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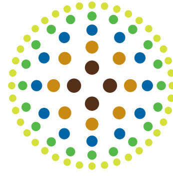

RURAL INDUSTRIES
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Value-adding to honey

MAY 2014

RIRDC Publication No. 13/123

Australian Government

Rural Industries Research and Development Corporation

Value-adding to Honey

by Dr Joan Dawes and Dr David Dall

May 2014

RIRDC Publication No. 13/123
RIRDC Project No. PRJ-005590

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ISBN 978-1-74254-616-2
ISSN 1440-6845

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Project No. PRJ-005590

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Electronically published by RIRDC in May 2014
Print-on-demand by Union Offset Printing, Canberra at www.rirdc.gov.au
or phone 1300 634 313

Foreword

At present the commercial value of Australian honeys primarily relates to taste quality, but stronger health awareness by consumers has created scope for adding value to Australian honeys by exploiting properties of the honeys that convey health benefits. This project has examined three such potential attributes of commercially-prepared Australian eucalypt honeys: Glycaemic Index; prebiotic properties; and therapeutic activity.

The project found that all the Australian eucalypt honeys tested were prebiotic food, stimulating the growth of gut bacteria that contribute to human health and reducing the growth of deleterious gut bacteria. Australian honey packers and marketers have already started to explore how to take advantage of this finding.

Although the honeys were found to be low to medium Glycaemic Index foods, the Index was also found not to be a useful parameter to apply to honeys. In the competitive market for honey products, the industry will need to consider the implication of this finding. No commercially useful antibacterial or antifungal activity was detected in the samples of commercial Australian eucalypt honeys tested.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Honeybee R&D program, which aims to secure a productive, sustainable and more profitable Australian beekeeping industry.

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Acknowledgments

The authors are extremely grateful to Ms Jodie Goldsworthy of Beechworth Honey Pty Ltd, who assembled, prepared, aliquoted, stored and distributed the honey samples for this project. They would also like to thank all the researchers for their input, as well as everyone from the Rural Industries Research and Development Corporation and participants in the Australian honey industry who attended and contributed to the project workshops.

Abbreviations

ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
BRS	Bureau of Rural Sciences
DHA	dihydroxyacetone
FAO	Food and Agriculture Organization of the United Nations
FSANZ	Food Standards Australia New Zealand
GC-MS	gas chromatography – mass spectrometry
GI	Glycaemic Index
HPLC	high performance liquid chromatography
IP	intellectual property
MGO	methylglyoxal
MIC	minimum inhibitory concentration
NMR	nuclear magnetic resonance
NPSC	nutrient profiling scoring criterion
PI	Prebiotic Index
R&D	research and development
SCFA	short chain fatty acids
TGA	Therapeutic Goods Administration
WHO	World Heath Organization

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Executive Summary

What the report is about

The long-term economic viability of the Australian honey industry is particularly important to Australia, not only for the honey industry itself but also in relation to the pollination services that the honeybees provide to the horticulture industries. This viability is intrinsically linked to the prosperity of the industry and its ability not only to compete with other natural and artificial sweeteners for dietary use, but also to differentiate Australian honey from the cheaper products marketed by international competitors.

This study focussed exclusively on the potential for ‘value-adding’ to Australian eucalyptus honey products delivered through the existing commercial supply chain, and the conclusions and recommendations of this report relate to such products. While alternative routes to honey sourcing, production and supply may offer other avenues to increased industry value and returns, they are inevitably associated with further costs and uncertainties, and were not considered in this study.

At present the commercial value of Australian honeys relates only to taste quality, but stronger health awareness by consumers has created scope for adding value to Australian honeys by exploiting any properties of the honeys that convey health benefits. Anecdotal evidence has identified three such potential functional properties of Australian eucalypt honeys: Glycaemic Index, prebiotic properties and therapeutic activity. This project provides in-depth analysis of the composition of twenty samples of commercially-prepared Australian eucalypt honeys, tests whether the honey samples do in fact exhibit health-related properties, and attempts to relate honey composition to its health benefits. Australian honey packers and beekeepers could benefit directly by using some of the results of the project to derive optimal returns for honey in an increasingly competitive market. Indirect benefits will flow through to the horticulture industries as a result of the increased security of supply of pollinators.

Who is the report targeted at?

The report is targeted at the Australian honey industry, particularly honey packers.

Where are the relevant industries located in Australia?

The honey industry is represented in all States of Australia, as are the horticulture industries. The strongest honey industry representation is in NSW, which frequently contributes over 40% of Australian honey production. Western Australia and Tasmania are important to the industry because of their endemic floral sources of honey, Jarrah (*Eucalyptus marginata*) and Leatherwood (*Eucryphia lucida*).

In 2011-12 the Australian honey industry had a gross value of honey and beeswax production of $79 million which was forecast to rise to $88 million in 2012-13 and $92 million in 2013-14 (ABARES, 2013). Furthermore it has been estimated that the industry contributes directly to between $4 billion and $6 billion worth of agricultural production. In 2006-7 1,700 commercial producers with more than 50 hives each accounted for more than 90% of Australia’s honey production. Australia is recognised for the premium quality of its honey. In 2004 about 30% of honey production was exported, mostly in bulk form, to over 38 countries.

There are only a small number of large Australian packers handling this honey. The largest is Capilano Honey Limited, which is based in Queensland and also packs honey in Victoria and Western Australia, but receives honey from many locations in Australia. The largest NSW-based honey packer

is Beechworth Honey in Corowa. There are also many other smaller honey-packing entities around Australia.

Australian honey packers and beekeepers could benefit directly by using the results of this research to derive optimal returns for honey in an increasingly competitive market. Indirect benefits will flow through to the horticulture industries as a result of the increased security of supply of pollinators. The general conclusions will benefit most of the sectors of the industry, and specific benefits can be generated for producers and suppliers of honeys from the floral sources tested: Jarrah (*Eucalyptus marginata*), Red Stringybark (*Eucalyptus macrorrhyncha*), Spotted Gum from southern New South Wales (*Corymbia maculata*) and Yellow Box (*Eucalyptus melliodora*). The beekeepers with access to these floral sources are located in Western Australia, Queensland, NSW and Victoria, and the honey packers sourcing these honeys are Capilano and the NSW packers.

Background

Anecdotal evidence and preliminary research raises the possibility that honey may confer health benefits. However, systematic studies to convincingly demonstrate such functional properties are lacking, and without them the commercial honey industry cannot make substantiated claims that would support premium pricing for honeys. Moreover, the honeys tested have not been rigorously characterised to determine whether specific physical or chemical properties contribute to functional characteristics.

Aims/objectives

The main objective of this project was to assist the Australian honey industry to maximise its revenues and enhance its public image by supply of honeys with reference to their highest-value properties. It was intended to address this objective by:

- generating high quality data examining the functional properties of these honeys as:
    - low-Glycaemic Index sweeteners,
    - prebiotic foods and/or
    - anti-fungal and antimicrobial agents;
- analysing honeys sourced from important Australian eucalypt species to link specific physical and chemical characteristics with these health-related functional properties;
- developing proprietary tests for these functional properties, which can be used by the Australian honey industry for identification and quality assurance testing of production batches of honey;
- using the datasets generated to support accreditation of appropriately identified honeys for commercial supply; and
- making data and intellectual property produced by this project available to support further research and development in other value-added contexts.

Achievement of these aims would also benefit the nation through improved recognition of the availability of choices of healthy food.

Methods used

This project analysed the chemical and functional properties of 20 unifloral Australian eucalypt honey samples of known provenance, using five samples originating from each of Jarrah (*Eucalyptus marginata*), Red Stringybark (*Eucalyptus macrorrhyncha*), Spotted Gum from southern New South

Wales (*Corymbia maculata*) and Yellow Box (*Eucalyptus melliodora*). Two other honeys, one from canola and the other a Canola/Stringybark blend, were analysed as controls. Honeys were selected for inclusion in this study after consideration of factors including general commercial availability, and prior indications of prospective but unrealised value characteristics. Honeys that already achieve premium value on the basis of characteristics such as unique flavour (eg Leatherwood honey) were not considered for inclusion.

The samples were sourced from Beechworth Honey Pty Ltd (Cowra, NSW). Jarrah honeys originated from Wescobee Limited (Bayswater, Western Australia) and were sent to Beechworth Honey for aliquoting, storage and distribution.

Groups with expertise in each field investigated were contracted to analyse the honey samples and assess their functional properties.

The floral sources of the honey samples were assigned by Beechworth Honey using the routine procedures in place at this large commercial packer, combining the information supplied on the beekeeper's vendor declaration form with sampling and tasting to examine its colour and flavour profile.

The colour, consistency, odour and taste of each sample were also examined by Intertek Food Services GmbH (Bremen, Germany), a company with an international reputation in honey analysis. They analysed the pollen content using microscopy and measured the electrical conductivity by an in-house method.

ChemicalAnalysis Pty Ltd (Croydon, Victoria) performed chemical analysis of the honey samples, including the water content, pH, refractive index, colour and opacity. The content of glucose, fructose, sucrose and maltose + oligosaccharides was measured using High-Performance Liquid Chromatography with Evaporative Light Scattering Detection. Methylglyoxal and dihydroxyacetone content were measured using High Performance Liquid Chromatography-Mass Spectrometry. Nuclear Magnetic Resonance spectroscopy was also carried out on one honey sample to identify oligosaccharides.

The Glycaemic Index (GI) values of seven selected honey samples were measured *in vivo.* The samples included one Jarrah honey, three Red Stringybark honeys, one Spotted Gum honey and one Yellow Box honey with a spread of glucose and fructose content to optimise attempts to relate these parameters to GI values. Each sample was tested in 10 normal human subjects by the Glycemic Index Research Service, University of Sydney (SUGiRS). The methodology is regarded as the 'gold standard' for measuring GI. Subjects consumed honey or a glucose control containing 50 grams of available carbohydrate, after which a 2-hour blood glucose response curve was used to calculate the GI value.

The Predictive GI was measured on 21of the honey samples used in this study. Next Instruments (Condell Park, Sydney) performed this test using the NutriScan G120 Glycemic Index Analyser, a high precision fully automated instrument that mimics the way carbohydrates are digested in the human gut.

The prebiotic potential of all 22 honey samples was assessed *in vitro* in the laboratory of Professor Patricia Conway (ProBiOz Pty Ltd), both before and after enzymic digestion and dialysis. Intestinal microcosms were derived from faecal material from two healthy human subjects. The effect of each honey sample on growth of total bacteria, the beneficial lactobacilli and bifidobacteria and the potentially harmful clostridia was determined. Short chain fatty acid metabolites produced during this process were quantified by gas chromatography.

Four honey samples were selected on the basis of their *in vitro* prebiotic effects for *in vivo* testing in a double blind crossover study in 20 healthy human subjects. The study by ProBiOz involved four

phases each of which was four weeks in duration. Phases 1 and 3 served as wash out periods to remove the effects of previously ingested honey, and during Phases 2 and 4 subjects consumed 20 grams of honey daily. Freshly voided faecal samples were collected at the beginning of Phase 1 and at the end of each phase, and the bacterial content of each faecal sample was analysed and the Prebiotic Index calculated. In addition butyrate levels in the faecal suspensions were determined by gas chromatography.

All 22 honey samples were tested in the laboratory of Associate Professor Dee Carter (University of Sydney) for antibacterial activity against *Staphylococcus aureus* and anti-fungal activity against *Candida albicans*. Samples were assessed both as received by Beechworth Honey and as prepared for market by warming and filtration. Standard growth inhibition assays were used for antibacterial testing and a microdilution technique for anti-fungal activity. The concentration of hydrogen peroxide in honey samples was determined using a colorimetric assay.

Results/key findings

The first objective of this study has been achieved, generating high quality data examining the functional properties of commercially-prepared honeys sourced from four important Australian eucalypt species as low-Glycaemic Index sweeteners, prebiotic foods and/or anti-fungal and antimicrobial agents. An intensive examination of the specific physical and chemical characteristics of these honeys is also reported. However, the project could not deliver a surrogate test for a health-related functional property because no characteristic of the honeys correlated sufficiently well with any functional property that it could be used as the basis for developing a surrogate test for that property.

The process for accreditation of honeys with health-related functional properties has changed completely since the beginning of this project, but we have identified a potential route for claiming such properties. The data and intellectual property produced by this project are available to support further research and development in other value-added contexts.

The key findings of the project are:

- No measured physical or chemical characteristic of the honeys contributed usefully to the assignment of floral source for Australian eucalypt honeys. It should specifically be noted that pollen analysis was not useful in this context, although both it and electrical conductivity are now extensively used in assessment and quality control of Northern hemisphere honeys. The paucity of available data and lack of expertise and experience in analysis of Australian eucalypt honeys contribute to inaccuracies in interpretation of pollen content and electrical conductivity.

- Australian eucalypt honeys are probably low to medium GI foods when consumed by the majority of individuals, but not necessarily of lower GI value than honeys from other floral sources. The automated *in vitro* Predictive GI test was highly reproducible, but the results did not correlate strongly with those from the *in vivo* analysis. The *in vivo* GI value of a honey could not be reliably predicted on the basis of its content of glucose, fructose or any other simple physical or chemical property measured in this study.

- Most of the Australian eucalypt honeys had significant prebiotic potential when tested *in vitro*. Results from *in vivo* clinical trials may be of commercial value. However, we were unable to identify a surrogate diagnostic for Prebiotic Index (PI). The *in vitro* data did not predict the *in vivo* result and none of the sugar contents or physical characteristics analysed correlated sufficiently strongly with the Prebiotic Indices to be useful as an indicator of PI.

- In *in vitro* studies most of the honeys, and all the Jarrah honey samples, elevated the levels of butyric acid, which at high concentrations is linked to a lowered risk of colon cancer.

- A few of the Australian eucalypt honeys had some antibacterial activity and low levels of anti-fungal activity, but both were entirely attributable to hydrogen peroxide, which is unstable on storage. Moreover, this is an attribute of honeys from many floral sources. There was no evidence from this study that any of the Australian eucalypt honeys tested contained stable antibacterial or anti-fungal components that could be of interest to the biotechnology or pharmaceutical industries.

- Food Standards Australia New Zealand (FSANZ) regulates health and nutritional claims in Australia. In January 2013 it released FSANZ Standard 1.2.7, under which honey, because it is almost entirely composed of sugars and water, is effectively prevented from being associated with health and nutritional claims. However, this barrier could be surmounted by identifying an independent expert not-for-profit organisation that would endorse such claims for Australian eucalypt honey.

Implications for relevant stakeholders

The implications for the Australian honey industry are:

- The present routine industry method for assigning floral source to Australian eucalypt honeys is adequate and appropriate. This study did not identify any physical or chemical characteristic, or combination thereof, that could be reliably used to differentiate between Australian eucalypt honeys sourced from different floral species.

- The Glycaemic Index, antibacterial activity and antifungal activity are not valuable properties of Australian eucalypt honeys.

- Prebiotic potential is the health-related property of Australian eucalypt honeys that is most likely to generate premium prices.

- No simple, cost-effective surrogate marker has been identified that could be used to analyse batches of honey and predict their prebiotic activity.

There are also implications of this research for policy makers. Standard FSANZ 1.2.7 does not address the regulation of health-related or nutritional claims for honeys in an appropriate manner. There should be the opportunity to address this matter and change the regulation.

Recommendations

The recommendations arising from this project are addressed to the Australian honey industry, particularly the honey packers.

- The current method of assigning floral sources to Australian eucalypt honey samples remains the best available and should not be modified to include pollen analysis or electrical conductivity, neither of which adds value to the present approach.

- Industry funds should not be expended on further analysis of the Glycaemic Index of Australian eucalypt honeys.

- Industry funds should not be expended on further analysis of the antibacterial activity or antifungal activity of Australian eucalypt honeys, which is unlikely to be productive.

- The industry should focus on prebiotic potential as the health-related property of Australian eucalypt honeys that is most likely to generate premium prices.

- We recommend that the Australian honey industry identifies an independent expert not-for-profit organisation to endorse Australian eucalypt honey as a prebiotic food.

Introduction

The honey industry is represented in all States of Australia, as are the horticulture industries that rely on honey bees for pollination. The strongest honey industry representation is in NSW, which frequently contributes over 40% of Australian honey production. However, it should be noted that beekeepers are highly mobile between states, typically moving their hives 500-600 kilometres to floral sources. In addition, production is highly dependent on weather events including droughts, floods and bushfires. Western Australia and Tasmania are important to the industry because of their endemic floral sources of honey, Jarrah (*Eucalyptus marginata*) and Leatherwood (*Eucryphia lucida*).

In 2011-12 the Australian honey industry had a gross value of honey and beeswax production of $79 million, which was forecast to rise to $88 million in 2012-13 and $92 million in 2013-14 (ABARES, 2013). It has also been estimated that honeybees contribute directly to between $4 billion and $6 billion worth of agricultural production annually (House of Representatives Standing Committee on Primary Industries and Resources 2008). In 2006-7 there were about 10,000 registered beekeepers with 572,000 hives, though it should be noted that registration was then not compulsory in Tasmania, the Northern Territory and the ACT. There were 1,700 commercial producers with more than 50 hives each, and these 17% of beekeepers accounted for more than 90% of Australia's honey production (Crooks 2008). Most commercial apiarists operate between 400-800 hives but some have more than 3,000 hives. Australia is recognised for the premium quality of its honey. In 2004 about 30% of honey production was exported to over 38 countries, with key markets in the United Kingdom, Indonesia and other South East Asian countries, North America and Saudi Arabia. Most honey was exported in bulk form, but there was a significant and increasing proportion of exports shipped as retail packs (Centre for International Economics 2005).

There are only a small number of large Australian packers handling this honey. The largest is Capilano Honey Limited, which is based in Queensland and also packs honey in Victoria and Western Australia, but receives honey from many locations in Australia. The largest NSW-based honey packer is Beechworth Honey in Corowa. There are also many other smaller honey-packing entities around Australia.

Honey has been an important part of the human diet from prehistoric times, and also has a long history of use as an active therapeutic. There is an extensive body of literature describing its physical, chemical and functional properties, and some of this discusses honeys from native Australian floral sources. There are indications that some Australian floral species may yield honeys with potentially valuable dietary attributes such as low glycaemic indices and prebiotic properties, and therapeutic attributes such as wound healing and anti-fungal and antibacterial properties (Conway et al. 2010, Carter et al. 2010).

At present the commercial value of Australian honeys relates only to taste quality. For example, Tasmanian leatherwood honey sells at a premium because consumers find its taste superior to most other Australian honeys. A recent study commissioned by the Rural Industries Research and Development Corporation (Kneebone 2010) indicates that a stronger health awareness by consumers has created scope for adding value to Australian honeys by exploiting any low Glycaemic Index, prebiotic potential and antibacterial and anti-fungal properties of the honeys. There is a clear precedent in the premium commercial value of New Zealand Manuka honey based on its antibacterial activity. However, existing demonstrations of the functional properties of Australian honeys are commonly only at 'proof-of-concept' level.

Previous studies have tested small numbers of honey samples, and there have been significant shortcomings in systematic supporting data such as sample provenance and physical and chemical characterisation of the test materials. Existing datasets are generally not sufficiently large to allow robust conclusions to be drawn. Lack of data describing the variation of physical, chemical and

functional properties between individual samples of the same type of honey further compounds this problem, and as a result a clear correlation between the functional attributes of Australian honey and its physical and chemical characteristics is lacking.

This study focussed exclusively on the potential for 'value-adding' to Australian eucalyptus honey products delivered through the existing commercial supply chain, and the conclusions and recommendations of this report relate to such products. While alternative routes to honey sourcing, production and supply may offer other avenues to increased industry value and returns, they are inevitably associated with further costs and uncertainties, and were not considered in this study.

Despite the repeatedly noted potential commercial value of certain properties of honeys, there has been general failure to capture these benefits for Australian honeys and the producer industry. As a consequence, Australian honeys are largely locked into the role of a general sweetener commodity in ever more competitive markets, thereby returning a potentially lower than possible benefit to the industry and the nation.

Objectives

As noted in the Introduction above, existing research provides some indication of various functional properties of Australian honeys that may hold significant commercial potential for the industry. The main objective of this 'Value-adding to Honey' project was to assist the Australian honey industry to maximise its revenues and enhance its public image by supply of honeys with reference to their highest-value properties.

At the outset of the project it was envisaged that this would be addressed by means such as:

- developing datasets that link specific physical and chemical characteristics with functional properties of honeys sourced from important Australian eucalypt species. The datasets are intended to support accreditation of appropriately identified honeys for commercial supply as:
    - low-Glycaemic Index (GI) sweeteners;
    - prebiotic foods and/or
    - anti-fungal and antimicrobial agents;

- developing proprietary assays for characteristics that are diagnostic of high-value functional properties, which can be used for identification and quality assurance testing of production batches of honey prior to their supply for accredited purposes. It is intended that the assays will be made available by licensing to the Australian honey industry (for example, honey packers). They in turn will access the assay methods through contracted sample testing by commercial laboratories. It is envisaged that packers will pass a proportion of premiums associated with sale of high-value honeys back to producers through individual contract supply arrangements;

- submitting the datasets linking physical or chemical attributes with functional properties to appropriate regulatory agencies to initiate a basis for accreditation of Australian honeys that conform with prescribed characteristics;

- licensing datasets linking physical or chemical attributes with anti-fungal and anti-microbial properties to biotechnology and/or biopharmaceutical companies to underpin the further testing processes required to capture honey values as therapeutic agents, or seeking external funding for this purpose;

- making data and intellectual property (IP) produced by this project available to support further research and development (R&D) required to enable use of honeys in other value-added contexts that are beyond the budgetary capacity of the present project. These might include advanced therapeutics such as anti-inflammatories and wound healing agents, functional foods including antioxidants and personal care products like shampoos and cosmetics.

The outcomes of this project for the Australian honey industry were intended to be:

- increased industry profitability;

- enhanced industry profile and social prominence through contribution to improved community health, and

- increased revenue for future R&D activities through exploitation of project IP.

Achievement of these aims would also benefit the nation through improved recognition of the availability of choices of healthy food.

Methodology

Identification and sourcing of honey samples

This project analysed the chemical and functional properties of 20 unifloral Australian eucalypt honey samples of known provenance, using five samples originating from each of:

- Jarrah (*Eucalyptus marginata*);
- Red Stringybark (*Eucalyptus macrorrhyncha*);
- Spotted Gum from southern New South Wales (*Corymbia maculata*) and
- Yellow Box (*Eucalyptus melliodora*).

These unifloral honeys were chosen because earlier studies indicated that Yellow Box and Stringybark honeys may be low GI foods (Holt et al. 2002; Arcot & Brand-Miller 2005), and that Jarrah and Spotted Gum honeys had antibacterial activity (Irish et al. 2011).

Two other honeys, one from canola and the other a Canola/Stringybark blend, were analysed as controls. These were chosen because canola honey was reported to have a relatively simple carbohydrate content and a glucose:fructose ratio higher than for most eucalypt honeys (Abell et al. 1996; Holt et al. 2002), and previous reports have indicated that they are likely to have mid- to high-GI and little prebiotic activity.

The samples were sourced from Beechworth Honey Pty Ltd (Cowra, NSW). Jarrah honeys originated from Wescobee Limited (Bayswater, Western Australia) and were sent to Beechworth Honey for aliquoting, storage and distribution. Each honey sample was a 100 kilogram single batch of honey, except for the Jarrah honeys from Wescobee, which were each 84 kilogram single batches. This amount ensured that enough honey was available for all the anticipated tests, and any additional research required during the course of the project or subsequent studies. This level of acquisition was therefore both a risk management exercise and a strategic investment.

As noted, the results of this project are intended to add value to some commercial Australian honeys. Honeys for use in the project were therefore processed according to standard industry practice. On receipt of the beekeeper's container at Beechworth Honey a subsample of 20 kilograms was removed and stored and the remaining 80 kilograms was warmed below 45°C for eight to ten hours and then filtered through a 100 micron filter. Jarrah honeys from Wescobee Limited were sampled from bulk containers in which they were delivered by beekeepers, and were not further processed before testing. Honeys were dispensed into 25 x 200 gram tubs and the remainder into 20 kilogram buckets and stored.

Samples were identified as shown in Table 1.

Table 0.1. Honey samples used in the project, with identifying codes and designations.

Sample No	Source	Packer's code
1	Jarrah 1	7843WES
2	Jarrah 2	7863WES
3	Jarrah 3	8012WES
4	Jarrah 4	8105WES
5	Jarrah 5	8113WES
6	Red Stringybark 1	7264DEN
7	Red Stringybark 2	7369HOL
8	Red Stringybark 3	7460EMM
9	Red Stringybark 4	7515BBN
10	Red Stringybark 5	7526BOM
11	Spotted Gum 1	3747RUT
12	Spotted Gum 2	3854DEN
13	Spotted Gum 3	3883SNO
14	Spotted Gum 4	4442BOM
15	Spotted Gum 5	5485BOM
16	Yellow Box 1	5735SPI
17	Yellow Box 2	7130SMI
18	Yellow Box 3	7141WRI
19	Yellow Box 4	7427RUT
20	Yellow Box 5	7626DEN
21	Canola 1	8168KLI
22	Canola/Stringybark 2	8193SNO

Composition of honey samples

Methodology specific to the analysis of the composition of the honeys is described in Chapter 1.

The honey samples were tested for:

- taste;
- water content;
- pH;
- refractive index;
- electrical conductivity;
- pollen content;
- content of individual monosaccharides, sucrose, maltose and oligosaccharides, and
- content of methylglyoxal (MGO) and dihydroxyacetone (DHA).

Functional properties of honeys

Methodology specific to the analysis of the functional properties of honey is described in the relevant chapters.

The honey samples were tested for:

- Glycaemic Index;
- Prebiotic Index;
- antimicrobial activity, and
- anti-fungal activity.

Chapter 1. Composition of honey samples

Introduction

The honey samples were analysed in detail with two specific objectives:

- to attempt to identify honey characteristics that correlate strongly with functional properties and
- to use these results to develop assays that allow commercially viable assessment of the functional properties of batches of honey by measuring surrogate parameters.

Floral source of honeys

Honeys are characterised in several ways. The honey source is traditionally assessed by experienced tasters using the organoleptic characteristics of taste, colour, and odour. This method is not completely accurate; it is difficult to identify nectar sources consistently and accurately by flavour and different individuals may recognise different sources for the same, or similar, products.

Qualitative and quantitative pollen analysis, which includes identification of the botanical species present as well as their relative abundance, has also been used to examine provenance and floral source and to provide a quantitative measure of floral origin. There is no direct correlation between the pollen found in honey and the nectar from which it is produced; the pollen and nectar content in a honey depend separately on floral structure, nectar secretion and pollen production by the source plants. Originating flora for some honey sources - such as thyme - are routinely under-represented in pollen analyses to such an extent that a unifloral thyme honey can contain as little as 20% thyme pollen. By contrast unifloral manuka honey must have a manuka pollen content of at least 70%, as manuka pollen is over-represented in honey (Moar 1985). Nevertheless, pollen analysis can be a useful approach to identifying the geographic and floral source of a honey, particularly when the characteristics of a particular unifloral honey have been established. Most of the nectar sources for a honey can be recognised by pollen analysis and it is a valuable objective approach that complements traditional methods of classifying honey. Not all honey is derived from floral sources, however. Some originates from ‘honeydew’, exudations from types of insect. In Australia the main source of honeydew honey is psyllid species such as *Psylla eucalypti*, which manufacture a protective shield of crystallised honeydew and are then known as lerps.

Chemical content of honeys

Honey is essentially a supersaturated solution of sugars, which also contains acids (including amino acids), vitamins, phenols, minerals and enzymes in small and varying amounts. The moisture content of Australian honeys is usually between 16 and 18%. The European Union standard for commercial honey requires a maximum moisture content of 21%, but several national standards have maxima of 18.0-18.5% and many buyers will not accept honey with a moisture content greater than 20%.

Sugars comprise 95.0-99.9% of the dry weight of honey, and the specific sugar content of a honey probably defines its Glycaemic Index and prebiotic properties. The monosaccharides fructose and glucose make up about 85% of honey dry weight, with small amounts of at least 22 other more complex sugars. Fructose, usually the dominant sugar, has the lowest Glycaemic Index (19 ± 2) of any naturally occurring monosaccharide, compared with 100 for glucose (by definition) and 68 ± 5 for sucrose (Atkinson et al. 2008). It is also often recommended for diabetics because it does not trigger the production of insulin by the pancreas (Melanson et al. 2007).

Sucrose, maltose, trehalose and turanose are the main disaccharides in honey, which can also contain isomaltose, isomaltulose (palatinose), nigerose, kojibiose, laminaribiose and gentiobiose. A range of trisaccharides can be present, including melezitose, 3-*a*-isomaltosylglucose, maltotriose, l-kestose, 6-kestose and panose. Isomaltulose, panose, 1-kestose and 6-kestose are nutritionally relevant (Bogdanov et al. 2008). These saccharides are low-glycaemic and low-insulinaemic, as their digestion by bacteria in the human intestine slowly releases the constituent glucose and fructose monosaccharides into the bloodstream (Holub et al. 2010). Two more complex sugars, isomaltotetraose and isomaltopentaose, have also been identified in honey samples. In most blossom honeys the great majority of sugars are reducing sugars, but many honeydew honeys have high amounts of non-reducing oligosaccharides such as melezitose, maltotriose and raffinose (Bogdanov et al. 2000).

Other compounds implicated in the functional properties of honey include DHA and MGO. MGO has been identified as the main non-peroxide antibacterial constituent of Manuka honeys (Mavric et al. 2009; Jervis-Bardy et al. 2011). MGO is produced from DHA in the honey during storage (Adams et al. 2009).

Methodology

Floral source of honeys

Routine assessment

The floral sources of the honey samples originating from Beechworth Honey, which comprised all except the Jarrah honeys, were assigned by the routine procedures in place at this large commercial packer. Thus, the source of a batch of honey was identified by the individual beekeeper on a vendor declaration form. Beechworth Honey cross-check this information with their own intelligence about the species that are flowering in each region. On receipt of the beekeeper's container at Beechworth Honey each lot of honey is sampled and tasted to ensure that its flavour profile and colour match the characteristics of the honey identified by the beekeeper.

In this study, the colour, consistency, odour and taste of each sample were also examined by Intertek Food Services GmbH (Bremen, Germany), a company with an international reputation in honey analysis.

Pollen analysis

Pollen analysis was carried out by Intertek using microscopy to perform qualitative pollen spectrum analysis and a quantitative assessment of the relative content of the different pollens in each sample.

Electrical conductivity

Electrical conductivity was measured by Intertek to detect the difference between blossom and honeydew honeys. The company's in-house method 3110.142 was used.

Chemical analysis of honeys

ChemicalAnalysis Pty Ltd (Croydon, Victoria) performed chemical analysis of the honey samples. The initial analysis measured the water content, pH, refractive index, colour, opacity and content of glucose, fructose, sucrose, maltose, total oligosaccharide, MGO and DHA. NMR spectroscopy was also carried out on one honey sample to identify oligosaccharides.

Water content

Samples were analysed by Karl Fischer titration against a Hydranal standard.

pH

Samples were diluted to 10% in deionised Milli-Q water for pH determination.

Refractive index

Samples were diluted with an equivalent mass of water before the refractive index was measured. The results were converted to percentage weight/weight glucose/fructose using Tables 8-59 (D-Fructose) and 8-60 (D-Glucose) in the CRC Handbook of Chemistry and Physics 87th Edition.

Individual sugar content

Samples were prepared in deionised Milli-Q water at a concentration of approximately 10 grams per litre. They were analysed using High-Performance Liquid Chromatography with Evaporative Light Scattering Detection. Calibration curves were generated for each sugar in the range 1.5-3.0 grams per litre for glucose, 2.0-5.0 grams per litre for fructose and 0.25-1.0 grams per litre for sucrose and maltose. The peaks generated by maltose and oligosaccharides overlapped; these results were therefore measured using the maltose calibration curve and reported as maltose + total oligosaccharides. The results for individual sugars are the mean of duplicate sample preparations.

MGO and DHA content

Samples and standards were prepared at 15% weight/volume in 0.5 M sodium phosphate (pH 6.5). They were then derivatised using 1% w/v orthophenylenediamine for approximately 24 hours before analysis by HPLC-Mass Spectrometry. Samples were quantified against individual calibration curves from 1 to 100 milligrams per litre. The results are the mean of duplicate sample preparations.

Nuclear Magnetic Resonance (NMR)

2D-NMR analytical procedures followed those described by Consonni et al. (2012). A solution of the honey sample was prepared at approximately 100 mg/ml in deuterated water, and analysed by proton (with and without pre-saturation of the water peak), carbon-13 and heteronuclear single quantum coherence NMR spectroscopy.

Statistical analysis

The chemical content of honeys from the four different eucalypt sources was compared using the 2-tailed Student's T-test assuming 2-sample unequal variance. A significance level of $P < 0.05$ was chosen.

Results

Floral source of honeys

The floral sources of the honey samples assigned by routine assessment are indicated by the sample names (see Tables).

Pollen analysis

Analyses by Intertek delivered the pollen content assessments shown in Table 1.1.

Table 1.1. Pollen content of honey samples.

Sample No	Packer's code	Source assigned by packer	Relative pollen type content (%)		
			Main (>45%)	**Relative (>15%)**	**Accompanying (≥3%)**
1	7843WES	Jarrah 1	85% eucalypt	none	12% *Echium*
2	7863WES	Jarrah 2	58% eucalypt	32% *Echium*	None
3	8012WES	Jarrah 3	87% eucalypt	none	6% *Echium*
4	8105WES	Jarrah 4	89% eucalypt	none	5% *Lotus*
5	8113WES	Jarrah 5	82% eucalypt	none	15% *Echium*
6	7264DEN	Red Stringybark 1	78% eucalypt	19% *Echium*	None
7	7369HOL	Red Stringybark 2	72% eucalypt	24% *Echium*	None
8	7460EMM	Red Stringybark 3	48% Trifolium	41% eucalypt	5% *Vicia* 4% Cruciferae
9	7515BBN	Red Stringybark 4	65% eucalypt	32% *Echium*	None
10	7526BOM	Red Stringybark 5	88% eucalypt	none	10% *Echium*
11	3747RUT	Spotted Gum 1	59% *Echium*	37% eucalypt	None
12	3854DEN	Spotted Gum 2	78% eucalypt	20% *Echium*	None
13	3883SNO	Spotted Gum 3	81% eucalypt	none	10% Cruciferae 7% *Echium*
14	4442BOM	Spotted Gum 4	54% eucalypt	38% Cruciferae	6% *Echium*
15	5485BOM	Spotted Gum 5	73% eucalypt	21% Cruciferae	5% *Echium*
16	5735SPI	Yellow Box 1	90% *Echium*	None	7% eucalypt
17	7130SMI	Yellow Box 2	50% eucalypt	44% *Echium*	None
18	7141WRI	Yellow Box 3	64% *Echium*	30% eucalypt	3% Cruciferae
19	7427RUT	Yellow Box 4	81% eucalypt	none	10% *Echium* 3% Trifolium
20	7626DEN	Yellow Box 5	76% eucalypt	20% *Echium*	3% Cruciferae
21	8168KLI	Canola 1	90% *Brassica*	0%	7% Fruit
22	8193SNO	Canola/Stringybark 2	57% eucalypt	26% *Brassica*	14% *Echium*

Electrical conductivity

Analyses by Intertek delivered the conductivity assessments and sample designations shown in Table 1.2.

Table 1.2. Electrical conductivity and designation of honey samples.

Sample No	Packer's code	Source assigned by packer	Electrical conductivity (milliSiemens/cm)	Designation by Intertek
1	7843WES	Jarrah 1	1.099	Eucalyptus honey
2	7863WES	Jarrah 2	0.716	Eucalyptus honey with honeydew honey
3	8012WES	Jarrah 3	1.124	Eucalyptus honey
4	8105WES	Jarrah 4	1.157	Honeydew honey with Eucalyptus honey
5	8113WES	Jarrah 5	0.935	Eucalyptus honey
6	7264DEN	Red Stringybark 1	0.521	Eucalyptus honey
7	7369HOL	Red Stringybark 2	0.479	Eucalyptus honey
8	7460EMM	Red Stringybark 3	0.297	Blossom honey
9	7515BBN	Red Stringybark 4	0.685	Eucalyptus honey
10	7526BOM	Red Stringybark 5	0.427	Eucalyptus honey
11	3747RUT	Spotted Gum 1	1.029	Eucalyptus honey
12	3854DEN	Spotted Gum 2	1.392	Eucalyptus honey
13	3883SNO	Spotted Gum 3	1.171	Eucalyptus honey
14	4442BOM	Spotted Gum 4	1.053	Eucalyptus honey with blossom honey
15	5485BOM	Spotted Gum 5	1.092	Eucalyptus honey
16	5735SPI	Yellow Box 1	0.359	Blossom honey
17	7130SMI	Yellow Box 2	0.530	Eucalyptus honey
18	7141WRI	Yellow Box 3	0.519	Blossom honey with Eucalyptus honey
19	7427RUT	Yellow Box 4	0.330	Eucalyptus honey
20	7626DEN	Yellow Box 5	0.260	Blossom honey with Eucalyptus honey
21	8168KLI	Canola 1	0.205	Canola honey
22	8193SNO	Canola/Stringybark 2	0.454	Blossom honey with Eucalyptus honey

The honey samples were designated by Intertek according to the criteria outlined in the European Community Council Directive 2001/110/EC together with information in the literature. The organoleptic characteristics, pollen analysis and electrical conductivity were all taken into account.

Intertek's analysis indicated that:

- Samples 1, 3, 5, 10, 13 and 19 were clearly eucalypt honeys, with >80% eucalypt pollen (Table 1.1) and no characteristics that suggest any other main source.

- Sample 21 was clearly canola honey, with 90% canola pollen.

- Nineteen of the 22 samples contained *Echium* (Paterson's Curse; Salvation Jane) pollen, but *Echium* is an over-represented pollen in honey (Intertek report) and as long as it did not affect the organoleptic characteristics of the honey this did not impact on sample designation, even in some cases when *Echium* was the main pollen species. Samples 6, 7, 9, 11 (59% *Echium*, 37% eucalypt pollen), 12 and 17 were designated eucalypt honey on this basis.

- The main pollen in sample 15 was eucalypt (73%) and the presence of 21% cruciferous pollen did not affect its designation as eucalypt honey.

- Sample 14 contained 54% eucalypt and 38% cruciferous pollen and was designated eucalypt honey with blossom honey.

- Sample 18 contained 64% *Echium* and 30% eucalypt pollen and was designated blossom honey with eucalypt honey, as was sample 22, the Canola/Stringybark control sample.

- The main pollen in sample 8 was Trifolium (48%); it also contained 5% *Vicia* and 4% cruciferous as well as 41% eucalypt pollen; this sample was designated blossom honey.

- Sample 16 contained 90% *Echium* and only 7% eucalypt pollen; it was also designated blossom honey.

- Sample 20 was designated blossom honey with Eucalyptus honey, despite containing 76% eucalypt pollen and most of the remainder being *Echium* pollen. In our view this is an incorrect assignment.

The electrical conductivity of honey depends on its ash and acid content. There is a linear relationship between the ash content and the electrical conductivity of a honey sample and the latter is now used in routine honey quality control procedures in Europe instead of determining the ash content.

In general for honeys produced in the northern hemisphere, blossom honeys and mixtures of blossom and honeydew honeys have conductivities of less than 0.8 and honeydew honeys have more than 0.8 milliSiemens/cm. However, eucalypt honeys provide exceptions to this relationship, with 181 samples giving a range of electrical conductivities of 0.19-1.33 milliSiemens/cm (Bogdanov et al. 2000). It can be seen from Table 1.2 that there is a very high variation between species in the conductivities of the honey samples. Three of the five Jarrah and all the Spotted Gum honey samples had electrical conductivities greater than 1.0, whereas the conductivities of all the Red Stringybark and Yellow Box samples were less than 0.7. The conductivities of the Jarrah honeys were highly significantly different from those of the Red Stringybark honeys ($P = 0.001$) and Yellow Box honeys ($P < 0.001$), as were the Spotted Gum honeys ($P < 0.001$ against both Red Stringybark and Yellow Box samples).

Despite this, Intertek designated Sample 2 as eucalypt honey with honeydew honey and Sample 4 as honeydew honey with eucalypt honey.

These results indicated that:

- from the pollen analysis, honey samples Red Stringybark 3 (7460EMM), Spotted Gum 4 (4442BOM), Yellow Box 1 (5735SPI) and Yellow Box 3 (7141WRI) may not be fully representative of eucalypt honey, and
- designation of honey samples Jarrah 2 (7863WES) and 4 (8105WES) as containing significant honeydew honey should be regarded with reservations.

Chemical analysis

pH, water content and refractive index

The results for pH and water content of the honey samples are given in Table 1.3. The refractive index measurements were not informative; although the refractive index of honey is measured as an indicator of the glucose:fructose ratios in the sample, the values obtained for the honeys assessed in this study did not reflect their sugar content measured by HPLC and the data are not included in this report.

Table 1.3. Water content and pH of honey samples.

Sample No	Packer's code	Source assigned by packer	Water content (%)	pH
1	7843WES	Jarrah 1	16.2	5.3
2	7863WES	Jarrah 2	16.4	5.1
3	8012WES	Jarrah 3	15.2	4.4
4	8105WES	Jarrah 4	16.2	5.2
5	8113WES	Jarrah 5	16.2	5.7
6	7264DEN	Red Stringybark 1	15.2	4.6
7	7369HOL	Red Stringybark 2	19.0	3.8
8	7460EMM	Red Stringybark 3	14.7	4.1
9	7515BBN	Red Stringybark 4	18.6	4.3
10	7526BOM	Red Stringybark 5	15.3	4.8
11	3747RUT	Spotted Gum 1	18.0	4.2
12	3854DEN	Spotted Gum 2	16.5	4.4
13	3883SNO	Spotted Gum 3	17.6	4.4
14	4442BOM	Spotted Gum 4	16.5	4.5
15	5485BOM	Spotted Gum 5	14.9	4.4
16	5735SPI	Yellow Box 1	15.3	3.3
17	7130SMI	Yellow Box 2	16.4	4.5
18	7141WRI	Yellow Box 3	15.7	4.3
19	7427RUT	Yellow Box 4	15.5	4.4
20	7626DEN	Yellow Box 5	14.7	4.1
21	8168KLI	Canola 1	16.6	3.9
22	8193SNO	Canola/Stringybark 2	17.9	4.3

These analyses indicated that:

- there was no significant difference between the water contents of the four groups of eucalypt honeys, and
- the Jarrah honey samples were significantly less acid ($P < 0.025$) than those of all the other eucalypt varieties.

Individual sugar content

Analyses by ChemicalAnalysis provided the sugar content assessments shown in Table 1.4.

Table 1.4. Individual sugar content of honey samples.

Sample No	Packer's code	Source assigned by packer	Weight/weight %				
			glucose	fructose	sucrose	maltose + total oligosaccharides	total saccharides
1	7843WES	Jarrah 1	22.9	40.7	7.3	3.8	74.8
2	7863WES	Jarrah 2	27.9	39.6	4.5	3.3	75.3
3	8012WES	Jarrah 3	23.6	42.1	6.8	3.1	75.6
4	8105WES	Jarrah 4	23.0	41.0	7.4	3.5	74.9
5	8113WES	Jarrah 5	20.5	35.3	5.4	3.8	65.0
6	7264DEN	Red Stringybark 1	21.2	42.7	6.2	3.3	73.5
7	7369HOL	Red Stringybark 2	28.9	38.3	5.6	1.7	74.4
8	7460EMM	Red Stringybark 3	27.1	41.2	7.1	3.6	79.0
9	7515BBN	Red Stringybark 4	24.7	42.6	4.5	2.3	74.0
10	7526BOM	Red Stringybark 5	24.5	43.5	5.2	1.9	75.2
11	3747RUT	Spotted Gum 1	25.6	43.1	3.4	2.2	74.3
12	3854DEN	Spotted Gum 2	24.4	45.4	3.0	2.2	75.0
13	3883SNO	Spotted Gum 3	25.6	43.8	3.4	2.4	75.2
14	4442BOM	Spotted Gum 4	27.5	42.7	2.7	2.2	75.1
15	5485BOM	Spotted Gum 5	27.9	43.2	2.7	2.0	75.9
16	5735SPI	Yellow Box 1	26.8	38.2	3.8	3.3	72.2
17	7130SMI	Yellow Box 2	24.2	43.2	5.2	3.5	76.1
18	7141WRI	Yellow Box 3	24.2	42.5	5.4	3.5	75.6
19	7427RUT	Yellow Box 4	24.2	42.0	6.3	2.8	75.3
20	7626DEN	Yellow Box 5	23.8	43.4	5.4	3.0	75.6
21	8168KLI	Canola 1	33.3	38.8	1.2	2.3	75.6
22	8193SNO	Canola/ Stringybark 2	26.1	42.1	4.4	2.0	74.7

The measured content of individual sugars was also calculated as 'grams per 25 grams of available sugar' (Table 1.5) because 25 grams of available sugar or multiples thereof are used as the basis for measuring the Glycaemic Index in human subjects (Holt et al. 2002; Chapter 2 this study).

Table 1.5. Normalised sugar content of honey samples.

Sample No	Packer's code	Source assigned by packer	Grams per 25 grams available sugars			
			glucose	fructose	sucrose	maltose + oligosaccharides
1	7843WES	Jarrah 1	7.7	13.6	2.4	1.3
2	7863WES	Jarrah 2	9.3	13.1	1.5	1.1
3	8012WES	Jarrah 3	7.8	13.9	2.2	1.0
4	8105WES	Jarrah 4	7.7	13.7	2.4	1.2
5	8113WES	Jarrah 5	7.9	13.6	2.1	1.5
6	7264DEN	Red Stringybark 1	7.2	14.5	2.1	1.1
7	7369HOL	Red Stringybark 2	9.7	12.9	1.9	0.6
8	7460EMM	Red Stringybark 3	8.6	13.0	2.2	1.1
9	7515BBN	Red Stringybark 4	8.3	14.4	1.5	0.8
10	7526BOM	Red Stringybark 5	8.1	14.5	1.7	0.6
11	3747RUT	Spotted Gum 1	8.6	14.5	1.1	0.7
12	3854DEN	Spotted Gum 2	8.1	15.1	1.0	0.7
13	3883SNO	Spotted Gum 3	8.5	14.6	1.1	0.8
14	4442BOM	Spotted Gum 4	9.2	14.2	0.9	0.7
15	5485BOM	Spotted Gum 5	9.2	14.2	0.9	0.7
16	5735SPI	Yellow Box 1	9.3	13.2	1.3	1.1
17	7130SMI	Yellow Box 2	8.0	14.2	1.7	1.1
18	7141WRI	Yellow Box 3	8.0	14.1	1.8	1.2
19	7427RUT	Yellow Box 4	8.0	13.9	2.1	0.9
20	7626DEN	Yellow Box 5	7.9	14.4	1.8	1.0
21	8168KLI	Canola 1	11.0	12.8	0.4	0.8
22	8193SNO	Canola/ Stringybark 2	8.7	14.1	1.5	0.7

The glucose:fructose ratios were also examined (Figure 1.1) to assess any variations that might make a sample unrepresentative of general honey composition, and thus unsuitable for use in further investigations. The corresponding glucose:sucrose ratios were also calculated (data not shown). It is considered that these analyses could also allow detection of unusual compositional characteristics that might be indicative of post-harvest manipulation of sugar content.

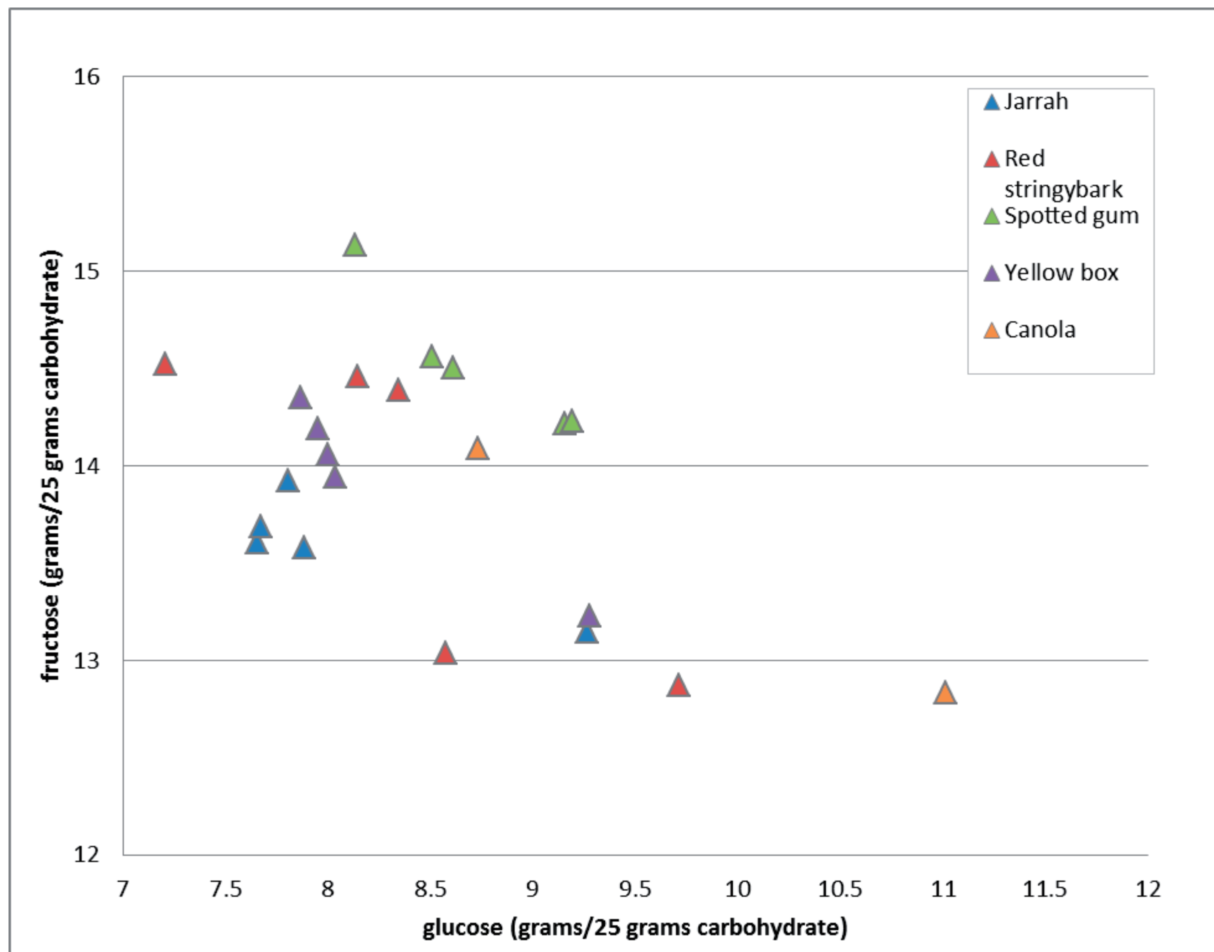

Figure 1.1. Glucose versus fructose content of honey samples.

Analyses indicated that:

- The concentrations of total saccharides in the Red Stringybark, Spotted Gum and Yellow Box honeys were extremely similar ($P > 0.8$), as were those of four of the Jarrah honeys.

- The fructose content of the Spotted Gum honeys was significantly higher than that of the Jarrah honeys ($P = 0.026$ when the raw data were compared, and $P = 0.002$ when the data had been normalised against sugar content). There were no other significant differences between fructose levels in the honeys from different eucalypt sources, and the fructose content of the Red Stringybark and Yellow Box honeys was very similar ($P > 0.8$).

- There were no significant differences between the glucose contents of any of the honeys from different eucalypt sources.

- The sucrose content of the Spotted Gum honeys was significantly lower than those of all the other eucalypt honeys (against Jarrah honeys $P = 0.004$ for the raw data and $P = 0.002$ for normalised data; against Red Stringybark honeys $P = 0.002$ for the raw data and $P = 0.001$ for normalised data; against Yellow Box honeys $P = 0.004$ for the raw data and $P = 0.002$ for normalised data).

- The amount of maltose + oligosaccharides in Spotted Gum honeys was also significantly lower than in Jarrah honeys (P = 0.004 for the raw data and P = 0.001 for normalised data) and in Yellow Box honeys (P = 0.001 for both the raw and normalised data). When the data were normalised the content of these saccharides in Jarrah honeys was higher than in Red Stringybark honeys (P = 0.029).

- The clustered scatter-plot distribution of glucose:fructose ratios (Figure 1.1) and the clustered scatter-plot distribution of glucose:sucrose ratios (data not shown) were consistent with the variabilities in composition noted above, but gave no indication of atypical composition for any honey sample. The outlier position of the canola-derived honey is consistent with the previously reported high relative glucose content (Abell et al. 1996).

- It should be noted that some of the individual sugar (glucose, fructose and particularly sucrose) contents of the Jarrah, Red Stringybark and Yellow Box honeys differ markedly from those reported in Chandler et al. (1974) and for individual samples of Stringybark and Yellow Box by Arcot & Brand-Miller (2005). This may be in part attributable to changes in analytical technology, but probably underlines the individual variation between samples from the same floral source.

MGO and DHA content

Analyses by ChemicalAnalysis provided the MGO and DHA assessments shown in Table 1.6.

Table 1.6. MGO and DHA content of honey samples.

Sample No	Packer's code	Source assigned by packer	MGO (mg/kg)	DHA (mg/kg)
1	7843WES	Jarrah 1	10	60
2	7863WES	Jarrah 2	120	179
3	8012WES	Jarrah 3	18	66
4	8105WES	Jarrah 4	14	62
5	8113WES	Jarrah 5	16	75
6	7264DEN	Red Stringybark 1	19	73
7	7369HOL	Red Stringybark 2	19	72
8	7460EMM	Red Stringybark 3	18	66
9	7515BBN	Red Stringybark 4	16	50
10	7526BOM	Red Stringybark 5	11	43
11	3747RUT	Spotted Gum 1	18	56
12	3854DEN	Spotted Gum 2	15	56
13	3883SNO	Spotted Gum 3	17	61
14	4442BOM	Spotted Gum 4	17	55
15	5485BOM	Spotted Gum 5	17	63
16	5735SPI	Yellow Box 1	22	66
17	7130SMI	Yellow Box 2	16	49
18	7141WRI	Yellow Box 3	19	57
19	7427RUT	Yellow Box 4	18	60
20	7626DEN	Yellow Box 5	15	58
21	8168KLI	Canola 1	5	25
22	8193SNO	Canola/Stringybark 2	6	15

Analyses showed that:

- There was no significant difference between either the MGO or the DHA contents of the four groups of eucalypt honeys.
- One of the Jarrah honey samples (2) had a high content of both MGO and DHA, which have been implicated in the antibiotic activity of some honeys.

Nuclear Magnetic Resonance

2D-NMR of Spotted Gum honey sample 3883SNO (Spotted Gum 3). The following saccharides were identified on the spectrum:

- monosaccharides: α-glucose, β-glucose;
- disaccharides: sucrose, palatinose, maltose, isomaltose, maltulose, nigerose, kojibiose, turanose, melibiose;
- trisaccharides: maltotriose, isomaltotriose, erlose, raffinose;
- tetrasaccharides: maltotetraose.

Several of these sugars may contribute to prebiotic activity (see Chapter 3).

Implications

This study did not identify any physical or chemical characteristic, or combination thereof, that could be reliably used to differentiate between Australian eucalypt honeys sourced from different floral species.

Recommendation

The current method of assigning floral sources to Australian eucalypt honey samples remains the best available.

Chapter 2. Glycaemic Index of honey samples

Introduction

The Glycaemic Index (GI) is a ranking of foods based on their overall effects on blood glucose levels. Foods with high GI values contain easily digested carbohydrates and produce a high, rapid rise and subsequent fall in blood glucose and insulin levels; those with low GI values contain carbohydrates that are digested more slowly and produce a slower, lower increase in blood glucose. The impact of the GI of a diet on a range of health outcomes is being increasingly recognised. Large-scale epidemiological studies and meta-analyses have shown that the long-term consumption of a high GI diet can increase the risk of developing diabetes, heart disease and some cancers (FAO/WHO Report 1997; Favero et al. 1999), and more recently that the GI of the diet may be the most important dietary factor in preventing type 2 diabetes (Barclay et al. 2008). Low GI diets have been shown to reduce the risk of these diseases, improve blood glucose control and insulin sensitivity in diabetics and reduce high blood lipid levels (Jenkins et al. 1985; Brand et al. 1991; Jarvi et al. 1999; Bouché et al. 2002). They are also recommended for weight control as in addition to reducing insulinaemia, low GI foods are associated with higher satiety than high GI diets (Roberts 2000).

The International Tables of Glycemic Index and Glycemic Load Values: 2008 (Atkinson et al. 2008) are based on 205 articles published between 1981 and 2007, as well as unpublished data where the data quality could be verified. A GI value of ≤ 55 is classed as low, from 56 to 69 as moderate and ≥ 70 as high. Fructose, usually the dominant sugar in honey, has the lowest GI (19 ± 2) of any naturally occurring monosaccharide, compared with 100 for glucose and maltose, and 68 ± 5 for sucrose. The GI values of different honeys compared with glucose (100) in healthy subjects ranged from 35 ± 4 to 87 ± 8. The GI values of different Australian honeys quoted in the Table were determined in a Rural Industries Research and Development Corporation study (Arcot & Brand-Miller 2005). All five types of eucalypt honey, Yellow Box, Stringybark, Red Gum, Ironbark and Yapunya, had low GI values (< 55). The lowest was Yellow Box at 35 ± 4. Using linear correlation analysis these authors reported that fructose content was significantly associated with the average GI values of the honeys ($r = -0.76$, $n = 9$, $p<0.05$) but the other individual sugars were not. An earlier publication by the same group reported that the glucose content of Australian honeys was significantly related to their mean GI, but that GI was not significantly related to the content of fructose, sucrose, maltose or organic acids (Holt et al. 2002).

The specific aims of this part of the study were to expand the preliminary studies quoted above to:

- confirm that Australian eucalypt honeys are low GI foods;
- determine whether it is possible to estimate the GI value of an Australian eucalypt honey from its content of individual sugars, and
- assess a new *in vitro* Predictive GI test for its potential value in testing commercial batches of honey.

Methodology

In vivo GI measurement

The GI values of seven selected honey samples were measured in normal human subjects by the Glycemic Index Research Service, University of Sydney (SUGiRS). The methodology was developed at this Centre and is recognised internationally. The procedures were approved by the Human Research Ethics Committee of the University of Sydney.

Subjects

The study was conducted in ten healthy, non-smoking subjects aged 18-45 years who were within the healthy weight range, not dieting, and who did not have impaired glucose tolerance. Seven were males and three were females.

Test foods

A workshop of project stakeholders and investigators selected seven of the 22 honeys to be tested for GI using the standard *in vivo* methodology. Six of these honeys (samples 3, 6, 7, 9, 12 and 17) were confirmed by pollen and conductivity analyses as being ‘eucalyptus honey’ in accordance with European Council Directive 2001/110/EC, and the other was confirmed as canola honey by the same criteria (see Chapter 1). The samples included one Jarrah honey, three Red Stringybark honeys, one Spotted Gum honey and one Yellow Box honey with a spread of glucose and fructose content (see Table 1.4) to optimise attempts to relate these parameters to GI values.

Test procedure

Pure glucose dissolved in water was used as the reference food. Glucose and the honey samples were administered in portions containing 50 grams of available carbohydrate, accompanied by 250 grams of water. Each subject completed 10 individual tests. After fasting overnight for 10-12 hours, every subject consumed each of the honey samples in random order on one occasion, and the reference glucose preparation in the first, sixth and tenth test sessions. At least one day was allowed between test sessions. For each test, two fasting blood samples were first obtained. The test food was then consumed, after which additional blood samples were taken at 15, 30, 45, 60, 90 and 120 minutes. The blood samples were centrifuged and the plasma frozen until analysis.

Sample analysis

The glucose concentration of the plasma samples was assayed in duplicate using a glucose hexokinase enzymatic method (Roche Diagnostic Systems) and a Roche/Hitachi 912® automatic centrifugal spectrophotometric analyser with internal controls.

Calculation of GI values

For each test session the glucose concentrations in the two fasting plasma samples were averaged to give a baseline. The incremental area under each 2 hour glucose response curve (iAUC) was then calculated. The ratio of the iAUC for a honey sample to the averaged iAUCs for glucose for that subject, expressed as a percentage (glucose = 100%), gave the GI value for the honey. If any individual subject’s GI value for a particular honey was either greater than the group mean value plus two standard deviations or less than the group mean value minus two standard deviations it was classified as an outlier and removed from the dataset.

Statistics

A power-based (90%) sample size calculation indicated that at least eight subjects would be required to generate statistically significant results (a difference of 1.0 standard deviation units in GI).

The researchers reported the GI value for each honey sample as the mean ± the standard error of the mean (SEM). They used analysis of variance and the Fisher PLSD test for multiple comparisons to determine whether there were significant differences between the GI values obtained.

In vitro Predictive GI test

Test foods

All 22 honey samples used in this study were tested by Next Instruments (Condell Park, Sydney), with the exception of Red Stringybark 7264DEN, which was omitted in error from the samples sent to the testing laboratory. The laboratory instead received two samples of Yellow Box honey 7427RUT, both of which were tested.

Test procedure

The *in vitro* test used the NutriScan G120 Glycemic Index Analyser, a high precision fully automated instrument that mimics the way carbohydrates are digested in the human gut. It uses 50 milligrams of carbohydrate per sample, which are analysed at 37°C under gentle agitation, with physiological pH maintained throughout. Samples are initially treated with an enzyme that mimics saliva, followed by a second enzyme that breaks down fats and proteins in the sample. A further enzyme converts the sugars to glucose, and aliquots are analysed in a glucose analyser 15, 60, 120, 180, 240 and 300 minutes after initiation of the reaction. Conversion is complete at 300 minutes.

Each honey sample was assayed in duplicate at the same time as duplicate samples of the control material, glucose. One sample, canola honey 8168KLI, was assayed in duplicate on two occasions. Two samples, Red Stringybark honeys 7369HOL and 7515BBN, were first assayed in duplicate and single samples were then re-assayed on two further separate days to assess the repeatability of the assay.

Calculation of GI values

The Predictive GI was calculated using the formula:

$$\text{Predictive GI} = \frac{\text{final glucose concentration (mg/ml) x final sample volume x 100}}{\text{total available carbohydrate in sample}}$$

The mean of the duplicate results was used as the final Predictive GI Value for the sample. The differences between duplicates were used to calculate the Standard Deviation of Differences (SDD).

Statistics

For both *in vivo* and *in vitro* tests the relationship between GI values and sugar content of the honey samples was examined using the Pearson correlation coefficient and the 2-tailed probability p value; a significance level of 5% ($p < 0.05$) was chosen.

Results

In vivo GI measurement

In vivo GI analyses of seven honey samples by SUGiRS resulted in the data provided in Table 2.1.

Table 2.1. GI values of honey samples.

Sample No	Packer's code	Source assigned by packer	Test subjects (n)	GI	
				GI value (mean ± SEM)	GI category
3	8012WES	Jarrah 3	10	54 ± 3	Low
6	7264DEN	Red Stringybark 1	10	58 ± 4	Medium
7	7369HOL	Red Stringybark 2	9	48 ±4	Low
9	7515BBN	Red Stringybark 4	9	60 ± 4	Medium
12	3854DEN	Spotted Gum 2	9	52 ± 5	Low
17	7130SMI	Yellow Box 2	9	57 ± 3	Medium
21	8168KLI	Canola 1	10	56 ± 4	Medium
Glucose reference			10	100 ± 0	High (reference)

The mean GI values for all the honey samples were significantly lower than that of the glucose reference and the difference was highly significant ($p < 0.001$) in all cases. The mean GI value for one Red Stringybark honey (7515BBN) was significantly higher than that for another (7369HOL), but there were no other significant differences amongst the mean GI values of the honey samples.

Using the mean GI value, three of the eucalypt honey samples (one Jarrah, one Red Stringybark and one Spotted Gum) were rated as being 'low' GI and the other three samples (two Red Stringybark and one Yellow Box) as being of 'medium' GI, as was the canola honey. Holt et al. (2002) and Arcot and Brand-Miller (2005) reported that all the Australian eucalypt honeys they tested, including a Yellow Box honey and a Stringybark honey, had low GI values. It should be noted that although all the *in vivo* GI tests were performed by the same research group, the carbohydrate load used in the earlier studies was only 25 grams, half that administered to subjects in the present project. This may have affected the results.

The data were analysed to detect any correlation between the mean GI value and sugar content of the honey samples (Table 2.2).

Table 2.2. Correlation between GI values and sugar content of honey samples.

Parameters	r	p
GI vs glucose	-0.311	0.50
GI vs fructose	+0.328	0.47
GI vs glucose/fructose	+0.339	0.46
GI vs sucrose	+0.049	0.92
GI vs maltose + oligosaccharides	+0.560	0.19
GI vs total saccharides	-0.134	0.77

There was no strong or significant correlation between any of the sugar contents analysed and the mean GI values for these honey samples. Similarly there was no strong or significant correlation of mean GI with the pH or content of MGO, DHA or water. The best correlation between mean GI value and any of the physical and chemical characteristics measured was with the combined maltose + oligosaccharide content. However, it was not sufficiently strong to form the basis for a valid surrogate marker of the GI value for a honey sample.

Multivariate analysis was considered as a potential means of deriving a significant correlation between GI values and two simple measurables of the honey samples, but the study did not yield sufficient data for this to be a useful approach.

Results from this study were not consistent with those of Holt et al. (2002), who previously reported a significant correlation between the GI value and glucose content of Australian honey samples. Moreover, there was no significant correlation between the GI value and glucose content of honey samples when the mean data from this study were combined with those of Holt et al.

We observe that the results reported by Holt et al. are disproportionately affected by a single test sample ('Commercial Blend 1'), for which the glucose content appears to have been calculated in error. If the GI result for this sample is amended their results do not show a significant correlation between GI value and glucose content. This is shown in Figure 2.1; the data from Holt et al. are labelled '2002', and the re-calculated result for 'Commercial Blend 1' as '2002 amended'.

Arcot and Brand-Miller (2005) subsequently reported a significant correlation between the GI values and fructose content of Australian honey samples; we are unable to reproduce this result from analysis of their data, and again the results of this study did not confirm that conclusion.

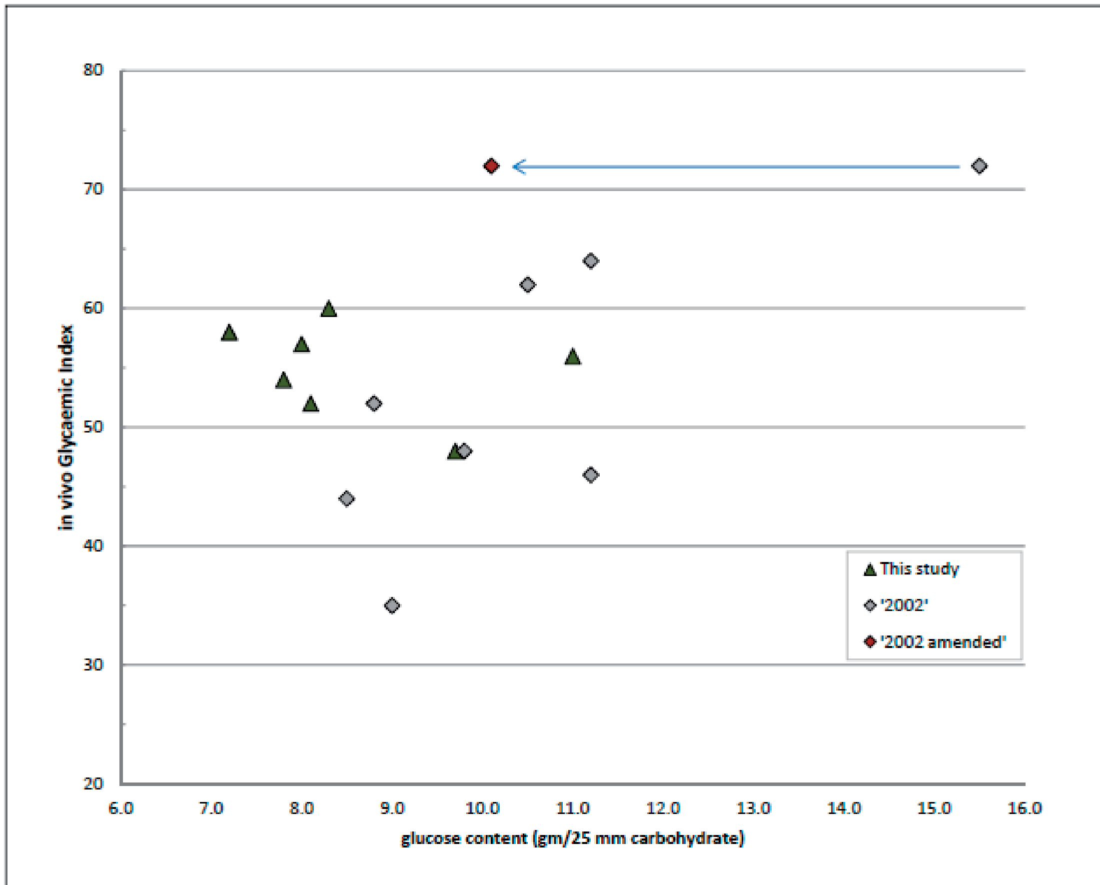

Figure 2.1. Glycaemic Index in relation to glucose content of honey samples.

Primary data from *in vivo* GI measurement

(Held in confidence)

In vitro Predictive GI test

In vitro measurements of the Predictive GI values for 21 honey samples by Next Instruments resulted in the data provided in Table 2.3.

Table 2.3. Predictive GI values of honey samples.

Sample No	Packer's code	Source assigned by packer	Predictive GI		
			Value	Mean	Category
1	7843WES	Jarrah 1	48.9; 47.8	48.4	Low
2	7863WES	Jarrah 2	58.3; 63.0	60.6	Medium
3	8012WES	Jarrah 3	50.6; 51.1	50.8	Low
4	8105WES	Jarrah 4	47.6; 48.0	47.8	Low
5	8113WES	Jarrah 5	55.2; 56.7	55.9	Medium
6	7264DEN	Red Stringybark 1	not assessed		
7	7369HOL	Red Stringybark 2	51.1; 48.0; 50.5; 51.4	50.3	Low
8	7460EMM	Red Stringybark 3	58.9; 56.5	57.7	Medium
9	7515BBN	Red Stringybark 4	61.1; 60.0; 62.7; 61.3	61.3	Medium
10	7526BOM	Red Stringybark 5	52.8; 51.9	52.3	Low
11	3747RUT	Spotted Gum 1	58.9; 58.1	58.5	Medium
12	3854DEN	Spotted Gum 2	56.9; 56.3	56.6	Medium
13	3883SNO	Spotted Gum 3	55.9; 56.6	56.3	Medium
14	4442BOM	Spotted Gum 4	60.4; 59.8	60.1	Medium
15	5485BOM	Spotted Gum 5	60.7; 60.1	60.4	Medium
16	5735SPI	Yellow Box 1	64.2; 63.1	62.7	Medium
17	7130SMI	Yellow Box 2	54.3; 54.0	54.2	Low
18	7141WRI	Yellow Box 3	55.855.7	55.7	Medium
19	7427RUT	Yellow Box 4	57.9; 56.7; 55.9; 56.1	56.7	Medium
20	7626DEN	Yellow Box 5	56.4; 56.0	56.2	Medium
21	8168KLI	Canola 1	65.2; 64.1; 66.8; 67.1	65.8	Medium
22	8193SNO	Canola/Stringybark 2	56.2; 56.5	56.3	Medium

The overall standard deviation of differences for these measurements was 1.1. Three honey samples were tested on three separate occasions and the estimated repeatability of the test was 2.0 GI units. The automated Predictive GI test is therefore highly reproducible.

Table 2.4. Correlation between Predictive GI values (PGI) and sugar content of honey samples.

Parameters	r	p
PGI vs glucose	+0.601	0.004
PGI vs fructose	-0.083	0.72
PGI vs glucose/fructose	+0.540	0.012
PGI vs sucrose	-0.763	<0.001
PGI vs maltose + oligosaccharides	-0.246	0.28
PGI vs total saccharides	-0.015	0.95

As shown in Table 2.4, the best correlation between mean Predictive GI value and any of the sugar contents of the honey samples was with the sucrose content. However, it was not sufficiently strong to form the basis for a valid surrogate marker of the Predictive GI value for a honey sample.

The correlation between the mean GI values from the *in vivo* test and the mean Predictive GI values was $r = 0.60$ ($p = 0.208$). This is not sufficiently strong for the Predictive GI test to be used as a surrogate for the *in vivo* test.

It should be noted that the Predictive GI values are not claimed to be the same as the *in vivo* GI. Although the automated system is designed around a simulated environment involving enzymes, pH, temperature and movement approximating the conditions in the human gut, other factors such as insulin release and gastric emptying are not included. Insulin release in particular could be a major factor in the *in vivo* catabolism of honey.

Implications

On the basis of the data and considerations provided above, we conclude that:

- Australian eucalypt honeys are probably low to medium GI foods when consumed by the majority of individuals, but not necessarily of lower GI value than honeys from other floral sources.

- The automated *in vitro* Predictive GI test is highly reproducible, but the results do not correlate strongly with those from the *in vivo* analysis. It should be noted that the Predictive GI test does not incorporate such *in vivo* parameters as insulin release and stomach emptying; the former in particular is likely to be highly relevant to individual responses to honeys. Although results for these honeys from the *in vivo* test are highly variable between subjects, this procedure is currently regarded as the 'gold standard' for measuring GI values, and the acceptance of any other form of testing is likely to pose major challenges.

- The *in vivo* GI value of a honey cannot be reliably predicted on the basis of its content of glucose, fructose or any other simple physical or chemical property measured in this study. The *in vivo* GI values of the honey samples were most clearly related to the measured content of maltose + oligosaccharides, and the Predictive GI values to the sucrose content. However, the correlation was not sufficiently strong for either of these properties to form the basis for a rapid surrogate assay of honeys that could be used instead of *in vivo* testing.

- The ability to describe Australian eucalypt honeys as having low or medium GI may have potential commercial value, but capture of any such benefits would require the GI value of each batch of honey to be measured. In considering this we note that:
    - The cost of the *in vivo* GI test is too high for this to be commercially viable.

- The automated Predictive GI test would be affordable for batch analysis. However, data for honeys from this assay do not correlate sufficiently strongly with the current ‘gold standard’ *in vivo* test for it to be automatically accepted as a surrogate assay.

Recommendation

We recommend that industry funds not be further expended on analysis of the Glycaemic Index of Australian eucalypt honeys.

Chapter 3. Prebiotic properties of honey samples

Introduction

Prebiotic foods promote the growth of beneficial bacteria in the human intestine with a positive impact on health. In recent years awareness of the role of intestinal bacteria and their complex interactions in human health has increased markedly, as evidenced by the publication of special issues of *Science* entitled 'The Gut Microbiota' and of *Nature Reviews Gastroenterology & Hepatology* named 'Gut Microbiota' in 2012. There is increasing evidence that the gut microbiota is intrinsically linked to our metabolic health and the number of disease states associated with dysbiosis of the gut microbiota is growing rapidly. These include gastrointestinal diseases such as inflammatory bowel disease, cancer, cardiovascular disease and obesity.

Prebiotic ingredients are not digested by human enzymes, but reach the large intestine intact and there act as a food source for beneficial bacteria including bifidobacteria and lactobacilli. Healthy populations of these bacteria can combat potentially deleterious species and increase resistance to invading pathogens. Transparency Market Research (2013) have recently reported that the market for prebiotic ingredients such as inulin and other complex oligosaccharides was worth USD 2.3 billion in 2012 and is estimated to reach USD 4.5 billion in 2018. Foods containing prebiotic ingredients are referred to as functional foods. In 2005 Sanz et al. reported that honey oligosaccharides had prebiotic properties, increasing the populations of bifidobacteria and lactobacilli. In 2010 Conway et al. indicated in a report to the Rural Industries Research and Development Corporation that some Australian honeys possess prebiotic properties. However, a recent report indicates that this is not the case for all honeys; Wallace et al. (2010) reported that neither a manuka honey with a high content of methylglyoxal nor a low-methylglyoxal multiflora honey altered the gut microbiota composition.

Gut microbiota can also synthesise short-chain fatty acids (SCFAs; Nicholson et al. 2012), which have known benefits in the body. Butyric acid is particularly interesting in this context as it is used as an energy source by colonic epithelial cells. It also has an important role in cell differentiation, with a highly proliferative effect on healthy intestinal cells and an anti-proliferative effect on cancerous cell lines (Hamer et al. 2008).
The specific aims of this study were to:

- assess *in vitro* the prebiotic potential of Australian eucalypt honeys;
- confirm *in vivo* the prebiotic properties of selected Australian eucalypt honeys;
- determine whether it is possible to relate prebiotic activity to the content of individual sugars and
- measure the microbial SCFA synthesis responses to the Australian eucalypt honeys.

Methodology

Assessment of prebiotic potential of the honey samples was carried out by ProBiOz Pty Ltd.

In vitro assessment of Prebiotic Index

Test samples

All the 22 honey samples were tested, both untreated (whole) and predigested to reflect the *in vivo* situation whereby the honey would be exposed to digestive enzymes and the simple sugars absorbed so that they were not available to intestinal microbes. Predigested samples of honeys and control media were prepared by treatment with acid and digestive enzymes followed by a dialysis step to remove the simple sugars, leaving only oligosaccharides larger than a pentasaccharide and polysaccharides. These were resuspended in the original volume of whole honey. Inulin and fructo-oligosaccharide were included in the assays as controls with high PI values. The PI values of fructose and glucose were also measured.

Experimental design

Intestinal microcosms were derived using faecal material from two healthy human subjects to allow examination of the effect of ingested honeys on the entire intestinal microbial population following the method of Conway et al. (2010). One subject was an adult female with a typical adult profile and the other a 12-month-old baby girl who was still being breast fed and who had high levels of bifidobacteria, as would be anticipated. Freshly voided faecal samples were collected and transferred to sterile specimen jars and stored at -20°C within1 hour to ensure maintenance of viability. Separate microcosms were established using suspensions of the adult and infant faecal samples and honey samples which were either untreated or had been predigested. After fermentation, samples were collected for culture evaluation using selective media and the plate count technique. Growth of the beneficial bacteria, lactobacilli and bifidobacteria, the potentially harmful clostridia and bacteroides and the total numbers of bacteria were determined. The assays were performed in duplicate on three separate days. Results were expressed as mean values (± 1 SD) and used to calculate a Prebiotic Index for each sample.

Short chain fatty acid (SCFA) metabolites were quantified by gas chromatography.

Prebiotic Index (PI)

The PI was calculated using the following equation (Palframan et al. 2003):

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

where:

Bif = final number of bifidobacteria /initial number;
Bac = final number of bacteroides/initial number;
Lac = final number of lactobacilli /initial number;
Clos = final number of clostridia/initial number;
Total = final total bacterial number/initial number.

In the *in vitro* study the PI refers directly to the effect of the honey samples. In the *in vivo* study, however, PI values are reported before and after honey consumption. The difference between these two values reflects the effect of the honey consumed.

Butyric acid analysis

Predigested honey samples incubated with adult and infant intestinal microcosms were analysed by gas chromatography – mass spectrometry (GC-MS) for short-chain fatty acid (SCFA) production as a result of bacterial fermentation. Samples from the microcosm were extracted with ether and analysed

by GC-MS using the internal standard method. The samples were run against a standard solution of volatile acids. Standard curves were generated and the levels of individual SFCAs in each sample were calculated by determining the area of the sample peak relative to the internal standard peak.

In vivo measurement of Prebiotic Index

Test samples

The Prebiotic Index of four honey samples, one from each eucalypt floral source, was measured *in vivo*. The samples were selected to reflect all four combinations of low and high PI and low and high butyrate generation when tested *in vitro*. These data are represented in Figure 3.1 with a halo indicating the samples chosen for the *in vivo* study; the detailed data are given in Tables 3.1 and 3.3.

Figure 3.1. ***In vitro*** **PI and butyrate generation after incubation with honey samples.**

Experimental design

The double blind cross-over study was conducted in 40 subjects aged 20-50 years of age and free of chronic diseases of the digestive tract or the cardiovascular system and not diabetic, obese, pregnant or allergic to honey. A few subjects dropped out during the trial and additional subjects were then recruited. The study was carried out in accordance with the guidelines laid down in the Declaration of Helsinki and approved by the University of NSW Human Research Ethics Committee. Subjects were randomised into two groups and two different honeys, one of high and the other of low *in vitro* PI, were sequentially tested in each group of 20 subjects. Group allocations ensured uniform distribution

of age, sex and diet. This design enabled effect sizes greater than 0·7 to be detected as statistically significant with 80% power.

The study was divided into four phases each of which was four weeks in duration:

Phase 1. Honey excluded from the diet

Phase 2: Daily consumption of 20g of honey A

Phase 3: No honey consumption

Phase 4: Daily consumption of 20g of honey B

Phases 1 and 3 served as wash out periods to remove the effects of previously ingested honey. Compliance was monitored at the end of each phase and major deviations from protocol resulted in subjects being discontinued.

Group 1 consumed honey sample Spotted Gum 3 as honey A, and Jarrah 4 as honey B; Group 2 consumed honey sample Red Stringybark 2 as honey A, and Yellow Box 2 as honey B.

Freshly voided faecal samples were collected at the beginning of Phase 1 and at the end of each phase, and stored at -80°C prior to analysis. The bacterial content of each faecal sample was analysed and the PI calculated as described above for the *in vitro* studies. In addition butyrate levels in the faecal suspensions were determined by gas chromatography. The effects of the honeys were calculated as the change from the beginning to the end of the 4 week honey consumption period.

Statistics

Relationships between PI values, butyric acid production and sugar content of the honey samples were examined using the Pearson correlation coefficient r and the 2-tailed probability p value; a significance level of 5% ($p < 0.05$) was chosen.

Results

In vitro assessment of Prebiotic Index

The majority of the honey samples promoted growth of the beneficial intestinal bifidobacteria and lactobacilli but not of the potentially deleterious clostridia and enterobacteria. PI values calculated from the data are summarised in Table 3.1.

Table 3.1. PI values of honey samples.

Sample No	Packer's code	Source assigned by packer	PI (mean ± 1 SD)			
			Whole honey		Predigested honey	
			Infant faecal sample	Adult faecal sample	Infant faecal sample	Adult faecal sample
1	7843WES	Jarrah 1	9.86 ± 0.56	8.94 ± 0.03	13.46 ± 0.05	8.32 ± 0.14
2	7863WES	Jarrah 2	2.68 ± 0.29	3.87 ± 0.42	5.88 ± 0.47	4.49 ± 0.03
3	8012WES	Jarrah 3	7.20 ± 0.16	5.26 ± 0.71	2.70 ± 0.07	3.63 ± 1.14
4	8105WES	Jarrah 4	4.12 ± 0.13	2.70 ± 0.40	13.27 ± 1.33	12.35 ± 0.34
5	8113WES	Jarrah 5	4.75 ± 0.09	3.28 ± 0.87	9.07 ± 0.86	9.61 ± 0.23
6	7264DEN	Red Stringybark 1	4.38 ± 0.30	3.27 ± 0.12	1.30 ± 0.01	2.31 ± 0.56
7	7369HOL	Red Stringybark 2	1.24 ± 0.19	1.23 ± 0.12	0.12 ± 0.03	0.41 ± 0.49
8	7460EMM	Red Stringybark 3	2.72 ± 0.73	2.56 ± 0.45	3.75 ± 0.41	2.42 ± 0.11
9	7515BBN	Red Stringybark 4	3.26 ± 0.07	3.89 ± 0.46	2.76 ± 0.29	1.97 ± 0.51
10	7526BOM	Red Stringybark 5	2.83 ± 0.31	3.93 ± 0.73	-0.13 ± 0.17	0.07 ± 0.08
11	3747RUT	Spotted Gum 1	3.21 ± 0.53	2.96 ± 0.05	0.61 ± 0.26	0.19 ± 0.13
12	3854DEN	Spotted Gum 2	3.14 ± 0.27	2.99 ± 0.25	0.23 ± 0.10	0.06 ± 0.1
13	3883SNO	Spotted Gum 3	1.34 ± 0.22	1.13 ± 0.05	0.76 ± 0.32	0.32 ± 0.05
14	4442BOM	Spotted Gum 4	0.90 ± 0.08	0.16 ± 0.10	0.21 ± 0.05	0.14 ± 0.11
15	5485BOM	Spotted Gum 5	2.39 ± 0.30	2.72 ± 0.30	0.18 ± 0.05	0.24 ± 0.06
16	5735SPI	Yellow Box 1	2.23 ± 0.23	3.85 ± 0.49	2.29 ± 0.22	4.59 ± 0.72
17	7130SMI	Yellow Box 2	3.71 ± 0.45	5.91 ± 0.34	4.03 ± 0.37	5.43 ± 0.98
18	7141WRI	Yellow Box 3	3.07 ± 0.70	2.52 ± 0.12	2.18 ± 0.08	2.54 ± 0.52
19	7427RUT	Yellow Box 4	1.11 ± 0.11	1.61 ± 0.38	0.66 ± 0.07	2.07 ± 0.45
20	7626DEN	Yellow Box 5	13.31 ± 0.06	11.77 ± 0.32	0.61 ± 0.12	2.89 ± 0.19
21	8168KLI	Canola 1	3.16 ± 0.44	1.89 ± 0.57	0.54 ± 0.10	0.63 ± 0.04
22	8193SNO	Canola/Stringybark 2	1.36 ± 0.50	1.38 ± 0.24	0.24 ± 0.30	0.54 ± 0.34
	controls	Inulin	12.74 ± 0.20	11.22 ± 1.23	15.16 ± 0.62	14.68 ± 0.05
		Fructo-oligosaccharide	5.41 ± 0.18	7.27 ± 0.16	4.67 ± 0.20	4.54 ± 0.06
		Fructose	1.76 ± 0.02	1.71 ± 0.18	-0.48 ± 0.32	-0.10 ± 0.06
		Glucose	5.26 ± 0.12	4.58 ± 0.50	-0.89 ± 0.68	-0.25 ± 0.06

The results of this study emphasise the complexity of the interaction of honeys with the intestinal microcosm. There was a very strong correlation between the PI values obtained with adult and infant faecal samples for both whole honey ($r = 0.930$; $p = 0$) and for predigested samples ($r = 0.931$; $p = 0$),

indicating that adult and infant intestinal flora responded similarly to honeys. However, the relationship between PI values for whole and predigested honey samples was much weaker with both adult (r = 0.348; p = 0.112) and infant faecal samples (r = 0.363; p = 0.097). Some whole honeys had higher PI value than the predigested material and for others it was the reverse; yet other samples gave similar PI values whether they were tested whole or predigested.

The individual PI values were not clearly related to the floral origin of the honey. Jarrah honey 7843WES was a very high PI food under all conditions, Jarrah honey 8105WES gave very high PI values after pre-digestion and whole Yellow Box honey 7626DEN had a higher PI than inulin, the highest positive control. At the other end of the scale, Spotted Gum honey 4442BOM and Red Stringybark honey 7369HOL were of negligible value as prebiotics either whole or predigested, and PI values for the Spotted Gum, Canola and Canola/Stringybark honeys were all low.

The data obtained with the adult faecal microbiota were analysed to ascertain whether PI values were related to the sugar content of the honey samples (Table 3.2).

Table 3.2. Correlation between PI values and sugar content of honey samples.

	Whole honey		**Predigested honey**	
	r	**p**	**r**	**p**
PI vs glucose	-0.376	0.085	-0.504	0.017
PI vs fructose	+0.097	0.668	-0.480	0.024
PI vs glucose/fructose	-0.351	0.109	-0.230	0.303
PI vs sucrose	+0.359	0.101	+0.582	0.004
PI vs maltose + oligosaccharides	+0.420	0.052	+0.782	<0.001
PI vs total saccharides	+0.032	0.888	-0.405	0.062

The PI values of whole honey do not correlate strongly or significantly with any of the sugar contents analysed. However, when the honeys have been predigested there is a strong significant positive correlation with the content of maltose + oligosaccharides. This is not surprising given that the pre-treatment mimics carbohydrate digestion in and absorption from the human gut before the contents interact with bacteria in the large intestine, but it does suggest that the oligosaccharides in honey cannot be digested by human enzymes. There is also a highly significant positive correlation between the PI values of predigested honeys and the sucrose content of the whole honey samples, and a significant negative correlation of predigested honey PI with both glucose and fructose content; this is harder to explain. It seems likely that although most honeys could be expected to deliver health benefits by their impact on the intestinal microbiota, they may not all do so by the same mechanism.

Despite the strong correlation between PI value and content of maltose + oligosaccharides, there are too many outliers for this parameter to be used to detect honey samples of high PI. Six of the 22 honey samples contain 3.5-3.8 mg/ml maltose + oligosaccharides (see table 1.4), but the PI values of these samples range from 2.42 to 12.35.

In vitro butyric acid production

Table 3.3. Butyric acid production (mM) with predigested honey samples.

Sample No	Packer's code	Source assigned by packer	Infant faecal sample	Adult faecal sample
1	7843WES	Jarrah 1	9.99	14.64
2	7863WES	Jarrah 2	6.47	12.42
3	8012WES	Jarrah 3	8.65	15.81
4	8105WES	Jarrah 4	4.53	8.62
5	8113WES	Jarrah 5	3.61	10.65
6	7264DEN	Red Stringybark 1	0.61	0.93
7	7369HOL	Red Stringybark 2	0.58	0.63
8	7460EMM	Red Stringybark 3	0.82	1.74
9	7515BBN	Red Stringybark 4	0.69	1.10
10	7526BOM	Red Stringybark 5	0.69	1.98
11	3747RUT	Spotted Gum 1	1.60	2.30
12	3854DEN	Spotted Gum 2	1.04	3.97
13	3883SNO	Spotted Gum 3	4.55	7.18
14	4442BOM	Spotted Gum 4	1.39	2.36
15	5485BOM	Spotted Gum 5	1.43	1.53
16	5735SPI	Yellow Box 1	1.93	1.81
17	7130SMI	Yellow Box 2	2.41	2.27
18	7141WRI	Yellow Box 3	3.18	3.74
19	7427RUT	Yellow Box 4	2.98	2.48
20	7626DEN	Yellow Box 5	3.15	3.23
21	8168KLI	Canola 1	1.09	2.07
22	8193SNO	Canola/Stringybark 2	2.20	1.91
	controls	Inulin	7.59	9.58
		Fructo-oligosaccharide	3.38	5.63
		Fructose	0.95	0.91
		Glucose	0.76	1.00

As with the PI value, there was a very strong correlation between the butyric acid production obtained with adult and infant faecal samples ($r = 0.934$; $p = 0$). There was a statistically significant relationship between PI value and butyric acid production ($r = 0.594$, $p = 0.004$ for adult samples; $r = 0.630$, $p = 0.002$ for infant samples). All the Jarrah honeys and one Spotted Gum honey were very effective in elevating levels of butyric acid. However, not all had high PI values, indicating that the factors influencing the two properties are not identical (Table 3.3).

Table 3.4. Correlation between butyric acid production and sugar content of honey samples when adult faecal microbiota were incubated with predigested honey.

	r	p
butyric acid vs glucose	-0.338	0.124
butyric acid vs fructose	-0.254	0.254
butyric acid vs glucose/fructose	-0.186	0.407
butyric acid vs sucrose	+0.388	0.074
butyric acid vs maltose + oligosaccharides	+0.510	0.015
butyric acid vs total saccharides	-0.214	0.339

As with the PI values, there was a significant positive correlation between butyric acid production and the content of maltose + oligosaccharides in the honey samples, but there were no other significant correlations with sugar content (Table 3.4).

The SCFA generated by intestinal bacteria include butyric acid, which at high concentrations is linked to a lowered risk of colon cancer (German 1999). Compared to the negative controls, most honeys elevated the levels of butyric acid. This highlights the potential for honey to deliver health benefits other than high PI. All the Jarrah honey samples generated high levels of butyric acid. The possibility that this is a definitive property of Jarrah honeys should be investigated further to determine whether generation of butyric acid can be a value-added claim for Jarrah honeys without the necessity for batch testing.

There was a moderate correlation between the PI values and butyric acid levels generated when the adult faecal sample was incubated with predigested honeys ($r = 0.59$; $p < 0.005$).

In vivo measurement of Prebiotic Index

(Results from *in vivo* clinical trials may be of commercial value. The details of these studies is therefore held in confidence.)

In vivo butyric acid production

(Results from *in vivo* clinical trials may be of commercial value. The details of these studies is therefore held in confidence.)

Implications

On the basis of these data we conclude that:

- The *in vitro* test probably underestimates the potential for honeys to raise the PI *in vivo*.
- We were unable to identify a surrogate diagnostic for PI. The *in vitro* data did not predict the *in vivo* result and none of the sugar contents or physical characteristics analysed correlated sufficiently strongly with the PI to be useful as an indicator.

Recommendation

We recommend that the Australian honey industry pursue the opportunity of promoting Australian eucalypt honeys as foods that will improve health by increasing the Prebiotic Index. We suggest the following steps should be included in this process:

- assess the need for additional research to adequately support this claim;
- conduct a cost: benefit analysis to determine whether pursuing the claim would be financially viable; and
- identify a not-for-profit, independent expert organisation that might endorse the claim and permit its use on labels and in advertising. Further consideration of this recommendation is provided in Chapter 5 of this report.

Chapter 4. Antimicrobial and anti-fungal properties of honey samples

Introduction

It has been recognised for centuries that honey has antiseptic properties. Hippocrates (about 460-370 BC), Aristotle (384-322 BC) and contemporary Arab physicians are among those noting the healing properties of honey. Its use as a wound dressing is summarised by Molan (2006). Some antibacterial effects are expected to arise from the high osmolarity and low pH of honey, and several chemical components contributing to this activity have been identified. Most of the antimicrobial activity of the majority of honey samples is thought to be due to generation of the antioxidant hydrogen peroxide (H_2O_2) by the bee-derived enzyme glucose oxidase (White et al. 1963). Frankel et al. (1998) reported that the water-soluble antioxidant activity of honey varied with its floral source. It has been reported that anti-fungal activity appears to be linked to H_2O_2 content (Irish et al. 2006).

However, other components in the honey, including MGO, bee defensin-1 (Kwakman et al. 2010) and other bee-derived compounds, florally derived phenolics (Estevinho et al. 2008), lysozyme and other yet unidentified compounds may modulate this activity. These components are together referred to as non-peroxide dependent activity. Because a range of compounds and properties contribute to its activity, honey is a broad-spectrum antimicrobial agent and can be active against a range of different bacteria and fungi (Efem et al. 1992) including clinically significant species such as *Staphylococcus aureus* (Chambers 2006) and *Candida albicans* (Irish et al. 2006) . The main antibacterial constituent of Manuka honey, now marketed as an antibacterial product, is MGO (Mavric et al. 2008; Jervis-Bardy et al. 2011).

The several antimicrobial activities in honeys respond differently to environmental conditions. Both prolonged storage and heating can inactivate the enzyme glucose oxidase and hence the level of H_2O_2 in the honey (White & Subers 1964; Irish et al. 2011). By contrast, MGO-based antibacterial activity can increase following heating and storage by the conversion of DHA, derived from nectar, to MGO in a non-enzymic chemical reaction (Adams et al. 2009). Routine treatment for commercial Australian honeys involves a process similar or identical to that to which the samples used throughout this study were subjected. The honeys were warmed below 45°C for eight to ten hours and then filtered through a 100 micron filter to remove wax and other debris and to minimise crystallisation. The effect of this process and of prolonged exposure to retail conditions on the antimicrobial activities of whole honey is unknown.

A recent study of the antimicrobial activity of 477 Australian honey samples found that some eucalypt honeys, particularly Marri and Jarrah honeys, had high antibacterial activity that was largely attributable to H_2O_2 production. A few others, including three of four Spotted Gum samples tested, had significant non-peroxide antibacterial activity (Irish et al. 2011).

The specific aims of this study were to:

- assess *in vitro* the antibacterial and anti-fungal potential of Australian eucalypt honeys before and after heating and filtration;
- determine whether it is possible to relate antibacterial and anti-fungal activities to the content of individual sugars and
- assess whether an as yet unidentified stable compound with commercial potential could contribute to these activities.

Methodology

Test samples

All the 22 honey samples were tested, both as received by Beechworth Honey (initial samples) and as prepared for market by warming and filtration; the latter are referred to as marketable samples.

A negative control of artificial honey (7.5 grams sucrose, 37.5 grams maltose, 167.5 grams glucose, and 202.5 grams fructose in 85 ml sterile water) that simulated the sugar levels found in honey was included as a negative control. Comvita UMF®18+ manuka honey was used as a positive control in the phenol equivalence assay.

Assessment of antibacterial activity

The antibacterial activity of honey samples against *Staphylococcus aureus* strain ATCC 25923 with reference to phenol was determined as described by Allen et al. (1991). Briefly, bioassay plates were seeded with a standardised culture of *S. aureus.* Wells were cut into the agar using a quasi-Latin square, which enabled duplicate samples to be placed randomly on the plate.

Freshly prepared, filter-sterilised 50 per cent (w/v) honey samples in water were mixed with either sterile deionised water for total activity testing, or with freshly prepared 5600 U/ml catalase solution for non-peroxide activity testing, to give a final concentration of 25 per cent (w/v) honey. Aliquots of 100 µL of each solution, and of phenol standards of 2%, 3%, 4%, 5%, 6%, and 7%, were placed into wells of the assay plate. Sterile deionised water and catalase solution were included as negative controls.

The plates were incubated at 37°C for 18 hours and the diameters of the zones of inhibition around the wells were measured using Vernier callipers. The mean diameter of the zone of inhibition around each well was squared, and a phenol standard curve was generated with phenol concentration against the mean squared diameter of the zone of inhibition. The activity of each honey sample was calculated using the standard curve. To account for the dilution and density of honey, this figure was multiplied by 4.69, based on a mean honey density of 1.35 grams/ml (Allen et al. 1991). The activity of the honey was then expressed as the equivalent phenol concentration (% w/v). Each honey sample was tested on at least three separate occasions, and the mean phenol equivalence calculated.

Assessment of anti-fungal activity

The minimum inhibitory concentration (MIC) for each honey against *Candida albicans* ATCC 10231 was determined using the microdilution method described by Irish et al. (2006). Briefly, standardised suspensions of *C. albicans* were incubated in microtitre plates at 35°C for 24 hours with filter-sterilised diluted honey samples at final honey concentrations in 1% (w/v) increments from 10% to 50%. Artificial honey was included as a control for the osmotic effects of the honeys. Growth controls without honey and sterility controls without *C. albicans* were included in each plate. Following incubation the MIC was recorded as the lowest concentration of honey that prevented visible growth. Each honey sample was tested in duplicate and the assays were repeated on at least three separate occasions, with the mean MIC of the six replicates recorded.

Hydrogen peroxide assay

The concentration of hydrogen peroxide (H_2O_2) in honey samples was determined using the colorimetric assay method of Kwakman et al. 2010. Filter-sterilised 50 per cent (w/v) honey samples in water were mixed with either sterile deionised water for total activity testing, or with freshly prepared 5600 U/ml catalase solution for non-peroxide activity testing, to give a final concentration of

25 per cent (w/v) honey. Hydrogen peroxide standards from 2.1 to 2200 µM and honey samples were added to wells of a microtitre plate. Sterile deionised water and catalase solution were included as negative controls. A fresh reagent mixture of 50 µg/ml of *O*-dianisidine and 20 µg/ml horseradish peroxidase type IV was added to the wells. After incubation for 5 minutes at room temperature, reactions were stopped by the addition of sulphuric acid. The absorbance at 560 nm was measured using a plate reader, and hydrogen peroxide concentrations were calculated using a standard curve derived from the hydrogen peroxide standards. Each honey sample was tested in triplicate and assays were repeated on three separate occasions, giving a total of nine measurements per honey sample.

Statistics

Statistical analysis was performed using IBM SPSS Statistics 19 software. Differences between the activities of different honey samples and between honeys and the artificial honey were evaluated using the independent samples t-test. Correlation analysis was done by the researchers using Spearman's Rank Correlation with an online tool available at http://www.wessa.net (Wessa 2011).

Relationships between antibacterial activity and H_2O_2 concentration were examined using the Pearson correlation coefficient r and the 2-tailed probability p value; a significance level of 5% ($p < 0.05$) was chosen.

Results

Antibacterial activity of honey samples

Table 4.1. Antibacterial activity of honey samples against *Staphylococcus aureus*.

			(mean ± 1 SD)			
Sample No	Packer's code	Source assigned by packer	Total antibacterial activity (% phenol equivalence)		Hydrogen peroxide concentration (µM)	
			Initial samples	Marketable samples	Initial samples	Marketable samples
1	7843WES	Jarrah 1	9.0 ± 0.8	Partial inhib[#]	0	37.6 ± 5.8
2	7863WES	Jarrah 2	0	0	0	0
3	8012WES	Jarrah 3	9.0 ± 0.8	Partial inhib[#]	0	4.7 ± 3.3
4	8105WES	Jarrah 4	0	0	3.5 ± 1.6	13.5 ± 3.4
5	8113WES	Jarrah 5	0	0	0	0
6	7264DEN	Red Stringybark 1	14.9 ± 0.9	11.9 ± 1.2	130.3 ± 6.2	137.0 ± 7.9
7	7369HOL	Red Stringybark 2	0	0	0	0
8	7460EMM	Red Stringybark 3	0	0	0	0
9	7515BBN	Red Stringybark 4	14.1 ± 0.9	11.4 ± 0.9	253.2 ± 2.7	155.7 ± 0.9
10	7526BOM	Red Stringybark 5	21.2 ± 1.1	14.0 ± 0.4	197.8 ± 0.3	183.7 ± 0.5
11	3747RUT	Spotted Gum 1	0	0	0	0
12	3854DEN	Spotted Gum 2	0	0	0	0
13	3883SNO	Spotted Gum 3	0	0	36.6 ± 0.3	29.3 ± 1.8
14	4442BOM	Spotted Gum 4	0	0	135.4 ± 4.9	0
15	5485BOM	Spotted Gum 5	0	0	70.0 ± 1.9	0
16	5735SPI	Yellow Box 1	0	0	0	0
17	7130SMI	Yellow Box 2	0	0	190.1 ± 1.8	0
18	7141WRI	Yellow Box 3	0	0	134.5 ± 1.9	68.5 ± 2.3
19	7427RUT	Yellow Box 4	0	0	121.0 ± 0.4	0
20	7626DEN	Yellow Box 5	0	0	0	0
21	8168KLI	Canola 1	0	0	187.5 ± 1.3	0
22	8193SNO	Canola/Stringybark 2	12.0 ± 1.6	0	228.0 ± 0.4	79.1 ± 2.4
	controls	Artificial honey	0	0	0	0

[#] partial inhibition; not possible to measure phenol equivalence

A percentage phenol equivalence of ≥ 10 is potentially useful therapeutically (Irish et al. 2011). As indicated in Table 4.1, only four honey samples had this level of antibacterial activity against *Staphylococcus aureus*, three of them Red Stringybark honeys and the fourth a mixed Canola/Stringybark honey; two Jarrah honeys had antibacterial activities slightly below 10 phenol equivalence units. Antibacterial activity was strongly associated with Stringybark or Jarrah as a floral source, but it was not found in all Red Stringybark or Jarrah honey samples. The majority of honeys, including all samples sourced from Spotted Gum, displayed no measurable antibacterial activity. These results differ from those of Irish et al. (2011), who found that 18 of 19 Jarrah honeys and all four Spotted Gum honeys tested had antibacterial activity. There is however no intrinsic contradiction between the two studies as it is clear from the present data that samples from the same floral source can have a wide range of antibacterial activities.

After treatment with catalase to destroy H_2O_2 none of the honeys had antibacterial activity (data not shown), indicating that all the detected activity was due to the H_2O_2 content of the honeys despite there being no meaningful correlation between the measured levels of H_2O_2 and the antibacterial activity. Although $r = 0.511$; $p = 0.015$ for the data set, the calculation is unduly influenced by the preponderance of zero values for both parameters. One of the Spotted Gum honeys, at least two of the Yellow Box samples and the Canola honey which had no antibacterial activity contained as much H_2O_2 as an active Red Stringybark honey.

It should be noted that the Jarrah 2 sample had no antibacterial activity at all despite its high MGO content (120 mg MGO/kg; see Chapter 1). According to recent views, the MGO content of honeys does not adequately explain non-peroxide antibacterial activity, and its effectiveness may depend on synergistic factors. If these are not present, dilution of the sample for testing could have reduced the concentration of MGO to below the level that would be expected to have conferred non-peroxide-dependent antibacterial activity (Molan 2008).

The samples were also tested after they had undergone the standard procedures of warming and filtration at the honey packer. These procedures could damage the relatively unstable enzyme glucose oxidase which produces H_2O_2, and may therefore reduce peroxide-dependent antibacterial activity. Nevertheless, in three instances marketable samples were observed to still possess antibacterial activity and high levels of H_2O_2 above 100 µM. The H_2O_2 concentration in the majority of the samples was decreased by 19-100% by processing. However, in three of the Jarrah honey samples processing increased the H_2O_2 concentration, which is hard to explain.

Table 4.2. Anti-fungal activity of honey samples against *Candida albicans*.

			(mean ± 1 SD)			
Sample No	Packer's code	Source assigned by packer	Minimum inhibitory concentration (% w/v honey)		Hydrogen peroxide concentration (µM)	
			Initial samples	Marketable samples	Initial samples	Marketable samples
1	7843WES	Jarrah 1	34.7 ± 0.58	35.0 ± 3.0	0	37.6 ± 5.8
2	7863WES	Jarrah 2	35.0 ± 0	36.8 ± 1.3	0	0
3	8012WES	Jarrah 3	32.3 ± 0.58	31.0± 1.4	0	4.7 ± 3.3
4	8105WES	Jarrah 4	35.0 ± 0	34.0 ± 0	3.5 ± 1.6	13.5 ± 3.4
5	8113WES	Jarrah 5	36.5 ± 0.71	36.3 ± 3.2	0	0
6	7264DEN	Red Stringybark 1	28.3 ± 0.58	18.0 ± 2.7	130.3 ± 6.2	137.0 ± 7.9
7	7369HOL	Red Stringybark 2	34.3 ± 0.58	38.3 ± 2.1	0	0
8	7460EMM	Red Stringybark 3	35.0 ± 0	35.0 ± 0	0	0
9	7515BBN	Red Stringybark 4	19.3 ± 0.58	18.0± 1.4	253.2 ± 2.7	155.7 ± 0.9
10	7526BOM	Red Stringybark 5	19.0 ± 0	20.0 ± 0	197.8 ± 0.3	183.7 ± 0.5
11	3747RUT	Spotted Gum 1	35.7 ± 2.1	40.7 ± 2.1	0	0
12	3854DEN	Spotted Gum 2	36.3 ± 1.2	38.0 ± 1.0	0	0
13	3883SNO	Spotted Gum 3	33.0 ± 1.0	29.0 ± 2.9	36.6 ± 0.3	29.3 ± 1.8
14	4442BOM	Spotted Gum 4	30.3 ± 0.6	36.3 ± 2.3	135.4 ± 4.9	0
15	5485BOM	Spotted Gum 5	30.3 ± 0.6	38.7 ± 2.9	70.0 ± 1.9	0
16	5735SPI	Yellow Box 1	35.0 ± 0	37.3 ± 4.0	0	0
17	7130SMI	Yellow Box 2	33.3 ± 1.2	33.3 ± 1.2	190.1 ± 1.8	0
18	7141WRI	Yellow Box 3	29.0 ± 0	27.5 ± 2.1	134.5 ± 1.9	68.5 ± 2.3
19	7427RUT	Yellow Box 4	31.7 ± 0.6	34.7 ± 0.6	121.0 ± 0.4	0
20	7626DEN	Yellow Box 5	38.0 ± 0	39.0 ± 1.0	0	0
21	8168KLI	Canola 1	38.3 ± 0.6	42.5 ± 1.0	187.5 ± 1.3	0
22	8193SNO	Canola/Stringybark 2	24.3 ± 1.2	28.5 ± 0.7	228.0 ± 0.4	79.1 ± 2.4
	controls	Artificial honey	40.7 ± 2.9	40.7 ± 0.6	0	0

The therapeutically useful minimum inhibitory concentration (MIC) for honey as an anti-fungal has not been determined, but the MICs for the majority of these honey samples when tested against *Candida albicans* were high. Only three Red Stringybark samples and the Canola/Stringybark honey recorded MIC below 30%. These were the same samples that displayed antibacterial activity. In addition one Yellow Box sample was recorded as having an MIC of 29.0%. There was a strong correlation between anti-fungal activity and hydrogen peroxide concentration in the initial samples ($r = -0.73$; $p < 0.001$), though there were some obvious outliers. The Canola honey, for example, was one of the least active anti-fungal samples, but contained a high concentration of H_2O_2. As with the antibacterial activity, there was a much lower correlation with H_2O_2 concentration when only the active samples were compared ($r = -0.57$; $p = 0.053$).

This work has been published (Chen et al. 2012).

Implications

We conclude that:

- A few Australian eucalypt honeys have some antibacterial activity. This is associated with Red Stringybark and Jarrah as floral sources, but samples from the same floral source can display a wide range of activity.
- All the antibacterial activity detected is based on production of H_2O_2 by the enzyme glucose oxidase contained in the honey samples.
- This activity is reduced by the standard procedures of honey packers, and as the enzyme is unstable it is unlikely to survive the storage at room temperatures that routinely occurs during the marketing of honey.
- The Australian eucalypt honeys tested displayed only low levels of anti-fungal activity, and this was strongly correlated with their H_2O_2 content.
- There was no evidence from this study that any of the Australian eucalypt honeys tested contained stable antibacterial or anti-fungal components that could be of interest to the biotechnology or pharmaceutical industries.

Recommendation

We recommend that pursuing antibacterial or anti-fungal activity as a value-adding property of Australian eucalypt honeys is unlikely to be productive.

Chapter 5. Regulation of health and nutritional claims in Australia and New Zealand

Introduction

Food Standards Australia New Zealand (FSANZ) controls nutritional claims and food-related health claims in these two countries. The Therapeutic Goods Administration (TGA) controls therapeutic claims in Australia. However, as this project did not indicate that Australian eucalypt honeys have valuable therapeutic properties, the approach of the TGA to therapeutic claims for honeys was not pursued.

Methodology

Professor Joan Dawes and Dr David Dall of Pestat Pty Ltd have

- analysed the relevant standards and regulations and
- conducted discussions with FSANZ personnel Dr Chris Schyvens, Senior Toxicologist/Risk Manager Product Safety Standards Section and Ms Jenny Hazelton, Manager, Labelling and Information Standards Section, to clarify the current regulatory position for nutritional and food-related health claims for honey.

Results

FSANZ Standard 1.2.7

Until recently, food claims in Australia have been industry-regulated. However, on 18 January 2013 a new standard known as FSANZ Standard 1.2.7 (‘Nutrition, Health and Related Claims’) came into force. Standard 1.2.7 permits pre-approved claims and allows for self-substantiated health claims, but these are subject to numerous conditions. A ‘claim’ is any statement about a food which is not mandatory under the *Australia New Zealand Food Standards Code.* Thus any voluntary statement about a honey would constitute a ‘claim’ under the Code. Crucially for the honey industry, a food cannot qualify to make health or nutrition claims unless it meets stringent eligibility criteria.

The new FSANZ Standard 1.2.7 was 10 years in preparation. The draft Standard, prepared under Proposal P293, has been the subject of an extensive review since 2008. The review included two rounds of public consultation, one in 2009 and a second in February 2012 that received 71 and 83 submissions respectively. FSANZ also conducted several targeted consultations addressed to key industry, consumer, public health and stakeholder groups. The honey industry does not appear to have made a submission to this process or to have been otherwise involved at any stage.

Health claims for honey under FSANZ Standard 1.2.7

Current situation

Value-adding claims for honey could be classed either as ‘health’ or ‘nutritional content’ claims. Under Standard 1.2.7, a health claim is one that “states, suggests or implies that a food or a property of food has, or may have, a health effect”. Any claim about prebiotic properties of honey would be

considered to be a health claim. A nutritional content claim is one that relates to the presence or absence of certain properties of a food. A claim about the Glycaemic Index of honey would constitute a nutritional content claim.

As noted above (Chapter 2) the authors do not recommend pursuit of claims relating to the glycaemic indices of eucalypt honeys. The remainder of this chapter is therefore focused on health claims as they relate to prebiotic properties of honeys.

Under FSANZ Standard 1.2.7 a 'high level' health claim specifically refers to a serious disease or a biomarker of a serious disease, such as mention of diabetes. All other health claims are 'general' health claims, and we consider that this type of claim is the most relevant to the honey industry. Any food about which a general level health claim is made must meet the 'nutrient profiling scoring criterion' (NPSC) that forms an integral part of Standard 1.2.7, and is intended to restrict the use of health claims on products considered to be of lower nutritional quality. The calculation of the numerical value of the NPSC for a food involves a baseline score derived from its content of components considered potentially damaging to health such as saturated fats, sodium and sugars. This is then offset by content of high nutritional quality such as protein, calcium and fibre.

Honeys, composed almost entirely of sugars and water, are by definition unable to meet the score requirements of the NPSC. The NPSC assessment procedure thus effectively disqualifies all honeys from making any health claims, including about prebiotic properties. This presents a major hurdle for all honeys in relation to making health claims in the context of Division 2 of the Standard.

Options for health claims for honeys

There will be a three-year transition period from January 2013 for the implementation of Standard 1.2.7. During the transition period FSANZ intends to continue to develop the way in which the Standard will be managed, by:

- establishing a Health Claims Committee to review procedures for high level claims;
- considering the use of authoritative sources for self-substantiation of food-health claims;
- completing the consideration of EU-approved claims for possible inclusion in Standard 1.2.7; and
- considering possible exemptions for certain foods.

This offers some potential opportunities to make health claims for honeys under the provisions of Division 2. Two possibilities are:

- proposal and adoption of pre-approved claims to allow specified honeys to be added to Schedule 3, which lists the general level health claims of the Standard, and
- the application of EU-approved claims.

However, under these mechanisms the fundamental inability of honey to meet the NPSC test would remain a major obstacle, which would need to be approached by seeking exemption of honey from the requirements of the Standard. Any such action is likely to be time-consuming, expensive and contentious, and might also benefit off-shore suppliers and importers of foreign honeys.

Another approach that would enable claims to be made about the prebiotic properties of Australian eucalypt honeys would be to obtain endorsement from an unrelated expert agency under the terms of Division 3 of the Standard.

Options for claiming prebiotic properties of honeys

The options identified above will be considered specifically in relation to the prebiotic activity of Australian eucalypt honeys, which is the only value-added property of these honeys that has been clearly identified during this project.

Pre-approved claims

There is increasing popular belief that honey has prebiotic properties and this is associated with promotion of honey as a prebiotic food, particularly in the electronic media. However, there do not appear to be any pre-approved claims for honey as a prebiotic in Australia and New Zealand. This route to approval is therefore not an option.

The application of EU-approved claims

The European Food Safety Authority, which controls food-related health claims in the EU, does not consider that increasing numbers of lactobacilli and/or bifidobacteria in the intestine is a beneficial physiological effect *per se*. It requires demonstration of specific beneficial consequences such as defence against pathogens in the intestine before it will authorise prebiotic health claims. No such claim has been substantiated to date (Flynn 2012). This route to approval is therefore also not currently available as an option.

We reiterate that each of these options is further impeded by the current terms of application of the NPSC.

Endorsement by an unrelated expert agency

Endorsement of the prebiotic properties of honey would require a statement by an independent expert not-for-profit organisation with no formal connection to the Australian honey industry. This endorsement should state that reliable data indicate that identified honeys possess the claimed prebiotic activity. Such endorsement is exempt from the necessity to meet the NSPC requirements, and we consider that this approach has real potential as a means of securing approval of the claim that honey is a prebiotic food.

Certain conditions are established by Division 3 of the Standard in relation to endorsements, and these would necessarily have to be met. We have already noted the requirement for independence of the endorsing party from the supplier of the endorsed foods, and more generally from the honey industry as a whole. The endorsing body must also be a not-for-profit organisation.

Necessarily the claims that are the subject of endorsement must be able to be substantiated (under terms of other instruments such as the *Trade Practices Act*); we consider that the data resulting from this project will be sufficient to allow this. Data must also be retained by suppliers during the term of supply or advertisement, and for a further two years after supply or advertisement ceases. Again, this condition should be able to be met without difficulty.

The process of endorsement would allow the promotion – by means including product labelling and advertising – of Australian eucalypt honeys as prebiotic foods. Agreement with the endorsing party could also result in a number of standard words or phrases such as ‘Australian eucalypt honeys are endorsed by [endorser] for good inner health’, whose adoption by multiple suppliers could mutually reinforce the value of the endorsement message.

Recommendation

We recommend that the Australian honey industry identifies an independent expert not-for-profit organisation to endorse Australian eucalypt honey as a prebiotic food.

Appendix 1

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Value-adding to honey

By Dr Joan Dawes and Dr David Dall

Pub. No. 13/123

This project examined three potential attributes of Australian eucalypt honeys: Glycaemic Index, prebiotic properties and therapeutic activity. Australian honey packers and beekeepers could benefit directly by using some of the results of the project to derive optimal returns for honey in an increasingly competitive market.

The most valuable finding from this project is that Australian eucalypt honey is a prebiotic food, stimulating the growth of gut bacteria that contribute to human health and reducing the growth of deleterious gut bacteria.

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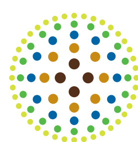

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