

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

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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

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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.

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

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

Ingredient	Concentration (mg mL ⁻¹)
Manuka honey UMF TM 20+	20.0
Bee pollen ^a	5.0
Rosehips	2.5
BroccoSprouts [®]	1.75
Blackcurrant seed oil ^b	0.21
Propolis ^b	0.6

^aIncludes DMSO.

^bIncludes ethanol.

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 (un-supplemented) 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 ($P < 0.05$) deviated further from the control value (zero) than the theoretical sum of the Δ_{growth} values of the individual extracts (eqn 2).

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

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 ($P < 0.05$) 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).

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

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 Δ_{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

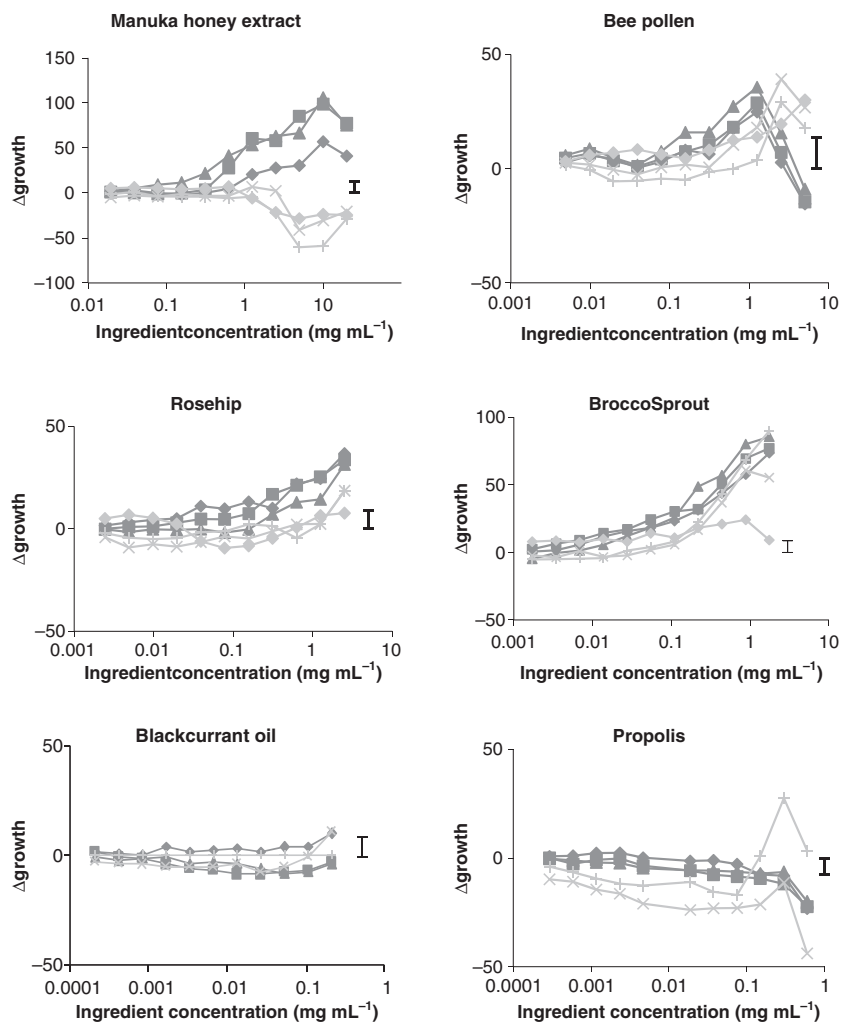


Figure 1 Δ growth values from bacterial cultures supplemented with increasing doses of functional food ingredients. Probiotic bacteria: *Lactobacillus reuteri* strain DPC16 (◆), *Lactobacillus rhamnosus* strain DR20 (▲) and *Bifidobacterium lactis* strain DR10 (■). Pathogenic bacteria: *Staphylococcus 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 ^{b,d}
DR10		+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}
2988		-43.4	-35.2 ^d	-43.0 ^c	-41.1	-39.0 ^d	-47.6 ^c
1772		-35.5	-42.4 ^{a,d}	-45.0 ^{a,d}	-47.3	-41.7 ^{a,d}	-50.5 ^{a,d}
25932		-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
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 ^{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
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 ^{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
2988					+0.1	+1.2 ^d	+6.0 ^{b,c}
1772					-8.5	+5.9 ^c	+11.5 ^{b,c}
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.

^aDesirable combined effect = increase (probiotic) or decrease (pathogen) of growth from the combination which (i) exceeds the sum (\pm LSD $P < 0.05$) of the contributing ingredient activities and (ii) exceeds the value (\pm LSD $P < 0.05$) of the most extreme of the contributing ingredient activities.

^bUndesirable combined effect = increase (pathogen) or decrease (probiotic) of growth from the combination which (i) exceeds the sum (\pm LSD $P < 0.05$) of the contributing ingredient activities and (ii) exceeds the value (\pm LSD $P < 0.05$) of the most extreme of the contributing ingredient activities.

^cGrowth exceeds the sum (\pm LSD $P < 0.05$) of the contributing ingredient activities.

^dGrowth less than the sum (\pm 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 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.

Conclusions

The high-throughput spectrophotometric microbial bio-assay 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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