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Discovery of novel anti-*Candida albicans* compounds from environmental bacteria

by
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Dissertation presented for the degree of Doctoral of Science (Biochemistry) in the Faculty of Science at Stellenbosch University

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DECLARATION

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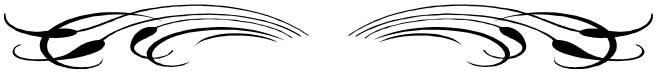
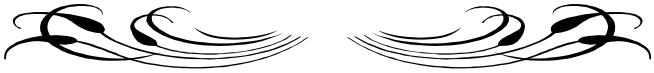
Bernice Barnard Jenkins *December 2023*



Greater even than the greatest discovery is to

keep open the way to future discovery

John Jacob Abel



SUMMARY

The opportunistic fungal pathogen, *Candida albicans* (*C. albicans*), has the ability to colonize different epithelial tissues (e.g. vaginal, intestinal and oral tissues) causing moderate to severe and fatal infections. The fatal infections are due to fungal cells that enter the bloodstream and circulate after being dispersed from their resistant biofilms. Organisms that have adapted to form biofilms are associated with improved tolerance to drugs, which was also found for some drugs in this study. A medium-throughput multiplex assay system (MPAS) has been developed for the in-parallel study of several fungal life-cycle stages of different strains of *C. albicans*. MPAS was designed to use conventional 96-well plates and 96-pin lids in four different assay layouts. In MPAS, the metabolic activity measurements were used to determine the effect of antimicrobial compounds in assay targeting planktonic cell susceptibility, biofilm prevention, biofilm eradication, as well as susceptibility of cells released from biofilms. MPAS detection limits were further optimized by testing the activity of antimicrobial culture extracts from known bacterial producers on the different *C. albicans* life stages. The MPAS data showed which producer extracts were more active against certain strains of *C. albicans*, as well as highlighted which fungal forms are more sensitive to certain culture extracts. This led to the successful application of MPAS to identify active culture extracts of unknown soil microbes against planktonic and biofilm forms of *C. albicans* for further characterization. Ultra-performance liquid chromatography linked to high resolution tandem mass spectrometry (HR UPLC-MS^e) was used to identify and characterize known antifungal lipopeptides (such as surfactins, iturins and fengycins), rhamnolipids, as well as active small quorum sensing compounds in the most active culture extracts. The overall success of the MPAS together with HR UPLC-MS^e was confirmed by the successful identification of known and unknown compounds, from soil sample bacteria, with activity against *C. albicans*. The study showed the potential of the medium throughput MPAS together with HR UPLC-MS^e to discover and characterize potential antibiofilm and antifungal compounds for further development.

OPSOMMING

Die opportunistiese swampatogeen, *Candida albicans* (*C. albicans*), het die vermoë om verskillende epiteelweefsels (bv. vaginale, derm- en mondweefsel) te koloniseer, wat matige tot ernstige en dodelike infeksies veroorsaak. Die dodelike infeksies is te wyte aan swamselle wat in die bloedstroom sirkuleer nadat hulle van hul weerstandbiedende biofilms vrygestel is. Organismes wat aanpas om biofilms te vorm, word geassosieër met verbeterde verdraagsaamheid teenoor medikamente, wat ook in hierdie studie vir sommige middels gevind is. 'n Medium-deurset multipleks essaï sisteem (MPAS) is vir die in-parallel studie van verskeie swam-lewensiklus-stadia van verskillende stamme van *C. albicans* ontwikkel. MPAS is ontwerp om gebruik te maak van konvensionele 96-putplate en 96-pendeksels in vier verskillende toets-uitlegte. In MPAS is die metaboliese aktiwiteitsmetings gebruik om die effek van antimikrobiese verbindings te bepaal in essaïs wat planktoniese sel vatbaarheid, biofilm voorkoming, biofilm uitwissing, sowel as vatbaarheid van selle vrygestel uit biofilms teiken. MPAS deteksielimiete is verder geoptimiseer deur die aktiwiteit van antimikrobiese kultuurekstrakte van bekende bakteriële produsente, op die verskillende *C. albicans* lewenstadia te bepaal. Die MPAS data het getoon watter produsent-ekstrakte meer aktief was teen sekere stamme van *C. albicans*, asook uitgelig watter swamvorme meer sensitief is vir sekere kultuurekstrakte. Dit het gelei tot die suksesvolle toepassing van MPAS om aktiewe kultuurekstrakte van onbekende grondmikrobes teen planktoniese en biofilmvorme van *C. albicans* te identifiseer vir verdere karakterisering. Ultradoeltreffendheid vloeistofchromtografie gekoppel met hoë resolusie tandem massaspektrometrie (HR UPLC-MS^e) is gebruik om bekende antifungiese lipopeptiede (soos surfaktiene, ituriene en fengisien), ramnolipiede, asook aktiewe klein kworumwaarnemingsverbindings, te identifeer en te karakteriseer in die mees aktiewe kultuurekstakte. Die algehele sukses van die MPAS saam HR UPLC-MS^e is bevestig deur die suksesvolle identifikasie van bekende en onbekende verbindings, vanaf grondmonsterbakterieë, met aktiwiteit teen *C. albicans*. Die studie het die potensiaal van die mediuim deurvloei MPAS tesame met HR UPLC-MS^e gewys om potensiële antibiofilm en antifungiese verbindings te ontdek en te karakteriseer vir verdere ontwikkelling.

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TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

$[M+H]^+$	singly charged monomeric molecular ion
$[M+2H]^{2+}$	doubly charged monomeric molecular ion
ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
AmB	Amphotericin B
AMP(s)	Antimicrobial peptide(s)
AFP(s)	Antifungal peptide(s)
ATCC	American type culture collection
B. aneurinolyticus	*Bacillus aneurinolyticus*
B. brevis	*Bacillus brevis*
B. laterosporus	*Bacillus laterosporus*
B. subtillis	*Bacillus subtillis*
Br. parabrevis	*Brevibacillus parabrevis*
BEC_{50}	Peptide concentration that results in 50% biofilm eradication
BIC	Peptide concentration that results in ≥90% biofilm prevention
BIC_{50}	Peptide concentration that results in 50% biofilm prevention
C. albicans	*Candida albicans*
CAS	Caspofungin
ESMS	Electrospray mass spectrometry
Fen(s)	Fengycidin(s)
Flu	Fluconazole
GS	Gramicidin S
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IC_{50}	Compound concentration leading to 50 % growth inhibition
Itn(s)	Iturin(s)
MOA(s)	Mode of action(s)
M_r	Relative molar mass
MS	Mass spectrometry
m/z	Mass over charge ratio
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agarose
PDB	Potato dextrose broth
R_t	Retention time
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
Srf(s)	Surfactin(s)
TOF-ESMS	Time-of-flight electrospray mass spectrometry
Trc mix	Tyrocidine mixture
TSB	Tryptone soy broth
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet

Standard 3-letter and 1-letter abbreviations were used for the natural amino acids, with uppercase 1-letter abbreviations for L-amino acid residues and lower case 1-letter abbreviations for D-amino acid residues in peptide sequences

PREFACE

Problem statement

The decrease in effectiveness of antibiotics due to their overuse and misuse, resulting in increased antimicrobial resistance, is a public health crisis of international concern, threatening modern medicine, animal health and food security. The opportunistic human fungal pathogen, *Candida albicans* (*C. albicans*), has the ability to colonize various epithelial tissues (i.e. vaginal, intestinal and oral tissues) causing superficial and systemic diseases. It has been observed that fatal systemic infections, caused by *C. albicans* biofilms, are due to planktonic forms liberated from the resistant biofilm fungi disseminating into the bloodstream. Life-threatening biofilms of a variety of micro-organisms are most often found in catheters, prosthetics and on implant surfaces, of which *C. albicans* is the most common. Additionally, the new plasmid mediated genes (mcr-1 and mcr-2) confer resistance against last resort antibiotics and have been estimated to cause 10 million deaths per annum by 2050 as well as costing an excess of $100 trillion from the world's economy. Alas, only non-ribosomally produced antimicrobial lipopeptides as a novel class of antibiotics has been discovered since the twentieth century due to the laborious and time-consuming antibiotic discovery methodologies. Therefore, there is an urgent need for rapid, repeatable and inexpensive methodologies to identify and characterize novel antifungal compounds from easily accessible environmental samples.

Rationale

Resistance has been found against every antibiotic on the market and this resistance is potentiated by drug-tolerant microbial biofilms, which cause 65% of treated infections in the developed world. Therefore, the discovery and development of novel antibiofilm antimicrobials is essential to combat multidrug resistant pathogens. A group of omnipresent natural antibiotics, namely non-ribosomal antimicrobial peptides (nrAMPs) and secondary antibiotic metabolites from environmental bacteria offer an array of candidates with potential as antimicrobial agents against biofilms. It is hypothesised that there are many undiscovered antifungal compounds. For this we needed to develop a rapid multiplex assays system that can be used to discover bacterial producers with antimicrobial activity directed towards all the life stages of biofilm forming organisms, with focus on those that inhibit the attachment and development of pathogenic biofilms.

Study Goal

This study's goal was to provide a rapid, reliable and repeatable medium- to high-throughput assay system for the discovery and characterisation of novel antimicrobials against *C. albicans*, both against its planktonic and biofilm stages. The identification and characterisation of a novel antibiofilm compounds may possibly lead to clinically important antibiofilm drugs.

Aims of this study

To discover novel producers and their antimicrobial compounds from a biomining survey of environmental samples, the following aims were completed:

Aim 1: Selection and characterisation of the life stages of five *C. albicans* strains that will be used as targets in the discovery of novel antimicrobial producers and their compounds (Chapter 2).

Aim 2: Development of a multiplex assay system using known antifungal compounds targeted to the different life stages, namely planktonic cells, young and mature biofilms and shed biofilm cells, of five selected stains of the fungal pathogen *C. albicans* (Chapter 2).

Aim 3: Validation and critical evaluation of the multiplex assay system for medium- to high throughput analysis by assessing extracted compounds from microcultures of eight selected bacteria with six of the bacteria producing known antifungal compounds. The multiplex assay system was combined with high resolution mass spectrometry to assess the production of compounds. (Chapter 3).

Aim 4: Biomining survey of environmental soil samples using the optimised multiplex assay system complemented by ultraperformance liquid chromatography linked to high resolution mass spectrometry for extract characterisation and comparison, as well as genetic analysis of producer organisms (Chapter 4).

Aim 5: Characterisation of the antifungal compounds in the top hits from the biomining survey advanced high resolution mass spectrometric methodology linked to ultraperformance mass spectrometry.

Thesis outlay

Chapter 1 gives an overview of drug discovery against not only bacterial biofilms, but also against fungal biofilms. Currently known antimicrobial and antifungal compounds are discussed in terms of their activity and structures, as well as some light shed on compounds that can be found in the antifungal pipelines. These include small antifungal compounds and antimicrobial peptides which are ribosomally and non-ribosomally synthesised. In Chapter 2, we designed a novel, multiplex assay system (MPAS) which can be used to study planktonic and biofilm forms of the test organism/fungus. The MPAS was then further challenged by testing the life-stage dependent antimicrobial sensitivity of five *C. albicans* strains isolated from various environmental and clinical sources, against known antimicrobial agents. The MPAS application to assessment of microbial culture extracts was validated in Chapter 3, where we used peptide producing bacterial strains to test the activity of known peptide compounds on the various cellular forms of the five *C. albicans* strains. This layout set the groundwork for the biomining study in Chapter 4, where soil isolates were cultured, antimicrobial compounds extracted and assessed for activity against *C. albicans* planktonic and biofilm forms, using the validated MPAS design. The antifungal compounds in seven broad spectrum hits were putatively identified by ultraperformance liquid chromatography linked to high resolution tandem mass spectrometry, reported in Chapter 4. Chapters 2-4 in this thesis were written as independent units to facilitate future

publication. This did lead to some repetition, however, it was attempted to this keep this to a minimum to allow readability. In Chapter 5 study conclusions and future studies were discussed.

OUTPUTS OF PHD STUDY

Barnard Jenkins B. (2019) Biomining and identification of novel antimicrobial peptides tailored to inhibit *Candida albicans* biofilm formation, PhD Progress talk, Biochemistry Departmental Forum, University of Stellenbosch

Barnard Jenkins B. (2023) Discovery of novel anti-*Candida albicans* compounds from environmental bacteria PhD oral defence, Biochemistry Departmental Forum, University of Stellenbosch

Barnard Jenkins B., Laubscher W.E., Rautenbach M. (May 2022) Multi-functional activity assay for discovering of antifungal peptides and compounds against planktonic and biofilm forms of Candida species, AC21 Workshop for Antimicrobial Peptides, University of Strasbourg, Strasbourg, France (poster presentation)

Masoudi Y, Van Rensburg W, Barnard-Jenkins, B, Rautenbach M. (2021) The influence of cellulose-type formulants on anti-Candida activity of the tyrocidines, Antibiotics, 10(5), 597, DOI: 10.3390/antibiotics10050597 (published article)

Barnard Jenkins B., Snoep J.L., Botha A., Rautenbach M. (2024) Comparison of planktonic and biofilm associated *Candida albicans* isolates using MPAS, an optimised multiplex assay system, *Biofilm* (manuscript submission from Chapter 2 in progress)

Barnard Jenkins B., Snoep J.L., Rautenbach M. (2024) Rapid assessment of bacterial micro-culture extracts towards different life-stages of *Candida albicans* (draft manuscript from Chapter 3; will be submitted after Chapter 2 article is accepted for publication)

Barnard Jenkins B., Laubscher, W. E, Rautenbach M. (2024) Utilising MPAS for discovering of anti-*Candida* compounds from soil samples (draft manuscript from Chapters 4; will be submitted after Chapters 2 and 3 articles are accepted for publication)

CHAPTER 1

Drug discovery targeting antifungal biofilms

Problem statement

Antimicrobial resistance has been found against every antibiotic on the market[1] and this resistance is potentiated by drug-tolerant microbial biofilms[2]. All facets of daily life are gravely affected by the global upsurge in antibiotic resistance, which is also commercially affecting the industrial, agricultural and medical industries. Particularly in developing countries (such as those in Africa) with high percentages of immunocompromised individuals and limited infra-structure, the increase in multi-resistant pathogens is apparent and deemed increasingly problematic. To further complicate the matter, some microbes have developed the ability to grow in a biofilm-form, ensuring their survival and resulting in enhanced tolerance to antimicrobial agents, which are often the cause of recurrent infections[3]. Currently, biofilms are the cause a high percentage of those infections needing treatment in the developed world, of which vascular-catheter biofilm-related bloodstream infections are the most serious and costly[4]. Therefore, the development of novel antibiofilm antimicrobials from easily accessible sources (such as soil samples), is essential to combat multidrug resistant pathogens.

Microbial biofilms

The existence of microbes (animalcules) was first confirmed by the invention of a rudimentary compound microscope designed by Antonie van Leeuwenhoek in the 17th century. This allowed for the initial visualization, illustration and labelling of bacteria scraped from teeth plaque. Following this discovery, Robert Koch identified the specific causative agents of tuberculosis, cholera and anthrax. Thereafter, his research led to the development of Koch's Postulates, a gold standard in medical microbiology in the 19th century. The microbial aggregates on the teeth of Antonie van Leeuwenhoek was the first mention of bacterial biofilms. He noted "The number of these animalcules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom"[5]. It was not until the 20th century that scientists changed their view, indicating that the planktonic pure culture method is not a true depiction of bacterial life-styles and/or microbial interactions. Thereafter, the word "biofilm" was coined by Bill Costerton in 1978[6]. A microbial

biofilm consists of a community of structured cells embedded in an extracellular polymeric matrix with the ability to attach to living and non-living surfaces [7].

The formation of a biofilm tracks the following steps: 1) the development of a surface conditioning film; 2) reversible and irreversible attachment of cells to a surface; 3) formation of microcolonies; 4) maturation and differentiation of the biofilm with expression of matrix polymers; 5) dispersal of cells from the biofilms (Fig. 1.1). In a bit more detail, the development of a surface conditioning film is a prerequisite for biofilm development as it improves the physical and chemical properties of the substratum. This film consists of various humic compounds, polysaccharides and glycoproteins, providing the necessary nutrients and trace elements for colonization. Interestingly, research has shown that tears, urine, components of blood and intravascular fluid contribute to the preparation of the conditioning film[8].

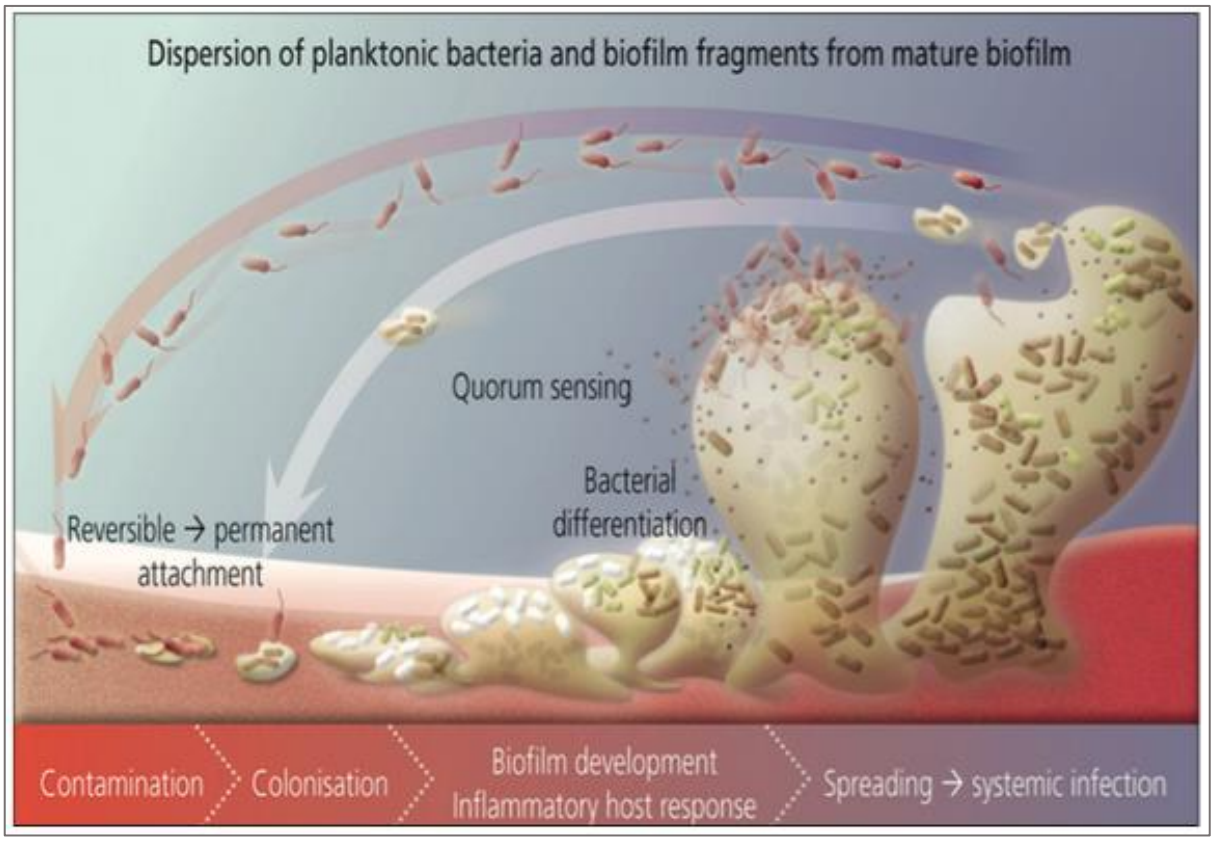

Figure 1.1 A demographic of biofilm formation. Progressive steps are followed, starting with the development of a surface conditioning film with reversible and irreversible attachment of cells to a surface. The micro-colony cells mature and continually express matrix polymers. After maturation, planktonic cells disperse from the biofilm[8] and the process resumes on an unaffected surface. (Image used with permission from author).

Microbial cells bind to the conditioned surface in two ways. Firstly, a weak reversible attachment forms between the microbial cells and a surface, which is followed by an irreversible, stable bond

between the microbes (binding of the long-axis of the cell) and the surface [9]. This attachment is a crucial step in biofilm formation due to the required activation of various extracellular genes resulting in the release of a "glue" to anchor the microbes to the various surfaces [8]. DNA microarray data has indicated that over 70 genes experience alterations in expression during biofilm formation/attachment. This attachment allows the microbial cells to form a biofilm by means of cell division as well as recruiting planktonic microbes using quorum sensing, which results in the formation of microcolonies (coherent cell-to-cell interactions) engulfed in a glycocalyx matrix containing aqueous channels. Quorum sensing molecules facilitate this cell-to-cell signalling, which is required for biofilm formation, microbial recruitment [9] and has also been shown to contribute to biofilm thickness[10]. However, new evidence might suggest that biofilms can still form in the absence of quorum sensing, which is possibly also later suppressed (in mature biofilms) as it might serve as a beacon to the host immune system[11].

Furthermore, the presence of aqueous channels, interstitial pores and conduit channels within biofilms allow for genetic acquisition and horizontal gene transfer as well as nutritional transfer in order for a biofilm to grow. Cells are dependent on nutrient exchange from neighbouring cells as well as substrate flux from the liquid phase [8]. Within a biofilm, the cells gather as micro-colonies and have been visualized and found to range from cylindrical pillars to filaments forming a "mushroom" shape, allowing for maximal nutritional availability (diffusion) and minimal exposure to waste[6]. Various nutrient signals allow for the differentiation and remodelling between a classic, differentiated biofilm structure (high nutrient concentrations) and an undifferentiated biofilm structure (low nutrient concentrations) [9].

The essential and final stage of biofilm development includes cell detachment and planktonic dispersion (erosion or sloughing) leading to 1) disease transmission; 2) biological dispersal (possibly to escape unfavourable living conditions); 3) bacterial survival and 4) genetic diversity. The dispersal process incorporates the detachment of microbes from the biofilm, translocation and attachment to substrate at a new site [8]. Interestingly, detached planktonic cells sometimes retain biofilm characteristics such as antibiotic resistance. Research has shown that during this auto-dispersal stage, there are signalling molecules (similar to quorum sensing), which induces this behaviour when above a threshold concentration[11]. A very interesting and pharmaceutically beneficial fact is that this molecule is universal across bacterial species, unlike the biochemical and biofilm components which differ among species [11].

After biofilm development, biofilms can be classified into five classes. Class I indicates a film constituting a single microbial species whereas Class II contains a few microbial species and is often associated with long-term prosthesis infections. Class III biofilms consist of a small consortium of species (±30 species) and this class is often found in environments where one specific substrate can be found in abundance. Classes IV and V biofilms are heterogeneous, high biodiversity microbial films with the ability to adjust to environmental changes, but they do tend to show signs of resistance to these perturbations. The normal human body microbiota is a good example, such as dental plaque or colon microbiota[12].

Biofilms are dynamic environments transitioning between planktonic and sessile cell modes interchangeably. They can be bacterial, fungal or a combination of both, but most research up to now has been focused on bacterial biofilms formed by species such as *Pseudomonas*[7], *Staphylococcus*[13], *Streptococcus*[14] and *Enterococcus*[15]. Bacterial biofilms adapt a pillar-and-mushroom shaped formation, which projects outwards[3,16]. They harbour microenvironments differing in pH, oxygen and nutrient availability, which results in metabolic heterogeneity with the biofilms. In bacteria, dispersal is frequently a terminal process, marking the end of the biofilm life cycle[3]. Due to the formation of hyphae after fungal cell adhesion, a more uniform and cohesive biofilm is formed and unlike bacterial biofilms, dispersal of cells occurs throughout the fungal biofilm life-cycle. Fungal biofilms have also been shown to undergo a dramatic release of the entire biofilm from a substrate, apart from the constant dispersal of cell. More research still needs to delve deeper into the reason for this as well as the mechanism associated to such a large event[3].

Fungal Biofilms

It has been speculated that there are more than 5 million fungal species on earth, of which 300 cause human diseases. Of these about 300 species, only 20-25 species cause human diseases frequently, and therefore are studied more intensely[17]. Many medically important fungi produce biofilms, including species from various genera such as Candida[18], Aspergillus[19], Cryptococcus[20], Saccharomyces[21] and Fusarium[22]. Furthermore, new fungal genera and species involved in pathogenic biofilms have been reported, as they are becoming more prevalent[23] and their specific biofilm characteristics need to be identified. These include species from genera such as Rhodotorula[24], Trichosporon[25], Coccidioides[26], Pneumocystis[27] and Malassezia[28]. Interestingly, research shows that a biofilm is the most natural and preferred form of fungi[23] and even though fungal biofilm infections in humans are less frequent than bacterial infections, they tend to be more serious and their occurrences are on the rise[29]. As a saprophytic fungus, *Aspergillus fumigatus* is

very widely distributed due to conidia spores and causes a range of between 30-90% mortality rates of systemic diseases in immunocompromised individuals[30]. *Aspergillus fumigatus* biofilms are common in patients with lung abnormalities such as cystic fibrosis patients[31] or chronic obstructive pulmonary disease. Their biofilms can affect various substrates (ranging from breast implants to catheters) and all clinical antifungal drugs are significantly less effective when this organism is present as a biofilm or spherical hyphae form[23]. This species seems to be more resistant towards itraconazole and to some extent to caspofungin[32] and as such is now the second most common fungal infection species in hospitals, after *Candida albicans*[33].

The opportunistic, ubiquitous human fungal pathogen, *C. albicans*, has the ability to colonize various epithelial tissues (i.e., vaginal, intestinal and oral tissues) causing superficial and systemic diseases. It has been observed that many fatal systemic infections, caused by *C. albicans* biofilms, are due to planktonic forms liberated from the resistant biofilm fungi disseminating into the bloodstream. Therefore, *C. albicans,* being the most common pathogenic fungus affecting the human population, is also the fourth most common cause of bloodstream infections in hospitalized patients[34]. These life-threatening biofilms are most often found in biomaterial-related substances like catheters, prosthetics and on implant surfaces, of which *C. albicans* is the most common, guilty fungal species[35], has the second highest colonization to infection rate and the overall highest crude mortality[2]. Therefore, biofilm characterization is key to solving health associated biofilm problems.

Candida albicans biofilms

Microscopically, a heterogeneous mosaic model (used to describe *C. albicans* biofilm formation), details it as stacks of microcolonies held together by extracellular polymeric substances, below which there is a basal layer attached to the substratum [34]. It was observed using light microscopy that we could visualize *C. albicans* as yeast cells, pseudohyphae and hyphae [36], which is known for a pleiomorphic fungus such as *Candida*[37]. This morphological plasticity is an important factor contributing to virulence[38]. Pseudohyphae are referred to as a series of elongated, joined yeast cells that have obvious constrictions at septal sites, where nuclear division (mitosis) occurs at the point of maximum constriction between mother and daughter cell[37]. In contrast, when true hyphal forms undergo mitosis, septin ring formation and septum formation occurs at the point of minimum cell diameter at the neck, some distance within the germ tube[37]. Hawser *et al.*[38] and Baille and Douglas [39] have shown that the hyphae are essential to a mature biofilm's architecture and structural integrity. The initial attachment is mediated by both non-specific factors (cell surface hydrophobicity and electrostatic forces), as well as specific adhesins which recognize ligands in the conditioning

film, such as serum proteins and salivary factors. Biofilms have been found to range from 25-450 μM in thickness [41]. The final thickness of a biofilm is affected by various factors, such as the penetrability of nutrients and gas, the substratum structure affecting biofilm attachment as well as the concentration of secreted enzyme necessary for efficient digestion of nutrients[42].

Once adhered to a surface, *C. albicans* cells are bound to this adhered life stage by coherence and attachment to the substratum. Coherence is facilitated with the extracellular matrix which keeps cells in place or by acting as an anchoring glue[42]. Importantly, biofilm formation correlates with cell surface hydrophobicity[41]. Mature *Candida* biofilms have been shown to have complex spatial heterogeneity, which is thought to facilitate optimal nutrient influx and waste disposal as well as the establishment of micro niches within the biofilm. However, the spatial architecture differs depending on which substrate it is formed, as well as the growth conditions. The cell wall and extra-cellular matrix are responsible for the proper maturation and formation of a candidal biofilm. The cell wall of *C. albicans* is composed of polysaccharides, mannan (±23%), glucans (40-60%) and chitins (0.6-9%) as well as proteins (6-25%) and lipids (1-7%)[43]. Some of these polysaccharides and related components are also found as extra-cellular polysaccharides in the biofilm matrix[43].

Not all *Candida* species and *C. albicans* strains have equal biofilm forming capacities[41]. Transitions between planktonic cells and sessile cell modes occur interchangeably, in response to various environmental cues. These include the lack, depletion or availability of nutrients, or a change in the composition of nutrients. However, the dispersed forms are planktonic counterparts which are mostly unbudded yeast forms showing enhanced pathogenicity[3], enhanced biofilm forming capabilities, enhanced adherence and filamentation but they still have many characteristics in common with their planktonic counterparts[16]. Interestingly, *C. albicans* has the ability to switch reversibly between yeast and filamentous forms (morphogenetic conversion) as well as co-aggregating with bacteria in a biofilm[41]. This complex morphology is difficult to observe, inhibit and assay.

Improved assay development is essential as current assays are time consuming, expensive and allow for only a single microbial life-stage (planktonic or biofilm) to be tested at a time. Two main biofilm studying methodologies are used: 1) cultivation of biofilms directly on objects in Petri-dishes and/or microtiter plates[44–46] and 2) or the cultivation of biofilms on pin-lids[15,47]. Biofilm assays include the Colony Biofilm Assay and Kadouri drip-fed assay, which are explained in more details in Chapter 2. The Colony Biofilm Assay is a static method that grows a biofilm colony on a semipermeable membrane, which can be easily moved to a new agar plate with the desired test conditions. The

stable Kadouri drip-fed assay uses two needles, which are inserted into a Petri-dish lid, adding and removing medium and waste as required. Furthermore, the air-liquid interface assays can be used for biofilm growth on practical components such as glass coverslips, catheters, denture strips[48] and contact lenses that are placed in 12-well plates[49]. Resulting biofilm growth and inhibition is assayed using fluorescent and luminescent dyes amongst other methods. There are also commercial systems, such as the Calgary Biofilm Device designed by Ceri *et al* in 1999[47], using standard 96-well plates with biofilm formation on peg lids. Some scientists have developed high-throughput, 384 pin-lid, luminescent attachment/detachment assays[15]. Alternatively, biofilms are often allowed to form on the bottom of standard 96-well plates[45]. Such microtiter assays are accurate, reproducible and inexpensive and, most importantly, can easily be reproduced in various laboratories with readily obtainable ingredients, such as media and consumables.

The medium used in biofilm experiments also affects the biofilm's cellular morphology. Media such as yeast-peptone-dextrose media (YPD) allowed for biofilms that contain mostly yeast forms (experimental data; Chapter 2) whereas Roswell Park Memorial Institute 1640 media (RPMI1640) supports more hyphal/filamentous formation, even though research shows that biofilm formation is not morphology specific[34]. This medium type (formulated at the Roswell Park Memorial Institute) is a high phosphate containing medium, with other formulants including glucose, a pH indicator (phenol red), salts (sodium chloride and sodium carbonate, potassium chloride, magnesium sulphate and calcium nitrate), amino acids (glutamine, arginine, asparagine, cystine, leucine and isoleucine amongst others) and vitamins (*para*-aminobenzoic acid, folic acid, *i*-inositol and choline chloride amongst others). It is typically formulated at a pH=8 and uses a bicarbonate buffering system, allowing for a stable pH to be maintained and preventing it from becoming acidic, but which would be affected by biofilm waste in the form of lactic acid and ketones. This medium has been shown to be able to support a variety of cells, including anchorage dependent cells, such as biofilm cells[50,51] and furthermore research also suggests that RPMI1640 stimulates hyphal development[34,52]. YPD medium is a complete, nutrient rich medium containing all amino acids required for yeast growth. It contains B-complex vitamins, peptones, dextrose, vitamins and minerals[53]. Research indicates that N-acetylglucosamine and proline, as well as cell numbers below 10^6 cells and a pH around neutrality all favour hyphal development[54]. These factors are more pronounced using RPMI1640 medium, supporting the results reported in this study. Furthermore, the dispersal of cells has been reported to be more prominent in RPMI1640 medium around 5 h, by Uppuluri *et al*.[16] observed that richer growth medium correlated to an increase in the number of

cells dispersed/shed. Although research is just starting to investigate what causes biofilm dispersal, results have shown that carbon sources and pH could be triggers for dispersal in fungal biofilms. An acidic pH has been shown to trigger dispersal whereas an alkaline pH curtails dispersal. Similarly, a richer medium (eg. glucose) has been associated with triggering dispersal whereas alternate sources (eg. galactose) limit detachment[16]. Once detached, these shed cells seem to be more resistant in comparison to their original planktonic counterparts. Furthermore, quorum sensing is also fundamental to microbial biofilm formation and has been indicated to be regulated by a two-component signal transduction protein known as Chk1p. Research shows it assists with preventing overpopulation, controlling nutrient competition, as well as communication required for dissemination and for the establishment of distal infection sites[41,55]. However, the resistance stage of the microbes within these biofilms as well as of the dispersed cells remains a worrisome topic, as treatment with antifungals is still crucial to overcome biofilm associated disease.

Antifungal and antibiofilm drugs

There are four major antifungal drug targets in *C. albicans,* as summarized in Table 1.1, namely: 1) 5-fluoro-cytocine which causes aberrant RNA synthesis and interferes with DNA replication; 2) the efflux of cations such as K^+, Na^+, Zn^{2+}, Ca^{2+} ions, which results when membrane pores are formed by the polyene drug class (examples include amphotericin B and nystatin); 3) the inhibition of the EGR11p gene by the azole antifungal class (examples include fluconazole and triazole) resulting in the accumulation of toxic sterol pathway intermediates, which inhibits growth, when ergosterol content is depleted in the membrane; 4) the inhibition of (1-3) β-D-glucan synthases by the echinocandin class of antifungal originally derived from soil samples (examples include caspofungin and micafungin). Therefore, current antifungals (Fig. 1.4A) affect *Candida*'s cell wall and cell membrane composition and integrity resulting in a stress-response[56]. Worryingly, the results from a combination of studies have shown that *Candida* biofilms are becoming more resistant to clinically important antifungals which are currently available and licenced for use; these include amphotericin B (AmB), fluconazole, flucytosine, itraconazole, caspofungin (CAS) and ketoconazole[57].

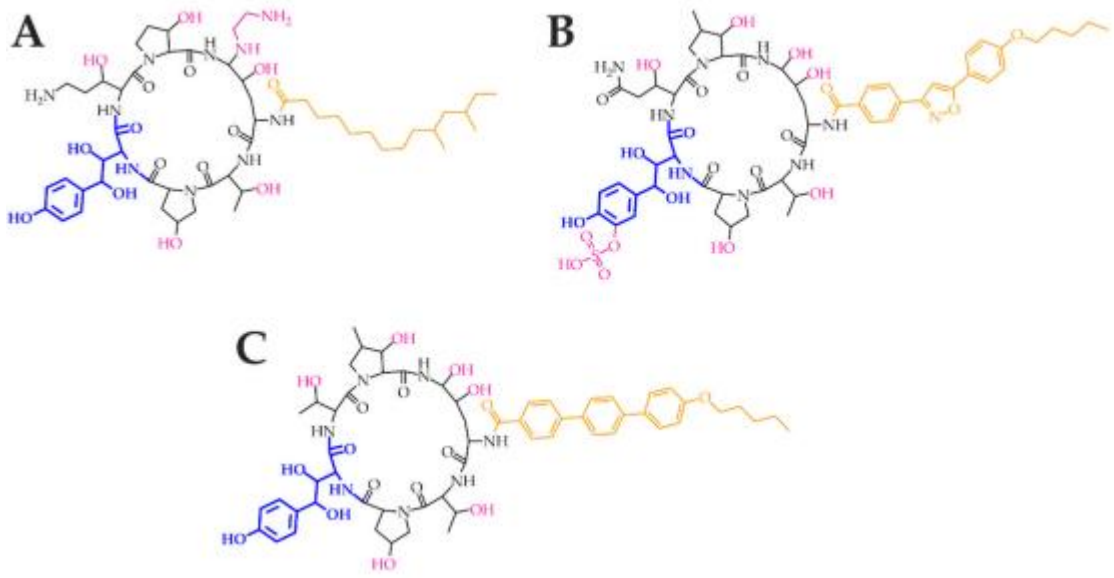

Figure 1.2 The primary structures of the echinocandins known as caspofungin (A), micafungin (B), and anidulafungin (C). Ethylenediamine, sulphate and hydroxyl groups contribute to water solubility (pink) whereas specific lipophilic side chains (orange) decrease the haemolytic activity. The inhibition of 1,3-β-D-glucan synthase is due to the homotyrosine amino acid shown in blue, whereas antifungal potency is linked to the amino acid core shown in black. Figure reproduced with permission from MDPI and Houšt' *et al*[58].

CAS (Fig. 1.2A), micafungin (Fig. 1.2B), anidulafugin (Fig. 1.2C) and enfumafungin are semi-synthetic, water-soluble lipopeptides which form part of the echinocandin class of antifungals, of which CAS as the first of this class was approved by the FDA in 2001[59]. They are structurally amphiphilic cyclic hexapeptides with an N-linked acyl lipid side-chain[60] and have been shown to inhibit (1-3) β-D-glucan synthase, which results in the disruption of the fungal cell wall as (1-3) β-D-glucan is a main structural component [34]. They are fast-acting but have a narrow spectrum of activity as they are only effective against *Candida spp.* (fungicidal) and *Aspergillus spp.* (fungistatic)[61]. Except for the oral administration of enfumafungin which is currently being developed[62], these compounds need to be administered intravenously. Resistance is due to point mutations in the β-glucan synthase subunit Gsc1p[56].

The polyene, nystatin, was the first antifungal drug discovered in 1950 but remains a topical agent due to its toxicity. However, the "gold-standard" AmB was originally made from *Streptomyces nodosus* in 1953[59,63]. It is fast acting and has a broad spectrum of activity[59,64]. Its mode of action (MOA) is the binding of its hydrophobic moiety to ergosterol (the fungal sterol), for which it has a slightly higher affinity compared to that of cholesterol (mammalian sterol). This bonding to sterols results in membrane pore formation, the leakage of monovalent ions, such as potassium and magnesium resulting in fungal cell death. It is due to the binding of AmB to mammalian and fungal sterols (both cholesterol and ergosterol) that toxicity is a problem associated with its use in the

pharmaceutical industry. However, it is one the World Health Organization's "List of Essential Medicines" and is mostly reserved for treatment of severe infections[65]. Unfortunately, certain *Candida* biofilms have been shown to be eight-fold more resistant to AmB than their planktonic counterparts [66]. Resistance to AmB is acquired through mutations in ERG3, decreasing the concentration of ergosterol in the cell membranes[56].

The pyrimidine flucytosine (Fig. 1.3B) was synthesized in 1957[64] as a potent antitumor drug and analogue of cytocine[58], after which it was discovered to have antifungal activity. Flucytosine is converted to 5-fluorouracil (Fig. 1.3C), which then inhibits DNA and RNA synthesis. There are many cases of intrinsic resistance against this compound in *Candida spp*., therefore it is often used in combination with azoles or polyenes[67]. Resistance is caused by the decrease in uptake or loss of enzymatic activity associated with the conversion of 5-fluorouracil to 5-fluorouridylic acid[64]. It is described as fungal specific because its target is absent in mammalian cells, but this results in a narrow spectrum of activity.

Fluconazole (Fig. 1.3D), a compound from the azole class of antifungals approved by the FDA in 1990, can be used to treat Candida infections with relatively low toxicity and high bioavailability. Azoles are fungistatic agents but have a slower onset of action than AmB and unfortunately, fluconazole has been found to be ineffective against biofilms[68]. Its MOA is the inhibition of the fungal Cytochrome P450 enzyme, 1,4α-demethylase. This prevents the conversion of lanosterol to ergosterol, which is essential for fungal cell membrane structure. Resistance to this compound is due to mutations in the ERG11 gene, which codes for 1,4α-demethylase, preventing the azole drug from binding the enzyme [69]. The resistance to the azole group can be caused by gene rearrangements or aneuploidy which affects the expression of transcriptions factors, drug targets and pumps [56]. Some studies have shown that the presence of β-glucans in the polymeric matrix play a role in antifungal resistance, possibly by hindering acid diffusion of the antimicrobial compounds[70].

Voriconazole (Fig. 1.3F) is a second-generation azole, the first of its kind to receive FDA approval. It displays broadened antifungal activity due to one of the triazole rings being replaced with a fluorinated pyrimidine and the addition of an α-methyl group. It is fungistatic towards yeasts and appears to be fungicidal towards filamentous fungi, showing inhibitory activity against all *Candida spp*. with MIC values 1-2 log lower than fluconazole MIC values[71].

Table 1.1 A summary the current antifungal compounds in the clinical use. For each compound, their antifungal class is specified, along with the year in which they were approved by the FDA and their associated advantages and disadvantages as antifungal compounds.

Antifungal	Antifungal Class	Year of FDA Approval	Fungicidal and/or Fungistatic	Advantage	Disadvantage
Nystatin	Polyene	1954	Both	Broad activity spectrum	Not available intravenously
Amphotericin B	Polyene	1958	Fungicidal	Broad activity spectrum and fast acting, low resistance	Nephrotoxic and expensive
Amphotericin B lipid complex	Polyene	1995			
Amphotericin B cholesteryl sulphate	Polyene	1996			
Liposomal amphotericin B	Polyene	1997			
Flucytosine	Pyrimidine	1972	Fungistatic	Good tissue distribution	Narrow activity spectrum
Ketoconazole	Imidazole	1981	Fungistatic	Broad activity spectrum	Toxicity
Fluconazole	Triazole	1990	Fungistatic	Low toxicity	Narrow activity spectrum with high rates of resistance
Itraconazole	Triazole	1992			Low bioavailability
Voricinazole	Triazole	2002			Narrow activity spectrum with high rates of resistance
Posaconazole	Triazole	2006			Expensive
Isavuconazole	Triazole	2015		Bioavailable	Cost-effective
Caspofungin	Echinocandin	2001	Fungicidal	Low toxicity	Narrow activity spectrum
Micafungin	Echinocandin	2005			
Anidulafungin	Echinocandin	2006			
Enfumafungin	Echinocandin	In Clinical Trials			

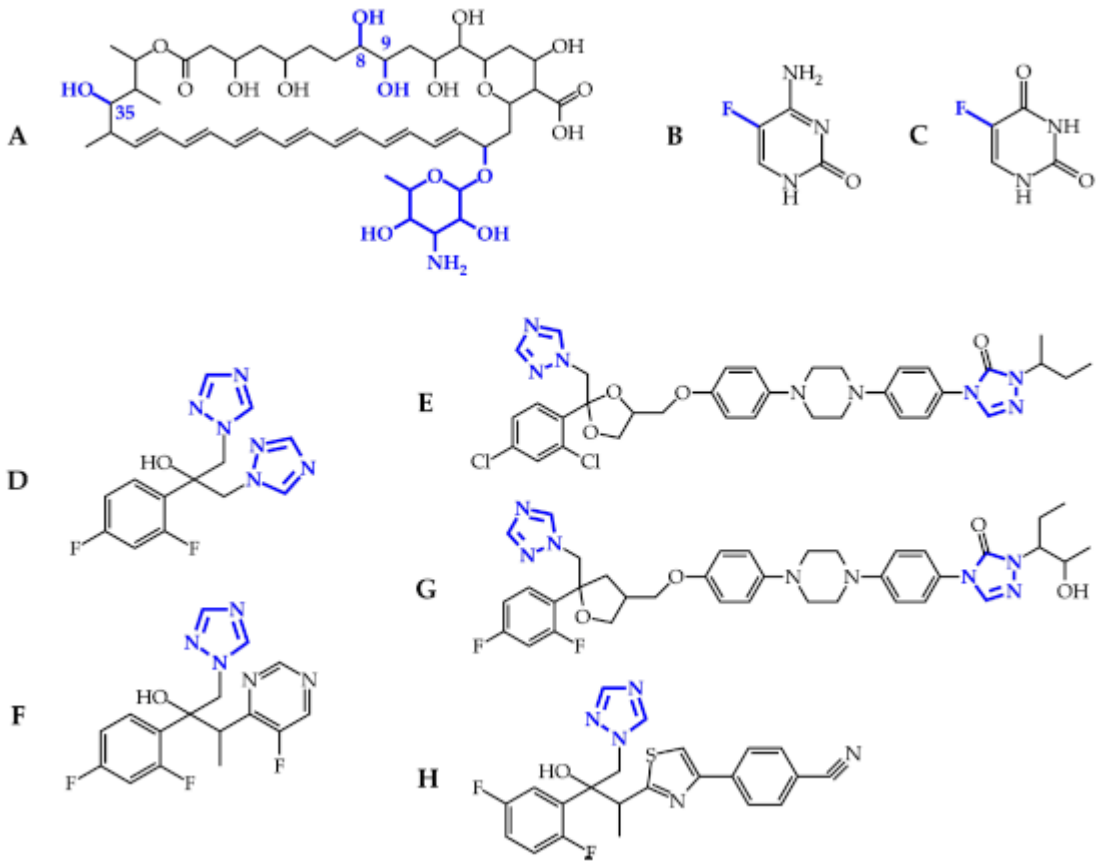

Figure 1.3 Chemical structures of selected antifungal drugs currently licenced, with each compound's active pharmacophore indicated in blue. Amphotericin B (A); flucytosine (B), and its deaminated product 5-fluorouracil (C); fluconazole (D); itraconazole (E); voriconazole (F); posaconazole (G); and isavuconazole (H). Reproduced with permission from MDPI and Houšt' *et al*[58].

The itraconazole (Fig. 1.3E) derivative, Posaconazole (Fig. 1.3H) is licenced as a prophylactic against invasive fungal infections showing broad activity against the majority of opportunistic yeasts and moulds. It displays MIC concentrations against *Aspergillus fumigatus* and *Candida albicans* at 0.12 mg/L and 0.06 mg/L[72] but showed erratic absorption profiles, especially with oral suspensions. Isavuconazole (Fig. 1.3G) on the other hand has excellent bioavailability for oral formulations with predictable pharmacokinetics and is available as a water-soluble intravenous formulation. It is registered for the use of invasive aspergillosis and mucormycosis. It is effective against nine strains of *Candida spp*. with MIC values ranging from 0.015-0.5 µg/mL[73].

The rise in resistance of various pathogens to currently available antibiotics and antifungals has resulted in an ongoing search for novel, non-toxic treatments, which are specific and have a low probability of inducing resistant microbes – such as AMPs discussed below. There is an ever-growing need for novel fungicides which will not only reduce the number of medical and veterinary fungal infections and protect agricultural produce, but which will not elicit harmful effects on the environment and consumers and have lowered risks of inducing resistance.

Biofilm Resistance Mechanisms

The mechanisms that protect biofilm organisms from antibiotics and biocides are still being elucidated. There are four suggested mechanisms under study: 1) slow penetration of the antimicrobial agent into the biofilm, 2) altered chemical micro-environments within the biofilms[35], which lead to zones of slow or no growth, 3) adaptive responses to environmental stress and 4) the existence of persister cells that are protected from all types of antimicrobial attacks[57]. It is likely that two or more mechanisms act together, resulting in biofilm resistance to antimicrobial and broad-spectrum biocidal compounds. These so-called persister cells are classified as a subset of cells that lie deep within the biofilm and these cells exhibit tolerance to multiple drug classes[66]. As mentioned earlier, biofilms can consist of various Gram-positive and Gram-negative bacteria, as well as fungi. Although bacteria in general are contributing to the increase in resistance, an alarming trend of fungal pathogen resistance against the established antifungals has been observed.

Unlike resistance in bacteria, there are no known transposon- and plasmid-like resistance mechanisms for antifungal drug resistance[74]. From research available, it can be deduced that antifungal resistance mechanisms are mostly due to genetic mutations of the genes encoding specific drug targets, transcription factors or enzymes. Such point mutations are seen as long-term stress responses because they take time to be acquired and are stable. However, some antifungals have been shown to stimulate the classic immediate stress responses. Typically, these are reversible phenotypic responses, which do not involve mutations or chromosomal rearrangement but that leads to drug tolerance.

Various resistance mechanisms that affect *Candida* biofilms specifically have been elucidated. Resistance is influenced by the roles of efflux pumps, persister cells, sterol synthesis, cell growth rate, decreased binding affinities to drug targets, increased target expression, lowered target contents as well as the contribution made by the extracellular matrix and the impact of cell density and quorum sensing[66] (Fig. 1.4B). The extracellular matrix is a polymeric matrix, which is essential for cell adherence, and it protects the biofilm cells from environmental insults. The matrix has been shown to impair drug delivery via steric hindrance or by actively binding/sequestering antifungals, such as AmB, some echinocandins and fluconazole among others. Research has linked *C. albicans* glucan synthase FKS1 to the biofilm-resistant phenotype as it affects β-1,3-glucan matrix production[66]. A stress response pathway affecting *Candida* biofilm resistance is the disruption of MKC1, which negatively affects hyphal formation as well as biofilm formation of *C. albicans*. Another

stress response pathway contributing to biofilm resistance is heat shock protein HSP90, which is involved with the destabilization of host proteins[66].

The over-expression of calcineurin is a possible antifungal drug target because calcineurin is central to many pathogenic fungal host stress responses[66]. It has also been shown to be central to virulence factors of pathogenic fungi such as *C. albicans* and *A. fumigatus*, and therefore is also essential for fungal survival and fitness. Interestingly, the heat shock protein HSP90 has been found to associate with calcineurin during planktonic conditions[66]. Currently, novel antifungals targeting calcineurin are being tested in animal models, but they have to show reduced immune suppressive activity as well as potent antifungal activity in order for calcineurin to be a viable target[74]. Another target for antifungals in *C. albicans* specifically, is the metabolic glyoxylate cycle, and specifically the enzyme isocitrate lyase. Inhibitors such as mohangamide A and B target this enzyme[75]. Alternate drug targets are the mitogen-activated protein kinase and the high-osmolarity glycerol pathways, which are essential for environmental queues. Three novel antifungal classes are currently being researched but do still require much work. These classes are first, the aminocandins that inhibit 1,3-β-glucan synthase[76], second, the sordarins that stabilise the ribosome elongation factor-2 inhibiting protein synthesis and third, the icofungipen group which is an isoleucyl-tRNA synthesis inhibitor[74].

Within biofilms, the cells which are deeply rooted tend to grow more slowly due to the lower concentrations of nutrients available to them. Subsequently, these cells have been found to be more resistant to antifungal drugs that rely on cell growth for their antimicrobial activity[15]. Furthermore, the transcription of efflux pumps MDR1 and CDR1 is increased in a young biofilm affecting cellular drug sensitivity but not in mature biofilms. The increased expression of efflux pumps during young biofilm formation has been shown to contribute to drug resistance. In terms of sterol synthesis, ERG25 (plays a role in C4-demethylation of ergosterol intermediates) and ERG11 (encodes azole drug target, 1,4α-demethylase) have been found to show altered transcription profiles during biofilm formation and young biofilms. However, mature biofilms have been shown to consist of less ergosterol and also require less ergosterol for fluidity, therefore being more resistant to ergosterol-targeting drugs than young biofilms[66]. Importantly, it must be noted that sometimes *Candida* biofilms can be many times more resistant than their planktonic counterparts[16,77] and as such they require drugs that are active at low concentrations and yet resistance must not be easily developed against these compounds.

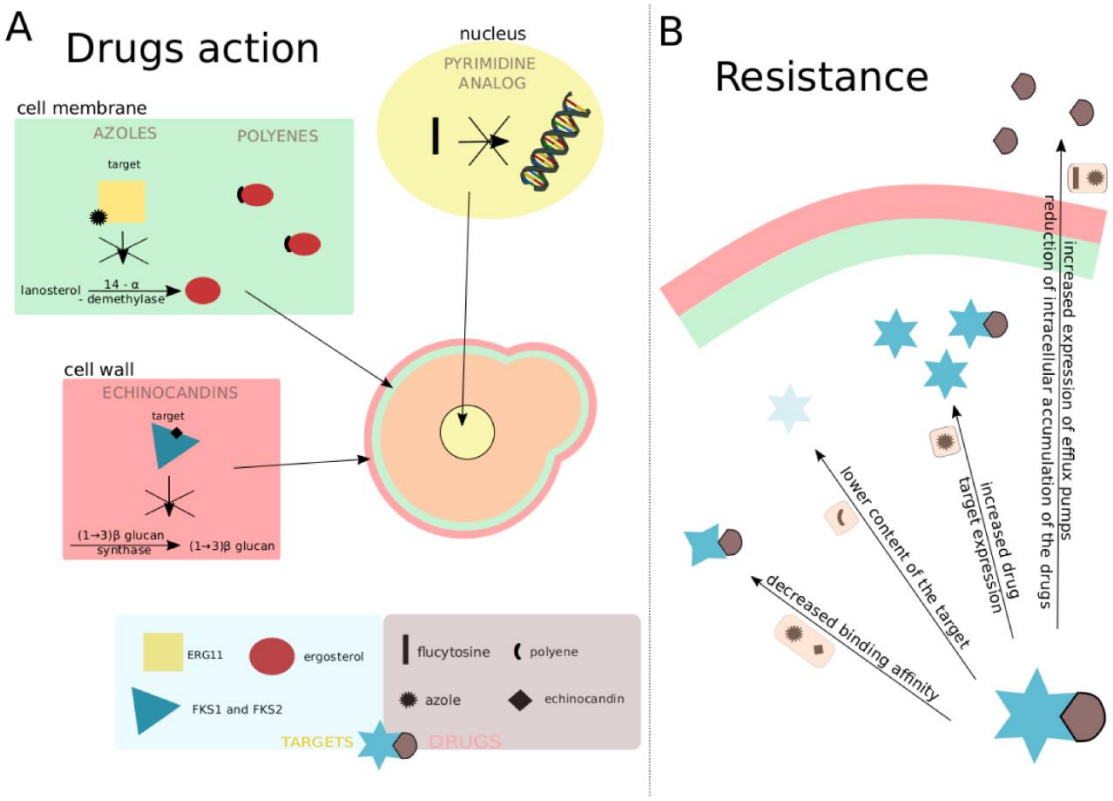

Figure 1.4 A graphical abstract of (A) the antifungal drug actions of azoles, polyenes, echinocandins, and the pyrimidine analogue. This image (B) also shows the resistance mechanisms in *Candida* species. (**A**) Target enzymes and/or molecules are shown using coloured shapes, with the blue box indicating the coding genes. Black shapes are used to show the various drug classes as well as flucytosine. Colours and arrows group the mechanisms of action, also indicating the main cellular location of the effect of the drugs. **B.** The image depicts the most common resistance mechanisms caused by mutations. Targets are characterized as blue stars and drugs as brown shapes. Light orange boxes indicate the type of drug and the arrows focus attention to the different mechanisms causing resistance. Drug shapes are as depicted in A. Image reproduced with permission from MDPI and Ksiezopolska *et al.*[78]

Antifungal drug discovery

Drug discovery is a lengthy process with the timeline from initial compound discovery via high-throughput screening to the launch of a new systemic antibiotic onto the market taking about 14 years[79]. Furthermore, it is estimated that of all compounds identified during drug discovery programs only 20% are sent for clinical trials and of these, only 10% are successful[80]. Still, the sparsity of the antibiotic pipeline lies at the feet of limited investment in novel antimicrobials as well as difficulty in finding new broad-spectrum compounds. Failures due to insufficient activity in patients, such as was the cause for the antibody against heat shock protein 90, Mycograb and the histone deacetylase inhibitor MGCD290[81], focuses attention on the fact that the discovery of new antimicrobial products is both time consuming and expensive. Synthetic and combinatorial

chemistry has provided rich sources of chemically diverse small molecules, which inherently increases the hit rate for discovering novel antimicrobials.

Virtual discovery pipelines as antifungal compound source

Ligand-based virtual screening uses known ligands as a template to search for novel, active ligands. This is based on the idea that chemically similar compounds would have similar biological activities, which is mostly true[82]. An example of what is available in the virtual discovery pipeline includes three coumarin derivatives, selected by Gidaro *et al*[83]. These compounds displayed anti-*Candida* activity similar to fluconazole with a MIC concentration ranging from 2-8 µg/mL. Their team used computational screening with reduced cost and time to identify *in silico* these promising hits from a library of 109 structurally similar to coumarin. Two new non-azole CYP51 inhibitors (CYP51, being an alias for ERG11) have also been discovered with activity against *Candida spp*. and *Aspergillus niger*[84], using a predictive pharmacophore model. NSC1215 and NSC1520 have been shown to have antimicrobial activity ranging from 2.57-15.36 µg/mL while results also confirmed that NSC1520 has high permeation across the intestinal wall, which is essential for the development phase of novel drugs[84]. Another source of promising novel antimicrobials are chemical libraries. Currently, there are two exciting compounds with novel mechanisms of action, namely nikkomycin Z[85], which targets chitin synthesis, and F901318, which has been shown to inhibit dihydroorotate dehydrogenase, an enzyme of *de novo* pyrimidine biosynthesis[81,86]. Nikkomycin Z has been shown to have protective activity against *C. albicans* with an MIC of 4 µg/mL as well as inhibitory activity against *Aspergillus flavus* with high MIC values and further activity against *Coccidioides*, *Histoplasma*, and *Blastomyces spp*. Although it was originally discovered in the 1970's, it started getting more attention when the MOA was discovered as chitin synthase inhibition[85]. Alternatively, F901318 is currently in clinical development for the treatment of invasive aspergillosis as it is the leading representative of a novel class of drug, the oromides[86]. Apart from virtual libraries, chemical libraries are also available consisting of numerous promising antimicrobial agents as will be elaborated on in the next section.

Chemical libraries as antifungal compound source

A library of chemical derivatives can be evaluated for antifungal activity and then molecular docking can be used to show binding affinity to a certain target. Five compounds from a series of 3-benzoyl imidazo[1,2-a] pyrimidines showed promising antifungal activity against seven *Candida spp*. With MIC values ranging between 0.0312-0.5 µg/mL, lower than that of fluconazole[82,87]. These compounds were docked in the active site of the lanosterol 14α-demethylase enzyme (CYP51),

which is essential for fungal growth, and the compounds also showed better binding energy than their reference counterparts[87]. The structures of these compounds alluded to the important role of the benzene ring with electron-withdrawing substituents to its association for biological activity. To further show the effectiveness of *in silico* approaches to antifungal drug discovery, two thiazolino-4-one derivatives showed activity at MIC values lower than those of fluconazole with two being 500-fold more active than fluconazole, tested against *C. albicans*, *C. parapsilposis* and *C. krusei*, from an initial library of 23 compounds[88,89]. They also target 14α-demethylase (or CYP51A1), which is an important enzyme for the synthesis of ergosterol, a key element of the cell membrane targeted by the azole antifungal class of drugs. Furthermore, compounds that show promise in the antifungal pipeline are fosmanogepix, ibrexafungerp, olorofim, opelconazole and rezafungin. Fosmanogepix falls in the N-phosphono-oxymethyl (pro)drug class with a mechanism of action associated with inhibition of Gwt1, which targets GPI-anchored protein maturation. It has activity against fungi such as *Candida spp.*, *Aspergillus spp.*, and *Fusarium spp.*, but no activity against *C. krusei* and variable activity against Mucorales. Ibrexafungerp is a glucan synthase inhibitor with an alternative binding site, showing activity against invasive candidiasis and aspergillosis but also no activity against Mucorales. Olorofim targets pyrimidine synthesis via the inhibition of dihydroorotate dehydrogenase. It is used against fungi with limited treatment options and has been shown to have antibiofilm activity. Opelconazole is a triazole used in the treatment of aspergillosis showing limited drug-drug interactions and high local concentration, beneficial for drug usage. Rezafungin is part of the echinocandin class of antifungals it has a prolonged half-life with limited drug-drug interactions and a favourable side effect profile[90].

Soil microflora as antifungal compound source – Successful methods and assays

An important natural source of promising, novel antifungal compounds is soil microbes and their metabolic products. Soil carries major populations of microbes of any habitat[91] and are still largely unidentified/unexplored. There are millions of strains of soil microbes and researchers in the field estimate that only about 1% of these strains have been identified[92–94], making soil microbes a very rich and promising platform for drug discovery. Furthermore, there are believed to be 61 bacteria phyla, of which 31 phyla have no cultivable representatives[92,95]. This lack of identification of soil microbes is due to difficulty in duplicating natural soil environments and stable growth conditions, required by these microbes in the laboratory[94,96,97]. Modified media and culturing conditions are often incapable of mimicking the endogenous abiotic and biotic conditions favouring bacterial growth. Therefore, we need to develop methods of cultivating, which allows for systems to mimic

natural growth conditions. These conditions include 1) the chemistry of the microbe's natural environment; 2) interactions of the biotic and abiotic factors; 3) the microbial diversity and inter-microbial interactions; 4) the effects of climate change and environmental changes at microbial level[92]. Various factors that need to be taken into account include temperature, growth medium, incubation time, pH, inoculation size, colony density and air conditions[98]. It is also important to note that microbes establish a relationship with other microbes in a community. These interactions can either result in competition for limited resources or co-operation through exchanges of metabolites and signalling molecules[92].

The "Great plate count anomaly" states that less than 1% of the soil microbiome can be cultured using traditional *in vivo* techniques. This includes the most common methods of working with soil microbes, which is the plate technique and direct counting techniques. These entail plating soil microbes on various agar media and selecting for culturable microbes and identifying them by means of various typing methods[99,100]. These are very selective types of methods, and it is suggested that only 1-10% of the microbial populations are estimated using these laborious techniques. As such are these methods are not very popular and encompassing[101].

Zengler *et al*.[102] devised a method of encapsulating 10^7 soil microbes into gel microdroplets by means of an agarose mixture and a CellMix emulsion mixture. The cell mixture is dispensed into a GK-16 chromatography column with filters on the outlet and inlet, to prevent microbial contamination and ensure gel microdroplet retention. Medium is continually pumped through the system and gel microcolonies allowed to incubate and grow for 5 weeks. Thereafter, colonies are sorted into 96-well plates by means of flow cytometry [102]. This method separates microbes from each other whilst simultaneously still allowing interaction/communication between the mircocolonies[102], it also boasts very sensitive growth detection and is extremely adaptable to various downstream requirements/characterization needs due in part to its ease of use and high-throughput design[102]. Applying this method to a sea water sample, subclasses of *Proteobacteria*, *Cytophaga–Flavobacterium–Bacteroides* and relatives *Planctomycetes* were identified using 16S rRNA sequencing[102].

Another novel type of technology, named iChip, led to the discovery of the new antibiotic known as teixobactin[103,104]. iChip is an assembly of plastic plates, which contains small holes forming diffusion chambers and allowing for the growth of only one microbe per chamber. The primary plastic plate was dipped into a diluted soil sample mixed with agar, attached to membranes as well as a top and bottom plate. The iChip was then placed in a larger sample of the same natural extract used

originally, supplying natural growth factors and nutrients. After incubation, the plate set-up is washed, and the primary plate inspected by means of microscopy for colonies. Thereafter, the agar plugs (from each chamber) are removed using sterile gauge paperclips and used for further studies[104]. The iChip technology allowed for a more diverse phylogenetic range of microbes to be isolated as well as showing that microbial recovery is more substantial than using traditional culturing techniques[104]. Teixobactin is a cyclic depsipeptide with activity against Gram-positive bacteria, such as methicillin resistant *Staphylococcus* and vancomycin resistant *Enterococcus*. Importantly, no resistance towards teixobactin could be generated in a laboratory setting, which bodes well for future use in the field[105].

A technique known as a soil substrate membrane system (SSMS) was shown to be more selective against oligotrophic bacteria. The samples undergo soil wetting, which allows the microbes to use the soil nutrients as the sole nutrient growth components, which is more efficient than alternate culture media[93]. A polycarbonate membrane, which is in direct contact with the wet soil, allows for microcolony formation on this membrane. As a membrane cultivation approach, it has the advantage of easy quantification of growing and nongrowing bacteria, it can be used for various viability assays and the bacterial integrity and morphology can be studied using data image analysis. However, a fluorescent microscopy approach can be followed to identify the microcolony cells using this technique. This specific study[94] resulted in the growth of uncharacterized bacteria as well as the discovery of candidate TM7, which was previously thought to be non-cultivatable. Furthermore, Ferrari *et al*., confirmed in this study that the unculturable constituents of the soil microbe community may have growth strategies that are not traditionally observed using known culturing conditions[94].

One idea to keep in mind being that most culture media are nutrient rich, which favours the growth of faster-growing bacteria, possibly at the expense of slower-growing bacteria. Therefore, an improved culturing method could include the use of nutrient-poor or half-strength medium to cultivate and incorporate slow-growing bacteria. These slow-growing bacteria would also require longer incubation times, such as 12-24 weeks of undisturbed growth. It is likely that during these longer incubation times, fast-growing bacteria would die-off, decreasing bacterial competition within the sample[95]. Another important growth factor is the incubation temperature, for which results have shown that soil bacteria grow better at 20-25°C than at higher temperatures[92,106].

Many of the oil derived compounds that were successfully identified in the last two decades using a variety of methods[105], are ribosomally and non-ribosomally synthesised antimicrobial peptides

(AMPs). An example of such a small AMP found in soil samples is bogorol A, which is produced by *Bacillus spp*[107]. It was originally isolated from a bacterium known as *Brevibacillus laterosporus*, isolated from the tissues of an unidentified tube worm. Bogorol A has been found to have antitumor activity and be active against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcal strains but showed no promising activity against drug resistant *Pseudomonas aeruginosa* or *C. albicans* (>200 µg/mL)[107,108]. AMPs are discussed in more details later in this chapter.

Most antibiotics and many of the antifungal drugs still in clinical use originated from soil organisms. Teixobactin and bogorol A were both isolated from soil microbes and illustrated the untapped potential of the soil expanse as a source of novel, small molecules that are yet still to be discovered! High-throughput screening of small antifungal compounds could identify hits that target multiple proteins, and these types of studies could bridge the gap between drug discovery and chemical biology. Globally speaking, the large number of small molecules identified with modern technology should enable for more hits to be identified and characterised more quickly, which should allow for improved lead optimization rates[59]. These small molecules are defined as synthetic or found in nature (such as those isolated from soil microbes) and are non-peptide organic compounds. Generally they have low molecular weights ranging from 200-500 Da, which allows them to cross the cell wall and membrane and bind to proteins and nucleic acids, then altering their normal functioning[59,109,110].

Potential novel antifungal compounds

Small antifungal compounds

Small molecules of interest to the antifungal pipeline are considered as diversity-orientated or target-orientated[59]. These small molecules are either diverse in structure, which should increase the probability of a lead compound being discovered, or are analogues of a specific structure, which should optimize the binding to the target site[111]. A literature study pointed to a few small antifungal molecules with promising *in vitro* results but *in vivo* data is either still being collected or not sufficient for lead development as of yet[59]. Three small molecules, namely ETYA, CGP-37157 and buhytrin A, have shown to be promising biofilm inhibitors[112]. All three were found to be yeast-to-hyphae inhibiting small molecules and were a combined collection from Toenjes *et al.* [113], one using ChemBridgeTM small molecule library and the other using BIOMOL Institute of Chemistry and Cell Biology Known bio-actives collection[114]. The most effective, ETYA, is an inhibitor of eicosanoid

synthesis whereas CGP-37157 affects calcium homeostasis and buhytrin A still has an unknown target[112]. Shearinines D and E suppress *C. albicans* biofilm formation by blocking yeast-to-hyphae transitioning. They were isolated from *Penicillium spp.* and have also been shown to work synergistically with AmB[115]. A small molecule, E1210[116] has been found by means of high-throughput screening of a chemical library from cell wall glycosylphosphatidylinositol biosynthesis inhibitors[117]. After some pharmacological optimizations, it has been described as a fungistatic small molecule, with low cytotoxicity, which targets the cell wall/membrane displaying potent activity against *Candida* spp. except *C. krusei*[59]. Two synthetic small molecules, UK-118005 and ML-60218 displayed broad spectrum antifungal activity by inhibition of RNA polymerase III[118], grouping them into small molecules with antifungal activity but novel mechanisms of action[59]. Another in the grouping is MGCD290, which inhibits Hos2 fungal histone deacetylase. It acts synergistically with azoles and this small molecule has been undergoing testing in Phase II clinical trials[119]. Another novel antifungal small compound known as SM21 has been shown to treat systemic and oral candidiasis in murine models, and is effective against a variety *Candida spp.* Its mechanism of action is still unknown[59,120,121]. Therefore, it is safe to say that the small molecule pipeline is progressing, albeit more slowly than would be ideal. However, the encompassing group known as antimicrobial peptides are a source of promising molecules to study further.

Antimicrobial Peptides

Known as “nature’s antibiotics”, the group of antimicrobial peptides (AMPs) consists of omnipresent, bio-degradable defence molecules. Antimicrobial peptides are produced by virtually all organisms on earth, from plants and insects to bacteria, fungi, fish, humans and animals. They have been shown to not only kill microbes directly but they play an important role in promoting elements of the innate immune system by acting as effector molecules[122]. Antimicrobial peptides have the startling ability to target a variety of microbes, including Gram-positive bacteria, Gram-negative bacteria, fungi, enveloped viruses as well as eukaryotic parasites and even some cancerous cells[122]. They are further beneficial, as they can be used in conjunction with antifungals, antibiotics and/or other antimicrobial compounds [123] and virtually no resistance has been developed against this class of compounds[124].

Unlike antibiotics with specific protein targets, AMPs have generalised targets which decreases the probability of microbial resistance by gene mutation[125]. Antimicrobial peptides have the ability to rapidly kill target cells[126], they exhibit a broad activity spectrum and have been shown to have activity against some of the more serious antibacterial-resistant pathogens[127]. Therefore, they are

a promising class to consider when looking for antifungal compounds to treat *C. albicans* biofilms. Although this group consists of a variety of structures and sequences, there are basic attributes that are common for all. Firstly, they are typically cationic as they consist of basic amino acids, such as arginine and lysine residues as compared to acidic residues. Secondly, they are made of 12-50 amino acids and contain ≥50% hydrophobic amino acids[122,127]. Owing to the presence of disulfide bridges or contact with cell membranes, they fold into 3-dimensional, amphiphilic structures that can be classified into four major structural classes[122,127]. These classes are β-sheets stabilized with 2/3 disulfide bonds, amphipathic α-helices, extended molecules and loops due to a single disulfide bond. It has been determined that a single host can contain up to 35 different AMPs from all structural classes. These structural classes have an effect on their various MOAs, of which three main types have been elucidated, namely, 1) targeting cell wall components/structures, 2) membrane disruption and 3) targeting of secondary, intra-cellular targets. Simultaneously, all AMPs can also be classified into two mechanistic classes: membrane disruptive and non-membrane disruptive.

Antimicrobial peptide targets and mode of action

Antimicrobial peptides (AMPs) have been shown to target the external cell wall components/structures, such as lipopolysaccharides. As per example, Gram-negative bacteria have the additional outer membrane barrier to cross when compared to Gram-positive bacteria, which led to th "self-promoted" uptake hypothesis. This hypothesis states that AMPs interact with the highly negatively charged, anionic glycolipid LPS of the outer membrane to neutralize charge over a patch of the outer membrane [122]. Research suggests that AMPs might have a higher affinity for the LPS than divalent cations, such as Mg^{2+} and Ca^{2+}. Therefore, these divalent cations are displaced from the negatively charged LPS leading to the local disturbance of the outer membrane [122]. Thereafter, the AMPs are inserted into and translocated across the bilayer.

Having crossed the outer membrane (Gram-negative bacteria) or the thick cell wall (Gram-positive bacteria), most AMPs interact electrostatically with the anionic surface of the bacterial cytoplasmic membrane (Fig. 1.5). This interaction results in the deposit of these AMPs parallel to the membrane, resulting in many different types of membrane disruptive/cellular death mechanisms (Fig. 1.5). The first membrane interaction model suggests that membrane permeability is compromised by detergent-like activity, and cell lysis is the cause of cell death. The second and third models suggest that when the peptides reach a high concentration at the outer surface of the cytoplasmic membrane, they are either re-orientated to sit perpendicularly to the cytoplasmic membrane,

forming channels and disrupting membrane integrity (barrel-stave model) or they cause breakdown of the cytoplasmic membrane integrity (the carpet model). This carpet model is applicable at high concentrations *in vitro* and is essentially an extreme extension of the toroidal pore model[125]. Last, the fourth popular model suggests a micellar aggregation of the AMPs creating informal micellar channels which allow for the movement of ions across the membrane. These micellar channels have been thought to vary in size and duration but can last as little as microseconds. When collapsing, theses micellar channels allow the AMPs to translocate to the inner or outer monolayer in a parallel pattern [122,128].

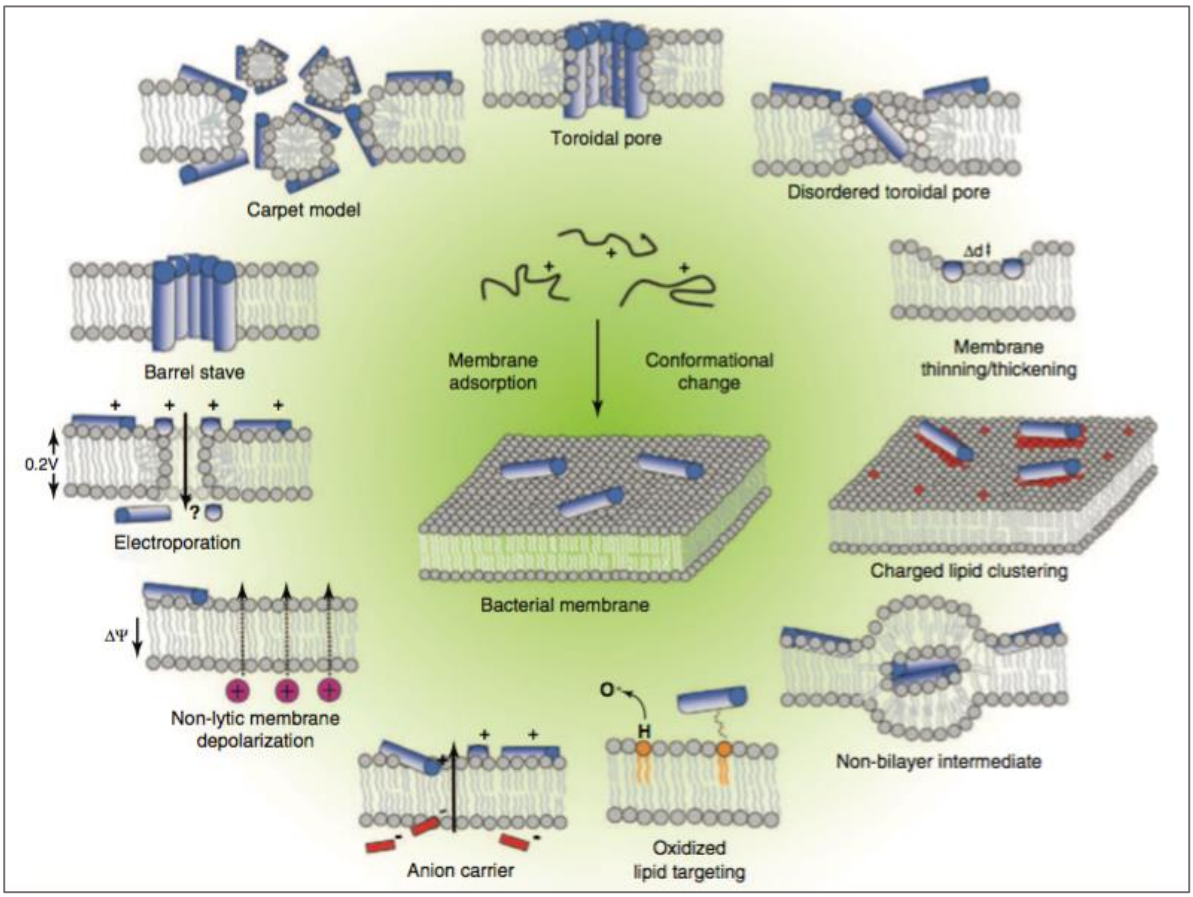

Figure 1.5 A schematic illustrating some of the mechanisms of action at the cytoplasmic membrane, associated with AMP mode of action (MOA). Image used with permission from Trends in Biotechnology and Nguyen *et al*. [125]

Membrane activity is only one of the AMPs MOA and many AMPs have been shown to have intracellular targets. Non-membrane disruptive AMPs translocate into the cytoplasmic membrane through the above-mentioned models without causing cell death. Once inside the bacterial cytoplasm, these cationic AMPs are thought to interact with DNA, RNA and/or cellular proteins to inhibit their synthesis[122]. Specific enzyme targets have also been elucidated, such as the binding to

DNAK (a heat shock protein) by the proline-rich insect peptide, pyrrhocoricin. This binding negatively affects chaperone-assisted protein folding. Mersacidin, a *Bacillus spp.* lanbiotic, binds to lipid II which leads to the inhibition of peptidoglycan biosynthesis[122]. Antimicrobial peptides from bacteria (referred to as bacteriocins) were among the first to be extracted and characterized. They assist with individual microbial survival by killing bacteria, which compete for nutrients in the same environment [129]. Due to their diverse structural range, bacteriocins are broadly classified into two categories namely: 1) lanthionine containing (lantibiotics) and non-lanthionine containing peptides. Lantibiotics require post-translational processing to acquire their active forms and consist of the unusual amino acid lantionine. The most extensively studies lantibiotic being nisin (produced by *Lactococcus lactis*), is commonly used as a food preservative and has potent activity in the low nano-molar range[130,131]. *Bacillus spp.* have also been shown to produce mersacidin with activity against methicillin-resistant *Staphylococcus aureus* in a mouse rhinitis model[132].

Production and classification of AMPs

AMPs can be classified into two groups, firstly according to their synthesis namely ribosomally synthesized and non-ribosomally synthesised[133], and secondly according to their chemical character and structure[133–135]. Peptides are ideal as antifungal agents due to their ability to be agonists or antagonists (as they can mimic natural ligands), they are well tolerated, efficient and highly selective[136]. Since their discovery in 1938, AMPs have shown antimicrobial activity against various bacteria, viruses, protozoa, fungi and some cancer cells[127]. However, fewer studies have been done to describe and investigate the antifungal spectrum of AMPs, and few mechanisms of action have been explained specifically in terms of fungicidal activities. It has been shown that a large number, but not all peptides have antifungal activity. Table 1.2 provides a summary of peptides from selected groups of AMPs that have been shown to have antifungal activity. These peptides are also referred to as antifungal peptides or AFPs[137].

When AMPs are designed, there is a focus on improving pharmacological properties, limiting side effects and possibly enhancing stability and bioavailability as well as lowering immunogenicity of natural peptides. Many factors affect the antifungal and antimicrobial activity of AMPs, namely peptide length, hydrophobicity, amphipathicity (important for cell membrane interactions), net charge, stereospecificity and secondary structure[136]. For synthetic AMPs, an increase in hydrophobicity and amphipathicity was shown to correlate with improved antifungal activity, but unfortunately is also associated with increased haemolytic activity. Targeting certain virulence traits has been found to be less efficient than wished, this is due to fungal cells expressing different

virulence traits. As the lysis of red blood cells is a known problem with the AMP group, the AFP echinocandin B was transformed to have lowered haemolytic activity by making side-chain adjustments to create cilofungin and anidulafungin[59,138]. Other examples of semi-synthetic AFPs include the KU2 and KU3, both of which have α-helix structures, are membrane active and effectively inhibit *C. albicans* with MIC values ranging from 8-16 mg/L[136,139]. The synthetic AMP known as L1 has been shown to effectively inhibit *C. albicans* (Table 1.2). L1 has a polyamidoamine dendrimeric structure and is intercalated into microbial DNA[136,140] and presented inhibitory activity against *E. coli* and *S. cerevisiae* too[140]. The hybrid peptide known as P18 (∝-helix) resulted due to the fusion of parts from magainin 2 and cecropin A, creating potent antifungal activity against *C. albicans*, *Aspergillus flavus*, *Trichosporon beigelii* and *Fusarium oxyspovrum* (Table 1.2). There is also a dimeric, β-sheet synthetic compound known as Killer peptide which has an unknown mode of action but has activity on a wide range of microbes[136]. This Killer peptide was found to be able to spontaneously and reversibly self-assemble, thereafter slowly releasing its active dimeric form over time[141]. There are a few synthetic and semi-synthetic compounds in literature displaying antifungal activity (Table 1.2), and these allow us to be more optimistic about the future pipeline of antifungal drugs. Likewise, AFP that are ribosomally synthesized are popular even though their production from natural sources is laborious and expensive.

Ribosomally produced and synthetic AFPs

From current research, it can be suggested that there is a unique structure-activity relationship between antifungal AMPs and certain residue types, as they are relatively rich in neutral and polar residues[129]. However, peptides with antifungal activity are also structurally diverse and vary substantially in sequences. As of January 2023, there were 1278 peptides with antifungal properties reported in the Antimicrobial Peptide Database (APD; AMP Database Search (unmc.edu)). Ribosomally synthesized peptides are gene-encoded which refers to one gene encoding for one peptides, and these peptides also generally have a narrower spectrum of activity[142]. The primary translational product for AMP in eukaryotes is a prepropeptide (a storage form of AMPs in cells), along with C-terminal peptides. These molecules consist of an N-terminal signal sequence (for endoplasmic reticulum processing), a pro segment and a C-terminal sequence, which is a cationic peptide showing activity after cleavage. The pro segment is mostly anionic in nature and is involved in intracellular trafficking or the correct folding of the C-terminus amongst others[142].

Examples include eucommia[137], which has a five-disulfide motif and indolicidin, which is an linear peptide. Indolicidin has been shown to exert its antifungal activity by means of disrupting the fungal

cell wall in a salt-dependent but energy-independent manner. At low concentrations the defensins (amphiphilic, Arg rich, β-sheet peptides) have been found to have antifungal, antibacterial and anti-viral properties[143].

Mammalian defensins have been grouped into β-defensins and α-defensins, which differ from each other not only in the number of residues but also in the location and connections at the cysteine double bonds. Another disulfide bonded AMP group is the cytolytic brevinins (Table 1.2). These compounds have broad spectrum antibacterial and antifungal activity[144,145], as does porcine protegrin with research indicating that only 16 residues are essential for antifungal activity whereas only 6 are essential for antibacterial activity[146]. The lantibiotic bacteriocin, nisin, is in use as a natural food preservative[143] due to its ability to inhibit pathogenic food borne bacteria such as *Listeria monocytogenes* and Gram-positive food associated bacteria[131]. Moreover, profound ultrastructural damage has been shown against the *C. albicans* cell wall with the use of bovine lactoferricin[147], which is an Fe-binding glycoprotein of the transferrin family, secreted by glandular epithelial cells and found in secondary granules of neutrophils[148,149].

Pletzer and colleagues[150] have shown that a human cathelicidin peptide (LL-37) had weak antiplanktonic activity but had antibiofilm activity at a concentration 16 times lower than its MIC value, against isolates of *S. aureus* and *E. coli* [150]. LL-37 was also found to be active in injured cells as it is found at sites of psoriasis, nickel contact dermatitis and lupus erythematosus[151]. A group of cyclic lipopeptides (isolated from *Pseudomonas* and *Bacillus*) have antimicrobial activity but have also been shown to regulate biofilm dynamics. It is from this group that Cubicin® (daptomycin injection) has been commercialized as a new antibiotic[152]. It is used to treat enterococcal infections in patients, especially due to the increase of resistance against vancomycin[153]. Furthermore, antifungal plant defensins, which are small cysteine rich peptides show activity against fungal biofilms. Radish defensin RsAFP2 (Table 1.2) and coral bell defensin FP1 have exhibited antibiofilm activity against *C. albicans* specifically[154]. Members of the *Bacillus* genus are often considered microbial factories for the production of a vast array of biologically active molecules[155], such as ribosomally and non-ribosomally synthesized peptides

Table 1.2 Examples of antifungal peptides from various sources, their origin, proposed targets and activity.

Structural class	Peptide	Origin	Proposed target(s)	Activity	Reference
Bacterial Peptides					
Lipopeptide	Iturin A	*Bacillus subtilis*	Lytic	*Saccharomyces cerevisea*	Rautenbach *et al*.[156]
Lipopeptide	Bacillomycin F	*Bacillus subtilis*	Lytic	*Aspergillus niger*	Mhamedi *et al*.[157]; Raaijmakers *et al*.[158]
Peptidyl nucleoside	Nikkomycin	*Streptomyces tendae*	Chitin synthesis	*Coccidiodes immitis, Blastomyces dermatitidis*	Larwood *et al*.[85]
Cyclodecapeptide	Gramicidin S	*Bacillus brevis*	Lytic	*C. albicans*	Rautenbach *et al*.[133]
Lanbiotic	Nisin	*Lactococcus lactis*	Lytic	Gram-positive bacteria, *C. albicans*	Breukink *et al*.[130]
Fungal Peptides					
Cyclic lipopeptide	Echinocadin B	*Aspergillus rugulosus*	Glucan synthesis	*C. albicans*	De Lucca *et al*.[159]; De Lucca *et al*.[160]
Lipopeptide	FK 463 (Micafungin)	*Coleophoma empetri*	Glucan synthesis	*C. albicans; A. fumigatus*	De Lucca *et al*.[159]; Tomishima *et al*.[161]
Lipopeptide	FR901379	*Coleophoma empetri*	Glucan synthesis	*C. albicans*	Tomishima *et al*.[161]
Lipopeptide	Pneumocandins	*Glarea lozoyensis*	Glucan synthesis	*C. albicans, Pneumocystis carnii*	Qin *et al*.[162]
Cyclic depsipeptide	Aureobasidin A	*Aureobasidium pullulans*	Actin assembly	*C. albicans*	De Lucca *et al*.[159]
Lipopeptide	LY 303366	*Aspergillus rugulosus*	Glucan synthesis	*Candida spp.*	de Lucca *et al*.[159]; De Ullivarri *et al*.[136]
Lipopeptide	Cilofungin	*Aspergillus*	Glucan synthesis	*C. albicans; A. fumigatus*	Pfaller *et al*.[119], de Lucca *et al*.[159]; De Ullivarri *et al*.[136]
Plant Peptides					
Cysteine rich	Thaumatin	*Thaumatococcus daniellii*	Lytic	*Saccharomyces cerevisea, Fusarium spp*	Thery *et al*.[163]
	CaThi	*Capsicum annum*	Lytic	*Saccharomyces cerevisea, C. albicans*	Taveira *et al*.[164,165]
	RsAFP2	*Raphanus sativus*	Apoptosis	*Filamentous fungi*	Thery *et al*.[163]; Aerts *et al*.[166]

Table 2.1 Continued

Structural class	Peptide	Origin	Proposed target(s)	Activity	Reference
Insect and Amphibian Peptides					
Cyclic	Apidaecin	*Apis mellifera*		Gram-negative bacteria	Casteels *et al*.[167]; Nissen-Meyer *et al*.[143]
α-helix	Mellitin	*Apis mellifera*	Permeabilisation	*C. albicans, HIV, Acinetobacter baumannii*	Park *et al*.[168]; Memariani *et al*.[169]
Linear	Cecropins	*Hyalopora cecropia*	Lytic	*Fusarium oxysporum, Aspergillus fumigatus*	De Lucca *et al*.[159]; Christensen *et al*.[170]
α-helix	Magainins	*Xenopus laevis*	Lytic	*C. albicans*; bacteria, fungi and protozoans	De Lucca *et al.*[159]
Disulfide bonded	Brevinin	*Rana brevipoda porsa*	Lytic		Morikawa *et al*.[145]; Nissen-Meyer *et al*.[143]
Mammalian Peptides					
α-helix	LL37	Human	Lytic	*C. albicans*	Delattin *et al*.,[171]
Cyclic	Lactoferricin B	*Bos Taurus*		*Staphylococcus aureus, C. albicans, C. tropicalis and C. neoformans*	Vorland *et al*.[172]; Ulvatne *et al*.,[173]
Cys rich	Gallinacin 1	Chicken leukocytes	Lytic	*Aspergillus fumigatus*	Harwig *et al*.[174]; De Lucca *et al*.[159]
α-helix	PMAP-23	Porcine	Membrane	*C. albicans*	Park *et al*.[175]
Synthetic and Semi-synthetic Peptides					
Linear-kaxins	dF21-10K	Synthetic	Membrane	*C. albicans, C. tropicalis*	Burrows *et al.*[176]; Bondaryk *et al.*[177]
α-helix decapeptide	KSL-W	Synthetic	Membrane	*C. albicans*	Bondaryk *et al*.[177]; De Ullivarri *et al*.[136]
Polyamidoamine dendrimer	L1	Synthetic	DNA intercalation	*Candida spp.*	Ottaviani *et al.*[140]; De Ullivarri *et al.*[136]
α-helix	P18	Synthetic	Unknown	*Candida spp., Cryptococcus neofromans, Paracoccidioides, Aspergillus fumigatus; Aspergillus flavus, Trichosporon beigelii and Fusarium oxyspovrum*	Magliani *et al*.[141]; De Ullivarri *et al*.[136]
α-helix	KU2	Synthetic	Membrane	*C. albicans*	Lum *et al*.[139]; De Ullivarri *et al*.[136]
α-helix	KU3	Synthetic	Membrane	*C. albicans*	Lum *et al*.[139]; De Ullivarri *et al*.[136]
Lipopeptide	L-693,989	Synthetic	Glucan synthesis	*C. albicans; P.carinii*	de Lucca *et al*.[159]

Non-ribosomallly produced AFPs

Non-ribosomally synthesized peptides are secondary metabolites produced by microbes, and are synthesized by multi-modular enzyme complexes[60,134]. Already used extensively in clinical settings, there are many examples of these peptides, such as cyclosporin which is widely used for immunosuppression and the tripeptide ACV is the precursor molecule for penicillin and cephalosporins[142]. Polymyxin B and colistin has been produced for topical applications along with the tyrocidine group. The tyrocidines and their analogues are a group of structurally related cyclodecapeptides that were discovered as part of the tyrothricin complex that was isolated from cultures of a soil bacterium, *Bacillus brevis*[178], later reclassified as *Brevibacillus parabrevis* (*B. parabrevis*)[179]. Tyrothricin is separated into two functional fractions namely 1) neutral, linear pentapeptides referred to as gramicidins and 2) basic, cyclic decapeptides referred to as tyrocidines[180]. This group has been shown to have micromolar activity against various bacterial species, some filamentous fungi [181], as well as inhibitory activity against *C. albicans* biofilms[182]. For the eradication of *C. albicans* biofilms, Troskie *et al.*, described that sub-haemolytic concentrations of tyrocidine A behave synergistically with the antifungal drugs AmB and CAS[182]. Furthermore, two tyrocidine analogues, phenycidine A (PhcA) and tryptocidine C (TpcC), respectively with a Phe and Trp in amino acid position 7 instead of the tyrosine residue found in the major tyrocidines, have both been shown to have a broader spectrum of antifungal activity than tyrocidine A, B or C[154]. The MOA for this antifungal group has not yet been fully elucidated. However, research has shown that membrane leakage and the generation of radical oxygen species in *C. albicans,* which would lead to membrane leakage and cell death is evident[154]. Tyrocidine A and tyrocidine B also show activity against the human pathogen *Aspergillus fumigatus* in the micromolar range[154].

The analogous compound, gramicidin S, is a cyclic decapeptide which primarily acts as a defence molecule but has been shown to have membranolytic activity[133]; it interacts with lipid bilayers, as well as mitochondrial and bacterial membranes[183,184] showing broad spectrum antibacterial activity[185]. It was first isolated by Gause and Brazhnikova[186] from the soil microbe, *Bacillus brevis* almost 80 years ago but is now easily and reproducibly produced by *Aneurinibacillus migulanus*[127]. Gramicidin S and the tyrocidines are membrane active. It inserts itself at the interface between phospholipid head groups and fatty acyl chains[124]. Therefore, it is believed to affect membrane fluidity, localization of peripheral membrane proteins and membrane potential. Gramicidin S has been shown to have strong inhibitory activity against the plant pathogen *Botrytis cinerea* and the human pathogen *Fusarium solani*[154]. Studies show that the multifunctional gramicidin S has

antibiofilm activity against biofilms of *S. aureus*, *Enterococcus faecalis* and *Enterococcus faecium* at concentrations ranging from 8-16 µg/mL[187] and anti-biofilm[182] activity against *C. albicans* ranging from 1.2-14.8 µg/mL (current study).

The Bacillus genus is well known for being able to produce a wide array of biologically active molecules, such as lipopeptides and is most often present in soil isolates. Lipopeptides have been shown to have antimicrobial activity, anti-tumour activity, immunosuppressant activity as well as having surfactant activities and being cytotoxic[60,158]. Unlike AMPs, lipopeptides are only produced by bacteria and fungi. The carbon source in cultivation play an integral role in the acyl chain length and which hydrophobic amino acids are incorporated into the lipopeptides [188]. They are synthesized non-ribosomally, using multi-enzyme complexes, which has allowed for heterogeneity among the sequence of amino acid residues, the nature of cyclization as well as length and branching of the fatty acid chain[155,189]. Lipopeptides from *Bacillus spp.* can be grouped into three families namely, 1) iturins, 2) surfactins and 3) fengycins[158]. Each family contains variants of the same peptide length but varying amino acid sequences. Additionally, each can have different lengths and isomers of their fatty-acyl chains. In the surfactin family, heptapeptide variants of esperin, lichenysin, pumilacidin and surfactin groups contain a peptide moiety linked to a β-hydroxyl fatty acyl residue with an ester bond. Their fatty acyl residue consists of 12-17 carbons either in a linear chain, iso (branched on penultimate carbon) or anteiso (branched on ante-penultimate carbon) branches. Surfactin (Srf) itself is a cyclic decapeptide with an anionic nature and it is associated with the sporulation and biofilm formation of *B. subtilis*[133]. This class has been shown to have antiviral, antimycoplasma and antibacterial activity but are also haemolytic and not fungitoxic[190]. They are powerful biosurfactins with emulsifying and foaming properties and they have the ability to anchor into the lipid layers, interfering with biological membrane integrity.

The iturin family consists of iturin A and C, bacillomycin D, F and L, and mycosubtilin. They showcase a heptapeptide attached to a β-amino fatty acid chain varying in carbon backbone length from 14-17 carbons. Interestingly, iturins show potent antifungal activity but only limited antibacterial activity and no antiviral activity[191]. It is suspected that this antifungal activity is due to the membrane permeabilization activity. However, the mechanism is based on osmotic perturbation due to the formation of ion-conducting pores[192]. Moyne *et al.*, described the successful antifungal activity of two bacillomycin D analogues against *Aspergillus flavus*, showing activity at 3µg[191]. Still, bacillomycin D, in conjunction with AmB has also shown synergistic activity against *C. albicans* biofilms[150], using

concentrations ranging from ¼ to $^{1}/_{64}$ of their individual MIC values[193] in the bacillomycin D-AmB combination.

The fengycin family comprises of fengycin A, B and C[194]. They are decapeptides linked to β-hydroxyl fatty acid chains (C14–C18) that can also be in linear, iso or anteiso forms and that may be saturated or not[158]. The synthesis of these natural defence molecules occurs later during the stationary growth phase. They have been found to be less haemolytic than iturins[195,196] and exhibit strong antifungal activity[195–198], as well as documented antibacterial activity[199]. The mechanism of action of fengycin is less well known than other lipopeptides, but they readily interact with lipid layers and are thought to some extent affect cell membrane structure and permeability[196,200,201]. Antifungal peptides with a narrow antifungal spectrum would have a greater therapeutic potential as they are less likely to disturb the host's natural microbiota[136]. Current research is concentrated on novel sources of AMCs, AMPs and AFPs. As an abundant and extensive source, soil microbes are being focused on, hopefully creating a novel library of AFPs, especially because there is virtually no resistance against this class of compounds[124]. Hence, the focus of this thesis becomes more evident.

Conclusion

The epidemiology of invasive fungal infections is changing, with less prevalent strains becoming more dominant and invasive, showing an increased frequency of resistance against currently available drugs and drug targets. This is due to the use of current antifungals for prophylaxis, therapy and as agricultural agents[90]. The current novel drug pipelines do have exciting compounds in the late clinical trial steps, some showing good pharmacokinetic profiles and broad-spectrum activity. The final FDA approval of drugs is a very slow and expensive process which curbs the current, urgent need for these new molecules. However, the current scientific drug discovery innovations allow quick and effective screening of virtual and chemical libraries of small compounds (such as peptides) for their antimicrobial activities. To assist in combatting with the global upsurge in antifungal resistance, the focus of this project was on the most common clinically relevant fungal pathogen, namely *C. albicans*, as the target in a biomining-search for antifungal compounds from soil samples. The overall aim was to contribute to the natural, small molecule antifungal pipeline by using a designed medium-high throughput multiplex assay system (chapters 2 and 3) to discover and characterise anti-Candida compounds (Chapters 4 and 5) from soil isolated microbes against planktonic and biofilm *C. albicans* cells.

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CHAPTER 2

Comparing the metabolic activity of planktonic and biofilm associated *Candida albicans* isolates using conventional and multiplex assays

Abstract

In this study, we describe a medium-throughput multiplex assay system (MPAS) that can be used to study the different life-stages of *Candida albicans* strains. Designed by using conventional 96-well plate and 96 pin-lid assay layouts, MPAS utilises metabolic activity to indicate planktonic cell susceptibility, biofilm prevention activity, biofilm eradication activity, as well as activity against the cells shed from biofilms, when treated with antimicrobial compounds. Growth lag times of planktonic cells from four *C. albicans* strains ranged between 3-4 hours, except for one clinical strain, which showed a shortened lag time of 1 hour. It is during these lag times that cells adapt to their new environment and proliferate, as well as reversibly bind to surfaces for biofilm initiation. MPAS results showed that planktonic inoculums initially formed biofilms on pin lids within 4 hours, followed by a planktonic counterpart shedding event around 24-48 hours (depending on the strain) and subsequent slow maturation of the biofilm with various inter-strain differences observed. The use of conventional broth assays, and our novel multiplex assay format, provided interesting data on C. *albicans* biofilm eradication and prevention activity of clinically relevant antifungal drugs such as caspofungin, amphotericin B and fluconazole. A high degree of resistance against fluconazole was observed, as well as some conceding loss in sensitivity against the polyketide, amphotericin B and the lipopeptide, caspofungin. The inter-strain differences in metabolic activity and sensitivity/resistance towards currently available antifungals on biofilm and planktonic cells, highlighted the importance of multiple parameters that should be considered for future assessments of antifungal efficacy.

Introduction

Approximately 300 fungal species have been implicated in clinical cases, but only 20-25 of these species are known to commonly cause mycosis [1]. The most well-known of these fungi, is the ascomycetous yeast *Candida albicans,* which is the most frequently isolated and the most clinically relevant fungus[2]. It is characterized by asporogenous yeast cells showing multilateral budding, with the ability to form pseudohyphae[3]. *Candida albicans* is an ubiquitous opportunistic pathogenic fungus with the ability to colonize various epithelial tissues, i.e. vaginal, intestinal and oral tissues, causing superficial and systemic mycoses[4]. It was observed that fatal systemic infections caused by *C. albicans* are due to planktonic cells, liberated from robust persistent Candida biofilms, that disseminate via the bloodstream[5]. This yeast is known to be one of most common causes of bloodstream infections in hospitalized patients[6]. Furthermore, *Candida* species are known to have one of the highest fungal colonization to infection rates compared to other opportunistic fungi, as well as the overall highest crude mortality rate when focussing on fungal infections[7]. A major virulence factor associated with *Candida albicans* is the ability to form life-threatening biofilms on medical devices such as catheters, prosthetics and on implants [8]

A Candida biofilm is known to comprise of a complex polymeric matrix consisting of >50% proteins and glycoproteins, lipids and nucleic acids[3]. The initial attachment is mediated by both non-specific factors (cell surface hydrophobicity and electrostatic forces), as well as specific adhesins which recognize ligands in the conditioning film (which anchors the biofilm), such as serum proteins and salivary factors. However, not all *Candida* species and all *C. albicans* strains have equal biofilm forming capacities[9]. Within the field of medical mycology, the development of effective antifungal drugs that target all stages of Candida colonisation and propagation, especially biofilm formation, is crucial. Some currently available antifungals, such as amphotericin B and fluconazole, display antibiofilm activity only at high concentrations, compared to their activity towards planktonic cells[10]. The mechanisms of drug resistance are not all understood, the most common hypothesis states that the polymeric matrix restricts the penetration of various drugs by means of a reaction-diffusion barrier. This results in only the surface layers of the *Candida* biofilms being exposed to therapeutic doses of antifungals[11,12]. Some studies have shown that the presence of β-glucans in the polymeric matrix play a role in antifungal resistance by sequestering antifungal compounds[13]. Alternate hypotheses suggest that the following factors might add to resistance namely: 1) the high density of cells within the biofilm; 2) nutrient limitations and a resulting decreased growth rate; 3) the expression of resistance genes, such as efflux pumps; and 4) the presence of persister cells. A

number of studies revealed that Candida biofilms are more resistant than their planktonic counterparts to currently available antifungal drugs; these include amphotericin B, fluconazole, flucytosine, itraconazole, caspofungin and ketoconazole[8,14,46].

For continued antifungal drug discovery there is a need for rapid and cost-effective assays that can discriminate between the efficacy towards the different phenotypes/life-stages of *C. albicans*. To study antifungal activity against planktonic cells there are various agar-based and broth-based assays[15]. A classic method entails the formation of inhibition zones caused by an antimicrobial compounds affecting a lawn of microbial growth on an agar plate[16]. Furthermore, there is also the use of agar dilution assays where a suspension containing a known concentration of microbial cells is spotted directly onto nutrient agar plates; each supplemented with different antibiotics and antibiotic dilutions. After the required incubation period, the presence and numbers of microbial colonies on the plates would indicate the level of resistance of the microorganism against the particular antibiotic[17]. As evidence accumulated, however, that agar is not suitable to test all antimicrobial compounds, broth dilution assays became more popular, especially using a 96-well assay plate layout. Broth dilution assays are based on using a liquid growth medium, containing dilutions of an antimicrobial agent, which is inoculated with a known concentration of microbial cells. After the required incubation period, microbial growth is indicated by increased turbidity (optical density) or a colorimetric result using a metabolic dye. Additionally, Du Toit and Rautenbach[18] developed a highly sensitive micro-gel well diffusion assay combining the best characteristics of both the inhibition zone assays in agar, and the micro-broth dilution assay in a 96-well format [18].

Compared to the various assays on the physiology of planktonic microbial cells, the repertoire of tests to study biofilms and anti-biofilm activity is surprisingly limited without a true standard assay for *in vitro* assessment of the activity of antifungal agents. In current research, two main biofilm studying methodologies are utilized: 1) cultivation of biofilms directly on objects in Petri-dishes and/or in wells of microtiter plates [2,19] or 2) the cultivation of biofilms on pin-lids [20,21]. However, the detection of the biofilms in terms of forming, growth and shedding remains a challenge. Biofilm assays are most popular in a microtiter plate format, which entails considering different aspects of the experimentation; use of different microplate types and designs, agitative or stationary attachment, rinsing of growth plates after biofilm attachment, fixation before quantification as well as the use of various fluorescent and luminescent dyes for quantification [22] and ultimately, the use

of different test organisms. Some of these biofilm assays and devices are discussed in more detail below.

In colony biofilm assays, where a biofilm colony is grown on a semipermeable membrane and placed onto an agar plate, the nutrient source and test compound can be easily changed by transferring the membrane to a fresh agar plate. Results, however, are recorded in a variety of manners, of which one is explained in more detail next. The Kadouri[23] drip-fed assay uses two needles, which are inserted into a Petri-dish lid, with one needle pumping in new growth medium and the second needle removing planktonic cells and waste – overcoming some of the static system's limitations. A possible shortcoming to this design would be the difficulty in accessibility to this apparatus and the inability to consider dispersed/shed planktonic cell resistance and sensitivity. Alternatively, known as air-liquid interface assays, various objects on which biofilms can form can be placed in a Petri-dish. Objects such as glass coverslips can also be placed in 12-well microtiter plates whereupon biofilms are allowed to form on the objects in a stationary assay[23]. Chandra *et al.*[24] placed denture strips, pre-treated catheters and treated contact lenses in 12-well microtiter plates to allow for *Candida* biofilm formation. The biofilm metabolic response was monitored using a dye reduction reaction to visualise and quantify biofilm formation via fluorescent microscopy[24]. They utilised the yellow dye, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5 [(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), is reduced to an orange formazan product.

For medium/higher through-put assays, the Calgary Biofilm Device or the MBEC Assay System designed by Ceri *et al*.[20] offers options. The MBEC assay system was primarily used to study biofilms of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.* This device utilises a two-part vessel that consists of a 96 peg-lid (pin-lid) which fits into channels of the device reaction vessel. Growth medium flows across the pegs/pins in the reaction vessel, resulting in comparable biofilm formation on each peg. Visualisation is accomplished by measuring the turbidity of the cultures at 650 nm[20]. Unfortunately, the Calgary Biofilm Device is not easily obtained or affordably accessible to all laboratories. An example of another high-throughput assay is the luminescent attachment/detachment assay developed by Junker and Clardy[21]. These attachment and detachment assays were originally employed to study biofilm formation by *P. aeruginosa* cultures growing on 384-well microtiter plates fitted with 384 pin-lids. Visualization was achieved via luminescence using BacTitre-Glo, which indicated either attachment or detachment, after the respective cultures were treated with various compounds[21]. A shortcoming to this design is primarily due to financial reasons, namely the costs of luminescent readers, luminescent dye kits

and 384-well pin-lids and plates. Alternatively, many biofilm assays for *C. albicans* are done in 96-well plates using a variety of dyes to detect metabolic activity. For example, Ramage et al.[2] cultivated *C. albicans* biofilm cells in flat-bottomed, polystyrene 96-well plates and after exposure to test compounds, the reduction of XTT was used to give a semi-quantitative result of biofilm metabolic activity[2]. In an alternative, conventional assay, Troskie *et al*.[25] and Delattin *et al*.[26] grew bacterial and fungal biofilms in 96-well microtiter plates for 24 hours, after which the biofilms were washed using phosphate-buffered saline. Thereafter, the cells were treated with antimicrobial compounds and incubated at 37°C for 24 hours. The antimicrobial activity was determined by measuring the fluorescence of a metabolic dye, resazurin, and its redox-dependent conversion to resorufin[27].

There is a need for efficient standardised antifungal/antibiofilm assays, as well as for safe, affordable and effective antifungal drugs to reduce the burden of systemic mycoses. The 96-well assay types represent medium to high throughput methodologies with multiple repeats which improve accuracy and reproducibility. In general, such assays are relatively inexpensive and simple, as well as being easily adaptable to all laboratories. They can be used to determine the biofilm-forming abilities of various micro-organisms and are easily adapted to test multiple parameters of interest[19].

In this study, we evaluated a multiplex medium-throughput assay system on both biofilm and planktonic cells of five *C. albicans* strains. We adapted the well-known conventional cell viability assay using resazurin-resorufin and combined it with a pin-lid type assay to monitor growth and metabolic activity of biofilm associated and planktonic yeast cells. This allowed us to monitor growth of five Candida strains differing in morphology or physiology. Our multiplex assay system (MPAS) was also used to test various antifungals against the five *C. albicans* strains in terms of 1) compound activity against planktonic *C. albicans* cells; 2) compound activity that can prevent the formation of *C. albicans* biofilms on pins; 3) the removal and/or eradication of established *C. albicans* biofilms from a colonized pin surface and 4) to determine the activity against the planktonic cell populations that had dispersed/shed from the biofilms (refer to Fig. 2.1). Live and resistant cells would continue to be metabolically active in the presence of antifungals and result in the reduction of the permeable and non-toxic dye, resazurin (oxidised, blue) to the reduced resorufin (red). This reaction is indicative of the reduction of the blue resazurin to the pink fluorescent resorufin occurring in the cytoplasm of living cells and is dependent on reduced metabolites/cofactors such as $FMNH_2$, $FADH_2$, NADH and NADPH[28]. Resorufin diffuses out of the cells and is highly fluorescent and proportional to the number of metabolically active cells in the reaction mixture[28].

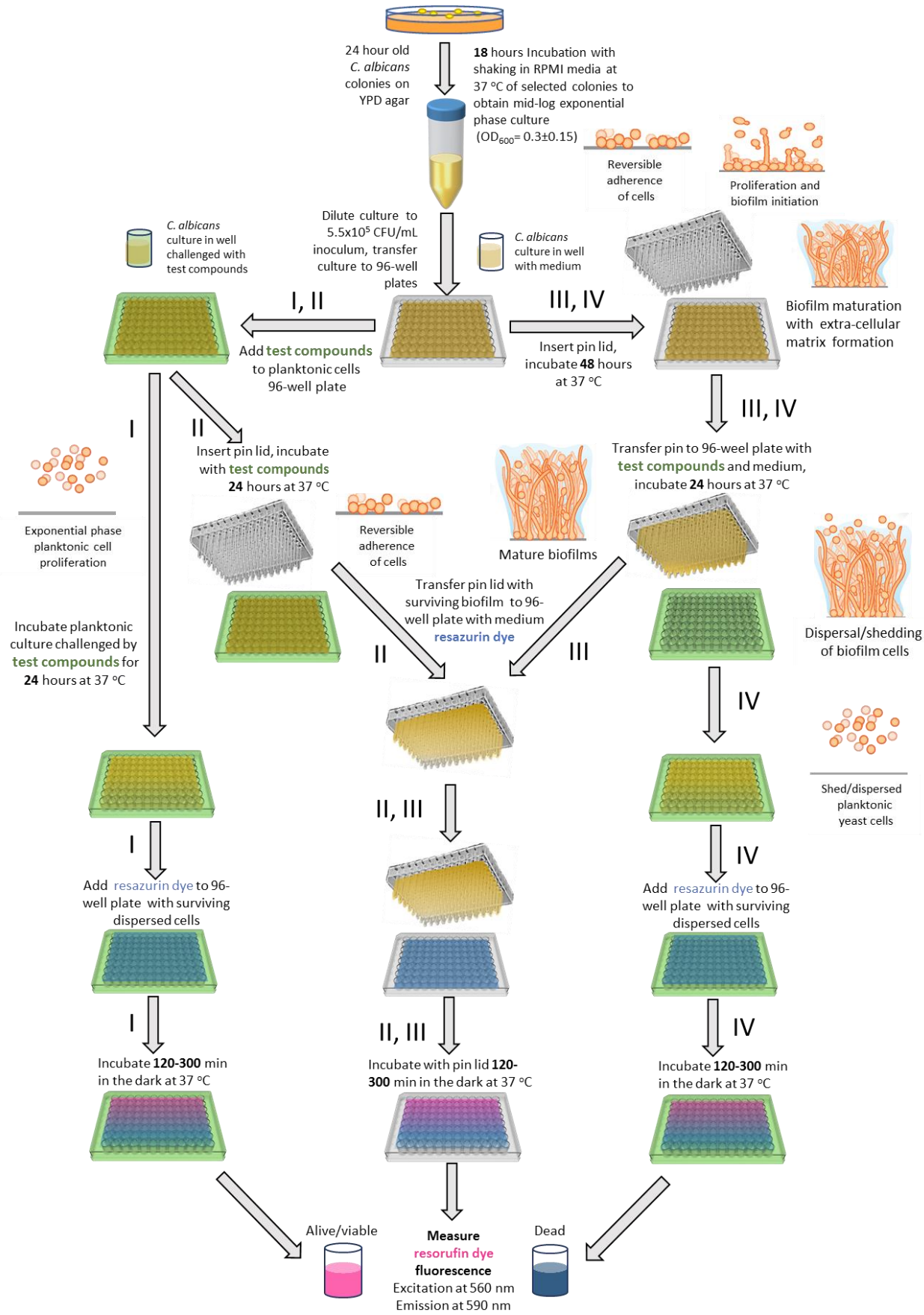

Figure 2.1 An informative demographic showing the various steps followed for each assay type (I, II, III, IV) in the MPAS design described in this study. Assay I diagram shows the steps followed to record planktonic cell data. Assay II diagram explains the steps followed to record biofilm prevention data. Assay III diagram shows the steps followed to record biofilm eradication data and Assay IV diagram shows the steps followed to record dispersed/shed planktonic cell results.

Materials and methods

Materials

Strains representing clinical, environmental and veterinary isolates of *C. albicans* were obtained from the yeast culture collection of the Department of Microbiology, University of Stellenbosch (Table 2.1). Gramicidin S (GS) and caspofungin (CAS) were purchased from Sigma Aldrich (Steinheim, Germany) and the Amphocil® formulation of amphotericin B (AmB) was purchased from Gibco® Life Technologies Corporation (New York, USA). Fluconazole (Flu) was purchased from Merck Chemicals (Pty) (Wadeville, Gauteng). Sterile water was prepared by reverse osmosis followed by filtration through a Millipore MilliQ® water purification system (Milford, USA). Ethanol (99.9%) was obtained from Merck Chemicals (Pty) (Wadeville, South Africa). The yeast extract powder, peptone powder, anhydrous D(+)-glucose and agar powder for the yeast peptone dextrose broth (YPDB) and yeast peptone dextrose agar were from Biolab Merck (Wadeville, South Africa). The non-sterile polystyrene plates were provided by Greiner bio-one (655101; Germany). The non-sterile NUNC polystyrene plates with fitted NUNC-TSP (Transferable Solid Phase Screening System) pin-lids were supplied by Thermo Fischer Scientific (Denmark). Sterile culture dishes and microtiter plates were obtained from Corning Incorporated (USA) and sterile Petri-dishes from Lasec (Cape Town, South Africa).

Table 2.1 The origins and GenBank accession numbers of the *C. albicans* yeast strains used in this study.

Strain	Origin	GenBank Accession Number
MRC8908[a]	Clinical isolate, Tygerberg Hospital, Cape Town, South Africa	KJ534504
MRC8912 [a]	Clinical isolate, Tygerberg Hospital, Cape Town, South Africa	KJ534505
CAB201	Veterinary isolate from a domestic pigeon, *Columba livia domestica*, South Africa	MK248726
CAB1653	Environmental isolate, Mosquito Larvae, South Africa	Not available
CAB1085 [b]	Environmental isolate, Plankenburg River, Stellenbosch South Africa	KJ534503

[a] Clinical isolates MRC8908 and MRC8912 were previously stored in the Program on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit culture collection of the South African Medical Research Council.
[b]Isolate obtained from a river that is known to be polluted with sewage.

C. albicans culturing conditions

Cultures of two clinical strains of *C. albicans,* MRC8908 and MRC8912, two environmental strains; CAB1085 (river isolate) and CAB1653 (mosquito larvae isolate), and one veterinary isolate, CAB201 (Table 2.1) were revived from glycerol stocks (30%) stored at -80˚C, by plating onto Yeast Peptone Dextrose (YPD) agar comprising of 1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 2% (*w/v*) dextrose and 15 % (*w/v*) agar contained in Petri-dishes. The resulting plate cultures were incubated at 37˚C for 24 hours, where after a single colony representing each *Candida* strain was used to inoculate 10 mL of YPD broth contained a 15 mL Falcon tube (Fig 2.1). The resulting pure cultures were incubated at 37 ˚C on an orbital shaker (150 rpm) for 18 hours, where after the cultures were sub-cultured by inoculation and allowed to grow in un-supplemented Roswell Park Memorial Institute 1640 (RPMI1640) medium (pH 7) to mid-log OD_{600nm} of 0.30, to cell concentrations required by the various assays and experiments.

Microscopy

Planktonic *C. albicans* cells were cultured in shake flasks for 18 hours in YPD broth as described above, and thereafter, sub-cultured in RPMI1640 to mid-log phase at OD_{600nm} of 0.30. Sub-cultures were diluted to an initial cell concentration of 5.5×10^5 CFU/mL, where after the cultures of all five strains were aliquoted into a UV-sterilized 96-well plate (Greiner Bio-one Microplate 655101). After an 8-hour incubation period in the 96-well plates, cells were unstained or stained with Hoechst dye (0.008 µM) and visualized using an Invitrogen Thermo Scientific EVOS FL Fluorescent Microscope.

Growth curves

Optical density monitored growth curves

Planktonic *C. albicans* cells were allowed to proliferate in RPMI1640 to mid-log phase with optical density at 600 nm (OD_{600nm}), after which they were diluted to an initial cell concentration of 5.5×10^5 CFU/mL. The negative controls contained 100 µg/mL GS and blank sterile controls only contained RPMI1640 medium without cells. The 96-well microplate with cultures and controls were incubated in a humidity chamber, which was placed in the Tecan Spark 10M multimode microplate reader, temperature controlled at 37˚C and the OD_{600nm} was recorded.

Biofilm growth assay

For each strain, an overnight culture was started in 3 mL YPD, from a single fungal colony on an YPD agar plate. The overnight culture was incubated for 18 hours at 37˚C while shaking on a rotating incubator at 150 rotations per minute (rpm). The overnight culture was grown to OD_{600nm} of 0.30

and 100 µL of the exponential phase planktonic culture was pipetted into a 96-well plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 442404) with a pin-lid (Thermo Fischer Scientific Sterile TSP Screening 445497). The plates were incubated and assessed at time intervals ranging from 12 to 120 hours. To detect the biofilms on pins, the pin-lids were removed and placed in fresh RPMI medium, and the metabolic activity measured by adding 10 µL resazurin solution (0.3 mg/mL) to each well. Simultaneously, for shed planktonic cell metabolic data, 10 µL resazurin solution was added to the original 96-well plate, in which the pin-lid with biofilm was allowed to proliferate and grown for the various time intervals. The cultures doped with the metabolically sensitive dye was then incubated for 20-30 min (shed planktonic cells) or 120-180 min (biofilm cells). Detection of conversion of resazurin to resorufin was done using a Tecan Spark 10M multimode microplate reader, temperature controlled at 37˚C and the fluorescence was recorded with excitation measured at 560 nm and emission measured at 590 nm. Refer to Fig. 2.1 for a flow diagram of the multiplex assay methodology for biofilms.

Multiplex Assay System (MPAS System)

Assay I: Planktonic growth inhibition

C. albicans cells in planktonic stage were sub-cultured in RPMI1640 to mid-log phase (OD_{600nm} at 0.30) and inoculations were diluted to an initial concentration of 5.5×10^5 CFU/mL. A 96-well "test compound" plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 655101) was set-up using 1.00 mg/mL GS as positive inhibition control with GS, CAS, Flu and AmB serially diluted from 1.00 mg/mL to 7.80 µg/mL. In a second 96-well plate (assay plate), 90 µL of planktonic cells in RPMI1640 was mixed with 10.0 µL of the above test compounds (10-fold dilution) and solvent growth controls, as the assay plate was incubated in a stationary humidity chamber at 37˚C. After a 24 hours incubation period 10 µL resazurin solution (0.3 mg/mL) was added to each of the wells and incubated in the dark (foil wrapped) at 37˚C for 15-30 minutes. Detection of the conversion of resazurin to resorufin was done as described above. Refer to Fig. 2.1 for a flow diagram of the MPAS methodology.

Assay II: Biofilm prevention

To determine the biofilm prevention ability of various antifungals, planktonic *C. albicans* cells were cultured as above to initial inoculation concentrations of 5.5×10^5 CFU/mL. A 96-well test compound plate was set up as above. In the second 96-well assay plate, 90 µL of planktonic cells in RPMI1640 was mixed with 10 µL of the test compounds, in which a 96 pin-lid was placed and incubated in a stationary humidity chamber at 37˚C. After a 24-hour incubation period, the 96-well assay plate pin lid was transferred to a new 96-well plate (biofilm prevention plate) containing 90 µL RPMI1640 and

10 µL resazurin solution (0.3 mg/mL). The prevention plate was incubated at 37˚C for 120-180 min (depending on strain), covered in foil and the absorbance and fluorescence results were recorded using a Tecan Spark 10M multimode microplate reader as described above. Refer to Fig. 2.1 for a flow diagram of the MPAS methodology.

Assay III: Biofilm eradication

To examine the biofilm eradication capability of known antifungals, planktonic *C. albicans* cells were sub-cultured in RPMI1640 to OD=0.30 at 600 nm resulting in an initial cell concentration of 5.5×10^5 CFU/mL. From this sub-culture 100 µL was pipetted into each well of a 96-well plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 442404) in which a 96 pin-lid was placed, as described above for the biofilm prevention assay. The 96 pin-lid and plate combination were incubated at 37˚C in a stationary humidity chamber for 48 h, allowing for biofilm formation on the pins. The test compounds were prepared as before and added (10 µL) to a second 96-well "assay" plate with 90 µL RPMI1640, into which the 96 pin-lid with 48-hour old biofilms on pins were placed. The biofilm cultures were incubated in a stationary humidity chamber at 37˚C for 24 hours, after which the 96 pin-lids and assay plates were transferred to a new 96 well plate with RPMI1640 medium, incubated for 24 hours and developed as before using resazurin solution (0.3 mg/mL). Absorbance and fluorescence measurements of the plates was recorded as described above. Refer to Fig. 2.1 for a flow diagram of the MPAS methodology.

Assay IV: Biofilm dispersed cells inhibition

To examine the biofilm shed cell inhibition of known antifungals, planktonic *C. albicans* cells were sub-cultured in RPMI1640 to OD=0.30 at 600 nm resulting in an initial cell concentration of 5.5×10^5 CFU/mL. From this sub-culture 100 µL was pipetted into each well of a 96-well plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 442404) in which a 96 pin-lid was placed, as described above for the biofilm prevention assay. The 96 pin-lid and plate combination were incubated at 37˚C in a stationary humidity chamber for 48 h, allowing for biofilm formation on the pins. The test compounds were prepared as before and added (10 µL) to a second 96-well "assay" plate with 90 µL RPMI1640, to which the 96 pin-lid with 48-hour old biofilms on pins were placed. The biofilm cultures were incubated in a stationary humidity chamber at 37˚C for 24 h, after which the 96 pin-lids were removed and assay plates containing the biofilm shed cells were developed as before, using resazurin solution (0.3 mg/mL at 37˚C for 20-30 min. Absorbance and fluorescence measurements of the plates was recorded as described above. Refer to Fig. 2.1 for a flow diagram of the MPAS methodology.

Conventional broth assays

Biofilm prevention

The biofilm prevention assay was performed as previously described.[25,26] *C. albicans* cells in RPMI1640 medium (5.5×10^5 cells/ml) were incubated with antimicrobial compounds as explained above, for 24 h at 37°C in round-bottomed 96-well plates. The biofilms were washed with phosphate-buffered saline, and biofilm formation was determined using resazurin solution (0.3 mg/mL). After a 1 hour incubation at 37°C, absorbance and fluorescence measurements of the plates was recorded as described above and previously[29].

Biofilm eradication

The biofilm eradication assay was performed as previously described. [25,26] *C. albicans* biofilms were grown in a round bottomed 96-well microtiter plates in RPMI1640 medium at 37°C for 1 hour[30]. Subsequent to a phosphate-buffered saline wash step, the biofilms were incubated at 37°C for 24 hours to mature in RPMI1640, followed by another phosphate-buffered saline wash step. Thereafter, the biofilms were incubated with antimicrobial compounds as explained above in RPMI1640 medium for 24 hours at 37°C. Biofilm survival was measured using resazurin solution (0.3 mg/mL). After a 1-hour incubation at 37°C, absorbance and fluorescence measurements of the plates were recorded as described above.

Data analysis

Sigmoidal curve fits with variable slope were used for analysing the dose response assay data to determine the inhibition parameters, according to Rautenbach *et al*.[31] The inhibition parameters in the four assays were the 50% minimum inhibitory concentration ($MIC_{50\ planktonic}$) for planktonic cells (Assay I) as well as dispersed planktonic cells ($MIC_{50\ dispersed}$) (Assay IV), 50% biofilm inhibitory concentration (BIC_{50}) (Assay II) and 50% biofilm eradication concentration (BEC_{50}) (Assay III). Refer to Fig. 2.1 for the detailed scheme of the multiplex assays system depicting assays I to IV.

Results and discussion

Candida biofilm formation can be represented by a heterogeneous mosaic model, , which describes stacking of microcolonies held together by extracellular polymeric substances, onto a basal layer that is attached to the substratum [6]. Using light microscopy we could visualize *C. albicans* cells as yeast cells, pseudohyphae and hyphae [32] within 8 hours. These cell types are typical for a pleiomorphic fungi belonging the genus *Candida* [33] (Fig. 2.2). After 8 hours, the cells had multiplied to such an extent that individual cell imaging became quite difficult.

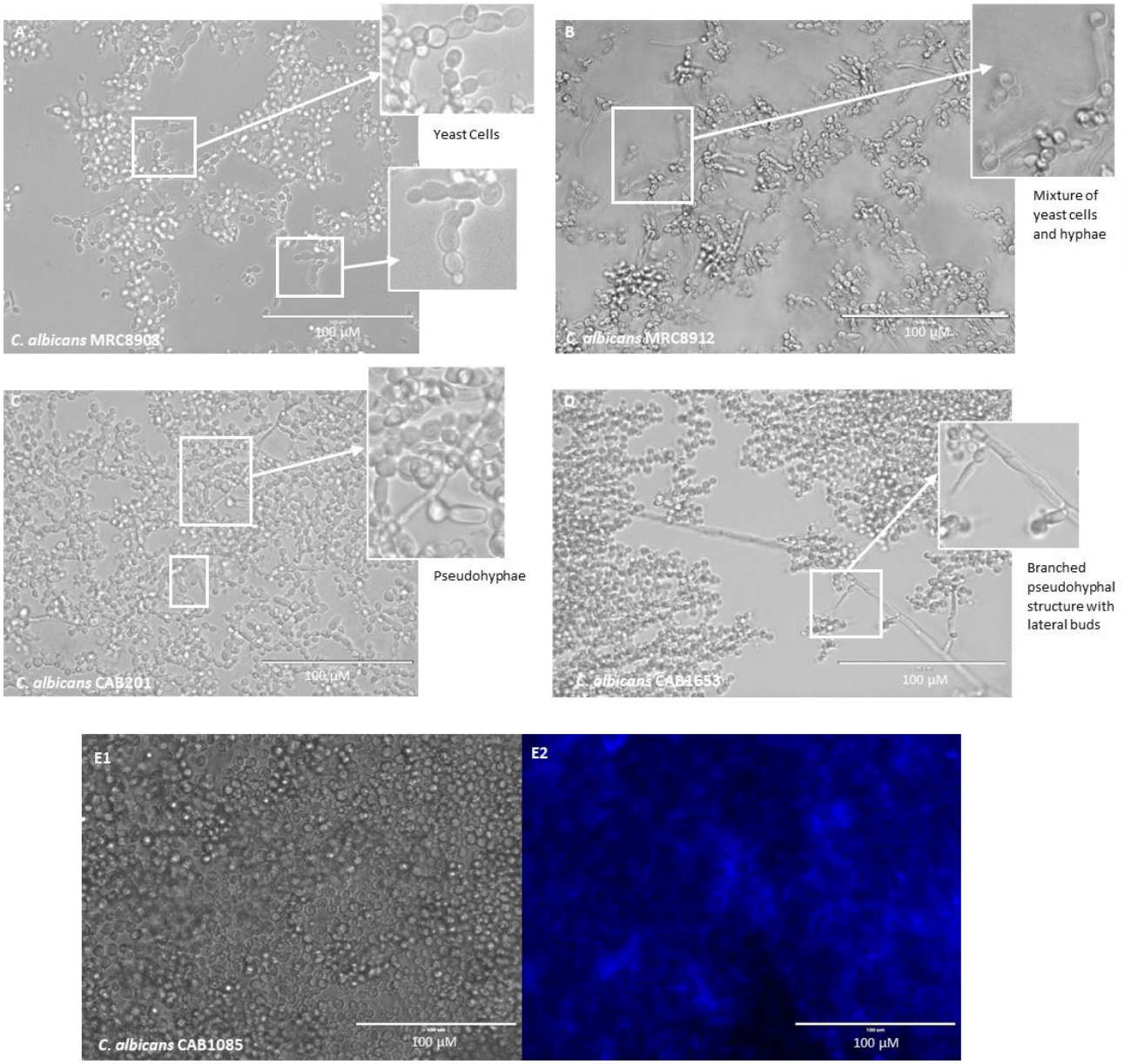

Figure 2.2 Microscopic images of the five unstained *Candida albicans* strains in this study showing their different morphologies at 8-hour of biofilm development. A and C shows a mixture of yeast cells and hyphae observed in strains MRC8908 and CAB201. B depicts the yeast cell population of strain MRC8912 and D depicts the presence of pseudohyphae (strain CAB1653). E1 shows 48-hour cultures of strain CAB1085 with the cells in the biofilm matrix (E2) fluorescently visible using Hoechst dye (0.008 µM; blue image) or unstained (grey images). The length of the bar per micrograph represents 100 µM. Images were recorded using an Invitrogen, Thermo Fischer EVOS FL Fluorescence microscope.

Merson-Davies *et al*.[34] noted that a fungus does not undergo abrupt changes of morphological phases but instead undergoes continual changes in morphology between spheroidal yeasts and true hyphae, which can be observed in the micrographs taken during this study. When studied using Hoechst dye which fluoresce when bound to dsDNA, a haze film covering the 48-hours old microcolonies was observed which shows that the extracellular matrix consists of yeast cells and most likely intertwined hyphal cells (Fig. 2.2 E2). Visibility of individual *C. albicans* cells was unclear even after 12 hours, as all five strains were already encased by the biofilm extra-cellular matrix [6]. However, the microscopic analysis confirmed that the strains cannot easily be distinguished from each other using cellular morphology as criterium, except for the differences in hyphal stage as was observed after 8 hours (Fig. 2.2).

Comparison of planktonic growth profiles

Planktonic growth was monitored by periodically measuring optical density (OD) over a 22-hour period in a 96-well plate. From the growth curves in Fig. 2.3 A-E, the veterinary and environmental strains (CAB201 and CAB1653 respectively), as well as clinical strain MRC8908 show an increased lag time compared to that of the environmental strain CAB1085 and clinical strain MRC8912. However, the lag time for the original inoculum sizes (10^4 – 10^7 CFUs) of all strains, ranged between 0-12 hours, indicated by the commencement of the exponential growth phase. This variation was expected, as strain vitality and cell numbers both influence detection and quorum sensing in culture. This inversely proportional relationship between lag time and inoculum size has been described for *Listeria monocytogenes*, where Robinson *et al*. [35] also showed an increased lag time when using exponential phase inoculum cells compared to stationary phase inoculum cells. Strain MRC8912 was different from the other strains in that it showed a short lag time of 0.5-1 hours at a range of inoculum concentrations, indicating quick initial growth, adaptation and multiplication of this strain (Fig. 2.3 E). However, we did find that this strain had similar biofilm maturation and stabilization, when compared to the other strains (refer to discussion below and Fig. 2.4). The difference in the initial rapid growth rate (Fig. 2.3 F) and biofilm formation could be related to the high susceptibility to antifungals with IC_{50} values of 0.4±0.2 µM towards Flu and 5.2±0.5 µM towards CAS (refer to discussion below, Table 2.2). The extended lag phases therefore could offer an advantage to organism survival, as well as offer tolerance against antimicrobials[36], specifically those that target rapidly growing cells. Furthermore, growth of all five strains plateaued into stationary phase within 10 hours of culturing at an $OD_{600\ nm} \leq 0.3$ ($\ln OD \leq 0.6$), independent of the number of cells in the inoculum.

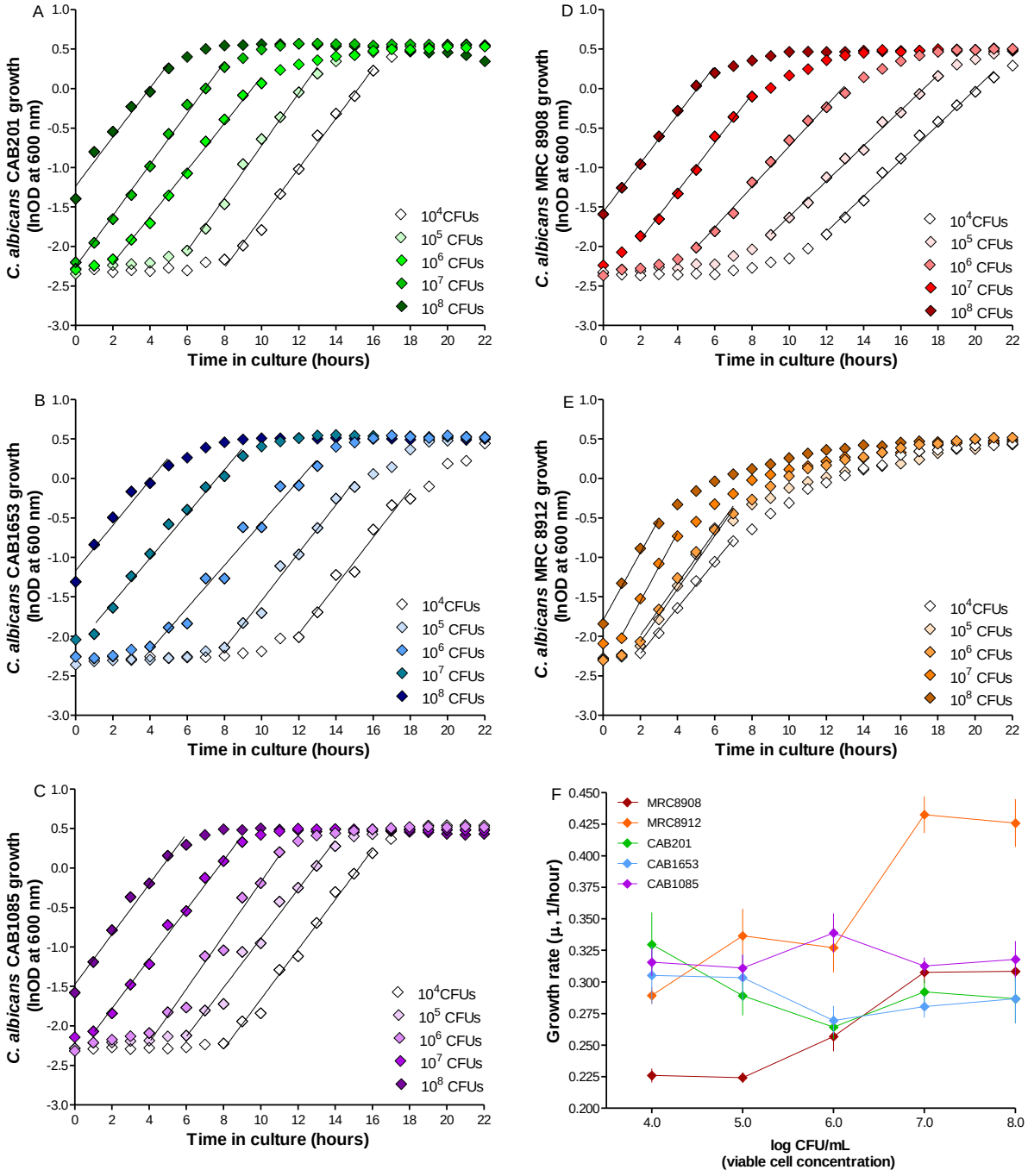

Figure 2.3 Planktonic growth curves of veterinary strain CAB201 (**A**), environmental strains CAB1653 and CAB1085 respectively (**B, C**) and clinical strains MRC8908 and MRC8912 respectively (**D, E**) of the *C. albicans* strains measured over 22 hours using optical density readings at 600 nm at different inoculum concentrations (colony forming units, CFUs). The growth data at each of the CFUs are the average of two biological repeats. The linear regression lines indicated the growth rate μ as 1/hour. In graph F the data is plotted against the cell number (log CFU/mL). The error bars show the mean ± standard deviation.

A significant variability in specific growth rate was observed, with SD of 10% for strains CAB201 and CAB1653 and 20% for strains MRC8908 and MRC8912, while strainCAB1085 had the lowest variability. The data in Fig. 2.3F shows that the growth rate of the two clinical strains was more sensitive to initial cell numbers, compared to the environmental strains that were assessed. This growth rate sensitivity was independent of lag times and the specific growth rates and biomass

yields for all strains are within a similar range (Fig. 2.3 F). The results shown in Fig. 2.3 also indicated that lag times must be correlated to initial cell numbers, as well as the strain and as such should be independently assessed for each strain. The genetic strain differences could result in differences in biofilm formation, that must also be considered (discussed below). With the practical time frame of 10-12 hours allowing for optimal cell growth of all strains, all further planktonic and prevention assays were performed at 5.5 x 10^5 CFUs for comparable results and improved ease of comparison to dose-response industry specifications[37].

Comparison of biofilm stability and metabolism

In order to conduct studies on the biofilms that formed on the pin lids it was necessary to ensure that there are indeed metabolising cells that adhered to the pins for assays II and III in our MPAS assay system, as well as a reservoir of cells that could disperse into the culture for assay IV (Fig. 2.1). Using the adherent cell medium (RPMI1640) we were able to compare inter-strain differences in biofilm formation and metabolism over a 12- to 120-hour timeframe as an indicator of adherence over time (Figs. 2.4 and 2.5).

All five strains formed biofilms on the pins within 24-hours displaying metabolic activity and average fluorescence of 20000-50000 RFUs above the background (Fig. 2.4A). If the distribution of data in each of the violin plots in Fig. 4A is considered, biphasic and even triphasic data distribution can be seen, indicating that some cultures have high metabolic activity, some mid-range and some low metabolic activity. The biphasic and triphasic distribution can be explained by the plasticity of *C. albicans* life-stages, with of *C. albicans* cultures having mixed life-stage populations. The life-stages vary in metabolic activity, as measured by resazurin to resorufin conversion. The observed fluorescence data would therefore be expected exhibit a biphasic or triphasic trend, depending on the life-stage composition and cell stress. However, the timed assays intended to focus on one major life-stage. As the cultures were not fully synchronised, they would include exponential phase planktonic cells and stationary phase planktonic cells in assays I and III, young and mature biofilm cells, as well as hyphae in assays II and IV. This plasticity may lead to differences in adherence to pins, with variability compounded on the pins by differences in biofilm metabolism, maturation and shedding. Variability is also due to reversible nature of the initial immature biofilms and loss of metabolising cells from some of the pins. The dispersal of cells has been reported to be more extensive in RPMI1640 medium by Uppuluri *et al*.[5], who noted that richer growth medium seems to correlate with an increase in the number of cells dispersed/shed.

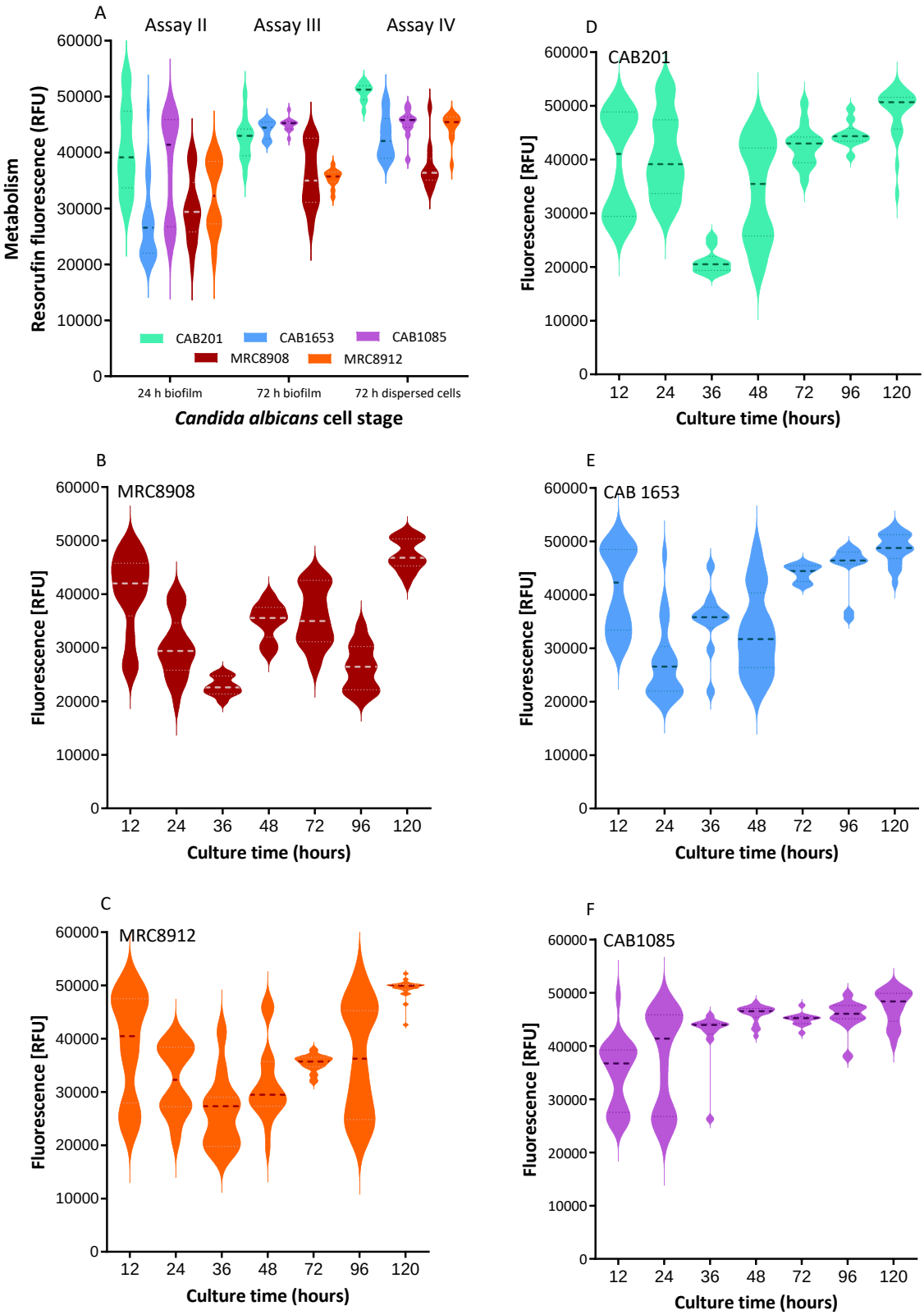

Figure 2.4 Metabolic activity during growth (resorufin fluorescence in RFU) of the biofilm formed on pins of five *C. albicans* strains (**B-F**) using the MPAS, as measured over 120 hours. Graph **A** indicates the variability within each strain of the three assay types and at the specific incubation periods, assay II referring to biofilm prevention, assay III referring to biofilm eradication and assay IV referring to shed/dispersed planktonic cell metabolism. Each violin plot shows the distribution of data from three cultures, each with at least six replicates, with the dotted line indicating the mean.

The phasic variability in measured biofilm metabolism in Fig. 4B-F is clearly illustrated for all the strains over the first 36-48 hours, with CAB1085 showing the least variability (Fig. 4B-F) .For the three environmental strains the biofilm attachment and metabolism stabilised after 48 hours with a steady increase in metabolism.

The metabolic response of the two clinical strains, MRC8908 and MRC8912, remained unstable or had variable metabolism on the pins over time, although their average metabolic response did increase over time indicating biofilm maturation (Fig. 2.4 B, C, Fig. 2.5). However, there was a high number of metabolising cells (30000-50000 RFU) and the variability was least at 72 hours (Fig. 2.4). This was ideal for the biofilm eradication control cultures in assay IV (refer to Fig. 2.1). The metabolism of dispersed cells was also checked at 72-hours and exhibited 35000-50000 RFU, that was suitable for the assay measuring dispersed cells (Fig. 2.4A, assay IV in Fig 2.1.

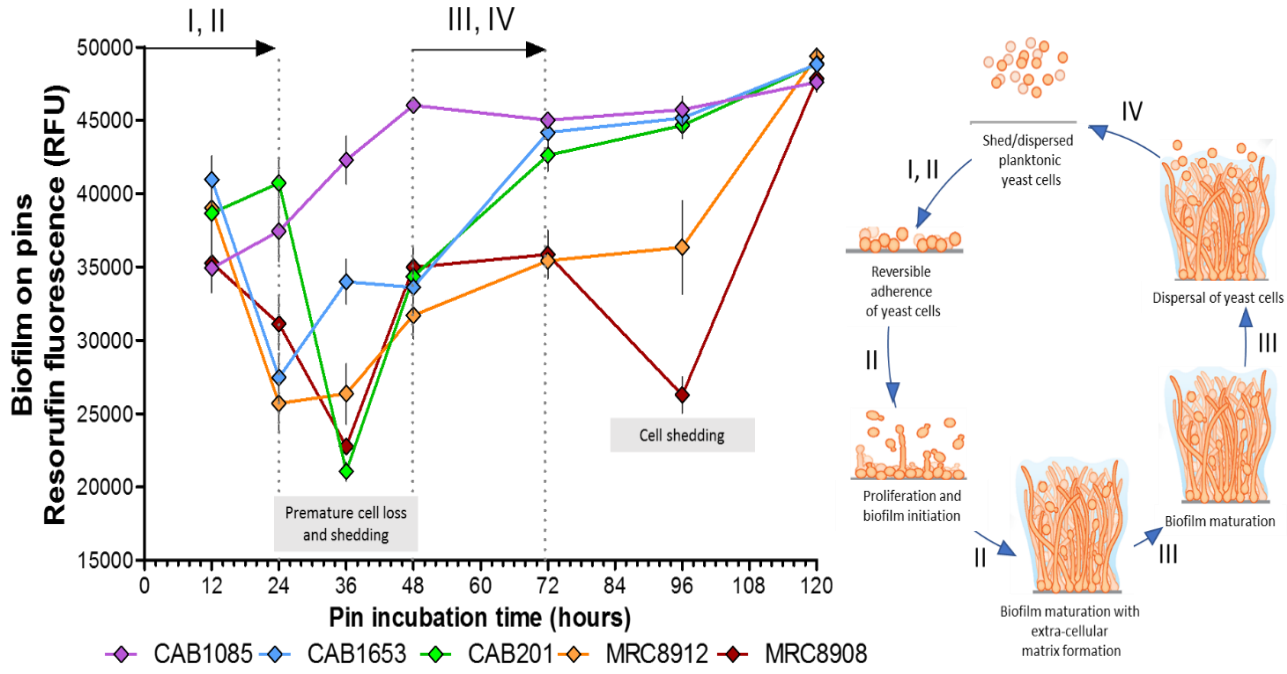

Figure 2.5 Averaged metabolism (resorufin fluorescence in RFU) of biofilms formed on pins by the five *C. albicans* strains, as measured from 12-120 hours of culturing. Each data point is the mean ± standard error (SEM) of 3 cultures with at least 6 replicates per culture. The cell cycle scheme on right is adapted from Nobile *et al*.[4] I, II, III and IV shows the correlation between the pin-attached biofilms' metabolism over time, a biofilm cell cycle and the biofilm assays performed as indicated in Fig. 2.1. Data is the averaged data depicted in Fig. 2.4.

Considering the good level of cell fluorescence above the background, depicted in Figs. 2.4 and 2.5, the decision was made to assess the metabolic response for assays I and II at 24 hours and assays III and IV at 72 hours after inoculation. Although it may not be the optimal time for all five of the *C.*

albicans strains, comparison of time-related response to antifungal compound is an important parameter in assessing activity.

Antifungal drug sensitivity of different *Candida* strains

We used the designed MPAS to assess the activity of commercial antimicrobial compounds (CAS, Flu and AmB as Amphocil® formulation) against the five strains and their different life-stages (Table 2.2, Fig. 2.6).

Table 2.2 Comparative summary of inhibitory parameters of caspofungin, fluconazole and Amphocil® against five strains of *C. albicans* as determined with the adapted resazurin assays. Prevention and eradication data from two conventional assays[25,26] are shown for caspofungin in brackets. The data is the average of 3-4 biological repeats with 2-3 technical repeats at each concentration in the dose response. Refer to Fig. 2.6 for typical dose response curves from which the parameters were calculated.

		***C. albicans* strains (values in μM ± Standard error of the mean)**				
Compound	Inhibition parameter	CAB201	CAB1653	CAB1085	MRC8908	MRC8912
Caspofungin	MIC_{50} Planktonic	2.5±0.4	4.3±0.3	2.5±0.1	6.3±0.2	5.2±0.5
	MIC_{50} Dispersed	8.2±2.2	6.8±0.9	7.5±0.0	13.7±1.1	6.9±1.8
	BIC_{50} (BIC_{50} [25,26])	18.3±1.7 (0.1±0.01)	23.7±1.6 (0.5±0.1)	19.2±2.6 (0.2±0.1)	10.7±1.0 (0.1±0.03)	4.2±0.3 (0.4±0.03)
	BEC_{50} (BEC_{50} [25,26])	6.1±1.6 (59.7±1.1)	6.9±1.5 (45.6±1.8)	21.1±2.3 (23.3±3.2)	10.2±2.5 (8.2±1.8)	3.3±1.6 (3.6±0.3)
Fluconazole	MIC_{50} Planktonic	>326	>326	~326	>326	>326
	MIC_{50} Dispersed	>326	~326	~326	~326	~326
	BIC_{50}	139±27	130±22	60.6±11	84.9±21	>326
	BEC_{50}	36.9±10	36.0±10	18.5±1.1	28.3±7.0	>326
Amphocil®	MIC_{50} Planktonic	5.4±2.7	3.4±1.3	1.2±0.2	1.8±1.4	6.1±1.4
	MIC_{50} Dispersed	1.7±0.6	1.6±1.5	3.0±1.4	4.6±3.6	7.1±6.0
	BIC_{50}	6.1±2.3	4.7±2.2	2.1±0.3	3.0±1.3	5.6±1.5
	BEC_{50}	5.6±2.2	0.9±0.2	3.1±1.0	1.5±0.9	1.8±0.9

MIC – minimal inhibitory concentration for planktonic cells or dispersed planktonic cells BIC – minimal biofilm inhibition (prevention) concentration at the biofilm forming growth stage

BEC – minimal biofilm eradication concentration of 48 h-old biofilm cells

When looking at the trends between compounds, it can be deduced that resistance seems to be most prevalent against Flu, especially against planktonic cultures. However, Flu still retained some biofilm prevention and biofilm eradication activity with eradication concentrations ranging from 19-37 μM. This is probably due to diffusion of smaller drugs into the biofilm, as well as a lower cell

count on the pins than in the well with planktonic cells. However, it could mean that planktonic cells and biofilm cells have a different profile of expression of resistance proteins, but this must be confirmed in future studies. BEC_{50} and BIC_{50} values higher than MIC_{50} values could indicate more resistant biofilms, as found for CAS against four of the five strains (Table 2.2). CAS showed good inhibitory activity against planktonic forms at MIC_{50} values ranging from 3 – 6 µM, while its BEC_{50} concentrations ranged from 3 – 21 µM, with higher BEC_{50} values raising some resistance concerns (Table 2.2). AmB showed potent inhibitory activity against planktonic forms at MIC_{50} values ranging from 1 – 6 µM and comparably low BEC_{50} concentrations also ranging from 1 – 6 µM (Table 2.2).

Overall, planktonic MIC_{50} values were all under 10 µM for AmB and CAS, with full resistance against Flu. The three compounds have different mechanisms of action. CAS is a semi-synthetic, water soluble lipopeptide which forms part of the echinocandin class of antifungals. It has been shown to inhibit (1-3) β-D-glucan synthase, which results in the disruption of the fungal cell wall as (1-3) β-D-glucan is a main cell wall structural component [6]. Resistance is due to point mutations in the β-glucan synthase subunit Gsc1p[38]. Our data extends previous research indicating susceptibility of *C. albicans* towards CAS and confirms that the planktonic MIC concentrations are insufficient against the biofilms [39]. The BEC_{50} of CAS ranged from 3.3 µM – 21.1 µM, which overall is higher than the planktonic IC_{50} values, as expected.

The planktonic stage of clinical strain MRC8908 exhibited the highest resistance against CAS, which is concerning as this a new generation antifungal (Table 2.2, Fig. 2.6A). The biofilm stage of environmental strain CAB1085 (a river isolate) had the highest biofilm eradication inhibition concentration of 21.1 µM (Table 2.2, Fig. 2.6E), which is as expected for a strain that evolved in an environment where the biofilm must be able to withstand mechanical forces. The clinical strain MRC8912 showed most sensitivity to CAS treatment with a BIC_{50} prevention value of 4.2 µM and a BEC_{50} value of 3.3 µM (Table 2.2, Fig. 2.6 C and E). The dispersed planktonic MIC_{50} values for CAS were overall higher than the normal planktonic MIC values, which confirms that dispersed biofilm cells may have a higher resistance to compounds such as CAS. This is concerning as CAS and the two other echinocandins (micafungin and anidulafungin) are currently the newest members of last resort antifungal drugs in clinical use[40]. However, one must consider that the cell numbers in the dispersed cell assay may be higher than that in planktonic assay leading to higher inhibition concentrations.

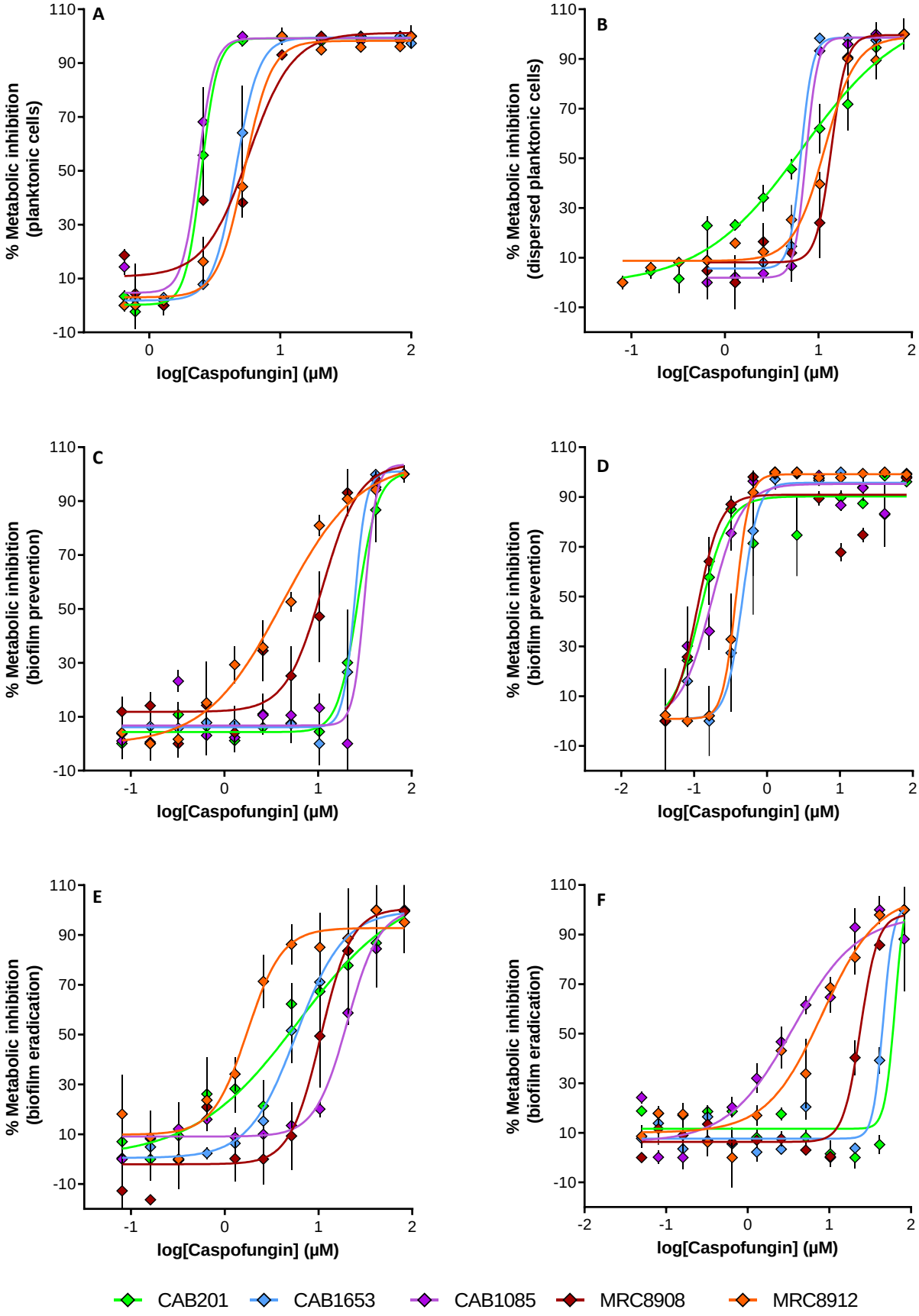

Figure 2.6 Dose response curves displaying the activity of CAS towards planktonic cell growth inhibition (**A**), dispersed planktonic cell growth inhibition (**B**), biofilm prevention (**C** and **D**) and biofilm eradication (**E** and **F**) of the five *C. albicans* strains. A, B, C and E were recorded using the MPAS (Fig. 2.2) whereas data for D and F were recorded using the conventional biofilm prevention and eradication assays [28]. The data show the average of six technical repeats ± SEM at each concentration.

The most concerning result of this study is some level of Flu resistance that was observed for both the environmental and clinical strains, especially towards planktonic cells (Table 2.2). Flu is a bis-triazole antifungal agent that binds to fungal cytochrome P-450, disrupting fungal membranes by interrupting the conversion of lanosterol to ergosterol[41]. Resistance to this compound is primarily due to mutations in the ERG11 gene, which codes for 1,4α-demethylase, preventing the azole drug from binding the enzyme[41]. The resistance to the azole group can also be caused by gene rearrangements or aneuploidy which affects the expression of transcriptions factors, drug targets and efflux pumps[38]. No planktonic and dispersed planktonic MIC_{50} values were found under 325 µM, but the data does indicate that biofilm forms are less resistant to Flu than their planktonic counterparts. This is an interesting finding and could be MPAS related. Similar planktonic cell resistance and biofilm sensitivity towards AmB was previously reported by Al-Fattani *et al*.[10,42], and could possibly also be the same phenomenon caused here by Flu where low planktonic growth rates can be linked to the observed drug resistance. The data could indicate that resistance is due to a very low growth rate of these planktonic cells compared to their biofilm counterparts. Alternatively, these planktonic cells could be expressing CDR1, CDR2 and/or MDR1 genes associated with efflux pumps, which have been found to cause drug resistance when employed during cellular detoxification in biofilms[43,46]. The MPAS assay must also be considered as the lower BEC_{50} and BIC_{50} compared to the Flu MIC_{50} could be due to the lower cell count on pins and better diffusion of the smaller Flu into the biofilms. Furthermore, drug resistance is a complex phenomenon and more than one resistance mechanism could be employed at one time[43]. There is much evidence supporting the resistance conferred from biofilms to their planktonic counterparts[43] as is evident by the high concentrations required to inhibit dispersed planktonic cell inhibition (Table 2.2).

We also assessed the sensitivity of the five strains towards the well-known polyketide antifungal drug, AmB, which was originally isolated from *Streptomyces nodosus* cultures in 1953[44,45]. The mechanism of action of AmB is membrane disruption by binding to ergosterol (the fungal sterol), for which it has a higher affinity compared to that for cholesterol (mammalian sterol)[44]. This binding to sterols results in membrane pore formation, leakage of monovalent ions, disruption of the proton gradient across fungal membranes and ultimately in cell death [46]. Resistance to AmB is acquired through mutations in ERG3, which leads to a decrease in the concentration of ergosterol in the cell membranes[38]. When considering the AmB data obtained during our study, the most resistant planktonic strain seems to be clinical strain MRC8912, followed by the veterinary strain CAB201. Furthermore, veterinary strain CAB201 required the highest inhibitory concentrations of AmB when it occurred in a biofilm, showing a BIC_{50} value of 6.1 µM and a BEC_{50} of 5.6 µM. Environmental

strains CAB1653 and CAB1085 were sensitive to AmB treatment with a BEC_{50} value of 3.1 µM and 1.5 µM, respectively. However, the activity of AmB was still high and confirmed the maintained potency of AmB to combat *C. albicans* infections by various strains.

On closer inspection of inhibition results on the five strains we found that compared to their planktonic counterparts, the biofilm and associated dispersed cell populations were generally more resistant to CAS and more sensitive to Flu for four of the five strains (Table 2). However, the five strains exhibited similar or higher sensitivity to AmB (Table 2). The observed higher CAS resistance of biofilms and similar sensitivities of the life-stages to AmB was comparable to trends found in other studies using a conventional assay[25,39]. This could relate to biofilm cells and dispersed cells having distinct phenotypic properties associated with increased virulence[5]. The drug concentration required to eradicate biofilms and that required to prevent biofilm formation vary from strain to strain due to variable metabolic states, biofilm cell numbers and biofilm stability (refer to Table 2). The planktonic cells increase in cell density and form young, reversible biofilms secreting extracellular matrix compounds[4] during the first 12-24 hours (refer to Fig. 3), whereas the mature biofilms shed planktonic cells[4] during the first 24-48 hours, which is strain dependent (refer to Fig. 5).

Comparison of MPAS Assays II and III with conventional biofilm prevention and eradication assays [25,26] revealed differences in the values obtained for CAS BIC_{50} and BEC_{50} as well as the for the dose response trend. It is postulated that these differences are due to the surface area of biofilm exposed to the antifungal compounds, as well as the incubation time for eradication (Fig. 6). Using the conventional assays[25,26], the biofilm is formed at the bottom of a round-bottom 96-well plate leading to a thicker biofilm layer than on the liquid suspended pins. The difference in biofilm thickness and stability on the pins versus the biofilms and shed cells that settled on the well bottom would influence the target concentration and accessibility, both of which influence the inhibition concentration. Furthermore, biofilms in our Assay III were 72 hours old at the time of growth inhibition recordings, but only 48 hours old when results were recorded in the conventional assay [25,26]. Both biofilms were 24 hours old when prevention data is recorded. Interestingly, the BIC_{50} values calculated using the conventional assays[25,26] on the same five *C. albicans* strains in these experiments showed low concentrations to prevent biofilm growth with significantly higher concentrations to eradicate biofilms. The trends of the conventional biofilm prevention assay correspond better with what we have determined in our planktonic growth inhibition assay. On the other hand the trends from the conventional biofilm eradication assay[25,26] correspond better with

that of our biofilm prevention assay (compare Fig. 2.6 C and F). The data collected comparing these two assay methods on the same strains highlights the importance of comparison and standardisation of assay methods for antibiofilm studies.

Conclusion

Our MPAS data on five *C. albicans* strains confirmed there are discernible differences between the strains in terms of growth and life-stage sensitivity to three of the current antifungal drugs in clinics. The most concerning result in this study was that all five the *C. albicans* strains in their planktonic life-stage showed pronounced azole resistance, although all were still sensitive towards AmB and CAS. The planktonic life-stages of two *C. albicans* clinical strains, compared to the veterinary strain and two environmental strains in this study, presented moderate resistance towards CAS. Moderate resistance towards AmB was also found for the planktonic life-stages of a clinical strain and the veterinary strain, compared to the other three strains. We hypothesise that this resistance is most probably due to these clinical and veterinary strains inhabiting ever-changing environments with continual changes in nutrition, oxygen, blood flow, microbial competition, and immune system interference. Interestingly, both environmental strains and the veterinary strain presented increased resistance against antifungals in their biofilm forms. It can be assumed that the more challenging environments in terms of mechanical removal, such as rivers that are also probably contaminated with fungicides resulting from agriculture, force these strains to survive optimally in biofilm forms and develop antifungal resistance.

Inter-strain differences and assay type, as well as mode of action(s) of various compounds should be considered individually when experimental data are assessed. Furthermore, biofilm surface areas, medium compositions, biofilm age and incubation periods all have effects that need to be accounted for and assessed before continuing with experiments associated with a specific strain or compound. From our results the combination of conventional planktonic assays with our multiplex antibiofilm assay provides a comparative activity profile, especially giving three parameters related to activity against biofilms in an environment closer to what is found in for example catheters. Our MPAS assay system can be used to follow the inhibitory concentration range trends of antifungal compounds towards different phenotypic and growth stages of *C. albicans*. We gained insight in life-stage sensitivity to drugs and how resistance may be alleviated by drug combinations. For example, if Flu is combined with CAS or AmB, resistance towards both planktonic and biofilm life-stages could be alleviated. Drug activity parameters towards the different phenotypes of biofilm forming organisms are important and MPAS would be able to easily identify effective drug combinations and

fast-track the compound hit-to-drug lead in drug discovery projects. Our adapted and multiplex assay combination can be used to give a quantitative, quick, reproducible, and reliable indication of activity with regards to antimicrobial compounds and their effect on both planktonic, dispersed planktonic and biofilm *C. albicans* forms. These assays in MPAS can facilitate more targeted, life-stage specific identification of novel compounds such as possible antibiotics and antifungals from large compound libraries.

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CHAPTER 3

Multiplex assay determination of micro-culture extract activity towards different life-stages of *Candida albicans*

Abstract

This study describes the optimisation of a multiplex assay system (MPAS) to utilise for reproducible assessment of *Candida albicans* planktonic cell and biofilm susceptibility to bacterial culture extracts from known antimicrobial producers. MPAS comprises of four medium-throughput assays that can be used to study various yeast life-cycle stages. MPAS is an inexpensive and reproducible 96-well pin-lid assay system, using a metabolic dye to indicate compound activity in terms of 1) planktonic cell susceptibility, 2) biofilm prevention, 3) biofilm eradication and lastly 4) dispersed planktonic cell susceptibility, once shed from the pins. MPAS for microbial extracts was optimised by testing the activity of extracted antimicrobial compounds from known producer organisms on the various *C. albicans* life-stages. Antimicrobial producer organisms were allowed to grow and produce for 10 days on nutrient agar, after which the active compounds were extracted using an organic solvent. After extraction, compounds were freeze-dried in 96-well plates and then assayed against planktonic and biofilm forms of *C. albicans* MPAS described in Chapter 2. Statistical parameters confirmed that the planktonic and biofilm eradication assay designs were more effective than the biofilm prevention assay design. However, multiple repeats allowed for representative population dispersions and the data from all four assay designs resulted in reproducible results. Furthermore, the obtained data showed not only which producer products were more active against certain strains of *C. albicans* but could also highlight which life-stages were more affected by certain producer compounds. This multiplex assay system allowed for a large amount of scientific information to be obtained simultaneously; 1) information about the sensitivity of the target organism being tested, 2) the antifungal sensitivity differences between various life-stages of the various target organisms, 3) extracted compounds from producer organisms and their activity on fungal stages, and 4) production differences between organisms producing similar compounds.

Introduction

Antonie van Leeuwenhoek noted “The number of these animalcules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom”[1]. However, the word “biofilm” was only coined by Bill Costerton in 1978 and is explained as a community of structured cells embedded in an extracellular polymeric matrix[2,3]. The formation of a biofilm is as follows: 1) the development of a surface conditioning film; 2) reversible and irreversible attachment of cells to a surface; 3) formation of microcolonies; 4) maturation and differentiation of the biofilm with expression of matrix polymers; 5) dispersal of cells from the biofilms[4]. The development of a surface conditioning film is a prerequisite for bacterial and fungal biofilm growth as it improves the physical and chemical properties of the substratum. This film consists of various humic compounds, polysaccharides and glycoproteins, providing the necessary nutrients and trace elements for colonization[4]. These surfaces to which biofilms attach, affect industries across the globe, such as the medical, veterinary, manufacturing and the agricultural industries[3,5,6].

Fungal biofilms are an escalating clinical problem, of which the most notorious pathogen is *Candida albicans* (*C. albicans)*, causing high mortality rates[7]. Fungal cells proliferate on a surface into filamentous forms (hyphae), resulting in a more cohesive and uniform biofilm[7]. Unlike their bacterial counterparts where dispersal is frequently a terminal process, marking the end of the biofilm life cycle[8], dispersal of fungal biofilm cells occurs throughout the life cycle and is not indicative of the end of the biofilm life cycle[8]. It has been observed that fatal fungal systemic infections are due to these planktonic forms dispersing from the resistant biofilm fungi, disseminating into the bloodstream[8]. These life-threatening *C. albicans* biofilms are most often found in biomaterial-related substances like catheters, prosthetics and on implant surfaces[7]. Although some currently available antifungals display antibiofilm activity at very high concentrations (amphotericin B and fluconazole)[9] compared to planktonic cells, *Candida* biofilms were observed to be resistant to the these clinically available and important antifungals[10]. Therefore, new antibacterial and antifungal compounds need to be discovered and developed, especially compounds with activity against *Candida* biofilms. An avenue that is being explored for these novel antifungal compounds is naturally occurring microbes found in soil, which have the potential for biomining exploration. In this study we included six such soil organisms, as test organisms producing known antimicrobial peptides for optimising the previously developed multiplex assay system (MPAS) in order to find anti-*Candida* hits in microbial extracts. These selected organisms and their antifungal compounds are given in Table 3.1.

Table 3.1 Summary of origin and possible antimicrobial compound production profiles of six bacterial producer strains used in assays in this study.

Name	Abbr	Origin	Antimicrobial metabolite
Brevibacillus parabrevis[11-19] ATCC8185; DSMZ362; DSMZ5618;	8185 362 5618	Soil organisms ATCC (Manassas, Virginia, USA) DSMZ (Braunschweig, Germany) DSMZ (Braunschweig, Germany)	Tyrothricin complex consisting of Tyrocidines (TrcA/A_1; TrcB/B_1; TrcC/C_1) Tryptocidines (TpcA/A_1; TpcB/B_1; TpcC/C_1) Phenycidine A/A_1 Gramicidins (VGA, IGA, VGB, IGB, VGC, IGC)
Aneurinibacillus migulanus[24-25] ATCC9999	9999	Soil organism ATCC (Manassas, Virginia, USA)	Gramicidin S and analogues with single and double Lys analogues
Bacillus subtilis[31] ATCC6633	6633	Soil organism ATCC (Manassas, Virginia, USA)	Surfactins and unknown antimicrobial peptide(s) and metabolites
Bacillus subtilis[31] ATCC21332 (*sfp*)	21332	Soil organism BGSC (Ohio State University, OH, USA) and ATCC (Manassas, Virginia, USA)	Surfactins (natural production)

The antimicrobial extracts from known bacterial producers, such as the tyrothricin complex containing the tyrocidine peptide complex/mixture (Trc mix), gramicidin S (GS) and the surfactin complex (Srf) was utilised to validate the multiplex assay system (MPAS) methodology due to their known activity against bacterial and/or fungal pathogens. One of the known antifungal mixtures is the tyrothricin complex, which is a combination of antibiotic cyclic and linear peptides[11]. These antifungal compounds from the soil bacterium *Brevibacillus parabrevis* (*Br. parabrevis,* formerly known as *Bacillus brevis*) were first discovered in 1939[12,13]. Tyrothricin is separated into two antimicrobial fractions namely neutral, linear pentapeptides referred to as gramicidins and basic, cyclic decapeptides referred to as tyrocidines, tryptocidines and phenycidines[11] or Trc mix. Since the discovery of tyrothricin AMPs[12,14], they have been shown to possess potent antimicrobial activity against various bacteria[15,16], *C. albicans*[17,18`] and filamentous fungi[19,20]. As tyrothricin has been shown to have potent broad spectrum antifungal activity we opted to use three tyrothricin producers in our multiplex assay validation[17–19] for using in bio-mining of soil organisms with anti-fungal and antibiofilm activity (Table 3.1), as well as GS producer and two Srf producers.

Gramicidin S was discovered after World war 2 in the USSR and even though it has strong haemolytic activity it is still used successfully in a topical applications to treat skin and throat infections[16,21–23].

GS is produced by the Gram-positive soil isolate[24] *Aneurinibacillus migulanus* ATCC9999, originally known as *Bacillus brevis*[25]. Gramicidin synthetase catalyses the biosynthesis of its peptide by means of a thiotemplate mechanism[26] and has growth inhibitory activity against pathogens such as *Escherichia coli* and *Pseudomonas aeruginosa*[27,28], as well as Gram-positive staphylococci and enterococci. Structurally, GS is a stable, antiparallel, amphiphilic β-sheet peptide that exhibits a polar and non-polar surface, which is proposed to be linked to its antimicrobial activity[29]. It is a cyclic decapeptide with a repeating pentapeptide, and has a primary structure of [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$]. Studies have shown that the basic nature of the two ornithine residues are essential for the antimicrobial action of GS, as well as the hydrophobic nature of the leucine and valine residues[29]. Research suggests that GS disrupts lipid packaging and leads to membrane collapse but it is not yet clear if it definitely forms pores or works in a detergent-like manner to destroy the membranes[16,29].

There are many examples of antifungal lipopeptides from soil organisms such as the iturins, fengycins and Srf, to name a few. However, one of the most studied and broadly detected lipopeptides in soil is the Srf complex[30]. The Srf complex contains analogous cyclic lipopeptides which are anionic and biodegradable also from bacterial origin (*Bacillus subtilis*) with Srf discovery dating back to 1968[31]. In solution, Srf's activity is largely thought to be linked to its horse-saddle conformation[31] and detergent-like action. It destabilises and disrupts membranes by dimerizing into the bilayer and the extent of perturbation of the bilayer can be correlated to the concentration of Srf[32].

With the potential of novel antifungal producers from soil and other microbiomes there is a need for high-throughput screening methodologies, specifically to find compounds with activity against all the life-stages of *C. albicans*, as well as a broad spectrum of activity against different *C. albicans* isolates, especially targeting their biofilms. Traditional techniques have resulted in a continuous search for better methods of culturing bacterial soil species due to the various limitations, such as replicating the soil environment[33,34]. Many hypothesize that less than 1% of the soil microbiome (amongst other microbiomes) can be cultured using these traditional *in vivo* techniques[34–36], which is referred to as the "great plate count anomaly"[37]. Briefly, this anomaly pertains to the difference in order of magnitude of colony forming cells from a natural environment, compared to the number of cells countable using a microscope. Soil carries the largest populations of microbes of any habitat. Furthermore, there are believed to be 61 bacteria phyla, of which 31 phyla have no cultivable representatives[35]. Modified medium and culturing conditions are often incapable of mimicking the endogenous abiotic and biotic conditions favouring bacterial growth[33,38,39]. Therefore, we need to

develop methods of cultivating, which allows for systems to mimic natural growth conditions. These conditions include 1) the chemistry of the microbe's natural environment; 2) interactions of the biotic and abiotic factors; 3) the microbial diversity and inter-microbial interactions; 4) the effects of climate change and environmental changes at microbial level. Various factors that need to be taken into account include temperature, growth medium, incubation time, pH, inoculation size, colony density and air conditions[33]. It is also important to note that microbes establish a relationship with other microbes in a community. These interactions can either result in competition for limited resources or co-operation through exchanges of metabolites and signalling molecules[33,35].

The main goal in this study is a robust multiplex assay system that allows for quick and efficient testing of micro-culture extracts to find compounds targeting *C. albicans* planktonic and biofilm life forms. Our methodology entails growth of micro-cultures in 96-well plates, extraction of cultures, electrospray mass spectrometry (ESMS) of culture extracts and assessment of activity with a classical broth assay for planktonic growth assessment, as part of a multiplex assay methodology that includes biofilm growth assessment (Fig. S3.1 in supplementary data, refer also to Chapter 2). The complexity of the data from MPAS and such multiplex assays must be considered, as there are various factors affecting the *Candida* cells and the antifungal producer microbes. In terms of the Candida-affecting factors, the stability, sensitivity to compounds produced by test-organisms and growth of each of the five strains in planktonic and biofilm life-stages need to be considered. Controllable factors include cell number, media type, incubation temperature and culture time. In terms of the producer-affecting factors, the number of cells, production success and extraction effectiveness are most difficult to consistently control. Controllable factors included medium type, incubation temperature and time, as well as extraction method.

MPAS (Fig. S3.1, Chapter 2) was developed to test various compounds against three *C. albicans* life-stages in parallel namely: 1) activity against planktonic *C. albicans* cells; 2) activity that can prevent the formation of *C. albicans* biofilms on a pin-lid; 3) the removal and/or eradication of established *C. albicans* biofilms from a colonized pin-lid surface as well as 4) the growth inhibitory effect of antimicrobial compounds on the dispersed biofilm cells shed from the pin-lids. This multiplex assay system utilises metabolic activity recordings to indicate if cell growth and biofilm colonization was affected by the activity of extracted compounds (Fig. S3.1). Unaffected cells would continue to be metabolically active and result in the mitochondrial reduction of the permeable and non-toxic dye, from the non-florescent (blue) resazurin (also known commercially as Alamar Blue®) to fluorescent (pink) resorufin[40,41]. This reaction in metabolically active cells occurs due to the reduction of

resazurin to resorufin, whose fluorescence is proportional to the number of metabolically active cells in the cell culture[40]. Alternatively, cells negatively affected by the extracted compounds will not reduce resazurin and therefore the dye will remain oxidised and blue/non-fluorescent.

In this study, we demonstrated the utilisation of 96-well micro-culture production and extraction of antifungal compounds, testing the extract activity using our novel 96 pin-lid MPAS (Fig. S3.1) to simultaneously test the susceptibility of planktonic and biofilm *C. albicans*. These classical and multiplex assays can be used to determine if test compounds can inhibit growth of planktonic cells, if test compounds can prevent biofilm formation on a pin-lid, if test compounds can eradicate biofilms from pin-lids and if biofilm cells eradicated from pin-lids, have experienced growth inhibitory activity due to the presence of extracted/test compounds (Jenkins *et al*., unpublished data; Chapter 2). Our results showed that this novel combined MPAS can be used to give quick, qualitative results that can be used for bio-mining exploration of soil samples in the discovery of potent anti-*Candida* compounds. MPAS is relatively inexpensive compared to the various other assay protocols and the assay system gives an indication of an extract's anti-planktonic and anti-biofilm activity. Furthermore, MPAS and associated methodology further allows direct analysis of an active extract using high resolution electrospray mass spectrometry.

Materials and methods

Materials

The fungal strains *Candida albicans* were obtained from the yeast culture collection, courtesy Prof Alf Botha, of the Department of Microbiology, University of Stellenbosch (Table 3.2). The bacterial producer strains tested in this study are listed in Table 3.1. *Bacillus subtilis* 168 and *B. subtilis* OKB120 (pheA1 sfp srfA:Tn917) were obtained from BGSC (Ohio State University, OH, USA). Gramicidin S, surfactin complex and tyrothricin complex were purchased from Sigma Aldrich (Steinheim, Germany). The gramicidin S and tyrocidine mixture was prepared as previously described[42–44]. Analytical quality water was prepared by reverse osmosis followed by filtration through a Millipore MilliQ® water purification system (Milford, USA). Ethanol (99.9%) were obtained from Merck Chemicals (Pty) (Wadeville, Gauteng). The yeast extract powder, peptone powder, D(+)-glucose anhydrous and agar powder for the yeast peptone dextrose broth (YPDB) and yeast peptone dextrose agar were from Biolab Merck (Wadeville, Gauteng). The non-sterile polystyrene plates were provided by Greiner bio-one (655101; Germany). The non-sterile NUNC polystyrene plates with fitted NUNC-TSP (Transferable Solid Phase Screening System) pin-lids were supplied by Thermo

Fischer Scientific (Denmark). The 96-well plates and pin-lids were sterilised by exposure to chloroform vapours from Merck Chemicals (Pty) (Wadeville, Gauteng) for 15 minutes before use or as described below. Sterile culture dishes and microtiter plates were obtained from Corning Incorporated (USA) and sterile Petri dishes from Lasec (Cape Town, South Africa).

Table 3.2 The origin and GenBank accession number of each of the *C. albicans* isolates used in this study as target organisms in the MPAS.

Species	Strain	Origin	GenBank Accession Number
Candida albicans	MRC8908[a]	Clinical Isolate, Tygerberg Hospital	KJ534504
Candida albicans	MRC8912 [a]	Clinical Isolate, Tygerberg Hospital	KJ534505
Candida albicans	CAB201	Veterinary Isolate, *Columba livia domestica*, South Africa	MK248726
Candida albicans	CAB1653	Environmental Isolate, Mosquito Larvae, South Africa	Not available
Candida albicans	CAB1085[b]	Environmental Isolate, Plankenburg River, South Africa	KJ534503

[a] Clinical isolates MRC8908 and MRC8912 were previously stored in the Program on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit culture collection of the South African Medical Research Council.

[b] Isolate obtained from a river that is known to be polluted with sewage.

Pure peptides and micro-culture extracts

Preparation of plates with pure peptides

Test compounds for the four assays were prepared using three known antifungal preparations, GS, Trc mix and Srf. GS was prepared to an initial stock concentration of 1 mg/mL by rehydration with MilliQ water. The initial stock solutions were used for eight doubling dilutions in triplicate from 100 µg/mL to 0.78 µg/mL, of which 10 µL of each added to the wells of the 96-well assay plates. Trc mix and Srf were prepared using 60% EtOH to stock solutions of 2 mg/mL, which was then further used as explained above with additional 1.5% EtOH (final EtOH concentration in assay) growth controls. The plates were sterilised by exposure to chloroform vapours for 15-20 minutes, followed by UV exposure for 30 min. Three to four empty wells were left for sterility controls (blank) and three to four growth controls (no inhibition). An aliquot of 10 µL GS at 100 µg/mL was added to three or four wells/plate as positive inhibition control after plate sterilisation.

Micro-culture extraction of antimicrobial peptides and compounds

B. subtilis 168 (abbreviated as 168) was used as negative soil isolate control. A secondary control for *Bacillus* species and Srf production used in this study was *B. subtilis* OKB120 (pheA1 sfp srfA: Tn917,

abbreviated as 120), a mutant of *B. subtilis* 168 with interrupted surfactin operon between modules 4 and 5. The six different peptide producing strains (Table 3.1), 168 and 120 were propagated by streaking onto 30-40^{o} slanted tryptone soy broth (TSB) agar in a 96-well plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 655101). Inoculated plates were incubated in a humidity chamber at 37˚C for 7-10 days for the production of antimicrobial peptides. After incubation the plates were placed in a freezer at -80˚C for 24 h. Once removed from the freezer, plates were allowed to adjust to room temperature (RT). To each micro-culture, 200 µL of 75% acetonitrile in analytical quality water (*v/v*) was added and the stationary plates incubated at 25˚C for 3 h, allowing for extraction. Plates were centrifuged at 2000 × *g* for 15 min and 150 µL supernatant transferred to a new 96-well assay plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 442404). The extracted micro-culture compounds (supernatant) were lyophilized in this 96-well assay plate overnight and stored at 4˚C until used for activity testing against *C. albicans* (method adapted from Laubscher and Rautenbach[45]). The plates were sterilised before use in chloroform vapours and under UV, as described above.

ESMS analysis of micro-culture extracts

The success of production for each micro-culture was evaluated by high resolution mass spectrometry of the culture extracts. The presence of the expected peptides and non-active background metabolites and contaminants in the extracts was confirmed using direct injections on a Waters Synapt G2 triple quadrupole time-of-flight mass spectrometer (Milford, MA, USA) with an electrospray ionisation source. Samples were exposed to a capillary voltage of 2.5 kV, a cone voltage of 15V and source temperature of 120°C. Desolvation was achieved with nitrogen gas (650 L/hour) and 275°C desolvation temperature. All higher resolution data was collected in positive mode with continuum scanning over an *m/z* range of 300-2000. Mass spectrometric data was analysed using MassLynx V4.2.

Anti-Candida multiplex assay system

C. albicans culturing conditions

Two clinical strains of *C. albicans,* MRC8908 and MRC8912, two environmental strains; CAB1085 and CAB1653 (mosquito larvae isolate), and one veterinary isolate, CAB201 (Table 3.1) were revived from glycerol stocks (30%) stored at -80˚C, by plating onto 1% yeast 2% peptone and 2% dextrose (YPD) agar Petri-dishes and the resulting Petri-dishes were incubated at 37˚C for 24 h before overnight cultures for each experiment were started. Overnight cultures were incubated at 37˚C in

YPD broth rotating on an orbital shaker at 150 revolutions per minute (rpm) for 14-18 hours. After overnight incubation, cultures were sub-cultured and diluted in un-supplemented Roswell Park Memorial Institute 1640 (RPMI1640) medium at pH 7 to mid-log OD_{600nm} and diluted to specific cell concentration for the various assays and experiments.

C. albicans planktonic cell inhibition *(Assay I)*

Candida albicans cells in planktonic form were sub-cultured in RPMI1640 to mid-log OD_{600nm} and diluted to an initial inoculum size of 5.5×10^5 CFU/mL. The 96-well assay plates (Thermo Fisher Scientific-Nunc 96 Flat Transparent 655101) with test compounds or micro-culture extracts were set-up as described above, including 100 µg/mL GS as positive inhibition control. After the 96-well assay plates with extracted compounds were allowed to adjust to room temperature and sterilised, assay I was started by rehydration of compounds in each well with 100 µL planktonic *C. albicans* culture in RPMI1640. Alternatively, 90 µL of planktonic cells in RPMI1640 were added to the 10 µL of the purified peptides prepared in the 96-well plates for dose response assessment. After a 24 h incubation at 37˚C in a humidity chamber, 10 µL resazurin solution was added to each of the wells and incubated in the dark (foil wrapped) at 37˚C for 15-30 minutes. Detection of conversion of resazurin to resorufin was done using a Tecan Spark 10M multimode microplate reader, temperature controlled at 37˚C and the fluorescence was recorded with excitation measured at 560 nm and emission measured at 590 nm (also refer to Chapter 2, Fig. 3.2).

C. albicans biofilm prevention *(Assay II)*

Planktonic *C. albicans* cells were cultured and the 96-well assay plates with extracted compounds or pure peptides were set up as described above. In the 96-well assay plate, 100 µL of planktonic cells in RPMI1640 was used to rehydrate the compounds in the wells and assay II was started by placing a 96 pin-lid on each assay plate. Alternatively, 90 µL of planktonic cells in RPMI1640 added to the 10 µL of the purified peptides prepared in the 96-well plates for dose response assessment in assay II. The plates with pin-lids were incubated in a stationary humidity chamber at 37˚C. After a 24 h incubation period, the 96-well assay plate pin lid was transferred to a new 96-well plate (biofilm prevention plate) containing 90 µL RPMI1640 and 10 µL resazurin solution. The prevention plate was incubated at 37˚C for 120-180 min (depending on *C. albicans* strain), covered in foil and the fluorescence in assay II was recorded as described above (also refer to Chapter 2; Fig. 3.2).

C. albicans biofilm eradication *(Assay III*) and biofilm shed cell inhibition *(Assay IV)*

To perform the eradication assay, planktonic *C. albicans* cells were sub-cultured in RPMI1640 to OD=0.30 at 600 nm. From this sub-culture 100 µL was pipetted into each well of a 96-well plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 442404) in which a 96 pin-lid was placed. The 96 pin-lid and plate combination were incubated at 37˚C in a stationary humidity chamber for 48 h, allowing for biofilm formation/maturation on the pins. The plates with compounds or peptides were prepared as before and hydrated with 100 µL RPMI1640 or mixed with 90 µL RPMI1640 respectively. To start assays III and IV the 96 pin-lid with 48-hour old biofilms on pins were placed into the plates with media, compound/peptides. The mature biofilms on pins (96 pin lids) were incubated in the compound plates in a stationary humidity chamber at 37˚C for 24 h. The pin-lid with remaining, treated biofilm was placed in a new 96-well plate containing 90 µL RPMI1640 media with 10 µL resazurin dye and incubated at 37˚C for 120-180 min to assess biofilm eradication (assay III). The compound plates with shed biofilm cells were incubated at 37˚C for 20-30 min, after addition of with 10 µL resazurin dye to each culture to assess the biofilm shed cell inhibition (assay IV). Fluorescence in both assay III and IV plates were recorded as described above (also refer to Chapter 2; Fig. 3.2).

Data analysis and statistics

Various statistical parameters were calculated to substantiate and use the collected producer data correctly and to the full benefit and improvement of the assay design. The Z-factor is a mathematical equation that is used to compare or regard in unison various high-throughput or screening assays. The equation to calculate the Z-factor is shown below (Equation 1).

Eq. 1	Z'-factor	$Z' = 1- [(3SD_+ + 3SD_-) / (Ave_+ - Ave_-)]$
Eq. 2	Signal-to-noise	$Median_+ - Median_-/Median\ Absolute\ Deviation_-$
Eq. 3	Signal-to-background	$Median_+ - Median_-$
Eq. 4	Coefficient of variation	$(Standard\ Deviation/Mean) \times 100$
Eq. 5	Assay Variability Ratio	$3\times (Standard\ Deviation_+ + Standard\ Deviation_-)/ Mean_+ - Mean_-$
Eq. 6	Signal Window	$[(Mean_+ – Mean_-) - 3\times (Standard\ Deviation_+ + Standard\ Deviation_-)] / Standard\ Deviation_+$

The signal-to-noise and signal-to-background ratios were calculated (Equation 2 and 3 respectively) as well as the assay variability ratio indicated by Equation 5, coefficient of variation (%) of equation 4 and signal window (Equation 6) calculated to substantiate the assay eligibility. To discern the cut-

off values of the assay, the inter-quartile ranges were calculated. The number of positive hits and negative samples were calculated from the recorded producer data (Table S3.1; Figs. S3.5-S3.9).

Results and discussion

Multiplex activity testing of known antifungal peptides from soil bacteria

Six bacterial producer strains were selected for testing micro-culture extracts with MPAS, because they produced three groups of antimicrobial peptides with known antifungal activity (Table 3.1). We tested the three purified antifungal peptides/complexes, GS[14,42], Trc mix[18,47] and Srf complex[31], with MPAS to assess their dose-dependent response against the five *Candida* strains (Table 3.3, Figures S3.2-4 in supplementary data).

Previous results obtained from our MPAS study on five *C. albicans* strains (Table 3.2), described in Chapter 2, showed that their biofilms are more resistant to eradication using known antifungals such as caspofungin, as also reported in literature[46]. Planktonic cells that have been shed from the biofilms by these strains have also shown a definite increase in concentration ranges required to inhibit their growth when working with the commercially available antimicrobials as also reported in Chapter 2. The activity of GS and Trc mix towards biofilms and shed planktonic cells showed similar trends (Table 3.3). For GS all inhibitory values against *C. albicans* (planktonic cells and biofilms) fell under 5 µM, except for MRC8908 with biofilm prevention concentration of 5.6 µM (Fig. S3.2C). Planktonic CAB201 had the highest planktonic MIC_{50} of 4.0 µM (Fig. S3.2A) and MRC8908 having the highest dispersed MIC_{50} value of 4.9 µM (Fig. S3.2B) and highest biofilm eradication concentration of 3.1 µM (Fig. S3.2C). From these results (Table 3.3) it can be deduced that MRC8908 was the most resistant to GS activity, when comparing data from these five strains. The biofilm inhibition parameters of GS were for some strains lower than those obtained with the conventional broth assays[17], specifically when considering the biofilm eradication (Table 3.3). This could because the MPAS has less biofilm surface area on the pins that come into contact with active compounds, when comparing the methodologies using a larger contact area of the side and bottom of a well as biofilm attachment area. Due to the potent activity of GS over a broad concentration range against all the *Candida* strains, we selected GS as a positive inhibitory control and its producer for testing the micro-culture extracts in our multiplex assay system (Table 3.3; supplementary data, Fig. S3.2).

The second group of peptides tested, were the Trc mix, with 50% sequence identity to GS and known activity against *Aspergillus fumigatus*[43] and *C. albicans*[17,47]. From the data shown in Table 3.3, it is evident that the Trc mix had potent activity against all five *C. albicans* strains. The Trc mix

MIC_{50} concentrations ranged from 2-4 µg/mL with the less sensitive strain being CAB201 against Trc mix (Fig. S3.3 A).

Table 3.3 Comparative summary of inhibitory parameters of pure gramicidin S, tyrocidine complex and surfactin complex against five strains of *C. albicans* as determined with the four assays in the MPAS. Prevention and eradication data using the same strains assessed with conventional biofilm prevention and eradication assays [17] is shown for GS in brackets. The data is the average of 3-4 biological repeats with 2-3 technical repeats at each concentration in the dose response. Refer to Figs. S3.2, S3.3 and S3.4 for typical dose response curves from which the parameters were calculated.

Compound	Inhibition parameter	*C. albicans* strains (values in µg/mL± SEM)				
		CAB201	**CAB1653**	**CAB1085**	**MRC8908**	**MRC8912**
Gramicidin S	MIC_{50} Planktonic cells	4.0±0.3	1.2±0.01	3.3±0.6	3.0±0.6	2.5±0.01
	MIC_{50} Dispersed biofilm cells	2.9±0.8	2.4±0.0	4.2±1.9	4.9±0.9	3.1±0.2
	BIC_{50}	1.0±0.6 (5.0±0.005)	4.4±1.4 (5.0±0.001)	2.2±0.8 (5.0±0.01)	5.6±0.4 (4.8±0.1)	4.0±0.6 (4.3±0.6)
	BEC_{50}	3.1±0.9 (13±4.5)	2.0±0.4 (4.2±0.6)	2.0±0.8 (9.8±4.1)	3.1±1.0 (14.8±3.1)	1.2±0.04 (7.2±2.1)
Tyrocidine complex	MIC_{50} Planktonic cells	3.9±0.6	1.7±0.4	2.6±0.4	3.2±0.4	2.5±0.5
	MIC_{50} Dispersed biofilm cells	16±1.4	28±1.8	22±4.8	16±2.5	40±8.1
	BIC_{50}	11±2.7	7.1±1.4	9.1±2.5	5.0±1.2	8.5±3.2
	BEC_{50}	26±2.0	2.8±1.9	4.0±2.2	11±4.3	18±6.1
Surfactin complex	MIC_{50} Planktonic cells	>200	>200	>200	>200	>200
	MIC_{50} Dispersed biofilm cells	>200	>200	>200	>200	>200
	BIC_{50}	>200	>200	>200	~60	0.44±1.3
	BEC_{50}	>200	5.0±1.3	>200	~60	>200

MIC – minimal inhibitory concentration for planktonic cells or dispersed planktonic cells
BIC – minimal biofilm inhibition (prevention) concentration at the biofilm forming growth stage
BEC – minimal biofilm eradication concentration of 48 h-old biofilm cells

We observed a decrease in sensitivity of the dispersed population of cells against Trc mix, with concentrations ranging from 16-40 µg/mL (Table 3.3). Eradication of some *C. albicans* biofilms necessitated high concentrations of Trc mix, ranging up to 26 µg/mL. The concentration range for biofilm eradication of GS was much lower and narrower, ranging from 2-3 µg/mL, indicating better activity. Of the five strains tested, the veterinary strain CAB201 was more resistant to Trc mix, followed by clinical strain MRC8912. Still, the results show that Trc mix had good inhibitory activity against all stages of *C. albicans*, although a higher concentration for biofilm inhibition was needed than those determined for GS, AmB or caspofungin (Chapter 2). For this reason, three tyrothricin producers (Table 3.2) with different production capacities (refer to discussion below) were included to test the multiplex assay system on micro-culture extracts (Table 3.3; supplementary data, Fig. S3.3).

Conversely to what was found for GS and Trc mix, we observed that biofilm life-stages were more sensitive than the planktonic stage to Srf (Table 3.3, Fig. S3.4). Srf had virtually no activity up to 200 µg/mL against the planktonic forms (Fig. S3.4A and D), while a proposed detergent/surfactant mode of action was observed against the biofilms of the environmental strains (Fig. S3.3 B and C). Srfs are anionic, cyclic lipopeptides with exceptional surfactant properties[32], which could disrupt biofilms because of detergent action. MRC8912 immature biofilms, as used in the biofilm prevention assay, was very sensitive to Srf with BIC_{50} at less than 1 µg/mL, while MRC8908 sensitivity levelled off at 60 µg/mL (Table 3.3, Fig. S3.4 B). CAB1653 and MRC8908 exhibited a BEC_{50} towards Srf at 5 µg/mL and 60 µg/mL (again levelled off), respectively (Table 3.3, Fig. S3.4 C). As Srf producers are abundant in soil as observed in our biomining studies (Chapter 4) and reported in literature[31,48] we included two Srf producers with variable production and a third producer of a truncated Srf for testing their culture extracts against the *Candida* strains.

Validating the multiplex assay methodology to test micro-culture extracts

Analysis of compounds in micro-culture extracts

To validate the broad application of our multiplex assay methodology, extracts of the selected soil derived bacilli were first analysed using high resolution mass spectrometry to confirm production of known peptides with antifungal activity such as Trcs, GS and Srfs (Fig. 3.1). We also utilised two non-producer organisms, *B. subtillis* 168 and a mutated Srf producer *B. subtillis* 120 to determine the background activity of culture extracts from bacilli.

Since the level of antifungal compound micro-production was expected to be unpredictable and variable, we assessed the production with electrospray mass spectrometry (ESMS). Fig. 3.1 depicts ESMS spectra of the bacterial extracts used in this study, where the intensity of spectral peaks related to peptide production in each extract is plotted for each unique bacterial culture extract. *B. subtilis* ATCC6633, OKB120 and 168 did not show appreciable ion signals indicating peptides (Fig. 3.1 A, B, C, I) and their anti-*Candida* activity was accordingly low and/or variable as discussed below.

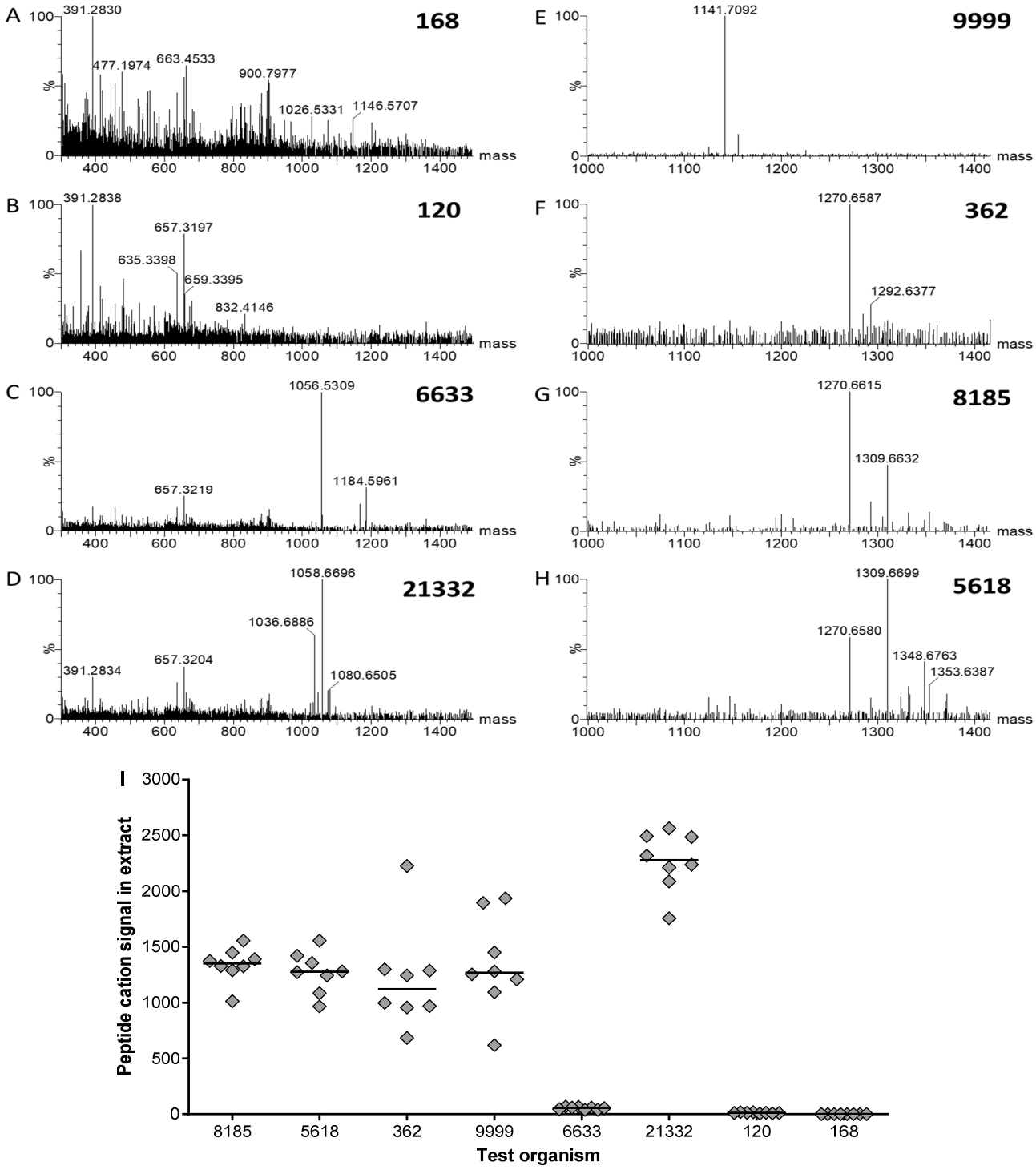

Figure 3.1 ESMS analysis results of 96-well micro-culture extracts from the eight selected bacteria in this study. Representative ESMS spectra of culture extracts of non-producers: *B. subtilis* 168 (A) and *B. subtilis* OKB120 (B), and from the producers: *B. subtilis* ATCC6633 (C), *B. subtilis* ATCC21332 (D), *A. migulanus* ATCC9999 (E), *B. parabrevis* DSMZ362 (F), *B. parabrevis* ATCC8185 (G) and *B. parabrevis* DSMZ5618 (H). In panel I, the intensity of spectral peaks related to peptide production in each extract was plotted for each unique micro-culture extract. The noisy spectra of *B. subtilis* 168 (A) and OKB120 (B) indicated the background of metabolites and media in extracts. The spectral data with *m/z* values ranging from 1141-1145 indicate the presence of antimicrobial GS (E), *m/z* values ranging from 1257-1395 indicate the presence of Trc mix (F-H), while *m/z* values ranging from 1044-1097 indicate the presence of Srfs (C-D).

The production is depicted with more variability for producers *B. parabrevis* DSMZ 362, *A. migulanus* ATCC9999 and *B. subtillis* ATCC21332, compared to that of *B. parabrevis* ATCC 8185 and DSMZ 5618, which are more constant (Fig. 3.1I). Interestingly, although the production of GS by *A. migulanus* 9999 (*m/z* values ranging from 1141-1145) was apparently variable (Fig. 3.1 E) the activity was constant with a nearly a 100% production (refer to discussion below). *B. parabrevis* 5618 and 8185 with *m/z* values ranging from 1257-1395 indicating the presence of Trc mix (Fig. 3.1, F, H) was a bit more variable but activity was also relatively constant with inhibition between 63-75%. The *m/z* values ranging from 1044-1097 indicated the presence of peptides in the Srf complex (Fig. 3.1 C, D) although the related activity we observed was lower than expected, indicating low production (see discussion below).

Activity determination of micro-culture extracts

With the confirmation that the antifungal peptides were indeed produced and extracted and after confirming the ability to use this assay methodology quantitatively over a broad concentration range for GS, Trc mix and Srf (Figs. S3.2-S3.4), the next step was to test the micro-culture extracts in our multiplex assay system with the five *C. albicans* strains as targets.

The fluorescent metabolic response, as determined in the MPAS for planktonic cells (assays I and IV) and biofilm cells (assays II, III) for five *C. albicans* strains (Table 3.1) when challenged by bacterial culture extracts containing Trc mix or GS, can be seen in Fig. 3.2.

The metabolic data depicted in Fig. 3.2 gives a comparative depiction of the variability in metabolism seen for the five *C. albicans* strains and their life forms, drawing attention to the increased variability of biofilm forms (Fig. 3.2 C and D) compared to the planktonic counterparts (Fig. 3.2 A and B). From this set off results we derived the expected range of resazurin fluorescence (metabolic activity) for observing the activity of a producing strain's extract and a non-producing strain's extract towards *C. albicans* growth compared to their non-inhibited (positive) survival growth and inhibited (GS-treated) metabolic activity.

A ≥50% cut-off of antifungal/inhibition metabolic activity threshold could be distinguished from 20000-25000 RFU whereas "positive" survival/growth metabolic activity ranged from 35000-50000 RFU. The MPAS ≥50% cut-off is based on the positive and negative growth control data collected from all experiments preformed in this study. The choice of this cut-off is also supported by the conventional use of 50% inhibitory concentration or IC_{50} as an antimicrobial parameter[55]. However, for fast identification of active extracts, a hard cut-off of 25000 RFU was chosen to ease the visual

identification of active extracts in a medium to high throughput MPAS experiment. Using this visual cut-off, it can be seen that the majority of data points of the most active extracts were under this cut-off, as illustrated in Fig. 3.2.

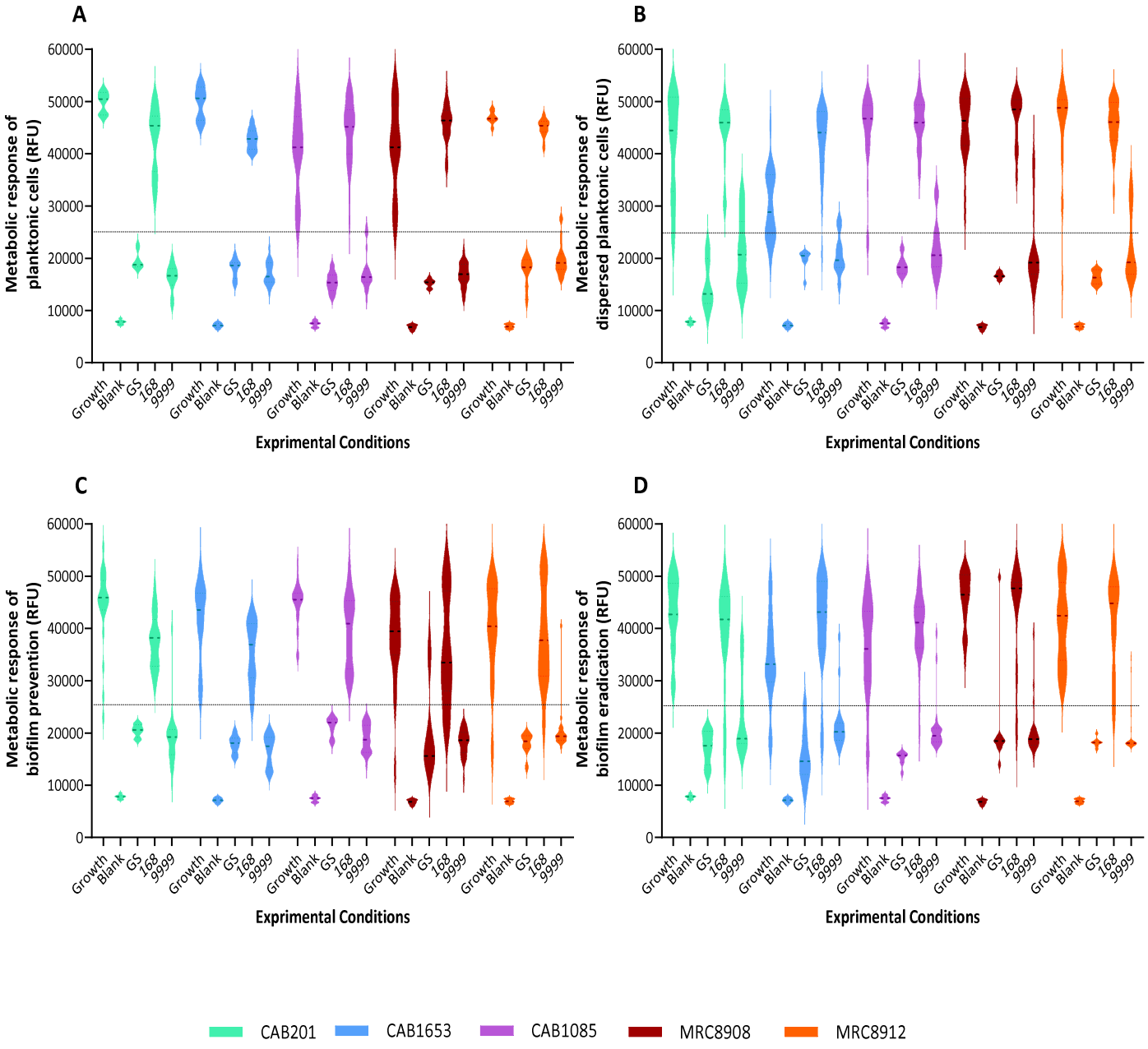

Figure 3.2 Comparative analysis of the metabolic response profile of the five *C. albicans* strains using the MPAS as relative fluorescent units measured over 24-72 hours. The violin plots (8-16 determinations), with width indicative of data density, represented the unchallenged fungal metabolic response (*C. albicans*), challenged metabolic response by GS (positive inhibition control) and by bacterial culture extracts of a non-producer (*B. subtilis* 168) and a known GS producer (*A. migulanus* ATCC9999). Figures show the metabolic response of planktonic cells in assay I (**A**), shed planktonic cells in assay IV (**B**), biofilms in assay II (biofilm prevention) (**C**) and in assay III (biofilm eradication) (**D**). The dotted line shows an arbitrary fluorescence cut-off of 25000 RFU to visually distinguish between survival (growth) and inhibition.

As depicted in Fig. 3.2, variability was more pronounced during biofilm eradication and biofilm prevention experiments. Once inhibited by micro-culture compounds or pure GS (positive inhibition control), the variability decreased amongst all four assay types and five strains. Furthermore, the blank media response was constant throughout all assay types (results not shown). It is clear from the MPAS data in Fig 3.2 that one can easily distinguish between survival (and consequent growth) and inhibition for planktonic cell stages, but for the biofilms there was a subset of cultures that did not show high metabolism (Fig. 3.2 C and D), indicating that assays II and III in MPAS could detect false inhibition. This trend was not strain specific for the biofilm stages (Fig. 3.2 C and D) and one could only derive a yes/maybe/no outcome in terms of extract activity against the *C. albicans* biofilm life-stages.

For assessing MPAS utilisation analysis of micro-culture extracts, it was important to also include known non-producers to eliminate false hits. *B. subtilis* 168 has been used as the model *B. subtilis* organism for many years[49,50]. It is a Gram-positive bacterium that does not produce any known antimicrobial compounds[24] and as such is was considered as ideal to use as control in conjunction with the other known producers in these experiments. A mutant strain of *B. subtilis* 168, *B. subtilis* OKB120, with a non-functional *Srf* operon was used as a second non-producing/test strain. The extracts from *B. subtilis* 168 and OKB120 showed no significant anti-candidal activity overall in the MPAS analysis (Fig. 3.2). In more detailed analyses the metabolic response to the culture control was considered in order to assess a relative inhibition giving a similar response that of the background (death) or above that of the unchallenged *C. albicans* culture response (stress). Following the detailed analysis of the anti-*Candida* activity exhibited in the micro-culture extractions (Fig. 3.3), it was evident that the GS producer *A. migulanus* ATCC9999[51] would be easily identifiable as a broad spectrum antifungal hit towards all the *Candida* strains and life-stages, as it's extract activity correlated with the activity profile of pure GS (Table 3.3). Extracts of two of the three *B. parabrevis* producers of tyrothricin containing Trc mix as the major antimicrobial contingent, strains ATCC8185 and DSMZ5618, also presented as clear anti-*Candida* hits against all of the *Candida* strains in assay I (planktonic cells), assay II (biofilm prevention) and less so in assay IV (shed planktonic cells) (Fig. 3.3). The lower activity in biofilm eradication (assay III) correlated with the high BEC_{50} values found for the Trc mix (refer to Table 3.3).

The results obtained for biofilm prevention and eradication in the presence of producer extracts showed that the biofilm forming cells were greatly disturbed/stressed by their presence, as was indicated by the lowered growth (Fig. 3.3 C and D respectively). However, this data could be due to

a high rate of false positives or severe stress induced by the producer strains, which was more evident when looking at the individual graphs Fig. 3.4 and Figs. S3.6-S3.9, where it was also easily observed that certain strains were more sensitive towards specific extracts. In Fig. 3.3, each strain has a coloured block/layer, of which the thickness depicts the growth of that strain in the presence of the extracts. As per example, the inhibition of strain MRC8908 by producer 8185's extracts are shown as a thin block/layer but much less to no inhibition was observed for producer 6633's extract (Fig. 3.3D; red blocks). These graphs give a general trend depiction and overview of what the producers' effect was on *Candida* growth. However, clear growth cut-off values are required to make informed decisions when collecting data.

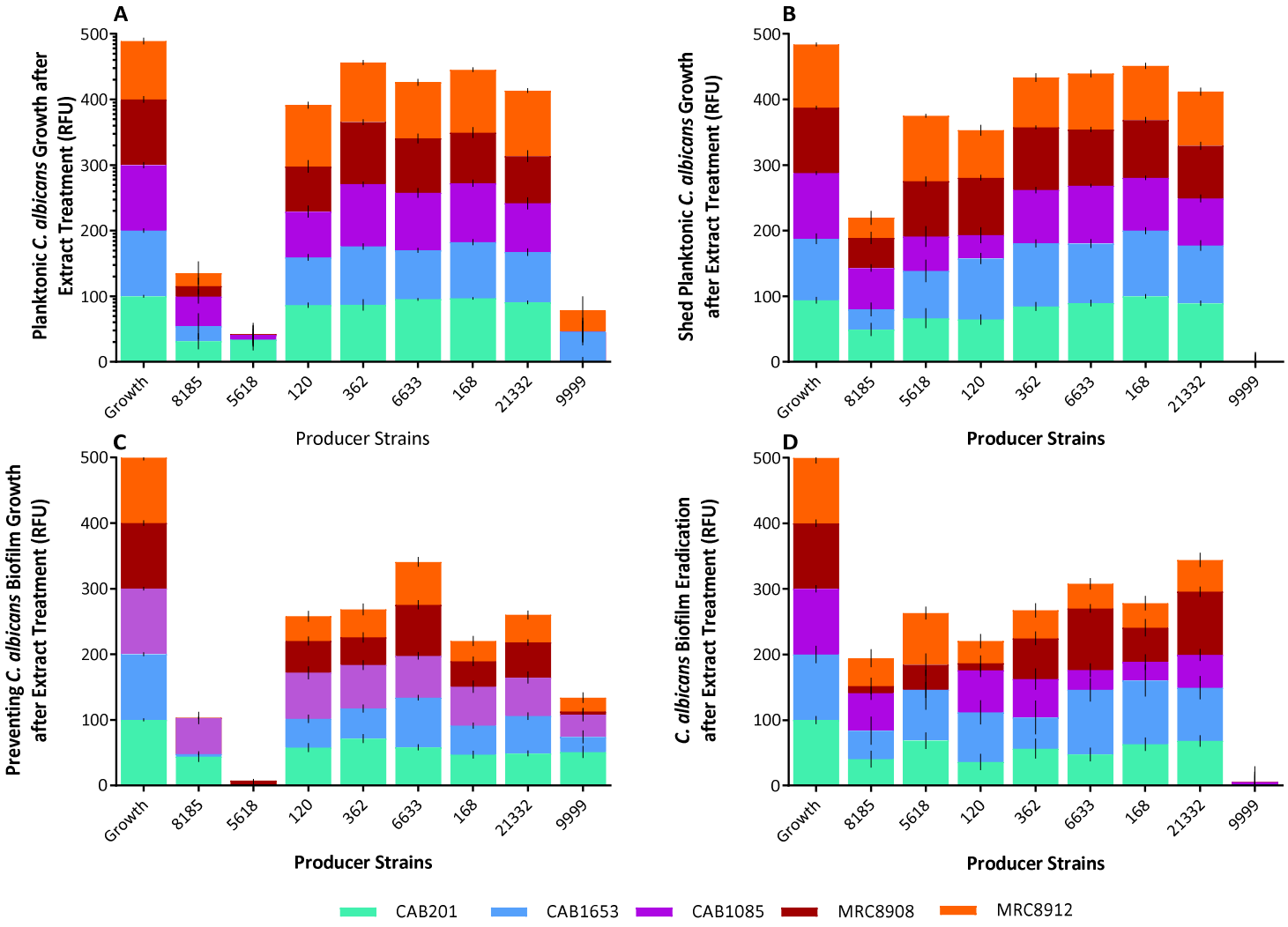

Figure 3.3 Comparison of normalised metabolic activity profiles of the five *C. albicans* strains using the MPAS system. The influence of microculture extracts on planktonic cell metabolism shown in **A (assay I)**, activity against shed planktonic cells is represented in **B (assay IV)**, biofilm prevention in **C (assay II)** and biofilm eradication in **D (assay III)**. Each data point is the mean ± standard error of the mean (SEM) of 4 biological repeats (n=3-8 technical repeats). Data were normalised to the control metabolism of each organism.

Defining the MPAS *cut-off limits for micro-culture extracts*

From the data depicted in Fig. 3.3 it was not possible to determine the performance of single culture extracts, as expected in a bio-mining scenario or to assess how a group of micro-culture extracts (biological repeats) performed. In order to determine, for example, if the third tyrothricin producer strain *Br. parabrevis* DSMZ362 or the Srf producer *B. subtilis* ATCC21332, could be potential hits, the data were scrutinised, but the active producing cultures could only be identified by defining cut-off limits indicating cell death and cell stress.

A benefit of the MPAS's design is the use of intermediate *C. albicans* growth values as an indication of partial inhibition by the culture extract of the producer and/or unknown soil microbe, as the active compound's concentration can vary from culture extract to extract. One micro-culture production set might have grown more prolifically than another and produced more inhibitory compounds, whereas another might have produced marginally less and slightly inhibited fungal growth but not completely inhibited growth. The method utilised to calculate absolute cut-off growth values for the various strains of the producer data was to work with ±1α of the standard deviation covering 68% of the strain's survival/growth metabolic activity data (absolute cut-off values). To visually illustrate the cut-off values, the MPAS results of *C. albicans* CAB 201 is depicted in Fig. 3.4 (also refer to Figs. S3.6-S3.9). Also refer to Figs. S3.6-S3.9 for similar graphs for the response of the other four strains. Median results below the absolute cut-off, grey section are putative or definite positive hits, which inhibit *C. albicans* growth. Median results above this grey section refer to *C. albicans* cells that have been placed in a stressed state due to the presence of production products with known or unknown activity but possibly at very low concentrations. This is specifically important for identifying bacterial producers of potent antifungal compounds with variable production. These various but strict/absolute cut-off values are necessary, especially for future experiments where the active compounds are unknown, and their activity needs to be confirmed with reasonable certainty and without wasting resources and time on false positive data. The collected producer data indicated that these absolute cut-off ranges are valid and appropriate for identifying hits, as described in more detail below.

When looking at data from the assay types, the most reliable data was collected using the planktonic cell assay (assay I) whereas the most variable results were obtained using the shed planktonic cells (assay IV) and biofilm eradication assay (assay III). This allows for the deduction that shed cell growth is more variable than planktonic growth and as such more biofilm and shed cell repeats (technical

and/or biological repeats) will allow for more definitive results. Having an absolute cut-off value for this assay allows it to be adapted for various uses.

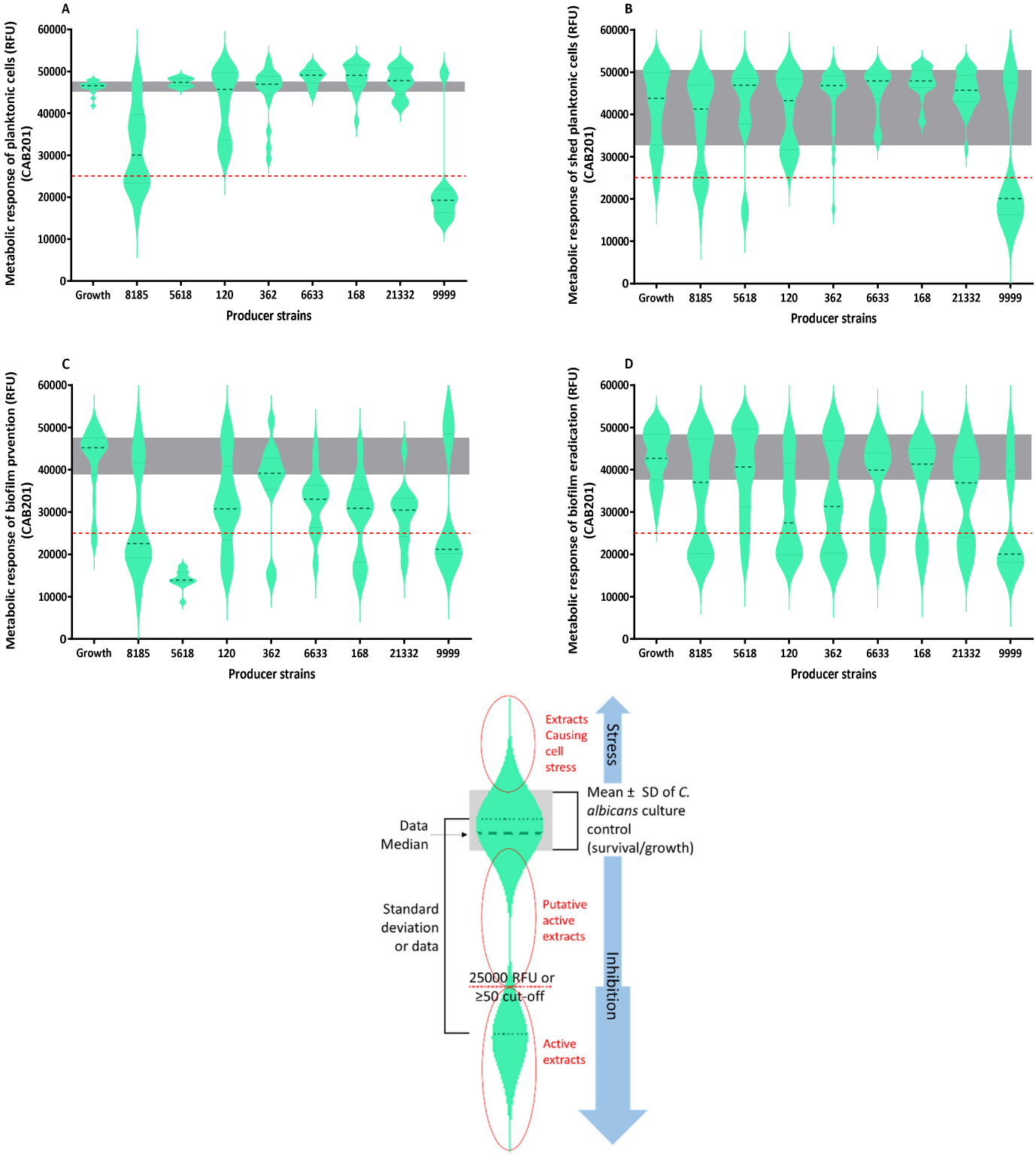

Figure 3.4 Influence on *C. albicans* CAB201 metabolic response of extracts from microcultures of selected bacterial species. Graphs represent the metabolic response of planktonic cells in assay I (A), shed planktonic cells in assay IV (B), biofilms in prevention assay II (C) and biofilms in eradication assay III (D). Each violin plot represents 24-40 determinations with width representing the density of the data. The different limits and statistics of the violin plots are depicted in the annotated cartoon below the graphs.

This limit allowed for identification of most putative hits, but this cut-off limit would include the selection of some false positives, specifically in assays II and III. The dotted red lines in Fig. 3.4 and Figs. S3.6-S3.9 indicates a strict 50% inhibition cut-off (data with fluorescent units indicating inhibition near or below 50% of the control culture) allowing for identification of only the most potent extracts with active compounds. This stringent cut-off decreases the likelihood of false positives. This will allow the user to test for activity on only the best hits without the workload from created by false negatives.

From the data collected for this study, it was evident that the tyrocidine and GS producers produce extracts with potent activity against *C. albicans* planktonic cells, as well as against *C. albicans* biofilms. *B. parabrevis* ATCC8185, *B. parabrevis* DSNZ5618 and *A. migulanus* ATCC9999 were observed as the producers with the highest number of cultures with >50% inhibitory activity against *C. albicans* planktonic cells and biofilms (Figs. 3.4, S3.6-3.9 and refer Fig. 3.3). We did find that some producers gave better production in microcultures than others with ATCC9999 > ATCC8185 > DSNZ5618 >> DSMZ362, although the last three all produce the same antifungal peptide complex, tyrothricin (refer to Fig 3.1 and S3.5). However, when the MPAS biofilm assays II and III data were considered, some of the micro-culture extracts of all the bacteria tested showed biofilm prevention and eradication activity (Figs. 3.3, 3.4, S3.6-S3.9), that could indicate a high rate of false positives if only results from these assays are considered.

Assessment of MPAS in micro-culture antifungal production

The micro-culture productions should be considered and examined per experiment done using this methodology. However, sufficient repeats of each production do create consistent results and this assay system also selects for good producers above weaker producers. For example, DSMZ362 is a known tyrothricin producer but showed no notable planktonic anti-candidacidal activity with low anti-biofilm activity, which could be due to consistently low production concentrations in a micro-culture environment. Furthermore, the *B. subtilis* ATCC21332 is a soil isolate that has a functional *sfp* gene and *srf* operon, had a 100% successful production rate (Fig. 3.1), but the extract did not inhibit planktonic or shed planktonic growth leading to false negative results (Figs. 3.3 and 3.4). However, it did prevent biofilms with a 93% success rate when correlated to the absolute cut-off values (Table 3.4). The eradication of biofilms was calculated at only 29% with an equal percentage of stressed cells (Table 3.1). The ≥50% inhibition cut-off, related to the generally used IC_{50} parameter, was also introduced for identifying cohorts of micro-culture extracts with good activity (indicated as positive in Table 3.4).

This assay design with stringent, absolute cut-off values classified strain ATCC21332 as a producer whose production is not significantly growth-inhibitory, possibly due to Srf production that is too low (< 100 µg/mL) for a potent inhibitory response (Fig. S3.4). This strain could require a different growth medium, higher nutrient stress or alternative culturing conditions to maximise its growth inhibitory production. However, these observations are again evidence that the MPAS is more selective towards good producers. Using more than one type of growth medium for culturing would be beneficial in the search for such producers.

Table 3.4 Summary of microculture extracts analysed with high resolution ESMS (refer to Fig 3.1) to determine the correlation of inhibitory (positive) non-inhibitory (negative) and stress-inducing productions (%) of the antimicrobial peptide's activities within cut-off limits for *C. albicans* CAB201. Data is representative of 2-4 biological repeats, each consisting of 4-8 extract repeats.

	Planktonic Growth Inhibition			Biofilm Prevention		
Strain	**Stress**	**Negative**	**Positive**	**Stress**	**Negative**	**Positive**
8185	0	17	83	4	25	71
5618	0	25	75	0	0	100
362	0	67	33	7	50	43
9999	0	4	96	21	11	68
168	0	71	29	7	21	71
120	0	58	42	7	21	71
6633	0	63	38	4	21	75
21332	0	46	54	0	7	93
	Biofilm dispersed cells growth inhibition			**Biofilm Eradication**		
Strain	**Stress**	**Negative**	**Positive**	**Stress**	**Negative**	**Positive**
8185	32	8	61	25	13	63
5618	36	5	59	31	38	31
362	55	11	34	25	13	63
9999	22	3	75	13	0	88
168	68	11	21	46	21	33
120	37	13	50	17	8	75
6633	58	13	29	29	42	29
21332	42	29	29	29	42	29

B. subtilis OKB120 is a *B. subtilis* 168 mutant with a mutation in the Srf operon, which results in putative production of a truncated Srf (tetrapeptide). The results show that the OKB120 extracts had a very low production of a truncated Srf according to ESMS results (Fig. 3.1). However, the OKB120 culture extracts prevented and eradicated biofilms, as indicated by the 71% and 75% positive hits respectively (Table 3.4). The antibiofilm response was mostly negative for strain MRC8912 and the planktonic/shed cells as targets. However, some inhibitory activity was observed

for planktonic and shed cells of strains CAB201 with 42% and 50% positive hits, respectively (Figs. 3.4 and Table 3.4). The activity of the truncated Srf is unknown, but there may be putative active components in the OKB120 extracts that may contribute to the observed activity.

As previously noted, the multiplex four-assay system allows for successful differentiation between weak producer organisms and good producer organisms, both for planktonic cell and for biofilm prevention (Fig. 3.3). Extracts from *B. parabrevis* ATCC8185, *B. parabrevis* DSNZ5618 and *A. migulanus* ATCC9999 were able to remove biofilms from pins (Figs 3.3, 3.4, S3.6-S3-9). This indicated that antifungal peptide production yielded at least 5-10 µM in the extract, as calculated from the dose-responses of pure peptides (refer to Fig. S3.2 and S3.3). Alternatively, the biofilms might have been prevented from forming due to strains experiencing inhibition of biofilm development by unknown metabolite(s) produced by these bacteria. However, even when there seems to be biofilm eradication, there is a very definite and clear trend of stress in terms of biofilm adhesion, even more so than distinct eradication (Figs. 3.3, 3.4, S3.6-S3.9).

Notably, the biofilm cells are easily stressed in the presence of both good, weak and non-producers, and as such it is more difficult to distinguish between stressed, inhibited and non-inhibited cells (Fig. 3.3 and 3.4). The growth of the biofilms on the pins was also variable (Fig. 3.4, Figs S3.6-3.9), which makes the cut-off more valuable as the results must be based on the population mean distribution. Even so, a valuable answer can still be gained from the data, especially if data from multiple repeats of the biofilm assay are available. The *C. albicans* biofilms were generally stressed in the presence of another organism's metabolites in the culture extracts, as was visible in population distribution depicted in Fig. 3.4C and Fig. 3.4D, as well as Figs. S3.6-S3.9. However, as shown in Table 3.4 the MPAS design does allow for successful identification of active extracts from producers with inhibition results above those of the negative control, *B. subtilis* 168. For planktonic cells, 83% active productions by ATCC8185 were found compared to 29% false positives for *B. subtilis* 168. This was a nearly three-fold higher success rate to identify inhibition above the *B. subtilis* 168 background. However, *B. subtilis* 168 produced compounds that inhibited initial biofilm attachment and as such was not a good negative/background control for the prevention assay II, with 71% active productions by ATCC8185 compared to 71% putative false-positive by *B. subtilis* 168. The population medians were very clear when considering the cut-off values, confirming their importance and relevance. Lastly, the eradication of cells was successful for 63% active productions by ATCC8185, compared to 33% false-positives from *B. subtilis* 168, which was still an appreciable two-fold above the background for such variable result.

When considering the data in Table 3.4, it could be deduced that the planktonic assay (assay I) was most successful showing no signs of stress and 83-99% positive hits (*B. parabrevis* ATCC8185 and *A. migulanus* ATCC9999) with 21-29% false positives (*B. subtilis* 168), but with no false negatives when considering the growth controls. The biofilm prevention assay (assay II) showed 11-50% false negatives, but the eradication assay (assay III) exhibited a much better 0-38% of false negatives (*B. parabrevis* ATCC8185 and *A. migulanus* ATCC9999 micro-culture extracts).

The dispersed cell data collected using Assay IV also presented stressed cells (68%; *B. subtilis* 168), which were not present in the planktonic cells but more associated with biofilm type cells. On a positive note, this assay showed low false negative results (3-8%; *B. parabrevis* ATCC8185 and *A. migulanus* ATCC9999 data) and also had a low false positive result (21%; *B. subtilis* 168). This suggests that assay IV gives a better idea of biofilm-associated planktonic cells with a different metabolism to normal planktonic cells, which is ideally what this assay layout and retrieved data was designed for.

During the design of a novel assay type, it is essential to validate the way the assay is used and applied, and one can use two different validation steps. The first validation factor will be the optimization of the dynamic range of signal and its deviations. The second validation will be the confirmation of reproducibility of the assay using concentrations of known compounds[52]. The validation results will indicate that the characterizations of the assay reagents and analytical formats have been well selected. To validate the current assay, all the micro-culture extract data was used to calculate the assay variability ratio resulting in a value of 0.48, which is below the recommended upper value of 0.6[52]. This indicates that the multiplex assay can be used for the desired identification of active hits. To validate our observed data, a signal to noise ratio (S/N) of 15.7 was calculated, which is well above the recommended S/N value of 10 and a signal to background (S/B) value of 3.9. The signal to noise ratio indicates solely if the signal can be confidently distinguished from the background noise. Since the S/B accounts for no variability in calculating the ratio, neither the S/N nor the S/B are ideal to determine assay confidence, as neither take into account variability of background and signal intensities, as well as considering the dynamic range of the readings[53].

The data collected from the producer activity was used to calculate a signal window value (SW) value of 5.4, which is indicative of a recommended assay with an SW value >2[52]. The signal window allows for the identification of novel entities with desired inhibitory activity in the presence of variability[54]. Some studies suggest that Z'-factor is a better validation value as it has a better bias

and precision than SW. This relates to in a lower coefficient of variation (CV), which indicates how spread-out variables are relative to the mean value. Our data resulted in a CV value below 20%. The Z-factor (Z') was calculated for this assay layout using the collected data to calculate the viability of *C. albicans* cells after drug and/or extract treatment. The Z-factor is regarded in unison with various high-throughput or screening assays. Values ≤ 0 indicate that positive and negative values could overlap, which results in the assay not being useful for screening purposes. A Z-factor between 0-0.5 is indicative of a marginal assay design whereas Z-factor values between 0.5-1 indicates that it is an excellent screening assay design. A Z-factor of 1 would indicate an ideal screening assay design. From the data collected above, our planktonic assay design has a Z-factor of 0.52, which is indicative of a marginal to excellent assay design. Our eradication assay design had a Z-factor of 0.48 and our prevention assay had a Z-factor of 0.02, both are indicative of marginal assay designs. As was evident in the data, overlap between positive growth and negative growth data points in the prevention assay were more frequently observed and the data more difficult to use, requiring more repeats for clear population groupings. However, the eradication data was more variable than planktonic data but much easier to analyse than prevention biofilm data, as was confirmed by a Z-factor. Although there will always be room for improvement, the growth and consequent metabolic activity of *C. albicans* biofilms on pins is variable due to shedding, therefore resulting in less reliable inhibition parameters than those obtained with pure planktonic cells and as such a lower Z'-factor can be expected. In this same regard, there will also be room for improvement in the assay layout, which could also be affecting the biofilm activity and recorded results. During this assay, the movement of the biofilm-containing pin-lid could result in variability of results due to more shedding of cells due to shaking of the lid. As such there are many factors affecting result variability, including natural shedding, stability and age of the biofilm and the surface to which the biofilm is attached. But from the data, the Z' factor indicates the assays fall within range of a good assay design overall, whereas the corresponding SW was indicative of a "recommended" assay. This is promising, especially since the MPAS adaptation for testing culture extracts was originally planned to provide a simple yes/no answer on a culture activity. However, a limitation is that the Z'-factor cannot evaluate the concentration dependent assay results with known compounds. In unison with data from Table 3.5 and statistical parameters calculated above, it is evident that the planktonic assays and the eradication assay are most efficient for easy use. The prevention assay is the most difficult to use. Furthermore, a multiplex assay protocol including this assay will benefit from more assay repeats to verify hits from this assay more easily.

One of the benefits of the data collected using this multiplex assay is that it could be deduced that certain extracts are more targeted towards certain *Candida* strains. This makes the assay easy to use with different strains of the same organism or different fungal and/or bacterial species as target organisms. The data also highlights that some extracts were more effective at eradication, some at prevention and/or some towards planktonic growth inhibition and clearly showed which extracts affect all four life-cycle stages. Overall, statistically the assay falls within range to be used as a validated assay layout for hit identification, especially when focusing on planktonic cell inhibition and biofilm eradication and can be used to further study unknown producer bacteria.

Conclusion

This multiplex assay system can be used to give a quick, reproducible, and reliable indication of activity with regards to antifungal compounds in micro-culture extracts and their effect on both planktonic and biofilm *C. albicans* forms. Concurrently, this assay system can give a medium-throughput result, indicating if an extracted compound's activity is targeting planktonic cells, preventing biofilm formation and/or eradicating biofilms. However, considering the methodology this assay system is easily adaptable to high throughput systems that can be employed using robotics. If successful, growth inhibitory activity results are observed, the specific compounds and their bacterial producers can be studied further. Therefore, this leads to quicker, improved possibilities of identifying novel antibiotics and antifungals from a complex microbiome, with which to aid the various industries struggling with resistant microorganisms. From data of peptides extracted from known peptide producer strains, it is clearly evident that *B. aneurinolyticus* ATCC8185, *B. parabrevis* DSNZ5618 and *A. migulanus* ATCC9999 produce compounds with potent inhibitory activity. The production of these compounds was also always at an appreciable concentration to give consistent and clear results in all the assays and with the five *C. albicans* strains as targets. This was observed using both conventional and absolute cut-off values for these producers. Notably, these micro-culture extracts of these three peptide producers prevented biofilm formation on the pin-lids, whereas none of the other extracts had good activity in preventing biofilm formation. From this study, the assay design, productions and micro-extractions can be considered a success with corresponding stringent and less stringent cut-off values to suit the needs for application of the multiplex assay system in antifungal drug discovery.

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SUPPLEMENTARY DATA

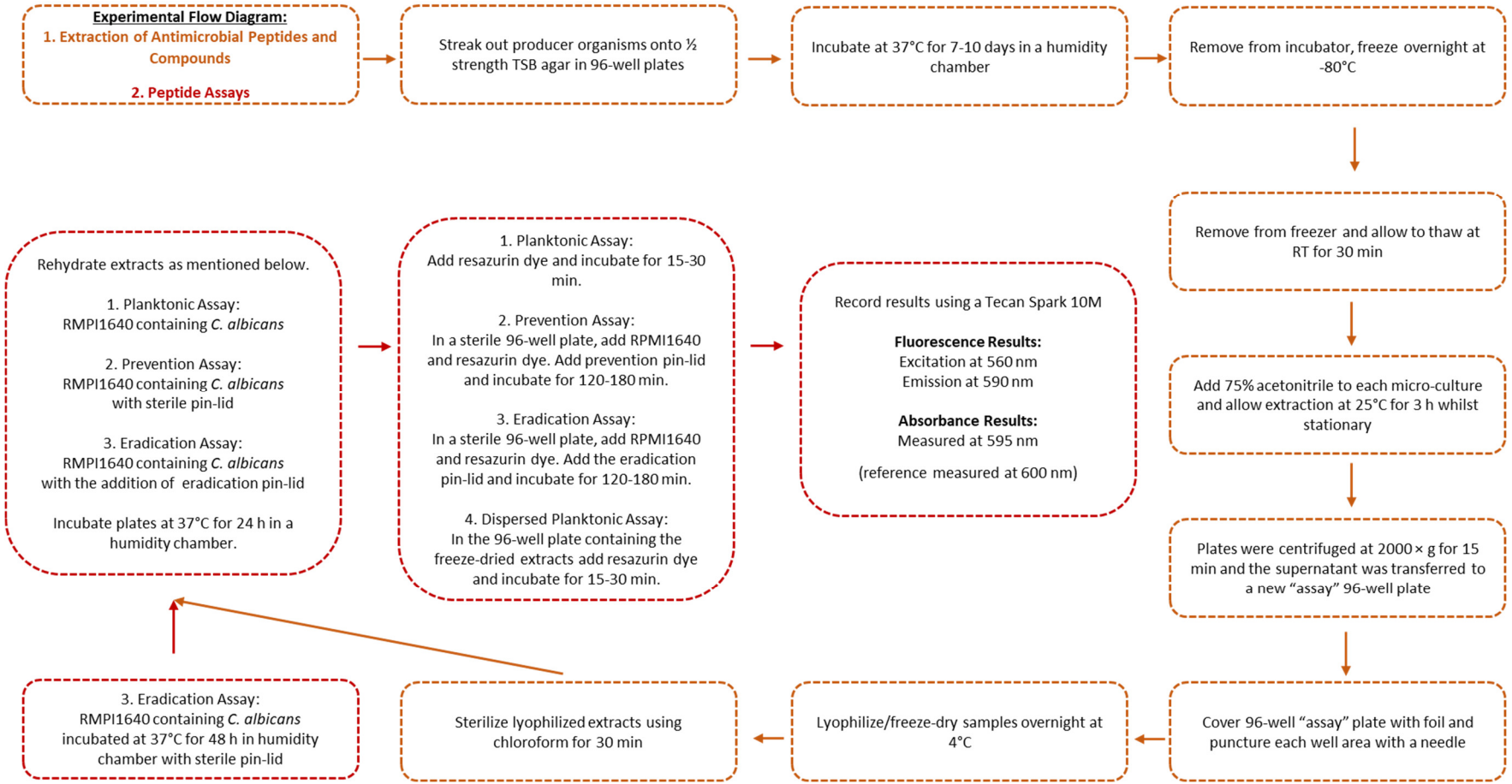


Figure S3.1 A flow diagram depicting the experimental steps followed to extract antimicrobial peptides from known producer micro-cultures and using the extracted peptides to test for anti-candida activity using the multiplex assay design.

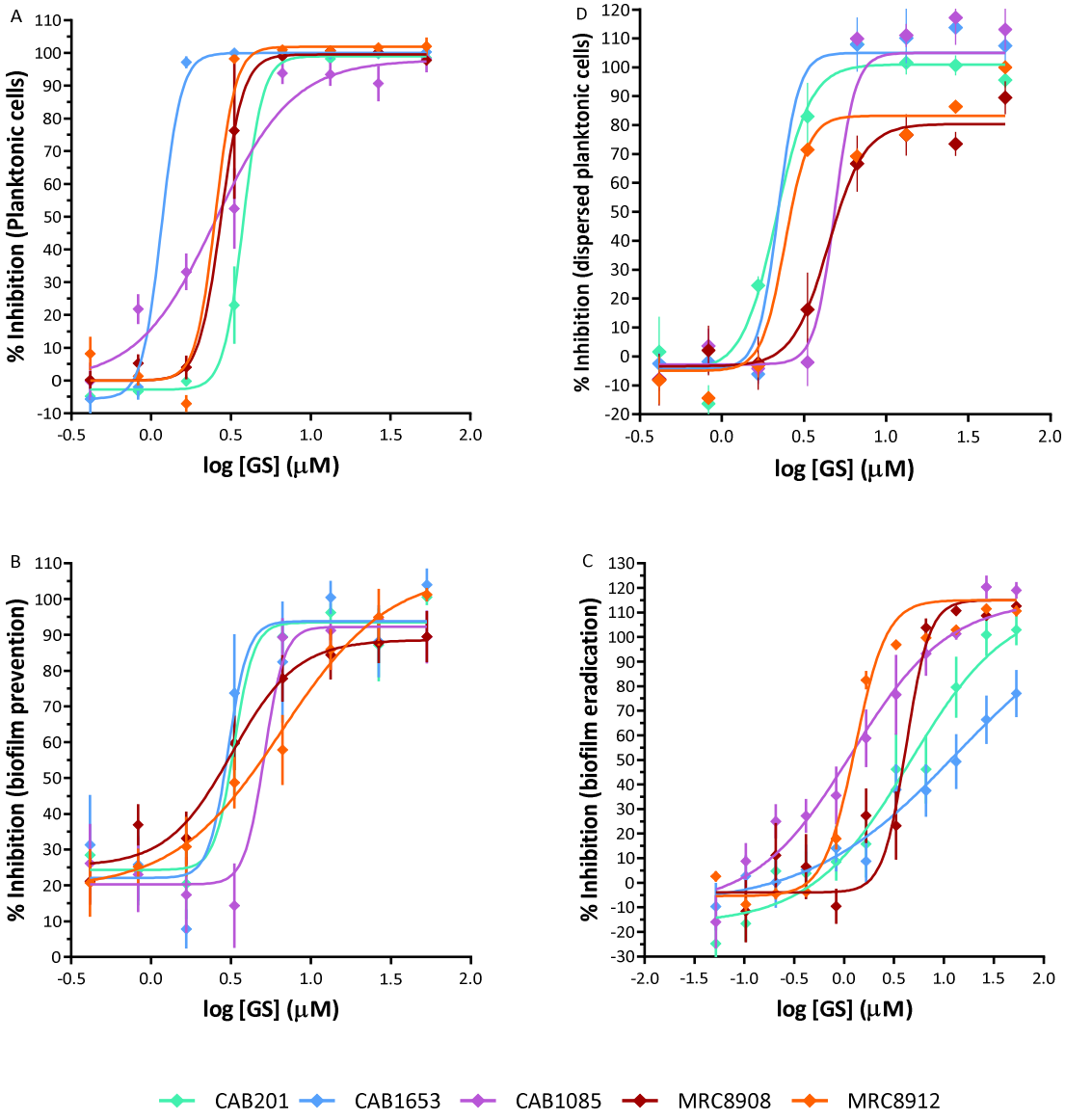


Figure S3.2 Representative dose response curves displaying the activity of pure gramicidin S (µM) towards the various life-stage forms of the five *C. albicans* strains. Data against planktonic cell inhibition shown in A and concentrations inhibiting shed planktonic cells shown in Figure S3.2D. Biofilm prevention data and biofilm eradication data are shown in Figures S3.2B and S3.2C respectively. The data is the average of 2 biological repeats with the mean of 8 technical repeats ± SEM at each concentration.

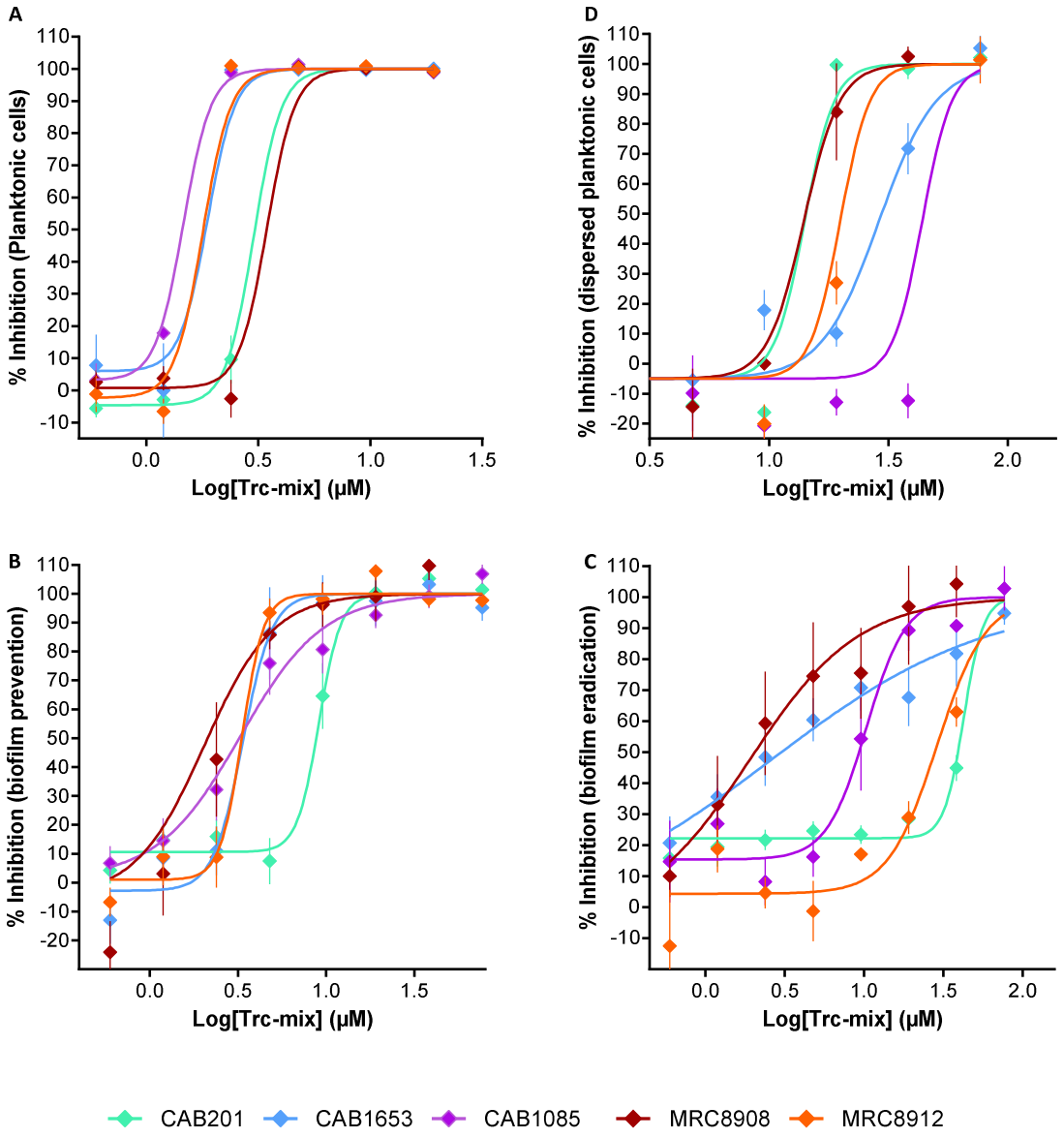


Figure S3.3 Representative dose response curves displaying the activity of pure Tyrocidine mix (µM) towards the various life-stage forms of the five *C. albicans* strains. Data against planktonic cell inhibition shown in A and concentrations inhibiting dispersed planktonic cells shown in Figure S3.3B. Biofilm prevention data and biofilm eradication data are shown in Figures S3.3C and S3.3D respectively. The data is the average of 2 biological repeats with the mean of 8 technical repeats ± SEM at each concentration.

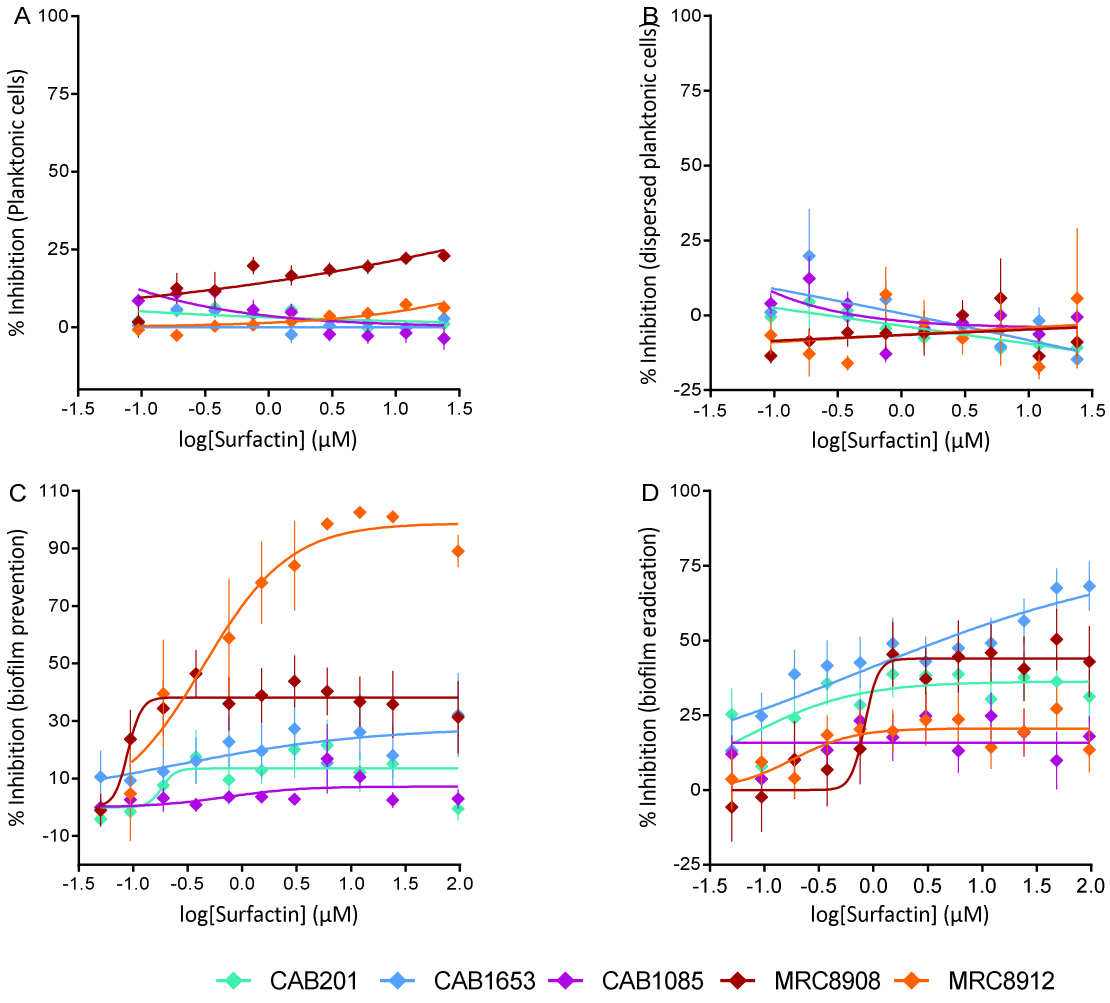


Figure S3.4 Representative dose response curves displaying the activity of surfactin complex (µM) towards the various life-stage forms of the five *C. albicans* strains. Data against planktonic cell inhibition shown in A and concentrations inhibiting dispersed planktonic cells shown in Figure S3.4B. Biofilm prevention data and biofilm eradication data are shown in Figures S3.4C and S3.4D respectively. The data is the average of 2 biological repeats with the mean of 8 technical repeats ± SEM at each concentration.

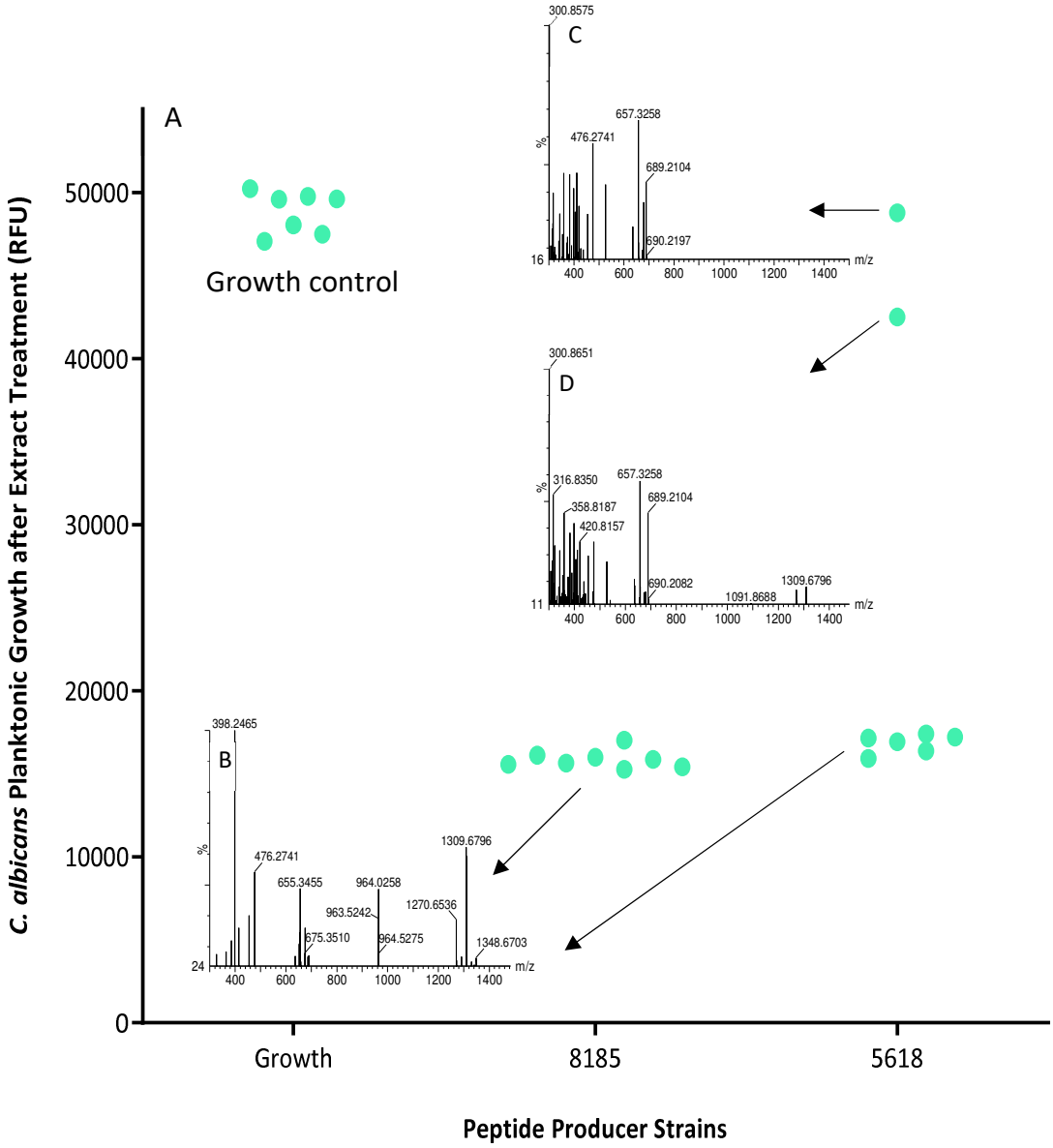


Figure S3.5 Correlation between production by tyrocidine producers, as determined via HRMS, and anti-Candida activity of the culture extracts A. The inhibitory activity against *C. albicans* strain CAB201 using known antimicrobial peptide producers *B. parabrevis* ATCC8185 and DSMZ5618, using the MPAS assay I. The tyrocidine production of each extract was confirmed by HR-ESMS spectra B-D). B. Mass spectrum of a good production showing TrcA (expected mass = 1270.6536) and TrcB (expected mass = 1309.6796), which correlated to *C. albicans* growth inhibition. C and D shows poor productions where active compound concentrations were low (D) or absent (C) and hence there was no inhibition of *C. albicans* growth. Data is representative of one biological repeat with 8 technical repeats (n=8).

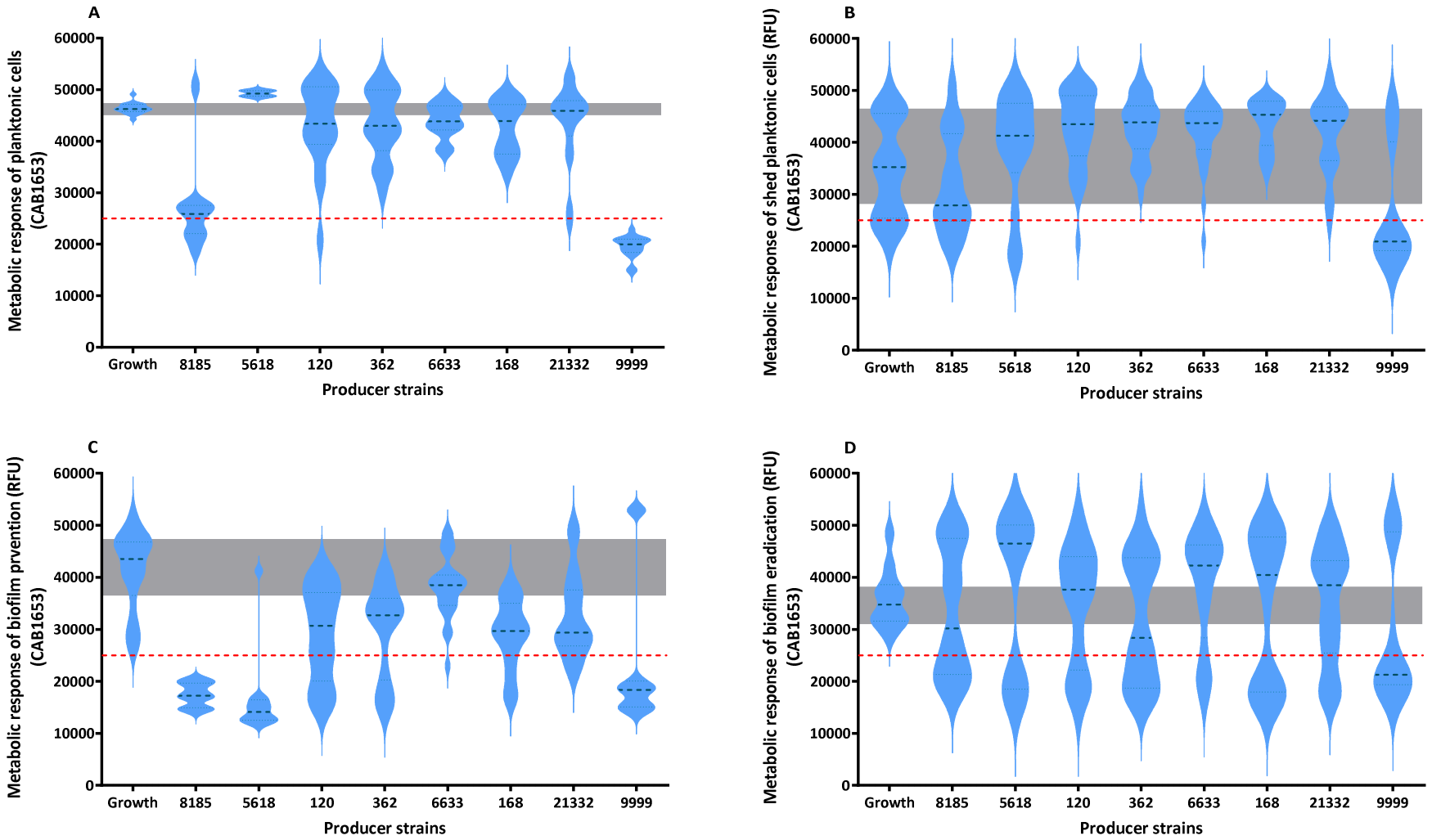


Figure S3.6 Influence on *C. albicans* CAB1653 metabolic response of extracts from micro-cultures of selected bacterial species. Graphs represent the metabolic response of planktonic cells in assay I (A), shed planktonic cells in assay IV (B), biofilms in prevention assay II (C) and biofilms in eradication assay III (D). Each violin plot represents 24-40 determinations with width representing the density of the data. The different limits and statistics of the violin plots are depicted in the annotated cartoon in Fig. 3.4.

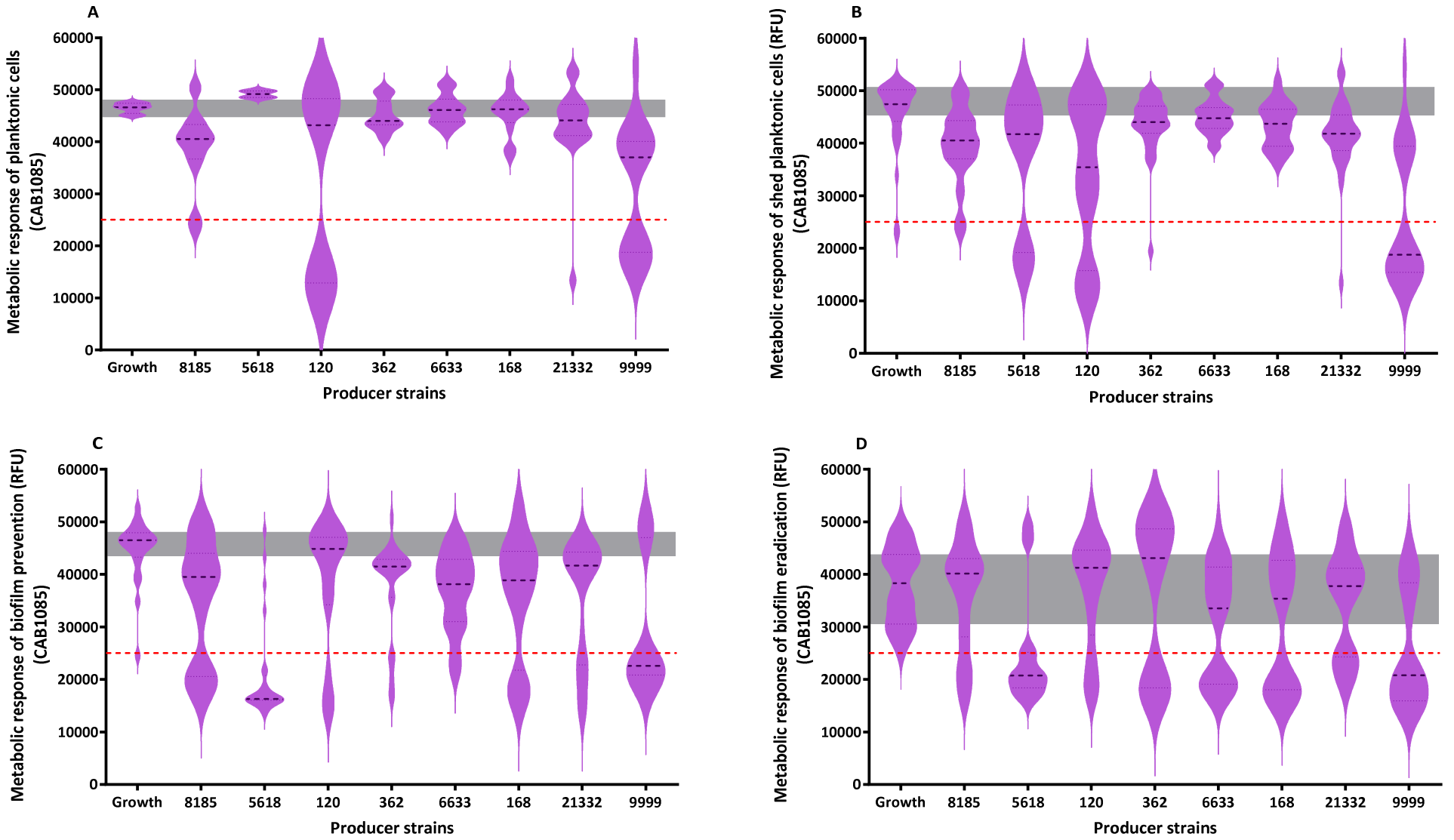


Figure S3.7 Influence on *C. albicans* CAB1085 metabolic response of extracts from micro-cultures of selected bacterial species. Graphs represent the metabolic response of planktonic cells in assay I (A), shed planktonic cells in assay IV (B), biofilms in prevention assay II (C) and biofilms in eradication assay III (D). Each violin plot represents 24-40 determinations with width representing the density of the data. The different limits and statistics of the violin plots are depicted in the annotated cartoon in Fig. 3.4.

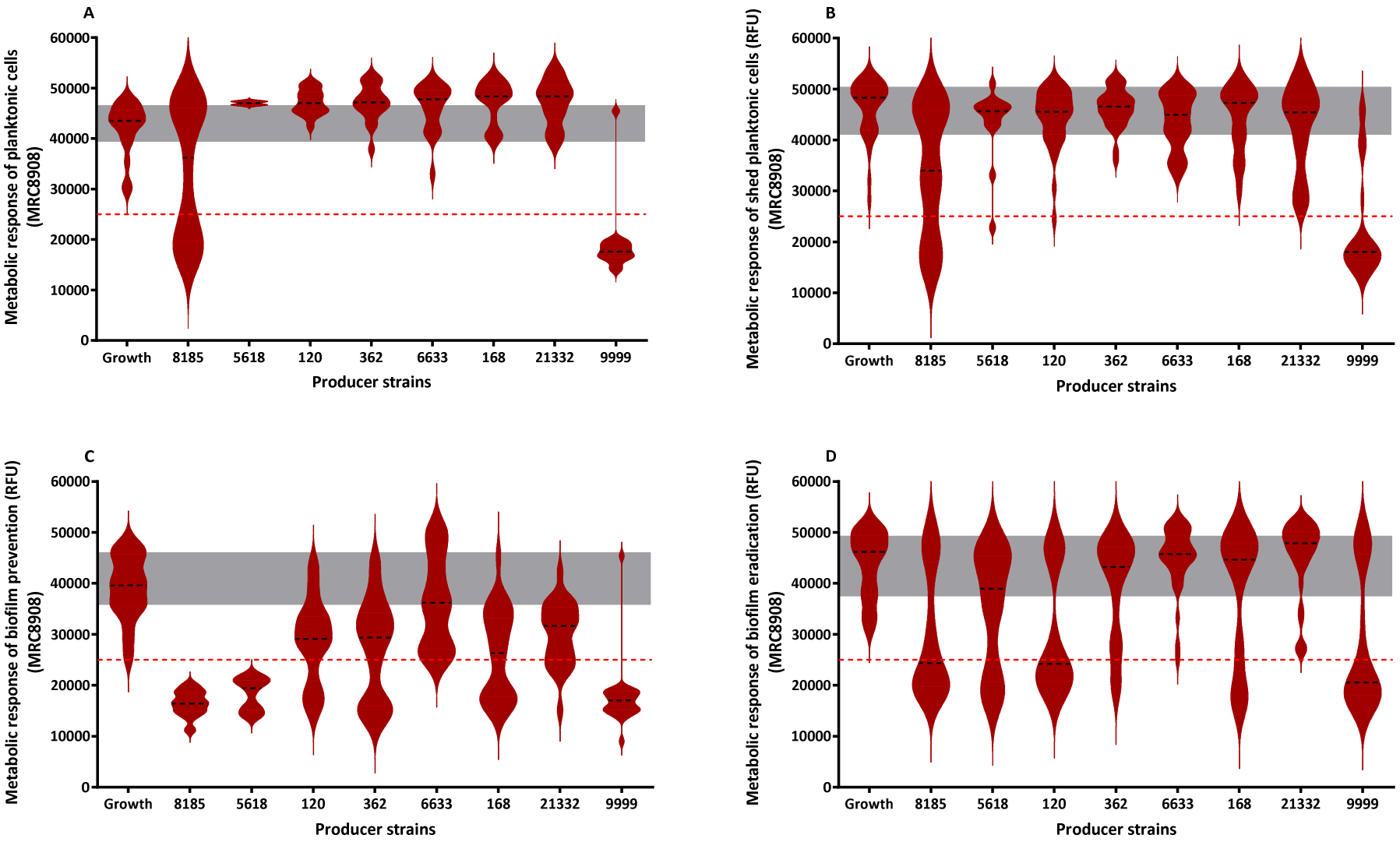


Figure S3.8 Influence on *C. albicans* MRC8908 metabolic response of extracts from micro-cultures of selected bacterial species. Graphs represent the metabolic response of planktonic cells in assay I (A), shed planktonic cells in assay IV (B), biofilms in prevention assay II (C) and biofilms in eradication assay III (D). Each violin plot represents 24-40 determinations with width representing the density of the data. The different limits and statistics of the violin plots are depicted in the annotated cartoon in Fig. 3.4.

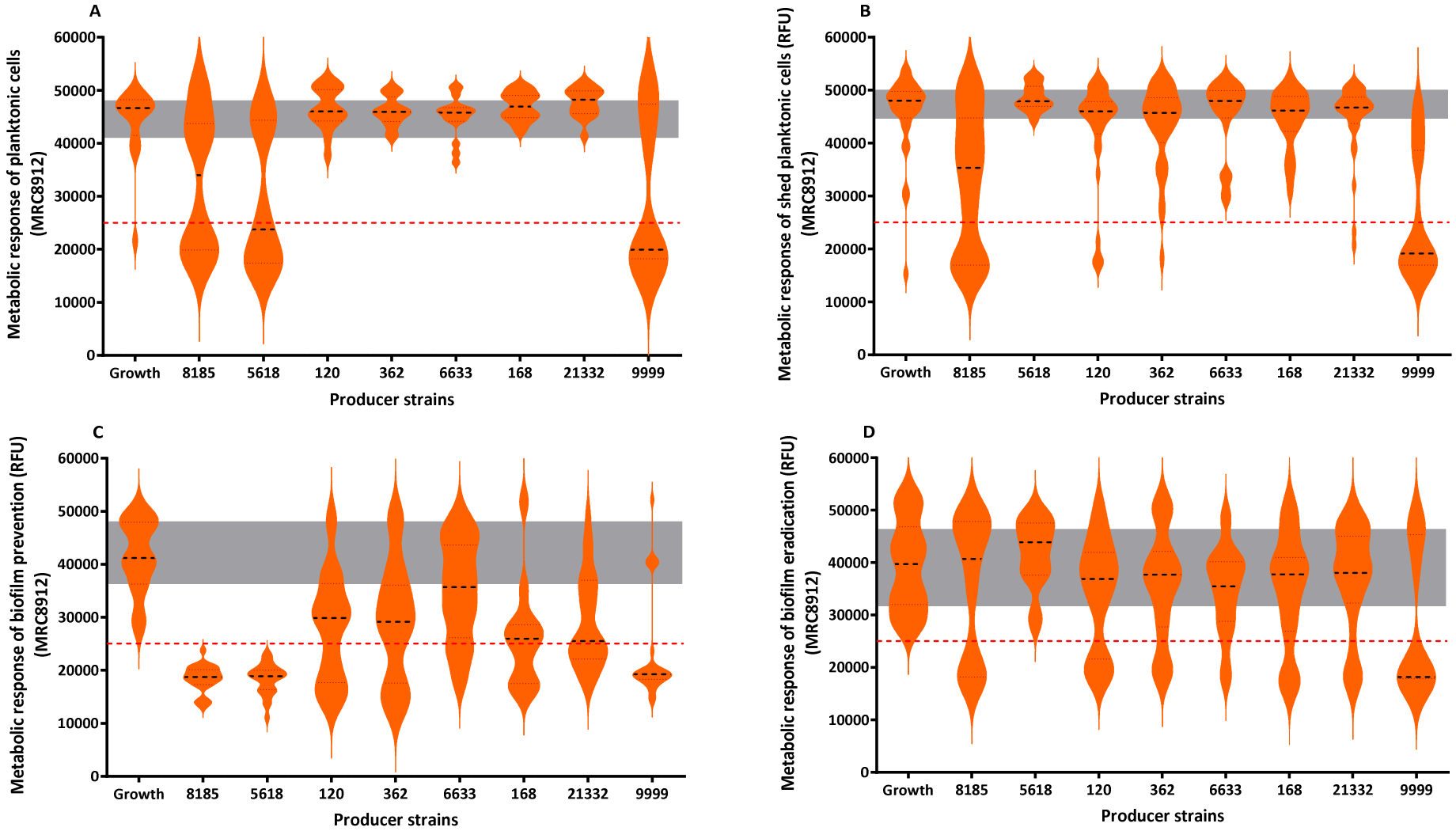


Figure S3.9 Influence on *C. albicans* MRC8912 metabolic response of extracts from micro-cultures of selected bacterial species. Graphs represent the metabolic response of planktonic cells in assay I (A), shed planktonic cells in assay IV (B), biofilms in prevention assay II (C) and biofilms in eradication assay III (D). Each violin plot represents 24-40 determinations with width representing the density of the data. The different limits and statistics of the violin plots are depicted in the annotated cartoon in Fig. 3.4.

CHAPTER 4

Utilising a multiplex assay system for discovering of anti-Candida compounds from soil samples

Abstract

The largest threat the world is currently experiencing, after global warming, is antibiotic resistance. This affects both first-world and second-world countries but is most prominent in poor areas with high numbers of immune incompetent individuals. Therefore, current research is focusing on the design, development and approval of novel drugs as well as the discovery and validation of novel drug targets within pathogens and cancer cells. To achieve the former, the discovery of new chemical entities, from synthetic and natural sources, is of importance. Therefore, using our known soil *Brevibacillus* producer strains, we have validated a rapid and reproducible method of extracting the *Brevibacillus* antimicrobial peptides, by testing the antimicrobial activity of extracts against *C. albicans*, as described in Chapter 3. In this chapter, we describe how this multiplex assay system (MPAS) was used to identify extracts of unknown compounds from soil bacterial samples with inhibitory activity, in the search for novel antimicrobial compounds to treat *C. albicans* biofilm infections. Soil bacteria were allowed to grow and produce for 10 days as micro-cultures in 96-well plates, after which the active compounds were extracted using an organic solvent. After extraction, compounds were dried in 96 well plates and then assayed against planktonic and biofilm forms of *C. albicans* using the MPAS described in detail in Chapter 3. Gene identification (16S) grouped the active soil bacteria into three main preliminary groups, namely *Bacillus spp.*, *Pseudomonas spp*., and *Citrobacter spp*. From the soil bacterial extracts, known lipopeptides, such as the iturins, fengycins and surfactins associated with inhibitory activity were identified by using ultra-performance liquid chromatography linked to high resolution tandem mass spectrometry. Furthermore, quorum sensing small molecules, and rhamnolipids associated with anti-*Candida* activity were also identified. The advantages of MPAS are the ease of adaptability for use in various laboratories to test a broad array of compounds and target organisms for the discovery of novel small molecule antifungal compounds.

Introduction

The ever-increasing global threat of common infections developing resistance to current therapeutics, is rapidly accelerating the onset of a primitive post-antibiotic era in medicine. If the current multidrug resistance (MDR) problem is not solved, it has been forecasted that by the year 2050, 10 million lives per annum will be at risk, amounting to an economic implication of 10 trillion dollars[1]. The mechanisms by which pathogens develop resistance are mostly understood. However, the prevention of further antimicrobial resistance development is unlikely due to the continued misuse of antibiotics, resulting in an increased need to discover novel antibiotics with novel mechanisms of action. The use of any new antibiotic naturally selects for resistant microbes, perpetuating the need for more novel antibiotics to fight the emerging and already identified resistant microbes[2].

Natural antibiotic discovery peaked between 1940 and 1950, thereafter novel antibiotic discovery took a steep plunge followed by a dramatic increase in the use and study of successive iterations of known scaffolds and their activity being tested against resistant microbes[2]. Much information can still be uncovered with regards to the old library of antibiotics (to which resistance is developing), as well as about the genetic arsenal making these antibiotics obsolete. However, over the last 30 years, many anti-bacterial and anti-infective agents that were discovered are natural products, most of which are secondary metabolites from microorganisms [3,4]. Development of the bio-active secondary metabolites has resulted in anticancer, anti-cholesterol and antimicrobial compounds amongst others[4–6].

Recent developments in synthetic medical chemistry made it possible to synthesize and screen large libraries of synthetic compounds and there are currently 127 natural products/natural product-derived compounds undergoing clinical trials for various indications[7,8]. Natural products for the antibiotic pipeline include tigecycline, which is a semi-synthetic derived product of tetracycline. Initial data shows activity of tigecycline against tetracycline resistant microorganisms[8]. Additionally, there are more naturally derived, semi-synthetic compounds such as dalbavancin, telavancin (vancomycin derived), retapamulin and tiacumicin to name a few[8]. There are also natural product antibiotics, for which the exact mechanism of action must still be fully elucidated. An example is the GE23077 family that are possibly novel cyclic heptapeptide inhibitors of bacterial RNA polymerase. This family was discovered using a target-screening campaign and is produced by *Actinomadura spp*., and is one example of many similar compounds such as muramycins and tripopeptins[8,9]. Natural products are a source of most currently used antibiotics, most originating from actinomycetes and

fungi isolated from easily accessible environmental samples. These microbes are mostly versatile, easily culturable and culturing conditions are easily upscale-able, they adapt easily to environments and challenges[10] and can be adapted to standard laboratory practices. These microbes are able to synthesize metabolites we can use for commerce, which aid their proliferation in nature, such as antibacterial, anti-viral, anti-fungal compounds and anti-cancerous compounds to name a few[10]. Furthermore, the advantage of focusing on actinomycetes and fungi is that they can be obtained from virtually any soil sample and are easy to cultivate, such is also the soil microbes from *Brevibacillus spp*. However, it is difficult to find antibiotics with new structures among all the known antibiotics that are produced by soil microorganisms due to the intricacy of their nature.

Soil is a very complex microhabitat, for four main reasons. First, the microbial population is extremely diverse with an estimated 6000 different bacterial genomes per gram of soil when normalising to the size of the *Escherichia coli* genome as one genome unit per gram of soil[11]. Second, the ecological characteristics of soil include a structured, heterogeneous system which is mostly poor in nutrients and energy, and it is subject to ever-changing environmental conditions. It can be described as poor in nutrients and energy, especially when compared to *in vitro* growth medium used in laboratory settings. Third, soil consists of a solid phase which adsorbs biological molecules[12]. Lastly, the soil surface mineral components can sometimes be cofactors to enzymes that catalyse reactions themselves, especially when looking at manganese and iron oxides[13]. These reactions are ones such as oxidation, reduction, polymerization, ring cleavage and deamination to mention a few[10].

Affecting natural processes and cycles above and below the ground, soil microbes play a much more pivotal role than given credit for, being one of the least well understood habitats in the world[14]. These microbes are very difficult to identify and experiment with as only about an estimated 1% of the population can be cultured using standard laboratory techniques[15], which is not enough to give us a proper picture of the microbial soil populations as a whole or of their phylogenetic diversity. Current research has indicated that there are about 61 distinct bacterial phyla, of which 31 are unculturable[16,17]. Various culturing factors are important to consider such as growth medium supplementation and restrictions, growth conditions (pH, temperature, humidity etc.), coculturing and community culture[16,18]. These community interactions result in the microbes interacting by competition for limited resources or cooperation by means of exchanging metabolites and signalling molecules[19], which cannot be duplicated in the lab. Therefore, improved methods for optimal microbial isolation are a necessity even though original methods still hold their ground in many ways.

Initially, the main method used to look at microbial soil diversity was plate counts. This technique allows for the culturing/growth of 1-10% of overall soil microbes and Bakken *et al*.[12] had hypothesized that the colonies visible were ecologically more important in the soil microflora due to their size and frequency of colony formation/numbers. As an alternative to plate counting, fluorescent microscopy increases counts by 100-1000 times when compared to plating, which is extremely laborious and dependent on agar specifications. There are many novel culturing methods for culturing soil microbes, these include methods such as trans-well plate assays[20], microfluid systems[21], the SimCell™ platform[22] as well as the diffusion growth system[23] and the multi-well microculture chip [24] to name a few[16]. Many of these systems are robotic, which are incredibly expensive, and they are specific to certain types of bacteria such as membrane growing bacteria, or they use experimental layouts that are not easily available in all laboratories. These were factors driving the layout of our methodologies wanting to design a method with high-throughput potential which can be easily adapted to most, if not all, laboratories. With the knowledge that, due to historical success and large diversity, antibiotics from natural sources are still promising alternatives we could proceed with our experimental plan of using soil microbes as a natural source. Factors that will be easy to test, for future experiments using the adapted assay, would be increased incubation periods of 5-12 weeks and supplementation of media with specific compounds to target specific bacterial groups. Furthermore, one can comparatively test the growth of isolated bacteria at 20°C and 25°C as well as adapting the pH of the growth medium to the specific soil sample from which the bacteria are isolated[16]. We strived to develop an easily adaptable and reliable methodology for bioprospecting, which focusses on the discovery of novel compounds with commercial value isolated from soil microbes[10].

In this study, soil was isolated from various sites (refer to Table 4.1) and the samples of bacterial microbiomes were cultured and extracted. The culture extracts were tested using the multiplex assay system (MPAS) for determining inhibitory activity on planktonic and biofilm life stages of five *C. albicans* strains. Once the activity of single bacterial colonies was confirmed using classical spread plates and MPAS against the *C. albicans* strains, extracts were analysed utilising high resolution tandem mass spectrometry linked to ultraperformance mass spectrometry (UPLC-MS^e) to probe the molecular composition. The specific bacterial colony related to an active extract was tested for preliminary identification using 16S rRNA gene sequencing. The extracts of the active hits were further analysed with UPLC-MS^e to fingerprint and determine the putative identity of the antifungal compounds.

Materials and methods

Materials

The fungal strains *Candida albicans* were provided by the Department of Microbiology, University of Stellenbosch. Gramicidin S (GS) was purchased from Sigma Aldrich (Steinheim, Germany). Sterile water was prepared by reverse osmosis followed by filtration through a Millipore MilliQ® water purification system (Milford, USA). Ethanol (99.9%) was obtained from Merck Chemicals (Pty) (Wadeville, South Africa). The tryptone soy broth (TSB) agar was purchased from Merck Chemicals (Pty) (Wadeville, South Africa), as was the yeast extract powder, agar and D(+)-glucose Anhydrous. The non-sterile polystyrene plates were provided by Greiner bio-one (655101; Germany). The non-sterile NUNC polystyrene plates with fitted sterile NUNC-TSP (Transferable Solid Phase Screening System) pin-lids were supplied by Thermo Fischer Scientific (Waltham, USA). Sterile culture dishes and microtiter plates were obtained from Corning Incorporated (USA) and sterile petri dishes from Lasec (Cape Town, South Africa). HPLC grade acetonitrile (>99.5%, UV cut-off 200 nm) was purchased from Microsep (Cape Town, South Africa).

Extract preparation and chemical analysis

Micro-culture extraction of antimicrobial peptides and compounds

Soil samples were collected from various locations (Table 4.1). One gram of each soil sample was suspended in 50 mL phosphate buffer saline (pH 7.0), syringe filtered using a 0.45 µm filter, allowing passage of most bacterial cells but not yeasts and filamentous fungi, and then serially diluted. Of the serially diluted inoculum, 10 µL was pipetted into wells of a sterile 96-well plate containing 80 µL half-strength tryptone soy in 10% agar (TSA). Inoculated plates were incubated in a humidity chamber at 37℃ for 7-10 days of culturing to allow the production of antimicrobial compounds. After incubation the plates were placed in a freezer at -80℃ for 24 hours. Once removed from the freezer, plates were allowed to adjust to room temperature (RT). To each micro-culture, 200 µL of 75% acetonitrile in analytical quality water (*v/v*) was added and the stationary plates incubated at 25℃ for 3 h, allowing for extraction. Plates were centrifuged at 2000 × *g* for 15 minutes and 150 mL supernatant transferred to a new 96-well assay plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 442404). The extracted micro-culture compounds (supernatant) were lyophilized in this 96-well assay plate overnight and stored at 4℃ until used for activity testing against *C. albicans* (method adapted from Laubscher and Rautenbach[25]). The plates were sterilised before use in chloroform vapours and under UV, as described in Chapter 3.

Table 4.1 The origins of soil samples, collected throughout the Western Cape, South Africa, in this study.

Soil Sample	Sampling Location	Co-ordinates
Sample 1	Cederberg 1, Western Cape, South Africa	-32.6508105, 19.2642092
Sample 2	Cederberg 2, Western Cape, South Africa	-32.6568068, 19.2569984
Sample 3.1	Wellington 1, Western Cape, South Africa (Fleury Trc)	-33.6317851, 19.0340407
Sample 3.2	Wellington 2, Western Cape, South Africa (Fleury Term)	-33.6317851, 19.0340407
Sample 3.3	Wellington 3, Western Cape, South Africa (Fleury Trc + Term)	-33.6317851, 19.0340407
Sample 4	Pot Plant, Stellenbosch, Western Cape, South Africa	-34.0180307, 18.7437413
Sample 5	Cederberg 3, Western Cape, South Africa	-32.6430857, 19.2563047
Sample 6	Malmesbury, Western Cape, South Africa	-33.4428428, 18.7105054
Sample 7	Malmesbury, Western Cape, South Africa	-33.4428428, 18.7105054
Sample 8	Stellenbosch Hiking Trail, Western Cape, South Africa	-33.9681524, 18.9380036
Sample 9	Stellenbosch University Tree Planter, Western Cape, South Africa	-33.9310268, 18.8655273

ESMS analysis of micro-culture extracts

The success of production for each micro-culture was evaluated by high resolution mass spectrometry (HR-MS) of the culture extracts. Extracted culture compounds in 96-well plated were resuspended in 150 µL ACN (75%), centrifuged at 150 ×g for 5 minutes and the supernatant (120 µL) transferred into 200 mL insert in 1 mL vials for mass spectrometry (MS) for analysis. The presence of the putative antimicrobial and non-active background metabolites and contaminants in the extracts were assessed using direct injections on a Waters Synapt G2 triple quadrupole time-of-flight mass spectrometer (Milford, MA, USA) with an electrospray ionisation source. Samples were exposed to a capillary voltage of 2.5 kV, a cone voltage of 15V and source temperature of 120°C. Desolvation was achieved with nitrogen gas (650 L/hour) and 275°C desolvation temperature. All higher resolution data was collected in positive mode with continuum scanning over an m/z range of 300-2000.

UPLC-MS and UPLC-MS^e analysis of micro-culture extracts

Extracted soil compounds were resuspended in 150 µL acetonitrile (75 %, *v/v*), centrifuged at 150 ×g for 5 min and the supernatant (120 µL) transferred to mass spectrometry (MS) vials for UPLC-MS and UPLC-MS^e analyses. The selected culture extracts sample was injected (3-5 µL) onto a Waters Acquity UPLC® HSS T3 C_{18} column (2.1 x 150mm, 1.8 µm particle size) using a 0.400 mL/minute flow rate. Separation was obtained with a gradient from solvent A (0.1% v/v formic acid) and solvent B (acetonitrile containing 0.1% *v/v* formic acid). The gradient was as follows: 95% A from 0 to 0.1

minutes, 0% to 100% B from 0.1 to 14 minutes, 14-15 min 100% B, 15-15.5 minutes from 100% B to 95%A, 15.5-17 minutes 95% A. In line HR- MS analysis was done the same as with direct injections, with a Waters Synapt G2 quadrupole TOF mass spectrometer (Milford, MA, USA) with an electrospray ionisation source. Samples were exposed to a capillary voltage of 2.5 kV, cone voltage of 15V and source temperature of 120°C. Nitrogen was selected as the desolvation gas (650 L/hour) at a desolvation temperature of 275°C, using leucine-enkephalin inline as lock mass *m/z* of 566.2771 Da (function 3). Data in UPLC-MS mode were collected scanning over an *m/z* range of 200-2000 Da in the positive mode (function 1) and monitoring UV absorption from 230-450 nm (function 4). HR-MS/MS was done simultaneously in the UPLC-MS^e mode using a collision energy gradient of 10 to 30 eV at 0.2 s MS/MS scan time. Data in UPLC-MS^e mode were collected in the second mass analyser (MS2) using centroid mode over *m/z* range of 40-2000 Da (function 2).

Anti-*Candida* multiplex assay system

C. albicans culturing conditions

Two clinical strains of *C. albicans,* MRC8908 and MRC8912, two environmental strains; CAB1085 and CAB1653 (mosquito larvae isolate), and one veterinary isolate, CAB201 (Table 4.2) were revived from glycerol stocks (30%) stored at -80℃, by plating onto 1% yeast 2% peptone and 2% dextrose (YPD) agar Petri-dishes and the resulting Petri-dishes were incubated at 37℃ for 24 hours before overnight cultures for each experiment were started. Overnight cultures were incubated at 37℃ in YPD broth rotating on an orbital shaker at 150 revolutions per minute (rpm) for 14-18 hours. After overnight incubation, cultures were sub-cultured and diluted in un-supplemented Roswell Park Memorial Institute 1640 (RPMI1640) medium at pH 7 to mid-log OD_{600nm} and diluted to specific cell concentrations for the various assays and experiments.

C. albicans planktonic cell inhibition (Assay I)

Candida albicans cells in planktonic form were sub-cultured in RPMI1640 to mid-log OD_{600nm} and diluted to an initial inoculum size of 5.5×10^5 CFU/mL. The 96-well assay plates (Thermo Fisher Scientific-Nunc 96 Flat Transparent 655101) with microculture extracts were set-up as described under “Micro-culture extraction of antimicrobial peptides and compounds”, including 100 µg/mL GS as positive inhibition control. After the 96-well assay plates with extracted compounds were allowed to adjust to room temperature and sterilised, assay I was started by rehydration of compounds in each well with 100 µL planktonic *C. albicans* culture in RPMI1640. After a 24-hour incubation at 37℃ in a humidity chamber, 10 µL resazurin solution was added to each of the wells and incubated in the dark (foil wrapped) at 37℃ for 15-30 minutes. Detection of conversion of resazurin to resorufin was

done using a Tecan Spark 10M multimode microplate reader, temperature controlled at 37℃ and the fluorescence was recorded with excitation measured at 560 nm and emission measured at 590 nm (also refer to Chapter 2, Fig. 2.1).

Table 4.2 The origins and GenBank accession numbers of the *C. albicans* yeast strains used in this study.

Strain	Origin	GenBank Accession Number
MRC8908[a]	Clinical Isolate, Tygerberg Hospital, South Africa	KJ534504
MRC8912[a]	Clinical Isolate, Tygerberg Hospital, South Africa	KJ534505
CAB201	Veterinary Isolate, *Columba livia domestica*, South Africa	MK248726
CAB1653	Environmental Isolate, Mosquito Larvae, South Africa	Not available
CAB1085[b]	Environmental Isolate, Plankenburg River, South Africa	KJ534503

[a] Clinical isolates MRC8908 and MRC8912 were previously stored in the Program on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit culture collection of the South African Medical Research Council. [b] Isolate obtained from a river that is known to be polluted with sewage.

C. albicans biofilm prevention (Assay II)

Planktonic *C. albicans* cells were cultured and the 96-well assay plates with extracted compounds were set up as described above. In a sterilised 96-well plate with pin lid, 100 µL of planktonic cells in RPMI1640 was incubated for 24 hours. After biofilms were formed in the pin lids, 100 µL RPMI1640 was used to rehydrate the extracted compounds the 96-well assay plate described above. Assay II was started by placing a 96 pin-lid on each 96-well assay plate. The plates with pin-lids were incubated in a stationary humidity chamber at 37℃. After a 24-hour incubation period, the 96 pin-lid was transferred to a new 96-well plate (biofilm prevention plate) containing 90 µL RPMI1640 and 10 µL resazurin solution. The prevention plate was incubated at 37℃ for 120-180 min (depending on *C. albicans* strain), covered in foil and the fluorescent results for assay II were recorded as described above (also refer to Chapter 2; Fig. 2.1).

C. albicans biofilm eradication (Assay III) and biofilm shed cell inhibition (Assay IV)

To perform the eradication assay, planktonic *C. albicans* cells were sub-cultured in RPMI1640 to OD=0.30 at 600 nm. From this sub-culture 100 µL was pipetted into each well of a 96-well plate (Thermo Fisher Scientific-Nunc 96 Flat Transparent 442404) in which a 96 pin-lid was placed. The pin-lid and 96-well plate combination were incubated at 37℃ in a stationary humidity chamber for 48 hours, allowing for biofilm formation/maturation on the pins. The plates with compounds were

prepared as before and hydrated with 100 µL RPMI1640. To start assays III and IV the pin-lid with 48-hour old biofilms on pins were placed into the 96-well plates with media, compound/peptides. The mature biofilms on pins (96 pin-lids) were incubated in the compound plates in a stationary humidity chamber at 37℃ for 24 hours. The pin-lid with remaining, treated biofilm was placed in a new 96-well plate containing 90 µL RPMI1640 media with 10 µL resazurin dye and incubated at 37℃ for 120-180 min to assess biofilm eradication (assay III). The compound plates with shed biofilm cells were incubated at 37℃ for 20-30 minutes, after addition of 10 µL resazurin dye to each culture to assess the biofilm shed cell inhibition (assay IV). Fluorescent measurements of both assay III and IV plates were recorded as described above (also refer to Chapter 2; Fig. 2.1).

Selection criteria for active extracts and broad-spectrum hits

An arbitrary cut-off limit of 25000 RFU was only used for visual inspection of data sets. For identification of the active extracts, the ≥50% inhibition cut-off was used in rounds 1 and 2 (refer to Chapter 3, for more details on MPAS cut-off limits). The final round of selection of top hits against all life stages of the five strains were based on broad spectrum activity and a strict ≥50% inhibition cut-off.

Spread Plate Analysis

Using a 1% inoculum volume, *C. albicans* strains MRC8912 and CAB1085 was inoculated into YPD agar and poured into square petri-dishes. According to labelled numbers, extracts from various soil microbes were rehydrated with 50 µL RPMI-1640 medium (for use in the MPAS) and 20 µL of each sample was spotted onto the agar plates. Petri-dishes were incubated at 25°C for 30 hour and observed for inhibition zones.

Bacterial Identification using 16S rRNA Gene Sequencing

Genomic DNA was extracted from bacterial cells supplied using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005, Irvine, USA), according to the manufacturer's instructions. Genomic DNA was further purified using the DNA Clean & Concentrate - 5 kit (Zymo Research, D4004, Irvine, USA) according to the manufacturer's instructions. The 16S rRNA gene sequences were amplified by means of PCR. DNA primers 8F and 1512R were used according to the method described by Neveling *et al*.[26]. The primers and primer sequences are displayed in Table 4.3.

The success and integrity of the amplicons was determined by gel electrophoresis on a 1.5% agarose gel (Lonza, Rockland, USA) stained with SafeView Classic (ABM, Richmond, Canada) to enable visualization. KAPA Universal DNA ladder (KAPA Biosystems, Wilmington, USA) was used to

determine the size of the amplicons. The amplicons were purified using the Zymo DNA Clean & Concentrate kit (Zymo Research, Irvine, USA), according to manufacturer's instructions. Primer 8F was used to sequence the 16S rDNA fragment. Blast analysis was performed, and sequences aligned using the ClustalW algorithm in MEGA version 6[27]. Phylogenetic tree was constructed using the 16S rDNA (500 bp) gene sequence. Phylogenetic analyses were done using the neighbour-joining method[28].

Table 4.3 Description of utilized primers Primer Target Sequence (5' to 3')

Primer	Target	Sequence (5'-3')
8F	16S DNA sequence	CACGGATCCAGACTTTGATYMTGGCTCAG
1512R	16S DNA sequence	GTGAAGCTTACGGYTAGCTTGTTACGACTT

The parameters for PCR reactions were as described below.

PCR parameters:

- NEB Q5 Hot Start High-Fidelity 2X Master Mix Genomic DNA (10-50 ng/µL)
- Forward Primer (10 µM)
- Reverse Primer (10 µM)
- Nuclease free water

PCR protocol:

- Initial denaturation: 98°C for 30 sec
- Denaturation: 98°C for 10 sec
- Annealing: 50°C for 30 sec
- Extension: 72°C for 30 sec
- Final extension: 72°C for 2 min
- Hold: 15°C.

Identifying active compounds using hierarchical clustering techniques

The cosine heatmap was constructed from the comparison of the direct injection mass spectra of 42 isolates. Direct injection flow grams were converted from the proprietary Water™ .RAW file format to the open .mzML format using the ProteoWizard software[29]. All subsequent injection pre-processing and analysis were done in the R statistical computation language[30]. Blank injection (75% ACN) scan spectra were subtracted from the sample injection scan spectra using interpolation. The scans of each sample injection were then summed into a single mass spectrum followed by baseline correction using locally weighted scatterplot smoothing with a span value set to 5% of the mass spectrum *m/z* range[31]. Subsequently, peak detection was done using an algorithm created by W. E. Laubscher (unpublished work) to obtain centroid spectra. Noise thresholds were calculated and subtracted from each centroid spectrum using the nonparametric regression estimator, Friedman's SuperSmoother[32]. Peaks were binned by summation using equally spaced bins of 0.02 *m/z*. All

preprocessing was done using the unpublished DiMS R package created by W.E Laubscher. Cosine similarity between all spectra were calculated using the coop R package to construct a cosine matrix[33] on which hierarchical clustering was performed using the complete agglomeration method[34]. Plotting was done using the base heatmap() function in the R statistical computation language[30].

Results and discussion

The isolation of soil bacteria for the novel identification of extracted compounds with antibacterial and antifungal activity has many difficult factors to consider. As discussed above, soil microbes interact with their environment and with each other in ways not fully comprehended yet. Culturing these microbes by mimicking their environment has been found to be difficult and as such biomining and discovery studies end up selecting for certain microbes without explicitly meaning to do so. The MPAS, used in this biomining study, can be easily employed in laboratories for an array of samples and it is relatively simple and inexpensive.

For this biomining survey soil samples were collected from various locations (Table 4.1). Micro-dilutions of suspended soil samples were cultured on nutrient agar and after 10 days of incubation the micro-culture of each soil isolate was extracted and tested for activity against two planktonic *C. albicans* strains, CAB1085 and MRC8912 (Fig. 4.1, refer to Fig. S4.1 for methodology scheme). Strain CAB1085 is an environmental strain from a river known to be contaminated with faecal matter, whereas strain MRC8912 is a clinical strain from human pus that has been shown to be resistant towards current antimicrobials such as fluconazole and amphotericin. The best hits were again confirmed by testing against planktonic strain MRC8912 of *C. albicans*. Once activity was confirmed by sieving through the large numbers using planktonic strains (as mentioned above), selected top hits from extracts showing activity were isolated and using the multiplex assay, again tested against all five strains of *C. albicans* (Table 4.2).

MPAS Screening and selection of active isolates from soil samples

From all the bacterial isolates, suspended and diluted in PBS and thereafter grown on 96-well agar plates, all collected and recovered from eight soil samples, 769 extracts from soil bacterium colonies were tested for activity against two strains of *C. albicans* using the assay I (planktonic cell inhibition) described here, as well as in Chapters 2 and 3. A global analysis of the activity data of the eight soil extracts (Table 4.1) against MRC8912 showed that there was indeed a subset of the extracts with

activity that fell within the hard cut-off limit (below 25000 RFU) of the planktonic cell assay in the multiplex assay system (Fig. 4.1).

This subset of active cultures was identified using a heatmap analysis (Fig. 4.2). From the data displayed in the heat maps of *C. albicans* strain CAB1085 and strain MRC8912, there is a distinct colour divide (separation) between active extracts (displayed as shades of blue) and non-active extracts (displayed as shades of red). The red shading in Fig. 4.2 indicates extracts that were not active enough to warrant further investigation. However, any shade of blue indicated an isolated extract had activity and was further investigated. Soil extracts were initially screened with MPAS assay I against planktonic life-stage of *C. albicans* MRC8912, followed by MPAS assay III assessing the eradication activity of *C. albicans* biofilms against two strains.

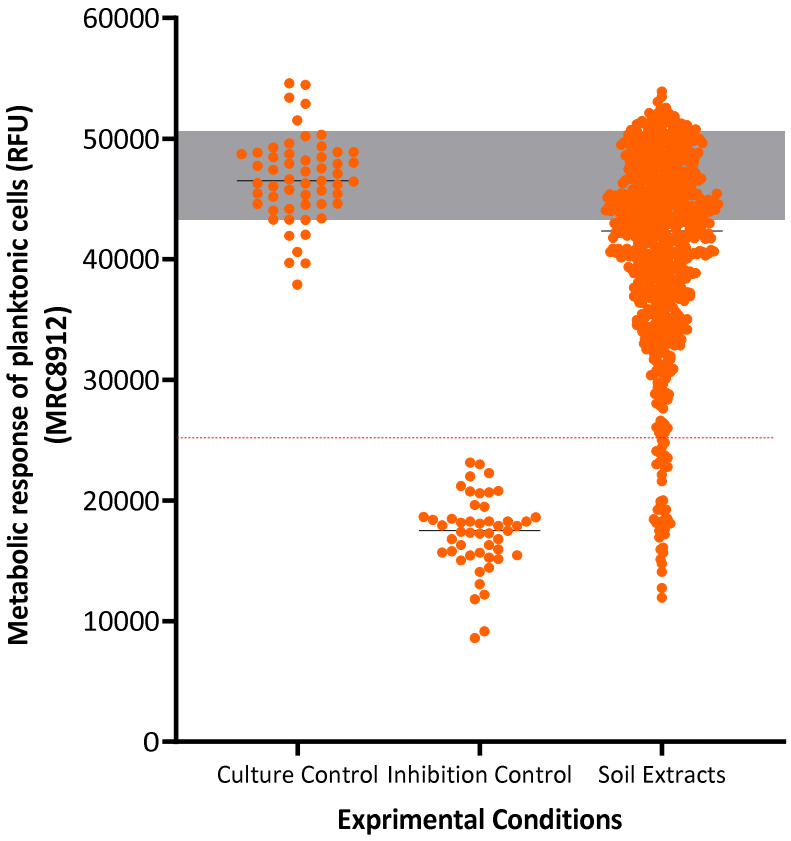


Figure 4.1 Global analysis of the percentage metabolic response profiles, using the MPAS assay I, of planktonic *C. albicans* MRC8912 towards the extracts of the eight soil samples. The data shows the pooled averaged planktonic metabolic activity of extracted compounds from eight soil samples and their cultured bacterial colonies. Gramicidin S (GS) was used as the positive inhibitory control. The lightly shaded grey area indicates absolute cut-off limits of mean ±1α standard deviation for identifying highly active extracts. The dotted red line depicts the 25000 RFU visual/hard cut-off for active extracts and results below this line indicate ≥50% inhibition. The medians of the data are demarcated with the dashed line in each violin plot.

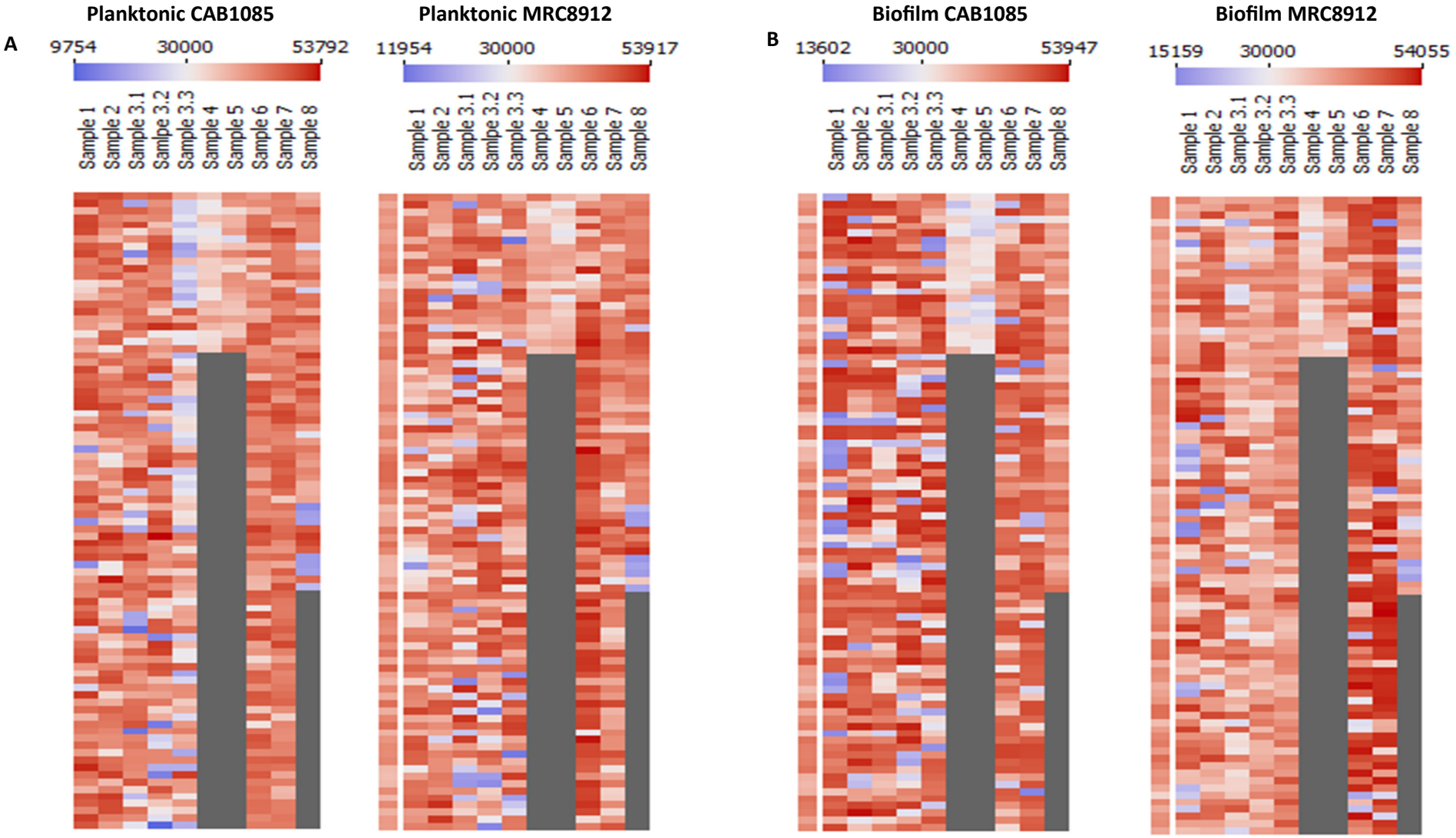


Figure 4.2 Metabolic activity data from assay I in the MPAS displayed as a heat map for the results towards two *C. albicans* strains (round 2 selection). The inhibitory activity against planktonic cells by 112 culture extracts from the soil isolates is shown in A. The biofilm eradication activity by 112 culture extracts from the soil isolates is represented in B. Heat map colour scales range from active (blue) to non-active (red). Each data point represents 1-3 determinations.

Using the heatmap-selection, depicted in Fig. 4.2, 77 active extracts form the bacterial isolates were selected at ≥50% inhibition cut-off and further assessed for activity with MPAS. From these 77 active extracts, 39 extracts were selected based on the statistical cut-off parameters calculated in Chapter 3 for strain CAB1085 and strain MRC8912.

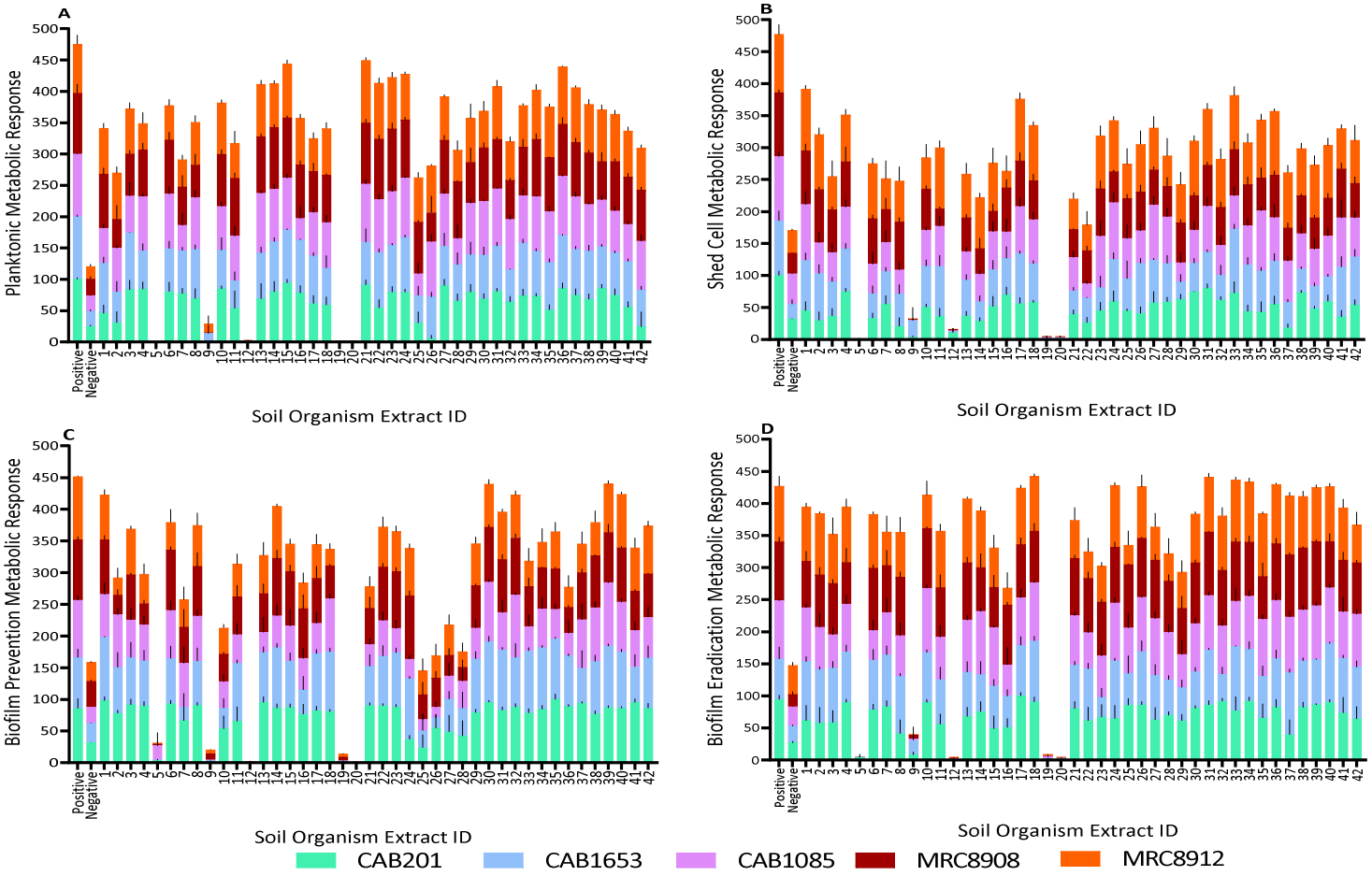
The activity of the 39 hits using the MPAS results was supported by results from a classical spread plate analysis of *C. albicans* CAB1085 and MRC8912 (Table S4.1). These two *C. albicans* strains were used as they are representative of one clinical and one environmental strain as well as being two more resistant strains according to data shown in Chapter 2. Of the active bacterial cultures (extracts 1A-39A), 32 bacterial colonies from these cultures were confirmed to have inhibition zones against *C. albicans* CAB1085 and/or MRC8912 using the classical spread plate analysis. There were only 32/39 active bacterial extracts according to the spread plate analysis (Table S4.1). Of these 32 active extracts, 22 extracts showed activity against the environmental strain CAB1085, whereas only 19 extracts showed inhibitory activity against clinical strain MRC8912. As expected from previous studies, most were more active against the environmental strain than the clinical strain. It must be considered that the agar environment could lead to false negatives as there may be an influence on the activity of the active compounds, as clear zones are dependent on diffusion without agar interaction. This solid medium could also influence the growth and life stage of the target organism leading either to false positives or false negatives. When considering only MRC8912 data (Table S4.1; Fig. 4.3), the comparison in spread plate activity and activity determined with MPAS, a resulting false negative rate of the classical spread plate assay was calculated as 49% and when considering activity against both strains on the spread plate analysis, a reduced false negative rate of 15% was calculated. This illustrated the power of the 20 parameter MPAS determinations above that of the labour-intensive classical spread plate assay, where only inhibition zones on agar are considered. Inhibition zones are furthermore dependent on the solubility, size and diffusion of the antimicrobial compound as well influence of agar matrix on activity, and differences in compound character can lead to false negatives. One limitation that must be kept in mind of the culture extraction procedure, is that each micro-extraction is limited to how well that bacterial isolate grows and produces during the 10-day culturing period used in this study (Chapter 3).

The 32 selected bacterial isolates were re-cultured on full-strength TSB agar plates in order to confirm culture purity and select for single colonies. Of the 32 bacterial isolates, 42 single colonies with anti-*Candida* inhibition zones were confirmed (single colony active extracts 1B-42B; Fig. 4.3A-D). A flow diagram depicting the process is shown in the supplementary data (Fig. S4.1). To

characterise and partially identify the organisms that produced the active extracts, the colony morphology and character was recorded (Table S4.2) and 16S DNA identification was performed on purified single colonies (Table S4.3). The data collected shows that from the 42 isolated colonies, 32 colonies were identified as belonging to *Bacillus* species, three to *Citrobacter* species, one *Pseudomonas aeruginosa* and five colonies having unsuccessful PCR results and one with an unsuccessful sequencing result. Of these 32 *Bacillus spp.*, 15 are thought to be *Bacillus pumilus*/ *zhangzhouensis* / *safensis* / *australimaris*, while the rest mapped to a variety of *Bacillus* species (Table S4.3). *Bacillus spp*. are Gram-positive, rod-shaped class *Bacilli* bacteria which are members of the phylum *Bacillota*. Originally, four species of the group were identified more than 40 years ago (*B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens*) after which the classifications have risen to about 377 species updated in 2019[35,36]. *Bacillus subtilis* members have the ability to form endospores, they have diverse physiological properties and they are able to produce a very wide range of antimicrobial products[35]. Stein *et al*.[35,37], postulate that 4-5% of any of the group of *B. subtilis*'s genome is devoted to antimicrobial product production, which are mainly antimicrobial peptides. These antimicrobial peptides have many functional and metabolic roles, are mostly cyclic and hydrophobic with moieties such as *D*-amino acids[35,38]. The main clades of antimicrobial compounds believed to be produced by the *B. subtilis* group are often non-ribosomally produced peptides, ribosomally-produced peptides, polyketides and volatiles[35]. *Bacillus spp*., such as *B. subtilis*[39] (possibly soil isolates 25, 32, 2, 11, 35, 4, 7, 14, 28 and 30) and *B. tequilensis*[40] (possibly soil isolates 11, 4, 14 and 35) are known to produce surfactants, including fengycin[41]. All three *Citrobacter spp*. were grouped as being *Citrobacter sedlakii / rodentium / werkmanii* (Table S4.3).

Broad spectrum MPAS assessment of 42 active hits

To further assess the broad-spectrum activity of the 42 extracts we used our comparative MPAS system with two clinical strains of *C. albicans*, MRC8908 and MRC8912, two environmental strains, CAB1085 and CAB1653 (mosquito larvae isolate), and one veterinary isolate, CAB201, as target organisms. With four assays and five target organisms our MPAS generates 20 inhibition parameters (% inhibition) per extract and therefore provides a broad-spectrum dataset on the activity of extracts (Fig. 4.3). Figs, S4.1-S4.4 in the supplementary data depict the effect of extracts 1B-42B on each strain using the MPAS assay to show planktonic inhibition, biofilm prevention and eradication, and inhibition of shed cell activity. In this data, all 42 soil isolates (1B-42B) were shown to be active to various degrees.



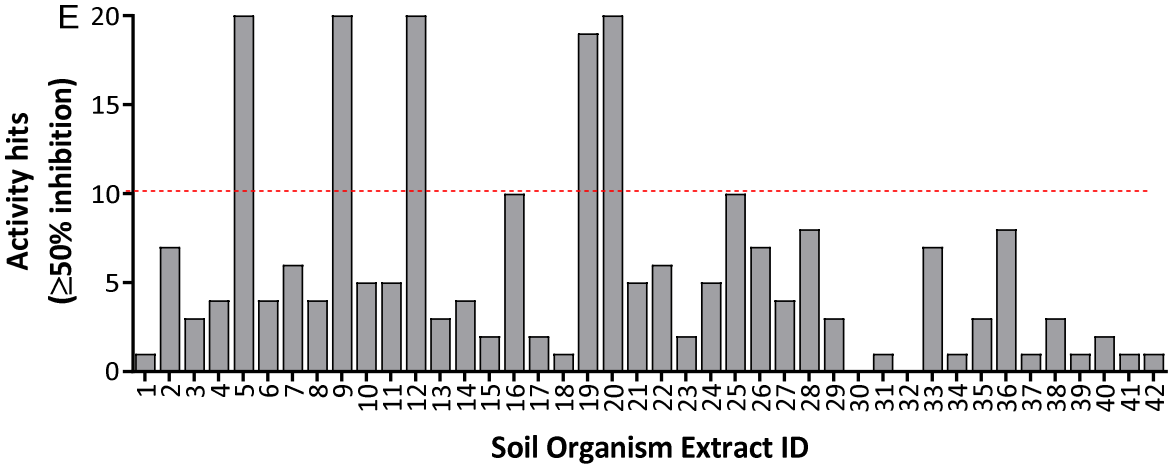


Figure 4.3 Comparative MPAS results (round 2) showing the normalised metabolic response of the five *C. albicans* strains by the selected 42 group B extracts from cultured soil organisms. The observed planktonic cell metabolism is shown in **A**, shed planktonic cell metabolism in B, biofilm metabolism with biofilm formation prevention activity represented in **C** and biofilm eradication represented in D. "Positive" and "Negative" A-D indicated the *C. albicans* culture controls and gramicidin S inhibition control, respectively. Each data point is the mean ± standard error of the mean (SEM) of four determinations. The bar graph in E shows the cumulative broad-spectrum activity of hits at ≥ 50% inhibition (number out of 20 assays) as found in the MPAS determinations for the 42 soil organism extracts (B samples). The dotted red line indicates 10/20 activity hits of ≥ 50% inhibition. Detailed data are provided in Figs. S2-S5.

Planktonic cell inhibition showed a general trend of only the five most active compounds above 50% inhibition (Fig. 4.3 A and Fig. S4.1 F) whereas shed cells seemed to be more affected by the presence of the soil microbes with only nine median values below the 25000 RFU inhibition cut-off (Fig. S4.4F). Biofilm prevention showed more general inhibition trends, although not all within absolute cut-off values but the biofilms were hindered by the presence of the microbes when considering the medians below the 50% cut-off value (Fig. 4.3 B and Fig. S4.2 F). From the data in Figs. S4.1-S4.4 and the derived stacked bar graphs in Fig. 4.3 A-D, it is evident that overall biofilm eradication was least affected by the soil microbes, except for the most active extracts (Fig. 4.3 C and Fig. S4.3F).

Overall, five extracts showed potent inhibitory activity (≥50% and within absolute cut-off values) towards the five *C. albicans* strains in all four assays encompassing the *C. albicans* life-stages (Fig. 4.3 E). In our MPAS this entails having ≥50% activity in 20 different assays highlighting that, extracts 5, 9, 12, 19 and 20 can be considered excellent broad-spectrum hits. Of the five extracts with clear inhibition activity, only colony 19 was identified as possibly *Citrobacter spp*., such as *(Citrobacter sedlakii/ rodentium/ werkmanii;* Table S4.4). The other four active colonies were not identified due to unsuccessful PCR results but were tentatively classified as Gram-negative bacteria (as confirmed using Gram staining) and colonies morphologically did appear to be extremely small, with all four possibly the same organism. Furthermore, there was a degree of broad-spectrum activity (10/20 determinations above 50% inhibition) observed for extracts 16 and 25. Extract 16 showed good biofilm prevention and eradication activity and was also identified as a *Citrobacter spp.* (*Citrobacter werkmanii/ rodentium*). Extract 25 showed good planktonic activity as well as biofilm prevention and eradication activity (Fig. 4.3 E) and was putatively identified as a *Bacillus spp*. (*Bacillus amyloliquefaciens/ siamensis/ velezensis/ subtilis*).

We utilised the UPLC-MS data to compare the molecular fingerprints of the 42 extracts with each other and correlated them with the genetic data of the producer soil organisms. Fig. 4.4 illustrates the similarities and differences in the UPLC-MS profiles of the seven most active extracts. A more detailed comparative analysis of the UPLC-MS profiles of the 42 extracts was done using a mathematical algorithm created in R (in collaboration with W.E Laubscher). In this a cosine map with associated hierarchical dendrograms was compiled (Fig. 4.5). From the cosine map of the extracts of the 42 producer bacteria revealed three major clusters, each with 2-5 sub-clusters and a total of 26 minor clusters. Cluster I consisted of extracts 23B, 19B, 9B, 12B, 5B and 20B. The majority of the producers of these extracts were genetically unidentified, except for extract 19B and 23B, which

were identified as *Citrobacter spp. (Citrobacter sedlakii/ rodentium/ werkmanii)*. This cluster analysis highlights the possibility that the other producers might also be from the same species.

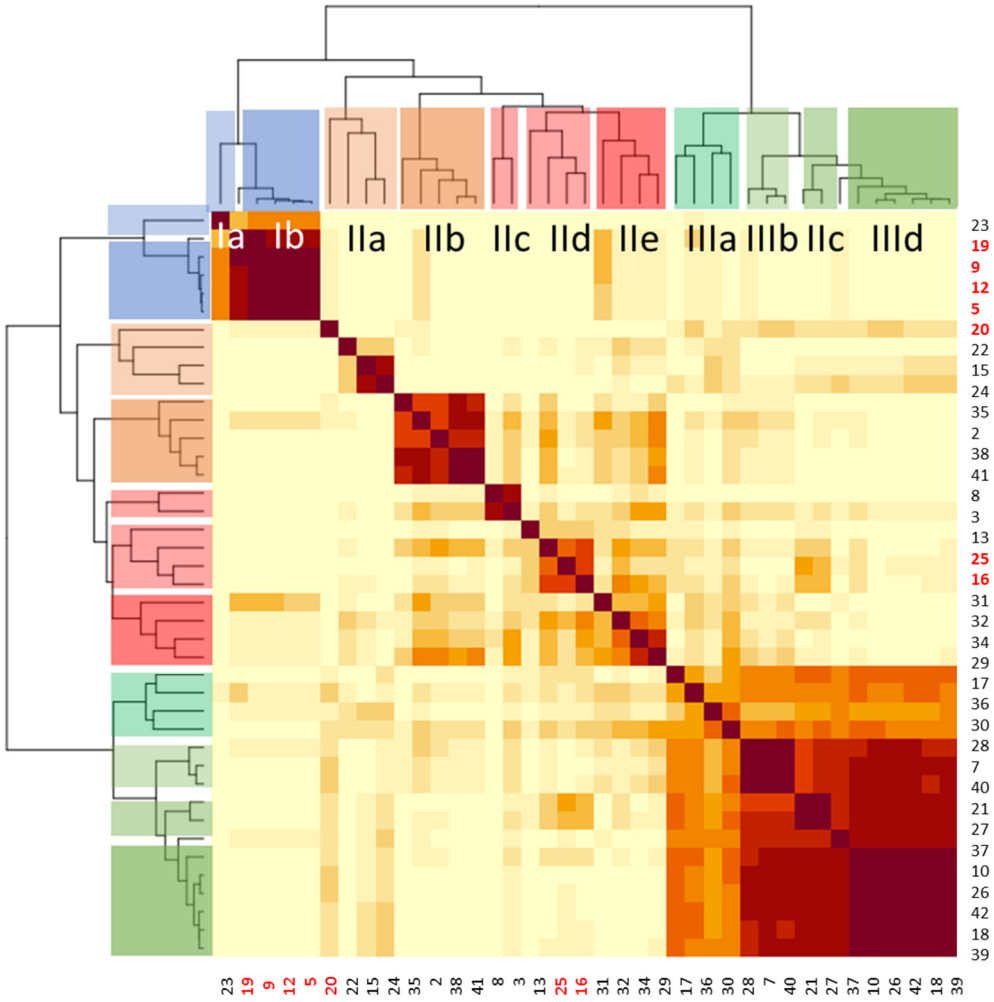


Figure 4.5 A hierarchical cluster dendrogram and cosine heat map of the 42 active extracts (B-samples) from high resolution UPLC-MS analyses (extracts 1B-42B). The coloured blocks highlight the major and sub-clusters that indicates correlating molecular profiles, therefor similar active compounds. The seven most active hits are shown in red. The map and dendrogram was compiled using R coded algorithm analysis (courtesy W Laubscher, unpublished R code from PhD study)

Cluster II consisted of *Bacillus spp.*, but they were grouped into a very wide array ranging between 16 possibilities; *Bacillus thuringiensis/ albus/ cereus/ tropicus/ anthracis/ paramycoides/ wiedmannii/ pumilus/ zhangzhouensi/ safensis/ australimaris/ amyloliquefaciens/ velezensis/ siamensis* to even possibly *Bacillus subtilis/ Bacillus tequilensis* (Refer to Table S4.3 for more details). More specific genetic correlations can be drawn between cluster III, which also consisted of *Bacillus spp*. but more specifically can be grouped into seven possibilities. These are *Bacillus pumilus/ Bacillus zhangzhouensis/ safensis/ australimaris/ amyloliquefaciens/ atrophaeus/ subtilis.* Genetically this procedure did select for *Bacillus spp*., which is a consideration that could be improved upon in future studies. Of the three larger clusters consisting of two or more pure colony

extracts, cluster Ib contained five of the most active bacterial extracts (5B; 9B; 12B; 19B; 20B). This cluster is associated to cluster IId of extract 25 and extract 16. This indicated that their production profiles had similar compounds, which could be related to the lipopeptides identified in the profile of extract 25 (refer to Fig 4.6 and the discussion below). As most of the extracts shared some compounds as indicated by the dendrogram linkages some of the detected activities could be because of their presence in the extracts.

Identifying active compounds in seven hit extracts

To follow up the activity results and observed clustering in cluster Ib, extracts of the seven most active hits from the pure isolates were analysed utilising UPLC-MS and UPLC-MS/MS (UPLC-MS^e). In order to putatively identify the compounds that may be related to the detected anti-Candida activity, accurate mass, elemental composition, and fragmentation data were used in conjunction with literature searches to putatively identify the compounds. An example of UPLC-MS profile of extract 25B with correlating high resolution mass spectra is presented in Fig. 4.6.

Isolates 9B, 12B, and 25B all produced fengycins (Fen). The fengycin family are cyclic, natural defence molecules containing variants of 10 α-amino acid residues linked to a C_{14}-C_{18} β-hydroxy fatty acyl residue. They display strong antifungal activity[35,42,43], as well as antibacterial activity[44] and it is thought that they affect membrane structure and permeability[42,45,46]. The fengycin family were traced in the extracts with a unique identifier at *m/z* 620.31 that maps to the sequence Glu-D-Orn-D-Tyr-D-alloThr-Glu in this lipopeptide family (Fig. S4.6). Isolate 25B, a *Bacillus* sp., produced the highest amount containing FenZ and FenA, B and D. Isolates 9B and 12B produced mostly of Fen D (Table 4.4).

Isolates 19A, 20A, and 25B all produced peptides from the iturin family. Isolates 19A, 20A were mixed cultures and the colonies were lost in the reculturing and selection of the 42 pure isolates. Iturins are a group of cyclic octalipopeptides with a conserved structure that contains a b-amino fatty acyl residue with varying chain length linked in a cyclic peptide moiety[38]. They have been known to affect surface tension leading to pore formation of lipopeptide-sterol aggregates[47], which results in cell death caused by ion leakage[48]. The family of iturins consists of iturin A, C, D and E[49], as well as bacillomycin D[50], F[51] and L[52], bacillopeptin[53] and mycosubtilin[54]. The bacillomycins have strong activity against yeasts and fungi such as *Aspergillus niger* and *Candida albicans*, with a narrow activity spectrum against bacteria[51,52]

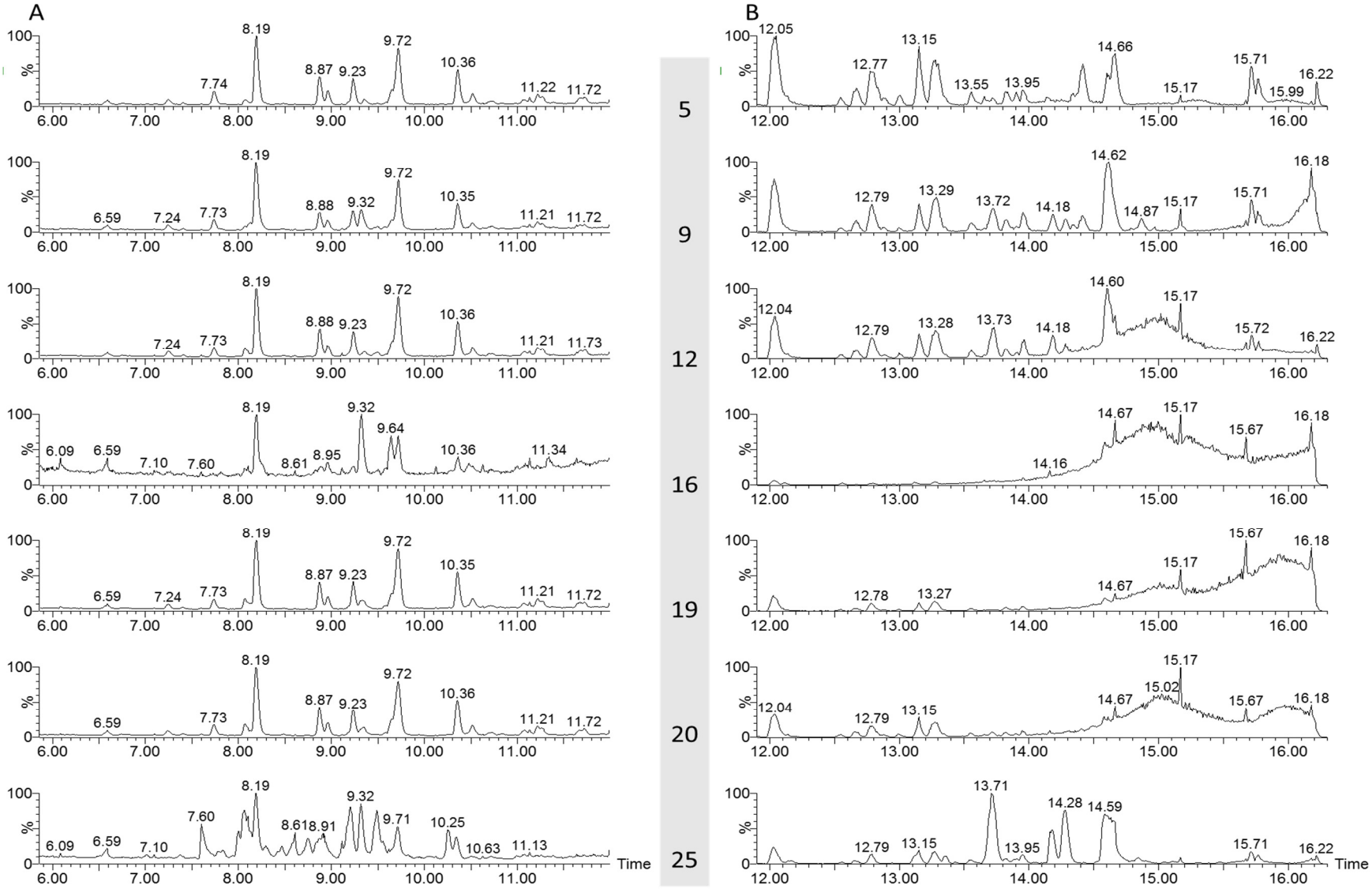


Figure 4.4 Comparison of the UPLC-MS profiles of the seven most active culture extracts that were identified after re-culturing (B samples). Panel A shows the comparative profiles of positive ions of the more amphipathic compounds eluting from 6 to 12 minutes from the C_{18} matrix. Panel B shows the comparative profiles of positive ions of the more hydrophobic compounds eluting from 12 to 17 minutes from the C_{18} matrix.

Iturin A, with identifier fragment *m/z* 212.10, mapping to Pro-D-Asn, with different chain lengths for the β-amino acyl residue was identified in isolate 19A extract (Fig. S4.8, Table 4.4). Bacillopeptin, with identifier fragment *m/z* 278.11, mapping to Asn- D-Tyr or D-Tyr-Asn, with different chain lengths for the β-amino acyl residue was identified in isolate 25B extract (Fig. S4.9, Table 4.4). Bacillomycin D, with identifier fragment *m/z* 314.12, mapping to Asn- D-Tyr or D-Tyr-Asn, with different chain lengths for the β-amino acyl residue was identified insolate 25A (Fig. S4.9, Table 4.4).

Isolates 9B, 12B, 19A, 20A, 20B and 25B all produced surfactins. Surfactins are acidic, cyclic heptalipopeptides consisting of six amino acids and a β-hydroxy fatty acyl (bHC_n) residue with varying chain length[37]. Their amino acid sequence is completely different to that of the iturin group[50]. There are four surfactin groups (I, II, III, IV), each sharing the same M_r in the surfactin complex. The peptides in the groups and within the groups differ from each other by the number of carbons in bHC_n chain and if there is a Val, Ile or Leu present at positions 4 and 7 in their sequences[55]. These lipopeptides have a moderate amphipathic character with pronounced hydrophobic component, which allows for the surfactant activity and hence their absorption at hydrophilic/hydrophobic interfaces[55,56]. The strong surfactant activity of this group of lipopeptides may lead to antibiofilm activity as well as antimicrobial activity. As the surfactin complex was observed to be produced with other lipopeptides in some of the extracts (isolates 9B and 25B), this could lead to synergistic activity against *C. albicans*. Synergistic activity against certain plant fungi was observed between the iturins and surfactin, as they form iturin-surfactin micelles[55,57]. This could be a beneficial combination of antimicrobial peptides which plays a role in the activity results observed during this study. The surfactins, with identifier fragment *m/z* 441.3, mapping to Leu/Ile-D-Leu/Ile-D-Val-Asp or D-Leu/Ile-D-Val-Asp Leu/Ile, were characterised in isolated 25B extract. Members of four surfactin groups I, II, III and IV were identified (Fig. S4.9, Table 4.4). The structure of surfactin IV was verified (Fig. S4.10).

Although lipopeptides were the major antimicrobial components identified, we also found other interesting components that may contribute to the antibiofilm activity. However, our search was not exhaustive. All the culture extracts, except extracts 16B and 25B produced a group of small compounds, all with a unique fragment at *m/z* 159.068 used as identifier, and a UV absorption peak at 325 nm (Fig. S4.9).

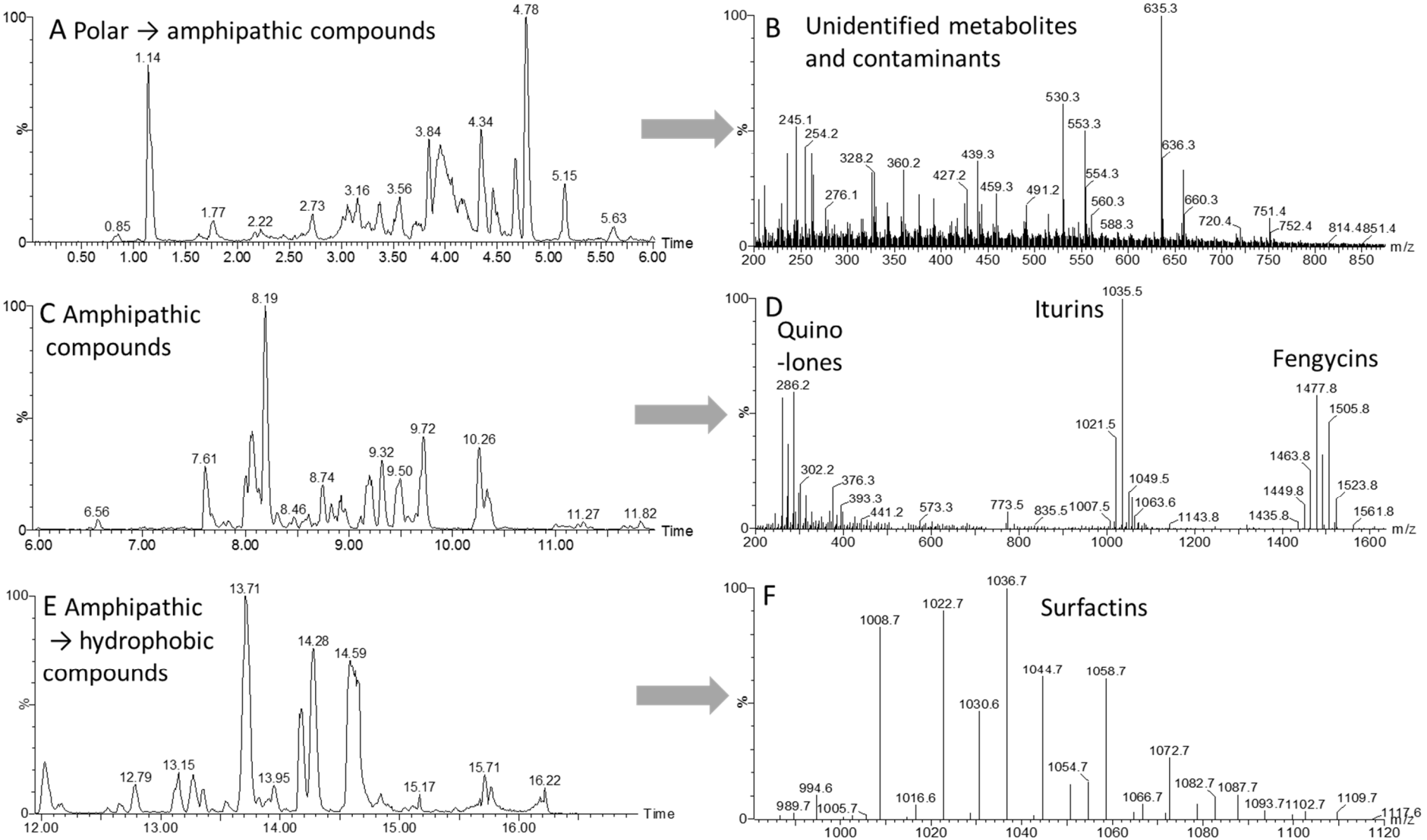


Figure 4.6 UPLC-MS analysis from a *Bacillus spp* colony (isolate 25B) illustrates the production profiles consistent with this species, namely polar molecules (A), amphipathic compounds (C), iturins and fengycins (D), amphipathic hydrophobic compounds (D) and surfactins (E). Furthermore, unidentified compounds and contaminants are seen in B. The UPLC-Ms profiles with positive ion detection are shown on the left, and spectra combined over 0-6 minutes (A), 6-12 minutes (B) and 12-17 minutes (C) are shown on the right.

Table 4.4 Summary of the major compounds identified in the seven active extracts with broad spectrum activity towards *C. albicans* strains and life stages. Compounds of which structures were elucidated are highlighted in the grey shaded cells and active extract indicated with bold font.

Retention time (minute)	Unique identifier fragment (*m/z*)	Putative Compound ID	Theoretical [M_r+H]	Experimental [M_r +H] (ppm error)	Active extract	Reference for structure
8.45		Fengycin Z	1449.7819	1449.7813 (4.7)		
8.88		Fengycin A	1463.7965	1463.7968 (4.7)		Villegas *et al*.[58]
9.19	620.31	Fengycin B	1477.8202	1477.8110 (5.7)	9B, 12B, **25B**	Li *et al*.,[59]
9.19	(Fig. S4.6)	Fengycin C	1491.1858	1491.8237 (7.6)		Price *et al*.,[60]
9.52		Fengycin D	1505.8382	1505.8413 (6.2)		Yang *et al*.,[61]
7.31		Iturin A (C_{14})	1043.5525	1043.5513 (1.2)		
7.76	212.104	Iturin A (C_{15})	1057.5795	1057.5871 (1.1)		Moyne *et al*.[50]
8.38	(Fig. S4.7)	Iturin A_L (C_{16})	1071.6065	1071.5809 (2.8)	**19A**	Yang *et al*.[62]
8.78		Iturin A_L(C_{17})	1085.6335	1085.5974 (1.9)		Rautenbach *et al.*[48]
7.75		Bacillopeptin (C_{14})	1021.5241	1021.5161 (4.4)		
8.14, 8.20	278.111	Bacillopeptin (C_{15})	1035.5511	1035.5325 (3.6)	**25B**	Hofemeister *et al*.[63]
8.75, 8.85	(Fig. S4.8)	Bacillopeptin (C_{16})	1049.5781	1049.5479 (3.8)		Kajimura *et al*.[53]
7.36		Bacillomycin D (C_{13})	1017.5292	1017.5253 (0.4)		
7.78	314.119	Bacillomycin D (C_{14})	1031.5562	1031.5417(-0.4)	**20A**	Volpon *et al*.[64]
8.24	(Fig. S4.9)	Bacillomycin D (C_{15})	1045.5832	1045.5574 (-0.4)		Peyoux *et al*.[65]
8.79		Bacillomycin D (C_{16})	1059.6102	1059.5721 (0.5)		

Table 4.4 Continued

Retention time (minute)	Unique identifier fragment (*m/z*)	Putative Compound ID	Theoretical [M_r + H]	Experimental [M_r+H] (ppm error)	Active extract	Reference for structure
13.27		Linear Srf IV	1054.7022	1054.7010 (0.5)		
13.50		Surfactin I	994.6439	994.6428 (1.2)		Rautenbach *et al*.[66]
13.86	441.271	Surfactin II	1008.6596	1008.6591 (0.5)	5B, 9B, 12B,	Ndlovu *et al*.[67]
14.03	(Fig. S4.10)	Surfactin IV + Na	1058.6728	1058.6708 (2.0)	16B, 20B, **25B**	Chen *et al*.[68]
14.32, 14.42		Surfactin III	1022.6752	1022.6734 (1.8)		Eyéghé-Bickong *et al*.[55]
14.74		Surfactin IV	1036.6909	1036.6897 (1.2)		
8.21		HQNO – OH, +H	244.1701	244.1696 (2.2)		
8.33	159.068	2-heptyl-4-quinolone-N-oxide	260.1651	260.1646 (1.7)	**5B**, 9B, 12B,	Andriole *et al*.[69]
9.05	(Fig. S4.11)	HQNO + 2CH_2	288.1964	288.1962 (0.5)	19B, 20B	Dubern *et al*.[70]
10.49		Novel Quinolone	NA	298.2163 (NA)		
10.51		Novel Quinolone	NA	314.2116 (NA)		
12.17		Rhamnolipid 2 (C_{12})	527.3188	527.3187 (0.2)		
12.92	153.128	Rhamnolipid 2 ($C_{14:2}$)	553.3353	553.3352 (-1.4)	**5B**, 16B, 25B	Rudden *et al*.[71] Haba *et al*.[72]
13.42	(Fig. S4.12)	Rhamnolipid 2 (C_{14})	555.3728	555.3506 (-0.9)		

The major peak at *m/z* 260.1645 and Rt 8.19 was identified as 2-heptyl-4-quinolone-N-oxide (HQNO, $C_{16}H_{21}NO_2$), a known quorum sensing compound, with a quinolone base ring structure[67]. The related detected cations ranged in *m/z* values from 244-314, which all have similar fragments to that of HQNO (Fig. S4.9). These small compounds are possibly a group of quorum sensing molecules associated with various quinolone variants. These quorum sensing molecules could have an influence on *C. albicans* behaviour by signalling microbial competitors. Some studies have shown the quinolones to have antimicrobial activity[69].

Another interesting group of compounds with antimicrobial activity was identified as rhamnolipids. Rhamnolipids are one of the groups of specialized metabolites produced by *Pseudomonas aeruginosa* and various other bacteria. They are a class of glycolipids which can act as bacterial surfactants and form part in a quorum sensing network[68,69]. Rhamnolipid 2 with $C_{12,}$ $C_{14:1}$ and C_{14} fatty acyl tails was identified in extracts of isolates 5B, 16B and 25B using a unique identifier at *m/z* 158.13, a putative fragment from the sugar moiety (Fig. S4.9).

From the data collected, it is evident that this MPAS assay can be used in a medium to high-throughput manner to identify novel bacterial isolates and their corresponding active extracts.

Conclusion

It can be considered that overall, that MPAS is a successful technique in biomining environmental samples for anti-*Candida* compounds. In future experiments, the producer bacterial selection could be changed by using different medium types and incubation temperatures and time frames. The added benefit of the selection criteria of MPAS allows the user to determine how stringent or relaxed the cut-off values are when considering antifungal activity against *C. albicans* or alternate fungal/bacterial targets. MPAS can be used to give a quick, reproducible and reliable indication of anti-*Candida* inhibitory activity of possible (novel) antifungal compounds in micro-culture extracts, from unknown soil bacterial isolates. From 77 bacterial isolates, the selection procedures resulted in 42 active, single bacterial isolates, preliminarily identified seven broad spectrum hits, but there are 35 more possible producer organisms according to MPAS and 27 according to the classical spread plate assay. The selection process of this study concentrated on *Bacillus spp.*, which could be improved upon in future biomining experiments, by tailoring culturing of producer organisms. However, the MPAS determination in our study did select for known lipopeptides with antifungal activity and there are possibly also novel compounds with inhibitory activity in the active cohort. The production and micro-extractions can be considered a success with corresponding cut-off values to suit the needs for this application in antifungal drug discovery. Overall, this work further improves

the possibility of identifying novel antibiotics and antifungals from a natural, complex microbiome and this system can be easily applied in any laboratory. With this work, we hope to aid in the discovery of novel small antimicrobials in the future, possibly adding to the antifungal pipeline of drugs to combat microbial resistance.

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Supplementary Data

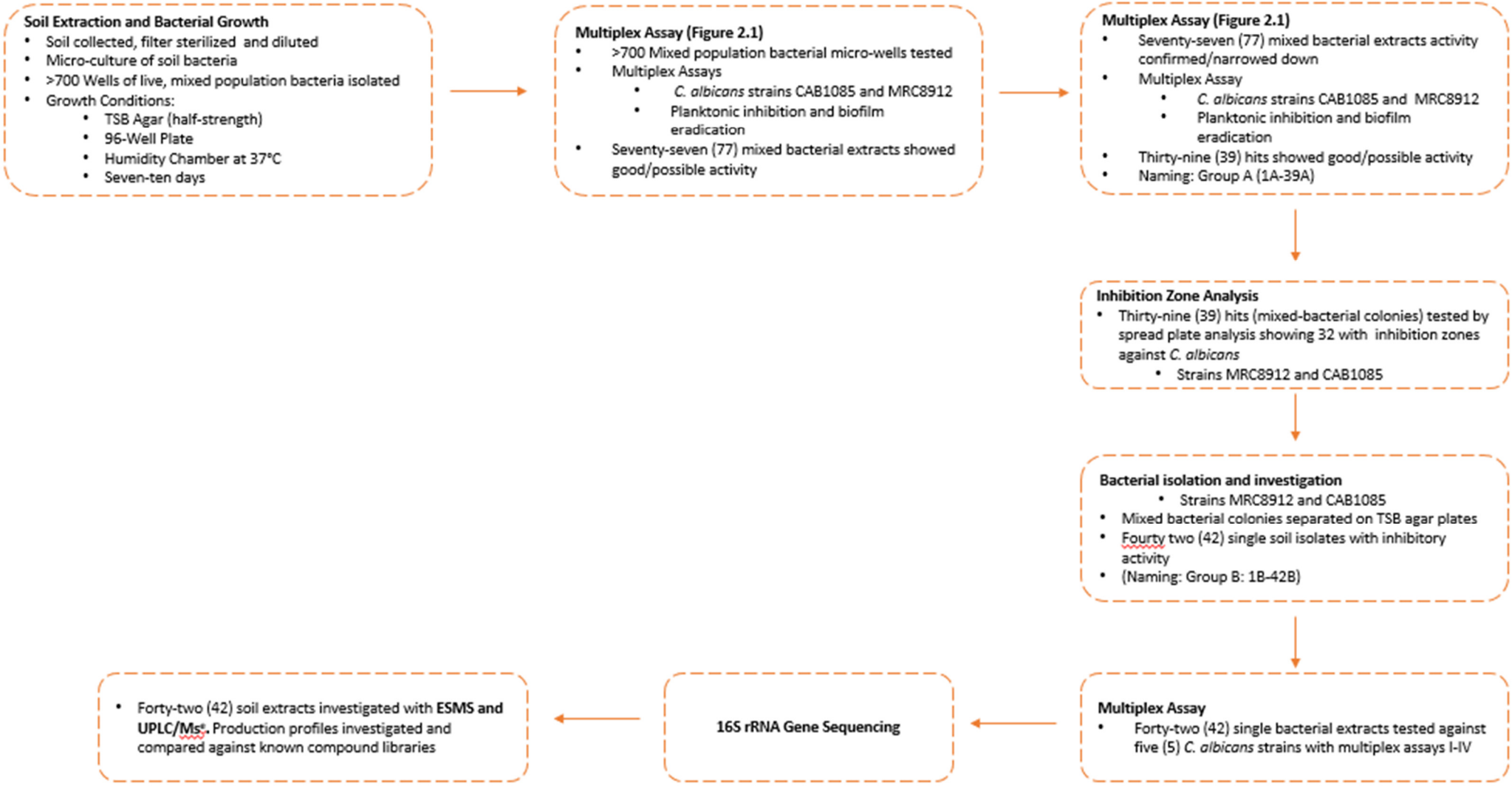

Figure S4.1. A flow diagram showing the process followed to isolate active bacterial colonies from soil extracts.

Table S4.1 Inhibition zone results from the classical spread plate assay to compare with MPAS results from extracted soil compounds, using two *C. albicans* strains. The grey shaded rows indicate the seven best hits from the MPAS determinations.

Soil Hits	Inhibition Zones at 30 h (mm)		
	MRC8912	**CAB1085**	**Both**
1A	-	17	-
2A	5	4	+
3A	3.5	4	+
4A	-	8	-
5A	-	9	-
6A	-	5	-
7A	-	7	-
8A	-	9	-
9A	-	9	-
10A	4	-	-
11A	-	-	-
12A	2.5	-	-
13A	2	1	+
14A	3	2	+
15A	-	-	-
16A	2	9	+
17A	-	-	-
18A	1	12	+
19A	-	8	-
20A	-	9	-
21A	-	2	-
22A	2	-	-
23A	-	-	-
24A	-	-	-
25A	3.5	12	+
26A	-	5	-
27A	1	2	+
28A	-	9	-
29A	2	1	+
30A	-	-	-
31A	6	-	-
32A	4	-	-
33A	1	-	-
34A	3.5	-	-
35A	4.5	-	-
36A	3	-	-
37A	-	1	-
38A	1	-	-
39A	-	-	-

Table S4.2 Morphological characteristics of soil isolates displaying definite/probable antimicrobial activity towards *C. albicans* as determined using the MPAS assay design and/or inhibition zones.

Isolate	Shape	Elevation	Size	Texture	Appearance	Pigmentation
1 B	Irregular	Flat	Small	Smooth	Dull	Milky yellow
2 B	Irregular	Raised globular centre, flat branches	Large	Smooth	Shiny	Milky white
3 B	Round	Flat	Small	Smooth	Shiny	Milky white
4 B	Irregular	Raised	Small	Smooth	Shiny	Milky white
5 B	Irregular	Flat	Moderate	Smooth	Shiny	Green/yellow
6 B	Irregular	Raised globular centre, flat branches	Moderate	Smooth	Shiny	Milky white
7 B	Irregular	Flat	Small	Rough	Dull	Milky yellow
8 B	Round	Raised	Moderate	Smooth	Shiny	Milky yellow
9 B	Irregular	Flat	Moderate	Smooth	Shiny	Green/yellow
10 B	Round	Flat	Small	Smooth	Dull	Milky white
11 B	Round	Raised globular centre, flat branches	Moderate	Smooth	Shiny	Milky white
12 B	Irregular	Flat	Moderate	Smooth	Shiny	Green/yellow
13 B	Irregular	Flat	Moderate	Rough	Dull	Milky white
14 B	Irregular	Raised	Moderate	Smooth	Shiny	Milky white
15 B	Irregular	Flat	Large	Rough	Dull	Milky white
16 B	Round	Raised	Small	Smooth	Shiny	Milky white
17 B	Round	Flat	Moderate	Rough	Dull	Milky white
18 B	Round	Flat	Small	Smooth	Dull	Milky yellow
19 B	Round	Raised	Small	Smooth	Shiny	Milky yellow
20 B	Irregular	Flat	Large	Smooth	Shiny	White
21 B	Round	Flat	Small	Smooth	Shiny	Milky yellow
22 B	Round	Flat	Moderate	Smooth	Shiny	Milky yellow
23 B	Round	Flat	Small	Smooth	Shiny	Milky white
24 B	Round	Flat	Moderate	Rough	Dull	Milky white
25 B	Irregular	Raised globular centre, flat branches	Large	Rough	Dull	Milky White
26 B	Irregular	Flat	Small	Smooth	Dull	Milky white
27 B	Irregular	Flat	Large	Rough	Dull	Milky White
28 B						
29 B	Irregular	Flat	Small	Smooth	Dull	Milky white
30 B	Irregular	Flat	Moderate	Rough	Dull	Milky White
31 B	Irregular	Flat	Moderate	Rough	Dull	Milky White
32 B	Irregular	Raised globular centre, flat branches	Large	Rough	Dull	Milky White
33 B	Irregular	Flat	Moderate	Rough	Dull	Milky White
34 B	Round	Flat	Small	Smooth	Shiny	Milky white
35 B	Round	Raised	Moderate	Smooth	Shiny	Yellow
36 B	Round	Flat	Moderate	Smooth	Shiny	Milky white
37 B	Round	Flat	Small	Rough	Dull	Milky white
38 B	Round	Raised globular centre, flat branches	Moderate	Rough	Dull	Milky White
39 B	Round	Flat	Small	Smooth	Dull	Milky White
40 B	Round	Flat	Small	Smooth	Shiny	Milky white
41 B	Round	Raised	Moderate	Smooth	Shiny	Brown

TABLE S4.3 Summary of the species and putative sub-species that were derived by 16S rDNA gene sequencing of the 42 active soil isolates[28].

Isolate	Possible organism identification
1 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
2 B	*Bacillus spp. (Bacillus amyloliquefaciens/ Bacillus velezensis/ Bacillus siamensis/ Bacillus subtilis)*
3 B	*Bacillus spp. (Bacillus safensis/ Bacillus australimaris/ Bacillus altitudinis/ Bacillus pumilus)*
4 B	*Bacillus spp. (Bacillus tequilensis/ Bacillus subtilis)*
5 B	PCR UNSUCESSFUL
6 B	PCR UNSUCESSFUL
7 B	*Bacillus spp. (Bacillus amyloliquefaciens/ Bacillus atrophaeus/ Bacillus subtilis)*
8 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
9 B	PCR UNSUCESSFUL
10 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
11 B	*Bacillus spp. (Bacillus subtilis/ Bacillus tequilensis)*
12 B	PCR UNSUCESSFUL
13 B	*Pseudomonas aeruginosa*
14 B	*Bacillus spp. (Bacillus subtilis/ Bacillus tequilensis)*
15 B	*Bacillus spp. (Bacillus thuringiensis/ Bacillus albus/ Bacillus cereus/ Bacillus tropicus/ Bacillus anthracis/ Bacillus paramycoides/ Bacillus wiedmannii)*
16 B	*Citrobacter spp. (Citrobacter werkmanii/ Citrobacter rodentium)*
17 B	*Bacillus spp. (Bacillus thuringiensis/ Bacillus cereus/ Bacillus wiedmannii/ Bacillus proteolyticus/ Bacillus albus*
18 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
19 B	*Citrobacter spp. (Citrobacter sedlakii/ Citrobacter rodentium/ Citrobacter werkmanii)*
20 B	PCR UNSUCESSFUL
21 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
22 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
23 B	*Citrobacter spp. (Citrobacter rodentium/ Citrobacter sedlakii)*
24 B	SEQUENCING UNSUCESSFUL
25 B	*Bacillus spp. (Bacillus amyloliquefaciens/ Bacillus siamensis/ Bacillus velezensis/ Bacillus subtilis)*
26 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
27 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
28 B	*Bacillus spp. (Bacillus amyloliquefaciens/ Bacillus atrophaeus/ Bacillus subtilis/ Bacillus nakamurai/ Bacillus vallismortis)*
29 B	*Bacillus spp. (Bacillus amyloliquefaciens/ Bacillus atrophaeus/ Bacillus subtilis/ Bacillus nakamurai/ Bacillus vallismortis)*
30 B	*Bacillus spp. (Bacillus velezensis/ Bacillus amyloliquefaciens/ Bacillus siamensis)*
31 B	*Bacillus spp. (Bacillus amyloliquefaciens/ Bacillus siamensis/ Bacillus velezensis/ Bacillus subtilis)*
32 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
33 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
34 B	*Bacillus spp. (Bacillus tequilensis/ Bacillus subtilis)*
35 B	*Bacillus spp. (Bacillus thuringiensis/ Bacillus cereus/ Bacillus paramycoides/ Bacillus anthracis/ Bacillus albus/ Bacillus tropicus)*
36 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
37 B	*Bacillus spp. (Bacillus amyloliquefaciens/ Bacillus velezensis/ Bacillus subtilis/ Bacillus siamensis)*
38 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
39 B	*Bacillus spp. (Bacillus thuringiensis/ Bacillus wiedmannii/ Bacillus proteolyticus/ Bacillus cereus/ Bacillus albus)*
40 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
41 B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*
42B	*Bacillus spp. (Bacillus pumilus/ Bacillus zhangzhouensis/ Bacillus safensis/ Bacillus australimaris)*

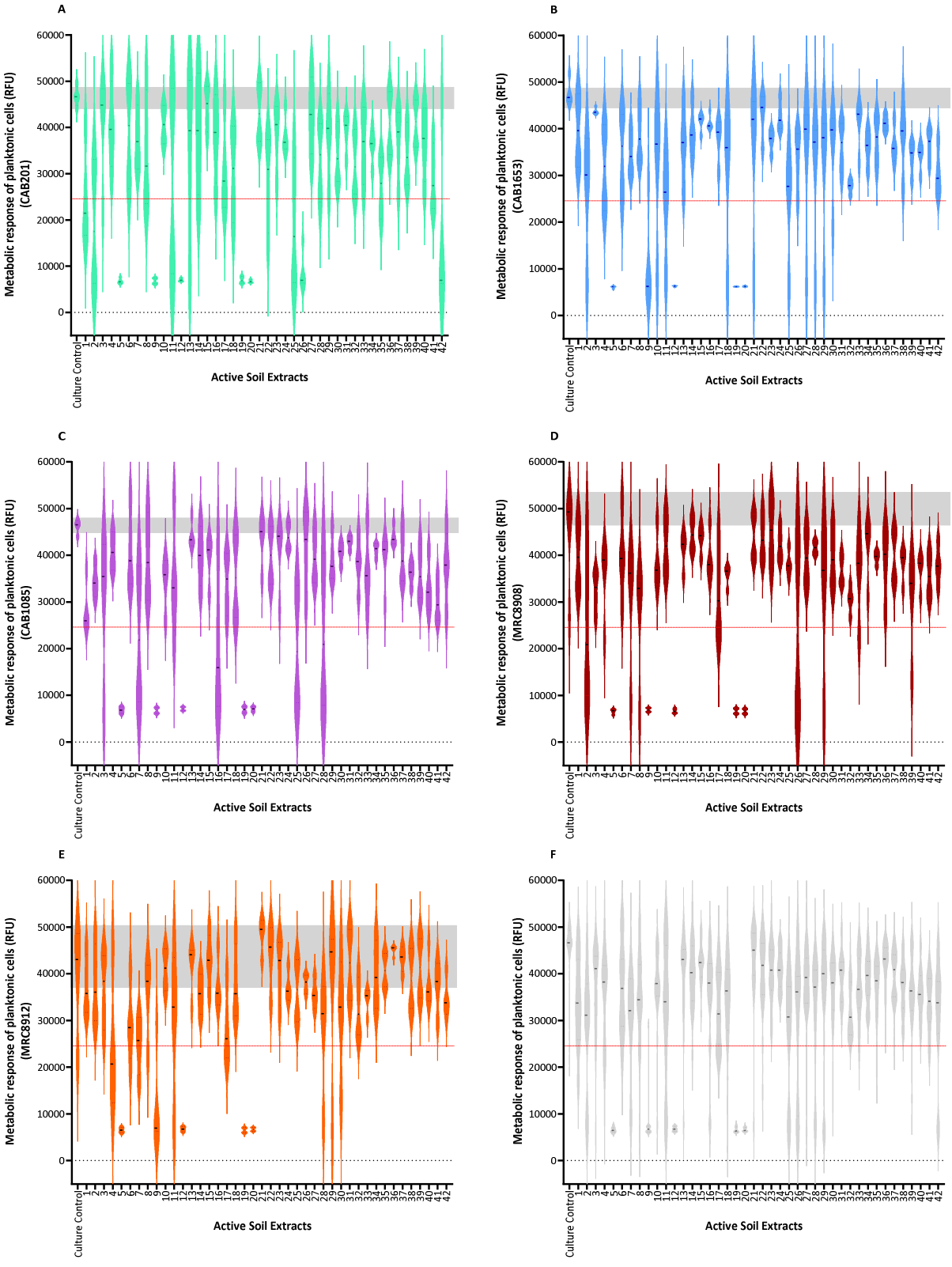

Figure S4.2 Comparative MPAS results showing the metabolic response in terms of culture control of the five *C. albicans* strains by the selected 42 group B extracts from cultured soil organisms. Activity against planktonic cell metabolism (assay I) shown against CAB201 (A), CAB1653 (B), CAB1085 (C), MRC8908 (D) and MRC8912 (E). Pooled planktonic inhibitory activity of all five strains against extracts shown in F. Each violin plot depicts four determinations. The shaded grey area indicates absolute cut-off limits of mean ±1α standard deviation for identifying putative extracts. The dotted red line depicts the 25000 RFU visual/hard cut-off for active extracts and results below this line indicates ≥50% inhibition.

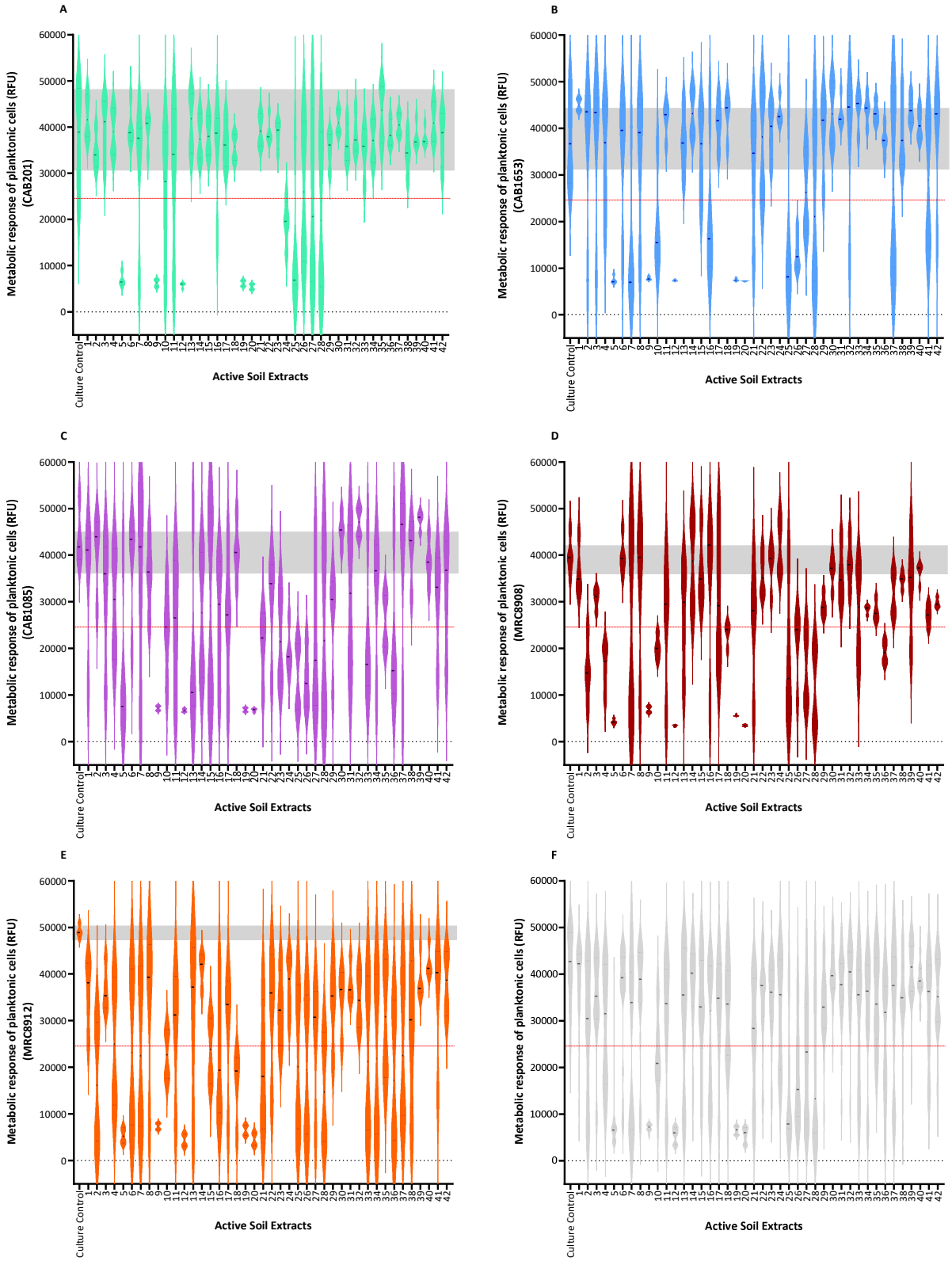

Figure S4.3. Comparative MPAS results showing the metabolic response of the five *C. albicans* strains by the selected 42 group B extracts from cultured soil organisms. Activity against preventing biofilms shown against CAB201 (A), CAB1653 (B), CAB1085 (C), MRC8908 (D) and MRC8912 (E). Pooled biofilm prevention metabolic activity of all five strains against extracts shown in F. Each violin plot depicts four determinations. The shaded grey area indicates absolute cut-off limits of mean ±1α standard deviation for identifying putative active extracts. The dotted red line depicts the 25000 RFU visual/hard cut-off for active extracts and results below this line indicates ≥50% inhibition.

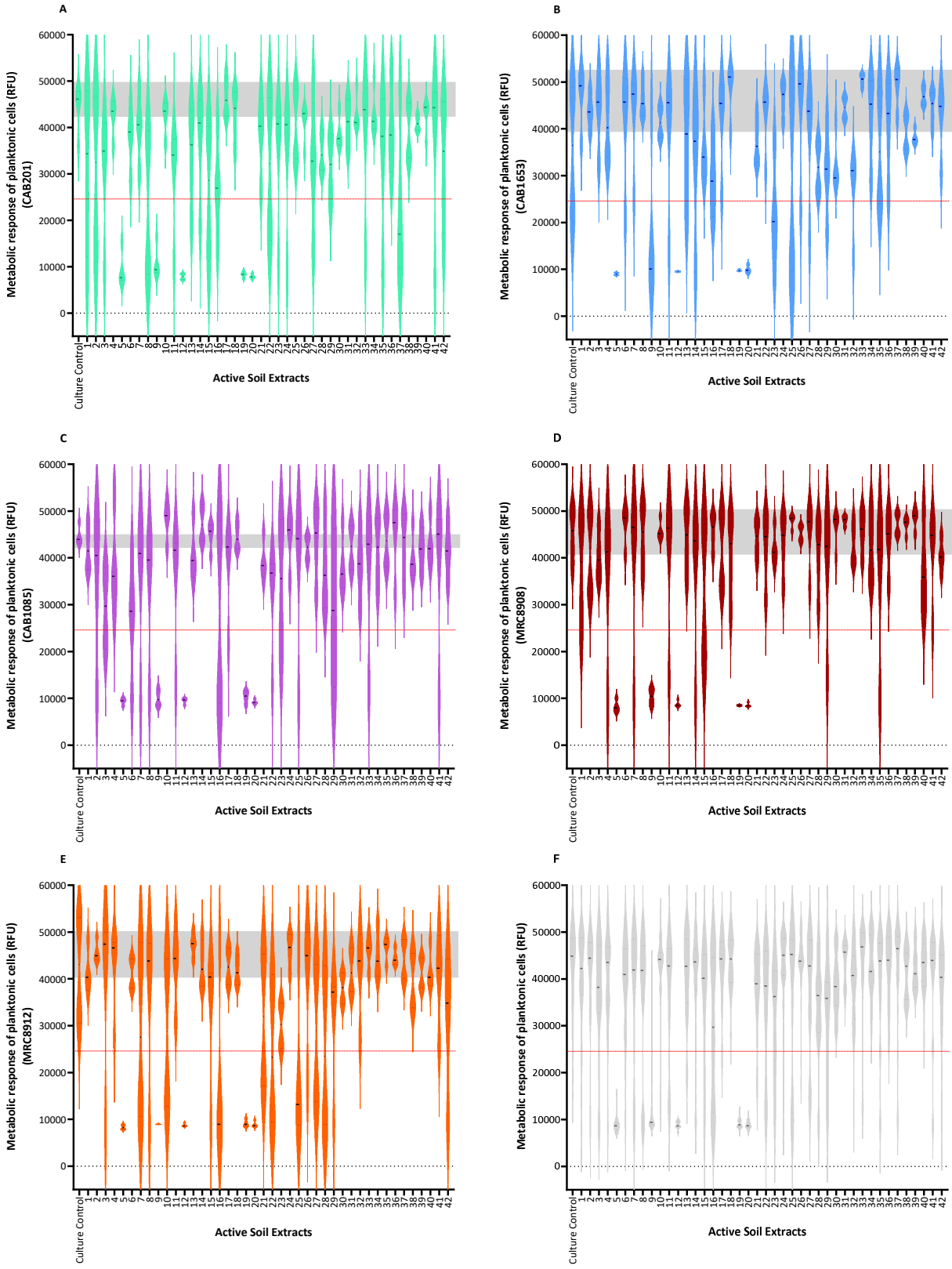

Figure S4.4. Comparative MPAS results showing the metabolic response of the five *C. albicans* strains by the selected 42 group B extracts from cultured soil organisms. Activity against eradicating biofilms shown against CAB201 (A), CAB1653 (B), CAB1085 (C), MRC8908 (D) and MRC8912 (E). Pooled biofilm eradication metabolic activity of all five strains against extracts shown in F. Each violin plot depicts four determinations. The shaded grey area indicates absolute cut-off limits of mean ±1α standard deviation for identifying highly putative extracts. The dotted red line depicts the 25000 RFU visual/hard cut-off for active extracts and results below this line indicates ≥50% inhibition.

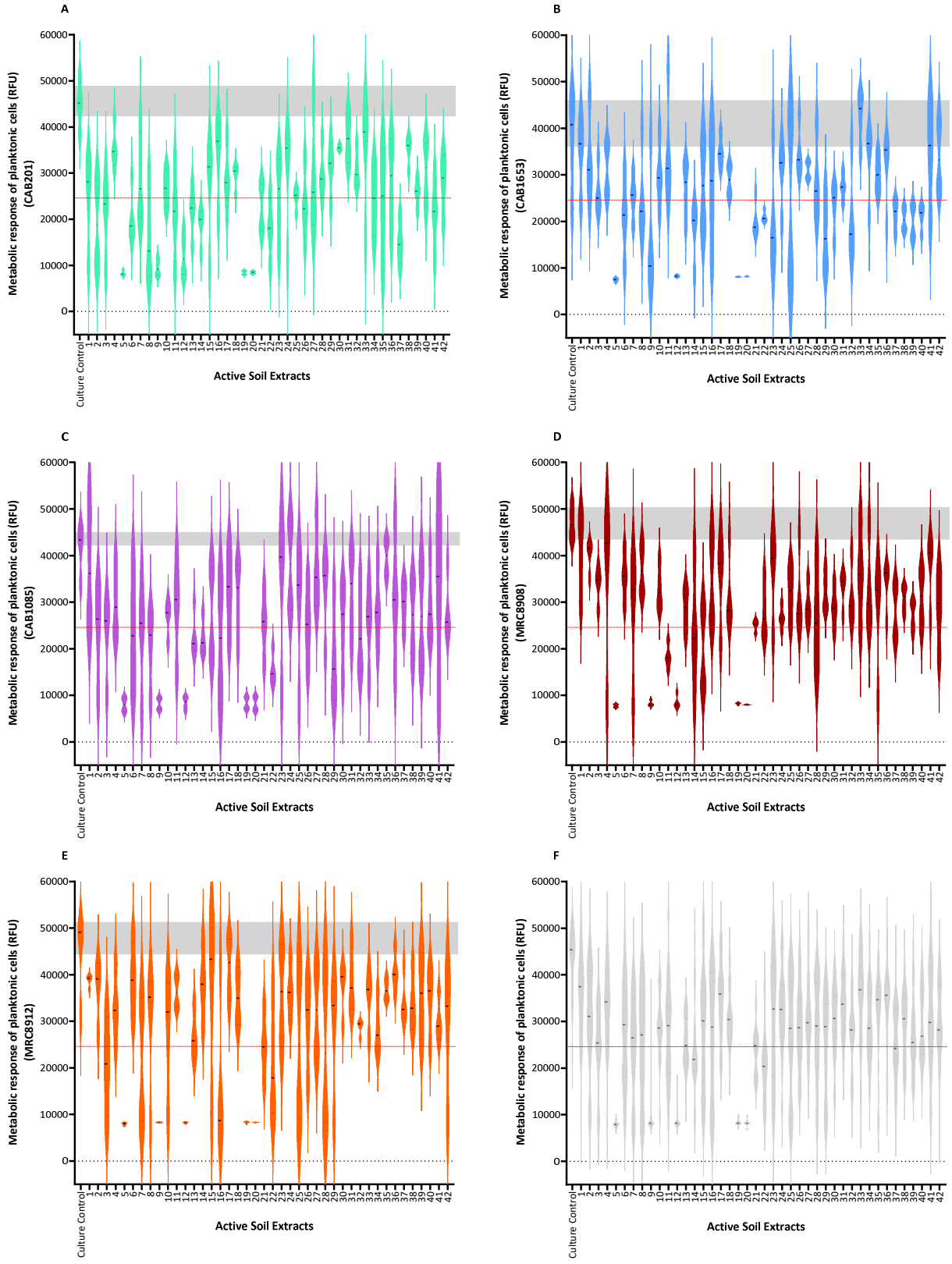

Figure S4.5 Comparative MPAS results showing the metabolic response of the five *C. albicans* strains by the selected 42 group B extracts from cultured soil organisms. Activity against shed planktonic cells shown against CAB201 (A), CAB1653 (B), CAB1085 (C), MRC8908 (D) and MRC8912 (E). Pooled planktonic metabolic activity of all five strains against extracts shown in F. Each violin plot depicts four determinations. The lightly shaded grey area indicates absolute cut-off limits of mean ±1α standard deviation for identifying highly putative extracts. The dotted red line depicts the 25000 RFU visual/hard cut-off for active extracts and results below this line indicates ≥50% inhibition.

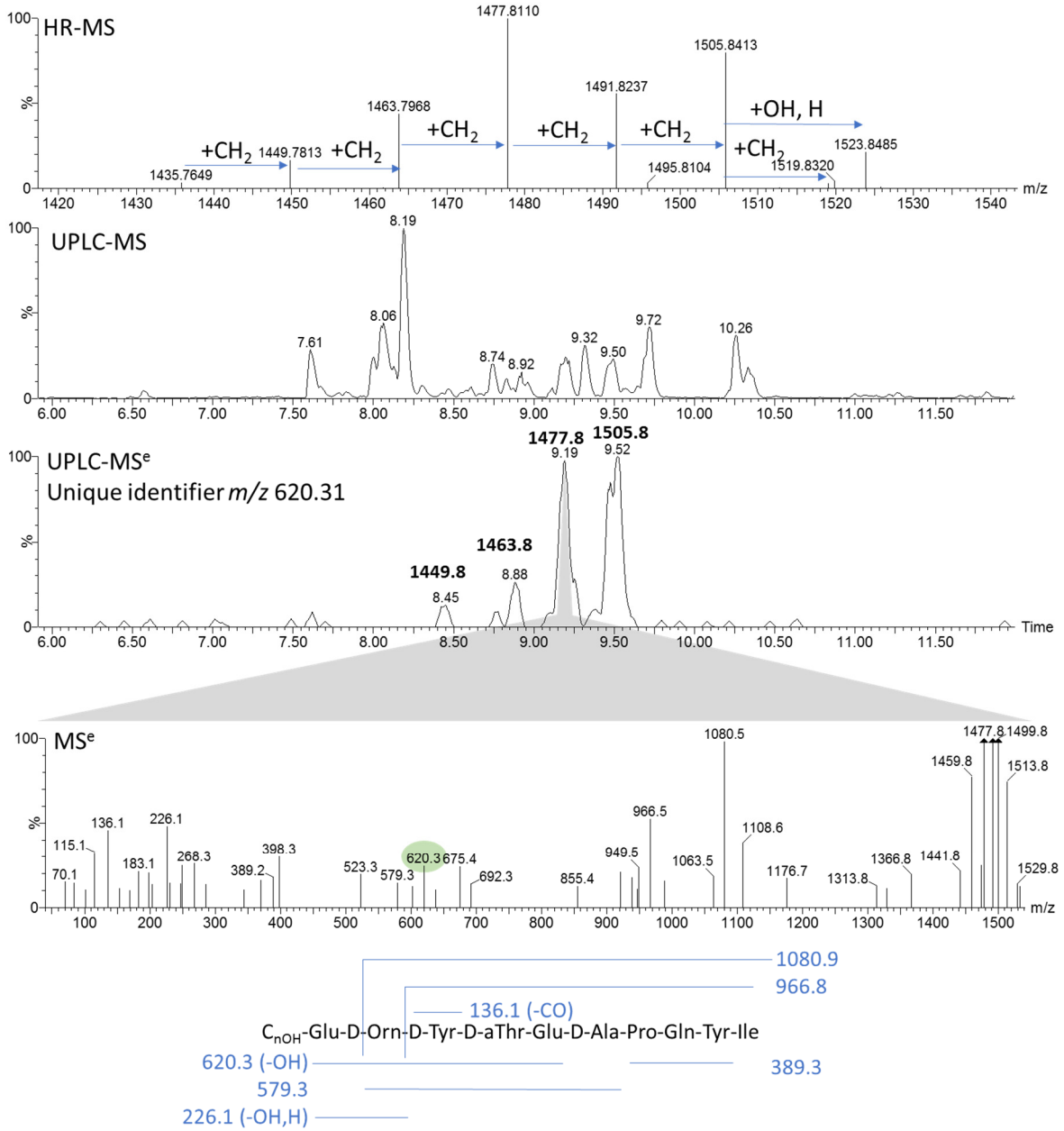

Figure S4.6 Structural elucidation of the fengycins with the unique identifier fragment ion *m/z* 620.31 from soil isolate extract 25B. The high-resolution MS (HR-MS) spectrum (top), collected from 6-12 minutes, show the compound group and their mass relationships. The UPLC-MS positive ion profile and UPLC-MS^e fragment ion of extract is shown in the following two chromatograms. UPLC-MS^e profile of a fragment ion at *m/z* 620.31 was used to track analogous compounds (*m/z* values of compound ions are indicated above each peak with the fragment ion. The CID spectrum (generated during MS^e) of compound with *m/z* 14677.8 at 9.19 minutes is shown, with the unique identifier fragment in the green circle The proposed structure is that of fengycin B. Structure elucidation was done by M Rautenbach. C_{nOH} = hydroxylated fatty acyl residue, structure shows linear fragment with ring opening between fatty acyl residue and Ile.

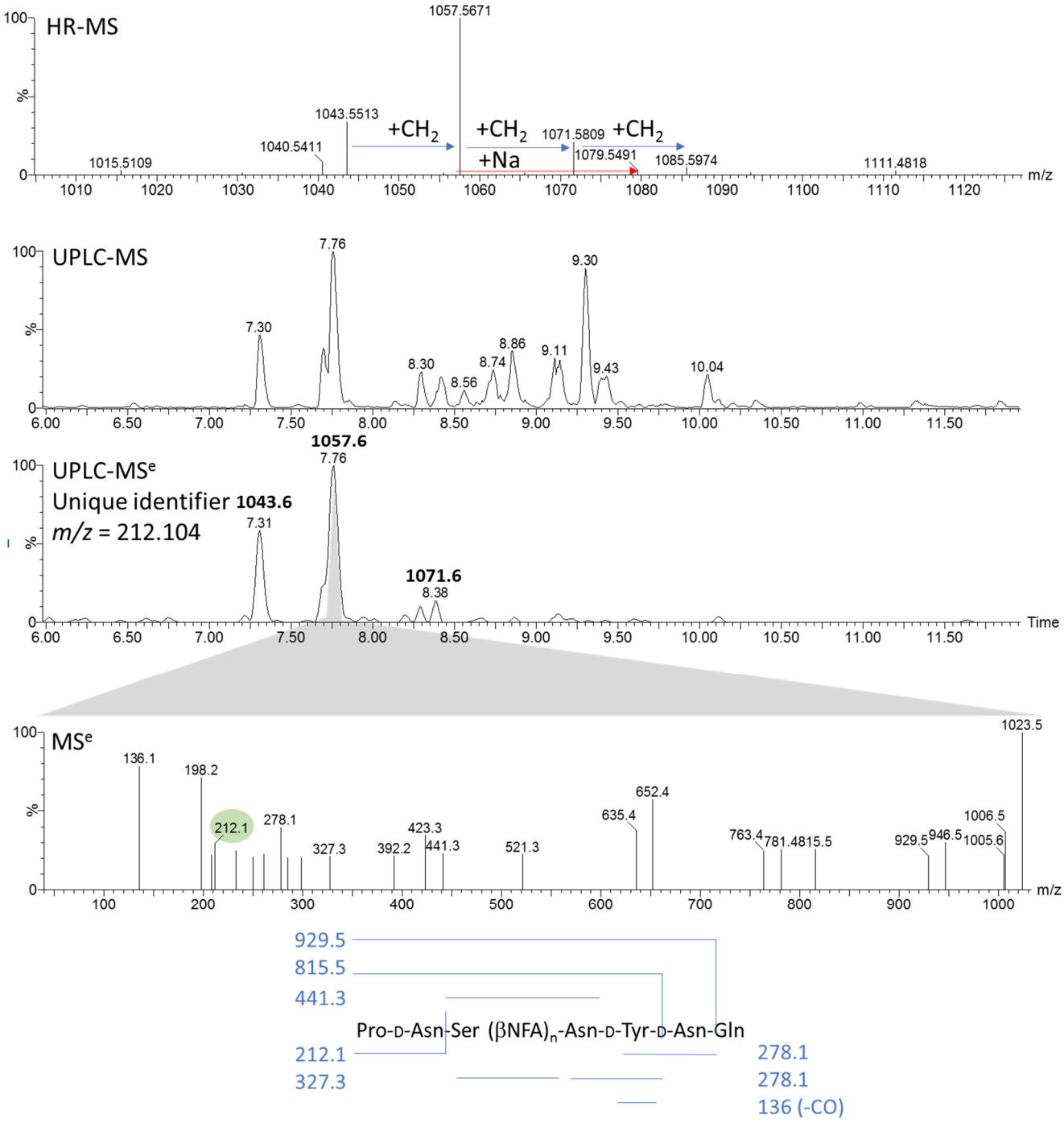

Figure S4.7 Structural elucidation of the iturin A group with the identifier fragment ion *m/z* 212.1 from soil isolate extract 19A. The high-resolution MS (HR-MS) spectrum (top), collected from 6-12 minutes, show the compound group and their mass relationships. The UPLC-MS positive ion profile and UPLC-MS^e fragment ion of extract is shown in the following two chromatograms. UPLC-MS^e profile of a fragment ion at *m/z* 212.1 was used to track analogous compounds (*m/z* values of compound ions are indicated above each peak with the fragment ion. The CID spectrum (generated during MS^e) of compound with *m/z* 1057.6 at 7.78 minutes is shown, with the unique identifier fragment in the green circle The proposed structure is that of iturin A (C_{15}). Structure elucidation was done by M Rautenbach. βNFA = β-amino fatty acyl residue, structure shows linear fragment with ring opening between Pro-Gln.

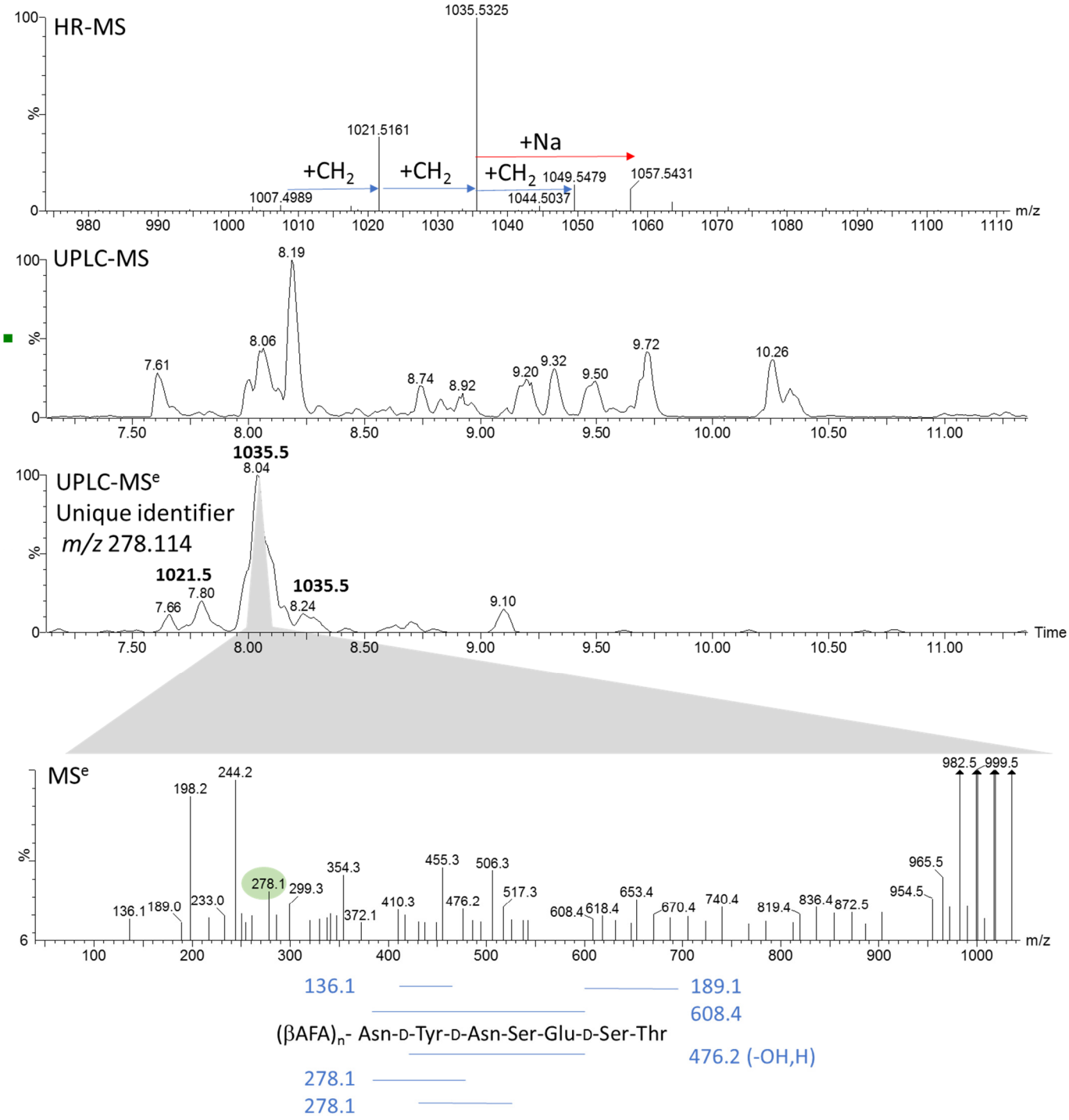

Figure S4.8 Structural elucidation of the bacillopeptins with the identifier fragment ion *m/z* 278.1 from soil isolate extract 25B. The high-resolution MS (HR-MS) spectrum (top), collected from 6-12 minutes, show the compound group and their mass relationships. The UPLC-MS positive ion profile and UPLC-MS^e fragment ion of extract is shown in the following two chromatograms. UPLC-MS^e profile of a fragment ion at *m/z* 278.1 was used to track analogous compounds (*m/z* values of compound ions are indicated above each peak with the fragment ion. The CID spectrum (generated during MS^e) of compound with *m/z* 1035.5 at 8.04 minutes is shown, with the unique identifier fragment in the green circle The proposed structure is that of bacillopeptin (C_{15}). Structure elucidation was done by M Rautenbach. βNFA = β-amino fatty acyl residue, structure shows linear fragment with ring opening between βNFA-Thr.

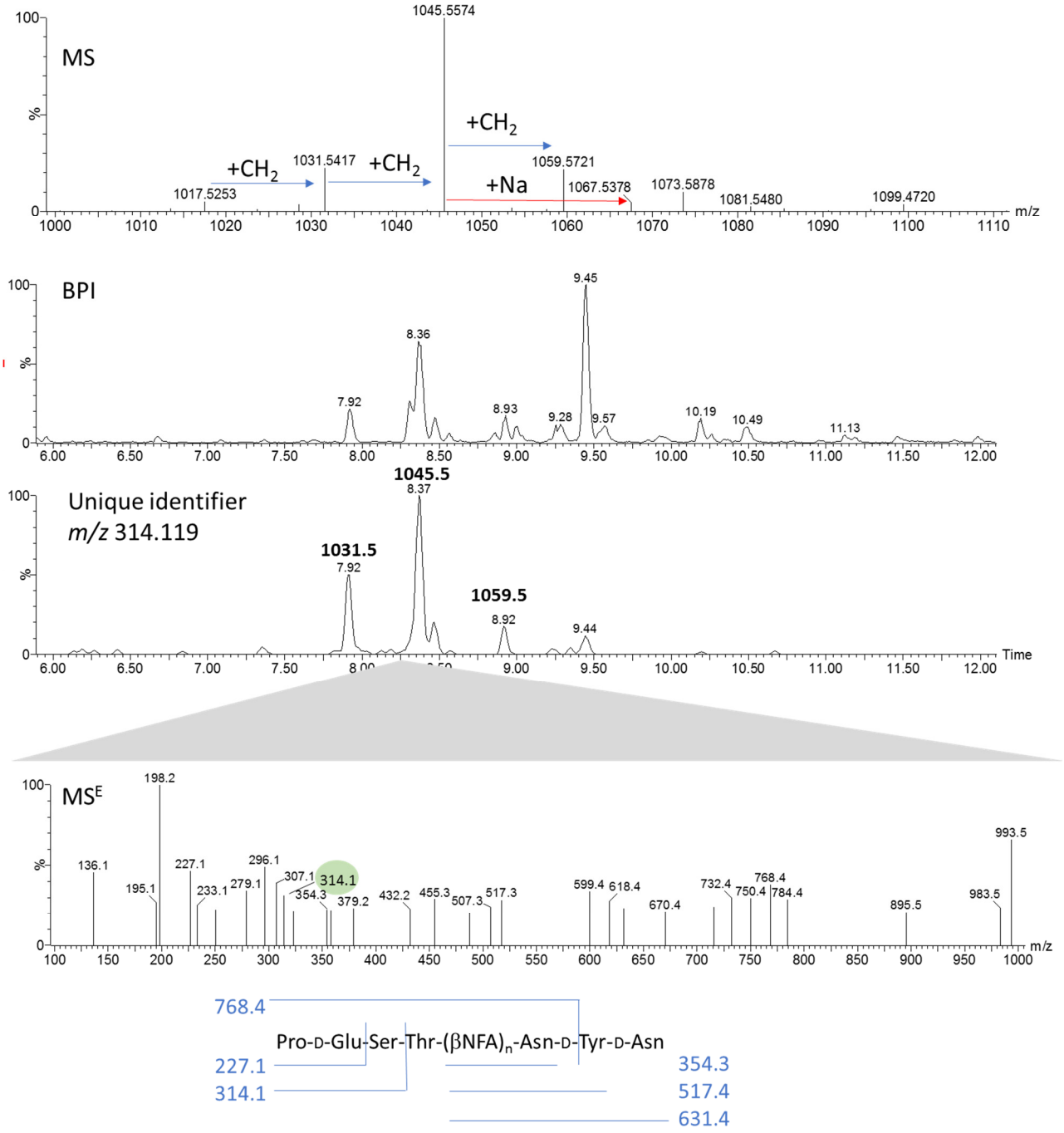

Figure S4.9 Structural elucidation of the bacillomycin Ds with the identifier fragment ion *m/z* 314.1 from soil isolate extract 20A. The high-resolution MS (HR-MS) spectrum (top), collected from 6-12 minutes, show the compound group and their mass relationships. The UPLC-MS positive ion profile and UPLC-MS^e fragment ion of extract is shown in the following two chromatograms. UPLC-MS^e profile of a fragment ion at *m/z* 314.1 was used to track analogous compounds (*m/z* values of compound ions are indicated above each peak with the fragment ion. The CID spectrum (generated during MS^e) of compound with *m/z* 1045.5 at 8.37 minutes is shown, with the unique identifier fragment in the green circle The proposed structure is that of bacillomycin D (C_{16}). Structure elucidation was done by M. Rautenbach. βNFA = β-amino fatty acyl residue, structure shows linear fragment with ring opening between Pro-D-Asn.

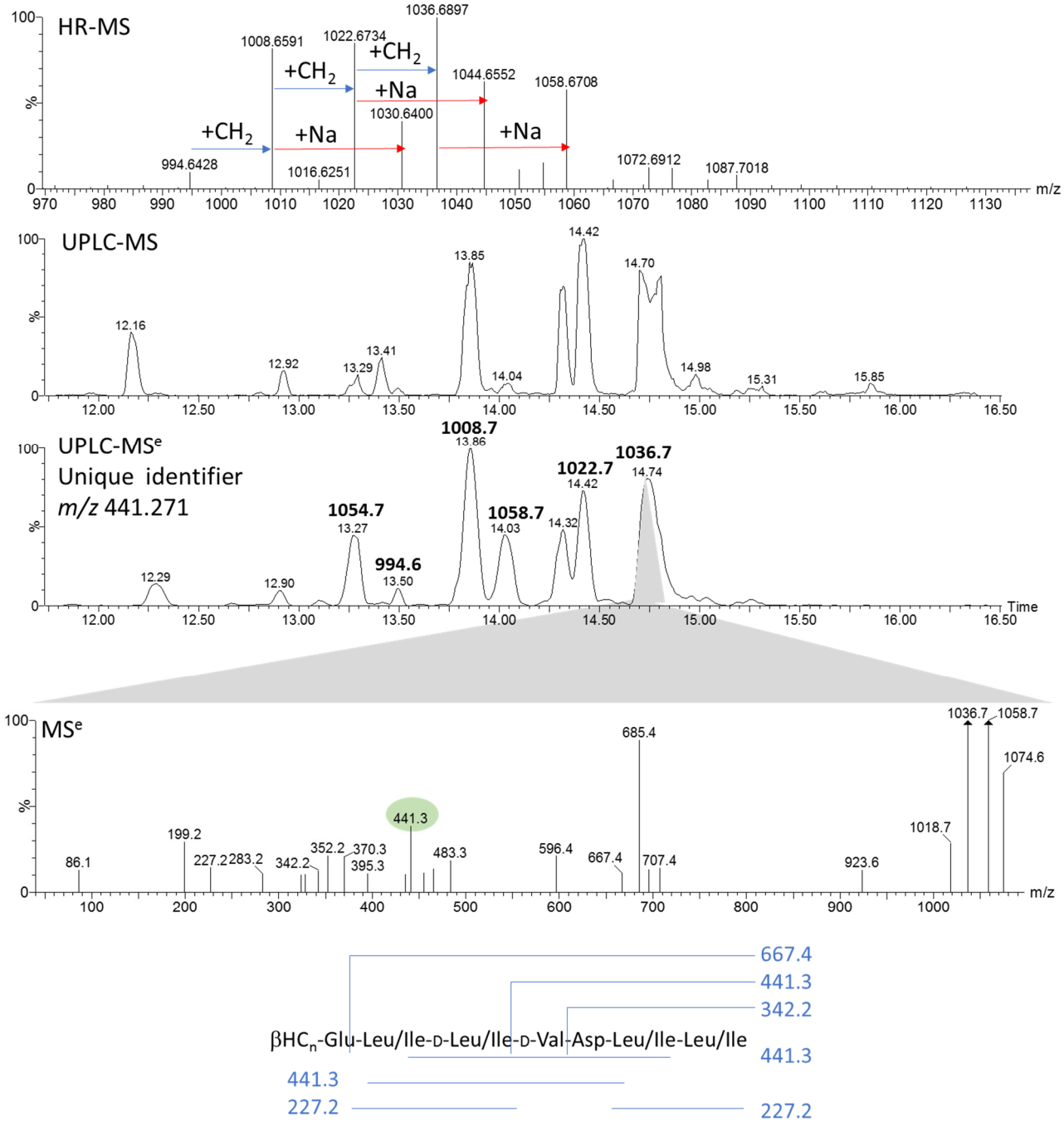

Figure S4.10 Structural elucidation of the surfactin family with the identifier fragment ion *m/z* 441.3 from soil isolate extract 25B. The high-resolution MS (HR-MS) spectrum (top), collected from 6-12 minutes, show the compound group and their mass relationships. The UPLC-MS positive ion profile and UPLC-MS^e fragment ion of extract is shown in the following two chromatograms. UPLC-MS^e profile of a fragment ion at *m/z* 441.3 was used to track analogous compounds (*m/z* values of compound ions are indicated above each peak with the fragment ion. The CID spectrum (generated during MS^e) of compound with *m/z* 1036.7 at 14.47 minutes is shown, with the unique identifier fragment in the green circle The proposed structure is that of surfactin IV. Structure elucidation was done by M. Rautenbach. βHC_n = β-hydroxy fatty acyl residue, coupled in cyclic structure with ester bond.

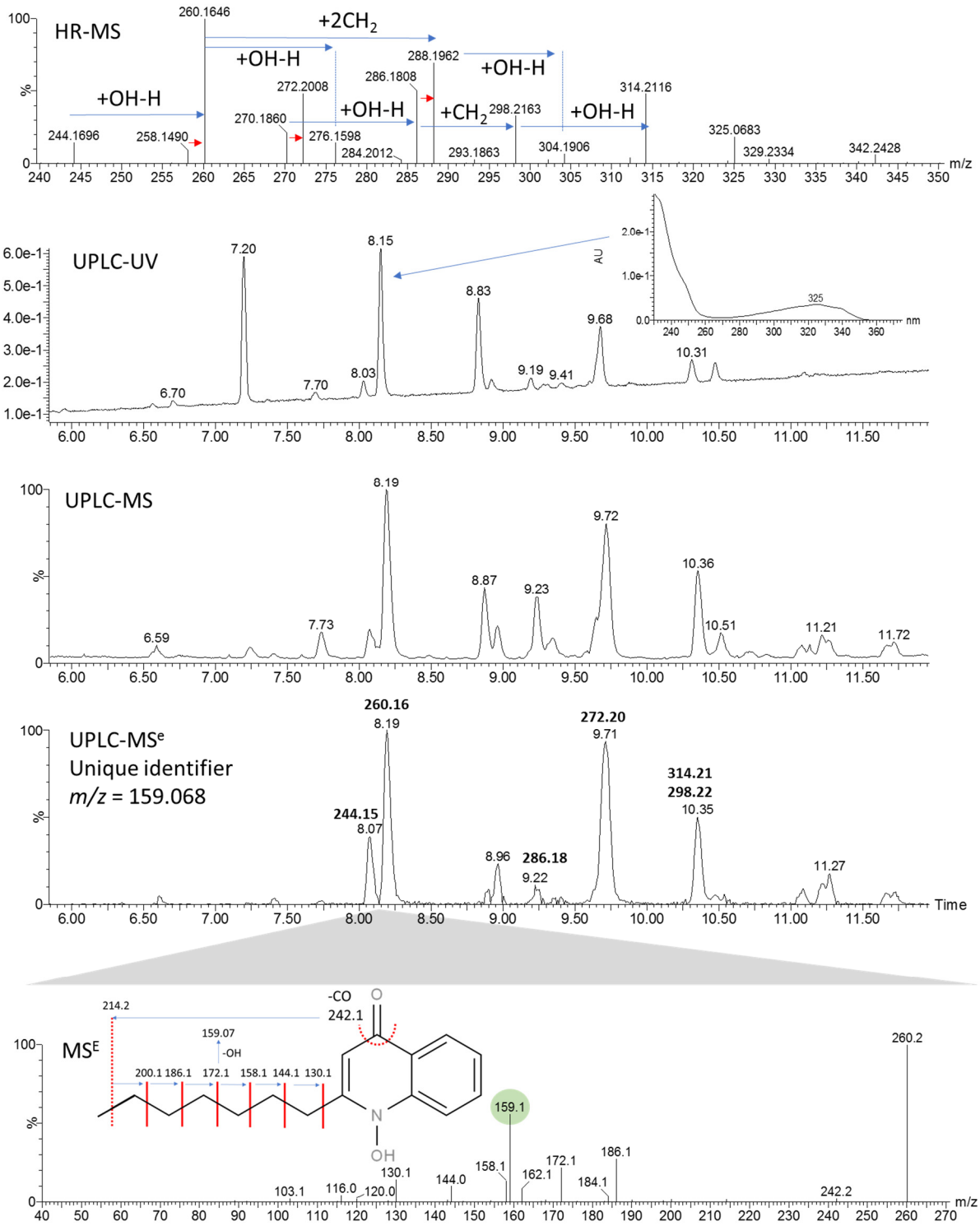

Figure S4.11 Structural elucidation of the UV absorbing compound with *m/z* 260.16 from culture extract 20B. The high-resolution MS (HR-MS) spectrum, collected from 6-12 minutes, show the small compound group and their relationships (red arrow indicate loss a double bond. The UPLC-UV and UPLC-MS positive ion profiles of extract 20-2 are shown in the following two chromatograms. The insert shows the UV spectrum of the compound eluting at 8.15 minutes in UV trace and 8.19 minutes in positive ion trace. UPLC-MS^e profile of a fragment ion at *m/z* 159.068 was used to track analogous compounds (*m/z* values of compound ions are indicated above each peak with the fragment ion. The CID spectrum (generated during MS^e) of compound with *m/z* 620.16 at 8.19 minutes is show with the unique identifier fragment in the green circle and the prosed structure as 2-heptyl-1,4-dihydroxyquinoline 1-oxide. Structure elucidation was done by M. Rautenbach.

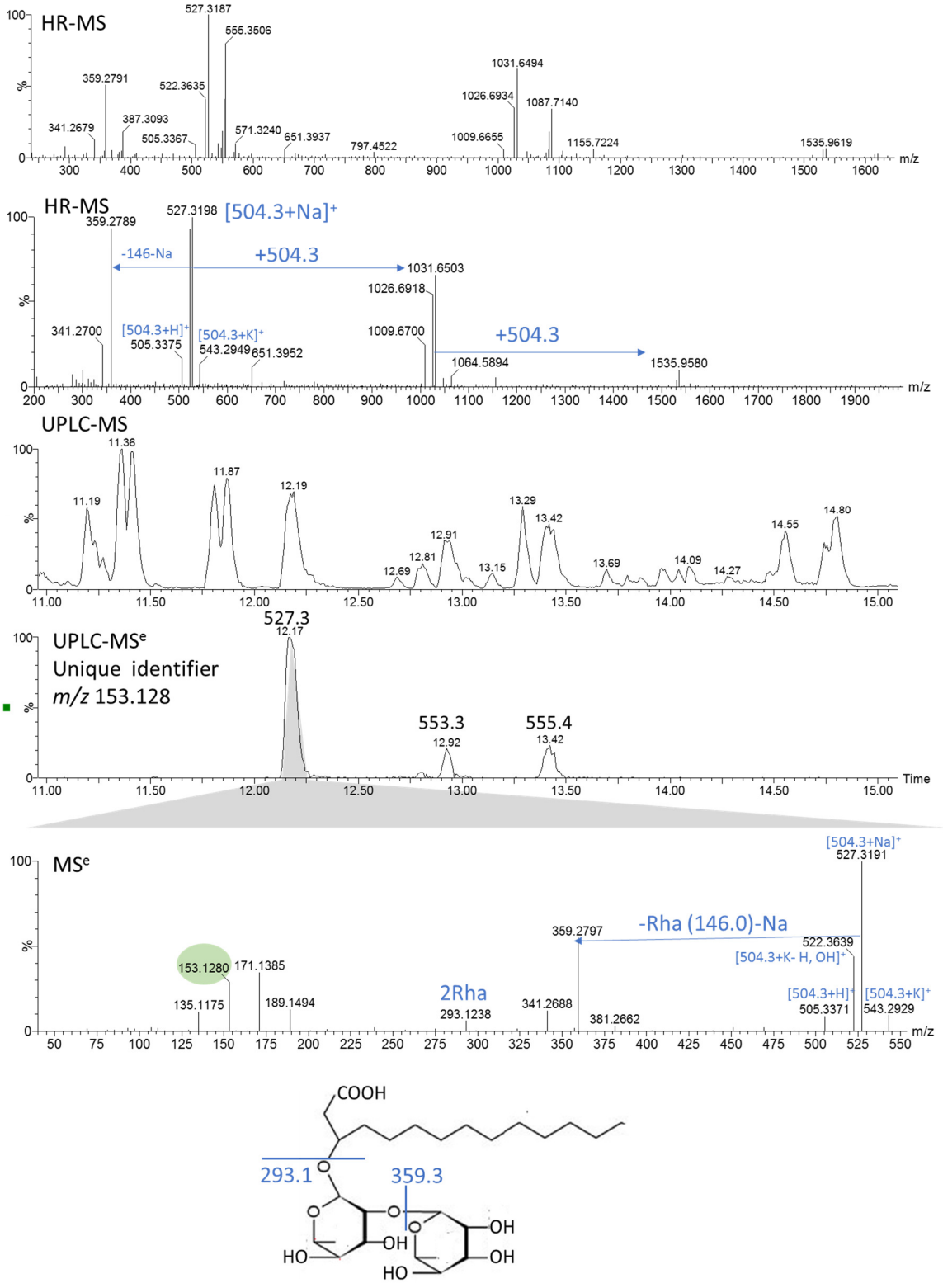

Figure S4.12 Structural elucidation of the rhamnolipid with the identifier fragment ion *m/z* 158.13 from soil isolate extract 5B. The high-resolution MS (HR-MS) spectrum (top), collected from the three peaks at 12.72, 12.92, 13.42 minutes, show the ion clusters. The HR-MS spectrum of the peak at 12.72 minutes the mass relationships is shown next. The UPLC-MS positive ion profile and UPLC-MS^e fragment ion of extract are shown in the following two chromatograms. UPLC-MS^e profile of a fragment ion at *m/z* 158.13 was used to track analogous compounds (*m/z* values of compound ions are indicated above each peak with the fragment ion. The CID spectrum (generated during MS^e) of compound with *m/z* 527.3 at 12.27 minutes is shown, with the unique identifier fragment in the green circle. The proposed structure is that of rhamnolipid 2 (C_{12}). Structure elucidation was done by M Rautenbach.

CHAPTER 5

CONCLUSIONS

Introduction

The decrease in effectiveness of antibiotics due to their overuse and misuse, resulting in increased antimicrobial resistance, is a public health crisis of international concern. It threatens modern medicine, animal health and food security. This resistance is potentiated by organisms that ensure their survival, by developing the ability to grow in a biofilm-form. These biofilm-associated forms result in enhanced tolerance to medications, such as antibiotics, and are often the cause of recurrent infections. The opportunistic and adaptable human fungal pathogen, *Candida albicans* (*C. albicans*), has the ability to colonize various epithelial tissues (i.e. vaginal, intestinal and oral tissues) causing moderate to severe infections[1–3]. Furthermore, it has been observed that *Candida* related fatal infections are due to fungal cells circulating into the bloodstream after being liberated from resistant biofilm fungi[4]. These life-threatening biofilms are most often found in catheters, prosthetics and on implant surfaces, of which *C. albicans* is the most common, causative fungal species[5,6]. Although some currently available antifungals display antibiofilm activity at very high concentrations (amphotericin B and fluconazole) compared to anti-planktonic counterparts, *Candida* biofilms have shown to be more resistant to the these clinically available and important antifungals[6,7]. Therefore, there is an urgent need for rapid, repeatable, and inexpensive methodologies to identify and characterize novel antifungal compounds, ideally from easily accessible environmental samples.

Study overview

Multiplexing to determine broad spectrum anti-*Candida* activity

In this study, we successfully developed a medium-throughput multiplex assay system (MPAS) that was used to study various fungal life-cycle stages of different strains of *C. albicans* simultaneously. This multiplex system was designed for conventional 96-well plate and 96 pin-lid assay layouts with tracked metabolic activity to indicate planktonic cell susceptibility, biofilm prevention, biofilm eradication, as well as activity against the cells shed from biofilms, when treated with antimicrobial compounds. General metabolic lag times of planktonic cells from four *C. albicans* strains ranged between 3-4 hours, except for one clinical strain, which showed a shortened lag time of 1 hour. It is

during these lag times that cells adapt to their new environment and proliferate[8], as well as reversibly bind to surfaces for biofilm initiation[1]. Results showed that planktonic inoculates initially formed biofilms on pin lids within 4 hours, followed by a planktonic counterpart shedding event around 24-48 hours (depending on the strain) and subsequent slow maturation of the biofilm with various inter-strain differences observed. The use of conventional broth assays and our novel multiplex assay format provided interesting data on *C. albicans* biofilm eradication and prevention activity ranges against antifungal drugs currently in the clinic, such as caspofungin, amphotericin B and fluconazole.

Our study generally showed that biofilms exhibited similar, if not increased resistance against the currently available repertoire of drugs when compared to their planktonic counterparts. The most concerning result in this study was that all five the planktonic *C. albicans* strains showed azole resistance, although all were still sensitive towards the polyketide, amphotericin B (AmB) and the lipopeptide drug, caspofungin (CAS). The high planktonic inhibitory concentrations (compared to their biofilm counterparts) against fluconazole (Flu) could be linked to lowered cell counts on the pins, improved diffusion of Flu into the biofilms and/or complex drug resistance mechanisms that would require further investigation. Caspofungin showed good planktonic inhibitory activity and decreased biofilm inhibition, whereas AmB had high inhibitory activity against both planktonic and biofilm forms of *C. albicans*.

Inter-strain differences and assay type, as well as mode of activity of various compounds should be considered individually when experimental data are assessed. Furthermore, biofilm surface areas, media compositions, biofilm age and incubation periods all have effects that need to be accounted for and assessed before continuing with experiments associated with a specific strain or compound. The assay time in the MPAS can easily be adapted for a specific *Candida* strain to optimise for studying a particular life-stage. This MPAS has broad application and can further be adapted for other biofilm forming organisms, by adjusting the cell numbers, and assay time and medium. Having used the MPAS for comparison of five *Candida* strains, we opted to standardize certain conditions, such as inoculation size, incubation and assay times that specifically influenced biofilms. Advantageously, from our results the combination of conventional planktonic assays with our multiplex antibiofilm assay provides a comparative activity profile, especially giving three parameters related to activity against biofilms in an environment closer to what is found in, for example catheters. Our MPAS can be used to follow the inhibitory concentration range trends of antifungal compounds towards different life-stages and growth forms of *C. albicans*. The knowledge

on concentration dependent activity towards the different cell cycle stages of biofilm forming organisms is important and our multiplex assay system would fast-track the compound hit-to-drug lead in drug discovery projects.

However, from the data collected, it was observed that the MPAS design could possibly be improved with the addition of a wash step before biofilm results are recorded, to further decrease the influence of planktonic cells or shed planktonic counterparts on final results. Future work will be very interesting, when the assay design is verified by using other biofilm-forming test organisms, such as on *Pseudomonas* species.

Validation of MPAS for identification of anti-*Candida* bacterial culture extracts

The MPAS was optimized by testing the activity of extracted antimicrobial compounds from known producer organisms on the various *C. albicans* forms. Known antimicrobial producer organisms were allowed to grow and replicate for 10 days, after which the active compounds were extracted using an organic solvent. After extraction, compounds were freeze-dried and then assayed against planktonic and biofilm forms of *C. albicans* using our optimised MPAS. The system gave a quick, reproducible, and reliable indication of activity, including a good signal/noise output, with regards to antifungal compounds in micro-culture extracts and their effect on both planktonic and biofilm *C. albicans* forms.

For each extract 20 inhibition parameters could be derived for the five strains with four assays in MPAS. The parameters were derived from the four assays in MPAS namely 1) % planktonic cell inhibition, 2) % biofilm prevention, 3) % biofilm eradication and 4) % shed planktonic cell inhibition, for each of the five *C. albicans* strains that were used as extract targets. From data of peptides extracted from known peptide producer strains, it was evident that *B. parabrevis* ATCC8185, *B. parabrevis* DSNZ5618 and *A. migulanus* ATCC9999 produce compounds with potent inhibitory activity (Chapter 3, Fig. 3.3). The antimicrobial compound production of these organisms is also at a high enough concentration to give consistent and clear results in all four the assays and with the five *C. albicans* strains as targets. This was observed using absolute cut-off and the conventional 50% inhibitory concentration values for these producers. Notably, the microculture extracts of these three peptide producers prevented biofilm formation on the pin-lids, whereas none of the other extracts had similarly good activity in preventing biofilm formation. Statistical parameters confirmed that the planktonic and biofilm eradication assay designs were more effective than the biofilm prevention assay design (Chapter 3, Table 3.4). However, multiple repeats allowed for accurate population dispersions and the data from all four assay designs resulted in precise results.

Furthermore, the obtained data showed not only which producer products were more active against certain strains of *C. albicans* but could also highlight which forms were more affected by certain production compounds (Chapter 3, Fig. 3.4 and Figs. S3.6-S3.9). This MPAS allows for a huge amount of scientific knowledge to be discovered at one time; 1) information about the organism being tested, 2) the differences between various forms of the organism, 3) compounds and their activity being produced by various producers and 4) subtle production differences between producer organisms.

Improvements to this design could include a step to measure the success of each micro-production before activity testing, by improved visual colony identification and preliminary mass spectrometric analyses. This could further decrease the number of similar or duplicate producer organisms, as well as eliminate know producers and compounds. However, this will increase the workload and analysis time. Using the MPAS for larger-scale soil extracts was manageable, especially, because one target organism and strain is utilised for sifting through the bulk extracts, followed by testing the selected active extracts using four more strains as targets. In this manner, our experimental plan was completed using the multiplex assay to produce, test and characterise extracts of unknown soil microbes against planktonic and biofilm forms of *C. albicans* in hope to identify new antimicrobial compounds.

Using MPAS in a biomining study on soil extracts

Biomining is a process involving the extraction of organic compounds from soil microbes and plants, which are then used in downstream processes. In some fields, these excretions can be used to extract metals from ores/solid materials. In other areas, these organic compounds can be used to study the inhibitory activity of these extracts on target organisms of interest. In this work, we collected soil samples from various areas in the Western Cape and isolated the microbes inhabiting the samples. The microbes were cultivated on agar samples and single colonies were identified and isolated. These single colonies were each cultivated in 96-well agar plates for 10 days and their special organic extracts collected and used to test for inhibitory activity against the life-threatening, biofilm and planktonic forms of *C. albicans*.

The results confirmed excellent planktonic and biofilm inhibition by extracts of seven soil microbes (Chapter 4, Fig. 4.3). Unfortunately, due to unsuccessful PCR results four were not identified by 16S rDNA sequence analysis but two were identified as a *Citrobacter spp.* and one identified as a *Bacillus spp.* (Chapter 4, Table S4.3). From their mass spectrometry profiles, their antimicrobial compounds were identified as lipopeptides from, surfactin, bacillomycin, iturin and fengycin groups (Chapter 4,

Fig. 4.6). Literature research further confirmed the design and use of this assay as all the lipopeptides obtained and mentioned here have been shown to have varying degrees of antifungal activity[9–13]. Additionally, low molecular weight products were identified as small, quinoline quorum sensing molecules[14] and unstable large molecules produced are in the process of being identified. Their possible contributing role to the activity observed will be further investigated in the future. The UPLC-MS^e data was used to set up a corresponding heat map of similar MS group clusters. Not only do these MS production profiles cluster, but their corresponding genomic clusters also indicated that these seven organisms with inhibitory activity are closely related to each other.

The seven final active extracts were identified with the strict activity cut-off values inflicted by our requirement outline, which this MPAS allows. More of the active culture extracts (identified in this experimental procedure) may have novel compounds and will be further investigated in the future. Our selection process also concentrated on amphipathic compounds, but there may be polar and non-polar compounds that may also have activity. Improvements to this design could include additional variations in type of medium used to broaden the soil species profile, from which colony selection could be improved. There could also be a change in the incubation period of soil microbes with short, medium and long incubation/production periods, which will allow for different production profiles and possibly more unknown products to be produced, as our production process overall selected for *Bacillus spp.* proliferation and hence associated lipopeptide production profiles. The workload is comparatively low for the quantity and significance of data that is collected during these experiments. The key to reliable data collection using our MPAS in active compound discovery lies in multiple repeats per experiment, as well as consistent cut-off values that must be used throughout the analysis process. Future studies will focus on further compound identification of the culture extracts already tested. This will be followed by further genetically identifying the top seven hit organisms. Global Natural Product Social Molecular Networking (GNPS)[15] using the UPLC-MS^e data is planned. This entails setting up biological networks which can be used to visualize and predict known and unknown natural products within associated chemical families[16,17]. Lastly, detailed compound and structural analysis using the mass spectrometry data collected from all 42 organisms has been initiated. The potential for identifying further active compounds from this study lies in a deepened study on the 42 organisms that were discovered. Furthermore, by adapting MPAS to different biofilm forming target organisms and continuing testing soil sample collections, could lead to the discovery of the next antibiotic lead.

Final word

Our optimised comparative MPAS gave a broad-spectrum medium-throughput results with 20 types of determinations on each extract. This rich dataset indicated if an extracted compound's activity was targeting planktonic cells, preventing biofilm formation and/or eradicating biofilms, as well as which one of the five *Candida* strains was the most resistant or sensitive. However, considering the methodology this assay system is easily adaptable to high-throughput systems that can be employed using robotics. If successful growth inhibitory activity results are observed, the specific compounds and their bacterial producers can be studied further. Therefore, this leads to quicker, improved possibilities of identifying novel antibiotics and antifungals from a complex, mostly untouched microbiome, with which to aid the various industries struggling with resistant microorganisms. Overall, the advantage of this inexpensive MPAS includes ease of adaptability to various laboratories, test compounds and target organisms.

The hope is that the seven hits from this study and possible future novel leads, which will be discovered by using or benefitting from our MPAS, could be developed for last resort drugs against resistant biofilms and eventually contribute to a healthy future of humankind.

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