GOVERNMENT OF WESTERN AUSTRALIA

ChemCentre
EXPERT SOLUTIONS

CRCHBP
FOR HONEY BEE PRODUCTS

Methods developed, certification established and its use by the industry

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Version 1.0

First published as BICWA Industry Quality Standard – Certified Western Australian Uni-Floral and Multi-Floral Honeys.

Written by Kenneth Dods, ChemCentre, under license from BICWA, ABN 68 609 634 059 as part of the project outcomes for the CRC Honey-Bee products.

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1. Methods developed, validated and certifications established.

A full list of methodology established during the research program to service industry is listed below:

1. pH
2. Electrical conductivity
3. Moisture
4. Ash
5. Optical rotation
6. Total acidity
7. HPLC simple sugars
8. Total phenolics, phenolics by LCMS TOF
9. Antioxidant potential (ORAC), (FRAP), (DPPH)

10. Total nitrogen
11. ICPMS and AES suite metals including heavy metals
12. Volatile signature
13. Amino acid analysis
14. Total phenolic assay (Folin Ciocalteu method)
15. Phenolic profile LCMS triple quad assay
16. Oligosaccharide profile assay
17. Antimicrobial radial diffusion antimicrobial assay (Phenol equivalence assay)
18. Antimicrobial Minimum Inhibitory Count (MIC) antimicrobial broth dilution assay - Commercial utilisation and adoption protocols in advanced development
19. Melissopalynology.

2. Legislative overview

Legislative and regulatory recommendations provided to industry.

International standards specify that,

"Honey may be designed according to floral or plant source if it comes wholly or mainly from that particular source and has the organoleptic, physico-chemical and microscopic properties corresponding with that origin" (CAC, 1989, 6.1.4; EEC Directive, 1974, 7.4.a)

Persano Oddo L et al (1995) "Characterisation of Uni-floral Honeys". *"Uni-floral honey may be described as a continuous series of intermediate possibilities that exists between a multifloral and a uni-floral honey. Decision is required for what point and on what basis does the uni-floral/multifloral discrimination take place?"*

In 1998 the International Honey Commission (IHC) established a Uni-floral Working Group comprised of 28 lead researchers from 11 countries and 20 international laboratories.

From this came a significant reference publication *"Main European uni-floral honeys: descriptive sheets*" (2004) Persano Oddo *et al; Apidologie* 35, S38-S81. Key elements were:

- 61,000 raw data, 6,719 honey samples from 21 countries were analysed.
- Output uni-floral descriptive sheets for 15 honey types were determined.
- Descriptive sheets include designations for Organoleptic, Physico-chemical and Microscopic to meet the needs of a "Botanical" uni-floral label identification.
- Results are presented as the range of Organoleptic Physico-chemical and Microscopic outcomes that then define a uni-floral honey.

In 2005 international legislative focus turned to the new labelling regulations that included determination of "Origin and Botanical Source" of honey due to new food regulations instituted in Europe. This was also adopted shortly after by FSANZ in their labelling requirements.

European Regulation (EC) No. 178/2002 – included important elements on the rules for traceability that required suitable certification and detection methods to enable the food control authorities to supervise compliance with labelling requirements.

In 2017 "*Honeygate*" – one of the largest food frauds in U.S. history with significant impact on world trade was reported. As summarised in the article "*International honey market*" by Garcia and Phipps in the *American Bee Journal* there was an urgent need to adopt a more comprehensive approach to honey certification in trade.

"Development of new methods and products used to adulterate honey have reached a magnitude that severely depresses price and challenges the survival of beekeeper operations". This is typified by:

- Rise in use of C3 syrup adulteration (rice syrups).

- Honey ranks number 3 amongst the most adulterated foods.
- The honey industry needs both more powerful scientific methodologies and greater integrity to overcome the adulteration and circumvention which have plagued and haunted the industry.

"*Entering an era of enhanced traceability and more sophisticated scientific methodology. While traceability is relevant, it is more important to employ more sophisticated scientific methodology to detect adulteration, transhipment, and circumvention*".

This led to a significant positional statement being made by Apimondia in 2019 "*Statement On Honey Fraud*" the context of this statement is outlined below;

It restates the Codex standard (1981) issued by the FAO:

"Honey is the natural sweet substance produced by honeybees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature".

And reinforces:

- No additional added ingredients, no changes to natural flavour, aroma, or taint, no fermented by-products, natural constituents – pollen, chemical attributes may not be added or removed.
- Heating or processing shall not change essential composition, chemical or biochemical treatments shall not be used to influence honey crystallisation.
- No Human intervention in maturation or drying.

"The current honey fraud problem has an extensive global magnitude and impacts on both the price of honey and the viability of many beekeeping operations."

Apimondia recommended the use of a multi-pronged approach strategy to combat honey fraud through:

- Traceability.
- Testing.

Apimondia highly recommends a choice of method(s) tailored to each specific situation and notes and recommends:

- A combination of targeted physico-chemical tests.
- Pollen and organoleptic testing.
- Use of both screening and certified methodologies.

Apimondia then stated it *"supports the development of new techniques to detect honey fraud, available at reasonable costs for the majority of stakeholders, and supports the constitution of an international database of original honeys with a more open exchange of analytical information between the different laboratories specialized in honey analysis".*

Based on these events (which occurred during the life of the CRC Honey Bee Products – CRC HBP Project), and their likely impacts on uni-floral standards for Jarrah and Marri, and the associated market access and regulatory impacts, these considerations were fundamental and needed to be included in certified uni-floral product that met customer expectations in these international markets.

3. Certifications and economic impact

There has been step-change in the WA honey industry. Key to the change process has been

increasing product value and volume with provenance and authenticity assurance. This is enabling movement into high-value export markets and enhancing the profitability of honey-based businesses.

Figure 1 Western Australian honey certified, packaged, and labelled for export markets around the world.

During the life of this Project, beekeepers have opened new international markets and differentiated their product from adulterated or falsely-branded premium honey found in many international markets using ChemCentre certification services. Working with suppliers to strengthen the supply chain by establishing deep relationships with distributors who have reinforced the value of certifications to their customers has been a positive outcome.

New international certifications that ensure market access and provide essential information to international distributors have been delivered in this Project. In 2019 over 150 International Bulk Containers (IBCs) of honey were certified by ChemCentre, (Figure 22), despite 2018 and 2019 being poor production seasons for beekeepers in Western Australia.

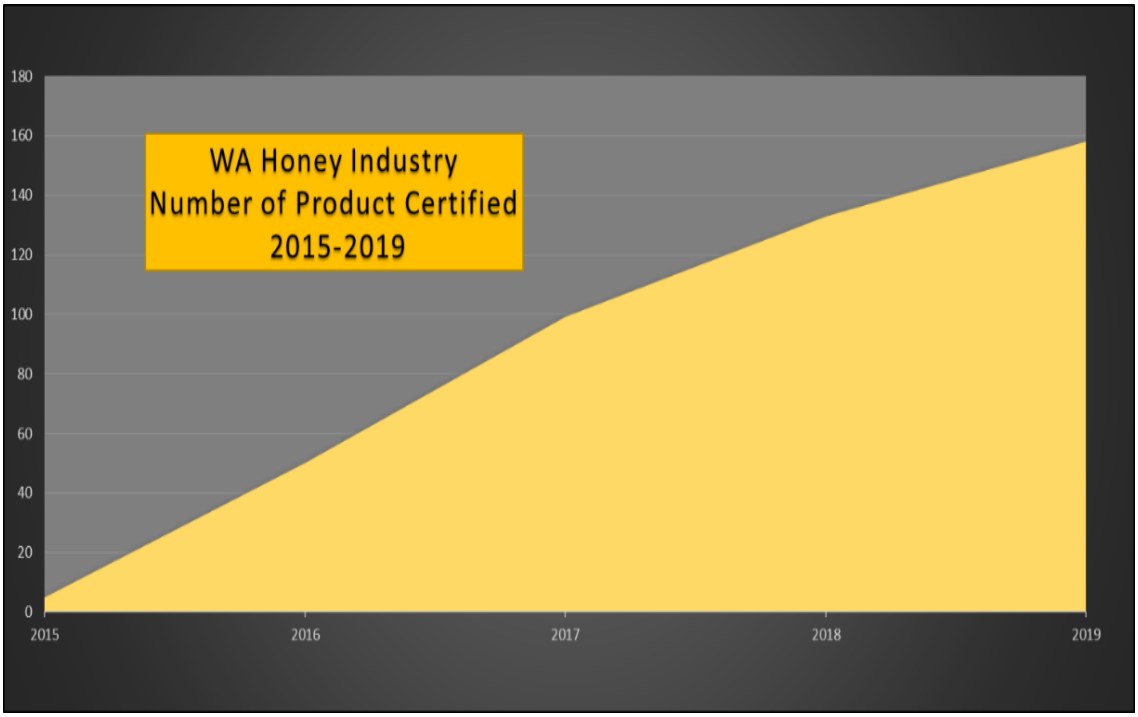

Figure 22 Number of products ISO certified for domestic or international sale at ChemCentre per year since formation of WAHRDG

New markets and international distributions of WA industry products have been enabled (Figure 33). Key export market destinations have included China, Malaysia and Japan with an emerging international trade in Middle Eastern countries.

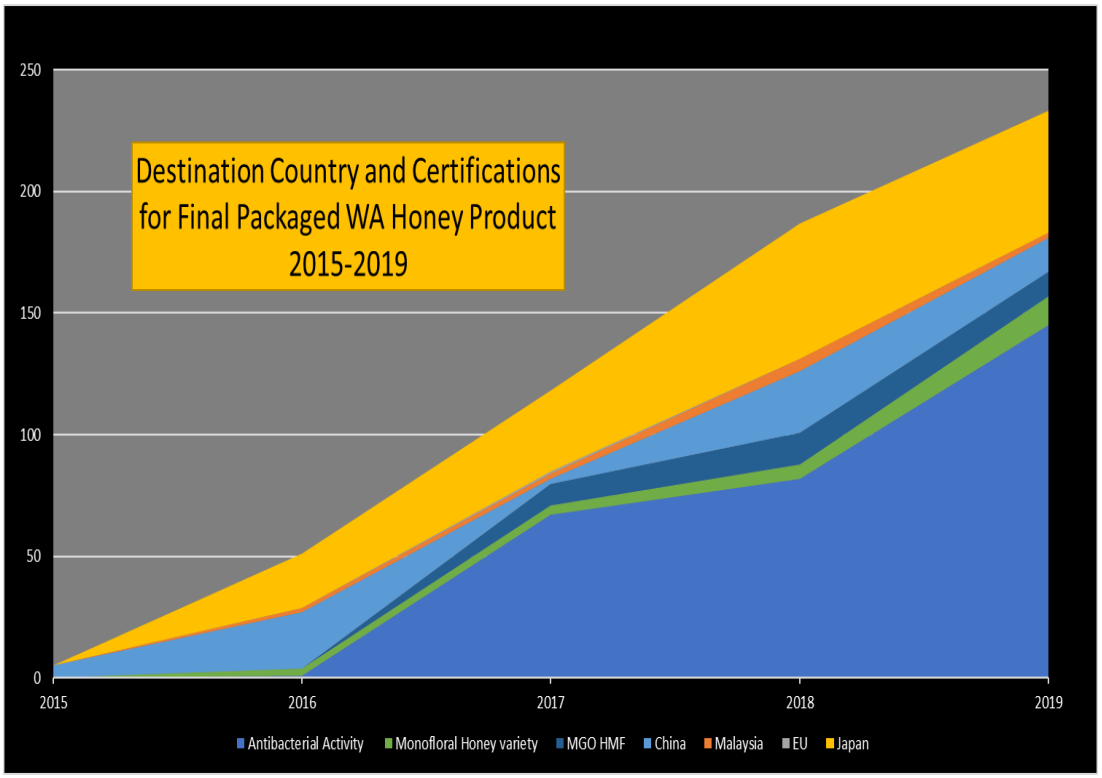

Figure 33 Products certified at ChemCentre – the Number of each ISO Certificates type issued per product (some products were issued with multiple certifications for different markets)

Capability was established to provide NATA certified hydroxymethylfurfural (HMF) analysis, which was critical for the industry to manage their supply and storage parameters and then certify their honey to meet the international requirements for this honey-ageing by-product.

The project also established new broth dilution assays for multiple bacteria, which expanded the currently limited applicability in antimicrobial interpretation of the radial diffusion assay which (by international standard) uses *Staph aureus* as its reference organism.

From this work the publication: Green, K. Dods, K. Hammer K. (2020) *Development and validation of a new microplate assay that utilises optical density to quantify the antibacterial activity of honeys including Jarrah, Marri and Manuka*, *PLOS ONE* 15(12): e0243246. https://doi.org/10.1371/journal.pone.0243246, was produced.

ChemCentre in collaboration with the National Measurement Institute (NMI) has established a NATA certified broth dilution assay which provides the industry with more options when seeking to evaluate the antimicrobial activity of their honeys and potentially define specific and optimal use of their honeys for a wider array of bacteria.

WA beekeeper exporters have been assisted with certification that differentiates their product in the international marketplace (Figure 44). Whilst the range of international destinations for WA honey continues to grow, so does the number of companies individually marketing their product assisted by the outcomes in this project.

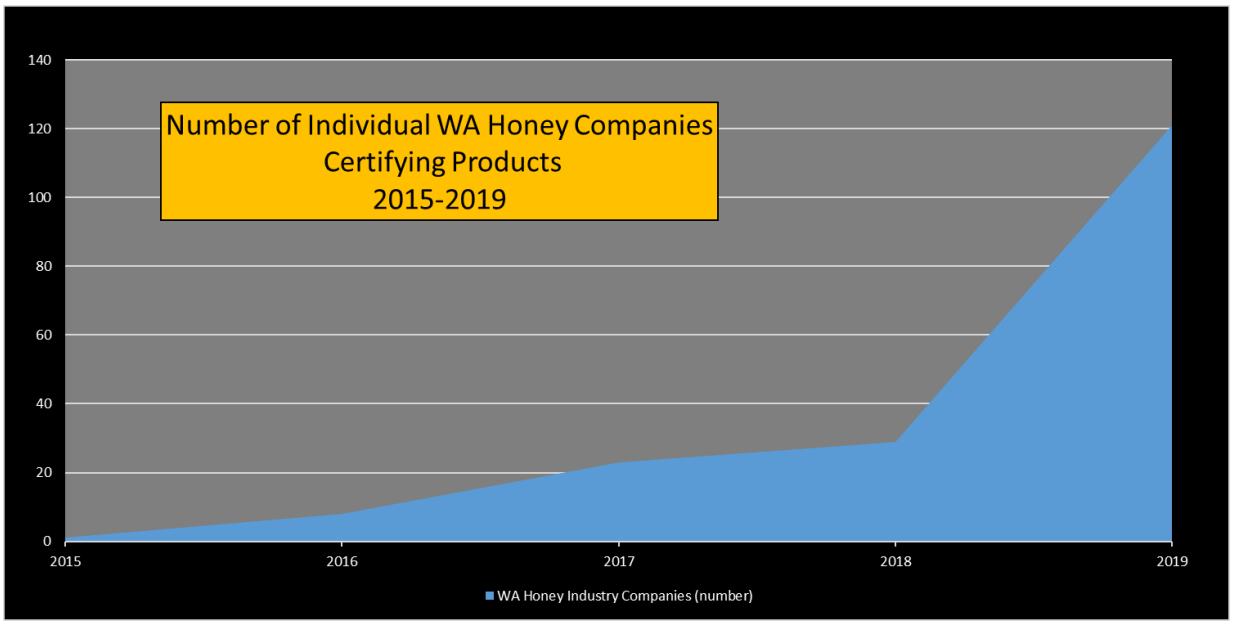

Figure 44 Growth in Number of WA companies exporting, producing their own label and producing ISO certified product.

Methods developed to inform labelling requirements include heavy metals analysis and nutritional panel analysis such as monosaccharide composition, and the general requirements by FSANZ for honey nutritional labels were also certified to a NATA standard. These align with the International Honey Commission (IHC) requirements that were also established and validated by ChemCentre as NATA certified analyses.

In 2017 the CRC HBP initiated and promoted antioxidant ratings for honeys that would enable newer markets to be accessed. ChemCentre established antioxidant analytical techniques to certify antioxidant activity, including total phenolic colorimetric assay results that were made available to industry to support this program.

4. Prebiotic potential of Jarrah, Marri and Powderbark uni-floral honeys

4.1. Summary

The following research describes the first quantification of soluble dietary fibre, fructan components in WA Jarrah, Marri and Powderbark uni-floral honeys. These components have been directly tied to *in-vitro* probiotic bacterial population promotion and short chain fatty acid production, and thus can be very useful to human nutrition and associated positive health outcomes.

Project research links fructo-oligosaccharide ratios to a proportional and positive probiotic population response. These are directly measured using ChemCentre NATA accredited methodology. This establishes an opportunity to work with Food Standards Australia and New Zealand (FSANZ) to advance specific health claims for these honey products by the industry, based on quantifiable levels of these compounds in these honeys.

Legislative and regulatory background

The WHO defines dietary fibre:

"Dietary fibre is that fraction of the edible part of plants or their extracts, or analogous carbohydrates, that are resistant to digestion and absorption in the human small intestine, usually with complete or partial fermentation in the large intestine. The term includes polysaccharides, oligosaccharides (degree of polymerisation (DP>2) and lignins. Dietary fibre promotes one or more of these beneficial physiological effects: laxation, reduction in blood cholesterol and/or modulation of blood glucose".

FSANZ recommended that under Standard 1.2.8 – *Nutrition Information Requirements*, a general definition of dietary fibre be inserted to include when:

1. fructans are the subject of a nutrition claim; or
2. fructans are declared as dietary fibre in a nutrition claim (including a content claim); or
3. fructans are simply declared in the Nutrition Information Panel.

4.2. Fructo-oligosaccharides as a soluble dietary fibre and human health

Dietary fibre is an important component of human nutrition, it is responsible for aspects of satiety and improves bacterial population and ecology in the large bowel. Inulin type compounds have been extensively associated with prebiotic health, especially as soluble dietary fibre type compounds. Fructo-oligosaccharides and malto-oligosaccharides have been identified in honey by previous authors.

4.2.1. Demonstrating prebiotic potential

FAO (Food and Agriculture Organization of the UN) defines a 'prebiotic' as *"a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota."* Essentially, prebiotics are carbohydrates that are not digested and absorbed by the host, and which therefore reach the large intestine where they are utilised by beneficial bacteria**.**

In this study, the prebiotic capacity of Jarrah, Marri, Powderbark and a multi-floral honey was studied to see if they could influence the levels of potentially beneficial bacteria in the gut microbiota and the resultant short chain fatty acids produced by these microbes, since these

have been shown to have beneficial effects on the host. An *ex-vivo* artificial gut system was used, which is referred to as a gut microcosm.

4.3. Experimental

ChemCentre developed NATA accredited fructo-oligosaccharide analytical methodology using Liquid Chromatography-Mass Spectrometry (LCMS) to establish fructo-oligosaccharide levels in the four WA honey samples used in this trial.

The *ex-vivo* laboratory model (referred to as a microcosm) has been used extensively for prebiotic studies, including the demonstration of the prebiotic properties of honeys. Prebiotic activity was analysed by measuring the impact on the Short Chain Fatty Acids (SCFAs), especially acetic acid, propanoic acid, and butyric acid, as well as modulation of the potentially beneficial lactobacilli and 10 bifidobacteria, in addition to the potentially undesirable Gram-negative enteric bacteria which includes the *E. coli* and *Salmonella* spp.

A concentration of 1% honey was used in the microcosms as this concentration has previously been used in similar studies with validation from *in vivo* findings. The same concentration was also used for the positive control, inulin, as this can be calculated to correspond to recommended doses of inulin in clinical studies.

4.3.1. Honey samples and the *in vitro* intestinal microcosms

The following honey samples were supplied by Chem Centre:

- 001, Marri
- 002, Powderbark
- 003, Multifloral
- 004, Jarrah
- Inulin (control).

Table 1 Oligosaccharide distribution in the four honey samples submitted in the trial.

Analyte	Method	LOR	Unit	001	002	003	004
DP3 Oligosaccharides	ORG181	30	mg/kg	300	4300	7800	11000
DP4 Oligosaccharides	ORG181	30	mg/kg	200	420	3200	2500
DP5 Oligosaccharides	ORG181	30	mg/kg	<30	<30	59	170

Microcosms were established using human faecal samples from a healthy human volunteer to allow examination of the effect of honeys and sugars on the complex intestinal microbial population. Anaerobic conditions were always maintained.

The microcosms were established using Wilkens-Chalgren Anaerobe (WCA) broth (Oxoid) containing faecal suspension (final concentration 10%) and either honeys or the positive control prebiotic, inulin (final concentration 1%). For the negative control, additional WCA was added instead of the honey. Samples were collected at 0 and 48 hours for enumeration of the lactobacilli, bidifobacteria and enteric bacterial groups using selective culture media. Short Chain Fatty Acid (SCFA) analysis was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS). Microcosms were set up in triplicate and subsequently each

microcosm was analysed in triplicate.

Standard curves were generated and SCFA levels in each sample were calculated by determining the 'relative area', i.e., area of the sample peak relative to the internal standard peak.

4.3.2. Enumeration of major viable bacterial groups and metabolites

The following selective media were used: Rogosa for lactobacilli, reinforced clostridial agar with aniline blue (RCA AD) (0.3 g/L) and dicloxacillin (2 mg/L) for bifidobacteria, and MacConkey for Gram negative enteric bacteria (e.g., salmonella and *E.coli*). All dehydrated media were obtained from Oxoid and prepared according to the manufacturer's instructions. Each microcosm was analysed in triplicate.

Samples from the microcosms were serially diluted, and aliquots plated using the micro-drop technique (10 µL drop) and spread plates (10 µL drop) using selective media. Plates were incubated according to the manufacturer's instructions. Counts were expressed as $\log_{10}$ CFU (colony forming units) per mL.

The amounts of SCFAs produced in the microcosms with the added substrates of honey, or control inulin were determined by Gas Chromatography-Mass Spectrometry (GC-MS) using an internal standard. Samples from the microcosm were extracted with ether and analysed by GC-MS using the internal standard method. Standard curves were generated and SCFA levels in each sample were calculated by determining the 'relative area', i.e., area of the sample peak relative to the internal standard peak.

4.3.3. Statistical analysis

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

4.4. Results

The addition of all honeys had a dramatic impact on the potentially harmful Gram-negative enteric bacteria which includes *E. coli* and *Salmonella*. All tested honeys had a significant negative effect on the enteric bacteria when compared to the no honey control and significantly better than the positive control, inulin (Figure 5). There was a variable impact on the lactobacilli (Figure 5), with the Powderbark honey increasing the potentially beneficial lactobacilli as much as the inulin and more than noted in the no honey control. The Marri honey had no significant effect on the lactobacilli. The multifloral honey reacted similarly to the no honey control, and had less effect than the positive control, inulin. The Jarrah honey had a larger effect than the no honey control but was not as effective as the inulin. For the potentially beneficial lactobacilli, the Powderbark honey had a far better effect than the other honeys. For the potentially beneficial bifidobacteria, all honeys and the inulin demonstrated no increase, with the bacterial numbers after incubation less than observed in the no honey control (Figure *7*).

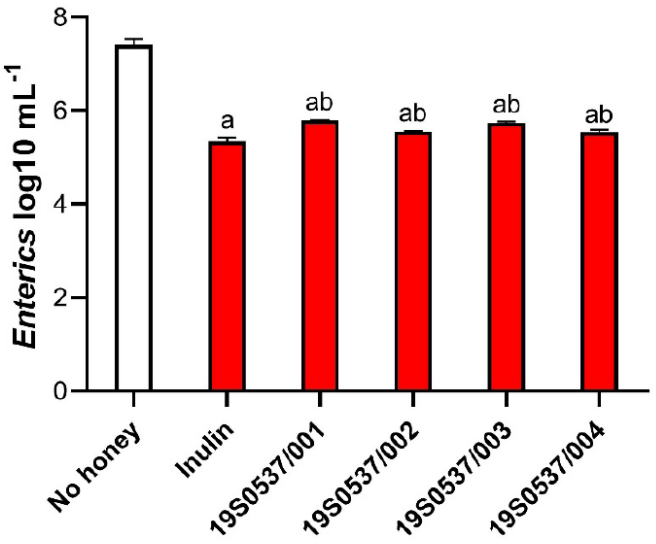

Figure 5 Viable count of Gram-negative enteric bacteria determined using MacConkey agar. Results are presented as the means±SEM; differences were assessed by ANOVA and denoted as follows: aP<0.05 compared with "No honey", bP<0.05 compared with "Inulin".

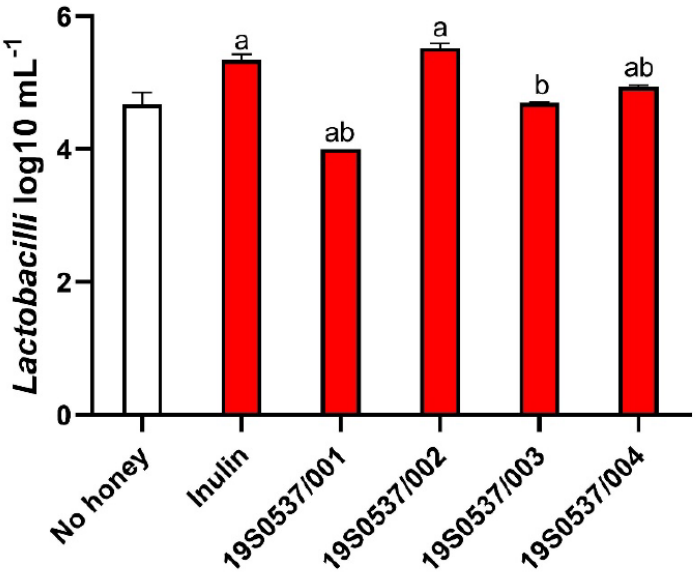

Figure 5 Viable count of lactobacilli assessed using on Rogosa agar. The results are expressed as the means±SEM; differences were assessed by ANOVA and denoted as follows: aP<0.05 compared with "No honey", bP<0.05 compared with "Inulin".

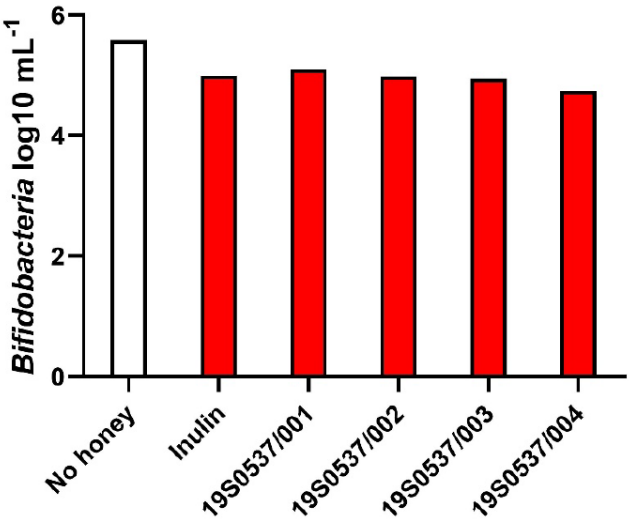

Figure 7 Viable count of bifidobacteria determined using reinforced clostridial agar supplemented with aniline blue and dicloxacillin. Results expressed as the colony forming units per mL of microcosm.

The analysis of the SCFAs (Figure 8) showed that all honeys increased levels of the acetic, propanoic and butyric acids, with Marri honey being comparable to the positive control, inulin. The other three honeys were not as effective at elevating these potentially beneficial SCFAs but were significantly better than the no honey negative control. With the valeric, iso-butyric and iso-valeric SCFAs, all honeys resulted in lower levels of all three acids, which were significantly higher in the no honey control.

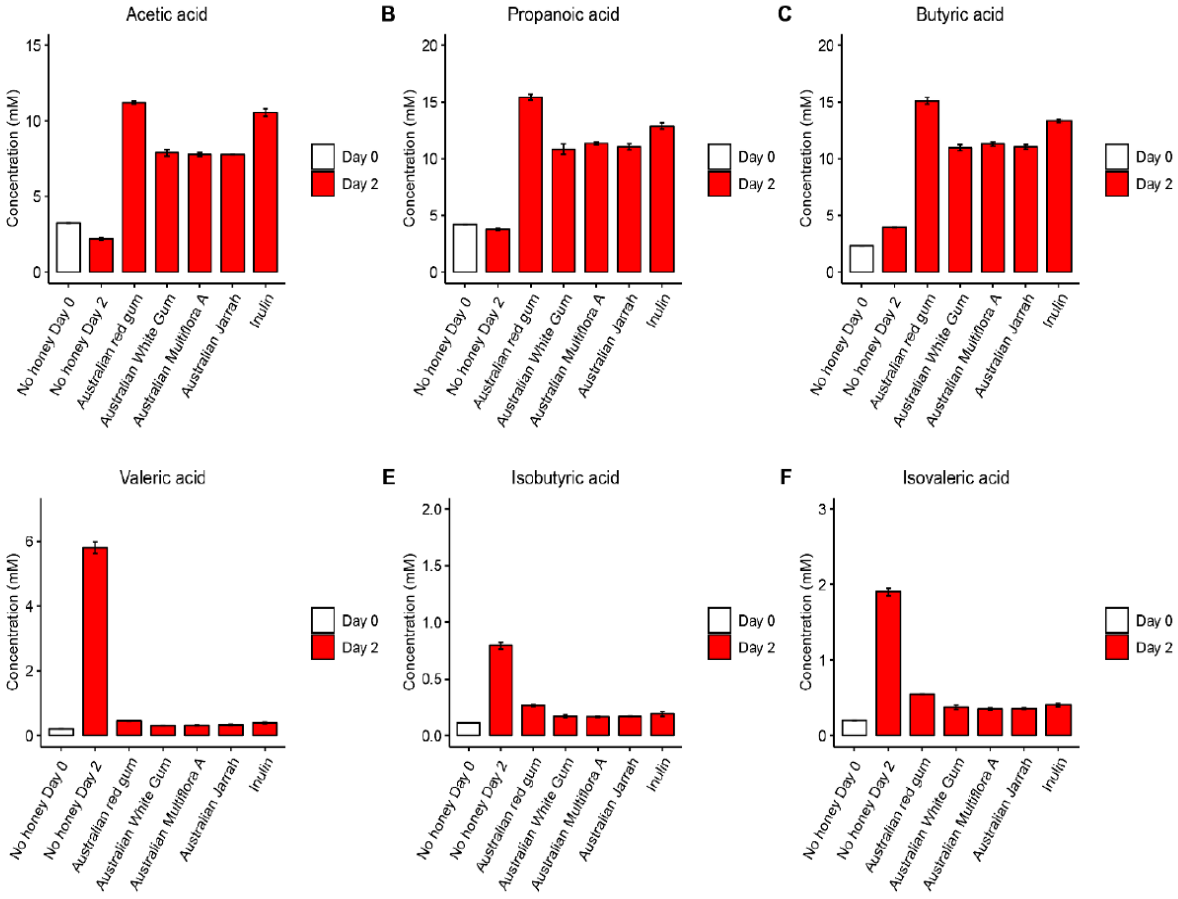

Figure 8 The effect of the four Australian honeys on short chain fatty acid (SCFA) production by the gut microbiota (1% w/v final concentration of honey). The no honey microcosm was used as the negative control; 1% inulin was used as the positive control Concentrations of SCFAs are expressed as mM.

4.5. Discussion and conclusions

The honeys tested had demonstrable prebiotic properties when examining the SCFA profiles, but in terms of the microbial profiles the Powderbark honey had the best prebiotic properties as it not only decreased potentially pathogenic enteric bacteria but also elevated levels of potentially beneficial lactobacilli. Although no beneficial impact was noted on the bifidobacteria, this is consistent with other honey studies. The least prebiotic activity was noted in the multifloral honey and the Marri honey.

The fructo-oligosaccharide profile was assessed among the four honeys. It was concluded that Marri honey (001) has the lowest oligosaccharides content among the four honeys. Jarrah honey (004) demonstrated the highest content of DP3 at 11,000 mg/kg; whilst all others contained less than 8,000 mg/kg of DP3. However, the prebiotic potential did not seem to depend solely on the content of this notable compound, as it did not agree with the prebiotic potential when only considering individual or total fructo-oligosaccharide measures.

The data indicate that the ratio of DP3:DP4 could be the key in explaining the prebiotic potential trend on the four honeys.

Table 2 Fructo-oligosaccharide profile of the four honeys used in this trial

Lab ID	Client ID and Description	Ratio of DP3:DP4
19S0537/001	**Marri**	**1.5**
19S0537/002	**Powderbark**	**10.2**
19S0537/003	**Multiflora**	**2.4**
19S0537/004	**Jarrah**	**4.4**

Powderbark honey (002) demonstrated the highest DP3:DP4 ratio compared to other four honeys, followed by Jarrah honey (004). This has strong agreement with the prebiotic potential findings where Powderbark honey (002) increased potentially beneficial lactobacilli as much as the inulin and no honey control, while Jarrah honey showed more effect than the no honey control but was not as effective as inulin.

Of all honeys tested, the Powderbark honey (002) demonstrated by far the most prebiotic benefits on the lactobacilli, whereas all honeys resulted in comparable short-chain fatty acid (SCFA) profiles. The least prebiotic activity was noted in the multiflora honey (003) and ANB honey (001).

In conclusion, the DP3:DP4 ratio could be useful in predicting the prebiotic potential of a honey. It is important to note that the conclusions/assumptions are evaluated based on the presented analysis data and this need not apply for other samples of Powderbark honey or other tested flora varieties.