Diversity and bioactivity of microorganisms associated with Australian stingless bee species

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DIVERSITY AND BIOACTIVITY OF MICROORGANISMS ASSOCIATED WITH AUSTRALIAN STINGLESS BEE SPECIES

A DISSERTATION SUBMITTED

BY

TOBY J T MILLS

B. Sc. (Hons.) M. Sci. Tech. UNSW

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR

THE AWARD OF

DOCTOR OF PHILOSOPHY

AT THE

SCHOOL OF CHEMISTRY

THE UNIVERSITY OF NEW SOUTH WALES

SYDNEY, AUSTRALIA

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The rapid emergence and transfer of antimicrobial resistance in pathogenic organisms has greatly reduced our ability to treat clinical microbial infections. To continue to treat clinical microbial infections in humans and animals it is imperative that we discover new antimicrobial compounds with novel modes of action. The aim of this thesis was to explore the antimicrobial potential of microorganisms associated with three Australian native stingless bees, *Tetragonula carbonaria*, *Austroplebeia australis* and *Tetragonula hockingsii.* Observations of antimicrobial activity from honey, hive materials and whole extracts of Australian stingless bees inspired the hypothesis that the microbiota associated with these stingless bee species could produce bioactive compounds. To address this aim, a comprehensive evaluation of the native bee whole gut microbiome was performed (Chapter 2), with the goal of identifying microorganisms exhibiting specific associations. Guided by these results bacteria and fungi were cultured from the gut and cuticle of three species of Australian stingless bees and screened by genetic and chemical methods to create a subset enriched for chemical diversity and biosynthetic potential (Chapter 3). Biosynthetic potential was correlated to the presence of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) gene clusters due to their historical success as therapeutic natural products and their potential to produce a wide array of bioactive chemical structures. Antimicrobial activity of the selected microbes was assessed by liquid culture bioassay and a successful candidate identified for bioactivity-guided fractionation and compound characterisation (Chapter 4).

The antimicrobial long chain fatty acid, 9-hexadecenoic acid, was isolated and characterised from the *Xanthomonas* sp., TMB - 122. Additional characterisation identified the production of 2,5-di-*tert*-butylphenol, a phenolic compound with antimicrobial activity, and tridec-1-ene, an acyclic olefin insect pheromone, by *Xanthomonas* sp., TMB - 122. This investigation confirmed our hypothesis that microbes associated with Australian stingless bees were bioactive. Furthermore the combined genetic and chemical analyses performed validated the selection of candidates possessing PKS and NRPS gene clusters as this methodology accounted for 75% of the chemical diversity observed by LC-MS profiling. The steps enclosed in this thesis are the practical application of the theory that modern drug discovery methods must build on the knowledge off the past whilst continuing to innovate and explore.

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Abstract

The rapid emergence and transfer of antimicrobial resistance in pathogenic organisms has greatly reduced our ability to treat clinical microbial infections. To continue to treat clinical microbial infections in humans and animals it is imperative that we discover new antimicrobial compounds with novel modes of action. The aim of this thesis was to explore the antimicrobial potential of microorganisms associated with three Australian native stingless bees, *Tetragonula carbonaria*, *Austroplebeia australis* and *Tetragonula hockingsii.* Observations of antimicrobial activity from honey, hive materials and whole extracts of Australian stingless bees inspired the hypothesis that the microbiota associated with these stingless bee species could produce bioactive compounds. To address this aim, a comprehensive evaluation of the native bee whole gut microbiome was performed (Chapter 2), with the goal of identifying microorganisms exhibiting specific associations. Guided by these results, bacteria and fungi were cultured from the gut and cuticle of three species of Australian stingless bees and screened by genetic and chemical methods to create a subset enriched for chemical diversity and biosynthetic potential (Chapter 3). Biosynthetic potential was correlated to the presence of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) gene clusters due to their historical success as therapeutic natural products and their potential to produce a wide array of bioactive chemical structures. Antimicrobial activity of the selected microbes was assessed by liquid culture bioassay and a successful candidate identified for bioactivity-guided fractionation and compound characterisation (Chapter 4).

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And congratulations to my write up buddy David, we (with a lot of help) did it!

"….cheer, cheer, the red and the white…"

Table of Contents

Abbreviations

A	adenylation
Acyl-CoA	acetyl coenzyme A
AT	acyltransferase
BG - #	bacterial group, this study
BHI10 +	10% brain heart infusion agar + chloramphenicol
BLAST	basic local alignment search tool
C	condensation
$CDCl_3$	deuterated chloroform
COSY	correlation spectroscopy
d	doublet
DMSO	dimethyl sulfoxide
DMSO-d_6	deuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	epimerase
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionisation
ELSD	evaporative light scattering detector
F#	fraction number
FG - #	fungal group, this study
GC-MS	gas chromatography mass spectrometry
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HRFTMS	high resolution Fourier transform mass spectrum

HSQC	heteronuclear single quantum coherence
KR	ketoreductase
KS	ketosynthase
LAB	lactic acid bacteria
LC	liquid chromatography
m	multiplet
ME25 +	25% malt extract agar + chloramphenicol
ME100 +	100% malt extract agar + chloramphenicol
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MRS25	25% de Mann, Rogosa and Sharpe agar
MRS100	100% de Mann, Rogosa and Sharpe agar
MT	methyltransferase
NA25	25% nutrient broth agar + cyclohexamide
NA100+	100% nutrient broth agar + cyclohexamide
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthetase
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PDA25	25% potato dextrose agar
PDA100	100% potato dextrose agar
PKS	polyketide synthase
q	quartet
RDBE	ring double bond equivalent
rRNA	ribosomal ribonucleic acid
s	singlet
t	triplet

T	thiolation
TE	thioesterase
TAE	tris acetic acid EDTA
TMF - #	fungal isolate this study
TMS	trimethylsilyl
TMB - #	bacterial isolate, this study
Tris	tris(hydroxymethyl)aminomethane
WYE	water, yeast extract and agar
XS	xanthogenate, sodium dodecyl sulfate
YECD	yeast extract, casamino acid and agar

List of Publications and Proceedings

PRESENTATIONS

Awarded IUPAC Poster Prize:

7th International Conference on Biodiversity & 27th International Symposium on the Chemistry of Natural Products, Brisbane 10-15th July 2011

For

"Symbiosis and Drug Discovery in Australian Native Bees" Toby J T Mills, Mark Brown & Brett Neilan.

http://www.raci.org.au/events/event/iscnp27-icob7

Poster presentation:

12th International Symposium on the Genetics of Industrial Microorganisms (GIM2013), Cancun, Mexico, 23-28th June 2013.

"Microbes, Drug Discovery and Australian Stingless Bees" Toby J T Mills, Brett A Neilan.

http://www.smbb.com.mx/GIM2013-Cancun/

List of Figures

CHAPTER 2

CHAPTER 3

List of Tables

CHAPTER 2

CHAPTER 3

CHAPTER 4

Chapter 1

Introduction

1.1. DRUG DISCOVERY AND NATURAL PRODUCTS

There exists a need to discover new bioactive compounds to treat diseases and infections of humans. This need has existed ever since man first identified the symptoms of disease. Today however, due to the emergence of resistance mechanisms in bacteria and fungi to previously successful medicines, we must tackle this task with innovative and creative approaches.

The dawn of modern drug discovery is said to originate from the work of Prof. Paul Ehrlich (1854-1915). His pioneering research into the use of dyes to specifically stain tissues and microorganisms developed from practical applications to his postulating the "magic bullet theory"; that specific chemical compounds could target disease causing organisms and leave the native tissues unharmed (Bosch and Rosich, 2008). This holy grail of drug discovery shaped the future of modern medicines.

Natural products have played a pivotal role in the history of drug discovery. Plant extracts documented in early *materia medica* have evolved from herbal remedies into some of the most well known and successful medical compounds, for example aspirin and the bark of the white willow *Salix alba* (Elwood, 2001), morphine and the opium poppy *Papaver somniferum* (Hamilton and Baskett, 2000) and digoxin and purple foxglove *Digitalis purpurea* (Somberg et al., 1985).

The history of modern drug discovery has been documented in countless articles and reviews however what is relevant to this study is the success of natural products and the

benefits in continuing to pursue natural products for future drug discovery (Newman et al., 2000).

Natural products are chemical compounds produced by living organisms and they can be grouped into two broad categories; primary metabolites and secondary metabolites (Dewick, 2002). As the nomenclature suggest; primary metabolites participate in the primary metabolism of the produces, for example carbohydrates, proteins, fats and nucleic acids (Dewick, 2002). In general, the pathways involved in the production of primary metabolites are common amongst all living things, with some minor variations. Secondary metabolites, on the other hand, serve either non-primary or as yet unknown functions (Demain and Fang, 2000) and are typically only found in select subgroups of organisms (Hanson, 2003). Many medicinal chemists are interested in secondary metabolites that possess a therapeutic value.

In their series of reviews, Newman, Cragg and Snedar (Cragg et al., 1997, Newman et al., 2003, Newman and Cragg, 2007, Newman and Cragg, 2012, Newman and Cragg, 2016) have outlined the importance of natural products and natural product inspired drugs as sources of new drugs in the 30 years from 1981 to 2010. Historically natural products have provided close to half of all new chemical entities that have successfully made their way to the market place and although the number of new drugs has declined from the glory days between 1940-1960, the influence of natural products on new drugs persists.

The converse approach to natural products drug discovery is compounds designed by combinatorial chemistry (Gordon et al., 1994, 2006). Synthesis of large libraries of compounds combined with high throughput screening was seen as a low cost high return model for drug discovery by many pharmaceutical companies however combinatorial chemistry although being successful in specific instances has up until now failed to produce significant results as compared to natural product based drug discovery approaches (Ortholand and Ganesan, 2004, David et al., 2015).

Natural products possess certain advantages over randomly generated synthetic compounds; firstly they occupy a distinct chemical space as a result of their biosynthesis (Ganesan, 2008). Secondly, as the products of biosynthesis they are more similar to existing metabolites and can be imported into cells via active transport mechanisms whereas many synthetic compounds rely on passive absorption across the cell membranes (Dobson and Kell, 2008).

The future of drug discovery is no longer a choice between natural products or combinatorial chemistry it is in fact both. The development of "drug like" libraries and the application of synthetic chemistry to develop analogues of successful natural product leads is required to capitalise on the success of the past and generate new treatments for the future (Ortholand and Ganesan, 2004, Boldi, 2006, Kumar and Waldmann, 2009, Pascolutti and Quinn, 2014).

1.2 NATURAL PRODUCTS AND BIOSYNTHESIS

Polyketides and non-ribosomal peptides represent a large group of natural products. Their structures can be found in many current and historically relevant drugs. These two classes of compounds are able to give rise to an astounding diversity of structural diversity, and therefore, are excellent targets for drug discovery.

1.2.1 Polyketide natural products

Polyketides represent a large class of bioactive natural products that possess distinctive repetitive alternating carbonyl and methylene groups or β-polyketones. Polyketides exhibit a variety of physiological and pharmacological effects including, antibiotic e.g. erythromycin (Cortes et al., 1990), antifungal e.g. nystatin (Brautaset et al., 2000), anticancer, e.g. epothilone B (Molnár et al., 2000) and immunosuppressant, e.g. rapamycin (Schwecke et al., 1995) activities. Additionally polyketides include a large number of toxins, in particular mycotoxins, that have negative effects on human health (Razzaghi-Abyaneh et al., 2014).

Polyketides are structurally diverse, and are classified based on their mode of biosynthesis as opposed to their final structure, or mode of action. Polyketides can be produced by most living organisms however their biosynthesis is most widely studied in bacteria and fungi.

The biosynthesis of polyketides is the result of repeated decarboxylative condensations of acyl-CoA monomers that is facilitated by large multidomain enzymes known as polyketide synthases (PKSs). The genes encoding these PKSs are clustered together with associated regulatory, export and resistance genes. The wide variety of final structures in polyketides is achieved by the varying specific starter and extender unit utilised by the PKSs and modifications to the polyketide chain achieved by tailoring enzymes e.g. cyclization, methylation (Hertweck, 2009).

PKSs may be grouped in three main classes, Type I, Type II and Type III. Common to these three groups is a fundamental β-ketosynthase (KS) functionality that catalyses the addition of acyl-CoA monomers to the growing polyketide backbone (Hertweck, 2009). As an essential domain the β-ketosynthase is used as a molecular target in genetic screening for PKS gene clusters (Miller et al., 2012)

1.2.1.1 Type I PKS

Modular type I PKS consist of enzymatic modules arranged sequentially. Each module is responsible for the incorporation of a single substrate into the growing polyketide chain. Each module is further organised into individual catalytic domains that facilitate a specific chemical reaction in the biosynthesis pathway. Three essential catalytic domains catalyse the elongation of the growing backbone, acyltransferase- (AT), thiolation (T) and ketosynthase (KS). The AT domain is responsible for the selection and attachment of acyl-CoA monomers to a phosphopantetheinyl "arm" present on the T domain and the KS domain catalyses attachment of this monomer to the growing

polyketide intermediate. In addition to the core catalytic domains, tailoring domains may be present, such as ketoreductases, dehydratases, methyltransferases and enoyl reductases, that catalyse specific modifications made to the growing polyketide backbone. These modifications give rise to the diversity present in polyketide structures. The final biosynthetic step is the cyclisation of the polyketide chain, catalysed by a thioesterase domain (TE), which simultaneously cyclises and releases the product from the enzyme complex (Staunton and Weissman, 2001).

1.2.1.2 Type II PKS and Type III PKS

In contrast to Type I PKS systems, type II PKS are organised as discrete monofunctional enzymes with a single catalytic function. Type III PKS systems utilise an iterative process similar to iterative type II PKS systems although the acyl-CoA monomers directly incorporated into the growing polyketide chain without the involvement of carrier protein. Additionally, Type III PKS are homodimeric enzymes, as compared to the Type II PKS (agglomerate of enzymes) and Type I PKS (single multi-functional enzyme)(Hertweck, 2009).

1.2.2 Non-ribosomal peptide natural products

Non-ribosomally synthesised peptides have a long and successful history as anti-infective agents. Tyrocidine and gramicidin, the active fractions of tyrothricin, were isolated from the *Bacillus brevis* (Hotchkiss, 1944) and were the first commercially manufactured antibiotics. They are biosynthesised by non-ribosomal peptide synthetases (NRPSs), which are large multimodular enzyme complexes that function similar to

PKSs. The primary difference is the use of amino acids as substrates in non-ribosomal peptide biosynthesis compared to acyl-CoA monomers used in PKS systems. NRPS modules consist of catalytic domains for chain elongation. The adenylation domain (A) selects and activates an amino acid starter unit as an aminoacyl adenylate, which is tethered to the thiolation domain (T). Following this, the condensation domain (C) catalyses formation of an amide bond between the tethered substrate and the growing peptide chain. Biosynthesis is completed by a thioesterase domain (TE), which hydrolyses the peptide chain from the catalytic machinery, and in many cases performs heterocyclisation. These fundamental domains may be complimented by additional catalytic functionalities including, acylation, halogenation, hydroxylation and epimerization to produce peptides with distinctive modifications (Fischbach and Walsh, 2006).

1.3 BEES

Bees are known to be a good source of antibacterial, antifungal, antiviral products (Jose Dardon and Enriquez, 2008, Zamora et al., 2013). Bee species belong to the insect order Hymenoptera, suborder Apocrita, infra order Aculeata. Aculeata contains all the social hymenoptera species and is comprised of three super families, Chrysidoidea -wasps, Vespoidea - ants and wasps, and Apoidea - wasps and bees. Within the super family Apoidea, bees represent a monophyletic lineage that together form the clade Anthophila.

All representatives of the clade Anthophila can be described as having one of three basic lifestyle habits: 1) eusocial, 2) communal and 3) solitary. Eusocial bees possess three key characteristics: reproductive division of labour, overlapping generations and co-operative care of offspring (Wilson, 1971, Crespi and Yanega, 1995). These characteristics result in what we know as bee hives or nests, which due to the overlapping of generations become a perennial ‘super-organism” (Moritz and Southwick, 1992). In contrast, communal and solitary bees live an annual existence that is punctuated by reproduction following which the females deposit an egg in to a nest stocked with provisions that then develops without any further care. The majority of bee species are either solitary or communal organisms.

Stingless bees belong to the Family Apidae, tribe Meliponini. They along with the honey bees (tribe Apini), bumble bees (tribe Bombini) and orchid bees (tribe Euglossini) are corbiculate bees, which possess specialized adaptations of the hind legs, called corbicula, used to collect provisions (Michener, 1999, Kawakita et al., 2008).

Stingless bees, are a highly diverse tribe of bees typically found in tropical and subtropical regions of the world, including areas in Africa, Central and South America, Indonesian, Malaysia and Australia (Jones, 2013). The size of the colonies and architecture of hives varies significantly between species. Hives are generally constructed from cerumen, a mixture of bee wax and plant resins, used to seal off naturally occurring cavities, within trees, rocks and occasionally underground. The typical stingless bee diet is comprised of pollen, nectar and other associated tree and plant products (Wille, 1983).

1.3.1 Australian native Meliponini

There are over 1650 species of native bees currently described in Australia with many classifications in need of revision. Australian native Apidae include two Meliponini genera, *Tetragonula* (Moure, 1961) and *Austroplebeia* (Moure, 1961) which account for all native eusocial bees in Australia. The genus *Tetragonula* recently underwent revision and was reclassified from *Trigona* to the current nomenclature (Rasmussen and Cameron, 2010). There are currently six described *Tetragonula* species, *T. carbonaria, T. hockingsii, T. mellipes, T. davenporti, T. sapiens* and *T. clypearis*, (Dollin et al., 1997) and nine described *Austroplebeia* species, *A. australis, A. symei, A. cassiae, A. cockerelli, A. essingtoni, A. ornate, A. percincta, A. websteri, A. cincta* (Cardale, 1993).

Meliponini hives are populated by two castes, queens and workers. The queen provides fertilized eggs that produce new bees, while the workers perform all the other hive duties. The queen of a hive mates only once in its life and the sperm from that encounter

is stored in a spermatheca and released into the oviduct to fertilize an egg (Bourke, 1999). Fertilized eggs are placed into a brood cell, constructed of cerumen, which is prepared by the specific workers and stocked with a mixture of pollen, honey and secretions from the hypopharyngeal gland, then sealed off (Drumond et al., 1999).

Hives are typically located within cavities inside dead trees or tree limbs and are comprised of a brood comb, honey and pollen "pots", and cerumen structural supports. With the exception of spaces for ventilation, the cavity that contains the hive is sealed off from the environment, with a mixture of cerumen and resins or mud, known as batumen (Roubik, 2006). The production of wax in stingless bees varies during the lifespan of the bee. Typically, wax production begins as the bee matures from a callow (juvenile) stage and diminishes as they mature (Landim, 1963).

1.3.1.1 Tetragonula carbonaria

T. carbonaria is the most widely distributed Meliponini species in Australia, found as far south as Bega in NSW, as far North as the Atherton Tablelands in QLD. They are commonly found in areas that receive high rainfall (Dollin et al., 1997). They are approximately 4 mm long, black, with white hairs. Unlike some stingless bee species, they do not construct an entrance tube to the hive and are known for smearing the entrance with resin. The entrance to the hive is guarded by bees that determine their hive mates from intruders by smell and protect the hive by ejecting and sometimes killing unfamiliar bees or other insects. If pests, such as parasitic hive beetle, manage to

enter the hive they are quickly entombed alive with drops of cerumen, wax and resin (Greco et al., 2010).

The brood comb of *T. carbonaria* is distinctively spiral shaped. Arranged in a regular fashion, one brood cell thick, newly added capsule shaped cells are placed on the outer rim and orientated vertically (Dollin et al., 1997).

Investigations of plant pollen collected by foraging *T. carbonaria* have revealed that although the hive as a whole exhibits non-specific pollen collection, individual foragers consistently collected pollen from a single source (White et al., 2001). This strategy is thought to preserve the quality of pollen collected and also to increase the success of pollination. Additionally a recent study by Leonhardt and colleagues determined that *T. carbonaria* foragers exhibit higher activity seeking pollen and nectar than their *A. australis* counterparts (Leonhardt et al., 2014).

1.3.1.2 Tetragonula hockingsii

T. hockingsii are hard to distinguish from *T. carbonaria*, as they are also black coloured with white hairs, though they are slightly larger. Despite the similarities in their gross morphology they can be distinguished as a separate species using genetic methods (Franck et al., 2004, Brito et al., 2012). Furthermore, unlike the spiral brood combs of *T. carbonaria*, *T. hockingsii* brood combs are constructed in horizontal planes interspersed with step like structures. Brood cells are hexagonal and are constructed in clusters of approximately 10 cells with vertical pillars linking different clusters. The hive entrance

is typically not adorned with either resins or any form of entrance tube. Most of the Australian *Tetragonula* species, *T. carbonaria, T. hockingsii, T. mellipes and T. davenporti* are described as a species complex known as the Carbonaria group (Brito et al., 2014).

1.3.1.3 Austroplebeia australis

A. australis are found along the east coast of Australia, from the tropical north of Queensland to Kempsey, NSW. In contrast to *T. carbonaria* they flourish in areas of low rainfall and can tolerate extreme ranges in temperature. They are approximately 4 mm long and black, with small yellow/cream coloured markings on their thorax and head. Hive entrances may be adorned with a tube, made of cerumen and plant resins, that projects out from the entrance up to 2 cm and is guarded by workers (Halcroft, 2007). As an additional defence strategy *A. australis* workers construct a thin curtain of cerumen over the entrance of the hive at night. As mentioned earlier, *A. australis* is thought to be a more selective forager, spending less time out of the hive collecting food and instead opting for a higher yield diet obtained from specific higher reward resources (Leonhardt et al., 2014).

The brood comb of *A. australis* is amorphous and composed of spherical brood cells constructed in clusters with the open cells facing outward from the growing comb. The brood comb may also contain pots of honey and pollen and is periodically covered with a layer of cerumen known as the involucrum. Production of trophic eggs by workers has been reported (Drumond et al., 1999)

1.3.2 Medicinal products from bees

1.3.2.1 Honey

The core component of honey is nectar collected from flowering plants, although other sugar rich substances may be collected including plant exudates or insect products such as honeydew. The range of plants visited by the foraging bees largely dictates the composition of the resultant honey. In addition to nectar, honey contains enzymes, produced by bees and added during the process of honey making (White and Doner, 1980).

Raw flower nectar is collected by bees using a proboscis and stored in a honey stomach, a specialised section of the gut that can expand to store nectar carried back to the hive. Nectar from flowers is composed of water and between 5% and 80% sugars as well as amino acids, lipids, antioxidants, alkaloids, phenolics and glycosides (Baker, 1977). During the process of nectar collection and honey production glandular bee secretions from the salivary and hypopharyngeal glands are added to the nectar (Ohashi et al., 1999). Upon returning to the hive the foraging bees pass the adulterated nectar, by regurgitating droplets, to the in hive worker bees (Crailsheim, 1998). The workers then dehydrate the adulterated nectar by passing it back and forth between their mandibles, during which more enzymes may be added.

1.3.2.2 Stingless bee honey

The honey from stingless bees is similar to that of honey bees although it typically has a higher water content, higher level of maltose, free acids and nitrogen and a lower pH (Souza et al., 2006)

Analysis of Stingless bees hypopharyngeal glands have revealed they produce a similar array of enzymes to those in honey bees although with differences in the levels detected (Costa and da Cruz-Landim, 2005). Levels of the enzymes, invertase, diastase and glucose oxidase vary between stingless bee honey from different stingless bee species though their presence has been detected in most studies (Sgariglia et al., 2010).

Flavonoid components of stingless bee honey are the result of source plant secondary metabolite production, the action of bee-produced enzymes and physical changes that occur during the honey ripening process within the hive. In general, stingless bee honey contains a higher ratio of flavonoid glycosides to flavonoid aglycones, as compared to honey bee honey, due to lower levels of diastase and the storage of honey in cerumen pots that contain plant resins (Oddo et al., 2008, Truchado et al., 2011).

Specific research on medicinal activity of Australian stingless bee honey is almost exclusively limited to the species *T. carbonaria*. The most extensive examination was by Boorn and colleagues (Boorn et al., 2010) who used agar diffusion, agar dilution, broth microdilution and time-kill viability assays to determine the antimicrobial activity of *T. carbonaria* honey obtained from 10 different hives located around Brisbane,

Australia. Medical grade Manuka honey, table grade honey and artificial honey (sugar solution) were also included in the experiments for comparison. The honeys were applied against a panel of 13 clinical isolates and 21 reference strains representing both bacteria and fungi. The results of this work showed that the *T. carbonaria* exhibited broad spectrum antibacterial activity but limited antifungal activity. There was variability in the results obtained for honey from different hives, though the minimum inhibitory concentrations (MIC) were between 6-8% honey for the agar dilution method and 4-16% for the broth dilution method. The MIC's for the *T. carbonaria* honeys were equivalent to those for medical grade honey with both being more active than the table grade and artificial honeys. The agar diffusion method used failed to produce any inhibition using 25 % w/v honey though some inhibition was demonstrated at 50% w/v honey. These results were congruent with a report by Kimoto-Nira and colleagues (Kimoto-Nira and Amano, 2008) that also failed to show any antibacterial activity from *T. carbonaria* honey using an agar diffusion method.

The results obtain by Boorn and colleagues were in agreement with an earlier study by Irish and colleagues (Irish et al., 2008) who tested *T. carbonaria* honey from 21 hives located around Brisbane against *Staphylococcus aureus* using an agar diffusion method, with 25 % w/v *T. carbonaria* in water, and Manuka honey as a positive control. The activity of the honeys was compared to zones of inhibition generated by varying percentages of phenol in water and the non-peroxide activity was determined by the addition of catalase, which degrades peroxide. The mean antibacterial activity of the *T. carbonaria* honey was equivalent to 26 % w/v phenol and the non-peroxide activity was 15 % w/v phenol equivalent as compared to the Manuka honey that was 18% w/v phenol equivalent for both the total and non-peroxide activities. It is unknown why this

study was successful using the agar diffusion method whereas others were not, the primary difference was the test organism was incorporated into the media in this study and the other study spread plate the test organism onto the media.

A more recent investigation by Massaro and colleagues characterised the phenolic components, peroxide content and antimicrobial activity of honey and phenolic concentrates from 6 hives of *T. carbonaria.* Agar diffusion and broth dilution methods were used to determine the antibacterial activity against *S. aureus* and *Klebsiella pneumoniae* with phenol equivalents used as the measure of activity. Neat honey was active against both test organisms using the agar diffusion method and produced an average inhibition equivalent to 3.8% phenol (w/v). Diluted honey was active against both test strains using the broth dilution method though only bacteriostatic not bactericidal effects were observed. Additionally, the antibacterial effects of *T. carbonaria* honey used in the study were determined to be due to both peroxide and non-peroxide components.

1.3.3 Bee digestive system

Honey bee nutrition is supplied by two primary sources, nectar that contains carbohydrates and pollen that contains protein and lipids. The carbohydrates in nectar are broken down by the action of bee enzymes in the both the honey stomach and the midgut to produce fructose and glucose. The remaining nutrient required for the successful maturation and growth is obtained from pollen, which includes, amino acids, fatty acids, cholesterol, phospholipids, minerals and vitamins.

The digestive system of all insects is constructed of three core elements, the foregut, the midgut and the hindgut. The primary function of the foregut is the ingestion and storage of food and its passage into the midgut. The midgut is where most digestion and absorption of nutrients occurs, aided by the enzymatic breakdown of complex food. The hindgut functions to absorb water and nutrients as well as providing a passage out of the digestive system. The foregut and hindgut are lined with cuticle, similar to structural material that forms the exoskeleton of insects but unsclerotised and flexible, it is shed with each successive moult through the life of an insect. Conversely, the midgut is lined with peritrophic membrane that is not shed with successive moults though it may undergo regeneration triggered by changes in food supply and age of the membrane. The alimentary tract is surrounded by and supported by muscle fibres and connective tissue, with the entire structure suspended in hemolymph. Hemolymph is analogous to human blood, however, it is not contained within vessels but fills the void between the insect exoskeleton and the internal organs. Hemolymph is composed of blood cells, hemocytes, plasma, proteins, enzymes, carbohydrates and lipids that provide the necessary requirements for all the insect tissues (Chapman et al., 2013).

The digestive system of bees is most extensively studied in the honey bee. There is no published research on Australian native eusocial bee digestive systems however studies of Meliponini from around the world have revealed that the general anatomy and function is very similar to that of honey bee. The following descriptions are of bees in general unless reference to specific bee species is made.

Entry of food into the bee digestive system begins with the mouth. The bee mouth has evolved to support two important functions; chewing/biting using the mandibles and sucking using the proboscis. These two functions allow bees to collect liquid nectar and also chew off plant resins and manipulate wax, resins and honey. Behind these mouthparts is the bucal cavity into which the salivary glands and hypopharyngeal glands drain. Following on is the pharynx, in the head, and esophagus, in the thorax, leading to an enlarged section of the esophagus known as the crop or honey stomach, in the abdomen. The pharynx and esophagus are slender tubes lined with cuticle and wrapped in muscle fibre. The honey stomach is constructed of multiple folds of tissue lined with cuticle and wrapped in muscle fibres that can expand to accommodate loads of nectar. At the ending of the foregut is a specialized apparatus that controls the passage of liquid and solid food into the midgut, the proventriculus. A combined filter and sphincter, the proventriculus is essential for the selective passage of pollen into the midgut whilst the crop is being used to store nectar or honey. In summary, the foregut of bee provides a passage from the mouth to the midgut, can be used to store nectar/honey for transport, is the entry point for substances produced by the salivary and hypopharyngeal glands and possess a specialized apparatus, the proventriculus, that is able to selectively permit the passage of solid food whilst maintaining the contents of the honey stomach (Chapman et al., 2013, Engel and Moran, 2013).

The midgut is the primary site of digestion and nutrient absorption. There are three types of cells that make up the epithelium of the midgut, digestive, endocrine and regenerative cells. The most abundant are digestive cells that produce enzymes and are responsible for the absorption of nutrients and water. Endocrine cells produce hormones implicated in the production of enzymes. Regenerative cells are undifferentiated cells

similar to stem cells and are responsible for the regeneration of digestive and endocrine cells. The midgut is composed of a hollow tube (the ventriculus), with blind invaginations (the midgut ceca), found primarily near the anterior end where much of the nutrient and water absorption occurs. Lining the ventriculus is the multilayered peritrophic membrane that separates the food mass from the delicate digestive cells. Composed of protein and chitin, the membrane selectively allows the transfer of enzymes out and partially digested food in where further breakdown occurs in the presence of digestive enzymes. Cells of the midgut epithelium are short lived and are constantly being differentiated from regenerative cells, producing enzymes and absorbing nutrients then degrading. The ventricular epithelium is also capable of secreting excess minerals, particularly calcium carbonate, into the ventriculus to be carried out of the gut. The pores in the peritrophic membrane are too small to allow most bacteria or fungi to pass thus keeping them contained within the ventriculus (Lehane, 1997, Chapman et al., 2013).

The hindgut continues from the ventriculus and is composed of the pylorus, Malpighian tubules, ileum and the rectum. The pylorus acts as a sphincter that separates the midgut from the hindgut. The Malpighian tubules, which are multiple long blind tubes that open into the pylorus, produce primary urine, a liquid waste filtered from the hemolymph. Primary urine is further processed to produce secondary urine that has water and other required elements removed. The Malpighian tubules are suspended in the bee abdomen, surrounded hemolymph, and form part of the bee excretory system. The ileum is a thin tube that allows the passage of solid digested food waste from the ventriculus and primary urine to the rectum where water and required nutrients and elements are

reabsorbed. The termination of the alimentary tract is the anus that permits the expulsion of both solid and liquid waste (Chapman et al., 2013).

1.4 INSECT MICROBIAL ASSOCIATIONS

Microorganisms associated with insects can be found on the outside of the insect on the exoskeleton or on the inside within organs and insect tissues. The associations of microorganism and insects can benefit the host insect in numerous ways. Much of the literature regarding gut associated microorganism has described the benefits bestowed on termites and other lignocellulose feeding insects by their gut microbiota. By degrading biomass to more simple and accessible biological building blocks microorganisms are essential to the host's existence, analogous to the biota of ruminant animals. Additionally microorganisms may produce nutrients, vitamins and other beneficial compounds, such as bio-available nitrogen, required by the host that may not be available from their diet. Prime examples being the fungus growing leaf cutter ants that cultivate mono cultured "gardens" of fungi that breakdown leaf and other plant materials and are in turn eaten by the ants (Currie et al., 1999). Most importantly, microbes have been found to be the true producers of numerous natural compounds that have been previously associated with their higher organism hosts. For example, numerous secondary metabolites from marine organisms have been traced to a microbial origin (Proksch et al., 2002).

1.4.1 Microbial associates of stingless bees

As is the case with honey bees, stingless bees have been shown to possess a core bacterial microbiota however there is significantly less research on these organisms. The primary bacteria found associated with stingless bee are *Bacillus* sp., detected in stored honey, pollen and also hypopharyngeal gland products (Gilliam et al., 1990). Interestingly similar *Bacillus* sp. were found in stingless bee from South America,

honey bees from America and a flesh feeding *Trigona* species, though it should be noted that these identifications were all based on enzymatic and morphological characteristics and not 16S rRNA gene identity (Gilliam et al., 1985).

Yeast species have also been identified in hive provisions and digestive tract of multiple stingless bee species (Teixeira et al., 2003). The primary yeasts found belong to a large clade of mostly Hymenoptera associated yeasts known as the *Starmerella* clade (Teixeira et al., 2003, Rosa et al., 2003). In addition, yeast from the genera *Aureobasidium, Cryptococcus, Rhodotorula, Pseudozyma* and *Kodamaea* have been isolated from stingless bees (Rosa et al., 2003). Some stingless bees have been observed to collected fungal spores and hyphae as a food source (Oliveira and Morato, 2000, Eltz et al., 2002). The interactions between bee and yeast can benefit the bees in terms of nutrient enrichment, production of enzymes and preservation of provisions. The benefits for the yeast in this arrangement may include, protection from competitors, predators and extreme environmental conditions, a method of dispersal, nutrient supply and increased reproductive success (Gonzalez, 2014).

Recent investigations into the bacterial associations of stingless bee have been limited. A few studies have included stingless bee associated bacteria as evidence of the core corbiculate bee microbiota. These include the examination of ants, honey bees and semi social Halictidae bees by McFrederick and colleagues (McFrederick et al., 2013) that showed the core bee specific microbiota is not present in other social hymenoptera. V**á**squez and colleagues (Vásquez et al., 2012) examined the crop of honey bee and

stingless bee and determined that the dominant organism, *Lactobacillus kunkeei*, was present in all the examined species except for a *Trigona* sp. from Borneo.

1.5 THESIS AIMS AND RATIONALE

The overall aim of this thesis was to explore the microbiota of three Australian stingless bee species, *T. carbonaria, T. hockingsi and A. australis*, and facilitate the isolation of biologically and chemically relevant compounds. The honey of Australian stingless bees has been shown to possess antibiotic and antifungal activity. In numerous other organisms, the interaction between microbes and higher organisms has been shown to be mediated by secondary metabolites. Furthermore, biological complexity has often been accompanied by chemical complexity (Strobel et al., 2004, T Ramesha et al., 2011). Therefore, microbiome of the Australian stingless bee was an ideal environment for the discovery of natural products.

This thesis is organised into three results chapters that contribute to this goal.

1) There has been no comprehensive study investigating the gut microbiota of Australian stingless bees. Therefore, a survey was performed to investigate the bacterial population present in three species of native Australian stingless bees (chapter 2). The results from this study would guide the design of downstream experiments to optimise the diversity of microbes cultured in subsequent chapters.

2) The second objective was to determine the biosynthetic (PKS/NRPS) and chemical diversity of microbial isolates from native Australian stingless bees. Our understanding of the bee microbiome (chapter 2) was used to guide the isolation of microbes from Australian stingless bees. Molecular screening and

chemical anlysis methods were combined to identify strains with biosynthetic potential and/or chemical uniqueness (chapter 3). Representative strains from this study were then selected for further bioactivity and chemical characterisation (chapter 4).

3) The final study (chapter 4) aimed to isolate compounds that had antimicrobial activity, or interesting chemistries, from representative strains of the bee gut microbiota. We performed bioactivity testing of extracts from representative strains of the bee microbiota (chapter 3) and used these results to select a single isolate for compound isolation. Spectral methods were then utilised to elucidate the structures of the purified natural products.

Chapter 2

Whole Gut Bacterial Associations of the Stingless Bee *Tetragonula carbonaria*

2.1.INTRODUCTION

Insects are one of the most diverse organisms on the planet with an estimated 1 million species worldwide (Chapman, 2009). Microbial associations in the insect gut have been shown to be fundamentally important to the health and development of a number of insects, including termites (Potrikus and Breznak, 1981), aphids (Douglas, 1998), cockroaches (Sabree et al., 2009) and bees (Evans and Armstrong, 2006, Yoshiyama and Kimura, 2009). These alliances can benefit the host in a number of ways including nutritional benefits, reproductive advantages, defence against parasites and a boosted immune system (Dillon and Dillon, 2004, Engel and Moran, 2013). Advances in sequencing technology and computing power have revitalized the study of insect associated microorganisms in recent years allowing researchers to characterize communities unseen with traditional culture based methods.

The Australian stingless bee *Tetragonula carbonaria* is found along the eastern coast of Australia where they flourish in the tropical climate of the northern regions. A hive forming, social insect they forage for nectar and pollen, which they store in wax pots within the hive to sustain the colony through the lean winter months when resources are scarce. Stingless beekeeping, or meliponiculture, in Australia has developed over the last 20 years driven by both public and academic interest (Halcroft et al., 2013). Stingless bee hives can be rescued from the environment when destructive action such as tree clearing and demolition reveal their existence. In such cases the hives can either remain in their existing cavity, such as within a log or tree branch, or they may be transferred into an artificial cavity within a specifically designed wooden box. Artificial

hives are also used to propagate stingless bee colonies by splitting an existing hive into two boxes.

The gut of *T. carbonaria* has yet to be described in the literature, however the general insect gut and the gut of the honey bee, though physically smaller in *T. carbonaria*, are considered an accurate representation. The honey bee gut is comprised of three main elements, the foregut (pharynx, oesophagus and crop), the midgut (midgut caecum and ventriculus), and the hindgut (Malpighian tubules, pylorus, ileum, rectum and anus) (Chapman et al., 2013). The foregut and the hindgut are lined with unsclerotised cuticle that is repeatedly shed as the insect develops and moults. The midgut is lined by peritrophic matrix that separates the food bolus from the midgut epithelial cells and is continually shed and replaced as required (Lehane, 1997). The moulting of the cuticle lining in the foregut and hindgut physically interrupts colonization of the gut by microorganisms as does shedding of the peritrophic matrix. The horizontal transfer of microorganisms by oral trophallaxis (mouth to mouth transfer via the proboscis) is one vector for transfer of microorganisms in highly social bees. Other routes of transfer include microbiota present in food stores (honey and pollen) as well as passive transmission due to communal living in the hive (Koch et al., 2013). Although vertical transmission of gut microorganisms between generations may occur via food deposited into the brood cell, the developing bees will lose these associations due to shedding of the gut lining. However newly emerged bees are quickly reinoculated by oral trophallaxis with their nest mates (Martinson et al., 2012).

Due to the economic importance and the current decline in commercial bee populations, the majority of research in the field of bee-associated bacteria has focused upon honey bees (genus *Apis*) and bumble bees (genus *Bombus*) (Colla et al., 2006, Cox-Foster et al., 2007, Pettis et al., 2013). Studies of gut, fecal material, hive materials and hive provisions from *Apis* species from varied geographic locations has revealed a consortium of bee specific bacteria including representatives of the Proteobacteria, Firmicutes, Bacteroides and Actinobacteria (Gilliam, 1997, Jeyaprakash et al., 2003, Babendreier et al., 2007, Mohr and Tebbe, 2006, Olofsson and Vásquez, 2008).

Observations of a core microbiota associated with honey bees from diverse geographic regions prompted questions as to the origin of these organisms, their mode of transmission and the evolutionary processes at work (McFrederick et al., 2012, Vásquez et al., 2012, Moran et al., 2012, McFrederick et al., 2014, Powell et al., 2014). Through the study of honey bees, bumblebees, stingless bees (highly social corbiculate bees), sweat bees (Halictidae), and their relatives, including ants and wasps, it has been shown that there is a core microbiota present only in honey bees with a subset in bumble bees (Martinson et al., 2011, Vásquez et al., 2012, McFrederick et al., 2013, Koch et al., 2013). The core honey bee microbiota is composed of the following phylotypes: Alpha1; a sister clade to the genus *Bartonella* which contains intracellular insect pathogens, Alpha 2; two clades within the *Acetobacteraceae*, Beta; a distinct clade within the *Neisseriaceae*, Bifido; 2 sister clades within the *Bifidobacterium*, Firm 4 and Firm 5 two clades within the genus *Lactobacillus*; and Gamma 1 and Gamma 2 sister groups to the *Pasteurellaceae* (Martinson et al., 2011).

Furthermore, surveys of diverse bee and wasp species have shown that a subset of these organisms, closely related to the genus *Lactobacillus,* the Firm 3, Firm 4 and Firm 5 Sensu (Babendreier et al., 2007), are found only in social corbiculate bee species, honey bees, bumblebee and some stingless bees (Vásquez et al., 2012, McFrederick et al., 2012). Two novel species of lactic acid bacteria (LAB)*, Snodgrassella alvi* and *Gilliamella apicola*, have now been identified as the dominant microorganism in the gut of both honey bees and bumble bees (Kwong and Moran, 2013) though they are currently thought to be absent from stingless bees (Koch et al., 2013).

Studies of the bacterial associates of Australian stingless bees are limited to a single report (Leonhardt and Kaltenpoth, 2014), where the bacterial community present in three native Australian stingless bee species, *T. carbonaria*, *Tetragonula hockingsi* and *Austroplebeia australis* was examined. The dominant microorganisms discovered were *Lactobacillales*, *Burkholderiales*, *Enterobacteriales* and *Rhodospirillales.* A novel monophyletic clade of LAB was described that was phylogenetically similar to LAB previously reported from Halictidae bees. This work was conducted on whole bees and as such there are no reports of the bacterial associations specifically focussing specifically on the gut of Australian native stingless bee.

In this chapter, we examined the gut microbiome of 13 *T. carbonaria* foragers sampled from both a rescued log hive and an artificial box hive. With only a single report in the literature regarding the *T. carbonaria* microbiome we aim to increase the depth of knowledge of these intriguing eusocial insects. Dissection of the whole gut from individual foragers from multiple hives allowed us to examine both the within hive

variation and differences that may exist between stable long-term hive structures and those transplanted into a man-made box. The hives examined were located in the same area and therefore had access to comparable foraging resources and were exposed to comparable climatic conditions and environmental stresses. We hypothesize that there should be no significant differences between the two hives examined, as the physical surroundings of the hive should not alter bacterial associations and once re-established the box hive colony was expected to quickly stabilize.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection

T. carbonaria individuals were collected from two hives located on the property of the Zabel family in Hatton Vale, QLD, Australia, latitude -27° 33' 5.3", longitude 152°, 29' 56.1". The first hive, denoted as Log hive, was located within a log section and the second hive was located within a wooden box and was denoted as Box hive. A total of 10 individual forager bees were collected from each hive by placing a sterile 50 mL tube over the entrance of the hive trapping the bees as they exited. The bees were immediately frozen by placing the sampling container in dry ice were they remained until returning to the laboratory where they were stored at -80°C.

2.2.2. Whole gut dissection

Each bee was surface disinfected by sonication for 45 seconds in 1 mL 70 % v/v aqueous ethanol then rinsed in 1 mL 0.15 M NaCl solution. The whole gut was removed using a sterile scalpel blade and a dissecting microscope and placed in a sterile 1.5 mL tube.

2.2.3. DNA extraction

Bacterial genomic DNA was extracted from individual bee guts using the Xanthogenate-SDS (XS) method (Tillett and Neilan, 2000). To each dissected gut, 1mL of XS buffer (1% w/v potassium ethyl xanthogenate, 800 mM NH_4OAc, 100 mM Tris-HCl (pH 7.4), 20 mM EDTA and 1% w/v sodium dodecyl sulphate (SDS)) was added and incubated at 65 °C for 2 h. The lysed cell suspensions were chilled on ice for 10

min and centrifuged at 12,000 g for 10 min. The resulting supernatants were transferred to sterile 2.0 mL tubes containing 1mL phenol/chloroform/isoamylalcohol (25:24:1). Samples were mixed via inversion and centrifuged at 12,000 g for 10 min. The upper aqueous layer was transferred to fresh 1.5 mL tubes and 2 volumes of 100% ethanol and 1/10th of a volume of 3 M sodium acetate were added. The tubes were incubated for 15 min at 4 °C and precipitated DNA was pelleted by centrifugation at 14,000 g for 20 min. DNA pellets were washed with 70% ethanol and centrifuged at 14,000 g for 10 min. Washed DNA pellets were air dried then resuspended in 20 µL of sterile milli-Q water. DNA yield and quality were checked using a Nanodrop® spectroscopy system (Thermo scientific) and final DNA concentrations were adjusted to ~30 ng/µL with sterile Milli-Q water.

2.2.4 16S rRNA pyrosequencing

Amplicon sequencing of partial 16S rRNA gene sequences was performed on extracted DNA at the Research and Testing Laboratory (Lubbock, Texas). Primers 27f and 519r (Weisburg et al., 1991) were used to PCR amplify a 500 bp product spanning the 16S rRNA variable regions V1-V3. Sequencing was performed using a Roche GS FLX Titanium machine. Sequence analysis was performed using Mothur version 1.33. Initial quality control measures used to ensure sequence fidelity included the removal of short sequences (<100 bp), any sequence with a discrepancy to the 5' primer, or any sequence containing an unresolved nucleotide (N). A 2% pre-clustering step was performed to remove other potential errors in sequence data (Huse et al., 2010). Sequences were aligned against the Mothur implementation of the SILVA 119 release 16S SSU alignment (Quast et al., 2013). Poorly aligned sequences were removed from the

analysis, and sequences were trimmed to ensure a consistent alignment space. The August 2013 release of the Greengenes reference dataset was used to derive the taxonomic identity of unique sequences using the Wang approach (Wang et al., 2007) with a cutoff score of 80. Contaminating sequences, annotated as c_chloroplast, k_Archeaea, or unknown were removed. Chimeric sequences were identified and removed using the chimera.slayer algorithm with minsnp = 100. Sub-sampling was performed at a level of 3430 sequences per sample. Pairwise distances between individual sequences were calculated and clustered into 1119 OTUs at a 0.03 distance as defined using the average neighbor algorithm. The taxonomic identify of each OTU was derived using a consensus approach based on the classification of individual sequences within each OTU; similarly a cutoff of 80 was assigned.

2.2.5 Phylogenetic analysis

For each of the 119 OTUs that were assigned to the family *Lactobacteriaceae* a representative sequence, identified as being the most abundant unique sequence following alignment to the SILVA alignment, was recovered. Representative sequences, along with an additional 620 reference sequences (Leonhardt and Kaltenpoth, 2014) were aligned using ClustalW (Larkin et al., 2007, Goujon et al., 2010). Phylogenetic inference of the resulting alignment, spanning 215 positions, was made with PhyML v3.0 (Guindon et al., 2010) using the Gamma Time Reversible model of nucleotide substitutions and 1000 bootstrap replicates. Trees were visualised and the final topology adjusted using Dendroscope v3.2.10 (Huson and Scornavacca, 2012).

2.3. RESULTS & DISCUSSION

2.3.1 T. carbonaria *gut bacterial composition*

PCR amplifiable DNA was obtained from six Log Hive individuals and seven Box Hive individuals. 16S rRNA gene pyrosequencing of the extracted DNA yielded at least 3430 sequences from each individual with the identification of 1119 distinct OTUs, defined at a 0.03 distance threshold. Sequence coverage, across the individuals was consistent and was considered to be sufficient. On average 136±24 OTUs were observed within each individual with an average Chao1 richness index of 410.43±62 and an average invSimpson index of 3.56±1.18.

The bacterial community composition of each *T. carbonaria* forager gut examined varied both within and between the two hives assessed (Figure 2.1). Variation in the phylotypes present and abundance of those phylotypes was observed. In seven of the 13 individuals sequence reads annotated as *Lactobacillaceae* were the most abundant, in two individuals *Acetobacteraceae* were the most abundant, in another two individuals *Halomonadaceae* were the most abundant and the final two individuals either *Neisseriaceae* or *Gammaproteobacteria* as the most abundant taxa (Figure 2.1).

Multivariate permutational analysis (PERMANOVA), as implemented on the total data matrix comprising 1119 OTUs, suggested that the average gut microbiome of forager bees between two hives could not be readily distinguished (Pseudo-F = 1.3737, p(Perm) = 0.145). The average *T. carbonaria* gut microbiome was dominated by sequence reads annotated as belonging to two bacterial orders; *Lactobacillales* (44.3% ± 21.3) and

Rhodospirillales (27.9% ± 24.5). Sequence reads annotated as *Oceanospirillales* (9.4% ±19.6), *Neisseriales* (5.3% ±14.7) and uncharacterized *Gammaproteobacteria* (5.9% ± 13.8) contributed a large proportion of the remainder of the stingless bee gut microbiome (Table 2.1).

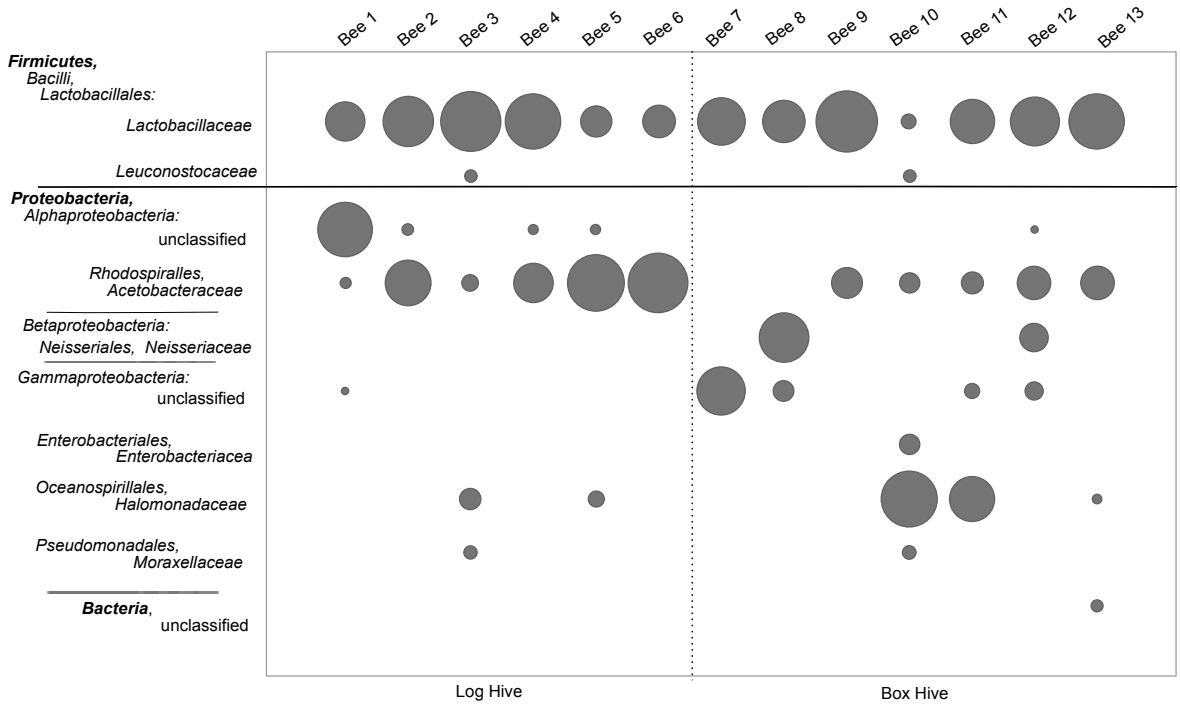

Figure 2.1: Abundance of bacterial phylotypes present at >1% abundance in *T. carbonaria* individuals from Log hive and Box hives. The size of each circle represents the relative abundance of each phylotype.

Table 2.1: Average abundance (%) of bacterial phylotypes >1% in *T. carbonaria* individuals from a Log, Box and combined hives, with associated relative standard deviation (%RSD).

Taxonomy	Log Hive	(n=6)	Box Hive	(n=7)	Hive's Combined	(n=13)
	%	RSD	%	RSD	%	RSD
Lactobacillales	43.4	22.9	45.1	21.7	44.3	21.3
Rhodospirillales	46.1	24.3	12.3	9.9	27.9	24.5
Oceanospirillales	2.6	3.8	15.3	25.8	9.4	19.6
Neisseriales	-	-	9.9	19.5	5.3	14.7
Gammaproteobacteria						
Unclassified	0.4	0.3	10.7	18.0	5.9	13.8
Pseudomonadales	1.7	2.2	1.5	3.3	1.6	2.7
Alphaproteobacteria						
Unclassified	2.7	1.3	0.7	0.6	1.6	1.4
Enterobacteriales	-	-	1.2	3.1	0.7	2.3
Other	3.1	1.0	3.2	2.9	3.2	2.1

This study represented the first attempt to examine the gut microbiome of multiple individual stingless bee foragers from multiple hives. Using a deep sequence methodology we were able to examine randomly selected foragers as they left the hive and the results obtained demonstrate the variability in individual gut communities. By applying culture-independent approaches, this study and that of Leonhardt and Kaltenpoth (2014), have highlighted the richness and diversity of stingless bee associated bacteria. On the basis of this extensive analysis, PERMANOVA was unable to differentiate the gut microbiome of each hive, as a consequence of the large variation that occurred within individual bees within each hive.

2.3.2 Core, hive specific, variable and unique communities

To further explore the gut community composition the core, variable and bee-specific communities that exist in the two hives were examined. The core community, defined as those OTUs that were found in at least 70% of the 13 individuals, was distinctive in that it comprised 1.6 % of the OTU's assigned and represented 56% of the total abundance (Tables 2.2 & 2.3).

These statistics provide an accurate representation of the community composition and enable the definition of the core community in both name and abundance without the diluting effect evident in the averaged results described above. A number of phylotypes in the core community, namely *Streptophyta, Xanthomonadales* and an unclassified *Bacilli,* were notably not present at substantial levels of abundance in any of the individual *T. carbonaria* gut microbiomes (Table 2.2, Figure 2.1). It is possible that these organisms are relevant to the biology of the bees without being highly abundant, though the presence of *Streptophyta* is unusual and the existence of algae or bryophytes in the gut of stingless bees may be a result of pollen-associated contamination.

Table 2.2: Core, variable and bee specific OTU's statistics for Log, Box and combined hives. OTUs present in the core community are defined by their presence within at least 70% of the individuals.

	# OTUs	OTUs (%)	Relative abundance (%)
Combined			
Core	18	1.6	56.0
Variable	221	19.7	38.6
Bee specific	880	78.6	5.4
Log			
Core	35	6.4	69.7
Variable	73	13.4	18.7
Bee specific	436	80.1	8.0
Box			
Core	15	2.1	71.4
Variable	125	17.2	24.2
Bee specific	587	80.7	4.3

Table 2.3: Core bacterial community OTU's and taxonomy. The core community is defined as being present in at least 70% of 13 *T. carbonaria* individuals collected from both the log and box hives.

OTU	Taxonomy
OTU 9	Alphaproteobacteria
OTU 11	Alphaproteobacteria
OTU 29	Bacilli
OTU 38	Bacilli
OTU 55	Bacilli
OTU 2	Lactobacillales
OTU 5	Lactobacillales
OTU 26	Lactobacillales
OTU 36	Lactobacillales
OTU 4	Oceanospirillales
OTU 21	Proteobacteria
OTU 34	Proteobacteria
OTU 15	Pseudomonadales
OTU 20	Pseudomonadales
OTU 7	Rhodospirillales
OTU 3	Rhodospirillales
OTU 63	Streptophyta
OTU 126	Xanthomonadales

Generally, the composition of the *T. carbonaria* gut microbiome, averaged across each or both hives (Table 2.1) is consistent with that previously reported.
(Table 2.4)(Leonhardt and Kaltenpoth, 2014). Sequence reads annotated as belonging to the order Lactobacillales, accounted for upwards of 40% of OTUs, with the remaining community typically comprising of members of the Enterobacteriales, Burkholderiales and Rhodospirillales. In three of the five hives studied by Leonhardt and Kaltenpoth (2014), Burkholderiales (10-20%) and Enterobacteriales (20-50%) contributed a greater proportion of the gut microbiome, with the Lactobacillales present at lower abundance. In the remaining two hives, however, where Lactobacillales contributed upwards of 80% of the microbiome, these two orders were mostly absent, with Rhodospirillales contributing to the majority of the remaining population (Leonhardt and Kaltenpoth, 2014).

Table 2.4: Average abundance (%) of bacterial phylotypes in *T. carbonaria* as determined from this study and that of Leonhardt and Kaltenpoth (2014).

Taxonomy	Relative Abundance (%)	
	This study	Leonhardt and Kaltenpoth (2014)
Lactobacillales	44.3	41.4
Enterobacteriales	0.7	30.5
Burkholderiales	-	10.2
Rhodospirillales	27.9	6.7
Pseudomonadales	1.6	3.7
Oceanospirillales	9.4	1.4
Other	16.1	6.1

In this study, averaged across all sampled individuals in the hives, the gut microbiome of the Log hive mirrored that of the latter two hives from the Leonhardt and Kaltenpoth (2014) study in that it was dominated by Lactobacillales and Rhodospirillales. Alternatively, when averaged across all sampled individuals the Box hive was distinct from those previously reported in that it contained a much higher abundance of

Oceanospirillales. As their name suggests the Oceanospirillales are mostly associated with marine environments where many of the members exhibit halotolerant/halophilic, psychrophilic and piezophilic adaptations (Garrity et al., 2005). As neither salt, low temperature or high pressure are conditions found in the bee gut, the high osmotic stress that accompanies concentrated sugars in honey may reflect similar mechanisms for osmotolerance and halotolerance in this species (Lages et al., 1999). Oceanospirillales have previously been detected as symbionts in bone-eating worms (Verna et al., 2010), moss bugs, (Santos-Garcia et al., 2014), ants (Eilmus and Heil, 2009), bumble bees (Lim et al., 2015) and stingless bees (Leonhardt and Kaltenpoth, 2014). The origins and functions of these bacteria as gut symbionts are unknown and require a more complete characterization of their taxonomy and physiology to elaborate their significance.

Variation in the bacterial composition between individual foragers was observed both within and between the two hives. In the Log hive Bee 2 and Bee 4 were similar, Bee 5 and Bee 6 were similar and Bee 1 and Bee 3 were different to all other bees from that hive. The Log hive individuals were primarily dominated by Lactobacillales or Rhodospirillales, with the exception being Bee 1 that differed from all other bees examined in that an unclassified Alphaproteobacteria was dominant. There was more variation between individuals in the Box hive than the Log hive. Bees 10 and 11, from the Box hive, had high levels of Oceanospirillales increasing the average of that taxa in the Box hive. Bee 7 had high levels of an unclassified Gamma Proteobacteria and Bee 8 had high levels of Neisseriaceae. This level of individual variation indicates that a pooled approach to sample analysis, as has been used for most studies of bee gut bacteria (Martinson et al., 2011, Leonhardt and Kaltenpoth, 2014), may overlook the diversity that exists between individuals. Our report raises questions as to the correct

number of bees that need to be sampled to correctly survey the microbiome of an insect gut if either a pooled or individual analysis is applied.

The variation observed between individuals may be the result of several factors. Unlike honey bees that only defecate outside of the hive, and as such maintain a hindgut full of food waste and bacteria, *T. carbonaria* individuals defecate within the hive and the waste is moved to waste dump sites and removed when required (Dollin, 1996). The difference in *T. carbonaria* community abundance observed between hives in both this study and the Leonhardt and Kaltenpoth (2014) study, and also between individuals in this study, may be the result of sampling the bees that have recently defecated and those that have not. It has been shown that in honey bees the majority of the bacterial community is located in the hindgut (Martinson et al., 2012). Emptying of the hindgut could be expected to remove hindgut specific bacteria resulting in a shift in the observed community structure. Furthermore, it has been demonstrated that diet has a distinct effect on the bacterial community of the insect gut (Colman et al., 2012). As bees transition from in-hive tasks at a younger age, to foraging as they mature, their diet typically switches from pollen-dominated to primarily nectar and honey-based as carbohydrates in these sources support the intense energy expenditure associated with flying to and from the hive collecting provisions. This change in diet could also result in shifts in the gut microbiome and although bees were collected exiting the hive (foragers) there is no way of determining how recently they shifted from in-hive to out-of-hive tasks.

There is no definitive answer as yet to the specific function of bacteria in stingless bees. The limited research into the bacterial associates of the stingless bee means we must

look to the work in *Apis* and *Bombus* species for clues. Current theories include; bacteria protect bees by outcompeting harmful microorganisms and occupy a niche that would otherwise be susceptible to the growth of pathogens (Koch and Schmid-Hempel, 2011), that bacteria increase the fitness of bees by priming their immune systems (Evans and Lopez, 2004) thus also providing protection against parasites and pathogens (Evans and Armstrong, 2006, Forsgren et al., 2010, Koch and Schmid-Hempel, 2011), and that bacteria can assist digestion and also preservation of food (Anderson et al., 2014).

2.3.3 Lactobacillaceae taxonomy

A phylogenetic analysis of Lactobacillaceae OTUs obtained from this study was made with reference to well-described bee associated bacterial communities. The alignment provided by Leonhardt and Kaltenpoth (2014) was primarily constructed by McFrederick et al. (2013) and included Meliponini associated lactobacilli sequences reported by Vasquez et al., (2012) in addition to those assigned in the Leonhardt and Kaltenpoth (2014). The Vasquez et al., (2012) sequences could not be aligned with the sequences generated from this study as each targeted a different region of the 16S rRNA gene.

A total of 111 OTUs from the order Lactobacillales (denoted as Mills OTU#) were identified within this study. The phylogenetic position of these sequences was inferred by comparison to 20 Meliponini associated LAB and 600 *Lactobacilli* sequences and outgroups (McFrederick et al., 2013, Leonhardt and Kaltenpoth, 2014). Visualization of the alignment produced two phylogenetic trees annotated in a manner consistent with a recent taxonomic revisions of the *Lactobacilli* (Figure 2.2) (Salvetti et al., 2012) and

with a recent investigation of LAB associated with stingless bees (Figure 2.3)(Leonhardt and Kaltenpoth, 2014). *T. carbonaria* associated LAB (red) formed four sub-groups, two located in the *L. delbrueckii group*, one in the *L. brevis* group and one stingless bee-associated group (Figure 2.2). The two *L. delbrueckii* sub-groups were the most diverse in that they contained the greatest number of individual OTUs from this study. It should be noted that the Firm 4 group was split into two sub-groups in this analysis as compared to the phylogeny created by McFrederick et al. (2013).

Each of the *T. carbonaria* gut associated LAB OTUs (this study) were phylogenetically proximal to other previously identified *T. carbonaria* associated OTUs (Figure 2.3). The mean abundance of the *T. carbonaria* associated OTU's (Figure 2.4) within each hive reveals that the relative contribution of each OTU is variable across individual foragers. *T. carbonaria* associated LAB OTUs 1, 2, 5 and 6 were considered dominant as the mean abundance of each was greater than 5%.

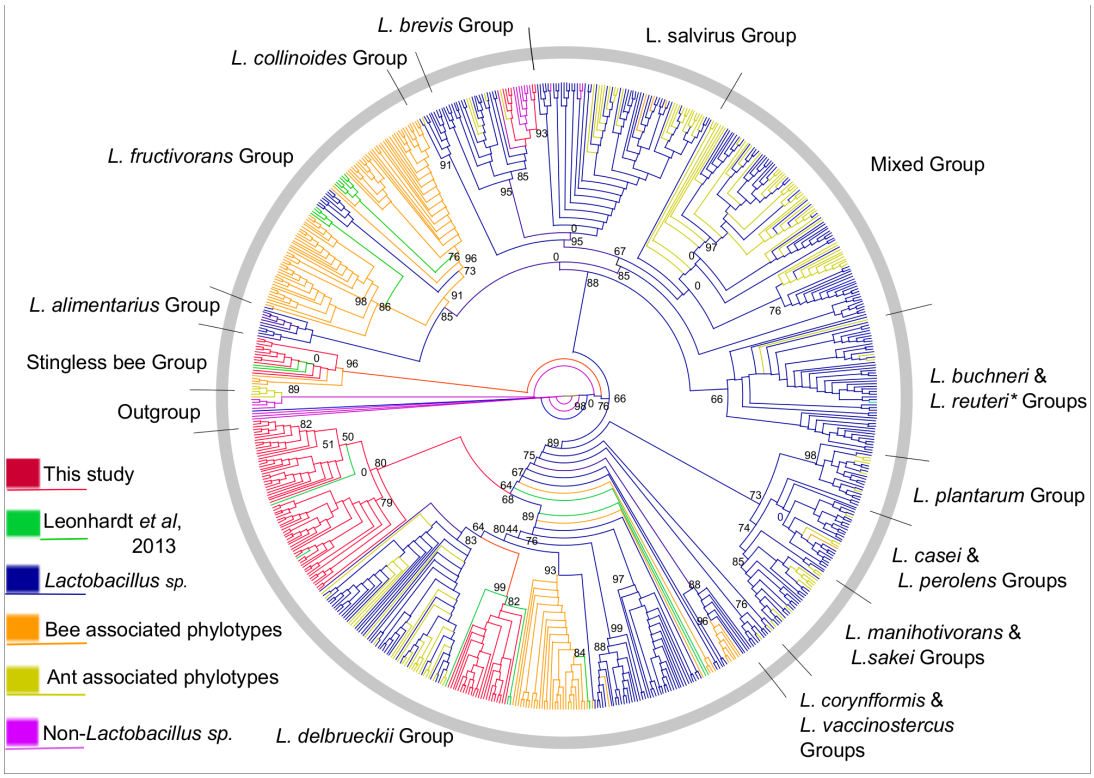

Figure 2.2: 16S rRNA gene phylogenetic placement of the Australian native stingless associated *Lactobacillus* OTU's (coloured Red and Green) within the genus *Lactobacillus.* Annotated with recent taxonomic revisions of the *Lactobacilli.* Numbers on tree nodes indicate % confidence values. OTU's assigned in this study are coloured red, OTU's assigned by Leonhardt and Kaltenpoth (2014) are coloured green and other bee associated phylotypes are coloured orange.

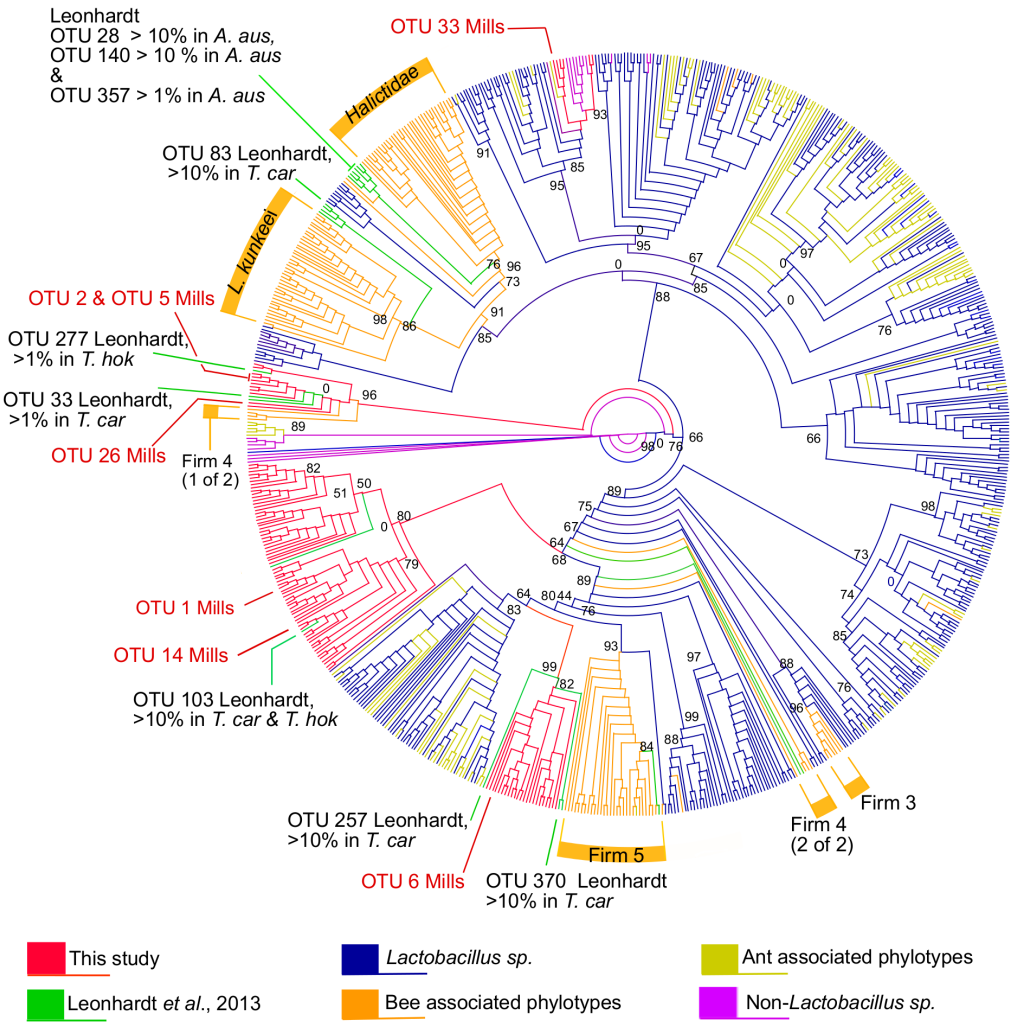

Figure 2.3: 16S rRNA gene phylogenetic placement of the Australian native stingless associated *Lactobacillus* OTU's (coloured Red and Green) within the genus *Lactobacillus.* Annotated with lactic acid bacteria associated with stingless bees. Numbers on tree nodes indicate % confidence values. OTU's assigned in this study are coloured red, OTU's assigned by Leonhardt and Kaltenpoth (2014) are coloured green and other bee associated phylotypes are coloured orange.

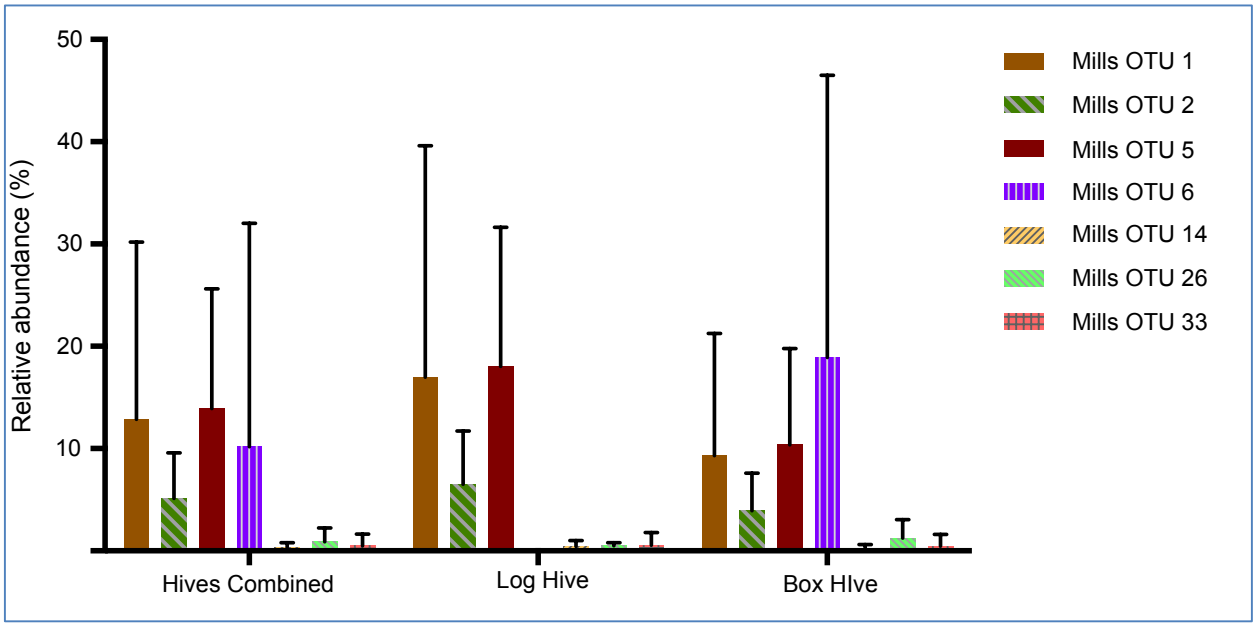

Figure 2.4: Relative abundance of the dominant *lactobacillus* OTU's assigned from the gut of *T. carbonaria.* Average abundance shown for individuals from the Log hive, Box hive and both hives combined.

The Lactobacillales sequences from our study formed four sub groups, however, the dominant OTU's 1, 2, 5 and 6, are located within only two of these, the *L. delbrueckii* group and the Stingless bee group (Figures 2.2, 2.3 & 2.4). The stingless bee group is interesting in that it contains sequences from both studies that examined *T. carbonaria* and a portion of the Firm 4 group, the corbiculate bee-specific Lactobacillaceae sequence (Leonhardt and Kaltenpoth, 2014). These sequences are unlike any other *Lactobacillus* found in the any other organism or environment. Each occurrence of *Lactobacillus* in this study was coupled with a similar sequence from the Leonhardt and Kaltenpoth (2014) study. However, the novel Halictidae-associated sequences reported by Leonhardt and Kaltenpoth (2014) were absent from our analysis. This may be a result of examining only the whole gut in this study indicating the Halictidae-associated sequences may originate from another body part of the bee. Similar to the results obtained by the V**á**squez et al. (2012) and Leonhardt and Kaltenpoth (2014) studies, our

Lactobacillus sequences were similar to the Firm 4 and Firm 5 groups, though no sequences similar to the Firm 3 group were detected.

Lactobacillus kunkeei is commonly found as one of the dominant organisms in the gut of the honey bee, bumble bee and some stingless bees (Vásquez et al., 2012, Corby-Harris et al., 2014). *L. kunkeei* is also found in the provisions of honey bee and in the environment associated with fructose rich niches such as the nectar of flowers. In this study we didn't identify any sequences similar to *L. kunkeei*. In contrast, Leonhardt and Kaltenpoth (2014) grouped their OTU83 closely with the *L. kunkeei* clade and this represented greater than 10% of the OTUs obtained from their *T. carbonaria* samples.

2.4 CONCLUSIONS

The data within this chapter demonstrates the individual variation that occurs within the gut microbiome of the Australian stingless bee *T. carbonaria*. The analysis highlights that each individual within a hive possesses its own distinct microbiome; the prospect that this can vary in response to changing environmental conditions, dietary limitations and life cycle has implications for the management of native stingless bees and ensuring their survivability.

The occurrence of specific lineages of stingless bee gut-associated LAB in this highly social Meliponini species adds to the understanding of corbiculate bee gut bacterial microbiome and the existence of distinct bacterial groups within the gut of eusocial bees. The absence of any *L. kunkeei* related sequences in this study is in contrast to the results obtained by Leonhardt and Kaltenpoth, (2014) and (Vásquez et al., 2012), suggests a role of this organism outside of the gut microbiome. Further studies examining the gut microbiome of Australian stingless bees are required to clarify the role of *L. kunkeei* and other microbial symbionts.

This chapter adds to the understanding of Australian stingless bee associated microbiota and highlights the variations between individuals and the similarities amongst box and log hives. The knowledge gained was used to design and develop sampling strategies and selection of appropriate media for culture based investigations of stingless bee microorganisms.

Chapter 3

Culturing, genetic screening and chemical analysis of microorganisms associated with the Australian stingless bees, *Tetragonula carbonaria, Austroplebeia australis* and *Tetragonula hockingsi*

3.1. INTRODUCTION

Stingless bee and their hive products have been used by humans for thousands of years (Crane, 1999). These products have been used for food, trade, tool and instrument construction, painting, religious ceremonies and as medicines (Jones, 2013). Numerous studies have identified antibacterial, antifungal, antiviral, anti-inflammatory and antiproliferative activity from stingless bee products (Kujumgiev et al., 1999, Jose Dardon and Enriquez, 2008, Liberio et al., 2011, Umthong et al., 2011, Massaro et al., 2011, Zamora et al., 2013, Massaro et al., 2014b, Massaro et al., 2014a).

Honey and propolis from the eusocial species, *T. carbonaria*, have been shown to exhibit antibacterial activity against clinical isolates and reference strains, including *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa Salmonella enterica* serovar Typhimurium*, Listeria monocytogenes* and *Klebsiella pneumoniae* (Irish et al., 2008, Boorn et al., 2010, Massaro et al., 2014a, Massaro et al., 2014b). Stingless bee honeys are also known to exhibit greater bioactivity, at lower concentrations, when compared to other bee products, such as medicinal Manuka honey (Boorn et al., 2010). Additionally, studies performed by Stow and colleagues demonstrated that ethanol extracts from *T. carbonaria* cuticle compounds possess inhibitory activity against *S. aureus* (Stow et al., 2007) and the fungal pathogen *Cordyceps bassiana* (Stow et al., 2010). Furthermore, they showed that more social bee species produce extracts with greater antibacterial activity as compared to bees with less social life cycles (Stow et al., 2007).

Many bee products have been investigated for isolation of bioactive compounds, including wax, honey, cerumen and the bees themselves. However, the origin of the

compounds in these products is challenging to identify because they are a mixture of numerous materials derived from different organisms. Despite this, one element that is common to all these environments is the presence of microorganisms. In many other sources, such as plant and animal products, the true origin of bioactive compounds has been attributed to microorganisms rather than the products themselves (Hildebrand et al., 2004, Piel, 2004). Furthermore symbionts of the honey bee gut have been shown to produce broad spectrum antimicrobials compounds (Olofsson et al., 2014) however the production of antimicrobial compounds by the microbiota associated stingless bees has never been investigated. These bee-associated microorganisms represent an, as yet, unexplored niche of microbial and chemical diversity.

The aims of this chapter were to 1) isolate microorganisms associated with the gut and cuticle of Australian stingless bees known to produce bioactive compounds, 2) assess their antimicrobial biosynthetic potential, 3) create groups of isolates with similar chemical profiles and 4) select suitable candidates for chemical extraction and bioactivity testing.

Bacteria and fungi from *Tetragonula carbonaria, Austroplebeia australis* and *Tetragonula hockingsi* were isolated and identified based on their respective 16S rRNA gene or 18S rRNA gene taxonomy. These isolates were then genetically screened for the presence of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes, two classes of biosynthetic enzymes responsible for the production of bioactive compounds. A PCR-based approach targeting the functionally and genetically conserved KS and C domains of both pathways was used for identification of biosynthetic clusters (Schwarzer and Marahiel, 2001). Finally, to assess the chemical

diversity present in the microbial library, chemical extracts of each isolate was analysed via liquid chromatography-mass spectrometry (LC-MS) to generate a metabolomic profile. The results of these genetic and chemical screening methods were used to select a subset of isolates with bioactive potential and a high degree of chemical diversity.

3.2. MATERIALS AND METHODS

3.2.1 Sample collection

Samples from one hive of *T. hockingsi* and three hives of *A. australis* were collected from Elanora, Australia (latitude 28°9'28.7" S, longitude 153°29'26.8 E) and samples from two hives of *T. carbonaria* were collected from Graceville, Australia (latitude 27°31'21.3" S, longitude 152°58'40.5" E). In an effort to capture the diversity present, bees at different stages of development were collected. Each developmental stage was visually distinguished and collected as described in Table 3.1.

All samples were immediately placed in a small portable cooler within another portable cooler containing 5 kg of ice to keep them cool but not frozen. Samples were transferred from the makeshift cool box to a 4°C cool room at UNSW within 10 h of collection. Processing of all samples took place within 2 weeks of collection.

Table 3.1: Stingless bee collection protocol.

Sample type	Description	Collection method
Foragers	Bees that exit the hive and collect provisions.	Collected first, before the hive was opened, by placing a sterile container over the entrance of the hive.
Callows	Juvenile bees found on the brood comb, pale brown in colour as compared to more mature bees, which are black.	Removed from the hive with tweezers and placed into sterile containers.
Mature eggs	Enclosed larval bees found in the brood comb.	Cut from the hive using a sterile scalpel.
Eggs	Undeveloped eggs found in the brood comb.	Cut from the hive using a sterile scalpel.

3.2.2 Microbial culturing

All bee and egg samples were surface disinfected by sonication in a water bath for 45 seconds in 1 mL 70% v/v aqueous ethanol; then rinsed in 1 mL 0.154 M NaCl solution. Bee samples were dissected on a sterilised glass slide with a sterile scalpel, forceps and dissecting microscope, using aseptic techniques. Dissection methods varied for each sample type, and are described in Table 3.2.

Each pooled sample was macerated with a sterile glass grinder and re-suspended in 400 µL PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Thirty microliter aliquots from each homogenised bee sample were spread plated onto 11 different solid media as listed in Table 3.3.

Table 3.2: Sample specific dissection methods.

Sample Type	Dissection method
Foragers	The whole gut was removed from five individuals from each hive and placed in sterile 1.5 mL tubes. The dissected bee bodies (without the gut) were also pooled and place in sterile tubes.
Callows	Processed in the same manner as the foragers however the number of callows used was limited by the number that could be found. A minimum of two callows were used from each hive.
Mature eggs	Five bee larvae were removed from their brood cells for each hive and placed in sterile 1.5mL tubes.
Eggs	Five bee eggs were removed from their brood cells for each hive and placed in sterile1.5mL tubes.

Table 3.3: Media used to isolate microorganisms from stingless bees

Acronym	Media and additives
WYE	Water, yeast extract and agar
YECD	Yeast extract, casamino acid and agar
ME100 +	100% Malt extract agar + Chloramphenicol
ME25 +	25% Malt extract agar + Chloramphenicol
BHI10 +	10% Brain heart infusion agar + Chloramphenicol
PDA100	100% Potato dextrose agar
PDA25	25% Potato dextrose agar
NA100+	100% Nutrient broth agar + Cyclohexamide
NA25	25% Nutrient broth agar + Cyclohexamide
MRS100	100% de Mann, Rogosa and Sharpe agar
MRS25	25% de Mann, Rogosa and Sharpe agar

Agar plates were incubated at 25°C and checked daily for microbial growth. Distinct colony morphologies were subcultured until pure colonies were obtained. Pure cultures were assigned a code denoting their origin and a unique number.

3.2.3 Colony morphology based dereplication

Colony morphology was used to gain an insight in the diversity present in the microbial library. The morphology of the each pure culture was determined by visual examination of parameters described in Table 3.4. Morphotypes parameters were converted into a numeric code and the statistical software Primer 6 (Clarke, 2006) used to the generated a resemblance matrix of organisms present. The resemblance matrix was assessed using an 80% similarity cut off to calculate the number of distinct morphotypes present. The colour parameter was excluded in the analysis, as different pigmentation can frequently occur when microorganisms are grown on different media.

3.2.4 Archiving

Pure cultures were preserved by preparation of glycerol stocks to be stored at -80°C. Biomass was harvested from freshly cultivated agar plates and aseptically transferred to 2 mL cryovials containing 750 µL of 80% aqueous glycerol. Each vial was then made up to a final volume of 1.5 mL with liquid media. Nutrient broth was used for bacterial isolates and Malt extract broth was used for eukaryotic samples.

Table 3.4: Parameters used to describe gross colony morphology

Form	**Size (mm)**	**Surface**	**Texture**	**Opacity**	**Elevation**	**Margin**
Circular	1	Dull	Butyrous	Opaque	Convex	Curled
Filamentous	2	Glistening	Dry	Translucent	Crateriform	Entire
Flat	3	Rough	Mucoid		Flat	Filiform
Irregular	4	Veined	Moist		Pulvinate	Lobate
Rhizoid	5	Wrinkled			Raised	Undulate
	6				Umbonate	
	7					
	8					
	9					
	10					

3.2.5 DNA extraction

Genomic DNA was extracted from pure cultures of representative strains. DNA was extracted using the XS method (Tillett and Neilan, 2000). Approximately 100 mg of biomass, harvested from agar plates, was placed in 1.5 ml tubes containing 1 mL of XS buffer (1% potassium ethyl xanthogenate, 800 mM NH_4OAc, 100 mM Tris-HCl (pH 7.4), 20 mM EDTA, 1% sodium dodecyl sulphate) and vortexed for 45 seconds. The tubes were incubated at 65°C for 2 h, vortexed and incubated for 1h at 65°C then chilled on ice for 10 min and finally centrifuged at 12,000 x *g* for 10 min. The resulting supernatants were transferred to clean 2 mL tubes and washed twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Aqueous layers were transferred to fresh 1.5 mL tubes and 2 volumes of ice cold 100% ethanol and 1/10th of a volume of 3 M sodium acetate were added then incubated at -20°C overnight. The precipitated DNA was pelleted by centrifugation at 14,000 x *g* for 20 min and washed with 70% ethanol. The resulting purified DNA was centrifuged at 14,000 x *g* for 10 min, air dried, then resuspended in 20 µL of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0). DNA yield and quality were checked using a Nanodrop ND-1000 spectrophotometer (Thermo scientific) and the 260/280 nm and 260/230 nm ratios recorded.

3.2.6 Polymerase Chain Reaction (PCR)

3.2.6.1 16S rRNA gene amplification

PCR amplification of 16S rRNA gene was performed using the bacterial-specific primers 27fl, 5'-(AGAGTTTGATCCTGGCTCAG)-3', and 1494rc, 5'-(GGTTACCTTGTTACGACTT)-3' (Neilan et al., 1997). Each 20 µL PCR reaction

mixture contained 2.5 mM $MgCl_2$, 0.15 mM each dNTP, 10 pmol each primer, 0.4 U of BioTaq DNA polymerase, the appropriate PCR buffer (Bioline) and approximately 10 ng genomic DNA. Thermal cycling conditions: 94°C for 2 min followed by 35 cycles of (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) then 72°C for 7 min.

3.2.6.2 18S rRNA gene amplification

PCR amplification of 18S rRNA gene was performed using the degenerate eukaryotic primers SS5 5'-(GGTTGATCCTGCCAGTAGTCATATGCTTG)-3' and SS3 5'-(GATCCTTCCGCAGGTTCACCTACGGAAACC)-3' (Rowan and Powers, 1992). Each 20 μL PCR reaction mixture contained 2.5 mM $MgCl_2$, 0.15 mM each dNTP, 25 pmol each primer, 0.4 U of BioTaq DNA polymerase, the appropriate PCR buffer (Bioline) and approximately 10 ng genomic DNA. Thermal cycling conditions: 94°C for 5 min followed by 35 cycles of (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) then 72°C for 7 min.

3.2.6.3 Screening PCR – PKS (DKF/DKR) and NRPS (CNDM/DCCR)

Genomic DNA from isolates that provided a positive result in either 16S rRNA or 18S rRNA gene PCR were further examined for the presence of PKS genes with the degenerate primers DKF 5'-(GTGCCGGTNCCRTGNGYYTC)-3' and DKR 5'-(GCGATGGAYCCNCARCARMG)-3' (Moffitt and Neilan, 2001) and the presence of NRPS genes with the degenerate primers MTF2 5'-(GCNGGYGGYGCNTAYGTNCC)-3' and MTR2 5'-(CCNCGDATYTTNACYTG)-3' (Neilan et al., 1999). Thermal cycling conditions for DKF/DKR were: 92°C for 2 min,

35 cycles of (92°C for 10 s, 55°C for 30 s, 72°C for 1 min) then 72°C for 7 min. Thermal cycling conditions for MTF2/MTR2 were: 92°C for 2 min, 35 cycles of (92°C for 10 s, 52°C for 30 s, 72°C for 1 min) then 72°C for 7 min.

3.2.7 Gel electrophoresis

PCR products were resolved from the reaction mixture using gel electrophoresis with 1% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA). Agarose gels were visualised with 0.5 μg/mL ethidium bromide solution and recorded with a Gel Doc XR system running Quantity One 4.6.1 software (BioRad).

3.2.8 PCR product clean up

PCR products from successful 16S rRNA and 18S rRNA reactions were precipitated with 2 volumes of 95% ethanol and 1/10 volumes 3M sodium acetate. Precipitated DNA was pelleted by centrifugation at 14, 000 x *g* for 10 min then washed with 70% ethanol, air-dried and resuspended in Milli-Q water. DNA yield and quality were checked using a Nanodrop ND-1000 spectrophotometer (Thermo scientific) and the 260/280 nm and 260/230 nm ratios recorded.

3.2.9 DNA sequencing and analysis

PCR products were sequenced using the automated sequencing Big Dye cycle sequencing protocol and ABI 3730 DNA analyser sequencer (Applied Biosystems) at the Ramaciotti Centre for Genomics. Reactions were carried out in a total volume of

20 µL, consisting of 1 µL of Big Dye, 20-50 ng of purified PCR product, 3.2 pmol of primer, 3.5 µL of 5× sequencing buffer and milli-Q water to a final volume of 20 µL. Thermal cycling: 96°C for 3 min, followed by 30 cycles of (96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 min).

Raw sequences were trimmed, to remove low quality regions, using BioEdit sequence alignment editor (Ibis Biosciences) and compared to sequences in the GenBank database using the BLASTn search tool (http://www.ncib.nlm.nih.gov/BLAST/)(Hou et al., 2012).

3.2.10 Chemical extraction

Approximately 200 mg of biomass was harvested from 2 week old agar cultures of individual isolates and stored at -20°C in 15 mL tubes. Freezing of the harvested biomass was an initial cell lysis mechanism and permitted the collection and processing of samples in manageable groups. Once frozen, for a minimum of 48 h, samples were resuspended in 5 mL of HPLC grade methanol and subjected to sonication at 60% amplitude, with three periods of 10 seconds pulse on, followed by 15 seconds of cooling (Branson Ultrasonics). Cells and solvent were incubated at 25°C for 3 h then centrifuged, 10,000 x *g* for 30 min, with the supernatant transferred to a clean 20 mL scintillation vial passing through a 0.22 µm syringe filter. Each extract was dried by rotary evaporation then resuspended in 600 µL HPLC grade methanol and transferred to a pre-weighed 2 mL glass HPLC vial and dried by rotary evaporation. The mass of each extract was recorded and resuspended in a 90:10 water:acetonitrile (v/v) at a

standardized concentration of 2 mg/mL. Extracts were transferred to Whatmann 0.2 µm regenerated cellulose Mini-UniPrep syringe-less filter units prior to LC-MS analysis.

3.2.11 Liquid chromatography–mass spectrometry

Reverse-phase LC separation was carried out on a Poroshell 120 column, (EC-C18, 2.7 µm, 2.1 x 150 mm, Agilent Technologies) with an inline Poroshell 120 UHPLC guard column, (EC-C18, 2.7 µm, 2.1 mm x 5 mm) attached to a Dionex Ultimate 3000 Binary RSLC system (Thermo Scientific) running a 1 mL/min gradient elution program over 30 minutes; 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile. The column compartment was maintained at 30 °C and the autosampler vials were maintained at 20°C. The injection needle was washed prior to and post injection with a 50:50 methanol:water solution. A blank run consisting of a 30 µL injection of HPLC grade methanol, utilizing the same run conditions as above, was performed after each extract run. Each analysis was initiated with the injection of 30 µL of 2 mg/mL extract onto the column. The solvent flow, post column, was reduced with a QuickSplit adjustable flow splitter (Analytical Scientific Instrument) from 1 mL/min to 70 µL/min running to the mass spectrometer, with the remainder to waste.

Mass spectra were acquired using a linear ion trap mass spectrometer (LTQ XL, Thermo Scientific) equipped with an electrospray (ESI) source. Each LC-MS spectrum was acquired for 30 min, starting at the point of LC injection and limited to a mass range of 50-1500 *m/z,* single stage full scan, scan rate: normal*,* data type: profile. Data collection was carried out over two separate periods, the first were four approximately

48 h time periods spaced 5 days apart and the second a further two 48 h time periods spaced 5 days apart. Prior to each 48 h acquisition the instrument was calibrated using the LTQ ESI positive ion calibration solution as per the manufacturer's instructions. Ion spray and ion optics settings were tuned to a 70 μL/min flow of 50:50 methanol:water spiked with diluted LTQ ESI positive tune solution, 1:15. Sheath gas settings were optimized for stable spray and ion optics settings for the maximum observation of the peptide MRFA (H-Met-Arg-Phe-Ala-OH) $[M+H]^+$ ion, 524.5 *m/z*.

3.2.12 Data analysis

3.2.12.1 MZmine 2

Processing of the acquired LC-MS data was performed using MZmine 2 (Pluskal et al., 2010). A total of 218 individual LC-MS runs were imported into MZmine 2 and processed using the specific steps and settings documented in Table 3.5. The resultant abundance matrix, peak list (*m/z*, retention time, and abundance) vs. sample, was exported as a comma separated values file. Eukaryotic and prokaryotic samples were processed separately, using identical settings.

3.2.12.2 Primer 6

Analysis of the abundance matrix generated with MZmine 2 was conducted using Primer 6 (Clarke, 2006). Cluster analysis was performed on a Bray Curtis dissimilarity resemblance matrix derived from square root transformed abundance matrix.

Table 3.5: MZmine 2 data processing options and settings used to generate data matrix from 95 crude microorganism extracts analysed by positive mode reverse-phase LC-MS

Menu tab	Processing step	Options	Settings	Units
Raw data methods	**Filtering**			
	Data set filtering			
		Crop filter		
		Retention time start	0	min
		Retention time finish	21	min
	Baseline correction			
		Chromatogram type	TIC	
		MS Level	1	
		Smoothing	1,000,000	
		Asymmetry	0.001	
		m/z bin width	0.05	
	Peak detection			
	Mass detection			
	Mass detector	Wavelet transform		
		Noise level	1.0 x E3	
		Scale level	40	
		Wavelet window size	15	%
	Chromatogram builder			
		Minimum time span	0.1	min
		Minimum height	1.0 x E3	
		m/z tolerance	0.5	*m/z*
		or	10	ppm
Peak list methods	**Peak detection**			
	Chromatogram deconvolution			
	Algorithm	Savitzky-Golay		
		Minimum peak height	1.0 x E3	
		Peak duration	0.1	min
		to	5	min
		Derivative threshold level	90	%
	Isotopes			

Isotopic peak grouper	*m/z* tolerance	0.5	*m/z*
	or	5	ppm
	Retention time tolerance	0.1	min
Monotonic shape	Monotonic shape	No	
	Maximum charge	20	
	Representative isotope	Lowest *m/z*	
Normalisation			
Retention time normaliser	*m/z* tolerance	0.5	*m/z*
	or	10	ppm
	Retention time tolerance	0.2	min
	Minimum standard intensity	1.0 x E3	
Alignment			
Join aligner	*m/z* tolerance	0.5	*m/z*
	or	10	ppm
	Weight for *m/z*	0.5	
	Retention time tolerance	0.2	min
	Weight for RT	1	
	Require same charge state	No	
	Require same ID	No	
	Compare isotope pattern	No	
Normalisation			
Linear normaliser			
	Normalization type		
	Average squared intensity	Yes	
	Peak measurement type	Peak area	
Export/Import			
Export to CSV file	Field separator	comma	
	Export common elements		
	Export row ID	yes	
	Export row *m/z*	yes	
	Export row retention time	yes	
	Export identity elements	yes	
	Export data file elements		
	Export peak area	yes	

3.3. RESULTS AND DISCUSSION

3.3.1 Microbial culturing media

The rationale behind the media selected for microbe isolation was as follows: Water-yeast extract agar and yeast extract-casamino acid-dextrose, both low nutrient media and as such they were different to the other media selected. Low nutrient media were included in an effort to capture those organisms that didn't flourish on the other nutrient media. In addition both WYE and YECD media have been successfully used to isolate Actinomycetes (Crawford et al., 1993). The same low nutrient approach was applied to all the media below which were made up at 25% of their normal concentrations.

Malt extract media is widely used for the cultivation of yeasts and moulds and is the recommended media for their enumeration in the U.S Food and Drug Administrations Bacteriological Analytical Manual (Tornas et al., 2001). Chloramphenicol was added to inhibit the growth of bacteria. A 10% concentration of brain heart infusion was used to specifically target Trichomycetes, gut-inhabiting fungi that are associated with insects and other arthropods (Lichtwardt, 1964). Potato dextrose agar is suitable for culturing fungi and bacteria and was not selective for any particular organisms, a general all purpose media.

Nutrient agar was originally developed for water testing though it is used to culture a wide range of non-fastidious bacteria. Cycloheximide was added to inhibit fungal

growth making the media bacteria selective. Developed by De Man, Rogosa and Sharpe, MRS media is selective for the cultivation of Lactobacilli (De Man et al., 1960).

3.3.2 Microbial culturing

A total of 41 individual stingless bees and eggs were dissected and spread plate onto 11 different media, producing 451 different spread plates. After incubation for one week, 212 of the spread plates displayed growth of one or more microorganisms. From the 212 successful spread plates, 559 isolates were identified and successfully subcultured.

3.3.3 Morphotype analysis

The morphotype analysis performed in PRIMER 6, was used to highlight the diversity of organisms present amongst the 559 cultured isolates. Applying an 80 % similarity cut off to the generated resemblance matrix identified a total of 95 distinct morphotypes. Representative isolates were selected for downstream genetic and chemical analysis and were assigned a unique alphanumeric code.

3.3.4 Genetic Analysis

3.3.4.1 16S rRNA gene analysis

Based on phylogenetic analyses of the 16S rRNA gene sequence fragments, bacterial isolates grouped into four clades, *Alphaproteobacteria, Gammaproteobacteria, Firmicutes* and *Actinobacteria,* Figure 3.1. These results are congruent with the

observations made in chapter one. Representatives of each bacterial order identified in the 16S rRNA gene metagenomic analysis of the *T. carbonaria* bee gut (Chapter 2) were successfully cultured and identified via Sanger dideoxynucleotide sequencing.

All three bees species examined were represented among the isolates identified, 22 of the bacteria isolated were from *T. carbonaria*, 18 from *A. australis* and 2 from *T. hockingsii*. The lower representation of *T. hockingsii* samples is likely due to only one *T. hockingsii* hive being sampled. Fifteen of the identified bacteria were sourced from the gut, 23 from the body and 4 from eggs/larvae, Table 3.6.

Comparison between the culture based (this Chapter) and non-cultured (Chapter 2) methodologies employed revealed one major difference, the absence of *Lactobacillus* strains obtained from the cultured methodology, despite their observed dominance in the stingless bee gut. This result is likely due to the aerobic incubation conditions used, whereas *Lactobacilli* are often cultured under anaerobic or microaerobic conditions. The decision to incubate agar plates in an aerobic environment was influenced by the results of the preliminary culturing experiments, and a desire to cultivate a diverse library of microorganisms and not only *Lactobacilli*. Furthermore, the large fermentations required to purify compounds down stream are technically challenging for anaerobic organisms. Despite the absence of *Lactobacillus* from the culture pool, a significant number of other Firmicutes, primarily *Bacilli,* were present.

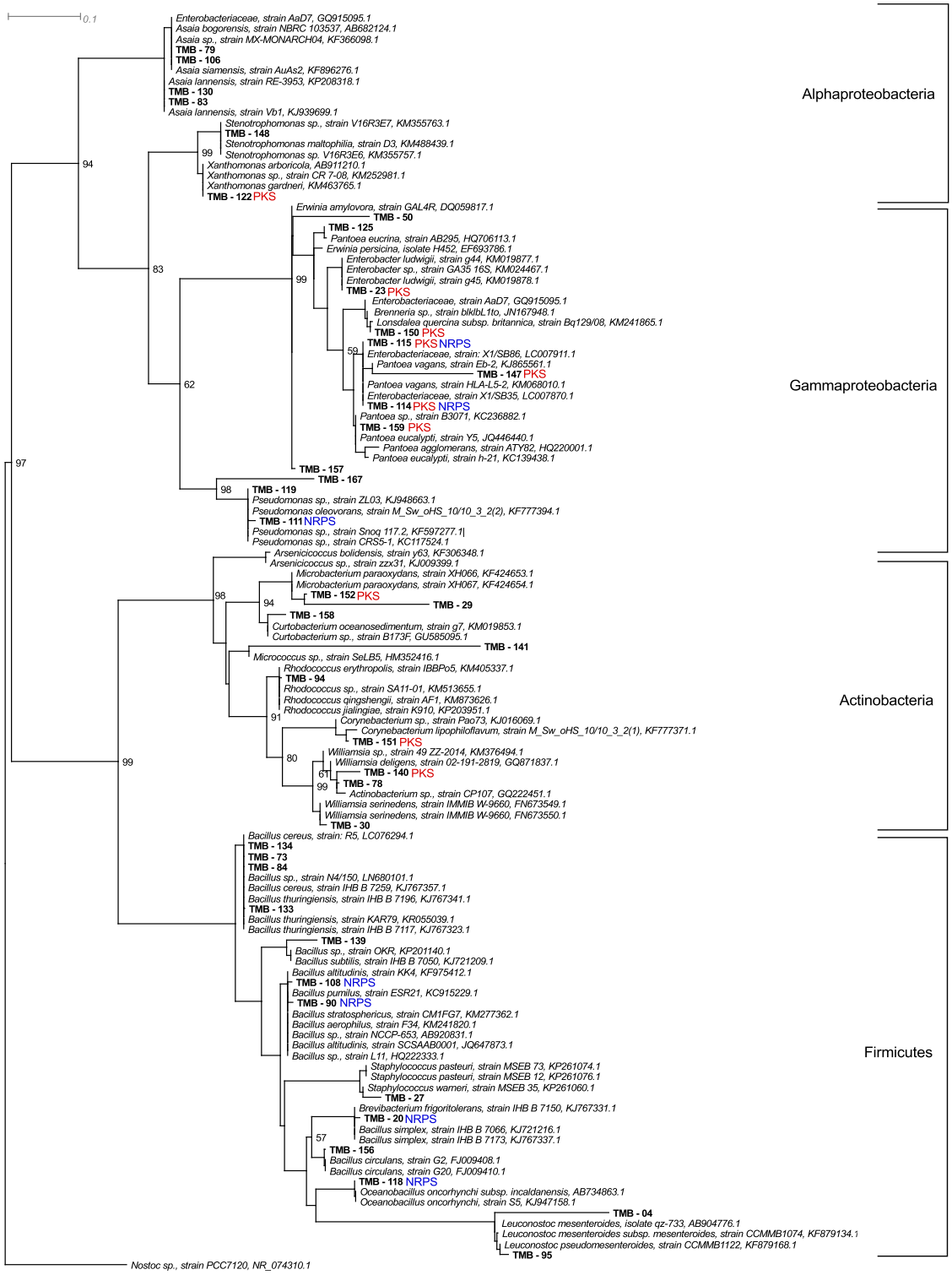

Figure 3.1. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences from bacterial isolates in this study and related reference sequences. PKS positive isolates are indicated in red and NRPS positive isolates are indicated in blue. The cyanobacterial *Nostoc* sp., PCC7120 16S rRNA gene sequence was used as the outgroup. Phylum level clustering is indicated on the right margin.

Table 3.6: Bacterial identification and screening for polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS) genes in bacterial isolates from three species of Australian stingless bee, *A. australis, T. hockingsi and T. carbonaria.*

TMB #	Origin species	Origin type *	PKS	NRPS	Closest BLAST match	Accession #	Identity %
04	*A. australis*	F, G	-	-	*Leuconostoc* sp.	KF879134.1	92
20	*T. carbonaria*	F, B	-	+	*Bacillus* sp.	KJ767337.1	100
23	*A. australis*	F, G	+	-	*Enterobacter* sp.	KM024467.1	100
27	*T. carbonaria*	F, B	-	-	*Staphylococcus* sp.	KP261076.1	97
29	*T. carbonaria*	C, B	-	-	*Microbacterium* sp.	KR906145.1	93
30	*T. carbonaria*	C, B	-	-	*Williamsia* sp.	FN673550.1	99
50	*A. australis*	M	-	-	*Erwinia* sp.	DQ059817.1	93
73	*A. australis*	F, G	-	-	*Bacillus* sp.	KJ767341.1	100
78	*T. carbonaria*	C, B	-	-	*Williamsia* sp.	GQ871837.1	99
79	*A. australis*	F, B	-	-	*Asaia* sp.	KF896276.1	100
83	*T. carbonaria*	F, G	-	-	*Asaia* sp.	KP208318.1	100
84	*T. carbonaria*	F, B	-	-	*Bacillus* sp.	KJ767341.1	100
90	*T. hockingsii*	F, B	-	+	*Bacillus* sp.	KF975412.1	100
94	*A. australis*	M	-	-	*Rhodococcus* sp.	KP203951.1	100
95	*A. australis*	F, G	-	-	*Leuconostoc* sp.	AB830328.1	100
106	*A. australis*	F, B	-	-	*Asaia* sp.	AB485745.1	99
108	*A. australis*	M	-	+	*Bacillus* sp.	HQ222333.1	99
111	*A. australis*	F, B	-	+	*Pseudomonas* sp.	KF777394.1	99
114	*T. carbonaria*	F, G	+	+	*Pantoea* sp.	KM068010.1	100
115	*T. carbonaria*	F, G	+	+	*Pantoea* sp.	KM068010.1	100
118	*A. australis*	C, B	-	+	*Oceanobacillus* sp.	KJ947158.1	99
119	*A. australis*	F, B	-	-	*Pseudomonas* sp.	KJ948663.1	100
122	*A. australis*	F, B	+	-	*Xanthomonas* sp.	KM463765.1	100

125	*T. carbonaria*	F, B	-	-	*Pantoea* sp.	KM068010.1	98
128	*T. carbonaria*	F, B	+	-	*Pantoea* sp.	KF202775.1	95
130	*T. carbonaria*	F, G	-	-	*Asaia* sp.	KP208318.1	100
133	*T. carbonaria*	F, B	-	-	*Bacillus* sp.	LN680101.1	99
134	*T. carbonaria*	F, B	-	-	*Bacillus* sp.	KJ767341.1	100
139	*T. carbonaria*	C, B	-	-	*Bacillus* sp.	JQ660687.1	98
140	*T. carbonaria*	C, B	+	-	*Williamsia* sp.	GQ871837.1	97
141	*T. carbonaria*	C, B	-	-	*Micrococcus* sp.	HM352416.1	98
147	*A. australis*	F, G	+	-	*Pantoea* sp.	KJ865561.1	90
48	*T. carbonaria*	M	-	-	*Stenotrophomonas* sp.	KM355763.1	100
150	*A. australis*	F, B	+	-	*Enterobacteriaceae* sp.	GQ915095.1	99
151	*A. australis*	M	+	-	*Corynebacterium* sp.	KJ016069.1	98
152	*T. hockingsii*	F, G	+	-	*Microbacterium* sp.	KF424654.1	99
157	*T. carbonaria*	F, G	-	-	*Erwinia* sp.	DQ059817.1	99
156	*A. australis*	C, B	-	-	*Bacillus* sp.	FJ009410.1	99
158	*T. carbonaria*	F, B	-	-	*Curtobacterium* sp.	GU585095.1	98
159	*T. carbonaria*	F, G	+	-	*Pantoea* sp.	KC236882.1	100
167	*A. australis*	F, G	-	-	*Pseudomonas* sp.	DQ910390.1	96

* F = forager, C = callow, M = mature egg, G = gut, B = body

Another factor contributing to the absence of *Lactobacilli* was the culture incubation temperature. In this study, 25°C was used as the incubation temperature, however, *Lactobacilli*-specific growth conditions are typically closer to 35°C (Reuter, 1985). The choice to incubate our cultures at 25°C was primarily based on a preliminary culturing experiment that produced a greater diversity of colony morphologies and also a greater number of colonies at 25 °C compared to plates cultured at 35°C. In addition, the 25 °C incubation conditions are more reflective of the isolate source environment, *T. carbonaria* bees have been observed to only exit the hive to forage when the external temperature is above 19 °C (Bartareau, 1996) and peak foraging activity was observed at 28 °C. The internal temperature of *T. carbonaria* hives are consistently around 26-28.5 °C. The lower growth temperature permitted a longer incubation period; suitable for slower growing organisms, without the agar plates drying out and resultant isolate death as well as reduced competition from faster growing organisms.

3.3.4.2 Bacterial Screening of PKS & NRPS genes by PCR

The historical success of PKS and NRPS derived natural products and their potential to produce a wide array of bioactive chemical structures makes them useful targets in the search for a new generation of bioactive compounds. Genetic screening of bacterial isolates for putative PKS and NRPS gene clusters revealed that 15 isolates, 36% of the total, were positive for PKS, NPRS or both genes with 24% of isolates PKS positive and 16% NRPS positive. These results are congruent with similar studies examining different environments, for instance; 24% of bacterial endophytes isolated from traditional Chinese medicine plants tested positive for either PKS or NRPS domains (Miller et al., 2012), 18% of sponge associated bacteria tested positive for PKS domains

(Zhang et al., 2009) and 14% of sponge associated tested positive for NRPS domains (Zhang et al., 2008). This result demonstrates that bacteria associated with the gut and cuticle of Australian stingless bees are promising targets for novel antimicrobial compound discovery.

A specific discussion of the PKS and NRPS positive isolates is presented below:

TMB -122 tested positive for a putative PKS gene and was most similar to *Xanthomonas gardneri* .The *Xanthomonads* are plant-associated bacteria known to cause diseases commonly known as spot, blight or canker. *Xanthomonas gardneri* is one of four *Xanthomonas* spp. that are causative agents of bacterial spot diseases in commercial crops (Jones et al., 1998, Ryan et al., 2011). The production of the toxin albicidin is believed to be biosynthesised by a hybrid, modular NRPS-PKS enzyme complex in *Xanthomonas albilineans* (Royer et al., 2004).

TMB - 150 tested positive for a putative PKS gene and was most similar to *Lonsdalea quercine* subsp. *britannica* one of the causative agents of drippy nut disease in oak trees also known as Oak canker. There have been no reports of *Lonsdalea* spp. containing PKS, however, a NRPS system is involved in the production of the red pigment rubrifacine in *Brenneria rubrifaciens* the causative agent of deep bark canker disease in English walnut trees (McClean and Kluepfel, 2009).

TMB - 23 tested positive for a putative PKS gene and was most similar to an *Enterobacter* sp. The next most similar organism, *Enterobacter ludwigii* is a clinically

relevant human pathogen, isolated from various human bodily fluids (Hoffmann et al., 2005). TMB-114 and TMB-115 both tested positive for putative PKS and NRPS genes and were most similar to two *Enterobacteriaceae* spp. isolates of insect origin (unpublished work by Hosokawa, T., and Fukatsu, T., Kyushu University, 2014)

Enterobacteriaceae are well known as human, plant and insect pathogens, in addition to being important symbionts (White and Torres, 2009, Petersen and Tisa, 2013). They have been shown to produce a number of toxic as well as antibiotic compounds via PKS, NRPS and hybrid mechanisms (Masschelein et al., 2013, Engel et al., 2014).

TMB -147 and TMB-159 tested positive for a putative PKS and were most similar to *Pantoea vagans* and *Pantoea eucalypti*, respectively. These strains were isolated and described in association with a bacterial blight disease of Eucalyptus trees in Uganda, Argentina and Uruguay (Brady et al., 2009). As is the case with other *Enterobacteriaceae, Pantoea* spp. have been isolated from a wide range of origins including plants, soil, water and clinical samples.

TMB -111 tested positive for a putative NPRS gene and was most similar to a cluster of *Pseudomonas* spp. The members of the genus *Pseudomonas* are found in diverse habitats and have been characterized as plant and insect pathogens as well as symbionts. The production of toxins and bioactive molecules is well documented from this genus. These features along with its utility as an expression system make it a powerful tool for

biotechnology (Gross and Loper, 2009, Rokni-Zadeh et al., 2011, Poblete-Castro et al., 2012).

TMB-118 tested positive for a putative NRPS gene and was most similar to two *Oceanobacillus* spp. As their name implies, they were first isolated from deep sea sediments as halotolerant, alkaliphilic bacteria (Lu et al., 2001). Novel type strains have now been identified from other deep sea sites, salt crusts, fermented and salted foods, insect eggs and algal mats (Romano et al., 2006, Raats and Halpern, 2007, Namwong et al., 2009, Amoozegar et al., 2014). These organisms have evolved to tolerate extreme osmotic pressure and would be capable of surviving within the gut of stingless bees where the high sugar content exerts osmotic pressure on fluid filled bacterial cells. To date no PKS or NRPS gene have been described from this genus.

TMB-140 tested positive for a putative PKS gene and was most similar to an *Actinobacterium* sp. isolated from a venomous cone shell snail (Peraud et al., 2009) and a number of *Williamsia* spp. (GQ871837.1 & KM376494.1) isolated from soils contaminated with oil and heavy metals (Yassin et al., 2007). The actinobacteria are well known and researched for their ability to produce toxins and bioactive compounds. Much of the modern research in actinobacteria is focused on developing the potential of silent or cryptic gene clusters, many of which are PKS and NRPS or hybrid clusters.

TMB-20, TMB-90 and TMB-108 tested positive for a putative NRPS gene and were most similar to *Brevibacterium frigoritolerans* and two strains of *Bacillus*. Species of

the genus *Bacillus* have seen renewed interest in the past decade with the identification of large number of PKS and NRPS clusters from numerous *Bacillus* spp. (Arguelles-Arias et al., 2009, Fickers, 2012). Furthermore *Bacillus simplex*, a human pathogen, has been shown to produce a heat stable emetic toxin (Taylor et al., 2005).

TMB-151 tested positive for a putative PKS gene and was most similar to two species of *Corynebacterium*, well known as human pathogens (Murphy, 1996, Funke et al., 1997) *Corynebacterium* spp. are also widely utilised for the production of enzymes and other compounds used in industry, in particular L-amino acids (Kalinowski et al., 2003). The presence of either PKS or NPRS gene clusters in *Corynebacterium* spp. is rare and typically secondary metabolite biosynthesis is driven by isoprenoid and terpenoid gene clusters (Doroghazi and Metcalf, 2013).

TMB-152 tested positive for a putative PKS gene and was most similar to two strains of *Microbacterium. Microbacterium* spp. can generally be found in soil, seawater and food.,A sponge associated, PKS positive, *Microbacterium* sp. has been isolated that exhibited antimicrobial compound production (Graça et al., 2015).

The PKS and NRPS positive isolates described in this section carry a common theme as the causative agents of plant bacterial disease. In addition to having diverse origins, functions and pathogenicity to humans, animals and insects. Many of these organisms can be isolated from the soil, humans and surface of plants. As such they are interesting

as a possible source of bioactive compounds and they may also be symbionts of the insect gut or cuticle under examination.

3.3.4.3 18S rRNA gene analysis

18S rRNA gene PCR products were successfully obtained from 53 fungal isolates. Results from the analysis of 18S rRNA gene PCR product sequences using the NCBI BLASTn algorithm are presented in Table 3.7; when two different genera were returned as the top hits both were listed in the table. All three bees species examined were represented among the isolates identified, 28 of the fungi isolated were from *T. carbonaria*, 25 from *A. australis* and 2 from *T. hockingsii*. The lower representation of *T. hockingsii* samples is likely due to only one *T. hockingsii* hive being sampled. Twenty-nine of the identified bacteria were sourced from the gut, 24 from the body and none from eggs/larvae.

Based on phylogenetic analyses of the 18S rRNA gene sequence fragments, bacterial isolates grouped into 9 classes, Saccharomycetes, Tremellomycetes, Eurotiomycetes, Urediniomycetes, Microbotryomycetes, Ustilaginomycetes, Agaricostilbomycetes and Exobasidiomycetes, Figure 3.2. The majority of isolates, 54%, were classified, as Saccharomycetes with Tremellomycetes, with 17 %, Urediniomycetes and Eurotiomycetes, with 15 % combined, making up the majority of the reminder. This is the first reported collection and taxonomic classification of fungal isolates associated with Australian native stingless bees though a number or studies have identified yeast as

being the dominant fungal isolates in stingless bees from other geographic regions (Teixeira et al., 2003) (Rosa et al., 2003).

3.3.4.4 Fungal screening of PKS & NRPS genes by PCR

Of the 53 fungal isolates examined 45% tested positive for PKS, NRPS or both genes, with 28% positive for the PKS gene cluster and 17% positive for the NRPS gene cluster. This figure was similar to the percentage obtained in the bacterial PKS and NRPS screening methodology and were congruent with studies examining different environments, e.g. Miller and colleagues isolated fungal entophytes from traditional medicine plants with 17% of samples NRPS positive (Miller et al., 2012). However reported figures for PKS positive fungi have been much higher, such as 63% PKS positive fungi from marine environments (Mayer et al., 2007) and 58% PKS positive fungi from the endophytes examined by Miller, et al, 2012. A detailed discussion of each positive isolate is included below.

Table 3.7. Identification and screening of fungal isolates for polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes from three species of Australian stingless bee, *A. australis, T. hockingsi and T. carbonaria.*

TMF#	Origin species	Origin type *	PKS	NRPS	Closest Blast match	Accession #	Identity (%)
16	*T. carbonaria*	F, G	-	-	*Cryptococcus* sp.	KF036626.1	100
21	*T. carbonaria*	F, G	-	-	*Cryptococcus* sp.	JX104120.1	100
41	*T. carbonaria*	F, G	-	-	*Cladosporium* sp.	KM582661.1	100
45	*T. carbonaria*	F, B	-	-	*Penicillium* sp.	KM582674.1	100
					Eupenicillium sp.	KM582670.1	100
46	*T. carbonaria*	C, B	-	-	*Penicillium* sp.	KM222259.1	100
47	*A. australis*	F, G	-	-	*Zygosaccharomyces* sp.	AB259006.1	99
61	*T. carbonaria*	F, G	-	+	*Cryptococcus* sp.	AB085796.1	98
63	*T. carbonaria*	F, G	+	+	*Cryptococcus* sp.	KF036657.1	97
80	*T. carbonaria*	F, G	+	-	*Rhodotorula* sp.	KJ708435.1	92
81	*T. carbonaria*	F, B	+	-	*Sporobolomyces* sp.	KJ534285.1	99
82	*T. carbonaria*	C, B	-	-	*Cryptococcus* sp.	KF036630.1	98
85	*T. hockingsii*	F, B	-	+	*Ustilago* sp.	X62396.1	99
					Melanopsichium sp.	HG529728.1	99
86	*T. carbonaria*	F, G	-	+	*Quambalaria* sp.	KF706440.1	97
					Volvocisporium sp.	DQ875384.1	97
87	*A. australis*	F, G	+	-	*Metschnikowia* sp.	AB023473.1	98
					Candida sp.	AY452054.1	98
88	*A. australis*	F, B	-	-	*Candida* sp.	AY520164.1	95
					Metschnikowia sp.	DQ437075.1	95
91	*A. australis*	F, G	-	-	*Metschnikowia* sp.	JQ698902.1	96

93	*T. carbonaria*	F, G	-	-	*Syzygospora* sp.	JN043559.1	83
					Cryptococcus sp.	KF036621.1	82
96	*A. australis*	F, G	-	-	*Candida* sp.	AY520164.1	99
					Metschnikowia sp.	DQ437075.1	98
99	*A. australis*	F, G	-	-	*Metschnikowia* sp.	DQ437075.1	99
					Candida sp.	AJ508272.1	99
100	*A. australis*	F, G	-	-	*Metschnikowia* sp.	DQ437075.1	99
					Candida sp.	AJ508272.1	99
101	*A. australis*	F, B	+	+	*Metschnikowia* sp.	AB023473.1	98
					Candida sp.	AY452054.1	98
102	*A. australis*	F, B	-	-	*Metschnikowia* sp.	DQ437075.1	94
					Candida sp.	AJ508272.1	94
103	*A. australis*	F, B	-	-	*Metschnikowia* sp.	DQ437075.1	96
					Candida sp.	AJ508272.1	96
104	*A. australis*	F, B	-	-	*Metschnikowia* sp.	DQ437075.1	99
					Candida sp.	AJ508272.1	99
105	*A. australis*	C, B	-	-	*Sterigmatomyces*	DQ985957.1	100
107	*T. carbonaria*	F, B	-	-	*Candida* sp.	AB628062.1	95
109	*A. australis*	C, B	-	-	*Cryptococcus* sp.	KF036651.1	100
110	*T. carbonaria*	F, G	-	-	*Rhodotorula* sp.	KM222308.1	97
112	*T. carbonaria*	F, G	-	-	*Metschnikowia* sp.	AY611606.1	100
					Candida sp.	AY611608.1	100
113	*A. australis*	F, B	-	-	*Metschnikowia* sp.	DQ437075.1	97
					Candida sp.	AJ508272.1	97
116	*A. australis*	F, G	-	-	*Metschnikowia* sp.	DQ266429.1	94
					Candida sp.	JX515974.1	93
121	*A. australis*	F, B	-	-	*Metschnikowia* sp.	DQ437075.1	92

123	*T. carbonaria*	C, B	+	-	*Leucosporidium* sp.	KF036682.1	97
					Sporobolomyces sp.	AB030319.1	97
126	*T. carbonaria*	F, B	-	-	*Metschnikowia* sp.	AY611608.1	100
					Candida sp.	AY611606.1	100
127	*T. carbonaria*	F, B	-	-	*Candida* sp.	AB628062.1	99
					Starmerella sp.	JQ698924.1	98
129	*T. carbonaria*	F, B	+	-	*Metschnikowia* sp.	AY611608.1	100
					Candida sp.	AY611606.1	100
131	*T. carbonaria*	F, G	-	-	*Rhodotorula* sp.	KM222308.1	99
132	*A. australis*	F, B	+	-	*Metschnikowia* sp.	DQ437075.1	98
					Candida sp.	AJ508272.1	98
135	*A. australis*	F, G	-	-	*Sporidiobolus* sp.	AB021697.1	92
					Leucosporidium sp.	KF036682.1	91
136	*T. hockingsii*	F, B	+	-	*Ustilago* sp.	X62396.1	98
					Melanopsichium sp.	HG529728.1	98
137	*A. australis*	C, B	-	-	*Penicillium* sp.	KM582674.1	95
138	*T. carbonaria*	F, G	+	-	*Syzygospora* sp.	JN043560.1	99
					Cryptococcus sp.	KF036621.1	99
142	*T. carbonaria*	C, G	-	-	*Zygosaccharomyces* sp.	CU928181.1	98
143	*A. australis*	F, G	-	+	*Candida* sp.	AY520279.1	99
					Clavispora sp.	KM516766.1	98
149	*A. australis*	F, G	-	+	*Metschnikowia* sp.	AB023473.1	99
					Candida sp.	AY452054.1	99
154	*T. carbonaria*	F, G	-	-	*Metschnikowia* sp.	AY611608.1	100
					Candida sp.	AY611606.1	100
155	*A. australis*	F, G	-	-	*Metschnikowia* sp.	DQ437075.1	98
					Candida sp.	AY520164.1	99
160	*T. carbonaria*	F, G	+	-	*Candida* sp.	AY611608.1	85

					Metschnikowia sp.	AY611606.1	85
161	*A. australis*	F, G	+	-	*Metschnikowia* sp.	DQ437075.1	100
					Candida sp.	AJ508272.1	100
162	*A. australis*	F, G	+	-	*Metschnikowia* sp.	DQ437075.1	99
					Candida sp.	AY520164.1	99
164	*A. australis*	F, G	+	-	*Metschnikowia* sp.	DQ437075.1	99
					Candida sp.	AJ508272.1	99
165	*T. carbonaria*	F, B	+	+	*Metschnikowia* sp.	AY611606.1	99
					Candida sp.	AY611608.1	99
166	*T. carbonaria*	F, B	+	+	*Metschnikowia* sp.	AY611606.1	100
					Candida sp.	AY611608.1	100

* * F = forager, C = callow, G = gut, B = body

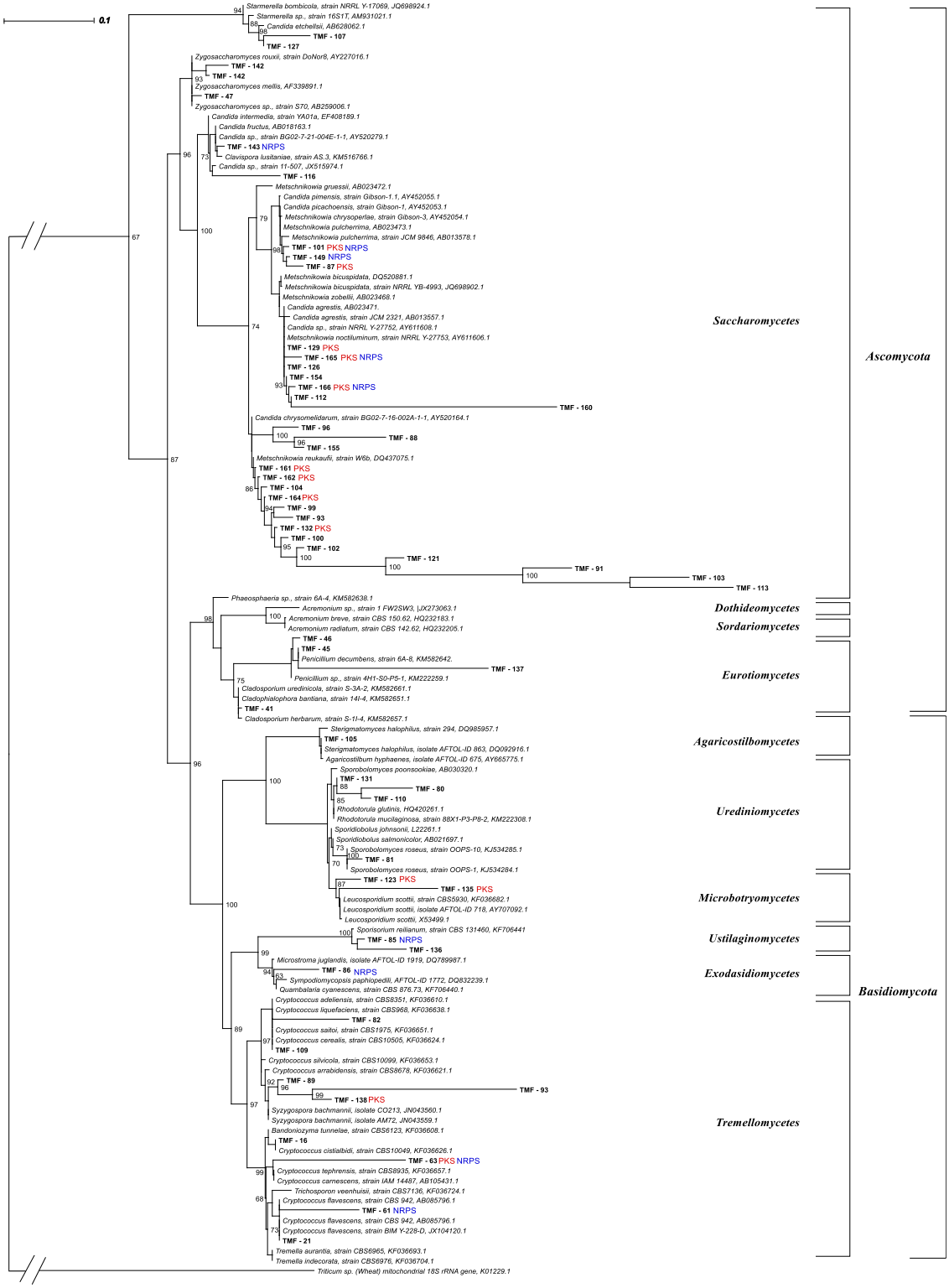

Figure 3.2. Maximum likelihood phylogenetic tree of 18S rRNA gene sequences from fungal isolates in this study and related reference sequences. PKS positive isolates are indicated in red and NRPS positive isolates are indicated in blue. The *Triticum* sp. (K01229.1) 18S rRNA gene sequence was used as the outgroup. Fungal divisions are labelled on the outer bars and fungal classes are labelled on the inner bars.

TMF - 81 was most similar to *Sporobolomyces roseus* (KJ534285.1) and tested positive for a putative PKS gene. *S. roseus* is one of the most common yeasts found on the surface of leaves in temperate regions (Nakase, 2000) and is a unicellular red yeast species. A recent survey of Basidiomycota genomes failed to detect any PKS genes in *S. roseus,* however PKS genes were common in the related Agaricomycotina and Ustilaginomycotina (Lackner et al., 2012).

TMF – 123 tested positive for a putative PKS gene and was most similar to *Leucosporidium scottii*. *L. scottii* is the heterobasidiomycetous stage of *Candida scottii* and has previously been isolated from Antarctic (Vishniac, 2006) and forest soils (Slávikova and Vadkertiová, 2000). No PKS genes have, as yet, been identified for *L. scottii* and their presence in related organism has not been reported (Lackner et al., 2012).

TMF – 85 tested positive for a putative NRPS gene and TMF – 136 tested positive for a putative PKS gene. Both isolates were most similar to a *Sporisorium* sp. (Feldbrügge et al., 2013) a parasitic plant pathogen, commonly known as smut fungi, that affects commercial crops including maize and sorghum. A related species *Sporisorium scitamineum* has been shown to possess both PKS and NRPS genes (Que et al., 2014). Members of the Ustilaginomycetes have potential as producers of secondary metabolites and enzymes as well as novel protein expression systems (Feldbrügge et al., 2013).

TMF – 86 tested positive for a putative NRPS gene and was most similar to *Sympodiomycopsis paphiopedili* and *Quambalaria cyanescens*. Both these organism have been reported as plant pathogens specifically affecting *Eucalyptus* and *Corymbia* species in Australia and South Africa (de Beer et al., 2006). The production of an antifungal glycolipid active against a wide variety of yeasts and yeast like fungi was reported for *S. paphiopedili* (Kulakovskaya et al., 2004) and crude extracts of *Quambalaria cyanescens* have been shown to exhibit antimicrobial activity (Padhi and Tayung, 2013). To date neither PKS nor NRPS gens have been demonstrated as being present in these genera.

TMF – 138 tested positive for a putative PKS genes and was most similar *Syzygospora bachmannii* (JN043560.1). A member of the jelly fungi *S. bachmannii* is a lichen parasite reported in a number of European countries including Portugal, Finland, Poland and the Czech Republic (Van den Boom and Giralt, 1996, Kocourková, 2000, Matwiejuk and Bohdan, 2011). There are no previous reports of PKS or NRPS genes in *Syzygospora* species or any antimicrobial activity.

TMF – 101,TMF – 165, TMF – 163 and TMF – 166 tested positive for putative PKS and NRPS genes. TMF – 149 tested positive for a putative NRPS gene. TMF – 87 and TMF – 129 tested positive for a putative PKS gene. These isolates were most similar to a number of *Candida* spp. and *Metschnikowia* spp. including *Candida picachoensis* and *Metschnikowia chrysoperlae*; novel yeast species isolated from the gut and eggs of *Chrysoperla* sp*.,* a green lacewing insect, collected in Arizona, USA (Suh et al., 2004). Similar yeasts have also been found associated with beetles (Suh et al., 2008). To date

there are no reports of PKS or NRPS genes in these yeast species. The association of these yeasts with other insect guts makes these isolates interesting candidates to carry through to the next screening stage.

TMF – 161, TMF – 162, TMF – 164 and TMF – 132 all tested positive for a putative PKS gene and were most similar to *Metschnikowia reukaufii* and *Candida chrysomelidarum*. *Metschnikowia reukaufii* is a flower inhabiting yeast that has also been isolated from insects and marine environments (Eisikowitch et al., 1990, Brysch-Herzberg, 2004, Li et al., 2009). *Candida chrysomelidarum* is a novel yeast species also isolated from *Chrysoperla* sp., green lacewing insect gut (Suh et al., 2008).

3.3.5 Chemical analysis – bacterial and fungal

Statistical treatment of the LC-MS analysis of 30 bacterial isolates, analysed in 2014, produced a data set that was clustered into 6 groups, labelled BG-1 to BG-6, using a cut off of 47% similarity, Figure 3.3. The second batch of 12 bacterial isolates analysed by LC-MS in 2015 clustered into an additional 6 groups, labelled BG-7 to BG-12, using a cut off of 37 % similarity, Figure 3.4. In addition, the dendrograms generated from the cluster analysis were overlaid with sample taxonomy at the division level and PCR screening results acquired from the genetic analyses, Figures 3.5 and 3.6.

Statistical treatment of the LC-MS analysis of 40 fungal isolates, run in 2014, produced a data set that was clustered into 11 groups, labelled FG-1 to FG-11 using a cut off of 57% similarity, Figure 3.7. The second block of 13 fungal isolates analysed by LC-MS in 2015 clustered into an additional 6 groups, labelled FG-12 to FG-17, using a cut off of 43 % similarity, Figure 3.8. In addition the dendrograms generated from the cluster analysis were overlaid with sample taxonomy at the division level and PCR screening results acquired from the genetic analyses, Figures 3.9 and 3.10.

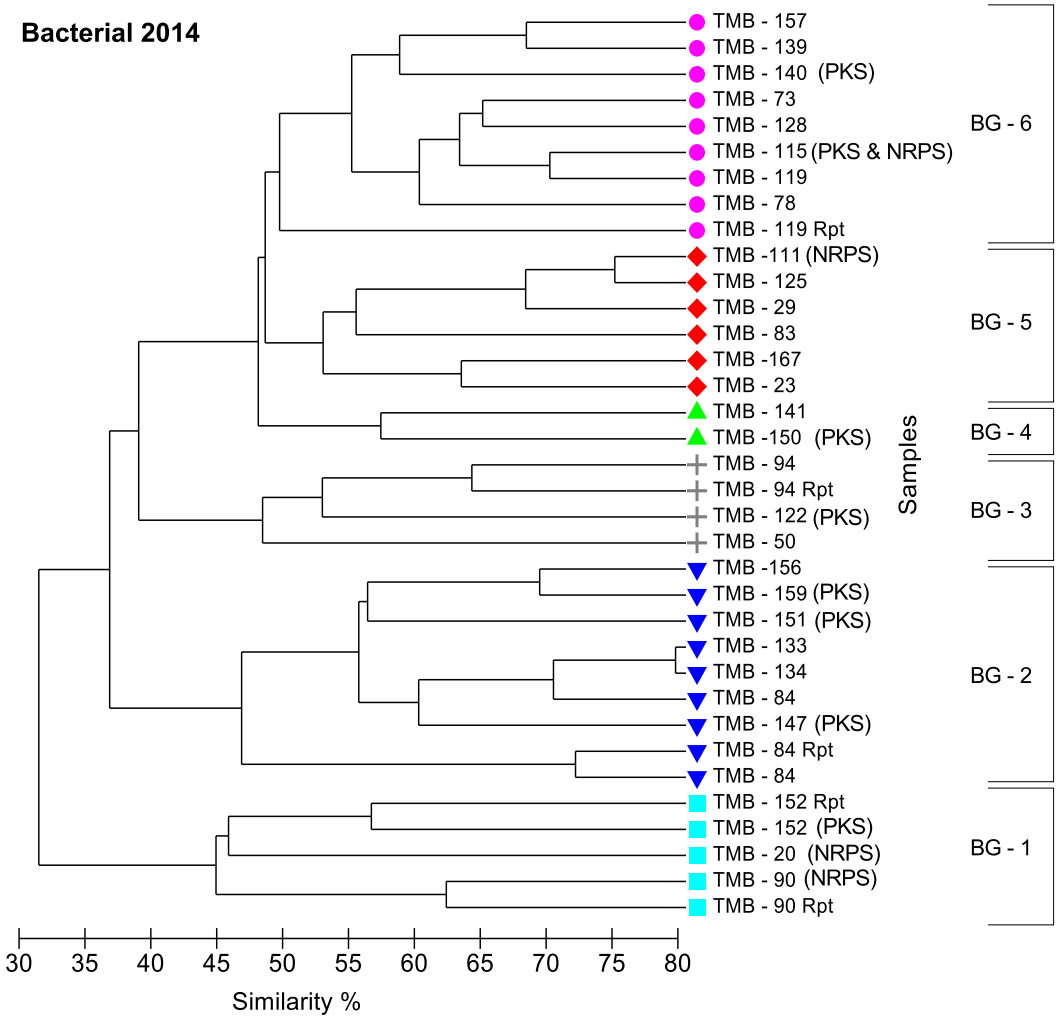

Figure 3.3. Dendrogram of chemical similarity generated from mass spectral data for 30 bacterial isolates from Australian stingless bees. Raw LC-MS data was processed using MZmine2 and the resultant matrix analysed using Primer 6+. Six groups BG-1 through BG-6 were assigned at 47 % similarity. PKS and NRPS positive isolates are indicated in brackets. Repeat LC-MS analysis of the same sample run on different days indicated by the suffix "Rpt".

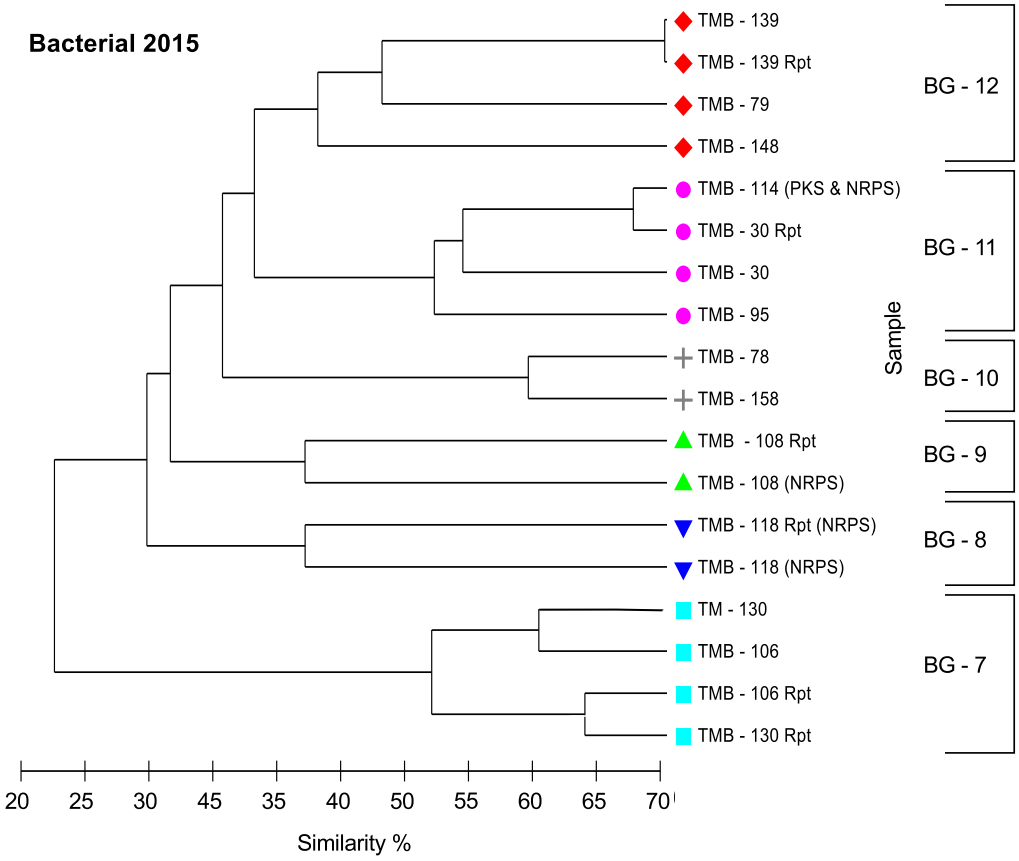

Figure 3.4. Dendrogram of chemical similarity generated from mass spectral data for 12 bacterial isolates from Australian stingless bees, 2015. Raw LC-MS data was processed using MZmine2 and the resultant matrix analysed using Primer 6+. Six groups BG-7 through BG-12 were assigned at 37 % similarity. PKS and NRPS positive isolates are indicated in brackets. Repeat LC-MS analysis of the same sample run on different days indicated by the suffix "Rpt".

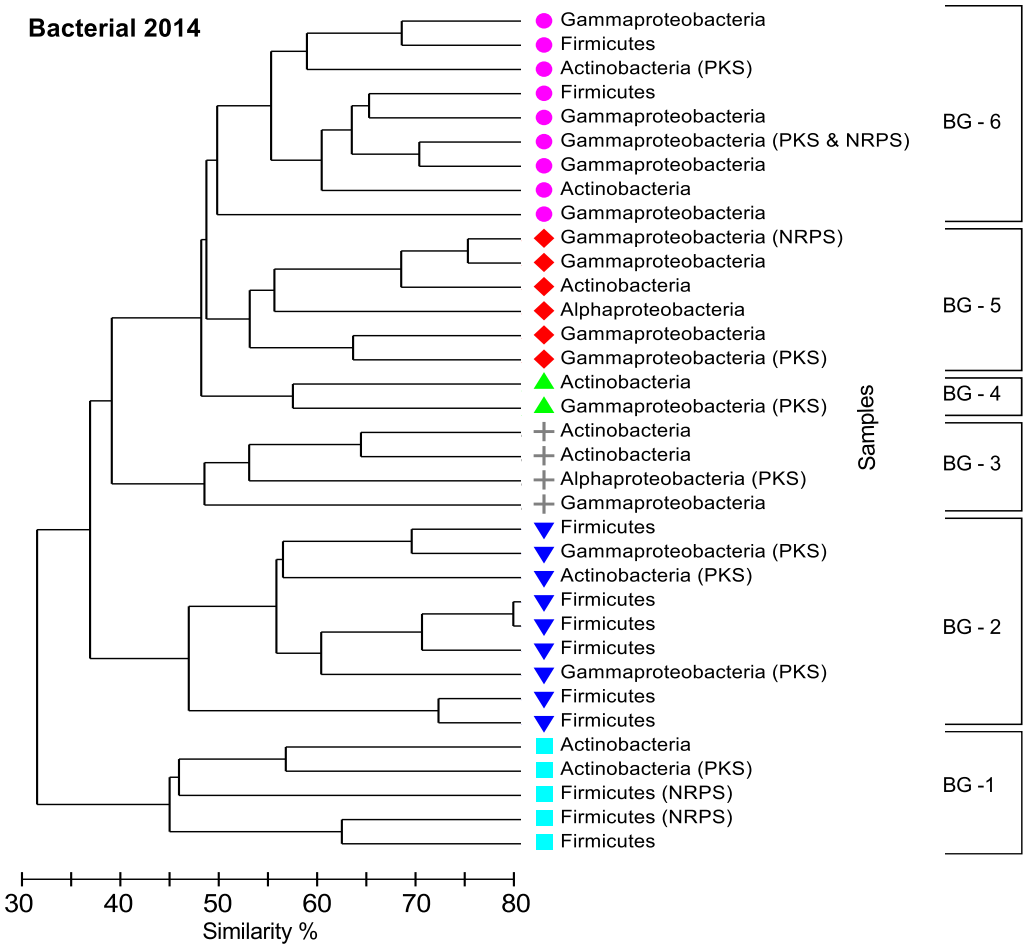

Figure 3.5. Dendrogram of chemical similarity generated from mass spectral data for 30 bacterial isolates from Australian stingless bees, labelled with isolate taxonomy at the division level. Raw LC-MS data was processed using MZmine2 and the resultant matrix analysed using Primer 6+. Six groups BG-1 through BG-6 were assigned at 47 % similarity. PKS and NRPS positive isolates are indicated in brackets.

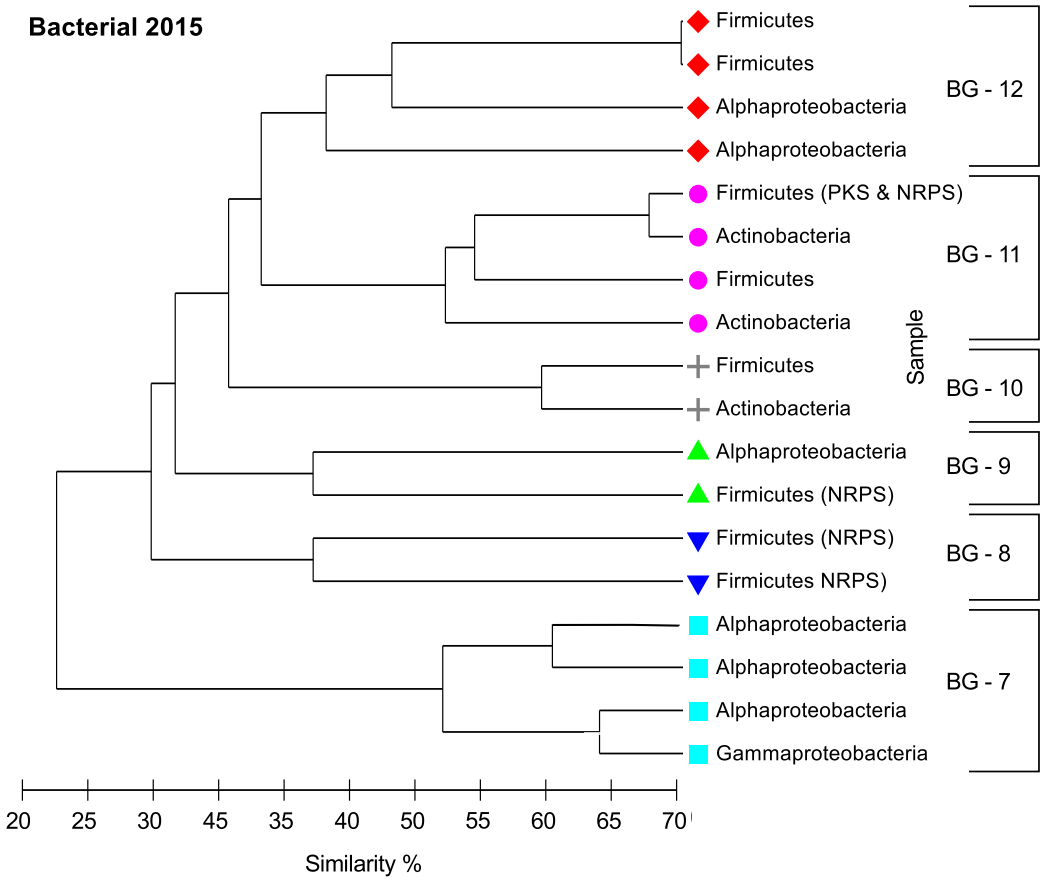

Figure 3.6. Dendrogram of chemical similarity generated from mass spectral data for 12 bacterial isolates from Australian stingless bees, 2015, labelled with isolate taxonomy at the division level. Raw LC-MS data was processed using MZmine2 and the resultant matrix analysed using Primer 6+. Six groups BG-7 through BG-12 were assigned at 37% similarity. PKS and NRPS positive isolates are indicated in brackets.

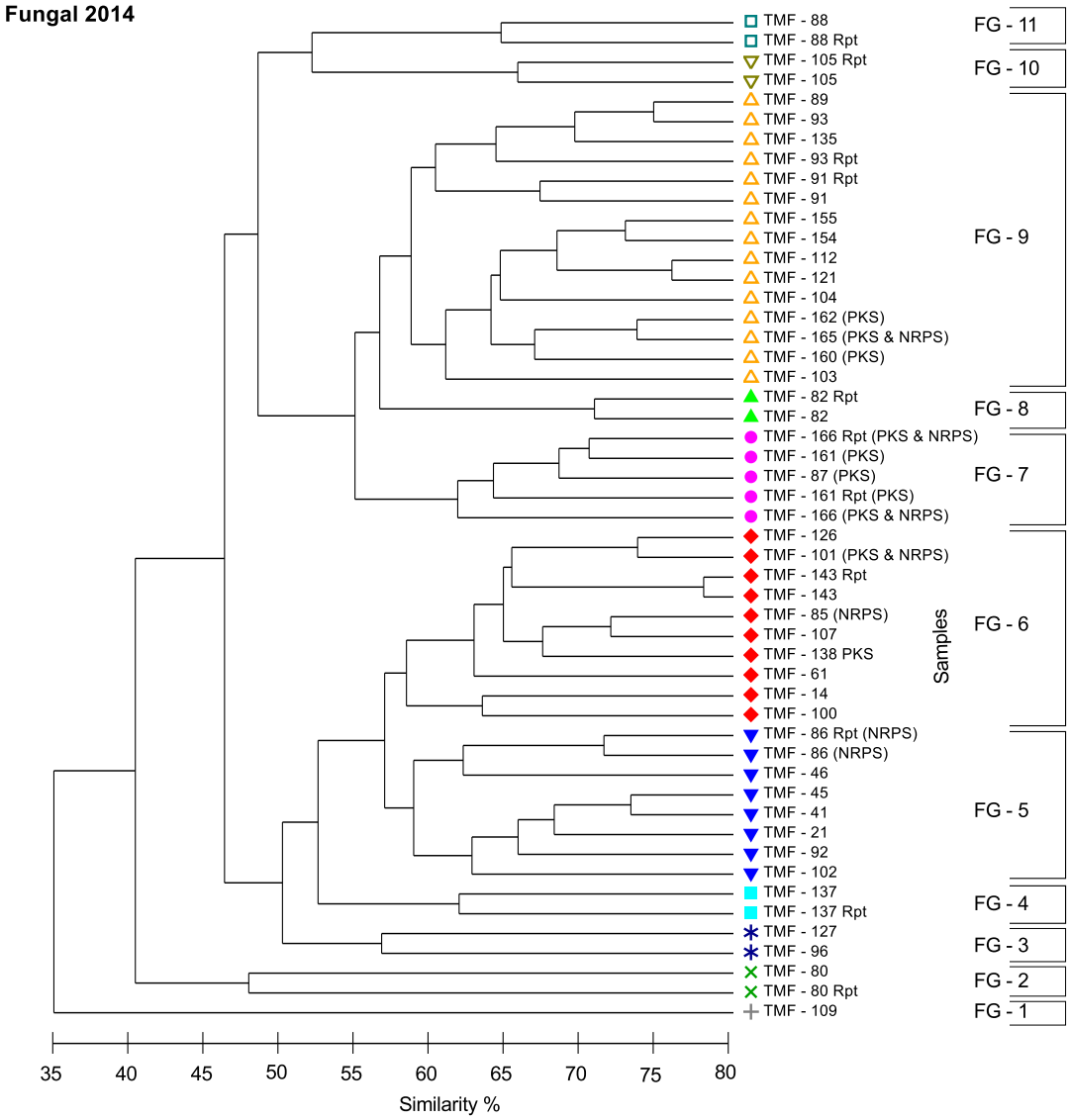

Figure 3.7. Dendrogram of chemical similarity generated from mass spectral data for 40 fungal isolates from Australian stingless bees. Raw LC-MS data was processed using MZmine2 and the resultant matrix analysed using Primer 6+. Eleven groups FG-1 through FG-11 were assigned at 57 % similarity. PKS and NRPS positive isolates are indicated in brackets. Repeat LC-MS analysis of the same sample run on different days indicated by the suffix "Rpt".

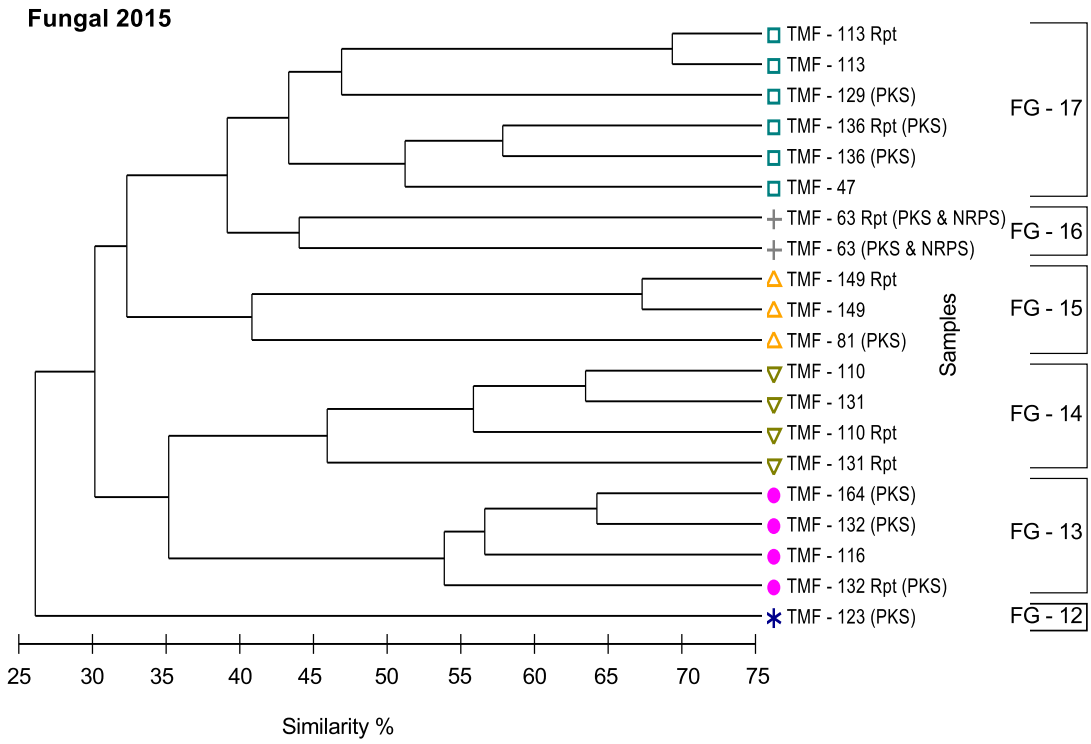

Figure 3.8. Dendrogram of chemical similarity generated from mass spectral data for 13 fungal isolates from Australian stingless bees, 2015. Raw LC-MS data was processed using MZmine2 and the resultant matrix analysed using Primer 6+. Six groups FG-12 through FG-17 were assigned at 43% similarity. PKS and NRPS positive isolates are indicated in brackets. Repeat LC-MS analysis of the same sample run on different days indicated by the suffix "Rpt".

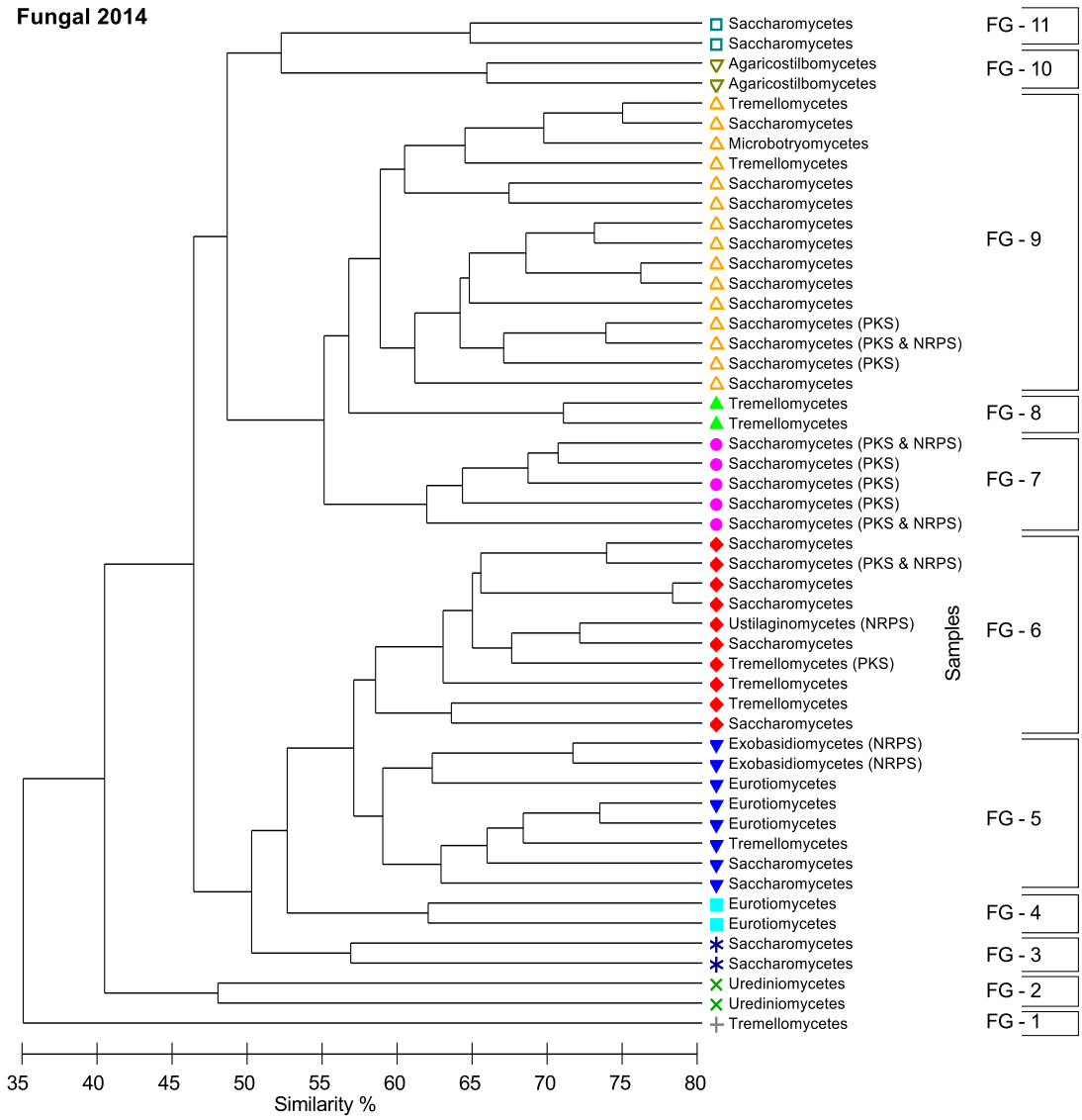

Figure 3.9. Dendrogram of chemical similarity generated from mass spectral data for 40 fungal isolates from Australian stingless bees, labelled with isolate taxonomy at the division level. Raw LC-MS data was processed using MZmine2 and the resultant matrix analysed using Primer 6+. Eleven groups FG-1 through FG-11 were assigned at 57% similarity. PKS and NRPS positive isolates are indicated in brackets.

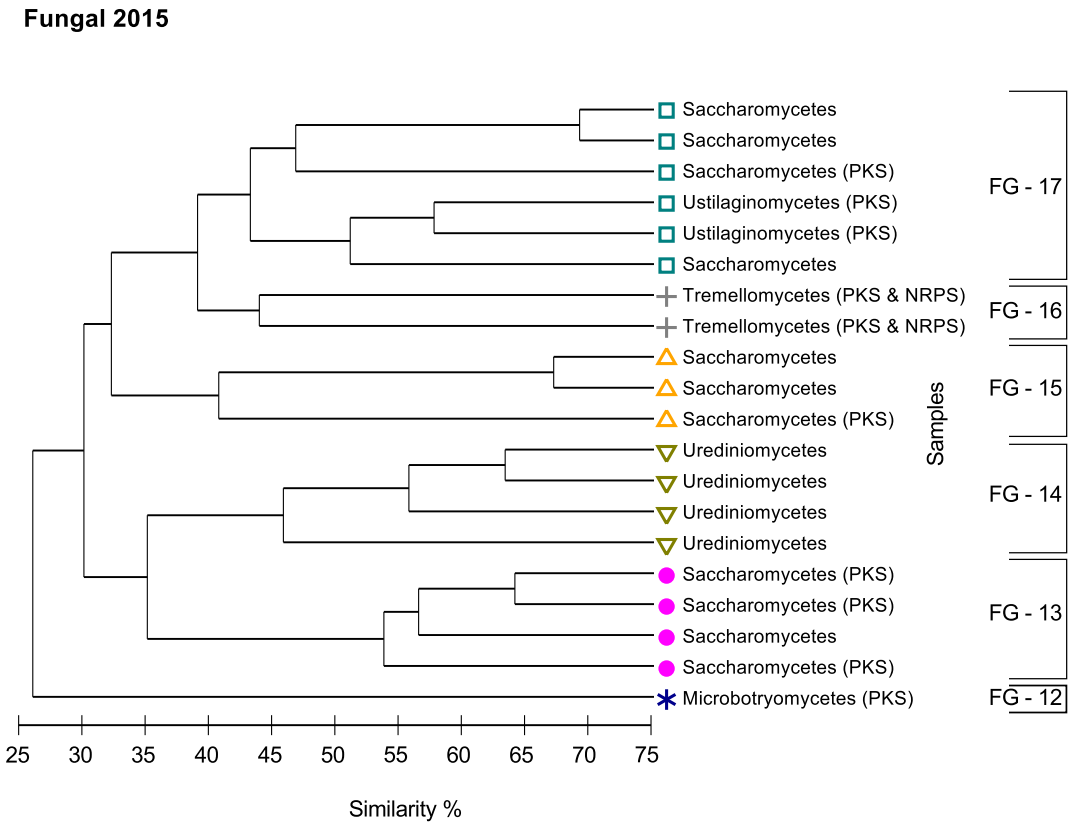

Figure 3.10. Dendrogram of chemical similarity generated from mass spectral data for 13 fungal isolates from Australian stingless bees, 2015, labelled with isolate taxonomy at the division level. Raw LC-MS data was processed using MZmine2 and the resultant matrix analysed using Primer 6+. Six groups FG-12 through FG-17 were assigned at 43% similarity. PKS and NRPS positive isolates are indicated in brackets.

Observation of the repeated LC-MS runs from the both bacterial and fungal isolates, Figures 3.3, 3.4, 3.7, & 3.8, demonstrates that the repeated analyses group together with their respective original isolate LC-MS run. For example in Figure 6A isolate TMF-136 groups together with isolate TMF-136 Rpt. This observation gives us confidence in both the LC-MS method and the robustness of the statistical analysis of the data. The percentage similarity cut off used in each instance were selected based upon the grouping present in each specific dendrogram.

Examination of the bacterial dendrograms overlaid with taxonomy data, Figures 3.5 and 3.6, reveals trends between the clustering of isolates based on their chemical similarity and the taxonomy of the isolates. For example, in Figure 3.5, the majority of the Firmicutes are clustered in the groups BG-1 and BG-2 and similarly the Gammaproteobacteria are clustered in groups BG-5 and BG-6. In addition, the Actinobacteria are scattered through each of the of the bacterial groups defined in Figure 3.5 as are the small number of Alphaproteobacteria present. However, there are exceptions to each of these generalisations, for instance the isolates TMB -147 and TMB -159 are Gammaproteobacteria that group within the Firmicutes dominated group BG-2 and isolates TMB -139 and TMB - 73 are Gammaproteobacteria that are group within the Firmicutes dominated group BG-6. Theses exception to the general grouping trends may be the result of a small number of dominate compounds in the chemical profile causing these isolates to group away from other taxonomically similar isolates. The isolates in Figures 3.6 and 3.7 show a less distinct taxonomy effect to the groupings with either Alphaproteobacteria or Firmicutes present in most of the groups, however this may simply be the result of the smaller sample cohort.

In Figures 3.7 and 3.8 the majority of the Saccharomycetes are clustered in groups FG-6, FG-7 and FG-9, the Eurotiomycetes are found primarily in FG-4 and FG-5 whilst the relatively small number of Tremellomycetes are found throughout the dendrogram. Again, there were exceptions to these observed trends such as the Saccharomycetes that group separately in FG-3 and FG-11. The smaller set of fungal isolates in Figures 3.9 and 3.10 were dominated by the Saccharomycetes interspersed with two distinct groups, the Tremellomycetes in FG-16 and the Urediniomycetes in FG-14.

Similarities in chemical profile between isolates with similar taxonomy may be the result of either their primary or secondary metabolites. The grouping of the isolates based on their taxonomy is to be expected as taxonomically similar microorganisms would have similar common ancestry and as such similar primary metabolite profiles. The differences in similarity between taxonomically similar isolates may be the result of differences in secondary metabolite profile or fundamental differences in their primary metabolites, such as the lipid bilayer. It is not possible to distinguish between primary and secondary metabolites in this analysis as the crude extracts contain both elements. It should also be noted that the power of this LC-MS analysis lies in the creation of a specific extract fingerprint for each isolate that can be statistically assessed for correlations. Determination of chemical structures from the extract LC-MS traces is not possible by measuring the *m/z* values of protonated molecules alone (Kind, T and Fiehn, O., 2007).

3.3.6 Dereplication of isolates

Using the combined genetic and chemical analysis results presented in Figures 3.3, 3.4, 3.7 & 3.8, the pool of 94 isolates was reduced to produce a short list of 46 isolates for further downstream analyses, Table 3.8. These 46 isolates were selected to maximise chemical diversity and biosynthetic potential. All isolates containing PKS or NPRS genes were included, as these genes are indicative of biosynthetic potential. Following on, a single representative from each bacterial or fungal group that did not include a PKS or NRPS-containing member was also included.

Looking at the shortlist of organisms created in this chapter versus their contributing factor we can see that the presence of putative PKS and NRPS gene clusters is the dominant deciding factor. Out of the 46 isolates in the subset 76% are included due to their biosynthetic potential and only 24% are included due to being chemically different from the total pool. However, without conducting the chemical profiling section of this work we would not know if we had included the majority of the chemical diversity present.

Table 3.8. List of isolates selected for bioactivity testing.

Bacterial Group	Taxonomy	Identifier	PKS	NRPS
BG-1	*Bacillus* sp.	TMB - 90	-	+
BG-1	*Bacillus* sp.	TMB - 20	-	+
BG-1	*Microbacterium* sp.	TMB - 152	+	-
BG-2	*Pantoea* sp.	TMB - 147	+	-
BG-2	*Corynebacterium* sp.	TMB - 151	+	-
BG-2	*Pantoea* sp.	TMB - 159	+	-
BG-3	*Xanthomonas* sp.	TMB - 122	+	-
BG-4	*Lonsdalea* sp.	TMB - 150	+	-
BG-5	*Pseudomonas* sp.	TMB - 111	-	+
BG-5	*Enterobacter* sp.	TMB - 23	+	-
BG-6	*Pantoea* sp.	TMB - 115	+	-
BG-6	*Williamsia* sp.	TMB - 140	+	-
BG-7	*Asaia* sp.	TMB - 106	-	-
BG-8	*Oceanobacillus* sp.	TMB - 118	-	+
BG-9	*Bacillus* sp.	TMB - 108	-	+
BG-10	*Williamsia* sp.	TMB - 158	-	-
BG-11	*Leuconostoc* sp.	TMB - 114	+	+
BG-12	*Stenotrophomonas* sp.	TMB - 78	-	-
Fungal Group				
FG-1	*Cryptococcus* sp.	TMF - 109	-	-
FG-2	*Rhodotorula* sp.	TMF - 80	-	-
FG-3	*Candida* sp.	TMF - 127	-	-
FG-4	*Penicillium* sp.	TM - 137	-	-

FG-5	*Microstroma* sp.	TMF - 86	-	+
FG-6	*Sygospora* sp.	TMF - 138	-	-
FG-6	*Sporisorium* sp.	TMF - 85	-	+
FG-6	*Metschnikowia* sp.	TMF - 101	+	+
FG-6	*Cryptococcus* sp.	TMF - 61	-	+
FG-6	*Candida* sp.	TMF - 143	-	+
FG-7	*Metschnikowia* sp.	TMF - 166	-	+
FG-7	*Metschnikowia* sp.	TMF - 161	+	-
FG-7	*Metschnikowia* sp.	TMF - 87	+	-
FG-8	*Cryptococcus* sp.	TMF - 82	-	-
FG-9	*Metschnikowia* sp.	TMF - 160	+	-
FG-9	*Candida* sp.	TMF - 165	+	+
FG-9	*Metschnikowia* sp.	TMF - 162	+	-
FG-10	*Sterigmatomyces*	TMF - 105	-	-
FG-11	*Candida* sp.	TMF - 88	-	-
FG-12	*Leucosporidium* sp.	TMF - 123	+	-
FG-13	*Metschnikowia* sp.	TMF - 132	+	-
FG-13	*Metschnikowia* sp.	TMF - 164	+	-
FG-14	*Rhodotorula* sp.	TMF - 110	-	-
FG-15	*Sporobolomyces* sp.	TMF - 81	+	-
FG-15	*Metschnikowia* sp.	TMF - 149	-	+
FG-16	*Cryptococcus* sp.	TM - 63	+	-
FG-17	*Sporisorium* sp.	TM - 136	+	-
FG-17	*Metschnikowia* sp.	TM - 129	+	-

3.4. CONCLUSIONS

Isolation of microorganisms associated with Australian stingless bees produced 559 microbial isolates. Classification of isolates by morphotype analysis indicated the presence of 95 unique individuals using an 80% similarity cut off. This figure was used to determine the approximate sample size required to cover the microbial diversity present. Complete genetic and chemical data was obtained from 94 isolates, 41 bacteria and 53 fungi.

The historical success of PKS and NRPS natural products and their potential to produce a wide array of bioactive chemical structures makes them useful targets in the search for a new generation of bioactive compounds. Using the presence of PKS or NRPS genes as the primary indicator of bioactive potential, a total of 35 isolates were initially selected; 15 bacterial and 20 fungal. By overlaying the results of the genetic analysis over the chemical analysis a further 11 isolates were selected to be carried forward, identified as being members of chemical groups not yet represented. The final result of this chapter is a short list of 46 isolates that will be examined further in the following chapter.

The large number of microbial isolates had its origin in the 11 different agar culture media used to isolate the bee-associated microorganisms. On one hand it is a good problem as we could be relatively confident that we cultured representatives of the microbial diversity present, that would grow under aerobic condition. However the multiple media process created a situation where a single organism could be present on many different media. Future studies on the stingless bee associated microorganisms

could utilise anaerobic culturing methods to select for other gut specific microbiota, such as the *Lactobacilli* that were absent from this study.

The morphological, genetic and chemical analyses permitted the logical reduction of isolates to a number that could be reasonably handled in the downstream processes, i.e. antimicrobial bioactivity testing. The PKS and NRPS screening method was effective as it simultaneously selected isolates with increased biosynthetic potential and also produced a subset of sample that accounted for approximately 75% of the chemical diversity present.

Following on from the work in this chapter it would be of interest to analyse the antimicrobial potential of the non PKS and NRPS positive representatives in the bacterial and fungal groups to determine classes of compounds not produced by these enzymes.

Chapter 4

Antimicrobial activity of Australian stingless bee associated microorganisms and the characterisation of compounds from a *Xanthomonas* sp.

4.1. INTRODUCTION

The development of resistance to front line antimicrobial compounds (Poole, 2002) combined with the scientific, regulatory and financial hurdles associated with novel antimicrobial drug discovery (Newman and Cragg, 2016) have created an urgent need for novel antimicrobial compounds. The discovery of novel chemical scaffolds to target microbial pathogens requires new approaches and methods that build on existing knowledge (Spellberg et al., 2015). Nature contains a wealth of untapped chemical and biological diversity and historically natural products have provided close to half of all new chemical entities that have successfully made their way to the market (Newman et al., 2000).

Symbiotic microorganisms represent an exciting field of research in the quest for novel bioactive compounds. For example, bacteria living on the cuticle of fungus-growing ants have been shown to produce antifungal compounds that help protect the ant's food system from infection by pathogenic fungal species (Sen et al., 2009). Additionally, studies have identified antibacterial (Sabaté et al., 2009, Forsgren et al., 2010, Vásquez et al., 2012) and antifungal activity (Gilliam, 1971, Gilliam et al., 1988, Reynaldi et al., 2004, Sabaté et al., 2009) from microorganisms isolated from the gut of bumble bees and honey bees.

Exploring the interactions between microorganisms and higher organisms is a growing field of research that has benefitted from the development of genetic and chemical analysis techniques. This study represents the first attempt to discover novel antimicrobial compounds produced by microorganisms associated with Australian stingless bees, an area of research inspired by the observation of antimicrobial activity

in extracts of the Australian stingless bee *Tetragonula carbonaria* and its products (Irish et al., 2008, Boorn et al., 2010, Massaro et al., 2014a, Massaro et al., 2014b). By combining knowledge of the biology and ecology of insects with cutting edge analytical techniques we strive to develop new approaches for novel antimicrobial compound discovery.

In this chapter we aimed to assess the antimicrobial activity of compounds biosynthesised by microorganisms isolated from three species of Australian stingless bees *T. carbonaria*, *A. australis* and *T. hockingsii* (Chapter 3). This chapter also describes the structural characterisation of chemical compounds responsible for the antimicrobial activity. Antimicrobial activity was assessed against a panel of pathogens using modified liquid culture protocol with the oxidative-reduction indicator Resazurin as a measure of cell viability. The origin of antimicrobial activity was determined by large-scale culture extraction and purification of extract compounds by liquid chromatography, NMR and mass spectrometry.

4.2. METHODS

4.2.1 Small-scale culturing

Small-scale cultures of each isolate where initiated by inoculating 50 mL of culture media in a 100 mL conical flask with a 1 cm x 0.5 cm slice of isolate inoculated agar plate. These starter cultures where incubated for 12 h at 25°C, shaking at 100 rpm, after which they were added to 100 mL of culture media in a 250 mL conical flask and incubated at 25°C, shaking at 180 rpm, for 6 days. Each isolate was grown in two media to account for nutrient effects on bioactive compound production.

The bacterial lean media, Yeast extract-Casamino acid-Dextrose (YECD), was composed of 0.1% (w/v) dextrose supplemented with 0.03% yeast extract and 0.03% casamino acids. In contrast, the bacterial rich media, Nutrient Broth No. 2 (NB) (Oxoid), was composed of 1% 'Lab-Lemco' powder, 1% peptone and 0.5% sodium chloride. The fungal lean media was also YECD, and a fungal specific rich media was used, Malt extract broth (MEB) (Oxoid), containing 1.7% malt extract and 0.3% mycological peptone.

4.2.2 Small-scale extractions

Each small-scale liquid culture was extracted separately by adding 150 mL of ethyl acetate into 150 mL of microbial culture in a 500 mL conical flask and incubating at 25°C, shaking at 180 rpm, for 24 hours. After incubation, the solvent was removed using a separating funnel. Each extract was dried by adding 10 g of sodium sulfate, shaken for 2 minutes and filtered through Grade 1 filter paper (Whatman) to remove

particulates. The extracts were taken to dryness by rotary evaporation in a pre-weighed 20 mL glass scintillation vial and dry mass of each extract determined.

4.2.3 Antibacterial and antifungal bioactivity assay

A general antibacterial and antifungal growth inhibition assay was used to test the bioactive potential of the crude extracts generated from each isolate. The assay was carried out in 96 well flat bottomed cell culture plates with a lid. Each plate included 4 controls; media only; media with test organism; media, test organism and commercial antimicrobial; media, test organism and DMSO. For the crude extracts, three different concentrations were assayed, 200, 100 and 10 µg/mL in media + 1% DMSO. All controls and test samples were performed in triplicate. A ring of wells on the outer edge of the plate was filled with 200 µL of sterile water to limit evaporation from the test wells during incubation. The test plates were sealed with Parafilm and incubated at 30°C for 20 h. After incubation, 50 µL of 0.05 mg/mL Resazurin (Sigma) solution, a cell viability reagent, was added to each well and incubated for a further 2 h at 30°C. Fluorescence readings were then measured using a CLARIOstar (BMG Labtech) fluorescence microplate reader with a 540-15 nm excitation filter and 590-20 nm emission filter.

Non-pathogenic representatives of human pathogens were chosen as test strains. Gram-negative (*Pseudomonas aeruginosa* NCTC 10490) and Gram-positive bacteria, (*Staphylococcus aureus* NCTC 6571) were grown in nutrient broth #1 (Oxoid) at 30°C until the cultures reached the mid-exponential phase of growth, as indicated by an

OD_{600} of 0.6. Ciprofloxacin (100 µg/mL; Invitrogen) was used as the antibacterial positive control. The unicellular fungus, *Candida albicans* ATCC 10231, was grown in RPMI-1640 Medium with 25 mM HEPES (Sigma-Aldrich) at 30°C until the cultures reached the mid-exponential phase of growth. Nystatin (50 µg/mL, Sigma) was used as the antifungal positive control.

Cell inhibition, as a percentage of the untreated control, was calculated using the formula:

$$\%\ \text{inhibition} = 1 - \left(\frac{F_{590\text{-}20}^{\text{test organism + extract+ DMSO}} - F_{590\text{-}20}^{\text{media + DMSO}}}{F_{590\text{-}20}^{\text{test organism only}} - F_{590\text{-}20}^{\text{media only}}}\right) \times 100$$

where $F_{590\text{-}20}$ was the measured fluorescence of the test organism + extract + DMSO, media + DMSO, test organism only or media only.

4.2.4 Large-scale culturing of TMB - 122

Bioactivity assays indicated that *Xanthomonas* sp. TMB - 122 was producing antimicrobial compounds. Therefore, a 9.6 L, large-scale culture of this isolate was conducted in twelve 2.5 L conical flasks, each containing 800 mL of Nutrient Broth #2 (Oxoid). Starter cultures where prepared as previously described in section 4.2.1. and added to 650 mL of culture media in 2.5 L conical flasks and incubated at 25°C, shaking at 180 rpm, for 6 days.

4.2.5 Large-scale extraction of TMB - 122

Extraction of the large-scale cultures was performed as described in section 4.2.2, however, 800 mL of ethyl acetate was added to 800 mL cultures in 2.5 L conical flasks and the flasks incubated at 25 °C, shaking at 180 rpm, for 36 h.

4.2.6 Fractionation of TMB - 122 large-scale extract

4.2.6.1 Flash chromatography

Fractionation of the large-scale extract from *Xanthomonas* sp. TMB - 122, was performed using the Reveleris X2 Flash Chromatography System (W.R Grace & Co). Dry loading cartridges were prepared with a mixture of 1.5 g of acid washed diatomaceous earth (Sigma) per 1 g of crude dry extract. Reverse-phase chromatography was augmented with normal-phase chromatography to achieve optimum separation.

Reverse-phase chromatography as conducted in two steps, using a Grace 40 g C18-WP (20 μ, 60 Å) flash cartridge (W.R Grace & Co). The first round consisted of a linear gradient elution program, of 100% MilliQ water (0.22 um filtered, FRP solvent A) to 100% acetonitrile (FRP solvent B). Compounds retained on the dry loading cartridge after this initial gradient were subject to a linear gradient elution program of 100% acetonitrile (FRP solvent B) to 100% isopropanol (FRP solvent C).

Fractions with limited affinity to the reverse-phase column were combined in a second dry pack cartridge and fractionated by normal-phase chromatography. Compounds were fractionated using a Grace 40 g HP-silica (40 μ, 60 Å) flash cartridge and linear gradient of 100% hexane (FNP solvent A) to 100% ethyl acetate (FNP solvent B). A final normal-phase separation was performed with the same 40g silica cartridge and linear gradient elution program of 100% ethyl acetate (FNP solvent B) to 100% methanol (FNP solvent C).

All flash chromatography runs were conducted at a flow rate of 40 mL/min and a 60 minute run time. Peak detection and automated fractionation were based on ELSD and UV detector channels, using the following settings: detector sensitivity = high, slope detection = off, threshold detection levels; ELSD = 40 mV and UV = 0.05 AU, UV wavelength; UV1 = 220 nm, UV2 = 254 nm, UV3 = 280 nm. Fractions corresponding to a single peak, but collected in separate tubes, were combined. These pooled fractions were dried by rotary evaporation and subject to bioactivity testing as described in section 4.2.3.

4.2.6.2 Semi-preparative HPLC

Bioactive fractions were further purified using normal-phase semi-preparative HPLC, on a Dionex Ultimate 300 Series semi-prep system configured with a Corona Ultra RS (charged aerosol detector), an Ultima MWD 3000 (UV-Vis detector) and an AFC-3000 (automated fraction collector). Separation was achieved on a GRACE Alltima Silica 5 μ 250 x 10 mm column, using a gradient of 100% hexane (solvent A) to 100% ethyl acetate (solvent B).

4.2.7 NMR analysis

Crude and purified fractions were redissolved in deuterated solvents, DMSO-d_6 or chloroform-d (Cambridge Isotope Laboratories). Proton-nuclear magnetic resonance (1H-NMR) and two-dimensional NMR correlation spectra (1H−1H COSY, and 1H−13C HSQC and HMBC) were recorded on a Bruker Avance III 600 (600 MHz for 1H, 150 MHz for 13C). Spectra were referenced to the relevant solvent signal using literature data (Gottlieb et al., 1997).

4.2.8 GC-MS analysis

GC-MS analysis of fractions TMB-122-F67/68-7 and TMB122-F7 were performed on a Thermo Scientific TSQ Quantum XLS equipped with a Thermo TR-50MS, 60 m x 0.25 mm, mid polarity column with a film thickness of 0.25 μm. The carrier gas was helium, at a flow rate of 1.8 mL/min. Column temperature was initially 60°C for 1 min, then increased to 130°C at 15°C/min, and finally increased to 315°C at 7°C/min and held for 15 min. Samples were introduced as 1.2 μL injections in splitless mode. GC-MS detection, 45 to 750 *m/z*, following positive mode electron ionization with a source temp of 200°C. Analysis performed at the Bioanalytical Mass Spectrometry Facility (BMSF), Mark Wainwright Analytical Centre, UNSW.

4.2.9 Accurate mass MS analysis

Accurate mass determination of fractions F5 and F6, and were obtained on Thermo Scientific LTQ FT ULTRA instrument using a HESI source with direct sample

infusion, at the Bioanalytical Mass Spectrometry Facility (BMSF), Mark Wainwright Analytical Centre, UNSW.

4.3 RESULTS AND DISCUSSION

4.3.1 Microbial fermentation

A total of 46 microbial isolates, (18 bacterial and 28 fungal), were selected for antimicrobial testing following combined genetic and chemical enrichment and dereplication (Chapter 3), however three isolates failed to revive from cryostocks. Each isolate was grown in both a rich (NA) and lean (YECD) media to maximise the diversity of secondary metabolites produced. Ethyl acetate extraction of the small-scale (150 mL) liquid cultures produced a total of 35 bacterial extracts (Table 4.1) and 52 fungal extracts (Table 4.2). On average, three times more extract mass was obtained from bacterial cultures grown on NA compared to YECD. This was also consistent in the media controls, which reflected the more complex composition of the NA media and indicated that a larger proportion of media components were present in the NA media extracts. Similarly, the average mass of the fungal ME extracts were four times greater than fungal YECD extracts. However, extracts obtained from the two media controls did not vary greatly (YECD = 1.1 mg, ME = 1.4 mg). This indicated that the increased abundance of extract in the ME grown cultures was due to an increase in microbial metabolite production.

Table 4.1: Mass of ethyl acetate extracts from small-scale bacterial cultures and media controls in lean media (YECD) and rich media (NB). NG = no growth.

Isolate	Media	Extract (mg)	Isolate	Media	Extract (mg)
YECD media	YECD	1.1	NB media	NB	3.6
TMB - 20	YECD	3.9	TMB - 20	NB	9.9
TMB - 78	YECD	6.3	TMB - 78	NB	7.6
TMB - 90	YECD	5.3	TMB - 90	NB	10.2
TMB - 106	YECD	NG	TMB - 106	NB	7.8
TMB - 108	YECD	2.4	TMB - 108	NB	10.6
TMB - 111	YECD	2.0	TMB - 111	NB	10.1
TMB - 115	YECD	2.3	TMB - 115	NB	15.4
TMB - 118	YECD	9.0	TMB - 118	NB	14
TMB - 122	YECD	14.1	TMB - 122	NB	15
TMB - 128	YECD	3.2	TMB - 128	NB	7.9
TMB - 140	YECD	2.8	TMB - 140	NB	6.4
TMB - 147	YECD	6.8	TMB - 147	NB	13.4
TMB - 150	YECD	3.3	TMB - 150	NB	7.8
TMB - 151	YECD	4.0	TMB - 151	NB	6.2
TMB - 152	YECD	2.2	TMB - 152	NB	5.8
TMB - 158	YECD	3.4	TMB - 158	NB	8.3
TMB - 159	YECD	2.6	TMB - 159	NA	7.7
	Average mass:	3.8		Average mass:	8.9

Table 4.2: Mass of ethyl acetate extracts from small-scale fungal cultures and media controls in lean media (YECD) and rich media (ME). NG = no growth.

Isolate	Media	Extract (mg)	Isolate	Media	Extract (mg)
YECD media		1.1	ME media		1.4
TMF - 61	YECD	2.6	TMF - 61	ME	9.5
TMF - 63	YECD	1.8	TMF - 63	ME	7.9
TMF - 80	YECD	5.9	TMF - 80	ME	8.1
TMF - 81	YECD	2.5	TMF - 81	ME	17.1
TMF - 82	YECD	5.3	TMF - 82	ME	11
TMF - 85	YECD	2.9	TMF - 85	ME	81.8
TMF - 86	YECD	2.8	TMF - 86	ME	21.3
TMF - 87	YECD	3.1	TMF - 87	ME	9.6
TMF - 88	YECD	7.5	TMF - 88	ME	9.1
TMF - 101	YECD	2.5	TMF - 101	ME	17.3
TMF - 105	YECD	12.6	TMF - 105	ME	NG
TMF - 108	YECD	5.4	TMF - 108	ME	11.9
TMF - 110	YECD	5.7	TMF - 110	ME	9.3
TMF - 123	YECD	5.4	TMF - 123	ME	28.1
TMF - 127	YECD	4.5	TMF - 127	ME	8.6
TMF - 129	YECD	2.7	TMF - 129	ME	7.6
TMF - 132	YECD	3.2	TMF - 132	ME	7.3
TMF - 135	YECD	4.2	TMF - 135	ME	16.6
TMF - 136	YECD	2.2	TMF - 136	ME	114.8
TMF - 138	YECD	2.2	TMF - 138	ME	8.8
TMF - 143	YECD	2.7	TMF - 143	ME	7.4
TMF - 149	YECD	5.2	TMF - 149	ME	8.3

TMF - 161	YECD	3.1	TMF - 161	ME	13.1
TMF - 162	YECD	3.4	TMF - 162	ME	6.1
TMF - 165	YECD	1.9	TMF - 165	ME	9.2
TMF - 166	YECD	3.1	TMF - 166	ME	7.1
	Average mass:	3.9		Average mass:	17.6

4.3.2 Small-scale antimicrobial bioactivity assay

Each ethyl acetate extract was tested for antimicrobial activity against a panel of three microorganisms, *Staphylococcus aureus* NCTC 6571, *Pseudomonas aeruginosa* NCTC 6571 and *Candida albicans* ATCC 10231. A selection of the top 30 antimicrobial activity results is presented below in Figures 4.1, 4.2 and 4.3.

Thirteen crude extracts exhibited antibiotic activity against *Staphylococcus aureus* NCTC 6571. At the top of the list were extracts from TMB -122 (*Xanthomonas* sp.) and TMB - 118 (*Oceanobacillus* sp.) that exhibited similar antibiotic activity across all three extract concentrations, (Figure 4.1). Furthermore, extracts obtained from both the rich and lean media showed very similar activity. These two isolates were considered the most promising for further examination.

Xanthomonas spp. are motile, gram-negative Gammaproteobacteria. Research focused on *Xanthomonas* spp. have been dominated by their behaviour as a plant pathogen of commercial crops (Rudolph, 1993) and the industrial production of the xanthan gum, a bacterial exopolysaccharide (Rosalam and England, 2006). The production of

antibacterial compounds by *Xanthomonas* spp. has been reported (Hu and Young, 1998) with the production of antibacterial proteins, bacteriocins, identified as the active compound in a recent study (Ghequire et al., 2012). As plant pathogens *Xanthomonas* spp. are known to produce a variety of phytotoxic compounds, a notable example being albicidin (Birch and Patil, 1985). Albicidin is a DNA gyrase inhibitor produced by *Xanthomonas albilineans* that also exhibits broad spectrum antibacterial activity at low concentrations (Hashimi et al., 2007). The biosynthesis of albicidin is facilitated by a polyketide–peptide synthetase and the final compound is constructed from p-aminobenzoic acids and cyanoalanine subunits (Huang et al., 2001, Cociancich et al., 2015).

Oceanobacillus spp. represents an interesting group of Bacilli found in extreme environments including high salt and alkalinity. The type strain *Oceanobacillus kimchii* was isolated from the fermented food product kimchi; subsequent species have been isolated from a variety of environments from the deep ocean to desert soils. One notable isolate was discovered as a bacterial symbiont of fungi, isolated from the gut of a dragon fly (Shao et al., 2015). There are currently no reports of antimicrobial activity from *Oceanobacillus* spp. although antimicrobial bacteriocins are common in related Bacilli.

The *Pseudomonas aeruginosa* NCTC 6571 assay demonstrated a consistent level of antibacterial activity amongst the all the extracts examined with a minimum of 20% to 30% inhibition observed amongst the top 30. Four isolates emerged from this base level, TMF - 161 (*Metschnikowia* sp.) in both YECD and ME media, TMF - 105

(*Sterigmatomyces* sp.) in YECD media, TMB - 147 (*Pantoea* sp.) in both YECD and NB media and TMB - 90 (*Bacillus* sp.) in YECD media. Antibacterial and antifungal compounds from the yeast species *Metschnikowia* and *Sterigmatomyces* have been identified in a number of recent studies (Sisti and Savini, 2013, Ali et al., 2014, Oro et al., 2014) with *Sterigmatomyces* spp. showing broad spectrum activity against both gram positive and negative organism. *Bacillus* spp. and *Pantoea* spp. represent well studied genus of organisms known to produce a variety of antibacterial and antifungal compounds with many bioactive compounds characterised including the bacteriocin herbicolin (Kamber et al., 2012) in addition to gramicidin and tyrocidine (Katz and Demain, 1977).

The *C. albicans* assay produced results similar to the *P. aeruginosa* assay, a base level of antifungal activity was present in most extracts examined. However in contrast the lower concentration extracts eliciting a greater response than the highest concentration extracts. The three extracts exhibiting the greatest antifungal activity across all three extract concentrations were TMF - 85 (*Rhodotorula* sp.), TMB - 115 (*Pantoea* sp.) and TMF - 136 (*Sporisorium* sp.) all from the YECD extracts. It should be noted that the YECD media control also demonstrated antifungal activity, however not to the level observed in the any of the isolate extracts. In general there was no major difference among the top 30 extracts examined.

In contrast to the *S. aureus* assay, neither the *P. aeruginosa* nor *C. albicans* assays were able to highlight particular isolates with consistent antimicrobial effects across the range of extract concentrations. For this reason, the results from the *S. aureus* assay were used

as the primary criteria for selecting the isolate for large-scale culturing, chemical extraction and structure elucidation. Two isolates, TMB - 122 (*Xanthomonas* sp.) and TMB - 118 (*Oceanobacillus* sp.) both displayed anti-*S. aureus* bioactivity in the small-scale crude extracts. However, *Xanthomonas* sp. TMB - 122 was able to grow faster on both solid and liquid media, while producing a similar quantity of crude extract. Therefore, this strain was carried forward for large-scale culturing, extraction and compound purification.

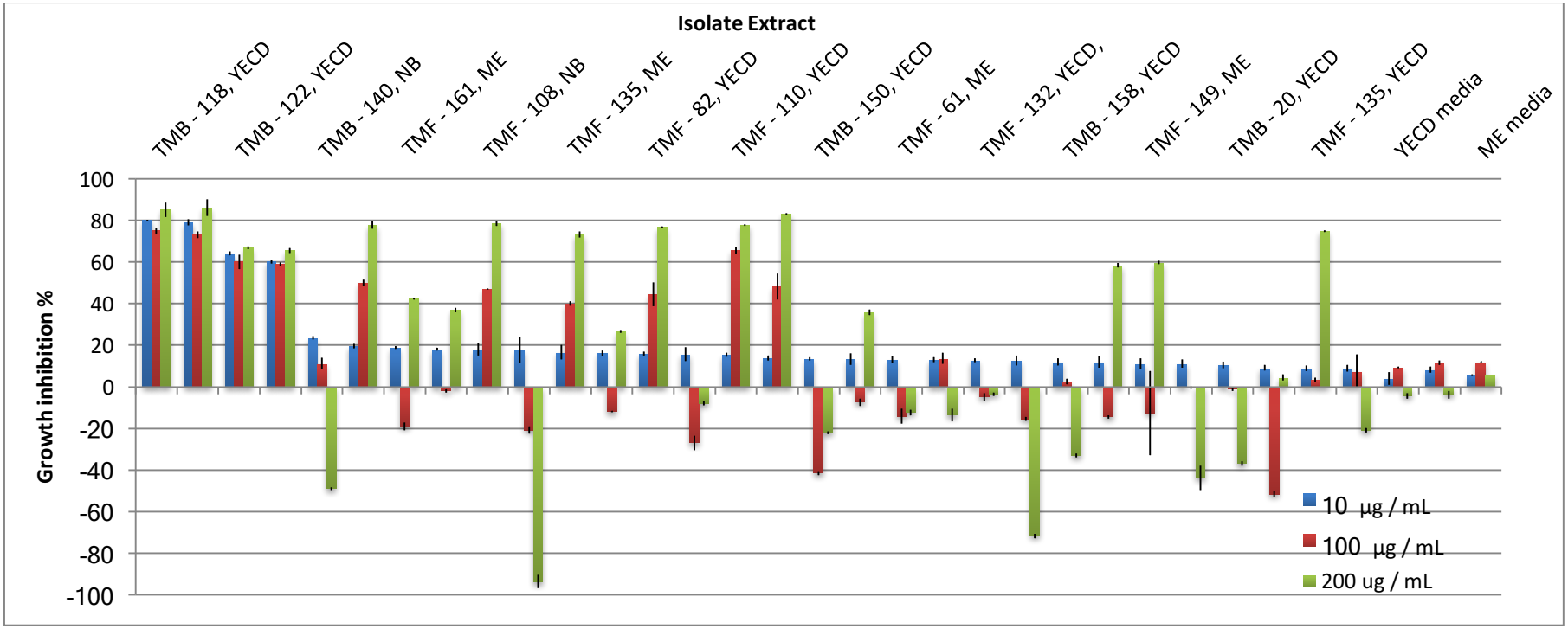

Figure 4.1: Top 30 bioactivity assay results for bacterial extracts, fungal extracts and media controls against *Staphylococcus aureus* NCTC 6571, black lines within columns indicate standard error of the mean (n=3). TMB = bacterial isolate #, TMF = fungal isolate #, YECD = Yeast extract-Casamino acid-Dextrose media, ME= Malt extract media, NB = Nutrient broth media.

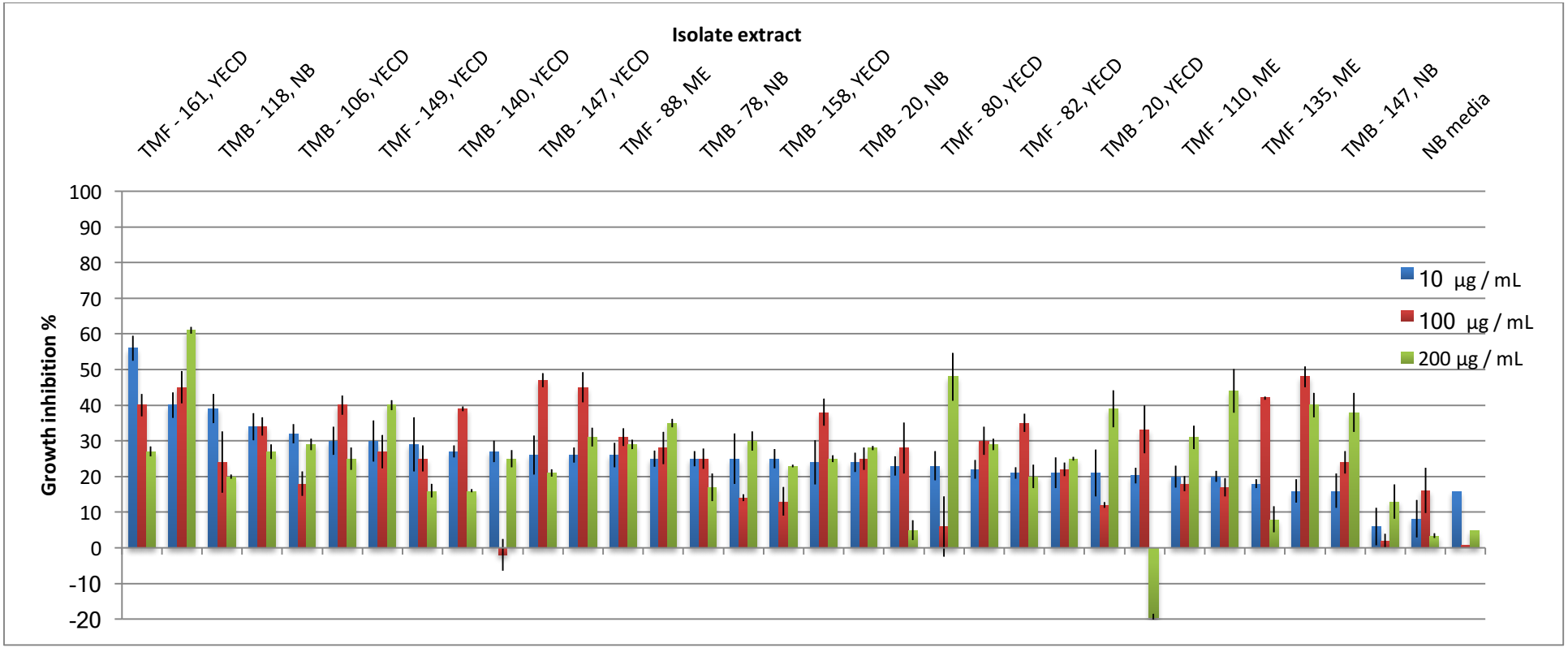

Figure 4.2: Top 30 bioactivity assay results for bacterial extracts, fungal extracts and media controls against *Pseudomonas aeruginosa* NCTC 6571, black lines within columns indicate standard error of the mean (n=3). TMB = bacterial isolate #, TMF = fungal isolate #, YECD = Yeast extract-Casamino acid-Dextrose media, ME= Malt extract media, NB = Nutrient broth media.

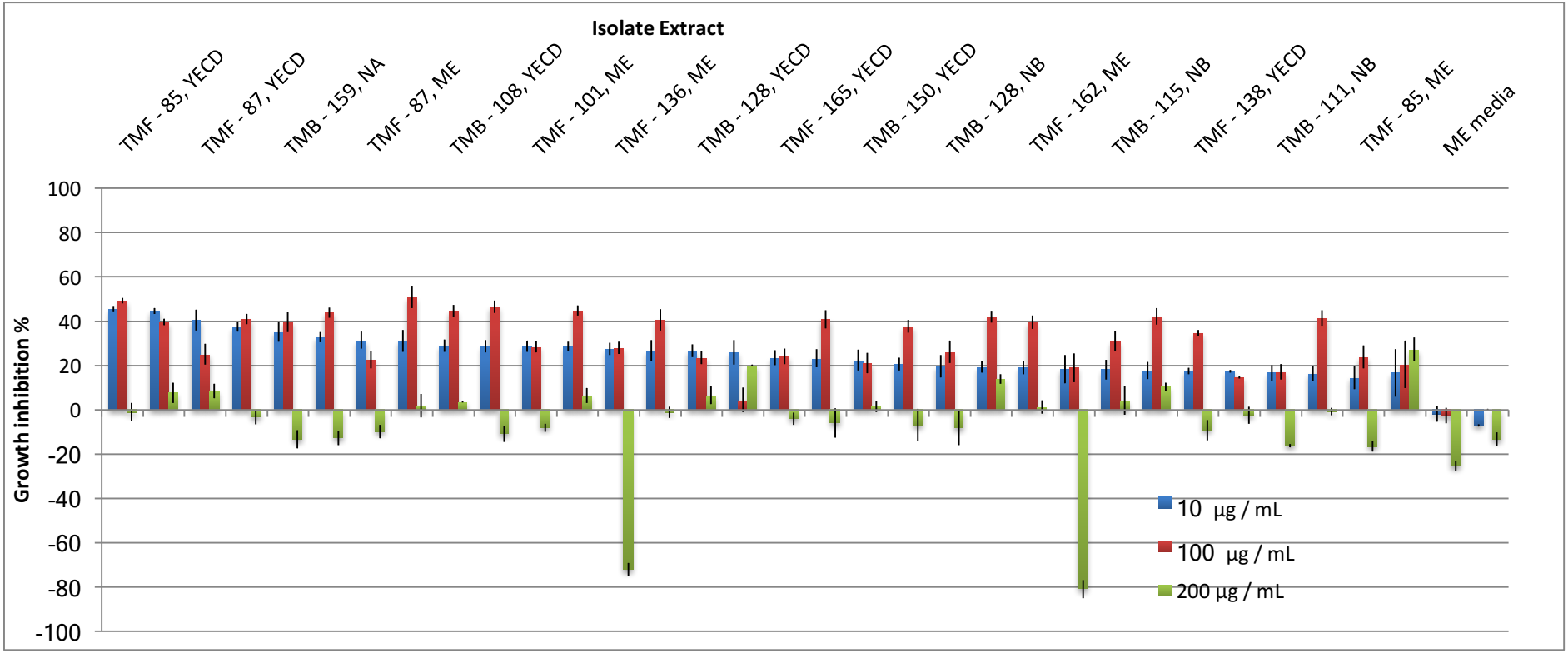

Figure 4.3: Top 30 bioactivity assay results for bacterial extracts, fungal extracts and media controls against *Candida albicans* ATCC 10231, black lines within columns indicate standard error of the mean (n=3). TMB = bacterial isolate #, TMF = fungal isolate #, YECD = Yeast extract-casamino acid- dextrose media, ME= Malt extract media, NB = Nutrient broth media.

4.3.3 Culturing, extraction, fractionation and antibacterial assay of TMB - 122, **Xanthomonas *sp.***

Ethyl acetate extraction of *Xanthomonas* sp. TMB - 122, grown in 9.6 L of NB media resulted in 1.6 g of crude extract. Reverse-phase and normal-phase flash chromatography of this extract resulted in a total of 80 fractions. Each fraction was tested for antibacterial bioactivity against *Staphylococcus aureus* NCTC 6571 at 100 µg/mL and 10 µg/mL concentrations. The top 30 anti-*S. aureus* bioactivity results are presented in Figure 4.4.

Four sequentially eluting fractions, TMB-122-F66, F67, F68 and F69, from the normal-phase chromatography exhibited the highest degree of antibacterial activity. The two most active fractions, F67 and F68 were combined and further purified by normal-phase HPLC producing 21 sub-fractions. Each sub-fraction was tested for antibacterial activity against *Staphylococcus aureus* NCTC 6571 at 100 µg/mL and 10 µg/mL. The seventh sub-fraction designated as TMB-122-F67/68-7 exhibited 84% growth inhibition of the test strain at the 100 µg/mL. This sub-fraction was selected for structure elucidation via NMR and MS.

Three other fractions, TMB-122-F5, TMB-122-F6 and TMB-122-F7 collected from the reverse-phase chromatography were selected for elucidation based on their purity and ^{1}H NMR spectrum data.

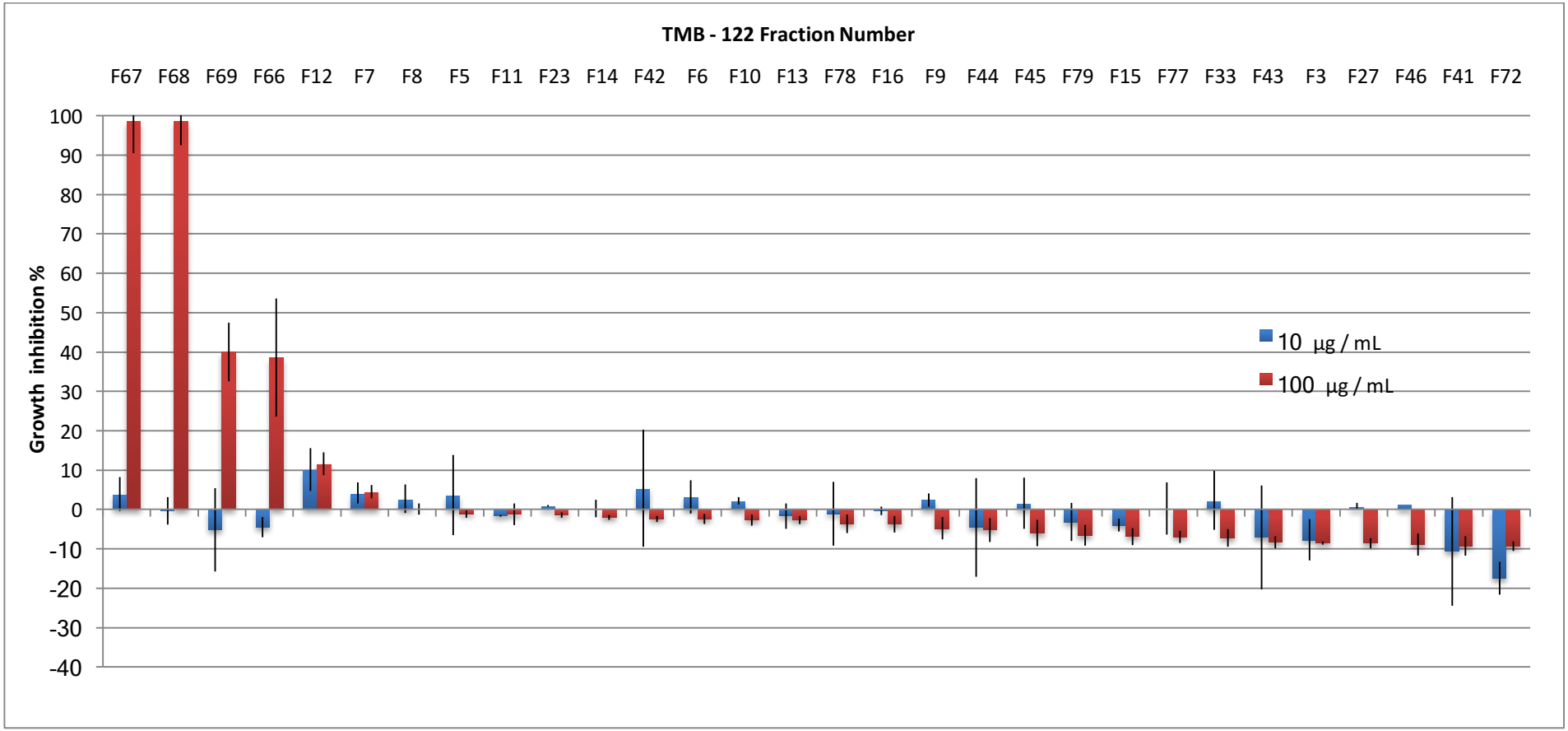

Figure 4.4: Top 30 bioactivity assay results for TMB - 122 fractions against *Staphylococcus aureus* NCTC 6571, black lines within columns indicate standard error of the mean (n=3).

4.3.4 NMR and structure elucidation

4.3.4.1 TMB-122-F67/68-7

TMB-122-F67/68-7 was purified as a colourless oily substance. The ^{1}H NMR spectrum in DMSO-d_6 indicated methine protons at δ 5.30 (H-9), and two methylenes at δ 1.97 (H-8) and δ 1.28 (H-7), (Table 4.3). The HSQC experiment showed correlations between the protons at δ 5.3 (m, 2H) and a carbon at 129.5 ppm (C-9) suggesting it was attached to a sp^2 hybrid (double bonded) carbon. The HSQC experiment showed correlations between the protons at δ 1.97 (td, 2H) and δ 1.28 (m) and the carbons at 26.7 ppm (C-8) and 29.2 ppm (C-7) respectively. The COSY experiment placed the protons at δ 5.3 three bonds from the protons at δ 1.97 which it turn were three bonds from δ 1.28 (m) allowing the partial structure A to be assigned, (Figure 4.5). Symmetry around the double bond at positions 9 was confirmed by correlations in the HMBC experiment between the protons at δ 5.3 and the carbon at 129.5 ppm. Extension from the partial structure A was difficult due to overlapping methylene signals in the ^{1}H NMR and 2D NMR experiments.

Table 4.3: NMR data for TMB-122-F67/68-7 (9-hexadecenoic acid) acquired in DMSO-d_6 at 600 MHz (^{1}H).

Position	**^{13}C**	**^{1}H (δ, mult, J_{ab}, J_{bc})**	**COSY**	**HMBC ($^2J_{CH}$, $^3J_{CH}$, $^4J_{CH}$)**
1	174.5			
1b (OH)		Not observed		
2	33.8	2.15 (td, J = 7.4, 1.7 Hz, 2H)	H-3	C-3, C-1
3	24.6	1.47 (m, J = 6.6, 3.6 Hz, 2H)	H-2, H-4	C-2, C-1
4	28.4	1.24 (m)	H-3	C-3, C-2
5				
6				
7a	29.2	1.28 (m)	H-8	C-8, C-5, C-4
7b	29.2	1.28 (m)	H-8	C-8, C-12
8a, 8b	26.7	1.97 (m, 4H)	H-7, H-9	C-7, C-9
9a, 9b	129.5	5.3 (m, 2H)	H-8	C-8, C-7
10				
11	31.3	1.24 (m)		
12	28.6	1.24 (m)	H-13	C-13, C-11
13	13.8	0.84 (m, 3H)	H-12	C-11

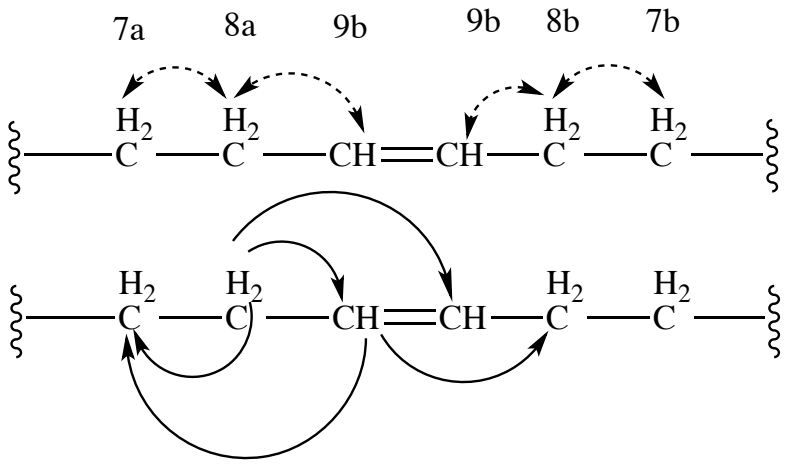

Figure 4.5: TMB-122-67/68-7 partial structure A, showing COSY correlations (dashed line) and HMBC correlations (7a, 8a, 9a only).

To gain further insight into the structure, a second fragment was generated (Figure 4.6). The ^{1}H NMR spectrum showed three methylenes at δ 2.15 (H-2), δ 1.47 (H-3) and δ 1.24 (H-4). The HSQC experiment showed correlations between the protons at δ 2.15 (td, 2H), δ 1.47 9 (dd, 2H) and δ 1.24 (m) and the carbons at 33.8 ppm (C-2), 24.6 ppm (C-3) and 28.4 ppm (C-4) respectively. Three bond COSY experiment correlations suggested the protons at δ 2.15 was adjacent the protons at δ 1.47 which is turn were adjacent to the protons at δ 1.24 allowing the partial structure B to be assigned, (Figure 4.6). HMBC correlations between the protons at δ 2.15 and the carbons at 174.5 ppm (C-1) and 24.6, the protons at δ 1.47 and the carbons at 174.5 and 33.8, and the protons at δ 1.24 and the carbons at 174.5 and 24.6 and 33.8 confirmed this assignment. The absence of the hydroxyl proton signal may have been the result of rapid intermolecular exchange with trace amounts of water present in the sample or the DMSO-d_6.

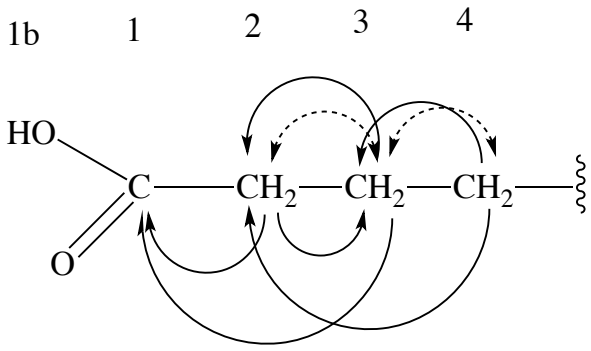

Figure 4.6: TMB-122-67/68-7 partial structure B, showing COSY correlations (dashed line) and HMBC correlations.

As above the overlapping methylene signals made extension from partial structure B difficult. A final partial structure was constructed from the remaining ^{1}H signals, methyl protons at δ 0.84 (H-13), and two methylenes at δ 1.24 (H-11 & H-12). The HSQC correlation indicated the protons at δ 0.84 (m, 3H) was attached the carbon at 13.8 ppm (C-13) and the two identical protons at position δ 1.24 (m) were attached to the carbons at 28.6 ppm (C-12) and 31.3 ppm (C-11) respectively. Three bond COSY correlations placed the methyl protons at δ 0.84 alongside protons at δ 1.24. The HMBC correlations between the protons at δ 0.84 and the carbon at 31.3 ppm and the protons at δ 1.24 (attached to the carbon at 28.6 ppm), and the carbon at 13.8 ppm and the carbon at 31.3 ppm suggested the assignment of partial structure C, (Figure 4.7).

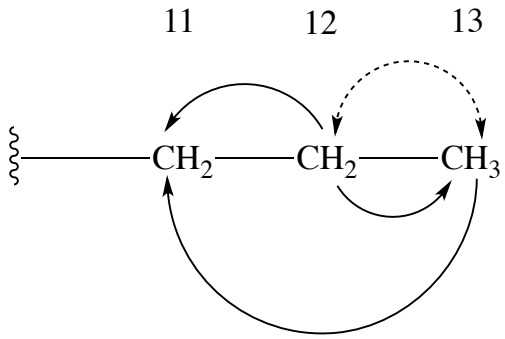

Figure 4.7: TMB-122-67/68-7 partial structure C, showing COSY correlations (dashed line) and HMBC correlations.

The three partial structures presented in Figures 4.5, 4.6 and 4.7 suggested that the final structure was most likely a fatty acid. Attempts were made to resolve the convoluted methylene region in the ^{1}H and 2D NMR experiments by running a homonuclear coupled HSQC experiment, to reduce the peak broadening due to coupling effects, and an INADEQUATE carbon experiment, to determine carbon-carbon bond attachments. However, these experiments afforded no further structural detail.

GC-MS analysis of the methyl ester derivative of TMB-122-F67/68-7 revealed a major peak at retention time 18.98 with an *m/z* 268, $[M]^{+}$ (Figure 4.8). The mass spectrum created was compared with the Wiley mass spectral library (Wiley W9N11.L and NIST 2.0) and a positive match made to 9-hexadecenoic acid methyl ester, with a match score of 980 and reverse match score 981. Match factors are cosine similarity calculations with the reverse match factor ignoring any peaks in unknown sample but not in the library sample. An exact match produces a score of 999 whilst a score of 0 indicates no ions in common, practically a match score above 900 is considered an excellent match.

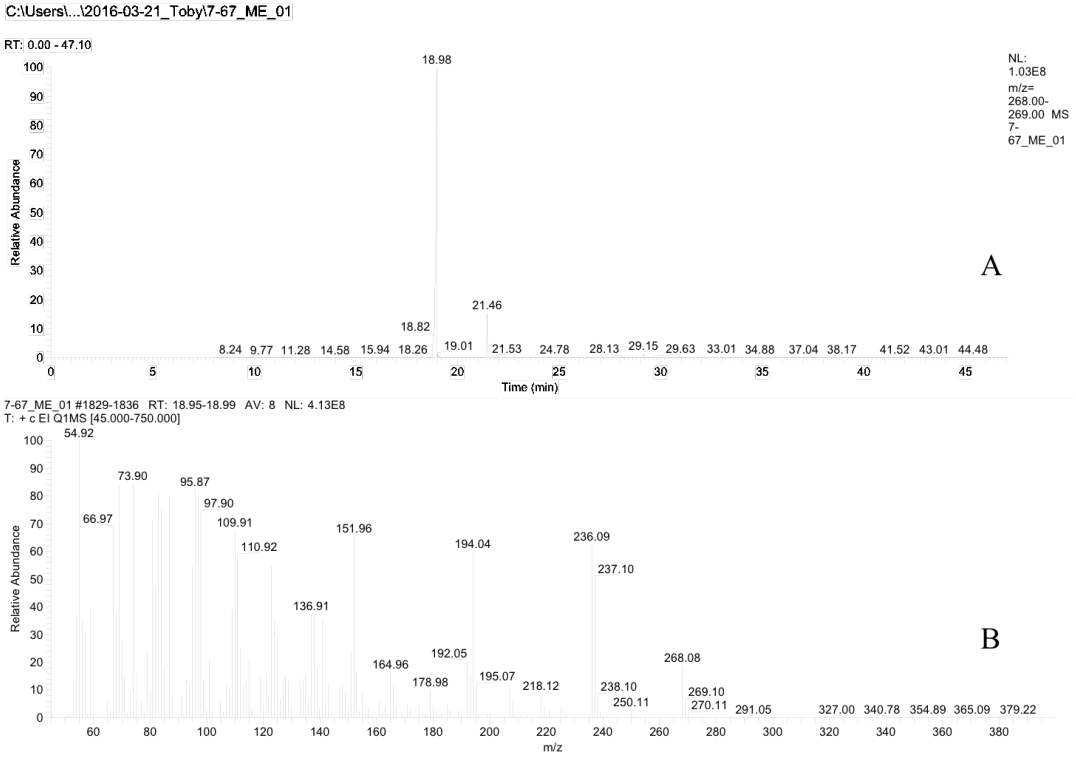

Figure 4.8: GC-MS chromatogram (labelled A) and mass spectrum (labelled B) of fraction TMB-122-F67/68-7 methyl ester. The mass spectrum displayed represents the peak eluting at retention time 18.98 minutes. A restricted mass range is presented to highlight the peak of interest from the methyl ester background.

A head to tail comparison of the mass spectrum obtained against the mass spectrum from the library is presented in Figure 4.9. To confirm this assignment an underivatised and trimethylsilyl (TMS) derivative were analysed and produced identical results. The elucidated structure TMB-122-F67/68-7 was determined to be of 9-hexadecenoic acid, $C_{16}H_{30}O_2$ is presented in Figure 4.10. Characterisation of the exact geometric isomer/s of 9-hexadecenoic acid was not possible from the acquired data and is indicated in the chemical structure.

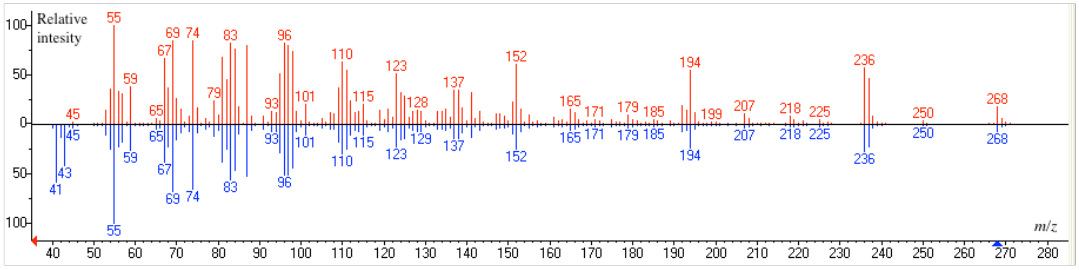

Figure 4.9: Head to tail MS spectrum comparison (NIST 2.0) between the major peak from the GC-MS analysis of TMB-122-F67/68-7 methyl ester (Red) and 9-hexadecenoic acid methyl ester (blue, Wiley library W9N11.L).

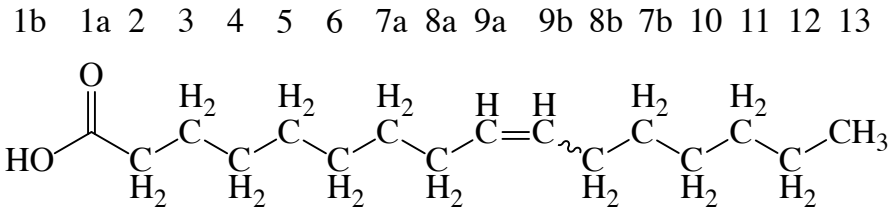

Figure 4.10: Assignment of TMB-122-F67/68-7, 9-hexadecenoic acid, labelled with positions.

Fatty acids from a wide variety of sources, including plant and animal and bacterial, have been shown to exhibit antimicrobial activity against important pathogenic microorganisms including *Mycobacteria sp.* (Sandoval-Montemayor et al., 2012) and Methicillin-resistant *Staphylococcus aureus* (Desbois et al., 2008, Desbois and Smith, 2009). In general, unsaturated fatty acids display greater antimicrobial activity than saturated fatty acids of the same length, furthermore increased length of the fatty acid chain is typically associated with greater antimicrobial activity (Desbois and Smith, 2009). The long chain unsaturated fatty acid, 9-hexadecenoic acid has previously been isolated from *Vibrio sp.* (Li et al., 2014), a marine diatom (Desbois et al., 2008), insects

(Urbanek et al., 2012) and numerous plant species (Gur et al., 2006). These compounds possess activity against gram-positive organisms, but Gram-negative bioactivity is most common for long chain unsaturated fatty acids like 9-hexadecenoic acid (Zheng et al., 2005), as was the case in this report.

The antibacterial activity observed from 9-hexadecenoic acid may be a result of a number of processes including, the inhibition of fatty acid synthesis by binding to the bacterial enoyl-acyl carrier protein reductase, FabI (Zheng et al., 2005), interfering with the electron transport chain and disrupting oxidative phosphorylation (Wojtczak and Więckowski, 1999), and the inhibition of nutrient uptake and cell lysis (Kenny et al., 2009).

The production of 9-hexadecenoic acid by the stingless bee-associated *Xanthomonas* sp. may protect the bee itself from opportunistic infection or protect the food stores accumulated in the hive as fatty acids have been shown to inhibit bacterial pathogens of honey bees including *Melissococcus pluton* and *Bacillus larvae* (Feldlaufer et al., 1993). Furthermore Australian *Eucalyptus* spp. have been shown produce low levels of fatty acids (0.59–1.9%) as compared to plant species that have evolved with European honey bees (Manning and Harvey, 2002) and as a result the stingless bee associated *Xanthomonas* sp. examined in this study may provide a fatty acid adjunct to that present in the local flora. Fatty acids have also been identified as components of the nest mate recognition in stingless bees and this may also be a role supported by the microbial production on these compounds (Buchwald and Breed, 2005).

The discovery of the antimicrobial fatty acid, 9-hexadecenoic acid, in the *Xanthomonas* sp. TMB-122, isolated from the cuticle of the stingless bee *A. australis* supports the hypothesis that antimicrobial compounds found in stingless bees and honey can have a microbial origin. It should be noted that the detected 9-hexadecenoic acid is likely the result of over expression by the *Xanthomonas* sp. TMB-122 as the strain was grown in isolation of the host bee species. However, a prior ongoing association with the stingless bee could influence the gene expression of the strain even when separated from the host.

The production of 9-hexadecenoic acid by *Xanthomonas* spp. has previously been reported, however the context of the research was not related to antimicrobial activity, instead the fatty acid profile was used for identification of similar species of plant pathogen (Peters and Chin, 2003). 9-hexadecenoic acid can also inhibit photosynthesis (Peters and Chin, 2003) and this may play a role in plant pathogenesis of some *Xanthomonas* sp.

4.3.4.2 TMB-122-F5

The RP-FPLC purified TMB-122-F5 was obtained as a white solid. The positive ion ESI high resolution Fourier Transform mass spectrum (HRFTMS) displayed a protonated ion $[M+H]^+$ at 207.17365 (Δ -3.3 ppm from the monoisotopic mass) which was consistent with the formula $C_{14}H_{22}O$ requiring 4 rings and double bond equivalents (RDBE).

The ^{1}H NMR spectra in $CDCl_3$ indicated the presence of three aromatic protons, δ 7.54 (H-3), δ 7.13 (H-4) & δ 7.36 (H-6), (Table 4.4). HSQC NMR experiment results indicated that these protons were attached to carbons at 119.1 ppm (C-3), 124.0 ppm (C-4) and 124.5 ppm (C-6) respectively. COSY NMR experiment results suggested that the protons at δ 7.13 and δ 7.54 were adjacent to each other. Integration of the ^{1}H NMR and HSQC experiment spectra indicated that a single proton was present on each of these carbons. The HMBC experiment indicated the presence of 5 quaternary carbons 34.6, 34.9, 138.5, 147.1 & 147.7 ppm. The aromatic carbons at 138.5 ppm (C-2), 147.1 ppm (C-5), 147.7 (C-1), 119.1 ppm, 124.0 ppm and 124.5 ppm suggested a six membered ring, which would satisfy the 4 RDBE established from the MS analysis.

Table 4.4: NMR data for TMB-122-F5 in $CDCl_3$ at 600MHz (^{1}H).

Position	**^{13}C**	**^{1}H (δ, mult, J_{ab}, J_{bc})**	**COSY**	**HMBC ($^{2}J_{CH}$, $^{3}J_{CH}$, $^{4}J_{CH}$)**
1	147.7			
2	138.5			
3	119.1	7.54 (dd, 8.6 Hz, 0.7 Hz, 1H	H-4, H-6	C-2, C-5, C-7
4	124.0	7.13 (dd, 8.6 Hz, 2.5 Hz, 1H)	H-3, H-6	C-5, C-6, C-9
5	147.1			
6	124.5	7.36 (t, 2.2 Hz, 1H)	H-4, H3	C-1, C-4, C-9, C-7
7	34.9	-		
8 a, b, c	30.2	1.34, (s, 9H)		C-7, C-2, C-8
9	34.6			
10 a, b, c	31.5	1.29 (s, 9H)		C-9, C-5, C-10
11 (OH)		Not observed		

Assignment of the position in the ring, and partial structure A, were suggested by the HMBC correlations between the proton at δ 7.54 and the carbons at 138.5 ppm and 147.1 ppm, between the proton at δ 7.13 and the carbons at 147.1 ppm and 124.5 ppm, and the proton at δ 7.36 and the carbons at 124.0 ppm and 147.7, (Figure 4.11).

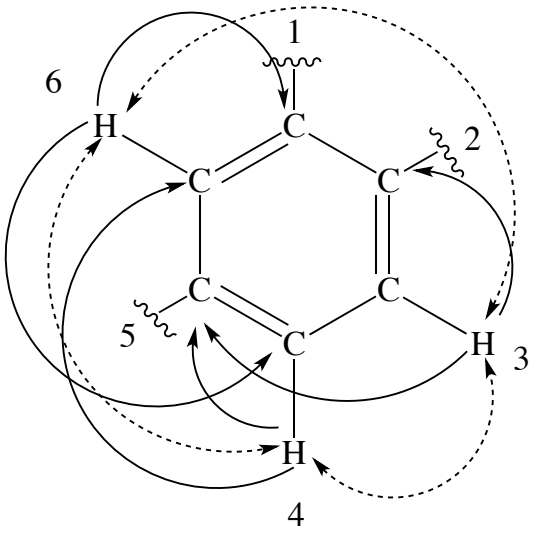

Figure 4.11: TMB-122-F5, Partial structure A, showing COSY correlations (dashed line) and HMBC correlations.

The HMBC correlation between the proton at δ 7.54 and the quaternary carbon at 34.9 ppm (C-7), the proton a δ 7.13 and the quaternary carbon at 34.6 ppm (C-9), and the proton at δ 7.36 and both quaternary carbons at 34.9 ppm and 34.6 ppm, suggested the assignment of the quaternary carbons 34.9 ppm and 34.6 ppm at positions C-7 and C-9 respectively (Figure 4.12). The ^{1}H NMR and HSQC experiments indicated tri-methyl protons at δ 1.34 (H-8) and δ 1.34 (H-9), attached to the carbons at 30.2 ppm (C-8) and 31.5 ppm (C-9) respectively. The HMBC correlations between the protons at δ 1.34 and the carbons at 30.2 ppm, 34.9 ppm and 138.5 ppm, in addition to the HMBC

correlations between the protons at δ 1.29 and the carbons at 31.5 ppm, 34.6 ppm and 147.1 ppm permitted the assignment of partial structure B, (Figure 4.12).

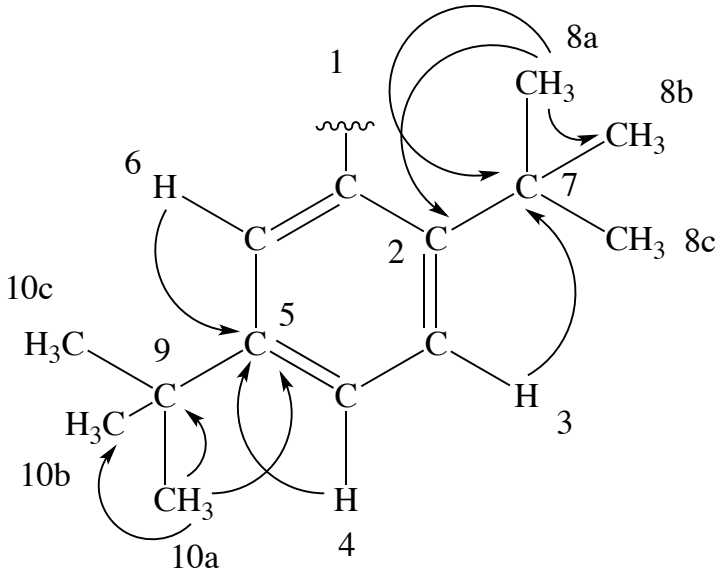

Figure 4.12: TMB-122-F5 partial structure B, showing COSY correlations (dashed line) and HMBC correlations.

The chemical shift of the carbon at 147.7 ppm (C-1) suggests the presence of a hydroxyl group that was not observed in the ^{1}H NMR spectrum. The elucidated structure of TMB-122-F5 as 2,5-di-*tert*-butylphenol is presented in Figure 4.13.

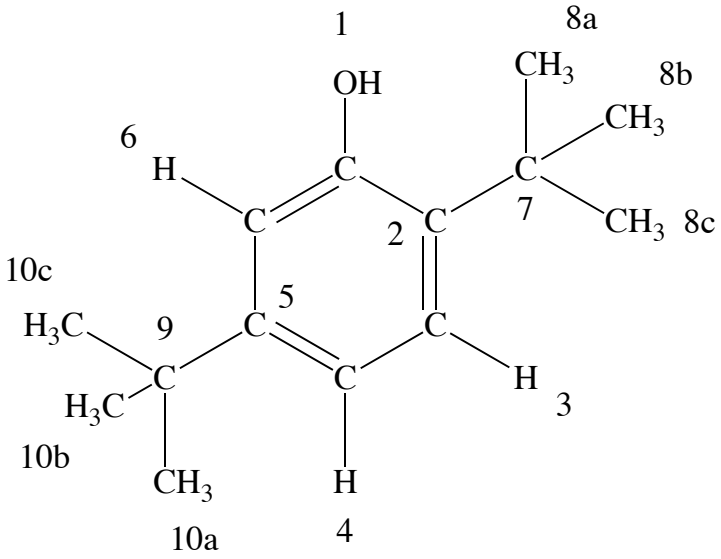

Figure 4.13: Assignment of TMB-122-F5, 2,5-di-*tert*-butylphenol, labelled with positions.

The phenolic compound 2,5-di-*tert*-butylphenol identified in this study has been previously isolated from a number of organisms, including a marine *Streptomyces* sp. (Nandhini, 2015) an ascidian (Ali H et al., 2015) and a number of plant species used as traditional medicines (Jemia et al., 2013). These studies identified antimicrobial, anti-inflammatory and antiproliferative activities from 2,5-di-*tert*-butylphenol. The production of phenolic compounds is primarily associated with plants (Matsuki, 1996) and some anaerobic bacteria (Smith and Macfarlane, 1996) with phenolic compounds similar to 2,5-di-*tert*-butylphenol identified as signalling molecules in plant-microbe interactions (Peters and Verma, 1990) and antifungal compounds (Prakash and Namasivayam, 2014). This is the first report on the production of 2,5-di-*tert*-butylphenol in a *Xanthomonas* species. The presence of this antimicrobial compound in microorganism isolated from the cuticle of a stingless bee is congruent with our hypothesis that some bioactive compounds from stingless bees are of microbial origin.

4.3.4.3 TMB-122-F6

The RP-FPLC purified TMB-122-F6 was obtained as a pale-yellow liquid. The positive ion ESI high resolution Fourier Transform mass spectrum (HRFTMS) displayed a protonated ion $[M+H]^+$ at 183.21137 (Δ 3.5 ppm from the monoisotopic mass) which was consistent with the formula $C_{13}H_{26}$ requiring 1 ring and double bond equivalents (RDBE).

Table 4.5: NMR data for TMB-122-F6 in $CDCl_3$ at 600 MHz (^1H).

Position	**^{13}C**	**^1H (δ, mult, J_{ab}, J_{bc})**	**COSY**	**HMBC ($^2J_{CH}$, $^3J_{CH}$, $^4J_{CH}$)**
1a	114.1	4.93 (ddt, J = 10.2, 2.3, 1.2 Hz, 1H)	H-2	C-2, C-3
1b	114.1	5.00 (dq, J = 17.1, 1.7 Hz, 1H)	H-2	C-2, C-3
2	139.3	5.82 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H)	H-1a, H-1b, H-3	C-3, C-4
3	33.9	2.05 (m, 2H)	H-2, H-4	C-2, C-4, C-1
4	29.0	1.38 (m, 2H)	H-5	C-3, C-5, C-2
5	29.3	1.29 (m)	H-4	
6 a, b, c, d, e	29.7	1.27 (m)		C-8, C-9, C-7, C-6a,C-6b, C-6c, C-6d, C-6e
7	32.0	1.27 (m)		C-8, C-9, C-6c, C-6b
8	22.8	1.31 (m, 2H)	H-9	C-7, C-9
9	14.1	0.89 (t, J = 7.0 Hz, 3H)	H-8	C-8, C-7

The ^{1}H NMR spectra in $CDCl_3$ indicated the presence of three alkenyl protons δ 4.93 (ddt, 1H), δ 5.00 (dq, 1H) and δ 5.82 (ddt, 1H), (Table 4.5). The HSQC spectra showed that the protons at δ 4.93 (H-1a) and δ 5.0 (H-1b) were attached to the same carbon at 114.1 ppm and both protons showed COSY NMR correlations to the proton at δ 5.82 (H-2). The coupling constants of the proton at δ 4.93, 10.2 Hz and 2.3 Hz, and the proton at δ 5.0 1b, 17.1 Hz and 1.7 Hz, indicate that the proton at δ 4.93 and the proton at δ 5.82 were in a vicinal, cis arrangement and the proton at δ 5.0 and the proton at δ 5.82 were in a vicinal, trans arrangement, which permitted the assignment of partial structure A, (Figure 4.14).

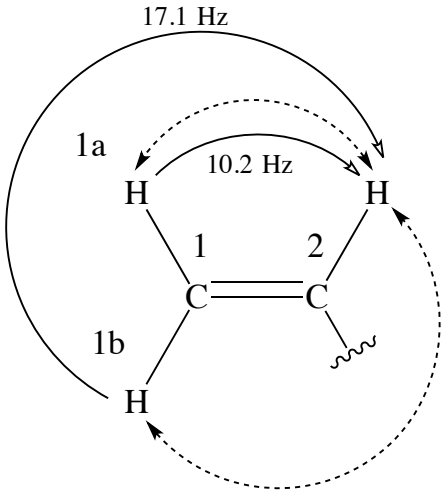

Figure 4.14: TMB-122-F6 partial structure A, showing COSY correlations (dashed line) and coupling constants $J_{1a,2}$ (10.2 Hz) and $J_{1b,2}$ (17.1 Hz) (solid line with hollow arrows).

The ^{1}H and HSQC NMR experiments indicated 3 methylene protons at δ 2.05 (H-3), δ 1.38 (H-4) and δ 1.29 (H-5) attached to carbons at 33.9 ppm (C-3), 29.0 ppm (C-4) and 29.3 ppm (C-5). COSY correlations of between the protons at δ 2.05 and δ 1.38,

and the protons at δ 1.38 and δ 1.29 allowed the assignment of partial structure B that was confirmed by the HMBC correlations, (Figure 4.15).

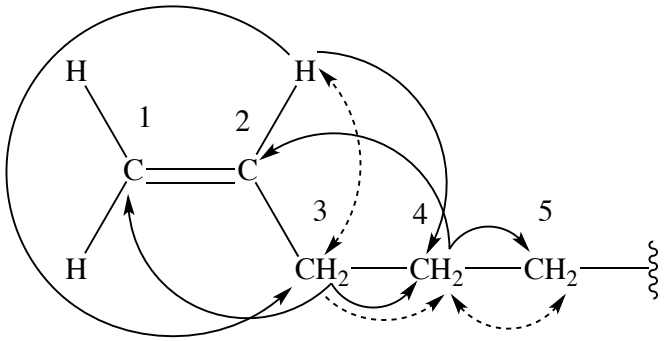

Figure 4.15: TMB-122-F6 partial structure B, showing COSY correlations (dashed line) and HMBC correlations.

Further extension from partial structure B was difficult due to overlapping methylene signals. The ^{1}H and HSQC NMR experiments indicated the presence of a methyl group, 3 protons at δ 0.89 (H-9) attached to a carbon at 14.2 ppm, and two methylene groups, two protons at δ 1.31 (H-8) attached to a carbon at 22.8 ppm in addition to two protons δ 1.27 (H-7) attached to a carbon at 32.0 ppm. The COSY experiment suggested that the protons at δ 1.31 was adjacent to the protons at δ 0.89, and the HMBC correlations suggested the protons δ 1.31 was adjacent to the carbon at 32.0 ppm (C-7), allowing the assignment of partial structure C, (Figure 4.16).

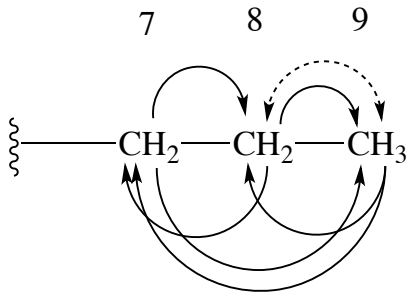

Figure 4.16: TMB-122-F6 partial structure C, showing COSY correlations (dashed line) and HMBC correlations.

Again, further extension from partial structure C was difficult due to overlapping methylene signals. Mass spectrometry data was required to complete the structure by correlating the mass of the compound ion with the partial structures A, B and C. The elucidated final structure of TMB-122-F6, tridec-1-ene, is presented in Figure 4.17.

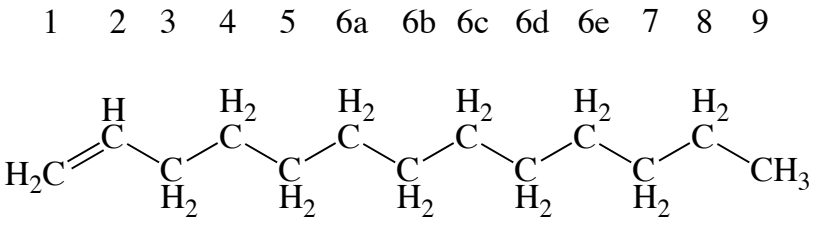

Figure 4.17: Elucidated structure of TMB-122-F6, tridec-1-ene, labelled with positions.

The acyclic olefine hydrocarbon, tridec-1-ene, can be found in a wide variety of organisms, including insects (Hurst et al., 1964, Walter et al., 1993, Hefetz et al., 1996, Fávaro and Zarbin, 2013), microorganisms (Rude et al., 2011) and plants (Verma et al., 2012). The microbial production of tridec-1-ene in some *E. coli* spp. is facilitated by

membrane bound desaturase-like enzymes that can convert fatty acids into 1-alkenes by oxidative decarboxylation (Rui et al., 2015). However, the production of alkenes in some *Xanthomonas* spp. occurs via enzymatic head-to-head condensation of fatty acids (Goblirsch et al., 2012). Tridec-1-ene plays a distinct role in the life cycle of a number of insects including bumble bees, ants and beetles where it is produced by the endocrine system with varying roles including sex pheromones, defensive allomones and recognition pheromones (Hurst et al., 1964, Walter et al., 1993, Hefetz et al., 1996, Fávaro and Zarbin, 2013). The production of a compound that plays an important role in insect chemical communication may benefit this *Xanthomonas* sp. by eliciting recognition reaction from the stingless bee thus preventing removal of the biomass from the insect cuticle or the hive during grooming processes. Also as many bacterial species, including *Xanthomonas* sp., can degrade hydrocarbons it is possible that the tridec-1-ene identified in this extract is a degradation product of a more complex compound.

4.3.4.4 TMB-122-F7

The RP-FPLC purified TMB-122-F7 was obtained as a white crystalline solid. The ^{1}H NMR spectrum in $CDCl_3$ in indicated the presence of three methylenes at δ 1.32 (H-12), δ 1.62 (H-11) and δ 4.08 (H-10) that were attached to carbons at δ 25.9 ppm, 28.7 ppm and 64.7 ppm respectively in the HSQC experiment, (Table 4.6).

Table 4.6: NMR data for TMB-122-F7 in $CDCl_3$ at 600 MHz (^1H).

Position	^{13}C	^1H (δ, mult, J_{ab}, J_{bc})	COSY	HMBC ($^2J_{CH}$, $^3J_{CH}$, $^4J_{CH}$)
1	152.1			
1b		5.08 (s, 1H)		C-1, C-2
2a, 2b	135.8			
3a, 3b	124.8	7.00 (s, 2H)		C-2, C-3, C-5, C-7
4	131.1			
5	34.3			
6a, b, c, d, e, f	30.3	1.44 (s, 18H)		C-5, C-6, C-2
7	31.0	2.88 (m, 2H)	H-8	C-4, C-8, C-3, C-9
8	36.6	2.60 (m, 2H)	H-7	C-7, C-9, C-4
9	173.4			
10	64.7	4.08 (t, J = 6.8 Hz, 2H)	H-11	C-11, C-9, C-12
11	28.7	1.62 (m, 2H)	H-10, H-12	C-10, C-12
12	25.9	1.32 (m)	H-11	
13a -> 13m	29.7	1.27 (m)		C-13
14	22.7	1.30 (2H)	H-15	
15	14.1	0.89 (t, J = 7.0 Hz, 3H)	H-14	C-14

The COSY NMR experiment indicated that the protons at δ 1.32 were adjacent to the protons at δ 1.62, which in turn were adjacent to the protons at δ 4.08. HSQC and HMBC correlations placed the protons at δ 4.08 within four bonds from a carbonyl carbon at 174.5 ppm (C-9). The ^1H NMR and HSQC experiments also indicated two methylene groups at δ 2.6 (H-8) and δ 2.88 (H-7) attached to carbons at 36.6 ppm and 31.0 ppm respectively. COSY and HMBC correlations indicated that the protons at

δ 2.60 were adjacent to the protons at δ 2.88 and both were within four bonds of carbonyl carbon at 174.5 ppm, permitting the assignment of partial structure A, (Figure 4.18).

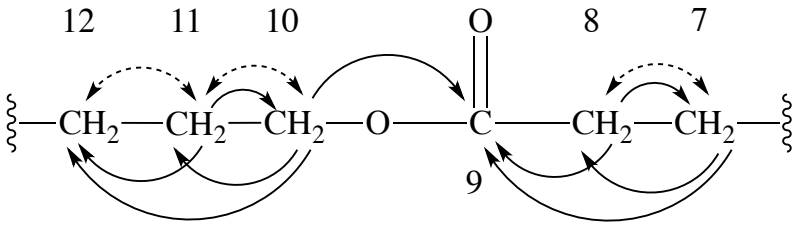

Figure 4.18: TMB-122-F7 partial structure A, showing COSY correlations (dashed line) and HMBC correlations.

The ^{1}H chemical shift and HSQC correlation of the protons at δ 7.0 (H-3) attached to the carbon at 124.8 ppm was indicative of a ring system. HMBC correlations from the protons at δ 7.0 to the carbon at 124.8 ppm suggested a symmetrical aromatic ring. The HSQC data indicated an additional 5 quaternary carbons at 173.4 ppm (C-9), 34.3 ppm (C-5), 131.1 ppm (C-4), 135.8 ppm (C-2) and 152.0 ppm (C-1) and all, except the carbons at 34.3 ppm and 173.4 ppm, were characteristic of aromatic carbons. The HMBC correlations together with the data above supported a ring system with symmetrical attachment of substituents, assigned as partial structure B, (Figure 4.19).

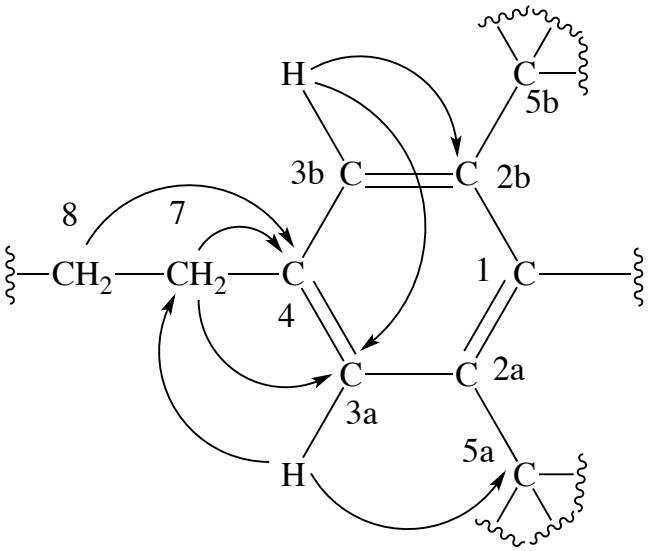

Figure 4.19: TMB-122-F7 partial structure B, showing HMBC correlations.

The proton at δ 5.08 (H-1b) was assigned as a hydroxyl group based on the ^{1}H chemical shift and the absence of an attached carbon in the HSQC experiment together with the HMBC correlation to the carbon at 152.0 ppm. Two tri-methyl groups attached to the ring at positions 2a and 2b were predicted based on the ^{1}H and ^{13}C shifts, the integration of the proton signal at δ 1.44 (H-6) and the HMBC correlations from the protons at δ 1.44 to the carbons at 30.3 ppm (C-6), 34.3 ppm (C-5) and 135.8 ppm (C-2), which permitted the assignment of partial structure C (Figure 4.20).

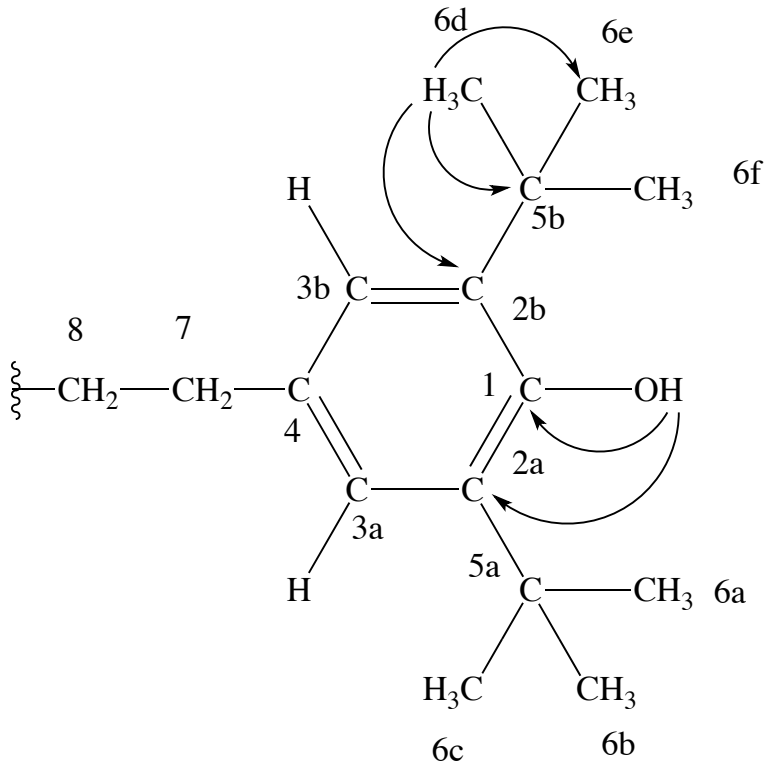

Figure 4.20: TMB-122-F7 partial structure C, showing HMBC correlations.

The remaining portion of the molecule consisted of a chain of methylene groups that were difficult to accurately account for due the overlapping of their signals in both the ^{1}H and ^{13}C NMR spectra, however it was possible to predict the methylene chain was terminated by a methyl group from the ^{1}H shift and HSQC data of the protons at δ 0.89 (H-15) attached to the carbon at 14.1 ppm (C-15).

With the majority of the molecule assigned the remaining portion could be elucidated with reference to the mass of the molecule. Attempts to obtain high resolution mass spectra using electrospray ionization were unsuccessful. A literature search of similar structures indicated a GC-MS approach might provide the information required. The

positive mode chemical ionization GC-MS mass spectrum displayed a major peak at retention time 40.04 minutes with a *m/z* 530, $[M]^+$ (Figure 4.21).

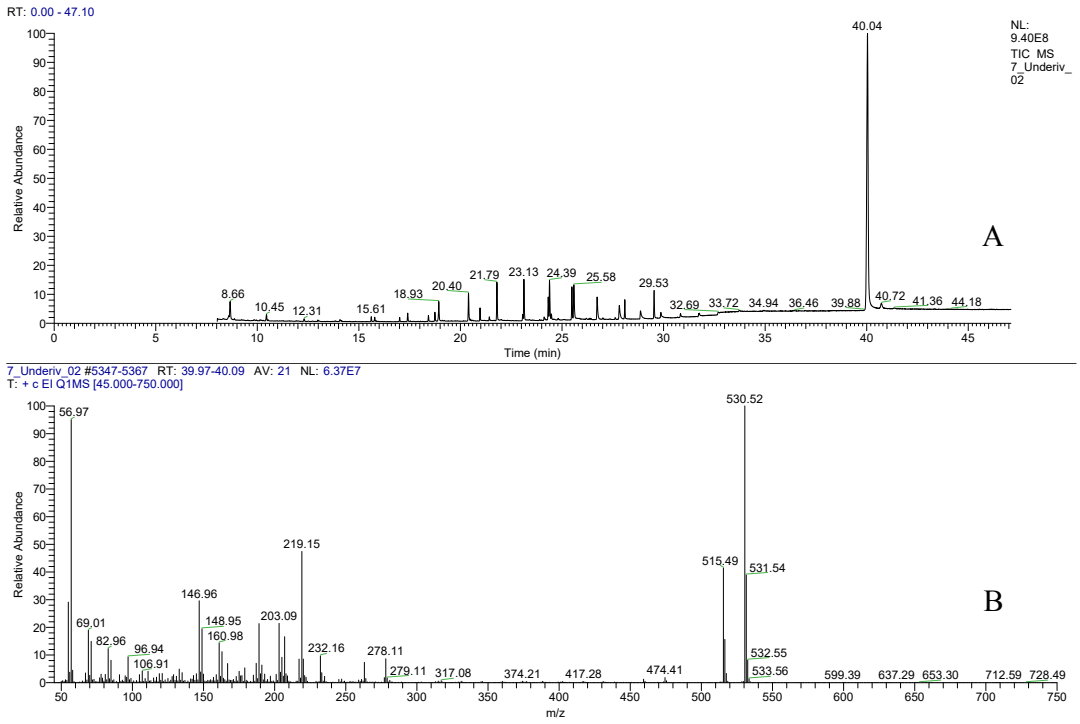

Figure 4.21: GC-MS chromatogram (labelled A) and mass spectrum (labelled B) of fraction TMB-122-F7. The mass spectrum displayed represents the peak eluting at retention time 40.04 minutes.

The mass spectrum created was compared with the Wiley mass spectral library (Wiley W9N11.L and NIST 2.0 version) and a positive match made to Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, $C_{35}H_{62}O_3$, with a match score 904 and reverse match score 906. Match factors are cosine similarity calculations with the reverse match factor ignoring any peaks in unknown sample but not in the library sample. A head to tail comparison of the mass spectrum obtained (red) against the mass spectrum from the library (blue) is presented in Figure 4.22. To confirm this assignment an underivatised

and trimethylsilyl (TMS) derivative were analysed and produced identical results. The elucidated structure of TMB-122-F7, Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, is presented in Figure 4.23.

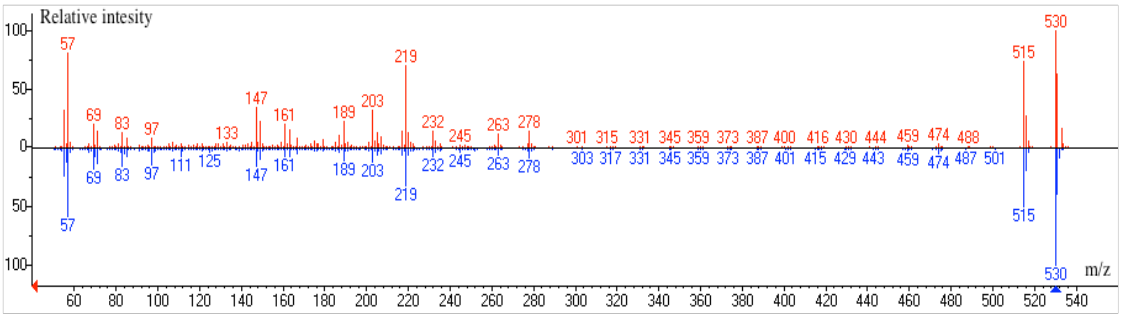

Figure 4.22: Head to tail MS spectrum comparison (NIST 2.0) between the major peak from the GC-MS analysis of TMB-122-F7 (red) and Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate (blue) (Wiley library W9N11.L).

Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate is an unusual compound to isolate from *Xanthomonas* sp. fermentation. Literature regarding this compound is dominated by use as an antioxidant and stabilizing compound in plastics and polymer manufacturing (Galotto et al., 2011, Yu et al., 2015); no bacterial production of this compound has been reported. However, due to the isolation of TMB-122-F5, 2,5-di-*tert*-butylphenol and the presence of fatty acids, e.g. 9-hexadecenoic acid, this type of structure is plausible. Similar structures have been identified from natural sources including an antifungal compound from a *Streptomyces* sp. 3-(3,5-di-tert-butyl-4-fluorophenyl) propanoic acid and 2,4-di-tert-butylphenol an antifungal compound isolated from a *Lactococcus* sp. (Yoon et al., 2006) and a *Pseudomonas* sp. (Dharni et al., 2014).

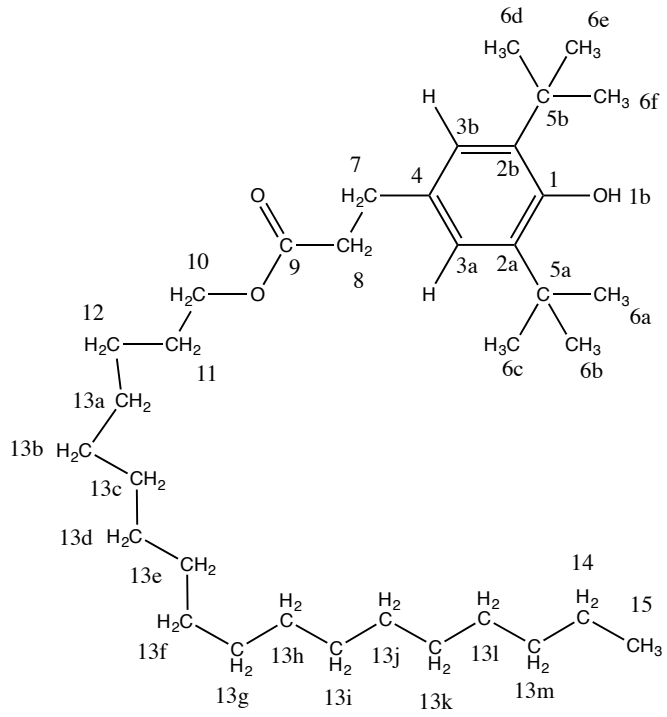

Figure 4.23: Elucidated structure of TMB-122-F7, Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, labelled with positions.

4.4 CONCLUSIONS

This chapter describes the selection of one microorganism, *Xanthomonas* sp. isolate TMB – 122, for compound characterisation, from a pool of 46 bacterial and fungal isolates based on antibacterial activity against *Staphylococcus aureus* NCTC 6571.

Bioassay guided fractionation of the crude ethyl acetate extract identified a bioactive fraction, which then identified as 9-hexadecenoic acid. Three additional compounds identified as, 2,5-di-*tert*-butylphenol, tridec-1-ene and octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate were also successfully purified from the *Xanthomonas* sp. extract. This study adds to the library of compounds isolated from *Xanthomonas* spp.

The *Xanthomonas* sp. TMB-122 was isolated from the cuticle of the stingless bee *A. australis* and of the three the compounds identified in this chapter, 9-hexadecenoic acid is a known antimicrobial fatty acid and 2,5-di-*tert*-butylphenol is an antimicrobial phenolic compound. The presence of these two bioactive compounds supports the hypothesis that antimicrobial compounds found in Australian stingless bees and bee products can come from a microbial origin. In addition, the third compound, tridec-1-ene is a common insect pheromone/allomone that plays a role in nest mate recognition in insect species and its presence in this bacterium may facilitate long term colonization of the insect.

Chapter 5

Conclusions and Future Directions

5.1. RESEARCH MOTIVATION AND OBJECTIVES

The rapid emergence and transfer of antimicrobial resistance in pathogenic organisms has greatly reduced our ability to treat clinical microbial infections (Poole, 2002). As a result antibiotics once considered drugs of last resort, many with serious side effects, are being increasingly used as primary treatment options (Huttner et al., 2012). Furthermore, in some cases, such as antifungal therapies, effective broad spectrum treatments have always been limited (Prasad et al., 2016). To continue to treat clinical microbial infections in humans and animals it is imperative that we discover new antimicrobial compounds with novel modes of action.

The encompassing aim of this thesis was to explore the antimicrobial potential of microorganisms associated with three Australian native stingless bees, *Tetragonula carbonaria*, *Austroplebeia australis* and *Tetragonula hockingsii.* Observations of antimicrobial activity from honey, hive materials and whole extracts of Australian stingless bees inspired the hypothesis that the microbiota associated with these stingless bee species could produce bioactive compounds. To address this aim, a comprehensive evaluation of the native bee whole gut microbiome was performed (chapter 2), with the goal of identifying microorganisms exhibiting specific associations. Guided by these results bacteria and fungi were cultured from the gut and cuticle of three species of Australian stingless bees and screened by genetic and chemical methods to create a subset enriched for chemical diversity and biosynthetic potential (chapter 3). Biosynthetic potential was correlated to the presence of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) gene clusters due to their historical success as therapeutic natural products and their potential to produce a wide array of bioactive

chemical structures. Antimicrobial activity of the selected microbes was assessed by liquid culture bioassay and a successful candidate identified for bioactivity-guided fractionation and compound characterisation (chapter 4).

In the quest for novel antimicrobial compounds new approaches must be developed that can build of the experience and knowledge of past discovery. The clinical success of natural product and natural product derived compounds (Newman and Cragg, 2016) underpinned this examination of a novel environmental niche for microorganisms with the capacity to produce bioactive compounds.

5.2. KEY FINDINGS

5.2.1. Whole gut microbiome of the stingless bee Tetragonula carbonaria

The gut microbiome of the stingless bee *Tetragonula carbonaria* was assessed by 16S rRNA gene pyrosequencing of whole guts from 13 *T. carbonaria* individuals, from a natural (Log) and man made hive (Box). Analysis revealed that gut bacterial compositions varied amongst the individual bees however on average no significant difference was observed between the two different hive populations. This result highlighted the importance of a robust sampling strategy when sampling stingless bee microbiota.

Analysis of the bacterial OTUs across all individuals revealed that 56% could be defined as being part of a core community that was present in at least 70% of the individuals with, 39% being variable and 5% specific to only one bee. The average *T. carbonaria* gut microbiome was dominated by sequence reads annotated as belonging to two bacterial orders; *Lactobacillales* (44.3%± 21.3) and *Rhodospirillales* (27.9%± 24.5). Sequence reads annotated as *Oceanospirillales* (9.4% ±19.6), *Neisseriales* (5.3%±14.7) and uncharacterized *Gammaproteobacteria* (5.9%± 13.8) contributed a large proportion of the remainder of the stingless bee gut microbiome.

This study was the first attempt to examine the gut bacteria found in individual Australia stingless bees. To date only one other report has characterised the bacterial community present in these native bee species. The results obtained in this study are overall in agreement with the previous work and phylogenetic examination of

Lactobacillaceae OTUs and confirmed the existence of Australian stingless bee specific Lactobacillaceae separate from all other Lactobacillaceae currently characterised.

5.2.2 Culturing, genetic screening and chemical analysis of microorganisms associated with the Australian stingless bees, **Tetragonula carbonaria, Austroplebeia australis** *and* **Tetragonula hockingsi**

Inspired by the observations of antimicrobial activity in stingless bee honey, hive provisions and whole bee extracts we hypothesised that the microbiota associated with these bees could contribute to the antimicrobial activities observed. Guided by the result obtained in Chapter 2 bacteria and fungi were isolated from the gut and cuticle of three species of Australian stingless bee. A total of 559 microbial isolates were collected from bees in varied stages of development. Dereplication according to colony morphology identified 95 distinct phenotypes that were then characterised by genetic and chemical analysis.

Taxonomic classification of 42 bacterial isolates revealed that the sampling strategy employed captured representatives of most bacterial classes identified in Chapter 2. With the exception of the Lactobacillales, which require microaerobic/anaerobic growth conditions. PKS and NRPS gene clusters were identified in 36% of the bacteria examined and their presence was used as an indicator of antimicrobial biosynthetic potential. The presence of these biosynthetic gene clusters is well correlated with the production of complex and diverse compounds.

The fungal species isolated from Australian stingless bees were dominated by yeast species, with 44% identified as part of the *Candida* sp., *Metschnikowia* sp. species complex. This result was congruent with previous cultured based examinations of bees and stingless bees from around the world. Seventy five percent of the fungal isolates examined tested positive for either PKS or NRPS gene clusters.

In parallel to the genetic screening, untargeted LC-MS metabolite profiling was employed to group the isolates based on their chemical similarity. Statistical analyses were used to bin the mass spectra using a similarity cut off between 37%-57%. A total of 12 bacterial and 17 fungal chemical groups were obtained.

The combined genetic and chemical analysis permitted the methodical selection of a subset of 46 isolates, from the initial 94, for antimicrobial compound assessment and bioassay guided compound purification and characterisation. Furthermore, the analyses performed revealed that Australian stingless bee species represent a highly rich source of both NRPS and PKS containing microbes.

5.2.3 Antimicrobial activity of Australian stingless bee associated microorganisms and the characterisation of compounds from a Xanthomonas sp.

Assessment of antimicrobial activity in chemical extracts from 46 microbes isolates, from Australian native stingless bees, prioritised two candidates TMB - 122, *Xanthomonas* sp., and TMB - 118, *Oceanobacillus* sp., with anti-*Staphylococcus aureus*

activity. Following a literature review of each organism and referencing experimental observations TMB - 122 was selected as the target organism for large-scale culture, extraction, and bioassay guided compound fractionation.

The antimicrobial long chain fatty acid, 9-hexadecenoic acid, was isolated and characterised from the *Xanthomonas* sp., TMB - 122. Additional characterisation identified the production of 2,5-di-*tert*-butylphenol, a phenolic compound with antimicrobial activity (Nandhini, 2015), tridec-1-ene, an acyclic olefin insect pheromone, and octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) by TMB - 122, *Xanthomonas* sp. The production of these antimicrobial compounds in a microorganism isolated from the cuticle of the Australian stingless bee *A. australis* supports the hypothesis that microbial associates of stingless bees contribute to the antimicrobial activity observed in stingless bees and their provisions.

Furthermore, this study adds to the library of compounds isolated from *Xanthomonas* sp. and further unravels the complex interplay between single cellular life and higher eukaryotes such as stingless bees.

5.3 FUTURE DIRECTIONS

This thesis has been successful in identifying secondary metabolites produced by cultured microbes obtained from the Australian stingless bee. However, a vast source of potentially unculturable bacteria remains to be explored. In pursuit of the uncultured bioactive potential DNA from the gut or whole bees could be used to create a metagenomic DNA library. These libraries could then be screened for clones containing novel NRPS/PKS gene clusters and expressed in a heterologous host to isolate their products. This method has the benefit of targeting gene clusters that display novel architectures, motifs or predicted products, which can eliminate the rediscovery of know bioactive compounds. However heterologous expression and identification of selected gene clusters is a complex and cryptic process that can sometimes require equal parts of skill and luck to achieve success.

From an ecological perspective there is a clear need to link the production of bioactive small molecules by endosymbiotic bacteria with the lifecycle of stingless and honey bees. Many bioactive small molecules exhibit antimicrobial properties but it is unclear whether this property is the primary role of the molecule for the producing organism or whether this is simply a secondary effect. Within the context of the host-microbe interaction, several small molecules have been shown to act as signalling molecules triggering stages within the lifecycle of the host. Typically, this process is critical for ensuring the survival of both the host and the microbial population such that there is active selective pressure to maintain production of these molecules. The production of the antimicrobial agent by a gut-associated symbiont of the Australian native stingless bee is important from a pharmacological aspect but it is unclear whether this would elicit a similar selective pressure. Given the differences between the nutrient environment of the stingless bee gut and that of the growth media used for cultivation of these microorganisms, it is possible that stimulation of these bioactive molecules was only permitted under laboratory conditions. Therefore, future work taking advantage of advances in imaging mass-spectrometry, should be directed towards establishing whether these molecules are produced *in situ*. In this regard, determining the factors that influence the occurrence and localisation of these molecules and their producers within

the cuticle, gut and honey of the native stingless bees may shed light on the true ecological role of such molecules.

5.4 CONCLUDING REMARKS

The aim of this thesis was to explore the biological and chemical diversity harboured with microorganisms associated with a complex, social organism, the Australian stingless bee. Practical applications of the knowledge gained drove an antimicrobial bioprospecting methodology that identified antimicrobial compounds in a *Xanthomonas* sp. isolate from the cuticle of the Australian stingless bee *Austroplebeia australis*.

This thesis began with an exploration the bacterial diversity present in the gut of a stingless bee species, known to exhibit antimicrobial activity against clinical microorganisms. We then focused on the bacteria and fungi found, in and on, three species of stingless bee targeting the whole bee gut and cuticle surface. A library of these organisms was created and curated by phenotypic, genetic and chemical profile. By targeting organism with PKS and NRPS gene clusters and chemical diversity we were able to methodically select organisms for further investigation. This approach resulted in the isolation and characterisation of antimicrobial compounds in large-scale culture.

This investigation confirmed our hypothesis that microbes associated with Australian stingless bee were capable of producing bioactive molecules. Furthermore, the combined genetic and chemical analyses performed validated the selection of candidates possessing PKS and NRPS gene clusters, as this selection criteria accounted for 75% of the chemical diversity observed by LC-MS profiling.

The steps enclosed in this thesis are the practical application of the theory that modern drug discovery methods must build on the knowledge of the past whilst continuing to innovate and explore. In practise this thesis has illuminated some of the intricate biological and chemical interactions that exist within this ancient continent we call Australia.

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