Coprophilous Fungi from Koala Faeces: 
A Novel Source of Antimicrobial Compounds

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Abstract

An urgent need for novel antimicrobial compounds is driven by the increased resistance of pathogens to current drugs and the rising incidence of opportunistic infections in immunosuppressed individuals. Natural products and their derivatives have long been exploited for their pharmaceutical potential, and fungi have provided numerous chemically and biologically diverse secondary metabolites. Coprophilous fungi remain relatively unexplored compared with fungi from other substrata and biological niches, despite the fact that they are prime candidates for the discovery of antimicrobials due to their ubiquity and their dominance in a highly competitive environment. This research presents, for the first time, the screening of coprophilous fungi from koala faeces for antibacterial, antifungal and anti-quorum sensing activity.

Fungi were isolated from the faeces of koalas living in Boho South and French Island in Victoria, Australia. The 31 fungal isolates were identified by DNA sequencing and submitted to the National Center for Biotechnology Information, where they represent only the second set of coprophilous fungi to have been isolated from koala faeces. All but one of the isolates were members of the phylum Ascomycota, a weighted diversity that is common in Ascomyceteous-dominated coprophilous collections in the literature.

Extracts were prepared by lyophilisation and liquid extraction of the fermentation liquors and mycelial biomass. Antibacterial activity was assessed against one Gram-positive bacterium \textit{Staphylococcus aureus} and three Gram-negative bacteria: \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa} and \textit{Klebsiella pneumoniae}. In plate-hole diffusion assays, 54.8\% of the fungi produced extracts that were capable of inhibiting at least one test bacterium. The lowest minimum inhibitory concentration values were attributed to the extracts from a \textit{Fusarium oxysporum} isolate (S6W2, 1.56 mg/mL against \textit{S. aureus} and \textit{K. pneumoniae} and 0.78 mg/mL against \textit{E. coli}) and a \textit{Sordaria alcina} isolate (F14P1, 3.16 mg/mL against \textit{P. aeruginosa}). Antifungal activity against \textit{Candida albicans} and \textit{Drechslera brizae} was tested, and 8.6\% and 38.7\%, respectively, of the coprophilous fungi produced extracts that inhibited the test fungi in
agiar assays. An extract from isolate S6W2 demonstrated the lowest minimum inhibitory concentration against *C. albicans* (0.52 mg/mL) and an extract from an *Aspergillus niger* isolate (S5P3) demonstrated the lowest minimum inhibitory concentration against *D. brizae* (6.25 mg/mL). In disk diffusion assays against the indicator strain *Chromobacterium violaceum*, 12.9% of the fungi produced extracts that exhibited anti-quorum sensing activity.

The liquor extract from isolate S6W2 was separated by activity-guided fractionation using XAD-16 resin and analytical and preparative reversed-phase high-performance liquid chromatography in conjunction with plate-hole diffusion and microdilution assays. Following analysis with nuclear magnetic resonance spectroscopy and mass spectrometry, the bioactive compound was identified as fusaric acid.

This research suggests that coprophilous fungi from koala faeces may represent a source of novel antimicrobials that warrant further exploration, especially given the paucity of research on this particular source.
Memorial

In memory of Sydney Melbourne Brisbane
(02.05.1915–25.02.2009)

with whom I shared a curious mind.

See you in the Spring.
Dedication

I dedicate this thesis to my parents, Julie and Douglas, for their unwavering support, love and belief in me.
Acknowledgements

I would like to thank my supervisor, Enzo Palombo, for fostering my interest in research during my undergraduate and honours studies. I have always appreciated his optimistic and enthusiastic approach to research and academia. With his open-door policy, he created an environment where I felt encouraged to discuss all matters of science. No question was considered too far-fetched or ingenuous, and his good sense of humour made it all the more enjoyable. I wish to thank my associate supervisor, Qi Yang, for her support and advice, particularly regarding the mass spectography and nuclear magnetic resonance data. Although we did not see each other very often during my candidature, I am grateful for her help and very kind words. I would like to express my appreciation for the time and assistance provided by Noel Hart. Retirement did not hold Noel back, and along with completing his PhD, he generously went out of his way to help me in the chemistry lab, read my thesis chapters and assist with Chapter Five. I would like to acknowledge the assistance and advice provided by the CSIRO instrument specialists, Stuart Littler, Jo Cosgriff, Carl Braybrook and Roger Mulder. I also acknowledge Kath Handasyde for the collection of koala faeces for this study.

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My PhD was brighter for having met Sarah, and I thank her for her support, advice and fantastic sense of humour. I also extend appreciation to all my other Swinburne friends for their camaraderie, Bita, Shanthi, Kaylass, Liz, Jun, Shahanee, Jiawey, Jafar, Rue, Azadeh, Dhivya, Snehal, Shaku, Vanu, Rashida and Rohan. I thank Justin for the encouragement, advice and time that he has given me since our very first class together in the undergraduate science program. I am very lucky to feel the love and support of many school friends, but I extend a special thank you to Ella for her unwavering belief in me and words of wisdom during challenging times and to Georgie for her life-long friendship and ability to bring me back to non-study life with our uncontrollable laughter. I would also like to acknowledge the quiet support from my two fluffy thesis companions, Smokey and Pepe.

Jacob arrived late in the PhD journey, making a dynamic and energetic entrance, and an overwhelmingly positive impact on my life and subsequently the progress of my thesis. I will forever cherish the love, patience, honesty and support he has provided, particularly during tough times. I cannot wait to start the next part of our lives together.

Finally, I am sincerely thankful to my family for encouraging me to pursue my goals. I dedicate this thesis to my Mum and Dad who have gone above and beyond over my (many) years of study, and without whom I would never have made it. All that I have achieved and all that I am is a testament to their love, sacrifices and belief in me. There have been some challenges along the way but I believe that they have brought us closer together, and for that I am truly grateful. I cannot thank them enough.
Declaration

I, Elisa Hayhoe, declare that this thesis is original work and contains no material that has been accepted for the award any other degree or diploma, except where due reference is made.

To the best of my knowledge, this thesis contains no material previously published or written by any other person except where due reference is made.

Where the work is based on joint research or publications, the relative contributions of the respective workers or authors has been disclosed.

Signature: ..............................................
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<tr>
<td>δ</td>
<td>chemical shift (ppm)</td>
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<tr>
<td>ACN</td>
<td>acetonitrile</td>
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<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<tr>
<td>cfu</td>
<td>colony-forming units</td>
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<tr>
<td>CHX</td>
<td>chlorohexidine</td>
</tr>
<tr>
<td>COSY</td>
<td>correlated spectroscopy (homonuclear)</td>
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<tr>
<td>D$_2$O</td>
<td>deuterium oxide</td>
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<td>EIMS</td>
<td>electron ionisation mass spectrometry</td>
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<td>electrospray ionisation</td>
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<td>sd</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction
1.1 Introduction

Humans have a long history of exploring and exploiting the natural world for the treatment and management of disease. But it was not until the 1940s, with the development of penicillin, that the enormous potential of microorganisms as a source of novel bioactive compounds was realised (Florey et al., 1949). Although the following three decades were very productive and saw the discovery of almost all groups of important antibiotics, pharmaceutical firms began to reduce or eliminate their natural product research programs believing that they were not compatible with recently developed technologies, such as high-throughput screening (Lam, 2007). What followed was a steady decline in the output of the pharmaceutical industry, suggesting that the dismissal of natural product research may have been misguided (Newman and Cragg, 2012).

New antimicrobial compounds are urgently needed to respond to increasing resistance to antibiotics and antifungal agents (WHO, 2014). There is renewed belief that the structural diversity found in nature, coupled with advances in technology, will provide new drug candidates for the treatment of many diseases (Bérdy, 2012; Cragg and Newman, 2013; Li and Vederas, 2009). Fungi harbour a tremendous capacity to produce diverse and complex secondary metabolites, and the vast majority of these remain unexplored (Bérdy, 2005; Brakhage, 2013; Chapman, 2009; Strobel, 2003). One group of fungi that has recently piqued the interest of researchers is the coprophilous fungi that thrive, if not dominate, in the highly competitive environment of faeces.

In the past, the majority of studies of coprophilous fungi have focused on their taxonomy and ecology. Recently, however, the potential of coprophilous fungi for natural product discovery has been demonstrated by a high frequency of bioactive secondary metabolites being isolated from relatively limited efforts (Bills et al., 2013; Essig et al., 2014; Ganesh Kumar et al., 2010; Jayanetti et al., 2015). The koala (Phascolarctos cinereus) has an unusual diet consisting exclusively of leaves from Eucalyptus spp., which are low in nutrients and are toxic to most animals (Moyal,
Chapter 1: Introduction

Koala faeces contain undigested cellulose, highly lignified fibre and tannin, and there are very few studies of the inhabiting fungi (Bell, 2005; Cribb, 1997; Peterson et al., 2009). The current study is the first to explore the capacity for the fungi found in koala faeces to produce antimicrobial compounds.

1.2 Aim

The major aims of this thesis were to:

i. isolate and identify fungi from koala faeces
ii. assess the antimicrobial activity of extracts prepared from the fungi
iii. identify the active compounds within the most active extract.

1.3 Thesis outline

Chapter Two provides a comprehensive review of the literature to discuss the major concepts relevant to the project. First, the history of natural product research and the need for new antimicrobial compounds is described. Second, the role of microorganisms in drug discovery, with a specific focus on filamentous fungi, is discussed. The literature review concludes with an overview of coprophilous fungi and an introduction to koala faeces as an unusual micro-environment for fungal colonisation.

Chapter Three outlines the isolation of fungi from the faeces of koalas living in Boho South and French Island in Victoria, Australia. The isolates were identified via DNA sequencing and comparison with the National Center for Biotechnology Information (NCBI) nucleotide database.

Chapter Four describes the preparation of extracts from the isolates and their screening against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida albicans and Drechslera brizae. The extracts were also assessed for their ability to quench quorum sensing (QS) signals, using the indicator strain.
Chromobacterium violaceum. The premise behind this work was that the fitness of coprophilous fungi is challenged by competing and invading organisms within koala faeces and, therefore, survival is enhanced by the production of chemicals to mediate these interactions.

Chapter Five presents the findings from the bioactivity-guided separation of the most active extract and the ultimate identification of the active constituent.

Finally, Chapter Six presents a summary of this project’s major findings as well as a brief discussion of the scope for further research.
CHAPTER 2

Literature Review
2.1 Natural products and secondary metabolites

The term *natural product*, in its broadest sense, refers to a chemical that is produced by a living organism and exerts a biological effect on other organisms (Colegate and Molyneux, 2008). This includes beneficial bioactivity, such as therapeutic activity for diseases of humans, plants and animals, as well as toxic bioactivity that is responsible for causing some diseases. In most cases, natural products appear to be non-essential for the organism’s everyday metabolism (Vicente et al., 2003). Because of this, the term *natural product* is often used interchangeably with *secondary metabolite* even though their starting materials invariably come from the major biosynthetic pathways of primary metabolism (Madigan et al., 2003).

Secondary metabolites were once hypothesised to be waste or detoxification products and, therefore, of little importance to an organism’s survival (Paech, 1950). However, it has become clear that such views were dismissive and inaccurate because many secondary metabolites (such as those involved in defence, mutualism and communication) are key components of complex mechanisms that contribute to the fitness of an organism (Bills et al., 2013; Cragg and Newman, 2013). The use of natural product as a simple descriptor was suggested almost 50 years ago (Zenk, 1967) to remove bias against any class of metabolite and to avoid the negative implications of the word secondary. Despite this, the use of secondary metabolite has continued (in addition to natural product) and it has become entrenched in the scientific lexicon. Thus, in the current work, both terms will be used.

2.2 History of natural products

2.2.1 Traditional medicine

Humans have a long history of exploring and exploiting the natural world for the treatment and management of disease. Almost all indigenous cultures have, at some time, made use of naturally derived medicines. Examples include the well-known
traditional Chinese medicine and Indian (Ayurvedic) medicine, which both date back to approximately 3,000 BC (Ng, 2005). While traditional medicines were prepared as crude extracts, tinctures and dried plant matter, the prescription of a therapeutic compound would have the obvious advantage of a quantified dosage. In 1805, the first pure pharmaceutical compound was isolated from a traditional medicinal plant: morphine from opium produced by cut seed pods of the poppy, *Papaver somniferum* (Sneader, 2005). This led to increased interest in phytochemical studies and the isolation of natural products (such as atropine, quinine and colchicine) from plants that were historically uses as medicines (Cragg and Newman, 2013).

### 2.2.2 The golden era

It was not until the late 1940s that the true potential of microorganisms as a source of novel bioactive compounds was realised. In 1928, Sir Alexander Fleming observed the accidental contamination of a bacterial culture (*Staphylococcus* spp.) by the filamentous fungus *Penicillium notatum* (Fleming, 1929). Later work by Howard Florey and his team resulted in the isolation of penicillin as the active compound as well as characterisation of its structure and observations of its broad therapeutic use (Florey et al., 1949). Research into microbial derived natural products was accelerated by the onset of World War II during which infectious diseases were a major problem. In Australia, the Commonwealth Serum Laboratories manufactured penicillin to supply the Australian forces and some of the American forces serving in the Pacific in 1944, and, in the same year, Australia became the first country in the world to supply penicillin freely to civilians (CSL, 2015). The following three decades are often referred to as the golden era of natural product research, which saw the discovery of almost all groups of important antibacterial antibiotics: tetracyclines, aminoglycosides, cephalosporins and macrolides (Bérdy, 2005).
2.2.3 A natural product decline

By 1990, about 80% of drugs were either natural products or analogues inspired by natural products (Li and Vederas, 2009). However, the rate of discovery and the introduction of new natural product drugs into clinical use began to decline. Many pharmaceutical firms began to reduce or eliminate their natural product research programs, believing that the protocols were not compatible with the high-throughput screening and combinatorial chemistry technologies that had been developed over this period (Lam, 2007). These new high-throughput screening techniques were not as successful as anticipated, with only one approved drug derived from combinatorial chemistry over the 20 years to 2010, namely the Bayer anti-tumour drug sorafenib. According to the review by Newman and Cragg (2012), there has been a steady decline in the output of the pharmaceutical industry since the late 1980s. At this time, over 60 small molecule new chemical entities were being described every year by pharmaceutical companies’ research and development programs but this reduced to an average of 23 new chemical entities per year over the decade from 2001 to 2010. It is noteworthy that this downturn coincided with the period of reduced interest in natural products by the major pharmaceutical companies, and suggests that their reliance on other techniques may have been misguided.

2.2.4 Rekindled interest today

Despite the reduction in natural product research by pharmaceutical companies, the majority of drugs used today are either natural products, semi-synthetically produced from natural products or chemically synthesised based on natural products. In the period from 1981 to 2010, a total of 1,073 small molecule drugs were approved, with only 387 (36%) being classified as truly synthetic and devoid of natural inspiration (Figure 2.1) (Newman and Cragg, 2012). Approximately half of the top-selling pharmaceuticals are derived from natural products; these include cholesterol lowering statins and many anti-cancer drugs and antibiotics (Demain 2009). In 2001, the worldwide market for
antibiotics was US$32 billion (Projan and Youngman, 2002) and the majority of these were (and are today) derived from natural products (Demain, 2009).

**Figure 2.1** Source of small-molecule drugs approved from 01/01/1981 to 31/12/2010 (Sourced from Newman and Cragg (2012). Note: The categories of sources are as follows: S, totally synthetic drug, often found by random screening/modification of an existing agent; S/NM, synthetic/natural product mimic; S*, made by total synthesis, but the pharmacophore is from a natural product; S*/NM, made by total synthesis, but the pharmacophore is from natural product mimic; N, natural product; NB, natural product botanical.

The overall decline in the number of new chemical entities in drug development pipelines and the ongoing need for new pharmaceuticals has rekindled interest in natural product research. There is renewed belief that the structural diversity offered by nature, coupled with advances in technology, will provide new drug candidates for many diseases (Cragg et al., 2014; Cragg and Newman, 2013; Li and Vederas, 2009; Milshteyn et al., 2014; Mishra and Tiwari, 2011).
2.3 The need for new antimicrobial compounds

New antimicrobial compounds are urgently needed to respond to increased resistance to antibiotics and antifungal agents.

2.3.1 Antibiotics

Most antibiotic classes were discovered before 1970, and a large majority of the compounds that have been approved since then are based on chemical modifications of existing scaffolds (Table 2.1) (Genilloud, 2014). All classes of antibiotics have seen the emergence of resistant bacteria. Combined with the lack of new antibiotics, this resistance represents a serious public health threat given that infectious diseases continue to be one of the leading causes of death worldwide (Livermore, 2009).

One of the major concerns is the increased prevalence of resistance among *K. pneumoniae*, *S. aureus* and *E. coli*. These bacteria cause infections that are common in hospitals and in the community, and their resistance to current antibiotics means that infections are becoming increasingly difficult to control. A recent report by the World Health Organization (WHO) states that, in many settings, more than 50% of these bacteria demonstrated resistance to commonly used antibacterial drugs (Table 2.2) (WHO, 2014). Another concerning finding was that all WHO regions reported the presence of *K. pneumoniae* that were resistant to carbapenems, which are usually the last line of available treatment. In the current research, antibacterial activity of the extracts was screened against these three bacteria and *P. aeruginosa*, which has also demonstrated multi-drug resistance (Yuhico and Blair, 2011).
## Table 2.1 Structural classes of antibiotics*

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Antibiotic</th>
<th>Year of discovery</th>
<th>Analogues developed after 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin (β-lactam)</td>
<td>Penicillin G</td>
<td>1928</td>
<td></td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Streptomycin</td>
<td>1943</td>
<td>Plazomycin (ACHN-490)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Chloramphenicol</td>
<td>1946</td>
<td></td>
</tr>
<tr>
<td>Cyclopeptides</td>
<td>Polymixin B</td>
<td>1947</td>
<td>NAB739</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Chlortetracycline</td>
<td>1948</td>
<td>Tigecyclines (Omadacycline, Eravacycline)</td>
</tr>
<tr>
<td>β-lactams</td>
<td>Cephalosporin C</td>
<td>1948</td>
<td></td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin</td>
<td>1948</td>
<td>Telithromycin</td>
</tr>
<tr>
<td>Pleuromutilin</td>
<td>Pleuromutilin</td>
<td>1952</td>
<td>Retapamulin</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Vancomycin</td>
<td>1953</td>
<td>Dalbavancin, Oritavancin, Telavancin</td>
</tr>
<tr>
<td>Streptogramins</td>
<td>Streptogramin B</td>
<td>1953</td>
<td></td>
</tr>
<tr>
<td>Rifamycins</td>
<td>Rifampicin</td>
<td>1957</td>
<td></td>
</tr>
<tr>
<td>Lincomycins</td>
<td>Lincomycin</td>
<td>1961</td>
<td></td>
</tr>
<tr>
<td>Quinolones</td>
<td>Fluoroquinolones</td>
<td>1962</td>
<td>Delafloxacin, Nemonoxacin</td>
</tr>
<tr>
<td>Macrolide</td>
<td>Fidaxomicin</td>
<td>1975</td>
<td></td>
</tr>
<tr>
<td>Carbapenem (β-lactam)</td>
<td>Imipenem</td>
<td>1976</td>
<td>Ertapenem, Doripenem</td>
</tr>
<tr>
<td>Monobactam (β-lactam)</td>
<td>Aztreonam</td>
<td>1981</td>
<td></td>
</tr>
<tr>
<td>Lipopeptides</td>
<td>Daptomycin</td>
<td>1986</td>
<td>CB-183,315</td>
</tr>
<tr>
<td>Oxazolidinone</td>
<td>Linezolid</td>
<td>1995</td>
<td>Radezolid, Tedizolid</td>
</tr>
</tbody>
</table>

* Sourced from Genilloud (2014)
Table 2.2 Resistance among bacteria that commonly cause infections*

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Examples of typical disease</th>
<th>Incidence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>≥50% resistance nationally&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Cephalosporins&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>Fluoroquinolones</td>
<td>92</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>Urinary tract infections, blood stream infections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Cephalosporins&lt;sup&gt;c&lt;/sup&gt;</td>
<td>87</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>Carbapenems&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71</td>
<td>2/6</td>
</tr>
<tr>
<td></td>
<td>Pneumonia, urinary tract infections, blood stream infections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>Methicillin</td>
<td>85</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>Wound infections, blood stream infections</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Adapted from the WHO global report on antimicrobial resistance and surveillance [WHO 2014]

<sup>a</sup> Reported by a WHO Member State (out of 194 providing data)

<sup>b</sup> The number of WHO regions (out of six) with national reports of 50% resistance or more

<sup>c</sup> 3rd generation drugs

2.3.1.1 **QS inhibition**

Bacteria are able to sense their population’s density through an intercellular QS communication system. This system regulates gene expression in response to cell density through the constant production and detection of signalling molecules. When the population density reaches a certain threshold, or quorum, specific sets of genes are collectively expressed, allowing the bacteria to act *en masse*. QS systems have been shown to coordinate a variety of physiological processes in bacteria including the production of antibiotics, bioluminescence, the release of virulence factors and biofilm production (Kociolek, 2009). Many pathogenic bacteria rely on this communication system for infection of their hosts and it is, therefore, a potential target for the discovery and development of new antibacterial compounds (Helman and Chernin, 2015). Furthermore, disruption of QS, rather than killing bacteria, may also reduce the development of resistant strains (Hentzer and Givskov, 2003). In the current study,
extracts were tested for anti-QS activity using a disk diffusion assay against the screening bacterium *C. violaceum*. In this wild type strain, the production of the purple pigment violacein is under N-acylhomoserine lactone QS control (McClean et al., 1997). When QS signals are quenched, non-pigmented bacteria grow and can be observed as a turbid halo of viable but colourless cells surrounding the extract impregnated disk. Growth inhibition by an extract results in a zone of inhibition similar to that observed in the conventional antibacterial plate-hole diffusion assay.

### 2.3.2 Antifungal agents

#### 2.3.2.1 Candida

Over 20 species of the yeast *Candida* are capable of causing infection, making the genus the most common cause of fungal infection worldwide (WHO, 2014). *Candida* cause superficial candidiasis, such as oral thrush, as well as invasive candidiasis, such as the bloodstream infection known as candidaemia. One common risk factor for candidiasis is the prior use of antibiotics because it alters the normal microbiota and can lead to dominance by *Candida* species (Ben-Ami et al., 2012). Therefore, candidiasis (particularly the invasive forms) is a major problem among patients in intensive care and in those receiving immunosuppressive therapy (Pfaller and Diekema, 2007).

Currently, there are only three classes of antifungal agents available to treat serious *Candida* infections: the azoles, the echinocandins and the polyenes (e.g., amphotericin B). Formulations of amphotericin B are available in many countries but it has higher toxicity compared with the other two agents and a few *Candida* species have been reported to develop resistance during the treatment period (WHO, 2014). The newer class of antifungals, the echinocandins, are the treatment of choice for *Candida* infections in developed countries, but they are not yet available for standard treatment in many developing countries and there has been an emergence of species not susceptible to the therapy (Kale-Pradhan et al., 2012; Pfaller et al., 2011). Azoles remain the most frequently prescribed antifungal class and are often the only therapy available. The
azole fluconazole is classified as a fungistatic agent and is listed on the WHO’s current Model List of Essential Medicines (WHO, 2015). Unfortunately, Candida species are also rapidly acquiring resistance to fluconazole and it is therefore imperative that new compounds with novel mechanisms of action are identified to attenuate Candida infections. C. albicans is both a member of the healthy human microbiome and a major pathogen in immunocompromised individuals. C. albicans was therefore chosen for use as a test fungus in the current study in screening for antifungal activity.

2.3.2.2 Phytopathogenic fungi

Phytopathogenic fungi are becoming increasingly problematic for important food crops, particularly in developing countries (Vurro et al., 2010). These plant diseases cause a reduced harvest yield (or post-harvest rot), which has serious implications in terms of food security and a sustainable economy (Fears et al., 2014). Resistance to current agrochemicals is an ongoing challenge, and concerns relating to the safety and environmental impact of agrochemicals have led to more stringent regulatory processes (Lamberth et al., 2013). Thus, there is now a demand for new, safer and more selective compounds, and it is important that natural products continue to be studied for their potential application in crop protection (Dayan et al., 2009; Olufolaji, 2010). The filamentous fungus D. brizae is a phytopathogen and was chosen for antifungal screening in the current study.

2.4 Bioactive metabolites isolated from microorganisms

Secondary metabolites are produced by almost all types of living organisms. They are produced by prokaryotes and eukaryotes of the Plant, Animal and Fungi Kingdoms, although there are variations in their production ability among the groups (Bérdy, 2005). Historically, plants have been a major focus for natural product discovery, owing to their structurally diverse secondary metabolites and their use in traditional medicine. Higher plants have provided a number of important drugs, such as the anti-cancer agent paclitaxel that was originally isolated in small quantities from the bark of the Pacific
yew tree, *Taxus brevifolia*. Fortunately, a paclitaxel precursor was also discovered to be produced in the needles of a different *Taxus* species; otherwise, an estimated 12,000 of the slow-growing Pacific yew tree would have needed to be destroyed to produce enough material to complete the clinical trials (Cragg and Boyd, 1996). Today, most of the clinical paclitaxel is produced either by semisynthesis or by plant cell fermentation using a *Taxus* cell line, both of which are expensive and time consuming processes (Gond et al., 2014). Microorganisms can provide a more ecologically friendly and potentially cheaper alternative to higher order plants because they can be artificially cultivated and require substantially less time for growth. For example, a number of fungi also produce paclitaxel and its precursors, and these fungi are being explored as a potential alternative to the reliance on *Taxus* trees (Gond et al., 2014; Zhou et al., 2010).

A recent review by Bérdy (2012) summarises the past, current and future aspects of antibiotic discovery and development. It includes a breakdown of the approximate number of known bioactive metabolites and their distribution according to organism origin. By the year 2012, an estimated 22,500 bioactive compounds had been discovered from microbes. Table 2.3 presents the distribution of bioactive compounds according to microbial origin; the three categories are discussed in the following sections.

<table>
<thead>
<tr>
<th>Source</th>
<th>Antibiotics</th>
<th>Other bioactive metabolites</th>
<th>Total bioactive metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>2,900</td>
<td>900</td>
<td>3,800</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>8,700</td>
<td>1,400</td>
<td>10,100</td>
</tr>
<tr>
<td>Fungi</td>
<td>4,900</td>
<td>3,700</td>
<td>8,600</td>
</tr>
<tr>
<td>Total</td>
<td>16,500</td>
<td>6,000</td>
<td>22,500</td>
</tr>
</tbody>
</table>

*Adapted from (Bérdy, 2012)*
2.4.1 Actinomycetes

In terms of microorganisms, the actinomycetes represent the largest producers of bioactive compounds. Actinomycetes are commonly isolated from soil and are closely related to bacteria in size and physiology but are similar in structure to fungi (Goodfellow, 2010). In the late 1930s, actinomycetes were the focus of the first established systematic screening protocols to detect antibiotics produced by microorganisms. This research by Selman Waksman and colleagues led to the discovery of many new antibiotics (a word that Waksman coined), including the first effective treatment for tuberculosis (streptomycin) for which he was awarded the Nobel Prize in 1952 (Waksman, 1954). It has been estimated that the actinomycetes produce over 10,000 bioactive compounds and account for about 45% of all microbial bioactive secondary metabolites, with 7,600 (80%) of these compounds being produced by Streptomyces species (Table 2.3) (Bérdy, 2012; Kurtböke, 2012). Predictive modelling has suggested that a further 150,000 bioactive compounds are yet to be discovered from Streptomyces species (Watve et al., 2001).

2.4.2 Bacteria

The total number of known bioactive compounds from bacteria is about 3,800, which represents 17% of all microbial metabolites (Table 2.3) (Bérdy, 2012). The Pseudomonas and Bacillus genera are the most well-known producers, but in recent years, myxobacteria and cyanobacteria species have garnered interest for their prolific production of structurally interesting natural products. For example, 16-membered ring macrolides, known as epothilones, that constitute a novel class of antimicrotubule-targeting agents have been isolated from various myxobacteria (Wang et al., 2009). These compounds have a mechanism of action similar to that of paclitaxel and are being extensively studied for their potential use in chemotherapy for tumours (Cragg and Newman, 2013; Hofle and Reichenbach, 2012).
2.4.3 Fungi

Fungi harbour a tremendous capacity to produce diverse and complex secondary metabolites. There are approximately 8,600 known bioactive secondary metabolites, representing 38% of all microbial products (Table 2.3) (Bérdy, 2012). Secondary metabolism in the fungi is almost exclusively associated with the filamentous fungi of the dikaryomycota – the Basidiomycetes and Ascomycetes. Filamentous ascomycete genomes encode a plethora of enzymes that synthesise secondary metabolites, including non-ribosomal peptide synthetases, polyketide synthases and terpene synthases. In contrast, the Basidiomycetes favour terpenoid biosynthesis for their secondary metabolite production, with polyketide synthases and non-ribosomal peptide synthetases occurring to a lesser extent (Bills et al., 2013; Wawrzyn et al., 2012). These enzymes build the core structural scaffolds of most fungal secondary metabolites and the genes that encode them are generally located in clusters. Genome mining of secondary metabolite gene clusters has demonstrated that many pathways remain silent under standard cultivation conditions, indicating that the capability of fungi to produce secondary metabolites is far greater than first thought (Brakhage, 2013; Spraker and Keller, 2014).

2.5 Filamentous fungi as a source of natural products

Fleming’s observation of antimicrobial activity in the filamentous fungus *P. notatum* and the subsequent development of the antibiotic penicillin by Florey and colleagues is arguably the most significant discovery of a bioactive compound from fungi. Penicillin was the first available broad spectrum antibiotic and its discovery set up the paradigm for future drug discovery research from fungi. Since then, thousands of compounds have been discovered that inhibit the growth of bacteria, fungi, protozoa, parasites, insects, viruses and even human tumour cells. Many other molecules with cytotoxic, mutagenic, carcinogenic, teratogenic, immunosuppressive, enzyme inhibitory, allelopathic and other biological effects have been found (Keller et al., 2005). The most famous fungal metabolites in clinical use today include the β-lactam antibiotics (e.g.,
penicillins G and V), the cholesterol lowering statin lovastatin and its derivatives, the immunosuppressant cyclosporin, and the migraine medication ergotamine. The statin drugs (such as lovastatin, mevinolin, compactin, pravastatin and atorvastatin) are the biggest selling pharmaceutical group today, providing enormous revenue to pharmaceutical companies and extending the lives of millions of people (Endo, 2010).

Filamentous Ascomycetes are the most prolific producers of bioactive compounds among the fungal species, with approximately 6,400 compounds having been isolated. The three common genera Aspergillus, Penicillium and Fusarium have produced approximately 950, 900 and 350 compounds, respectively, and several hundred have been isolated from species of Trichoderma, Phoma, Alternaria and Acremonium (Bérdy, 2005).

With the return to natural product drug discovery, there has been a recent increase in interest towards secondary fungal metabolites, and the belief that they may provide the leads and scaffolds for the development of desperately needed drugs for a multitude of diseases (Bills et al., 2013; Brakhage, 2013; Prakash, 2015).

2.6 Enhancing natural product discovery from fungi

Fungal natural product research is most effective when it takes a multidisciplinary approach that respects the complexity and diversity of nature and also embraces new technologies and approaches (Cragg et al., 2014; Demain, 2009). The renewed interest in compounds derived from natural products has led to rapid development in diverse research disciplines. The following sections describe three key areas of research that are enhancing and accelerating natural product discovery from fungi.

2.6.1 Genome mining and engineering

Advances in microbial genomics have led to a greater understanding of the biosynthetic gene clusters in fungi that are responsible for the production of important secondary
metabolites (Milshteyn et al., 2014; Zotchev et al., 2012). Since the first genome of a filamentous fungus (*Neurospora crassa*) was published in 2003 (Galagan et al., 2003), approximately 1,204 full genome sequences of fungi have become available (Genilloud, 2014). The number of sequenced genomes is expected to grow dramatically in the near future, and with automated gene prediction technology, this will provide a preview of the biosynthetic pathways within a fungus, for both known and unknown natural products (Bills et al., 2013). Activation of silent or unproductive biosynthetic pathways can result in the isolation of novel bioactive compounds, superior analogues of previously known compounds or increase the yield of natural products (Brakhage, 2013). Furthermore, manipulation of the pathways via metabolic engineering (also known as combinatorial biosynthesis) can allow microbes to produce compounds that would not normally exist in nature (Krivoruchko and Nielsen, 2015; Wong and Khosla, 2012; Wu et al., 2012). For example, Fisch et al. (2011) used combinatorial biosynthesis of fungal polyketide synthases to produce bassianin in *Aspergillus oryzae*. This metabolite had already been isolated but the original strain no longer exists and the production of bassianin had not been observed since. Thus, bassianin was effectively an extinct metabolite. Rational domain substitutions between polyketide synthases that encode the biosynthesis of closely related compounds was used to create hybrid synthetases. Combined with the coexpression of two cytochrome P450 encoding genes, the biosynthesis pathway for bassianin was resurrected.

### 2.6.2 Analytical chemistry platforms for metabolomics

The improvement of analytical chemical platforms combined with the benefits of high-throughput screening appears to be one area of focus for future success in natural product discovery from fungi. Bioactivity-guided separation is a technique commonly used in natural product drug discovery and was the methodology employed in the current study. The aim of the process is to isolate compounds with particular biological activities from crude extracts. To achieve this, the abundant and often diverse compounds in the extract must be separated into fractions that are then screened for the biological activity of interest. The fractions without bioactivity are disregarded and
those that demonstrate bioactivity are further separated, often using a variety of separation techniques, until the bioactive constituent has been isolated and can be identified.

Given the complexity of the initial natural extract, bioactivity-guided separation is often a complicated, labour-intensive and time-consuming process. The complexity of the starting material does not make it amenable for the rapid high-throughput screening protocols that are favoured by pharmaceutical research and development programs. An ideal solution is automated separation of the crude extract into individual components, coupled with full spectroscopic identification prior to high-throughput screening (Li and Vederas, 2009). Alternatively, the crude extract can undergo partial purification by pre-treatment (to remove promiscuous materials such as tannins) or pre-fractionation prior to high-throughput screening (Cragg and Newman, 2013). The development of modern hyphenated chemical analytical techniques, such as high-performance liquid chromatography (HPLC) coupled online to nuclear magnetic resonance (NMR) spectroscopy, has helped to address this challenge by enabling the rapid separation and identification of potential drug leads from an extract (Brkljača and Urban, 2011). Techniques such as this often include high-resolution mass spectrometry analysis and comparison of hits to a compound database that provides an assessment of a secondary metabolite’s novelty and its potential as a drug candidate (El-Elimat et al., 2013; Nielsen and Larsen, 2015).

Recently, a new method was described by Sica et al. (2015) that eliminates the need for traditional extraction processes of a fungus, and subsequent activity-guided separation. The in situ technique achieves identification of secondary metabolites directly on the surface, or surrounding, a fungal culture in a Petri dish. Continued development of analytical chemistry platforms for metabolomics studies will contribute to the improvement of drug discovery from fungi and aid researchers in finding novel compounds.
2.6.3 Bioprospecting

The two previous approaches are concerned with enhancing natural product discovery, but once a producer has been established, bioprospecting efforts aim to expand the diversity of fungi available for exploitation. The number of described fungal species in the world is varyingly estimated from 45,000 to up to 300,000. This represents only a small proportion of the predicted total diversity, with several studies estimating that there may be as many as 1.5 million species (Chapman, 2009). Therefore, there is a largely untapped reservoir of fungi awaiting discovery, and given their proven potential for producing structurally complex and diverse secondary metabolites, isolation of new species will increase the likelihood of discovering novel bioactive compounds.

In the search for bioactive compounds, biodiverse environments are more likely to play host to increasingly diverse microbiota, which in turn, may produce chemically novel metabolites (Bérdy, 2005). Australia has been described as a megadiverse nation, placing it in a group of countries that have less than 10% of the global surface but support more than 70% of the biological diversity on earth (Kumar et al., 2015). Although this statement may have been in response to the myriad of flora and fauna in Australia, the diversity of macroorganisms in an environment is likely mirrored by the diversity of microorganisms (Strobel, 2006). Thus, the various biotopes within Australia represent an ideal location to search for new species of fungi.

2.6.3.1 Biological niches of interest

Further to exploring biodiverse geographical locations for fungi, interest has grown in three unique biological niches that have emerged as natural product hot spots. First, the intracellular spaces of many higher plants support the growth of a group of fungi known as endophytes. These usually exist as symptomless inhabitants, suggesting a long-standing and complicated symbiotic relationship with the plant (Strobel and Daisy, 2003). Adaptation to the micro-environment has included the uptake of some plant DNA into their genomes, which has allowed certain endophytes to synthesise
phytochemicals originally associated with the host (Zaferanloo et al., 2012). For example, Stierle et al. (1993) reported that an endophytic fungus (*Taxomyces andreanae*) isolated from *T. brevifolia* was capable of producing the same paclitaxel compound for which the host plant was well known. Since then, many endophytic fungi have been found to produce paclitaxel, both from *Taxus* species and non-*Taxus* species (Kumaran et al., 2009; Liu et al., 2009; Strobel et al., 1996; Zhou et al., 2010). The relatively low yield from these fungi needs to be improved before they can be considered a viable alternative for commercial production (Gond et al., 2014).

Numerous compounds with antimicrobial activity have also been isolated from endophytes, including those of the alkaloid, peptide, steroid, terpenoid, phenol, quinone, flavonoid and aliphatic chemical classes (Zaferanloo et al., 2012).

The second biological niche that has recently garnered interest is faeces, particularly from herbivorous mammals, that support the growth of coprophilous fungi. These fungi are the focus of the present study and will be discussed in greater depth in Section 2.7.

Finally, marine fungi have recently emerged as a biological niche that is capable of producing a diverse range of natural products. Although the majority of the world’s surface is oceans, the marine environment has not been studied as extensively as the terrestrial environment due to the difficulty in collecting samples. Early research into marine natural products was limited to the study of organisms from shallow waters until improvements were made in the areas of scuba diving and trawling (Cragg and Newman, 2005). One study conducted by Giuseppe Brotzu in 1945 investigated seawater samples from a sewage outlet in Sardinia and led to the discovery of the β-lactam antibiotic cephalosporin C from *Acremonium chrysogenum* (Abraham and Loder, 1972). Since then, marine fungi have been isolated from various sources including sponges, algae, wood, tunicates, sediment, molluscs, coral, plants and fish, and have led to the discovery of over 1,000 natural products (Gomes et al., 2015; Rateb and Ebel, 2011).
2.7  Coprophilous fungi

Coprophilous fungi are fungi that inhabit or are associated with the faeces of animals, including soil contaminated with faeces. Coprophilous fungi play an important role as recyclers in the ecosystem by degrading the contents of faeces and subsequently returning micronutrients into the ecosystem. The majority of studies of coprophilous fungi have focused on their taxonomy and ecology. Recently, however, the potential of coprophilous fungi for natural product discovery has been supported by a high frequency of bioactive secondary metabolites isolated from relatively limited efforts (Bills et al., 2013).

2.7.1  Life in the dung environment

The spores of some coprophilous taxa will only germinate on faeces if they have previously travelled through the digestive track of the animal, whereas other species are introduced to the faeces via air dispersal of spores or contact with other surfaces (Misra et al., 2014). Following growth on the faeces, the fungi are dispersed to nearby vegetation by rain, arthropods, mammals, or by their spores being forcefully discharged into the air. The latter method forms part of a cyclic relationship that some species have developed, whereby their spores are mucilaginous and adhere to the vegetation that is eaten by the animal whose faeces they colonise. The spores are then ingested by the animal and germination, growth and sporulation occur on the freshly excreted faeces (Krug et al., 2004). Other coprophilous fungi, such as *Mucor hiemalis*, produce a sticky droplet around their spores that sticks to the bodies of insects that visit the dung. The insects then inadvertently disperse the spores to a new environment. A similar method is employed by some cleistothecial Ascomycetes whose fruiting bodies have modified appendages for attachment to fur. The structures are particularly useful for fungi that inhabit the faeces of rodents because they deposit dung at the entrance to their burrow and thus act as a vector between different dung deposits (Krug et al., 2004). In Australia, some species of Mallee moth feed on koala faeces and lay their eggs on the surface. The caterpillars then complete their development in a single dung pellet, spin
their cocoons and emerge as adult moths Figure 2.2 (Van Dugteren, 1999). These moths help to recycle nutrients back into Australia’s nutrient-poor soils, and their ingestion of the faeces may also aid in disseminating coprophilous fungi.

![Image of a mallee moth](image)

**Figure 2.2** The mallee moth, *Telanepsia stockeri*, feeds on koala faeces and was named in honour of Australia’s chief scientist, Dr John Stocker. Photo: Van Dugteren (1999).

### 2.7.2 Succession of fungi on dung

The succession of fungi that appear on dung at various stages of degradation appears to follow a generalised pattern (Richardson, 2002). Fast-growing Zygomycetes are frequently observed early in succession, especially on fresh dung, and use the easily metabolised simple sugars, starch and protein. Ascomycetous species are the most commonly identified coprophilous fungi. These appear when the simple carbon sources are depleted because they can use the hemicellulose and cellulose material. Basidiomycetes usually appear later in the succession and are able to metabolise both cellulose and lignin (Krug et al., 2004; Sarrocco et al., 2015a; Wicklow et al., 1980). The order of appearance is also likely to be affected by the time taken to form fruiting bodies. The simple sporangia of the Zygomycetes can develop more quickly than the more complex Ascomycete fruiting body, which requires less energy than the even
larger Basidiomycete fructification (Krug, 2004). Furthermore, the activities of competitors such as bacteria, insects, worms and other fungi can influence succession (Krug et al., 2004). The challenge that these competitors pose to the fitness of coprophilous fungi is met, at times, with the production of bioactive compounds by the fungi, and this forms the premise behind bioprospecting of coprophilous fungi for natural products.

2.7.3 Distribution and diversity

The majority of research on coprophilous fungi is concerned with identification, usually by morphology, and a high proportion of the studies are from Europe and North America. There are approximately 52 genera of Zygomycetes, 169 genera of Ascomycetes and 33 genera of Basidiomycetes that are known to use dung as a substrate (Misra et al., 2014) and new species continue to be discovered (Fukiharu et al., 2015; Goh et al., 2013; Kruys, 2015). The literature on coprophilous fungi describes species from a large variety of animal dung, including sheep, cow, horse, camel, llama, deer, tapir, rabbit, hare, dormouse, hedgehog, chamois, grouse, goose, goat, buffalo, zebra, elephant, giraffe, rhinoceros, tiger, kangaroo, wombat and koala (Bell, 2005; Bills and Polishook, 1993; Cribb, 1988, 1989, 1997; Doveri et al., 2012; Doveri et al., 2010; Ganesh Kumar et al., 2010; Goh et al., 2013; Gupta, 2010; Melo et al., 2012; Pandey, 2009; Richardson, 2001c; Sarrocco et al., 2015c; Watling and Richardson, 2010). In almost all surveys of coprophilous fungi, species of the phylum Ascomycota dominate the collection. For example, Watling and Richardson (2010) studied the fungi from dung samples of sheep, cattle, horse, rabbit and goose living in the Southern Atlantic archipelago of the Falkland Islands. They isolated 97 taxa, 60 of which were Ascomycetous species, 28 were Basidiomycetes and five were Zygomycetes.

2.7.4 Antimicrobial compounds from coprophilous fungi

The fitness of coprophilous fungi in the dung environment is challenged by the presence of bacteria, protists, invertebrates and other fungi competing for a nutrient-rich,
transient resource. Within the dung microcosm there are antagonistic and defensive interactions that may require the production of bioactive compounds that affect prospective competitors or predators (Gloer, 1995). Although competitive interactions had long been observed among fungi (Ikediugwa and Webster, 1970; Wicklow, 1981), it was the formative research of J. B. Gloer and colleagues into the chemistry associated with these interactions that exemplified their potential for producing bioactive compounds. Since their isolation of an antifungal diphenyl ether from *Preussia fleischhakii* in 1988 (Weber and Gloer, 1988), Gloer has co-authored at least 23 papers that describe the identification of one or more novel bioactive metabolites from coprophilous fungi (see references in Table 2.4).

In comparison with the fungal endophytes, reports of novel antimicrobial compounds from coprophilous fungi have been limited and come mainly from a few laboratories. However, the efforts that have been undertaken have shown that coprophilous fungi are indeed a rich source of structurally diverse metabolites, with several demonstrating antibacterial activity and even more inhibiting fungi. The increased occurrence of antifungal activity may be biased because of the dependence on fungal phenotypic assays in the initial screening process (Bills et al., 2013). It is therefore important to also test extracts and compounds for antibacterial activity.

Table 2.4 lists some of the known species of coprophilous fungi that are reported to have produced antifungal or antibacterial compounds. Compounds from all major biosynthetic classes are represented, and all but one of the examples (*Coprinopsis cinerea*) are members of the Ascomycota phylum. The coprophilous ascomycetes have emerged as the prominent phylum for producing antimicrobial compounds due to their higher frequency of isolation and their broader array of biosynthesis enzymes (Bills et al., 2013).
Table 2.4 Antibacterial (AB) and antifungal (AF) compounds isolated from coprophilous fungi*

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Compounds</th>
<th>Biological activity (AF/AB)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podospora</td>
<td>araneosa</td>
<td>Sordarins</td>
<td>AF</td>
<td>(Hauser and Sigg, 1971a; Odds, 2001)</td>
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<td></td>
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<td>(Bloch and Tamm, 1981; Breitenstein et al., 1981)</td>
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<td></td>
<td>(Fehlhaber et al., 1988; Mukhopadhyay et al., 1998; Roy et al., 1992)</td>
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<tr>
<td>Pseudeurotium</td>
<td>ovalis</td>
<td>Pseurotins</td>
<td>AB</td>
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<tr>
<td>Pseudoarachniotus</td>
<td>roseus</td>
<td>Aranorosins</td>
<td>AB</td>
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<tr>
<td>Preussia</td>
<td>isomera</td>
<td>Preussomerins</td>
<td>AF</td>
<td>(Weber et al., 1990)</td>
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<tr>
<td>Sporormiella</td>
<td>similis</td>
<td>Similins</td>
<td>AF</td>
<td>(Weber et al., 1992)</td>
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<tr>
<td>Podospora</td>
<td>appendiculata</td>
<td>Appendolides</td>
<td>AF</td>
<td>(Wang et al., 1993)</td>
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<td>Scopulariopsis</td>
<td>Unknown</td>
<td>Restricticins</td>
<td>AF</td>
<td>(O'Sullivan et al., 1993)</td>
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<tr>
<td>Apiospora</td>
<td>montagnei</td>
<td>Apiosporamide</td>
<td>AF</td>
<td>(Alfatafta et al., 1994)</td>
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<tr>
<td>Coniochaeta</td>
<td>saccardoi</td>
<td>Coniochaetones</td>
<td>AF</td>
<td>(Wang et al., 1995a)</td>
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<tr>
<td>Petriella</td>
<td>sordida</td>
<td>Petriellins</td>
<td>AF</td>
<td>(Lee et al., 1995)</td>
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<tr>
<td>Polytolypa</td>
<td>hystricis</td>
<td>Polytolypin</td>
<td>AF</td>
<td>(Gamble et al., 1995)</td>
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<tr>
<td>Sporormiella</td>
<td>australis</td>
<td>Australifungin</td>
<td>AF</td>
<td>(Hensens et al., 1995)</td>
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<tr>
<td>Sporormiella</td>
<td>intermedia</td>
<td>Zaragozic acid B</td>
<td>AF</td>
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<tr>
<td>Sporormiella</td>
<td>teretispora</td>
<td>Terezines</td>
<td>AF</td>
<td>(Wang et al., 1995b)</td>
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<tr>
<td>Cercophora</td>
<td>areolate</td>
<td>Cercophorins</td>
<td>AF</td>
<td>(Whyte et al., 1996)</td>
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<tr>
<td>Cercophora</td>
<td>sordarioides</td>
<td>Arthrinones</td>
<td>AF</td>
<td>(Whyte et al., 1997)</td>
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<tr>
<td>Podospora</td>
<td>anserine</td>
<td>Anserinones</td>
<td>AF/AB</td>
<td>(Wang et al., 1997)</td>
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<tr>
<td>Ascodesmis</td>
<td>sphaerospora</td>
<td>Arugosin F</td>
<td>AF/AB</td>
<td>(Hein et al., 1998)</td>
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<tr>
<td>Preussia</td>
<td>fleischhakii</td>
<td>Diphenyl ethers</td>
<td>AF</td>
<td>(Weber and Gloer, 1988)</td>
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<tr>
<td>Sporormiella</td>
<td>vexans</td>
<td>Sporovexins</td>
<td>AF/AB</td>
<td>(Soman et al., 1999)</td>
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<td>Bombardioidea</td>
<td>anartia</td>
<td>Bombardolides</td>
<td>AF/AB</td>
<td>(Hein et al., 2001)</td>
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<tr>
<td>Nigrosabulum</td>
<td>globosum</td>
<td>Pseudodestruxins</td>
<td>AB</td>
<td>(Che et al., 2001)</td>
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<td>Stilbella</td>
<td>aciculosa</td>
<td>Fusidic acid</td>
<td>AB</td>
<td>(Kuznetsova et al., 2001)</td>
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<td>Podospora</td>
<td>decipiens</td>
<td>Decipenin A</td>
<td>AF/AB</td>
<td>(Che et al., 2002)</td>
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<td>Coniochaeta</td>
<td>ellipoidea</td>
<td>Coniosetin</td>
<td>AF/AB</td>
<td>(Segeth et al., 2003)</td>
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<tr>
<td>Podosordaria</td>
<td>tulasnei</td>
<td>Tulasnein</td>
<td>AF</td>
<td>(Ridderbusch et al., 2003)</td>
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</tbody>
</table>

* Coprophilous fungi are fungi that thrive in the gut of grazing animals.
### Chapter 2: Literature Review

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Compounds</th>
<th>Biological activity (AF/AB)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Podospora</em></td>
<td>communis</td>
<td>Communiols</td>
<td>AB</td>
<td>(Che et al., 2005;</td>
</tr>
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<td><em>Podospora</em></td>
<td>curvicolla</td>
<td>Curvicollides</td>
<td>AF</td>
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<td>minimoides</td>
<td>Sporminarins</td>
<td>AF</td>
<td>(Mudur et al., 2006)</td>
</tr>
<tr>
<td><em>Stilbella</em></td>
<td>erythrocephala</td>
<td>Antiamoebins</td>
<td>AF</td>
<td>(Lehr et al., 2006)</td>
</tr>
<tr>
<td><em>Cuprinopsis</em></td>
<td>cinerea</td>
<td>Copsin</td>
<td>AB</td>
<td>(Essig et al., 2014)</td>
</tr>
<tr>
<td><em>Hypocopra</em></td>
<td>rostrata</td>
<td>Hypocoprins</td>
<td>AB</td>
<td>(Jayanetti et al., 2015)</td>
</tr>
</tbody>
</table>

* Modified from Bills et al. (2013)

#### 2.8 Koala faeces: a unique environment for fungi

The koala (Figure 2.3) is an arboreal marsupial inhabiting the bushland of eastern and south-eastern Australia. The koala has an unusual diet consisting exclusively of the leaves from *Eucalyptus* spp., which are low in nutrients and are toxic to most animals due to the presence of phenolics (principally tannin) and essential oils (Cork and Foley, 1997).

![Koala feeding](image)

**Figure 2.3** Koalas feed exclusively on *Eucalyptus* leaves. Photo: E. Hayhoe.
Due to their nutrient-poor diet, koalas spend most of their time sleeping to conserve energy (Cork and Hume, 1983). Koalas are able to obtain nutrition from *Eucalyptus* leaves by hindgut fermentation using their highly developed caecum, which is proportionately the largest of all animals. The finer, more nutritious leaves are retained in the caecum and colon (the hindgut) and fermented by anaerobic bacteria to detoxify the material and release enough nutrition to maintain the koala’s slow metabolism (Moyal, 2008; Osawa et al., 1993). The larger particles, consisting of undigested cellulose, hemicellulose, highly lignified fibre and tannin, are compressed in the rectum and excreted rapidly as firm, dry and fibrous faecal pellets (Figure 2.4) (Moyal, 2008; Tyndale-Biscoe, 2005).

![Figure 2.4](image)

**Figure 2.4** Koala faeces present a unique substrate for fungal colonisation, consisting of highly fibrous remnants of *Eucalyptus* foliage (www.abc.net.au/science/scibblygum).

The diet and digestive system of an animal can influence the diversity of coprophilous fungi in the dung microcosm (Kruys and Ericson, 2008). The unusual feeding habits and hindgut digestion of the koala lends weight to the hypothesis that interesting coprophilous fungi will be isolated from their faeces. There has been very little research into the fungi that grow on koala faeces, and the early studies were conducted from a taxonomic perspective only (Bell, 2005; Cribb, 1997). Recently, Peterson et al. (2009) screened fungi from koala faeces for the production of hydrolytic enzymes on the premise that, to survive, they must be capable of degrading the lignocellulose-rich material that the faeces contain. They isolated 37 fungi: 18 Ascomycetous genera, three Basidiomycetes and one Zygomycete. Their research represents the one other instance,
besides the current study, in which fungi from koala faeces were identified by internal transcribed spacer (ITS) sequencing. The following chapters present, for the first time, the systematic isolation of fungi across a succession period from koala faeces and their identification by ITS sequencing for the principal purpose of screening their secondary metabolites for antimicrobial activity.
CHAPTER 3

Isolation and Identification of Coprophilous Fungi
3.1 Introduction

Fungi are an important but often overlooked member of the Australian biota. Estimates of the number of described species in Australia range from 5,672 to 12,500, and the total diversity is varyingly estimated from 50,000 up to 250,000 (Chapman, 2009). Studies of coprophilous (dung-inhabiting) fungi often focus on their taxonomy and their ecological aspects rather than investigating their secondary metabolites for bioactivity. Coprophilous fungi have shown promise as a source of antimicrobial compounds (see Section 2.7.4), but the majority of research has taken place in the northern hemisphere, leaving Australian coprophilous fungi as an underexplored arena primed for discovery (Bell, 2005; Krug et al., 2004).

The koala has an unusual diet consisting solely of eucalyptus leaves, which are extremely toxic to most animals. The koala is able to extract enough nutrients from eucalyptus leaves to maintain metabolism and excretes faeces consisting of undigested cellulose, highly lignified fibre and phenolic compounds (Peterson et al., 2009). The digestive system of an animal may influence the species composition and richness of the dung microcosm (Kruys and Ericson, 2008), and therefore, the unusual feeding habits and hindgut digestion of the koala lends weight to the hypothesis that interesting coprophilous fungi will be isolated from their faeces.

To the best of my knowledge, this is the first time that fungi have been systematically isolated across a succession period from koala faeces and identified by ITS sequencing for the principal purpose of screening their secondary metabolites for antimicrobial activity. The ITS sequences obtained in this research have been submitted to the NCBI and represent only the second collection of coprophilous fungi from koala faeces in their database (Peterson et al., 2009). This chapter describes the isolation and identification of coprophilous fungi from the faeces of koalas living in Boho South and French Island in Victoria, Australia.
3.1.1 Chapter aims

The major aims of this chapter were to:

i. isolate coprophilous fungi from a collection of koala faeces

ii. identify fungal isolates via sequencing of the ITS rDNA regions

iii. determine the phylogenetic relationship between the isolates.

3.2 Materials and methods

3.2.1 Isolation media

Culture media were purchased from Becton Dickinson (NJ, USA). All media were prepared using distilled water and sterilised by autoclaving at 121 °C for 15 minutes prior to use. Potato dextrose agar (PDA) consisted of 4 gL\(^{-1}\) potato starch, 20 gL\(^{-1}\) glucose and 15 gL\(^{-1}\) agar with a natural pH of 5.6 ± 0.2. Potato dextrose broth (PDB) was used for liquid cultivation of isolates and consisted of 4 gL\(^{-1}\) potato starch and 20 gL\(^{-1}\) glucose. Water agar consisted of 15 gL\(^{-1}\) agar. Agar plates were prepared by aseptically by pouring 15–20 mL of molten (~50 °C), sterilised media into circular (80 mm diameter) Petri dishes (Techno-Plas, Australia).

3.2.2 Collection of faeces

Koala faeces were obtained in Victoria, Australia from areas surrounding Boundary Hill Rd in Boho South and Bayview Rd on French Island (Figure 3.1). The faeces were collected by Dr Kath Handasyde (Department of Zoology, University of Melbourne, Australia) within 15 minutes of falling to the ground using clean disposable gloves and they were then lightly brushed to remove any materials adhering to the surface. This was to reduce the isolation of organisms that had originated in the soil and leaf debris adjacent to the faeces rather than being present in and on the faeces themselves. The faeces were then stored in clean paper envelopes where they were allowed to dry at room temperature until further use. This
drying process minimised the number of bacteria that may grow when the faeces are later incubated (Krug, 2004).

**Figure 3.1** The faeces were collected in Boho South and French Island in Victoria, Australia.

### 3.2.3 Isolation of fungi from faeces

Faeces-in-agar and ethanol-faeces-agar (pour-plate and spread-plate) plates were prepared from surface sterilised koala faeces. All plates were incubated in the dark at 25 °C for up to 6 weeks. The plates were inspected daily and fungi were isolated by either transferring hyphae as they appeared or by using a sterile inoculating loop and blade to transfer small slices of agar to a separate PDA plate. The isolates were then incubated at 25 °C and monitored to ensure the absence of contamination.

#### 3.2.3.1 Surface sterilisation

To sterilise the surface of the koala faeces, the pellets were soaked in a weak bleach solution (0.01% v/v NaClO) for 1 minute, rinsed twice with MilliQ water and blotted dry with sterile filter paper (Bradner et al., 2000).
3.2.3.2 Faeces-in-agar plates

Using a sterile blade and forceps, the surface-sterilised faecal pellets were cut in half lengthways and each half was placed in the centre of an empty Petri dish. Then, 20 mL of cooled, molten water agar was poured into one Petri dish to surround the pellet and 20 mL of PDA was poured into the other Petri dish.

3.2.3.3 Ethanol-faeces-agar plates

Faecal pellets were cut into small pieces (approximately 2 mm³) with a sterile blade and then placed in 60% ethanol and shaken by hand for 4 minutes. First, 1 mL of the faeces suspension was removed and added to a sterile Petri dish. Then 20 mL of PDA was poured into the dish and moved gently to evenly mix the suspension and the agar, thus achieving a pour-plate (Bills and Polishook, 1993). A further 1 mL of the suspension was spread evenly onto the surface of a PDA plate with a sterile glass spreader to produce a spread-plate for isolation of fungi.

3.2.4 Cultivation of fungal isolates

All fungal subculture and inoculation processes were carried out in a Class 1 ultraviolet biological safety cabinet (Email Westinghouse Pty Ltd.) with adherence to aseptic techniques.

Fungal isolates were grown on PDA plates after inoculation from agar slant stock cultures. A small portion of the mycelium that was growing at the slant perimeter was removed and transferred to the centre of a PDA plate. The cultures were incubated at 25 °C until radial growth approached the Petri plate edges.
3.2.5 Storage of fungal isolates

Medium-term storage of fungal isolates was achieved using agar slants. McCartney bottles were filled with 15 mL of PDA then sterilised and allowed to solidify on an angle of approximately 30° to produce a slant. A segment of mycelia from an isolate growing on a Petri plate was placed on the surface of the agar slant and incubated until growth had extended approximately 30 mm. Agar slants were then stored upright at 2 °C to 8 °C.

For long-term storage of fungal isolates, glycerol stocks were prepared. Microcentrifuge tubes containing 180 µL of liquid fungal culture and 20 µL of 80% glycerol (at a final concentration of 8%) were stored at −80 °C for up to 24 months.

Deep freeze stocks for the purpose of DNA extraction were also prepared. Slices of fungi were removed from the surface of the PDA plates and then transferred to microcentrifuge tubes and stored at −80 °C without the addition of a glycerol solution. Preliminary experiments suggested that this was an adequate form of storage for the later extraction of DNA.

3.2.6 Identification of fungal isolates

Pure cultures of each fungal isolate were established on PDA and photos were taken and general observations were recorded. Identification of the isolates was performed via amplification and direct sequencing of the ITS1, 5.8S and ITS2 regions of the fungal rDNA and comparison with the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.6.1 Extraction of fungal DNA

Genomic DNA was isolated from the fungi using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp., USA) with the following minor adjustments to
the manufacturer’s protocol. Each sample was introduced to a lysis tube in the form of a slice (approximately 5 mm × 15 mm) taken from the perimeter of actively growing fungi on a PDA plate. Prior to DNA extraction, these mycelia samples had been stored at −80 °C according to the long-term storage protocol (see Section 3.2.5). After the addition of the DNA elution buffer, the column matrix was allowed to rest for 4 minutes. The isolated DNA was assessed, in terms of quantity and quality, by 1% (w/v) agarose gel electrophoresis.

### 3.2.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse extracted DNA and fragments generated by polymerase chain reaction (PCR). A 50 × stock solution of Tris-acetate-EDTA was prepared with (per 1 L) 2 M Tris base and 6.5 M EDTA disodium salt at a pH of 8. Ethidium bromide (at a final concentration of 0.5 µg/mL) was incorporated into the 1% (w/v) agarose gels to allow for detection of DNA bands. A loading dye was added to each sample lane and a 1 Kb ladder was added to one lane for estimation of DNA length. Samples were electrophoresed in a Mini-Sub cell GT tank with an attached PowerPac Mini (Bio-rad) in 1 × Tris-acetate-EDTA for 40 minutes at 120 V and 30–40 mAmperes.

### 3.2.6.3 PCR amplification and direct sequencing of fungal ITS regions

PCR was performed to amplify the ITS1, 5.8S and ITS2 regions of the extracted fungal DNA. Table 3.1 lists the primers used in the PCR (White et al., 1990) and Figure 3.2 shows the location of the two primers on nuclear ribosomal DNA. The ITS1 forward primer (ITS1 fwdpr) locates upstream (5’) from the ITS1 region and the ITS4 reverse primer (ITS4 revpr) locates immediately downstream (3’) from the ITS2 region (White et al., 1990).
Table 3.1 PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
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</thead>
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<tr>
<td>ITS1</td>
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</tr>
<tr>
<td>ITS4</td>
<td>TCC TCC GCT TAT TGA TAT GC</td>
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</tbody>
</table>

Figure 3.2 Schematic representation of the ITS regions of fungal DNA and the binding sites of the ITS1 forward primer (ITS1 fwdpr) and ITS4 reverse primer (ITS revpr).

Note: PCR amplification using these two primers results in an amplicon spanning the 5.8S rDNA, ITS regions 1 and 2 and immediately adjacent areas of the 18S and 28S rDNA.

Each 50 μL reaction contained 25 μL of GoTaq Green Master Mix (Promega, NSW, Australia), 10 μmol of each primer (Sigma, NSW, Australia), 100 ng of DNA template and 21 μL of nuclease-free water. PCR was performed in a MyCycler™ thermal cycler (Bio-Rad, Hercules, USA) as described in Table 3.2.

Amplified DNA was purified using the centrifugation procedure of the Wizard SV Gel and PCR Clean-up System kit (Promega, Madison, USA) in accordance with the manufacturer’s protocol. Purified samples were visualised via electrophoresis to assess the quality and quantity for preparation of samples for direct sequencing.
Table 3.2 Thermal cycle for PCR

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<th>Time (s)</th>
</tr>
</thead>
<tbody>
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<td>180</td>
</tr>
<tr>
<td>Amplification</td>
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<td>95</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55</td>
<td>45</td>
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<td>120</td>
</tr>
<tr>
<td>Extension</td>
<td>1</td>
<td>72</td>
<td>420</td>
</tr>
</tbody>
</table>

The DNA concentration in each sample was determined via spectrophotometry (GeneQuant Pro Amersham, Biosciences) with reference to distilled water, and approximately 20 ng of DNA was added to 1 μL of ITS4 primer (the total volume was 12 μL). Samples were submitted to the Australian Genome Research Facility (http://agrf.org.au) in Melbourne for DNA sequencing and capillary separation on the AB 3730xl DNA analyser.

3.2.6.4 DNA sequence analysis

The DNA sequence data provided by the Australian Genome Research Facility was compared with sequences in the NCBI database using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identifications were assigned to each isolate based on the similarity of their sequences to database entries. Pairwise alignment of the ITS regions of the isolated fungi and the NCBI database entries was completed with MEGA software version 5 (Tamura et al., 2011) and multiple sequence alignment used the ClustalW alignment method (blosum matrix; http://www.ebi.ac.uk/clustalw/). Results were displayed using the Jalview workbench software (Waterhouse et al., 2009).

The multiple sequence alignment of the ITS regions of the isolated fungi was used to construct a phylogenetic tree with the MEGA software. The neighbour-joining method (Saitou and Nei, 1987) was used to infer the evolutionary history of the fungal isolates and bootstrapping was performed with 1,000 replications.
3.3 Results and discussion

3.3.1 Collection of koala faeces

Observations of the faecal pellets were consistent across the collection with lengths ranging from 11 mm to 29 mm, medium to dark brown colour, pleasant eucalypt-like scent, very dry and dense consistency, visible undigested fibre, and in some of the samples, the presence of a small amount of animal fur. Figure 3.3 shows a representative faecal pellet from the French Island collection.

![Figure 3.3](image)

**Figure 3.3** A koala faecal pellet (a) intact and (b) cut in half, showing the dense and dry interior.

There were no discernible differences in the appearance of the faeces from koalas residing in French Island and the faeces from koalas in Boho South. Individual faecal pellets were chosen at random to be incubated for the isolation of fungi.

3.3.2 Incubation of koala faeces

The koala faeces were incubated using two techniques: Faeces-in-agar (water agar and PDA) and ethanol-faeces-agar (pour and spread) plates. The faeces-in-agar method required the faecal pellet to be sliced in half so that one half could be
incubated in the presence of water agar and the other with PDA. However, due to the very dry and densely packed nature of the faecal pellets, it was difficult to cut them evenly in half. Therefore, some plates had one larger piece while other plates contained smaller segments (see Figure 3.4b). It is possible that the degree of fragmentation of the sample may have affected the quantity or variety of fungi isolates because it changed the internal surface area of the faeces that was in contact with the agar.

Faecal fragment size was more consistent on the ethanol-faeces-agar plates because the samples were first cut into small pieces and then shaken in ethanol. Ethanol was included because it is a recognised method for reducing the growth of rapidly growing zygomycetous and ascomycetous species, and to maximise the isolation of ascomycetous and basidiomycetous species that usually appear later in the succession (Krug et al., 2004; Peterson et al., 2009; Warcup, 1950). No zygomycetous species were isolated from the koala faeces, which could be due to the ethanol or because they prefer fresh dung as a substrate and may have been affected by the drying period (Bell, 2005).

In some studies, antibiotics such as chloramphenicol and ampicillin are included in the PDA to reduce or eliminate bacterial competition (Krug, 2004; Peterson et al., 2009). Antibiotics were not used in this study, and no bacterial growth was observed at any point during incubation of the koala faeces. Although there was no bacterial competition, some of the faeces-in-agar plates resulted in the dominance of one fungal species, which made isolation of emerging species difficult. One such fungus, later identified as *Sordaria alcina*, belongs to an almost exclusively coprophilous genus and was identified on six occasions in this project. Many of the fungi belonging to the order Sordariales (although not exclusively) are capable of secreting antifungal compounds such as sordarin and its derivatives. This may have been produced during incubation in this study and inhibited the growth of competing fungal species (Bell, 2005; Bills et al., 2009; Hauser and Sigg, 1971b), as shown in Figure 3.4a.
An alternative incubation method that is commonly used to isolate coprophilous fungi is the moist chamber method. In this approach, the dung is placed inside a Petri dish (or another appropriate container) with filter paper that is periodically moistened with water to create a humid environment to encourage growth on the surface of the faeces (Krug, 2004). This technique reveals fungi that are able to rely solely on the faeces for nutrition and is commonly used among coprophilous fungi enthusiasts because it requires no specialised equipment or media. However, because this research required the fermentation of the isolates in PDB to produce secondary metabolites, it was more appropriate for the koala faeces to be incubated in PDA so that the isolated fungi are more likely to be cultivable with PDB.

![Image of faeces-in-agar plates](image)

**Figure 3.4** a) An isolate later identified as *Sordaria alcina* dominated this faeces-in-agar plate. The faecal pellet in this instance was able to be cut in half so that one larger piece was present for fungal growth and isolation; b) this faeces-in-agar plate contains fragments of a faecal pellet that broke apart upon slicing.

### 3.3.3 Isolation of fungi

Every faecal sample produced visible fungi upon incubation, and most were successfully transferred to PDA for continued growth and further study. A selection of isolated fungi growing on PDA plates is shown in Figure 3.5.
Some isolates did not survive subculturing beyond the original faecal agar plate, which suggests the presence of symbiotic relationships among the mixed culture or dependence by the isolate on the presence of the koala faeces. Some isolates that survived subculturing exhibited difficulty in producing confluent growth. In these cases, PDA may not have been the ideal nutrient medium and 25 °C may not have been the optimum temperature for incubation.

**Figure 3.5** Images of a selection of fungi isolated from koala faeces, growing on PDA plates.

It is likely that there were fungal species present in the faeces that were unable to be cultured under laboratory conditions. These species may be identified by sequencing the DNA extracted directly from the faeces using a method that is usually employed when the objective of the research is to report on the distribution and diversity of specific fungi taxa within a substratum (Anderson and Cairney, 2004; Bastias et al., 2006; Herrera et al., 2011). For example, researchers in Australia have used this
technique by sampling DNA from soil to study the effects of fire on fungal communities in eucalypt forests in New South Wales and Victoria (Chen and Cairney, 2002).

This study is not an exhaustive survey of the coprophilous fungal community found in koala faeces. The fungi that were isolated were those that survived the drying process, were able to undergo sporulation or spore germination under the prescribed incubation method, and could be grown on PDA. In addition, these isolates were not significantly restricted by antagonistic behaviour of competing fungi, such as the production of diffusible antibiotics or hyphal interference (Wicklow, 1981).

The collection of fungi in this research represents an unknown fraction of the species present in a collection of faeces from 12 individual koalas living in two locations at one time of the year. Given these considerations, an expanded survey of the coprophilous fungi population could address experimental variables, including adjustment of the drying time for faeces, changes to incubation temperature, the introduction of light and dark cycles, and the use of a different medium such as malt extract agar. In terms of variables relating to the collection of faeces, some studies have suggested that season and location are likely to affect the diversity of the coprophilous fungal community (Krug et al., 2004; Kruys and Ericson, 2008; Melo et al., 2012; Richardson, 2001c). Therefore, although time intensive, a more comprehensive survey would include many faecal pellets collected at different times of the year from numerous koalas residing in various locations. This approach may identify fungal species that were not found in this research and therefore increase the opportunities for discovery of novel bioactive compounds produced as secondary metabolites.

3.3.4 Identification of fungi by ITS sequencing

Genomic DNA was extracted from the isolated fungi using the Zymo Fungal DNA MiniPrep after the isolates had been stored as agar slices at −80 °C. The
manufacturer’s protocol suggests that fungal cells be added in the form of a suspension in water or isotonic buffer; however, the addition of frozen mycelia on a very thin slice of agar directly to the lysis solution did not appear to negatively affect the extraction and sufficient DNA yields were achieved from all isolates.

Amplification of the ITS regions of the fungal isolates resulted in PCR products ranging from 513–603 base pairs in length. Figure 3.6 shows the gel electrophoresis image of PCR products from 20 isolates that were later identified as belonging to the phyla Ascomycota.

![Figure 3.6](image.jpg)

**Figure 3.6** Agarose gel electrophoresis of PCR products following the amplification of the ITS regions of rDNA of a selection of fungi isolated from koala faeces. Note: 5 µl of DNA was loaded per lane. Lane M is the 1kb DNA ladder; lanes 1–20 are samples: F14P1, S5P5, S4P1, F14W1, F3P1, F13P1, S4P2, F14W2, F3W3, S5P3, S3P1, S3P2, S3P3, F11EP1, F16EP1, F16EP2, F16EP1, F16ES1, F4P1, F2P1 and F2P2.

The PCR products were sequenced at the Australian Genome Research Facility and resulted in DNA sequences spanning the 5.8S rDNA, ITS regions 1 and 2, and the adjacent 18S and 28S rDNA. The sequence data chromatogram for each isolate was analysed with particular attention paid to the beginning and end of the sequences where peaks can be of poor resolution. Sequences were edited at residues where the peaks were incongruent with nucleotide allocation (see Figure 3.7). These sequences were searched against the NCBI database using BLAST and identifications were considered based on their similarity to sequences in the database (see Table 3.3).
Pairwise alignment of the ITS regions of the isolated fungi and their NCBI database matches revealed points of difference between the sequences (see Figure 3.8).

**Figure 3.7** A section of the DNA sequence chromatogram showing the ITS2 region and adjacent 28S rDNA of the isolate S4P4, KT071741, *Trichoderma pseudokoningii*. Note: Comparison of the chromatogram with a pairwise alignment of the sequence with its top BLAST match (X93970) highlighted two residues (cytosine, blue and adenosine, green) that were apparent in the peaks but not the sequence. The sequence was edited at these two points. Sequence chromatograms from the Australian Genome Research Facility show reverse complement sequences as the reverse primer, ITS4 was used for sequencing.

The ITS sequences were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/) and were assigned the accession numbers KT071713–KT071744 (KT071743 has been omitted). To date, this submission was only the second collection of ITS sequences from fungi isolated from koala faeces present in the Genbank database. The sequences form the GenBank PopSet (population sequence data) with the identifier 914704626. All of the isolates returned sequence matches of at least 95% (E = 0) with known fungal species, and 19 of the 31 isolates were identified with matches of at least 99%. Identification based on comparison with GenBank accessions requires that other users submit accurate sequencing results with correct assignment of taxonomic groups (Bellemain et al., 2010). Moreover, the non-coding ITS regions are subject to relatively fast evolutionary rates that may lead
Chapter 3: Isolation and Identification of Coprophilous Fungi

to difficulties in achieving perfect sequence alignment at high taxonomic levels (Huang et al., 2009). The GenBank database has numerous environmental isolates that are listed as unidentified fungi or have not been identified at the species level, which can further impede identification (McMullan-Fisher et al., 2011). For example, isolate F5A4 (HQ406811) was identified as *Penicillium spinulosum* in this work, but it also shared maximum sequence homology with an accession listed as “Unverified – Uncultured Penicillium clone” (HQ211519) in the database (Deslippe et al., 2012).

When research such as this requires the comparison of sample ITS sequences with GenBank accessions, there is no agreed upon percentage of sequence similarity that definitively indicates that the two sequences represent the same species (Bruns et al., 2007; Hughes et al., 2009). However, a number of studies have used ≤3% ITS sequence divergence to indicate conspecificity in fungi (Morris et al., 2008; O’Brien et al., 2005; Ryberg et al., 2008; Smith et al., 2007; Walker et al., 2008). All but two of the fungi isolated from the koala faeces (S4W1, *Neurospora intermedia*, KT071742, 96% and F16EP1, *Anthostomella leucospermi*, KT071725, 95%; Table 3.3) meet this cut-off. An alternative method which may provide a more robust identification at the species level is multilocus sequence typing (MLST). MLST measures the variation in DNA sequences of a number of genes such as those encoding beta-tubulin and calmodulin (Hong et al., 2014; Perrone et al., 2014).

Almost all of the fungi isolated from the koala faeces were identified as species belonging to the phylum Ascomycota. One isolate (F3W1, KT071734) identified as *Bjerkandera adusta* is from the phylum Basidiomycota. No members of the phylum Zygomycota were isolated. The prevalence of ascomycetous species in the collection of fungi is consistent with previous studies of coprophilous fungi, in which members of the phylum Ascomycota have dominated the dung mycota (Bell, 2005; Doveri et al., 2013; Gupta, 2010; Melo et al., 2012; Pandey, 2009; Peterson et al., 2009; Richardson, 2001a, c; Watling and Richardson, 2010).
Figure 3.8 Pairwise alignment of the ITS1 and ITS2 regions and adjacent rDNA of the isolate S4P4, KT071741, *Trichoderma pseudokoningii*, isolated from koala faeces to that of the sequence with the greatest similarity in the NCBI database (at the time of analysis) with the accession number X93970.

Note: The two edited residues (Figure 3.7) are indicated in yellow and the one discrepancy between the two sequences appears in red.
Table 3.3 Identification of fungi isolated from koala faeces determined by BLAST

<table>
<thead>
<tr>
<th>Sample*</th>
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<th>Accession number</th>
<th>Similarity (%)</th>
<th>Sequence length (bp)</th>
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<td>EU440778</td>
<td>98</td>
<td>572</td>
</tr>
</tbody>
</table>

* Sequences from each sample were submitted to the NCBI database in this order and assigned ascending accession numbers KT071713–KT071744 (KT071743 was omitted).
A number of the genera are recorded in the literature as being found on koala faeces; *Sordaria*, *Aspergillus* and *Penicillium* species were identified by morphology only (Bell, 2005; Cribb, 1997), and *Aspergillus*, *Penicillium*, *Sordaria*, *Fusarium*, *Neurospora* and *Trichoderma* were identified by DNA sequencing in the formative research by Peterson et al. (2009). Some of the Ascomycetes isolated in this work have not been definitively recorded in the literature as having been observed on koala faeces. However, it would be rash to assume that this is the first occasion of their discovery on koala faeces. The majority of morphological identifications that have been made during dung surveys in Australia have not been submitted to peer-reviewed journals. Rather, the samples were often collected by keen naturalists and identifications, if published, were limited to club newsletters and the like.

One survey that was more systematic in nature is the illustrated guide compiled by Ann Bell (2005), in which she identified 176 species of Ascomycetes from approximately 300 dung samples (including koala) from Australia that were collected mostly by volunteers. Bell also makes reference to two other mycologists who studied coprophilous fungi in Australia, Dade and Cribb, the former identifying both *Sordaria* and *Fimetariella* species on dung collected in Victoria, Australia. Of the remaining genera isolated and identified in this research, the Ascomycetes *Thielavia* and *Anthostomella* and the Basidiomycete *Bjerkandera* are known to occur as coprophilous fungi (Baker et al., 2013; Misra et al., 2014; Reina et al., 2013). The genus *Neurospora* is closely related to *Sordaria*; however, while the latter contains predominantly coprophilous species, the majority of species within the sister genus *Neurospora* are found primarily on other substrates such as soil (Krug et al., 2004; Raju, 2002). Finally, several *Plectosphaerella* species are pathogens of plant species in Australia, causing root rot (Jahromi, 2007; Tesoriero, 2014), but to the best of my knowledge, this genus has not been reported to occur on faeces in Australia.
3.3.5 Comparison of the ITS regions of the fungal isolates

A multiple sequence alignment of the ITS regions of the fungal isolates was created using ClustalW to explore the number and location of differences in nucleotide bases between selected isolates. Jalview software produced a visualisation of the analysis including the construction of the consensus graph shown in Figure 3.9.

The ITS regions, as expected, demonstrated a higher degree of difference between the isolates compared with the highly conserved sequences in the adjacent 18S, 5.8S and 28S rDNA regions. In terms of the ITS regions, similarities were most common between isolates of the same genus (e.g., Neurospora intermedia, F4P1, KT071718 and Neurospora pannonica, S3P1, KT071728; Table 3.3 and Figure 3.9), and then between isolates belonging to the same phylum (N. intermedia, F4P1, KT071718; N. pannonica, S3P1, KT071728; Trichoderma pseudokoningii, S4P4, KT071741; all Ascomycetes; Figure 3.9). In comparison, further differences can be seen between the three representative Ascomycete isolates and the isolate Bjerkandera adusta (F3W1, KT071734; Figure 3.9) from the phylum Basidiomycota. Insertions in the ITS regions of the T. pseudokoningii isolate accounted for its sequence length being larger than that of the other Ascomycetes (Table 3.3, final column). B. adusta also contained insertions, notably within the mostly conserved 5.8S rDNA region, accounting for its second largest sequence length.

The multiple sequence alignment of the ITS regions of the fungi was used to construct a phylogenetic tree with MEGA software version 5. The neighbour-joining method with bootstrap re-sampling performed over 1,000 replicates was used to infer evolutionary history of the fungal isolates. As shown in Figure 3.10, this method placed the thirty Ascomycete isolates into five different orders (Sordariales, Hypocreales, Incertae sedis, Xylariales and Eurotiales), all belonging to the Sordariomycetes class; the single Basidiomycete isolate, B. adusta, is shown on a separate branch.
Figure 3.9 Jalview representation of ClustalW multiple alignment of ITS1, 5.8S rDNA, ITS2 and adjacent 18S rDNA and 28S rDNA of four fungi isolated from koala faeces. *Neurospora intermedia* (F4P1) KT071718, *Neurospora pannonica* (S3P1) KT071728 and *Trichoderma pseudokoeningii* (S4P4) KT071741 from the phylum Ascomycota; *Bjerkandera adusta* (F3W1) KT071734, a representative of the Basidiomycete phylum. The black bar graph represents the percentage of bases matching the consensus sequence at each residue.
Figure 3.10 Phylogenetic position of fungi isolated from koala faeces.
Note: The evolutionary history was inferred using neighbour-joining method (Saitou and Nei, 1987) and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (≥50%, 1,000 replicates) is shown next to the branches (Felsenstein, 1985). Five orders within the Sordariomycetes class are annotated and the one Basidiomycete isolate is positioned at the lowest branch.
3.4 Summary

Coprophilous fungi are an important part of the Australian ecosystem, but there is little published data describing these communities compared with their Northern hemisphere counterparts. In this chapter, a collection of coprophilous fungi were isolated from koala faeces and identified via ITS sequencing. All but one of the 31 isolates were members of the phylum Ascomycota, a weighted diversity that is common in numerous Ascomyceteous dominated coprophilous collections in the literature. This work did not reveal any previously unknown species, but the findings are of interest because they add to the very small amount of research into Australian coprophilous fungi. The isolates were assigned accession numbers KT071713–KT071744 and lodged with the NCBI database, where they represent only the second set of DNA sequences of coprophilous fungi isolated from koala faeces.

Coprophilous fungi have shown potential as a source of antimicrobial compounds in the literature, and this bioactivity will be explored in the following chapter. Chapter Four describes the preparation of extracts from liquid fermentations of isolated coprophilous fungi and the screening of these extracts against selected bacteria and fungi.
CHAPTER 4

Screening of Coprophilous Fungi for Antimicrobial Activity
4.1 Introduction

Natural products and their derivatives have long been exploited for their pharmaceutical potential, and it is estimated that more than 70% of the antimicrobial drugs currently in clinical use are derived from natural products (Qadri et al., 2014). However, new drug approvals are declining and it has been suggested that a discovery void exists (Li and Vederas, 2009). Given the increased resistance of pathogens to current drugs and the rising incidence of opportunistic infections in immune-compromised individuals, it is essential that previously untapped biological niches are explored for novel microbes and natural products. An alternative system of bacterial control is through the disruption of QS signals. An ideal quenching compound would reduce the virulence of, rather than kill, the target bacteria, and in turn, may reduce the development of resistant strains (Hentzer and Givskov, 2003).

Phytopathogenic fungi are becoming increasingly problematic for important food crops, particularly in developing countries (Vurro et al., 2010). These plant diseases cause a reduced harvest yield (or post-harvest rot), which has serious implications in terms of food security and a sustainable economy (Fears et al., 2014). Resistance to current agrochemicals is an ongoing challenge, and concerns relating to the safety and environmental impact of agrochemicals have led to a more stringent regulatory process (Lamberth et al., 2013). Thus, there is now a demand for new safer and more selective compounds, and it is important that natural products continue to be studied for their potential application in crop protection (Dayan et al., 2009; Olufolaji, 2010).

Fungi have provided numerous chemically and biologically diverse secondary metabolites that have proved immensely useful in medical, agricultural and industrial settings. Coprophilous fungi remain a relatively untapped source compared with fungi from other substrata and biological niches. However, coprophilous fungi are prime candidates for discovery of antimicrobials due to their ubiquity and their dominance in a highly competitive environment. The limited research to date has
revealed a number of bioactive secondary metabolites and further reinforces their potential for antibiotic discovery (Bills et al., 2013).

Australian coprophilous fungi have yet to be explored in terms of their potential to produce antimicrobial secondary metabolites. This chapter presents, for the first time, the screening of coprophilous fungi from koala faeces for antibacterial, antifungal and anti-QS activity.

4.1.1 Chapter aims

The experiments described in this chapter were designed to evaluate the bioactivity of coprophilous fungi from koala faeces. More specifically, this work aimed to assess the antimicrobial potential of their liquid cultivated extracts against:

i. the Gram-positive bacterium *S. aureus*

ii. three Gram-negative bacteria: *E. coli*, *P. aeruginosa* and *K. pneumoniae*

iii. the filamentous fungus *D. brizae*

iv. the yeast *C. albicans.*

In addition, the research sought to establish whether the extracts were capable of anti-QS activity using the indicator bacterium *C. violaceum.*

4.2 Materials and methods

4.2.1 Bacterial cultures

Antibacterial testing was performed against *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 9721) and *K. pneumoniae* (Australian Collection of Microorganisms: accession no. 90), which were part of the culture collection at Swinburne University of Technology, Victoria, Australia. Anti-QS activity was tested using the Gram-negative indicator strain *C. violaceum* (University of New South Wales collection: accession no. 040100).
Agar slants (Section 3.2.5) were prepared for medium-term storage of bacterial cultures. Overnight cultures were subcultured onto Mueller–Hinton agar (MHA; Oxoid, Basingstoke, UK) slants and incubated at 37 °C for 24 hours prior to storage at 4 °C for up to six months. Recovery of bacteria was achieved by subculture to Mueller–Hinton broth (MHB) and incubation for 18 hours at 37 °C. Storage and maintenance of C. violaceum used Luria–Bertani agar (Merck, Germany) and Luria–Bertani broth. For long-term storage of cultures, the Protect Bead storage system (Technical Service Consultants, Lancashire, UK) was used to enable frozen storage at −80 °C for several years.

The preparation of a standardised bacterial inoculation was required for the antibacterial and anti-QS assays. One colony was removed from a plate culture and then suspended in 2 mL of the appropriate liquid medium and incubated at 37 °C (30 °C for C. violaceum) for approximately 24 hours with shaking (at 220 rpm). Following incubation, the turbidity of the solution was adjusted with sterile saline to visually match a 0.5 McFarland standard, thus producing an inoculum with an approximate cell density of $1.5 \times 10^8$ colony forming units (cfu)/mL that could be further diluted if required.

### 4.2.2 Fungal cultures

Antifungal testing was performed against one filamentous phytopathogenic fungi, D. brizae (Phillips et al., 2011), and one yeast strain, C. albicans (FRR5580), which were both part of the culture collection at Swinburne University. D. brizae and C. albicans were both maintained on PDA and PDB with incubation at 25 °C for 7 days and 30 °C for 18 hours, respectively. Long-term storage of C. albicans used the Protect Bead system, whereas glycerol stocks were prepared for D. brizae (Section 3.2.5).

A standardised fungal inoculum of C. albicans was prepared following the protocol used for bacteria (Section 4.2.1) with the use of PDA and PDB media and incubation
at 30 °C. A 0.5 McFarland standard equates to $1 \times 10^6$ yeast cells/mL (Espinel-Ingroff and Canton, 2007d). Standardised suspensions of *D. brizae* were created from cultures that were grown in PDB for 7 days at 25 °C with shaking (at 180 rpm). Mycelia were vortexed vigorously and repeatedly drawn and aspirated through a 25 gauge needle (Terumo Corp., Macquarie Park, Australia) to separate cells. The resulting solution was adjusted to a concentration of $0.4 \times 10^6$ mycelia fragments/mL ($\text{OD}_{600} = 0.15–0.17$) (Espinel-Ingroff and Canton, 2007a).

### 4.2.3 Fungal fermentation for preparation of crude extracts

Extracts were produced from liquid fermentations of the isolated coprophilous fungi described in Chapter Three. For each isolate, a 1 cm$^2$ agar plug of fungi was placed in an Erlenmeyer flask containing 100 mL of PDB. The flask was stoppered with cotton wool and incubated at 25 °C in the dark with shaking at 150 rpm for 22 ± 2 days. Following incubation, the contents were filtered through Whatman (No. 1) filter paper under vacuum to separate the bulk of the mycelium from the fermentation liquor. The remaining fungal cells were removed from the liquid by membrane filtration through a 0.22 μm filter (Merck Millipore, MA, USA) under vacuum. This resulted in approximately 100 mL of sterile liquor and the separated mycelial biomass for each sample.

#### 4.2.3.1 Mycelia extraction

Mycelial biomass that had been separated from the fermentation broth was transferred to weighed sterile round-bottom flasks and frozen at −80 °C in preparation for lyophilisation. Following freeze-drying in a Cryodos-50 (Telstar, Spain), the mycelia were weighed and resuspended in 100% ethanol to a concentration of 100 mg/mL. Extracts were stored at 4 °C until use. When the mycelial extracts were tested for antimicrobial activity, the control consisted of an equivalent volume of ethanol.
4.2.3.2 Liquor extraction

The filtered liquor was extracted twice with equal volumes of ethyl acetate (EtOAc, Merck) in a separating flask for a total of 22 hours. The organic phases were combined and dried with anhydrous sodium sulphate (Sigma), evaporated to approximately 2 mL on a rotary evaporator at 45 °C (Buchi, Switzerland) and then transferred to a microcentrifuge tube and evaporated to dryness in a micro-rotary evaporator (John Morris Scientific, Chatswood, Australia) with centrifugation at 40 °C. The dried extracts were then resuspended in methanol (Merck) to a concentration of 100 mg/mL and stored at 4 °C until use (Corrado and Rodrigues, 2004). When the EtOAc extracts were tested for antimicrobial activity, the control consisted of equivalent volumes of methanol.

The aqueous phase of the liquid–liquid separation was rotary evaporated to remove any residual EtOAc; it was then frozen and lyophilized to dryness. The aqueous extracts were resuspended in MilliQ water to a concentration of 100 mg/mL and stored at 4 °C until use.

4.2.4 Antibacterial testing

Coprophilous fungal extracts were tested against one Gram-positive bacterium, (S. aureus) and three Gram-negative bacteria (E. coli, P. aeruginosa and K. pneumoniae).

4.2.4.1 Plate-hole diffusion

Screening of all extracts was performed using a modification of the plate-hole diffusion assay described by Rahman et al. (2001). Molten MHA (15 mL, 45 °C) was inoculated with 200 µL of a bacterial suspension (1.5 × 10^8 cfu/mL), mixed and then poured into a sterile petri dish. Once set, a sterile stainless steel cork borer (6 mm diameter) was used to make wells in the agar and 20 µL of extract was added to each
well. Control wells contained ethanol for mycelial extracts and methanol for organic liquor extracts. A chlorhexidine (CHX) gluconate solution (0.5% w/v in 70% v/v ethanol) was included for comparison (J & J Medical, USA). The plates were kept at room temperature for 30 minutes prior to incubation to allow for absorption of the extracts and controls. After incubation for 18 hours at 37 °C, the zones of inhibited growth were measured to the nearest millimetre (inclusive of well diameter) and the average of triplicate results was recorded.

4.2.4.2 Microdilution assay

Minimum inhibitory concentrations (MIC) were determined for the fungal extracts that produced a zone of inhibited bacterial growth that was greater than 8 mm in the plate-hole diffusion assays. A microdilution method was employed in accordance with a modification of the Clinical Laboratory Standards Institute document, M7-A7 (CLSI, 2006). In sterile round-bottom microtitre plates (Corning, USA), 150 µL of MHB was added to each of the test wells and 50 µL of each extract (5 mg) was added to the wells in the first column. The contents were mixed via gentle aspiration with a micropipette and a two-fold serial dilution was prepared by sequentially transferring 100 µL aliquots (with mixing) across each row. Bacterial inocula were prepared by diluting a 0.5 McFarland standardised suspension 1:200 in MHB to a final concentration of $7.5 \times 10^5$ cfu/mL. One hundred microlitres of the suspension was added to each of the test wells, producing an extract concentration range of 12,500–98 µg/mL. Each extract was tested in triplicate against each bacterium. A medium sterility control well contained only MHB, a growth control well contained bacteria in the presence of MHB only, and a positive control comprised two-fold dilutions of 0.5% CHX in addition to the bacterial inoculum and MHB. The plates were incubated overnight at 37 °C. After incubation, the wells were inspected for turbidity and the MIC for each extract was determined as the lowest concentration that inhibited visible growth.
Minimum bactericidal concentrations (MBC) were also deduced via a growth test (Qaiyum, 2007) by sub-culturing a 100 µL aliquot from the microtitre wells that had no visible growth onto MHA. Following incubation, the MBC was defined as the lowest concentration of the extract that resulted in zero growth on the MHA plate.

### 4.2.4.3 Time-course growth assay

To visualise the antibacterial activity of the coprophilous fungal extracts over 24 hours, a modification of the time-course assay described by Verma (2007) was used. Two extracts were selected for the assay based on their performance in the plate-hole diffusion and MIC assays. Inocula of *E. coli* and *S. aureus* were prepared by diluting a 0.5 McFarland standardised suspension 1:100 in MHB to a final concentration of $1.5 \times 10^6$ cfu/mL. For each bacterium, 1 mL of inoculum was added to six sterile tubes containing 4 mL of MHB. Fungal extract was then added to two tubes (duplicate) to a final concentration of 1 × MIC and to two tubes (duplicate) at a final concentration of 2 × MIC. The final two tubes served as duplicate growth controls. Two aliquots of 100 µL were removed from each tube and spread onto the surface of two MHA plates. A further two 100 µL aliquots were removed from each tube and duplicate ten-fold serial dilutions were performed from $10^{-1}$ to $10^{-6}$; 100 µL aliquots from each dilution were spread onto MHA plates. These plates represented the time=0 viable count. The time-kill tubes and all plates were incubated at 37 °C. This process was repeated at 0.5, 1.5, 1, 3, 6, 12 and 24 hours. Following overnight incubation of all plates, the viable cell numbers were counted for dilutions containing 30–300 cfu and the number of viable bacteria at each time point was calculated.

Each assay was performed in triplicate and a two-way analysis of variance with the Bonferroni’s post-hoc correction was performed using GraphPad Prism v.6 (GraphPad, La Jolla, CA) to assess statistical differences across conditions at each time interval. Values of $p < 0.05$ were considered statistically significant. Data are presented as the means ± standard deviation.
4.2.5 Antifungal testing

Coprophilous fungal extracts were tested against the pathogenic yeast strain *C. albicans* and the phytopathogenic fungus *D. brizae*.

4.2.5.1 *C. albicans inhibition*

Antifungal activity against *C. albicans* was assessed using modifications of the two methods that were used for the antibacterial testing. Plate-hole diffusion assays were carried out in PDA with the addition of 200 µL of a 1–5 × 10^6 cells/mL (McFarland standard 0.5) inoculum, and the plates were incubated at 30 °C (see Section 4.2.4.1). Extracts that exhibited anti-candidal activity were tested in a microdilution assay to determine their MIC and growth tested to assign a minimum fungicidal concentration (MFC). Modifications of this method included the use of PDB, addition of 100 µL of a 1–5 × 10^3 cells/mL inoculum to each test well and incubation of the 96-well microtitre plate at 30 °C (see Section 4.2.4.2).

4.2.5.2 *D. brizae inhibition*

Four methods were used to evaluate the inhibitory potential of the extracts against the filamentous fungus *D. brizae*.

*Spore germination assay*

To establish if the fungal extracts were able to inhibit spore germination by *D. brizae*, bioautographic assays were performed using the method of Quiroga et al. (2009) with some adjustments. Silica gel aluminium plates (0.2 mm thick, Sigma) were cut to size and then placed into empty sterile Petri plates and spotted with 15 µL of each extract and controls. After the plates had dried, 12 mL of semi-solid PDA (0.6% agar) inoculated with 0.3 mL of a *D. brizae* spore suspension (0.4–5 × 10^6 spores/mL) was poured over the surface of the plates. Following incubation
at 25 °C for 3 days, 100 µL of a 0.25% (w/v) thiazolyl blue tetrazolium bromide (MTT, Sigma) solution was spread over the surface of the plates and they were incubated for 1 hour. Plates were observed for the presence of a colourless zone surrounding an extract, which signified inhibition of spore germination. Each extract was tested in triplicate and results are presented as the mean clear zone diameter ± standard deviation.

Radial growth assay

In this antifungal assay, extracts and controls were incorporated into PDA prior to inoculation with the test fungus *D. brizae* to assess the effect of the extracts on radial growth (Boyraz and Ozcan, 2005; Quiroga et al., 2009; Zhao et al., 2010).

Extracts were mixed evenly with 10 mL of molten (45 °C) PDA to a final concentration of 5 mg/mL and poured into Petri plates. The agar was allowed to set and a fungal plug (8 mm diameter) of *D. brizae* was placed in the centre of the plate. All plates were incubated at 25 °C until the growth of the control approached the plate edge. Following incubation, the diameter of growth was measured in two directions and growth reduction was calculated using the following formula:

$$\text{Growth reduction} (\%) = \left( \frac{\text{growth in control} - \text{growth in extract}}{\text{growth in control}} \right) \times 100$$

Plates containing appropriate solvent (without extracts) were included as controls. Data are presented as the average growth reduction (%) from triplicate assays ± standard deviation.

Hyphal extension assay

The ability of the extracts to inhibit hyphal extension of *D. brizae* was assessed by disk diffusion assays using the method of Quiroga et al. (2009) with some
modifications. A fungal plug (8 mm diameter) of *D. brizae* was placed in the centre of a PDA plate. Sterile paper disks (Oxoid, 6 mm diameter) were impregnated with 20 µL of each extract. The discs were allowed to dry in a sterile dish and were then placed on the PDA surface, approximately 2 cm from the fungal plug with a maximum of 4 disks per plate. Controls included disks impregnated with 20 µL of an appropriate solvent, including water for the aqueous liquor extracts. Plates were inverted and incubated in the dark for 5 days at 25 °C. The assay was performed in triplicate, and any inhibition of fungal growth was visualised as a crescent-shaped inhibitory zone at the mycelial front adjacent to the disk.

**Microdilution assay**

Extracts that exhibited positive results in two or more of the antifungal tests were selected for inclusion in a microdilution assay to determine their MIC against *D. brizae* (Espinel-Ingroff and Canton, 2007a).

The assay was performed in 96-well sterile polystyrene plates with low evaporation lids (Corning). Wells were filled with 200 µL of PDB and then 100 µL of each extract was added to the first well of each row and a 2-fold serial dilution was carried out across the row. A mycelial suspension of *D. brizae* was prepared (see Section 4.2.2) and diluted 1:50 in PDB to a final concentration of 0.8–10 × 10⁶ mycelial fragments/mL and each well was inoculated with 50 µL of the suspension. The final concentration of the extracts was within the range of 25–0.78 mg/mL and all extracts were tested in triplicate. Growth control wells did not contain extract, and wells containing 0.5% CHX were included as an antifungal control. The wells at the plate perimeter were filled with 200 µL of MilliQ water to reduce evaporation of the test wells and the plates were incubated in the dark for 7 days at 25 °C. Following incubation, the plates were placed on a light box and the wells were inspected for fungal growth. The MIC was defined as the lowest concentration of extract to completely inhibit fungal hyphal growth (Espinel-Ingroff and Canton, 2007a).
4.2.6 Anti-QS testing

For each extract, QS inhibition was tested via the standard antibacterial disk diffusion assay (Bauer and Kirby, 1966) using the indicator strain C. violaceum following the method described by Adonizio et al. (2006) with some modifications. Each extract (20 μL) was loaded onto a sterile paper disk (6 mm diameter), allowed to dry and then placed on the surface of Luria–Bertani agar plates spread with 100 μL (approximately 1.5 × 10^8 cfu/mL) of C. violaceum culture. Gentamicin disks (10 μg, Oxoid) were used to compare QS inhibition with an antibiotic effect and appropriate solvents were loaded onto disks for a negative control. Plates were incubated at 30 °C for 40 hours. QS inhibition was detected by a halo of colourless but viable cells around the disk, whereas antibacterial activity, when present, produced a zone devoid of cells.

Additionally, the ability of cells to recover from the anti-QS activity and regain production of violacein was established. A small sample of colourless colonies was removed from around an active extract and then subcultured onto Luria–Bertani agar and incubated for 40 hours at 30 °C. Following incubation, the plates were inspected for the presence of purple pigmented growth, which signals QS ability. Assays were performed in triplicate and measurements (to the nearest half millimetre) were taken from the edge of the disk to the edge of the halo.

4.3 Results and discussion

4.3.1 Production of mycelial extracts

Following incubation of the coprophilous fungi and separation of the mycelia from the fermentation liquor, the mycelia were freeze dried and dissolved in ethanol to produce 100 mg/mL mycelial extracts.
Mycelial extracts were produced in this manner from four fungi (F2P1, F13P1, S6W2 and S4P1). For the remaining isolates, only a liquor extract was created. The decision to omit the mycelium extract protocol for the remaining isolates was due, in part, to the difficult and time-consuming nature of working with the separated mycelia (compared with working with the sterile liquid filtrate). This decision was supported by numerous published studies describing the preparation liquor extracts for the screening of bioactive compounds (Bhagobaty and Joshi, 2012; Ganesh Kumar et al., 2010; Hoffman et al., 2008; Lin et al., 2005; Nath et al., 2012; Swathi et al., 2013; Xing et al., 2011).

4.3.2 Production of EtOAc and aqueous extracts from liquor

The fermentation liquor from each coprophilous fungus was separated from the mycelia and then extracted with EtOAc, thus producing an aqueous extract and an EtOAc extract. This protocol was followed with four fungi (F2P1, F13P1, S6W2 and S4P1), but it was decided to omit the liquid–liquid separation step for the remaining fungi and simply lyophilise the fermentation liquor to produce the liquor extract. The reason for this change in the protocol was that preliminary studies indicated that there was little difference in the bioactivity if the liquor was extracted with EtOAc, and this was further supported by the results of the screening assays (see Sections 4.3.3, 4.3.4 and 4.3.5. Given that the liquid–liquid partitioning, drying and screening of the extracts was a very time-intensive process, omitting this step meant that more of the fungi could be screened. Although extraction of the fermentation liquor with EtOAc is a common method in the literature (Gamble et al., 1995; Swathi et al., 2013; Weber and Gloer, 1991; Whyte et al., 1997), there are many published examples where the liquor is used in screening for bioactivity, either as the only approach or as a preliminary screening tool that precedes further extraction and separation (Ganesh Kumar et al., 2010; Liu et al., 2012; Zhang et al., 2012b).

In one such study, Phongpaichit et al. (2006) isolated endophytic fungi from *Garcinia* plants and screened them for antimicrobial activity. The fermentation liquor
was used for preliminary screening via plate-hole diffusion assays, and EtOAc extracts were produced from the liquor (and mycelia) for determining the MIC. The study found that of the 18.6% of fermentation liquors that exhibited antimicrobial activity, only 0.9–1.9% of the corresponding EtOAc extracts inhibited the same test organism. These results suggest that testing the fermentation liquor is a useful approach for evaluating antimicrobial activity, at least as the preliminary phase of screening. Therefore, for the remaining coprophilous fungi, EtOAc extraction was not performed and the fermentation liquor was lyophilised to produce extracts for screening assays. Liquor extracts were produced from the four aforementioned fungi, so that a comparison could be made in relation to the bioactivity potential of the different extraction methods.

4.3.3 Antibacterial activity of coprophilous fungi extracts

4.3.3.1 Plate-hole diffusion screening of extracts

The coprophilous fungal extracts were screened for antibacterial activity via the plate-hole diffusion assay against *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. The final amount of extract added to each well of bacteria-seeded agar was 2 mg. Following incubation, the appearance of a clear zone surrounding a well, as shown in Figure 4.1, indicated antibacterial activity for the extract. The zone diameter (inclusive of the well diameter, 6 mm) was measured and the mean values from triplicate assays are presented in Table 4.1.
Figure 4.1 *S. aureus* plate-hole diffusion assay, following incubation. Clear zones indicate inhibition of *S. aureus* by liquor extracts from the fungi S4P4 and F14P1 and by the positive control CHX. The extracts from fungi F13P1 and F14W1 did not inhibit growth of the bacterium.

Seventeen (54.8%) of the 31 coprophilous fungi produced extracts that were capable of inhibiting the growth of at least one test bacterium. Six fungi demonstrated broad-spectrum antibacterial activity, and three fungi (F16EP1, F14P1 and S5P3) were able to inhibit the growth of every bacterium to some degree. The Gram-positive bacterium *S. aureus* was more sensitive to the fungal extracts than the three Gram-negative bacteria. *S. aureus* was affected by the extracts from 14 fungi, whereas *E. coli*, the second most susceptible bacterium, was inhibited by nine. Only three of the extracts were capable of inhibiting the growth of *P. aeruginosa*. Similar observations have been reported from the screening of extracts from other fungal species, whereby *S. aureus* was shown to be the most sensitive of the test bacteria (Buatong et al., 2011; Ganesh Kumar et al., 2010; Hoffman et al., 2008; Phongpaichit et al., 2006). The differential sensitivity to the extracts by Gram-negative and Gram-positive bacteria are likely to be due to differences in their cell walls; Gram-negative bacteria have an outer polysaccharide membrane that acts as a barrier to environmental substances, including antibiotics (Madigan et al., 2003).
Table 4.1 Antibacterial activity of coprophilous fungal extracts

<table>
<thead>
<tr>
<th>Coprophilous fungus isolate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S. aureus</th>
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</tr>
<tr>
<td>F2P1 (EtOAc)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F2P1 (mycelium)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F2P2</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F4P1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F5A1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F5A2</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F5A3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F5A4</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F11EP1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F13P1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F13P1 (aqueous)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F13P1 (EtOAc)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F13P1 (mycelium)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F16EP1</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F16EP2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F16ES1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S3P1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S3P2</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>S3P3</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S6W2</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>S6W2 (aqueous)</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>S6W2 (EtOAc)</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S6S2 (mycelium)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S5P5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F3P1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F3W1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F3W3</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F14P1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>F14W1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F14W2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P1 (aqueous)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
The mycelium from liquid cultivation of four coprophilous fungi (F2P1, F13P1, S6W2 and S4P1) was used to produce extracts. However, as discussed in Section 4.3.1, mycelium extracts were not produced from the remaining 27 fungi. The decision to focus on producing extracts from the fermentation liquor rather than mycelium was validated by the results obtained in the plate-hole diffusion assay. The four mycelia extracts did not demonstrate any antibacterial activity, whereas the liquor extracts from two of the isolates, F2P1 and S6W2, were effective against two and three of the test bacteria, respectively. However, it should be noted that alternative extraction methods, such as soaking the mycelia in methanol for 24 hours, may have yielded extracts with antibacterial activity (Tong et al., 2011).

The fermentation broth from the four fungi (F2P1, F13P1, S6W2 and S4P1) underwent extraction with EtOAc to produce an aqueous and an EtOAc extract. This protocol was not used for the remaining fungi for reasons that are addressed in Section 4.3.2. In contrast with the mycelium extracts, the plate-hole diffusion assay revealed antibacterial potential from half of the EtOAc and aqueous extracts. In each case, the active extracts were produced from fungi whose crude liquor also demonstrated antibacterial activity. This makes sense in theory because when the fermentation broth was mixed with EtOAc and then separated, secondary metabolites

<table>
<thead>
<tr>
<th>Coprophilous fungus isolate(a)</th>
<th>S. aureus</th>
<th>E. coli</th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4P1 (EtOAc)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P1 (mycelium)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P4</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4W1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S5P3</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CHX</td>
<td>+++++</td>
<td>+++++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

\(a\) Extracts produced from these isolates were liquor extracts except where otherwise stated.

++ Indicates zone 9–11 mm
++++ Indicates zone 12–14 mm
+++++ Indicates zone 15–17 mm
++++++ Indicates zone ≥18 mm
— No clear zone

++ Indicates zone 9–11 mm
++++ Indicates zone 12–14 mm
+++++ Indicates zone 15–17 mm
++++++ Indicates zone ≥18 mm
— No clear zone
were either extracted in the organic phase or retained in the aqueous phase. Therefore, the EtOAc and aqueous extracts should encompass all the components of the liquor extract, but their distribution between the two phases will differ. Both the EtOAc and aqueous extracts from F2P1 and S6W2 produced zones of inhibition against one or more test bacteria, but the aqueous extracts demonstrated greater bacterial inhibition compared with the EtOAc extracts. When fungi produced antibacterial aqueous extracts, the resulting clear zone measurement was the same diameter as the corresponding liquor extract, or, as in the case of the E. coli assays, it was larger than the crude liquor extract. A possible explanation for this is that the compounds within the fermentation liquor that were responsible for the inhibition were retained in the aqueous phase during partitioning with EtOAc. Extraction of non-active material into the EtOAc phase would therefore increase the concentration of active metabolites in the aqueous phase. All extracts were tested at the same concentration (2 mg in 20 µL) and, therefore, this would result in a larger inhibitory effect.

The plate-hole diffusion assay is ideal for screening extracts for antibacterial activity because it is easy to perform and relatively inexpensive. However, the size of a zone of inhibition depends on a number of variables relating to the inoculum, media and extract. Therefore, the assay is generally considered to be an indirect qualitative method that provides categorical results rather than quantitative values (Wanger, 2007). Essentially, the size of a clear zone is determined by the competing interactions of two dynamic processes: first, the growth of the test bacteria throughout the agar, and second, the diffusion of the fungal extract compounds from the well into the surrounding agar. Diffusion of the extract from the well produces a descending concentration gradient radiating outwards from the well. The growth of the test bacteria (and consequently the zone size) is influenced by variables including the initial inoculum density, the nutritional composition of the agar, and the temperature and length of incubation for the assay (Jorgensen et al., 1999). For reproducible zone sizes, all of the assay parameters must be kept constant.
To address the above variables, a standardised inoculum density (1.5 × 10⁸ cfu/mL) was used and all plates were incubated for 18 hours at 37 °C. MHA was used for the assay because it is a commonly used media for agar diffusion methods and is suitable for the cultivation of a range of non-fastidious aerobic Gram-negative and Gram-positive bacteria (Reade, 1994). The diffusion of the fungal extract compounds is also affected by the agar composition, the incubation conditions and the depth of the agar in the plate (Wanger, 2007). Therefore, a measured volume (15 mL) of MHA was added to every plate. Furthermore, every extract was added to a well as a 20 µL aliquot (100 mg/mL) and the wells were consistently 6 mm diameter. It is important to remember that the diffusion of an antibacterial compound through the agar is also influenced by its molecular properties such as weight, size, ionic charge and aqueous solubility (Wanger, 2007). Given that the diffusion rate of the antibacterial compound directly affects the size of the zone of inhibition, it cannot always be assumed that a larger zone signifies greater susceptibility of the bacteria to the extract.

More than half of the isolated fungi produced an extract that elicited a visible zone of inhibition against one or more species of pathogenic bacteria. These results support the hypothesis that coprophilous fungi from koala faeces are a possible source of antibacterial compounds. Further studies of the fungus collection could include testing the extracts against additional clinically relevant bacteria such as methicillin-resistant strains of *S. aureus*.

### 4.3.3.2 Determination of MIC and MBC for most active extracts

The MIC is the lowest concentration of extract that is required to inhibit the growth of the test bacterium. Compared with the plate-hole diffusion assay, this provides a quantitative measurement of the efficacy of an antibiotic against a particular bacterium. The MIC and MBC were established for the fungal extracts that were capable of eliciting a zone of inhibition greater than 8 mm for one or more test bacteria in the plate-hole diffusion assay (see Table 4.1). The extracts were tested
using a 96-well microdilution assay at a concentration range of 12,500–98 µg/mL. Following incubation, the test wells were inspected for turbidity, with comparison to the growth control and sterility control wells, and the MIC was determined for each extract. MBC was assigned to the lowest concentration of extract that prevented regrowth of the bacteria following subculture to MHA plates. Table 4.2 presents the observed MIC and MBC values for the 11 liquor extracts and the two aqueous extracts that were tested.
Table 4.2 MIC and MBC of coprophilous fungal extracts against Gram-positive and Gram-negative bacteria*

<table>
<thead>
<tr>
<th>Coprophilous fungus isolate(^a)</th>
<th>Genus(^b)</th>
<th>(S., aureus)</th>
<th>(E., coli)</th>
<th>(K., pneumoniae)</th>
<th>(P., aeruginosa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2A1</td>
<td>Aspergillus</td>
<td>6.25</td>
<td>&gt;12.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>F2P1</td>
<td>Fimetariella</td>
<td>6.25</td>
<td>&gt;12.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>F2P1 (aqueous)</td>
<td></td>
<td>6.25</td>
<td>12.5</td>
<td>3.125</td>
<td>6.25</td>
</tr>
<tr>
<td>F2P2</td>
<td>Fimetariella</td>
<td>6.25</td>
<td>12.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>F16EP1</td>
<td>Anthostomella</td>
<td>—</td>
<td>—</td>
<td>3.125</td>
<td>3.125</td>
</tr>
<tr>
<td>S3P2</td>
<td>Neurospora</td>
<td>3.125</td>
<td>6.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>S3P3</td>
<td>Neurospora</td>
<td>3.125</td>
<td>6.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>S6W2</td>
<td>Fusarium</td>
<td>3.125</td>
<td>&gt;12.5</td>
<td>1.56</td>
<td>6.25</td>
</tr>
<tr>
<td>S6W2 (aqueous)</td>
<td></td>
<td>1.563</td>
<td>&gt;12.5</td>
<td>0.78</td>
<td>6.25</td>
</tr>
<tr>
<td>F3W3</td>
<td>Thielavia</td>
<td>3.125</td>
<td>6.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>F14P1</td>
<td>Sordaria</td>
<td>3.125</td>
<td>6.25</td>
<td>3.125</td>
<td>12.5</td>
</tr>
<tr>
<td>S4P4</td>
<td>Trichoderma</td>
<td>6.25</td>
<td>&gt;12.5</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Values represent the average concentration (mg/mL) of triplicate experiments and sd = (±0)
\(^a\) Extracts produced from these isolates are liquor extracts, except where otherwise stated
\(^b\) Identification of fungi was achieved by comparison of ITS regions of fungal rDNA with the GenBank database (see Section 3.3.4)
– Not tested.
The MICs and MBCs of the tested extracts were in the range of 6.25–0.78 mg/mL and 12.5–3.125 mg/mL, respectively. The positive control, CHX, had an MIC and MBC below the lowest concentration that was tested (4.88 µg/mL) for each bacteria. In general, the relative antibacterial activity that was observed between the extracts in the plate-hole diffusion assay was mostly consistent with the MIC values. For example, the extracts that produced the largest clear zones (S6W2 liquor and aqueous extracts, F14P1 and S5P3; see Table 4.1) also exhibited the lowest MIC values. Although there was an overall relationship between zone size and MIC, there were variations between the results from the two methods for each zone diameter range. For example, in the twelve instances that an extract produced a zone of inhibition that was 9–11 mm (++) in diameter, seven of the corresponding MICs were 6.25 mg/mL and five were 3.125 mg/mL. These differences could have been influenced by the diffusion rate of the active compounds through the agar in the plate-hole diffusion assay, a factor that is not a consideration in the microdilution MIC assay. Two aqueous extracts were tested, and similar to the plate-hole diffusion results, they performed equally or better than their liquor extract counterparts. In particular, the aqueous extract from S6W2 was capable of inhibiting the growth of both *S. aureus* and *E. coli* at half the MIC of the S6W2 liquor extract for each bacterium.

The MIC of an extract is capable of limiting bacterial growth without necessarily killing it. Therefore, it is possible that if the extract is removed, the bacteria will recover and begin to grow again (Qaiyumi, 2007). To ascertain which concentration of extract was bactericidal, 100 µL was taken from the wells showing no growth and subcultured onto MHA plates. The lowest concentration of extract that led to the inability of the bacteria to recover and produce visible growth was reported as the MBC. One study into the antibacterial effects of natural compounds found that MBC values for natural product extracts and compounds are commonly twice the MIC, and sometimes this is used rather than an experimentally determined value (Furiga et al., 2008). This was true for more than half of the experiments in this study; of the 24
extracts and bacteria combinations tested, 13 displayed MBCs that were twice the corresponding MIC.

Although the MIC is a quantitative assessment of antibacterial activity compared with the plate-hole diffusion assay, it is not a constant. It is affected by parameters such as the inoculum size, the composition of the medium and the incubation conditions (Madigan et al., 2003). To address this, these variables were kept consistent throughout the study, which meant that it was possible to compare the extracts and determine which was the most effective against each organism (see Table 4.3).

**Table 4.3** Summary of the most effective extracts against each test bacteria

<table>
<thead>
<tr>
<th>MIC (mg/mL)</th>
<th>S. aureus</th>
<th>E. coli</th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>S6W2</td>
<td>S6W2</td>
<td>S6W2</td>
<td>S6W2</td>
</tr>
<tr>
<td></td>
<td>aqueous</td>
<td>aqueous</td>
<td>liquor</td>
<td>aqueous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>liquor</td>
</tr>
</tbody>
</table>

Comparisons of MICs of extracts, fractions and compounds between different research studies are only meaningful if conditions are rigorously standardised within each study, and if conditions are identical between studies. Nonetheless, reporting of, and comparisons between, fungal extract MIC values is considered standard practice. The research into bioactive coprophilous fungi is scarce despite their proven potential for antibiotic discovery. This is thought to be because they have not yet been incorporated in screening programs at levels competitive with fungi from other substrata and niche groups (Bills et al., 2013). The vast majority of studies detail the structural elucidation and antibacterial activity of isolated compounds, and very few report the early stages of research and state the activity of fermentation broths or crude extracts like those that were tested in this chapter.
In contrast, there are numerous studies of endophytic fungi that report the antibacterial activity of broths and crude extracts, many of which have not yet ascertained the MIC and instead rely on agar diffusion methods (Gond et al., 2012; Hazalin et al., 2009; Tolulope et al., 2015; Zhang et al., 2012a; Zhang et al., 2015). MIC values were ascertained by Phongpaichit et al. (2006), who studied the antibacterial activity of fermentation broths of endophytic fungi isolated from *Garcinia* plants. The EtOAc extracts of 10 fungal broths demonstrated inhibition of *S. aureus*, with MICs in the range of 32–512 µg/mL. Similar MIC values (31.25–250 µg/mL) were obtained by Arivudainambi et al. (2011) for the extracts of the endophyte fungus *Colletotrichum gloeosporioides* against 10 clinical strains of *S. aureus*. In comparison, the lowest MIC against *S. aureus* observed in this study was 1.563 mg/mL, which is between three and 50 times larger.

The results presented in this chapter represent preliminary screening efforts and must not be disregarded. First, the extracts prepared in this chapter are crude in nature. Therefore, it is likely that any bioactive compounds that are present make up only a small percentage of the extract by weight, which lessens the observed antibacterial affect. Fractionation of the extracts, guided by antibacterial assays, would isolate active constituents for identification and provide an accurate assessment of their antibacterial potential. Second, there has been no attempt to optimise the fermentation conditions to improve antibacterial activity. Changes to the liquid medium (e.g., variations to carbon and nitrogen sources and pH) and the fermentation temperature could affect the secondary metabolite profile of an isolate and potentially upregulate the production of an antibacterial compound and decrease the observed MIC (Thammajaruk et al., 2010; Xu et al., 2010).

### 4.3.3.3 Time-course growth analysis of liquor extracts F14P1 and S6W2

While the plate-hole diffusion and MIC methods provided an end-point assessment of an extract’s ability to inhibit bacterial growth, the time-course assay allowed the
evaluation of the antibacterial effect over 24 hours. The method is sometimes referred to as a time-kill assay because it allows for the observation of a bactericidal effect of a treatment (if present) over time. Bactericidal activity is defined as a reduction of 99.9% ($\geq 3 \log_{10}$) of the total number of cfu/mL in the original inoculum (CLSI, 1999).

The liquor extracts from two fungi, S6W2 and F14P1, were chosen for this assay because they demonstrated the lowest MIC of a liquor extract against both Gram-negative and Gram-positive bacteria. It is standard practice to test the compound or extract at concentrations equal to or multiples of its MIC value (Verma, 2007). In this study, $1 \times$ MIC and $2 \times$ MIC were evaluated and Table 4.4 lists the final concentrations of each extract. Although the aqueous extract from S6W2 exhibited the lowest MIC against *S. aureus* and *E. coli*, it was not used in the assay so that a comparison could be made between two liquor extracts.

**Table 4.4** Summary of extract concentrations used in time-course assay (mg/mL)

<table>
<thead>
<tr>
<th></th>
<th>S6W2</th>
<th>F14P1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$1 \times$ MIC</td>
<td>$2 \times$ MIC</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.56</td>
<td>3.125</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>3.125</td>
<td>6.25</td>
</tr>
</tbody>
</table>

Standardised cultures of *S. aureus* and *E. coli* were subjected to S6W2 and F14P1 liquor extracts at a final concentration of $1 \times$ MIC and $2 \times$ MIC and viable counts were carried out for 0, 0.5, 1.5, 3, 6, 12 and 24 hours of incubation at 37 °C. Cultures without extract were also incubated under the same conditions to provide a growth control curve. Figure 4.2 to Figure 4.5 display the antibacterial effect of the extracts on growing bacterial cells of *S. aureus* and *E. coli* for S6W2 and F14P1, respectively.
Figure 4.2 Time-course growth assay of *E. coli* in the presence of S6W2 liquor extract. Data points represent mean ± standard deviation.
Figure 4.3 Time-course growth assay of *S. aureus* in the presence of S6W2 liquor extract. Data points represent mean ± standard deviation.
Figure 4.4 Time-course growth assay of *E. coli* in the presence of F14P1 liquor extract. Data points represent mean ± standard deviation.
Figure 4.5 Time-course growth assay of *S. aureus* in the presence of F14P1 liquor extract. Data points represent mean ± standard deviation.
The S6W2 extract exhibited a significant reduction in viability of \textit{E. coli} from 1.5 hours onwards compared with the growth control (Figure 4.2). However, the difference between the original inoculum (at time = 0) and the \(1 \times \text{MIC}\) and \(2 \times \text{MIC}\) treated cells after 24 hours was only approximately +0.5 \(\log_{10}\) and \(-1.0 \log_{10}\) units, respectively. Because this represents less than a 3 \(\log_{10}\) reduction, the S6W2 extract cannot be described as bactericidal against \textit{E. coli}. The \(1 \times \text{MIC}\) treatment did cause an initial decline in viable cells during the first three hours of incubation, but the bacteria were able to recover and grow to a density greater than the original inoculum, although not significantly. In the presence of the two-fold concentrated S6W2 extract, \textit{E. coli} was also able to recover growth following an initial steep decline, but not to a level beyond the original inoculum. In conclusion, the S6W2 extract can be described as bacteriostatic against \textit{E. coli} at both 1.56 mg/mL and 3.125 mg/mL. Bactericidal activity may have been observed for the extract using the MBC (6.25 mg/mL) in the assay, but this was not performed.

The S6W2 extract demonstrated significant inhibitory activity against \textit{S. aureus} from 3 hours onwards compared with the growth control (see Figure 4.3). At 24 hours, the number of surviving cells represented approximately a 0.7 \(\log_{10}\) and 1.0 \(\log_{10}\) reduction from the original inoculum upon treatment with \(1 \times \text{MIC}\) and \(2 \times \text{MIC}\), respectively. According to the definition, extract S6W2 did not exhibit bactericidal activity against \textit{S. aureus} within 24 hours. The antibacterial effect of the \(2 \times \text{MIC}\) extract (6.25 mg/mL) was not significantly different to the inhibition caused by the \(1 \times \text{MIC}\) extract (3.125 mg/mL) at every time point. This is not surprising given that, in the microdilution assay, an MBC was not assigned because the highest concentration of the extract (12.5 mg/mL) was not able to prevent regrowth of \textit{S. aureus} in the subsequent growth test (see Table 4.2).

The F14P1 extract exhibited a significant reduction in viability of \textit{E. coli} from 1.5 hours onwards compared with the growth control (see Figure 4.4). Similar to the S6W2 extract, the \(1 \times \text{MIC}\) treatment resulted in an initial decline in viable \textit{E. coli} cells, but the bacteria were able to recover and grow to a density approximately
0.7 \log_{10} \text{ units greater than the original inoculum, which represents a significant increase. The two-fold concentrated F14P1 extract caused an initial steep incline in viability followed by a plateau of growth inhibition at approximately 1.2 \log_{10} \text{ units lower than the original inoculum. In conclusion, the F14P1 extract can be described as bacteriostatic against } E. \text{ coli} \text{ at both 3.125 mg/mL and 6.25 mg/mL. Bactericidal activity may have been observed for the extract using the MBC (12.5 mg/mL) in the assay, but this test was not performed.}

Figure 4.5 shows the significant reduction in viable } S. \text{ aureus} \text{ cells treated with } 1 \times \text{MIC and } 2 \times \text{MIC F14P1 extracts from 1.5 hours onwards compared with the growth control. The } 1 \times \text{MIC extract exhibited a bacteriostatic effect that inhibited growth of the organism, but this did not reduce the viability by more than } 3 \log_{10} \text{ units. In contrast, at 12 hours the } 2 \times \text{MIC extract had produced an approximate } 3.8 \log_{10} \text{ reduction in viable cells, thus demonstrating bactericidal activity against } S. \text{ aureus}. \text{ This result was expected because the } 2 \times \text{MIC extract equated to 6.25 mg/mL, which was the MBC value ascertained in the microdilution and growth test.}

Overall, the results of the time-course experiments correlated with the MIC and MBC values that were determined for both extracts in the microdilution method (see Table 4.2). The S6W2 extract exhibited bacteriostatic inhibitory activity against both test organisms at both concentrations. The F14P1 extract also demonstrated bacteriostatic activity against } E. \text{ coli} \text{ at both concentrations. For these tests, the } 2 \times \text{MIC represented a concentration that was half the experimentally determined MBC, or as was the case with S6W2 against } S. \text{ aureus}, \text{ an MBC could not be determined. The F14P1 extract exhibited bacteriostatic activity against } S. \text{ aureus} \text{ at } 1 \times \text{MIC and bactericidal activity at } 2 \times \text{MIC that was equal to the MBC. It would be interesting to conduct the time-course assay using the MBC for all extracts. This work could be carried out in the future, but it would be more useful to perform these additional experiments with an extract that has undergone activity-guided separation rather than with the crude extracts.
4.3.4 Antifungal activity of coprophilous fungi extracts

The coprophilous fungal extracts were screened for antifungal activity against the yeast *C. albicans* and the filamentous fungus *D. brizae*.

4.3.4.1 *C. albicans* inhibition

Plate-hole diffusion assays were performed with all extracts to determine which were capable of inhibiting the growth of *C. albicans*. The total amount of extract within each well was 2 mg and the presence of a clear zone surrounding a well indicated inhibitory activity by the respective extract, such as in Figure 4.6.

Eight (18.6%) of the 31 coprophilous fungi produced extracts that were capable of inhibiting the growth of *C. albicans*. Extracts that produced a clear zone greater than 8 mm were tested in a microdilution assay (with a concentration range of 12.5–0.098 mg/mL) to ascertain their MIC and MFC. Table 4.5 summarises the results of these assays from the extracts that demonstrated anti-candida activity.

In the plate-hole diffusion assay, *C. albicans* exhibited a similar sensitivity to *E. coli* against the collection of fungal extracts. For both microorganisms, eight fungi produced extracts that produced a zone of inhibition that was greater than 6 mm. In general, the MICs of the active extracts against *C. albicans* were approximately twice the MICs against *E. coli*. However, the aqueous extract of the S6W2 liquor was capable of preventing visible growth at a concentration of 0.52 mg/mL, which was lower than any MIC obtained against the test bacteria.
Figure 4.6 *C. albicans* plate-hole diffusion assay following incubation. Clear zones indicate inhibition of the yeast by the liquor extract from the fungus S5P3 (left) and by the positive control CHX (right).

Table 4.5 Inhibition of *C. albicans* by coprophilous fungi extracts

<table>
<thead>
<tr>
<th>Coprophilous fungal extracts</th>
<th>Genus</th>
<th>Plate-hole diffusion assay</th>
<th>Microdilution assay (mg/mL)</th>
<th>MIC</th>
<th>MFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11EP1</td>
<td><em>Penicillium</em></td>
<td>++</td>
<td>12.5 ± 0.0</td>
<td>&gt;12.5± 0.0</td>
<td></td>
</tr>
<tr>
<td>S3P1</td>
<td><em>Neurospora</em></td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S6W2</td>
<td><em>Fusarium</em></td>
<td>+++</td>
<td>0.78 ± 0.0</td>
<td>1.56 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>S6W2 (aqueous)</td>
<td></td>
<td>++++</td>
<td>0.52 ± 0.23</td>
<td>1.04 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>S5P5</td>
<td><em>Plectosphaerella</em></td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P1</td>
<td><em>Sordaria</em></td>
<td>++</td>
<td>6.25 ± 0.0</td>
<td>12.5 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>S4P2</td>
<td><em>Sordaria</em></td>
<td>++</td>
<td>12.5 ± 0.0</td>
<td>&gt;12.5 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>S4P4</td>
<td><em>Trichoderma</em></td>
<td>++</td>
<td>6.25 ± 0.0</td>
<td>12.5 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>S5P3</td>
<td><em>Aspergillus</em></td>
<td>++</td>
<td>6.25 ± 0.0</td>
<td>&gt;12.5 ± 0.0</td>
<td></td>
</tr>
</tbody>
</table>

*a* Extracts produced from these isolates were liquor extracts except where otherwise stated.

*b* Identification of fungi was achieved by comparison of ITS regions of fungal rDNA with the GenBank database (see Section 3.3.4)

- Not tested.

*c* Zone of inhibition diameter includes 6 mm disk:

+ Indicates zone ≤ 8 mm

+++ Indicates zone 12–14 mm

++++ Indicates zone 15–17 mm
Four of the seven extracts demonstrated an MFC that was twice the corresponding MIC, which suggests that they may be good candidates for further separation and the identification of a fungicidal constituent. A time-course growth assay would provide a better indication as to whether these extracts were fungicidal or fungistatic, but this test was not performed. Treatment with a fungicidal (as opposed to a fungistatic) drug is often important for the treatment of immunosuppressed individuals suffering from invasive infections caused by *C. albicans* (Lewis and Graybill, 2008). Fungistatic drugs are still immensely useful, as demonstrated by one of the most commonly administered drugs for *Candida* infections, fluconazole. This azole compound is classified as a fungistatic agent and is listed on the WHO’s current Model List of Essential Medicines (WHO, 2015). Unfortunately, *Candida* species are rapidly acquiring resistance to fluconazole, and therefore the identification of novel compounds that can attenuate *Candida* infections would be beneficial. Extracts produced from the fungus S6W2 represent ideal candidates for further exploration of this activity and were chosen to undergo bioassay-directed separation (see Chapter Five).

In addition to discovering new stand-alone anti-candidal compounds, current research efforts are working to potentiate the efficacy of fluconazole via drug synergism, with the aim of affecting previously resistant strains and improving fluconazole’s activity from fungistatic to fungicidal (Fiori and Van Dijck, 2012; Liu et al., 2014; Spitzer et al., 2011; Zhai et al., 2010). This was achieved by Phaopongthai et al. (2013) who combined fluconazole with a secondary metabolite that they isolated from an endophytic fungus *Alternaria alternata* that was obtained from a Thai medicinal plant. Screening of the coprophilous fungal extracts for synergistic activity is an alternative approach to exploring their therapeutic potential, although it would be more beneficial to use isolated compounds rather than crude extracts.
4.3.4.2  *D. brizae inhibition*

The ability of the extracts to inhibit the growth of *D. brizae* was evaluated using three agar-based assays and one broth microdilution assay. This methodology was employed because it assessed the effect of the extract on the fungus at different stages of growth. The spore germination assay detected antifungal activity in the earliest stages of growth, the radial growth assay assessed inhibition during the first phases of filamentous growth and the hyphal extension assay analysed the effect on active growth (Quiroga et al., 2009). The extracts that inhibited *D. brizae* during two or more stages of growth were subsequently included in the microdilution assay to determine their MIC. Table 4.6 presents the results of the three agar assays for each extract.

Twelve (38.7%) of the coprophilous fungi produced extracts that exhibited antifungal activity in one or more of the agar assays. Three extracts (S6W2, S6W2 aqueous and S4P2) were able to inhibit the test fungus in all three assays. The hyphal extension assay recorded the highest number of extracts demonstrating inhibition (15), followed by the radial growth (10) and spore germination (five) assays. The variation in inhibition between the methods may reflect the effect that the *D. brizae* growth stage had on the antifungal capabilities of the extracts. For example, of the 15 extracts that inhibited hyphal extension, only four were also able to inhibit spore germination, which suggests that this early stage of growth may be more resistant to interference from the secondary metabolites present in the extracts.
### Table 4.6 Inhibition of *D. brizae*

<table>
<thead>
<tr>
<th>Coprophilous fungal extract&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Agar assay</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyphal extension&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Radial growth (% growth inhibition ± sd)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>F2A1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>F2A2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>F2A3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F2P1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F2P1 (aqueous)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>F2P1 (EtOAc)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F2P1 (mycelium)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F2P2</td>
<td>+ 14.60 ± 2.53</td>
<td>–</td>
</tr>
<tr>
<td>F4P1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>F5A1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F5A2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F5A3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F5A4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F11EP1</td>
<td>+ 11.16 ± 2.11</td>
<td>–</td>
</tr>
<tr>
<td>F13P1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F13P1 (aqueous)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F13P1 (EtOAc)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F13P1 (mycelium)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F16EP1</td>
<td>+ 15.82 ± 2.46</td>
<td>–</td>
</tr>
<tr>
<td>F16EP2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>F16ES1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S3P1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S3P2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S3P3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S6W2</td>
<td>+ 17.85 ± 3.80</td>
<td>17.67 ± 0.76</td>
</tr>
<tr>
<td>S6W2 (aqueous)</td>
<td>+ 31.03 ± 2.88</td>
<td>20.50 ± 1.80</td>
</tr>
<tr>
<td>S6W2 (EtOAc)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S6W2 (mycelium)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S5P5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F3P1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F3W1</td>
<td>+ 14.00 ± 3.06</td>
<td>–</td>
</tr>
<tr>
<td>F3W3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F14P1</td>
<td>+ 18.86 ± 2.74</td>
<td>–</td>
</tr>
<tr>
<td>F14W1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F14W2</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
### Table 4.6: Agar assay results for the Hyphal extension, Radial growth, and Spore germination assays.

<table>
<thead>
<tr>
<th>Coprophilous fungal extract*</th>
<th>Hyphal extensionb</th>
<th>Radial growth (% growth inhibition ± sd)c</th>
<th>Spore germination (mm ± sd)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4P1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P1 (aqueous)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P1 (EtOAc)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S4P1 (mycelium)</td>
<td>–</td>
<td>14.40 ± 3.67</td>
<td>–</td>
</tr>
<tr>
<td>S4P2</td>
<td>+</td>
<td>15.82 ± 3.12</td>
<td>13.33 ± 1.04</td>
</tr>
<tr>
<td>S4P4</td>
<td>+</td>
<td>–</td>
<td>13.33 ± 1.04</td>
</tr>
<tr>
<td>S4W1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S5P3</td>
<td>+</td>
<td>10.55 ± 1.61</td>
<td>–</td>
</tr>
<tr>
<td>CHX</td>
<td>+</td>
<td>38.13 ± 3.00</td>
<td>28.17 ± 1.04</td>
</tr>
</tbody>
</table>

* Extracts produced from these isolates were liquor extracts except where otherwise stated.

b (+) indicates the presence of an inhibition crescent at the mycelial front approaching the disk, in two or more of the triplicate assays.

c Growth control plate represented 100% growth.

d Diameter of the colourless zone.

– No inhibition.

The high number of extracts demonstrating antifungal activity in the hyphal extension assay may also be due in part to the methodology of the assay. *D. brizae* was inoculated in the centre of a PDA plate and paper disks impregnated with either the extract or the control were placed on the surface of the plate, approximately 2 cm from the fungus. Following incubation, antifungal activity was observed as a zone of restricted hyphal extension adjacent to the paper disk. As with the published method, the size of the inhibition zone was not measured (Quiroga et al., 2009). Rather, only the presence or absence of an inhibition zone was recorded, which, on occasion, proved difficult to interpret. The extracts that produced a zone in two or more of the triplicate assays are listed in Table 4.6, but there were several extracts that produced an inhibition zone in only one assay (not shown). In five of the assays, a negative control disk produced a small inhibition zone. The reason for this observation is not known; it may be possible that the disk itself exerted physical inhibition, but this has not been described in the literature.
Unlike the method of Quiroga et al. (2009) this study included a positive control that consisted of disks impregnated with 20 µL of CHX. However, this positive control could not generate a reproducible inhibition zone and in some assays it did not exhibit any inhibition at all (see Figure 4.7). CHX is a commonly used biocidal agent for testing against bacteria and yeast, and one study has confirmed its antifungal activity against some filamentous fungi (Sandle et al., 2014). CHX was capable of inhibiting D. brizae in the radial growth, spore germination and microdilution assays, but an alternative positive control (such as Derosal Plus®; Bayer, Germany) may better serve the hyphal extension assay. For future studies, further modifications could be made to this assay to improve the quality of the data. For example, a similar but quantitative method was employed by Flores et al. (2013) in their evaluation of the antifungal activity of extracts prepared from the fermentation liquor of the endophytic fungus Phomopsis longicolla. Disks impregnated with extract were placed 4 cm from the fungal pathogen plug (one extract per PDA plate), and on day seven, the distance between the disk and the mycelial front was measured with comparison to the negative and positive controls. Because the inhibitory zones were measured, the mean diameter could be compared with those produced by the controls and a statistical test (the Tukey method) was used to determine if statistically significant inhibition had occurred.
Figure 4.7 Example of a hyphal extension assay where the positive control (CHX) disk demonstrated only very minimal inhibition of *D. brizae*.

In the radial growth assays, 10 extracts were capable of reducing the growth of *D. brizae* compared with the growth control. The S6W2 aqueous extract demonstrated the greatest inhibition, producing a $31.03 \pm 2.88\%$ reduction in radial growth. The mycelium extract of S4P1 exhibited a $14.40 \pm 3.67\%$ reduction in radial growth and was the first and only occurrence of antimicrobial activity from a mycelium extract.

This assay required the incorporation of the extracts into the agar at a final concentration of 5 mg/mL. For each plate, 10 mL of PDA was poured and 500 µL of each extract stock solution (100 mg/mL) had to be added to each replicate assay. This agar-dilution method is a common approach to antifungal testing; however, it became apparent during this research that it is more appropriate for testing extracts or compounds when supply is in abundance. Although the supply of the extracts was not strictly limited, their production was a time-intensive process. Thus, it was
important to consider the efficient use of the extracts and it was decided that any subsequent antifungal testing of fractions or compounds that were separated from the extracts would not be carried out via the radial growth test.

In the spore germination assay, MTT was spread onto the surface of the agar to increase the clarity of the zones of inhibition surrounding the antifungal extracts. Viable fungi cells are able to reduce the MTT, which leads to the intracellular accumulation of a purple formazan compound that can be seen macroscopically (Meletiadis et al., 2000). Non-viable cells cannot reduce the MTT and therefore remain colourless. Hence, an extract that is capable of inhibiting spore germination will produce a colourless zone in the surrounding agar, such as in Figure 4.8 Five extracts inhibited spore germination in the bioautographic assay, and the S6W2 aqueous extract was again the most effective, producing a colourless zone that measured 20.50 ± 1.80 mm.

Ten extracts demonstrated inhibition of D. brizae in two or more of the agar assays and these were subsequently included in the microdilution assay to determine their MIC. The MIC of the extracts ranged from 8.33 ± 3.6 to 25.00 ± 0.0 mg/mL (see Table 4.7).
Figure 4.8 Spore germination plate following incubation and treatment with MTT. A clear zone surrounds the S6W2 aqueous extract.

Table 4.7 MIC of coprophilous fungal extracts against *D. brizae*

<table>
<thead>
<tr>
<th>Coprophilous fungal</th>
<th>Genus(^{b})</th>
<th>MIC (mg/mL ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2P2</td>
<td><em>Fimetariella</em></td>
<td>12.5 ± 0.0</td>
</tr>
<tr>
<td>F4P1</td>
<td><em>Neurospora</em></td>
<td>&gt;25.00 ± 0.0</td>
</tr>
<tr>
<td>F11EP1</td>
<td><em>Penicillium</em></td>
<td>25.00 ± 0.0</td>
</tr>
<tr>
<td>F16EP1</td>
<td><em>Anthostomella</em></td>
<td>20.83 ± 7.22</td>
</tr>
<tr>
<td>S6W2</td>
<td><em>Fusarium</em></td>
<td>16.67 ± 7.22</td>
</tr>
<tr>
<td>S6W2 (aqueous)</td>
<td></td>
<td>8.33 ± 3.61</td>
</tr>
<tr>
<td>F3W1</td>
<td><em>Bjerkandera</em></td>
<td>12.50 ± 0.0</td>
</tr>
<tr>
<td>F14P1</td>
<td><em>Sordaria</em></td>
<td>10.42 ± 3.61</td>
</tr>
<tr>
<td>S4P2</td>
<td><em>Sordaria</em></td>
<td>25.00 ± 0.0</td>
</tr>
<tr>
<td>S5P3</td>
<td><em>Aspergillus</em></td>
<td>6.25 ± 0.0</td>
</tr>
</tbody>
</table>

\(^{a}\) Extracts produced from these isolates were liquor extracts except where otherwise stated.

\(^{b}\) Identification of fungi was achieved by comparison of ITS regions of fungal rDNA with the GenBank database (see Section 3.3.4)
The F4P1 liquor extract did not inhibit *D. brizae* at 25 mg/mL despite demonstrating inhibitory activity in the hyphal extension and spore germination assays. However, as discussed previously, the hyphal extension results may have been unreliable given the presence of some inhibitory zones surrounding the negative controls. The clear zone that this extract produced in the spore germination assay (11.00 ± 0.50 mm) was the smallest of those recorded.

The lowest extract MIC against *D. brizae* (8.33 ± 3.6 mg/mL) was attributed to the aqueous extract from coprophilous isolate S6W2. CHX was able to prevent visible growth of the fungus at the lowest concentration tested, 39.06 µg/mL, which is approximately 200-fold lower than the MIC of the S6W2 aqueous extract. Although the MICs of the extracts appear modest when compared with CHX, there are two important factors that need to be considered. These factors have been comprehensively addressed in the discussion of the antibacterial MIC assays (see Section 4.3.3.2). Briefly, the extracts tested in this chapter are crude and the research represents a preliminary exploration of their antifungal potential prior to activity-guided separation. Furthermore, optimisation of fermentation conditions was not carried out, and it is likely that the bioactivity of the crude extracts can be enhanced. Given that there has been very little research into the exploitation of coprophilous fungi against plant pathogens (Sarrocco et al., 2015a), the extracts warrant further evaluation as possible control agents.

### 4.3.5 Anti-QS activity of coprophilous fungi extracts

The coprophilous fungal extracts were tested for anti-QS activity using a disk diffusion assay against the screening bacterium *C. violaceum* (Figure 4.9). In this wild type strain, the production of the purple pigment violacein is under N-acylhomoserine lactone QS control (McClean et al., 1997). When QS signals are quenched, non-pigmented bacteria grow and can be observed as a turbid halo of viable but colourless cells surrounding the extract impregnated disk. Growth inhibition by an extract results in a zone of inhibition similar to those observed in the
antibacterial plate-hole diffusion assay (see Section 4.3.3.1, Figure 4.1). Thus, *C. violaceum* is an excellent screening tool to detect the presence of anti-QS activity by an extract.

![Image](image_url)

**Figure 4.9** Anti-QS activity using *C. violaceum* in a disk diffusion assay. Disks were impregnated with 2 mg of liquor extracts from fungi S4P4, F13P1, F14W1 and F14P1.

Note: A 20µg gentamicin disk was included (centre) for comparison to an antibacterial zone of inhibition. When the bacteria grow to a threshold density, N-acylhomoserine lactone molecules are produced and detected, resulting in the production of purple violacein pigment.

Following incubation of the disk diffusion assay, the extracts were inspected for the presence of anti-QS or antibacterial activity, and zones were measured to the nearest millimetre. For the purpose of this thesis, photos were taken with the plate on top of printed text to demonstrate the difference in appearance between the turbid anti-QS zone and the clear antibacterial zone. In Figure 4.10a, the aqueous extract of fungus S6W2 produced an anti-QS halo. Colourless *C. violaceum* colonies were observed surrounding the disk and caused the underlying text to appear blurred. In comparison, the gentamicin disk completely inhibited the growth of the bacterium,
resulting in a clear halo and well-defined text (see Figure 4.10b). The liquor extract of S4P4 did not demonstrate anti-QS or antibacterial activity and therefore was surrounded by confluent purple growth (see Figure 4.10c).

![Figure 4.10](image_url)

**Figure 4.10** Example of results obtained in anti-QS disk diffusion assays. In each photo the disk is 6 mm: a) colourless, turbid anti-QS halo, b) clear antibacterial halo and c) no halo.

<table>
<thead>
<tr>
<th>Coprophilous fungi extract a</th>
<th>Genus b</th>
<th>QS inhibition (mm ± sd) c</th>
<th>Growth inhibition (mm ± sd) d</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6W2</td>
<td><em>Fusarium</em></td>
<td>1.8 ± 0.29</td>
<td>0.5 ± 0</td>
</tr>
<tr>
<td>S6W2 (aqueous)</td>
<td></td>
<td>3.5 ± 0</td>
<td>0.67 ± 0.29</td>
</tr>
<tr>
<td>F3W3</td>
<td><em>Thielavia</em></td>
<td>2.8 ± 0.29</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>F14P1</td>
<td><em>Sordaria</em></td>
<td>3.0 ± 0</td>
<td>0.5 ± 0</td>
</tr>
<tr>
<td>S5P3</td>
<td><em>Aspergillus</em></td>
<td>2.0 ± 0</td>
<td>0.17 ± 0.29</td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td>0 ± 0</td>
<td>5.17 ± 0.29</td>
</tr>
</tbody>
</table>

a Extracts produced from these isolates were liquor extracts except where otherwise stated.

b Identification of fungi was achieved by comparison of ITS regions of fungal rDNA with the GenBank database (see Section 3.3.4)

c Measurements of turbid, colourless zones were taken from the edge of the purple growth to the perimeter of the disk or growth inhibition (if present).

d Measurements of clear zones were taken from the edge of the disk to the boundary of clear and turbid zones.

Four (12.9%) of the coprophilous fungi produced extracts that exhibited anti-QS activity in the disk diffusion assays. The largest zone of colourless *C. violaceum* growth was produced by the aqueous extract of S6W2. Table 4.8 presents the widths
of the turbid colourless anti-QS zones and clear antibacterial zones produced by the active extracts.

QS systems regulate diverse physiological processes in bacterial populations, including several functions that are associated with increased virulence. Disruption of QS, rather than killing bacteria, represents an alternative approach to bacterial control that may also reduce the development of resistant strains (Hentzer and Givskov, 2003). The potential of an extract to be an effective anti-QS agent is also predicted by its absence of antibacterial activity in the assay. All but one of the active extracts (F3W3) produced very small antibacterial clear zones immediately adjacent to the disk, preceding turbid anti-QS halos. As described in Section 4.3.3.1, assays that rely on diffusion of an extract through agar result in a descending concentration gradient radiating outwards from the disk or well. Hence, the area closest to the disk contained the highest concentration of extract. This begs the question: was the observed anti-QS halo simply the result of incomplete inhibition by the extract (at decreasing concentrations) that prevented C. violaceum from reaching the density threshold required to initiate QS and produce violacein? Although this conclusion cannot be entirely disregarded, it was not observed in the disk diffusion results for gentamicin. A halo completely devoid of bacteria surrounded the antibiotic disk and there were no colourless cells preceding the purple growth, despite a similarly decreasing concentration gradient (see Figure 4.11). The same observation has been reported with another common antibiotic, tetracycline (Adonizio et al., 2006).
Additionally, the ability of cells to recover from the anti-QS activity and regain production of violacein was established. A sample of colourless colonies that were surrounding an active extract was subcultured onto Luria–Bertani agar alongside a sample of pigmented cells from the same plate for comparison. Following incubation, the previously colourless bacteria were purple, indicating that their viability had not been affected by the extract and that there had been recovery of QS communication among the cells (Figure 4.12).
Figure 4.12 Colourless cells were able to grow upon subculture and incubation and regained QS directed production of violacein (right). A sample of purple cells from the same plate was also subcultured for comparison (left).

The results of the anti-QS screening in this chapter are promising, particularly as there is currently no published research describing anti-QS activity by coprophilous fungi or their extracts. In particular, the liquor extract of isolate F3W3 warrants further exploration because it did not exhibit any growth inhibition. The fungi S6W2 and F14P1 were chosen for bioactivity-guided separation (Chapter Five) with the aim of identifying the compound responsible for the observed QS inhibition.

4.4 Summary

The premise behind this work was that the fitness of coprophilous fungi is challenged by competing and invading organisms within koala faeces and, therefore, to survive they must produce chemicals to mediate these interactions. In accordance with this premise, many of the isolates were, indeed, found to exhibit antimicrobial activity. This was the first work, to my knowledge, in which the antibacterial, antifungal and anti-QS potential of Australian coprophilous fungi have been explored.
Lyophilised fermentation liquors of 31 fungi were produced in addition to 12 extracts that were made from the mycelial biomass and liquid separation of the fermentation broth from four isolates. All 43 coprophilous fungi extracts were screened for antibacterial, antifungal and anti-QS activity.

Antibacterial activity was assessed against *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. In plate-hole diffusion assays, 54.8% of the coprophilous fungi produced extracts that were capable of inhibiting at least one test bacterium. Six fungi demonstrated broad-spectrum antibacterial activity, and three fungi (F16EP1, F14P1 and S5P3) were able to inhibit the growth of all four bacteria. The MIC and MBC were determined for the 13 extracts that produced inhibition zones greater than 8 mm in the plate-hole diffusion assay. The most effective extracts were produced by isolates S6W2 and F14P1. Time-course growth assays suggested that the antibacterial activity of S6W2 against *S. aureus* and *E. coli* at 2 × MIC was bacteriostatic. Under the same conditions, F14P1 demonstrated bacteriostatic inhibition against *E. coli* and bactericidal activity against *S. aureus*.

Antifungal activity was tested for against *C. albicans* and *D. brizae* and 8.6% and 38.7%, respectively, of the coprophilous fungi produced extracts that inhibited the test fungi in agar assays. The MIC was ascertained for extracts against both fungi, and there were six (19.4%) that were capable of inhibiting visible growth in both the yeast and filamentous fungi.

The extracts were screened for their ability to inhibit the production of violacein in *C. violaceum*, which is under QS control mediated by N-acylhomoserine lactone. In disk diffusion assays, four (12.9%) of the coprophilous fungi produced extracts that inhibited pigment production, thus exhibiting anti-QS activity. This is the first time, to my knowledge, that coprophilous fungi have been screened for anti-QS activity.

The antimicrobial activity exhibited by the extracts in this chapter, while modest, are encouraging given the high percentage of bioactive isolates from one fermentation
method and the production of only crude extracts. Optimisation of the fermentation conditions and the extraction methodology would likely improve the efficacy of the extracts and could be considered for further research. This work has suggested that coprophilous fungi from koala faeces may represent a source of novel antimicrobials and they warrant further exploration, especially given the paucity of research on this particular source.

Chapter Five describes the selection of an extract and the scaled-up fermentation process and bioactivity-guided separation to identify its active compound.
CHAPTER 5

Bioactivity-Guided Separation of Extract S6W2
5.1 Introduction

Bioactivity-guided separation is a technique commonly used in natural product drug discovery. The aim of the process is to isolate compounds with a particular biological activity from crude extracts. To achieve this, the abundant and often diverse compounds in the extract must be separated into fractions that are then screened for the biological activity of interest. Fractions without bioactivity are disregarded and fractions that demonstrate bioactivity are further separated, often using a variety of techniques, until the bioactive constituent has been isolated and can be identified.

It is important to note that a standardised nomenclature does not exist when describing bioactivity-guided separation (Weller, 2012). The separation component of the phrase may also be referred to as fractionation, which is then guided or directed by the bioactivity, activity or bioassay (Giltrap et al., 2009; Rodríguez-Algaba et al., 2015; Seida et al., 2015). These interchangeable terms must all be used when performing literature searches to ensure that all relevant research is identified.

In this chapter, the scaled-up fermentation liquor of S6W2 was separated using adsorption to Amberlite® XAD-16 resin, to isolate and enrich the active substance in solution so that it is more suitable for further purification by preparative reversed-phase HPLC. The fractions obtained from these isolation and purification procedures were screened for antibacterial, anti-candidal, antifungal and anti-QS activity using a combination of the plate-hole diffusion, microdilution and disk diffusion assays. Identification of the active compound was achieved using ultraviolet (UV) spectroscopy, mass spectrometry (MS) and NMR spectroscopy analyses.

5.1.1 Chapter aim

The aim of this chapter was to use bioactivity-guided separation to isolate and identify the active compound from the S6W2 extract.
5.2 Materials and methods

5.2.1 Liquid fermentation of fungi

Two Erlenmeyer flasks containing 400 mL of PDB were each inoculated with four 1 cm$^2$ agar plugs of the fungal isolate. The flasks were then stoppered with cotton wool and incubated at 25 °C in the dark with shaking at 150 rpm for 27 days. Following incubation, the bulk of the mycelium was removed by filtration through Whatman (No.1) filter paper under vacuum. The fermentation liquor then underwent membrane filtration using a 0.22 µm filter under vacuum to remove any remaining fungal cells. The filtrate was dried by lyophilisation to determine its mass, and bioassays were performed to confirm the presence of the bioactivity that was originally observed in the smaller scale fermentation liquid.

5.2.2 Amberlite XAD-16 resin

Amberlite® XAD-16 (Rohm and Haas, USA) is a non-ionic polymeric macroporous adsorbent resin that is supplied as insoluble off-white beads. This is regularly used in natural product research for the removal or recovery of compounds from an aqueous solution, such as microbial fermentation liquor, manufacturing waste water or plant extracts (Li et al., 2008; Pu et al., 2013; Scoma et al., 2012).

Prior to use, one volume of XAD-16 resin was soaked in two volumes of methanol in a five-volume beaker with occasional stirring for 30 minutes. Distilled water was then slowly added with stirring to the XAD-16 in methanol. When the beaker had filled the resin was allowed to settle. Two-thirds of the aqueous methanol supernatant was then decanted and the addition of distilled water continued until the beaker was again filled. Two-thirds of the aqueous methanol supernatant was again decanted, and the process of adding distilled water and decanting was repeated three more times, so that the aqueous solution was mostly distilled water. The XAD-16 resin should settle to the bottom of the beaker when the stirring stops. If this procedure of
wetting the XAD-16 resin is not followed, the resin floats and would not adsorb hydrophobic material from the aqueous extract very efficiently. The XAD-16 resin was transferred to a glass chromatography column and further washed with another 10 bed volumes of distilled water, to ensure that all methanol had been removed from the resin. It was vital that all methanol had been removed from the XAD-16 resin to ensure the efficient adsorption of hydrophobic compounds from the aqueous extract.

The extract was loaded slowly onto the XAD-16 resin in a glass chromatography column at a flow rate of approximately one bed volume per hour. The flow-rate was not allowed to exceed two bed volumes per hour to ensure efficient adsorption of weakly hydrophobic compounds from the solution. The adsorption of weakly hydrophobic compounds of interest to the resin can be improved by increasing the ionic strength of the extract solution through addition of sodium chloride or ammonium formate to produce a 3–5% salt solution (N. Hart, personal communication). Following collection of the column loading effluent, the XAD-16 resin was rinsed with distilled water to wash the remaining non-adsorbed material from the resin. The adsorbed hydrophobic compounds were then eluted from the XAD-16 resin using 3 to 4 bed volumes of methanol at a flow rate of approximately 5 bed volumes per hour. A final methanol rinse ensured that all adsorbed material had been eluted from the column. The fractions were collected in glass screw-top vials, capped and kept at 2–8 °C until they had been analysed by HPLC and concentrated for bioassays.

The small-scale separation of S6W2 liquor included a preliminary capture step with the XAD-16 resin in a beaker before pouring the resin into the glass column. About 10 mL of XAD-16 resin was added with gentle stirring to a beaker with 10 mL of aqueous solution containing 100 mg of the extract. The suspension was gently stirred at room temperature for one hour to ensure complete adsorption to the XAD-16 resin before being poured into a glass chromatography column. Elution of the XAD-16 resin was then performed using the procedure for the scaled-up experiment, with the first distilled water rinse of the beaker being used to transfer any remaining XAD-16
resin into the column. Rinsing the XAD-16 resin in the column with distilled water washed the remaining non-adsorbed liquor extract from the resin column effluent. The adsorbed material was eluted with methanol.

5.2.3 Analytical and preparative reversed-phase HPLC

Fractions were analysed by reversed-phase HPLC using a Waters 600 gradient pump and the Waters Millennium v4.0 Chromatography Manager Software with an Alltech® Alltima C18 column (150 × 4.6 mm, 5 µm). Isocratic and gradient elution with acetonitrile/water (ACN/H₂O) mobile phase at a flow rate of 1 mL/min was used. Peak detection was made with a Waters 2996 Photodiode Array UV detector.

Extracts were separated by preparative reversed-phase HPLC using a Waters 600E gradient pumping system with an Alltech® Alltima C18 column (150 × 4.6 mm, 5 µm). Peaks were detected with a Waters 2487 UV detector at λ 254 nm and recorded on a Curken 250-2 model chart recorder.

5.2.4 Evaporation and drying of fractions

The XAD-16 fractions and the bioactive fractions collected from the preparative reversed-phase HPLC were evaporated to dryness to determine their mass. Organic solvents were removed from the fractions using a Heidolph Laborota 4000 rotary evaporator (Germany) equipped with a dry ice cold trap connected to a Vacuubrand CVC 2000 II vacuum pump (Germany). The water bath was kept below 40 °C to avoid the possible degradation of compounds, and the vacuum pressure was regulated to avoid bumping and loss of product. While water could be removed from the fractions using the dry ice cold trap attached to the rotary evaporator, large volumes of water were removed from the aqueous solutions in round-bottom flasks by lyophilisation on a Telstar Cryodos-50 freeze dryer.
Following evaporation of fractions to smaller volumes in a round-bottom flask on the rotary evaporator, the concentrated solutions were transferred to pre-weighed 10 mL glass screw-top vials. A custom-made adaptor allowed for attachment of the vials directly to the rotary evaporator vapour duct. This meant that the fractions could be evaporated to dryness in the vials and immediately capped and weighed when removed from the rotary evaporator.

5.2.5 Bioassays

Bioassays were carried out to guide the separation of the extract. Fractions that exhibited bioactivity were further chromatographed to purify the active material. Antibacterial and anti-candidal activity was assessed using plate-hole diffusion assays (Sections 4.2.4.1 and 4.2.5.1), anti-QS was determined by disk diffusion (Section 4.2.6) and antifungal activity was assessed using microdilution assays (Section 4.2.5.2). When the mass of a fraction was known (after drying in pre-weighed glass vials; Section 5.2.4), it was reconstituted in MilliQ water or aqueous methanol to a concentration of 12.5 mg/mL for the bioassays. Aqueous methanol was used as the control. The final concentration range in the antifungal microdilution assays was 12.5–0.39 mg/mL. All bioassays were performed in duplicate.

The preparative reversed-phase HPLC fractions were tested after they had been concentrated to approximately 2 mL. In these bioassays, negative controls consisted of 5% ACN/H$_2$O and 50% ACN/H$_2$O that had undergone the same drying process as the fractions. Antifungal activity of the fractions against *D. brizae* was assessed using the microdilution assay described in 4.2.5.2, with the following adjustment. To duplicate wells, 100 µL of PDB and 50 µL of each fraction were combined with 50 µL of the *D. brizae* suspension. Following incubation, the growth of fungi in the presence of each fraction was compared with growth control wells that did not contain any fraction.
The MIC was determined for the final isolated compound against the three susceptible test bacteria and the yeast using the antibacterial and anti-candidal microdilution assays described in Sections 4.2.4.2 and 4.2.5.1, respectively. To reduce the amount of isolated material that was needed in these assays, the method was modified as follows. Instead of adding 50 µL of a 100 mg/mL solution of the extract (5 mg) to the first microtitre well, 50 µL of a 12.5 mg/mL solution of the fraction (625 µg) was added. This produced a final concentration range of 1,560–12 µg/mL. The MIC of the isolated compound against *D. brizae* was determined using the microdilution method described in Section 4.2.5.2.

### 5.2.6 Identification of active compound

#### 5.2.6.1 Mass spectrometry

Gas chromatography mass spectrometry (GC-MS) was performed with a ThermoQuest TRACE DSQ GC mass spectrometer in the positive ion mode with an ionisation energy of 70 eV. GC was performed with a SGE BPX5 (15 m × 0.1 mm ID, 0.1 µm film thickness) using a temperature program of 40 °C for 2 minutes, then increasing the temperature by 25 °C per minute to 300 °C after which the temperature was held for 17.6 minutes with a split ratio of 10, an injector temperature of 280 °C and the transfer line was set to 280 °C. High-purity helium was used as carrier gas with a flow rate of 1 mL/min.

Liquid chromatography mass spectrometry (LC-MS) electrospray ionisation (ESI) analyses were conducted on a Shimadzu liquid chromatography mass spectrometer–2010EV quadrupole mass spectrometer equipped with a solvent pump and Mercury MS C18 column (3 µm, 20 × 2.0 mm) using a gradient solvent of 5% to 100% ACN in water (0.1% formic acid) with a flow rate of 0.3 mL/min. The analyses were conducted in both positive and negative ionisation modes with a cone voltage of 50 V and the source maintained at 80 °C.
5.2.6.2 NMR spectroscopy

The structure of the isolated compound was determined using NMR spectroscopy. NMR spectra were obtained on a Bruker Av400 NMR spectrometer using the Topspin software package and deuterium oxide (D$_2$O) as the solvent. Gradient versions of the 2-dimensional $^1$H-$^1$H correlated spectroscopy (COSY) and $^1$H-$^{13}$C heteronuclear single quantum coherence (HSQC) experiments were performed using the standard pulse sequences as supplied by Bruker. The $^1$H peaks were referenced to residual solvent resonances (4.81 ppm for D$_2$O) and the $^{13}$C NMR chemical shifts were externally referenced using trimethylsilyl propionic salt in D$_2$O prior to the sample analysis.

The sodium salt of fusaric acid was prepared as a reference compound for NMR studies. Fusaric acid (Sigma; 10 mg) was dissolved in D$_2$O (0.5 mL) containing sodium deuteroxide (NaOD) (Sigma; 2.23 mg).

5.3 Results and discussion

5.3.1 Selection of extract for bioactivity-guided separation

The preparation of extracts from 31 coprophilous fungi and the antimicrobial screening of the extracts are described in Chapter Four. The extracts produced from isolates S6W2 and F14P1 demonstrated the greatest inhibitory activity. However, the yield of lyophilized liquor from F14P1 was small and S6W2 was therefore chosen for bioactivity-guided separation to isolate the active constituent. Table 5.1 shows a summary of the bioactivity of the liquor extract from S6W2. The extract exhibited broad spectrum antibacterial activity and was capable of inhibiting the test yeast and filamentous fungi. In addition, the extract inhibited violacein production in C. violaceum, which signifies QS quenching.
The extraction of the fermentation liquor from isolate S6W2 with EtOAc to produce an organic extract and an aqueous extract was described in Section 4.2.3.2. The aqueous extract exhibited enhanced inhibition of *S. aureus*, *E. coli*, *C. albicans* and *D. brizae* compared with the crude liquor. Section 5.3.2.1 describes the separation of both the aqueous extract and fermentation liquor with XAD-16 resin, to determine if the extraction with EtOAc was necessary for the scale-up of the separation procedure.

### Table 5.1 Summary of the antimicrobial activity of the liquor extract from the *F. oxysporum* isolate S6W2 (100 mg/mL)*

<table>
<thead>
<tr>
<th></th>
<th>S6W2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibacterial activity</strong>&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>3.125</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.56</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>1.56</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>–</td>
</tr>
<tr>
<td><strong>Anti-candidal activity</strong>&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>0.78</td>
</tr>
<tr>
<td><strong>Antifungal activity</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>D. brizae</em></td>
<td>16.67</td>
</tr>
<tr>
<td><strong>Anti-QS activity</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>C. violaceum</em></td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Values represent the average of triplicate assays
<sup>a</sup> MICs (mg/mL) were determined via microdilution assays (Sections 4.3.3.2, 4.3.4.1 and 4.3.4.2)
<sup>b</sup> Zone values represent the diameter of inhibition (mm) recorded in plate-hole diffusion assays (Sections 4.3.3.1 and 4.3.4.1)
<sup>c</sup> Anti-QS zone values represent the width of the zone of colourless cells from the perimeter of pigmented cells to the disk or zone of inhibited growth (Section 4.3.5).

### 5.3.2 Bioactivity-guided separation of S6W2

The scale of fermentation of S6W2 was increased to eight times the original fermentation and yielded 1,522 mg of lyophilised material. In the isolation of natural products from fungi, it is not uncommon to encounter a lack of reproducibility by an isolate, in terms of its physiological and metabolic instability. This may manifest
itself in a change in bioactivity after a scaled-up fermentation or even result in the complete loss of activity (Martin et al., 2006). Therefore, before any separation efforts, the bioactivity of the scaled-up fermentation liquor was tested to confirm that it had reproduced a similar level of inhibitory activity that was observed by the original smaller scale fermentation (Table 5.2). Both fermentation liquors were assayed at 12.5 mg/mL because this was the concentration of the fractions in the bioassays that guided their separation.

Isolation of the active compound from the scaled-up S6W2 liquor was carried out using adsorption to XAD-16 resin and preparative reversed-phase HPLC. A summary of the separation is shown in Figure 5.1.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Original S6W2 liquor</th>
<th>Scaled-up S6W2 liquor</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.33</td>
<td>9.0</td>
</tr>
<tr>
<td>E. coli&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.0</td>
<td>11.33</td>
</tr>
<tr>
<td>K. pneumoniae&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>P. aeruginosa&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C. albicans&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.67</td>
<td>7.67</td>
</tr>
<tr>
<td>D. brizae&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.67</td>
<td>16.67</td>
</tr>
<tr>
<td>C. violaceum&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Results are presented as the average of triplicate assays
<sup>a</sup> Values represent the zone of inhibition (mm) in plate-hole diffusion assays
<sup>b</sup> Antifungal activity against D. brizae was determined by microdilution assays and the values represent the MIC (mg/mL); The MIC of the original liquor was presented in Chapter Four (Table 4.7)
<sup>c</sup> Anti-QS activity against C. violaceum measured the width (mm) of the zone of colourless cells from the perimeter of pigmented cells to the disk or zone of inhibited growth.
Figure 5.1 Bioactivity-guided separation and chromatography of S6W2 liquor from the scaled-up fermentation.

5.3.2.1 Preliminary investigation of separation methodology

Before the scaled-up fermentation of isolate S6W2, smaller amounts of the crude fermentation liquor and the aqueous extract following extraction with EtOAc were applied to XAD-16 resin to assess the adsorption of their active constituents. XAD-16 resin was used to separate each extract into two fractions: the non-adsorbed inorganic salts and highly polar compounds in the effluent, and the hydrophobic compounds that were adsorbed by the XAD-16 resin and subsequently eluted with methanol.

The fermentation liquor and aqueous extract (100 mg in 10 mL of water) were both chromatographed on XAD-16 resin in a glass chromatography column (70 × 15 mm, 12 mL bed volume). Fraction collection was guided by observing the colour of the
effluent coming from the column; there appeared to be no difference in this respect between the liquor and the aqueous extract. The initial dark pink effluent (8 mL) was denoted the column effluent, followed by elution with distilled water (15 mL) that rinsed the remaining non-adsorbed material from the XAD-16 resin. Elution of the column with methanol produced a yellow eluate (13 mL) that contained most of the adsorbed material; further elution with methanol (23 mL) ensured all the adsorbed material was stripped from the XAD-16 resin. The difference in colour between the effluent and eluate fractions can be seen in Figure 5.2.

![Figure 5.2](image)

**Figure 5.2** Fractions collected from the separation of the aqueous extract of isolate S6W2 on XAD-16 resin: a) Initial column effluent that passed through the XAD-16 resin without interaction; b) first water wash; c) second water wash; d) methanol eluate; e) final methanol eluate.

The effluent fractions were combined and dried by lyophilisation. The methanol eluate fractions were combined and evaporated to dryness in a pre-weighed screw-top vial using the rotary evaporator. The dried methanol eluate residues were dissolved in 5% (v/v) methanol/water and the lyophilised effluent residues were reconstituted in MilliQ water. Bioassays of the column effluent and eluate of both the liquor and the aqueous extracts showed that the active compounds had been adsorbed by the XAD-16 resin and were eluted by the methanol in the eluate fractions (Table 5.3). The original extracts (prior to separation with XAD-16) were also assayed at
12.5 mg/mL for comparison of their zones of inhibition. The 5% methanol/water solution used to reconstitute the eluate fractions did not exhibit any antimicrobial activity (data not shown).

**Table 5.3** Bioactivity of XAD-16 fractions from the separation of S6W2 liquor and aqueous extracts (12.5 mg/mL)*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Liquor</th>
<th></th>
<th>Aqueous</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XAD-16 separated</td>
<td></td>
<td>XAD-16 separated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Effluent</td>
<td>Eluate</td>
<td>Original</td>
<td>Effluent</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>7.5</td>
<td>16.5</td>
<td>9.5</td>
<td>7.5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>–</td>
<td>17.5</td>
<td>11.0</td>
<td>–</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>–</td>
<td>14.0</td>
<td>8.0</td>
<td>–</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>–</td>
<td>15.0</td>
<td>7.5</td>
<td>–</td>
</tr>
<tr>
<td><em>D. brizae</em></td>
<td>–</td>
<td>6.25</td>
<td>16.67</td>
<td>–</td>
</tr>
<tr>
<td><em>C. violaceum</em></td>
<td>–</td>
<td>3.0</td>
<td>1.0</td>
<td>–</td>
</tr>
</tbody>
</table>

* Results are presented as the average of duplicate assays
* Values represent the zone of inhibition (mm) in plate-hole diffusion assays
* Antifungal activity against *D. brizae* was determined by microdilution assays and the values represent the MIC (mg/mL); The MIC of the original extracts were presented in Chapter Four (Table 4.7)
* Anti-QS activity against *C. violaceum* measured the width (mm) of the zone of colourless cells from the perimeter of pigmented cells to the disk or zone of inhibited growth

The methanol eluate fractions demonstrated antibacterial activity against the same three organisms as the original extracts, and the antifungal and anti-QS activities were also retained in the eluate. In both cases, the eluate fractions exhibited greater inhibition than the original extract, which suggested that separation using XAD-16 resin was a suitable method for concentrating the active constituent. A flow diagram for the fractionation of each extract is shown in Figure 5.3.

The highly polar compounds in the original extracts passed through the column of XAD-16 resin with minimal interaction and were contained in the column effluent fractions. The effluent would have included more components of the PDB nutrient medium such as carbohydrates, buffer, inorganic salts and other inactive polar
material, which would account for the comparably large mass of the effluent fractions.

Most of the bioactivity was contained in the methanol eluate from the XAD-16 resin; however, the column effluent also demonstrated some inhibition of the Gram positive bacterium *S. aureus*, albeit to a lesser degree. This suggested the presence of other less hydrophobic active compounds in the fermentation liquor that had not been fully retained by the XAD-16 resin and were also present in the column effluent. The column effluent was not investigated further because the extent of *S. aureus* inhibition was much greater in the methanol eluate.

![Figure 5.3](#) Preliminary bioactivity-guided separation of S6W2 fermentation liquor.
The testing of the fractions for antimicrobial activity also revealed that, although the aqueous extract demonstrated greater inhibition than the liquor, the XAD-16 eluates from both experiments had comparable levels of bioactivity. Analytical HPLC of the two eluates showed that they were also very similar in terms of composition (Figure 5.4).

**Figure 5.4** HPLC chromatograms of methanol eluate fractions from the XAD-16 separation of S6W2 liquor and aqueous extracts, in 5–80% ACN/H₂O/0.1% trifluoroacetic acid (TFA), peak detection λ 254 nm. Isocratic elution to 15 minutes followed by a 30 minute gradient to 80% ACN.

Therefore, it was not necessary to perform the EtOAc extraction of the liquor from the scaled-up fermentation prior to XAD-16 separation. Omission of this step provided an improvement to the isolation process, given that the liquid-liquid extraction was time consuming and required the use of an organic solvent.
5.3.2.2 **Stage 1 Separation – Amberlite® XAD-16 resin**

Having established that extraction of the S6W2 liquor with EtOAc was not necessary to concentrate the bioactivity, the first step of the separation was solid phase extraction using XAD-16 resin.

XAD-16 resin in a glass chromatography column (70 × 30 mm, bed volume 49 mL) was equilibrated with 10 bed volumes of distilled water before loading the liquor extract (1,350 mg in 100 mL). The resulting effluent (80 mL) displayed the same pink colour that was observed in the smaller scale separation, and this was combined with the effluent from a water rinse (200 mL). The adsorbed material was eluted with methanol (400 mL), yielding 204 mg of crude product on evaporating the eluate to dryness using a rotary evaporator.

**Table 5.4** Bioactivity of the effluent and eluate fractions from the separation of S6W2 liquor using XAD-16 resin (12.5 mg/mL)*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Effluent</th>
<th>Eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>7.0</td>
<td>16.0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>–</td>
<td>17.5</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>–</td>
<td>14.0</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>–</td>
<td>15.5</td>
</tr>
<tr>
<td><em>D. brizae</em></td>
<td>–</td>
<td>6.25</td>
</tr>
<tr>
<td><em>C. violaceum</em></td>
<td>–</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Results are presented as the average of duplicate assays

a Values represent the zone of inhibition (mm) in plate-hole diffusion assays

b Antifungal activity against *D. brizae* was determined by microdilution assays and the values represent the MIC (mg/mL)

c Anti-QS activity against *C. violaceum* measured the width (mm) of the zone of colourless cells from the perimeter of pigmented cells to the disk or zone of inhibited growth.
Bioassays of the methanol eluate confirmed that the activity was contained in this adsorbed fraction and the strength of inhibition was equivalent to the XAD-16 eluate from the smaller scale separation of the S6W2 liquor. A sample of the effluent was also tested to verify that it contained only inactive material, with the exception of minor antibacterial activity against *S. aureus* (Table 5.4).

### 5.3.2.3 Stage 2 chromatography of S6W2 – preparative reversed-phase HPLC

The XAD-16 eluate of S6W2 was further separated by reversed-phase HPLC using a preparative Alltima C18 column that had been pre-equilibrated with 5% ACN/H₂O. The sample (185 mg) was dissolved in 5% ACN/H₂O (2 mL) and loaded onto the column at a flow rate of 8 mL/min. The column was eluted 5% ACN/H₂O at a flow rate of 8 mL/min for 5 minutes, after which the flow-rate was increased to 10 mL/min for a further 15 minutes. The column eluate was monitored using a UV detector at λ 254 nm and was collected in 25 mL screw top vials. The fraction collections were marked on the chart paper recording the detector response. After 20 minutes, a linear solvent gradient to 100% ACN over 60 minutes at flow rate 10 mL/min was applied, which gradually eluted all the material injected onto the column. A total of 65 fractions were collected. Each fraction was concentrated to approximately 2 mL by rotary evaporation and tested for antimicrobial activity in duplicate. To conserve material, antibacterial assays were performed against only two bacteria, *S. aureus* and *E. coli*. In addition, an adjusted antifungal assay (see Section 5.2.5) was carried out against *D. brizae* that required a smaller volume of fraction compared with the serial dilution method. Fractions 12 and 13 were the only fractions that exhibited bioactivity, and both demonstrated antibacterial, antifungal and anti-QS activity (Table 5.5).
Table 5.5 Bioactivity of fractions 12 and 13 from the Stage 2 Separation of S6W2 liquor using preparative reversed-phase HPLC*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Fraction 12</th>
<th>Fraction 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.0</td>
<td>8.0</td>
</tr>
<tr>
<td>E. coli&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.0</td>
<td>8.5</td>
</tr>
<tr>
<td>C. albicans&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.5</td>
<td>9.0</td>
</tr>
<tr>
<td>D. brizae&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>C. violaceum&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Unknown concentration; results are presented as the average of duplicate assays
<sup>a</sup> Values represent the zone of inhibition (mm) in plate-hole diffusion assays
<sup>b</sup> The fractions were tested without serial dilution by an adjusted antifungal assay
<sup>c</sup> Anti-QS activity against C. violaceum measured the width (mm) of the zone of colourless cells from the perimeter of pigmented cells to the disk or zone of inhibited growth.

The collection of fraction 12 corresponded with the detection of a broad peak at retention time 35 minutes, which was initially thought to be a group of poorly resolved peaks. Fractions 13 and 14 were collected at the tail end of the broad peak. Analytical HPLC of fractions 12, 13 and 14 showed that they all had a major peak at retention time 24.5 minutes, which is the same as that of the XAD eluate peak in the analytical HPLC chromatogram of the methanol eluate from the XAD-16 separation shown in Figure 5.4. The overlay of the HPLC chromatograms of fractions 12, 13 and 14 presented in Figure 5.5, shows that fraction 12 contained the same compounds as present in fractions 13 and 14, and that most of the material had been eluted in fraction 12. Fraction 13 demonstrated the same pattern of bioactivity as fraction 12, albeit on a lower scale of activity. It is likely that fraction 14 would have produced similar results upon further concentration. HPLC analysis of the other main fractions did not reveal a peak with retention time 24.5 minutes. Thus, fractions 12, 13 and 14 were pooled, evaporated to dryness in a pre-weighed glass screw-top vial on the rotary evaporator and weighed (42.2 mg). The combined fractions were designated F12–14.
Figure 5.5 An overlay of analytical HPLC chromatograms of fractions 12, 13 and 14 from the preparative reversed-phase HPLC of S6W2 liquor, at λ 215 nm. Isocratic elution with 5% ACN/H₂O/0.1% TFA for 15 minutes followed by a 30 minute gradient to 80% ACN/H₂O/0.1% TFA. The three fractions were combined and designated F12–14.

The bioactivity of F12–14 was assayed at a concentration of 12.5 mg/mL against all microorganisms (Table 5.6), for comparison of bioactivity with the XAD-16 eluate fraction and original liquor activity (Table 5.3). In addition, the MIC was also determined.

The inhibition zones in the antibacterial and anti-candidal assays were slightly reduced compared with those produced by the concentrated preparative HPLC fraction 12, which suggested that the concentration of the active compound was higher in the 2 mL fraction than in the 12.5 mg/mL solution. This was consistent with the recording of a combined weight of fractions 12, 13 and 14 of 42.2 mg, and the vast majority of material being eluted into fraction 12. Bioactivity-guided
separation of the S6W2 liquor increased the antibacterial inhibition zones by an average of 62.4% (standard deviation 2.5). The anti-candidal inhibition zone was increased by 40%, and the anti-QS zone, which was measured on only one side of the paper disk, was increased by 36%. The MICs against the test bacteria and yeast were reduced more than fifteen-fold and the fraction was capable of inhibiting the growth of *D. brizae* at a concentration of only 2% of the original liquor MIC against the fungus.

**Table 5.6** Bioactivity of F12–14 from the preparative chromatography of S6W2*

<table>
<thead>
<tr>
<th>Organism</th>
<th>F12–14</th>
<th>Zone (mm)</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>µ</td>
<td>24.5</td>
<td>195</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>µ</td>
<td>28.0</td>
<td>98</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>µ</td>
<td>22.0</td>
<td>98</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>µ</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>µ</td>
<td>35.0</td>
<td>49</td>
</tr>
<tr>
<td><em>D. brizae</em></td>
<td>µ</td>
<td>n/a</td>
<td>390</td>
</tr>
<tr>
<td><em>C. violaceum</em></td>
<td>µ</td>
<td>5.0</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*Results are presented as the average of duplicate assays

a Antibacterial and anti-candidal activity were determined by plate-hole diffusion assays

b Anti-QS activity against *C. violaceum* measured the width (mm) of the zone of colourless cells from the perimeter of pigmented cells to the disk or zone of inhibited growth

c The fraction was tested at 12.5 mg/mL.

HPLC analysis of F12–14 under isocratic conditions showed a strong sharp peak at 20.15 minutes followed by two weaker broad shoulders of decreasing absorbance at 21.9 minutes and 23 minutes (Figure 5.6). The spectral data of signals from the photodiode array detector were collected, and the UV profile of the main peak was compared with the profile of the broad shoulder at 21.9 minutes (Figure 5.7). Both peaks had absorption maxima at λ 200.1, 227.1 and 272.0 nm, which strongly suggested that they were the same compound.
Figure 5.6 Analytical reversed-phase HPLC chromatogram of F12–14 from the preparative reversed-phase HPLC of S6W2 liquor, using 10% ACN/H₂O/0.1% TFA at λ 254 nm.

Figure 5.7 Diode array UV profile (λ 190–400 nm) of the main peak of F12–14 at 20.15 minutes and of the first shoulder at 21.9 minutes.
Ultraviolet–visible spectroscopy can be helpful in the analysis of natural products if they contain functional groups with absorption frequencies in the easily accessible region of the UV spectrum (190–400 nm). Absorption in this region usually signifies the presence of polyunsaturated chromophores in the compound, such as carbonyl, carboxyl, carboxylic acid, amide functional groups and aromatic ring systems (Larsen and Hansen, 2008). The strong absorbance at λ 272 nm in the UV spectrum of F12–14 indicated the presence of a carboxyl group in conjugation with a double bond or attached to an aromatic ring.

The reproducible broad shoulders on the tail of the main peak seen in the HPLC analysis of F12–14 (Figure 5.6) are typical of a compound such as a weak acid that is in a state of partial protonation or ionisation in the acid and salt forms in the acidic mobile phase being used (N. Hart, personal communication). A smaller injection volume (5 µL instead of 20 µL) of F12–14 produced only the two broad peaks and was missing the sharp, faster eluting peak (Figure 5.8), which indicated that the acidic moiety in the smaller injected amount had been fully protonated and the faster eluting salt form was absent.

HPLC analysis of fraction F12–14 using an alkaline mobile phase (10% ACN/0.02M disodium phosphate (Na₂HPO₄), pH 9) resulted in the compound eluting much faster as a single peak at 12 minutes (Figure 5.8). Because the salt forms of ionisable compounds elute faster than the protonated forms, this observation confirmed the presence of an acidic group in F12–14.
5.3.2.4 Identification of active compound in S6W2

Fraction F12–14 was analysed by GC-MS and the resulting electron ionisation mass spectrometry (EIMS) spectrum was compared with those of the compound library using the National Institute of Standards and Technology search program. The spectral fingerprint returned an 82.4% probability of the compound being 3-butylpyridine and contained the molecular ion at \( m/z \) 135 (Figure 5.9). However, its chromatographic behaviour (which does not result in the fragmentation of the compound during analysis that occurs in a mass spectrometer) indicated that the compound had an acidic group. This was confirmed by the strong absorbance at \( \lambda \) 272 nm in the UV spectrum (Figure 5.7), suggesting that F12–14 had a carboxyl group on an aromatic ring.

Figure 5.8 The effect of different mobile phase pH and injection volumes on the elution of F12–14 at \( \lambda \) 272 nm.
Figure 5.9 The EIMS of F12–14 and of the top match chemical structure 3-butylypyridine in the National Institute of Standards and Technology spectral database.

LC-MS (ESI) analysis of F12–14 produced a different molecular ion and fragmentation pattern (Figure 5.10) to that of the EIMS (Figure 5.9). It was determined that the peak at $m/z$ 179.9 [M]$^+$ represented the aforementioned 3-butylypyridine with an attached carboxylic acid functional group.
Analysis of the $^1$H and $^{13}$C NMR spectra of F12–14 (Appendices 1 and 2, respectively), showed the presence of an n-butyl group attached to an aromatic ring to which a carboxyl group was also attached. The $^1$H NMR and $^{13}$C NMR spectra resonance assignments are summarised in Table 5.7. The triplet at $\delta 2.70$ in the $^1$H spectrum, which integrated for two protons, was typical of the methylene of an aliphatic chain attached to an aromatic ring (i.e., the methylene at C-1’ of the n-butyl group). The multiplets at $\delta 1.62$ and $\delta 1.32$, which each integrated for two protons, are typical of methylene moieties at C-2 and C-3 of an n-butyl group, with the three proton triplet at $\delta 0.90$ being a characteristic end of chain methyl group. The presence of only five aromatic carbon atoms in the $^{13}$C NMR spectrum indicated that the compound had a pyridine ring as predicted by the National Institute of Standards and Technology software from the library of EIMS spectra. There were only three
aromatic proton signals in the $^1$H spectrum: The doublets at $\delta$ 7.79 and $\delta$ 7.90 were coupled and therefore adjacent to each other, and the singlet at $\delta$ 8.45 is indicative of a proton on a carbon atom adjacent to a pyridine nitrogen and a quaternary carbon (i.e., it is the methine proton at C-6). This being the only signal in the $^1$H spectrum of a proton on a carbon next to the pyridine N atom, it indicated that the carboxyl group must therefore be attached to the pyridine ring at C-2.

The COSY spectrum of F12–14 (Appendix 3) confirmed the proton–proton connectivity in the n-butyl chain. There was connectivity between the signals at $\delta$ 7.90 and $\delta$ 7.79 of the protons on C-3 and C-4 of the pyridine ring, although it was difficult to see because of their close proximity to one another and the poor peak shape of the C-3 proton signal. The connectivity between the proton on C-4 of the pyridine ring at $\delta$ 7.79 to a proton on C-1′ of the n-butyl group at $\delta$ 2.70, was evidence of long range allylic coupling between these two protons, which showed that the n-butyl group was attached to the pyridine ring at C-5. The HSQC spectrum (Appendix 4) confirmed the connectivity between the assigned carbon and proton atoms in the n-butyl chain and the connectivity between the carbon and proton atoms at C-4 of the pyridine ring. These assignments led to the presumptive identification of the active compound F12–14 as 5-butylpyridine-2-carboxylic acid, which is better known as fusaric acid (Figure 5.11). Fusaric acid is not very soluble in water, but its solubility can be improved under acidic or alkaline conditions.

![Figure 5.11 Structure of fusaric acid with assigned atom numbering.](image-url)
The $^1$H and $^{13}$C NMR spectra of the reference fusaric acid in NaOD/D$_2$O are shown in Appendices 5 and 6, respectively. Comparison of the $^1$H and $^{13}$C NMR spectra of F12–14 with those of fusaric acid (Figure 5.12 and Figure 5.13, respectively) confirmed the identification of F12–14 as fusaric acid. A summary of the $^1$H and $^{13}$C NMR spectral data of the reference fusaric acid are included in Table 5.7. The small differences in the chemical shifts and peak multiplicity are due to the differences in concentration of the NMR samples and to the reference fusaric acid spectra being acquired in D$_2$O containing NaOD. Although F12–14 appears to be present in a salt form in the NMR analysis, the bioactive constituent of the S6W2 liquor extract was identified as fusaric acid. Fusaric acid is an amphoteric compound that is capable of chelating metals and can also form a salt with monovalent ions, such as a sodium ion (Na$^+$), available in the fermentation media or in solution during the isolation process (Bochner et al., 1980; Stack Jr et al., 2004).

### Table 5.7 Summary of the $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR spectral data of F12–14 and FA $^{\text{Na}}^+$ in D$_2$O

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta^{^{13}}$C in ppm</th>
<th>$\delta^1$H in ppm, Mult.* ($J$ in Hz)</th>
<th>$\delta^{^{13}}$C in ppm</th>
<th>$\delta^1$H in ppm, Mult.* ($J$ in Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>148.26, qC</td>
<td>–</td>
<td>148.51, qC</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>123.70, CH</td>
<td>7.90, d (8.0)</td>
<td>123.53, CH</td>
<td>7.86, d (8.0)</td>
</tr>
<tr>
<td>4</td>
<td>137.80, CH</td>
<td>7.79, d (8.0)</td>
<td>137.60, CH</td>
<td>7.76, dd (8.0, 1.8)</td>
</tr>
<tr>
<td>5</td>
<td>141.20, qC</td>
<td>–</td>
<td>141.04, qC</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>150.30, CH</td>
<td>8.45, bs</td>
<td>150.55, CH</td>
<td>8.41, bs</td>
</tr>
<tr>
<td>7</td>
<td>173.10, qC</td>
<td>–</td>
<td>173.18, qC</td>
<td>–</td>
</tr>
<tr>
<td>1’</td>
<td>31.73, CH$_2$</td>
<td>2.70, t (7.5)</td>
<td>31.67, CH$_2$</td>
<td>2.68, t (7.6)</td>
</tr>
<tr>
<td>2’</td>
<td>32.37, CH$_2$</td>
<td>1.62, m</td>
<td>32.35, CH$_2$</td>
<td>1.60, m</td>
</tr>
<tr>
<td>3’</td>
<td>21.45, CH$_2$</td>
<td>1.32, m</td>
<td>21.44, CH$_2$</td>
<td>1.31, m</td>
</tr>
<tr>
<td>4’</td>
<td>13.06, CH$_3$</td>
<td>0.90, t (7.4)</td>
<td>13.07, CH$_3$</td>
<td>0.90, t (7.4)</td>
</tr>
</tbody>
</table>

* Mult. means multiplicity; bs = broad singlet; d = doublet; dd = doublet of doublets; m = multiplet; t = triplet; qC = quaternary carbon; CH = methane; CH$_2$ = methylene; CH$_3$ = methyl.
Figure 5.12 Comparison of $^1$H NMR spectra (400 MHz) of (a) F12–14 in D$_2$O and (b) Fusaric acid in D$_2$O/NaOD.
Fusaric acid is a well-documented fungal toxin that was first isolated by Yabuta et al. (1934) from Fusarium heterosporum as a compound inhibiting the growth of rice seedlings. Fusaric acid has subsequently been implicated in a number of plant diseases and has been isolated from many Fusarium species including F. moniliforme, F. crookswellense, F. subglutinans, F. sambucinum, F. napiforme, F. oxysporum, F. solani, F. proliferatum, F. verticillioides, F. sacchari and
*F. fujikuroi* (Bacon et al., 1996; Brown et al., 2012; Ghannam et al., 2014; Niehaus et al., 2014). The production of fusaric acid by pathogenic *Fusarium* strains plays a role in the pathogenesis of various wilt, rot and blight diseases in commercially important plants such as barley, corn, wheat, rice, banana and cottonseed. These lead to reduced crop yields and contamination of the harvested product, particularly those used for animal feed (Arias, 1985; Barna et al., 2011; Dong et al., 2012; Rofiat et al., 2015; Saremi and Okhovvat, 2006; Shimshoni et al., 2013; Stipanovic et al., 2011).

In addition to phytotoxic activity, fusaric acid exhibits diverse biological activities including anti-oomycete and antifungal (Son et al., 2008), antibacterial (May et al., 2000; Son et al., 2008), anti-mycobacterial (in metal complexes) (Pan et al., 2011), acanthamoebicidal (Boonman et al., 2012), nematicidal (Kwon et al., 2007), anti-hypotensive (Hidaka et al., 1969), dopamine β-hydroxylase inhibition (Goodwin and Sack, 1974) and anti-tumour activity (Jaglowski and Stack Jr, 2006). Anti-QS activity has been attributed to fusaric acid, in the repression of the production of QS signal N-hexanoyl-L-homoserine lactone in *Pseudomonas chlororaphis* (Van Rij et al., 2005). Recently, Martín-Rodriguez et al. (2014) identified a marine endophytic isolate of *F. oxysporum* that demonstrated QS inhibition against *C. violaceum*, and it is likely the active compound was fusaric acid. The compound has also been used to screen for tetracycline-resistant mutants of *E. coli* and *Salmonella typhimurium* because they are more susceptible to fusaric acid than wild type strains (Herrin Jr et al., 1982). In this thesis, *P. aeruginosa* was not susceptible to the extracts from the *F. oxysporum* isolate S6W2 or to the isolated fusaric acid. This may be explained by the presence of a fusaric acid resistance gene in the bacterium, similar to that reported by Utsumi et al. (1991) in *Pseudomonas cepacia* and by Ruiz et al. (2015) in *Pseudomonas protegens*.

*F. oxysporum* are commonly isolated from soil, although they have previously been isolated from faeces (Krug et al., 2004; Peterson et al., 2009). They are considered a facultative coprophile that can take advantage of the variable nutrients provided by dung from different herbivores. As discussed in Chapter Three, koalas feed
exclusively on eucalyptus leaves and their faeces consist of undigested cellulose, highly lignified fibre and phenolic compounds. *Fusarium* species are known producers of the enzymes cellulase, hemicellulase, ligninase and tannase, and are therefore prime candidates to use the recalcitrant substrate for nutrition (Demain et al., 2005; Kikot et al., 2009; Murugan et al., 2007). The production of fusaric acid by the fungus may also confer a competitive advantage to the species by inhibiting the growth of other fungi in the dung as the compound exhibited antifungal activity in the bioassays performed in this thesis. Interestingly, antifungal activity by fusaric acid has only been reported in the literature once. Son et al. (2008) demonstrated growth inhibition of two plant pathogenic fungi by fusaric acid in an antifungal assay similar to the radial growth assay described in Section 4.2.5.2.

The isolation of fusaric acid from extract S6W2 supports the hypothesis that coprophilous fungi are present in the koala faeces and that these fungi are capable of producing bioactive compounds. Bérdy (2005) summarises the difficulties of bioactivity-guided separation as follows: “The identification of active compounds from fermentation samples (extracts, whole broths) is one of the most complicated, labour intensive, time consuming steps of the screening protocols” (p.23). Since fusaric acid is a known compound, any efforts to identify active compounds within the remaining active extracts should be focused on the lesser studied fungi genera, such as *Fimetariella* and *Anthostomella*, to increase the chances of identifying a novel compound. Furthermore, if resources permit, dereplication strategies that provide fast and unambiguous identification of previously described compounds without reference standards should be used (Cordell, 1999). For example, this could include ultra-HPLC coupled to photodiode array and high-resolution MS in combination with searching a microbial compound database such as AntiBase (Wiley-VCH, Germany) with additional in-house reference standards from previous screening efforts, if available (El-Elimat et al., 2013; Klitgaard et al., 2014; Nielsen and Larsen, 2015). Recently, a new method was described by Sica et al. (2015) that eliminates the need for any extract preparation from a fungus, by achieving identification of secondary metabolites directly on the surface, or surrounding, a fungal culture in a Petri dish. Continued development in technology will contribute
to the improvement of natural product drug discovery and aid researchers in finding novel compounds.

5.4 Summary

Australian coprophilous fungi have yet to be explored in terms of their potential to produce antimicrobial secondary metabolites. In this chapter, the extract from the fermentation liquor of *F. oxysporum* isolate S6W2 was separated to identify the compound responsible for the observed antibacterial, antifungal and anti-QS activities. Separation of the extract was achieved by bioactivity-guided fractionation using adsorption to Amberlite® XAD-16 resin and preparative reversed-phase HPLC in conjunction with plate-hole diffusion, microdilution and disk diffusion assays. Following analysis with UV spectroscopy, mass spectrometry and NMR spectroscopy, and after comparison with a reference standard, the bioactive compound was identified as fusaric acid.

Fusaric acid is a well-documented mycotoxin of the *Fusarium* genus and is implicated in various wilt, blight and rot diseases in commercially important plants. The compound has also demonstrated a range of biological activities since its isolation in 1934. In natural product drug discovery research, the main objective is to find new compounds. The isolation of a known compound in this work highlights the importance of employing dereplication protocols (if available) to reduce the costly and time consuming efforts of bioactivity-guided separation. Nonetheless, the isolation of fusaric acid from extract S6W2 supported the hypothesis that coprophilous fungi capable of producing bioactive compounds were present in the koala faeces.
CHAPTER 6

Conclusion
6.1 Introduction

Fungi have provided numerous chemically and biologically diverse secondary metabolites that have proved useful in medical and agricultural settings. Given the increasing resistance of human and plant pathogens to current treatments, it is essential that previously untapped biological niches are explored for novel microbes and natural products.

The preceding chapters described the studies undertaken to address the major aims of the thesis, which were to:

i. isolate and identify fungi from koala faeces
ii. assess the antimicrobial activity of extracts prepared from the fungi
iii. identify the active compounds within the most active extract.

This chapter provides an overview of the major findings of this project as well as identifying the scope for further research.

6.2 Summary of findings

6.2.1 Chapter Three

Very little research has been carried out on coprophilous fungi in Australia, despite the important role they play in the ecosystem and their potential for producing bioactive secondary metabolites. Chapter Three broadened our knowledge about the community of coprophilous fungi that inhabit koala faeces. Thirty-one fungal isolates from koala faeces were identified using ITS sequencing, representing only the second time that fungi from koala faeces have been identified by molecular means (Peterson et al., 2009). The ITS sequences now form a Popset (population sequence data) in the NCBI database and serve as a good reference for studying coprophilous fungi communities in the future. All but one of the isolates were members of the
phylum Ascomycota, a weighted diversity that is common in numerous Ascomyceteous dominated coprophilous collections in the literature (Bell, 2005; Gupta, 2010; Melo et al., 2012; Pandey, 2009; Peterson et al., 2009; Richardson, 2001a, c; Watling and Richardson, 2010). Although this work did not reveal any previously unknown species, the findings are important because they add to the paucity of research into Australian coprophilous fungi.

6.2.2 Chapter Four

Studies of coprophilous fungi often focus on their taxonomy and their ecology rather than investigating their secondary metabolites for bioactivity. The limited studies that do exist suggest that coprophilous fungi are capable of producing bioactive compounds, and that these are are potentially a source of bioactive compounds that is on par with their more well-documented counterparts such as the marine and endophytic fungi (Bills et al., 2013). The reason for this capability and the premise behind this work is that the fitness of coprophilous fungi is challenged by competing and invading organisms within koala faeces. To survive, the coprophilous fungi must produce chemicals to mediate interactions with other organisms. In accordance with this premise, many of the fungi isolated in this project were, indeed, found to exhibit antimicrobial activity.

In plate-hole diffusion assays, 54.8% of the fungi produced extracts that were capable of inhibiting at least one of the test bacteria: *S. aureus, E. coli, K. pneumoniae* and *P. aeruginosa*. Extracts from the fermentation liquor of *A. leucospermi* (F16EP1), *S. alcina* (F14P1) and *A. niger* (S5P3) isolates were able to inhibit the growth of all four bacteria. The MICs of the most effective extracts were determined via microdilution assays and the lowest MIC was exhibited by an extract of *F. oxysporum* (S6W2, aqueous). This extract inhibited the growth of *S. aureus, E. coli* and *K. pneumoniae* at a concentration of 1.56 mg/mL, 0.78 mg/mL and 1.56 mg/mL, respectively. Time-course growth assays provided an evaluation of the antibacterial effect over 24 hours and suggested that the inhibition of *S. aureus* and
E. coli by S6W2 was bacteriostatic at 2 × MIC. Antifungal activity was assessed against C. albicans and D. brizae with 8.6% and 38.7%, respectively, of the coprophilous fungi producing extracts that inhibited the test fungi in agar assays. Six extracts were capable of inhibiting both fungi in microdilution assays, and the lowest MICs against C. albicans and D. brizae were produced by F. oxysporum (0.52 mg/mL) and A. niger (6.25 mg/mL), respectively. An alternative system of bacterial control is through the disruption of QS signals. In this work, 12.9% of the coprophilous fungi produced extracts that inhibited violacein production in the screening bacterium C. violaceum, thus indicating anti-QS activity. This is the first time that coprophilous fungi have been screened for anti-QS activity.

The antimicrobial activity exhibited by the extracts in this thesis, while modest, are encouraging given the high percentage of bioactive isolates from one fermentation method and the production of only crude extracts. This was the first work in which the antibacterial, antifungal and anti-QS potential of Australian coprophilous fungi have been explored.

6.2.3 Chapter Five

Isolate S6W2 (F. oxysporum) was chosen to undergo bioactivity-guided separation based on its adequate extract yield and its moderate antibacterial, antifungal and anti-QS activity. Separation of the scaled-up extract was achieved via adsorption to XAD-16 resin and preparative RP-HPLC in conjunction with antimicrobial screening assays. Following analysis with UV spectroscopy, MS, NMR spectroscopy and comparison with a reference standard, the bioactive compound was identified as fusaric acid.

Fusaric acid is produced by many Fusarium species and is most notable for its implication in the pathogenesis of various wilt, blight and rot diseases in plants of commercial importance. Additionally, fusaric acid has been reported to exhibit diverse biological activities including antibacterial, antifungal and anti-QS activity.
The isolation of fusaric acid from extract S6W2 supported the hypothesis that coprophilous fungi that were capable of producing bioactive compounds were present in the koala faeces.

6.3 Scope for further research

6.3.1 Immediate source of additional study

Several fungal isolates that warrant further investigation were identified from the koala faeces. Isolates that exhibited antimicrobial activities but were not studied further could be cultivated in a scaled-up fermentation and undergo bioactivity-guided separation to identify the active compounds. The most likely candidates include F2P2 (F. rabenhorstii), F16EP1 (A. leucospermi), F3W3 (T. hyrcaniae), F14P1 (S. alcina) and S5P3 (A. niger).

6.3.2 Broadening of the fungal survey

Attempting to increase the diversity of fungi isolated from koala faeces would more comprehensively address the research aims in two ways. First, a larger collection would further broaden our understanding of the coprophilous community in koala faeces, and second, an increased number of fungi for bioactivity screening would improve the likelihood of uncovering novel bioactive secondary metabolites.

The fungi isolated in this research represent an unknown fraction of the species present in a collection of faeces from 12 koalas living in two locations at one time of the year. Given these considerations, an expanded survey of the coprophilous fungi population could address experimental variables, including adjustment of the drying time for faeces, changes to incubation temperature, the introduction of light and dark cycles, and the use of a different medium, such as malt extract agar. For example, some isolates had difficulty producing confluent growth and did not survive multiple subculturing, which may have been resolved by changes to the growth medium or to
the incubation temperature. Moist chamber incubation was not performed in this study because the aim was to isolate fungi that are more likely to be cultivable with PDB. However, because moist chamber incubation identifies isolates that are able to rely solely on the faeces for nutrition (Krug, 2004), it may allow the isolation of different species to those isolated in the agar methods and, therefore, may be worth performing. In terms of variables relating to the collection of faeces, some studies have suggested that season and location are likely to affect the diversity of the coprophilous fungal community (Krug et al., 2004; Kruys and Ericson, 2008; Melo et al., 2012; Richardson, 2001c). Therefore, although time intensive, a more comprehensive survey would include many faecal pellets collected at different times of the year from numerous koalas residing in various locations. These approaches may identify fungal species that were not found in the current study and may increase the opportunities for discovery of novel bioactive compounds produced as secondary metabolites.

6.3.3 Optimisation of fermentation conditions

Modern genome sequencing has shown that many fungi are only capable of producing a small fraction of their secondary metabolites under standard laboratory conditions (Spraker and Keller, 2014; Takahashi et al., 2013). Therefore, in this work, the true potential for natural product discovery in the isolated fungi may have been underestimated. Activation of silent secondary metabolite gene clusters may yield novel compounds with interesting chemistries. One method of triggering production is to systematically alternate cultivation parameters such as the media composition, temperature, aeration, and addition of enzyme inhibitors or biosynthetic precursors (Kurtböke, 2012). This technique was termed the OSMAC (one strain – many compounds) approach by Bode et al. (2002), and it has proven to be a simple and effective tool to diversify metabolite production and to produce compounds from silent metabolic pathways (Hewage et al., 2014; Wang et al., 2013; Wei et al., 2010). Fungi are also capable of photosensing, and the presence (or absence) of light at differing intensities and wavelengths has been shown to affect the production of
secondary metabolites (Bayram et al., 2008; Castrillo et al., 2013; Schmidt-Heydt et al., 2010). Another consideration is to take advantage of the role that secondary metabolites play in interspecies communication, and co-cultivate the target fungus with another fungus or bacterium also known to inhabit koala faeces. This would mimic the physiological conditions of the dung related to communication and competition, and could activate silent gene clusters, leading to the production of different compounds (Brakhage, 2013; Netzker et al., 2015).

The cultivation parameters in this thesis were consistent for every fungus (PDB in a flask stoppered with cotton wool and incubated at 25 °C in the dark with shaking at 150 rpm), and it would therefore be valuable to investigate if alternative conditions could lead to the production of novel antimicrobial compounds.

### 6.4 Conclusion

The results from the current study provide compelling evidence that coprophilous fungi from koala faeces produce antimicrobial compounds. Further study should be carried out to identify the active constituents in the active extracts that did not undergo bioactivity-guided separation. More broadly, this thesis encourages greater investigation of fungi from minimally explored biological niches, such as animal faeces.
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Appendix 1: $^1$H NMR spectrum (400 MHz) of F12–14 in D$_2$O
Appendix 2: $^{13}$C NMR spectrum (100 MHz) of F12–14 in D$_2$O
Appendix 3: COSY Spectrum of F12–14 in D₂O
Appendix 4: HSQC Spectrum of F12–14 in D$_2$O
Appendix 5: $^1$H NMR spectrum (400 MHz) of fusaric acid in D$_2$O/NaOD
Appendix 6: $^{13}$C NMR spectrum (100 MHz) of fusaric acid in D$_2$O/NaOD
Appendix 7: Publications arising from this thesis

1. Book chapter


2. Conference poster presentation