
AQ	absolute quantification
atm	atmospheres
ATP	adenosine tri phosphate
a_w	water activity
BCA	bichinchoninic acid
BHI	Brain Heart Infusion
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD4(+)	immune T cell lineage
CD8(+)	immune T cell lineage
CD86	immune T cell lineage
CO_2	carbon dioxide
Ct	crossing threshold
DC	dendritic cell
DHA	dihydroxyacetone
$DiSC_35$	3,3-dipropylthiacarbocyanine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
DPC16	*L. reuteri* strain Drapac # 16
DR10	*B. lactis* HN001
DR20	*L. rhamnosus* HN019
EDTA	ethylene diamine tetraacetic acid
EHEC	enterohaemorrhagic *E. coli*
Em.	emission (wavelength)
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
Ex.	excitation (wavelength)

FAE	follicle-associated epithelium
FAE	follicle-associated epithelia
FAO/WHO	World Health Organisation
Fc	fraction crystallisable (region of antibody molecule)
FCS	fetal calf serum
Fe	iron
Fe^{III} TPTZ	ferric-tripyridyltriazine
fMLP	formyl-methionine-leucine-phenylalanine
FOSHU	foods for specified health uses
FRAP	ferric reducing/antioxidant power
FRST	Foundation for Research, Science and Technology
FSANZ	Food Standards Australia New Zealand
GAE	gallic acid equivalents
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
H^+	Hydrogen ion/proton
HBSS	Hank's Buffered Salt Solution
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
IBD	inflammatory bowel disease
IBD	inflammatory bowel disease
IELs	intraepithelial lymphocytes
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1, IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13	interleukins (cytokines)
IFN-γ	interferon gamma (cytokine)
IRAK	IL-1 receptor-associated kinase
K+	potassiuim ion
kDa	kilo Daltons
Kdp	turgor-sensitive transporter
KefB and KefC	proton antiporter
LAB	lactic-acid producing bacteria
LD	lethal dose
LDH	lactate dehydrogenase
LP	effector lamina propria
LP	lamina propria
LPLs	LP lymphocytes
LPS	lipopolysacharide
LSD	least significant difference
M cells	microfold cells
MAC	membrane attack complex
MALT	mucosa-associated lymphoid tissue

MAPK	mitogen-activated protein kinase
MGO	methylglyoxal
MHC II	major histocompatability complex class II
MICs	minimum inhibitory concentrations
MLN	mesenteric lymph nodes
mRNA	messenger ribose nucleic acid
MRS	de Man Rogosa and Sharp media
MRSA	multidrug resistant *Staphylococcus aureus*
MTBSTFA	N-methyl-N-E-butyldimethylsilytrifluoracetamide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride salt
NADPH	nicotinamide dinucleotide phosphate
NaH_2PO_4	sodium dihydrogen phosphate
NER	net experimental reading
NF-kB	nuclear factor kappa B
NK	natural killer
NO	nitric oxide
NPR	net positive reading
O_2	oxygen
OD	optical density
OTC	over the counter
PAMPs	pathogen-associated molecular patterns
PBFI	potassium-binding benzofuran isopthalate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
pIgR	polymeric immunoglobulin receptor
PMNs	polymorphonuclear cells
PPARg	peroxisome proliferator-activated receptor g
pWBC	pig white blood cells
RNIs	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
SCFA	short chain fatty acid
SEM	standard error of the means
TBDMSCl	tert-butylmethylsilyl chloride
TGF-b	transforming growth factor beta
T_H	T helper cells
T_H1, T_H2, T_H3	T helper cell lineages
TLRs	toll-like surface receptors
TNF-α	tumour necrosis factor alpha
Treg	regulatory T cells

TRI	6-hydroxy-2,4,5-triaminopyrimidine
trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSB	tryptic soy broth
U	units (enzyme activity)
UMF	unique manuka factor
USFDA	United States Food and Drug Administration
VSPs	variant-specific surface proteins
w/v	weight per volume
Δgrowth	change in growth (relative to control 100% growth)

CHAPTER ONE

INTRODUCTION

Overview

The work outlined in this thesis focuses on investigating the efficacy and mechanisms-of-action of food which may contribute to the maintenance of the healthy gut function by affecting and controlling the gastrointestinal (GI) microflora.

As part of the worldwide study of the GI tract and its complex microflora-host interactions, there is an interest in traditional and non-conventional medicines such as functional foods, i.e. foods which are "satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Scientific Concepts of Functional Foods in Europe Consensus Document, 1999). Foods containing ingredients or ingredient combinations that can moderate the GI microflora by encouraging the growth of probiotic organisms (prebiotics) or by inhibiting the growth of pathogens, thereby promoting or maintaining healthy GI microbial homeostasis, are potentially an alternative to pharmaceutical intervention for people with pathogen-related GI disorders, as well as a potential preventative for symptom-free people. Food ingredients or ingredient combinations are likely to have multiple modes of action, in contrast to the 'silver bullet' pharmaceutical approach, so their use carries the additional benefit that development of microbial resistance to them is unlikely. This work provides the basis for the future work of a multidisciplinary team as part of a larger project to develop concept functional foods with the goal of moderating the GI flora of healthy potential consumers.

1.1 The Gastrointestinal Tract

The primary function of the GI tract is the digestion of food and absorption of nutrients to satisfy the body's biochemical requirements. The GI lumen is essentially a region of the external environment, a muscular tube continuous with the outside of the body, specialising as a portal for environmental nutrients to gain access to the circulatory system. As a measure of the relative importance and activity of this large and complex organ in the context of the whole body, the tissues in the GI wall have a higher metabolic activity compared with other organs, manifesting as higher contribution of total body heat production as a fraction of body weight than other organs, a higher fractional protein synthesis rate than other organs and a protein degradation rate higher than other organs (Bannink et al., 2006). Furthermore, the GI tract is the largest of the body's endocrine and immune organs. Finally, the GI tract is unique amongst organs in that it is colonised by a rich and diverse commensal microflora that depends upon nutrition derived ultimately from dietary residues or, alternatively, from host-derived (secreted) sources. These microflora collectively and inextricably contribute to the development and functioning of the organ, and hence the supply of nutrients to the rest of the body, to the extent that the microbe-mammalian symbiosis constitutes a 'superorganism' (Lederberg, 2000).

To fulfil the functions of digestion of food, absorption of nutrients, fermentation, storage and disposal of dietary residues and defend against assault by potentially harmful gut constituents, the GI tract needs to perform a number of roles: secretion of enzymes, mucus, ions and defensive (immune and antimicrobial) compounds; selective absorption of water, ions and nutrients across the epithelia and into the blood; and motility, the contractions of smooth muscle in the walls of the tube to propel ingesta. Each part of the GI tract performs at least some of these functions, and is coordinated by a combination of electrical and chemical messages which originate either from within the lumen, the GI nervous and endocrine systems, or from the central nervous system and endocrine organs. Regulation of healthy gut function is by a complex, multi-layered and multiple redundancy approach, and is reasonably robust, yet the GI mucosa is continuously challenged by exposure to food and microorganisms. Food contains a wide variety of chemical species, many of which are biologically active, and the passage of food through the GI tract can result in shear forces

and abrasion. The body's own digestive processes generate the requirement for a continual process of regeneration and renewal of the GI barrier functions. The commensal microflora possess additional degradative enzymes and other damaging agents, and there is a continual risk of challenge by transient food-associated pathogens, or even from commensal organisms turned opportunistic pathogens upon perturbation of the GI tract by external factors such as surgery. Consequently, the correct functioning of the GI tract is important to the health of the whole body.

1.2 Gastrointestinal Microflora

Whilst the adult human skin may cover some 2 m^2 surface area, the epithelial surfaces of the gastrointestinal, urogenital and respiratory tracts collectively cover greater than 200 m^2 (DeSesso and Jacobson, 2001). Furthermore, in the GI tract especially, the high moisture content, stable temperature and abundant nutrients provide an ideal environment for microorganisms. The GI tract, particularly the distal regions, has a rich and diverse microflora which outnumbers the total host cells of the body by at least an order of magnitude (Turnbaugh et al., 2007). It has been proposed that there are in excess of 1000 different species (7000 strains) of bacteria present in the colon alone (Ley et al., 2006), as well as yeast, fungi and protozoa. The bacterial microbiota consists of a complex resident population of aerobes, facultative and obligate anaerobes (Conway, 1995), with nonsporulating anaerobes predominating, and has a variable number and proportion of transients which sometimes influences, but is unable to compete with or displace residents in the synergistic associations and complex food chains which, in part, determine the composition and stability of the flora (Hudson and Marsh, 1995). The colonic ecosystem in particular consists primarily of phylogenetic clostridial clusters XIVa and IV of low G+C Gram-positive bacteria and the Gram-negative *Bacteroides* class. Clusters XIVa and IV, alongside the commensal and probiotic lactobacilli and bifidobacterium, are reported to be of benefit to health and, although XIVa and IV are more abundant than the beneficial lactobacilli and bifidobacteria, they have received far less attention. Whilst current knowledge is largely based upon studies of faecal diversity, it has become evident that the mucosa-associated community is significantly different from lumenal and faecal communities, and is host-specific (Zoetendal et al., 2004).

The structure and function of the microbiota are regulated by environmental conditions such as nutrient availability, pH, redox potential and microbial-microbial (Conway, 1995) and microbial-host interactions. The latter pair of interactions are of importance because a stable ecosystem is of pivotal importance in maintaining the wellbeing of the host. Many of the indigenous microbial population have low pathogenic potential, and may exclude pathogenic organisms on the basis of outcompeting them for food or stable niches within the ecosystem. Furthermore, a stable ecosystem of commensal organisms is required for the development and maintenance of gut immunity, homeostasis of the intestinal surface and robust repair in response to injury (Madara, 2004; Kelly et al., 2005). There is a battery of bacterial (Table 1.1) and host (Table 1.2) factors which influence establishment and maintenance of bacterial communities, promote epithelial integrity, enhance GI immunity, control the microbial population balance and defend against pathogenic assault. In addition, host nutrition is supplemented by the metabolic flora, whereupon the host epithelium is able to capture and metabolise bacterial byproducts of undigested dietary substrates (Cummings and Macfarlane, 1991). Furthermore, there are a number of both positive and negative associations inherent in the close microbe-host interactions, such as the etiology of certain diseases, the development of the host's metabolic phenotype, and thus the outcome of certain drug interventions (Table 1.3)

The balance of flora can be disrupted by low concentrations of antimicrobial drugs and, in a few instances, changes in diet, with a commensurate rise in pathogenic activity (Hudson and Marsh, 1995) and/or the reduced ability of the GI epithelia to withstand insult or effect repair.

Table 1.1 Commensal microbial elements contributing to maintenance of mucosal integrity and resistance to pathogenic assault. (Adapted from Acheson and Luccioli, 2004; and Salzman et al., 2007))

Commensal Microbial Factor	Effect
Competition	• Deplete sources of essential nutrients
Exclusion	• Occupy, modify or block bacterial receptors
Restriction	• Creation of unfavourable physiological environment (pH, redox potential)
Antimicrobial	• Secretion of antibacterial compounds (bacteriocins, lactic acids)
Dietary modification	• Chemical modification of bile salts and dietary fats • Fermentation of host digestion-resistant dietary residue and synthesis of vitamins and amino acids
Induction of host responses	• Upregulate protective IgA response • Influence inflammatory responses • Induce oral tolerance and regulatory T cell polarization • Stimulate peristalsis • Modulate mucin synthesis and structure

Table 1.2. Host elements contributing to maintenance of mucosal integrity. (Adapted from Acheson and Luccioli, 2004)

Host Factor	Function
Epithelium, glycocalyx, villi	• Innate immune response • Antigen presentation • Block penetration intestinal antigens
Defensins	• Antimicrobial peptides • Immune signalling
Trefoil factors	• Protection from damaging bacterial, or chemical agents or toxins • Restitution after mucosal injury
Mucus/mucins	• Protection from abrasion • Physical barrier vs host digestive agents • Block penetration of ingested antigens • Binding and clearance of ingested antigens • Glycan-associated anti-microbial activity
Gastric acid (pH)	• Breakdown of ingested antigens
Digestive enzymes	• Breakdown of ingested antigens
Bile	• Breakdown of ingested antigens
Secretory IgA	• Block adhesion to epithelial surface by antigens
GALT-associated IgA, IgG(1), IgM(1) (serum)	• Clear antigens penetrating barrier • Systemic immunity • Assist opsonization and phagocytosis of antigens
Peristalsis	• Clearance of ingested antigens
Lymphoid follicles Lamina Propria	• Clear antigens penetrating barrier
Intraepithelial cells Mesenteric lymph nodes	• Phagocytosis and antigen presentation

Table 1.3. Elements of microbe-host interactions. (Adapted from Martin et al., 2008, and Salzman et al., 2007)

Microbe-host interaction	Result
Disease etiologies	• Insulin resistance • Crohn's disease • Irritable bowel syndrome • Food allergies • Gastritis and peptic ulcers • Obesity • Cardiovascular disease • Gastrointestinal cancer
Metabolic	• Determination host phenotype • Corresponding drug intervention outcomes • Availability of nutrients (host:bacterial)
Developmental	• GI organ maturation (cell differentiation and proliferation) • GI immune maturation (IgA induction, tolerance) • GI functional maturation (villi length/crypt depth, tight junction modification)
Protective	• Competitive exclusion for environmental niches

1.3 The Gastrointestinal Defences

The primary function of the epithelia is providing a physical deterrent whilst allowing the uptake of nutrients. Whilst the villous epithelia, for example, possess microvilli on their upper surfaces accompanied by a filamentous brush border glycocalyx which aids in preventing penetration by foreign antigens, they simultaneously express major histocompatability complex class II (MHC II) receptors to facilitate antigen presentation to immune cells, earning them the term 'non-professional immune cells'. A class of MHC II receptors, the toll-like surface receptors (TLRs), recognise conserved bacterial antigenic determinants known as pathogen-associated molecular patterns (PAMPs), which can initiate signalling cascades leading to induction of the adaptive immune response. Furthermore, epithelial cells produce mucins, trefoil proteins and defensins which further protect the host (Table 1.1).

Interspersed with the villous epithelial monolayer is the follicle-associated epithelium (FAE), which overlies and provides access to a network of organised mucosa-associated lymphoid tissue (MALT). This specialised tissue is distributed throughout the GI tract as part of the gut-associated lymphoid tissue (GALT), and is predominantly found in regions at particular

risk from, or high incidence of exposure to, foreign antigens. The GALT contains regulatory and immune cells such as lymphocytes equipped to mount rapid and selective immune responses, and phagocytes which play a role in sample presentation and destruction of pathogens. The anatomy of the FAE illustrates its role in facilitating the association between the immune system and antigens from the gut lumen, and these interactions are of crucial importance to gut health by distinguishing between harmful or beneficial food or microbial antigens through the process of tolerance (Nagler-Anderson, 2001, Cario and Podoski, 2005).

The GI immune system is covered in further detail in Appendix C.

1.3.1 Gastrointestinal Physical Defences

1.3.1a. Mucins

The viscoelastic mucus gel barrier plays a variety of protective roles dependent upon its location within the GI tract. In the sparsely microbially-colonised region of the stomach the water-insoluble mucus (50-450 μm thickness (Allen and Hoskins, 1988)) protects against hypo- or hyper-osmotic conditions, abrasion by food particles and host digestive secretions such as acid and pepsin. It protects the underlying epithelia by maintaining a pH gradient of bicarbonate secreted by the underlying epithelia through the thick gel layer. In the lightly colonised (approx 10^5 total bacteria) small intestine the mucus layer (10-250 μm (Allen and Hoskins, 1988)) protects against extremes of pH, large molecular weight toxins and pancreatic proteases, whilst in the large intestine (60-150 μm in the proximal colon, 60-200 μm in the distal colon and 100-200 μm in the rectum (Pullan et al., 1994)) it protects against toxins and digestive enzymes which have survived passage from the small intestine, and is believed to form the main physical defence against the dense and varied colonic microbiota.

Other roles played by the mucus barrier include enhanced fat digestion (Shiau et al., 1990), hydrophobicity (Goddard et al., 1990) mediated by association with lipids (Witas *et al*., 1983) and configuration of mucin oligosaccharide chains (Sundari et al., 1991) which is thought to maintain a barrier against diffusion. This diffusion is thought to be bidirectionally selective, in that it allows passage of nutrients (such as SCFA from the lumen to colonocytes) whilst, for example, allowing diffusion of bicarbonate from the stomach epithelia, or diffusion of immunomolecules such as secretory IgA or antimicrobial defensins. The mucus barrier also has an indirect protective role, by acting as a nutrient source for some commensal microbes,

speculated to be especially important during circumstances such as when the host is in a starved state (Miller et al., 1984). These mucus-degrading microbes could subsequently act to prevent damage from pathogens by competitively excluding the pathogen from establishing themselves in environmental niches. Thus, the integrity of a continuous mucus barrier depends upon a balance between secretion by specialised epithelial cells and removal by bacterial degradative enzymes or peristaltic shear forces. Trefoil proteins associated with the epithelial surface are thought to contribute to preservation of the mucus barrier through amelioration of mucosal damage, wound healing and mucosal restitution by mitogenic activity (Sands and Podolski, 1996, Dignass *et al*., 1994).

The primary structural components of the mucus gel are the high molecular weight mucin glycoproteins, which exist in two classes; anchored or secreted. The concentration of these gel-forming macromolecules varies according to the GI location, from 50 mg/mL in the stomach through 40, 30 and 20 mg/mL in the duodenal, small intestinal and large intestinal mucus, respectively (Allen and Hoskins, 1988). The structure of the mucus gel is influenced by the structure of the mucin glycoprotein via the covalent polymeric structure of the glycoprotein, involving polymer-polymer and polymer-solvent interactions (Carlstedt *et al*., 1985) and non-covalent interdigitation of the carbohydrate side chains (Sellars *et al*., 1988). Isolated and purified mucins are able to reconstitute the gel when dissolved at similar concentrations to the region from which the mucins originated, although this structure may collapse upon mercaptoethanol-induced reduction or from proteolysis. However, the gel is sufficiently dense that exposure to the latter will only result in the collapse of the surface of the gel (Bell et al., 1985), and it is somewhat protected from proteolysis by the heavy glycosylation of the mucins (Variyam and Hoskins, 1983).

The mucins forming the mucus gel are thought to linearly polymerise end-to-end into a “coiled thread” (Sheehan and Carlstedt, 1984) mediated both by the interactions mentioned above, and by hydration caused by charge-repulsion of the negative carbohydrate side chains sufficient to enable gel formation. Monomers of 0.25 – $2x10^3$ are thought to comprise polymeric structures of up to $3x10^4$ kDa, although polymeric size has been difficult to quantify. The mucin monomer essentially consists of a protein core and attached O-linked oligosaccharide chains which radiate from the core akin to the bristles on a bottle brush. The densely glycosylated and protease-resistant regions of the molecule consist of a core of tandemly repeated amino acid sequences rich in serine and threonine oligosaccharide attachment sites flanked by protease-sensitive regions rich in cysteine and responsible for polymerisation via disulfide bridges.

The mucin oligosaccharides, consisting of core (attachment) modules of sugar residues, a repeated series of backbone or elongation sugar modules and peripheral sugars terminating in antigenic determinants such as ABH blood group-related sugar sequences and a range of sialylated or sulfated units (Schachter and Brockhousen, 1992; Hounsell et al., 1996), are believed to confer the primary protective benefits against degradation by host or bacterial enzymes. The peripheral regions, being those most exposed and thus the first to be subject to bacterial degradative enzymes (Hoskins *et al*., 1985) have a regulatory role in mucin breakdown in addition to their properties as ligands for cellular receptors. As can be expected, the modular nature of the mucin oligosaccharide chains is dependent upon the specific glycosyltransferases present in the secretory cells (Podolski et al., 1985a, *ibid*, 1985b), indicating functional specificity conferred by the tissue of origin (Corfield and Warren, 1996; Roberton et al., 1989) which implies that the degree of protection from degradative enzymes is tissue specific.

Protection of the mucin oligosaccharide from bacterial glycosidase degradation is thought to be conferred by the sulfated terminal sugars. Under normal conditions a large bacterial population in a region of the GI tract coincides with the presence of highly sulfated mucin, providing teleological evidence that the latter may be necessary to control the former. In addition, some sulfated sugar residues have been observed to inhibit bacterial glycosidases such as *Clostridium perfringens* neuraminidase activity (Mian et al., 1979) due to steric hindrance of the sulfate group. Furthermore, in patients with *Pseudomonas aeruginosa* lung infections, relative increases in the sulfated salivary mucin content when the sputum was infected were observed (Houdret et al., 1989), implying that non-sulfated mucins were preferentially degraded. Finally, addition of a sulfatase (capable of removal the sulfate group from the mucin oligosaccharides) resulted in a five-fold increase in mucin degradation by faecal extracts (Tsai et al., 1992), implying that removal of sulfate exposed formerly inaccessible carbohydrate to faecal glycosidase action. This evidence collectively indicates that sulfated mucin, present in densely microbially populated regions of the GI tract, limits the rate and extent of mucin degradation by bacteria, and hence suggests that the mucin forming the mucus barrier is integral in protecting the underlying epithelia from bacterial infection.

1.3.1b Epithelial Glycocalyx

The glycocalyx is a layer of heavily glycosylated membrane-proteins found on the apical surface of epithelial cells, underlying the mucus layer, and comprised of membrane-associated mucin macromolecules as well as other cell-signalling glycoproteins. The structures of these membrane-associated oligosaccharides influence a wide variety of interactions, including cell-cell recognition (Varki 1997); binding and internalisation of pathogens, toxins (Varki, 1997); and viruses (Niles and Cohen, 1991; Zimmer et al., 1995; Keppler et al., 1998). The negatively charged glycoside moieties contribute to and buffer an acidic pH at the apical epithelial surface (Dzekunov and Spring, 1998) and this alters the transport of some ionizable solutes (Holtug et al., 1992). Parallels between the functional roles of the glycocalyx layer and the overlying mucus barrier can also be drawn.

1.3.1c Defensins

One of the major constituents of primary granules in phagocytic leukocytes are defensins (Ganz and Lehrer, 1997): cationic 3-4 kDa peptides characterised by six cysteine residues in characteristic tri-disulfide array (Pardi et al., 1988; Selsted and Harwig, 1989; Hill et al., 1991). These antimicrobial peptides perform the non-oxidative killing of phagocytosed micro-organisms by cells of myeloid origin (Lehrer et al., 1991). In addition, various epithelia secrete defensins (also known as cryptdins in mice) on their mucosal surface (Diamond and Bevins, 1998), but in the GI tract defensins and additional antimicrobial proteins are abundant constituents unique to Paneth cell secretory granules (Porter et al., 1997; Selsted et al., 1992), which are secreted apically into the gut lumen. Dose-dependent secretion of defensins has been shown to occur upon exposure to bacteria and bacterial antigens, although not by live fungi or protozoa, and the microbial peptides are discharged at accordingly effective concentrations (Ayabe et al., 2000). Paneth cells are found adjacent to the stem cells in the intestinal crypts. Protection of these stem cells is essential for long-term maintainance of the intestinal epithelium, and the location of Paneth cells adjacent to stem cells suggests that they play a critical role in defending epithelial cell renewal. In addition, faulty defensin production is implicated in the aetiology of the IBD Crohn's Disease (Fellerman et al., 2006), demonstrating their underlying role in protecting the epithelia from microbes.

1.4 Breakdown of Gut Defensive Function

Modification, breakdown or disregulation of the network of physical, physiological, immune and endogenous microflora leads to ineffective clearance, sequestration or degradation of harmful antigens and/or disruption of regulatory function, and results in mucosal damage, increased gut permeability and overgrowth of harmful pathogens. Factors which may contribute to this include unanswered pathogenic challenge, dietary toxins, antimicrobial compounds (antibiotics) and modifications due to age and/or overall immune competency.

1.4.1 Bacterial Pathogens

Many pathogens are able to subvert the gut defences and immune system in order to enhance their survival, resulting in persistent infections which, in some cases, subsist by upregulating host inflammatory responses in order to use host-derived material of inflammatory or tissue damage-origin as growth substrates. Other pathogens are able to downregulate host responses, ensuring their unhindered invasion of the epithelia, with subsequent risk of systemic infection.

1.4.1a *Helicobacter pylori*

Helicobacter pylori is a Gram-negative bacterium which has adapted to the harsh gastric environment through expression of urease to neutralise the acidic pH through establishment of a higher local pH gradient, and by existing within the gastric and duodenal mucus through selective binding to exposed fucosylated or sialyl-dimeric-Lewis x blood group antigens using its BabA and SabA adhesins (Mahdavi *et al*., 2002). Such antigens are upregulated in gastric epithelial cells during inflammation, aided by the *H. pylori cag* pathogenicity island-encoded type IV secretion system which stimulates host cell receptors resulting in upregulated IL-8 production and maintenance of the inflammatory response (Fischer et al., 2001). Subsequent recruitment of polymorphonuclear cells (PMNs) and *H. pylori* urease-induced upregulation of the PMN nitric oxide production and release of more inflammatory mediators further contribute to mucosal damage (Gobert et al., 2002). Coupled with the *H. pylori* ability to downregulate immune responses such as inhibition of T cell activation (Gebert et al., 2003) and inhibition of APC antigen degradation/processing (Molinari *et al*., 1998), and the gastric mucosa's lack of TLR4 (Backhed et al., 2003), this leads to the gastric mucosa being largely non responsive with regard to *H. pylori* removal, allowing chronic colonisation.

1.4.1b *Escherichia coli*

Many different types of *Escherichia coli* have been linked to intestinal diseases (Acheson and Luccioli, 2004), including enterohaemorrhagic (EHEC), enteropathogenic (EPEC), enterotoxigenic (ETEC) and enteroaggregative (Eagg) *E. coli.* Some of these produce stable virulence factors such as the Shiga toxins of EHEC, the heat stable, labile toxins of ETEC, and others. Briefly, these toxins/virulence factors lead to inhibition of IL-2, IL-4, IFN-γ and other regulatory cytokine production leading to a decreased host response (Malstrom and James, 1998), combined with upregulated chemokine production leading to increased toxin uptake by underlying host cells (Hurley et al., 2001) and further propagation of the condition.

1.4.1c *Salmonella* and *Yersinia*

Salmonella and *Yersinia* species have also adopted immunosuppresive strategies to ensure survival, although their approach is somewhat different. Both cross the epithelia at the M cells. There, *Salmonella* adopts a long term survival within cells by means of altered PAMPs (Acheson and Luccioli, 2004) and an ability to downregulate NADPH oxidase and NO-mediated ROI formation in vacuoles (Hornef et al., 2002). *Yersinia* survives by impairing internalisation by disruption of host cytoskeletal structure and resisting opsonisation and complement-mediated phagocytosis (Hornef et al., 2002). *Yersinia* and some *Salmonella* are also able to inhibit inflammatory responses and upregulate IL-10 production with subsequent immunosuppressive and host tolerance effects (Sing et al., 2002; Rhen et al., 2003).

1.4.1d *Listeria* and *Shigella*

In contrast to *Salmonella* and *Yersinia*, *Listeria monocytogenes* and *Shigella*, both of which avoid host responses by existing within host phagocytes through disruption of the endosome or phagosome membranes resulting in cytosolic colonisation, activate NF-kB leading to increased IL-8 production and other inflammatory mediators (Dramsi and Cossart, 2002; Philpot et al., 2001). This benefits *L. monocytogenes* by recruiting more phagocytes to ensure a constant supply of host cells, and benefits *Shigella* species through the inflammatory disruption of epithelial membranes and further bacterial invasion.

1.4.1d *Staphylococcus*

Staphyloccocus aureus is a common or commensal skin or foodborne organism with extreme medical significance due to the consequences of wound or nosocomial infection (Safdar and Maki, 2002). Its risks are due to multi-drug resistance, and because it is a producer of toxins including superantigens. Superantigens induce potent T-cell stimuli implicated in the pathophysiology of autoimmune and inflammatory disease (Benjamin et al., 1998). *S. aureus* is the test organism used by the Active Manuka Honey Association to assay manuka honeys for antimicrobial activity.

1.4.1e *Clostridium*

Clostridium difficile is a Gram-positive sporulating anaerobe which predominantly exists in spore form in the colon but can enter its vegetative state upon perturbations of the gut microflora, whereupon toxic forms can produce an enterotoxin (toxin A) and cytotoxin (toxin B). Toxin A can cause epithelial cell apoptosis (Brito et al., 2005), whilst both toxins can result in upregulated IL-8 and ICAM-1 expression, leading to inflammation, necrosis and protein loss, whilst increasing peristalsis and capillary permeability, which collectively may result in diarrhoea and intestinal perforation (Durai, 2007). Multiple drug-resistant *C. difficile* is an increasingly prevalent pathogen worldwide. Its adaptability and drug resistance can be correlated to a highly mobile and mosaic genome (Sebaihia et al., 2006). This versatile genome content includes conjugative transposons, phage, IS elements and IStrons, providing resistance, virulence factors and a very adaptable metabolic capability with a preference for carbohydrate growth substrates allowing the vegetative form to effectively compete for GI environmental niches (Sebaibia et al., 2006).

1.4.2 Parasites

Major parasites of the GI tract which damage the mucosal epithelia upon infection include helminths and protozoans. Helminths attach to the mucosal epithelia by buccal attachment (hookworms)(Loukas and Prociv, 2001) or by lectin-mediated binding (nematodes)(Loukas and Maizels, 2000), and generally stimulate a T_H2 response similar to an allergy (Maizels and Yazdanbakhsh, 2003). Hookworms appear to be resistant to proteolytic host defences (Loukas and Prociv, 2001), whilst nematodes inhibit inflammatory responses and mimic

immunosuppresive cytokines (Maizels and Yazdanbakhsh, 2003; Schonemeyer et al., 2001; Gomez-Escobar et al., 1998; Pastrana et al., 1998), thus engendering stable infection. Protozoan infection begins upon cyst digestion by host digestive enzymes to release trophozoites, which adhere to the epithelia (Katz and Taylor, 2001) and cause mucosal damage. *Giardia* resist host defences by expression of variant-specific surface proteins (VSPs) which are protease-resistant and impair immunoglobulin recognition (Nash, 2002), whilst *Entamoeba* evade complement-mediated assault by mimicking the membrane attack complex (MAC) of the complement cascade (Braga et al., 1992). *Cryptosporidium* is unusual amongst protozoa in that it remains bound at the lumenal suface, and is commonly found in immunocompromised patients (Acheson and Luccioli, 2004).

1.4.3 Dietary Compounds

A vast number of harmful and potentially harmful compounds are ingested alongside compounds of nutritious value in food, and their effects depend largely upon the dose of available compound within that food. For example, plants contain a broad spectra of toxins and antinutrients including cyanogenic glycosides, glucosinolates, glykoalkaloids, lectins, oxalate, phenolics, phytate, protease inhibitors, saponins and tannins, many of which may lead to gastrointestinal inflammation and damage, as well as myriad of other ill-effects (Novak and Haslberger, 2000). In addition, plant material may contain contaminants such as quinolones, tetracyclines, sulfonamides, dithiocarbamates, polar-P containing compounds (glyphosate etc), organophosphorus, urea-derived compounds, triazine and acidic herbicides (Juan-Garcia et al., 2005).

Some plant compounds, such as lectins, are ingested daily in appreciable amounts, survive digestion, and bind to gastrointestinal epithelial membrane glycosyl groups and initiate a series of harmful local and systemic reactions (Vasconcelos and Oliveira, 2004). Lectin-induced reactions which harm the gastrointestinal epithelia are as follows: Lectins cause inhibition of parietal secretion of gastric acid (Kordas et al., 2000; *Ibid*, 2001). They cause shedding of brush border membranes, with accelerated cell loss and shortened, sparse and irregular enterocyte microvilli (Bardocz et al., 1995; Herzig et al., 1997). Lectins stimulate alterations in the microbial balance by reducing efficiency of food digestion whilst increasing mucin secretion, epithelial cell loss and serum protein leakage, which has the result of providing a source of nutrients for overgrowth of coliform bacteria such as *E. coli* (Grant, 1999). Lectins also modulate the GI immune system by triggering antibody production, histamine secretion by basophils, and upregulation of the inflammatory T_H2 response by

stimulating IL-4 and IL-13 secretion (Haas et al., 1999). They might also be implicated in facilitation of translocation of both dietary and gut-derived pathogenic antigens to peripheral tissues (Cordain et al., 2000).

1.4.4 Antibiotics

Antibiotics may contribute to the breakdown of GI defensive function by eliminating vulnerable organisms from the commensal flora which act to inhibit or suppress the growth of pathogenic organisms. Antibiotics which preferentially target anaerobes compared to facultative organisms pose a higher risk of promoting pathogenic overgrowth and thus a higher prevalence of infection. Furthermore, prolonged use of antibiotics may favour colonisation by antibiotic-resistant strains, which can pose serious problems if those strains are pathogenic.

1.4.5 Alterations in Immune Competency

Immune competency can be altered by drugs such as steroids, extreme youth or old age, acquired disease states such as infections or malignancies, stressors such as trauma or surgery, and germline immunodeficiency. The end result is naïve, delayed or defective IgA, phagocytic or T cell effector immune responses with commensurate increases in penetration of pathogen into the epithelia, decreased elimination of penetrative pathogens and reduced elaboration of cytokines and other chemokines which regulate the complex cell-cell interactions, respectively, leading to microbial disease and chronic persistence of tissue pathogens resulting in malignancies, inflammatory disorders and other systemic illness (Acheson and Luccioli, 2004).

1.5 Promoting Gut Health

1.5.1 Probiotics

The term ‘probiotics’ has been variously defined over the years (Kollath, 1953; Vergin, 1954; Kolb, 1955; Rusch, 1956; Lilly and Stillwell, 1965; Parker, 1974; Sanders, 1996; Conway, 1995; ibid, 1996a; *ibid*, 1996b; Fuller, 1989; *ibid*, 1991; *ibid*, 1992; Havenaar and Huis in’t Veld, 1992; Guarner and Schaafsma, 1998; Salminen et al., 1998; Hanson and Yolken,

1999; Ouwehand et al., 1999a; Tannock, 1999, Schrezenmeir and de Vrese, 2001, and others), and defined into categories of probiotics by Rusch (2002). Published definitions have been reviewed by Sanders (2003). However, for the purposes of this work, the definition 'live micro-organisms which when administered in adequate numbers confer a health benefit on the host' (FAO/WHO, 2001) will suffice.

Desirable properties of a probiotic organism have been summarised (Zeimer and Gibson, 1998) to include easy propogation and maintenance of viability during processing and storage; confer no pathogenic, toxic, mutagenic or carcinogenic reaction to the presence of the organism, its cell components or fermentation products; be genetically stable with no plasmid transfer mechanisms; be able to survive passage through the extreme conditions of the upper gastrointestinal (GI) tract; be able to proliferate and/or colonise in the gut; and be antagonistic towards pathogenic organisms. It is also desirable that the probiotic species originate from the same species as the host, as some benefits may be species-specific (Vaughan and Mollet, 1999).

Mechanisms by which probiotic organisms can improve health include production of acids, peroxides or bacteriocidal compounds active against organisms that negatively impact upon health; competitive exclusion, that is, competition for mucosal binding sites and competition for growth substrates; and stimulation of the immune system (Scheinbach, 1998). Administration of oral lactic acid bacterial culture can alter the growth and diversity of commensal lactic acid bacteria, contributing to the defence attributes of the established microflora (refer Table 1.1, Section 1.3, and see below).

1.5.1a Lactic Acid Bacteria

A major group of probiotic microorganisms consists of Gram-positive bacteria which ferment carbohydrate to energy and lactic acid, conferring the group term lactic-acid producing bacteria (LAB). A number of genera from the phylum Firmicutes, including *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* and the phylum Actinobacteria including *Aerococcus*, *Microbacterium* and *Propionibacterium* as well as *Bifidobacterium* have been recognised as LABs (Ercolini et al., 2001; Holzapfel et al., 2002; Stiles and Holzapfel; 1997; Gibson and Fuller, 2000). LABs were first isolated from milk by Metchnikoff in 1908, and have since been found in foods and fermented products including meat, vegetables, beverages, milk and bakery products (Caplice and

Fitzgerald, 1999; Liu et al., 2003a; *ibid*, 2003b; *ibid*, 2003c; Lonvaud-Funel, 2001; O'Sullivan et al., 2002); in soil, manure, sewage and water (Holzapfel and Schillinger, 2002); in humans (Carroll et al., 1979; Boris et al., 1998; Ocana et al., 1999a; *ibid*, 1999b; *ibid*, 1999c; Reid, 2001; Schrezenmeir and de Vrese, 2001; Martin et al., 2003) and animals (Sandine et al., 1962; Fuller and Brooker, 1974; Fuller et al., 1978; Klijn et al., 1995; Fujisawa et al., 1990; Fujisawa and Mitsuoka, 1996; Schrezenmeir and de Vrese, 2001).

LABs used as probiotics, predominantly of the genus *Lactobacilli* and *Bifidobacterium*, have a long and safe history in the manufacture of dairy products. They are established primarily during labour and later from the environment (Fuller and Gibson, 1997; Fuller, 1989; Edwards and Parrett, 2002), and dominate the microbiota of the full-term neonate (Hall et al., 1990), especially when breast-fed (Edwards and Parrett, 2002). Bifidobacteria are the first anaerobic group to establish themselves in high numbers, comprising over 99% of the total population, following a brief bloom of facultative anaerobes of matriarchal vaginal and faecal origin, and growth of this LAB genus creates a low pH environment which may protect the neonatal gut ecosystem against growth of potentially pathogenic Gram-negative facultative anaerobes (Scheinbach, 1998). The GI ecosystem develops further so that, by two years, *Bacteroides* spp., clostridia, streptococci and other anaerobes establish themselves, the Bifidobacterium population drops to under 25%, and the gut ecosystem approaches that of adults, whereupon it is developed enough to act as a physical and competitive barrier to potential pathogens. During the course of this early establishment and later microbial progression, these beneficial gut bacteria engage in direct communication with host epithelial cells, leading to the maturation and maintenance of the gut microanatomy (Madara, 2004; Kelly et al., 2005), and establishment of this community leads to the gut health benefits outlined in Section 1.3, above. However, the gut microbiota are not immutable, and some people harbour non-overlapping strains and exhibit shifts over time (McCartney et al., 1996). The observation that major stressors such as antibiotic therapy or pathogen colonisation result in changes supports the concept that the microbiota play a large role in their own regulation of stability. Certainly, it is difficult for microbes to establish themselves in an already established community (Tannock, 2000). This is reinforced by the observation that consumption of probiotics has little measurable effect on the dominant microbiota, but instead effects the diversity of commensal LAB (Heilig et al., 2002). Evidence suggests that LAB, whilst surviving passage through the gut (Donnet-Hughes et al., 1999; Yuki et al., 1999), do not adhere or multiply (Isolauri et al., 1994; Sarem-Damerdji et al., 1995; Alander et al., 1997; *ibid*, 1999), and that continual ingestion of probiotic cultures is required for exogenous probiotic effect to be maintained (Bezkorovainy, 2001). In light of the *in vitro* and clinically observed benefits of LAB (Section 1.6.1.2, below) this suggests that the health

benefits of administered LAB can be attributed to both temporary effects (immune stimulation and the introduction of antimicrobial compounds), and the stimulation of diversity in an existing LAB population, by the effects of transient LAB strains.

1.5.1b Health Benefits from Administered LAB

Species from the genera *Lactobacillus* and *Bifidobacterium* have been observed to positively contribute to a variety of gut disorders (Table 1.4). Some functions of these probiotic organisms have been recently reviewed by Sanders (2003), Saxelin et al. (2005) and others.

The mechanisms by which probiotic organisms exert their effects on pathogens and the host are still speculative (Kopp-Hoolihan, 2001). They may antagonise pathogens directly through production of antimicrobial compounds, act by competitive exclusion processes, stimulate the host immune system, and/or stimulate growth, induction of enzyme activity or diversification of commensal microbiota to achieve the effects noted in Table 1.1 (section 1.3, above).

1.5.1c Immunomodulation by LAB

Early establishment of the neonatal microbiota engenders a transient immune response characterised by increased IgA production (Jiang et al., 2004; Ibnou-Zekri et al., 2003), and further application of LAB engenders a dose-dependent upregulation of IgA-producing lymphocytes (Perdigon et al., 1995). LAB upregulation of IgA production has been shown to reduce the impact of pathogenic infection in the murine model (Shu and Gill, 2001). The neonatal gut is initially characterised by a T_H2 response based upon the cytokine profile, but upon emerging bacterial stimuli it is driven towards a more finely balanced T_H1/T_H2 response.

Commensal or administered transient LAB can lead to the upregulation of phagocytic activity (Schiffrin *et al.*, 1995) or other inflammatory responses via TLR2 signalling-induced upregulation of NF-κB and MAPK leading to increased IL-6 and TNF-α production (Ruiz et al., 2005; Matsuguchi et al., 2002). In addition, some LAB can downregulate the inflammatory response (including downregulating the responses induced by other LAB strains) by inhibiting NF-κB translocation and TNF-α induced IL-8 synthesis (Ma et al., 2004). T_H1 or T_H2 cytokine profiles induced by LAB have continued to display IL-10

production, indicating continuation of T_H3 regulation, and this continued exposure to IL-10 inducing probiotic or commensal organisms is regarded as important for continued regulation of the T_H1/T_H2 balance (the "Hygiene Hypothesis")(Rook and Brunet, 2005).

Clearly LAB play a role in immune modulation, and the response has been shown to be strain-specific. LAB have been shown to differentially stimulate these responses by upregulation of dendritic cell (DC) MHC II and CD86 receptors, indicative of DC maturation, and influencing cytokine production, indicating that the lumenal sampling capability of DCs potentially allows commensal organisms and administered LAB to drive $T_H1/T_H2/T_H3$ responses (Christensen, 2002).

1.5.1d Antagonisation of Pathogens by LAB

LAB are thought to interfere with microbial (pathogen) growth through the production of metabolic products such as lactic acid (Ogawa et al., 2001), peroxide (Brashears et al., 1998) and antimicrobial compounds such as bacteriocins against Gram-positive bacteria (Jack et al., 1995); heat-stable, protease-resistant low molecular weight compounds active against a variety of Gram-negative pathogens (Vescovo et al., 1993; Benjamin et al., 1998) and low molecular weight protease-resistant compounds found in the spent culture supernatant active against a range of both Gram-positive and negative pathogens, but inactive against normal gut flora such as LAB (Bernet-Camard et al., 1997; Coconnier et al., 1997). A large number of bacteriocins have been described for LAB, particularly *Lactobacillus* species (refer Vaughan and Mollet, 1999, and references therein).

LAB, shown by *in vitro* studies to possess the ability to adhere the GI mucosa (Servin and Coconnier, 2003, and references therein), even when heat-killed (Coconnier et al., 1993a) have been shown to inhibit pathogen binding in a strain and dose-dependent manner (Forestier et al., 2001; Chauviere et al., 1992; Horie et al., 2002; Lee and Puong, 2002; Coconnier et al., 1993a; *Ibid*., 1993b); and thus prevent invasiveness (Lievin et al., 2000; Gopal et al., 2001; Bernet et al., 1994), by the postulated mechanism of steric hindrance through competition for pathogen receptors (Reid et al., 2001b), and upregulation of mucus secretion to prevent pathogen translocation across the mucosal epithelia (Mack et al., 1999; Tuomola et al., 1999).

Table 1.4. Examples of the probiotic effects of lactic acid bacteria in human and animal health. LAB = undefined lactic acid bacteria.
(Adapted from Sanders, 2003 and Saxelin et al., 2005)

Condition	Organism
Prevent food allergy	*L. rhamnosus* GG
Block formation of biogenic amines	*L. lactis* ESI 561
Overcome lactose intolerance	*L. acidophilus*
Prevent diarrhea (antibiotic-induced, rotavirus, travellers, community acquired, *Clostridium difficile* colitis)	LAB *L. rhamnosus* GG *L. acidophilus* LB
Reduce intestinal disorders and pouchitis	LAB *L. rhamnosus* GG
Suppress side effects of *Helicobacter pylori* medication with antibiotics.	*L. acidophilus*
Treat Crohn's disease, ulcerative colitis and inflammatory bowel disease (IBD)	*L. rhamnosus* GG *B. infantis* UCC35624 LAB
Stimulate anticarcinogenic activity	LAB *L. acidophilus*
Treat coronary heart disease and anticholesterolaemic effects	*L. acidophilus*
Control of human urinary tract infection and vaginosis	*L. rhamnosus* GG *L. rhamnosus* GR-1
Prevent kidney stones	*L. acidophilus* *L. plantarum* *L. brevis* *B. infantis*
Treat atopic disease	*L. rhamnosus* GG
Prevent caries formation	*L. rhamnosus* GG
Protection against tetanus toxin	*L. plantarum*
Treat chronic fatigue syndrome	LAB
Inhibit pathogens causing bovine mastitis	*L. lactis* DPC3147
Feed supplement for growth promotion in animals	*L. brevis* C10
Inhibit enteropathogens in small intestine of animals	*L. acidophilus* LA1

1.5.2 Prebiotics

Compared with probiotics, which introduce exogenous bacteria into the GI tract, a prebiotic is a 'nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve host health' (Gibson and Roberfroid, 1995). Prebiotics are not hydrolysed or absorbed in the small intestine but are available as substrates for commensal (and transient probiotic) organisms in the large intestine (Molis et al., 1996). Human breast milk oligosaccharides are considered the proteotypic prebiotic (Duggan et al., 2002) because they facilitate the preferential growth of LAB in the colon of exclusively breast-fed neonates (Rotimi and Duerden, 1981; Dai and Walker, 1999; Gnoth et al., 2000). Commercial prebiotic compounds used specifically to encourage or support LAB growth include fructo-oligosaccharides such as inulin; transgalactosyl oligosaccharides; or lactulose derived from isomerised whey lactose.

1.5.3 Synbiotics

A synbiotic has been defined as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare' (Gibson and Roberfroid, 1995).

In addition to the anti-pathogenic and immunostimulatory activities conferred by the probiotic organism (Section 1.5.1, above), there are benefits to the consumer which are not a direct induced response, where the action of microorganisms in either the preparation of foods, or in the GI tract, can somewhat improve the digestibility of some dietary nutrients (Kopp-Hoolihan, 2001) and produce byproducts beneficial to the host (Stanton et al., 2005). For example, food-grade LAB are known to release enzymes into the lumen which alleviate the symptoms of digestive malabsorption; increase free amino acid production; produce short chain fatty acids (SCFA) such as lactic acid, propionic acid and butyric acid which contribute to the colonocyte nutrient pool (Rombeau and Kripke, 1990, Cummings and Macfarlane, 1991); maintain an appropriate gut pH for bacterial enzyme function and foreign/carcinogenic compound catabolism (Mallet et al., 1989); release secondary metabolites such as vitamins B2, B11 and B12 (Stanton et al., 2005); and release bioactive peptides with properties as deverse as opiate, cholesterol-lowering, mineral-utilising, anti-hypertensive and increasing

osteoblastic bone formation (Meisel, 1997; Yoshikawa et al., 2000; Minervini et al., 2003; Seppo et al., 2003; Narva et al., 2004a, Narva et al., 2004b).

1.5.4 Functional Foods

1.5.4.a Marketing functional foods

Foods have long been used as means of contributing to health in addition to a source of nutrients. Whilst the primary function of food is "to provide sufficient nutrients to meet the metabolic requirements of an individual and to give the consumer a feeling of satisfaction and wellbeing through hedonistic attributes (such as taste)" (Asp and Contor, 2003) there is a growing interest in the use of functional foods, those foods which were described earlier but reiterated here as "satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Scientific Concepts of Functional Foods in Europe Consensus Document, 1999). Proposed gut health benefits from functional foods and means of determining and/or proving these benefits have been extensively detailed in the PASSCLAIM series of publications (e.g. Asp and Contor, 2003; Cummings et al., 2003; *ibid*, 2004; Aggett et al., 2005). Current trends in the food industry involve capitalising on this functional role of food as a part of their "differentiate or die" marketing strategies, wherein failure to innovate product formulation, delivery and add value with scientific benefits can lead to failure to have their product recognised as different and 'better' than the alternatives from other manufacturers/suppliers. Means of differentiating product involve innovation of existing products (delivering in innovative manner, e.g. 'chews'), value added (supplementation of existing product with functional components (e.g. adding fibre to beverages), competitive contrast, cost, and by functional benefits (e.g. more readily digestible) and health benefits (e.g. antimicrobial).

1.5.4.b Regulating functional food claims

Health benefit claims are regulated by various authorities worldwide. Europe is governed by European Regulations governed by the Codex Alimentarius, a joint WHO FAO commission with 171 member countries, whilst the United States claims are governed by the Food and Drug Administration Regulations (USFDA), Japan by the Ministry of Health, Labour and Welfare's Foods for Specified Health Uses (FOSHU) and New Zealand uses the Food Standards Australia New Zealand (FSANZ) agency. These agencies all allow general health

claims supported by scientific evidence, but generally forbid claims about disease prevention (with the general exception of folate and neural tube defects) or cure (Saldanha, 2008; Tapsell, 2008) (http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html, Corrigendum to Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods (**OJ L 404, 30.12.2006**)) (accessed 17th March 2009). The USDA outright forbids food claims involving "use in the cure, treatment, mitigation, prevention or diagnosis of a disease or condition", as these claims are the preserve of drugs (Heimbach, 2008). Drugs, of course, are defined as "an article (other than food) intended to affect the structure or any function of the body" (Heimbach, 2008). This does recognise that foods can affect the structure or function of the body, claims which are allowed, so long as they do not represent affecting disease, and are confined to maintenence of health (the absence of disease). The FSANZ health claims framework will allow a series of claims involving risk reduction in health conditions where medical assistance is not a requirement, or more specific statements relating to disease risk reduction and associated biomarkers where there may be overlap with medical care (Tapsell, 2008). This reflects and acknowledges that food delivers nutrients and bioactives which potentially prevents or manages early disease development, and that therapeutics are not required.

1.5.4.c Functional foods programme of which this thesis forms a part

In 2003, The New Zealand Institute for Crop & Food Research Ltd (now Plant & Food Research Ltd), in partnership with The University of Otago Christchurch School of Medicine and Health, was funded by the New Zealand Foundation for Research Science and Technology (Contract No C02X0402) with co-investment from Comvita New Zealand Ltd to develop functional foods from New Zealand raw ingredients to ameliorate *H. pylori* related symptoms as an alternative to therapeutic or more traditional, over the counter (OTC), pharmaceutical products. Rather than seeking a 'silver bullet' ingredient they aimed to develop concept products containing synergistic combinations of ingredients with multiple modes of action, ensuring a greater chance of product efficacy. Potential modes of action included reducing inflammation and combating free radical damage – the primary effects of *H. pylori* infection. Key aims of the research programme were to: (1) develop novel ingredient combinations with synergistic *in vitro* efficacy against *H. pylori*, reducing the effects of infection; (2) deliver stable concept food products (a spread, beverage and a confectionary item), containing efficacious ingredient combinations, with desirable sensory characteristics; and (3) prove *in vivo* efficacy and safety of ingredients and concept products. The eventual

goal of the project, as outlined in Fig. 1, was to produce functional foods of proven efficacy so that general or high-level health claims for these potential products could be supported.

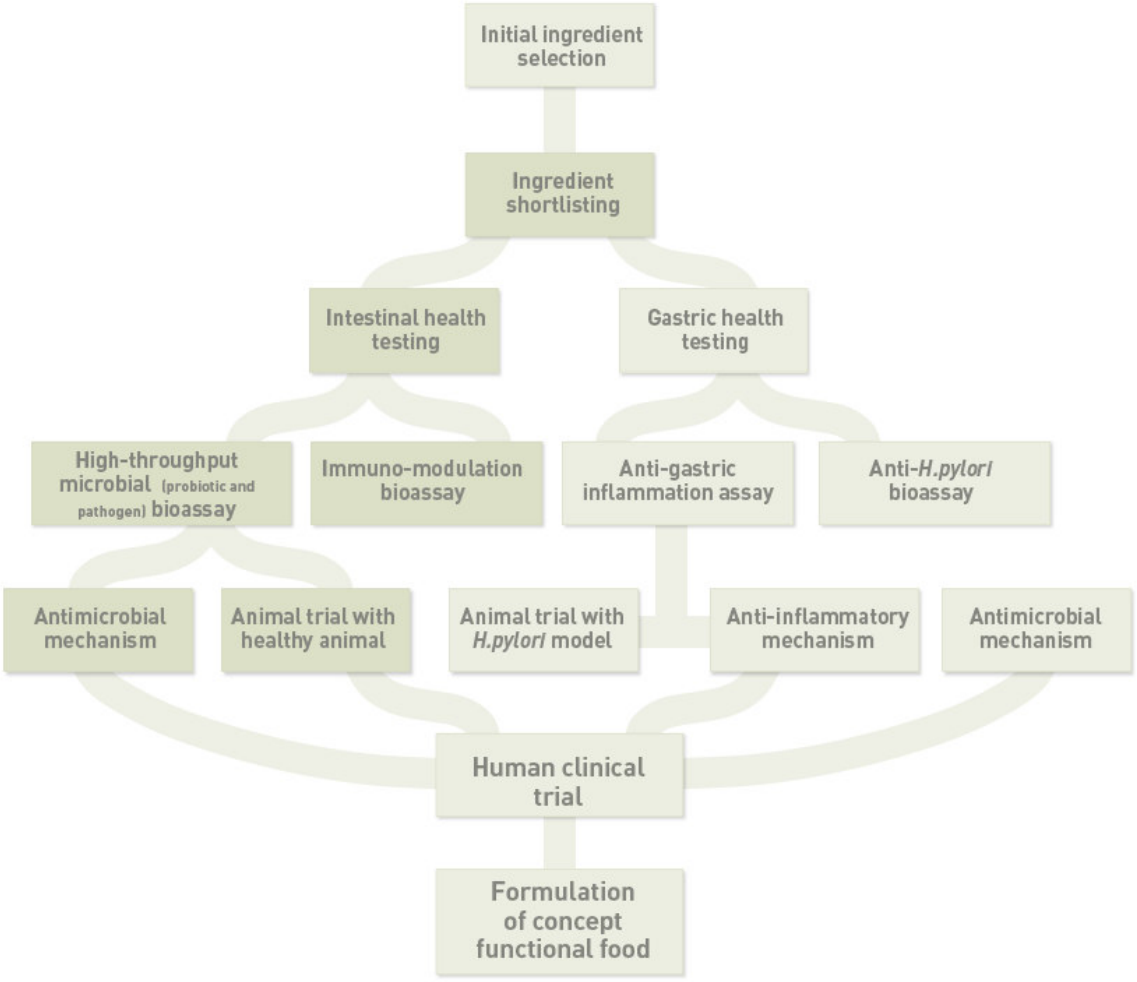


Figure 1. Workflow of the Foods for *H. Pylori* programme. Work to be conducted during the course of this research shown in black font.

1.5.4.d Functional Food Ingredients Used in this Study

Appropriate functional food ingredients were examined by literature review by Lister and Kerkhofs (2005) as part of the FRST-funded program 'Foods for *Helicobacter pylori*' to develop a list of potential ingredients that could be used for testing and product formulation. The list was compiled from searching on a number of biological activities that have relevance for eradicating or controlling *H. pylori* and gastric conditions associated with infection. These activities were: anti-*H. pylori* (*in vitro* and *in vivo*), other antibacterial, antioxidant, anti-inflammatory and immunomodulation. The literature review arrived at an extensive list of potential materials that displayed one or more relevant activities. This list was narrowed down further for initial screening, and a subsequently refined list of six ingredients were selected for more detailed study, consisting of manuka honey UMF™ 20+, bee pollen, BroccoSprouts®, rosehips, blackcurrant seed oil and propolis. These ingredients were chosen for a number of reasons including: multiple activities, complementary activities, diversity of phytochemicals or other constituents responsible for activities, and provision of fewer sensory and or safety challenges than some other ingredients. Further ingredient selection criteria involved practical aspects such as solubility testing and ease of handling for microbial and immune bioassays (Keenan and Salm, pers. comm.), finally resulting in a shortlist of candidate functional food ingredients fulfilling all requirements.

1.6 Mechanisms of action of natural antimicrobial agents

Mechanisms of action of natural antimicrobial compounds have been comprehensively reviewed (for example, Brul and Coote, 1999; Cowan, 1999; Tiwari et al., 2009)(Table 1.5), and are accompanied by reviews detailing the antimicrobial mechanisms of antibiotics (for example, O'Grady 1971, Dzidic et al., 2007)(Table 1.5). Antimicrobial agents of natural (plant) origin have been of increasing interest for over 10 years, and many antimicrobial mechanisms have been incorporated into screening exercises to extract value from plants (Cowan, 1999). Alongside antimicrobials of microbial origin, these compounds act via indiscriminant perturbation of the cell wall or membrane leading to loss of cellular metabolites and/or ions, loss of macromolecules, loss of membrane potential (respiration, electron transport chain), nutrient uptake or membrane transporter function and inactivation of other membrane or membrane-associated proteins, or selectively through degradation (lysozyme), inhibition of cell wall synthesis (some classes of antibiotics), or formation of pores (antimicrobial peptides).

Table 1.5 Classes of antimicrobial compounds, modes of action and targets (Adapted from O'Grady, 1971; Cowan, 1999; Tiwari et al., 2009; Dzidic et al., 2007 and Brul and Coote, 1999).

Broad target	Mechanism	Class of compound
Cell wall/membrane	Permeabilisation/perturbation	Plant phenolics Plant isothiocyanates Lipids Peroxides and radicals Cation (metal) chelators Weak acids Antimicrobial peptides (lysozyme, defensins, cryptdins, nisins etc.) Antibiotics (polymyxins, cyclic lipopeptides)
	Synthesis inhibition	Antibiotics (β-lactams, glycoproteins)
Proteins	Synthesis inhibition	Ribosome-binding antibiotics (chloramphenicol, tetracycline, glycylglycines, oxazolidonines)
	Structural disruption	Isothiocyanates (sulforaphane etc.), aldehydes (reuterin, methylglyoxal etc.), reactive anions, radicals etc.
Nucleic acids	Disruption/perturbation	Anions, aldehydes, radicals
	Synthesis inhibition	Antibiotics (rifampicin, quinolones)
Sequestration/loss resources	Cations/cofactors	Chelators
	Specific inhibition metabolic pathways	Folate synthesis inhibitors (sulfonamides, trimethoprim)
Non-specific reactions		Anions, aldehydes, radicals

Other compounds exert direct cytotoxic effects by either binding to or reacting with vital metabolites, cofactors or enzymes/enzyme active sites, disrupting enzyme structure (disulfide bridges, hydrogen bonds) either indiscriminately (reactive aldehydes, anions, radicals) or selectively (some antibiotics), thereby interfering with metabolic pathways or macromolecular synthesis.

Inhibition of cell wall synthesis, as characterised by β-lactams and glycopeptides, works by preventing cross-linking or incorporation of subunits of cell wall peptidoglycans of Gram negative and Gram positive bacteria, respectively. This alone does not kill the bacteria, but the lack of cell wall renders the resulting spheroplast susceptible to osmotic effects. Phenolics and lipids affect membrane fluidity, with effects ranging from disruption of embedded protein complexes through to loss of integrity leading to leakage or complete lysis (Cowan, 1999). Peptide antibiotics such as the polymixins also act at the membrane with similar effects, and are particulalrly disruptive (directly causing cell death) compared to the less urgent effects of the cell wall effectors or protein synthesis inhibitors or inhibitors of resource uptake mechanisms (below)(O'Grady, 1971). Antimicrobial cationic peptides function by being attracted to the negatively charged bacterial walls, where they assemble a pore structure which causes cell leakage.

Protein synthesis inhibition or structural/active site disruption. Inhibition of protein synthesis primarily occurs by binding of ribosomal subunits as reviewed by O'Grady (1971). For example, tetracyclines block access of amino acids to the ribosomes, whilst chloramphenicol blocks the enzyme which adds the nascent peptide chain to the next amino acid to be added, fusidic acid and macrolides such as erythromycin block movement of the mRNA in relation to the ribosome which determines the next amino acid to be added. For example, streptomycin-induced RNA positional modification can cause single amino acid mismatches leading to the production of defective proteins. Interestingly, with streptomycin-dependent mutant strains, this may actually repair an existing mismatch and yield functional proteins (O'Grady, 1971). Structural damage is more direct: specific residues may form covalent links with reactive compounds (for example, methylglyoxal with arginine, lysine and cysteine residues, Ferguson et al., 1998; Kalapos, 1999) thereby perturbing tertiary structure and enzyme function. Phenolic compounds such as quinones may be free radical producers as well as functioning in a flavone-, flavenoid- and flavol-like manner and complexing irreversably with nucleophilic amino acids, or tannins binding through hydrophobic interactions, hydrogen- or covalent bonds to inactive surface proteins, receptors and transport proteins (Cowan, 1999).

Nucleic acids can be disrupted or perturbed by reactive chemical species, resulting in modification of bases and/or cleavage of the phosphodiester backbone. Base modification can result in misreading leading to functionally inactive proteins, or cause mismatch repair mechanisms to cleave the DNA in an attempt to excise the damaged portion. This particular role of the mismatch repair mechanisms has been examined in *E. coli* for the aldehyde methylglyoxal which reacts with guanine and to a lesser extent cytosine and adenine resulting in cleavage by the UvrA subunit of the Uvr(A)BC exonuclease (Ferguson et al., 1998; Ferguson et al., 2000), and will be discussed in more detail in Chapter 5 of this thesis. Other mismatches can result in misreading due to intercalation between bases, such as by aromatic planar molecules such as some alkaloids (e.g. berbarine) or coumarins (e.g. warfarin)(Cowan, 1999).

Sequestration or loss of resources occurs through chelation of cations required for structural or co-factor roles, or modification of metabolites (or their uptake mechanisms) required for the synthesis of macromolecules. For example, tannins (e.g. ellagitannin) may complex with metal ions (Cowan, 1999), while sulphonamides prevent the uptake of *p*-aminobenzoic acid required to synthesis folate, a nucleic acid precursor (O'Grady, 1971).

Collectively, these examples illustrate the range and breadth of antimicrobial activities which may be found from natural products or potential functional food ingredients, such as those selected during the process outlined in Section 1.5.4.d., above.

1.7 Aims of this thesis

Regulation of GI health is by a complex network of interrelationships and communication between the GI structure, immune system, and microbiota. In the context of the larger project of which this work is a part, benefits to GI health will be studied by examining the effects of functional food ingredients which potentially exert antimicrobial effects against pathogens whilst supporting or stimulating the growth and activity of probiotic organisms and providing beneficial immune stimuli.

The best of the ingredients identified during the course of this research will be used by others within the project to examine shelf-life and other storage parameters, and eventually be formulated into concept functional foods.

Within this work, these successful ingredients will be further examined for efficacy *in vivo*, and the mechanisms of action against GI microorganisms studied in greater detail, both to maximise efficacy in any functional foods which might incorporate this food ingredient, and to support any risk/disease reduction claims. The information gained during the course of this work has the higher goal of expanding our understanding of how certain food ingredients interact with gut bacteria, and how these interactions contribute to maintenance of gut health in the normal healthy individual.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Materials used during the course of this research are included with suppliers' details for the purpose of informing others who may wish to repeat or extend this work.

2.1.1 Chemicals and media

Chemicals were obtained from the following manufacturers/suppliers:

Ajax Chemicals, Auburn, NSW: Dimethylsulfoxide (DMSO).

BDH Laboratory Supplies, Poole, England: Disodium hydrogen phosphate (AnalaR); Glucose (AnalaR); Hydrochloric acid (AnalaR), Propan-2-ol (AnalaR), Sodium acetate (AnalaR); Ethanol.

Invitrogen, Auckland, NZ: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)(MTT)(Molecular Probes); Hank's Buffered Salt Solution (HBSS)(Gibco), PBFI ammonium salt, impermeant (Molecular Probes); Phenol; Phosphate buffered saline (PBS)(Gibco).

Mallincredt Baker BV, Deventer, The Netherlands: Methanol, HPLC grade.

Merck KGaA, Darmstadt, Germany: Agar; Glycerol; Sodium hydroxide.

Pierce, Rockford, IL: Surfacil.

Reidel de Haen, Seelze, Germany: Sodium dihydrogen phosphate

Scharlau Chemie S.A., Barcelona, Spain: n-hexane 96% multisolvent.

Sigma-Aldrich Inc., St Louis, MO: Betaine, Bichinchoninic acid solution, cupric (II) sulphate pentahydrate Reagent Plus, Lipopolysaccharide, Histopaque 1077, methylglyoxal 40% (v/v) solution, trypan blue, fructose, 6-hydroxy-2,4,5-triaminopyrimidine.

Sigma-Aldrich Rare Chemicals, Milwaukee, WI.: Triton X-100.

Growth media were obtained from the following manufacturers/suppliers:

Becton Dickinson and Co., Sparks, MD.: Bacto™ tryptic soy broth, de Man Rogosa Sharpe broth and agar.

Invitrogen, Auckland, New Zealand (Gibco): Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, Fetal Calf Serum (FCS).

Merck KGaA, Darmstadt, Germany: Agar

Oxoid Ltd., Basingstoke, England: Brain Heart Infusion broth.

Sigma-Aldrich Inc., St Louis, MO.: Penicillin-streptomycin solution, RPMI-1640 medium.

2.1.2 Enzymes

Enzymes were obtained from the following manufacturers/suppliers:

Sigma-Aldrich Inc., St Louis, MO.: Catalase

2.1.3 Organisms

2.1.3.1 Animals

Healthy male C57BL/6J mice were obtained from Animal Resources Centre, Murdoch, WA 6125, Australia, with approval from the AgResearch Grasslands Animal Ethics Committee #11163 and 11493.

2.1.3.2 Bacteria

Bacteria, media and growth conditions, and source are listed in Table 2.1

Table 2.1 Probiotic and pathogenic bacteria and culture media.

Bacteria	Growth/assay media
Lactobacillus reuteri DPC16 [a]	MRS + 0.05% (w/v) cysteine
Lactobacillus rhamnosus HN001 (DR20™) [b]	MRS + 0.05% (w/v) cysteine
Bifidobacterium lactis HN019 (DR10™) [b]	MRS + 0.05% (w/v) cysteine
Salmonella enterica serovar Typhimurium ATCC 1772[a]	Tryptic Soy Broth
Escherichia coli O157:H7 strain 2988 [a]	Tryptic Soy Broth
Escherichia coli Nissle strain 1917 [c]	Tryptic Soy Broth
Staphylococcus aureus ATCC 25932 [d]	Brain Heart Infusion Broth (plated on Tryptic Soy Agar)

[a] From Bioactives Research New Zealand Limited, Auckland, New Zealand.
[b] From the New Zealand Dairy Research Institute, Palmerston North, New Zealand.
[c.] From Auckland medical School, The University of Auckland, Auckland, New Zealand.

[d] From the Functional Microbiology Laboratory, New Zealand Institute for Crop and Food Research Limited, Auckland, New Zealand.

2.1.3.3 Mammalian Cell Culture

Mammalian cell lines, media and source are listed in Table 2.2

Table 2.2 Mammalian cell lines and culture media

Cell line	Medium
Murine Abelson murine leukemia virus-induced tumour macrophage (RAW 274.6) (ATCC TIB-71) [a]	DMEM + 10.0% (v/v) FCS (growth)
RAW 274.6 [a]	DMEM (assay)
Murine Moloney leukemia virus-induced lymphoblast (YAC-1) (ATCC TIB-160) [a]	RPMI-1640 + 10% (v/v) FCS (growth)
YAC-1 [a]	RPMI-1640 (assay)
Porcine white blood cells [b]	RPMI-1640 + 10% (v/v) FCS + Penicillin/streptomycin (growth)
Porcine white blood cells [b]	RPMI-1640 + Penicillin/streptomycin (assay)

[a] From ATCC, Cryosite Distribution Pty Ltd., Lane Cove NSW 2066, Australia.
[b] Source animal from Auckland Meat Processors Ltd, Otahuhu, Auckland, New Zealand, isolated according to Section 2.2.2.5..

2.1.4 Reagent kits

Reagent kits were obtained from the following manufacturers/suppliers:

Becton Dickinson and Co., Sparks, MD. (Difco): Gram's crystal violet, Gram's Iodine, Gram's safranin.

Invitrogen, Auckland, New Zealand (Molecular Probes): LIVE/DEAD BacLight Bacterial Quantification Assay Kit, Vybrant Phagocytosis Assay Kit

Promega Corp., Madison, WI.: Bactiter-Glo™ Microbial Cell Viability Assay

2.1.5 Gases

Carbon dioxide gas and liquid nitrogen were obtained from BOC, Auckland, New Zealand.

2.1.6 Other materials

Specific materials were obtained from the following manufacturers/suppliers:

Alltech Associates Inc., Deerfield, IL.: Nylon solvent filtration 0.45 um membrane filters

Becton Dickinson and Co., Sparks, MD.: GasPak™ EZ Anaerobic container system, GasPak™ EZ gas generating sachets.

Microgon Inc., Laguna Hills, CA.: Dynagard 0.2 um syringe filters

Werner Reifferscheidt GmbH, Langerwehe, Germany: La-Pha-Pack®.

Other routine equipment, consumables and disposables such as pipette tips, serological pipettes, cell scrapers, inoculating loops, multichannel pipettors, culture vessels, 96-well plates, pipettors, 0.2, 0.45 and 0.8 um filters, barrier tips and filter paper were obtained from manufacturers or suppliers including the following: Axygen Inc., Beckton Dickonson and Co., Sparks, MD; Biolab, Auckland, New Zealand; CAPP, Denmark; Corning Inc., Corning, NY.; Eppendorf; Nunc; Roskilde, Denmark; Sartorius AG, Goettingen, Germany; Sorenson BioScience Inc., Salt Lake City, Utah and Whatman International Ltd., Kent, England.

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Sterilisation

Sterilisation of glassware, growth media, water and solutions for use with microorganisms was achieved by autoclaving at 121℃ for a minimum of 15 min. Solutions requiring sterilisation other than by autoclaving were filter sterilised by passing through 0.22 µm Millipore filters. Where possible, disposable gamma sterilised plasticware (loops, spreaders) was used for manipulation of pathogenic organisms.

2.2.1.2 Storage of Bacteria

Aerobic and anaerobic organisms were stored at -80°C in broth containing 15 % (v/v) glycerol.

2.2.1.3 Recovery of Bacteria

Frozen cultures (section 2.2.1.2) were revived by scraping a loop across the frozen stock and streaking on an agar plate to produce single colonies upon overnight incubation under conditions of appropriate aerobicity. Purity was assessed microscopically by Gram staining. Single colonies were used to inoculate broth (section 2.2.1.4). A minimum of four subcultures in broth, performed daily, was used to ensure organisms were fully adapted to the medium prior to use for any microbial experiments.

2.2.1.4 Broth Culture

Cultures were routinely grown or passaged by inoculating a loopful (10 µL) into glass screwcap tubes containing growth medium (10 mL). Caps were not fully tightened, to ensure exposure to the correct atmosphere. Cultures were grown at 37°C in a Contherm Digital Series Incubator (Contherm Scientific Ltd, Wellington, NZ).

2.2.1.5 Maintenance of Anaerobic Conditions

Anaerobic bacteria were grown in Gas-Pak™ EZ Incubation Chambers (BD, Auckland, NZ) containing Gas-Pak™ EZ Gas Generating Sachets which, according to the manufacturer's literature, contain inorganic carbonate, activated carbon, ascorbic acid and water that become activated upon exposure to air, reducing the oxygen concentration whilst releasing CO_2. Where necessary, vessels were monitored with anaerobic indicator strips.

2.2.2 Mammalian cell culture methods

2.2.2.1 Sterilisation

Sterilisation of glassware, water and solutions for use with cultured cells was achieved by autoclaving at 121°C for a minimum of 15 min. Solutions requiring sterilisation other than by autoclaving were filter sterilised by passing through 0.22 µm Millipore filters. Where possible, disposable gamma sterilised plasticware (cell scrapers) was used.

2.2.2.2 Storage

RAW 264.7 murine macrophage cells or YAC-1 murine lymphoblasts were centrifuged at 200 x g and resuspended to 2 x 10^6/mL for storage in appropriate growth medium containing 10 % (v/v) fetal calf serum (FCS) and the cryoprotectant 10% (v/v) DMSO. Cryotubes containing cells were gradually frozen by successively placing at -20°C, -80°C and finally in liquid N_2.

2.2.2.3 Recovery

Cryotubes (section 2.2.2.2) were placed in a 37°C waterbath with continual monitoring to ensure complete thawing did not occur, prior to the contents being added dropwise to DMEM growth media containing 10% (v/v) FCS to dilute out the DMSO.

2.2.2.4 Growth

Cells were grown in a Sanyo Model MCO-20AIC CO_2 incubator at 37°C with humidity control and CO_2 elevated to 5%. Cultures were monitored microscopically, refreshed with new media when appropriate, and passaged prior to confluence by scraping the cells from the wall of the vessel, centrifuging (200 x g, 5 min) and resuspending to approx. 4 x 10^5/mL. Where necessary, cells were counted using a haemocytometer, with an equal volume of 0.4% (w/v) trypan blue to assess viability.

2.2.2.5 Isolation of Pig White Blood Cells (pWBCs)

Blood (~450 mL) from a freshly slaughtered pig was collected in a suitable vessel (containing EDTA anticoagulant) by exsanguination via the neck blood vessels immediately after death by captive bolt, according to standard Auckland Meat Processors protocols. These samples were collected under the Request for Category C Samples (Educational and other samples, ME 103-PRO-QAL-05, Auckland Meat Processors, Otahuhu, Auckland) and approved by the resident supervising veterinarian. The blood was maintained at room temperature whilst awaiting animal sign off by the meat inspectors and whilst being transported back to the laboratory.

Blood was diluted in equal volume Hank's Buffered Salt Solution (HBSS) and gently overlaid on equal volume Histopaque 1077 and centrifuged at 400 x g for 35 min at room temperature (20-25°C) with minimal braking. pWBCs were collected from the interface using Pasteur pipettes, and collected in 15 mL tubes containing HBSS. These cells were washed by centrifuging at 290 x g for 35 min at room temperature and resuspended in HBSS. Cells were washed again, by centrifuging under the same conditions and aseptically resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin. pWBCs were maintained at 37°C under 5% CO_2 in air with constant humidity until use.

2.2.3 General methods

2.2.3.1 Extraction of Functional Food Ingredients

Manuka honey UMF™ 20+, bee pollen granules (mixed floral source) and propolis (80% tincture) were supplied by the manufacturer (Comvita New Zealand Ltd, Bay of Plenty, New Zealand). Blackcurrant seed oil was purchased from Nutrizeal Ltd and supplied sealed under N_2 gas, and rosehips (Sweet Briar, *Rosa rubiginosa*) and BroccoSprouts® were purchased from local (Christchurch, New Zealand) suppliers and freeze-dried within 5 days of purchase.

All samples except manuka honey, propolis and blackcurrant oil, were extracted using 25 mM sodium phosphate buffer pH 7.4. Bee pollen samples also contained 0.2% DMSO in the buffer to aid solubilisation. These suspensions were homogenised, filtered through Whatman No. 4 filter paper, centrifuged to remove particulate matter, and sterilised through a series of

0.8, 0.4 and 0.2 µm filters. Manuka honey was extracted either by the above method (noted hereafter as filtered manuka honey), or simply by resuspension in deionised water. Propolis and blackcurrant oil were solubilised in ethanol or DMSO, respectively, and then diluted in deionised water for use.

Extracts were dispensed in aliquots and frozen at -80°C, and aliquots used only once, to prevent any freeze/thaw-induced denaturation of active components. The blackcurrant seed oil extract aliquots were frozen sealed under N_2 gas to minimise oxidation.

Extracts were thawed to room temperature and diluted as required immediately prior to each assay.

2.2.3.2 Ingredient Extract Concentration

An aliquot of the aqueous extracts was dispensed (0.5 mL) into preweighed tubes immediately after filtration, then lyophilised, and the dry weight of the extract calculated, including corrections for the buffer salt weight, to establish the concentration of soluble material in the extract.

2.2.4 Analytical methods

2.2.4.1 Antimicrobial Assays

For single extract analyses, the first column of a 96-well plate was loaded with the appropriate bacterial growth medium supplemented with an extract (Section 2.2.3.1). Multiple plates were prepared, each containing a different extract. Extract concentrations were halved at each dilution from the maximum concentration for each extract, forming a two-fold dilution series, resulting in 11 dilutions each containing eight replicate wells (50.0 µL). Eight replicate control wells containing medium without extract were included in the last column of the 96-well plate.

Plates were inoculated with an equal volume (50.0 µL) of bacteria (Table 2.1) at a density of 10^3 cells/mL (counted with a haemocytometer) and the optical density (OD) of the plate was immediately measured at a wavelength of 620 nm using a Thermo Multiscan EX 96 well

plate reader to determined the blank (zero growth) value. Plates were incubated at 37°C for 16 h, and then the OD was determined to measure the growth of the cultures.

The effects of the extracts on the growth of the bacteria were compared by converting the OD of the extract-supplemented culture to a percentage of the control, unsupplemented culture, and then subtracting 100 (Eq. 1). This resulted in a positive or negative value representing increased or decreased growth, respectively, where the magnitude of deviation from the control (zero) was a measure of relative efficacy.

$$\Delta\text{Growth} = \left(\frac{(\text{Extract OD} - \text{blank OD}) \text{ x } 100}{(\text{Control OD} - \text{blank OD})} \right) - 100 \quad (1)$$

Combined extract assays were performed exactly as described for single extract assays except that maintaining the same assay volume meant that extracts were present at half the concentration of the highest dose used when tested individually.

To estimate the bacterial viability counts, extracts were assayed for antimicrobial activity exactly as described above, except that resulting cultures were serially diluted in medium and spread plated on agar plates. After incubation, colonies were counted and the CFU/mL of the original well culture was determined and compared to the control (unsupplemented) cultures.

To estimate antimicrobial activity using well diffusion, agar petri dishes were inoculated by spread-plating bacterial suspensions of sufficient density to form a confluent lawn upon overnight incubation. After inoculation the plates were allowed to dry for 1 h, and then holes were bored in the agar using a sterile implement. Extract was pipetted into the hole to the level of the agar, and the plate was incubated face upwards. Exclusion zones were photographed, and the radius measured at two points for calculation of area of inhibition.

2.2.4.2 Protein Estimation

Protein was estimated using the bichinchoninic acid (BCA) reagent kit, according to the manufacturer's instructions, with a BSA standard curve from 0-20 µg.

2.2.4.3 Measurement of Phagocytosis

Phagocytosis by macrophages was measured using the Vybrant™ Phagocytosis Assay Kit (V-6694)(Section 2.1.4) according to the manufacturer's instructions (MP 06694; 18 March 2001).

In summary, cells were pre-incubated with food ingredient extracts, and then incubated with fluorescent *E. coli* bioparticles to measure phagocytic uptake. Unphagocytosed fluorescent particles were quenched using a trypan blue wash, allowing measurement of the internalised particles using a fluorospectrophotometer.

Details of the procedure are as follows:
Macrophages were resuspended to a concentration of $2x10^5$ cells/mL in growth medium. Medium without supplements was used as diluent (assay medium) for all subsequent steps. Cells (0.1 mL) were allowed to adhere to the floor of the wells in a 96-well microplate by incubating overnight in the cell culture incubator. In the case of the naïve RAW264.7 murine macrophage cells, LPS was added during this step, to a final concentration of 50 ng/mL in the cell suspension, in order to prestimulate them and thus avoid an excessive response upon exposure to 'anything'. Following adherence, blanks (zero fluorescent uptake, background controls) were prepared by adding assay medium (0.1 mL), whilst negative controls were prepared by treating a subset of the positive control (untreated) cells with 1.0% Triton X-100 in assay medium (0.1 mL). Suitably diluted ingredient extracts (0.1 mL) were added and the plates were immediately incubated for 2 h. The supernatants were removed by aspiration, and fluorescent bioparticles (0.1 mL) were added to all wells and the plate was incubated again for 2 h. The remaining fluorescent particle solution was removed from the incubated cells by aspiration and the cells were washed with the trypan blue quenching solution (0.05 mL) by adding to all wells and then removing by aspiration after exactly 1 min at room temperature. Fluorescence was measured with a Bio-TEK Synergy HT fluorescent plate reader at ~480 nm (494) excitation and ~520 (518) emission.

The response to the extracts was expressed as the net fluorescence of the effector cells as a percentage of the net fluorescence of the untreated cells. Negative control and blank values were calculated on the basis of cells lysed with detergent and no addition of cells, respectively. The Net Positive Reading (NPR): average fluorescence of positive control wells minus negative control wells and the Net Experimental (extract) Reading (NER): average fluorescence of extract wells minus negative control wells were calculated. The phagocytosis response was expressed as NER as a percentage of NPR: (NER x 100)/NPR

2.2.4.4 Determination of Methylglyoxal

The amount of methylglyoxal (MGO) present in samples was measured by derivatising the MGO in reaction with 6-hydroxy-2,4,5-triaminopyrimidine (TRI) to form a stable fluorescent derivative, 6-methylpterin (6-MPT)(Fig. 2.1), which can be separated and quantified by HPLC using a fluorescence detector. This assay was based on methods developed for the routine detection of MGO in cattle rumen (Lodge-Ivey et al., 2004).

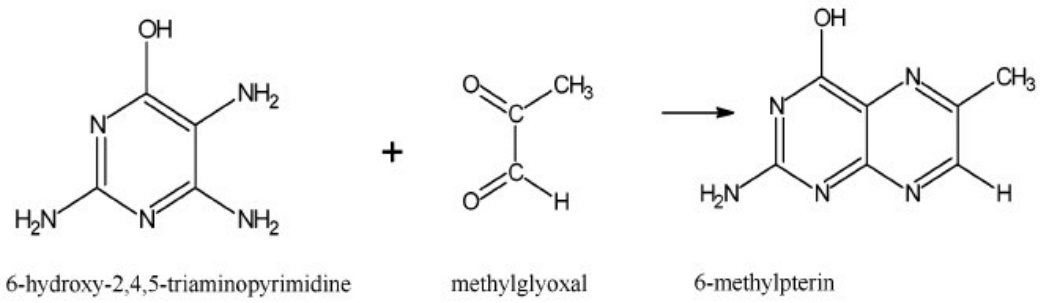


Figure 2.1. Derivatisation of MGO with TRI to form 6-MPT. From Lodge-Ivey et al., (2004).

Samples (0.1 mL) and standard solutions of 0.004% (v/v) MGO (0.025 – 0.1 mL) in sodium actetate buffer (20 mM, pH 4.05) were dispensed into siliconized (7% (v/v) SurfaSil in hexane) 32 mm amber HPLC sample vials. Sodium acetate buffer (20 mM, pH 4.05) was added to 0.8 mL final volume. TRI (4.0 mg/mL, 0.2 mL), freshly solubilised by raising the pH to 10 using NaOH (0.25 M) and then acidifying to the sodium acetate (20 mM) buffered pH of 4.05, was added to begin the derivatisation process, conducted at 60°C for 45 min. This resulted in a MGO range of 10 - 40 µg/mL in the derivatisation standards. The derivatised solutions were immediately filtered through 0.2 µm syringe filters into siliconized La-Pha-Pack® HPLC inserts and placed in vials in the HPLC autosampler sample holder at 4°C.

The pterdinic derivative of MGO, 6-MPT was separated and detected by high performance liquid chromatography (HPLC). The chromatographic system comprised a Dionex Ultimate 3000 HPLC (Dionex, Sunnyvale, CA) coupled to a Shimadzu-10A XL fluorescence detector (excitation and emission wavelengths of 352 and 447 nm, respectively). Samples (10 µL) were auto-injected into the mobile phase (flow rate 1.5 mL/min) of sodium acetate buffer (20 mM, pH 4.05) and were separated using a Spherisorb pH Stable C18 (5 µm, 250x4.6 mm) column (Alltech, Cat. No. 82055) at 30°C. HPLC management and data capture were performed using a Chromeleon Chromatography Management System (v. 6.8, June 2006, (Dionex)).

2.2.4.5 Determination of Short Chain Fatty Acids

Short chain fatty acids (SCFA) from mouse caecum digesta were analysed by a process of solubilisation of organ/digesta in buffer, extraction of the SCFA in ether and derivatisation with a silylating agent for detection by gas chromatography (GC).

The half caecum sample was weighed and then homogenised using a hand held glass-glass homogeniser, aided by vortex mixing, in phosphate buffered saline (PBS, pH 7.4))(10 mL/g sample) containing the SCFA internal standard 2-methyl butyrate to a final concentration of 5 mM. The resulting homogenate was clarified by centrifugation (3000 x g, 5 min, 4°C) and stored at -80°C. SCFAs were subsequently extracted by adding an aliquot of the sample (0.5 mL) to conc. HCl (0.25 mL) followed by the addition of diethyl ether (1.0 mL), thorough mixing, and then the upper organic phase was collected, with centrifugation (3000 x g, 5 min, 4°C) used to compact the interface between the phases and increase the recovery volume. Diethyl ether extracts were stored at -80°C prior to derivatisation. Extracts (0.1 mL) were then combined with N-methyl-N-E-butyldimethylsilytrifluoracetamide (MTBSTFA) containing 0.1% *tert*-butylmethylsilyl chloride (TBDMSCl) (0.02 mL) and heated to 80°C for 20 min, followed by a 48 h incubation at room temperature, to generate silylated SCFA derivatives. SCFAs were then quantified by comparison to a standard curve of a two-fold serial dilution of the highest standard concentration (10 mM each) of a cocktail of derivatised SCFAs; formic, acetic, propionic, *iso*-butyric, butyric, lactic and succinic acids, with the 2-methyl butyric acid internal standard retained at a consistent 2.5 mM. The instrument used was a Shimadzu GC-17A gas chromatography system equipped with a Shimadzu AOC 20i auto injector and Shimadzu AOC 20s autosampler, controlled using the Shimadzu GC Solutions software package.

2.2.4.6 Measurement of Water Activity (a_w)

This was measured using a temperature-stable AQUA LAB series 3TE Water Activity Meter v3.4 (Decagon Devices Inc., Pullman, WA) which uses chilled-mirror dewpoint measurements with thermoelectric (Peltier) internal temperature control (25°C). The instrument was operated and calibrated according to the manufacturer's instructions (Operaters Manual version 1.5, 2000) using the manufacturer's standard solutions and steam-distilled H_2O.

All test solutions were equilibrated to the temperature of the room (25+/-1°C) prior to measurement. Two-fold serial dilutions of samples in growth media (mimicking sample measurement in the antimicrobial assay) were poured (4.0 mL) into sample cups immediately prior to measurement. Samples were inserted into the instrument and the a_W determined, with three measurements per sample.

2.2.4.7 Assay of Cell Viability or Respiration

The viability of cells was determined by comparing cellular metabolic activity, as determined by the formation of the insoluble crystalline formazan dye product of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) breakdown by mitochondrial or cytoplasmic membrane dehydrogenases of cell culture or microorganisms, respectively). The response to the effectors was expressed as the net absorbance of the affected cells as a percentage of the net absorbance of the untreated cells.

For tissue culture cells, pWBCs or RAW264.7 murine macrophage cells were resuspended to a concentration of $5x10^5$ or $2x10^5$ cells/mL, respectively, in growth medium. Medium without supplements was used as diluent (assay medium) for all subsequent steps. Cells (0.1 mL) were allowed to adhere to the floor of the wells in a 96-well microplate by incubating overnight in the cell culture incubator. Following adherence, the culture supernatant was removed by aspiration and the assay medium (0.1 mL) was added to the control wells. Suitably diluted ingredient extracts were added (0.1 mL) and plates were immediately incubated for 1 h, after which supernatants were removed by aspiration. MTT reagent (0.1 mg/mL, 0.1 mL) was added and plates were incubated for 2 h, as above. Crystal product formation was monitored using microscopy. Upon visual confirmation of crystal formation, the supernatant was removed by aspiration. DMSO (0.1 mL) was added to lyse the cells and dissolve the insoluble formazan dye crystals. The absorbance was then read at 550 nm using a Thermo Multiscan EX 96 well plate reader.

For bacteria from the 96-well antimicrobial assays (section 2.2.4.1), metabolic activity was measured by adding 0.5 mg/mL MTT reagent (0.01 mL) to the culture wells and otherwise following the monitoring, solubilisation and absorbance methods outlined above.

2.2.4.8 Statistical Analyses

Data was analysed using Microsoft Excel or using the Genstat program (Genstat Release 8.2, Lawes Agricultural Trust, Rothamsted Experimental Station). Values were presented as mean of n replicates, accompanied with either the standard error of the means (SEM)(Excel), or by using the ANOVA function (Genstat) to calculate the least significant difference (LSD) at the 95% confidence interval ($p < 0.05$).

CHAPTER THREE

SCREENING OF FOOD INGREDIENTS FOR THEIR EFFECTS ON GROWTH OF SELECTED BACTERIA

3.1 Introduction

This chapter describes the screening of six potential functional food ingredients of New Zealand origin, and their combinations; manuka honey UMF™ 20+, propolis, bee pollen, BroccoSprouts®, rosehips and blackcurrant seed oil. These ingredients were selected for use as either a stand-alone food ingredient or as part of a combination with other ingredients, on the basis of potential or known ability to impact upon microbial growth. The testing of ingredients in combination with each other is as important, if not more so, than the ability to act alone, given that ingredients would be consumed as a part of a complex food matrix. Furthermore, a simple process for the identification of synergistic interactions between ingredients, whereupon a combination generates an effect greater than the sum of the individual ingredients, would be of value to the food industry and to the consumer looking to maximise the benefits of functional food for the purposes of managing the gut ecosystem. Finally, determination of whether unwanted interactions or antagonisms could result from the mixing of individually efficacious ingredients was required. Such knowledge would impact heavily upon the selection of ingredient combinations made by both the food industry and the consumer.

The ability of these ingredients to impact positively or negatively on the growth of a panel of well-studied strains of probiotic and pathogenic bacteria was to be assessed using *in vitro* microbial assays. The bacterial isolates used were chosen on the basis of ease of use and relevancy: the probiotics, *L. reuteri*, *L. rhamnosus* and *B. lactis* and pathogens *E. coli*, *S. enterica* and *S. aureus* grew under similar respective culture conditions, important because isolates requiring different growth conditions or significantly differing nutrient supplementation or growth rates (e.g. *H. pylori*) would hinder high throughput analysis of growth; and relevance from the perspective of contributing to (probiotic strains) or impacting negatively upon (pathogenic strains) the intestinal wellbeing of the normal healthy individual (c.f. *Listeria monocytogenes*).

Thus the three aims of this chapter are summarised as follows:

First, to establish a rapid spectrophotometric microbial growth assay and a manner of expressing the efficacy of the functional food ingredients. These simple tests may be applied during subsequent investigation of the efficacy of other potential functional food ingredients intended to manage the gut microflora. Thus this thesis was initiated by determining the pro- or antimicrobial dose-response profiles of the selected ingredients in detail, and by identifying which ingredient combinations are capable of generating synergistic (or unwanted antagonistic) effects against a panel of both probiotic and pathogenic bacteria.

Secondly, this chapter was to form a first step in confirming the viability of New Zealand functional food ingredients as an alternative, non-pharmaceutical approach to maintaining human gut health and wellbeing through promotion of gut microbial homeostasis.

Finally, ingredients or ingredient combinations selected on the basis of the results of this chapter could later be examined for *in vivo* effects in an animal gut health model, and to determine prospective active constituents and explore potential mechanisms of action, to support claims made on the efficacy of the ingredients or ingredient combinations, and to provide the knowledge to underpin future research efforts.

3.2 Ingredients

3.2.1 Ingredient Preparation

Food ingredient extracts were prepared according to Section 2.2.3.1 with a starting concentration of 100 mg/mL. Extract concentrations were determined according to Section 2.2.3.2, and the dose used for microbial assays is presented in Table 3.1.

3.2.2 Ingredient Analysis

An aliquot of the aqueous extracts was dispensed (0.5 mL) into preweighed tubes immediately after filtration, lyophilised, and the dry weight of the extract was calculated, including corrections for the buffer salt weight, to establish the concentration of soluble material in the extract.

The concentration of the soluble material in the extracts (Section 2.2.3.2) was determined to ensure that comparisons of the effects of different extracts could be made. The preparation and extraction conditions all started at 100 mg/mL, but because the amount of insoluble material left in the tubes after the initial centrifugation step with the aqueously extracted ingredients (Section 2.2.3.1) varied, measurement of the final concentration was judged necessary for comparison between extracts. It is acknowledged that the concentration of soluble material in solution does not necessarily reflect the concentration of bioactive components within that solution.

Table 3.1 Extracts and concentrations at the highest dose used for the Single Extract Assay.

Extract	Extract concentration (mg/mL)
Filtered Manuka honey UMF20+	20.0
Bee pollen [a]	5.0
Rosehips	2.5
BroccoSprouts®	1.75
Blackcurrant seed oil [a]	0.21
Propolis [b]	0.6

[a] Includes DMSO (<0.2% (v/v)).

[b] Includes ethanol (<2.0% (v/v)).

3.3 Screening of individual food extracts

3.3.1 Methods

Ingredients were screened for antimicrobial activity using the single extract analysis (section 2.2.4.1). There are a variety of techniques available for measuring the antimicrobial activity of natural compounds outlined by Patton et al. (2006), who investigated manuka honey antimicrobial activity. These researchers outlined the advantages and disadvantages of the three primary methods: disc diffusion, well diffusion and spectrophotometric analysis, with the demonstrable conclusion that the latter method was more accurate, sensitive, reproducible, faster and cheaper. The possibility for extensive kinetic studies with lower concentrations than possible with well diffusion assays was claimed (Patton et al., 2006). Thus, a spectroscopic microplate assay method is suitable for measuring changes in microbial growth during a screening program of potentially antimicrobial ingredients, where the format (microplate layout), number of replicates and control well designs could be optimised for rapid, accurate and consistent high throughput screening and for ease of subsequent statistical analyses. In addition, the spectrophotometric measurement of optical density (OD) has the added benefit of being a more suitable index of final microbial biomass concentration than the use of viable cell counts (Krist et al., 1998), as viable cell counts do not necessarily represent biomass due to differences in cell mass and shape, where the same biomass concentration can be contained within several smaller cells or few larger cells. OD closely correlates with biomass concentration except in extreme cases (Krist et al., 1998).

Thus, a spectroscopic method was adopted for this screening of potentially antimicrobial ingredients, with the format (plate layout), number of replicates and control well designs designed in consultation with a New Zealand Institute for Crop & Food Research Ltd biometrician (Duncan Hedderly, Palmerston North, New Zealand).

Initial experiments to optimise the process involved examining the plate reader outputs for reproducibility and to ensure potential plate reader ‘hot spots’ (where wells of the 96-well plate or plate reader might consistently read higher or lower than the surrounding wells) were accounted for. The same brand of 96-well microassay plate (Costar, Corning, NY) was used throughout to eliminate inter-brand plate variation. After ensuring the

spectrophotometer operated appropriately, the bacterial growth conditions were tested and optimised to ensure the growth media, culture revival and handling, inoculum concentrations and incubation times could be standardised to consistently generate optical densities that would potentially allow deviations from the theoretical control value (ie cultures unsupplemented by extracts) to still fall well within the plate reader's useful absorbance range (an absorbance of 0.1 – 1.0) at late log phase growth under standard handling conditions (data not shown). These conditions thus developed, as outlined in Chapter 2, were adopted for all experiments to maintain consistency and allow comparison with assays conducted at different times.

The screening of the food ingredient extracts was performed on three occasions on different days, with fresh dilutions of the extracts and freshly grown inocula. Separate days ensured that potential fluctuations in growth conditions, growth of the organisms, unknown power outages or the like would all have reduced impact. Anecdotal evidence discussed by a number of scientists, including Jacqui Keenan, Christchurch School of Medicine, Otago University, Christchurch, New Zealand, has suggested that bacteria can just grow differently on different days, so repeated experiments would ensure minimisation of variation. Fresh dilutions of the extracts, all aliquots from the same preparation, would ensure that variation in extraction conditions could not play a role. Freshly grown inocula were all produced by the same system (a minimum three days acclimatisation to the medium, passaging each day at the same time, etc.) as developed above, according to the principles outlines in Chapter 2.

Data were expressed as Δgrowth values, as described in Section 2.2.4.1, Eq. 1, which is a representation of how well they grew relative to the unsupplemented control cultures. Plates were read immediately after inoculation (t = 0 h) and then at 16 h growth, during late log phase-early stationary phase growth of the organisms. The 0 h reading was subtracted from the 16 h end-point reading to eliminate all changes in optical density not due to growth. Therefore potential variations in plate density, media colour or any other unknown factors could be accounted for. Eight replicates were used, for statistical analyses and for practical considerations: that number can easily be pipetted and serially diluted lengthways (columnwise) across a 96-well plate with an 8-channel pipettor. The latter allows the preparation of 11 dilutions, with one remaining (control) column.

Data were subjected to statistical analysis using the Genstat program (Genstat Release 8.2, Lawes Agricultural Trust, Rothamsted Experimental Station) because it allowed pooling of results for comparison across extracts, and across bacterial strain, from multiple determinations, by determining analysis of variance using the two-tailed ANOVA function.

This method of analysis meant that the least significant difference (LSD) of the means could be expressed at the 95% confidence interval ($p < 0.05$) as a single bar (rather than multiple "detonator bars", one per time point) on the graph. If the ∆growth value deviated from zero by more than the length of the bar then there was a greater than 95% chance that this difference was significant, or 'real'.

3.3.2 Results and discussion

A total of 36 dose-response curves were generated. Figure 3.1 displays the effects of the six ingredients, each against six bacterial species. Limitations on the quantity of two of the ingredients, blackcurrant oil and propolis, meant that only preliminary dose-response screening of these ingredients against *S. aureus* was performed (data not shown).

Manuka honey (Fig. 3.1) increased probiotic growth and decreased pathogen growth in a dose-dependent manner. There was a clear difference in the effect on the pathogens and probiotics, validating both the means of expressing the data (∆growth values) on the same chart for immediate visual recognition, and in the choice of model ingredient to trial the system.

The decrease in pathogen growth was expected. This result can be explained by a contribution of factors: Manuka honey, derived from *Leptospermum* spp., has known wound-healing and antimicrobial properties (Molan, 2001). Honey antimicrobial properties have been largely attributed to the presence of residual peroxide (White *et al.*, 1963) arising from bee-derived glucose oxidase upon dilution. Honey also contains a high sugar content, sufficient to lower the water activity (a_w) enough to prevent microbial growth through osmotic shock. Other factors include acidic pH and the presence of plant-derived phenolic compounds (Molan, 1992). Manuka honey also has non-peroxide activity (Molan and Russel, 1998), the Unique Manuka Factor (UMF™), suggested to include very high levels of the 1,2-dicarbonyl compound methylglyoxal (MGO) (Adams et al., 2008; Mavric, 2008; Weigel et al., 2004).

Increases in probiotic growth, a prebiotic effect attributed to manuka honey, have been reported in faecal cultures by Sanz *et al.* (2005). This confirmation of honey prebiotic ability is a significant finding, although the mechanisms responsible for this outcome remain to be elucidated. Similar results for other organisms have been reported for honey, such as for

the yeast *Candida albicans* (Patton et al., 2006), but the mechanism is currently unknown. Whilst honey is predominantly sugar, and could conceivably contribute to increased growth of the honey-supplemented cultures due to greater nutrient (honey oligosaccharides) than present in the media of the control cultures (Sanz et al., 2005), the probiotic growth medium MRS is rich in glucose, thus lack of sugar limiting the growth of the control cultures relative to honey samples is unlikely.

Changes in the medium pH might be expected to affect bacterial growth, whereas buffering of the media could increase probiotic growth relative to the assay control wells. This is because without buffering, accumulating acidic byproducts in the media inhibit growth. No ingredient-induced pH changes were observed (data not shown). In the case of the probiotic organisms, the expected lowering of pH after growth, induced by the acidic by-product, was observed. Essentially, growth (OD) was proportional to the pH, and thus proportional to the production of acidic by-products. Thus the observed increases in probiotic growth could be attributed to factors other than the ingredients influencing the pH value of the medium.

Bee pollen (Fig. 3.1) showed a biphasic response, with the most statistically significant effect manifesting as increased growth of bacteria at the second (pathogens) and third (probiotics) highest doses, 2.5 and 1.25 mg/mL, respectively. The highest doses appeared to inhibit the growth of the probiotics, although the significance of this is questionable. Currently, there are no reports in the literature discussing an antimicrobial function of bee pollen, and there are no available mechanisms to explain the increases in growth observed. The absence of a linear dose-dependent response implies that either (i) more than one component is involved in generating the observed effects, or (ii) a single active component behaves differently at different concentrations - an explanation not without precedent in the field of antimicrobial plant compounds (Inoue et al., 2005b). DMSO, at the doses used to solubilise this ingredient (<0.2% (v/v)), has been tested and has no effect on the growth of these organisms (data not shown).

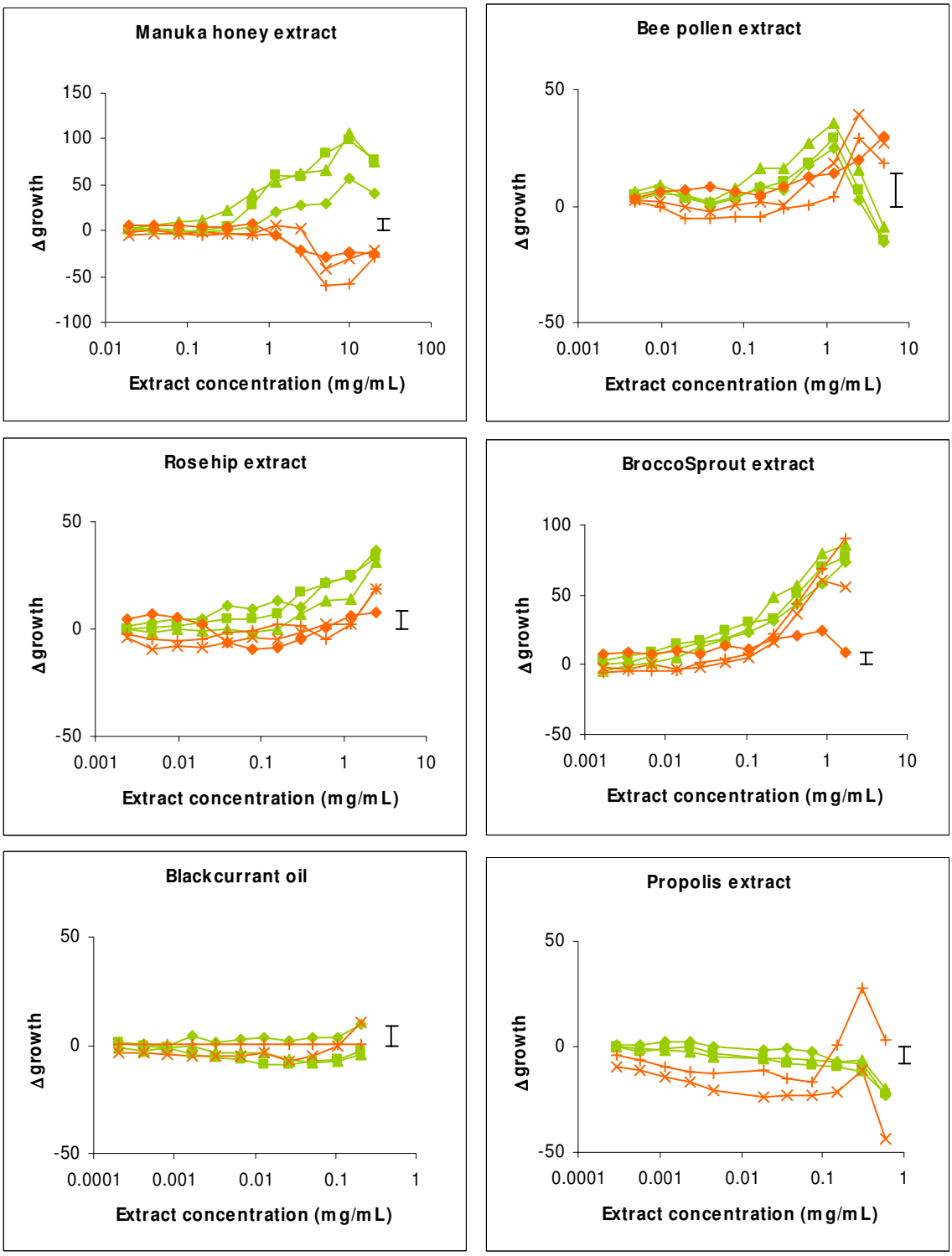


Figure 3.1 Δgrowth values from bacterial cultures supplemented with increasing doses of functional food extracts. Probiotic bacteria: *Lactobacillus reuteri* strain DPC16 (◆), *Lactobacillus rhamnosus* strain DR20 (▲) and *Bifidobacterium lactis* strain DR10 (■). Pathogenic bacteria: *Staphylococcus aureus* (●), *Escherichia coli* strain O157:H7 (+) and *Salmonella typhimurium* (x). Data points show the mean (n=24) 16 h growth at 37°C, obtained over 3 separate experiments with 8 replicates per experiment. The bar displays the Least Significant Difference at $P < 0.05$.

Rosehip and BroccoSprouts® solutions (Fig. 3.1) encouraged bacterial growth in a dose-dependent manner, although the probiotics were the most responsive to rosehips, whilst *S. aureus* was least responsive to the BroccoSprouts®. Rosehips (*Rosa* spp. fruit) have significant antioxidant activity (Gao et al., 2000) and whilst numerous clinical trials have reported a variety of benefits, a meta-analysis of trials conducted with *Rosa canina* subspecies reveals that perhaps only benefits against osteoarthritis are valid (Chrubasik et al., 2006). Rosehips have been reported to have antimicrobial activity associated with the phenolic fractions (Yi et al., 2007). In addition, tellimagrandin I, a hydrolysable tannin extracted from the petals of the plant *Rosa canina*, inhibits *H. pylori* growth, although not growth of *E. coli* (Funatogawa et al., 2004). Interestingly, tellimagrandin I synergistically increases methicillin-resistant *S. aureus* (MRSA) susceptibility to β-lactam antibiotics (Shiota et al., 2000). The possibility that this compound may be present in rosehips is interesting and warrants further investigation, although not during the course of this thesis. Cruciferous vegetables, specifically young plants such as BroccoSprouts® (*Brassica oleracea* var. *italica*), contain antioxidants and high levels of isothiocyanates, particularly sulforaphane, converted by myrosinase from glucosinolates (Shapiro *et al*., 2001), which is active against *H. pylori* (Fahey et al., 2002). Clearly these components were either not present in levels sufficient to negatively impact upon the growth of the tested organisms, or the organisms tested may have simply not been susceptible to these compounds. It could be postulated that *S. aureus* was the organism least susceptible to the growth-encouraging effects observed with the other strains because of an increased susceptibility to antimicrobial components, but without quantitative analyses of these compounds and/or knowledge of the mechanisms involved, it remains speculative. The mechanism by which bacterial growth was enhanced has not been determined, but the ability of these ingredients to buffer the pH of the media has been examined and found to provide no significant buffering ability. Finally, the sugar content of these ingredients remain unknown, but again, as in the case of the manuka honey, the glucose content of the growth media should be more than enough to exclude sugar as a growth-limiting step which ingredient contributions could overcome.

Blackcurrant seed oil (Fig. 3.1) had little effect against any of the bacterial strains used in this work. Blackcurrant seed oil (*Ribes nigrum*) has been reported to affect the membrane of pathogens such as *H. pylori* to the extent that the oil has been suggested as an ideal adjuvant during the use of other antimicrobial agents (Frieri et al., 2000). Thus, despite the lack of significant growth promoting or, alternatively, antimicrobial effects, blackcurrant oil is an ideal candidate for further investigation into possible synergies from ingredient extract combinations.

Propolis (Fig. 3.1) has anti-oxidant, anti-inflammatory, anti-tumour, immune-stimulating and hepato-protective activities (Bankova, 2005; Burdock, 1998) and antimicrobial activity against *H. pylori* (Banskota et al., 2001; Boyanova et al., 2003), *Campylobacter* (Boyanova et al., 2003) and *Staphylococcus* (Miorin et al., 2003). It has also been observed to synergistically increase the effects of some antibiotics (Scazzocchio et al., 2006). The present results indicate that propolis tended to decrease bacterial growth of all strains as the concentration in the media increased, except for an apparent growth peak at 0.3 mg/mL, which was sufficient to increase growth of *E. coli* only at this concentration. This phenomenon of decreased growth from the propolis is likely to be due to perturbation of the cell membrane by phenolic compounds (Sikkema et al., 1994). The similarities in the shape of the dose-response profile of pathogen growth on propolis and bee pollen have been noted, and could reflect the presence of the same or a similar active compound(s).

OD data were compared to viable counts where it was found that increases or decreases in OD were accompanied by increases or decreases in plate counts (data not shown). Whilst statistical analyses were not performed, this assessment was considered sufficient to discount potential factors such as cell clumping or settling or, alternatively, major changes in cell morphology - factors which might have respectively increased or decreased the incident light reaching the photomultiplier and thus given apparent OD changes unrelated to growth or biomass.

The minimum inhibitory concentrations (MICs) of the ingredients were not calculated from the OD data, for several reasons. Firstly, where food ingredients have unknown or poorly defined active components, presenting the data as Δgrowth for a given amount of solubilised ingredient material would avoid misrepresention of the knowledge of the quality and quantity of active components. Secondly, perhaps, the use of MICs is more suitable for compounds intended for pharmaceutical-based eradication procedures than management of gut microorganisms by dietary intervention. Finally, the expression of dose-response curves might shed some light on the nature of the Δgrowth values observed. Thus, one could speculate on the presence and effects of putative active components whilst the chemical determination and quantitative analyses required to establish their presence and concentration, and thus determine useful MICs, remained outside the scope of this chapter.

Whilst the rosehip and blackcurrant oil largely failed to exhibit significant antimicrobial effect, they were selected on the basis that they potentially contribute to the antimicrobial effect of the extracts that were expected to be active. Thus, all the extracts would require testing in combination, where these expected contributions could be measured.

3.4 Synergies

3.4.1 Introduction

In keeping with the aims of this chapter to identify food ingredients which exhibit multiple modes of action to control the growth of probiotic and pathogenic bacteria rather than seeking a 'silver bullet' or pharmaceutical approach, the ingredients were combined and tested for potential synergistic activities. As outlined in Section 3.3, some of the ingredients were selected on the basis of probable contribution to synergistic antimicrobial activity, that is, they would synergistically increase the efficacy of other antimicrobial ingredients. As mentioned in Section 3.3.1, rose petals have been shown to possess the compound tellimagrandin I, which synergistically increases MRSA susceptibility to β-lactam antibiotics (Shiota *et al*., 2000). Thus, despite displaying no significant antimicrobial activity, and encouraging the growth of all the bacteria, probiotic or pathogen, (increased growth which may or may not be nutrient-related), rosehips remain a likely choice for a contributor to potential synergies with other, active extracts. Blackcurrant seed oil has also been reported to affect the membrane of pathogens such as *H. pylori* (Frieri et al., 2000), and render them more susceptible to the entry or activity of other compounds, effectively acting as an adjuvant. Thus, blackcurrant oil is another candidate for further investigation into possible synergies from ingredient extract combinations. Propolis and bee pollen were not unlikely to act in a similar manner, by virtue of their high phenolic content, as illustrated by the nature and antimicrobial properties of the former, and the antioxidant properties of the latter, potentially impacting upon membrane structure or fluidity. It is acknowledged that simply possessing a high phenolic content alone does not constitute having the same antimicrobial and antioxidant activity, but was judged sufficient to warrant further investigation.

3.4.2 Methods

Ingredient combinations were assessed for synergistic antimicrobial activity using the antimicrobial Combined Extract Assay (2.2.4.1), except that maintaining the same assay volume meant that ingredients were present at half the concentration of the highest dose used when tested singly. The raw data were expressed as ΔGrowth using Equation 1 (Section 2.2.4.1).

However, to compare the efficacy of these combinations with those obtained using the extracts independently, and to mine the data for the most efficacious combinations, additional processing of the data was required (as described below). This would serve to identify potential synergistic or unwanted antagonistic interactions in a rapid and simple manner consistent with the aims of this work: to identify those combinations worth pursuing further, and to recognise combinations to be avoided.

Two simple comparisons were performed, in order to assign the terms of effect (desirable, good, poor, undesirable) introduced previously, and calculated according to the following methods:

Comparison 1. Combination Δgrowth values were noted that significantly (P<0.05) deviated further from the control value (zero) than the theoretical sum of the Δgrowth values of the individual extracts (Eq. 2).

$$\text{Does } \Delta\text{growth}_{AB} \text{ exceed } (\Delta\text{growth}_{A} + \Delta\text{growth}_{B})? \quad (2)$$

This immediately identified Δgrowth values which were potentially synergistically acting to exert prebiotic or antimicrobial effects, as the activity of the combination was more than the sum of the effect of the ingredients acting independently. The term ‘exceed’ (Eq. 2) is used because this equation is designed to compare like values with like (positive Δgrowth with positive Δgrowth, negative Δgrowth with negative Δgrowth). This simplistic comparison does not take into account the effect of adding unlike values, ie. a weak positive growth value added to a strong negative growth value, or *vice versa*, in effect cancelling out a part of the apparent effect of the stronger contributor. Thus a second comparison was introduced:

Comparison 2. Combination Δgrowth values were noted that significantly (P<0.05) deviated further from the control (zero) value than the most extreme of the Δgrowth values from either one of the ingredients independently (Eq. 3).

$$\text{Does } \Delta\text{growth}_{AB} \text{ exceed } \textbf{\textit{either}} \ \Delta\text{growth}_{A} \ \textbf{\textit{or}} \ \Delta\text{growth}_{B}? \qquad (3)$$

This identified ingredients displaying an apparently increased efficacy despite an artificial lowering of the sum of the activities obtained from Eq. 2. For example, an ingredient providing an extreme ∆growth such as a 100% increase in growth over the control culture (a ∆growth value of 100) combined with an ingredient inhibiting the growth to only 80% of the growth of the control (a ∆growth value of -20) would have an apparent $\Delta growth_{AB}$ value of 80 using Eq. 2. Should the growth of the combination of ingredients yield a number such as a 90% increase in growth (∆growth value of 90) then Eq. 2. would identify a potentially synergistic effect, which was, in fact, less than the activity of the first ingredient acting independently. However, should the combination yield, for example, a 120% increase, or ∆growth of 120, then that would satisfy the comparisons drawn from both Eq 2. and Eq. 3. and represent a synergistic combination.

Ingredient combinations which met Comparison 1 were termed ‘good’, that is, the combination performed better than mathematically predicted. Those which did not meet Comparison 1 were termed ‘poor’. Again, good combinations did not necessarily outperform single ingredients acting independently.

Ingredient combinations which met both requirements, that is, had an effect greater than either ingredient alone or predicted in combination (synergistic), were termed ‘desirable’ or ‘undesirable’, based upon their activity relative to the bacteria (probiotic or pathogen) being tested, as described earlier.

3.4.3 Results and discussion

The effects of the combinations of extracts on the growth of the bacteria are shown in Table 3.2. Combinations were noted that fulfilled the criteria 'desirable', 'undesirable', 'good' or 'poor'.

Desirable combinations included manuka honey combined with bee pollen (suppressed *S. enterica*), with rosehips (suppressed *S. enterica*, promoted *L. rhamnosus*), with BroccoSprouts® (suppressed *S. aureus*, promoted *B. lactis*), with blackcurrant oil (suppressed *S. enterica*, promoted *B. lactis*) and with propolis (suppressed *S. enterica* and *S. aureus*).

Undesirable combinations, in which combined activities promoting pathogen growth or suppressing probiotic growth exceeded the contribution from the component ingredients, included manuka honey combined with rosehip (suppressed *B. lactis*) or propolis (suppressed all three probiotic strains), or propolis or blackcurrant oil combined with rosehip or BroccoSprouts®.

Some ingredient combinations, such as manuka honey combined with propolis, or propolis combined with BroccoSprouts®, yielded both desirable and undesirable effects, depending upon which strain of bacteria was used. Thus, the response of the bacteria to any given combination of ingredients was often strain-specific. Mathematical analysis failed to show any significant relationship or pattern involving combination, strain and effect (D. Hedderley, Pers. Comm). This is not unexpected, given the complex mixtures of potentially bioactive compounds present in the ingredients used in this chapter.

Manuka honey was particularly effective at increasing probiotic growth and reducing pathogen growth, both alone and in combination with other food ingredients.

Table 3.2 Microbial synergy assay results, expressed as Δgrowth.

		Δgrowth					
Strain	Extract (mg/mL)	Manuka honey 10.00	Bee pollen 2.500	Rosehip 1.750	Brocco-Sprouts® 0.525	Black-currant oil 0.105	Propolis 0.300
DPC16	Manuka	+60.3	+88.7 [d]	+52.6 [c]	+92.2 [d]	+49.1 [d]	-88.2 [b,d]
DR10	honey	+29.6	+74.1 [d]	+3.4 [b,d]	+87.2 [a,c]	+49.6 [a,c]	-64.5 [b,d]
DR20		+20.4	+57.5	+27.9 [a,c]	+54.4 [d]	+14.6 [d]	-89.0 [b,d]
E. coli		-43.4	-35.2 [d]	-43.0 [c]	-41.1	-39.0 [d]	-47.6 [c]
S. ent.		-35.5	-42.4 [a,d]	-45.0 [a,d]	-47.3	-41.7 [a,d]	-50.5 [a,d]
S. aur.		-6.1	+24.0	-16.8[c]	-1.0 [a]	+4.8	-49.0 [a,d]
DPC16	Bee pollen		+80.0	+41.3 [d]	+71.6 [d]	+47.9 [d]	+12.8 [d]
DR10			+76.7	+37.1 [d]	+65.3 [d]	+55.8 [d]	-2.0 [d]
DR20			+37.3	+4.80 [d]	+18.9 [d]	+23.8 [d]	-23.1 [d]
E. coli			+10.2	+20.7	+2.9 [d]	+7.2 [d]	-1.8
S. ent.			+22.5	+25.2 [d]	+18.3	+26.3	+10.0 [d]
S. aur.			+30.0	+40.4	+30.0	+14.6 [d]	-4.5 [a,d]
DPC16	Rosehip			+17.1	+57.6	+31.1 [a,c]	-30.4 [b,d]
DR10				+28.1	+29.9 [d]	+26.4 [c]	-15.5 [d]
DR20				+12.8	+48.0 [d]	-10.6 [d]	-43.9 [d]
E. coli				+12.6	+6.8 [d]	+5.5 [d]	-5.2
S. ent.				+14.4	+12.1 [c]	+15.3 [d]	+3.5 [d]
S. aur.				+5.4	+22.9 [b,c]	+13.8	-86.7 [a,d]
DPC16	Brocco-Sprouts®				+48.1	+29.8 [d]	+21.0
DR10					+20.1	+34.8 [a,c]	+31.9 [a,c]
DR20					+45.6	+45.3	+10.8 [c]
E. coli					+0.1	+1.2 [d]	+6.0 [b,c]
S. ent.					-8.5	+5.9 [c]	+11.5 [b,c]
S. aur.					+6.9	+6.1	-0.7 [c]
DPC16	Black-currant oil					+6.3	-13.6
DR10						-2.4	-17.1
DR20						+6.1	-37.4
E. coli						+6.9	-8.20
S. ent.						+8.3	+6.0
S. aur.						+6.3	-13.6
DPC16	Propolis						-19.3
DR10							-8.1
DR20							-45.4
E. coli							-13.5
S. ent.							-3.6
S. aur.							-19.3

[a] 'desirable' combined effect = increase (probiotic) or decrease (pathogen) of growth from the combination which (i) exceeds the sum (+/- lsd P <0.05) of the contributing extract activities and (ii) exceeds the value (+/- lsd P <0.05) of the most extreme of the contributing extract activities.

[b] 'undesirable' combined effect = increase (pathogen) or decrease (probiotic) of growth from the combination which (i) exceeds the sum (+/- lsd P <0.05) of the contributing extract activities and (ii) exceeds the value (+/- lsd P <0.05) of the most extreme of the contributing extract activities.

[c] 'good' = growth exceeds the sum (+/- lsd P <0.05) of the contributing extract activities.

[d] 'poor' = growth less than the sum (+/- lsd P <0.05) of the contributing extract activities.

The less soluble compounds, propolis and blackcurrant oil, and the plant ingredients rosehips and BroccoSprouts®, tended to generate the most interesting effects in combinations, and thus appear suitable for use as adjuvants or mitigants to moderate the effects of other active ingredients. It is feasible that this may be attributable to fatty acid or phenolic compound(s) perturbing bacterial membranes. Plant compounds (phenolics, polyphenolics, flavones, flavanoids, tannins, coumarins, terpenes, and alkaloids) are known antimicrobial agents with a variety of mechanisms of action including reacting with proteins or perturbing membranes thereby increasing permeability, depending on the lipophilicity of the compounds (reviewed by Cowan (1999)). The generation of synergistic responses may be a consequence of the low concentration of the ingredients, where at higher concentration they may have exerted direct antimicrobial activity. Some plant compounds have demonstrated synergistic effects in combination (for example, essential oils and flavanoids have been shown to contribute to synergies (Williamson, 2001)). The ability of the rosehip to synergise with other ingredients is interesting in regard to the synergistic activities of the rose-derived Tellimagrandin I (Shiota et al., 2000) mentioned earlier.

3.5 Conclusions

The high-throughput spectrophotometric microbial bioassay refined and used during the course of this chapter was proven to be simple, robust, sensitive, accurate, highly reproducible, and easily amenable to use with multiple potential active compounds at a variety of doses and against a number of bacterial species. Much information can be derived from the data generated here and, from a commercial food development perspective, it is fast and inexpensive, ensuring cost does not limit use in an industrial setting.

The manuka honey was the most promising candidate for the inclusion into a concept functional food intended to manage gut bacteria for the purposes of maintaining and increasing gut health. Although the mechanisms of honey action on bacteria, both alone and in combination with other potentially bioactive ingredients were still to be fully explored, the data were entirely consistent with the effects of manuka honey in the literature. Further investigations on the effects of this ingredient were carried out in Chapters 4-7 of this thesis.

As observed with the manuka honey in particular, the bee products in general, and to a lesser extent with the rosehip solution, there appears to be a division between the effects of the ingredients on probiotic organisms compared with the effects on pathogens. The inclusion of the

Gram positive pathogen *Staphylococcus* in some of the assays dispels the potential theory that the results were dictated by a Gram positive or Gram negative-specific element. The role played by the differing media requirements, and the lactic acid production and anaerobic respiration of the probiotics versus the aerobic pathogens, has not yet been explored. Furthermore, manuka honey or other ingredient-derived factors contributing to increased growth of the probiotic organisms have not been unequivocally identified (although honey oligosaccharides (Sanz et al., 2005) remain primary candidates), nor their mechanisms established. Currently, this phenomenon of increased probiotic growth in the presence of food ingredients (which are not conventional prebiotics such as oligosaccharides) is not prominent in the literature, and was an exciting development.

Collectively, these *in vitro* investigations into potential synergistic interactions between ingredients illustrate a potential for combining food ingredients to modify components of the gut flora to a degree not achieved by a single ingredient alone. Although combinations of ingredients yield unusual results, sometimes desirable and sometimes undesirable from a health perspective, specific combinations such as manuka honey extract combined with BroccoSprouts® or to a lesser extent, bee pollen, rosehip and blackcurrant oil, show immediate potential as an ingredient combination in, for example, a yoghurt containing *B. lactis* DR10, which is specifically and synergistically encouraged to grow by three of those four combinations. It is recognised that the effects of these ingredients or ingredient combinations may perform differently with mixed populations of bacteria than with single strains tested in isolation. In addition, they may perform very differently in a more complex food matrix, or as conditions change during consumption and digestion. Food synergy is the basis for modern nutrition science. It is an extremely complex area and is composed not only of interactions between compounds in ingredients but between them and the general food matrix. Also, whilst some of these ingredients may well retain their efficacy when incorporated into foods, other factors such as safety, toxicity and organoleptic impact would need to be considered. It is acknowledged that documented allergic responses have been observed with bee products (for example, refer Menniti-Ippolito et al. (2008)), which may limit their usefulness to some manufacturers or potential consumers.

Finally, in regard to the adverse synergies observed, a thorough screening programme should be considered as an essential part of functional food development to avoid any undesirable synergies between functional food ingredients.

This work was presented as a poster at the 40th Anniversary Convention of the Australian Institute of Food Science and Technology, Melbourne, Australia (Appendix A).

This work has been published (Rosendale et al., 2008)(Appendix B).

Additional screening of the extracts for effects on host innate immunity were conducted (Appendix C). At this stage of the work, it was intended to screen the food extracts for their effects on host innate immunity. Unfortunately, considerable difficulties were encountered with the methodology involved, and a decision was made to not pursue this work any further. However, the fact that this work was done did lead to decisions regarding the animal trial that is reported in Chapter 4 of this thesis. Hence the results are reported in an Appendix, to demonstrate the difficulties involved and the preliminary results that were obtained, rather than in the main body of the text where it would hinder the natural flow of the more significant results

To examine whether the results observed *in vitro* may be applicable *in vivo*, manuka honey and broccosprouts extracts were tested *in vivo* (Chapter 4).

CHAPTER FOUR

ANIMAL FEEDING TRIAL

4.1 Introduction

As shown previously (Chapter 3), data from single extract and combined extract analyses demonstrated that manuka honey could inhibit pathogen growth and encourage probiotic growth *in vitro*. In addition, BroccoSprout® extract was shown to synergistically increase probiotic growth in combination with manuka honey. Furthermore, other researchers within the larger project of which this thesis forms a part have shown that manuka honey inhibited *H. pylori* growth, and that BroccoSprout® extracts were effective at managing *H. pylori* growth and/or *H. pylori*-induced gastric inflammation (Yanaka et al., 2009, J. Keenan and N. Salm, University of Otago, pers. comm., 2005). Experiments using the sulforaphane standard compound (*ibid*) have suggested that the reported BroccoSprout®-induced effects were due to that molecule, which is found in cruciform vegetables (Shapiro et al., 2001), and known to be active against *H. pylori* (Yanaka et al., 2009, Fahey et al., 2002).

On the basis of the Chapter 3 manuka honey findings, and on the evidence from others in the project, a pilot human clinical trial was commissioned to explore the effects of UMF™ manuka honey and compare it with a non-UMF™ control (clover, mixed floral) honey (Sutherland et al., 2007). This randomised, double-blind crossover pilot trial was conducted by Crop & Food Research (C&FR, now Plant & Food Research) scientists in Palmerston North and Christchurch. Twenty individuals were divided into cohorts of 10, asked to abstain from all honey products for two weeks, and then one cohort supplemented their normal diet with 20 g manuka honey and the other with 20 g control honey every day for four weeks. All subjects then abstained from honey products for a two week 'washout' period, and then swapped to the other honey for four weeks. Blood and stool samples were collected, the former at the beginning of the 10 week trial and then weekly throughout the periods of dietary intervention, whilst the latter was collected at the beginning and end of the four week intervention periods.

Real-time PCR of important colonic microbial groups was conducted with the faecal samples, revealing that no significant changes occurred post-intervention with either UMF™ manuka honey or control honey (Fig. 4.1.1).

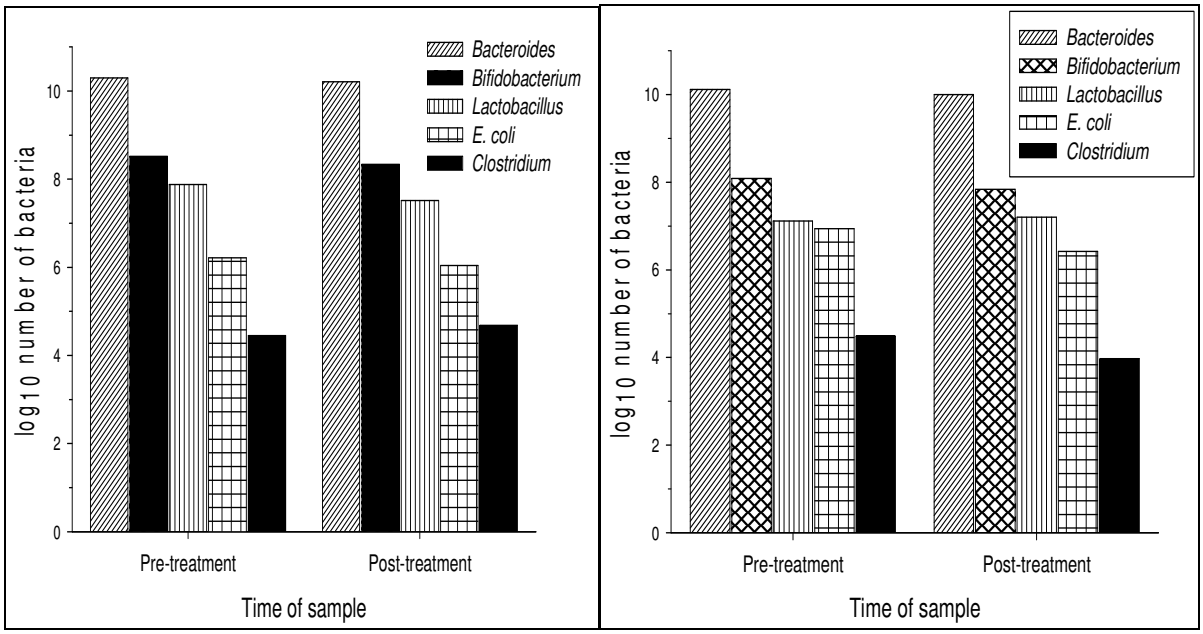


Figure 4.1.1.: Log_{10} number of bacteria in the 5 groups pre- and post-treatment with manuka UMF®20 honey (left) or control honey (right). Reproduced from Sutherland et al. (2007).

The human clinical trial had low sample numbers (n=20). Whilst participant compliance was apparently good it is never as controlled as in an animal trial, with dietary intervention *versus* controlled diet. Recommendations arising from that trial for establishing the effects of UMF™ manuka honey on the gut health of subjects included an animal trial on the basis of using genetically identical subjects and a greater degree of dietary control. An animal trial would have the added benefit of allowing invasive testing of efficacy of the chosen ingredients.

Indices of gut health to be examined in an animal trial were selected on the basis of the pilot clinical trial and from the literature. Analysis of the gut microbiota by PCR was to be restricted to those population groups thought to best represent probiotic organisms (Lactobacilli and Bifidobacteria), other commensal organisms (Bacteroides) and potential pathogens (Clostridia, pathogenic strains of *E. coli*)(Sutherland et al., 2007). These data would be supported by short chain fatty acid (SCFA) analysis as a representation of the metabolic activity or profile of the gut microbiome (Gråsten et al., 2002). Previous trials indicated that the region of the mouse gut exhibiting the greatest microbial population density and propensity to change when subject to dietary intervention was the caecum (J. Sutherland and G. Paturi, C&FR, pers. comm., 2007),. This organ is a well defined anatomic structure situated at the junction of the small and large intestines, the concents can be readily and easily removed, and the mouse caecum harbours a dense and well-studied microbial population (Rawls et al., 2006). Thus, microbial profiling was to be restricted to this organ.

Phagocytosis and macrophage proliferation in the blood and peritoneum, and analysis of liver, spleen and mesenteric lymph nodes (MLN) for translocated pathogens have been reported as indices of changes to the innate immune functions involved in gut health (Shu and Gill, 2001; Schiffrin et al., 1995)(also refer Section 1.5 and Appendix C). However, seeking changes in systemic innate immunity (blood phagocytic ability or leukocyte proliferation) or bacterial translocation to some visceral organs (liver, spleen) was judged of less immediate value in normal healthy animals (cf. Shu and Gill, 2001, 2002). Thus markers of innate immune function were restricted to peritoneal macrophage proliferation and phagocytic ability, and microbial quantification of the MLN. The process followed is summarised in Fig. 4.1.2.

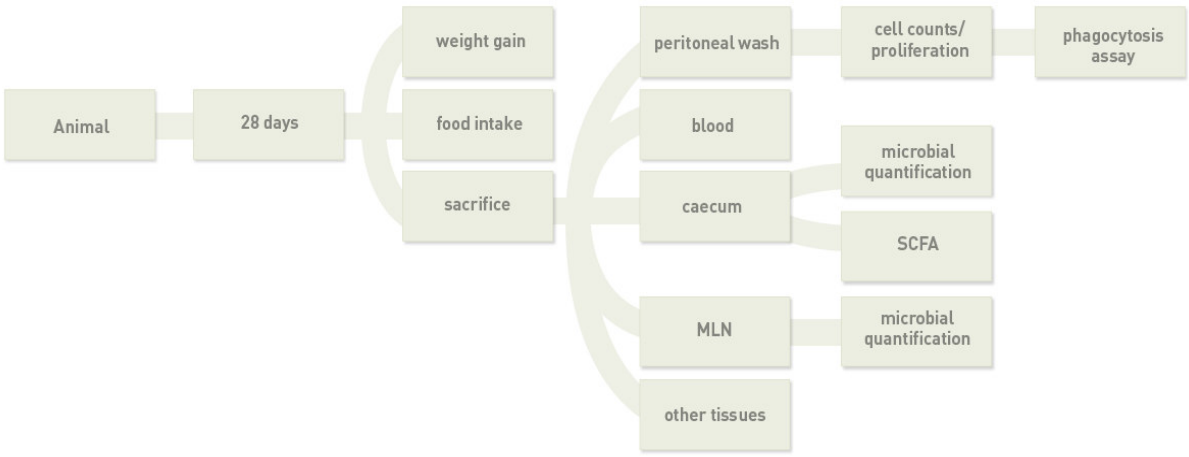


Figure 4.1.2. Process work flow depicting sample collection and fate of samples during course of animal trial.

Thus the aims of this trial were to determine the gastrointestinal effects of BroccoSprouts® and UMF™ 20+ manuka honey as dietary supplements in C57BL/6 mice by measuring weight gain, peritoneal macrophage numbers and phagocytic ability, quantification of selected MLN and caecal microbial groups, and caecal SCFAs. The results would indicate the ability of these ingredients to moderate innate gut immunity, gut microbiota and gut microbial metabolic activity.

4.2 Methods

4.2.1 Trial Conditions

The feeding trials were approved by the AgResearch Grasslands Animal Ethics Committee (New Zealand Pastoral Agriculture Research Institute Ltd (AgResearch), Palmerston North), Ethics Applications 11493 (Trial 1, all ingredients *versus* control) and 11163 (Trial 2, UMF™ 20+ manuka honey *versus* controls), respectively. The trials were carried out under PC2 conditions in an accredited animal trial facility based at The New Zealand Institute for Crop & Food Research Ltd (now Plant and Food Research), Palmerston North, in a room maintained at a temperature of 22±1°C, with humidity of 60±5%, air exchange of 12 times/hour, and a 12 hour light/dark cycle. Healthy male C57BL/6J mice (Section 2.1.3.1) were housed individually under conventional rodent housing conditions and were fed standard rodent diet AIN-76A (Appendix D) *ad libitum* with drinking water available at all times.

After 1 week of settling into the facility (approx 5-6 weeks old) the mice were randomly assigned into 2 treatment groups of N=20 per group, where sample numbers were chosen on the basis of a power analysis conducted by a Crop & Food Research Ltd biometrician (Duncan Hedderly, C&FR, pers. comm., 2007). Treatment diets consisted of modified AIN-76A with ingredient supplement replacing an equivalent weight of the sucrose component (Table 4.1).

Two trials were performed, the first comparing five supplemented diets (Table 4.1) with control diets, and a subsequent trial comparing manuka honey UMF™ 20+ supplemented diet with controls. These controls were separate, and differed because the first trial, containing BroccoSprout® extract alone and in combination with manuka honey, was to involve use of metabolism cages to collect and analyse urine and faeces, and additional samples would be collected at dissection for the study of the metabolic fates of sulforaphane and other glucosinolates or isothiocyanates, by other researchers in the project of which this thesis forms a part. Thus, as a part of the first trial, groups were also fed with diets containing UMF™ 20+ manuka honey, a probiotic bacterial strain (*L. rhamnosus* DR20), manuka honey + probiotic, and honey + BroccoSprout®, but due to unavailability of metabolism cages for these animals, the data from these treatments was of limited use, such as establishing parameters for the second trial, as controls were not included.

Fresh diets were prepared daily and food consumption was recorded throughout the trial. Mice were weighed three times a week and checked daily for the presence of loose stools or blood in faeces, to monitor the unlikely possibility of gut inflammation. In Trial 1, after 21 days of feeding the mice from the control (unsupplemented) diet and Broccosprout® groups were placed in metabolism cages to obtain and quantify excreted metabolites for the use of other researchers within the project. In Trial 2, where the metabolism cage data were not required, mice were kept in their original cages throughout. After 28 days of feeding the mice from both trials were humanely euthanased via CO_2 asphyxiation.

Table 4.1. Animal treatment diets, where the functional food supplement replaced an equivalent weight of the sucrose component of the diet (Appendix D). Treatment groups focused on in this chapter are in black font. Other groups unused (grey font).

#	Treatment	Supplement
1	Control diet 28 days (**Trial 1**) Metabolism cage days 22-28 (**Trial 2**) Normal cages 28 days	unmodified
2	Broccosprouts® 28 days (**Trial 1**) Metabolism cages days 22-28	approx. 60 mg/day(1)
3	Manuka honey UMF20+ **(Trials 1 and 2**) Normal cages 28 days	2 g/kg animal(2) (approx. 60 mg/day, 20 g mouse)
4	*L. rhamnosus* (**Trial 1**) Normal cages 28 days	approx. 40 mg/day (3) or 10^{11} cfu/g
5	Manuka honey + *L. rhamnosus* (**Trial 1**) Normal cages 28 days	as treatments 3 and 4, above.
6	Manuka honey + Broccosprouts® (**Trial 1**) Metabolism cages days 22-28	as treatments 3 and 2, above.

(1) Based upon values used during anti-inflammatory *H. Pylori* mouse trial (A. Wallace, C&FR, J. Keenan and N. Salm, University of Otago, pers. comm., 2005).

(2) Based on the value used in honey anti-inflammatory trial by (Nasuti et al., 2006), and also an approximation of the dose used in the human clinical trial (Sutherland et al., 2007). Amount in diet (15 g/kg diet) based upon average intake of 4 g/day/animal (C. Butts, C&FR, pers. comm., 2007).

(3) Sufficient to exceed the recommended daily dose of $10^{9\text{-}10}$ cfu (Sanders and t Veld, 1999), weight based upon recovery (cfu/g) from freeze-dried stocks(Danisco Howaru™ Lacto HN001, Danisco, Deutchland, GmbH)(data not shown).

4.2.2. *Ex vivo* sample collection

Immediately after asphyxiation a blood sample was collected by direct heart puncture, and a peritoneal wash was immediately performed using phosphate buffered saline (5 mL) introduced by syringe and collected in tubes to collect macrophages, which were stored and transported at 37℃ to the laboratory for counting and, with Trial 1 samples, for immediate conduction of a phagocytosis assay.

Following peritoneal wash the mice were dissected and the following samples were collected and snap frozen in liquid nitrogen: Trial 1: mesenteric lymph nodes, urine, faeces, stomach, jejunum, ileum, intact caecum, colon tissue, and liver. Trial 2: caecum. The whole intact caecum was cut in two and half reserved for short chain fatty acid analysis and the other half for quantification of microbial groups by Real Time qPCR. Samples were stored at -80℃.

Subsequent analyses conducted with these samples for this work have been confined to using the macrophages for measuring phagocytosis (Section 4.2.3), and using the caecum for SCFA analysis and microbial quantification.

4.2.3. Phagocytosis assay

Peritoneal macrophages were immediately transported to the laboratory, counted using a haemocytometer to quantify possible macrophage proliferative effects which had occurred within the animal, standardised to a concentration of $1x10^6$ cells/mL with PBS for the phagocytosis assay, and maintained throughout at 37℃. The phagocytosis assay was performed according to Section 2.2.4.3., without addition of food extract.

4.2.4. Short Chain Fatty Acid Analysis

This analysis was conducted as described in Section 2.2.4.5.

4.2.5. Microbial Quantification

The half-caecum sample was weighed and then homogenised using a hand held homogeniser in a lysis buffer (ALS buffer from Qiagen DNA stool kit) to prevent degradation of the samples, according to the manufacturer's instructions, with the optional 95°C lysis step included as part of this extraction to ensure that DNA was obtained from both Gram-positive

and Gram-negative microbes. Real Time PCR was performed using a standard SYBR-green based assay and a Roche Lightcycler 480. Results were obtained in the form of Ct values, being the number of amplification cycles required to generate sufficient fluorescent product to reach an arbitrary fluorescence threshold. Absolute quantification (AQ) for five microbial groups was performed, calculated as copy number of template DNA per gram of caecal material by comparing the means of three Ct determinations for each sample to a standard curve produced using DNA from a reference strain belonging to each microbial group. Primers used for each microbial group are given in Table 4.2. These primers have been assessed for specificity as a part of their routine use within the research group where this work was performed (C-E. de Guzman and J. Sutherland, pers. comm.).

Table 4.2. Microbial group primers used to quantitatively amplify gut microbial populations from the mouse caecum using RT-PCR with primers for 16S rRNA genes.

Strain	Primer	Reference	Sequence (5' to 3')
***E.coli* group**	E.coli -Huijsdens F	Huijsdens et al., 2002	CATGCCGCGTGTATGAAGAA
***E.coli* group**	E.coli-Huijsdens R	Huijsdens et al., 2002	CGGGTAACGTCAATGAGCAAA
Bifidobacteria group	Bifid group-F	Rinttila et al. 2004	TCGCGTCYGGTGTGAAAG
Bifidobacteria group	Bifid group-R	Rinttila et al. 2004	CCACATCCAGCRTCCAC
Lactobacillus group	Lact-g-F	Fu et al. 2006	CGATGAGTGCTAGGTGTTGGA
Lactobacillus group	Lact-g-R	Fu et al. 2006	CAAGATGTCAAGACCTGGTAAG
Clostridium perfringens		Rinttila et al. 2004	ATGCAAGTCGAGCGA(G/T)G
Clostridium perfringens		Rinttila et al. 2004	TATGCGGTATTAATCT(C/T)CCTTT
Bacteroides-Prevotella-Porphyromonas	BPP -f	Rinttila et al. 2004	GGTGTCGGCTTAAGTGCCAT
Bacteroides-Prevotella-Porphyromonas	BPP-r	Rinttila et al. 2004	CGGA(C/T)GTAAGGGCCGTGC

4.3 Results and Discussion

4.3.1 Animal feeding and weight gain

The weight gain by the mice during the course of the trial is shown in Fig. 4.3.1. The diet consumption is shown in Fig. 4.3.2. Only controls and BroccoSprout® (Trial 1) or controls and manuka honey (Trial 2) results are shown.

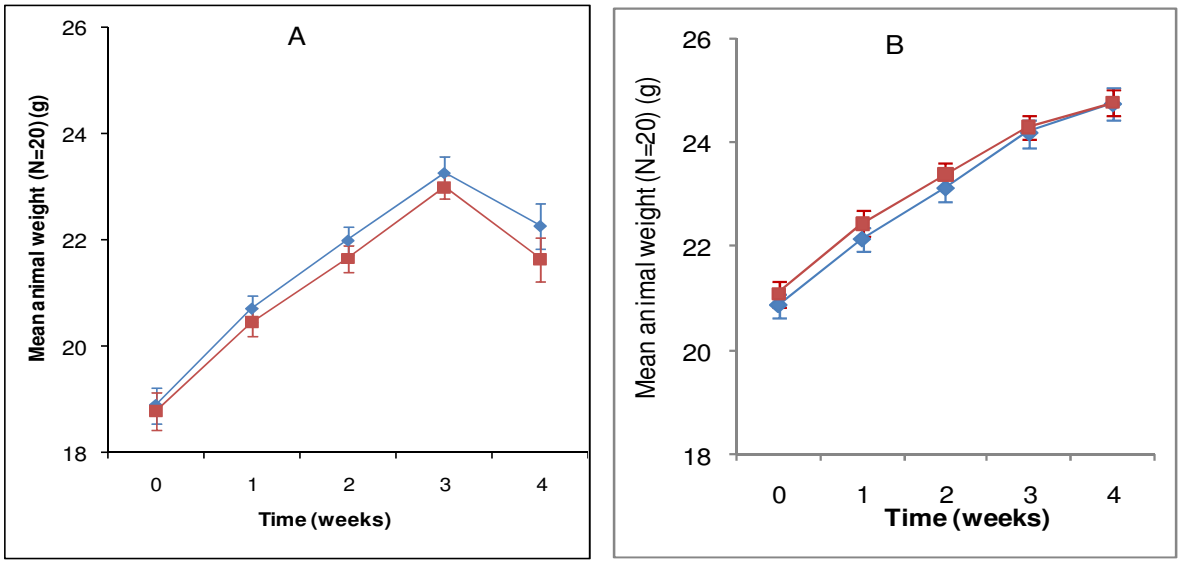


Figure 4.3.1. Mouse weight gains during course of 28 day feeding Trial 1 (A) on control (◆) and broccoSprout® (■)diets or Trial 2 (B) on control (◆) and manuka honey UMF™ 20+ (■) diets. Data mean of 20 animals, with SEM shown.

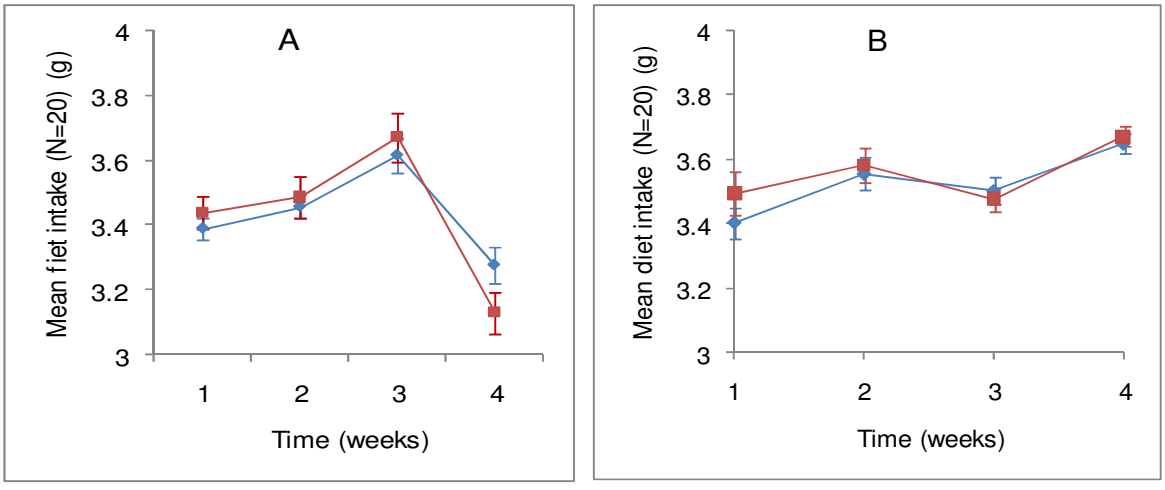


Figure 4.3.2. Average weekly food intake for (A) Trial 1 control (◆) and BroccoSprout® (■), and (B) Trial 2 control (◆) and manuka honey UMF™ 20+ (■) fed C57BL/6 mice during 28 day feeding trial. Data mean of 20 animals, with SEM shown.

Within both trials mice ate the same on an average per treatment group per day basis, irrespective of diet. However, the Trial 2 animals had higher starting weights, and there was a contrast between Trial 1 and 2 over the fourth week of the 4 week trial. All mice gained weight up to the 3 week point. In Trial 2, the control and manuka honey-treated mice continued to eat and gain weight over the fourth week. In Trial 1, all of the metabolism cage animals started eating less and lost weight over the course of the fourth week. Discussion with facility staff (Sheridan Martell and Hannah Smith, pers. comm.), and similarly experienced animal facility researchers from other institutes, revealed that agoraphobia- and stress-induced loss of weight results from placement of rodents into metabolism cages, and is to be expected.

Stress associated with placement in metabolism cages has been shown to lead to sustained changes in cardiovascular (and potentially renal) function in C57BL/6 mice (Hoppe et al., 2009). The impact of stress on gastrointestinal function is widely known, and can involve development of gastric ulcers, altered gastrointestinal motility and ion secretion (Caso et al., 2008). Stress can also result in increased intestinal permeability leading to bacterial translocation to inguinal and mesenteric lymph nodes (Bailey et al., 2006), although this has been reported to be abrogated somewhat by probiotic treatment (Zareie et al., 2006). Stress can also synergize with other pathogenic factors such as *Helicobacter pylori*, non-steroidal anti-inflammatory drugs or colitis-inducing chemicals to produce gastrointestinal disease (Caso et al., 2008).

On this basis it was decided to exclude further analyses involving samples from the Trial 1 manuka honey, manuka honey + BroccoSprout®, probiotic and probiotic + manuka honey treatments due to the lack of appropriately similar controls for the relevant components, and this led to the commissioning of Trial 2 to (re)investigate potential *in vivo* effects of manuka honey. As a further consequence, further processing of some of the samples collected, such as MLN bacterial analyses, was not performed at this stage.

4.3.2 Phagocytosis assay

Cell counts from the first animal trial are shown in Fig. 4.3.3A and B, which displays the number of peritoneal macrophages recovered/mL peritoneal wash from Trials 1 and 2, respectively.

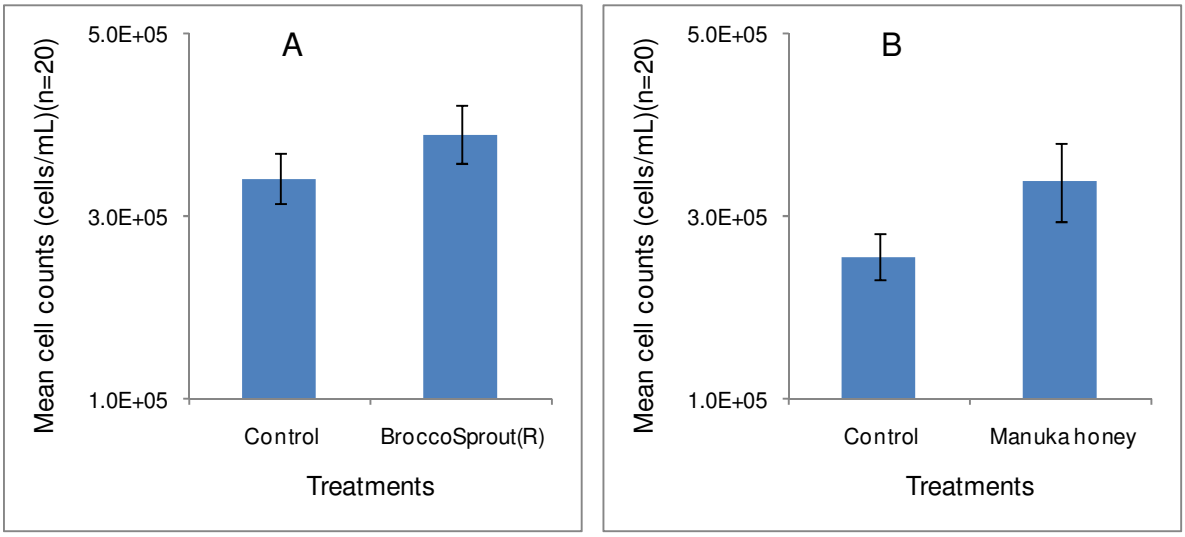


Figure 4.3.3 Peritoneal macrophages from C57BL/6 mice treated with different functional food dietary supplements from Trial 1 (A) and Trial 2 (B). Data are expressed as cells/mL peritoneal wash recovered. SEM shown (N=20).

Trial 1 did not result in a significantly different mean population of peritoneal macrophages in the BroccoSprout®-fed animals compared to the control animals, indicating that supplementation of the diet with that ingredient was neither significantly beneficial nor detrimental regarding macrophage proliferation of numbers.

Trial 2 showed an increased average macrophage count in the manuka honey-fed animal group, indicating that additional proliferation of macrophages had occurred, although statistical analysis indicated this was only significant at a level of $P<0.10$.

Phagocytosis from macrophages collected from Trial 1 animals is shown in Fig. 4.3.4. No significant changes in peritoneal macrophage phagocytic ability were observed. This is in contrast with the findings of Shu and Gill (2001, 2002) upon *E. coli* O157:H7 challenge and subsequent probiotic intervention. Furthermore, no significant changes in phagocytic ability were observed for any of the other treatments from Trial 1, including manuka honey (data not shown). The latter observation, in the context of the degree of gastrointestinal perturbation some of the animals should have suffered as they underwent a degree of stress sufficient to cause appetite and weight loss (Section 4.3.1, above), indicated that changes to phagocytic ability, even upon perturbation of that magnitude, were not observable with this measurement system.

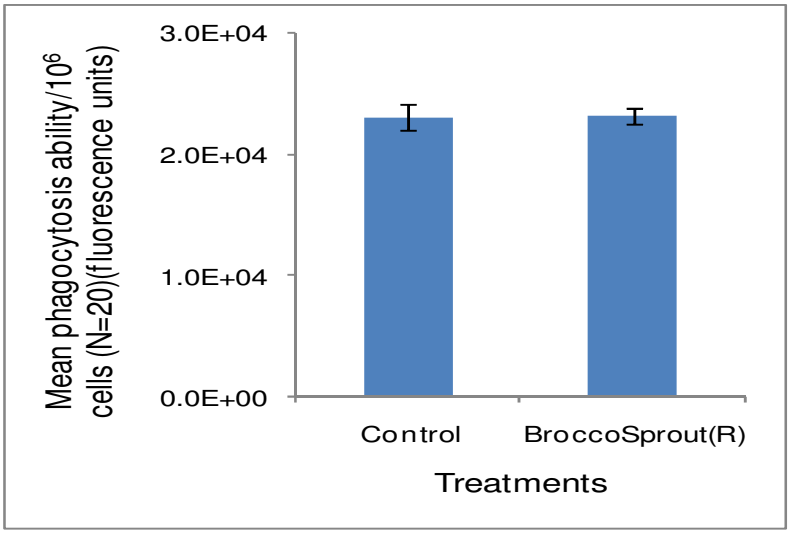


Figure 4.3.4. Macrophage phagocytosis from Trial 1 animals. Data mean of 20 animals, SEM shown.

Furthermore, the changes in phagocytic ability observed by Shu and Gill (2001, 2002) upon challenge by *E. coli* O157:H7 and with probiotic intervention were measured after 7 days, not the 28 days dietary intervention in ostensibly healthy animals conducted in this trial. This may indicate that innate immune responses of this nature are attenuated by continual exposure over long (28 day) periods of time. However, other work by Gill and co-workers has revealed increased phagocytosis upon probiotic treatment after both 10 and 28 days (Gill et al., 2000). Furthermore, measurement in the stressed animals in this study occurred 7 days after the onset of the metabolic cage stress, indicating that sampling time was not a factor.

On these bases, phagocytosis was not measured on the peritoneal macrophages collected from the Trial 2 animals.

4.3.3 Short chain fatty acids

The results of the SCFA analyses are presented in Fig. 4.3.5.

In Trial 1, supplementation of the diet with BroccoSprout® resulted in a decrease in mean formic acid levels [control 0.685 (SEM 0.198) mM to BroccoSprout® 0.123 (SEM 0.059) mM] and increase in succinic acid levels [from 0.258 (SEM 0.073) mM to 0.654 (SEM 0.073) mM]. None of the other SCFA levels changed significantly ($P<0.05$).

Succinic acid is a major byproduct of *Bacteroides* fermentation (Rotstein et al., 1985). Thus increases in levels of this SCFA may reflect either an increased population of these commensal organisms, or increased metabolism of a population with unchanged numbers. Interestingly, succinic acid production has been suggested as a possible virulence mechanism for *Bacteroides*, where it has been reported to inhibit neutrophil function and prevent phagocytic killing under certain conditions (Rotstein et al., 1985). However, succinic acid is also a minor fermentation product of Bifidobacteria (Doleyres and Lacroix, 2005), and so changes in numbers or activity of these organisms cannot be discounted. It is also possible that the increased succinic acid was of plant origin.

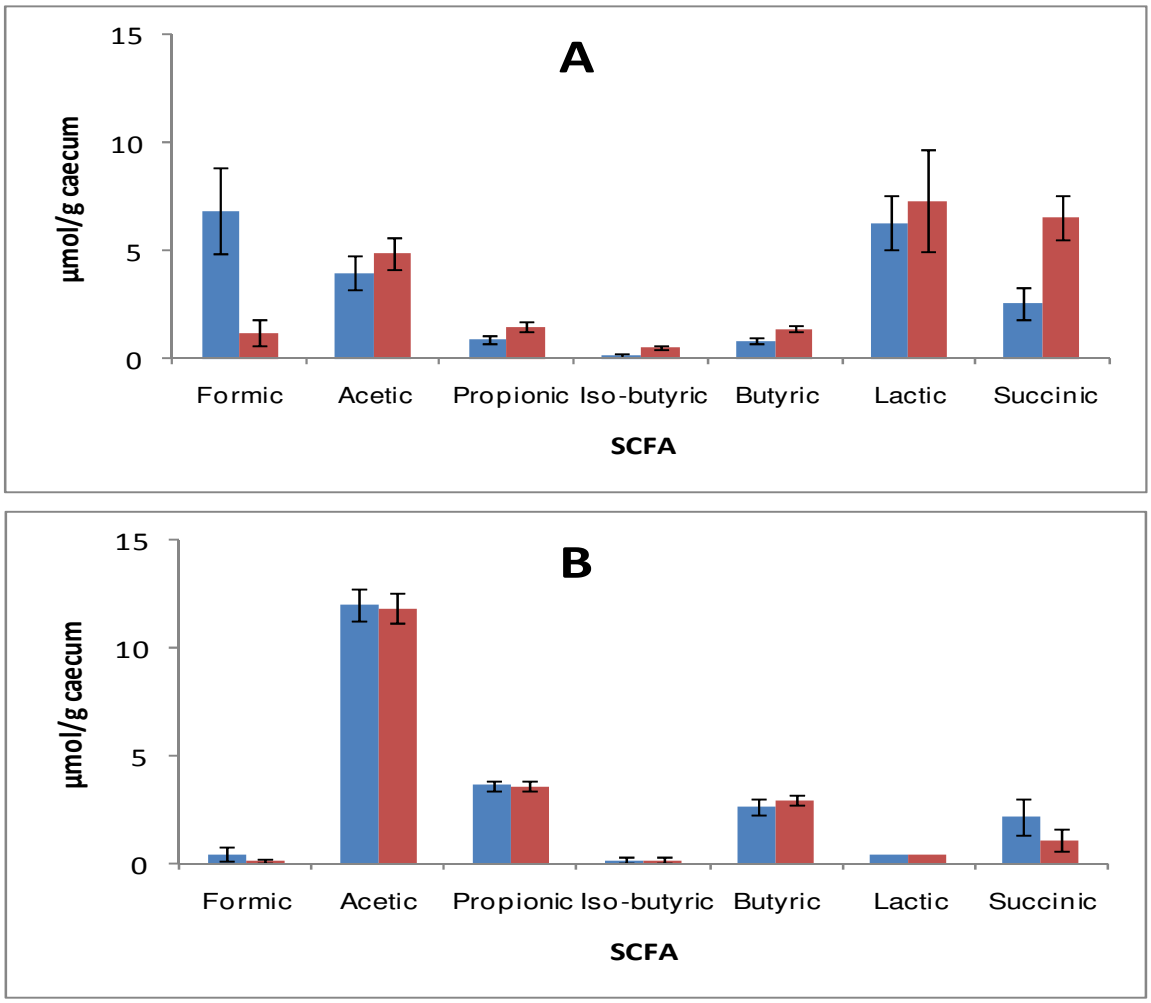


Figure 4.3.5 SCFA analyses of mice caecal contents, Trial 1, A (control, ■; BroccoSprout®, ■) and 2, B (control, ■; manuka honey, ■).

Formic acid is produced at moderate levels upon fermentation of carbohydrate by gut organisms such as Lactobacilli and Bifidobacteria (Pompei et al., 2008). Interestingly, it does not appear to be a major fermentation product of *Bacteroides*, Clostridia or Eubacteria (Pompei et al., 2008). The difference in formic acid between control animals and those fed

BroccoSprout®, together with the increase in succinic acid detected, may indicate a shift in metabolic activity from commensal probiotic organisms towards the other commensal and opportunistically pathogenic organisms residing in the gut.

In Trial 2, supplementation of the diet with manuka honey UMF™ 20+ did not result in any significant changes in SCFA levels.

The two trials could not be compared due to the disparity between SCFA levels measured. This suggests that either the two groups of mice used in the different trials had quite different microbial metabolic activity and/or bacterial numbers, or that the stresses induced by the metabolism cages in Trial 1 had profound effects.

4.3.4 Microbial quantification

The results for the bacterial group analysis are presented in Fig. 4.3.6. The values represent the DNA copy number, or number of organisms, measured and presented as number recovered per gram of caecal material from which the DNA was extracted.

With the Trial 1 animals there does not appear to be any difference in *Bifidobacterium*, *Lactobacillus*, *Bacteroides* or *Clostridium* group numbers between the control or BroccoSprout®-fed mice. A lower level of *E. coli* was observable with BroccoSprout® treatment. This indicates that BroccoSprout® treatment at the dose used barely improved gut health from the perspective of decreasing levels of one potential pathogen, whilst not increasing the levels of probiotic or commensal organisms examined. Further work would be required to determine whether the relatively high levels of *E. coli* and *Clostridium* measured (equal to the commensal *Bacteroides*) represented the flora of a normal, healthy animal when not exposed to stress from metabolism cages.

With the Trial 2 animals there was not any difference in *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *E. coli* or *Clostridium* group numbers between the control or manuka honey-fed mice. This is a similar result to that obtained during the human pilot clinical trial (Section 4.1) and is in contrast to the *in vitro* results observed with manuka honey on probiotics in Chapter 3. This may represent a dilution effect of the honey, whereupon potential growth-regulating effects were at too low concentration to yield the same effects as observed in pure culture. Alternatively, the honey component(s) which generated this effect may have been susceptible to degradation by host and/or bacterial processes (digestion) prior to exerting an

effect in the caecum of the animal. Neither treatment was shown to be detrimental to the gut health of the organism.

The observation of higher numbers of bacteria in the Trial 2 animals compared to the Trial 1 animals, particularly the *Bifidobacterium* and *Bacteroides* groups, was interesting. Whilst this might be attributed to differences between the batches of animals, it bears repeating that the Trial 2 animals were unstressed whilst the Trial 1 animals were highly stressed, and these differences may be (i) an indication of the impact of stress on gut bacterial species, particularly the important commensal Bifidobacteria and *Bacteroides* groups (Section 1.2), and (ii) further evidence that the Trial 1 stress was a perturbation of significantly greater magnitude than the dietary intervention attempted in this study.

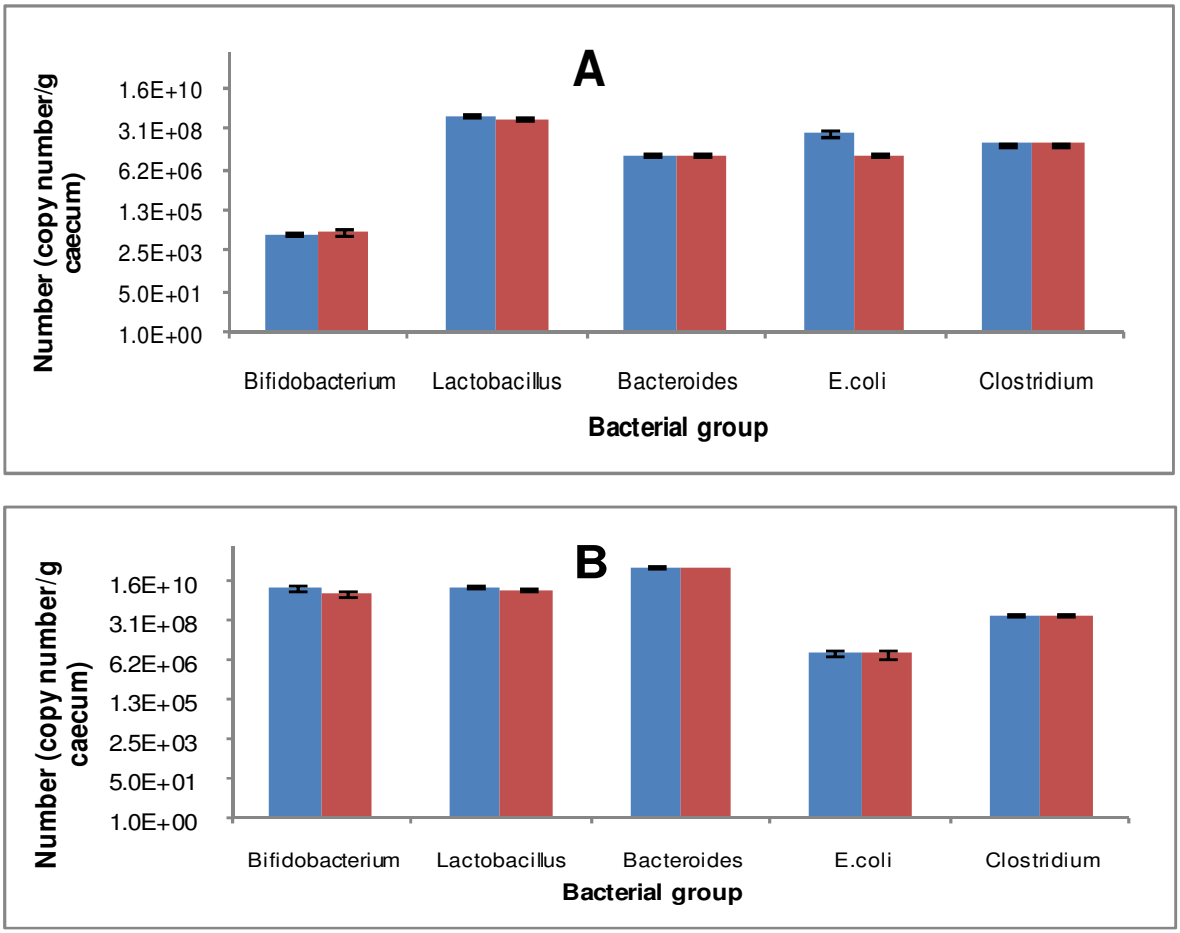


Figure 4.3.6. Bacterial group numbers from the caecum of mice fed unmodified (A and B)(■) or BroccoSprout-supplemented (A, Trial 1) or manuka honey-supplemented (B, Trial 2)(■) diets for 28 days. SEM values shown.

4.4 Conclusions.

This research was intended to be based upon the pilot human clinical trial outlined in Section 4.1, expanded with the details outlined in Fig. 4.1.2 and Table 4.1. However, as outlined in Section 4.2.1 and noted in Table 4.1, a large part of the first animal trial was subsequently discovered to be unusable due to lack of an appropriate control group. The second trial (Trial 2) was envisaged as a means of potentially recreating the missing control group for the first trial (Trial 1), on the basis that if the Trial 2 animals with identical manuka honey-supplemented diets under conditions replicating Trial 1 generated similar results this might be sufficient to attempt comparison (Duncan Hedderly, 2008, pers. comm.). Accordingly, Trial 2 was attempted, but differences became apparent. The animals received were of different weights at the beginning of the trial, consumed their diets at a different rate, displayed different numbers of peritoneal macrophages, and displayed markedly different group DNA copy number profiles (Trial 1 manuka honey data not shown), collectively indicating that no comparison would be appropriate. Thus the usable data (control and BroccoSprout®) from Trial 1 were kept as a completely separate body of work from Trial 2 (control and manuka honey UMF™ 20+), and whilst they were presented and discussed alongside each other, comparisons between the trials were drawn only for the purposes of illustrating the differences made by subjecting mice to metabolism cages.

Collectively, the data for each trial indicated that dietary supplementation with the respective functional food ingredients had minimal or no observable effects on any of the gut health parameters tested under the conditions used.

The BroccoSprout® dietary supplementation resulted solely in alteration of the SCFA profile, with decreased formic acid and increased succinic acid concentrations in the caecal contents. This was not accompanied by a change in bacterial group numbers, suggesting that the ingredient either altered the metabolic activity of the *Bacteroides* and possibly *Bifidobacteria* groups, and in the case of the increased succinic acid, the elevated level may have been due to plant-derived material reaching the caecum. It is entirely feasible that indigestible fibre enters the caecum relatively intact, potentially associated with small molecules (such as Vitamin E, a succinate salt). As mentioned previously (Section 4.1 and Chapter 3, Section 3.3.2) cruciferous vegetables such as BroccoSprouts® contain high levels of isothiocyanates, particularly sulforaphane, converted from glucosinolates by myrosinase (Shapiro et al., 2001). Data indicating that colonic bacterial myrosinase activity can result in isothiocyanates liberation and subsequent detection in faeces (Rouzaud et al., 2004;

Rungapamestry et al., 2008) shows that plant-derived compounds survive passage to the distal gut. Thus BroccoSprout®-derived chemicals could be expected to be measured in the caecum if of sufficient concentration.

The manuka honey UMF™ 20+ was only shown to induce a slight increase in peritoneal macrophage numbers harvested. The lack of effect on gut bacterial numbers or SCFA production is in contrast to the *in vitro* microbial screening performed in Chapter 3, where the honey increased probiotic growth and decreased pathogen growth in a dose-dependent manner. This dose-dependency may illustrate the reason that no intestinal changes were evident – dilution or digestion may have rendered any manuka honey factors that survive passage to the gut at a concentration too low to exert significant measurable effects.

However, data in this chapter confirm the findings from the pilot human study by Sutherland et al. (2007), whereupon no beneficial or detrimental effects of the honey could be observed. Sutherland et al. (2007) recommended two main avenues of investigation to determine the effects of manuka honey on the gut flora: (i) investigate the metabolic activity of gut constituents in order to detect effects unrelated to changes in population density, and (ii) examine the effects in a disease model. The first point has been addressed in this chapter, with SCFA analysis, whilst the second avenue of investigation, using a disease model, involves ongoing investigations by others within the project. Further work involving unused samples from Trial 1, such as examining the stored MLN for evidence of changes in the degree of bacterial translocation that could be attributed to the functional food ingredients remains an option for future researchers.

The nature of the compound(s) that potentially contribute to the *in vitro* dose-dependent effects on gut bacteria of the UMF™ 20+ manuka honey, and yet are either susceptible to digestion or too dilute at doses used here and by Sutherland et al. (2007) to exert effects *in vivo,* was intriguing and was subject to further investigation (Chapter 5).

While the sample analyses for this animal trial were being conducted, an abstract describing the preliminary results, and the work from the preceding chapter, was entered into Stage I of the 2008 MacDiarmid Young Scientist of the Year Awards (May 2008) (Appendix A), successfully leading to entry into Stage II (communications entries: poster (Appendix A) and short film), with the poster being displayed at the awards ceremony (Auckland, 14th August 2008).

CHAPTER FIVE

MANUKA HONEY ANTIMICROBIAL ACTIVITY: Contributions by Potential Active Factors

5.1 Introduction

The most suitable antimicrobial functional food ingredient as defined by the screening and synergy assays (Chapter 3) was manuka honey. Honey has long been used as a traditional medicine in a number of roles, including wound healing (Lusby et al., 2002; Molan, 1998) due to its antimicrobial properties (Molan, 1992; Molan, 2001). As discussed in Section 3.3.2, a number of these antimicrobial properties have previously been investigated: (i) hydrogen peroxide, (ii) osmotic effects due to the high sugar content, and (iii) the low pH value of the honey. In addition, manuka honey possesses a further contributing factor, the Unique Manuka Factor (UMF™), believed to be the reactive electrophile methylglyoxal (MGO) (Weigel et al., 2004; Adams et al., 2008; Mavric, 2008), produced in manuka honeys by the breakdown of manuka nectar dihydroxyacetone (DHA) (Adams et al., 2009). Finally, honeys are rich in phenolic compounds (Yao et al., 2003; Inoue, 2005a; Henriques et al., 2006; Bertoncelj et al., 2007; Weston et al., 1999), and investigation of the antimicrobial activity of these compounds has been performed but levels of activity are not thought to be sufficiently high to significantly contribute to the overall effect of the honey (Weston et al., 1999).

The purpose of the work described in this chapter was to investigate these predominant contributing factors to determine the extent of their contribution to the overall antimicrobial activity of the manuka honey observed in Chapter 3. This would enable directed enquiry into possible mechanisms of antimicrobial effect in the target organisms (Chapter 6). In the case of the antimicrobial factor MGO, the purpose was to confirm, supplement and extend the newly published information on the potential role of this toxic electrophile by examining it in the context of the other factors contributing to the antimicrobial properties of honey.

To investigate the predominant contributing factors to the antimicrobial activity of the manuka honey, factors were selectively eliminated (e.g. peroxide, MGO), compared to standard compounds (e.g. MGO, sugar content), or modified in order to establish their influence on other factors (e.g. pH). Whilst every effort has been taken to present information in this

chapter as a series of sections devoted to each contributing factor, such division is essentially artificial, and it was inevitable that information on some factors would be presented under the section devoted to a different factor due to the multiple overlapping, interacting, complementary and competing modes of action of factors in a complex mixture. The approach taken is summarised in Fig.5.1. The placement of Section 5.5 dealing with the effects of pH on the antimicrobial activity of the manuka honey is due to a focus on how pH modifies the contributions from the osmotic and MGO antimicrobial factors.

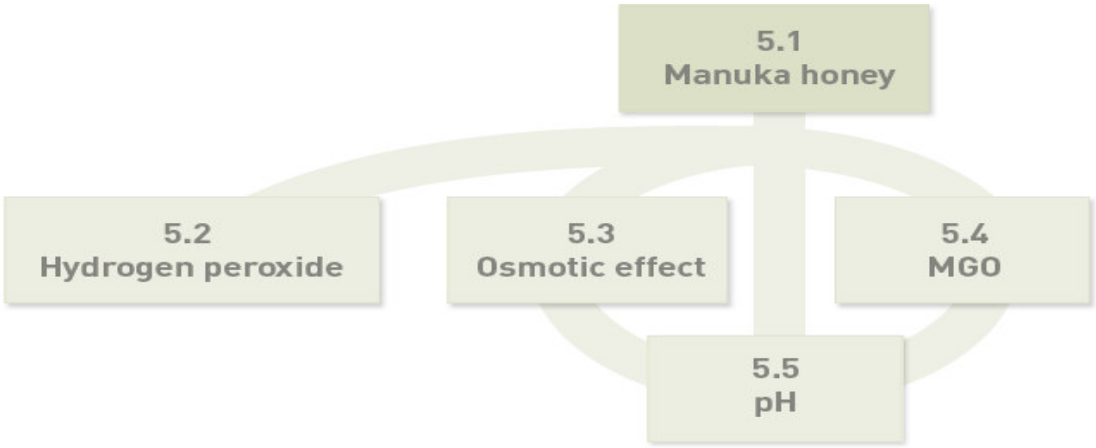


Figure 5.1. Organisational chart of this chapter.

Escherichia coli was selected as the test microorganism of choice, due to the extensive literature coverage of tests with the above antimicrobial factors, and the coping mechanisms used by *E. coli* in response to these factors. In many cases *E. coli* O157:H7 was tested in parallel with the non-pathogenic *E. coli* Nissle, to ensure that appropriate comparisons of responses could be used in those circumstances where the experimental techniques lacked the containment stringency that the use of *E. coli* O157 required, and it was determined prudent to use the less harmful Nissle strain. In support, the literature shows that *E. coli* O157 is likely to have the same growth rate as non-pathogenic strains, but not necessarily the same optimum growth conditions such as temperature, etc. (Gonthier et al., 2001).

5.2 Hydrogen peroxide.

5.2.1 Introduction

The antimicrobial compounds present in honey were initially termed 'inhibines' (White et al., 1963), light and heat-sensitive compounds that at that time were not chemically characterized. It soon became apparent that the most prominent inhibine was hydrogen peroxide, produced by the action of bee-derived glucose oxidases upon dilution of the honey in water.

Exogenous peroxide can inhibit *E. coli* growth at concentrations as low as 30 µM (Seaver and Imlay, 2001a), although 60 µM may constitute a sublethal dose (Mukhopadhyay and Schellhorn, 1997), and honeys are believed to be able to produce peroxide at up to 1-2 mM (Bang et al., 2003). Exogenous peroxide does not diffuse freely through membranes, but forms a concentration gradient with as much as an order of magnitude lower concentration on the inside of an *E. coli* membrane (Seaver and Imlay, 2001b). However, oxidative membrane damage, after an initial period of decreasing fluidity, can increase membrane fluidity, and increased fluidity increases the permeability of peroxide (Branco et al., 2004) . The *E. coli* catalases are located in the periplasm (Brunder et al., 1996) where they may scavenge the peroxide prior to it reaching the cytoplasm, effectively forming a 'defensive layer'.

Peroxide antimicrobial effect includes oxidative damage (strand breaks or aldehydic lesions) to DNA (Nakamura et al., 2003), peroxidation of lipids creating cell lesions and oxidation of thiols causing inactivation of enzymes with active site sulfhydryl residues (Seaver and Imlay, 2001b). In addition, peroxide contributes to creation of sulfoxide adducts of protein methionine residues (Moskovitz et al., 1995) and selective creation of protein carbonyls leading to problems with protein elongation and maintaining protein and DNA architecture (Dukan and Nystrom, 1999). Reaction of peroxide with intracellular iron creates hydroxy radicals which attack DNA leading to mutation (Seaver and Imlay, 2001a), and causes loss of Fe ions from iron-dependent enzymes (Halliwell et al., 2000).

Conventionally, the antimicrobial properties of honey are investigated through the use of well diffusion assays, where the extent of the inhibition zone is related to the concentration or efficacy of the antimicrobial ingredients, primarily peroxide. After pre-treatment with catalase, the inhibition zone was not observed for most honeys, confirming peroxide as the

primary antimicrobial compound. The concentration of honey solutions at which peroxide was shown to accumulate was 30-50% (w/v) (Bang et al., 2003).

However, some honeys, predominantly New Zealand manuka honey and some Australian *Leptospermum* honeys, have shown significant non-peroxide inhibition zones after catalase treatment. This non-peroxide inhibition was termed the Unique Manuka Factor (UMF™). It has been suggested that UMF™ was merely a consequence of insufficient catalase to remove all the residual peroxide present (Weston, 2000), but doubt was later cast on that theory (Snow and Manley-Harris, 2004), and subsequent data have proven it incorrect. Manuka honeys have been shown to possess sufficient antioxidant potential to scavenge any peroxide that may be produced (Inoue, 2005a; Henriques et al., 2006). In addition, honeys may possess plant-derived catalases (Weston, 2000). These observations strongly suggest that peroxide is unlikely to be one of the manuka honey factors exerting antimicrobial effects.

This section attempts to confirm this observation regarding hydrogen peroxide, and thus discount peroxide as a significant contributing antimicrobial factor in the growth inhibition observed in Chapter 3 with the manuka honey used in this work.

5.2.2 Methods

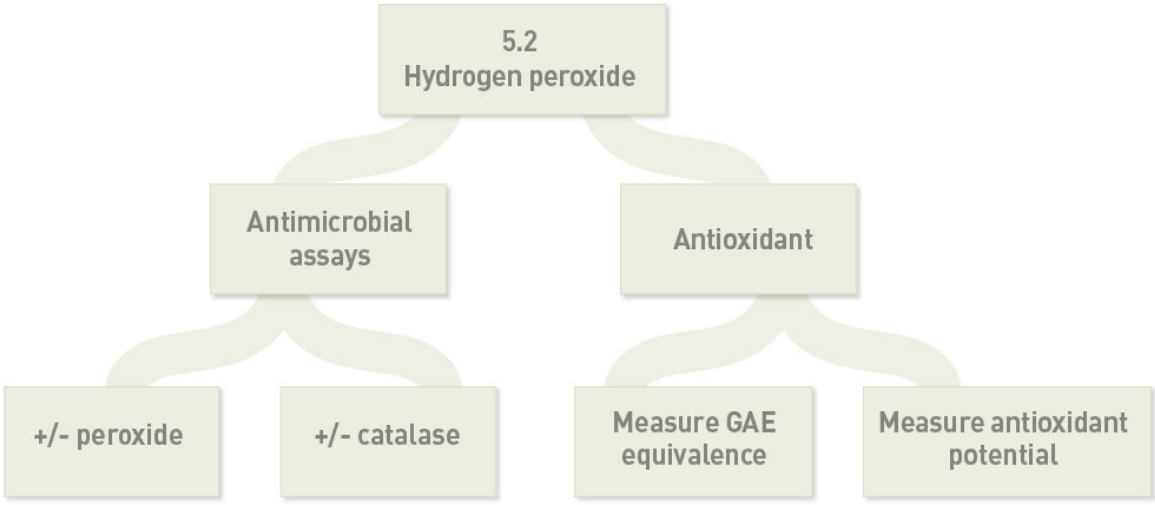


Figure 5.2. Summary (work flow) of the approach used to investigate role of hydrogen peroxide as a potential manuka honey antimicrobial factor.

The potential contribution of peroxide to the previously observed antimicrobial activity of the manuka honey was investigated (Fig. 5.2) using both the well diffusion and microassay

(broth) methods (Section 2.2.4.1), using a process of elimination. Catalase (500 U/mL) was added to the honey to remove any potential peroxide, which may be present, by adding the enzyme prior to the assay, in order to see if a resulting drop in antimicrobial activity was observed. In addition, manuka honey was spiked with a known amount (starting at 4 mM) of peroxide, (i) to take into account a simulation of the ability of peroxide to exert its effects in a higher honey concentration than used in this work, (ii) to display the expected antioxidant (Inoue, 2005a; Henriques et al., 2006) or potential native catalase (Weston, 2000) activity of the honey, and (iii) to control for the possibility of potential catalase inhibition by the honey. The well diffusion assay treatments, and controls are detailed in Table 5.2.1. Clover honey was used as a non-UMF™ control honey.

In addition (Fig. 5.2), manuka and control ("clover" (mixed floral)) honey samples were submitted to the Phytochemicals Research Team of The New Zealand Institute for Crop and Food Research Ltd, Lincoln, Christchurch, New Zealand, to measure their total phenolic content using the Folin assay (Folin and Ciocalteu, 1927, with modifications by Lister and Wilson, 2001, as described in Djeridane et al., 2006) as an indication that the manuka honey possessed potential antioxidant compounds.

Finally (Fig. 5.2), the manuka and control honeys were subjected to a Ferric Reducing Ability [of Plasma], or Ferric Reducing/Antioxidant Power (FRAP) assay (Benzie and Strain, 1996; Bertoncelj *et al.*, 2007) to measure "antioxidant potential" to determine whether the honeys should be capable of destroying peroxide. This assay compares the ability of the samples to reduce ferric-tripyridyltriazine (Fe^{III} TPTZ) complex to the intensely blue ferrous (Fe^{II}) form at low pH with the reducing ability of the powerful antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), a water soluble Vitamin E analogue.

5.2.3 Results and discussion

The microassay results are displayed in Fig. 5.2.1. Antimicrobial well diffusion results are detailed on Table 5.2.1. .

In the microassay, both manuka and the control honey displayed dose-dependent antimicrobial activity. Manuka honey displayed greater inhibition than the control honey at the top microassay doses. Peroxide-only controls displayed an abrupt antimicrobial activity profile under the doses tested – it inhibited growth completely, until it was diluted below the level of effect, upon which it failed to inhibit growth at all. Addition of catalase completely abolished this peroxide activity. Addition of catalase did not affect antimicrobial activity of the

honey samples. Addition of hydrogen peroxide to the honey samples failed to show any antimicrobial activity. This indicated that catalase could abolish the antimicrobial activity of peroxide, but not the growth inhibition by honey, and that honey could also abolish the peroxide activity. The difference in degree of inhibition between the two *E. coli* strains over the honey concentrations 6.25 – 0.1 were interesting, and were further explored in subsequent sections of this chapter.

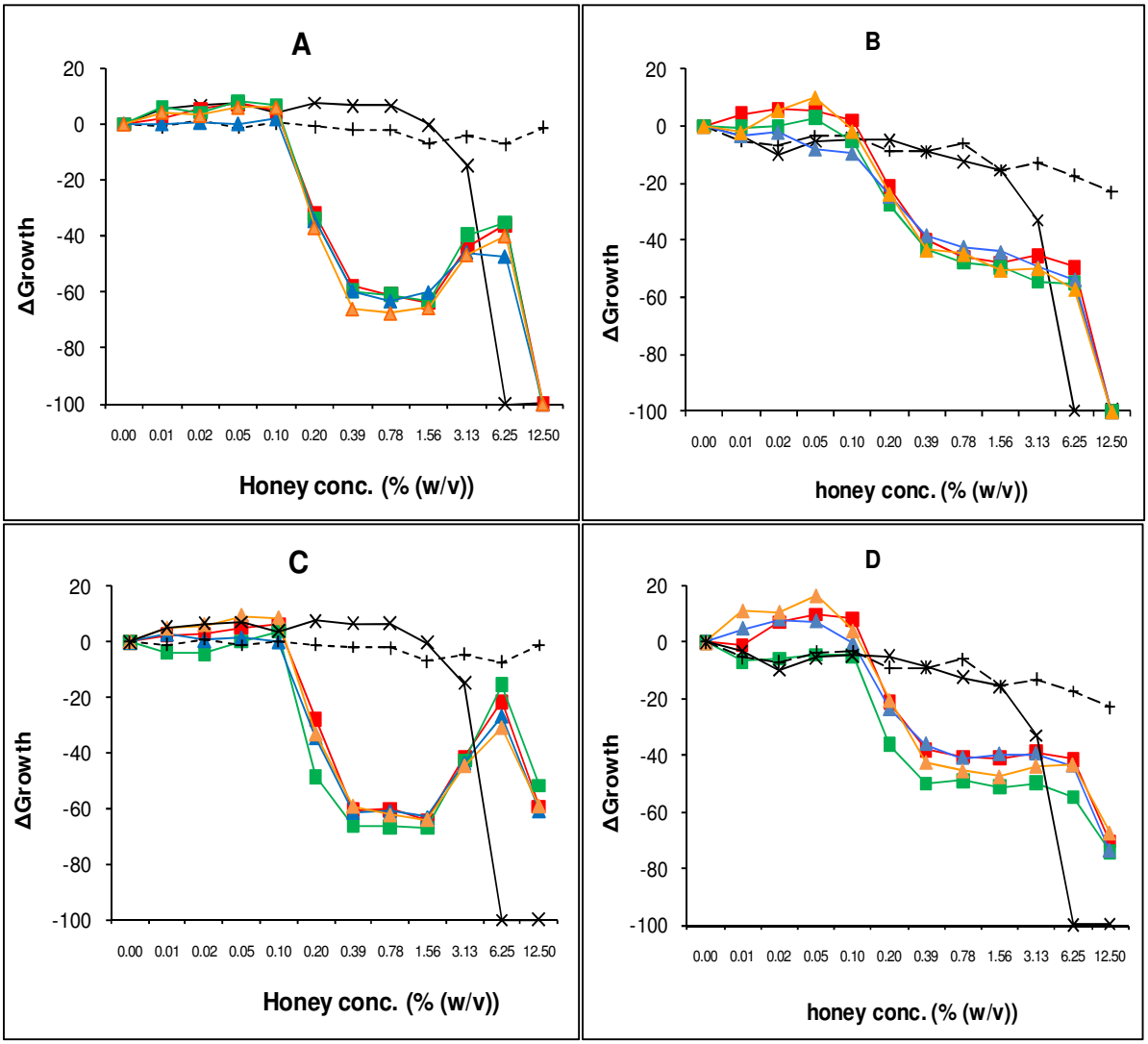


Figure 5.2.1 Microbial assay results measuring effects of manuka honey and clover honey with and without peroxide and catalase. Growth of *E. coli* strains O157:H7 (A, C) and Nissle (B, D) after 16 h incubation at 37℃ in presence of manuka honey (A, B) and clover control honey (C, D) with and without peroxide and/or catalase. Legend: Peroxide, (4.0 mM)(**x**); peroxide (4.0 mM) + catalase, (500 U/mL)(**+**); honey, (■); honey + peroxide, (4.0 mM)(▲); honey + catalase, (500 U/mL)(■); honey + peroxide (4.0 mM) + catalase, (500 U/mL)(▲). SEM as percentage of the mean ΔGrowth = 1.83 (range 3.33 – 0.012).

By using the well diffusion assay, the peroxide control had an inhibition zone of 3.3 mm (table 5.2.1). This inhibition zone was not observed in the presence of catalase (0.0 mm).

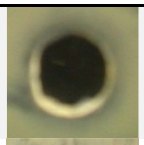
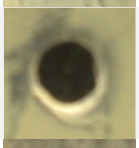
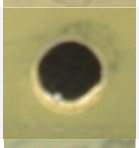
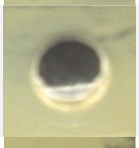
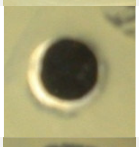
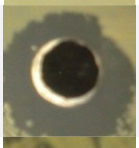
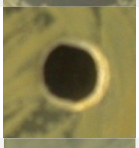
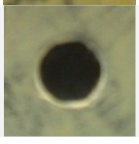
Manuka honey alone generated a 1.1 mm inhibition zone, clearly indicating antimicrobial activity. If the manuka honey did not scavenge the peroxide then an inhibition zone in excess of 3.3 mm would have been expected. However, manuka honey supplemented with the peroxide control generated only an 0.6 mm inhibition zone, clearly indicating loss of peroxide activity attributable to the honey.

Interestingly, manuka honey combined with catalase (1.0 mm) or peroxide and catalase (1.2 mm) was more antimicrobial than manuka honey and peroxide alone. Whilst this might suggest a possible manuka honey active factor being oxidised by the peroxide during the course of peroxide scavenging, no loss of activity was seen for this combination in the microplate assay (Figs. 5.2.1 A and B), suggesting that this phenomenon is unique to the agar diffusion assay.

Furthermore, the control clover honey did not display a clear zone of inhibition either in the presence or absence of catalase, and an insignificant amount (1.0 mm, relative to peroxide alone (3.3 mm)) in the presence of the added peroxide control. This shows that clover honey was also able to somewhat prevent peroxide-induced inhibition of growth. Given the similarity of clover honey antimicrobial dose-response profile with manuka honey at all but the highest doses of the microassay, these data also imply that, under the conditions used, the only observable antimicrobial activity that could be detected with this assay was that present in high manuka honey concentrations, of non-peroxide origin The clover honey did not possess this compound at sufficient concentration at the doses of honey used. This result highlights the UMF$^{(TM)}$ activity possessed by manuka honey.

These data collectively confirmed that the honey samples demonstrated prevention of peroxide inhibition of the bacteria. As stated in Section 5.2.1, this could potentially be attributed to catalases present in the honey, as some are purported to exist (Weston, 2000), the expected antioxidant potential of the honey by phenolic (Inoue, 2005a; Henriques et al., 2006), or other compounds, or by some other protective mechanism.

Table 5.2.1. Well diffusion assay comparing manuka honey and clover honey with addition of peroxide and/or catalase to examine possible contribution of peroxide to antimicrobial activity, as defined by the size of the inhibition zone in a lawn of *E. coli* Nissle.

Honey sample (30% (w/v))	Peroxide (mM)	Catalase (U/mL)	Mean inhibition zone (n=3) (mm) [1]	Well diffusion
Manuka	-	-	1.1	
Manuka	36.7	-	0.6	
Manuka	-	1000	1.0	
Manuka	36.7	1000	1.2	
Clover	-	-	0.0	
Clover	36.7	-	1.0	
Clover	-	1000	0.1	
Clover	36.7	1000	0.1	
-	36.7	-	3.3	
-		1000	0.0	
-	36.7	1000	0.0	

[1] Mean standard deviation 0.189 (range 0 – 0.577)

Manuka and control honeys were shown to have 841 and 656 Gallic Acid Equivalents (GAE) mg/kg, respective total phenolic content (Table 5.2.2), and FRAP activity of 0.804 and 0.701 mM Trolox equivalents, respectively (Table 5.2.3).

Table 5.2.2. Estimation of phenolic content of manuka and clover control honey in Gallic Acid Equivalents. Data are the mean of two determinations, each conducted in triplicate.

Sample	GAE (mg/kg)
Manuka honey	841.4 +/- 0.3
Clover (control) honey	656.2 +/- 17.9

These GAE results are significantly higher than observed for European honeys, which range from 45 +/- 15 (Acacia honey) to 241 +/- 40 (Fir honey)(Bertoncelj et al., 2007). Bertoncelj and coworkers showed a correlation between phenolic content, colour measurement and antioxidant potential, where darker, more phenolic-rich honeys displayed higher antioxidant activity in FRAP or 1,1-diphenyl-2-picrylhydrazyl (DPPH) antiradical assays. Interestingly, the comparatively low phenolic-containing light honey, acacia, has also been compared and shown to possess significantly less free radical scavenging ability relative to manuka honey (Inoue, 2005a). However, Inoue and coworkers also demonstrated that manuka honey predominantly scavenged superoxide rather than hydroxyl radicals, due to the presence of methyl syringate, which elsewhere has been shown to be in excess of 45% of the total phenolic content (Weston et al., 1999). Whilst methyl syringate has been postulated as a contributor to UMF™, this has been disputed by Weston et al. (1999) who claim that levels of methyl syringate are not particularly antimicrobial, that they are found in equivalent concentrations in non-active honey, and that the antimicrobial factor is associated with the carbohydrate fraction (Weston et al., 1999).

Furthermore, the high antioxidant ability of the control honey, nearly that of manuka honey observed during this work (Table 5.2.3), implies that neither honey was going to exhibit peroxide-derived antimicrobial activity, irrespective of the nature or potential antimicrobial activity of the antioxidant.

Table 5.2.3. Estimation of FRAP activity in 25% (w/v) manuka and clover control honey as millimolar Trolox Equivalents. Data are the mean of two replicates.

Sample	FRAP (Trolox mM Eq.)
Manuka honey	0.804 +/- 0.004
Clover (control) honey	0.701 +/- 0.002

In conclusion, with the assay systems and manuka honey samples and honey concentrations used during the course of this work, and in support of the literature, the observed antimicrobial activity of this manuka honey cannot be attributed to peroxide.

5.3 Osmotic effects.

5.3.1 Introduction

Honey is predominantly sugar, primarily fructose and supersaturated glucose (Zamora and Chirife, 2006) , as well as trehalose, isomaltose, kojibiose and turanose (Zamora and Chirife, 2006; Weston and Brocklebank, 1999, and others). Even when significantly diluted in water, the high sugar content results in a water activity (a_w) value below that which supports bacterial growth. This lowering of a_w is believed to be primarily due to the fructose and glucose, with a minor contribution by the disaccharides maltose and sucrose, and an insignificant contribution by the other sugars (Zamora and Chirife, 2006). Whilst a_w is primarily of importance for antimicrobial activity during application as a wound dressing rather than as a food in the gut, any contribution to the antimicrobial profile of the honey determined earlier in this work needed to be addressed.

A low a_w, or high osmotic potential, is thought to exert its effects by hyperosmotic shock. This involves a sudden efflux of water from the cytoplasm, concomitant decrease in cytoplasmic volume (plasmolysis) which leads to impairment of cellular functions such as nutrient uptake, DNA replication and an increase in ATP thought to be due to impairment of macromolecular synthesis (reviewed by Csonka, 1989). Such impairment of cellular function is attributed to orientation of water molecules into high density regions of disordered, reactive water close to charged moieties, surrounded in turn by low density, energetically unavailable ordered water (Wiggins, 1990). This orientation of water can impair chemical function, induce structural changes in protein and exert pressure of up to 1000 atm (Wiggins, 1990). Recovery from non-lethal hyperosmotic shock by the process of osmotic adaption occurs by increasing the cytoplasmic concentration of a limited number of solutes, such as K^+ ions and “compatible solutes” such as betaine, trehalose or glutathione, which lead to an influx of water and reinstatement of cytoplasmic volume. Charged moieties such as K^+ ions are balanced by raising the pH of the cytoplasmic medium to maintain membrane polarisation (Csonka, 1989), whilst the compatible solutes reorient extremes of water molecule structure towards normal (Wiggins, 1990).

Whilst much is known of the effect of osmotic shock on bacteria, and *E. coli* in particular, much of the work has been conducted with NaCl or sucrose, and information on the mechanism or response to glucose and/or fructose solutions, or indeed any humectant other than NaCl (Buchanan and Bagi, 1997) is scarce. As mentioned above, the osmotically active ingredients of honey are thought to be glucose and fructose, but mechanisms have not been investigated.

Finally, any contributions of the osmotic effects of honey to the dose-response profile obtained in Chapter 3 of this thesis need to be determined.

5.3.2 Methods

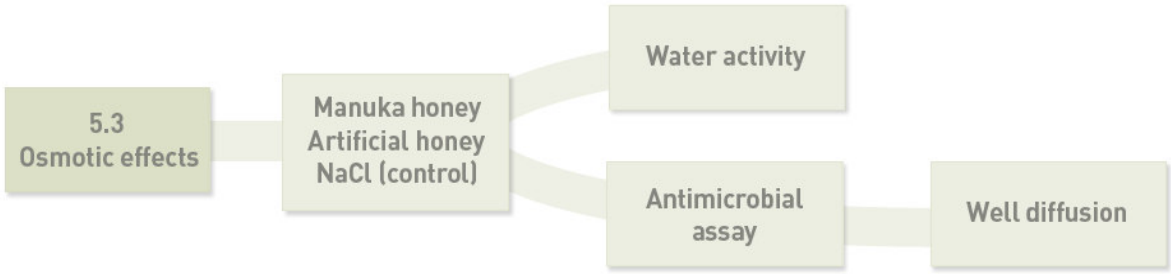


Figure 5.3 Summary of approach (work flow) used to measure the contribution of osmotically active factors to manuka honey antimicrobial activity.

The effect of adding the honey's high sugar concentration to the antimicrobial assays (Section 2.2.4.1) was examined (Fig. 5.3) by preparing an artificial honey solution. Fructose and glucose were made up to 4.0 g and 3.5 g per 20 mL deionised H_2O, respectively, equivalent to the dose found in a 50% (w/v) honey solution. The humectant properties of the honey and artificial honey solutions were calibrated by measuring a_w (section 2.2.4.6) at each dose used in the antimicrobial assay. Both antimicrobial effect and a_w were compared to NaCl, the most studied humectant in the literature.

The contributions of high concentrations of soluble, diffusible and potentially osmotically-active honey sugars were also examined by well diffusion assay (Section 2.2.4.1).

5.3.3 Results

5.3.3.1 Antimicrobial activity and a_w

The a_w and antimicrobial effects of the honey and artificial honey solutions over the normal two-fold serially diluted assay doses are presented in Fig. 5.3.1. The a_w values of the honey and artificial honey solutions were very similar, confirming the role played by fructose and glucose in determining the osmotic or water-withdrawing effects of honey (Zamora and Chirife, 2006).

Interestingly, the growth inhibition dose response profiles for honey and artificial honey were similar for all but the highest dose in the range of concentrations tested. This initially appeared to confirm the osmotic effect of the honey as a major contributor to the dose response profile observed with the honeys tested during the course of this work, as the a_w value below which the bacteria were inhibited corresponds to below the optimum a_w value for *E. coli* growth (0.995)(AQUA LAB series 3TE Water Activity Meter v3.4 Operators Manual version 1.5, 2000, Decagon Devices Inc., Pullman, WA).

However, the results obtained by growing the cells in the presence of NaCl do not correspond to those obtained with manuka honey or the artificial honey solution. At the highest dose, the growth of the culture supplemented with NaCl was comparable to the growth in the artificial honey solution, despite a much lower a_w (0.978 vs 0.983, respectively). As the a_w of the salt solution approached 0.995, increases in ΔOD values above the value of the unsupplemented control cultures were observed. This showed an increase in growth under challenging but non-lethal ("permissible") a_w values, until a "crash threshold" was reached, upon which marked inhibition was observed. In contrast, the honey and sugar solutions consistently inhibited growth at similar permissible a_w values. This suggests that either (i) the osmotic effects of the sugar-based solutions were of a significantly greater magnitude than osmotic effects of a salt-based solution, or (ii) the mechanisms of action of the sugar solutions could not be solely attributed to a_w.

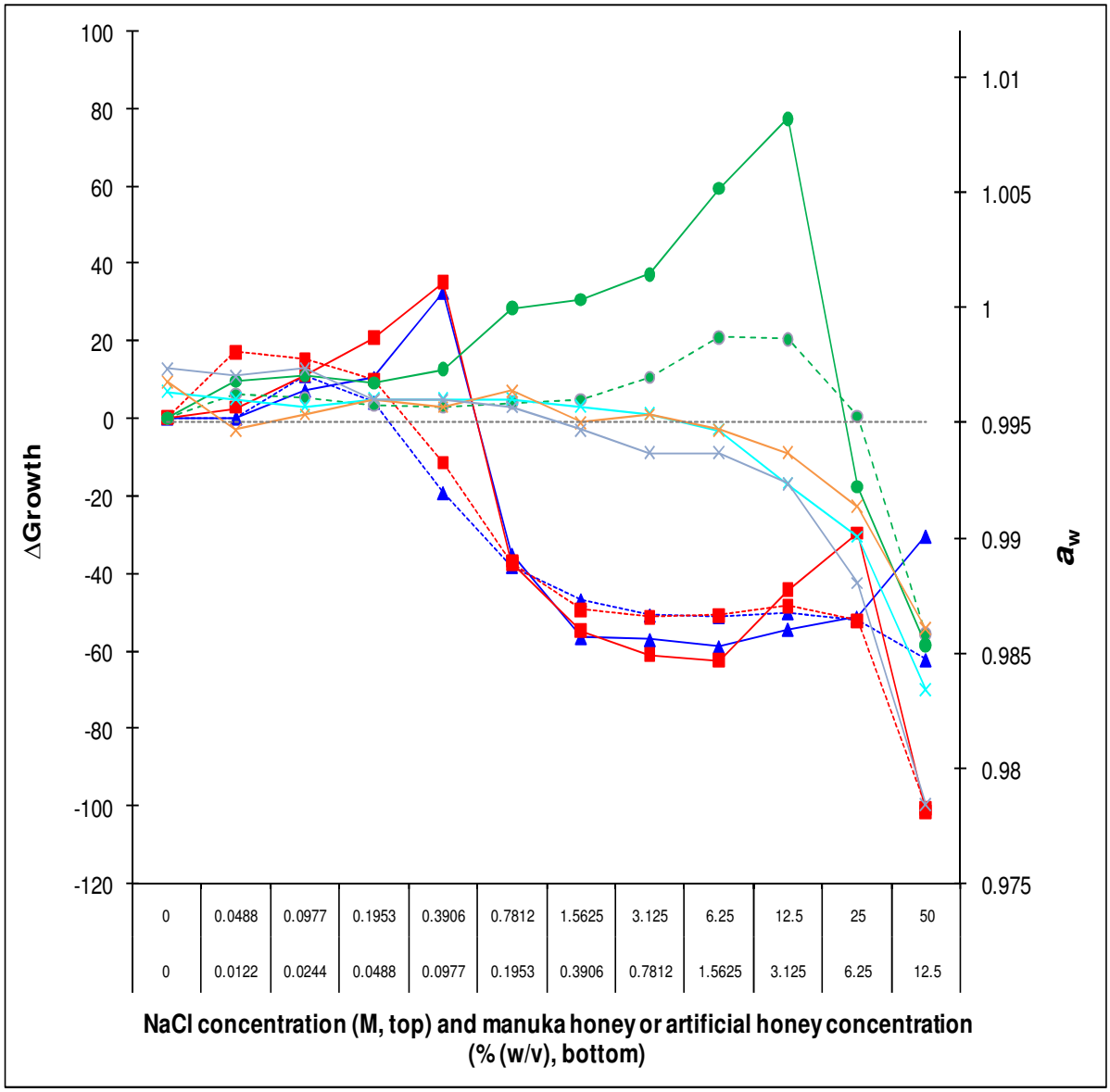


Figure 5.3.1. Water activity of manuka honey (x), artificial honey (x) and NaCl (x), antimicrobial activity of manuka honey (■), artificial honey (▲) and NaCl (●)against *E. coli* strains O157:H7 (solid line) and Nissle (dotted line). Horizontal dashed line demonstrates position of 100% (control) growth (0 ΔGrowth) and the theoretical optimum a_w for *E. coli* growth (a_w 0.995). Data mean of 4 or 3 determinations of *E. coli* growth or a_w, respectively, and representative of two experiments. LSD (P<0.05) as percentage of the means are as follows: *E. coli* O157 ΔGrowth, 18.58; *E. coli* Nissle ΔGrowth, 10.69; a_w, 0.119.

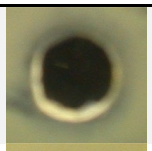
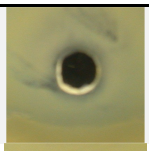
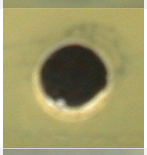
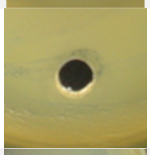
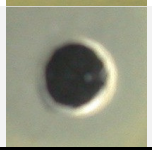
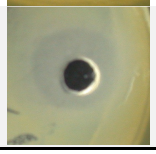
5.3.3.2 Well Diffusion

The results of the well diffusion assays (Table 5.3.1) displayed a diffuse zone of less dense growth, or halo of slightly translucent growth, present surrounding the wells of all honey and sugar samples, particularly prominent in the manuka and artificial honey samples. This zone corresponds to the partial inhibition observed over the first 6-7 dilutions of the microplate assays. In addition, manuka honey displayed a clear zone of no growth in the immediate

vicinity of the wells, suggesting a low concentration of highly antimicrobial compound of some type was present in that honey.

These results provide confirmation of the validity of the microassay data in comparison to the commercially accepted UMF™ well diffusion assay commonly used with *S. aureus* as the test organism.

Table 5.3.1. Well diffusion assay comparing manuka honey and clover honey with artificial honey (sugar solution) to examine possible contribution of sugars to antimicrobial activity, as defined by the size of the inhibition zone in a lawn of *E. coli* Nissle.

Honey sample (30% (w/v))	Mean inhibition zone (n=3) (mm)[1]	Well diffusion (close up)	Well diffusion (showing diffuse halo of growth)
Manuka	1.1		
Clover	0.6		
Artificial	1.0		

[1] Mean standard deviation 0.189 (range 0 – 0.577)

5.3.4. Discussion

The identity of humectants used to induce osmotic shock has not been observed to alter susceptibility to a_w except under multiple growth-limiting conditions (temperature, pH < 5.5)(Buchanan and Bagi, 1997). The conditions used (temperature optimal at 37°C, pH within the acceptable range for growth (Section 5.5)) were not growth- limiting under the range of doses where the inhibition was observed. Apart from the highest dose tested, it appears unlikely that other honey molecules at sub-lethal doses (including phenolics, sugars, methylglyoxal) acted to render the cultures more susceptible to the a_w due to the correlation between the manuka honey and artificial honey solutions in both inhibition and a_w values.

Increases in biomass concentration in the presence of osmotically challenging levels of NaCl have been reported in the literature. For example, Krist et al. (1998) used up to 2% NaCl (~0.5 M) and showed that the final biomass was greater in the presence of NaCl. However, the rate of growth was significantly lessened by amounts proportional to the reduction in a_w.

This illustrated a general effect of low but permissible a_w slowing growth rate but increasing final biomass concentration. In addition, the same researchers reported that significant inhibition of *E. coli* biomass concentration was only observed at an a_w "threshold", below which the organism would not grow (Krist et al., 1998). This observation supports the NaCl data above.

Interestingly, Krist et al. (1998) supplemented their NaCl-grown cultures with the compatible solute betaine (2 mM), and showed that the final biomass concentration was slightly lessened but the rate of growth was increased at the same a_w levels, and that the a_w 'crash threshold' below which the organisms failed to grow was significantly lowered. This illustrates the general effect of betaine effectively "raising" the a_w, and suggests that betaine could be used to see if it ameliorates the effects of the honey and sugar solutions, thereby reintroducing osmotic shock as a mechanism of action.

In addition, the *E. coli* cellular response to osmotic shock is not energetically demanding and does not result in lower conversion of glucose to biomass, just reduced growth rate (Krist et al., 1998), whilst the response to acidity is energy demanding (due to changing energy requirements and altered metabolic end products (ter Steeg et al., 1995)) and lowers biomass concentration (Krist et al., 1998). A time course of growth described in the literature by Shadbolt and co-workers (1999) described a biphasic response – an initial drop in growth rate and cell numbers due to osmotic shock, followed by survival of 'resistant' cells which grew at a slightly increased rate. These points are relevant here because the growth experiments described in this chapter consisted of fixed assay end points. Thus, the possibility that reduced biomass concentration merely reflected a decrease in growth rate needs to be explored.

Exploration of the effects of osmotically active solutes or humectants (NaCl, sucrose, ethanol, polyethylene glycol) on *Staphylococcus aureus* (Ballesteros et al., 1993) have shown that, where specific direct membrane-affecting solvent characteristics (eg. ethanol) could be excluded, humectants characteristics such as altered oxygen diffusion and solubility, media viscosity and changes in the dielectric constant were of less significance than a_w in preventing growth. Thus most humectants directly affect growth through high osmotic potential without impairing diffusion of vital media constituents.

The potential that the high glucose (or fructose) concentrations might have exerted an inhibitory metabolic or genetic regulatory effect to generate the observed inhibition must be considered. Despite the high correlation between a_w and honey/sugar inhibition, the NaCl

data suggests that osmotic effects are not the prime candidate for the inhibition observed. It is acknowledged that use of NaCl has introduced an extra variable, in that NaCl is readily ionised whilst the sugars are not. Thus, despite their mid-placing on the Hofmeister series, they are likely to make ion-macromolecule- or hydration shell-interactions rather than affecting bulk water structure (Zhang and Kremer, 2006). Further work with non-ionisable humectants such as polyols might shed further light on this a_w-inhibition relationship.

The decreased growth observed at the highest honey doses compared to the artificial honey solution was also interesting. Given the similarity of effect at the lower doses, it seems that this observed antimicrobial activity at the highest dose could not be attributed to the fructose/glucose present in honey. Thus one or more other factors must contribute to this antimicrobial activity. It is not known whether this might be due to a contribution from the other sugars, which were not thought to significantly contribute to honey a_w (Zamora and Chirife, 2006), but it is interesting that the greatest deviation in respective a_w values was observed at this high concentration. . The varying inhibition at the highest dose of artificial honey used suggests that the relationship between a_w and inhibition with sugars is not straight forward.

This high concentration of manuka honey contains MGO at doses reported to inhibit *E. coli* (Section 5.4). Hence, this factor needs to be addressed before drawing any conclusions regarding what it is in the manuka honey that effects bacterial growth at this concentration.

In conclusion, and irrespective of the actual mechanism of inhibition, the inhibition observed for all but the highest dose of honey tested corresponded to the concentration of glucose and fructose present, as determined by comparing a_w.

5.4 Methylglyoxal

5.4.1 Introduction

The antimicrobial effects of the honey at the highest doses tested cannot be attributed to osmotic affects (Section 5.3) or peroxide (Section 5.2). Thus the UMF™ remains a candidate. During the course of this work, scientists in Dresden, Germany, investigating the presence of 1,2-dicarbonyl compounds in foods turned their attention towards honey, where the high sugar content could be a source of these compounds. It was found that methylglyoxal (MGO) is present in high concentrations in honey, and in particularly high

concentrations in manuka honey (Weigel, 2004; Weigel et al., 2004) . The concentration of MGO in these honey samples was reported to correspond to the non-peroxide (UMF™) inhibition zones observed with the honey (Mavric, 2006; Mavric, 2008), and addition of MGO to non-UMF™ honeys was shown to provide a similar affect (Mavric, 2006; Mavric, 2008). Removal of MGO abolished the antimicrobial effect (Adams et al., 2008). These reports have been used as proof that MGO is the major contributing non-peroxide antimicrobial factor (the UMF™) in manuka honey.

However, the effect of manuka honey-derived MGO in liquid cultures has not yet been investigated. Furthermore, the reports of manuka honey-derived MGO antimicrobial activity have so far mostly been explored with the bacterium *Staphylococcus aureus*, the standard organism for UMF™ testing according to the Active Manuka Honey Association (AMHA, Hamilton, New Zealand, http://www.umf.org.nz/Unique-Manuka-Factor.cfm). In addition, it has been claimed that direct application of the chemical MGO to *S. aureus* cultures failed to exhibit the same degree of antimicrobial activity as manuka honey containing an equivalent level of MGO (R. Schlothauer, Comvita NZ Ltd, Te Puke, New Zealand, pers. comm). Whilst the effects of standard compounds and bacterial metabolically-produced MGO have been studied in *E. coli*, where mechanisms of MGO antimicrobial action and cellular coping mechanisms have been determined (Ferguson, 1999; Ferguson et al., 2000; Ferguson and Booth, 1998; Ferguson et al., 1996; Ferguson et al., 1998; Kalapos, 1999), the work described here is the first reported study of the impact of manuka honey-derived MGO on this organism.

MGO is also a product of metabolism. It is found in *E. coli*, for example, as a highly regulated product synthesised by MGO synthase when there is an excess of the glycolytic intermediates dihydroxyacetone phosphate and glyceraldehyde-3-phosphate as a result of accumulation of sugar phosphates in an imbalance of glycolysis events, involving high cAMP and a low phosphate pool (reviewed by Ferguson et al., 1998; Kalapos, 1999). The purpose for which it is thought to be synthesised is as a bypass regulator of glycolysis in nutrient-limiting environments, which have resulted in the aforementioned conditions (Kalapos, 1999). Under extreme conditions, bacteria may excrete up to millimolar quantities. Inhibition of *E. coli* occurs at 0.3 mM, and death at above 0.6 mM (Ferguson et al., 1998).

MGO binds macromolecules and inhibits replication and protein synthesis (Kalapos, 1999) by degrading nucleic acids (Ferguson et al., 2000; Kalapos, 1999). It forms Maillard reaction protein adducts (Kalapos, 1999), generates reactive oxygen or other anion radicals and causes lipid peroxidation (Kalapos, 1999). In addtition, MGO causes changes in intracellular

pH and K+ levels (Ferguson and Booth, 1998). It is also involved in the modification of the glycolysis pathways within which it plays an intimate role. MGO degrades nucleic acids by reacting with guanine and, to a lesser extent, cytosine and adenine (Ferguson et al., 1998), resulting in cleavage of DNA by the UvrA subunit of the DNA repair enzyme Uvr(A)BC exonuclease (Ferguson et al., 2000). MGO affects proteins by binding to and modifying the amino acid residues arginine, lysine and cysteine (Ferguson et al., 1998; Kalapos, 1999). The actual mechanism of cell death is unclear (Kalapos, 1999), but respiration appears not to be inhibited (Ferguson et al., 1998; Kalapos, 1999). Generation of radical species is thought to play only a minor role, and cell death has still been observed even when DNA degradation has been prevented (Ferguson et al., 2000). It seems likely that an accumulation of affects contributes to cell death. The effects of MGO are dose-dependent (Kalapos, 1999). One of the mechanisms *E. coli* uses to survive exposure to MGO is to lower the cytoplasmic pH with K+ efflux and concomitant proton influx. This mitigates the toxic effects of MGO by an unknown mechanism whilst it is being detoxified by the glyoxalase system (Ferguson, 1999; Ferguson et al., 2000; Ferguson and Booth, 1998; Ferguson et al., 1996).

The aim of this section initially was to measure the MGO content in the UMF™ 20+ manuka honey and compare it to other honeys of known UMF™ values and MGO content. This information would enable subsequent investigations to include MGO controls of appropriate concentrations.

First, the aim was to adapt an HPLC method that was initially developed for the routine measurement of MGO in cattle rumen (Lodge-Ivey et al., 2004) to measure MGO in honey.

The second aim was to measure the MGO concentration in a number of different honey samples which included: UMF™ 20+ manuka honey; two samples of manuka honey (UMF™ 16.1 and 18.1) (supplied by Comvita NZ Ltd.) with known MGO concentrations; and in clover honey which has no UMF™ activity.

Finally, the antimicrobial activities of the aforementioned honeys against *E. coli* O157:H7 were assayed and compared to (i) establish if there was a correlation between the MGO content and UMF™ antimicrobial indices, and (ii) to determine whether MGO was responsible for the increased antimicrobial activity previously observed with UMF™ 20+ manuka honey at the highest microbial assay doses.

5.4.2 Methods

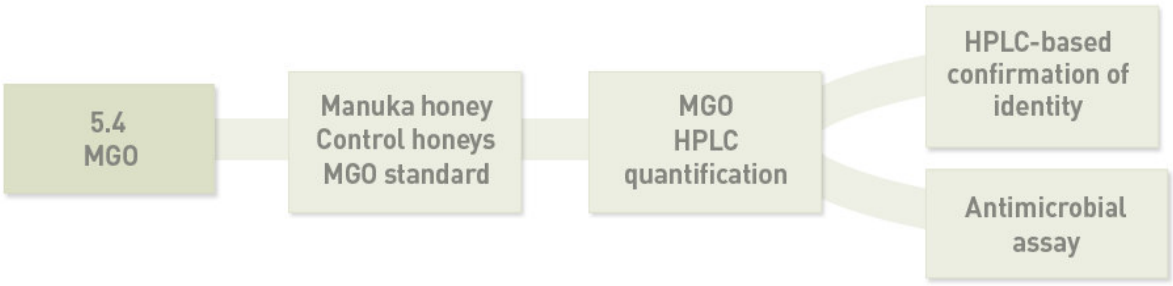


Figure 5.4. Summary of the approach (work flow) used to measure the contribution of MGO to manuka honey antimicrobial activity.

MGO was measured (Fig. 5.4) by derivatising the MGO in reaction with 6-hydroxy-2,4,5-triaminopyrimidine (TRI) to form a stable fluorescent derivative, 6-methylpterin (6-MPT) that can be separated and quantified by HPLC using a fluorescence detector (Section 2.2.4.4). Others have used HPLC protocols including conversion with *ortho*-phenylenediamine (OPD) to form a quinoxaline derivative (Mavric, 2006; Mavric, 2008; Weigel, 2004; Weigel et al., 2004), or by direct HPLC measurement using a refractive index detector (Adams et al., 2008). The TRI method used in this study was selected because (i) the available HPLC equipment was not equipped with a refractive index (RI) detector, and (ii) generation of a fluorescent product eliminates the enormous levels of background RI "noise" that was observed and attributed to the honey sugars (Adams et al., 2008), and (iii) was significantly faster than the OPD method, which has a 9 h incubation *versus* the TRI method with an incubation period of 45 min.

The quantification of MGO by this method was calibrated by including manuka honey samples of known MGO and UMF™ content (Comvita, Te Puke, New Zealand), the former being quantified using the alternative OPD derivatisation method. The samples and MGO values were kindly provided by Comvita NZ Ltd.

Antimicrobial assays (Fig. 5.4) against *E. coli* strain O157:H7 were conducting according to the methods outlined in Section 2.2.4.1.

5.4.3 Results

5.4.3.1 Measurement of MGO standard compound

A standard curve (Fig. 5.4.1) of varying amounts of derivatised MGO (0.1 – 0.4 µg/injection) was constructed, and the amount of MGO in samples was determined by comparing 6-MPT peak areas (mV.min). The retention time of 6-MPT was 10 min (Fig. 5.4.2 a). By varying the concentration of MGO derivatised it was possible to demonstrate that the amount present was proportional to the sample concentration (Fig. 5.4.1.) Derivatisation and HPLC of the MGO standard compound yielded consistent, reproducible quantities of 6-MPT, with a variation of less than 5% between replicate determinations.

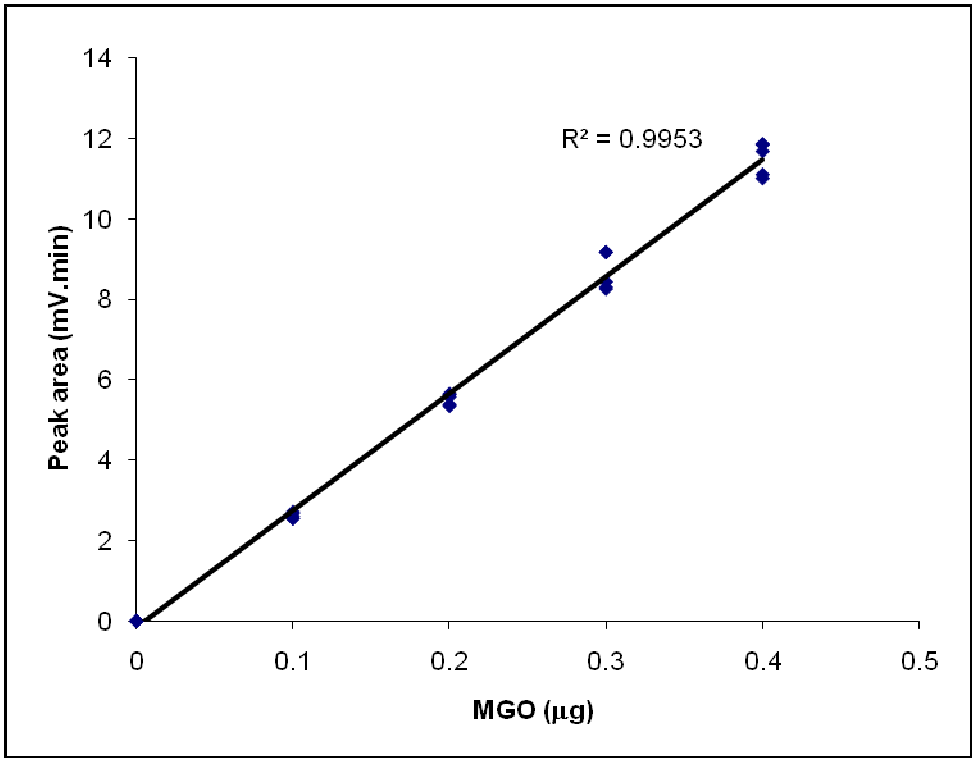


Figure 5.4.1. MGO standard curve, calculated by comparing the peak area (mV.min) of fluorescent 6-MPT to the amount of MGO derivatised (µg). Five data points per concentration were used, obtained from three separate HPLC determinations conducted on different days, with two of the three determinations consisting of duplicate injections. All data points shown.

Due to the unavailability of a 6-MPT standard, the 6-MPT peak was identified by running controls consisting of TRI alone (Fig. 5.4.2 d), and MGO without TRI (Fig. 5.4.2 e) that were heated at 60°C for 45 minutes as per the derivatisation procedure.

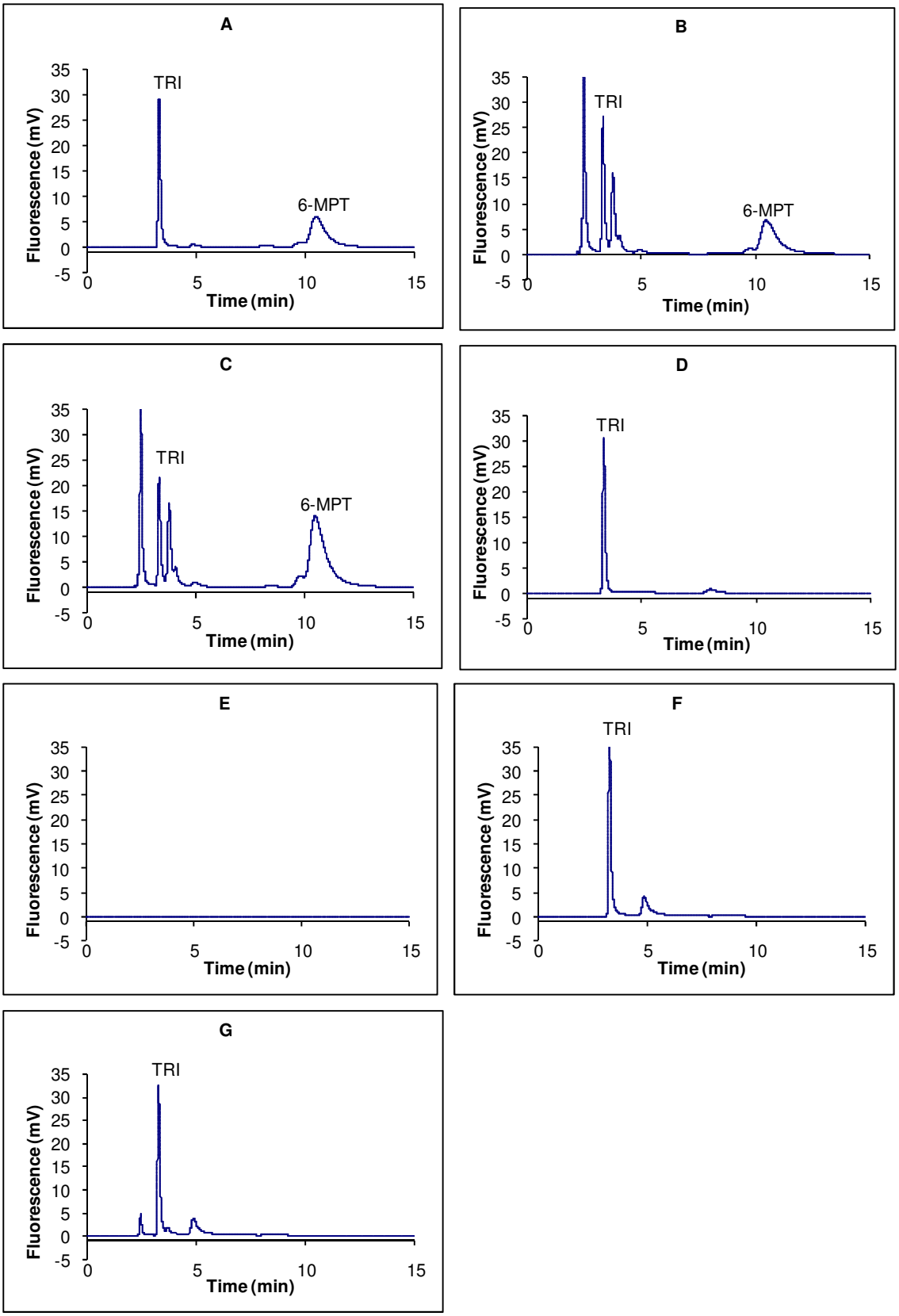


Figure 5.4.2. HPLC chromatograms of various MGO derivatisations. (a) 0.2 µg derivatised MGO; (b) 0.4 % (w/v) derivatised UMF™ 20+ manuka honey; (c) 0.4 % (w/v) derivatised UMF™ 20+ manuka honey plus 0.2 µg derivatised MGO; (d) TRI alone; (e) MGO alone; (f) 0.2 µg pH13-treated derivatised MGO; (g) 0.4 % (w/v) pH13-treated derivatised UMF™ 20+ manuka honey.

Further confirmation of peak identity was achieved by selective removal of the 6-MPT. A standard MGO solution (0.02 mg/mL) was given a 5 min exposure to a pH of 11, 12 or 13 by adjusting the pH with 0.25 M NaOH to convert the MGO to lactate. This solution was then returned to the buffered pH of 4.05 using 0.5 M HCl, and diluted to normal derivatisation concentrations to undergo the derivatisation process. The reduction in the resulting 6-MPT peak areas was proportional to increase in pretreatment pH (Table 5.4.2), consistent with conversion of the MGO to lactate (Friedemann, 1927). Complete loss of MGO was observed at pH 13 (Fig. 5.4.2 f).

The TRI-only control HPLC trace revealed the presence of a major TRI-derived peak, which eluted at 3.2 minutes, completely distinct from the 6-MPT peak area. Comparison of the TRI-only run with the standard compound further confirmed that the peak at 10 min was 6-MPT, consistent with reduction in TRI peak area upon increase in 6-MPT peak area (Figs. 5.4.2 b and c), and reinforced by the absence of the 6-MPT peak upon alkaline treatment of the standard MGO to yield lactate (Fig. 5.4.2 f).

5.4.3.2 Measurement of MGO in manuka honey

A 6-MPT peak was found in all honey samples (for example Fig.5.4.2 b, manuka honey UMF™ 20+) and Table 5.4.1 displays a summary of the MGO content of the different honey types used in this work. The concentration of MGO in the UMF™ 20+ manuka honey was 609 mg/kg, whilst the other honeys, UMF™ 16.1 and 18.1 had MGO contents of 616 and 484 mg/kg, respectively, and the non-UMF™ clover honey had a much lower (167.7 mg/kg) MGO concentration (Table 5.4.1). The MGO concentrations of the UMF™ 16.1 and 18.1 honeys were both within 6% of the MGO concentration previously reported (Wright and Eyskens, Comvita NZ Ltd., pers.comm, 2007) who used an alternative derivatisation process based on OPD (Weigel et al., 2004). Interestingly, the MGO concentrations of the UMF™ honeys did not exactly correlate with their UMF™ value.

The HPLC analysis of derivatised honey yielded two other major peaks which eluted at 2.3 and 3.6 minutes, which were not observed with the MGO standard compound. These additional fluorescing compounds were completely resolved from the 6-MPT peak, and did not interfere with the analysis. To further confirm the correct peak was identified in the honey samples, a 0.04 % (w/v) UMF™ 20+ manuka honey sample was given the same 5 min exposure as the MGO standard compound above (Section 5.4.3.1) to a pH of 11, 12 or 13 prior to the derivatisation process. The reduction in the resulting 6-MPT peak areas was proportional to increase in pre-treatment pH (Table 5.4.2), with the honey sample undergoing

greater loss (complete conversion to formate and acetate) than the MGO standard compound. This difference between honey and MGO standard solution is consistent with greater susceptibility of MGO to conversion by a combination of alkali and hydrogen peroxide (Friedemann, 1927), with the assumption that the latter was only likely to be present in the honey solutions. Complete loss of MGO was observed for samples at pH 13 (Fig. 5.4.2 g), the same result as achieved with the MGO standard (Fig. 5.4.2 f).

Table 5.4.1 MGO quantity in honey samples, including honey "spiked" with a known amount of MGO standard compound, measured according to the methods outlined in Section 2.2.4.4)

Sample	Peak Area (mV.min)	MGO (µg)	MGO (mg/kg honey)
UMF™ 20+ manuka honey (4.0 % (w/v))	6.12	0.22	609.8
UMF™ 20+ manuka honey (4.0 % (w/v))+MGO (0.2 µg)	12.84	0.44[(i)]	Not applicable
UMF™ 16.1 manuka honey (4.0 % (w/v))	6.20	0.22	615.8[(ii)]
UMF™ 18.1 manuka honey (4.0 % (w/v))	4.77	0.17	483.7[iii)]
Clover (mixed floral, non-UMF™) honey (12.5 % (w/v))	4.98	0.18	167.7

(i) Within 4.8% of the expected additive value of 0.42 µg MGO
(ii) Within 3.8% of Comvita's independently measured value of 593 mg/kg (M. Eyskens, pers.comm.)
(ii) Within 5.8% of Comvita's independently measured value of 457 mg/kg (M. Eyskens, pers.comm.)

Table 5.4.2 Effect of alkali treatment on recovery of the MGO standard compound and from manuka honey according to the methods outlined in Section 2.2.4.4.

Sample	pH treatment	Peak Area (mV.min)	MGO (ng)	Recovery (%)
MGO (0.2 µg)	11	2.8029	110.63	47.28
UMF™ 20+ manuka honey (0.04 % (w/v))	11	0.0043	0.17	0.64
MGO (0.2 µg)	12	0.9566	37.76	16.14
UMF™ 20+ manuka honey (0.04 % (w/v))	12	0.0003	0.01	0.04
MGO (0.2 µg)	13	0.0000	0.00	0.00
UMF™ 20+ manuka honey (0.04 % (w/v))	13	0.0000	0.00	0.00

To eliminate the possibility that honey compounds could interfere with the reaction, sequester MGO, or use MGO in the formation of the non-6-MPT peaks mentioned above, MGO recovery from the honey solution was tested by spiking UMF™ 20+ manuka honey with a known amount (0.2 µg) of MGO (Fig. 5.4.2 c). The MGO was fully recovered (Table 5.4.1), indicating that no significant interference, or loss of MGO, occurred during the derivatisation process.

5.4.3.3 Antimicrobial activity of MGO and honey

The antimicrobial assay results are presented in Fig. 5.4.3. The manuka honeys UMF™ 20+, 16.1 and 18.1 all caused complete inhibition of growth at 12.5% (w/v), the highest concentration used. The MGO standard at 1.0 mM caused 90% inhibition of growth. The non-UMF™ clover honey caused 50% inhibition of growth at 12.5 % (w/v).

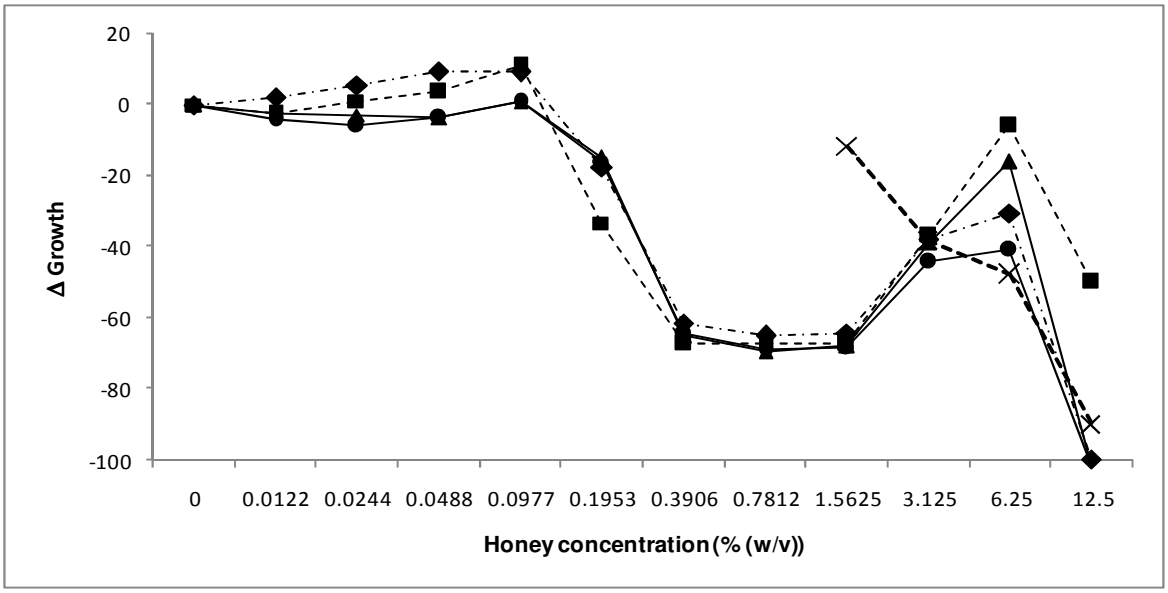


Figure 5.4.3. Antimicrobial dose response profile of honeys and MGO standard compound (n=4) against *E. coli* O157:H7 after 16 h incubation at 37°C, using the standard microassay protocol (Section 2.2.4.1). Manuka honey UMF™ 20+ (▲, solid line), UMF™ 16.1 (◆, dashed line), UMF™ 18.1 (●, solid line), clover honey (■, dashed line) and MGO (x, heavy dashed line). MGO concentrations 1.0 mM, 0.5 mM, 0.25 mM and 0.125 mM positioned at 12.5, 6.25, 3.125 and 1.5625 % (w/v) honeys, respectively, approximating the MGO concentration of the UMF honeys. The LSD (P<0.05) as a percentage of the total mean *E. coli* O157 ΔGrowth for all honeys and MGO standard compound was 6.86.

The UMF™ 20+ manuka honey solution with an MGO concentration of 609 mg/kg equates to 1.05 mM at 12.5% (w/v) honey in solution, a concentration demonstrated to prevent growth of *E. coli* O157:H7, and exceeding the published lethal MGO dose of 0.6 mM (Ferguson et

al., 1996). The 12.5% (w/v) solutions of the UMF™ 16.1 and 18.1 manuka honeys, equating to 1.07 mM and 0.94 mM, respectively, also exceed the published lethal dose for *E. coli*. Interestingly, the clover honey with 168 mg/kg MGO, equal to 0.3 mM MGO at 12.5% (w/v), below the concentration required to kill *E. coli*, but sufficient to inhibit growth (Ferguson et al., 1996), was only observed in this assay to inhibit growth without apparently eliminating it.

The MGO standard compound significantly inhibited, but did not completely inhibit the growth of *E. coli* at 1.0 mM, in contrast to the results of Ferguson and coworkers (1996) where the lethal dose was 0.6 mM. Preliminary work with MGO at 1.5 and 2.0 mM showed 100% inhibition of bacterial growth (data not shown). However, the complete inhibition by the UMF™ honey samples with MGO at the reported lethal dose may indicate honey factors generate some sort of synergistic or additive effect. A subsequent experiment extending the dilution series has confirmed that MGO at honey-equivalent concentrations does not fully inhibit either *E. coli* O157 or Nissle strains (Fig. 5.4.4). However, according to Fig 5.4.3., it does appear to inhibit slightly more at the equivalent of a 6.25% (w/v) honey solution, although the significance of this is unknown.

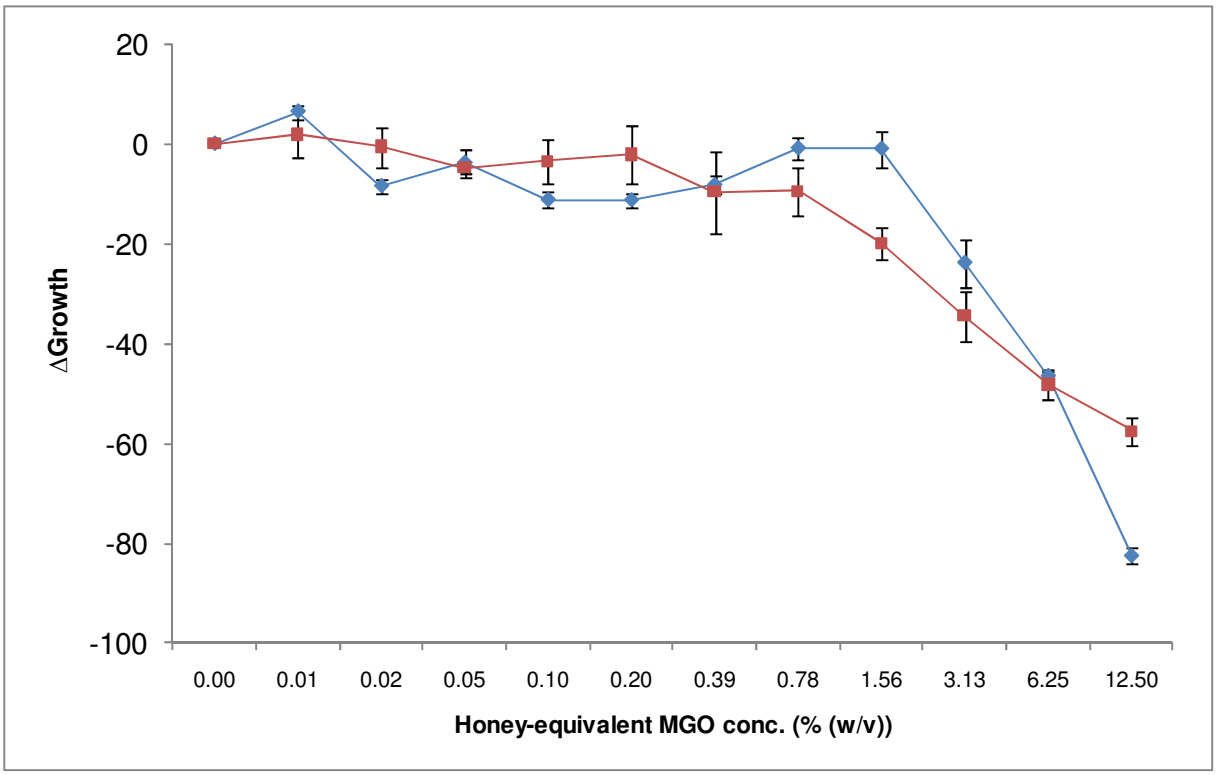


Figure 5.4.4. Antimicrobial dose-response profile of MGO standard compound (n=4) against *E. coli* O157 (◆) and *E. coli* Nissle (■) strains, after 16 h incubation at 37℃ using the standard microassay (Section 2.2.4.1). MGO solutions at equivalent manuka honey concentrations (1 mM = 12.5% (w/v) manuka honey UMF™ 20+). SEM shown.

5.4.4 Discussion

The TRI-based method of MGO derivatisation and HPLC detection was shown to be robust and accurate. Nemet et al. (2006) claimed that commercial MGO was impure and advised further purification, and specified that biological samples needed to be deproteinised to maximise availability of the MGO. They also claimed that the formation of other products from sample compounds might occur. The work presented here demonstrated that a single major MGO-derived peak was observed, and spiking of the honey samples (shown in Chapter two to contain protein) revealed that recovery was acceptable. Although additional derivatisation products were observed, they were clearly separate from the MGO-derived peak, and recovery of MGO was within acceptable limits. Presumably the quantity of MGO measured during the course of this work, which was in the mg/kg range, was so much higher than usual biological quantities (picomoles per gram (Nemet et al., 2006)) that impurities in the standard compound and minor losses by protein binding in the sample were insignificant.

The UMF™ manuka honeys were shown to have high levels of MGO, whilst non-UMF™ clover honey was shown to have a lower concentration, consistent with previously published observations (Mavric et al., 2008). The supplied UMF™ values supplied by Comvita for 20+, 16.1 and 18.1 (M. Eyskens, pers. comm.) do not exactly correlate to their MGO content (609, 616 and 484 mg/kg, or 1.05, 1.07 and 0.95 mM assay concentrations (with 25% (w/v) honey solutions), respectively). Similarly, the UMF™ 20+ honey has a similar MGO concentration to the UMF™ 16.1 manuka honey designated by the supplier as possessing an apparent lower antimicrobial activity (609 mg/kg versus 616 mg/kg, respectively, for honeys apparently displaying inhibition equivalent to >20 % (v/v) and 16.1% (v/v) phenol solutions, respectively). Differences in UMF™ values and observed activities with the assay reported in this thesis may be due to the different assay systems, as one is conducted in broth culture, whilst the other relies upon diffusion of antimicrobial compound through agar seeded with a lawn of bacteria. Alternatively, and highly likely, the differences may be attributed to different susceptibilities of the respective test organisms (*E. coli* versus *S. aureus* in the UMF™ assay). Further work examining the threshold concentration required for the UMF™ manuka honeys to kill *E. coli,* using intermediate doses between 12.5% and 6.25% (w/v), would be required to be able to determine if the MGO concentration measured in the honey samples corresponded to their UMF™ numbers.

The UMF™ manuka honeys were all shown to possess sufficient antimicrobial activity to prevent growth of *E. coli*, as befits the high levels of MGO measured. However, further work would be required to establish why 100% cell death was not observed with MGO standard

compound at an equivalent concentration, given that the MGO concentration is over the threshold that Ferguson and coworkers (1996) claim is sufficient to kill *E. coli*.

It is interesting that the decreased inhibition of *E. coli* O157 (not *E. coli* Nissle) in the presence of honey samples observed consistently throughout this work, at the concentration of 6.25% (w/v), has been shown to not be attributable to the honey sugars glucose and fructose (Section 5.3, Fig. 5.3.1). The honey at this concentration allows higher growth than should be observed at the apparent concentration of MGO in the manuka honey samples (Fig. 5.4.3). *E. coli* Nissle appears to suffer the same degree of inhibition from the MGO standard solution at this dose, indicating that O157 is perhaps responding to some manuka honey component that mitigates the inhibition to a degree, in a manner that the Nissle strain is unable to duplicate. This phenomenon is unusual and the mechanism unexplained.

Collectively these data have shown that the MGO concentration in manuka honey is comparable to the UMF™ value (although does not correlate exactly), is present at concentrations apparently sufficient to account for the increased inhibition observed at the highest assay doses, but is not solely responsible for the degree of inhibition observed.

5.5 Acidity

5.5.1 Introduction

Honey solutions contain numerous aryl-aliphatic and aromatic carboxylic acids. These include hydroxyl and methoxy derivatives of benzoic and cinnamic acids (Dimitrova et al., 2007), and minor components which include malic, maleic, citric, fumaric and succinic acids (Suarez-Luque et al., 2002). Composition and concentration of components vary considerably amongst honeys, and contribute to the sensory aspects (Anklam, 1998). These components also render the honey to pH 4-5, an obvious candidate antimicrobial factor. One of the effects of acid stress is that it increases the energy requirements of the cell, resulting in lowered growth rate and final biomass concentration (Krist et al., 1998). Growth rate and biomass concentration are not necessarily linked. A lower growth rate due to temperature or a_w may still result in the same final biomass concentration, as energy requirements of the cell have not been altered. In contrast, the energy requirements of the cell due to acidic conditions are more demanding to cope with, and observed reductions in growth rate will correlate with reductions in final biomass concentration (Krist et al., 1998). Thus, if honey

solutions are acidic, bacteria would be expected to have a lower growth rate and final biomass concentration.

However, the acidity of honeys has been reported not to be associated with inhibitory activity (Garcia et al., 2001). Nevertheless, this would need to be confirmed in this study. The effect of pH upon bacterial growth in culture would also need to be examined. In addition, whilst the growth inhibition observed in this work could be related to the sugar and MGO present in the honey, the degree of inhibition by standard solutions of these factors did not exactly match that observed with the manuka honey. Whilst the influence of other factors (e.g. phenolics) could not be studied, the degree to which pH influenced the activity of these factors alone and in combination needed to be determined.

5.5.2 Methods

The ability of pH to influence microbial growth was investigated as shown in Fig. 5.5:

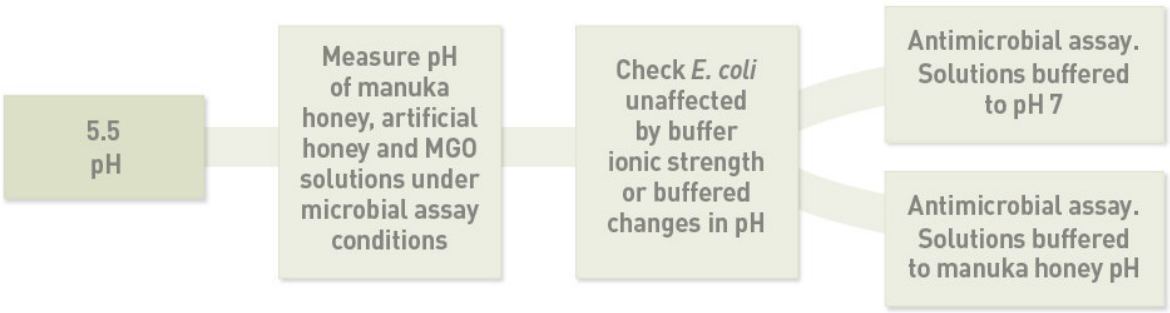


Figure 5.5. Summary of the approach used to measure the effect of pH on the antimicrobial activity of manuka honey and the contributing antimicrobial factors sugar (artificial honey) and MGO.

Firstly, the pH of the honey solutions in growth media across the assay dilution series was measured using a Shindengen ISFET KS701 pH meter, calibrated using the supplied standard, and operated according to the manufacturer's instructions. The pH of the honey was compared to an equivalent pH of artificial honey solution in order to eliminate pH as a factor contributing to the increased antimicrobial activity observed with manuka honey *versus* artificial honey at the top doses (Section 5.3).

Secondly, the *E. coli* strains were checked to ensure that growth over that pH range with a variety of buffer strengths would not be affected. This was carried out over a 16 h incubation period using the standard broth microassay method (Section 2.2.4.1) with a range of

Na_2HPO_4-NaH_2PO_4 buffer solutions (Dawson et al., 1986) substituted for samples (0-200 mM, resulting in 0-100 mM in the assay at pH 7.4, or 100 mM in the assay with a pH range of 5.8 – 7.4). This ensured that buffer of sufficient concentration to effectively modify the pH of solutions containing the strongly buffering honey and TSB would not detrimentally affect the growth of the organism. This buffer solution could then be used to standardise or control the pH of the honey and MGO solutions, to measure the contribution of pH to the antimicrobial activity at the highest concentrations.

Finally, the influence of pH on the activity of the methylglyoxal standard compound was examined and compared to the unbuffered manuka honey solutions, by assaying MGO at the same pH as measured for the equivalent honey concentration.

5.5.3 Results and discussion

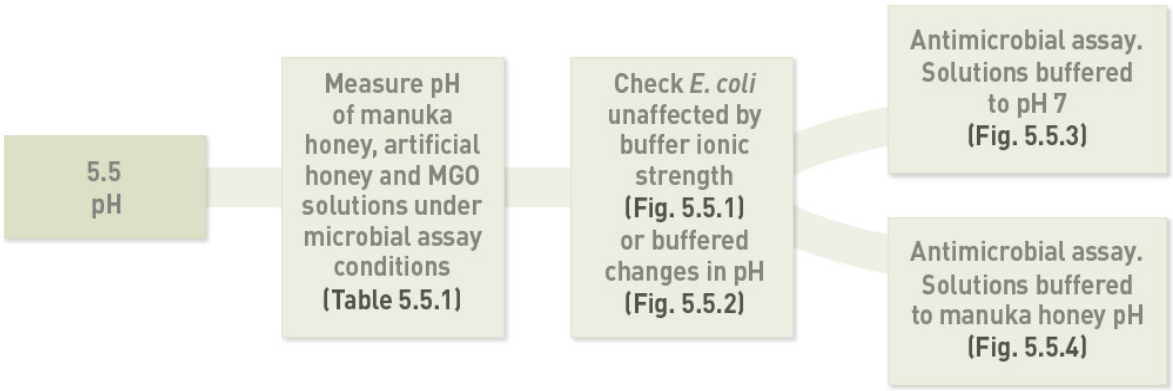


Figure 5.5.b. Summary of the results obtained during the measurement of the effect of pH on the antimicrobial activity of manuka honey and the contributing antimicrobial factors sugar (artificial honey) and MGO.

The pH values of the manuka honey, control honey and artificial honey solutions are presented in table 5.5.1. MGO solutions did not alter the pH of the growth medium (data not shown) from pH 7.4. Artificial honey only slightly dose-dependently decreased the pH in the presence of the buffering medium relative to manuka honey, and only at the four highest doses. Whilst manuka honey had a greater dose-dependent effect on the pH over the highest doses, the pH of the growth medium was still maintained at levels expected not to impact upon growth of *E. coli* (Buchanan and Bagi, 1997; Buchanan and Klawitter, 1992; Garcia et al., 2001). Manuka honey in water alone (12.5% (w/v)), not buffered by growth media, has a pH of 4.1 (measured in the same manner as above). The effect of pH on growth of *E. coli* O157 is thought to be of little importance between pH 5.5-7.5. (Buchanan

and Klawitter, 1992; Buchanan and Bagi, 1997), when under otherwise optimum growth conditions, but dropping the pH to 4.5 suppresses growth (Buchanan and Bagi, 1997).

Table 5.5.1. pH of manuka honey and artificial honey sugar solutions in TSB medium over the assay range of concentrations.

	Honey concentration (% (w/v))										
	12.50	**6.250**	**3.125**	**1.563**	**0.781**	**0.391**	**0.195**	**0.098**	**0.049**	**0.024**	**0.000**
Manuka honey	6.11	6.91	7.17	7.30	7.36	7.39	7.41	7.42	7.42	7.42	7.42
Artificial honey	7.10	7.25	7.31	7.35	7.38	7.39	7.41	7.41	7.42	7.42	7.42

The growth of *E. coli* O157 and Nissle strains on media containing sodium phosphate buffers of fixed (neutral) pH but varying concentration (ionic strength) are presented in Fig. 5.5.1, and of fixed ionic strength but with varying pH in Fig. 5.5.2. The growth of neither strain was detrimentally effected by the concentration of the buffer. *E. coli* O157 actually grew better in the presence of 25, 50 and 100 mM buffer. This indicated that buffered media could be used in subsequent experiments without risk of inhibition due to the concentration of buffer salts. The buffering effect of the medium is shown in Table 5.5.2, illustrating how the medium tends to be higher in pH, as observed with the manuka honey samples in Table 5.5.1.

Table 5.5.2. pH of 100 mM Na_2HPO_4-NaH_2PO_4 buffer solutions in TSB.

Buffer	**5.8**	**6.0**	**6.2**	**6.4**	**6.6**	**6.8**	**7.0**	**7.2**	**7.4**	**H_2O**
Buffered media (TSB)	6.22	6.32	6.43	6.56	6.76	6.91	7.11	7.29	7.45	7.48

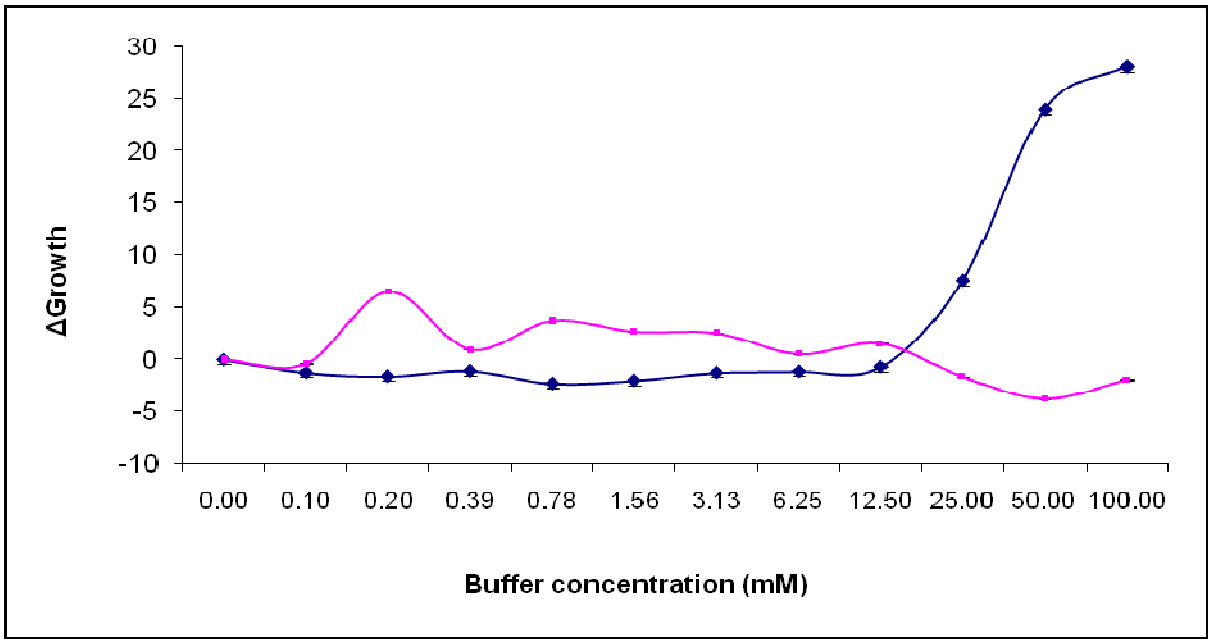


Figure 5.5.1. Growth (n=4) of *E. coli* O157 (◆) and Nissle (■) strains on Na_2HPO_4-NaH_2PO_4 buffer solutions pH 7.0, in TSB medium, over a range of buffer concentrations (0-100 mM), after 16 h incubation, performed using the standard microassay (Section 2.2.4.1). SEM shown.

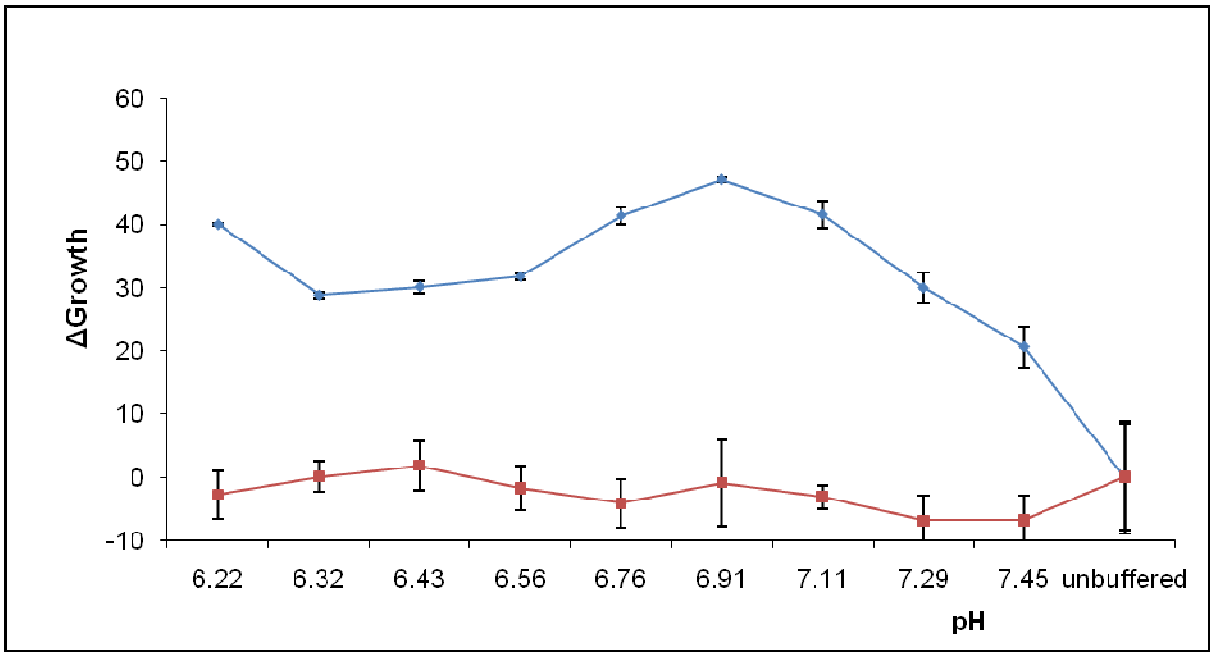


Figure 5.5.2. Growth (n=4) of *E. coli* O157 (◆) and Nissle (■) strains on Na_2HPO_4-NaH_2PO_4 buffer solutions (100 mM), further buffered by the TSB growth medium, over a range of pH (5.2-7.5), after 16 h incubation, performed using the standard microassay (Section 2.2.4.1). SEM shown.

The increased growth of *E. coli* O157 at different pH values is partially due to the buffer (100 mM) as observed in Fig. 5.5.2, although the peak at pH 6.9 may indicate a preferred growth condition of this organism. Interestingly, this pH is the same as that of the 6.25% (w/v)

dilution of the honey solution, which has consistently decreased inhibition (Figs 5.2.1, 5.3.1 and 5.4.3).

The increased growth of *E. coli* O157 at pH 6.2 may indicate that a more acidic pH was preferable, but lower pH values were not tested, as these would approach values below those experienced by the bacteria during the course of this thesis. These data confirm the claims of Buchanan and co-workers. (Buchanan and Bagi, 1997; Buchanan and Klawitter, 1992), and indicate that pH as a stand-alone factor should not have significantly inhibited the growth of the *E. coli* strains.

The antimicrobial activities of the honey solutions and MGO standard at buffered pH (7) are presented in Fig. 5.5.3.

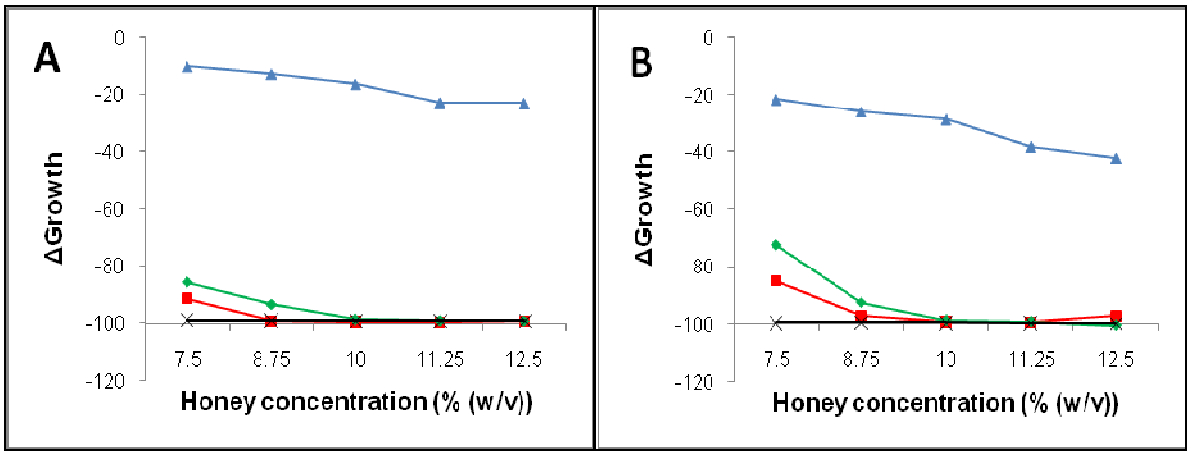


Figure 5.5.3. *E. coli* O157 (A) or Nissle (B) microassay showing Δgrowth in the presence of varying pH 7-controlled doses of manuka honey (■), artificial honey (▲), manuka-honey equivalent dose of MGO (♦), and artificial honey spiked with manuka honey-equivalent MGO (**x**). Control cultures had Δgrowth values of zero (not shown). SEM as percentage of the mean ΔGrowth = 1.5 (range 2.9 – 0.53).

The activity of the MGO and artificial honey, buffered to the pH of equivalent doses of manuka honey, are presented in Fig. 5.5.4.

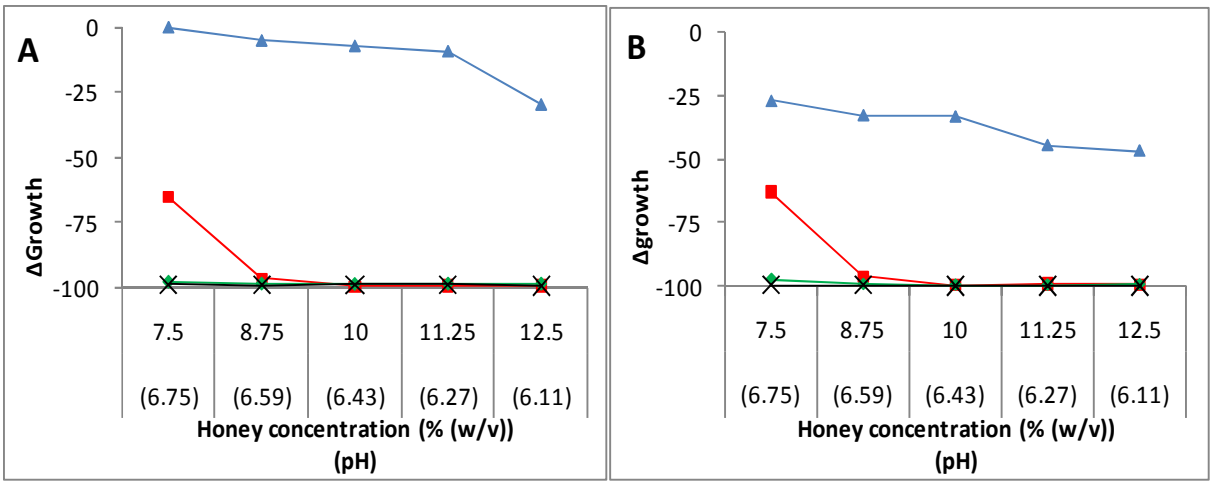


Figure 5.5.4. *E. coli* O157 (A) or Nissle (B) microassay showing Δgrowth in the presence of varying doses of manuka honey (■), and the following solutions all buffered to the same pH (also shown (abscissae)) as the manuka honey: artificial honey (▲), manuka-honey equivalent dose of MGO (♦), and artificial honey spiked with manuka honey-equivalent MGO (**x**). Control cultures had Δgrowth values of zero (not shown). SEM as percentage of the mean ΔGrowth = 1.5 (range 2.9 – 0.53).

There does not appear to be a difference in the threshold lethal dose for the bacterial population (LD_{100}) of the manuka honey at either the native (buffered by medium) pH (Fig. 5.5.4) or buffered to pH 7 (Fig. 5.5.3): inhibition appears to be complete at concentrations of 8.75 % (w/v) and above. However, the manuka honey does appear to display greater inhibition (lower Δgrowth value) when buffered at pH 7 than when unbuffered at the permissive (i.e. not completely inhibiting; allows growth) concentration of 7.5% (w/v). The previously observed phenomenon of increased *E. coli* O157 growth at pH 6.9 (Fig. 5.5.2), which also corresponds to a relative increase in Δgrowth value, or relative decrease of inhibition, at 6.25% (w/v) honey (Figs. 5.2.1, 5.3.1 and 5.4.3, which is pH 6.9 under assay conditions) may suggest that the bacterium is more tolerant of adverse conditions at this pH than at the higher pH 7.0.

Alone, when buffered to the pH of the manuka honey, the artificial honey sugar solution did not appear to display inhibition commensurate with a_w (cf. Fig 5.3.1) although the observed inhibition increased at pH 7 at the lower doses tested. Not only did the levels of inhibition fail to approach those obtained by the sugar solutions in the absence of buffer, but they also failed to match the inhibition obtained by the manuka honey shown in Section 5.3. However, given that both experiments involved buffering the sugar solutions using the same ionic strength buffer solution, in contrast to Fig. 5.5.1, these results suggest that the decreased inhibition may have been due to the presence of buffer salts/ionic strength.

The MGO solution showed greater inhibition when buffered to the pH of the manuka honey solution in media than when buffered to pH 7. At pH 7, this inhibition was still less than observed with the manuka honey at the same concentration. When buffered to the honey pH, MGO was more effective than when not buffered (Figs. 5.5.3 and 5.5.4), indicating that pH, or possibly sublethal stresses caused by ionic strength of the buffer, were able to modify the efficacy of this compound as an inhibitor of *E. coli*. At the more acidic honey-equivalent pH, MGO caused complete inhibition even at 7.5% (w/v) where the actual honey failed to completely inhibit growth. Subsequent experiments (data not shown) confirmed that increasing acidity (pH 6.0) increased inhibition from both manuka honey and MGO. In addition, in the presence of the artificial honey solution, the MGO-sugar solution caused complete inhibition under both pH conditions. Again, this was a greater effect than that obtained with the actual manuka honey at that same concentration.

Collectively, these data indicate that the direct effect of pH and ionic strength, as determined by sodium phosphate-buffering the growth medium, does not appear to inhibit the growth of *E. coli*, confirming previous reports (Buchanan and Bagi, 1997; Buchanan and Klawitter, 1992). However, using these factors in combination with manuka honey or manuka honey constituents (sugar and MGO) alters either the efficacy of these compounds, or the susceptibility of the bacteria.

5.6 Conclusions

The antimicrobial properties of honey have been previously attributed to hydrogen peroxide (White *et al*., 1963), osmotic effects and acidity (Molan, 2001; Molan, 1992), whilst the UMF™ activities of manuka honey has also been attributed to MGO (Adams et al., 2008; Mavric, 2008; Weigel et al., 2004).

The potential contribution of hydrogen peroxide to the antimicrobial activity of manuka honey observed with the assay used in this work was discounted (Section 5.2) due to a combination of (i) lack of prevention of antimicrobial activity by addition of catalase, (ii) the demonstration of the ability of the honeys to negate the antimicrobial effects of added peroxide at known antimicrobial concentrations, and (iii) the presence of high levels of antioxidant phenolic compounds.

The inhibition observed with the highest dose used in the microassays (12.5% (w/v)) was demonstrated to be compatible with the presence of MGO, although it did not precisely correspond with either UMF™ rating or MGO concentration. This inhibition was altered upon modification of the pH (and ionic strength), indicating that the activity is somewhat pH-dependent.

The remaining inhibition, at lower (0.19 – 6.25% (w/v)) honey concentrations, was demonstrated to be comparable to that of an artificial honey sugar solution, and suggests that a_w was a contributing factor.

These results indicate a complex network of interacting and overlapping effects which combine to create the antimicrobial activity of manuka honey (Fig. 5.6.1). Essentially, the combination of osmotic effects, pH, MGO and, where relevant, peroxide and phenolic compounds, combine (when their effective threshold concentrations are reached) to present a natural hurdle antimicrobial effect, with the MGO and osmotic effects predominating. The effects of some of these factors are investigated in more detail in the next chapter.

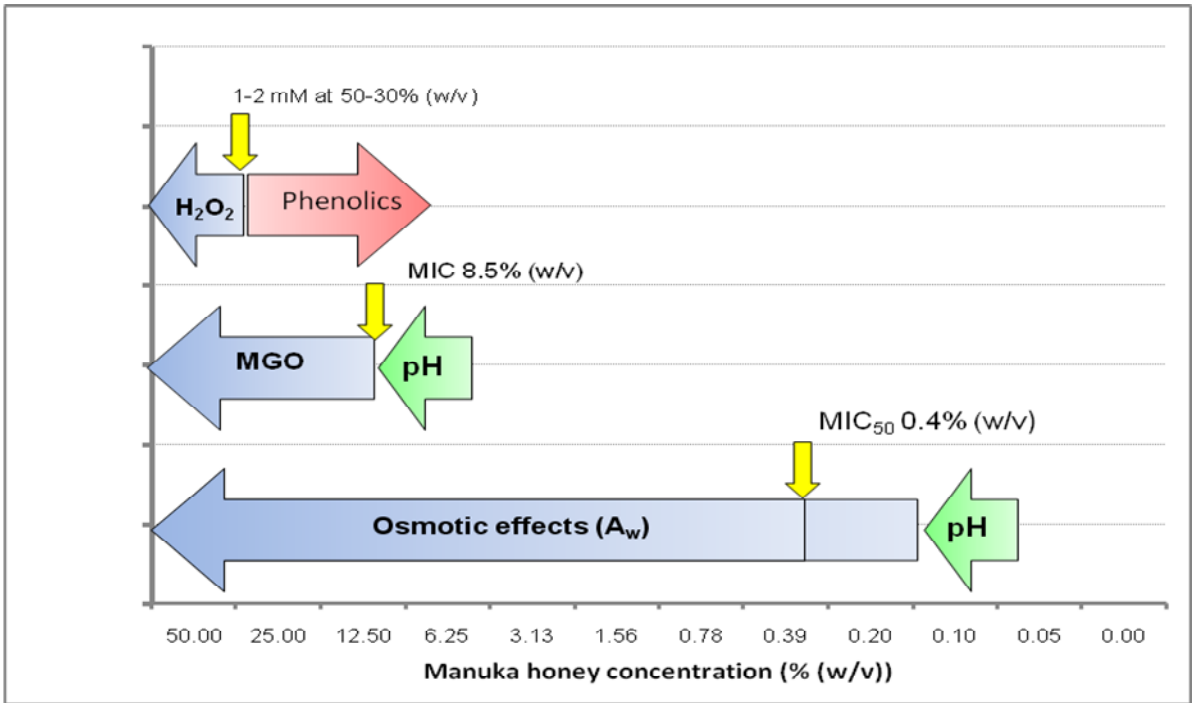


Figure 5.6.1. Proposed summary of degree of contribution of manuka honey antimicrobial components (blue arrows), with the direction of these arrows representing increased antimicrobial effect and also increasing concentrations.

Yellow downwards-pointing arrows mark, from top to bottom, the concentration of maximum production (hydrogen peroxide)(Bang et al., 2003); the minimum inhibitory concentration required to cause any significant inhibition (MICs)(MGO); and minimum dose required to cause 50% inhibition (MIC_{50})(a_w).

Factors which influence the efficacy of the antimicrobial components are shown: phenolics (red) and pH (green). The orientation of arrows representing these factors relative to the blue antimicrobial component arrows represents the direction in which they are proposed to influence the efficacy of the antimicrobial components. Essentially they drive the antimicrobial component effect towards that of a lower (phenolics vs peroxide) or higher (pH vs MGO or aw) apparent concentration, respectively.

Notes:

(1) unlike the position of the blue (antimicrobial component) arrows relative to the x-axis, the placement of the factor (red or green) arrows does not necessarily reflect either the value/concentration at that honey concentration or the change in value/concentration (increase or decrease) of the factor required to effect that change.

(2) Any potential interactions between the antimicrobial factors have not been illustrated.

(3) The values for hydrogen peroxide were drawn from claims in the literature (Bang et al., 2003), and have not been demonstrated to be effective.

(4) Any direct antimicrobial effects due to phenolic compounds have not been established, and thus are not illustrated.

CHAPTER SIX

MANUKA HONEY ANTIMICROBIAL ACTIVITY: Effects on rate and extent of *E. coli* growth

6.1 Introduction

The individual contributions of manuka honey antimicrobial factors osmotic potential, MGO and pH were described in Chapter 5, and comparisons to existing literature have been made. Some of this is summarised below:

Increase in osmotic potential (using NaCl) has been reported (Krist et al., 1998) to result in decreased nutrient uptake, decreased DNA replication and increased ATP accumulation, the latter thought to be due to impairment of macromolecular synthesis. Recovery is associated with increase in cytoplasmic levels of compatible solutes (betaine, trehalose, glutathione), increase in K+ and increase in pH commensurate with K+ in order to maintain membrane polarisation. High osmotic potential is not thought to result in increased energy demands for the bacterial cell. At permissive (non-lethal) concentrations of NaCl, decreases in growth rate but overall increase in final biomass concentration were reported (Krist et al., 1998). Addition of betaine resulted in a slightly increased growth rate and decreased biomass concentration relative to the presence of NaCl alone (Krist et al., 1998). These reports were supported by the observations in Chapter 5, where permissive NaCl a_w levels resulted in increased biomass, but once past the a_w threshold, NaCl inhibited biomass formation as measured by OD. However, the effects of the compatible solute betaine were not investigated, and growth rates were not determined. Furthermore, the inhibition caused by manuka honey and artificial honey solutions was greater at comparable a_w than that caused by NaCl, and significantly inhibited growth even at permissive a_w levels. This suggests that the mechanism of inhibition was not proportional to a_w (or at least not the same as NaCl), despite the corresponding a_w levels between manuka honey and artificial honey solutions. This indicates that the inhibition was by the osmotically active sugars.

Decreases in pH have been reported to result in increased cellular energy requirements, decreased growth rates and decreased biomass concentration (Krist et al., 1998; Shadbolt et al., 2001). However, the observations in Chapter 5 indicated that decreases in pH resulted in

greater inhibition by the manuka honey and MGO standard compound, whilst decreased inhibition by an artificial honey solution was observed. Furthermore, decreases in pH using phosphate buffer (without manuka or artificial honey) did not inhibit *E. coli* Nissle biomass accumulation (OD), and actually increased *E. coli* O157 biomass. Growth rates were not determined.

For these reasons, it was decided that the effect of the manuka honey antimicrobial factors would be investigated in greater detail, with growth rates being measured to accompany the biomass data, in order to determine if the bacterium is reacting to these factors together in the honey, and in isolation (controls), as expected from the literature.

As mentioned in section 5.1, *E. coli* Nissle was used in experiments which lacked the containment stringency required for the pathogenic *E. coli* O157:H7 strain.

6.2 Manuka honey osmotically active solutes

6.2.1 Introduction

In Chapter 5 the effects of osmotically active solutes or humectants on the growth of *E. coli* have been described. However, the design of the research described in Chapter 5 was based on literature focusing on the effects of NaCl (e.g. (Cayley et al., 2000; Krist et al., 1998)). Osmotically active sugars are scarcely mentioned in the literature, and when they are, the reports are usually confined to sucrose. Comparatively little information is available on the osmotic effects of glucose or fructose. Thus it is not known how glucose and fructose (or other honey sugars) affect the growth kinetics of *E. coli*. Previous growth experiments reported in this thesis have focused on end-point reactions, whereupon a specific time-of-growth (16 h) was used to compare the growth of honey-supplemented cultures to unsupplemented controls. In this chapter the growth kinetics of *E. coli* Nissle were investigated and compared to those described in the literature (Krist et al., 1998; Shadbolt et al., 2001). In addition, the effects of the osmoprotective compatible solute glycinebetaine (betaine) were examined, to establish whether it could lower the sugar-associated inhibitory effects of the honey.

6.2.2 Methods

The effects of manuka honey and the artificial honey sugar solution on *E. coli* growth were measured using a modified version of the assay outlined in Section 5.3.2. First, the effects of varying concentrations of the compatible solute betaine alone (0 - 100 mM) were examined to verify that they did not interfere with the growth of the *E. coli.* Then *E. coli* cultures were grown with honey solutions in the presence or absence of betaine and growth assessed by measuring changes in OD.

6.2.3 Results and discussion

Betaine alone did not change the growth of *E. coli* Nissle (data not shown). Further assays were then conducted with manuka honey and artificial honey-supplementation of the cultures in the presence and absence of betaine in the culture medium. No significant differences in antimicrobial activity were observed over the ranges of betaine concentrations (0.25 – 100.0 mM) used here (Fig. 6.2.1).

There was no difference in biomass concentration (OD) of cultures after 16 h growth in the presence of betaine with either manuka honey or artificial honey. However, the effects of compatible solutes such as betaine are not pronounced (Krist et al., 1998) at the relatively high but inhibitory a_w values (>0.98, Section 5.3, Fig. 5.3.1) experienced by the bacteria during the course of this study. Furthermore, the effects of adding compatible solutes are likely to be less readily observed when the organism is not under nutrient limiting conditions (Buchanan and Bagi, 1997), as more substrate is available for compatible solute synthesis. However, the osmoprotective ability of betaine is more manifest upon examination of growth rate than OD (Krist et al., 1998), so this would require investigation.

The growth rate of *E. coli* Nissle was measured in the presence of a range of concentrations of manuka honey, artificial honey, and the humectant control NaCl, in the presence and absence of betaine (2 mM)(Fig. 6.2.2).

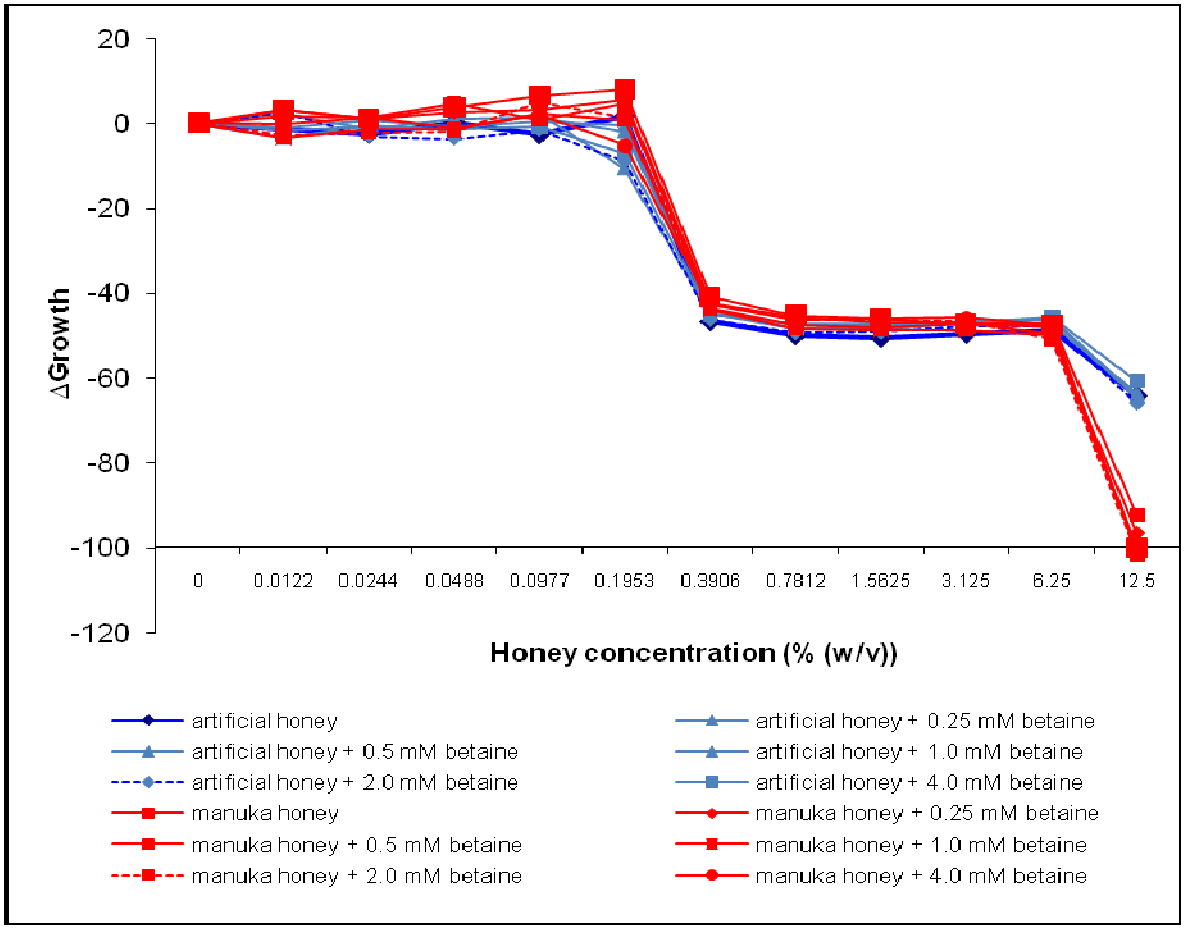


Figure 6.2.1. Effect of varying concentrations of either manuka honey or artificial honey (0 – 12.5% (w/v)) combined with betaine (0 – 4.0 mM) on growth of *E. coli* Nissle after 16 h incubation. Data from manuka honey and artificial honey in the presence of the range 5.0 – 100.0 mM betaine are not shown. Data are the mean of 8 replicates. LSD (P<0.05) as percentage of the mean ΔGrowth is 5.40.

A number of observations can be made from the data. First, at the highest doses tested (12.5% (w/v) honeys or 0.5 M NaCl), the onset of log phase growth was slightly delayed (artificial honey and NaCl) or highly delayed (manuka honey). Second, the initial rates of log phase growth for all conditions (except the highest manuka honey concentration) were largely indistinguishable from the control culture. In the presence of the highest manuka honey concentration, 12.5% (w/v), growth was observed, but after 24 h, at a much lower rate, and to a much lesser extent compared to the controls. Third, the NaCl solutions appeared to slightly inhibit the final OD of the culture in a dose-dependent manner, whilst the honey solutions at inhibitory concentrations limited the growth of the *E. coli* to a final OD commensurate with concentrations reported earlier (Fig. 6.2.1, and Chapters 3 and 5). These data correspond to the a_w values displayed in Fig. 5.3.1 of Chapter 5. Below these inhibitory concentrations (or above the inhibitory a_w values), log-phase growth briefly resulted in slightly increased biomass (honey solutions at concentrations 0.4 – 0.2 % (w/v)), which

may indicate the increased amounts of non-inhibitory sugar substrate available for growth. Interestingly, above the manuka and artificial honey concentrations of 0.39-0.78% (w/v) the cultures appeared to achieve a second proliferation phase, which occurred earlier at the lower of the two doses. At 0.2% (w/v) honey concentration, this growth occurred as part of the initial log phase. This suggests that the degree to which the cells may overcome the inhibition by the osmotically active factors is time and concentration-dependent.

The presence of betaine did not significantly affect the rate of growth, although its presence appears to have slightly increased the final biomass concentration (OD) over the inhibitory a_w range. This confirms the statement by Buchanan and Bagi (1997) that the effects of compatible solutes are likely to be less pronounced under nutrient rich conditions. These results appear to contrast with the findings of Krist and coworkers (1998), who reported that humectants (e.g NaCl) decrease growth rate whilst increasing final biomass, and that betaine would thus decrease the biomass (relative to NaCl) and increase the growth rate (relative to NaCl). The data in Fig. 6.2.2 appear to contradict this report, and indicate that the onset of log phase growth is delayed marginally, while the growth rate is only marginally decreased at the top assay dose (0.5 M) of NaCl used here, and the final biomass concentration was much lower under the conditions used. Interestingly, others (Natesan et al., 2000) have found that NaCl at similar concentrations to those used in this experiment resulted in decreased growth rate (0.31 M break-point, or the threshold concentration below which growth is permissable), respiration (O_2 consumption)(0.57 M break-point), ß-galactoside transport (0.54 M break-point) and increased turgor (swelling)(0.57 M break-point).

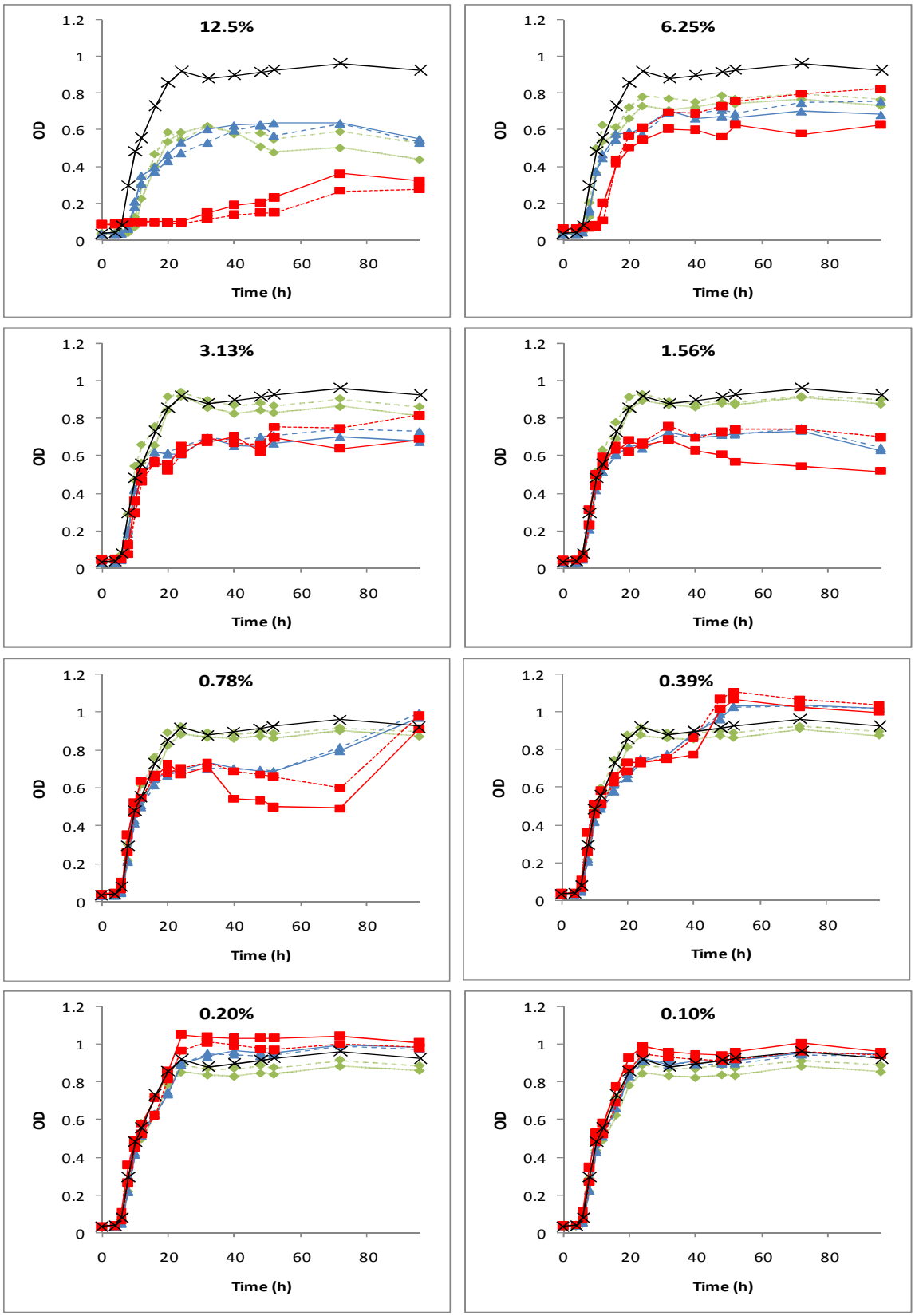


Figure 6.2.2. Growth kinetics of *E. coli* Nissle supplemented with manuka honey (■) or artificial honey (▲) (12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195 and 0.098 % (w/v)), or NaCl (◆) (0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008 and 0.004 mM) in the presence (dotted line) and absence (solid line) of betaine (2 mM). Unsupplemented (control, black **x**) growth shown. Graphs labelled with honey concentrations. Data are the mean of 8 replicates, with a mean standard deviation of 0.029 (range 0.001 – 0.054).

The biomass-limiting potential of the sugars glucose and fructose, which is greater than the NaCl-equivalent a_w, has been remarked upon earlier (Section 5.3.4), and may reflect their ability to act as metabolites, or in some other manner. Whilst the honey solutions were not buffered to a controlled pH to exclude the effects of acidity at the top assay doses used (and it is acknowledged that this will affect the growth kinetics of the bacterium), earlier data suggest that pH as a stand-alone factor is not significantly detrimental to the growth of the organism, although it has also been conceded earlier that it is likely to act as additional stress (Buchanan and Klawitter, 1992; Buchanan and Bagi, 1997; Ross et al., 2003) compounding the osmotic effects of these honey solutions.

Finally, the phenomenon of measurable growth, although it occurred after a long lag phase (late onset) and resulted in a decreased OD (limited extent), in the presence of manuka honey samples possessing known (Section 5.4) inhibitory MGO concentrations (1.0 and 0.5 mM for 12.5 and 6.25 % (w/v) manuka honey, respectively), further confirms the inhibitory role played by MGO, and also indicates that this inhibition can be overcome by time. This may suggest (i) the presence of a surviving population of bacteria (not resistant, as cells which survive MGO treatment are still susceptible to subsequent MGO treatments (Ferguson *et al.*, 1993)), (ii) that MGO damages but does not lyse cells, allowing bacteria to recover through glyoxalase or other MGO-detoxification mechanisms, or (iii) that MGO is a bacteriostatic, not bactericidal, molecule. Interestingly, it has been observed that non-viable cells were still metabolically active (Ferguson et al., 1996), and MGO detoxification does not require protein synthesis (Ferguson et al., 2000). This may allow those members of a cell population that have already succumbed to MGO-induced damage to still detoxify MGO and thus eventually reduce the local MGO concentration, further supporting hypotheses (i) and (ii), or even (iii) if considered in entire population terms.

6.2.4 Summary

Both honey solutions, the manuka honey and the artificial honey sugar solution, appeared to inhibit growth over the concentration range that has been demonstrated previously to possess inhibitory a_w levels. This inhibition largely takes the form of limiting the final biomass concentration to below that reached by the control cultures. At low concentrations of these osmotically active sugars, the inhibition may be overcome by the cells in a time- and dose-dependent fashion, which occurs earlier when the inhibitory concentration is lower. The humectant control, NaCl, lowered final biomass concentrations in a dose-dependent fashion, commensurate with the a_w values measured previously. Neither the honeys nor NaCl modified the growth rate or final biomass concentration in the manner reported in the

literature, as determined by either OD (Krist et al., 1998) or viable counts (Natesan *et al*., 2000).

At the highest dose of manuka honey used here (12.5% (w/v)) inhibition of growth was much greater than that observed for the artificial honey or NaCl. This inhibition could be attributed to MGO according to earlier work in this thesis (Chapter 5). However, this inhibition still allowed a gradual increase in population biomass, suggesting that MGO did not exert bactericidal effects under these assay conditions, despite being at a concentration (1 mM) reported to be lethal (>0.6 mM) to *E. coli* (Ferguson et al., 1998; Ferguson, 1999; Ferguson et al., 2000; Kalapos, 1999).

6.3 Manuka honey methylglyoxal

6.3.1 Introduction

As described above, *E. coli* populations are able to overcome inhibition by concentrations of manuka honey possessing reportedly lethal doses of MGO. The role of MGO in this inhibition needs to be confirmed by use of a standard compound, and the delays in onset of log-phase growth accounted for. Standard (10^3) and high (10^8) inoculums concentrations were used to explore how cell numbers impacted on the ability of MGO to inhibit growth.

6.3.2 Methods

The effect of manuka honey and MGO were measured by a modified version of the assay outlined in Section 5.3.2. Cultures (10^3 and 10^8 cells/mL of *E. coli* Nissle inocula in TSB) were grown with or without honey (12.5% and 6.25% (w/v)) or MGO (2, 1 and 0.5 mM) solutions, and OD was measured periodically throughout the incubation time to determine the growth rate. MGO solutions at 1 and 0.5 mM correspond to the MGO concentration in 12.5 and 6.25% (w/v) honey, respectively. Honey and MGO solutions were not balanced for pH.

6.3.3 Results and discussion

The results of the manuka honey and MGO growth experiments are presented in fig. 6.3.3.

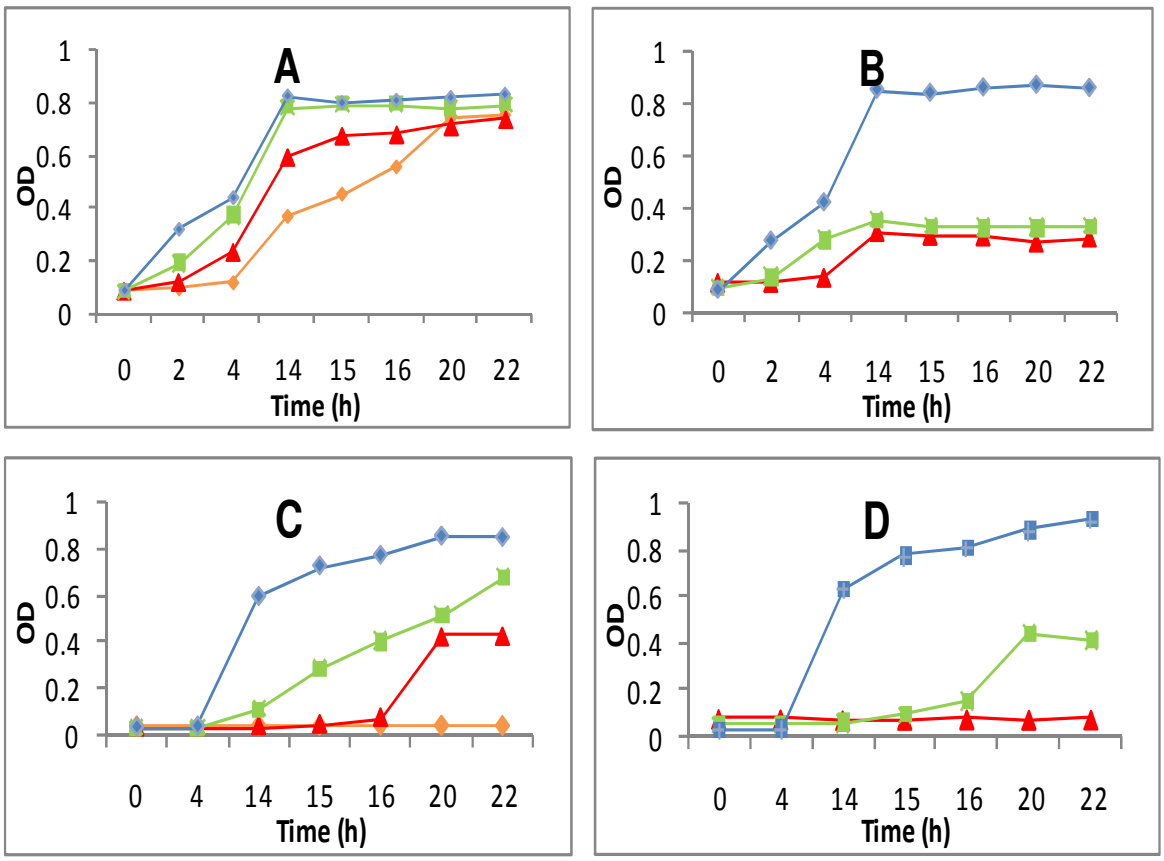


Figure 6.3.3. Mean growth (OD, n=8) vs time (h) for *E. coli* Nissle at 10^8 (A, B) or 10^3 (C, D) cfu/mL initial concentration, supplemented with either MGO (A, C) or manuka honey (B, D) of varying (0 mM, ♦; 0.5 mM, ■; 1.0 mM, ▲; 2 mM (A and C), ♦) concentration of MGO or MGO-equivalent conc. Mean standard devation 0.022 (range 0.001 – 0.085).

The presence of MGO in the media (A, C) delayed the onset of growth in a dose-dependent manner. Manuka honey (B, D) delayed the onset of log-phase growth in a similar fashion, but at lower dose (1 mM manuka honey MGO comparable to 2 mM MGO alone, 0.5 mM manuka honey MGO comparable to 1 mM MGO). In both cases, the degree of inhibition, i.e, the delay of onset of growth and the final biomass (OD) achieved, depended on the starting inoculum density: the inocula of 10^8 cell/mL were less susceptible to the MGO than were the 10^3 cell/mL inocula. This may be due to a greater MGO concentration being required to achieve the same intracellular concentrations with the larger inoculum due to greater number of cells/biomass, or it may be that the denser cell populations are more able to detoxify the MGO faster. This explanation fits within the three hypotheses proposed in Section 6.2.3, above, whereupon the greater cell mass allows (i) larger surviving population of bacteria, (ii) greater numbers of cells capable of detoxifying MGO or (iii) dilution of any bacteriostatic effects by greater cell biomass, or a combination thereof.

Relative to MGO, the manuka honey-induced delays prior to onset of log phase growth were more pronounced. This could be due to a number of factors, including the acidic pH (solutions weren't adjusted for same pH), the osmotically active sugars, or other compounds such as phenolics. The maximum growth achieved by *E. coli* in the presence of honey (B, D) can be attributed to the limiting presence of the sugars (Section 6.2.3, above, and Ch. 5), which appears to limit the growth of the bacteria irrespective of their starting inoculum density, whilst the pronounced effects at high concentrations appear to be due to MGO in combination with one or more other factors, as discussed below:

Previous experiments have shown that manuka honey at 1 mM MGO-equivalent (12.5% (w/v)) has a pH value of 6.6 in TSB, whilst manuka honey at 0.5 mM MGO equivalent (6.25% (w/v)) has a pH value of 6.8. Medium or medium + MGO has a value of pH 7.2. Previous experiments (Section 5.5) have shown that MGO inhibited growth to a greater extent when adjusted to an equivalent manuka honey pH, either alone, or (to an even greater extent) when mixed with an artificial honey solution. This was attributed, in part, to increased buffer ionic strength combining with pH and the osmotically active sugars to provide multiple stresses to the bacteria (Figs. 5.5.3 and 5.5.4). Thus, increased inhibition by MGO in manuka honey is likely to be attributable to the osmotically active honey solutions and acidic pH.

MGO analyses have been reported to underestimate the concentration in the presence of proteins which may sequester the MGO (Nemet et al., 2006). The protein content of manuka honey has been measured using the Bradford (coomassie) protein estimation method and shown to be as much as 0.15% (w/w)(Swapna Gannabathula, Plant & Food Research Ltd., pers. comm.). This could be a possible explanation for the apparent increased MGO-like delay in onset of log-phase growth of the *E. coli*. However, the HPLC-based MGO analysis (Ch. 5) was able to recover all the MGO from a honey sample "spiked' with added MGO (Section 5.4), indicating that sequestration of MGO by honey proteins was unlikely to be the cause of this increase.

6.3.4 Summary

In conclusion, MGO appeared to delay the onset of log phase growth and this delay was dose-dependent. The manuka honey also demonstrated this effect, but to a greater extent than MGO alone, indicating the cooperation of one or more other honey factors. *E. coli* appears to be able to overcome this inhibition in a time-, concentration- and cell density-dependent fashion.

6.4 Discussion

Collectively, these data show that manuka honey at inhibitory a_w concentrations inhibited the cultures from reaching the same final biomass concentration (OD) as the control cultures, until a concentration threshold had been reached, after which the culture was observed to grow to the same extent as the control. At effective MGO concentrations, the honey delayed the rate and onset of log-phase growth, and this delay was dependent on the dose of MGO. With both a_w and MGO-based inhibition the cultures were able to recover and continue to grow. The time taken before the onset of this secondary growth phase was proportional to both the concentration of the inhibitory factors and the size of the starting inoculum.

These mechanisms of antimicrobial action appear to allow the population (if not individual cells) to recover from these inhibitory factors and continue to grow, after sufficient time has elapsed. These results suggest that either the effects are not lethal to the entire population, and a subset survives to replicate, as discussed above, or that the individual cells are damaged and/or reproductively quiescent whilse they use tolerance and repair mechanisms to overcome the inhibition.

The molecular mechanisms for inhibition and cell death from osmotic shock and MGO have been discussed previously (Sections 5.3.1 and 5.4.1, respectively). *E. coli* response mechanisms to these stresses have been briefly mentioned, but it is pertinent to further expand on the MGO response mechanisms here.

MGO detoxification methods, conserved from bacteria to man, but most studied in *E. coli*, primarily involve autoreaction with glutathione forming hemithiolacetal followed by glyoxalase I and II-catalysed conversion, via S-lactoylglutathione, to lactose and glutathione. This system does not appear to require protein synthesis to be engaged, as determined by the fact that MGO detoxification can take place in cells even after chloramphenicol addition. Other detoxification systems such as aldo-keto reductases and non-glutathione-dependent glyoxalases exist, but they do not appear to be as significant in preventing inhibition/death from MGO, although some bacteria have been suggested to possess aldo-keto reductases as a quite important detoxification method (Booth et al., 2003, Nemet et al., 2006).

Mitigation of MGO effects occurs through S-lactoylglutathione derepression of the partially glutathione-repressed KefB and KefC K^+ efflux systems, leading to K^+ efflux and subsequent

proton influx, resulting in a lowering of the intracellular pH which protects the cells (Ferguson et al., 1996). This intrinsic capability for cells to protect and detoxify explains the dose- and cell density-dependence. Media dependence involves K^+ concentration (increased K^+ in media slows K^+ efflux with commensurate decrease in lethal MGO dose) and presence of MGO binding constituents, such as amino containing molecules, for example Tris or other possible constituents of complex media. Cells unable to acidify the cytoplasm (such as those lacking the KefB and KefC efflux systems) are sensitised to MGO and die more easily. A slight drop in pH (ca. 0.1 pH unit) appears to be all that is required for this protection, and it has been mimicked by addition of weak acid to *E. coli* cultures. Interestingly, while weak acids (e.g. acetic acid) might be able to dissociate and freely enter the cell, thus protecting the cell from MGO-induced damage as it undergoes the detoxification process (Ferguson et al., 1993; Ferguson et al., 1996; Ferguson, 1999; Ferguson et al., 2000), the acidic pH-contributing factors from manuka honey (Section 5.5.1) may not necessarily be able to freely enter the cytoplasm, and thus could be hypothesised to confer no protection. Furthermore, the osmotic response to the honey in order for the cell to maintain turgor is likely to involve the Kdp K+ uptake mechanism. Whilst the cell conventionally retains a fairly stable pH despite the external pH (Booth, 1985), the turgor-sensitive Kdp transporter would raise cytoplasmic K+ levels, resulting in raised cytoplasmic pH (~8) to balance electroneutrality and maintain the membrane polarisation required for respiration (Csonka, 1989). This rise in intracellular pH would not allow the cells to lower the pH sufficiently to protect themselves, and thus sensitise the cells to the effects of MGO (Ferguson et al., 1996; Ferguson, 1999).

These data suggest that the antimicrobial effects of manuka honey may be bacteriostatic rather than necessarily bactericidal. This is an interesting development, because none of the current literature imply anything other than manuka honey is antibacterial, and that the combined battery of inhibitory factors (peroxide, pH, osmotic effects, MGO, potentially antimicrobial phenolics etc.) collectively lead to either (i) lack of growth or (ii) cell death. Clearly what actually happens to the bacterial cell during the course of exposure to manuka honey and its constituent antimicrobial factors requires further investigation.

CHAPTER SEVEN

Mechanisms of Manuka Honey Antimicrobial Activity

7.1 Introduction

High MGO concentrations are associated with protein and DNA damage and other effects that accumulate and eventually contribute to cell death. However, metabolism is reported to continue even when the cell is no longer viable (Ferguson et al., 1993). As mentioned previously, recovery of the cells proceeds by K^+ efflux, proton influx and commensurate decrease in cytoplasmic pH, the increased acidity making the MGO less reactive/more stable whilst the cell undergoes detoxification by reaction with glutathione *via* the glyoxalase system. MGO was shown previously to be inhibitory at high concentrations, and to have no effect at low concentrations. The inhibitory activity of the honey solutions was shown previously to correspond to the presence of reportedly toxic MGO concentrations. Interestingly, the protective effect of the lowered intracellular pH has formerly been shown in the literature to be mimicked by addition of weak acid. However, this response to weak acid is dependent upon protein synthesis. In addition, the normal cellular response can be overwhelmed by (i) an increase in the MGO concentration to overwhelm the K^+ efflux system, (ii) by environmental K^+ levels then providing an osmotic barrier to K^+ efflux, and (iii) Kdp-caused K^+influx to maintain turgor resulting in an increase in the cytoplasmic pH to levels at which the K^+ efflux system cannot respond quickly enough.

Collectively these data indicate that multiple and conflicting responses are expected to be generated by the bacterial cell upon exposure to manuka honey. Two important conflicting or competing mechanisms, discussed earlier, are summarised below:

- Increased osmotic potential results in impairment of macromolecular synthesis (which would result in ATP accumulation) compared with increased energy requirements from extracellular acidic pH, presumably relating to increased metabolic activity and/or protein synthesis.
- Increased osmotic pressure tolerance mechanism (increasing K+ and intracellular pH) conflicts with the MGO tolerance mechanism (decreasing K+ and intracellular pH).

The work reported in this chapter attempted to investigate and resolve these apparent conflicts, or at least determine if one of the response mechanisms (osmotic or MGO) takes

precedence, or whether the manuka honey induces an inappropriate response, thus explaining its greater inhibition than that observed with osmotic or MGO controls.

7.2 Cellular K+ response of *E. coli* to manuka honey exposure

Potassium-binding benzofuran isopthalate (PBFI) is an insoluble, monovalent cation-binding fluorescent compound (Fig. 7.1). The dye is cell permeable (MP 01262, Molecular Probes), so can either measure the total K^+ content of a culture plus broth, or separately measure the culture supernatant and cell pellet to determine leakage vs retention of K^+, and thus indicate (i) the membrane permeability of the cell, or (ii) the cell's current metabolic state regarding potassium ions.

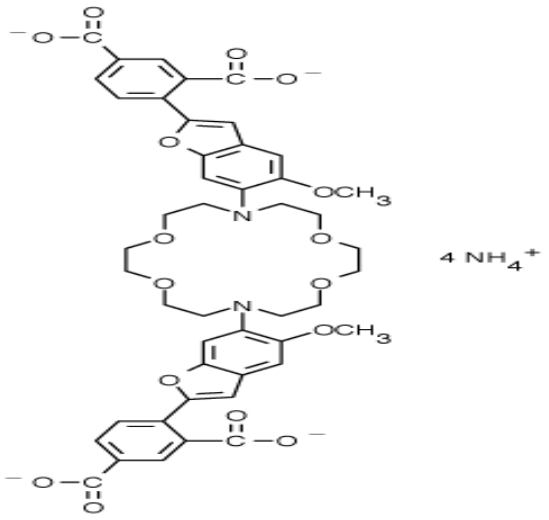


Figure 7.1 PBFI structure (non-ester form): two benzofuran isophthalate fluorophores linked to the nitrogens of a diazacrown ether with a cavity size that confers selectivity for K^+ (affinity 1.5 times that for Na^+)(Meuwis *et al.*, 1995).

The hypothesis being tested was that manuka honey may perturb membranes, either directly, or as a consequence of other mechanisms resulting in cell death. Membrane perturbation will also affect K^+ leakage, transport or retention. In addition, the enterobacterial response to MGO is to pump K^+ out of the cell with the KefB and KefC proton antiporter system, thus acidifying the cytoplasm to protect from MGO's effects. The response to osmotic pressure/a_w is to import K^+ into the cell using the Kdp transporter and also alkalinise the cytoplasm in response to the K^+ charge to retain electroneutrality and thus maintain membrane polarization. Thus a change should be observed in K^+ levels in the cells and culture supernatant, depending upon membrane damage and the priority/severity of the cells' response to MGO and osmotic pressure.

7.2.1 Methods

A culture of *E. coli* Nissle (10^8 cfu/mL) was centrifuged, and washed three times by centrifugation and resuspension in water to remove culture media components before being resuspended in water (to minimise excess background K^+ present in the media) containing manuka honey or artificial honey (12.5% (w/v)), or MGO at manuka honey equivalent concentration (1 mM). A culture resuspended in water was used as a control. After a 30 min incubation period, samples were rapidly prepared to ensure that changing the osmotic state upon removal of the humectants (honey solutions) minimised the impact on intracellular K+ concentration. Cultures were briefly centrifuged at maximum *g* in an eppendorf microfuge, and cells were resuspended in water. Aliquots (0.025 mL) were further diluted into water (0.075 mL) in a microplate and 0.01 mL of PBFI (100 µM in 80% DMSO) was added to give a final concentration of approximately 10 µM in 8% DMSO. Fluorescence was measured after 3 min (Fig. 7.2), and again at 5 min, using a Bio-TEK Synergy HT fluorescence plate reader set to an excitation wavelength of 340 nm and emission wavelength of 520 nm.

7.2.2 Results and discussion

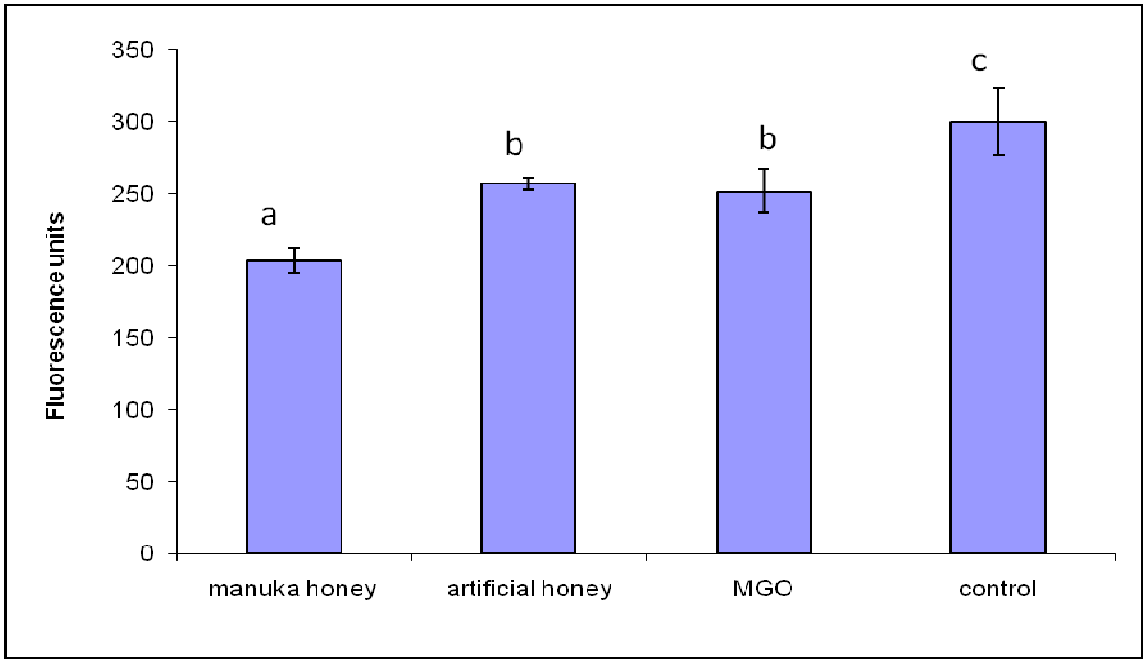


Figure 7.2. PBFI determination of intracellular K^+ measured in *E. coli* cells treated with manuka honey (12.5% (w/v)), artificial honey (12.5% (w/v)), or MGO (1.0 mM, equivalent to 12.5% (w/v) manuka honey) solutions and compared to unsupplemented (control) cells. Data are expressed as fluorescence units (Ex. 340 nm; Em. 520 nm) after 3 min and are the mean of 3 replicates. SEM shown. Data which are not significantly ($P<0.05$) different are marked with the same letter.

There was no difference between fluorescence readings after 3 (fig. 7.2) or 5 min (data not shown). All three treatments led to significantly ($P<0.05$) lower K^+ levels in the cells than in the control group. Artificial honey and MGO treatments led to approximately the same K^+ content, less than the control. The manuka honey treatment resulted in the lowest K^+ levels. In addition, the K+ levels in the cells treated with manuka honey were significantly lower than in those treated with artificial honey or MGO. The latter two treatments were not significantly different from each other. The decrease in intracellular K^+ in the MGO- and manuka honey-treated cells was expected. However, the decrease in K^+ in the artificial honey-treated cells was not the expected osmotic response. This may indicate that earlier comments on alternative modes of action of the osmotically active solutes glucose and fructose may be responsible for the inhibition of cells grown under these conditions. Alternatively, all three treatments may have compromised cells to the extent that they all lost K^+ ions through damaged membranes, and that manuka honey caused the greatest loss by virtue of being more antimicrobial than either the artificial honey or MGO alone. This hypothesis would have to be further tested by inclusion of controls containing a mixture of artificial honey and MGO. In addition, whilst the washing and incubation steps were conducted as rapidly as possible to minimise changes in cytoplasmic K^+ levels due to changes in the osmotic state of the cells, the impact of this must be considered.

Separation of cells from the growth medium has been performed by centrifugation through silicon oil (Bakker and Mangerich, 1981) to minimise cation or other solute loss from the cytoplasm due to changes in the osmotic environment. However, given that K^+ levels were lower than the controls, which must be considered the normal state, and that returning the treated cells to the same state as the controls would, at worst, reduce the difference between the control and treated cells, then still observing changes induced by the treatment indicates that the experiment was successful in terms of indicating divergent K^+ levels. Certainly, making claims on the actual value in terms of molar concentration of intracellular K+ could not be determined by the method used. Finally, levels of cytoplasmic water have been shown to be unlikely to change within the time period used to wash the samples (~1 min)(Cayley et al., 2000).

7.2.3 Summary

Treatment of a washed cell suspension (10^8 cells/mL) with manuka honey, artificial honey or MGO all resulted in decreased levels of cytoplasmic K^+ compared to untreated controls. This

may indicate that the MGO-tolerance response (decrease in K+ with commensurate increase in H^+) is the more likely mechanism than the osmotic response (increase K^+, decrease H^+). This non-osmotic response, even upon artificial honey treatment, either reinforces earlier suggestions that these sugars exert a non-osmotic effect to account for their inhibition, or suggests that all the treated cells have perturbed membranes causing leakage of cytoplasmic K^+.

7.3 Manuka honey effects on membrane integrity and respiration.

Previous data in this thesis have shown that manuka honey inhibits the growth of bacteria by two means: (i) MGO increases lag time (delaying onset of log-phase growth) in a dose-dependent manner, but is dependent upon media, cell density and pH, and (ii) osmotically active sugars restrict extent of growth (cell biomass) in a non-dose-dependent manner which correlates with inhibitory a_w levels but may not necessarily be due to osmotic effects. In both cases the cells recover, dependent on cell density and time. This recovery could be due to tolerance mechanisms returning the cells to favourable states for growth and replication, or could be due to repair of damage suffered as a result of the stressors. The data described in the previous section (Section 7.2) possibly allows for membrane perturbation as a symptom of the inhibition.

Bacterial respiration is performed at the inner cell membrane envelope (Melo et al., 2004). Therefore conditions which perturb membranes would be expected to affect bacterial respiration. Thus membrane perturbations could be measured, both directly, and in terms of potential changes in *E. coli* respiration.

7.3.1 Membrane integrity

The ability of the membrane to withstand or allow access of membrane-impermeable dye after manuka honey treatment was examined using the LIVE/DEAD *Bac*light kit (Molecular Probes, Eugene, OR). This commercial preparation contains two nucleic acid-binding fluorescent dyes, SYTO9 and propidium iodide (PI). The former, a green fluorescent dye, is membrane permeable; able to enter cells and stain them, irrespective of the condition of their membranes. In contrast, the latter, a red fluorescent dye, is membrane impermeable, so may only enter damaged/dead cells ("comprehensively dead"), where it outcompetes SYTO9 for DNA binding. Thus live, intact cells fluoresce green, and damaged or dead cells

fluoresce red. Control cells ("LIVE" cells and isopropanol-killed "DEAD" cells) were prepared and mixed, and the green:red ratio was used to qualify the degree of staining by each dye.

The hypothesis that was examined in this section is that manuka honey treatment results in cells staining positive for PI, either through damaging the membrane, or causing the cells to die, thus resulting in leaky membranes. The proportion of the population affected should relate to the ratio of green: red fluorescence.

7.3.1.1 Methods

E. coli Nissle (~10^8 cfu/mL) was added to 96-well microtitre plate wells containing varying concentrations of manuka honey (12.5 – 2.5 % (w/v)) and LIVE/DEAD *Bac*light staining was immediately performed for microplate assay as described by the manufacturer (MP 07007, 15 Jul 2004). "LIVE" and "DEAD" control cells were prepared separately from the honey samples but stained as part of the same batch. Fluorescence was determined for each dye with excitation wavelength 485 nm, emission 528 nm (SYTO9) and 645 nm (PI) using a Bio-TEK Synergy HT fluorescent plate reader and the green/red ratio (Emission 285/20 (S=50)/Emission 640/40 (S=50)) was calculated. The standard curve, showing proportion of SYTO9 and PI-staining cells of known live:dead ratios, is displayed in Fig. 7.3.1. The effects of varying concentrations of manuka honey are displayed in Fig. 7.3.2.

Staining was confirmed by fluorescence microscopy. *E. coli* Nissle (~10^8 cfu/mL), in the presence or absence of 12.5% (w/v) manuka honey, was added to an equal volume of H_2O containing 1 µL/mL SYTO9 (manufacturer's specification, used in Section 4.1-2, above) and 3 µL/mL PI (three times recommended concentration). Drops were placed on a glass slide with cover slips and viewed under the fluorescent microscope to confirm the presence of green and red fluorescence.

7.3.1.2 Results and discussion

Fig 7.3.1 shows that with increasing numbers of dead cells, the PI staining increased, accompanied by a commensurate decrease in SYTO9 staining, indicating that the assay accurately reflects the status of the membrane for the cell.

Whilst the controls (Fig. 7.3.1), and media blanks etc., performed exactly to the manufacturer's descriptions, Fig 7.3.2 shows that the manuka honey gave an unusual result.

This result was unusual because there was no increased PI staining with increased manuka honey concentration to accompany the expected decreases in SYTO9 staining (and the line on the graph would reach through the no-honey control, were it shown). This result indicated that the green/red ratio bore no relevance to the likely proportion of dead cells. Possible explanations include (i) manuka honey does not perturb cell membranes and PI entry into the cytoplasm is prevented, (ii) complete cell lysis of the affected cells had occurred, thus proportionately decreasing the live, green staining population but leaving no material for the PI to stain, or (iii) manuka honey interfered with the staining process, and PI was more susceptible to this interference than was SYTO9.

Despite the reason being unclear, the degree of LIVE/DEAD staining did not appear to relate to the degree by which manuka honey inhibited growth.

The experiment was repeated, where LIVE/DEAD controls were prepared as part of the same treatment as the honey. This ensured similar cell densities and allowed comparable fluorescence units to accompany the green/red ratio. The above experiment did not allow comparison of the SYTO9 or PI staining with proportion of respective live or dead control cells. The results (not shown) were similar to Fig. 7.3.2. SYTO9 stained in a dose-dependent manner with a profile similar to that obtained by OD growth experiments, whilst PI stained at a consistent level equivalent to an approx. 40% LIVE/DEAD ratio. This appeared to indicate that SYTO9 staining might be proportional to live or growing cells, whilst PI was staining at a consistent high background level in the presence of honey, irrespective of the actual concentration – if honey was there, PI only stained at that level.

Interestingly, the SYTO9 results indicated that the effects of the manuka honey were not reversed by washing the cells to remove the honey solution: decreases in SYTO9 staining were proportional to manuka honey concentration, implying (i) cell lysis, (ii) irreversible (in that time scale) damage, or (iii) binding/accumulation of honey factors (such as possible formation of a sugar capsule) which resisted the washing step. Alternatively, MGO-induced DNA damage was not accompanied by lysis, resulting in decreased SYTO9 staining due to lower nucleic acid levels within the cell, whilst PI was still prevented from entering the bacterial cytoplasm. This is briefly discussed below.

Fluorescence microscopy with green and red filters indicated that there were more green fluorescing cells in the control sample than in the honey sample (results not shown), whilst numbers of PI-stained cells were the same between honey-treated and control samples (and significantly less than the number of green-stained cells). Interestingly, whilst some of the cells stained with SYTO9 were also stained with PI, as viewed by changing the filter, there were some PI-stained cells that were notably negative for SYTO9. This result appears to suggest a degree of differential staining based on the "deadness" of the cell. It may be that cells only staining with PI were totally dead, whilst those cells staining with both dyes were compromised but not yet dead. Those cells staining with SYTO9 only were, of course, neither dead nor compromised.

Of further interest, when viewed by switching to brightfield microscopy, it was revealed that there was approximately the same number of cells in each sample, but only a fraction of the population were stained in the manuka honey sample versus the control sample. This indicates that manuka honey reduces staining in some way, perhaps by coating the bacteria and restricting access, or alternatively adsorbing or chelating the stains, rather than by causing lysis and removing the staining target.

One could speculate that the presence of MGO, which is known to degrade DNA whilst allowing cells to remain metabolically active (Ferguson, 1999; Ferguson et al., 2000), could be responsible for these observations. Intact, but non-viable cells would result in decreased SYTO9 staining proportional to the MGO-induced DNA damage, whilst still excluding PI from entry to the cell. The use of pH, MGO and the artificial honey sugar solution, as controls or test compounds to further investigate this or other mechanisms would be required.

In addition, examining the staining properties over a time course of manuka honey exposure, over an interval which spanned the period where bacteria had begun to enter the previously observed 'recovery phase' after 24 h incubation (Figs. 6.2.2, 6.3.3), would be interesting, as this might shed light on the potential mechanism, as well as indicate if this recovery was due to a surviving population, or cells that have undergone repair.

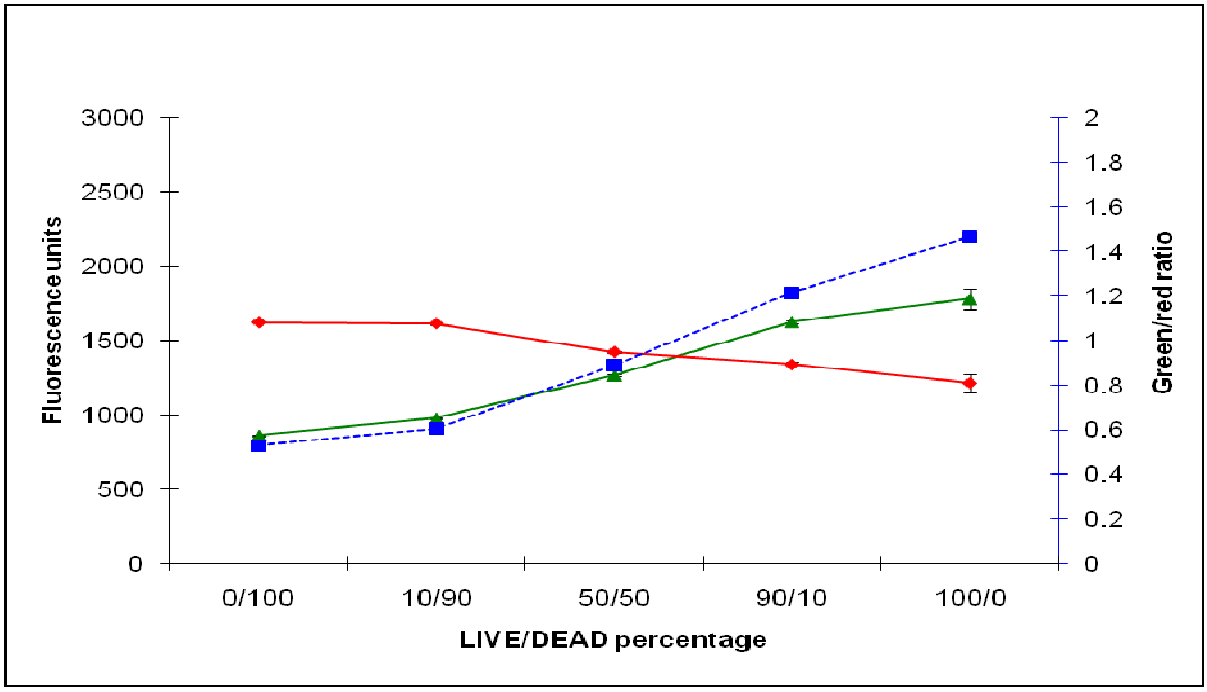


Figure 7.3.1. LIVE/DEAD *Bac*light fluorescent membrane integrity assay of controls with decreasing PI positive staining (Ex. 485 nm, Em. 645 nm, ◆) and increasing SYTO9 positive staining (Ex. 485 nm, Em. 528 nm, ▲) staining as a result of increasing the proportion of intact LIVE *E. coli* Nissle cells compared to leaky DEAD *E. coli* Nissle cells (N=8, standard deviations shown). The green/red (PI/SYTO9) ratio (■) (blue axis) indicates staining corresponds to ratio of LIVE:DEAD cells by passing through the centre (50:50, green/red ratio = 1) at the same point that the SYTO9 and PI staining profiles cross.

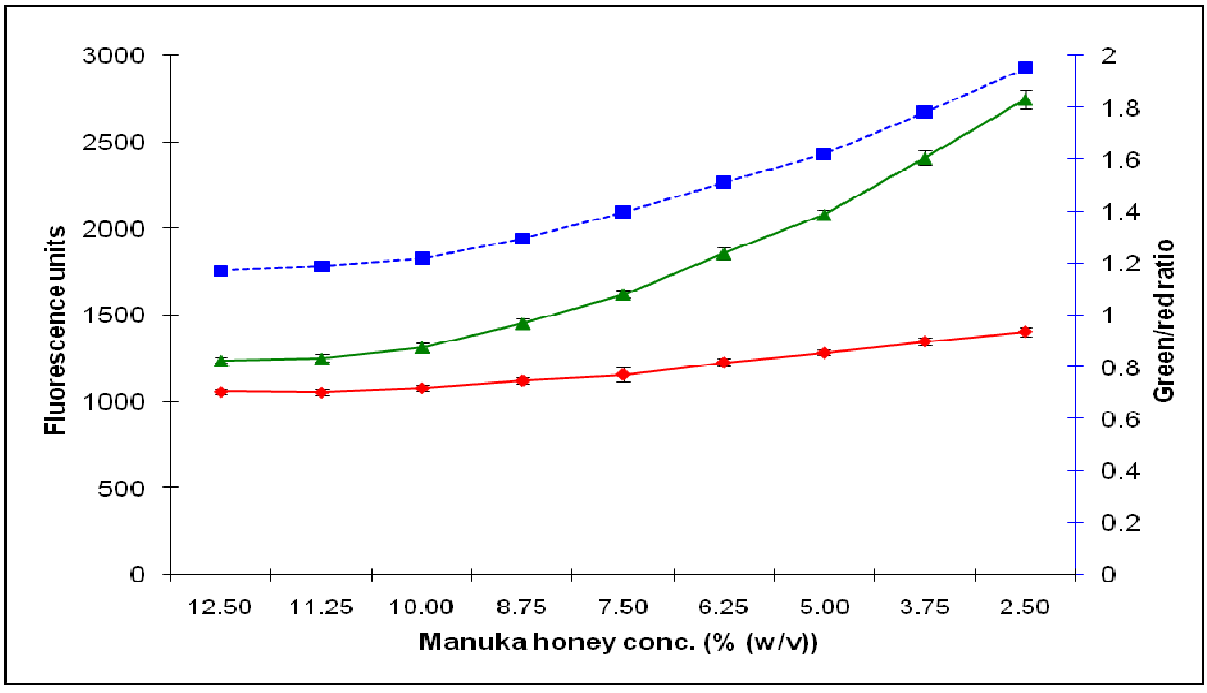


Figure 7.3.2. LIVE/DEAD *Bac*light fluorescent membrane integrity assay of *E. coli* Nissle subjected to varying concentrations of manuka honey. PI positive (DEAD) fluorescence (Ex. 485 nm, Em. 645 nm, ◆), SYTO9 positive (LIVE) fluorescence (Ex. 485 nm, Em. 528 nm, ▲) (N=8, standard deviations shown) and LIVE/DEAD ratio (■) (green/red ratio, blue axis) shown.

7.3.1.3 Summary

It appears that manuka honey interferes with the LIVE/DEAD *Bac*light staining of *E. coli* Nissle cells by SYTO9 in a dose-dependent manner, and PI in a dose independent manner. Destruction of DNA by MGO is a potential explanation for this phenomenon, but further work would be required to establish the role of MGO here.

7.3.2 Respiration (Aerobic vs anaerobic)

E. coli Nissle is able to grow to a limited extent under anaerobic conditions. Under these conditions it can be hypothesised that dependence on membrane integrity in terms of the electron transport chain is lessened, and thus growth of the bacterium would be less affected by manuka honey factors that mildly compromise the membrane or cause loss of membrane potential. Thus growth of *E. coli* under aerobic and anaerobic conditions was compared in the presence and absence of manuka honey.

7.3.2.1 Methods

Anaerobic growth was maintained as outlined in Section 2.2.1.5. Aerobic conditions were maintained as normal, with shaking at >250 rpm to enhance aeration. The effects of manuka honey and the MGO standard compound were measured by standard antimicrobial assay conditions as described previously (Section 2.2.4.1), except that cells were grown under aerobic, anaerobic and semi-anaerobic (non-shaking) conditions.

7.3.2.2 Results and discussion

The results of varying the aeration conditions are shown in Fig. 7.3.3.

Growth of *E. coli* Nissle supplemented with 12.5% and 3.125% (w/v) manuka honey and ensuring full aeration by bubbling air aseptically through cultures was not significantly different from the growth of cultures aerated by rotary shaking (data not shown).

The manuka honey doses which conventionally limit growth over the inhibitory a_w range (0.1 – 3 % (w/v)) show that the degree of growth inhibition is proportional to the degree of

aeration in the following manner: aerobic with shaking -> aerobic without shaking -> anaerobic. Thus it appears that, unlike MGO-derived inhibition, “osmotic” inhibition is linked to respiration and/or O_2 consumption or redox conditions. Others (Natesan et al., 2000) have found that increased (aerobic) respiration is linked to increased membrane permeability, that may include greater access of molecules such as hydrogen peroxide, into the cell. Interestingly, this does not appear to apply to pure MGO.

The top honey dose used here (12.5% (w/v)) inhibited *E. coli* growth irrespective of the aeration conditions. In contrast, the degree of MGO inhibition was proportional to lack of aeration – i.e. MGO was more inhibitory at lower doses in the following manner: anaerobic > non-shaken aerobic > shaken aerated. Rather than suggesting that MGO affects the cytoplasmic membrane, these data suggest that either (i) lack of aerobic respiration renders the bacterium more susceptible to MGO-derived inhibition, or (ii) the more reducing nature of the anaerobic medium results in either greater effects of MGO or less resistance to MGO. Manuka honey does not display this changed dose-dependent inhibition, despite possessing more stressors which would be expected to contribute to increased inhibition. This may indicate that the observed results more reflect the effects of the conditions on the MGO standard compound than on the bacterium. One may speculate that the anaerobic conditions may render the MGO more stable, and simultaneously increase its half-life (and thus ability to exist to inhibit growth). This would result in lowered ability to dissociate from whichever honey compounds normally harbour it and/or limit access to the cytoplasm of the bacterial cell. Work described in previous sections within this thesis chapter has suggested that manuka honey alters the access of compounds to the cytoplasm of the bacterial cell. This effect may relate to the actual respiratory condition of the cell and warrants further investigation.

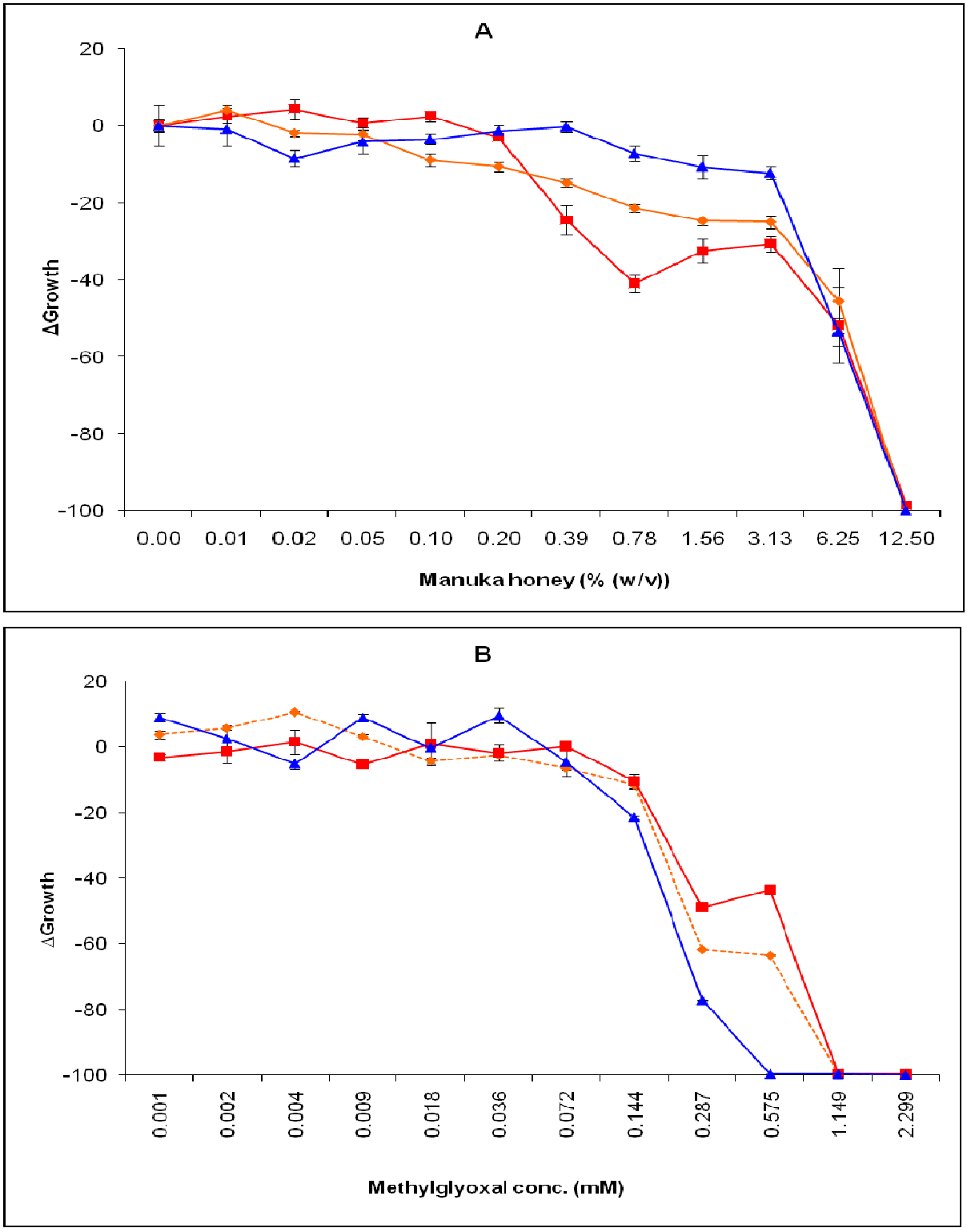


Figure 7.3.3 Effects of a range of concentrations of manuka honey (A) and MGO (B) on *E. coli* Nissle grown after 16 h at 37℃ under different aeration conditions: anaerobic (▲), aerobic (◆) and aerobic with shaking (■). Data are the mean of 8 replicates. SEMs shown.

7.3.2.4 Summary

Inhibition of *E. coli* growth by manuka honey osmotically-active sugars increases with increasing aeration, whilst inhibition by the MGO standard compound increases with decreasing aeration. The literature suggests that the respiratory state of the cell determines the permeability of the membrane (Natesan et al., 2000). Further work is required to determine whether manuka honey has any effects on respiration, and this was investigated in the next section.

7.3.3 Respiration (MTT)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is absorbed into cells and metabolised by respiratory enzymes to form an insoluble formazan dye, so is used as a measure of metabolic activity and "usually" represents live cell numbers. Generation of formazan dye is usually proportional to cell density and respiratory levels of the cells. Cell death or inhibition of respiration through membrane perturbation, loss of membrane polarisation, or any other mechanism, would reduce formazan dye production.

7.3.3.1 Methods

The MTT assay was conducted as described in Section 2.2.4.7, with modifications as follows:

First, *E. coli* Nissle cultures of varying inoculum densities (range 10^3-10^8 cells/mL) were exposed to manuka honey under standard assay conditions and their OD was determined after 16 h growth (Section 2.2.4.1). The cells were then incubated in the presence of MTT (0.01 mL of 0.5 mg/mL) for 30 min, then lysed with DMSO to solubilize the dye prior to determining the absorbance.

Second, *E. coli* Nissle (~10^8 cfu/mL) was incubated in the presence of 0.01 mL of 0.5 mg/mL MTT (0.5 mg/ml). Honey (12.5% (w/v) final conc.) was added to wells at 10 min intervals, giving a series of wells with increased incubation time with MTT (0-90 min) prior to honey addition.

Finally, *E. coli* Nissle cultures (~10^8 cfu/mL) had manuka honey (12.5% (w/v) final conc.) added to their microplate wells at 10 min intervals, giving a series of wells with increased exposure to the honey (0-90 min) prior to MTT addition. MTT was then added, incubated a further 30 min, and the cells were lysed, the formazan was solubilised and the absorbance was measured.

7.3.3.2 Results and discussion

Although results were obtained from a complete range of cell inoculum densities, only data from the lowest and highest (10^3 and 10^8) inocula are presented (Fig. 7.3.4). Unexpectedly, little formazan dye colour was generated by cells in the presence of inhibitory concentrations of manuka honey, despite the OD indicating that some 40-60% of growth of cells had occurred in all but the highest inhibitory dose. This indicates that cell death had not occurred, but that either (i) metabolic or respiratory arrest had occurred, perhaps due to perturbation of the membranes preventing respiration without causing cell lysis, or a repressive effect was induced by the high concentration of metabolically active glucose present, or (ii) that MTT was not able to freely diffuse into the cytoplasm (c.f. PI, Section 7.3.1.2). As the colour is not proportional to cell density, the cells may no longer be actively metabolizing or respiring, perhaps due to a depressive effect of the glucose present at those concentrations.

To examine whether manuka honey causes metabolic arrest in grown cells, effectively limiting their growth to a certain threshold and explaining the unusual MTT results, manuka honey (12.5% (w/v)) was added at staggered 10 min intervals to cells already loaded with MTT, giving a series of wells with increased incubation with MTT (0-90 min) prior to honey addition. Formazan dye was produced in similar (+/- ~5%) quantities across all time points (data not shown). This inhibition was some 10-15% less than the control culture (not incubated with honey). Either manuka honey does not cause arrest of respiration (which would be reflected in lower OD for those wells where honey had been added earlier), or the honey takes longer than 90 min to exert its inhibitory effects.

A subsequent experiment examined whether the manuka honey inhibition of formazan dye production was sensitive to the length of time cells were pre-incubated with honey (Fig. 7.3.5).

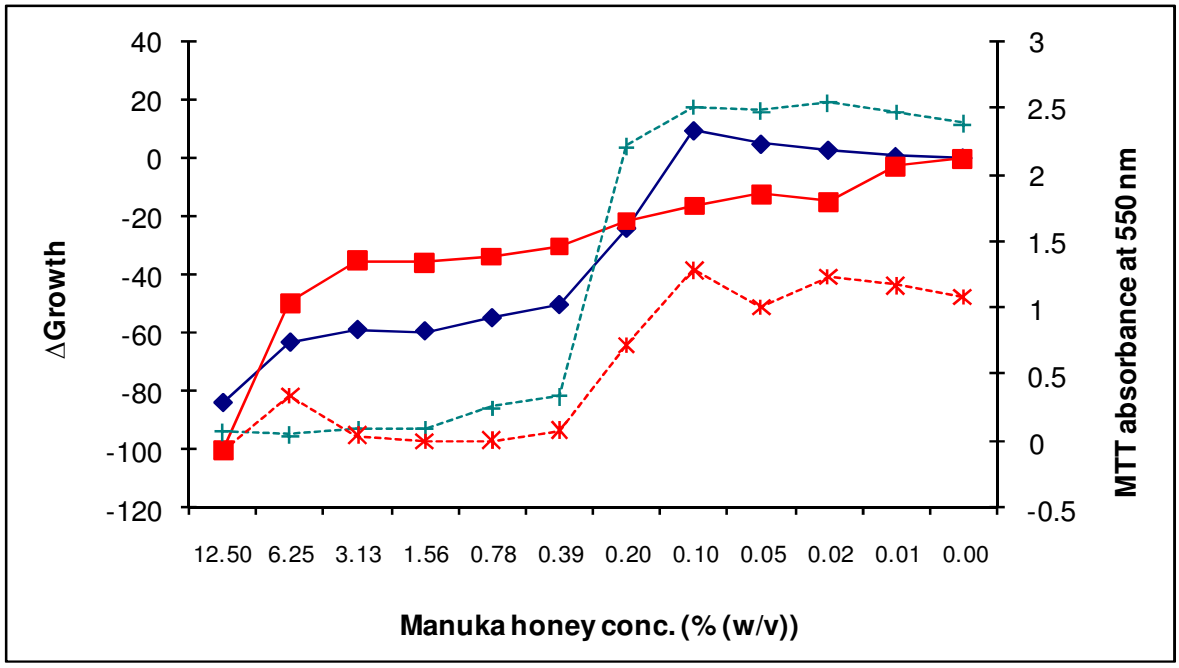


Figure 7.3.4. The effect of manuka honey on the growth and respiratory activity of *E. coli* Nissle. Two cultures of *E. coli* Nissle, with different inoculation densities (10^3 cfu/mL, ■ and 10^8 cfu/mL, ◆), were grown for 16 h at 37°C in the presence of varying concentrations of manuka honey. Respiratory activity (formazan dye colour generation from MTT metabolism) (10^8 cfu/mL inoculum, +; 10^3 cfu/mL inoculum, x) is shown as dashed lines. Data are the mean of 8 replicates. Standard deviation as percentage of the mean ΔGrowth is 5.01 (range 0.76 – 18.82).

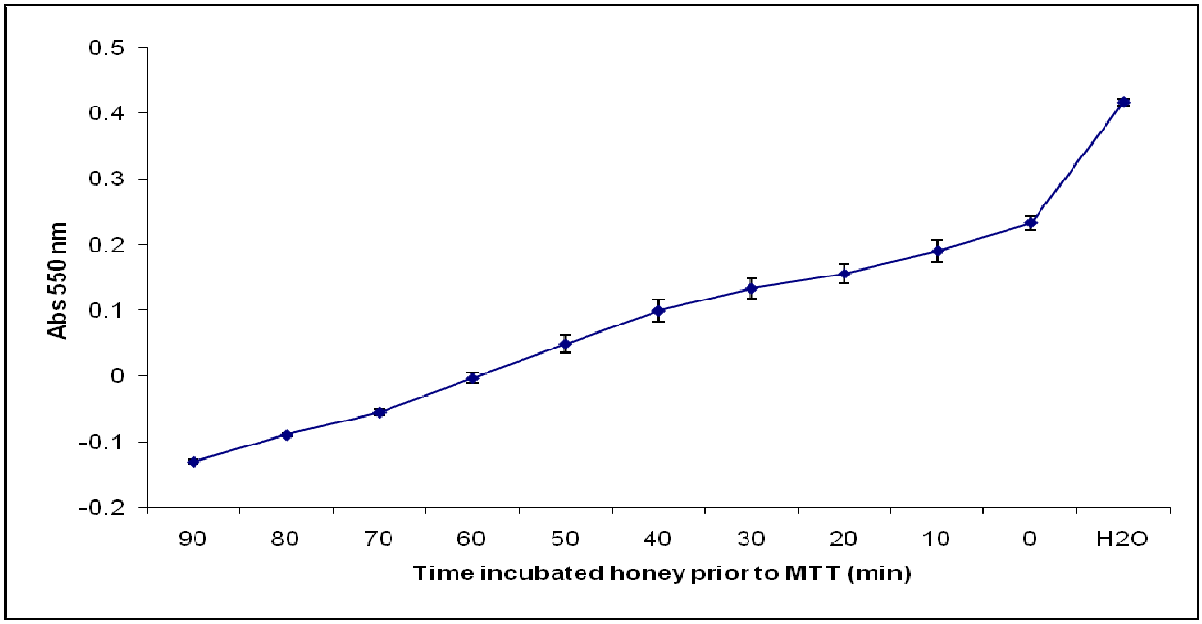


Figure 7.3.5. Respiratory activity (formazan dye generation from MTT) of *E. coli* Nissle cultures (10^8 cfu/mL inoculum) after varying pre-incubation periods (0-90 min) with manuka honey (12.5% (w/v)). Data are the mean of 8 determinations. SEMs shown.

Interestingly, colour development was inversely proportional to honey incubation time. This indicates that the hypothesis was correct, and that manuka honey exhibits increased effect with increased incubation time. This may be inhibiting more cells, or giving a greater degree of inhibition to individual cells.

7.3.3.3 Summary

Pre-incubation of cells with manuka honey inhibits production of formazan dye from MTT by respiratory enzymes. The degree of inhibition is proportional to time, not manuka honey concentration (over the threshold concentration required for inhibition of growth). Addition of manuka honey after addition of MTT (when, presumably, it has established a stable concentration within the cytoplasm) does not result in inhibition of formazan dye production. This result is similar to that observed previously (Section 7.3.1) where SYTO9 and PI staining was altered by treatment with manuka honey. Collectively, these data suggest that manuka honey prevents cytoplasmic access by cell-permeable chemicals (MTT, SYTO9) and/or prevents respiration (or access to chemicals used to measure respiration) by some form of membrane-acting mechanism, which may be a perturbation, or by forming a physical capsule.

7.3.4. Cellular ATP levels

Previous attempts to measure membrane integrity conducted within this thesis have relied upon access to the cytoplasm by chemical markers. Manuka honey may interfere with access by these chemicals. To further examine respiration/membrane-affecting manuka honey activity, cellular ATP levels were measured, as these levels are dependent upon the respiratory levels of the cell, and are measured after cell lysis using assay reagents. Thus this approach attempts to avoid any confounding effect on measurement by the manuka honey.

7.3.4.1 Methods

The determination of cellular ATP levels was performed using a Bactiter-Glo$^{(TM)}$ Microbial Cell Viability Assay kit (Promega, Madison, WI) in a 96-well microtitre plate according to the manufacturer's protocols (Promega technical bulletin TB337), with the modification that cells were centrifuged, resuspended in 0.1% peptone water to 10^8 cfu/mL, and exposed to

manuka honey, artificial honey or MGO for 1 h prior to addition of the BacTiter-Glo$^{(TM)}$ reagent. Data were expressed as ΔluminescenceΔ, calculated using the percentage of the control luminescence in the same manner as used for absorbance values in the spectrophotometer-based antimicrobial assay (Section 2.2.4.1, Eq. 1).

7.3.4.2 Results and discussion

The results are shown in fig. 7.3.6.

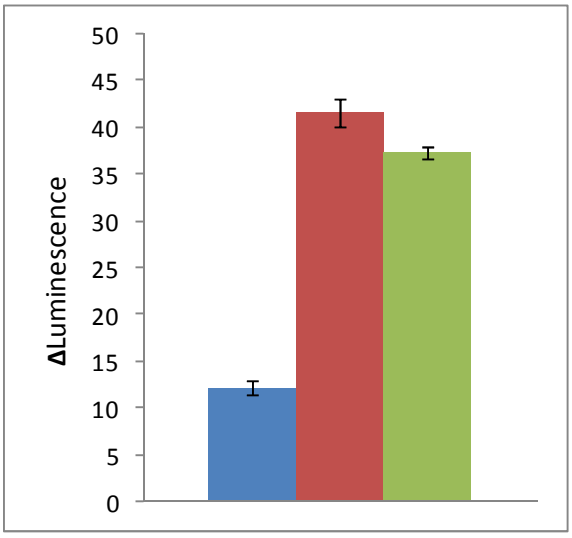


Figure 7.3.6. ΔLuminescence relative to unsupplemented control culture (zero) of ATP levels of log-phase *E. coli* Nissle culture (10^7 cells/mL) supplemented with manuka honey (12.5%, ■), artificial honey (12.5% (w/v), ■) and MGO (1 mM, 12.5% manuka honey-equivalent, ■). Data are the mean of 4 replicates. SEM shown.

The manuka honey-treated cells resulted in a 12% increase in ATP levels of the cells relative to the control (untreated) cells. The artificial honey and MGO standard solutions showed 42% and 37% increases, respectively. The increase in ATP levels in the presence of the osmotically active glucose and fructose artificial honey solution is supported by reported increases in levels of ATP in osmotically challenged cells (Krist et al., 1998), potentially due to lower levels of macromolecular synthesis. The increase in ATP levels observed with the MGO-challenged cells supports the observation that cells remain metabolically active and respiring despite accumulating DNA and protein damage from the labile electrophile (Ferguson, 1999; Ferguson et al., 2000). The increase in ATP levels in the manuka honey-supplemented cells could be a combination of the above factors. This could be investigated by determining the effects of a mixture of artificial honey and MGO. However, the

significantly lower level than the artificial honey and MGO solutions might also be a function of the lower pH in these unbuffered solutions. Manuka honey is acidic under these conditions (Section 5.5, Table 5.5.1), and acidic conditions are energy-demanding (Krist et al., 1998), thereby requiring greater utilisation of the ATP sources than under the other two conditions.

7.3.4.3 Summary

Manuka honey treatment resulted in a 12% increase in cellular ATP levels, while artificial honey and MGO treatment resulted in a 40% increase in cellular ATP levels compared to untreated controls. This could be due to lower growth and/or energy requirements in the treatment groups, with these requirements being higher in the presence of manuka honey than in the standard solutions due to its lower pH value. These elevated levels of ATP relative to the control may suggest that the cell membranes were not perturbed by manuka honey, MGO or the osmotically active sugars. However, it is not known whether the elevated levels of ATP are due to lower energy requirements than in control cells respiring at an equal rate, or that they result from much lower energy requirements in non-respiring cells which outweighs the amount produced by respiring controls cells with higher energy demands.

7.4 Conclusions

Manuka honey has been shown to decrease or prevent access of some permeable dye compounds to the cytoplasm. This suggests that bacterial membranes remain intact in the presence of the honey. Previous experiments have suggested that MGO and manuka honey may inhibit bacterial growth without lysing cells or loss of membrane integrity. Respiration apparently continues even during inhibition of growth. However, perturbations of the membrane have not been conclusively confirmed. Perturbation of the bacterial inner membrane by antimicrobial agents would result in loss of membrane potential. Future work could involve monitoring the membrane potential by the use of the fluorescent dye 3,3-dipropylthiacarbocyanine ($DiSC_35$). $DiSC_35$ is a cyanine dye that inserts into the cytoplasmic membrane under the influence of the membrane potential gradient and quenches its own fluorescence. Upon disruption of the membrane potential the dye is released and fluoresces. However, as illustrated by the MTT assay, preincubation of the dye would be required before treatment with manuka honey.

The main conclusion which can be drawn from this work is that cell lysis and/or cell death do not appear to occur, even at apparently “lethal” doses of manuka honey (and MGO). Thus inhibition appears to be bacteriostatic rather than bactericidal. This distinction has never before been pointed out in the existing literature describing the antimicrobial effects of manuka honey, and has important implications with regards to the perceived status of manuka honey as a functional food for influencing gut health. These and other implications for the non-dietary use of manuka honey are discussed in the final chapter.

CHAPTER EIGHT

General discussion/conclusion

8.1 Summary

The aim of this project was to identify and assess possible gut health benefits of functional food ingredients or ingredient combinations of New Zealand origin. Ingredients were pre-selected by scientists from The New Zealand Institute for Crop & Food Research Ltd (now Plant & Food Research) for their known or suspected antimicrobial activity, primarily targeted against *H. pylori*, but also with known or suspected antioxidant or other activities conducive to health. The selection process for the ingredients was detailed in Chapter 1. A systematic approach of *in vitro* assays for antimicrobial activity (Chapter 3) and immunomodulatory activity (Appendix 3) was performed, in order to select the best ingredient(s) for further study and for incorporation into a concept functional food by members of the larger project of which this work forms a part.

Manuka honey was identified as an outstanding candidate, with BroccoSprouts® also selected for further investigation due to performance in the *in vitro* assays together with results achieved by others in the larger project (working on *H. pylori* and associated gastric inflammatory markers). Manuka honey, already widely known to posses antimicrobial (UMF™) and wound-healing properties, was shown to increase probiotic growth and decrease pathogen growth in mono-cultures of those organisms, and to partake in synergistic interactions with some of the other ingredients tested.

Manuka honey and BroccoSprouts® were then tested in a small animal *in vivo* model to confirm that the result that was observed in the test tube was also observed in the whole body. Results of this animal trial (and a previous pilot clinical trial by other researchers) failed to demonstrate any changes to the parameters examined (macrophage numbers of phagocytosis activity, bacterial populations, SCFA concentrations) by manuka honey. This was attributed, in part, to the likelihood that the manuka honey would be digested prior to reaching the colon (or caecum of the mice in the animal trial) where effects were being sought. Nevertheless, the efficacy of the manuka honey *in vitro* was undeniable, and the active constituent(s) and antimicrobial mode of action were still unknown.
Subsequent work focused on these elements, with a fortuitous discovery by researchers overseas, identifying the putative active UMF™ compound, MGO, at the time that this

exploratory part of the thesis was barely underway. During the course of this work it was established that the main contributors to the observed *in vitro* manuka honey activity against the test organism *E. coli* were MGO and the osmotically active sugars, both modulated by the honey acidic pH, in a complex web of interacting, and perhaps overlapping, activities. A common honey antimicrobial factor, hydrogen peroxide, was eliminated, whilst honey phenolics were not investigated here due to lack of support in the literature and the need to focus this research on the factors which appeared to play greater antimicrobial roles.

Further work examining the response of the bacterium *E. coli* to the manuka honey and primary antimicrobial factors revealed for the first time that manuka honey appears to act primarily in a bacteriostatic fashion, not necessarily bactericidal as had been previously implied by the existing literature.

8.2 Implications

Firstly, the discovery of MGO by Prof. Henle's food chemistry group at the University of Dresden has revolutionised New Zealand's UMF™ manuka honey industry. The Active Manuka Honey Association, which monitors and maintains the status and value of the UMF™ brand, now contends with alternate branding, such as Manuka Health's MGO™ brand, and associated publicity, based upon quantification of the MGO in the honey by HPLC (http://www.manukahealth.co.nz/main.cfm?id=105).

Secondly, manuka honey research has been strengthened by the addition of another bioactive compound to investigate, and an existing field of research already focusing on antimicrobial MGO has been incorporated into the manuka honey research area (Adams et al., 2008; Mavric, 2008).

Finally, this thesis, and the larger project of which this work forms a part, has revealed that manuka honey appears to confer no *in vivo* large intestinal benefits. This thesis suggests that manuka honey may not kill pathogenic bacteria, but merely delay the onset and/or extent of growth of bacteria. The duration and extent of this effect depends upon the concentration of the manuka honey, and is mitigated by the density of the bacterial biomass, the respiratory state and/or redox environment of the bacteria, the pH of the environment, and sundry other factors.

The rich, diverse and numerous gut microbiome (or metabolome, as the host must be taken into account), with its robustness, redundancy, competition for nutrients and environmental niches, is likely to suffer little impact from ingested manuka honey. The sheer numbers and mass of the microflora coupled with this extreme competition and the adaptability of the microbiome, confounded by the host's own digestive processes, means that manuka honey was always unlikely to both reach the colon at concentrations capable of exerting an effect, and to actually induce significant changes. The results in this thesis support this hypothesis.

Thus, from a functional food perspective, manuka honey possesses little value in terms of managing the gut microbiota, given consumption of reasonably normal manuka honey portions, unless the honey is formulated in a way that protects it from digestion.

However, it is still not known what, exactly, is the effect of a honey solution (which may or may not reach the colon at an adequate concentration to exert effects) upon a complex and robust ecosystem, or even a biofilm of a single strain. Furthermore, manuka honey is likely to still exert effects in the comparatively early regions of the GI tract, such as the mouth, oesophagus and stomach, with potential roles against dental caries and/or gastric *Helicobacter* or other more opportunistic pathogens. Finally, manuka honey is likely to still retain its value as an antibacterial agent during topical wound healing, where bacterial numbers are comparatively fewer, inhibition of bacterial growth may oppose microbial prevention of the wound healing process, and the manuka honey can be controlled, maintained and refreshed at effective concentrations. Moreover, investigators are still uncovering various immunostimulatory and wound healing properties (Timm et al., 2008; Tonks et al., 2001; Tonks et al., 2003; Tonks et al., 2007) and antioxidant activities (Henriques et al., 2006; Inoue, 2005a) of manuka honey, which continue to stimulate research, generate unanswered questions, and provide potential health benefits to the consumer.

8.3 Future Work

This thesis has opened some new avenues for exploration of the effects of manuka honey, osmotically active sugars, and MGO, on bacteria such as *E. coli*. The precise mechanisms of action, optimum conditions for activity, apparently contradictory responses of the bacterium, and structural integrity of the bacterial membrane (and nucleic material) still require investigation.

Furthermore, additional work on the characterisation of the manuka honey constituents, apparently bioactive and inactive, is still required. The source of the extremely high MGO concentrations in manuka honey, and the reason why it is unique to this variety, has only very recently started being elucidated (Adams et al., 2009). The role that honey phenolics, proteins and sugars may play in stabilising or binding this reactive electrophile, and contributing to its activity in the complex honey matrix still requires substantial investigation. The antioxidant and immunostimulatory properties are still essentially uncharacterised and unexplained, and the role of the newly discovered MGO in those functions, if at all, requires further investigation.

8.4 Conclusion

In conclusion, manuka honey is a complex matrix of bioactive compounds which exert an inhibitory, but not necessarily lethal, effect on pathogenic bacteria, by mechanisms still to be clarified, and no doubt to be identified. Manuka honey also possesses a number of other bioactive functions derived from its numerous constituents, and much further investigation of its composition, formation, storage and activities awaits.

APPENDIX A

Poster 1: AIFST 40th Annual Convention, Melbourne, Australia, June 2007

CROP & FOOD RESEARCH

Mana Kai Rangahau

Douglas Rosendale
Email: RosendaleD@crop.cri.nz

Juliet Sutherland SutherlandJ@crop.cri.nz
Lynn McIntyre Lynn.McIntyre@esr.cri.nz
Institute of Environmental Science and Research.
Ian Maddox I.S.Maddox@massey.ac.nz
College of Sciences, Massey University.
Margot Skinner MSkinner@hortresearch.co.nz
The Horticulture and Food Research Institute of NZ.

Combining extracts of functional food ingredients for managing the growth of good and bad gut bacteria

D Rosendale, L McIntyre, I Maddox, M Miles, M Rodier, M Skinner, J Sutherland

Introduction

The development of concept functional foods, made from raw ingredients extracted from New Zealand food sources, is an interesting alternative to pharmaceutical products to improve gastrointestinal health. We aimed to promote and maintain a healthy gut ecosystem by identifying food ingredients or combinations of ingredients with a prebiotic mode of action, increasing the growth of good, probiotic gut bacteria whilst decreasing the growth of bad, pathogenic bacteria. Rather than seeking a single 'silver bullet' compound, we were interested in ingredients and synergistic ingredient combinations with multiple modes of action. Functional food ingredients were extracted and subjected alone or in combination to a panel of probiotic and pathogenic gut bacteria. Dose-dependent effects of single extracts on the growth of the organisms were measured. Single-dose combinations of the ingredients were tested, revealing that some specific combinations exerted desirable or undesirable effects.

Methods

Single extract assay

Extracts screened for the ability to increase or decrease growth of broth cultures (2-fold dilution series, 8 reps, 3 independent tests), with appropriate controls. Effects determined by measuring OD before and after 16 h growth, and expressed as Δgrowth:

$$\Delta\text{growth} = \left(\frac{(\text{extract OD-blank OD}) \times 100)}{(\text{control OD - blank OD})} \right) - 100$$

This resulted in positive or negative values representing increased or decreased growth, respectively. Deviation from zero (control cultures) was a measure of relative efficacy.

Combined extract assay

Combined extract assays were performed as described above. Extracts used at half the concentration of the top single extract dose.

Data compared to determine 'desirable' combinations of extracts. In a functional food context, desirable combinations result in increased probiotic growth and decreased pathogen growth relative to either single extract, and an undesirable combination achieved the opposite. Two simple comparisons were performed:

1. Does ΔgrowthAB exceed (ΔgrowthA + ΔgrowthB)?
2. Does ΔgrowthAB exceed **either** ΔgrowthA **or** ΔgrowthB?

Extract combinations which met Comparison 1 were termed 'good', as they performed better than mathematically predicted. Those which did not were termed 'poor'. Good combinations did not necessarily outperform single extracts acting alone.

Extract combinations which met both requirements, having a superadditive effect greater than either extract alone or predicted in combination, were termed 'desirable' or 'undesirable'.

Materials

Organisms

Probiotic organisms *Lactobacillus reuteri* DPC16, *Lactobacillus rhamnosus* HN001 (DR20™) and *Bifidobacterium lactis* HN019 (DR10™) grown anaerobically at 37°C in MRS plus 0.05% (w/v) cysteine.

Pathogenic organisms S*almonella enterica* serovar *Typhimurium* ATCC 1772, *Escherichia coli* O157:H7 strain 2988 and *Staphylococcus aureus* ATCC 25932 grown aerobically at 37°C in TSB or BHI broth (*S. aureus*).

Appropriate steps were taken to maintain the purity, age and acclimatisation of the cultures.

Extracts

Food ingredients as follows. Bee products kindly supplied by Comvita NZ Ltd. Plant compounds freeze-dried after purchase from local (Christchurch, NZ) suppliers.

- Manuka honey UMF™20+ (known anti-pathogenic activity), bee pollen granules (mixed floral source, anti-oxidant), rosehips (Sweet Briar, *Rosa rubiginosa*, antioxidant, anti-microbial/adjuvant) and BroccoSprouts® (anti-oxidant, anti-*H. pylori* activity) solubilised in buffer, homogenised, coarse-filtered, centrifuged and sterile-filtered.
- Propolis (80% tincture, anti-oxidant, anti-inflammatory, anti-bacterial) and blackcurrant oil (anti-inflammatory, anti-microbial/adjuvant) solubilised in ethanol or DMSO, respectively, then diluted in water.
- Concentrations determined by freeze-drying known quanitities of extract to determine dry weight of soluble components.

Results and discussion

Single Extracts

Growth of bacteria with single extracts presented in **Figure 1**.

- Manuka honey increased probiotic growth and decreased pathogen growth. Decreased pathogen growth expected. Increased probiotic growth not observed elsewhere in the literature by these authors.
- Bee pollen had a biphasic response, with initial inhibition followed by region of increased bacterial growth. Activity presumably due to unknown compounds of plant origin.
- Rosehip and BroccoSprout® extracts generally encouraged or had little effect on bacterial growth.
- Blackcurrant oil and propolis tended to have little effect or decrease bacterial growth. Activity presumably due to non-polar compounds perturbing bacterial membrane.
- Differences in bacterial susceptibility based upon whether probiotic or pathogen.

Combinations

Growth of the probiotic and pathogenic bacteria with the combinations of extracts is summarised in **Table 1**.

- Desirable combinations included manuka honey combined with bee pollen, rosehips, BroccoSprouts®, blackcurrant oil and propolis. Propolis, blackcurrant oil, rosehip or BroccoSprouts® extracts in combination with each other also tended towards desirable effects.
- Undesirable combinations included manuka honey combined with rosehips or propolis, or propolis, blackcurrant oil, rosehips or BroccoSprouts® combinations.
- Less soluble compounds, propolis and blackcurrant oil, and the plant extracts rosehips and BroccoSprouts®, appear to be useful as adjuvants to increase or decrease effects of other active ingredients.
- Manuka honey activities appear to be particularly susceptible to presence of other extracts.
- Response of the bacteria to the combinations of extracts appears to be strain-specific.

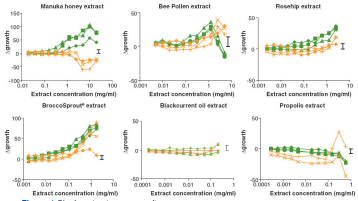


Figure 1 Single extract assay results.
Δgrowth of bacterial cultures supplemented with increasing doses of functional food extracts.
Probiotic bacteria:
Lactobacillus reuteri strain DPC16 (◆),
Lactobacillus rhamnosus strain DR20 (▲)
and *Bifidobacterium lactis* strain DR10 (■).
Pathogenic bacteria:
Staphylococcus aureus (◆),
Escherichia coli strain O157:H7 (+)
and *Salmonella typhimurium* (x).
Data points show the mean (n=24) 16 h growth at 37°C, obtained over three separate experiments with 8 replicates per experiment.
The bar displays the Least Significant Difference ($P < 0.05$).

Table 1 Combined Extract Assay Results.
+ or – denotes positive or negative Δgrowth value, with multiples denoting increasing deviation from the control.

Extract	Manuka honey	Bee pollen	Rosehip	Brocco Sprout®	Blackcurrent oil	Propolis	Strain
Manuka honey	+++	++++	+++	++++	+++	----	DPC16
	++	++++	+	++++	+++	---	DR10
	++	+++	++	+++	+	----	DR20
	---	--	---	---	--	---	E. coli
	--	---	---	---	---	---	S. ent.
	-	++	-	+	+	---	S. aur.
Bee pollen		++++	+++	++++	+++	+	DPC16
		++++	+++	++++	+++	-	DR10
		++	+	+	++	--	DR20
		++	++	+	+	-	E. coli
		++	++	+	++	+	S. ent.
		++	+++	++	+	-	S. aur.
Rosehip			+	+++	++	--	DPC16
			++	++	++	-	DR10
			+	+++	-	---	DR20
			+	+	+	-	E. coli
			+	+	+	+	S. ent.
			+	++	+	----	S. aur.
Brocco Sprout®				+++	++	++	DPC16
				++	++	++	DR10
				+++	+++	+	DR20
				+	+	+	E. coli
				-	+	+	S. ent.
				+	+	-	S. aur.
Blackcurrant oil					+	-	DPC16
					-	-	DR10
					+	--	DR20
					+	-	E. coli
					+	+	S. ent.
					+	-	S. aur.
Propolis						-	DPC16
						-	DR10
						---	DR20
						-	E. coli
						-	S. ent.
						-	S. aur.

Desirable combination
Good combination
Poor combination
Undesirable combination

Conclusions

- Manuka honey is particularly effective, both alone and in combination with other food ingredient extracts.
- Plant-derived and/or less soluble extracts generated the most interesting effects in combinations.
- Combinations of extracts yield unusual results, sometimes desirable and sometimes undesirable, and in a strain-dependent fashion.
- Caution and testing advised before combining functional food ingredients in food products – two 'good' ingredients combined could give rise to a 'bad' or undesirable effect.

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Crop & Food Research
Private Bag 4704
Christchurch, New Zealand
Tel. +64 3 325 6400
Fax +64 3 325 2074

Research Summary: MacDiarmid Young Scientist of the Year Awards, Auckland, March 2008

Douglas. I. Rosendale

Functional food ingredients for managing the growth of good or bad gut bacteria

Research Summary.

Introduction

Gastrointestinal health is a major factor in human well-being and quality of life. A major contributing factor to the maintenance of gut well-being and health is the state of the gut ecosystem. The aim of my research is to develop non-pharmaceutical means of promoting a favourable gut ecosystem using food ingredients in collaboration with Comvita, a New Zealand health food company. I have screened ingredients or ingredient combinations to promote probiotic bacterial growth whilst controlling pathogen growth. The particular target is multiple modes of action acting synergistically on gut bacteria rather than seeking a single "silver bullet" compound. The best performing ingredients have been tested in an animal feeding trial. Results indicate that particular food ingredients do favourably impact on the gut microflora, and test tube results are corroborated in the live gut. The most promising ingredient is being examined to determine the mechanism of action: how bacteria react to it at the cellular or molecular level.

Methods

New Zealand sourced ingredients, comprising bee- and plant-derived products, selected on the basis of their probable antimicrobial activity or potential to increase the effects of antimicrobial compounds. These ingredients, including UMF™ manuka honey, were assessed at different concentrations. Effects determined by measuring optical density before and after probiotic or pathogen growth, and expressed as change (Δ) in growth values. These indicated positive or negative deviations from control culture growth – an index of relative efficacy.

Combined extract assays were performed as described above. Data were compared to determine 'desirable' combinations of extracts. In a functional food context, desirable combinations result in increased probiotic (good bacteria) growth and decreased pathogen (bad bacteria) growth relative to either given as a single extract. An undesirable combination achieved the opposite effect. Identification of synergistic activity was mathematically calculated from Δgrowth values and ingredient concentrations.

Probiotic organisms *Lactobacillus* and *Bifidobacterium* and pathogenic organisms *Salmonella*, *Escherichia coli* O157:H7 and *Staphylococcus aureus* were grown under favourable aeration conditions, with steps taken to maintain the purity, age and acclimatisation of the cultures.

Normal, healthy mice were fed diets incorporating the selected ingredients. The health and growth of the animals was monitored and documented throughout the trial. The effects of the ingredients on gut health were determined by comparing changes in specific bacterial populations using molecular biology techniques (Real-Time Polymerase Chain Reaction

(RT-PCR)). Changes in overall gut microbial metabolism were compared by measuring bacterial metabolites (Short Chain Fatty Acids (SCFAs)) that are known to reflect gut microflora composition and influence host gut tissue metabolism. Numbers and activity of host peritoneal macrophage cells were measured as indicators of innate gut immune function.

Results.

A food ingredient, UMF™ manuka honey, both increased probiotic growth and decreased pathogen growth in culture, in a dose-dependent manner. This effect is extremely interesting and has hitherto not been reported. Other ingredients uniformly increased or decreased growth of all bacteria tested. Combinations of ingredients synergistically increased growth, whilst undesirable combinations were also observed. Interestingly, certain non-polar and/or plant-derived ingredients synergistically increased the efficacy of other ingredients, particularly UMF™ manuka honey, despite being relatively inactive when tested alone. Differences in bacterial susceptibility towards the single ingredients tended to reflect whether the strain was probiotic or pathogenic, whilst response to combinations was bacteria strain-specific.

Results from the animal feeding trial indicated that UMF™ manuka honey increased the observed numbers of probiotic populations whilst decreasing pathogens, confirming the results of the screening experiments performed on pure bacterial cultures. No differences in gut immune health or function were observed, confirming that the ingredients had no detrimental effect on gut health. Data revealing potential effects on microbial metabolism (SCFA production) are imminent.

Additional data on the contribution of various UMF™ manuka honey factors that inhibit *E. coli* O157:H7 growth confirm multiple modes of action (pH, UMF™, osmotic pressure) interact to inhibit growth. These modes of action are still being explored in depth.

Conclusions

UMF™ manuka honey favourably altered the mouse gut microflora, human clinical trial planning is underway, and the mechanisms of action are being explored. The novel finding that UMF™ manuka honey acts as a prebiotic is a fascinating and important result. Other ingredients in combination have the potential to contribute in a synergistic manner.

Collectively, these data confirm the viability of New Zealand functional food ingredients as an alternative, non-pharmaceutical approach to maintaining human gut health and well-being. This work provides the scientific basis which underpins development by Comvita and Crop & Food Research's Food Concepts Unit of a new functional food aimed at entry into the global food market.

Poster 2: MacDiarmid Young Scientist of the Year Awards, Auckland, August 2008

APPENDIX B

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Original article

High-throughput microbial bioassays to screen potential New Zealand functional food ingredients intended to manage the growth of probiotic and pathogenic gut bacteria

Douglas I. Rosendale,[1,2]* Ian S. Maddox,[2] Michelle C. Miles,[1] Maroussia Rodier,[1] Margot Skinner[3] & Juliet Sutherland[4]

1 Nutrition and Health Group, New Zealand Institute for Crop and Food Research Limited, Mt Albert Research Centre, 120 Mt Albert Road, Sandringham, Auckland 1025, New Zealand
2 College of Sciences, Massey University, Albany Campus, Albany, Auckland 1311, New Zealand
3 The Horticulture and Food Research Institute of New Zealand Limited, Mt Albert Research Centre, 120 Mt Albert Road, Sandringham, Auckland 1025, New Zealand
4 Nutrition and Health Group, New Zealand Institute for Crop and Food Research Limited, Palmerston North Research Centre, Batchelar Road, Palmerston North 4474, New Zealand

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Summary A spectrophotometric bioassay was used to screen selected food ingredients intended for development of functional foods designed to influence the growth of gut bacteria. Dose–response profiles displaying Δ_{growth}, the magnitude of deviation from growth of controls, were generated for probiotics *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Bifidobacterium lactis* and pathogens *Escherichia coli*, *Salmonella* Typhimurium and *Staphylococcus aureus*. Ingredients were manuka honey UMF™20+ (dose-dependently increased probiotics and decreased pathogens); bee pollen (biphasic growth effects against all); Rosehips and BroccoSprouts® (increased all dose-dependently); blackcurrant oil (little effect) and propolis (inhibited all strains). Ingredients were also bioassayed in pairs to assess desirable or undesirable synergistic interactions. Observed synergies included manuka honey (predominantly desirable); rosehips or BroccoSprouts® (desirable and undesirable); blackcurrant oil (desirable) and propolis (tended towards synergies reinforcing its antimicrobial effects), collectively revealing a complex web of interactions which varied by ingredient and bacterial strain. Manuka honey was particularly effective at influencing gut bacteria. The surprising frequency of undesirable synergistic interactions illustrates the importance of pre-testing potential ingredient combinations intended for use in functional foods.

Keywords Bioassay, functional food, New Zealand, pathogen, probiotic, screening, synergies.

Introduction

The gastrointestinal (GI) tract is the body's largest tissue boundary, essentially continuous with the outside of the body, which interacts with nutrients, exogenous compounds and gut microflora in a complex interplay of environmental factors and genetic elements. The host and complex microbial communities coexist, interact and compete in conditions that are often far from optimal.

Microorganisms colonise the GI tract heavily, primarily in the distal gut, to the extent that they vastly outnumber the cells forming the human body (Madara, 2004). These resident bacteria form complex ecosystems with enormous diversity, and more than 50% are believed to be unculturable by conventional techniques (Tlaskalova-Hogenova *et al.*, 2004). The commensal (normal, indigenous) microflora coexists with their host in a mutually beneficial arrangement and although they are able to exert pathological effects, it is rare for them to do so. Pathogenic organisms, including members of the genera *Escherichia*, *Salmonella* and *Staphylococcus*, are generally believed to be transient inhabitants, although some pathogens (such as *Helicobacter*) are able to form stable infections, where they colonise the host over a longer term without the manifestation of symptoms associated with the presence of the organism (Acheson & Luccioli, 2004).

Commensal organisms are one of the main factors that prevent the establishment of pathogenic organisms

*Correspondent: Fax: +64 9 845 9029;
e-mail: rosendaled@crop.cri.nz

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in the gut, via a number of mechanisms including the production of antimicrobial compounds, competition for food or excluding the pathogens from environmental niches (Rolfe, 2000; Reid & Burton, 2002; Saxelin *et al.*, 2005). The study of the interactions between commensal organisms, pathogenic organisms and the host is a complex and growing research field, particularly in terms of probiotic organisms – defined strains of commensal bacteria such as members of the genus *Lactobacillus* and *Bifidobacteria*, that show specific, beneficial functions in gut health, with positive influences on the gut microbiota and anti-pathogenic activity as well as influencing human health and nutritional status (Collins & Gibson, 1999; FAO/WHO, 2001; Rusch, 2002; Sanders, 2003; Tannock, 2004; Rastall *et al.*, 2005; Martin *et al.*, 2008).

There is an interest in traditional and non-conventional medicines either to treat imbalances or to maintain a healthy balance of complex microflora–host interactions in the gut. This includes the use of functional foods, foods which provide more than nutritional benefits to the consumer (Roberfroid, 2000). We are developing potential functional food ingredients that modulate the gut microflora for the promotion and maintenance of healthy GI microbial homeostasis. Such modulation may take the form of prebiotics; and food ingredients (usually oligosaccharides) indigestible by the host that are utilised by and encourage the growth of probiotic organisms and beneficially modify the health of the host (Gibson & Roberfroid, 1995; Collins & Gibson, 1999; Schrezenmeir & de Vrese, 2001; Rastall *et al.*, 2005; Parracho & Gibson, 2007). Focusing on prebiotics avoids viability issues with probiotic supplementations, whilst ensuring that organisms native to the gut are targeted (Gibson & Roberfroid, 1995). Other food ingredients may act through antimicrobial compounds which prevent or discourage the growth or establishment of pathogens. Food ingredients, or ingredient combinations, are likely to have multiple modes of action, so their use may carry the additional benefit that the development of microbial resistance to them is unlikely. Collectively, these effects could be a potential alternative to pharmaceutical intervention for people with pathogen-related disorders as well as preventatives for symptom-free people.

This work describes high-throughput bioassay screening of the potential antimicrobial and/or prebiotic effects of six potential functional food ingredients of New Zealand origin; manuka honey UMF™ 20+, propolis, bee pollen, BroccoSprouts®, rosehips and blackcurrant seed oil. These ingredients were selected as trial ingredients for the development of a rapid selection system for use as either a stand-alone food ingredient or as part of a combination with other ingredients, on the basis of potential or known ability to impact upon microbial growth. We regard the testing of ingredients in combination with each other as important, if not more so, than the ability to act alone, given that ingredients will be consumed as a part of a complex food matrix. Furthermore, a simple process for the identification of synergistic interactions between ingredients, whereupon a combination generates an effect greater than the sum of the individual ingredients, would be of value to the food industry and to the consumer looking to maximise the benefits of functional food for the purposes of managing the gut ecosystem. Finally, we aimed to determine whether unwanted interactions or antagonisms could result from the mixing of individually efficacious ingredients. Such knowledge would impact heavily upon the selection of ingredient combinations made by both the food industry and the consumer.

To identify and effectively communicate combinations useful for the development of concept functional foods, the combinations of ingredients were to be categorised on the basis of how they performed relative to the efficacy of the ingredients tested independently, against any given bacteria. Two categories were established, termed 'desirable' combinations and 'undesirable' combinations. A 'desirable' combination was defined as an increase in probiotic growth or a decrease in pathogen growth compared to the growth achieved using either extract independently, whilst an 'undesirable' combination was defined as decreased or increased probiotic or pathogen growth, respectively.

There are a variety of techniques available for measuring the antimicrobial activity of natural compounds outlined by Patton *et al.* (2006) who investigated manuka honey antimicrobial activity. These researchers outline the advantages and disadvantages of the three primary methods: disc diffusion, well diffusion and spectrophotometric analysis, with the demonstrable conclusion that the latter method was more accurate, sensitive, reproducible, faster and cheaper. The possibility for extensive kinetic studies with lower concentrations than possible with well diffusion assays was claimed (Patton *et al.*, 2006). Thus, a spectroscopic microplate assay method is suitable for measuring changes in microbial growth during a screening program of potentially antimicrobial ingredients, where the format (microplate layout), number of replicates and control well designs could be optimised for rapid, accurate and consistent high-throughput screening and for ease of subsequent statistical analyses. In addition, the spectrophotometric measurement of optical density (OD) has the added benefit of being a more suitable index of final microbial biomass than use of viable cell counts (Krist *et al.*, 1998), as viable cell counts does not necessarily represent biomass due to differences in cell mass and shape, where same biomass can be contained within several smaller cells or few larger cells. OD closely correlates with biomass except in extreme cases (Krist *et al.*, 1998).

Thus the two aims of this work could be summarised as follows:

Firstly, to establish a rapid spectrophotometric microbial growth assay and a manner of expressing the efficacy of the functional food ingredients. These simple tests may be applied during subsequent investigation of the efficacy of other potential functional food ingredients intended to manage the gut microflora. Thus this investigation was initiated by determining the pro- or antimicrobial dose–response profiles of the selected ingredients in detail, and by identifying which ingredient combinations are capable of generating synergistic (or unwanted antagonistic) effects against a panel of both probiotic and pathogenic bacteria.

Secondly, this work was to form a first step in confirming the viability of New Zealand functional food ingredients as an alternative, non-pharmaceutical approach to maintaining human gut health and wellbeing through promotion of gut microbial homeostasis.

Methods

Microbial methods

Organisms used in this study included *Lactobacillus reuteri* DPC16, *Lactobacillus rhamnosus* HN001 (DR20™), *Bifidobacterium lactis* HN019 (DR10™), *Salmonella enterica* serovar Typhimurium ATCC 1772, *Escherichia coli* O157:H7 strain 2988 and *Staphylococcus aureus* ATCC 25932. *Lactobacillus reuteri*, *S.* Typhimurium and *E. coli* were supplied by Bioactives Research New Zealand Ltd, Auckland, New Zealand. *Lactobacillus rhamnosus* and *B. lactis* were from The New Zealand Dairy Research Institute, Palmerston North, New Zealand. *Staphylococcus aureus* belonged to the Functional Microbiology Laboratory collection, The New Zealand Institute for Crop and Food Research Ltd, Auckland, New Zealand.

For this work we confined the choice of bacterial isolates used for the testing to a select few. Probiotic organisms selected were *L. reuteri*, *L. rhamnosus* and *B. lactis*. Pathogenic organisms selected were *E. coli*, *S.* Typhimurium and *S. aureus*. These probiotics and pathogens grew under similar respective culture conditions, which were deemed important because isolates requiring different growth conditions or significantly differing nutrient supplementation or growth rates (e.g. *H. pylori*) would hinder high-throughput analysis of growth. The strains were relevant to the purposes of the investigation based upon their ability to contribute to (probiotic strains) or impacting negatively upon (pathogenic strains) the intestinal wellbeing of the normal healthy individual (cf. *Listeria monocytogenes*).

Aerobic and anaerobic organisms were stored at −80 °C in broth containing 15% (v/v) glycerol. Frozen cultures were revived by scraping a loop across the frozen stock and streaking on an agar plate to produce single colonies upon overnight incubation under conditions of appropriate aerobicity. Purity was assessed microscopically by Gram staining. Single colonies were used to inoculate broth. Broth cultures were routinely grown or passaged by inoculating a loopful (10 μL) into glass screwcap tubes (10 mL). Caps were not fully tightened, to ensure exposure to the correct atmosphere. Cultures were grown at 37 °C in a Contherm Digital Series Incubator. A minimum of four subcultures on broth, performed daily, were used to ensure organisms were fully adapted to the media prior to use for any microbial experiments.

The aerobes *S.* Typhimurium and *E. coli* were grown on tryptic soy broth (TSB), whilst *S. aureus* was grown on brain heart infusion (BHI) broth. The anaerobic bacteria *L. reuteri*, *L. rhamnosus* and *B. lactis* were grown on de Mann Rogosa Sharpe (MRS) broth supplemented with 0·05% (w/v) cysteine in Gas-Pak™ EZ Incubation Chambers containing Gas-Pak™ EZ Gas Generating Sachets. Where necessary, vessels were monitored with anaerobic indicator strips.

Bacterial inoculums for the microbial assays were prepared by estimating the culture density with a haemocytometer and adjusting to that required for the assay (10^2 cells mL^{-1} for *L. reuteri*, 10^3 cells mL^{-1} for all others). Viable counts of the inoculum were routinely performed by spread plating and calculating colony forming units (CFU) mL^{-1} to confirm the haemocytometer counts.

Food ingredient preparation

Manuka honey UMF™ 20+, bee pollen granules (mixed floral source) and propolis (80% tincture) were supplied by the manufacturer (Comvita New Zealand Ltd, Bay of Plenty, New Zealand). Blackcurrant seed oil was purchased from Nutrizeal Ltd (Nelson, New Zealand) and supplied sealed under N_2 gas, and rosehips (Sweet Briar, *Rosa rubiginosa*) and BroccoSprouts® were purchased from local suppliers (Christchurch, New Zealand) and freeze-dried within 5 days of purchase.

All samples except propolis and blackcurrant oil were solubilised using 25 mM sodium phosphate buffer pH 7·4. Bee pollen samples also contained 0·2% DMSO in the buffer to aid solubilisation. These suspensions were homogenised, filtered through Whatman No. 4 filter paper, centrifuged to remove particulate matter, and sterilised through a series of 0·8, 0·4 and 0·2 μm filters. Propolis and blackcurrant oil were solubilised in ethanol or DMSO, respectively, and then diluted in deionised water for use. To establish the concentration of soluble material in the ingredient solution an aliquot of the solution was dispensed (0·5 mL) into pre-weighed tubes immediately after filtration, lyophilised and the dry weight of the soluble material calculated, including

corrections for the buffer salt weight. The ingredients and their concentrations used in the microbial growth assay (below) are given in Table 1.

Ingredient solutions were dispensed in aliquots and frozen at −80 °C, and aliquots used only once, to prevent any freeze/thaw-induced denaturing of active components. The blackcurrant seed oil extract aliquots were sealed under N_2 gas to minimise oxidation and then frozen. Ingredients were thawed to room temperature and diluted as required immediately prior to each assay. All ingredients used during this work were solubilised as part of a single batch, so that batch-specific variation could be avoided.

Microbial growth assays

A ninety-six-well microplate growth bioassay measuring optical density (OD) was used throughout this work. To compare ingredient effects on a group of diverse bacterial strains which achieve quite different ODs, a standardised value expressing the change in growth of cultures in the presence of ingredient(s) relative to the growth of unsupplemented control cultures, or Δ_{Growth}, was calculated and used to represent the magnitude of effect. This value was calculated by the simple method of converting the OD to a percentage of the control OD and then subtracting 100, effectively normalising the control growth to a baseline value of zero (eqn 1).

$$\Delta\text{Growth} = \left(\frac{(\text{Extract OD} - \text{blank OD}) \times 100}{(\text{Control OD} - \text{blank OD})}\right) - 100 \quad (1)$$

This represents the magnitude of deviation of the growth of a culture from the growth of the control culture, which had not been supplemented with ingredient. This resulted in a positive or negative Δ_{growth} value representing increased or decreased growth, respectively, where the magnitude of deviation from the control Δ_{growth} value was a measure of the ingredient's relative efficacy. This was found to be the best method of graphically comparing the effects of varying concentrations of the same ingredient against multiple bacterial strains.

Table 1 Ingredients and concentrations at the highest dose for the single ingredient assay

Ingredient	Concentration (mg mL^{-1})
Manuka honey UMF™ 20+	200
Bee pollen[a]	5·0
Rosehips	2·5
BrocoSprouts®	1·75
Blackcurrant seed oil[b]	0·21
Propolis[b]	0·6

[a]Includes DMSO.
[b]Includes ethanol.

Single ingredient assay

For single ingredient analyses, the first column of a ninety-six-well microplate was filled with the appropriate bacterial growth medium supplemented with an ingredient. Multiple microplates were prepared, one microplate per strain of bacteria, and with separate microplates for separate ingredients. A range of ingredient concentrations was examined by conducting a two-fold dilution series across the microplate, moving left to right from the first column, containing the highest concentration (Table 1). This resulted in eleven dilutions each containing eight replicate wells (50 μL), each dilution half the concentration of the previous one. Eight replicate control wells containing medium without ingredient were always included in the last column of the ninety-six-well microplate.

Microplates were inoculated with an equal volume (50 μL) of bacterial inoculum, and the optical density (OD) of the microplate was immediately measured at a wavelength of 620 nm using a Thermo Multiscan EX ninety-six-well plate reader to determine the blank (zero growth) value. Microplates were incubated at 37 °C for 16 h, and then the OD was determined to measure the growth of the cultures at late log phase-early stationary phase growth of the organisms. The 0-h reading was subtracted from the 16-h end-point reading to eliminate all changes in optical density not due to growth. Therefore, potential variations in plate density, media colour or any other unknown factors could be accounted for. Furthermore, un-inoculated ingredient controls were routinely included to confirm the sterility of the ingredients.

Data were subjected to statistical analysis using the Genstat program (Genstat Release 8·2, Lawes Agricultural Trust, Rothamsted Experimental Station, UK) because it allowed pooling of results for comparison across ingredients, and across bacterial strain, from multiple determinations, by determining analysis of variance using the two-tailed ANOVA function. The least significant difference (LSD) of the means ($n = 8$), at the 95% confidence interval ($P < 0·05$) for all of the strains tested for a particular ingredient, was shown on a single graph per ingredient. This allowed simple comparison of the effects of an ingredient on multiple organisms, which was particularly useful when comparing the effects on probiotics vs. pathogens.

The pH of the culture medium, before and after incubation with bacteria, was measured using an ISFET KS701 pH meter (Shindengen, Tokyo, Japan), both in the presence of the maximum ingredient concentration used, and in the control cultures. These measurements were to exclude either changes in pH, or buffering of the

media, as reasons for any observed increases or decreases in bacterial growth.

To estimate the bacterial viability counts where necessary, ingredients were assayed for antimicrobial activity exactly as described above, except that resulting cultures were serially diluted in medium and spread plated on agar plates. Colonies were counted and the CFU mL^{-1} of the original well culture was determined and compared to the control (unsupplemented) cultures.

Combined ingredient assay

Combined ingredient assays were performed exactly as described for single ingredient assays above, except maintaining the same assay volume meant that ingredients were present at half the concentration of the highest dose used when tested singly.

However, to compare the efficacy of these combinations with those obtained using the extracts independently, and to mine the data for the most efficacious combinations, additional processing of the data was required. This would serve to identify potential synergistic or unwanted antagonistic interactions in a rapid and simple manner consistent with the aims of this work: to identify those combinations worth pursuing further, and to recognise combinations to be avoided.

Two simple comparisons were performed, in order to assign the terms of effect (desirable, good, poor, undesirable) introduced previously, and calculated according to the following methods:

Comparison 1: Combination Δ_{growth} values were noted that significantly ($P < 0{\cdot}05$) deviated further from the control value (zero) than the theoretical sum of the Δ_{growth} values of the individual extracts (eqn 2).

$$\text{Does } \Delta_{GrowthAB} \text{ exceed } (\Delta_{GrowthA} + \Delta_{GrowthB})? \quad (2)$$

This immediately identified Δ_{growth} values which were potentially synergistically acting to exert prebiotic or antimicrobial effects, as the activity of the combination was more than the sum of the effect of the ingredients acting independently. The term 'exceed' (eqn 2) is used because this equation is designed to compare like values with like (positive Δ_{growth} with positive Δ_{growth}, negative Δ_{growth} with negative Δ_{growth}). This simplistic comparison does not take into account the effect of adding unlike values, i.e. a weak positive growth value added to a strong negative growth value, or *vice versa*, in effect cancelling out a part of the apparent effect of the stronger contributor. Thus a second comparison was introduced.

Comparison 2: Combination Δ_{growth} values were noted that significantly ($P < 0{\cdot}05$) deviated further from the control (zero) value than the most extreme of the Δ_{growth} values from either one of the ingredients independently (eqn 3).

$$\text{Does } \Delta_{GrowthAB} \text{ exceed } \textit{either } \Delta_{GrowthA} \textit{ or } \Delta_{GrowthB}? \quad (3)$$

This identified ingredients displaying an apparently increased efficacy despite an artificial lowering of the sum of the activities obtained from eqn 2. For example, an ingredient providing an extreme Δ_{growth} such as a 100% increase in growth over the control culture (a Δ_{growth} value of 100) combined with an ingredient inhibiting the growth to only 80% of the growth of the control (a Δ_{growth} value of –20) would have an apparent $\Delta_{growthAB}$ value of 80 using eqn 2. Should the growth of the combination of ingredients yield a number such as a 90% increase in growth (Δ_{growth} value of 90) then eqn 2 would identify a potentially synergistic effect, which was, in fact, less than the activity of the first ingredient acting independently. However, should the combination yield, for example, a 120% increase, or Δ_{growth} of 120, then that would satisfy the comparisons drawn from both eqns 2 and 3 and represent a synergistic combination.

Ingredient combinations which met Comparison 1 were termed 'good', that is, the combination performed better than mathematically predicted. Those which did not meet Comparison 1 were termed 'poor'. Again, good combinations did not necessarily outperform single ingredients acting independently.

Ingredient combinations which met both requirements, that is, had an effect greater than either ingredient alone or predicted in combination (synergistic), were termed 'desirable' or 'undesirable', based upon their activity relative to the bacteria (probiotic or pathogen) being tested, as described in the introduction to this paper.

Results and discussion

Single ingredient assay

A total of thirty-six dose–response curves were generated. Figure 1 displays the effects of the six ingredients, each against six bacterial species. Limitations on the quantity of two of the ingredients, blackcurrant oil and propolis, meant that only preliminary dose-response screening of these ingredients against *S. aureus* was performed (data not shown).

Manuka honey (Fig. 1). increased probiotic growth and decreased pathogen growth in a dose-dependent manner. There was a clear difference in the effect on the pathogens and probiotics, validating both the means of expressing the data (Δ_{growth} values) on the same chart for immediate visual recognition, and in the choice of model ingredient to trial the system.

The decrease in pathogen growth was expected. This result can be explained by a contribution of

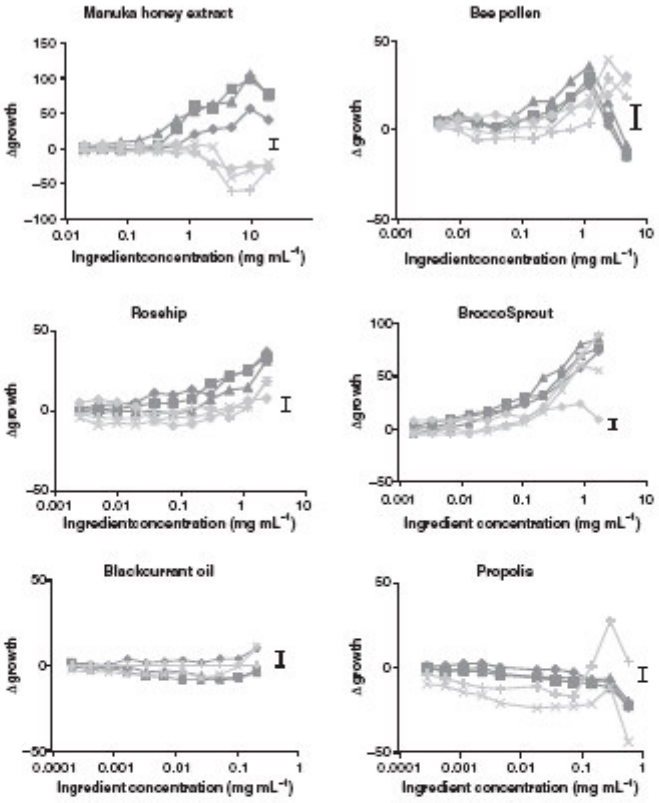

Figure 1 Δgrowth values from bacterial cultures supplemented with increasing doses of functional food ingredients. Probiotic bacteria: *Lactobacillus reuteri* strain DPC16 (♦), *Lactobacillus rhamnosus* strain DR20 (▲) and *Bifidobacterium lactis* strain DR10 (■). Pathogenic bacteria: *Staphylococcus aureus* (♦), *Escherichia coli* strain O157:H7 (+) and *Salmonella* Typhimurium (×). Data points show the mean (n = 24) 16 h growth at 37 °C, obtained over 3 separate experiments with 8 replicates per experiment. The bar displays the Least Significant Difference at $P < 0.05$.

factors: Manuka honey, derived from *Leptospermum* spp., has known wound-healing and antimicrobial properties (Molan, 2001). Honey antimicrobial properties have been largely attributed to the presence of residual peroxide (White *et al.*, 1963) arising from bee-derived glucose oxidase upon dilution. Honey also contains a high sugar content, sufficient to lower a_w enough to prevent microbial growth through osmotic shock. Other factors include acidic pH and the presence of plant-derived phenolic compounds (Molan, 1992). Manuka honey also has non-peroxide activity (Molan & Russel, 1998), the Unique Manuka Factor (UMF™), suggested to include very high levels of the 1,2-dicarbonyl compound methylglyoxal (MGO) (Weigel *et al.*, 2004; Adams *et al.*, 2008; Mavric *et al.*, 2008). Subsequent data (D. Rosendale, unpublished results) suggests that the inhibition of pathogen growth shown in this work is almost entirely due to the honey sugars lowering the a_w of the assay solution. The honey was used at a dose containing MGO at less than 0·3 mM. This concentration was below the limit required to inhibit *E. coli* growth (Ferguson *et al.*, 1996).

Increases in probiotic growth, a prebiotic effect attributed to manuka honey, have not been reported in the literature. This is a significant finding, and the mechanisms responsible for this outcome remain to be elucidated. Similar results against other organisms have been reported for honey such as against the yeast *Candida albicans* in the literature (Patton *et al.*, 2006), but the mechanism is currently unknown. Whilst honey is predominantly sugar, and could conceivably contribute to increased growth of the honey-supplemented cultures due to greater nutrient than present in the media of the control cultures, the probiotic growth media MRS is rich in glucose, thus lack of sugar limiting the growth of the control cultures relative to honey samples is unlikely.

Changes in the medium pH might be expected to affect bacterial growth, whereas buffering of the media could increase probiotic growth relative to the assay

control wells. This is because without buffering, accumulating acidic byproducts in the media inhibit growth. No ingredient-induced pH changes were observed (data not shown). In the case of the probiotic organisms, the expected lowering of pH after growth, induced by the acidic by-product, was observed. Essentially, growth (OD) was proportional to the pH, and thus proportional to the production of acidic by-products. Thus, the observed increases in probiotic growth could be attributed to factors other than the ingredients influencing the pH value of the medium.

Preliminary experiments suggest that sub-lethal (<0·3 mM) doses of MGO, such as those determined by HPLC to be present over the growth-promoting concentrations of manuka honey used in this work (D. Rosendale, unpublished results), might encourage increased growth of probiotics (D. Rosendale, unpublished results). This effect requires further investigation.

Bee pollen (Fig. 1) showed a biphasic response, with the most statistically significant effect manifesting as increased growth of bacteria at the second (pathogens) and third (probiotics) highest doses, 2·5 and 1·25 mg mL^{-1}, respectively. The highest doses appeared to inhibit the growth of the probiotics, although the significance of this is questionable. Currently, there are no reports in the literature discussing an antimicrobial function of bee pollen, and we have no mechanism yet to explain the increases in growth observed. The absence of a linear dose-dependent response implies that either (i) more than one component is involved in generating the observed effects, or (ii) a single active component behaves differently at different concentrations – an explanation not without precedent in the field of antimicrobial plant compounds (Inoue *et al.*, 2005). DMSO, at the doses used to solubilise this ingredient, has been shown to have no effect on the growth of these organisms (data not shown).

Rosehip and BroccoSprouts® solutions (Fig. 1) encouraged bacterial growth in a dose-dependent manner, although the probiotics were the most responsive to rosehips, whilst *S. aureus* was least responsive to the BroccoSprouts®. Rosehips (*Rosa* spp. fruit) have significant antioxidant activity (Gao *et al.*, 2000) and whilst numerous clinical trials have reported a variety of benefits, a meta-analysis of trials conducted with *Rosa canina* subspecies reveals that perhaps only benefits against osteoarthritis are valid (Chrubasik *et al.*, 2006). Rosehips have been reported to have antimicrobial activity associated with the phenolic fractions (Yi *et al.*, 2007). In addition, tellimagrandin I, a hydrolysable tannin extracted from the petals of the plant *Rosa canina*, inhibits *H. pylori* growth, although not growth of *E. coli* (Funatogawa *et al.*, 2004). Interestingly, tellimagrandin I synergistically increases methicillin-resistant *S. aureus* (MRSA) susceptibility to β-lactam antibiotics (Shiota *et al.*, 2000). The possibility that this compound may be present in rosehips is interesting and warrants further investigation. Cruciferous vegetables, specifically young plants such as BroccoSprouts® (*Brassica oleracea* var. *italica*), contain antioxidants and high levels of isothiocyanates, particularly sulforaphane, converted by myrosinase from glucosinolates (Shapiro *et al.*, 2001), which is active against *H. pylori* (Fahey *et al.*, 2002). Clearly these components were either not present in levels sufficient to negatively impact upon the growth of the tested organisms or the organisms tested may have simply not been susceptible to these compounds. It could be postulated that *S. aureus* was the organism least susceptible to the growth-encouraging effects observed with the other strains because of an increased susceptibility to antimicrobial components, but without quantitative analyses of these compounds and/or knowledge of the mechanisms involved, it remains speculative. The mechanism by which bacterial growth was enhanced has not been determined, but the ability of these ingredients to buffer the pH of the media has been examined and found to provide no significant buffering ability. Finally, the sugar content of these ingredients remain unknown, but again, as in the case of the manuka honey, the glucose content of the growth media should be more than enough to exclude sugar as a growth-limiting step which ingredient contributions could overcome.

Blackcurrant seed oil (Fig. 1) had little effect against any of the bacterial strains used in this work. Blackcurrant seed oil (*Ribes nigrum*) has been reported to affect the membrane of pathogens such as *H. pylori* to the extent that the oil has been suggested as an ideal adjuvant during the use of other antimicrobial agents (Frieri *et al.*, 2000). Thus, despite the lack of significant growth promoting or, alternatively, antimicrobial effects, blackcurrant oil is an ideal candidate for further investigation into possible synergies from ingredient extract combinations.

Propolis (Fig. 1) has anti-oxidant, anti-inflammatory, anti-tumour, immune-stimulating and hepato-protective activities (Burdock, 1998; Bankova, 2005) and antimicrobial activity against *H. pylori* (Banskota *et al.*, 2001) (Boyanova *et al.*, 2003), *Campylobacter* (Boyanova *et al.*, 2003) and *Staphylococcus* (Miorin *et al.*, 2003). It has also been observed to synergistically increase the effects of some antibiotics (Scazzocchio *et al.*, 2006). We observed that propolis tended to decrease bacterial growth of all strains as the concentration in the media increased, except for an apparent growth peak at 0·3 mg mL^{-1}, which was sufficient to increase growth of *E. coli* only at this concentration. This phenomenon of decreased growth from the propolis is likely to be due to perturbation of the cell membrane by phenolic compounds (Sikkema *et al.*, 1994). The similarities in the shape of the dose–response profile of pathogen growth on propolis and bee pollen have been noted, and

could reflect the presence of the same or a similar active compound(s).

OD data were compared to viable counts where it was found that increases or decreases in OD were accompanied by increases or decreases in plate counts (data not shown). Whilst statistical analyses were not performed, this assessment was considered sufficient to discount potential factors such as cell clumping or settling or, alternatively, major changes in cell morphology – factors which might have respectively increased or decreased the incident light reaching the photomultiplier and thus given apparent OD changes unrelated to growth or biomass.

Consistency of OD across experiments was assured by conducting initial experiments to optimise the process involved examining the microplate reader outputs for reproducibility and to ensure potential plate reader 'hot spots' (where wells of the ninety-six-well microplate or microplate reader might consistently read higher or lower than the surrounding wells) were accounted for. The same brand of ninety-six-well microplate (Costar, Corning, NY, USA) was used throughout to eliminate inter-brand microplate variation. After ensuring the spectrophotometer operated appropriately, the bacterial growth conditions have been tested and optimised to ensure the growth media, culture revival and handling, inoculum concentrations and incubation times could be standardised to consistently generate optical densities that would potentially allow deviations from the theoretical control value (i.e. cultures unsupplemented by extracts) to still fall well within the plate reader's useful absorbance range (an absorbance of 0·1–1·0) at late log phase growth under standard handling conditions (data not shown). These conditions were adopted for all experiments to maintain consistency and allow comparison with assays conducted at different times.

We elected not to calculate minimum inhibitory concentrations (MICs) of the ingredients from the OD data. Firstly, where food ingredients have unknown or poorly defined active components, presenting the data as Δ_{growth} for a given amount of solubilised ingredient material would not misrepresent our knowledge of the quality and quantity of active components. Secondly, perhaps the use of MICs is more suitable for compounds intended for pharmaceutical-based eradication procedures than management of gut microorganisms by dietary intervention. Finally, the expression of dose–response curves might shed some light on the nature of the Δ_{growth} values observed. Thus, we could speculate on the presence and effects of putative active components whilst the chemical determination and quantitative analyses required to establish their presence and concentration, and thus determine useful MICs, remains outside the scope of this paper. That is not to say that findings from this work would not be used to prompt such work in the future.

Combined ingredients assay

The effects of the combinations of ingredients on the growth of the bacteria are shown in Table 2. Combinations were noted that fulfilled the criteria 'desirable', 'undesirable', 'good' or 'poor'.

Desirable combinations included manuka honey combined with bee pollen (suppressed *S.* Typhimurium), with rosehips (suppressed *S.* Typhimurium, promoted *L. rhamnosus*), with BroccoSprouts® (suppressed *S. aureus*, promoted *B. lactis*), with blackcurrant oil (suppressed *S.* Typhimurium, promoted *B. lactis*) and with propolis (suppressed *S.* Typhimurium and *S. aureus*).

Undesirable combinations, in which combined activities promoting pathogen growth or suppressing probiotic growth exceeded the contribution from the component ingredients, included manuka honey combined with rosehip (suppressed *B. lactis*) or propolis (suppressed all three probiotic strains), or propolis or blackcurrant oil combined with rosehip or BroccoSprouts®.

Some ingredient combinations, such as manuka honey combined with propolis, or propolis combined with BroccoSprouts®, yielded both desirable and undesirable effects, depending upon which strain of bacteria was used. Thus, the response of the bacteria to any given combination of ingredients was often strain-specific. Mathematical analysis failed to show any significant relationship or pattern involving combination, strain and effect (D. Hedderley, personal communication). This is not unexpected, given the complex mixtures of potentially bioactive compounds present in the ingredients used in this study.

Manuka honey was particularly effective at increasing probiotic growth and reducing pathogen growth, both alone and in combination with other food ingredients.

The less soluble compounds, propolis and blackcurrant oil, and the plant ingredients rosehips and BroccoSprouts®, tended to generate the most interesting effects in combinations, and thus appear suitable for use as adjuvants or mitigants to moderate the effects of other active ingredients. It is feasible that this may be attributable to fatty acid or phenolic compound(s) perturbing bacterial membranes. Plant compounds (phenolics, polyphenolics, flavones, flavanoids, tannins, coumarins, terpenes and alkaloids) are known antimicrobial agents with a variety of mechanisms of action including reacting with proteins or perturbing membranes thereby increasing permeability, depending on the lipophilicity of the compounds [reviewed by Cowan (1999)]. The generation of synergistic responses may be a consequence of the low concentration of the ingredients, where at higher concentration they may have exerted direct antimicrobial activity. Some plant

Table 2 Microbial combined ingredient assay results displaying changes in growth from combinations of ingredients

Δ_{growth}

Strain	Ingredient (mg mL^{-1})	Manuka honey 10·00	Bee pollen 2·500	Rosehip 1·750	Brocco-Sprouts® 0·525	Black-currant oil 0·105	Propolis 0·300
DPC16	Manuka honey	+60·3	+88·7^{d}	+52·6^{c}	+92·2^{d}	+49·1^{d}	−88·2^{A,d}
DR10		+29·6	+74·1^{d}	+3·4^{A,d}	+87·2^{A,c}	+49·6^{A,c}	−64·5^{A,d}
DR20		+20·4	+57·5	+27·9^{Ac}	+54·4^{d}	+14·6^{d}	−89·0^{A,d}
2988		−43·4	−35·2^{d}	−43·0^{c}	−41·1	−39·0^{d}	−47·6^{c}
1772		−35·5	−42·4^{Ad}	−45·0^{A,d}	−47·3	−41·7^{A,d}	−50·5^{Ad}
25932		−6·1	+24·0	−16·6^{c}	−1·0^{A}	+4·8	−49·0^{Ad}
DPC16	Bee pollen		+80·0	+41·3^{d}	+71·6^{d}	+47·9^{d}	+12·8^{d}
DR10			+76·7	+37·1^{d}	+65·3^{d}	+55·8^{d}	−2·0^{d}
DR20			+37·3	+4·80^{d}	+18·9^{d}	+23·8^{d}	−23·1^{d}
2988			+10·2	+20·7	+2·9^{d}	+7·2^{d}	−1·8
1772			+22·5	+25·2^{d}	+18·3	+26·3	+10·0^{d}
25932			+30·0	+40·4	+30·0	+14·6^{d}	−4·5^{Ad}
DPC16	Rosehip			+17·1	+57·6	+31·1^{A,c}	−30·4^{b,d}
DR10				+28·1	+29·9^{d}	+26·4^{c}	−15·5^{d}
DR20				+12·8	+48·0^{d}	−10·6^{d}	−43·9^{d}
2988				+12·6	+6·8^{d}	+5·5^{d}	−5·2
1772				+14·4	+12·1^{c}	+15·3^{d}	+3·5^{d}
25932				+5·4	+22·9^{A,c}	+13·8	−86·7^{A,d}
DPC16	Brocco-Sprouts®				+48·1	+29·8^{d}	+21·0
DR10					+20·1	+34·8^{A,c}	+31·9^{Ac}
DR20					+45·6	+45·3	+10·8^{c}
2988					+0·1	+1·2^{d}	+6·0^{Ac}
1772					−8·5	+5·9^{c}	+11·5^{Ac}
25932					+6·9	+6·1	−0·7^{c}
DPC16	Black-currant oil					+6·3	−13·6
DR10						−2·4	−17·1
DR20						+6·1	−37·4
2988						+6·9	−8·20
1772						+8·3	+6·0
25932						+6·3	−13·6
DPC16	Propolis						−19·3
DR10							−8·1
DR20							−45·4
2988							−13·5
1772							−3·6
25932							−19·3

Lactobacillus rhamnosus, DPC16; *Bifidobacterium lactis*, DR10; *Lactobacillus rhamnosus*, DR20; *Escherichia coli*, 2988; *Salmonella* Typhimurium, 1772; *Staphylococcus aureus*, 25932.

[A]Desirable combined effect = Increase (probiotic) or decrease (pathogen) of growth from the combination which (i) exceeds the sum (±LSD $P < 0·05$) of the contributing ingredient activities and (ii) exceeds the value (±LSD $P < 0·05$) of the most extreme of the contributing ingredient activities.

[b]Undesirable combined effect = Increase (pathogen) or decrease (probiotic) of growth from the combination which (i) exceeds the sum (±LSD $P < 0·05$) of the contributing ingredient activities and (ii) exceeds the value (±LSD $P < 0·05$) of the most extreme of the contributing ingredient activities.

[c]Growth exceeds the sum (±LSD $P < 0·05$) of the contributing ingredient activities.

[d]Growth less than the sum (±LSD $P < 0·05$) of the contributing ingredient activities.

compounds have demonstrated synergistic effects in combination [for example, essential oils and flavanoids have been shown to contribute to synergies (Williamson, 2001)]. The ability of the roschip to synergise with other ingredients is interesting in regard to the synergistic activities of the rose-derived Tellimagrandin I (Shiota *et al.*, 2000) mentioned earlier.

Conclusions

The high-throughput spectrophotometric microbial bioassay refined and used during the course of this work has proven to be simple, robust, sensitive, accurate, highly reproducible, and easily amenable to use with multiple potential active compounds at a variety of

doses and against a number of bacterial species. Much information can be derived from the data generated here and, from a commercial food development perspective, it is fast and inexpensive, ensuring cost does not limit use in an industrial setting.

The manuka honey was the most promising candidate for the inclusion into a concept functional food intended to manage gut bacteria for the purposes of maintaining and increasing gut health. Although the mechanisms of honey on bacteria, both alone and in combination with other potentially bioactive ingredients are still to be fully explored, the antimicrobial data are entirely consistent with the effects of manuka honey currently in the literature. Further investigations on the beneficial effects of this ingredient are currently being carried out by the authors, both *in vitro* and *in vivo*.

As observed with the manuka honey in particular, the bee products in general, and to a lesser extent with the rosehip solution, there appears to be a division between the effects of the ingredients on probiotic organisms compared with the effects on pathogens. The inclusion of the Gram positive pathogen *Staphylococcus* in some of the assays dispels the potential theory that the results were dictated by a Gram positive or Gram negative-specific elements. The role played by the differing media requirements, and the lactic acid production and anaerobic respiration of the probiotics versus the aerobic pathogens, has not yet been explored. Furthermore, manuka honey or other ingredient-derived factors contributing to increased growth of the probiotic organisms have not been unequivocally identified, nor their mechanisms established. Currently, this phenomenon of increased probiotic growth in the presence of food ingredients (which are not conventional prebiotics such as oligosaccharides) is not prominent in the literature, and is an exciting new development.

Collectively, these *in vitro* investigations into potential synergistic interactions between ingredients illustrate a potential for combining food ingredients to modify components of the gut flora to a degree not achieved by a single ingredient alone. Although combinations of ingredients yield unusual results, sometimes desirable and sometimes undesirable from a health perspective, specific combinations such as manuka honey extract combined with BroccoSprouts® or to a lesser extent, bee pollen, rosehip and blackcurrant oil, show immediate potential as an ingredient combination in, for example, a yoghurt containing *B. lactis* DR10, which is specifically and synergistically encouraged to grow by three of those four combinations. It is recognised that the effects of these ingredients or ingredient combinations may perform differently with mixed populations of bacteria than with single strains tested in isolation. In addition, they may perform very differently in a more complex food matrix or as conditions change during consumption and digestion. Food synergy is the basis for modern nutrition science. It is an extremely complex area and is composed not only of interactions between compounds in ingredients but between them and the general food matrix. Also, whilst some of these ingredients may well retain their efficacy when incorporated into foods, other factors such as safety, toxicity and organoleptic impact would need to be considered. It is acknowledged that documented allergic responses have been observed with bee products (for example, refer Menniti-Ippolito *et al.*, 2008), which may limit their usefulness to some manufacturers or potential consumers.

Finally, in regard to the adverse synergies observed, a thorough screening programme should be considered as an essential part of functional food development to avoid any undesirable synergies between functional food ingredients.

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APPENDIX C

Screening food ingredients for their effects on innate immunity

C.1 Introduction

The work presented in this thesis focuses on examining the effects of selected food ingredient extracts on the gut microbiota. However, the GI tract has a variety of physical and immune properties required for the maintenance of the health of the organ. The GI immune system is extensive and complex, and deserves consideration whilst examining potential gut effects of potential functional foods.

C1.1 Gastrointestinal Immune System

C1.1.2 The Follicle-Associated Epithelia

Interspersed with the villous epithelia monolayer are regions known as follicle-associated epithelia (FAE) . The FAE cells have physiology which reflects a specialised role in recognition and uptake of lumenal antigens. Within the FAE are M cells, characterised by apical (lumenal) microfolds interspersed with clathrin-coated microdomains to facilitate the endocytosis of docked antigen, aided by the expression of surface glycosylation patterns to enhance their interaction and engulfment of microbes (Giannasca et al., 1999). In addition, the basolateral membrane of M cells posessess a pocket which presses close to the apical surface, allowing subpopulations of lymphocytes to remain close to the lumenal surface (Kraehenbuhl and Neutra, 2000). Thus the FAE, through M cells, is specialised for epithelial transport and delivery of foreign antigens to the underlying GALT, with the commensurate penalty that they are more readily accessible to pathogens.

C1.1.3 The gut-associated lymphoid tissue

The GALT which underlies the GI epithelia is divided into four discrete lymphoid compartments; inductive Peyer's patches, intraepithelial lymphocytes (IELs), effector lamina propria (LP) sites and the mesenteric lymph nodes (MLNs). The inductive Peyer's patches

are aggregates of lymphoid follicles found primarily in the distal ileum of the small intestine. The microfold (M) cells, located within the dome like structure of the FAE, deliver antigens to antigen presenting cells (APCs) in the Peyer's patch subepithelial dome (Neutra, 1998), aided by dentritic cells (DCs) which extend dendrite-like processes through tight junctions and sample lumenal antigen directly (Rescogno et al., 2001). Peyer's patches lymphoid follicles have aggregates of immature B-cells and CD4(+) T helper cells sitting within the basal pocket of the M cells. These lymphoid aggregates come into contact with antigens from the gut lumen and act as inductive sites for intestinal immune reponse. In addition, these sites are rich in IgA, which is produced in large quantity at the mucosal surface but poorly elsewhere in the body, and is a non-inflammatory form of immunoglobulin, as it barely binds complement (Kaetzel et al., 1991). Mucosal secretory IgA (sIgA) is usually found as a dimer, bound to a portion of the polymeric immunoglobulin receptor (pIgR) at the Fc region known as the secretory component, which is thought to confer resistance to lumenal protease activity. Specific interactions with the CD4(+) cells and the APCs with the follicles results in increased TGF-β production and inducing B cells class switching to IgA. The B cells then migrate out of the follicles and into the epithelia where they release sIgA into the lumen where it can function as pathogen binding and inhibit pathogen-epithelial attachment. The sIgA also functions as shuttle, binding free antigen in the epithelial space and transporting it back to the lumen by the action of the pIgR.

The lamina propria (LP) is the layer of cells surrounding the lymphoid follicles and below the FAE, and contains numerous T cells, DCs, macrophages, mast cells and other polymorphonuclear leukocytes as well as enormous numbers of terminally differentiated B cells (Brandtzaeg, 2002). This effector site primarily prevents the entry and systemic spread of pathogens and for destroying invading pathogens. LP lymphocytes (LPLs) largely consist of IgA-producing B cells and T effector cells. There are three types of T helper (T_H) cell functions of T effector cells, mostly of the CD4(+) lineage but also including CD8(+) cells with cytotoxic as well as effector responses. The variety of cytokine-producing T_H cell is dependent upon cellular responses to antigen and the prevailing cytokine milieu, which is determined by TLR ligand binding (Table C.3) and subsequent downstream effects.

T_H1 cell inflammatory responses occur where IL-12 is released by APCs, inducing the production of IFN-γ, responsible for cell mediated responses such as upregulated macrophage production of bacteriocidal reactive oxygen and nitrogen intermediates (ROIs and RNIs). IL-1 and TNF-α in this cascade activate macrophages, and IL-8 recruits other phagocytes such as neutrophils. IL-12 is also responsible for inducing T cell production of

natural killer (NK) cell cytotoxic function. T_H2 immunergic responses occur in response to multicellular pathogens and secrete cytokines IL-4, IL-5 and IL-13 to induce B cell activation and differentiation, and recruitment of eosinophils and mast cells. T_H2 reponses are also often equated with oral tolerance, although features which differentiate the 'tolerant' from 'immunergic' responses are not yet clear (Nagler-Anderson, 2001). T_H3, also known as regulatory T (Treg) cells, express immunosuppressive cytokines IL-4, IL-10 and TGF-β, and are involved in the active immune suppression required for oral tolerance to food antigens and commensal organisms.

Toll-like Receptor(s)	**Recognised PAMP Ligand**	**References**
TLR 1 and TLR2	Triacylated bacterial peptides	Alexopoulou et al., 2002
TLR1 and (TLR2 or TLR6)	• Peptidoglycan • Yeast cell wall zymosan • Phenol soluble modulin,	• Ozinsky et al., 2000 • Ibid • Hajjar et al., 2001
TLR2	Gram-positive and mycobacterial PAMPs, including: • Lipopeptide • Lipoteichoic acid • Peptidoglycan	Takeuchi et al., 1999 Underhill et al., 1999 • Aliprantis et al., 1999 • Underhill et al., 1999 • Ibid
TLR3	Double stranded viral RNA	Alexopoulou et al., 2001
TLR4	Lipopolysaccharide	Shimazu et al., 1999 Arbour et al., 2000 Rhee et al., 2000
TLR5	Flagellin	Hayashi et al., 2001
TLR9	Bacteria CpG DNA	Hemmi et al., 2000

Table C.3. Toll-like receptor ligands (adapted from Furrie et al., 2005).

IELs, present in the interdigiting spaces between the epithelial cells above the basement membrane are rich in cytotoxic T cells and rarer species of NK-like lymphocytes. Significant differences between IELs and and LPLs exist, including the ability to respond directly to antigen without antigen presentation, supports their poorly elucidated but proposed role of functioning in the innate defence against pathogens, and in tumour surveillance in the gut.

Macrophages and DCs possessing ingested antigen can migrate into the underlying MLN, and their antigens are presented to T cells in the node, whereupon antigen-sensitised T cells can migrate out through efferent lymphatics and ultimately enter circulation through the thoracic duct. These T cells also aid B cell differentiation into IgA producing plasma cells, and induce a systemic response to the antigen. These plasma cells can also be recruited back to the MLN where they are needed to release IgA into the lumen in response to epithelial challenge. Pathogens able to avoid immune responses and gain access to the MLNs, which also possess pathogen killing and removal ability, have increased likelihood of spreading systemically through the bloodstream or lymphatics.

C1.1.4 Oral Tolerance

The main sources of potential antigenic determination for the GALT are from commensal bacteria and food proteins. However, a large body of evidence shows that oral administration of soluble antigen induces systemic non-responsiveness to antigen challenge, known as oral tolerance. This is of crucial importance because exaggerated response in the absence of pathogenic antigen is deleterious, and is a factor in the inflammatory response during IBD. Tolerance is largely mediated by suppression of TLRs, by induction and location.

Suppression of TLRs from tolerance has been associated with a number of factors. One is suppression of TLR expression (Nomura et al., 2000; Wang et al., 2002). Another is negative interregulation with the nucleotide-binding oligomerisation domain (NOD) proteins NOD1 and NOD2 which interfere with downstream TLR signalling at NF-κB activation (Cario and Podolski, 2005). High expression of the TLR-signalling suppressor molecule Tollip has been observed (Melmed et al., 2003; Otte et al., 2004), which is mediated through suppression of IL-1 receptor-associated kinase (IRAK) after TLR2/4 activation (Burns et al., 2000; Zhang and Ghosh, 2002). Tollip expression directly correlates with lumenal bacterial load (Otte et al., 2004) and is upregulated during repeated TLR-ligand signalling to produce a hyporesponsive state (Zhang and Ghosh, 2002; Otte et al., 2004). TLR signalling pathways have been suppressed by induction of the peroxisome proliferator-activated receptor γ (PPARγ) by commensal organisms. PPARγ acts by uncoupling NF-κB-dependent target genes in a negative feedback loop (Dubuquoy et al., 2003). In addition, dendritic cells carrying live commensals (Macpherson and Uhr, 2004) produce sIgAs which bind TLR-ligand-like antigens such as endocytosed LPS, and thus prevent activation of NF-κB

(Fernandez et al., 2003). Finally, lumenal or host factors can modify TLR ligands, such as by the action of host or bacterial digestive enzymes, and further reduce TLR signalling.

Intracellular location of the TLRs, and the distribution of TLR expression amongst intestinal epithelia are also regarded as factors in promoting tolerance to commensal organisms. For example, TLRs 2 and 4 are thought to be expressed at higher levels in the walls of the small intestinal crypts, whilst TLR3 is expressed at the tips of the villi (Furrie et al., 2005). This reflects the distribution of their ligands within the gastrointestinal tract: TLR2 and TLR4 ligands of commensal bacterial origin are expected in the gut lumen, and therefore tolerance can be maintained whilst maintaining the sanctity of the epithelial stem cells so that epithelial cell renewal can be maintained, whilst mature epithelial cells on the apical villi surfaces are able to immediately respond to viral pathogens through TLR3-recognition, as viruses are not part of the gut commensal microbiota. In addition, subcellular compartmentalisation of the TLRs correlates with the epithelial cells state of differentiation which is directly related to immune reponsiveness. For example, TLR2 and TLR4 are present at the apical surface of polarised, confluent epithelial cells, thus readily responding to stimulation by ligands of bacterial origin (Cario et al., 2000; ibid 2002; Otte et al., 2004), whilst being sequestered and compartmentalised within the cytoplasm of undifferentiated epithelial cells, and expressed at lower levels, correlating with ligand tolerance (Cario et al., 2000; ibid 2002).

In order to investigate potential immunomodulatory effects of the food ingredients, *in vitro* assays were developed, based on the *in vivo* work of Shu and Gill (Gill et al., 2000; Shu and Gill, 2001; *ibid*, 2002).

C.2 Methods

C.2.1 Lymphocyte proliferation

Proliferation of B-cell lymphocytes can be induced by the B-cell mitogen lipopolysacharide (LPS). Thus assessment of mitogenic ability might indicate the degree of LPS (bacterial) contamination of samples, and this would influence subsequent immune assays.

White blood cells from pigs (pWBCs) were isolated according to Section 2.2.2.5. pWBCs in RPMI 1640 medium supplemented with 10% Fetal Calf Serum (FCS), 2 mM glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin were diluted to $5x10^5$ cells/mL and their viability

was confirmed using trypan blue dye exclusion and haemocytometer counting. This cell suspension (0.9 mL) was added to the wells of a 96-well flat bottomed microassay plate that contained extracts (0.1 mg/mL in complete RPMI 1640 medium)(10 µL/well). Plates were incubated under standard mammalian culture conditions (Section 2.2.2.4) for 72 h to allow proliferation. Proliferation was measured using the MTT assay of cell viability described in Section 2.2.4.7.

C.2.2 Natural Killer Assay

Natural killer (NK) cells are an important part of the innate immune system which recognise and kill cells lacking MHC class I markers, such as tumours or virus-infected cells. The NK assay was based on the ability of the NK cells to kill sensitive target cells such as human K562 cells and mouse YAC-1 lymphocytes, where upregulated NK activity from food ingredients would result in increased cell lysis and release of cytoplasmic lactate dehydrogenase (LDH).

The optimum YAC-1 (target) cell concentration for measuring LDH release in the NK assay was determined by comparing unlysed (spontaneous release) versus detergent-lysed (maximum release) samples at varying concentrations, where the greatest difference in values at any given concentration remained within the useful range of the spectrophotometer. After incubation for 4 h the YAC-1 cells were centrifuged and the culture supernatant was assayed for LDH activity using a commercial kit. The LDH activity was expressed as the net absorbance of the LDH product and a graph of activity versus concentration was drawn. Blank values were calculated on the basis of media minus cells. Three replicates of each cell concentration were used.

Target cells were prepared as follows. YAC-1 cells were diluted to2 x 10^6 cells/mL in RPMI supplemented with 10% FCS + 1% glutamine, penicillin (100 U/mL) and streptomycin (0.1 mg/mL). RPMI without supplements was used as diluent (assay medium) for all subsequent steps. The LDH Cytotoxicity Detection Kit reagents were prepared according to the manufacturer's instructions (Cytotoxicity Detection Kit (LDH), Version July 2005, Cat. No. 11 644 793 001, Roche Applied Science)

To determine the optimum YAC-1 cell concentration for use in the assay, YAC-1 cells (0.1 mL, diluted across the range from 2 x 10^6 to 5 x 10^3 cells/mL) were added to maximum release and spontaneous release wells of a round-bottom 96-well microplate. Assay medium was added to low control wells (0.1 mL) and blank wells (0.2 mL), whilst assay medium

containing 2% Triton X-100 to make a final concentration of 1% was added to maximum release wells (0.1 mL). The plate was incubated under standard mammalian cell culture conditions (Section 2.2.2.4) for 4 h. The plate was then centrifuge at 290 x g for 10 min at room temperature to pellet the YAC-1 cells. The culture supernatant was transferred to a flat-bottom microplate to which reagent (0.1 mL) was added to all wells. Absorbance was measured in a plate reader at 490 nm and blank values were subtracted.

To optimise the ratio of effector pBWCs to YAC-1 target cells, the optimum YAC-1 cell concentration determined by the above method was subjected to a range of pBWC concentrations and LDH release was measured. This was done by adding effector pBWCs (prepared according to Section 2.2.2.5)($1x10^7$ – $2.5x10^4$ cells/mL, 0.1 mL) to experimental and effector blank wells, and the assay was conducted exactly as described above.

C.2.3 Phagocytosis

The role of phagocytic cells (blood PMN, gut macrophages) in the body is to remove foreign material, bacteria and cell debris by a combination of phagocytosis and proteolysis. Phagocytosis can be accompanied by release of specific mediators, cytokines and Reactive Oxygen Intermediates (ROIs). These make macrophages one of the first lines of defence in the gut innate immunity. In addition, dendritic cells and macrophages from the FAE are implicated in tolerance mechanisms (Ch. 1).

Measuring increased phagocytosis from macrophages is an assay of innate immune stimulation, and is used to examine passive immune upregulation. Other alternatives either developed but not used included measurement of ROIs, whilst those not developed or used included cytokine release, specifically TNF-α, IL-6 and IL-1ß.

Assays were conducted according to Section 2.2.4.3.

C.3 Results and discussion

C3.1 Lymphocyte proliferation

The proliferation assay results with functional food extracts standardised to concentrations of 0.1 and 0.01 mg/mL (Fig. C.3.1) indicate that none of the extracts, or the LPS positive

control, significantly influenced the proliferation of the pBWCs. Interestingly, propolis extract slightly decreased proliferation, which may correlate to the likely higher concentration of membrane-perturbing phenolic compounds than in the other ingredients. However, this assumption is based purely on the relative insolubility of propolis versus the other ingredients, and has not been further investigated.

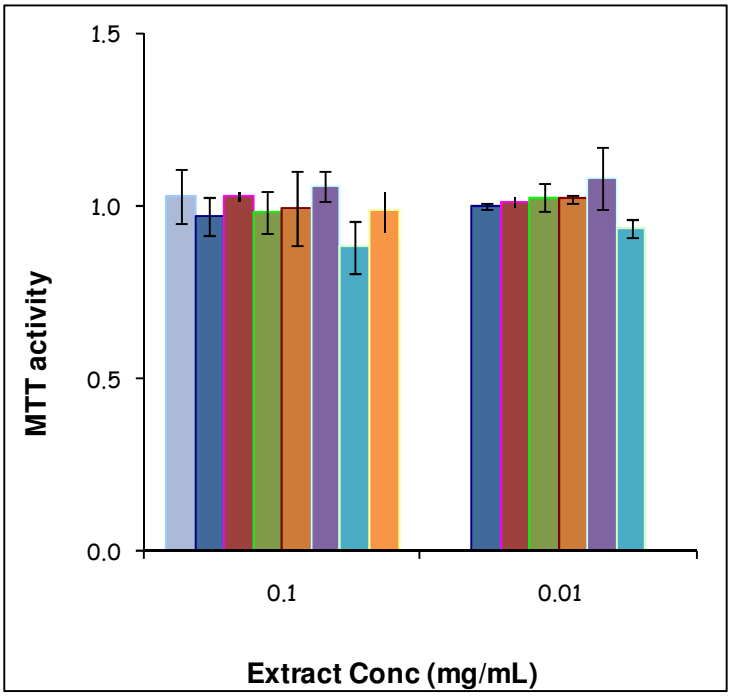

Figure C.3.1. Cell viability assay on pBWCs subjected to manuka honey (■), bee pollen (■),rosehip (■), BroccoSprout® (■), blackcurrant oil (■) and propolis (■) extracts to assess proliferation. B-cell mitogen LPS positive control (■), unsupplemented cell negative control (■). Data mean of 4 determinations, standard deviations shown. Representative of >3 determinations.

The failure of LPS to significantly stimulate proliferation above the control cells may suggest that the cell preparation consisted of either (i) existing high concentrations of LPS and/or bacteria, or (ii) the B-cells component were not present in sufficient numbers or were not amenable to stimulation with LPS. Multiple experiments under different conditions aimed at improving the B cell response to LPS carried positive outcomes (data not shown) with no significance.

Were this experiment to be repeated, mouse or purified human B-cells would be used.

C3.2 Natural Killer Assay

The optimal YAC-1 target cell concentration yielding the best ratio of high LDH from lysed cells (maximum release) to low LDH leakage from unlysed cells (spontaneous release) was determined to be 5.0 x 10^5 cell/mL (Fig. C.3.2). This concentration of target YAC-1 cells was used to establish the ratio of effector (pBWC natural killer) cells for subsequent assay (Fig. C.3.3).

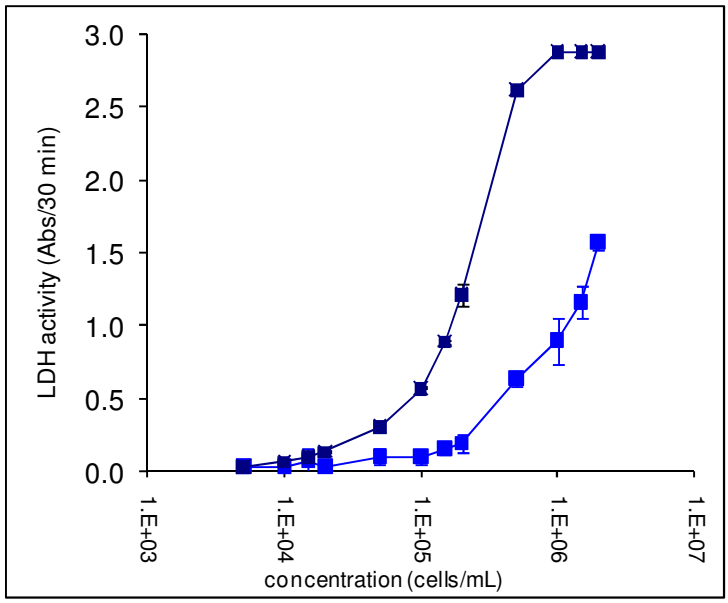

Figure C.3.2 LDH release from lysed cells (maximum release)(■) and unlysed cells (spontaneous release)(■). Data mean of 3 determinations. Standard deviations shown.

From the results shown in Fig. C.3.3 it was obvious that the effector cells were compromised, as shown by the high levels of LDH released. By subtracting the effector blanks from the effector + target cell mixes it was obvious that all the LDH was derived from the effector pBWC population.

To confirm this unsuitability a greater range of effector cells was tested, to check whether the measured LDH was proportional to effector cell concentration (Fig. C.3.4).

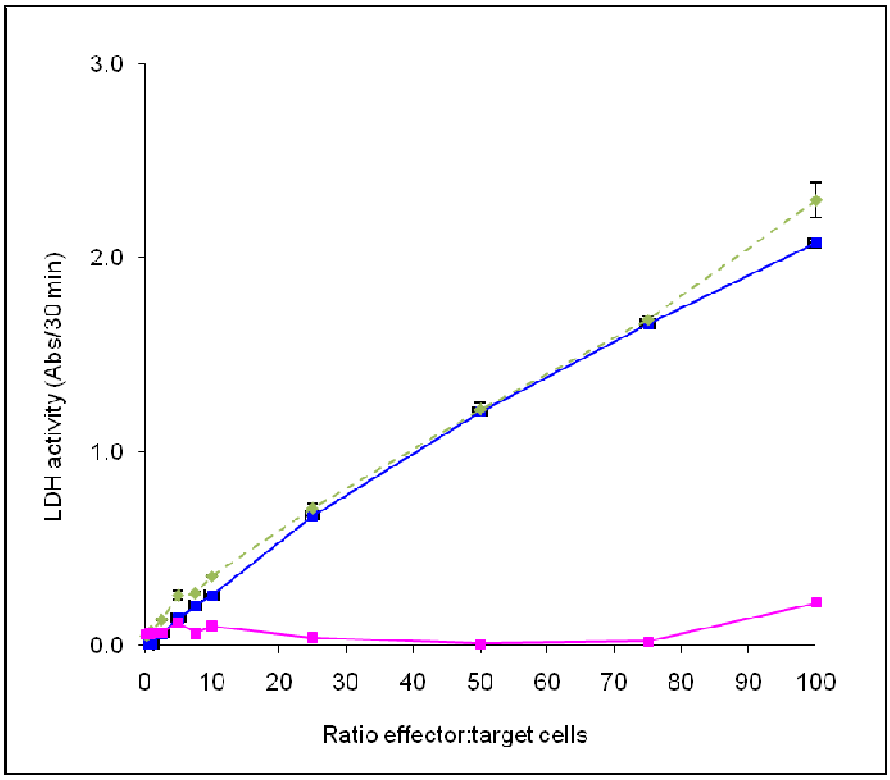

Figure C.3.3 Optimising the effector (pBWC (NK)) cell to target (YAC-1) cell ratio for best release of LDH. Effector + target LDH release (◆), Effector control (■), Target minus effector control (■). Data mean of 3 replicates, representative of >3 determinations. SEMs shown.

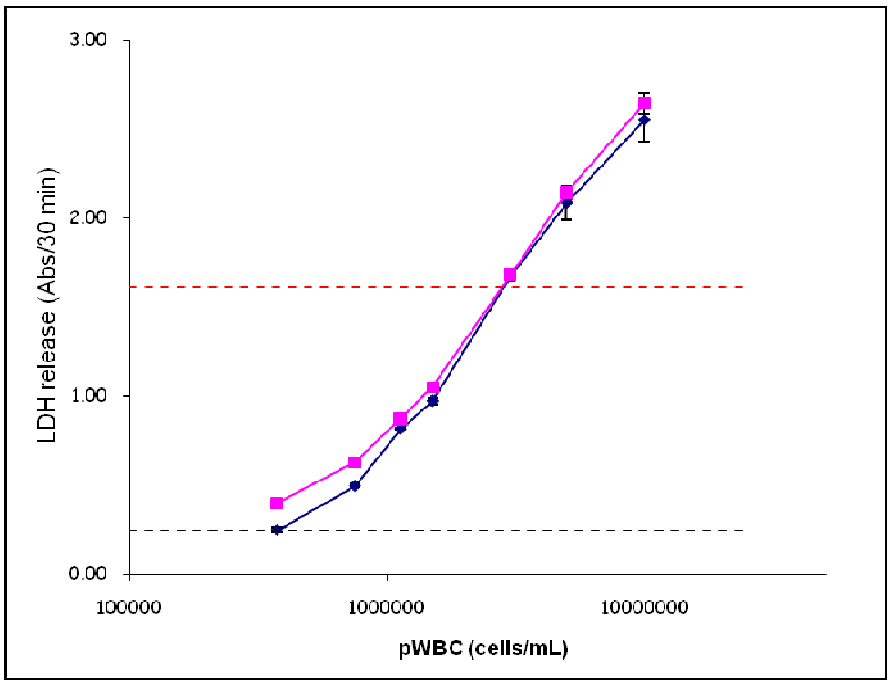

Figure C.3.4 Comparing target + effector cell LDH release (■) to effector cell leakage (■) under conditions of unchanged target (YAC-1) cell numbers and varying effector (NK) cell numbers. Target cell maximum release (---) and spontaneous release (---) controls shown. Data mean of 3 replicates. SEMs shown.

The results of Fig. C.3.4 showed that the LDH release from high (>10^7 cells/mL, or 100 effector:1 target cell ratio) produced slightly more LDH than was released from the maximum release target cell control. Clearly, NK cells present in pWBCs were not suitable for use in this assay. Multiple variations of the pBWC preparation were attempted, including freshly prepared cells collected the same day from the abattoir, but the LDH leakage consistently proved to be too high.

Thus the assay of NK activity from pBWCs was abandoned prior to testing any of the functional food ingredient extracts. Were this work to be continued, a human NK cell line (NK96) would be used.

C3.3 Phagocytosis

The results of the phagocytosis assays with the food ingredients are shown in Figs C.3.5 and C.3.6, below.

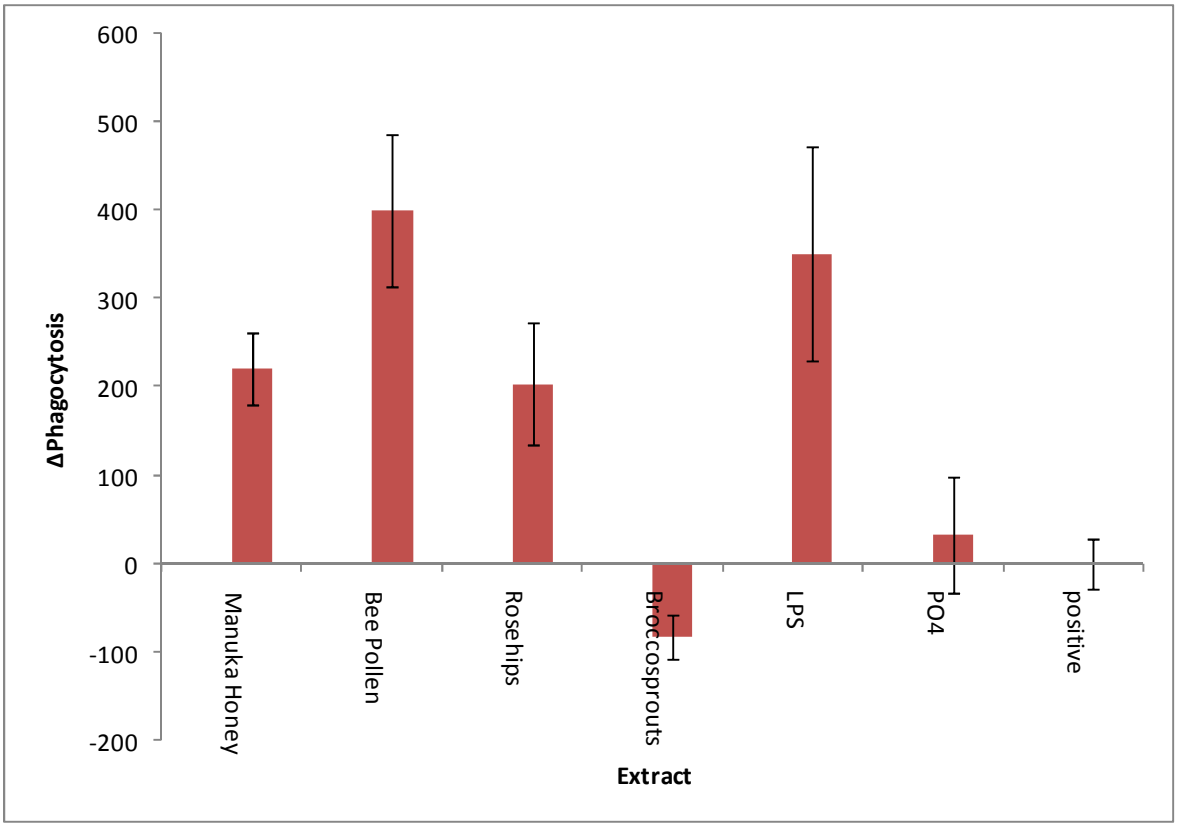

Figure C.3.5 ΔPhagocytosis activity of RAW264.7 mouse macrophage cells after exposure to food ingredients (0.1 mg/mL). An LPS positive control (0.001 mg/mL) was included. Data are mean of 7 replicates and expressed as change relative to the phagocytic activity of control cells (100%). SEM shown.

These data indicate that manuka honey, bee pollen and rosehips stimulate phagocytosis by the mouse macrophages. Bee pollen stimulates to a similar level as LPS. BroccoSprouts® appears to inhibit phagocytosis somewhat, whilst the phosphate buffer extract blank has very little effect compared to the unsupplemented controls.

Previous experiments using pBWCs had failed to give consistent results, thus requiring the use of a defined cell line of known activity. The high degree of increased phagocytosis of the macrophage cell line may be due to naïve cells reacting to 'anything', so a subsequent experiment was performed after prestimulating the cells with LPS according to Section 2.2.4.3. A stimulator of phagocytosis, formyl-methionine-leucine-phenylalanine (fMLP) was included as a positive control.

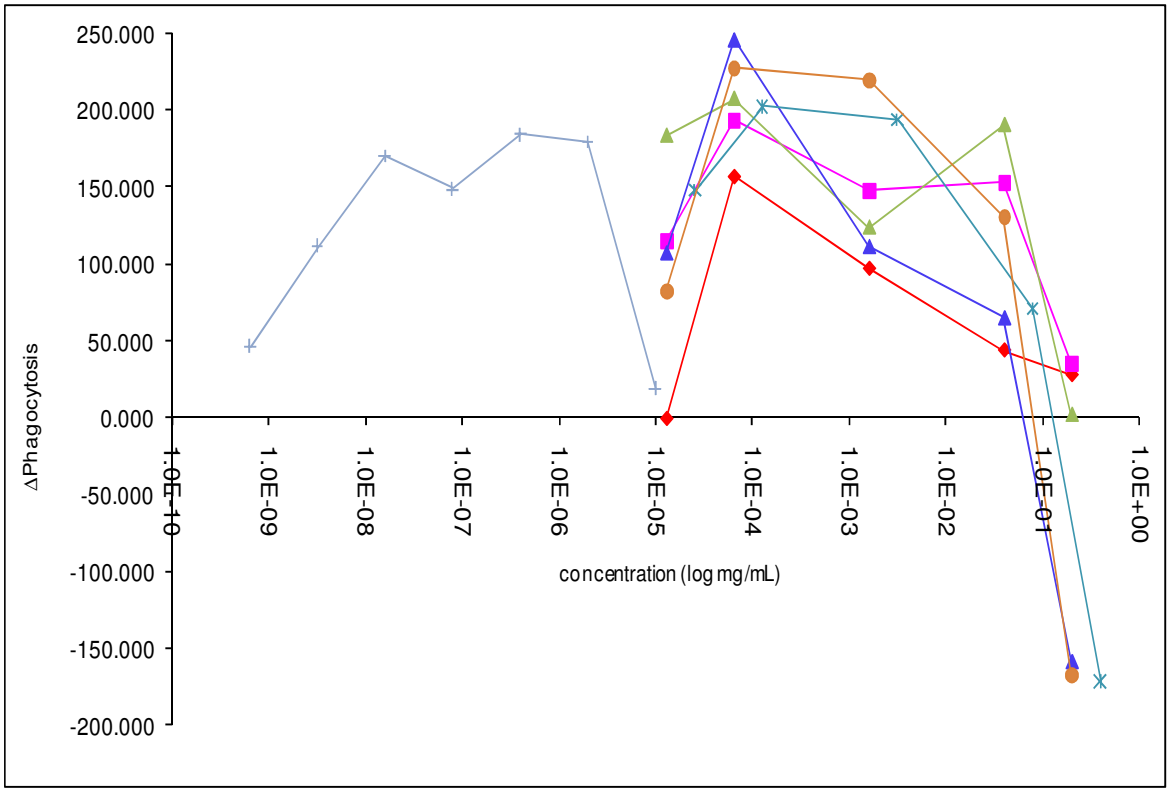

Figure C.3.6 ΔPhagocytosis activity of RAW264.7 mouse macrophage cells after exposure to various food ingredients (manuka honey, ◆; bee pollen, ■; rosehip, ▲; BroccoSprouts®, ▲; blackcurrant oil, x, and propolis, ●, and the phagocytosis upregulator fMLP, +, at varying concentrations, after pre-incubation with LPS. Data are mean of 7 replicates and expressed as change relative to the phagocytic activity of control cells (100%). SEM shown.

The dose response profiles of the food ingredients indicate that stimulation occurs above and beyond that achieved by pre-stimulation by LPS. The fMLP was particularly efficacious at very low doses. However, the nature of the response curves indicates that the assay reacted unusually to the highest and lowest doses tested, probably indicating an assay layout

problem or technical flaw. Interestingly, the BroccoSprout® extract similarly inhibited phagocytosis at 0.1 mg/mL, suggesting consistent performance. The blackcurrant oil and propolis extracts also inhibited at this concentration, but were not included in the previous assay, and thus a comparison could not be made.

C.4 Conclusions

The pBWCs proved to be generally inadequate for use in immune assays which relied upon B- or T-cell function. This work was abandoned in favour of the more promising antibacterial manuka honey work. Were this subject to be revisited, different cells such as commercially available cell lines or fresh human blood cells would be used.

The phagocytosis assay proved to be more useful, and indicated some positive effects. The work described here was used as method development for use of the assay in subsequent *in vivo* work (Chapter 4).

APPENDIX D

Rodent Purified Diet AIN-76A

INGREDIENT	g/kg
Lactic casein	200.00
DL-methionine	3.00
Sucrose	500.00
Dextrin	150.00
Arbocel	50.00
Corn oil	50.00
Choline bitartrate	2.00
AIN-76A Mineral mix	35.00
AIN-76A Vitamin mix	10.00
Ethoxyquin	0.10

AIN-76A Mineral Mix

INGREDIENT	g/kg
Calcium Phosphate Dibasic	500.00
Sodium chloride	74.00
Potassium citrate monohydrate	220.00
Potassium sulphate	52.00
Magnesium oxide	24.00
Manganous carbonate (43-48% MN)	3.50
Ferric citrate (16-17% Fe)	6.00
Zinc carbonate (70% Zn)	1.60
Cupric carbonate (53-55% Cu)	0.30
Potassium iodate	0.01
Sodium selenite	0.01
Chromium potassium sulphate	0.55

AIN-76A Vitamin Mix

INGREDIENT	mg/kg
Thiamine Hydrochloride	600
Riboflavin	600
Pyridoxine hydrochloride	700
Nicotinic acid	3000
Calcium pantothenate	1600
Folic acid	200
Biotin	20
Cyanocobalamin CFR premix	100
Vitamin A (Retinol acetate) 400,000 IU	0.64
Vitamin E (alpha-tocopherol) 5,000 IU	10
Vitamin D3 (Cholecalciferol 100,000 IU	0.04
Vitamin K (Menadione	50

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