INVESTIGATING NOVEL ANTIMICROBIAL STRATEGIES TO TARGET *MYCOBACTERIUM ABSCESSUS* INFECTIONS

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Mycobacterium abscessus is an opportunistic pathogen of increasing importance, especially for individuals with pre-existing lung conditions such as bronchiectasis and cystic fibrosis. The current drug regimen for pulmonary *M. abscessu*s infections requires a lengthy course of multiple antibiotics with severe side effects, usually resulting in poor patient outcomes. Therefore, new and novel strategies to combat these infections are urgently required. New areas of interest are the natural product Manuka honey, as well as metal-ion complexes, such as ruthenium based compounds. We have explored the efficacy of manuka honey against *M. abscessus in vitro*, as well as against a panel of clinical *M. abscessus* isolates. Building upon this activity, we assessed the interactions between manuka honey and the front-line antimicrobials amikacin, tobramycin and azithromycin in combination against *M. abscessus*. The synergy found between manuka honey and amikacin has led to the development of an *in vitro* nebulisation assay, as a potential new therapy option. We have also investigated the components of manuka honey to identify the active compounds against *M. abscessus*. This led us to the development of a modified vegan ‘honea’, which gained antimicrobial activity through the addition of the precursors that give rise to the antimicrobial compounds found in honey. Finally, we investigated the potential of novel ruthenium based complexes against *M. abscessus* and a variety of other bacterial pathogens as a potential new set of antimicrobial compounds. Overall, the work within this thesis demonstrates new antimicrobials and novel strategies to combat severe *M. abscessus* infections.

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Publications Associated with this Work

Nolan, V.C., Harrison, J. and Cox, J.A.G. (2019) 'Dissecting the antimicrobial composition of honey', *Antibiotics*, 8(4), pp. 1–16. Available at: https://doi.org/10.3390/antibiotics8040251.

Nolan, V.C. *et al.* (2020) 'Clinical significance of manuka and medical-grade honey for antibiotic-resistant infections: A systematic review', *Antibiotics*, 9(11), pp. 1–24. Available at: https://doi.org/10.3390/antibiotics9110766.

Nolan, V.C. *et al.* (2022) 'Indole-containing arene-ruthenium complexes with broad spectrum activity against antibiotic-resistant bacteria', *Current Research in Microbial Sciences*, 3(October 2021), p. 100099. Available at: https://doi.org/10.1016/j.crmicr.2021.100099.

Nolan, V.C., Harrison, J. and Cox, J.A.G. (2022) 'In vitro synergy between manuka honey and amikacin against Mycobacterium abscessus complex shows potential for nebulisation therapy', *Microbiology*, 168(9), pp. 1–9. Available at: https://doi.org/10.1099/mic.0.001237

Nolan, VC., Harrison, J. and Cox, J.A.G. (2022) 'Manuka honey in combination with azithromycin shows potential for improved activity against *Mycobacterium abscessus*', *The Cell Surface*, 17(8), pp. 1-5. Available at: https://pubmed.ncbi.nlm.nih.gov/36452962/

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List of Abbreviations

% – percent

< – less than

°C – degrees Celsius

β – beta

µg – microgram

µL – microlitre

µm – micrometre

µM – micromolar

ABC – ATP binding cassette

AMR – antimicrobial resistance

ANOVA – analysis of variance statistical test

ATCC – American Type Culture Collection

ATP – adenosine triphosphate

Bla_{Mab} – *M. abscessus* β-lactamase

CF – cystic fibrosis

CFTR – cystic fibrosis conductance regulator gene

CFU/mL – colony forming units per millilitre

DAT – diacyl trehalose

DHA – dihydroxyacetone

ESBL – extended spectrum beta lactamase

g – gram

g/mL – gram per millilitre

g/mol – gram per mole

h – hour

H_2O – water

I.V. – intravenous

KatG – gene encoding catalase-peroxidase enzyme

$KatG^{Mabs}$ – *M. abscessus* catalase-peroxidase enzyme

$KatG^{Mtb}$ – *M. tuberculosis* catalase-peroxidase enzyme

KPC – *Klebsiella pneumoniae* carbapenemase producing bacteria

L – litre

M – molar

mM – millimolar

MBC – minimum bactericidal concentration

mg – milligram

mg/kg – milligram per kilogram

mg/L – milligram per litre

mg/mL – milligram per millilitre

MGO – methylglyoxal

MIC – minimum inhibitory concentration

min – minute

mL – millilitre

NCTC – National Collection of Type Cultures

nm – nanometres

NTM – non-tuberculous mycobacteria

OD – optical density

PAT – pentaacyl trehalose

PDIM – phthiocenol dimycocerosate

PIMs – phosphatidylinositol mannosides

rpm – revolutions per minute

w/v – weight to volume

Chapter 1: Introduction: Mycobacteria, *Mycobacterium abscessus* and the potential solution of manuka honey in the fight against antimicrobial resistance

1.1 Mycobacteria and *Mycobacterium abscessus*

1.1.1 Overview of Mycobacteria

Mycobacteria are a genus of rod shaped bacterial pathogens that cause a multitude of diseases (Jones *et al.*, 2019). Unlike most bacteria, they cannot be classified as either Gram positive or Gram negative due to differences in the cell wall composition that are unique to this genus (Brown *et al.*, 2020). The main differences are due to the presence of several structural components including various outer membrane lipids, mycolic acids, glycolipids, arabino-galactan, lipomannan and phosphatidylinositol mannosides (Figure 1.1).The presence of these components in the cell wall contribute to the multidrug resistant nature of these bacterial pathogens because they are highly impenetrable and extremely hydrophobic (Primm, Lucero and Falkinham, 2004).

The most well-known mycobacteria are *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, respectively (Keragala *et al.*, 2020). Lesser known mycobacteria that are also responsible for causing a plethora of diseases include: *Mycobacterium chelonae, Mycobacterium fortuitum, Mycobacterium avium* complex and *Mycobacterium abscessus* complex, amongst many others. The mycobacteria that do not cause tuberculosis are described as nontuberculous mycobacteria (NTM), which encompasses a large amount of mycobacterial species (Figure 1.2) (Ratnatunga *et al.*, 2020). NTM are often opportunistic pathogens, causing infections to those who are immunocompromised, have undergone surgery or trauma, have a catheter or injection site, new tattoo or acupuncture as well as exposure to hot springs and spas (Lee *et al.*, 2015). This wide ranging pathogenicity is due to the extensive spread of these bacteria that are found within the environment including water reservoirs and taps, sewage, soil and dust (Hruska and Kaevska, 2012). This is also compounded by the types of infections that they cause, including: pulmonary infection, skin and soft tissue infections, central nervous system infections, bacteremia and disseminated diseases (Nishiuchi, Iwamoto and Maruyama, 2017). The treatment for these infections can be complicated, due to intrinsic drug resistant mechanisms, such as efflux pumps and resistance genes, the hydrophobic cell wall preventing drug penetration into the cell and the bacteria being able to evade the host immune responses (Lopeman *et al.*, 2019). This results in poor patient outcomes and can assist in the spread of these infections in a clinical setting. This makes infections caused by mycobacteria problematic and a cause for concern. One group of mycobacteria that is of growing importance, especially for patients with cystic fibrosis and bronchiectasis, is *M. abscessus* complex (Bronson *et al.*, 2021).

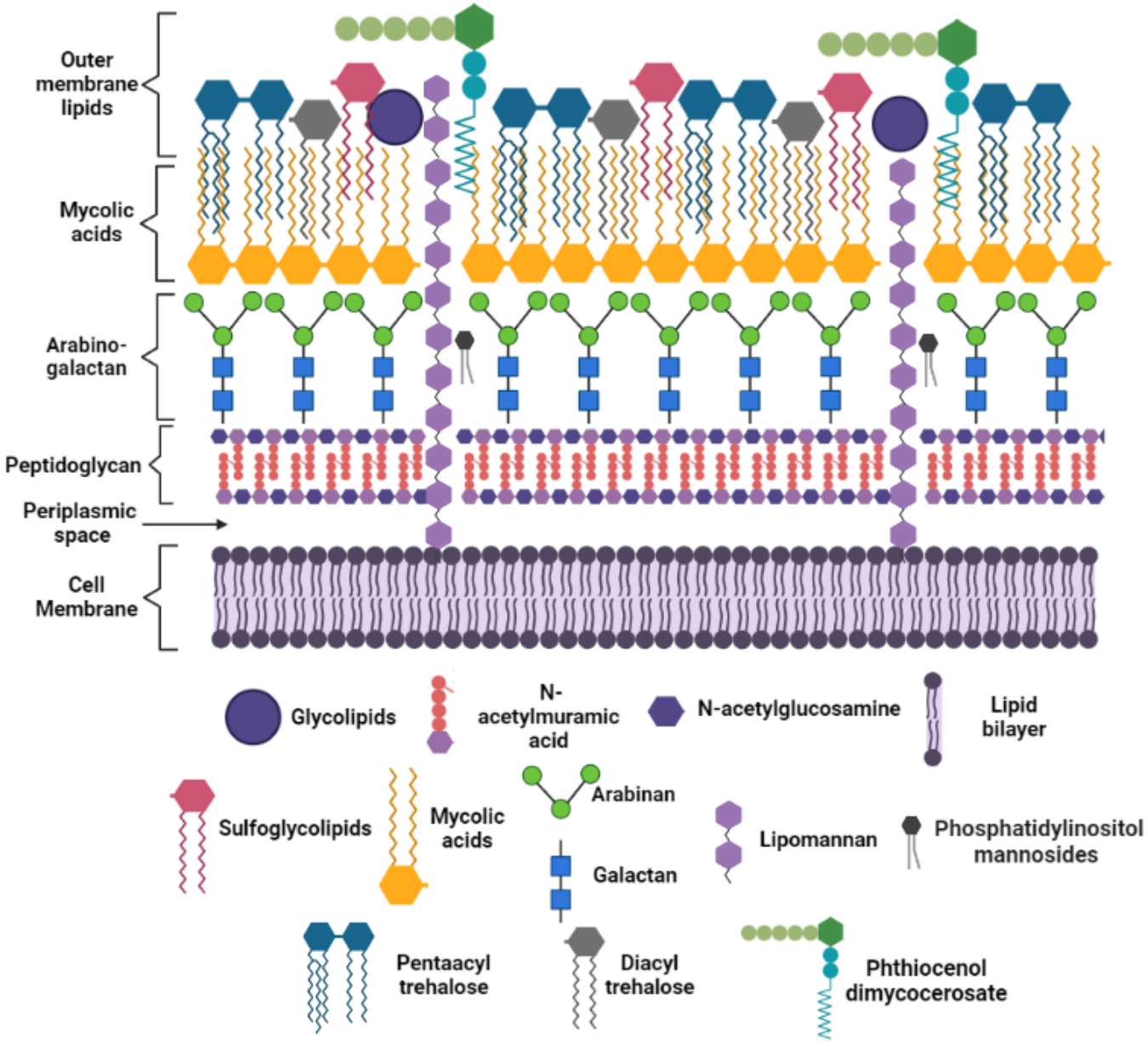

Figure 1.1 Cell wall composition of mycobacteria. The cell wall of mycobacteria consists of an outer layer of intercalated lipids, including pentaacyl trehalose (PAT), sulfoglycolipids, diacyl trehalose (DAT), phthiocenol dimycocerosate (PDIM) and glycolipids. This is followed by a layer containing mycolic acids. Beneath the mycolic acids are a layer of arabino-galactan, made up of arabinose and galactan, with interspaced phosphatidylinositol mannosides (PIMs). These are attached to a layer of peptidoglycan, consisting of N-acetylmuramic acid and N-acetylglucosamine. A long chain of lipomannan spans the cell, from the outer lipids to the lipid bilayer. A periplasmic space is present between the peptidoglycan and the internal cell membrane.

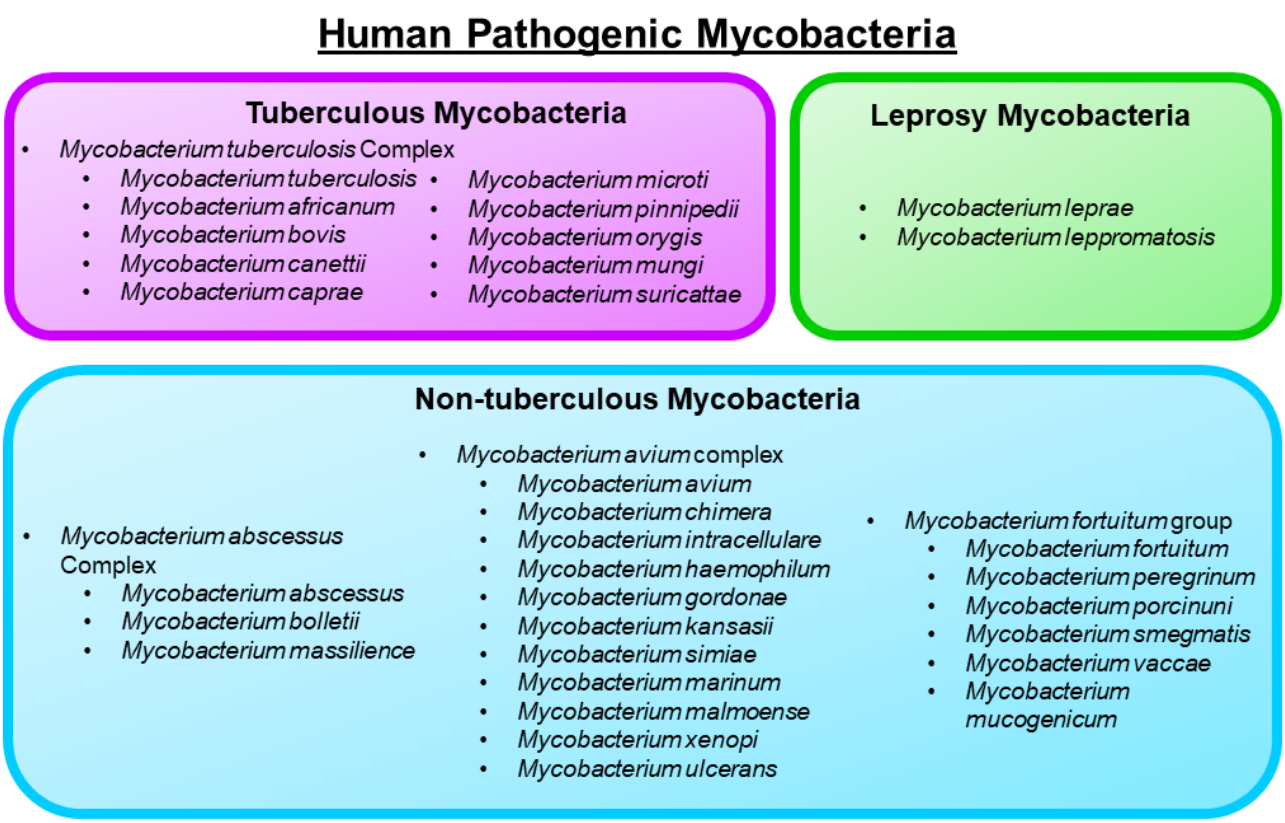

Figure 1.2 Groups of human pathogenic mycobacteria. An outline of the different mycobacterial species that cause tuberculosis and leprosy in humans, as well as the nontuberculous mycobacteria. This list is not exhaustive and there are many other species of mycobacteria (To *et al.*, 2020).

1.1.2 *M. abscessus* Infections

M. abscessus was first isolated and characterised from a wound abscess in 1952. It was considered a *Mycobacterium* of low virulence because it did not appear as a progressive disease and eventually disappeared with no lasting damage (Moore and Frerichs, 1953). However, *M. abscessus* is now established as an opportunistic pathogen with increasing prevalence worldwide (Osmani *et al.*, 2018). Most noted for its ability to cause lung infection, *M. abscessus* also causes skin and soft tissue infections, often infecting open wounds or patients who have undergone cosmetic surgery where contaminated water or equipment was used (Desai and Hurtado, 2018). *M. abscessus* is of significant clinical importance to the cystic fibrosis community. Cystic fibrosis is a genetic condition, caused by a mutation in the CF transmembrane conductance regulator gene (CFTR), resulting in the production of mucus in the lungs and pancreas (Prayle *et al.*, 2010). This increased mucus provides an environmental niche for bacterial pathogens. Therefore, cystic fibrosis patients are highly vulnerable to bacterial infections, often contracting pulmonary infections and ultimately resulting in mortality due to chronic bacterial infection (Lyczak, Cannon and Pier, 2002). The instance of NTM infections in cystic fibrosis patients is on the rise, from 3.3% to 22.6% within the last 20 years in the United States of America (Degiacomi *et al.*, 2019). The majority of these infections are caused by *M. abscessus* complex and *M. avium* complex (Skolnik, Kirkpatrick and Quon, 2016). It was originally considered that infection occurred through environmental contamination and could be managed through reduced exposure (Chmiel *et al.*, 2014). However, there is evidence that patient-to-patient transmission could be occurring, further compounding the problem of these infections (Aitken *et al.*, 2012; Bryant *et al.*, 2021).

1.1.3 *Mycobacterium abscessus* complex

The taxonomic classification of *M. abscessus* has been largely debated over the years due to poor sequencing and identification techniques as well as several distinct subspecies (Lopeman *et al.*, 2019). Upon discovery of *M. abscessus* it was originally classified as *M. chelonae*, a different species of NTM (Lee *et al.*, 2015). In 1972, it was designated as a subspecies of *M. chelonae* before being identified as its own individual species of mycobacteria (Kubica *et al.*, 1972). The discovery of the subspecies belonging to the *M. abscessus* complex was made through polymerase chain reaction sequencing of the *rpoB* gene. The *rpoB* gene encodes the β subunit of RNA polymerase in bacteria, which is used in the transcription of genes and the production of proteins (Adékambi, Drancourt and Raoult, 2009). This is considered a housekeeping gene, one necessary for bacteria, and evolves more rapidly than other genes used for sequencing, such as the 16S ribosomal subunit, a more commonly used sequencing site (Ogier *et al.*, 2019). Through the sequencing of the *rpoB* gene of a distinct mycobacteria isolated from a patient with hemoptoic pneumonia, along with 59 clinical isolates of rapidly growing mycobacteria, that two species of mycobacteria with remarkable similarity to *M. abscessus* were identified. These were *Mycobacterium massiliense* and *Mycobacterium bolletii* (Adékambi *et al.*, 2006). It was not until 2013, through whole genome sequencing, that *M. massiliense* and *M. bolletii* were to be amended

as subspecies of *M. abscessus*, along with the originally identified *M. abscessus*, to form the *M. abscessus* complex (Bryant *et al.*, 2013). One main distinction between the subspecies is the presence of a functional and inducible *erm*(41) gene, which encodes macrolide resistance. This gene is present in *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* but is non-functional in *M. abscessus* subsp. *massiliense* (Lee *et al.*, 2015). These differences between the subspecies become of importance when considering antibiotic therapy to treat an *M. abscessus* infection, as two of the subspecies will remain unaffected by macrolide based antibiotics. This reduces the options for treatment of infections and requires additional testing to identify the subspecies causing the infection or antibiotic susceptibility testing before prescribing any drugs. The discovery of a treatment that can inhibit all subspecies equally, regardless of subspeciation, would be highly beneficial, removing the need to identify isolate susceptibility and identification which can be costly and time consuming.

1.1.4 Rough and smooth morphologies

Another characteristic of *M. abscessus* is the ability to grow in two different morphologies on solid agar (Figure 1.3). The smooth morphological variant has been largely associated with *M. abscessus* isolated from the environment or during early infection and has increased sliding mobility and biofilm formation compared to the rough variant (Ryan and Byrd, 2018). The rough morphological variant is more commonly associated with an established *M. abscessus* infection and can replicate within macrophages (Aulicino *et al.*, 2015). It has since been identified that the transition from the smooth to the rough morphology is largely due to the loss of glycopeptidolipids on the outer cell membrane (Kim *et al.*, 2020). This loss of glycopeptidolipids exposes phosphatidyl-myo-inositol mannosides (PIMs) on the outer membrane of the mycobacterial cell. The exposure of the PIMs allows for macrophage recognition through toll-like receptor 2, resulting in phagocytosis (Gutiérrez *et al.*, 2018). Unlike the smooth variant, upon phagocytosis the rough variant triggers apoptosis which re-releases the *M. abscessus* cells back into the host for re-infection (Figure 1.4) (Kim *et al.*, 2019). Additionally, the loss of GPLs and switch from smooth to rough is permanent due to mutations in the GPL production gene, therefore selecting for improved cell survival (Pawlik *et al.*, 2013).

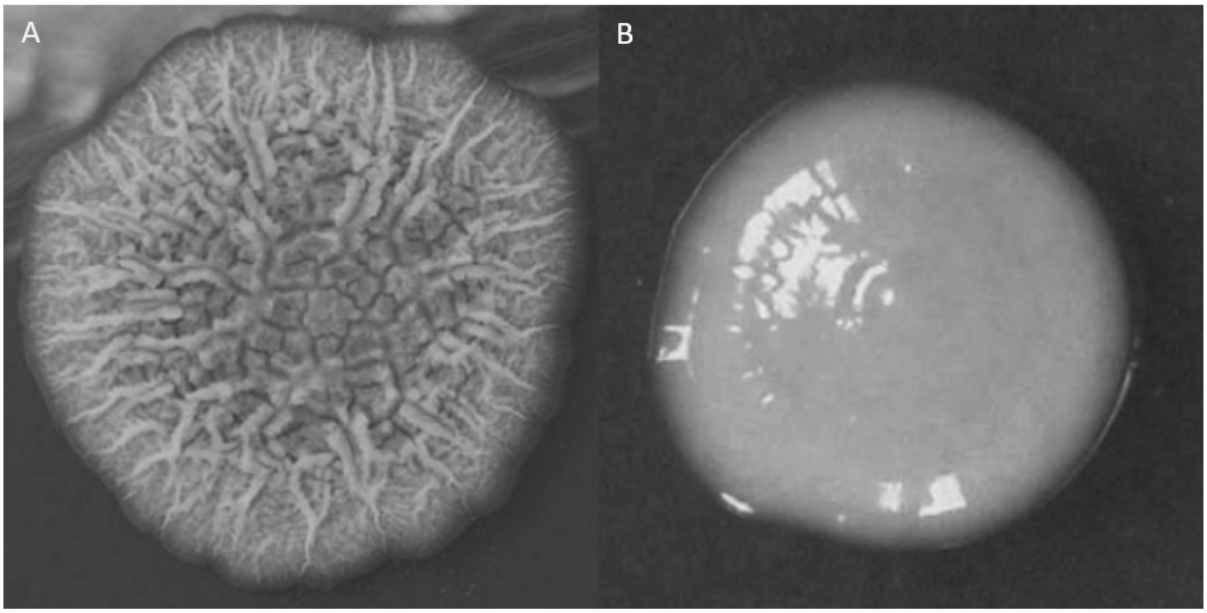

Figure 1.3 Rough and smooth morphological variants of *M. abscessus* after growth on solid media.
A, the rough morphological variant which appears more textured and waxy and B, the smooth variant which appears glossy and more rounded (Rüger *et al.*, 2014).

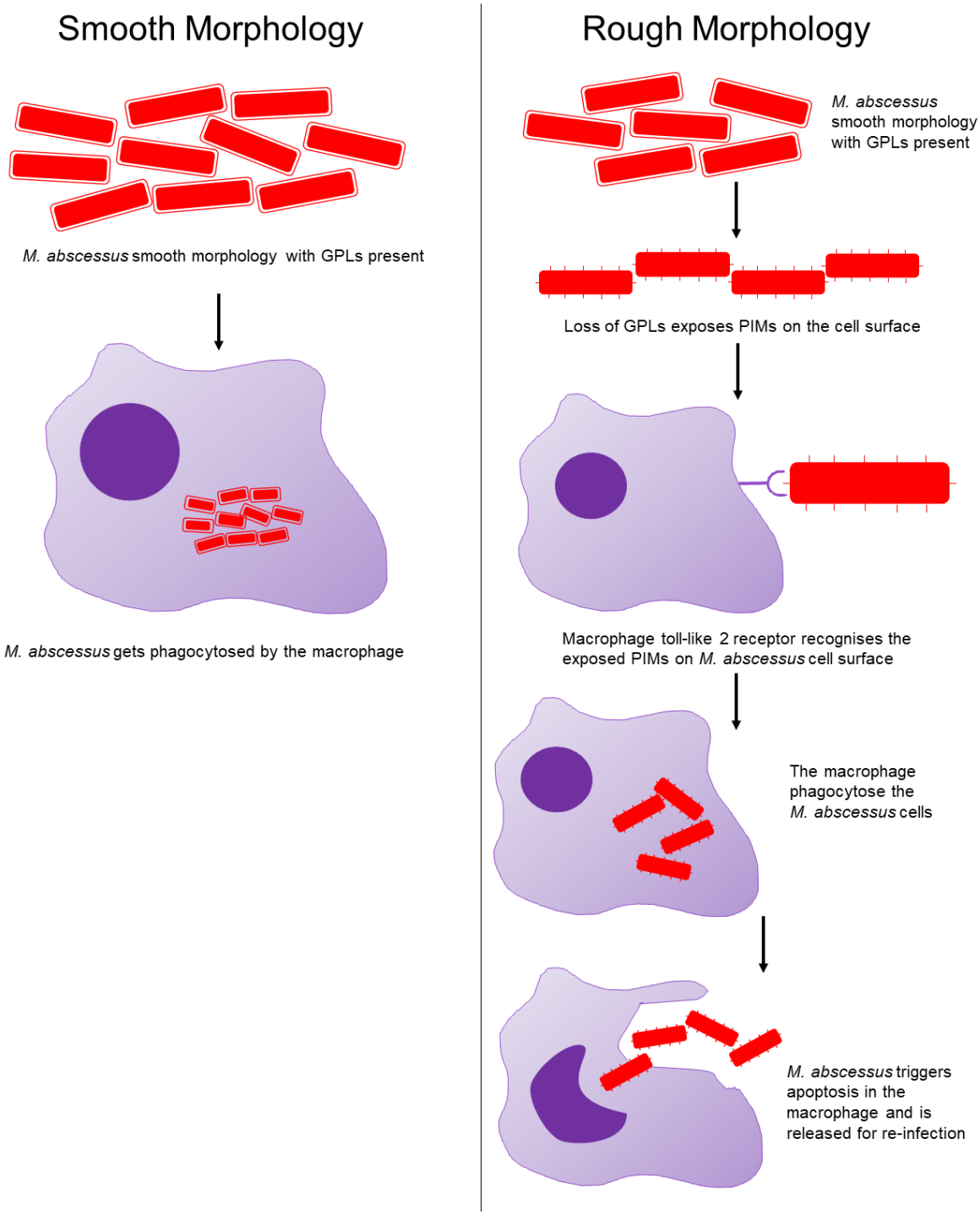

Figure 1.4 Smooth and rough morphological variants of *M. abscessus* and how these variants interact with the host's immune response. The smooth morphological variant retains glycopeptidolipids on the mycobacterial cell membrane and become phagocytosed by macrophages. The rough morphological variant has a loss of glycopeptidolipids on the outer membrane, exposing phosphatidyl-myo-inositol mannosides. These are recognised by macrophage toll-like 2 receptors and become phagocytosed. The rough variant is then able to trigger apoptosis of the macrophage, being re-released into the host.

1.1.5 Drug resistance and current treatments

Further to the presence or absence of a functional macrolide resistance gene, *M. abscessus* has other intrinsic drug resistance mechanisms, including an impermeable cell wall structure, inducible resistance genes for other antibiotic classes, cell export systems and plasmid mediated resistance (Nessar *et al.*, 2012). The cell wall structure is highly hydrophobic, due to the large quantity of free intercalated lipids and mycolic acids on the surface, as well as arabinogalactan, peptidoglycan, lipoarabinomannan and lipomannan, resulting in a membrane which is impermeable to hydrophilic and lipophilic molecules (Figure 1.1) (Jarlier and Nikaido, 1994). Further to this, the cell membrane of *M. abscessus* contains porins, which allow for the transport of small solute molecules into the cell and preventing the entry of larger molecules (Lopeman *et al.*, 2019). This is not the only mechanism employed by *M. abscessus*, efflux pumps actively transport molecules out of the cell, including ATP-binding cassette (ABC) transporters and Mycobacterial membrane protein Large (MmpL) proteins, although not all MmpLs are efflux plumps (Ripoll *et al.*, 2009). ABC transporters have been identified in all domains of life and are responsible for the transmembrane transport of antibiotics, proteins, sugars and more, out of the cell through hydrolysis of ATP, making them active transporters (Greene *et al.*, 2018). MmpL proteins are transmembrane proteins belonging to the resistance-nodulation cell division proteins, involved in the transport of lipids or lipophilic molecules out of the cell (Viljoen *et al.*, 2017; Dubois *et al.*, 2018). These, and other efflux pumps present in *M. abscessus* have the ability to transport a variety of antibiotics out of the cell, including clarithromycin, linezolid, clofazamine and bedaqualin (Gutiérrez *et al.*, 2019; Vianna *et al.*, 2019; Ye *et al.*, 2019). Further to these mechanisms and as previously mentioned, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* both possess the functional *erm*(41) gene for macrolide resistance (Brown-Elliott *et al.*, 2015). Additionally, although the occurrence of plasmid mediated resistance is uncommon for mycobacteria, there have been findings of plasmid mediated kanamycin resistance in *M. abscessus* subsp. *bolletii*, indicating more potential routes for resistance to antimicrobials (Matsumoto *et al.*, 2014). Due to these drug resistant mechanisms employed by *M. abscessus*, treatment options are therefore limited and often ineffective.

The current treatment regimen for *M. abscessus* depends on the isolate's susceptibility to macrolide antibiotics (Table 1.1) (Haworth *et al.*, 2017). The overall treatment regimen constitutes a 1 month phase of intravenous (I.V) amikacin, tigecycline and imipenem. If the isolate is sensitive to macrolide antibiotics, treatment will also consist of clarithromycin or azithromycin. This is followed by 12 months of nebulised amikacin, and if macrolide sensitive the continuation of clarithromycin and/or azithromycin. In addition, a combination of clofazimine, linezolid, minocycline, moxifloxacin and co-trimoxazole may be used. This is a combination based therapy because each one of these drugs is from a different antibiotic class, each with a differing target, or mechanism of action (Figure 1.5). This increases the likelihood of success during treatment, however it also increases the side effects experienced by the patient.

Table 1.1 Current drug treatment regimen for pulmonary *M. abscessus* infections as detailed by the British Thoracic Society (Haworth *et al.*, 2017)

	Macrolide-Sensitive	Macrolide Resistant
Initiation Phase (1 month)	• I.V. amikacin (15mg/kg daily) • I.V. tigecycline (50 mg twice daily) • I.V. imipenem (1 g twice daily) • Oral clarithromycin (500 mg twice daily) or oral azithromycin (250 – 500 mg daily)	• I.V. amikacin (15 mg/kg daily) • I.V. tigecycline (50 mg twice daily) • I.V. imipenem (1 g twice daily)
Continuation Phase (12 months)	• Nebulised amikacin (250 – 500 mg twice daily) • Oral clarithromycin (500 mg twice daily) or oral azithromycin (250 – 500 mg daily) Based on susceptibility testing 1 to 3 of the following: • Oral clofazimine (50 – 100 mg daily) • Oral linezolid (600 mg twice daily) • Oral minocycline (100 mg twice daily) • Oral moxifloxacin (400 mg daily) • Oral co-trimoxazole (960 mg twice daily)	• Nebulised amikacin (250 – 500 mg twice daily) Based on susceptibility testing 2 to 4 of the following: • Oral clofazimine (50 – 100 mg daily) • Oral linezolid (600 mg twice daily) • Oral minocycline (100 mg twice daily) • Oral moxifloxacin (400 mg daily) • Oral co-trimoxazole (960 mg twice daily)

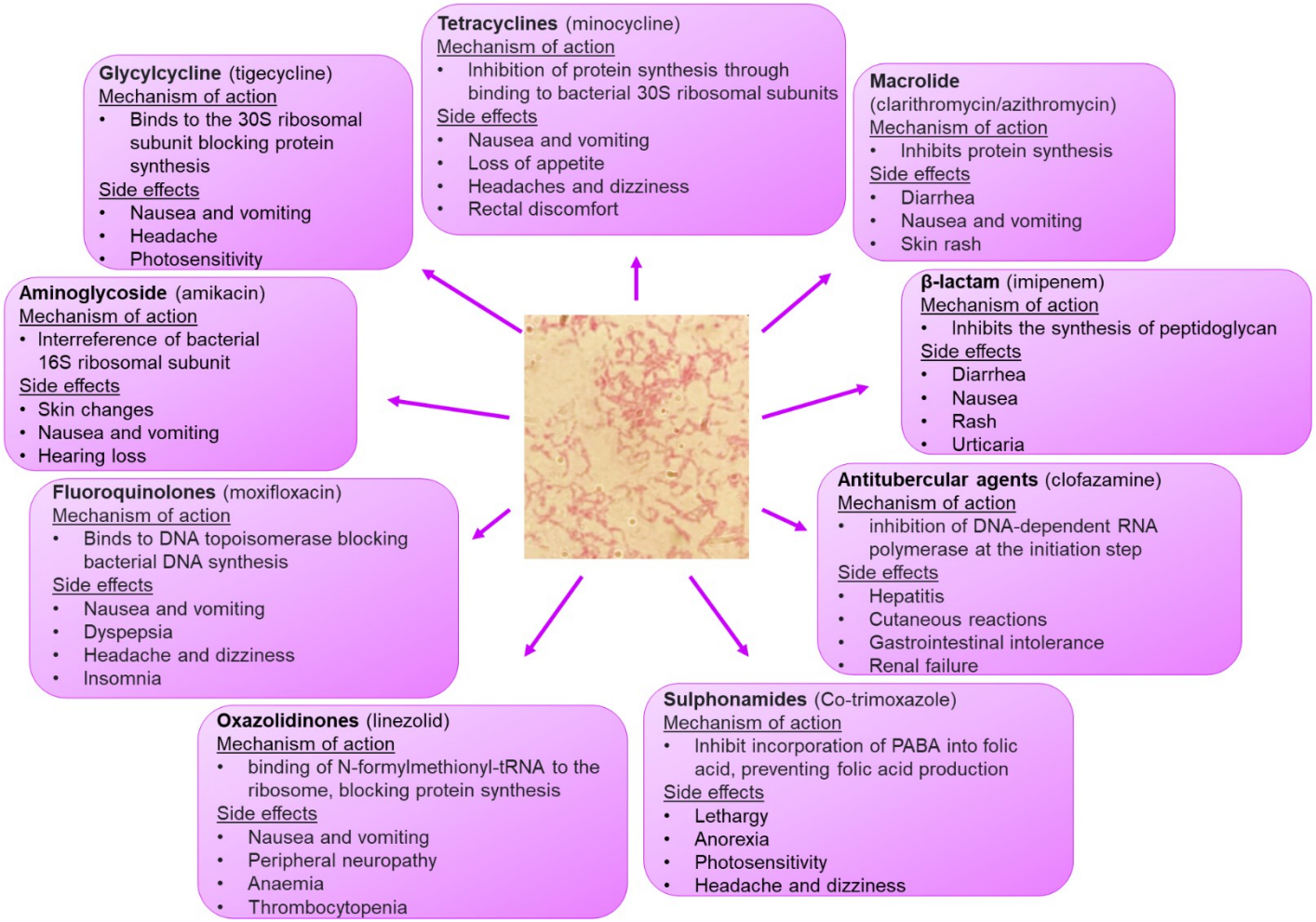

Figure 1.5 The main antibiotics prescribed for the treatment of *M. abscessus* complex infections. A variety of different classes of antibiotics are used in the treatment of *M. abscessus* infections. These are all based on different mechanisms of action. They all have a variety of side effects associated with them, which can result in patients opting to stop treatment.

1.1.5.1 Amikacin

Amikacin is the only antibiotic administered initially through I.V and then in a nebulised form for the treatment of *M. abscessus* pulmonary infections (Table 1.1). It is an aminoglycoside antibiotic based around the deoxystreptamine or streptidine ring (Figure 1.6). The aminoglycoside antibiotics were initially isolated from *Streptomyces griseus* and have been developed into several different antibiotics, such as streptomycin, tobramycin and kanamycin (Krause *et al.*, 2016a). Alongside the use of amikacin, ciprofloxacin has also been administered in a nebulised form to treat non-tuberculous mycobacterial pulmonary infections (Maselli, Keyt and Restrepo, 2017). The mechanism of action of aminoglycosides is through the blocking of protein synthesis by ribosomal interference (Figure 1.3) (Krause *et al.*, 2016b). This is achieved by binding to the A site of the bacterial 30S ribosomal subunit through a specific interaction between L-(−)-γ-amino-α-hydroxybutyryl group of amikacin and the RNA at specific glycine-cysteine pairs (Ramirez and Tolmasky, 2017). This reduces the proof reading capabilities of the ribosome resulting in protein mistranslation (Chulluncuy *et al.*, 2016). Furthermore, mistranslated proteins can become embedded into the cytoplasmic membrane, creating holes by reducing cell membrane integrity and ultimately accelerating cell death (Ramirez and Tolmasky, 2010). Resistance to aminoglycosides is usually through aminoglycoside modifying enzymes, such as acetyltransferase, but mycobacteria also gain resistance to amikacin due to modifications in the ribosomal RNA, preventing binding (Ramirez and Tolmasky, 2017). Side effects associated with amikacin include nephrotoxicity and ototoxicity which can result in kidney disease and permanent hearing loss (Prayle *et al.*, 2010).

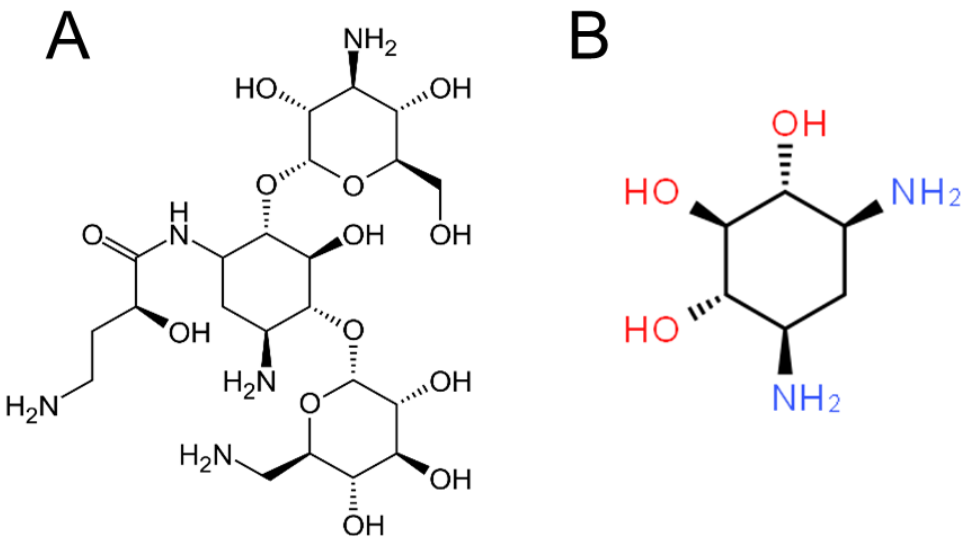

Figure 1.6 Chemical structure of amikacin. A, the chemical structure of amikacin. B, the chemical structure of deoxystreptamine, the basis for aminoglycoside antibiotics.

1.1.5.2 Minocycline

Minocycline is a tetracycline based antibiotic administered orally in the continuation phase of *M. abscessus* treatment (Table 1.1) (Figure 1.7). The tetracycline class of antibiotics were first isolated from actinomycetes bacteria and were approved for clinical use in 1948 (Duggar, 1948). The widespread use of these antibiotics, due to broad spectrum of activity and little side effects, resulted in resistance to this class of antibiotic rather rapidly (Roberts, 2003). The tetracycline antibiotics bind to the A-site of the 30S ribosomal subunit, a highly conserved region in the bacterial 16S ribosome, and block the binding of aminoacyl-tRNA (Grossman, 2016). This ultimately inhibits protein synthesis (Figure 1.5). The bacterial resistance mechanisms employed to evade this class of antibiotic are through efflux pumps, enzyme inactivation by the *tet*(X) gene product and ribosomal protection by proteins that bind to the ribosome causing a confirmational change and prevent binding of the tetracycline (Chopra and Roberts, 2001). The use of efflux pump inhibitors administered alongside tetracycline antibiotics has been considered as one solution to overcome bacterial resistance, which could improve the use of tetracycline antibiotics in a clinical settings (Blanchard *et al.*, 2014; Opperman and Nguyen, 2015). Side effects associated with minocycline are nausea, vomiting, diarrhoea, dizziness, photosensitive rash, pruritus, urticarial and hyperpigmentation of the skin and mucous membranes (Asadi *et al.*, 2020).

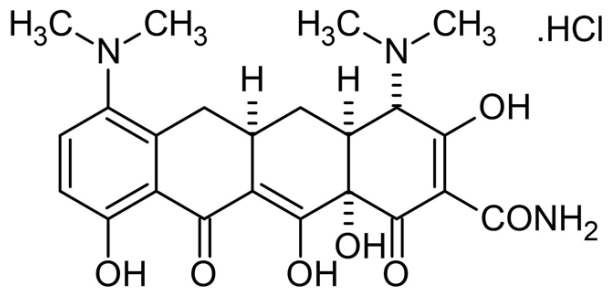

Figure 1.7 Chemical structure of minocycline. Minocycline is a tetracycline antibiotic used for its broad spectrum of activity.

1.1.5.3 Tigecycline

Tigecycline is a glycylcycline antibiotic used in the initiation phase of *M. abscessus* treatment (Table 1.1) (Figure 1.8). The glycylcycline antibiotics are derived from the structure of tetracycline antibiotics (Greer, 2006). The development of the glycylcycline antibiotics was to overcome some of the drug resistance mechanisms employed by bacteria against the tetracycline antibiotics, namely efflux pumps and ribosomal protection (Stein and Craig, 2006). The mechanism of action of glycylcycline antibiotics is the same as for tetracycline antibiotics (Figure 1.5), however they have higher binding affinity to the ribosomal target, thus can overcome the ribosomal protection mechanisms by preventing the gene employed by bacteria from interacting with the ribosome (Greer, 2006). It has also been suggested they can overcome export out of the bacterial cell by efflux pumps due to either not being recognised by the efflux proteins or not being able to transport the glycylcycline across the cell membrane (Chopra, 2002). The side effects associated with tetracyclines are nausea, vomiting and diarrhoea, as well as tooth discoloration, photosensitivity and coagulopathy (Kaewpoowat and Ostrosky-Zeichner, 2015; Cui *et al.*, 2019).

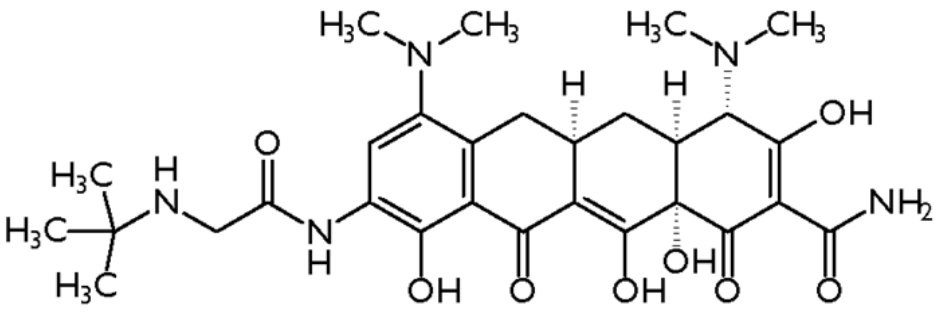

Figure 1.8 Chemical structure of tigecycline. Tigecycline is a glycylcycline antibiotic, derived from the tetracycline antibiotics.

1.1.5.4 Imipenem

Imipenem is a carbapenem based β-lactam antibiotic that is prescribed in the initiation phase of *M. abscessus* treatment (Table 1.1). The β-lactam class of antibiotics are based around a β-lactam ring (Figure 1.9) and include: carbapenems, cephalosporins, monobactams and penicillin derivatives (De Rosa *et al.*, 2021). The β-lactams act by binding to the penicillin-binding proteins (PBP) which are essential in cell wall synthesis (Bush and Bradford, 2016). The carbapenem β-lactam antibiotics have the ability to bind to several different PBPs including PBP1a, PBP1b, PBP2 and PBP3 (Sumita and Fakasawa, 1995). The multiple targets that carbapenems can bind to reduce the instance of resistance to this class of antibiotic (Bush and Bradford, 2016). The resistance mechanisms employed by bacteria to overcome β-lactam antibiotics are through the production of β-lactamase enzymes which inactivate the β-lactam antibiotic or through altered PBPs, preventing binding of the β-lactam antibiotic to the target site (Worthington and Melander, 2013). Resistance to β-lactam antibiotics through the production of a β-lactamase, specifically the Bla_{Mab} gene in *M. abscessus*, can be overcome by the addition of a β-lactamase inhibitor, rendering the β-lactamase enzyme ineffective and thus allow binding of the β-lactam antibiotic to its target site (Drawz and Bonomo, 2010). A major side effect that is associated with β-lactam antibiotics is anaphylactic shock due to allergic reaction. However it has been shown recently that the majority of individuals labelled as β-lactam allergic, may in fact be β-lactam tolerable, suggesting further assessment may be required (Jeimy *et al.*, 2020). Other side effects include fever, urticaria and angioneurotic edema (Prescott, 2013).

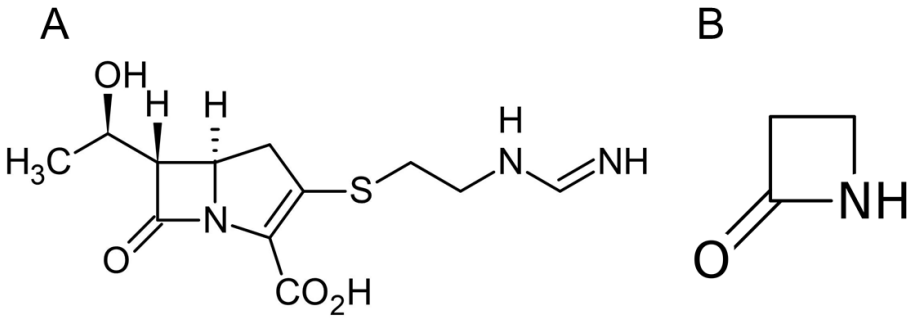

Figure 1.9 Chemical structure of imipenem. A, the chemical structure of imipenem. B, the chemical structure of a β-lactam ring, the basis for the β-lactam antibiotics.

1.1.5.5 Clarithromycin and azithromycin

Clarithromycin and azithromycin are macrolide antibiotics that are used in the treatment regimen of *M. abscessus*, if the isolate is macrolide sensitive (Table 1.1). Macrolide sensitivity in *M. abscessus* can be dependent on the subspecies of *M. abscessus* causing the infection (Section 1.1.3). This class of antibiotic has been widely used as an alternative to β-lactam and cephalosporin antibiotics (Leclercq, 2002). Macrolides are used mainly for Gram positive bacterial infections and pulmonary infections. This is because concentrations of macrolides have been observed to be 10-fold higher in concentration in epithelial lung fluid than serum (Kanoh and Rubin, 2010). The macrolide antibiotics are based on a macrocyclic lactone ring (Figure 1.10) and are modified versions of erythromycin (Seiple *et al.*, 2016). They inhibit protein synthesis by targeting the bacterial ribosome (Vázquez-Laslop and Mankin, 2018). The mechanisms employed by bacteria to overcome macrolides include ribosomal modification, enzyme inaction and efflux pumps (Leclercq, 2002). The main side effects associated with macrolides are hepatotoxicity, a rash and ototoxicity (Altenburg *et al.*, 2010).

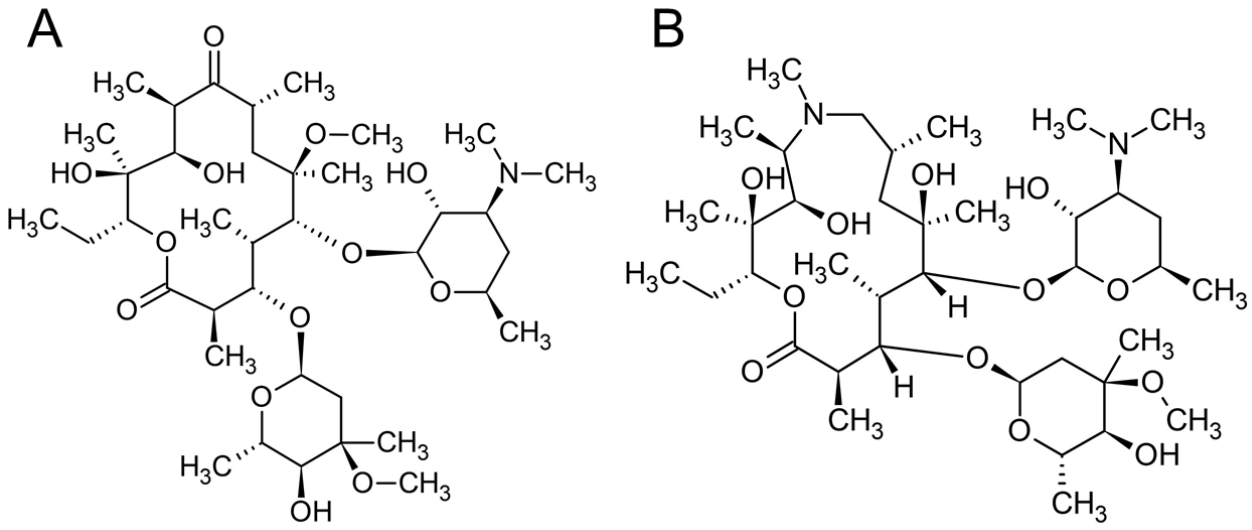

Figure 1.10 Chemical structure of clarithromycin and azithromycin. A, the chemical structure of clarithromycin. B, the chemical structure of azithromycin.

1.1.5.6 Clofazimine

Clofazimine is an antitubercular antibiotic used to treat mycobacterial infections and is used during the continuation phase of treatment for pulmonary *M. abscessus* infections (Haworth *et al.*, 2017). The structure of clofazimine is based on a phenazine dye (Figure 1.11). The antimycobacterial action has not yet been fully elucidated, but it is thought to bind preferentially to mycobacterial DNA and therefore prevents DNA replication (NCBI, 2021). The side effects associated with clofazimine are gastrointestinal toxicity, red skin colouration, phototoxicity and cardiotoxicity (Queiroz *et al.*, 2002).

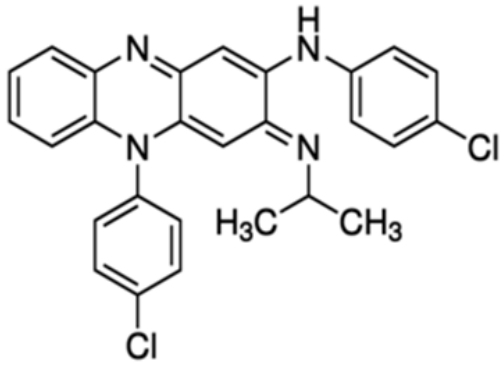

Figure 1.11 Chemical structure of clofazimine. Clofazimine is an antitubercular antibiotic.

1.1.5.7 Linezolid

Linezolid is an oxazolidinone antibiotic based on a five membered ring containing nitrogen and oxygen (Figure 1.12) (Pandit, Singla and Shrivastava, 2012). It can be prescribed as one of the antibiotics in the continuation phase of *M. abscessus* pulmonary infection treatment. It inhibits protein synthesis, but unlike other protein inhibition antibiotics it does not target the elongation stage of protein synthesis, it targets the initiation of protein synthesis (Ament, Jamshed and Horne, 2002). This is done by binding to the 50S ribosomal subunit near to the interface of the 30S subunit, preventing formation of the 70S initiation complex (Diekema and Jones, 2001). Due to this novel mechanism of action, microbial resistance to other protein synthesis inhibitors does not result in resistance to linezolid (Hashemian, Farhadi and Ganjparvar, 2018). However, resistance to linezolid can be achieved by small mutations in the bacterial ribosome that interfere with the binding of linezolid (Long and Vester, 2012). Moreover, linezolid is bacteriostatic rather than bactericidal, therefore other antibiotics with bactericidal action might be more preferential for treatment as they would eradicate the infectious bacteria, rather than only prevent growth (Rafailidis *et al.*, 2009). The side effects most commonly associated with linezolid are peripheral neuropathy, anaemia and thrombocytopenia (Schecter *et al.*, 2010).

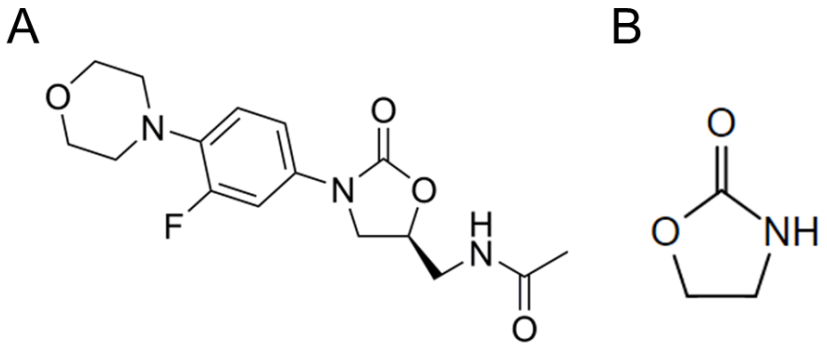

Figure 1.12 Chemical structure of linezolid and oxazolidinones. A, chemical structure of linezolid. B, the chemical structure of oxazolidinone antibiotics.

1.1.5.8 Moxifloxacin

Moxifloxacin is a fluoroquinolone antibiotic, based around a bicyclic ring with the addition of a fluorine (Figure 1.13) (Kocsis, Domokos and Szabo, 2016). Fluoroquinolones can bind to two bacterial DNA enzymes, DNA gyrase and topoisomerase IV, depending on if the bacteria is Gram positive or Gram negative. For Gram negative organisms, it binds to the DNA gyrase but for Gram positive organisms it binds to the topoisomerase IV (Rusu *et al.*, 2021). At low concentrations, fluoroquinolones can block DNA replication and translation by binding to the topoisomerase-DNA cleavage complex responsible for repairing damaged DNA. Whereas, at high concentrations they can result in chromosome fragmentation by preventing DNA repair occurring (Bush *et al.*, 2020). Resistance to fluoroquinolones can be achieved through single amino acid changes in either DNA gyrase or topoisomerase IV to prevent binding or through the use of efflux pumps and altered porins (Redgrave *et al.*, 2014). Side effects associated with moxifloxacin are nausea, dizziness, hepatotoxicity and skin reactions (Tulkens, Arvis and Kruesmann, 2012). Moreover, fluoroquinolones are often not prescribed to paediatric patients due to the possibility of severe side effects, including those affecting the central nervous system, gastrointestinal, muscular and cardiovascular (Valerio *et al.*, 2003). However, moxifloxacin is preferential to other fluoroquinolones such as sparfloxacin due to lowered toxicity to the skin (Valerio *et al.*, 2003).

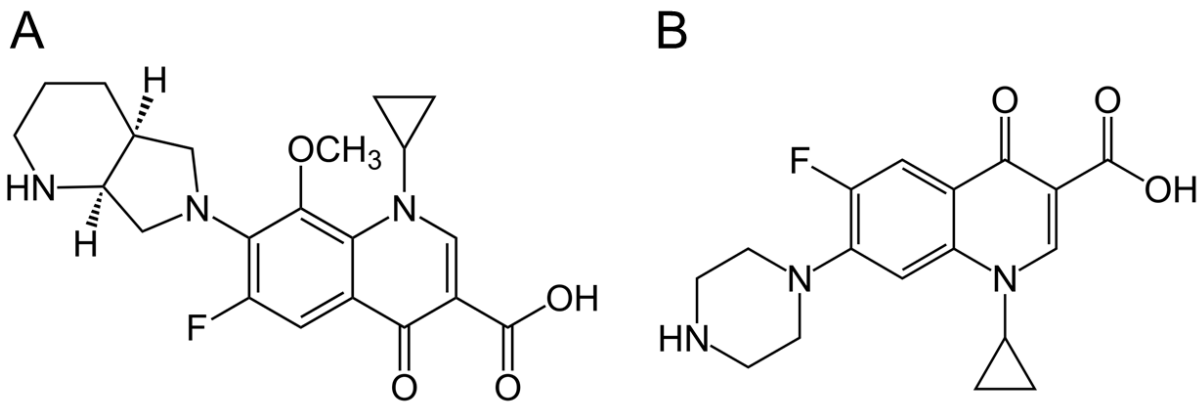

Figure 1.13 Chemical structure of moxifloxacin and fluoroquinolones. A, the chemical structure of moxifloxacin. B, the chemical structure for the basis of fluoroquinolone antibiotics.

1.1.5.9 Co-trimoxazole

Co-trimoxazole is a combination of two antibiotics, trimethoprim and sulfamethoxazole, that act synergistically to block the production of folic acid (Church *et al.*, 2015). Trimethoprim is a general antibiotic and sulfamethoxazole is a sulphonamide antibiotic. The general structure of sulphonamide antibiotics are based around a sulphur with two double-bonded oxygen's, a nitrogen and a carbon ring (Figure 1.14). The structure of sulfamethoxazole is important because it is a structural analogue of para aminobenzoic acid (PABA), an essential step in the folic acid synthesis pathway (Yousef, Mansour and Herbali, 2018). During folic acid synthesis, PABA binds to dihydropteroate synthetase to form dihydropteroate diphosphate. With the addition of sulfamethoxazole, the sulfamethoxazole binds to dihydropteroate synthetase, blocking the binding of PABA (Estrada, Wright and Anderson, 2016). This then halts the production of folic acid. Trimethoprim acts downstream of sulfamethoxazole, by disrupting the production of tetrahydrofolate. This is done in a similar way to sulfamethoxazole, by which it competitively binds to dihydrofolate reductase, inhibiting the production of tetrahydrofolate and preventing the formation of folate (Church *et al.*, 2015). The production of folate is important as it cannot be taken up from the environment and is essential for the synthesis of purines and thymidine's, which are crucial for the synthesis of DNA and proteins, making the folate pathway essential (Zheng and Cantley, 2019). The two antibiotics alone only act as bacteriostatic agents, however when administered together they have bactericidal activity because they disrupt two steps in a biosynthetic pathway that ultimately impacts the production of DNA and proteins (Kester, Karpa and Vrana, 2012). The side effects associated with co-trimoxazole are nausea, dizziness, vomiting, headaches and rashes (D Mwambete and Kamuhabwa, 2013).

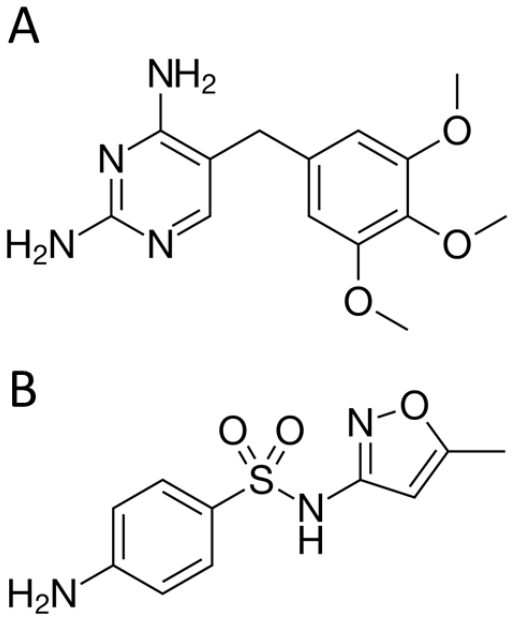

Figure 1.14 Chemical structure of co-trimoxazole which is comprised of 2 different antibiotics.
A, the chemical structure for trimethoprim. B, the chemical structure of sulfamethoxazole.

1.1.6 Treatment Failure

The antibiotics currently available for *M. abscessus* antimicrobial therapy cover a vast array of antimicrobial classes. However, drug resistance mechanisms employed by mycobacteria have resulted in these drugs being largely unsuccessful at treating infections. Coupled with severe side effects, patient outcomes are extremely poor and most patients choose to not complete the course of treatment. With limited new antibiotic drugs in the pipeline and approaching clinical trials, new strategies are urgently required to combat these infections. Some of these novel approaches include the implementation of β-lactamase inhibitors to improve efficacy of β-lactam antibiotics or new combinatorial therapies and the repurposing of existing drugs (Harrison *et al.*, 2021). More novel approaches include the use of artificial intelligence to identify new possible drugs, such as halicin (Stokes *et al.*, 2020). Another rather under explored area for antimicrobial therapy is metal based compounds, which have the ability to make 3D structures that are not achievable with the current antibiotics (Frei, 2020). One such area that has been explored for other bacterial infections, but not yet mycobacteria, is the antimicrobial activity of manuka honey.

1.2 Honey

1.2.1 History of honey

Honey has been established as an effective antimicrobial, anti-inflammatory and antioxidant for millennia (Cokcetin *et al.*, 2016). Throughout history, honey has been used in a variety of cultures, with differing applications. The ancient Egyptians used honey as a topical ointment, a wound dressing and for embalming their dead, whereas the ancient Greeks used it as a remedy for gout, pain, fever and also wound healing (Eteraf-Oskouei and Najafi, 2013). The first observations of the antimicrobial activity of honey were made in 1892, and since then honey has been observed to have a broad spectrum of activity, inhibiting both Gram positive and Gram negative organisms, including: *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis* and *Listeria monocytogenes* and their multidrug-resistant counterparts (Dustmann, 1979; Laallam *et al.*, 2015). Despite this, the initial interest into honey as an antimicrobial therapy was drastically diminished upon the discovery and implementation of antibiotics. However, with the alarming rise in the prevalence of antimicrobial-resistant organisms, in particular the increase in multi-drug resistance (MDR), the number of effective antibiotic compounds is shrinking at a greater rate than new drugs are being developed (Davies and Davies, 2010; Simpkin *et al.*, 2017). This grave predicament has many researchers looking back to the pre-antibiotic era for solutions, sparking more recent interest into honey as an antimicrobial (McLoone, Warnock and Fyfe, 2016). Since then, the majority of research has focused on the diverse organisms that can be inhibited by honey as well as what makes honey such an effective antimicrobial agent. Initial studies into honey outlined some key factors contributing to its antimicrobial action, these include high sugar content, low pH, hydrogen peroxide, polyphenolic compounds and the identification of an inhibine (Dustmann, 1979; Molan, 1992; Albaridi, 2019). Further studies exploring why honey is a powerful antimicrobial identified that the inhibine was a 1,2-dicarbonyl compound in the form of methylglyoxal, a potent antimicrobial, found mainly in manuka honey (Mavric *et al.*, 2008). More recent studies have also identified a bee-derived protein, bee defensin-1, as a potential antimicrobial component within honey (Bucekova *et al.*, 2019). This has given rise to the idea that the antimicrobial activity of honey cannot be attributed to a single antimicrobial agent, it is a combination of all these factors working together that makes honey such a powerful antimicrobial.

There have since been developments of honey into medical grade products that are mainly used for the treatment of surface wounds, burns and inflammation (Saranraj and Sivasakthi, 2018; Albaridi, 2019). These medical grade honey's are all based on different elements of honey's antimicrobial activity and have been shown to have a range of activity against a variety of organisms, regardless of drug resistance (Nolan *et al.*, 2020).

1.2.2 Composition and Classification

Honey is a complex food substance, produced by the honey bee (*Apis mellifera*), comprised of 180 to 200 different substances, including sugar, water, proteins, vitamins, minerals, polyphenolic compounds and plant derivatives (El Sohaimy, Masry and Shehata, 2015; Bucekova *et al.*, 2019). Depending on origin, honey can be classified as honeydew or blossom. Honeydew honey is produced by the collection of living plant, aphid and insect secretions (Vasić *et al.*, 2020), whereas blossom honey is produced by the collection of flower nectar and characterised by pollen content. Blossom honey can be further divided into unifloral, where the botanical origin is predominantly from one flower species, or multifloral, where multiple sources of flower species can be identified (Manivanan, Rajagopalan and Subbarayalu, 2018). The botanical origin of honey can have the biggest influence on its antioxidant and antimicrobial activity (Silici, Sagdic and Ekici, 2010). One honey that has been of great significance, due to its broad spectrum of antimicrobial activity, is manuka honey, derived from *Leptospermum sp.* (Cokcetin *et al.*, 2016). This unifloral honey is used within the pharmaceutical industry and has been developed into medical grade honey. Its antimicrobial activity has been attributed to phytochemicals produced by the *Leptospermum sp.* plant and subsequently transferred to the honey (Nolan, Harrison and Cox, 2019). Recently however, honeydew honey has been investigated as a more potent antimicrobial than unifloral honey, furthering the importance of honey origin (Pita-Calvo and Vázquez, 2017). Furthermore, the composition of active compounds present within plant nectar can vary, depending on geographical location and climate conditions (Silici, Sagdic and Ekici, 2010). All of these different components can influence the quality of the honey and, subsequently, the antimicrobial activity.

1.2.3 Antimicrobial Components of honey

There are currently 4 main components that give rise to honey's antimicrobial activity through direct inhibitory mechanisms, along with several components that act indirectly (Figure 1.15). Those that act through direct inhibitory mechanisms are polyphenolic compounds, hydrogen peroxide, bee defensin-1 and methylglyoxal (Nolan, Harrison and Cox, 2019). These act by causing DNA damage, disrupting membrane permeability and altering the physical structures of the microbial cell (Figure 1.15). Those that act through indirect inhibitory mechanisms are low pH, high sugar content and high osmotic pressure. These have a wider ranging impact on the bacterial cell, as opposed to the direct inhibitory factors affecting cellular mechanisms (Figure 1.15). These all influence the bacterial cell by exerting physical pressure, such as increasing the water potential or altering activity of enzymes through ionisation. This can especially impact bacteria that are not osmotolerant and often inhibits bacterial growth (Szweda, 2017).

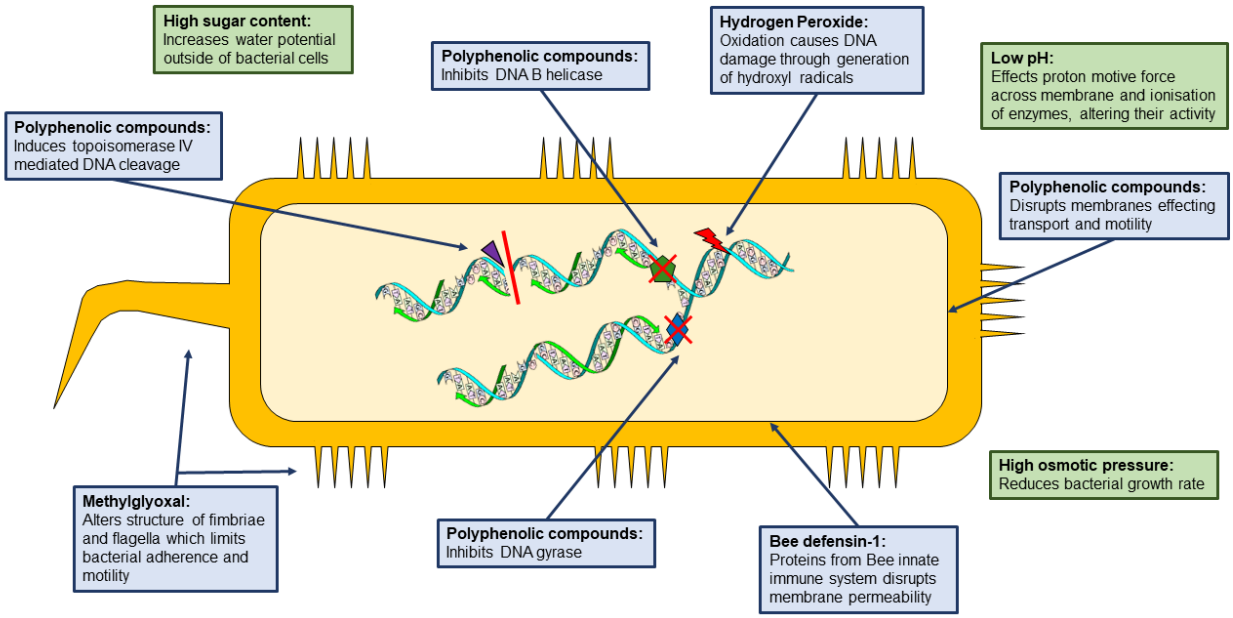

Figure 1.15 The main constituents attributed to honey's antimicrobial activity and their mechanism of action. Direct inhibitory factors that affect cellular mechanisms (**blue**) and indirect inhibitory factors that have a wider ranging effect on the bacterial cell (**green**) (reviewed in Nolan, Harrison and Cox, 2019).

The route by which the 4 main components are present within honey also differs, with some being directly transferred into the honey from the flower nectar, some through addition of secretions by the bee and others being converted whilst in the honey (Figure 1.16). The presence of polyphenolics is through direct transfer from the flower nectar into the honey, whereas hydrogen peroxide is produced through addition of bee derived enzymes (Figure 1.14). Bee defensin-1 is produced as an innate immune protein and methylglyoxal is converted over time. Meaning that not all honeys are equal, and therefore each honey has a unique antimicrobial activity profile.

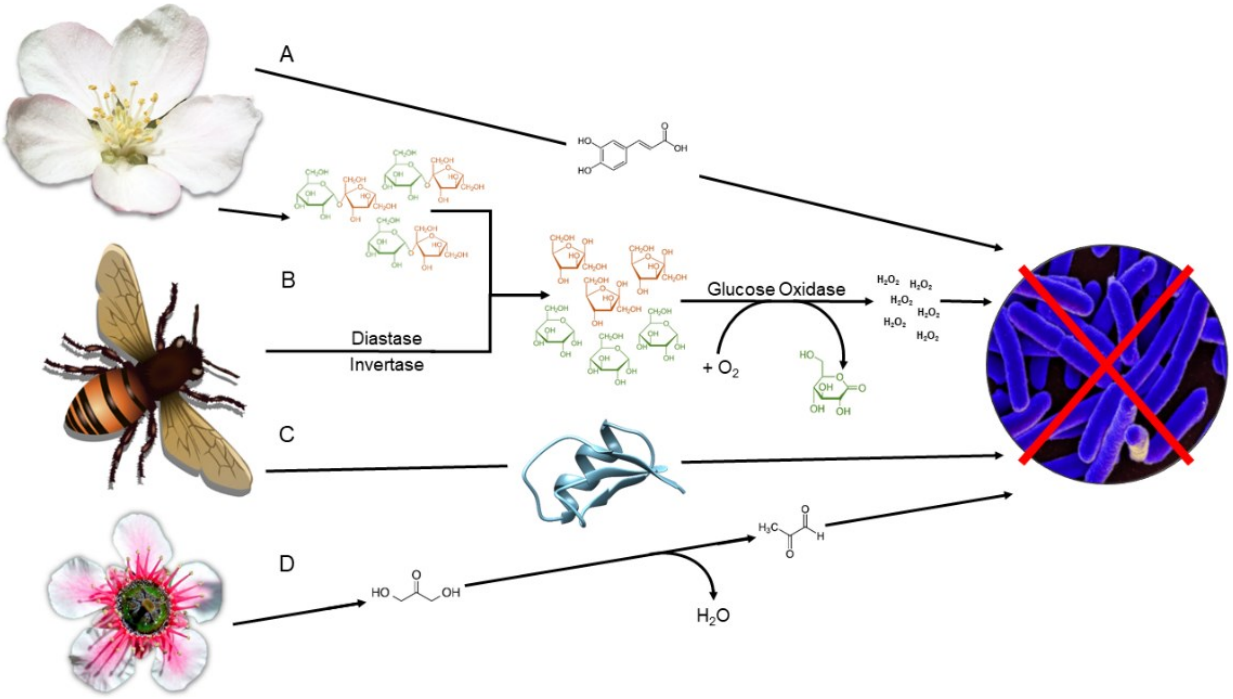

Figure 1.16 Acquisition of antimicrobial compounds within honey. A) Polyphenolic compounds derived from the plant are transferred by the bee. B) Sucrose from the flower is ingested by the bee and broken down into glucose and fructose upon addition of diastase and invertase by the bee. The glucose is oxidised by glucose oxidase upon the addition of oxygen, producing D-glucono-δ-lactone and hydrogen peroxide. The hydrogen peroxide has antimicrobial activity. C) Bee defensin-1 is added to honey by the bee (Swissmodel 6mry.5.A). D) Dihydroxyacetone is harvested from *Leptospermum sp.* and converted non-enzymatically to methylglyoxal through dehydration reaction (Nolan, Harrison and Cox, 2019).

1.2.3.1 Polyphenolics

Polyphenolic compounds are a diverse group of chemicals that include flavonoids and phenolic acids (non-flavonoids), defined by the presence of phenolic structures (Cianciosi *et al.*, 2018). These are produced as plant secondary metabolites, and are bioactive compounds that are transferred directly from the plants flower nectar to the honey (Figure 1.16). These have been identified as a major component of the health-promoting properties of honey due to their ability to scavenge free radicals in the body preventing damage to cells caused by oxidation (Güneş *et al.*, 2017). Additionally, the phenolic acids identified in honey have been used to trace the botanical and geographical origin of a given honey sample (Escuredo *et al.*, 2012). Therefore, the botanical origin of honey is highly significant because it can influence the phytochemicals present, and consequently impact the antimicrobial and antioxidant capacity (Sherlock *et al.*, 2010; Vasić *et al.*, 2020).

The polyphenolic compounds identified in honey have been shown to have antimicrobial activity and the mechanisms of action have largely been elucidated. However, the concentrations at which these polyphenolic compounds are active are much lower than the levels found within honey (Nolan, Harrison and Cox, 2019). However, due to the role of

polyphenols in destroying free radicals and inhibiting oxidation, it is suggested that they could play a role in the generation of hydrogen peroxide within honey (Estevinho *et al.*, 2008; Bucekova *et al.*, 2018, 2019; Jibril, Hilmi and Manivannan, 2019). Furthermore, when testing polyphenolic extracts taken from honey for antimicrobial activity, inhibition of *S. aureus, E. coli,* and *K. pneumoniae* was observed (Estevinho *et al.*, 2008). Suggesting that the polyphenolic compounds could still be impacting the antimicrobial potential of honey. Further investigations into the role of polyphenolic compounds and their direct antimicrobial impact on honey are required.

1.2.3.2 Hydrogen peroxide

The presence of hydrogen peroxide within honey has been well established and is considered one of the main antimicrobial constituents in honey. It is produced as a by-product during nectar harvest by the honey bee (*Apis mellifera*) (Brudzynski *et al.*, 2011). Upon harvest, bee-derived enzymes are added, including diastase, invertase and glucose oxidase. The diastase and invertase break down the larger disaccharides, mainly sucrose, into the monosaccharides, glucose and fructose (Ball, 2007). Upon the addition of oxygen, glucose oxidase catalyses the oxidation of glucose to D-glucono-δ-lactone and hydrogen peroxide, the latter of which has antimicrobial activity (Figure 1.15) (Brudzynski *et al.*, 2011). Interestingly, the antimicrobial effect of hydrogen peroxide in honey increases upon dilution of the honey, enabling the glucose oxidase enzyme to bind to glucose more readily, resulting in a continuous production of hydrogen peroxide (Brudzynski, 2006). It has also been suggested that molecular crowding could play a role in hydrogen peroxide production, provided the concentration of glucose was high enough (Brudzynski *et al.*, 2017). The levels of hydrogen peroxide in honey vary between samples and are dependent on two factors: the amount of glucose oxidase added and the presence of pollen-derived catalase (Taormina, Niemira and Beuchat, 2001). Since glucose oxidase catalyses the reaction, it is assumed that higher levels of glucose oxidase result in more hydrogen peroxide production. This can be influenced by honey bee health and diversity of foraged diet (Weston, 2000). Conversely, more recent research has suggested that the levels of glucose oxidase present are not directly related to the volume of hydrogen peroxide produced, although these non-enzymatic methods of production are yet to be elucidated (Bucekova *et al.*, 2019). Additionally, catalase is known for the breakdown of hydrogen peroxide into water and oxygen, therefore it is of no surprise that catalase concentration is proportionate to hydrogen peroxide content (Taormina, Niemira and Beuchat, 2001).

1.2.3.3 Bee defensin-1

Bee defensin-1 is an antimicrobial peptide identified in bee hemolymph (the bee blood system) and hypopharyngeal glands (McLoone, Warnock and Fyfe, 2016). It is one of four antimicrobial peptides, others include apidaecin, abaecin, hymenoptaecin and defensin (Ilyasov *et al.*, 2012). Their role within the bee is as an innate immune response, exhibiting activity against fungi, yeast, protozoa and both Gram positive and Gram negative bacteria (Oryan, Alemzadeh and Moshiri, 2016). Importantly, bee defensin-1 is mainly effective against Gram positive bacteria, most notably *Bacillus subtilis, S. aureus* and *Paenibacillus larvae*, however, it has limited effectiveness against multidrug-resistant organisms (Kwakman and Zaat, 2012). Levels of bee defensin-1 vary between honey samples, this is a result of its production from glands of individual bees, whose production of antimicrobial peptides varies (Kwakman *et al.*, 2011). Although the full mechanism of action for bee defensin-1 has not been elucidated, defensin proteins from other species have been shown to create a pore within the bacterial cell membrane, resulting in cell death (Ganz, 2003). Furthermore, bee defensin-1 has been shown to be important in the role of wound healing, through stimulation of MMP-9 secretions from keratinocytes (Bucekova *et al.*, 2017). Therefore, honey containing high levels of bee defensin-1 would be good candidates for medicinal honey.

1.2.3.4 1,2-dicarbonyls

Antimicrobial activity observed in honey with low levels of hydrogen peroxide, or after the removal of hydrogen peroxide, are defined as honey's with non-peroxide activity. The non-peroxide activity of honey has been attributed to a variety of different substances, one of which is a group of compounds known as 1,2-dicarbonyls. The 1,2-dicarbonyls are highly reactive compounds, generated in carbohydrate-rich foods through caramelization or Maillard reactions (Degen, Hellwig and Henle, 2012). These are achieved through heat treatment or prolonged storage and are associated with aroma, colour and taste (Arena *et al.*, 2011). 1,2-dicarbonyls are formed as an intermediate of a non-enzymatic reaction with glucose and free amino groups, resulting in the formation of advanced glycation end products (Schalkwijk *et al.*, 1999). Those formed by hexoses include 3-deoxyglucosone (3-DG) and glucoson; formation by disaccharides and oligosaccharides results in 3-deoxypentosone (3-DP) (Degen, Hellwig and Henle, 2012). Breakdown products of 3-DG result in the generation of 5-hydroxymethalfurfural, indicating honey freshness (Laallam *et al.*, 2015). Other breakdown products of antimicrobial significance are methylglyoxal and glyoxal.

Methylglyoxal (MGO) has been identified as the main antimicrobial component of manuka honey (Adams, Manley-Harris and Molan, 2009). The MGO content of Manuka honey has been directly correlated to the MGO rating or 'Unique Manuka Factor' rating, indicating the amount of MGO present within the manuka honey (Johnston *et al.*, 2018). The presence of MGO in manuka honey is determined by the concentration of dihydroxyacetone (DHA). The nectar of the manuka flower contains varying levels of DHA and no measurable MGO

(Adams, Manley-Harris and Molan, 2009). Further investigation into the presence of DHA and MGO revealed that upon addition of DHA to clover honey, derived from Trifoluim species, MGO was later observed. Furthermore, the addition of arginine and lysine resulted in greater production of MGO, consistent with findings that the non-enzymatic production of MGO requires these amino acids (Majtan *et al.*, 2012).

Once the flower nectar has been harvested by the bee and stored within the hive, low levels of MGO can be detected, but high levels of DHA are present. Upon honey harvest, the conversion of DHA into MGO takes place, resulting in increased MGO levels and a reduction in DHA (Grainger, Manley-harris, *et al.*, 2016a). Interestingly, heating of the honey to 37 °C results in increased MGO, however, heating to 50 °C causes a loss of both MGO and DHA (Adams, Manley-Harris and Molan, 2009). It has been observed that MGO has the ability to alter the structure of bacterial fimbriae and flagella, as well as causing damage to the cell membrane resulting in shrinking and rounding of the bacterial cell (Rabie *et al.*, 2016).

1.2.8 Antimicrobial activity

The efficacy of honey against a variety of microorganisms is dependent on the honey used, due to variations in botanical origin, bee health, geographical location and the processing of honey (Sherlock *et al.*, 2010; El Sohaimy, Masry and Shehata, 2015; Cokcetin *et al.*, 2016). This is due to the influence these factors have upon the chemical makeup of the honey and therefore, the antimicrobial potential. This results in honey having a broad spectrum of activity, being able to inhibit a variety of both Gram positive and Gram negative organisms, with some honey samples having a larger inhibitory impact on Gram positive bacteria and others having a greater inhibitory impact on Gram negative bacteria (Table 1.2).

Table 1.2 Geographical variation in honey's antimicrobial activity. The minimum inhibitory concentrations indicate how geographical location and botanical source can result in differing inhibition of microorganisms,

Geographical Variation in Honey's Antimicrobial Activity		
Country of Origin	Honey Sample	Organisms and Antimicrobial Activity
Australia		
New Zealand (Deng *et al.*, 2018)	Manuka	*Staphylococcus aureus* (zone of inhibition 25 mm with 10 % honey), *Pseudomonas aeruginosa* (zone of inhibition 8 mm with 10 % honey)
New Zealand (Girma, Seo and She, 2019)	Manuka	*S. aureus* (MIC range of <5 to >15 %), MRSA (MIC range of <5 to >15 %), MSSA (MIC range of <5 to >15 %), coagulase-negative *Staphylococcus epidermidis* (MIC range of <5 to >15 %), *P. aeruginosa* (<9 to >27 %), ESBL *E. coli* (21 to 33%)
North America		
Canada (Brudzynski, 2006)	Canadian Honey	*E. coli* (MIC range of 6.25 % to 25%), *Bacillus subtilis* (MIC range of 12.5% to 50%)
Cuba (Alvarez-suarez *et al.*, 2010)	Christmas vine, Morning glory, Black mangrove, Linen vine, Singing bean	*S. aureus* (minimum active dilution range of 2.5 % to 5%), *P. aeruginosa* (minimum active dilution range of 7.2 % to 16%), *E. coli* (minimum active dilution range of 4.7 % to 12.5 %) and *B. subtilis* (minimum active dilution range of 4.7 % to 10.4 %)
South America		
Chile (Sherlock *et al.*, 2010)	Ulmo Honey	MRSA (MIC range of 3.1 % to 6.5 %), *E. coli* (MIC 12.5%) and *P. aeruginosa* (MIC 12.5 %)
Argentina (Isla *et al.*, 2011)	Algarrobo, citrus and multifloral honey	*S. aureus* (MIC range of 0.1 to 0.2 g/mL), *Enterococcus faecalis* (MIC range of 0.2 g/mL to 0.25 g/mL), *E. coli* (MIC range of 0.1 g/mL to 0.2 g/mL), *Morganella morganii* (MIC range of 0.1 g/mL to 0.2 g/mL)and *P. aeruginosa* (MIC range of 0. g/mL to 0.15 g/mL)
Europe		
Scotland (Fyfe *et al.*, 2017)	Blossom, heather, Highland, Portobello Orchard	*Acinetobactor calcoaceticus* (75 % honey reduced growth from 10 $\log_{10}$ CFU/mL to 4 $\log_{10}$ CFU/mL or less), *S. aureus* (75 % honey reduced growth from 10 $\log_{10}$ CFU/mL to 6 $\log_{10}$ CFU/mL or less), *P.*

		aeruginosa (75 % honey reduced growth from 10 $\log_{10}$ CFU/mL to 6.2 $\log_{10}$ CFU/mL or less) and *E. coli* (75 % honey reduced growth from 10 $\log_{10}$ CFU/mL to 5.8 $\log_{10}$ CFU/mL or less)
Northwest Spain (Escuredo *et al.*, 2012)	Rubus Honey	*S. aureus* (MIC range of 62.5 mg/mL to 125 mg/mL), *S. epidermidis* (MIC range of 31.3 mg/mL to 125 mg/mL), *Micrococcus luteus* (MIC range of 15.6 mg/mL to 62.5 mg/mL), *E. faecalis* (MIC range of 15.6 mg/mL to 31.3 mg/mL), *B. cereus* (MIC range of 7.8 mg/mL to 31.3 mg/mL), *Proteus mirabilis* (MIC range of 7.8 mg/mL to 31.3 mg/mL), *E. coli* (MIC range of 15.6 mg/mL to 31.3 mg/mL), *P. aeruginosa* (MIC range of 15.6 mg/mL to 31.3 mg/mL) and *Salmonella. typhimurium* (MIC range of 15.6 to 31.3 mg/mL)
Denmark (Matzen *et al.*, 2018)	Heather, Raspberry, Rapeseed, Hawthorn and White Clover	*S. aureus* (zone of inhibition 0.6 cm to 1.2 cm), *P. aeruginosa* (zone of inhibition 0.6 cm to 0 cm) and *E. coli* (zone of inhibition 0.6 cm to 0 cm)
Slovakia (Bucekova *et al.*, 2019)	Honeydew Honey	*P. aeruginosa* (MIC range of 8 % to 40 %) and *S. aureus* (MIC range of 6 % to 45 %)
Asia		
China (Deng *et al.*, 2018)	Buckwheat Honey	*S. aureus* (zone of inhibition 25 mm with 10 % honey) and *P. aeruginosa* (zone of inhibition 8 mm with 10 % honey)
Africa		
Algeria (Laallam *et al.*, 2015)	Astragalus, Wall-rocket, Eucalyptus, Legume, Peach, Juniper, Buckthorn and multifloral	*Clostridium perfringens* (zones of inhibition range of 0 mm to 20 mm), *S. aureus* (zones of inhibition range of 6 mm to 10.5 mm), *E. coli* (zones of inhibition range of 11.6 mm to 15 mm) and *B. subtilis* (zones of inhibition range of 0 mm to 20 mm)
Nigeria (John-isa, Adebolu and Oyetayo, 2019)	Wildflower and Bitter leaf Honey	*S. typhimurium* (zone of inhibition range of 10 mm to 23.67 mm), *Shigella dysenteriae* (zone of inhibition range of 10 mm to 16.3 mm), *E. coli* (zone of inhibition range of 10 mm to 22.67 mm), *B. cereus* (zone of inhibition range of 0 mm to 20.33

		mm) and *S. aureus* (zone of inhibition range of 10 mm to 21 mm)
Egypt (El-Borai *et al.*, 2018)	Cotton, Blackseed, Orange, Eucalyptus, Sidr and Clover Honey	*E. coli* (MIC up to 50 %), *S. aureus* (MIC up to 100 %), *Streptococcus mutans* (MIC up to 50 %), *P. mirabilis* (MIC range of 25 % to 50 %), *P. aeruginosa* (MIC up to 50 %) and *K. pnuemoniae* (MIC range of 25 % to 50 %)

Manuka honey has been identified to inhibit the Gram positive organisms *S. aureus*, including methicillin resistant and methicillin susceptible isolates, and *Staphylococcus epidermidis*, as well as the Gram negative *P. aeruginosa, K. pneumoniae* and *E. coli* (Table 1.2) (Deng *et al.*, 2018; Cokcetin *et al.*, 2019; Girma, Seo and She, 2019). However, the susceptibility of these organisms vary and numerous studies have explored the activity, often identifying the Gram positive organisms being inhibited at lower concentrations than the Gram negative organisms. Observations of one Gram positive, *Enterococcus faecalis*, and one Gram negative, *E. coli*, identified that the Gram negative *E. coli* was more resistant to manuka honey treatment than *E. faecalis* (Kumar *et al.*, 2014). Furthermore, in a larger study observing the efficacy of 3 manuka honey's with varying MGO rating against a wide range of bacterial pathogens, the lowest minimum inhibitory concentration (MIC) was observed for the Gram positive *S. aureus* (Girma, Seo and She, 2019). Similar results of linen vine honey, void of MGO, showed *S. aureus* was more susceptible to honey treatment than *P. aeruginosa* (Alvarez-suarez *et al.*, 2010). Another study observing the effectiveness of honey from a variety of botanical origins identified greater susceptibility overall towards the Gram positive organisms, *S. aureus* and *S. epidermidis*, and either no effect or reduced susceptibility to the Gram negative organisms, *E. coli* and *P. aeruginosa* (Matzen *et al.*, 2018). Further to this, observations of the antimicrobial activity of Polish honey against *S. aureus* found an MIC of only 1.56% (*v/v*) of honey was required (Grecka *et al.*, 2018). However, other studies have demonstrated that Gram positive bacteria are more resistant to honey (Isla *et al.*, 2011; Escuredo *et al.*, 2012; Fyfe *et al.*, 2017). One study identified that Gram negative organisms were more susceptible to honey than Gram positives, suggesting this could be due to the higher hydrogen peroxide content and osmolarity of the samples (Mohapatra, Thakur and Brar, 2011). In regards to Rubus honey, from Southwest Spain, *Proteus mirabilis* was the most susceptible organism tested, exhibiting an MIC range of 7.8 to 31.3 mg/mL, yet *S. aureus* had an MIC range of to 125 mg/mL (Escuredo *et al.*, 2012). Further to this, honey's of both monofloral origin (algarrobo and citrus) and mulitfloral origin exhibited greater efficacy against the Gram negative organisms than the Gram positive organisms, with *P. aeruginosa* having an MIC of 100 mg/mL, whereas *S. aureus* MIC was 250 mg/mL and *E. feacalis* ranged from 200 to 250 mg/mL with some honey samples having

no effect on the Gram positive organisms tested (Isla *et al.*, 2011). Moreover, a study observing the effect of Egyptian honey identified the only effective honey against *S. aureus* was Sidr honey at an MIC of 100% and only 4 out of 6 honey samples were effective against *Streptococcus mutans*. All honey samples tested were effective against *P. mirabilis* and *K. pneumoniae* with MIC values of 50% or less. Only one honey was not effective against *E. coli* and three out of six were not effective against *P. aeruginosa,* but the MIC values for those that were inhibitory were 50% or less (El-Borai *et al.*, 2018). Furthermore, it has been identified that *Acinetobactor calcoaceticus* was the most affected organism, compared to *E. coli, P. aeruginosa* and *S, aureus,* when treated with a range of Scottish honey samples (Fyfe *et al.*, 2017). This variety of results suggests that not all honeys are equal and their effectiveness is largely variable, further outlining the significance of botanical origin and geographical location on the antimicrobial activity exhibited by a specific honey.

1.2.9 Antibiotic Synergy

Observing the broad spectrum of activity exhibited by honey, especially against drug-resistant organisms, has led to investigations of honey–antibiotic synergistic relationships. A variety of antibiotics and honey combinations have now been explored, with some promising results. The pairing of Manuka honey with tetracycline exhibited an increased antimicrobial effect against *P. aeruginosa* and *S. aureus*. The broad spectrum activity of tetracycline, and the increase in its activity upon addition of manuka honey, make the combination a strong candidate for wound healing (Jenkins and Cooper, 2012). Another combination, in which sub-inhibitory concentrations of Medihoney, a medical grade manuka based honey, were used alongside rifampicin, showed synergistic activity against *S. aureus* and also appeared to prevent rifampicin resistance (Müller *et al.*, 2013). This is not the first instance of honey reversing resistance to antibiotics. Jenkins and Cooper (2012) identified that sub-inhibitory concentrations of honey, with the addition of oxacillin, restored the susceptibility of MRSA to oxacillin (Rowena. Jenkins and Cooper, 2012). These previous findings provide a strong basis for the use of honey in a clinical setting, especially for persistent or chronic infections. Additionally, combinations of honey and antibiotics have been shown to have synergistic and additive actions against biofilms. This was demonstrated by the combination of vancomycin with manuka honey against *S. aureus,* and gentamicin with manuka honey against *P. aeruginosa* (Campeau and Patel, 2014). Furthermore, one study has observed the synergistic effects of Portuguese honey and phage therapy, identifying that 25% (*w/v*) honey paired with phage was equally as effective in *E.* coli biofilm destruction as 50% (*w/v*) honey alone (Oliveira *et al.*, 2017). This highlights the exciting potential and possibilities of the use of honey, and the need for further research into its synergistic effects and clinical applications.

1.2.10 Honey in Medical Settings

The main applications of honey within a medical setting are for the treatment of surface wounds and burns. Several distinct types of honey have been developed into medical grade honey, including: Medihoney, Revamil and Surgihoney. The activity of these honey's are based on the different antimicrobial components of honey. Medihoney has been developed from Manuka honey with activity based around MGO, whereas Revamil honey is produced in greenhouses under standardised conditions with bee defensin-1 as the main component (Kwakman *et al.*, 2011; Carter *et al.*, 2016). However, the activity of Surgihoney has been based primarily on enhanced reactive oxygen species activity (Cooke *et al.*, 2015).

Medical honey can be applied directly to the surface of a wound as a balm, this provides a physical barrier between the wound and the environment, preventing contamination (Molan, 2002). The secondary effects provided by application are the antimicrobial properties, including both bacteriostatic and bactericidal activity, further preventing wound contamination (Kwakman *et al.*, 2011). Additionally, an osmotic gradient is generated due to the high sugar content and low water activity, generating a flow of bacteria, necrotic tissue and debris out of the wound (Minden-Birkenmaier and Bowlin, 2018). Finally, the phenolic content in honey aids in inflammation, helping to improve wound healing (Samarghandian, Farkhondeh and Samini, 2017). Overall, this has been observed to improve both the healing of the wound, and the time taken to heal and reduce scarring as well as reducing infection at the wound site (Molan, 2006). This can reduce the use of antibiotics, while still aiding wound treatment.

The use of honey to treat wounds has identified that daily treatment with manuka honey showed re-epithelisation of the wound with full healing in 6 weeks, highlighting the ability of honey to promote angiogenesis (Dunford *et al.*, 2000). Another medical use of manuka honey was to treat a haematoma that had become infected with *P. aeruginosa* and *S. aureus* that had failed to heal. Upon treatment with manuka honey the infection was cleared and healed within 8 weeks (Dunford *et al.*, 2000). Another study explored the use of honey to aid the healing of skin grafts identifying increased healing and reduced pain in comparison to the vaseline control (Subrahmanyam, 2015). Additionally, honey can be used to heal burns. In a study observing the effects of honey and 1% silver sulfadiazine, they found that honey reduced the healing time and cleared the burn of infection and pain quicker than the 1% silver sulfadiazine (Mashhood, Khan and Sami, 2006). Interestingly, another application of honey, not relating to antimicrobial activity, is in a nebulised form to alleviate the symptoms of asthma (Kamaruzaman *et al.*, 2014). These case studies outline the different uses of honey within a clinical setting, outlining that honey can be implemented in a variety of wound healing applications, not only to prevent infection, but also to reduce healing times and patient discomfort.

1.3 Aims and Objectives

The first aim of this study is to explore the possibility of manuka honey as an antimicrobial against *M. abscessus* and a panel of clinical isolates. This will be achieved through broth microdilution assays with subsequent transfer to solid media for assessment of bactericidal activity. An exploration into storage times and conditions will also be explored to determine any impacts these may have on the antimicrobial activity.

The second aim of this study is to investigate the possible relationship between manuka honey and 3 antibiotics against *M. abscessus*. The antibiotics selected are currently used for nebulisation therapy for *M. abscessus* pulmonary infections. The relationship between manuka honey and antibiotics will be further developed into an *in vitro* nebulisation assay as potential new therapy reducing the antibiotic burden on patients by the addition of manuka honey.

The third aim will explore the role of the main components present within honey on their antimicrobial activity against *M. abscessus*. This will be achieved through assays detecting the components, broth microdilution testing of the components alone and in combination and removal of the components and re-examining antimicrobial activity. A vegan honea will also be used to determine the impact of indirect mechanisms, such as high sugar content and low pH, as well as modifying the vegan honea with the precursors of the major components and exploring this impact on antimicrobial activity.

Lastly, an exploration into novel antimicrobial compounds will be conducted. This will include testing a range of bacterial pathogens to assess antimicrobial activity.

Chapter 2: Assessment of the antimicrobial activity of manuka honey against *Mycobacterium abscessus*

2.1 Introduction

2.1.1 *M. abscessus*

Mycobacterium abscessus is a highly drug resistant opportunistic pathogen, commonly isolated from the environment (Johansen, Herrmann and Kremer, 2020). Established as an opportunistic pathogen it causes skin and soft tissue infections as well as pulmonary infections, and has increasing prevalence worldwide (Osmani *et al.*, 2018). *M. abscessus* infections of skin and soft tissue are often observed in individuals with open wounds, such as those who have undergone cosmetic surgery with contaminated equipment and those with new tattoos, as well as new piercing sites and injection sites (Lee *et al.*, 2015; Desai and Hurtado, 2018). Pulmonary infections caused by *M. abscessus* are often in individuals with pre-existing respiratory conditions such as cystic fibrosis or bronchiectasis (Vande Weygaerde *et al.*, 2019). The highly drug resistant nature of *M. abscessus* has resulted in infections being notoriously difficult to treat, with lengthy drug regimens that patients often do not complete (Haworth *et al.*, 2017). The lack of antibiotics and treatments for these infections has resulted in the requirement for new and novel treatments. One area that has not yet been explored in the fight against *M. abscessus* is the use of manuka honey, a well-established antimicrobial agent.

2.1.2 Manuka honey

The antimicrobial activity of honey has been well established and documented since the 1940s (Abdalla, Elbassiony and Mahfouz, 2021). Manuka honey in particular has gained increased interest due to its broad spectrum of activity against a range of bacterial pathogens. The microorganisms thoroughly explored include *E. coli, S. aureus* and *P. aeruginosa.* Typically the minimum inhibitory concentrations required range from <5% to 15% for *S. aureus*, between 10% and 35% for *P. aeruginosa* and 5% to 30% for *E. coli* with other bacterial isolates being less than 30% (Nolan *et al.*, 2020). This improved efficacy over other honey has been attributed to the presence of methylglyoxal and it has become a desirable alternative to persistent infections that are unresponsive to antibiotic treatments (Dunford *et al.*, 2000). Furthermore, it has been noted that resistance to antibiotics does not impede honey activity, and honey treatment can be more effective in drug resistant microorganisms than their non-drug resistant counterparts (Nolan *et al.*, 2020). Additionally, to date there has been no recorded instance of resistance to honey therapy, further highlighting its potential (Cooper *et al.*, 2010). Although a vast majority of clinically relevant pathogens has been reported to be susceptible to honey therapy, one genus that has not yet been explored is mycobacteria. This is in part due to a report in 1988 that *Mycobacterium ulcerans* was unresponsive to honey therapy in a patient with a persistent infection (Efem, 1988). Which led to the belief that honey is ineffective against all mycobacterial species (Dunford *et al.*, 2000). However, without thorough investigation into honey as an antimycobacterial agent, this conclusion is premature. Therefore, this chapter aims to establish if manuka honey could successfully inhibit *M. abscessus* and provide a basis for the use of manuka honey in treating mycobacterial infections.

2.1.3 Aims and Objectives

Initially, the antimicrobial efficacy of manuka honey against *M. abscessus* and clinical isolates will be determined. This will be achieved using the broth microdilution assay to assess growth inhibition, coupled with determination of bactericidal activity by plating onto solid media. Then, the impact of culture temperature upon the efficacy of manuka honey against *M. abscessus* and clinical isolates will be assessed as described above. Lastly, the storage conditions and length of storage of manuka honey in regard to its antimicrobial activity will be assessed against *M. abscessus* and clinical isolates.

2.2 Materials and Methods

2.2.1 Media Preparation

All chemicals and reagents were purchased from Sigma-Aldrich or Melford, unless otherwise stated. For growth of *Mycobacterium abscessus* cultures, Middlebrook 7H9 broth and Middlebrook 7H11 agar were selected. The 7H9 broth was made by adding 2.35 g of 7H9 to 450 mL of distilled H_2O and supplemented with 4 mL of 50% (w/v) glycerol before autoclaving (121 °C for 15 min). Once cooled, 1.25 mL of 20% (w/v) filter sterile Tween80 was added (final concentration of 0.055%). The 7H11 agar was made by adding 10.25 g of 7H11 to 450 mL distilled H_2O and supplemented with 5 mL 50% (w/v) glycerol before autoclaving (121 °C for 15 min). The agar was subsequently poured into petri dishes.

2.2.2 *M. abscessus* strains and culture

The *M. abscessus* strains used were the type strain NCTC 13031 (also called ATCC 19977) and a panel of 16 clinical isolates obtained from Brighton and Sussex Medical School, isolated from patients with *M. abscessus* pulmonary infection. The antibiotic susceptibility of the clinical isolates can be seen in Appendix 8.1.1. All organisms were grown for 72 h in 7H9 broth before being stored in 25% (w/v) glycerol stock solutions and stored at -80 °C. Prior to testing, *M. abscessus* isolates were grown in 10 mL 7H9 broth, prepared as described in 2.2.1, for 72 h at either 30 °C or 37 °C, depending on the experiment, with orbital shaking at 180 rpm.

2.2.3 Honey sample storage and preparation

The 4 manuka honey samples selected for this study were all of differing MGO concentrations. The lowest grade selected was MGO40 (Manuka Doctor, UK), followed by MGO55 (ManukaPharm, UK), MGO70 (Manuka Doctor, UK) and the highest grade selected was MGO83 (Comvita, UK). The MGO rating determines that each manuka honey will have certain levels of MGO, for example the MGO40 will contain 40 mg/kg MGO. All honey jars were stored in the dark at room temperature prior to testing and not used beyond any use by dates. For each experiment honey stocks were made up to 1 g/mL in distilled H_2O (w/v) and filter sterilised in a two-step filtration process using 0.8 µm filter and 0.22 µm filter (Sartorius), unless otherwise stated. No further sterility testing was conducted, such as testing for *Bacillus* spores.

2.2.4 Broth microdilution of manuka honey with *M. abscessus* culture at 30 °C

The broth microdilution assay was used to screen the honey samples for antimicrobial activity against *M. abscessus* NCTC 13031 and clinical isolates cultured at 30 °C. Initially, 100 µL of 7H9 broth, prepared as described in 2.2.1, was added to all experimental wells, this was rows A to F and columns 1 to 8 (Figure 2.1). Then 100 µL of manuka honey MGO40, prepared as described in 2.2.3, was added to row A1-A4 and manuka honey MGO55 was added to row A5-A8, mixed and serially diluted down the plate. This was

repeated for MGO70 and MGO83 respectively, on subsequent plates. On row F, the excess 100 µL was removed and discarded. The plates were inoculated with 5 µL of optical density (OD) $_{600\ nm}$=0.1 *M. abscessus* culture, prepared as described in 2.2.2, down columns 1-3 and 5-7. The two control columns, column 11 and 12, (Figure 2.1) had 100 µL of 7H9 broth added and the *M. abscessus* only control (row 11) was inoculated with 5 µL of $OD_{600\ nm}$=0.1 *M. abscessus* cells. Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 30 °C for a total of 96 h, with OD $_{570\ nm}$ reads every 24 h. After 96 h, the final OD $_{570\ nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using a 1 µL loop and spreading onto 7H11 agar plates, prepared as described in 2.2.1. The agar plates were incubated for a further 72 h at 30 °C and the minimum bactericidal concentration (MBC) was determined as the minimum concentration where no bacterial growth was visually observed.

	MGO40				MGO55							
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL			*M. abscessus*	Broth
B	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL			*M. abscessus*	Broth
C	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL			*M. abscessus*	Broth
D	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL			*M. abscessus*	Broth
E	0.02975 g/mL *M. abscessus*	0.02975 g/mL *M. abscessus*	0.02975 g/mL *M. abscessus*	0.02975 g/mL	0.02975 g/mL *M. abscessus*	0.02975 g/mL *M. abscessus*	0.02975 g/mL *M. abscessus*	0.02975 g/mL			*M. abscessus*	Broth
F	0.0148 g/mL *M. abscessus*	0.0148 g/mL *M. abscessus*	0.0148 g/mL *M. abscessus*	0.0148 g/mL	0.0148 g/mL *M. abscessus*	0.0148 g/mL *M. abscessus*	0.0148 g/mL *M. abscessus*	0.0148 g/mL			*M. abscessus*	Broth
G												
H												

Figure 2.1 Plate map of manuka honey broth microdilution with *M. abscessus* culture at 30 °C. The broth microdilution was prepared by adding 100 µL of 7H9 broth to all experimental wells followed by 100 µL manuka honey from a stock concentration of 1 g//mL (w/v) to wells A1 to A8, with MGO40 being added to wells A1 to A4 and MGO55 being added to A5 to A8. This was serially diluted from row A to row F. Columns 1, 2, 3, 5, 6, 7 and 11 were inoculated with OD adjusted culture of *M. abscessus*. Columns 4, 8 and 12 were used as controls and therefore not inoculated.

2.2.5 Broth microdilution of manuka honey with *M. abscessus* culture at 37 °C

The antimicrobial activity of manuka honey against *M. abscessus* NCTC 13031 and clinical isolates was also tested at 37 °C using the broth microdilution method, with modifications to both culture temperature and plate layout. In brief, 100 µL of 7H9 broth, prepared as described in 2.2.1, was added to all wells of the 96 well plate. Then 100 µL of manuka honey MGO40, prepared as described in 2.2.3, was added to row A1-A6 and manuka honey MGO55 was added to row A7-A12, mixed and serially diluted down the plate (Figure 2.2). This was repeated for MGO70 and MGO83 respectively, on subsequent plates. On row G, the excess 100 µL was removed and discarded. The plates were inoculated with 5 µL of optical density (OD) $_{600\ nm}$=0.1 *M. abscessus* culture, prepared as described in 2.2.2, down columns 1-3 and 7-9. This included control wells of H1-3 and H7-9 for *M. abscessus* only and H4-6 and H10-12 for 7H9 broth only. Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C for a total of 96 h, with OD $_{570\ nm}$ reads every 24 h. After 96 h, the final OD $_{570\ nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using 5 µL aliquots and spotting onto 7H11 agar plates, prepared as described in 2.2.1. The agar plates were incubated for a further 72 h at 37 °C and the minimum bactericidal concentration (MBC) was determined as the minimum concentration where no bacterial growth was visually observed.

	MGO40						MGO55					
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL	0.476 g/mL	0.476 g/mL	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL	0.476 g/mL	0.476 g/mL
B	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL	0.238 g/mL	0.238 g/mL	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL	0.238 g/mL	0.238 g/mL
C	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL	0.119 g/mL	0.119 g/mL	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL	0.119 g/mL	0.119 g/mL
D	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL	0.0595 g/mL	0.0595 g/mL	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL	0.0595 g/mL	0.0595 g/mL
E	0.0297 g/mL *M. abscessus*	0.0297 g/mL *M. abscessus*	0.0297 g/mL *M. abscessus*	0.0297 g/mL	0.0297 g/mL	0.0297 g/mL	0.0297 g/mL *M. abscessus*	0.0297 g/mL *M. abscessus*	0.0297 g/mL *M. abscessus*	0.0297 g/mL	0.0297 g/mL	0.0297 g/mL
F	0.014 g/mL *M. abscessus*	0.014 g/mL *M. abscessus*	0.014 g/mL *M. abscessus*	0.014 g/mL	0.014 g/mL	0.014 g/mL	0.014 g/mL *M. abscessus*	0.014 g/mL *M. abscessus*	0.014 g/mL *M. abscessus*	0.014 g/mL	0.014 g/mL	0.014 g/mL
G	0.007 g/mL *M. abscessus*	0.007 g/mL *M. abscessus*	0.007 g/mL *M. abscessus*	0.007 g/mL	0.007 g/mL	0.007 g/mL	0.007 g/mL *M. abscessus*	0.007 g/mL *M. abscessus*	0.007 g/mL *M. abscessus*	0.007 g/mL	0.007 g/mL	0.007 g/mL
H	*M. abscessus*	*M. abscessus*	*M. abscessus*	Broth	Broth	Broth	*M. abscessus*	*M. abscessus*	*M. abscessus*	Broth	Broth	Broth

Figure 2.2 Plate map of manuka honey broth microdilution with *M. abscessus* culture at 37 °C. The broth microdilution was prepared by adding 100 µL of 7H9 broth to all 96 wells followed by 100 µL manuka honey from a stock concentration of 1 g//mL (w/v) to wells A1 to A12, with MGO40 being added to wells A1 to A6 and MGO55 being added to well A7 to A12. This was serially diluted from row A to row F. Columns 1, 2, 3, 7, 8 and 9 were inoculated with OD adjusted culture of *M. abscessus*. Columns 4, 5, 6, 10, 11 and 12 were used as controls and therefore not inoculated.

2.2.7 Broth microdilution of manuka honey with short term storage at 37 °C

To identify if honey temperature or *M. abscessus* culture temperature impacts the efficacy of honey, a broth microdilution assay was conducted using *M. abscessus* NCTC 13031, altering these variables. Manuka honey stocks were prepared as described in section 2.2.3, with the addition of storage in the dark at 37 °C for 1 h, 2 h, 3 h, 4 h, 5 h and 6 h prior to testing. For each time point selected for testing, the broth microdilution assay was set up and conducted as described in 2.2.5 with *M. abscessus* culture grown at 30 °C and 37 °C.

2.2.9 Broth microdilution of manuka honey stored for 16 days with *M. abscessus* cultured at 37 °C

To assess if storage conditions and duration impact the efficacy of the antimicrobial activity of manuka honey, a time and temperature study was designed using *M. abscessus* NCTC 13031. All 4 of the honey samples were prepared as described in 2.2.3 and stored at 3 different temperatures, 4 °C, 20 °C and 37 °C, in the dark for a total of 16 days. To assess the difference in antimicrobial activity due to storage conditions, the broth microdilution method was used, as described in 2.2.5. The days selected for testing were: 0, 2, 4, 7, 9, 11, 14 and 16 days after the 4 manuka honey samples were made. On each day selected for testing, the broth microdilution assay was set up and conducted as described in 2.2.5.

2.2.11 Broth microdilution of manuka honey matured for 30 days with *M. abscessus* cultured at 37 °C

To determine if storage of longer than 16 days impacted the efficacy of manuka honey against *M. abscessus* NCTC 13031 and clinical isolates, a broth microdilution was conducted with honey matured for 30 days. To do this, manuka honey samples were prepared as described in section 2.2.3 with the addition of storage at 4 °C for 30 days prior to testing. The broth microdilution assay was conducted as described in 2.2.5, with *M. abscessus* culture at 37 °C.

2.2.12 Broth microdilution of manuka honey matured for 112 days, with *M. abscessus* cultured at 37 °C

To observe the effects of long term storage of the manuka honey samples, a broth microdilution assay was conducted on manuka honey after maturation for 112 days against *M. abscessus* NCTC 13031. Honey samples were prepared as described in section 2.2.3, with honey storage for 112 days prior to testing at 4 °C, 20 °C and 37 °C. The broth microdilution assay was used, prepared as described in 2.2.5, including MBC plate out.

2.2.13 Data processing and statistical analysis

All data collected were n=3 technical replicates and 2 biological replicates, the broth microdilution assays were processed in Microsoft Excel 2016 and subsequently analysed using GraphPad Prism 8. Prior to data analysis, blank control values were deducted from experimental values. The data was then analysed for normal distribution using Shapiro-Wilk test and subsequently analysed using a One-Way ANOVA. A Dunnett's multiple comparisons analysis was also conducted. For each experiment conducted, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The MIC was defined as the lowest concentration required to inhibit the growth of *M. abscessus* and the MBC was defined as the lowest concentration required for no visible growth after transfer onto solid media.

2.3 Results

2.3.1 Efficacy of manuka honey against *M. abscessus* cultured at 30 °C

M. abscessus NCTC 13031 and 16 clinical isolates were treated with 4 different manuka honey samples in a broth microdilution assay (section 2.2.4) and cultured at 30 °C to assess antimicrobial activity. Growth curves were generated for *M. abscessus* NCTC 13031 (Figure 2.3) and MICs and MBCs determined for all mycobacterial isolates tested (Table 2.2 and 2.3). Inhibition of growth was observed for all mycobacterial isolates when treated with manuka honey, regardless of MGO strength.

All 4 of the manuka honey samples tested were inhibitory against *M. abscessus* NCTC 13031 at the highest concentration of 0.476 g/mL (Figure 2.3). The lower concentrations of 0.238 g/mL and 0.119 g/mL also reduced the growth of *M. abscessus* compared to the control of no honey treatment but did not fully inhibit growth (Figure 2.3). End point data, taken at 96 h, had normal distribution and was analysed using a one-way ANOVA. The one-way ANOVA identified a statistical significance between the treatments for all 4 honey's, P=<0.0001 (Figure 2.3). A Dunnett's multiple comparison was then conducted, comparing the growth of *M. abscessus* alone with the growth of *M. abscessus* at each concentration of honey (Table 2.1). Regardless of honey, or concentration, a significant difference was observed between the growth of *M. abscessus* with the addition of manuka honey and *M. abscessus* alone, P=0.0003 or less (Table 2.1).

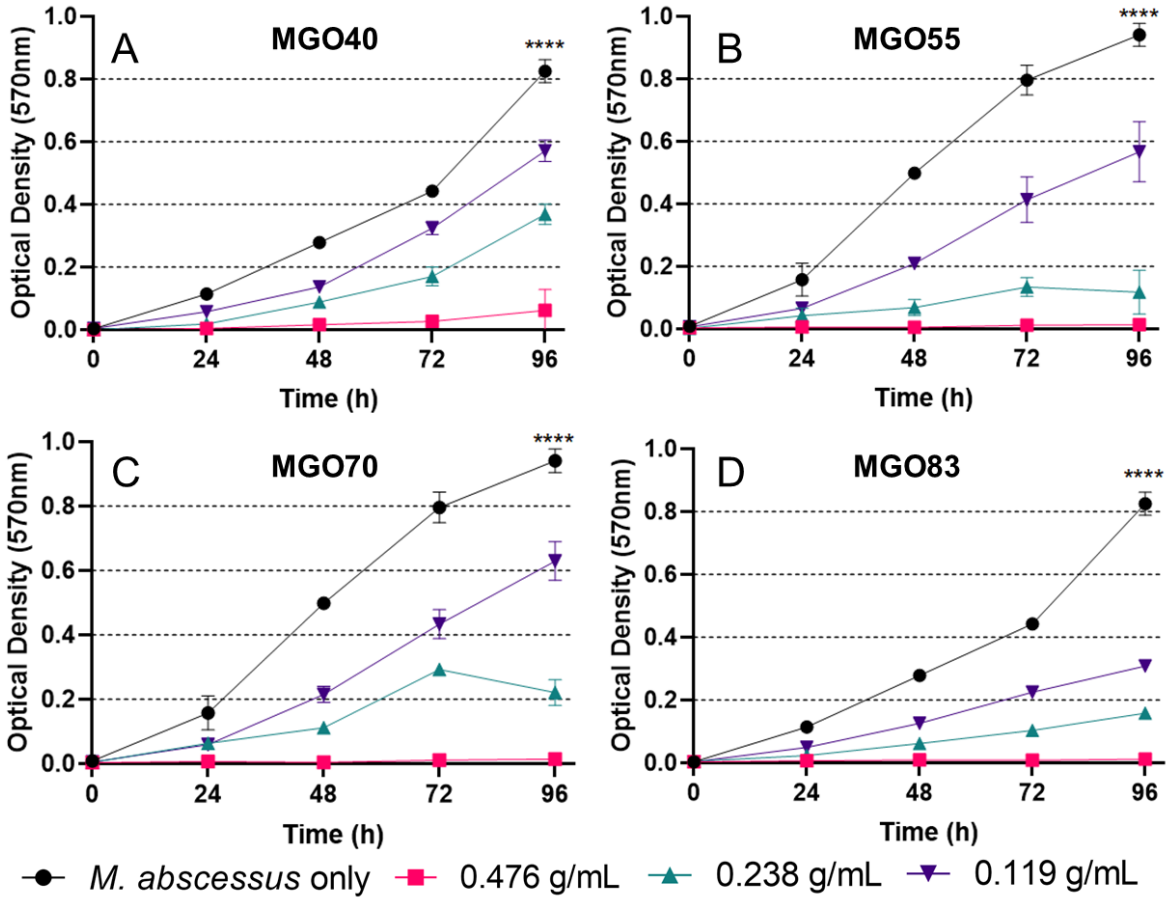

Figure 2.3 Growth Curves of *M. abscessus* NCTC 13031, cultured at 30 °C treated with 4 different manuka honeys. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honey samples inhibited *M. abscessus* at 0.476 g/mL, with a reduction in growth for 0.238 g/mL and 0.119 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a significant difference was observed for all honey treatments P=<0.0001. C) MIC of 0.476 g/mL for MGO70. A significant difference was observed between all honey treatments, P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, P=<0.0001.

Table 2.1 *Post-hoc* analysis of end point data, taken at 96 h, comparing all manuka honey treatments to the growth of *M. abscessus* alone at 30 °C. The Dunnett's multiple comparison P values are shown for all 4 manuka honey samples at the concentrations tested.

	Honey Concentration		
	0.476 g/mL	**0.238 g/mL**	**0.119 g/mL**
MGO40	<0.0001	<0.0001	0.0003
MGO55	<0.0001	<0.0001	0.0002
MGO70	0.0001	0.0001	0.0001
MGO83	<0.0001	<0.0001	<0.0001

All of the clinical isolates tested were inhibited by all 4 manuka honey samples, with some variation in MIC (Table 2.2). The MIC observed for MGO40 and MGO83 was 0.476 g/mL for all the clinical isolates tested. MICs observed for MGO70 were 0.476 g/mL for 15 of the clinical isolates, with isolate 186144 having a lower MIC of 0.238 g/mL. MGO55 exhibited the largest range in MIC with 13 of the isolates having a lower MIC of 0.238 g/mL and only 5 isolates inhibited at the higher concentration of 0.476 g/mL (Table 2.2). All raw data for the clinical isolates can be seen in Appendix 8.1.2, Figures 8.1 to 8.16.

Table 2.2 Minimum inhibitory concentrations for *M. abscessus* clinical isolates cultured at 30 °C treated with manuka honey (n=3)

30°C	Minimum Inhibitory Concentration (g/mL)			
***M. abscessus* strain**	**MGO40**	**MGO55**	**MGO70**	**MGO83**
NCTC 13031	0.476	0.476	0.476	0.476
137071	0.476	0.238	0.476	0.476
147028	0.476	0.238	0.476	0.476
159544	0.476	0.238	0.476	0.476
186144	0.476	0.238	0.238	0.476
186154	0.476	0.238	0.476	0.476
186433	0.476	0.238	0.476	0.476
189961	0.476	0.238	0.476	0.476
194891	0.476	0.238	0.476	0.476
199277	0.476	0.238	0.476	0.476
211666	0.476	0.238	0.476	0.476
DC088A	0.476	0.238	0.476	0.476
DC088B	0.476	0.238	0.476	0.476
DC088C	0.476	0.476	0.476	0.476
DC088D	0.476	0.238	0.476	0.476
DC088E	0.476	0.476	0.476	0.476
DC088ref	0.476	0.476	0.476	0.476

The bactericidal activity of the manuka honey against *M. abscessus* NCTC 13031 and clinical isolates was also determined. Overall a variation in MBC was observed. For *M. abscessus* NCTC 13031 only 2 of the honey samples were bactericidal at the concentrations tested, MGO55 and MGO70. No MBC was determined at the concentrations tested for MGO40 and MGO83 (Table 2.3). Out of the clinical isolates tested, 6 had an MBC for all of the manuka honey samples explored (Table 2.3). MGO55 exhibited improved bactericidal activity over the other honey's, with an MBC of 0.476 g/mL for all of the clinical isolates. MGO70 had an MBC of 0.476 g/mL for 14 of the clinical isolates. Both MGO40 and MGO83 had MBCs of 0.476 g/mL for the same 7 clinical isolates and no MBC observed for the rest of the clinical isolates tested (Table 2.3).

Table 2.3 Minimum bactericidal concentrations for *M. abscessus* clinical isolates cultured at 30 °C treated with manuka honey (n=3)

30°C	Minimum Bactericidal Concentration (g/mL)			
***M. abscessus* strain**	**MGO40**	**MGO55**	**MGO70**	**MGO83**
NCTC 13031	>0.476	0.476	0.476	>0.476
137071	>0.476	0.476	0.476	>0.476
147028	>0.476	0.476	0.476	>0.476
159544	>0.476	0.476	0.476	>0.476
186144	0.476	0.476	>0.476	0.476
186154	>0.476	0.476	0.476	>0.476
186433	>0.476	0.476	0.476	>0.476
189961	0.476	0.476	0.476	0.476
194891	>0.476	0.476	>0.476	>0.476
199277	0.476	0.476	0.476	0.476
211666	0.476	0.476	0.476	0.476
DC088A	0.476	0.476	0.476	0.476
DC088B	0.476	0.476	0.476	0.476
DC088C	>0.476	0.476	0.476	>0.476
DC088D	0.476	0.476	0.476	0.476
DC088E	>0.476	0.476	0.476	>0.476
DC088ref	>0.476	0.476	0.476	>0.476

2.3.2 Efficacy of manuka honey against *M. abscessus* cultured at 37 °C

To determine if culture temperature would impact the efficacy of manuka honey against *M. abscessus*, the broth microdilution assay was repeated with culturing at 37 °C (section 2.2.5). All 4 of the manuka honey samples inhibited *M. abscessus* NCTC 13031 at 0.476 g/mL, with a reduction in growth for 0.238 g/mL and 0.119 g/mL (Figure 2.4). Growth curves were generated for *M. abscessus* NCTC 13031 (Figure 2.4) and MICs and MBCs determined for all mycobacterial isolates tested (Table 2.5 and 2.6). Inhibition of growth was observed for all mycobacterial isolates when treated with manuka honey, regardless of MGO strength.

The highest concentration of manuka honey, 0.476 g/mL was inhibitory against *M. abscessus* NCTC 13031 for all 4 honey samples tested (Figure 2.4). The lower concentration of 0.238 g/mL also reduced the growth of *M. abscessus* compared to the control. The concentration of 0.119 g/mL reduced the growth of *M. abscessus* treated with MGO40 and MGO55, almost no inhibition was observed at 0.119 g/mL for MGO70 and no growth inhibition was observed for MGO83 at 0.119 g/mL (Figure 2.4). End point data, taken at 96 h, had normal distribution and was analysed using a one-way ANOVA (n=3). The one-way ANOVA identified a statistical significance between the treatments for all 4 honey's, P=<0.0001 (Figure 2.3). A Dunnett's multiple comparison was then conducted, comparing the growth of *M. abscessus* alone with the growth of *M. abscessus* at each concentration of honey (Table 2.4). MGO40 and MGO55 had a significant difference for all concentrations tested, P=0.0004 or less. MGO70 also had a significant difference for all concentrations, P=<0.0001 at 0.476 g/mL, P=<0.0.001 at 2.38 g/mL and 0.0345 at 0.119 g/mL. MGO83 had a significant difference for 0.476 g/mL and 0.238 g/mL, P=<0.0001, but no significance was observed for 0.119 g/mL, P=0.9996 (Table 2.4).

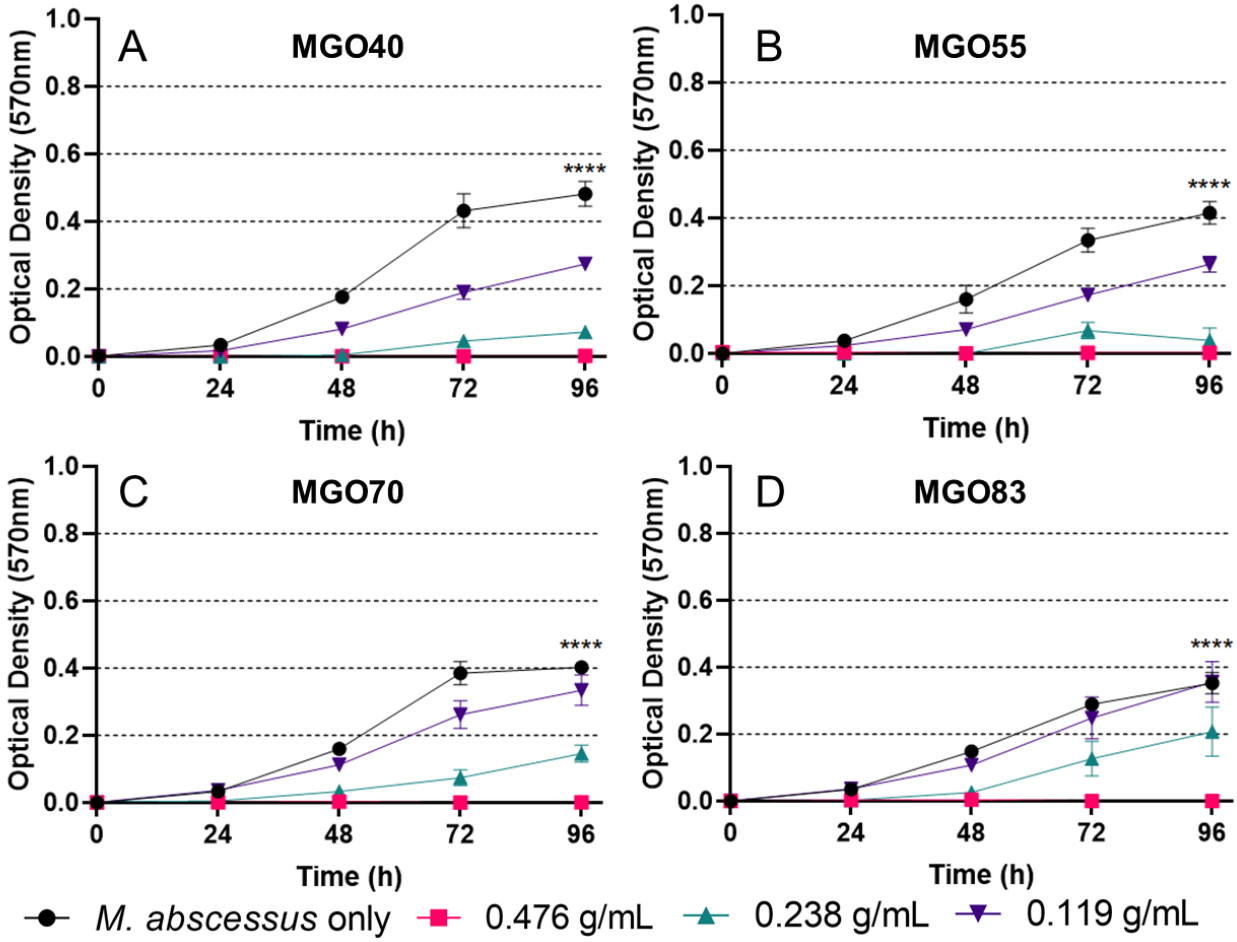

Figure 2.4 Growth Curves of *M. abscessus* NCTC 13031, cultured at 37 °C treated with 4 manuka honey sampless. Data shown are mean + SD for n=3 technical replicates All 4 manuka honeys inhibited *M. abscessus* at 0.476 g/mL, with a reduction in growth for 0.238 g/mL and 0.119 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) MGO40 growth curve. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve, a significant difference was observed for all honey treatments P=<0.0001. C) MGO70 had a significant difference between all honey treatments, P=<0.0001. D) MGO83 a significant difference was observed for honey treatments, P=<0.0001.

Table 2.4 *Post-hoc* analysis of end point data, taken at 96 h, comparing all manuka honey treatments to the growth of *M. abscessus* alone at 37 °C. The Dunnett's multiple comparison P values are shown for all 4 manuka honeys at the concentrations tested.

	Honey Concentration		
	0.476 g/mL	**0.238 g/mL**	**0.119 g/mL**
MGO40	<0.0001	<0.0001	<0.0001
MGO55	<0.0001	<0.0001	0.0004
MGO70	<0.0001	<0.0001	0.0345
MGO83	<0.0001	<0.0001	0.9996

A variation in MICs was observed for all 16 clinical isolates tested at 37 °C, with the majority being inhibited at 0.476 g/mL (Table 2.5). Only one isolate, 186154, had an MIC of 0.238 g/mL for all honey samples tested and only 2 isolates, 186433 and 194891 had an MIC of 0.238 g/mL for MGO40 and MGO55 and 186144 had an MIC of 0.238 g/mL for MGO55 and MGO70. Another isolate, DC088A had a lower MIC of 0.238 g/mL for MGO70 and MGO83. The rest of the lower MICs of 0.238 g/mL were observed in response to exposure to MGO83, the highest grade of manuka honey used in this study (Table 2.5). All raw data for the clinical isolates can be seen in Appendix 8.1.3, Figures 8.17 to 8.32.

Table 2.5 Minimum inhibitory concentrations for *M. abscessus* clinical isolates cultured at 37 °C treated with manuka honey (n=3)

37°C	Minimum Inhibitory Concentration (g/mL)			
***M. abscessus* strain**	**MGO40**	**MGO55**	**MGO70**	**MGO83**
NCTC 13031	0.476	0.476	0.476	0.476
137071	0.476	0.476	0.476	0.238
147028	0.476	0.476	0.476	0.476
159544	0.476	0.476	0.476	0.476
186144	0.476	0.238	0.238	0.476
186154	0.238	0.238	0.238	0.238
186433	0.238	0.238	0.476	0.238
189961	0.476	0.476	0.476	0.476
194891	0.238	0.238	0.476	0.476
199277	0.476	0.476	0.476	0.476
211666	0.476	0.476	0.476	0.238
DC088A	0.476	0.476	0.238	0.238
DC088B	0.476	0.476	0.476	0.238
DC088C	0.476	0.476	0.476	0.238
DC088D	0.476	0.476	0.476	0.238
DC088E	0.476	0.476	0.476	0.476
DC088ref	0.476	0.476	0.476	0.238

Bactericidal activity was observed for *M. abscessus* NCTC 13031 for all 4 manuka honey samples when cultured at 37 °C (Table 2.6). Not all of the clinical isolates tested had an MBC in response to honey treatment. Two of the isolates tested, DC088E and DC088ref, had no MBC for any honey tested, despite both isolates having an MIC of 0.476 g/mL for each honey (Table 2.5 and 2.6). The isolates DC088B and 186433 had no MBC for 3 of the honey samples tested, yet MGO40 exhibited bactericidal activity against DC088B, and MGO83 exhibited an MBC for 186433, both at 0.476 g/mL. Out of the 16 clinical isolates tested, bactericidal activity was observed for 10 clinical isolates (Table 2.6).

Table 2.6 Minimum bactericidal concentrations for *M. abscessus* clinical isolates cultured at 37 °C treated with manuka honey (n=3)

37°C	Minimum Bactericidal Concentration (g/mL)			
***M. abscessus* strain**	**MGO40**	**MGO55**	**MGO70**	**MGO83**
NCTC 13031	0.476	0.476	0.476	0.476
137071	>0.476	0.476	>0.476	0.476
147028	0.476	0.476	0.476	0.476
159544	0.476	0.476	0.476	0.476
186144	0.476	0.476	0.476	0.476
186154	0.476	>0.476	0.476	>0.476
186433	>0.476	>0.476	>0.476	0.476
189961	0.476	0.476	0.476	0.476
194891	0.476	0.476	0.476	0.476
199277	0.476	0.476	0.476	0.476
211666	0.476	0.476	0.476	0.476
DC088A	0.476	0.476	0.476	0.476
DC088B	0.476	>0.476	>0.476	>0.476
DC088C	0.476	0.476	0.476	0.476
DC088D	0.476	0.476	0.476	0.476
DC088E	>0.476	>0.476	>0.476	>0.476
DC088ref	>0.476	>0.476	>0.476	>0.476

2.3.3 Efficacy of manuka honey preheated to 37 °C against *M. abscessus* cultured at 30 °C and 37 °C

Due to the differences observed in MIC when *M. abscessus* was cultured at 30 °C and 37 °C, a study into the short term storage of manuka honey at 37 °C with culturing at these temperatures was conducted. This was only done for *M. abscessus* NCTC 13031. Depending on the honey, variations in both MIC and MBC were observed after incubating the honey. Differences were also observed when *M. abscessus* was cultured at the different temperatures.

2.3.3.1 MGO40

The MGO40 honey maintained similar MICs to those observed previously when cultured at 30 °C, regardless of honey incubation time (Figure 2.5). A change in MIC was observed for *M. abscessus* cultured at 37 °C after 3 h and 5 h of honey incubation (Figure 2.6). After 1 h of honey incubation, the MIC was 0.476 g/mL for both culture conditions and the growth of *M. abscessus* was reduced when treated with honey at 0.238 g/mL and 0.119 g/mL, one-way ANOVA P=0.0002 for culture at 30 °C and P=<0.0001 for culture at 37 °C (Figure 2.5 A and Figure 2.6 A).

When MGO40 honey was incubated for 2 h prior to testing, the MIC remained at 0.476 g/mL for both culture temperatures and growth reduced overall for honey treatment compared to *M. abscessus* only, with a one-way ANOVA P=<0.0001 for both culture temperatures. A reduction in growth at 0.238 g/mL was seen for both culture temperatures in comparison to the control of *M. abscessus* only (Figure 2.5 B and Figure 2.6 B). A Dunnett's multiple comparison showed a significant difference between 0.238 g/mL and *M. abscessus* only, P=<0.0001 (Table 2.7 and 2.8). At 0.119 g/mL honey, the growth of *M. abscessus* was very similar to the control when cultured at 30 °C, however a Dunnett's multiple comparison showed a significant difference between the growth of *M. abscessus* only and *M. abscessus* treated with 0.119 g/mL, P=0.0074 (Table 2.7). Suggesting that 0.119 g/mL still had an impact on the growth of *M. abscessus*.

After 3 h of MGO40 incubation, *M. abscessus* cultured at 30 °C maintained the same MIC of 0.476 g/mL (Figure 2.5 C), but *M. abscessus* cultured at 37 °C had an improved MIC of 0.238 g/mL (Figure 2.6 C). A reduction in growth was maintained for 0.119 g/mL for both culture conditions compared to the control of *M. abscessus* only, one-way ANOVA P=<0.0001. This improved MIC seen after 3 h incubation and culturing at 37 °C was lost at 4 h honey incubation, but the growth of honey treated *M. abscessus* was still reduced, one-way ANOVA P=<0.0001 (Figure 2.6 D). The MIC was maintained at 0.476 g/mL for 4 h incubated honey and *M. abscessus* cultured at 30 °C, with a reduction of growth for honey treated *M. abscessus* compared to the control, one-way ANOVA P=<0.0001 (Figure 2.5 D).

The increased MIC of 0.238 g/mL was observed again for *M. abscessus* cultured at 37 °C with honey incubated for 5 h (Figure 2.6 E). A reduction in growth at 0.119 g/mL was also maintained, one-way ANOVA P=<0.0001. The MIC remained at 0.476 g/mL for *M. abscessus* cultured at 30 °C with 5 h incubated honey, with a reduction in growth for 0.238 g/mL and 0.119 g/mL compared to *M. abscessus* only, one-way ANOVA P=<0.0001 (Figure 2.5 E). After 6 h of honey incubation the MIC for both 30 °C and 37 °C cultured *M. abscessus* remained at 0.476 g/mL with a reduction in growth of honey treated *M. abscessus*, one-way ANOVA P=<0.0001 for both (Figure 2.5 F and Figure 2.6 F).

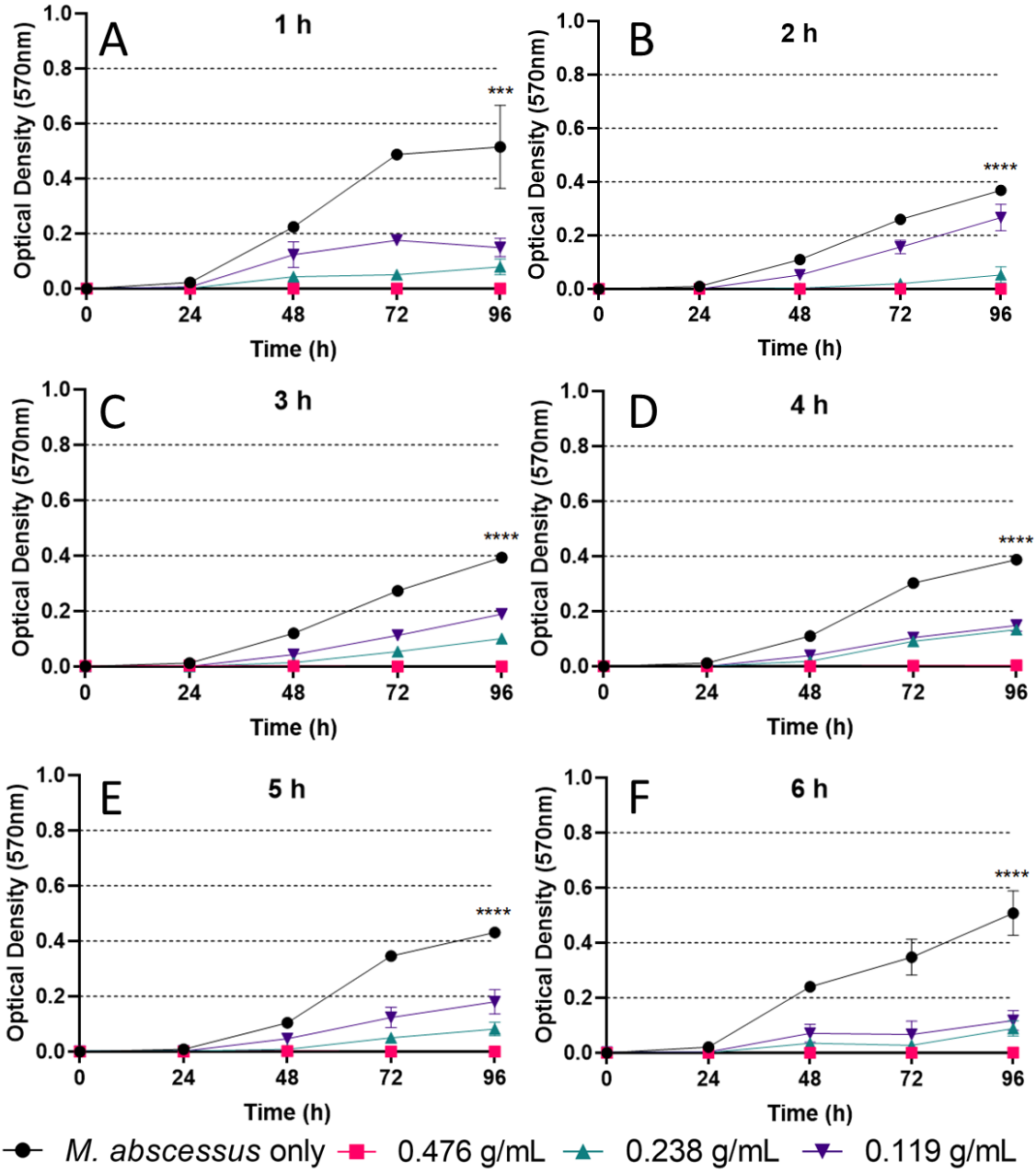

Figure 2.5 Growth Curves of *M. abscessus* NCTC 13031 cultured at 30 °C and treated with MGO40 honey stored at 37 °C for 1 h to 6 h. Data shown are mean ± SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) Honey incubation for 1 h showing an MIC of 0.476 g/mL. A significant difference was observed for all treatments, one-way ANOVA P=0.0002. B) Honey incubation after 2 h maintaining an MIC of 0.476 g/mL with a reduction in growth for 0.238 g/mL. A one-way ANOVA identified a significant difference, P=<0.0001. C) Honey incubation for 3 h had an MIC of 0.476 g/mL, one-way ANOVA showed a significant difference, P=<0.0001. D) An MIC of 0.476 g/mL for manuka honey incubated for 4 h, one-way ANOVA identified a significant difference, P=<0.0001. E) Honey incubation for 5 h exhibiting an MIC of 0.476 g/mL, one-way ANOVA P=<0.0001. F) Honey incubation for 6 h maintaining the MIC of 0.476 g/mL, one-way ANVOA P=<0.0001.

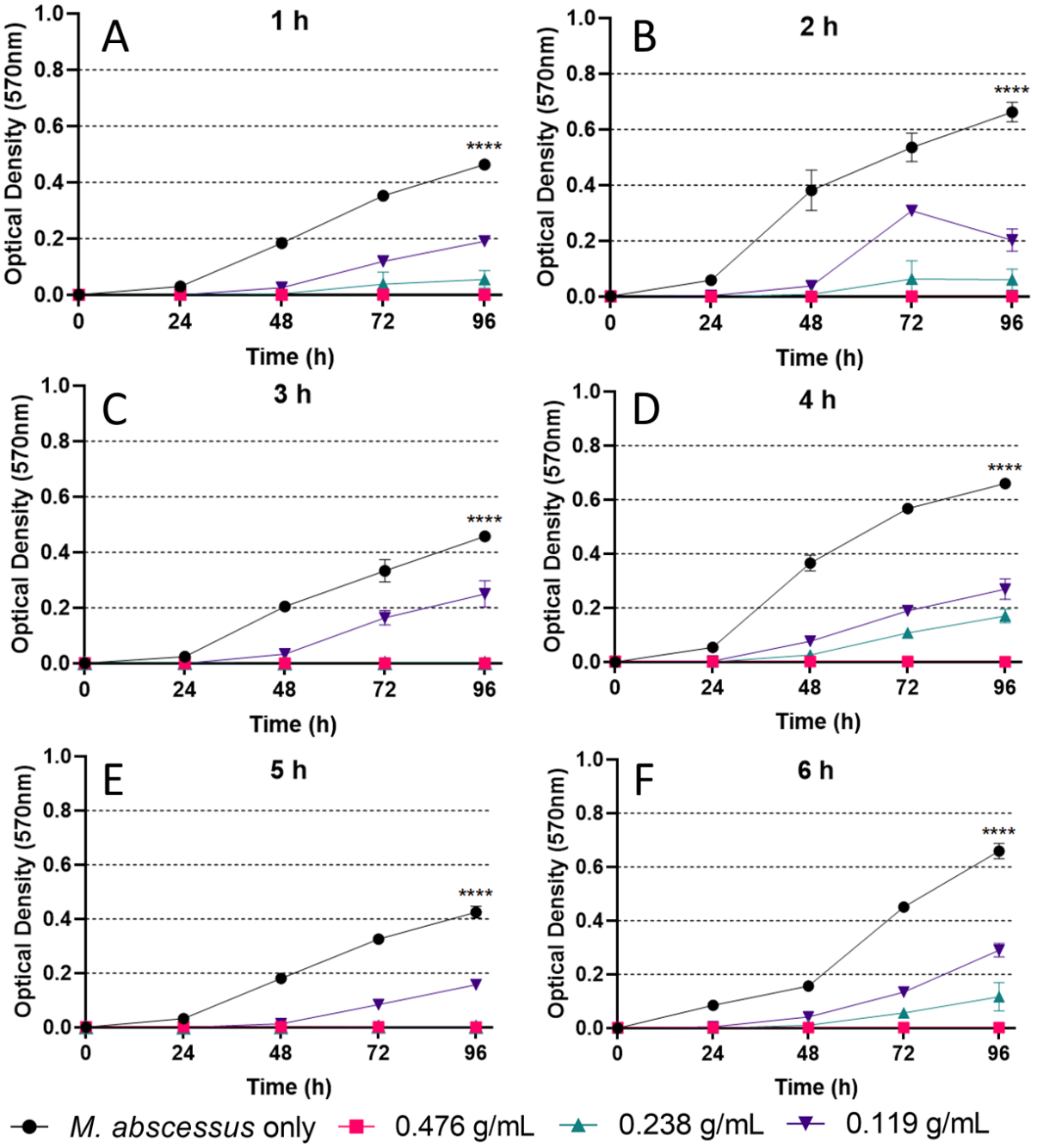

Figure 2.6 Growth Curves of *M. abscessus* NCTC 13031 cultured at 37 °C and treated with MGO40 honey stored at 37 °C for 1 h to 6 h. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) Honey incubation for 1 h, one-way ANOVA P=<0.0001. B) Honey incubation after 2 h, one-way ANOVA P=<0.0001. C) Honey incubation for 3 h, one-way ANOVA P=<0.0001. D) Honey incubation for 4 h, one-way ANOVA P=<0.0001. E) Honey incubation for 5 h, one-way ANOVA P=<0.0001. F) Honey incubation for 6 h, one-way ANVOA P=<0.0001.

Table 2.7 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO40 time points to the growth of *M. abscessus* cultured at 30 °C with no treatment. The Dunnett's multiple comparison P values are shown for the 3 concentrations tested.

30 °C	MGO40 Treatment		
Honey Preheating (h)	0.476 g/mL	0.238 g/mL	0.119 g/mL
1	0.0001	0.0004	0.0012
2	<0.0001	<0.0001	0.0074
3	<0.0001	<0.0001	<0.0001
4	<0.0001	<0.0001	<0.0001
5	<0.0001	<0.0001	<0.0001
6	<0.0001	<0.0001	<0.0001

Table 2.8 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO40 time points to the growth of M. abscessus cultured at 37 °C with no treatment. The Dunnett's multiple comparison P values are shown for the 3 concentrations tested.

37 °C	MGO40 Treatment		
Honey Preheating (h)	0.476 g/mL	0.238 g/mL	0.119 g/mL
1	<0.0001	<0.0001	<0.0001
2	<0.0001	<0.0001	<0.0001
3	<0.0001	<0.0001	<0.0001
4	<0.0001	<0.0001	<0.0001
5	<0.0001	<0.0001	<0.0001
6	<0.0001	<0.0001	<0.0001

The bactericidal activity fluctuated depending on how long the honey was incubated prior to testing and the culture temperature used (Table 2.9). There was no MBC observed for *M. abscessus* cultured at 30 °C for the concentrations tested. MBCs were observed for honey incubated for 2 h, 3 h, 4 h and 6 h prior to testing against *M. abscessus* cultured at 37 °C. No MBC was observed for *M. abscessus* cultured at 37 °C with honey incubated for 1 h and 5 h prior to testing.

Table 2.9 The MBCs observed for *M. abscessus* treated with MGO40 preheated to 37 °C prior to testing and culturing at either 30 °C or 37 °C for all time points tested.

Minimum Bactericidal Concentration for MGO40		
Honey storage time	**Culture 30 °C**	**Culture 37 °C**
1 h	No MBC	No MBC
2 h	No MBC	0.476 g/mL
3 h	No MBC	0.476 g/mL
4 h	No MBC	0.476 g/mL
5 h	No MBC	No MBC
6 h	No MBC	0.476 g/mL

2.3.3.2 MGO55

MGO55 maintained the same MIC of 0.476 g/mL observed previously when cultured at 30 °C, regardless of honey incubation time (Figure 2.7). The same MIC was also maintained for *M. abscessus* cultured at 37 °C, apart from for one of the incubation time points (Figure 2.8). After 1 h of honey incubation, both culture temperatures had the same MIC of 0.476 g/mL and similar inhibition at the other concentrations of 0.238 g/mL and 0.119 g/mL that was seen previously when compared to *M. abscessus* only, one-way ANOVA P=<0.0001 for both.

The 2 h incubation maintained an MIC of 0.476 g/mL for *M. abscessus* cultured at 30 °C and greatly impacted the growth of *M. abscessus* treated with 0.238 g/mL (Figure 2.7 B). At 0.119 g/mL the growth of *M. abscessus* was reduced compared to the control, one-way ANOVA P=<0.0001, Dunnett's comparison for all concentrations P=<0.0001 (Table 2.10). The reduction of growth for 0.238 g/mL was not an MIC but was very similar to that of 0.476 g/mL. To see if there was a significant difference between the two concentrations, a Dunnett's comparison was conducted using 0.476 g/mL as the comparison. This identified no significant difference between the two concentrations, Dunnett's comparison P=0.1231 (Table 2.12). This reduction in inhibition was not observed for the 2 h incubation cultured at 37 °C, and the MIC remained at 0.476 g/mL with a reduction in growth for 0.238 g/mL and 0.119 g/mL, one-way ANOVA P=<0.0001 (Figure 2.8 B).

A similar occurrence was observed for *M. abscessus* cultured at 30 °C after 3 h incubation, as was seen after 2 h incubation (Figure 2.7 C). The MIC remained at 0.476 g/mL with a drastic reduction in growth at 0.238 g/mL and an overall reduction in growth at 0.119 g/mL, when compared to the control of *M. abscessus* only, one-way ANOVA P=<0.0001. However, a Dunnett's comparison comparing all concentrations to 0.476 g/mL showed the difference in growth between 0.476 g/mL and 0.238 g/mL was significant, Dunnett's comparison P=0.0353 (Table 2.12). Whereas, for *M. abscessus* cultured at 37 °C against 3 h incubated honey there was a new MIC of 0.238 g/mL (Figure 2.8 C). This was further confirmed by a Dunnett's

multiple comparison, comparing 0.476 g/mL and 0.238 g/mL, P=0.9983 (Table 2.13). A reduction in growth at 0.119 g/mL compared to the control of *M. abscessus* only was maintained, one-way ANOVA P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *M. abscessus* only, identified a significant difference between all treatments and *M. abscessus* only, P=<0.0001 (Table 2.11).

The reduction in growth of *M. abscessus* cultured at 30 °C when treated with 0.283 g/mL was also seen after 4 h honey incubation (Figure 2.7 D). The MIC still remained at 0.476 g/mL and growth was reduced for 0.238 g/mL and 0.119 g/mL, one-way ANOVA P=<0.0001. To see if there was a significant difference in growth between 0.476 g/mL and 0.238 g/mL, a Dunnett's comparison was conducted, comparing all treatments to 0.476 g/mL (Table 2.12). This identified a significant difference between 0.476 g/mL and 0.238 g/mL, P=0.0314. The lower MIC of 0.238 g/mL observed for *M. abscessus* cultured at 37 °C after honey incubation for 3 h, was not maintained when the honey was incubated for 4 h, the MIC was 0.476 g/mL (Figure 2.8 D). The lower concentrations did reduce the growth of *M. abscessus*, one-way ANOVA P=<0.0001. This was also significantly reduced for all concentrations of honey when compared to the control of *M. abscessus* only, Dunnett's comparison P=<0.0001 for all (Table 2.11).

M. abscessus cultured at 30 °C had very similar inhibition for honey incubated for 5 h and 6 h with MICs remaining at 0.476 g/mL (Figure 2.7 E and F). Both 0.238 g/mL and 0.119 g/mL reduced the growth of *M. abscessus*, with similar inhibition patterns, one-way ANOVA P=<0.0001. For *M. abscessus* cultured at 37 °C, both 5 h and 6 h incubated honey showed similar inhibition to 1 h, 2 h and 4 h incubated honey, one-way ANOVA P=<0.0001 for 5 h and 6 h (Figure 2.8). A Dunnett's comparison, comparing all treatments to *M. abscessus* only, identified a significant difference between all treatments, P=<0.0001 for all (Table 2.11).

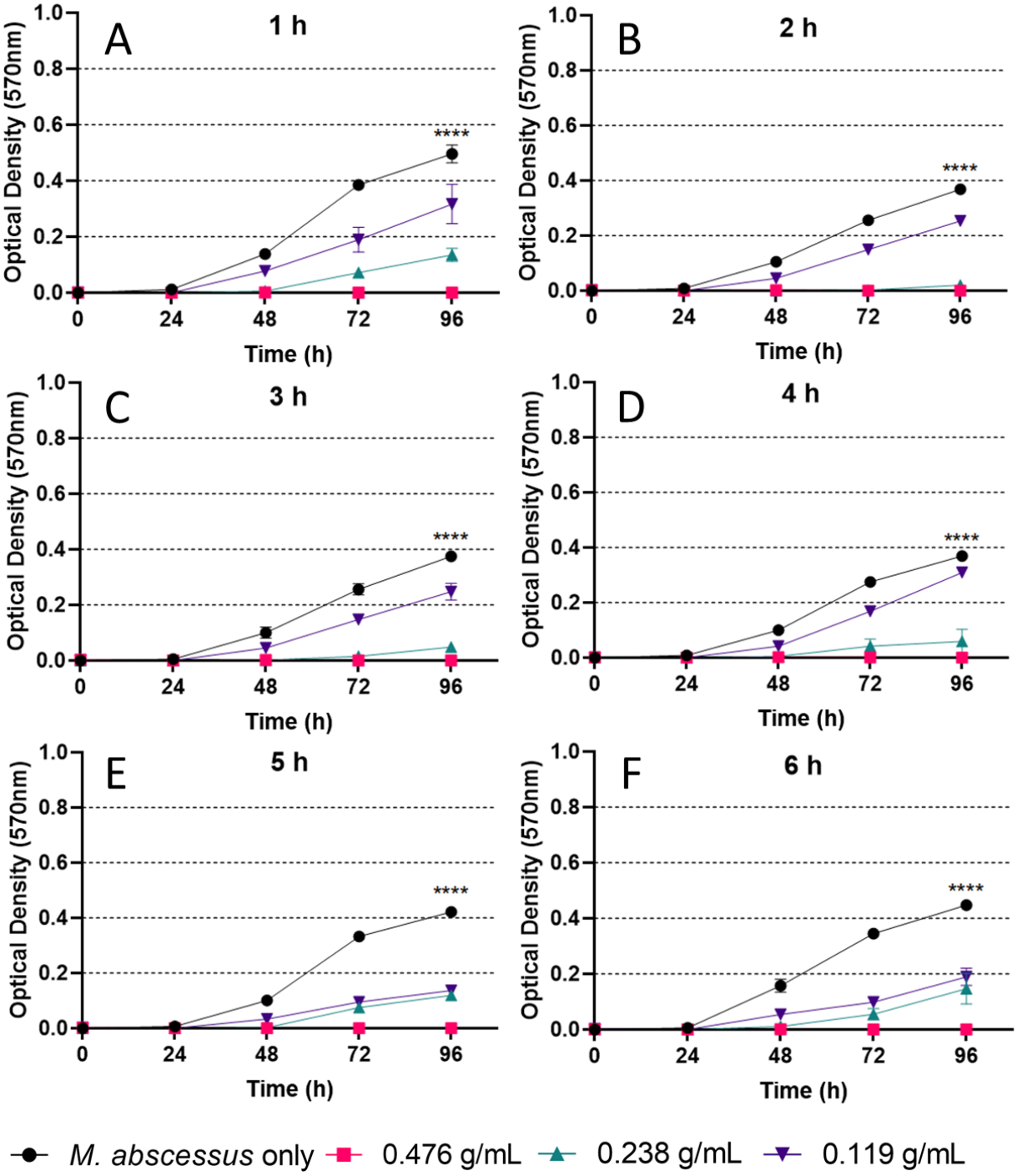

Figure 2.7 Growth Curves of *M. abscessus* NCTC 13031 cultured at 30 °C and treated with MGO55 honey stored at 37 °C for 1 h to 6 h. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) Honey incubation for 1 h, one-way ANOVA P=<0.0001. B) Honey incubation after 2 h, one-way ANOVA P=<0.0001. C) Honey incubation for 3 h, one-way ANOVA P=<0.0001. D) Honey incubation for 4 h, one-way ANOVA P=<0.0001. E) Honey incubation for 5 h, one-way ANOVA P=<0.0001. F) Honey incubation for 6 h, one-way ANVOA P=<0.0001.

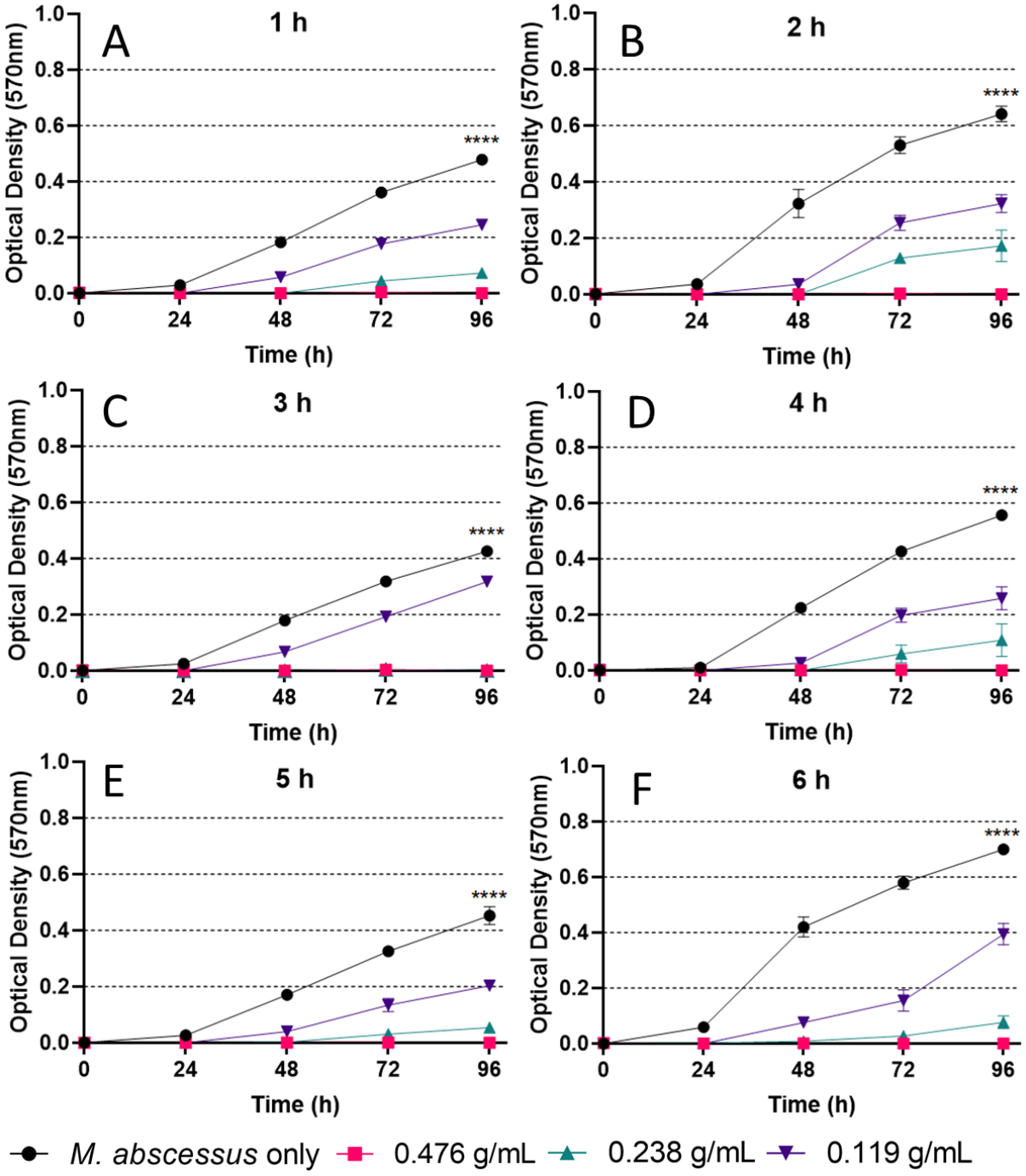

Figure 2.8 Growth Curves of *M. abscessus* NCTC 13031 cultured at 37 °C and treated with MGO55 honey stored at 37 °C for 1 h to 6 h. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) Honey incubation for 1 h, one-way ANOVA P=<0.0001. B) Honey incubation after 2 h, one-way ANOVA P=<0.0001. C) Honey incubation for 3 h, one-way ANOVA P=<0.0001. D) Honey incubation for 4 h, one-way ANOVA P=<0.0001. E) Honey incubation for 5 h, one-way ANOVA P=<0.0001. F) Honey incubation for 6 h, one-way ANVOA P=<0.0001.

Table 2.10 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO55 time points to the growth of *M. abscessus* cultured at 30 °C with no treatment. The Dunnett's multiple comparison P values are shown for the 3 concentrations tested.

30 °C	MGO55 Treatment		
Honey Preheating (h)	0.476 g/mL	0.238 g/mL	0.119 g/mL
1	<0.0001	<0.0001	0.0016
2	<0.0001	<0.0001	<0.0001
3	<0.0001	<0.0001	0.0001
4	<0.0001	<0.0001	0.0298
5	<0.0001	<0.0001	<0.0001
6	<0.0001	<0.0001	<0.0001

Table 2.11 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO55 time points to the growth of *M. abscessus* cultured at 37 °C with no treatment. The Dunnett's multiple comparison P values are shown for the 3 concentrations tested.

37 °C	MGO55 Treatment		
Honey Preheating (h)	0.476 g/mL	0.238 g/mL	0.119 g/mL
1	<0.0001	<0.0001	<0.0001
2	<0.0001	<0.0001	<0.0001
3	<0.0001	<0.0001	<0.0001
4	<0.0001	<0.0001	<0.0001
5	<0.0001	<0.0001	<0.0001
6	<0.0001	<0.0001	<0.0001

Table 2.12 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO55 time points to the growth of *M. abscessus* cultured at 30 °C treated with 0.476 g/mL MGO55. The Dunnett's multiple comparison P values are shown for the 2 concentrations tested and *M. abscessus* alone.

30 °C	MGO55 Treatment		
Honey Preheating (h)	*M. abscessus*	0.238 g/mL	0.119 g/mL
1	<0.0001	0.0088	<0.0001
2	<0.0001	0.1231	<0.0001
3	<0.0001	0.0353	<0.0001
4	<0.0001	0.0314	<0.0001
5	<0.0001	<0.0001	<0.0001
6	<0.0001	0.0013	0.0002

Table 2.13 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO55 time points to the growth of *M. abscessus* cultured at 37 °C treated with 0.476 g/mL MGO55. The Dunnett's multiple comparison P values are shown for the 2 concentrations tested and *M. abscessus* alone.

37 °C	MGO55 Treatment		
Honey Preheating (h)	*M. abscessus*	0.238 g/mL	0.119 g/mL
1	<0.0001	0.0007	<0.0001
2	<0.0001	0.0008	<0.0001
3	<0.0001	0.9983	<0.0001
4	<0.0001	0.0146	<0.0001
5	<0.0001	0.0189	<0.0001
6	<0.0001	0.0114	<0.0001

The bactericidal activity for MGO55 was largely different to that of MGO40. There was no bactericidal activity observed for any of the 30 °C cultured *M. abscessus* with honey incubated prior to testing (Table 2.14). Only one of the incubated honey time points, 3 h, had an MBC for *M. abscessus* cultured at 37 °C.

Table 2.14. The MBCs observed for *M. abscessus* treated with MGO55 preheated to 37 °C prior to testing and culturing at either 30 °C or 37 °C for all time points tested.

Minimum Bactericidal Concentration for MGO55		
Honey storage time	**Culture 30 °C**	**Culture 37 °C**
1 h	No MBC	No MBC
2 h	No MBC	No MBC
3 h	No MBC	0.476 g/mL
4 h	No MBC	No MBC
5 h	No MBC	No MBC
6 h	No MBC	No MBC

2.3.3.3 MGO70

The MGO70 honey maintained an MIC of 0.476 g/mL for all of the honey incubation time points when cultured at 30 °C (Figure 2.9). This was also observed for all incubation time points when cultured at 37 °C (Figure 2.10). After both 1 h and 2 h honey incubation, with culturing at 30 °C the MIC was 0.476 g/mL with a reduction in growth at 0.238 g/mL. The growth at 0.119 g/mL was similar to that of *M. abscessus* only, with a slight reduction in growth (Figure 2.9 A and B). For both of these time points the one-way ANOVA was P=<0.0001. A Dunnett's multiple comparison also showed there was a significant difference between all treatments and *M. abscessus* only (Table 2.15). A similar occurrence was observed for 1 h and 2 h honey incubation for *M. abscessus* cultured at 37 °C (Figure 2.10 A and B). The MIC remained at 0.476 g/mL with a reduction in growth at 0.238 g/mL and only a slight reduction in growth at 0.119 g/mL, one-way ANOVA P=<0.0001 for both. A Dunnett's multiple comparison showed a significant difference between all treatments and *M. abscessus* only (Table 2.16).

The same trend continued for 3 h honey incubation and *M. abscessus* cultured at 30 °C. The MIC remained at 0.476 g/mL with reductions in growth observed for 0.238 g/mL and 0.119 g/mL, one-way ANOVA P=<0.0001 (Figure 2.9 C). However, this was not observed for 3 h honey incubation and *M. abscessus* cultured at 37 °C. The MIC remained at 0.476 g/mL but the growth at 0.238 g/mL was significantly reduced (Figure 2.10 C). The growth at 0.119 g/mL was reduced compared to the control and a one-way ANOVA identified a significant difference between the treatments, P=<0.0001. A Dunnett's multiple comparison was conducted for all concentrations and identified a significant difference between the growth of

M. abscessus treated with 0.476 g/mL, 0.238 g/mL and 0.119 g/mL honey compared to the control, P=<0.0001, P=<0.0001 and P=0.0002, respectively (Table 2.16). To see if there was a difference between 0.476 g/mL and 0.238 g/mL, a Dunnett's multiple comparison was conducted for all treatments compared to 0.476 g/mL. This identified no significant difference between 0.476 g/mL and 0.238 g/mL, Dunnett's comparison P=0.6212. Furthermore, the growth of *M. abscessus* treated with 0.238 g/mL honey only occurred in the last 24 h of the 96 h experiment. A significant difference between 0.476 g/mL compared to 0.119 g/mL and *M. abscessus* only was observed, Dunnett's comparison P=0.0002 and P=<0.0001, respectively.

Honey incubated for 4 h and cultured at 30 °C maintained the MIC of 0.476 g/mL with reductions in growth at 0.238 g/mL and 0.119 g/mL, one-way ANOVA P=<0.0001 (Figure 2.9 D). This was also the same for 4 h honey incubation and culturing at 37 °C, one-way ANOVA P=<0.0001 (Figure 2.1 D). After 5 h honey incubation and culturing at 30 °C, the MIC was maintained at 0.476 g/mL (Figure 2.9 E). The growth of *M. abscessus* was reduced for 0.238 g/mL and 0.119 g/mL but there was a small difference in inhibition between these concentrations. The one-way ANOVA identified a significant difference, P=<0.0001, and the Dunnett's multiple comparison of honey treated *M. abscessus* compared to the control of *M. abscessus* only identified a significant difference between all honey treatments and *M. abscessus* only, P=<0.0001 for all (Table 2.15). A Dunnett's comparison was also conducted comparing all honey treatments to 0.119 g/mL to see if there was a difference between the growth of *M. abscessus* at 0.238 g/mL and 0.119 g/mL. A significant difference was identified between 0.238 g/mL and 0.119 g/mL, Dunnett's comparison P=0.0010, identifying that these concentrations were significantly different from each other. Similar observations were made for honey incubated for 5 h and *M. abscessus* cultured at 37 °C (Figure 2.10 E). The MIC remained at 0.476 g/mL and a reduction in growth for 0.238 g/mL and 0.119 g/mL was observed, one-way ANOVA P=<0.0001. However, the growth of *M. abscessus* at 0.238 g/mL and 0.119 g/mL were almost the same. A Dunnett's comparison was conducted comparing all treatments to 0.119 g/mL. This identified no significant difference between 0.238 g/mL and 0.119 g/mL, P=0.9978, suggesting that there is no difference between these two concentrations. The honey incubated for 6 h for both culturing temperatures had an MIC of 0.476 g/mL with reductions in growth for 0.238 g/mL and 0.119 g/mL, one-way ANOVA P=<0.0001 for both (Figure 2.9 F and Figure 2.10 F).

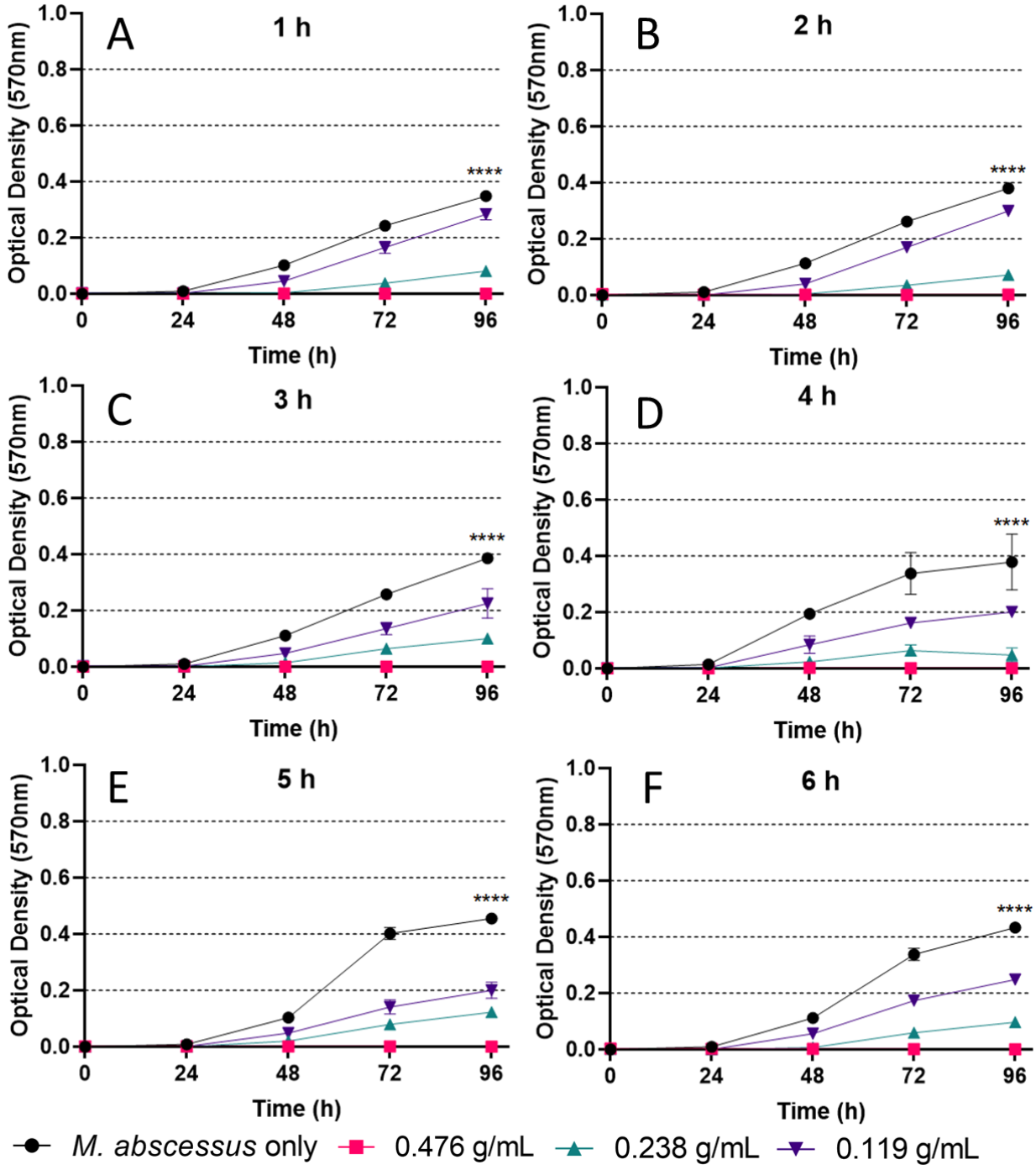

Figure 2.9 Growth Curves of *M. abscessus* NCTC 13031 cultured at 30 °C and treated with MGO70 honey stored at 37 °C for 1 h to 6 h. Data shown are mean ± SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) Honey incubation for 1 h, one-way ANOVA P=<0.0001. B) Honey incubation after 2 h, one-way ANOVA P=<0.0001. C) Honey incubation for 3 h, one-way ANOVA P=<0.0001. D) Honey incubation for 4 h, one-way ANOVA P=<0.0001. E) Honey incubation for 5 h, one-way ANOVA P=<0.0001. F) Honey incubation for 6 h, one-way ANVOA P=<0.0001.

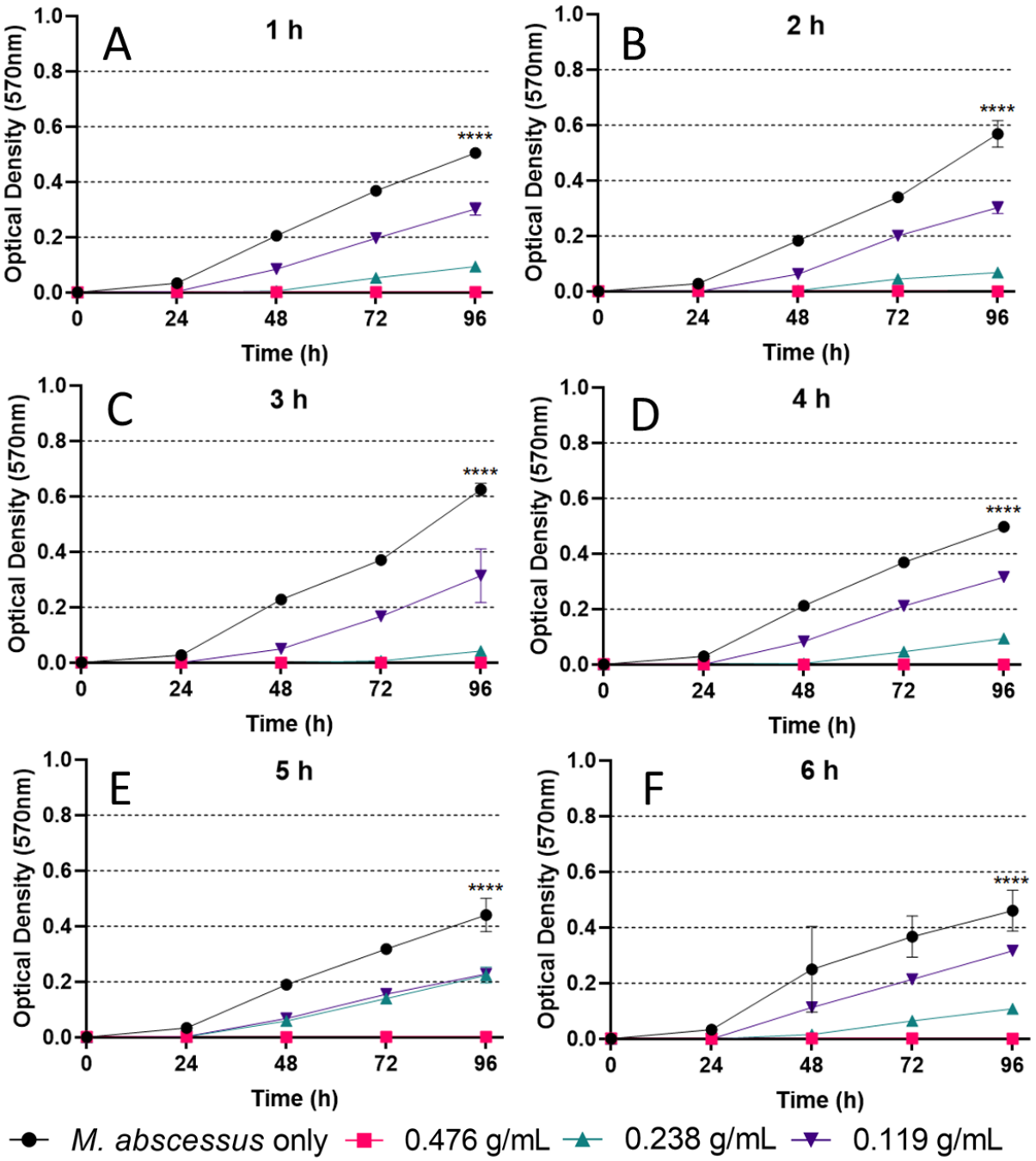

Figure 2.10 Growth Curves of *M. abscessus* NCTC 13031 cultured at 37 °C and treated with MGO70 honey stored at 37 °C for 1 h to 6 h. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) Honey incubation for 1 h, one-way ANOVA P=<0.0001. B) Honey incubation after 2 h, one-way ANOVA P=<0.0001. C) Honey incubation for 3 h, one-way ANOVA P=<0.0001. D) Honey incubation for 4 h, one-way ANOVA P=<0.0001. E) Honey incubation for 5 h, one-way ANOVA P=<0.0001. F) Honey incubation for 6 h, one-way ANVOA P=<0.0001.

Table 2.15 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO70 time points to the growth of *M. abscessus* cultured at 30 °C with no treatment. The Dunnett's multiple comparison P values are shown for the 3 concentrations tested.

	MGO70 Treatment		
Honey Preheating (h)	**0.476 g/mL**	**0.238 g/mL**	**0.119 g/mL**
1	<0.0001	<0.0001	0.0010
2	<0.0001	<0.0001	<0.0001
3	<0.0001	<0.0001	0.0003
4	<0.0001	0.0001	0.0071
5	<0.0001	<0.0001	<0.0001
6	<0.0001	<0.0001	<0.0001

Table 2.16 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO70 time points to the growth of *M. abscessus* cultured at 37 °C with no treatment. The Dunnett's multiple comparison P values are shown for the 3 concentrations tested.

	MGO70 Treatment		
Honey Preheating (h)	**0.476 g/mL**	**0.238 g/mL**	**0.119 g/mL**
1	<0.0001	<0.0001	<0.0001
2	<0.0001	<0.0001	<0.0001
3	<0.0001	<0.0001	0.0002
4	<0.0001	<0.0001	<0.0001
5	<0.0001	0.0002	0.0002
6	<0.0001	<0.0001	0.0047

The bactericidal activity observed for MGO70 was similar to that observed for MGO55 (Table 2.17). No bactericidal activity was observed for any of the honey incubation time points and *M. abscessus* cultured at 30 °C. Bactericidal activity was observed for two of the time points when cultured at 37 °C. These were 3 h and 6 h of honey incubation.

Table 2.17 The MBCs observed for *M. abscessus* treated with MGO70 preheated to 37 °C prior to testing and culturing at either 30 °C or 37 °C for all time points tested.

Minimum Bactericidal Concentration for MGO70		
Honey storage time	**Culture 30 °C**	**Culture 37 °C**
1 h	No MBC	No MBC
2 h	No MBC	No MBC
3 h	No MBC	0.476 g/mL
4 h	No MBC	No MBC
5 h	No MBC	No MBC
6 h	No MBC	0.476 g/mL

2.3.3.4 MGO83

MGO83 maintained MICs of 0.476 g/mL for all honey incubation time points against *M. abscessus* for both 30 °C and 37 °C culturing temperatures (Figure 2.11 and 2.12). After 1 h honey incubation and culturing at 30 °C, the MIC was 0.476 g/mL with a reduction in growth observed for 0.238 g/mL and 0.119 g/mL, one-way ANOVA P=<0.0001 (Figure 2.11 A). The same occurrence was observed for 2 h and 3 h when cultured at 30 °C (Figure 2.11 B and Figure 2.11 C). A Dunnett's multiple comparison showed a significant difference between all concentrations (Table 2.18). This was also observed for honey incubated for 1 h and 2 h with culturing at 37 °C, one-way ANOVA P=<0.0001 for all (Figure 2.12 A and Figure 2.12 B). A Dunnett's multiple comparison identified a significant difference between all treatments compared to *M. abscessus* only (Table 2.19). For 3 h incubation the MIC was maintained at 0.476 g/mL, however a reduction in growth was observed for 0.238 g/mL (Figure 2.12 C). A Dunnett's multiple comparison identified a significant difference between all treatments and *M. abscessus* only (Table 2.19). To see if there was a difference between 0.476 g/mL and 0.238 g/mL, a Dunnett's comparison was conducted comparing all treatments to 0.476 g/mL. This identified no significant difference between 0.476 g/mL and 0.238 g/mL, Dunnett's comparison P=0.1352, suggesting there is no difference between these treatments.

After 4 h honey incubation and culturing at 30 °C, the MIC remained at 0.476 g/mL with a reduction in growth observed for 0.238 g/mL and 0.119 g/mL compared to the control of *M. abscessus* only, one-way ANOVA P=<0.0001 (Figure 2.11 D). For 4 h honey incubation with culturing at 37 °C, a similar occurrence to 3 h honey incubation was observed. The MIC was 0.476 g/mL with almost no growth observed for 0.238 g/mL and a reduction in growth for

0.119 g/mL compared to *M. abscessus* only, one-way ANOVA P=<0.0001 (Figure 2.12 D). To see if there was a difference between 0.476 g/mL and 0.238 g/mL, a Dunnett's comparison was conducted comparing all treatments to 0.476 g/mL. This identified no significant difference between 0.476 g/mL and 0.238 g/mL, Dunnett's comparison P=0.0742, suggesting there was a difference between the treatments.

For both 5 h and 6 h honey incubation, and both culture temperatures the MIC remained at 0.476 g/mL with reductions in growth observed for 0.238 g/mL and 0.119 g/mL when compared to the control (Figure 2.11 E and F, 2.12 E and F). A one-way ANOVA was conducted for 5 h and 6 h honey incubation with *M. abscessus* cultured at 30 °C, P=<0.0001 for both. A one-way ANOVA was conducted for 5 h and 6 h honey incubation with *M. abscessus* culture at 37 °C, P<0.001 for both.

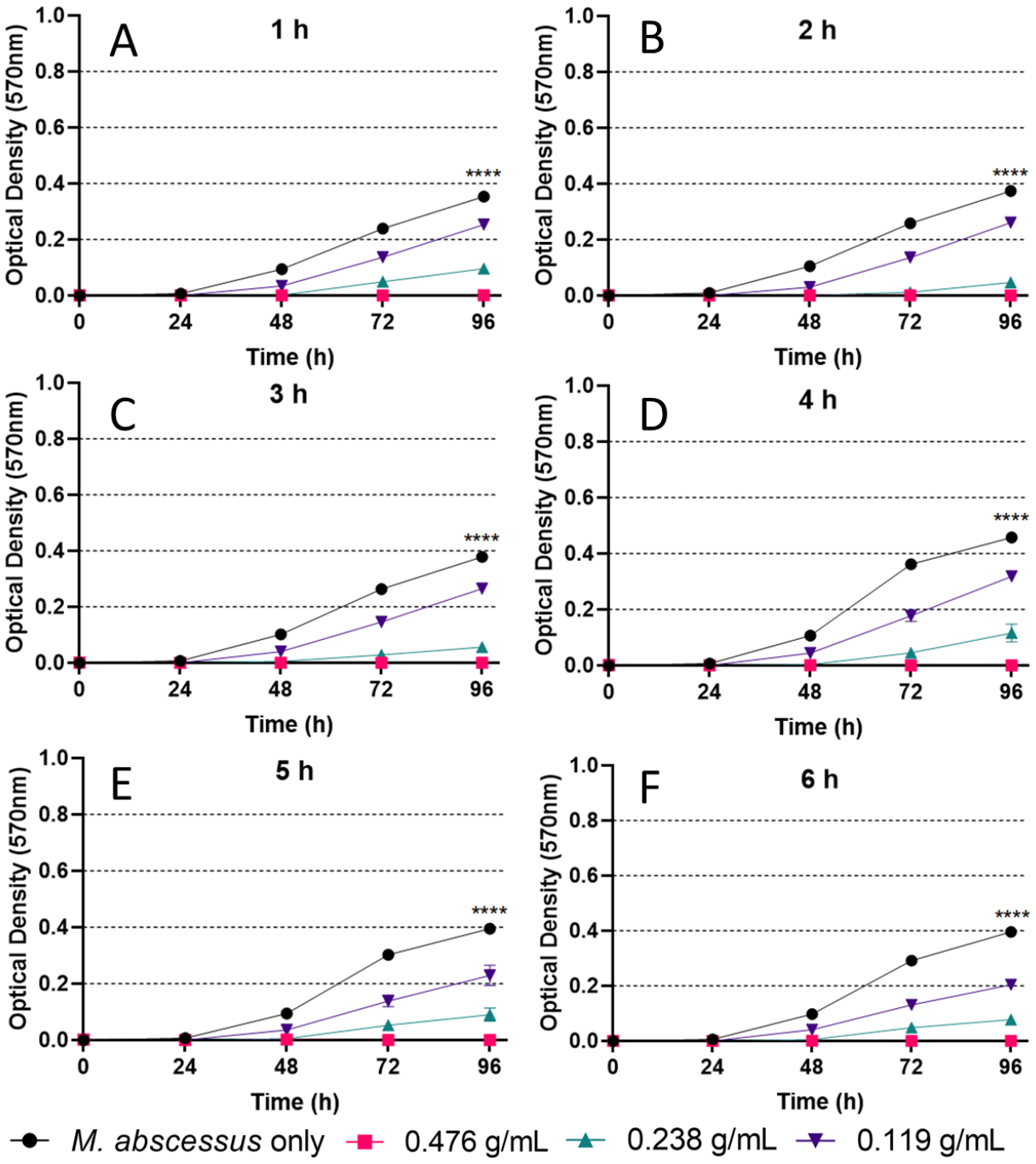

Figure 2.11 Growth Curves of *M. abscessus* NCTC 13031 cultured at 30 °C and treated with MGO83 honey stored at 37 °C for 1 h to 6 h. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) Honey incubation for 1 h, one-way ANOVA P=<0.0001. B) Honey incubation after 2 h, one-way ANOVA P=<0.0001. C) Honey incubation for 3 h, one-way ANOVA P=<0.0001. D) Honey incubation for 4 h, one-way ANOVA P=<0.0001. E) Honey incubation for 5 h, one-way ANOVA P=<0.0001. F) Honey incubation for 6 h, one-way ANVOA P=<0.0001.

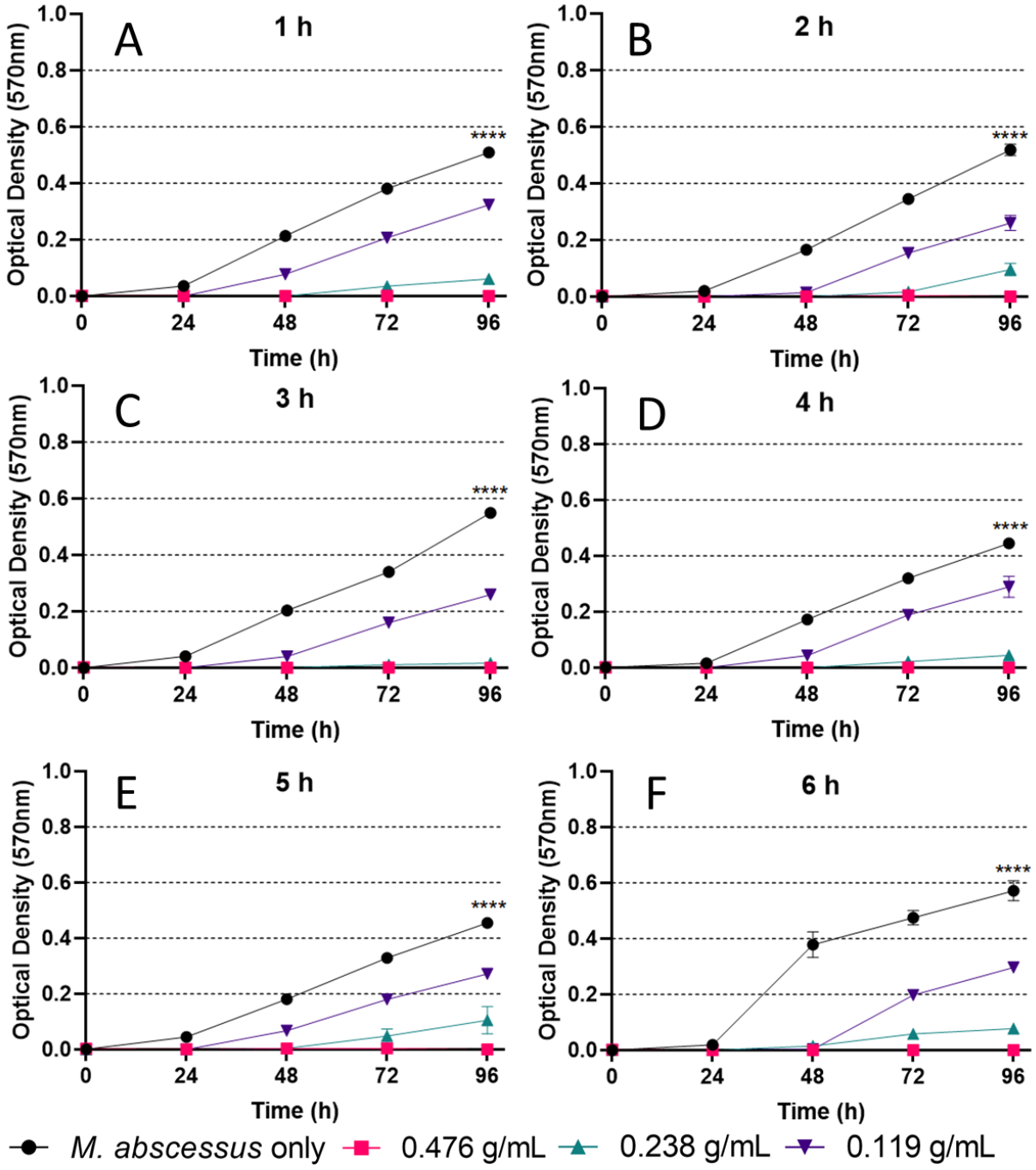

Figure 2.12 Growth Curves of *M. abscessus* NCTC 13031 cultured at 37 °C and treated with MGO83 honey stored at 37 °C for 1 h to 6 h. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) Honey incubation for 1 h, one-way ANOVA P=<0.0001. B) Honey incubation after 2 h, one-way ANOVA P=<0.0001. C) Honey incubation for 3 h, one-way ANOVA P=<0.0001. D) Honey incubation for 4 h, one-way ANOVA P=<0.0001. E) Honey incubation for 5 h, one-way ANOVA P=<0.0001. F) Honey incubation for 6 h, one-way ANVOA P=<0.0001.

Table 2.18 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO83 time points to the growth of *M. abscessus* cultured at 30 °C with no treatment. The Dunnett's multiple comparison P values are shown for the 3 concentrations tested.

	MGO83 Treatment		
Honey Preheating (h)	**0.476 g/mL**	**0.238 g/mL**	**0.119 g/mL**
1	<0.0001	<0.0001	<0.0001
2	<0.0001	<0.0001	<0.0001
3	<0.0001	<0.0001	<0.0001
4	<0.0001	<0.0001	<0.0001
5	<0.0001	<0.0001	<0.0001
6	<0.0001	<0.0001	<0.0001

Table 2.19 *Post-hoc* analysis of end point data, taken at 96 h, comparing all 37 °C preheated MGO83 time points to the growth of *M. abscessus* cultured at 37 °C with no treatment. The Dunnett's multiple comparison P values are shown for the 3 concentrations tested.

	MGO83 Treatment		
Honey Preheating (h)	**0.476 g/mL**	**0.238 g/mL**	**0.119 g/mL**
1	<0.0001	<0.0001	<0.0001
2	<0.0001	<0.0001	<0.0001
3	<0.0001	<0.0001	<0.0001
4	<0.0001	<0.0001	<0.0001
5	<0.0001	<0.0001	<0.0001
6	<0.0001	<0.0001	<0.0001

The bactericidal activity observed for MGO83 was similar to that observed for MGO 55 (Table 2.20). No bactericidal activity was observed for any of the honey incubation time points and *M. abscessus* cultured at 30 °C. Bactericidal activity was only observed for one of the time points when cultured at 37 °C. This was for 6 h of honey incubation.

Table 2.20 The MBCs observed for *M. abscessus* treated with MGO83 preheated to 37 °C prior to testing and culturing at either 30 °C or 37 °C for all time points tested.

Minimum Bactericidal Concentration for MGO83		
Honey storage time	**Culture 30 °C**	**Culture 37 °C**
1 h	No MBC	No MBC
2 h	No MBC	No MBC
3 h	No MBC	No MBC
4 h	No MBC	No MBC
5 h	No MBC	No MBC
6 h	No MBC	0.476 g/mL

2.3.4 Efficacy of manuka honey stored at different temperatures for 16 days against *M. abscessus* cultured at 37 °C

To assess if storage time and temperature conditions impact the efficacy of the honey's antimicrobial activity, a time and temperature dependant study was conducted as described in 2.2.6. The end point data, taken at 96 h was used to determine MICs and subsequent culture onto solid media after 96 h was used to determine MBCs, to assess any changes in antimicrobial activity (Table 2.21, 2.22, 2.23 and 2.24). Manuka honey stored at 4 °C maintained the same MIC of 0.476 g/mL, regardless of length of time stored for MGO40, MGO55 and MGO70. Variations in MIC were observed for MGO83. All 4 manuka honey samples stored at 20 °C had more variation in MIC between 0.476 g/mL and 0.238 g/mL. For storage at 37 °C, all of the manuka honey samples maintained an MIC of 0.476 g/mL. Variations in MBCs were also observed, depending on storage temperature and length of time, with some conditions resulting in no MBC at all.

MGO40 exhibited slight variation in MIC depending on honey storage duration and temperature (Table 2.21). The MIC for honey stored at 4 °C remained at 0.476 g/mL, regardless of how many days it was stored for prior to testing. However, for days 2, 4 and 7 of testing, no MBC was observed. From day 9 onwards, the MBC remained at 0.476 g/mL. The MICs observed for MGO40 stored at 37 °C were very similar to that of storage at 4 °C. The MIC remained at 0.476 g/mL for all days of testing, but variations were observed in MBC. An MBC of 0.476 g/mL was observed for all days tested apart from day 4. Storage at 20 °C had the most variation in MIC, with the majority of days testing maintaining the 0.476 g/mL MIC. Whereas day 7, 11 and 16 of testing had a lower MIC of 0.238 g/mL. This was not reflected in the MBC, with the MBC remaining at 0.476 g/mL for all days of testing apart from day 4. Interestingly, on day 4 of testing, no MBC was observed for any of the storage temperatures.

Table 2.21 MICs and MBCs of *M. abscessus* NCTC 13031 for MGO40 stored at either 4 °C, 20 °C and 37 °C from 2 days to 16 days.

	MIC MGO40 (g/mL)			MBC MGO40 (g/mL)		
Storage day	4 °C	20 °C	37 °C	4 °C	20 °C	37 °C
2	0.476	0.476	0.476	No MBC	0.476	0.476
4	0.476	0.476	0.476	No MBC	No MBC	No MBC
7	0.476	0.238	0.476	No MBC	0.476	0.476
9	0.476	0.476	0.476	0.476	0.476	0.476
11	0.476	0.238	0.476	0.476	0.476	0.476
14	0.476	0.476	0.476	0.476	0.476	0.476
16	0.476	0.238	0.476	0.476	0.476	0.476

MGO55 had some variation in MIC, depending on storage duration and temperature (Table 2.22). For storage at 4 °C, the MIC remained at 0.476 g/mL, regardless of day of testing. However, variation in MBC was observed, with no MBC for days 4, 7, and 14. The MBC remained at 0.476 g/mL for all other days of testing. Storage at 37 °C also maintained an MIC of 0.476 g/mL, regardless of day of testing. The MBC also remained at 0.476 g/mL, apart from day 4 of testing. Storage at 20 °C had some variation in MIC, with the majority of days tested having an MIC of 0.476 g/mL, but days 11 and 16 had a lower MIC of 0.238 g/mL. This was not reflected in the MBC, with no MBC observed for days 2, 4 and 7, and an MBC of 0.476 g/mL observed for days 9, 11, 14 and 16.

Table 2.22 MICs and MBCs of *M. abscessus* NCTC 13031 for MGO55 stored at either 4 °C, 20 °C and 37 °C from 2 days to 16 days.

	MIC MGO55 (g/mL)			MBC MGO55 (g/mL)		
Storage day	4 °C	20 °C	37 °C	4 °C	20 °C	37 °C
2	0.476	0.476	0.476	0.476	No MBC	0.476
4	0.476	0.476	0.476	No MBC	No MBC	No MBC
7	0.476	0.476	0.476	No MBC	No MBC	0.476
9	0.476	0.476	0.476	0.476	0.476	0.476
11	0.476	0.238	0.476	0.476	0.476	0.476
14	0.476	0.476	0.476	No MBC	0.476	0.476
16	0.476	0.238	0.476	0.476	0.476	0.476

MGO70 maintained the same MIC for almost all storage conditions but had more variation in MBC (Table 2.23). Storage at 4 °C maintained an MIC of 0.476 g/mL, regardless of day of testing. However, this was not the case for MBCs. No MBC was observed for days 4, 7, 14 and 16. On days 2, 9 and 11 the MBC remained at 0.476 g/mL. For storage at 37 °C the MIC also remained at 0.476 g/mL for all days tested. This was also not the same for MBCs, with no MBC observed for days 4, 7 and 14 of testing. MBCs remained at 0.476 g/mL for days 2, 9, 11 and 16 of testing. The storage of honey at 20 °C maintained an MIC of 0.476 g/mL, apart from on day 16 where the MIC reduced to 0.238 g/mL. This was not reflected in the MBC, with no MBC observed for days 4 and 7 of testing, and an MBC of 0.476 g/mL for all other days of testing.

Table 2.23 MICs and MBCs of *M. abscessus* NCTC 13031 for MGO70 stored at either 4 °C, 20 °C and 37 °C from 2 days to 16 days.

	MIC MGO70 (g/mL)			MBC MGO70 (g/mL)		
Storage day	4 °C	20 °C	37 °C	4 °C	20 °C	37 °C
2	0.476	0.476	0.476	0.476	0.476	0.476
4	0.476	0.476	0.476	No MBC	No MBC	No MBC
7	0.476	0.476	0.476	No MBC	No MBC	No MBC
9	0.476	0.476	0.476	0.476	0.476	0.476
11	0.476	0.476	0.476	0.476	0.476	0.476
14	0.476	0.476	0.476	No MBC	0.476	No MBC
16	0.476	0.238	0.476	No MBC	0.476	0.476

MGO83 had more variation in MIC than the other honey samples, depending on storage duration and temperature (Table 2.24). For storage at 4 °C, the MIC was 0.476 g/mL for most days of testing, apart from days 7 and 11, where the MIC was reduced to 0.238 g/mL. However, this was not reflected in the MBC, with no MBC for days 4, 7 and 14. The MBC remained at 0.476 g/mL for all other days of testing. Storage at 37 °C maintained an MIC of 0.476 g/mL for all days of testing. The MBC also remained at 0.476 g/mL, apart from for day 4 of testing. Storage at 20 °C had some variation in MIC, with most days maintaining an MIC of 0.476 g/mL, apart from days 7, 11 and 16 of testing which had a lower MIC of 0.238 g/mL. There was also variation in MBC, with no MBC observed for days 4, 7 and 14 of testing, and an MBC of 0.476 g/mL for all other days of testing.

Table 2.24 MICs and MBCs of *M. abscessus* NCTC 13031 for MGO83 stored at either 4 °C, 20 °C and 37 °C from 2 days to 16 days.

	MIC MGO83 (g/mL)			MBC MGO83 (g/mL)		
Storage day	4 °C	20 °C	37 °C	4 °C	20 °C	37 °C
2	0.476	0.476	0.476	0.476	0.476	0.476
4	0.476	0.476	0.476	No MBC	No MBC	No MBC
7	0.238	0.238	0.476	No MBC	No MBC	0.476
9	0.476	0.476	0.476	0.476	0.476	0.476
11	0.238	0.238	0.476	0.476	0.476	0.476
14	0.476	0.476	0.476	No MBC	No MBC	0.476
16	0.476	0.238	0.476	0.476	0.476	0.476

2.3.6 Efficacy of manuka honey stored for 30 days at 4 °C against *M. abscessus* cultured at 37 °C

The storage of manuka honey at 4 °C for 30 days prior to testing improved the antimicrobial efficacy against *M. abscessus* NCTC 13031 and clinical isolates (Table 2.25). The MIC was reduced to 0.014 g/mL for MGO40, MGO55 and MGO83. For MGO70 the MIC was reduced to 0.029 g/mL (Figure 2.13). All 4 of the manuka honey samples showed a significant difference between treatments, one-way ANOVA P=<0.0001 for all.

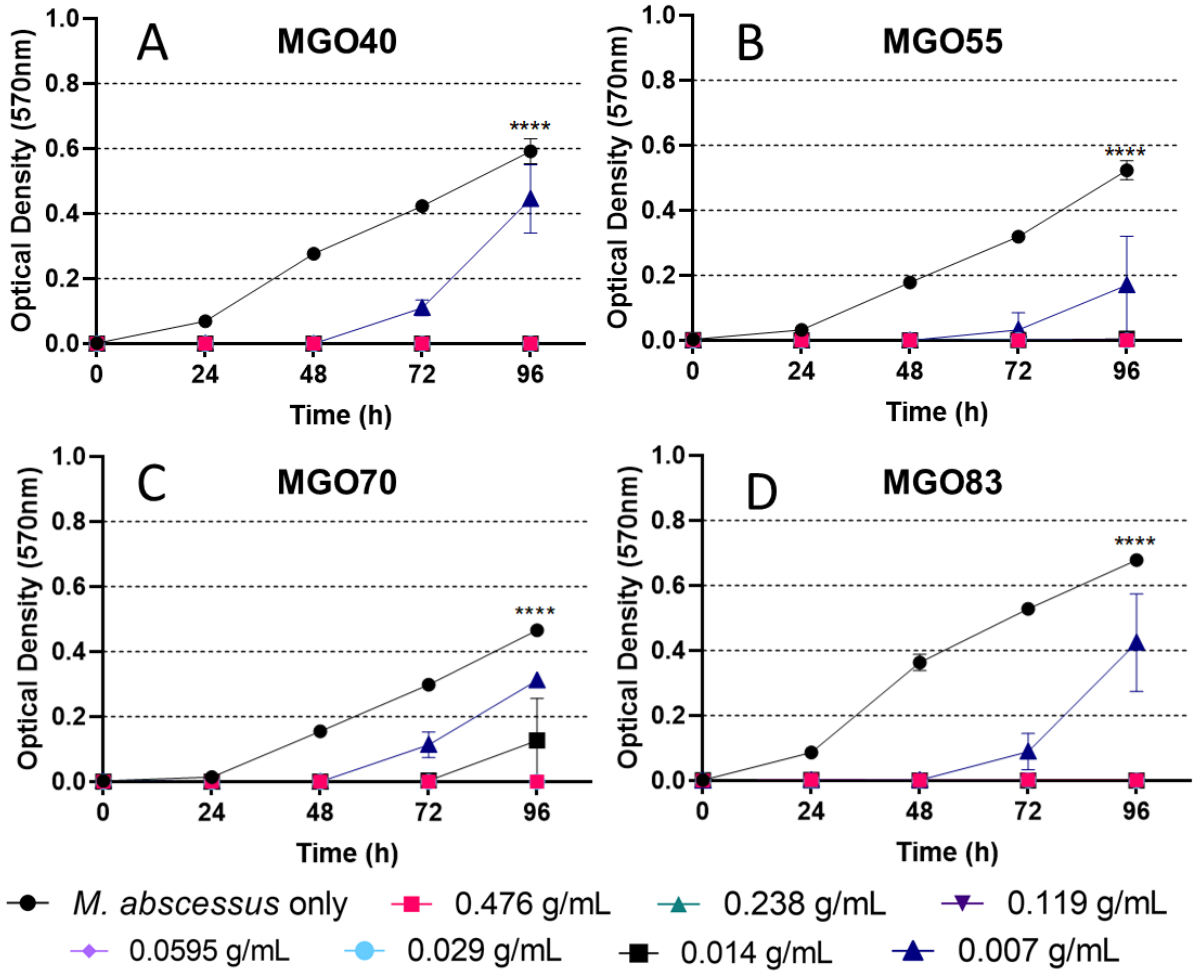

Figure 2.13 Growth Curves of *M. abscessus* NCTC 13031, cultured at 37 °C treated with 4 manuka honeys stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. The manuka honeys inhibited *M. abscessus* at lower concentrations than previously observed. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) MGO40 growth curve showing an MIC of 0.014 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve exhibiting an MIC of 0.014 g/mL, a significant difference was observed for all honey treatments P=<0.0001. C) An MIC of 0.029 g/mL was observed for MGO70 and a significant difference between all honey treatments was identified, P=<0.0001. D) MGO83 MIC of 0.029 g/mL, a significant difference was observed for honey treatments, P=<0.0001.

For the clinical isolates, the MICs were reduced for honey stored at 4 °C for 30 days prior to testing (Table 2.25). The highest MIC observed was 0.238 g/mL for isolates 189961, 194891, 199277, 211666 and DC088E when treated with MGO40 and MGO83. Interestingly, this higher MIC was not observed in response to either MGO55 or MGO70. The next highest MIC was 0.119 g/mL, in response to all 4 manuka honey samples tested for 6 of the clinical isolates. One isolate, DC088A had an MIC of 0.0595 g/mL for all of the manuka honey's tested. A further 8 clinical isolates were also inhibited by 0.0595 g/mL, these were 159544 for MGO40 and MGO83, 186144 for MGO70, 186154 for MGO40, 186433 for MGO83, DC088B for MGO70, DCO88C for MGO40, MGO55 and MGO70, DCO88D for MGO70 and MGO83 and DCO88ref for MGO40 and MGO83, showing variation depending on isolate and honey. MICs of 0.029 g/mL and 0.014 g/mL were also observed and this was dependent on isolate tested and manuka honey used. All raw data for the clinical isolates can be seen in Appendix 8.1.4, Figures 8.33 to 8.48.

Table 2.25 Minimum inhibitory concentrations for *M. abscessus* clinical isolates cultured at 37 °C treated with manuka honey stored for 30 days at 4 °C prior to testing (n=3)

Honey stored for 30 days, culture at 37°C	Minimum Inhibitory Concentration (g/mL)			
***M. abscessus* strain**	**MGO40**	**MGO55**	**MGO70**	**MGO83**
NCTC 13031	0.014	0.014	0.029	0.014
137071	0.029	0.014	0.014	0.014
147028	0.029	0.014	0.014	0.029
159544	0.0595	0.029	0.029	0.0595
186144	0.029	0.029	0.0595	0.029
186154	0.0595	0.029	0.029	0.029
186433	0.029	0.029	0.029	0.0595
189961	0.238	0.119	0.119	0.238
194891	0.119	0.119	0.119	0.238
199277	0.238	0.119	0.119	0.238
211666	0.238	0.238	0.238	0.238
DC088A	0.0595	0.0595	0.0595	0.0595
DC088B	0.029	0.029	0.0595	0.029
DC088C	0.0595	0.0595	0.119	0.0595
DC088D	0.029	0.014	0.0595	0.119
DC088E	0.238	0.119	0.119	0.238
DC088ref	0.0595	0.029	0.029	0.0595

The MBCs observed for manuka honey stored at 4 °C for 30 days prior to testing were either the same or lower than those observed previously for all *M. abscessus* clinical isolates (Table 2.26). Interestingly, an MBC was observed for all of the clinical isolates tested, which was not observed previously when the manuka honey was made fresh on the day of testing. However, some of the MBCs remained at 0.476 g/mL, this was seen for isolates 186154, 189961, 211666 and DC088A, regardless of the lower MICs observed for these isolates. A range of MBCs lower than 0.476 g/mL were observed for all other clinical isolates tested. This was also not observed for any of the clinical isolates tested previously with fresh honey. The lowest MBC observed was 0.029 g/mL manuka honey, for *M. abscessus* NCTC 13031, 137071 and 147028, depending on the honey tested. These isolates also had the lower MICs of 0.014 g/mL and 0.029 g/mL, depending on manuka honey.

Table 2.26 Minimum bactericidal concentrations for *M. abscessus* clinical isolates cultured at 37 °C treated with manuka honey stored for 30 days at 4 °C prior to testing (n=3)

Honey stored for 30 days, culture at 37°C	Minimum Bactericidal Concentration (g/mL)			
***M. abscessus* strain**	**MGO40**	**MGO55**	**MGO70**	**MGO83**
NCTC 13031	0.029	0.029	0.029	0.0595
137071	0.119	0.029	0.029	0.0595
147028	0.029	0.029	0.029	0.029
159544	0.119	0.0595	0.119	0.0595
186144	0.119	0.119	0.238	0.119
186154	0.238	0.238	0.476	0.238
186433	0.119	0.0595	0.0595	0.119
189961	0.476	0.238	0.476	0.476
194891	0.238	0.238	0.238	0.238
199277	0.238	0.238	0.238	0.238
211666	0.476	0.476	0.476	0.476
DC088A	0.238	0.476	0.238	0.476
DC088B	0.119	0.119	0.119	0.119
DC088C	0.238	0.119	0.119	0.238
DC088D	0.119	0.119	0.238	0.119
DC088E	0.238	0.119	0.119	0.238
DC088ref	0.119	0.0595	0.0595	0.238

2.3.6 Efficacy of manuka honey stored at different temperatures for 112 days against *M. abscessus* cultured at 37 °C

To assess if long term storage at different temperatures would impact the efficacy of the honey's antimicrobial activity, manuka honey stocks were prepared as described in section 2.2.3 and stored for 112 days at either 4 °C, 20 °C or 37 °C. The broth microdilution assay, described in section 2.2.12, was used to assess the antimicrobial activity against *M. abscessus* NCTC 13031. Growth curves were generated for each storage temperature (Figure 2.14, 2.15 and 2.16) and MICs and MBCs determined for all treatment conditions (Table 2.27). Overall, the MICs observed for honey stored for 112 days were lower than those identified for either no storage, short term storage or storage for up to 16 days prior to testing, regardless of storage temperature. However, the MICs after 112 days of honey storage were higher than that of storage for 30 days.

Manuka honey stored at 4 °C for 112 days prior to testing exhibited an MIC of 0.0595 g/mL or less, for all of the honey samples tested (Figure 2.14). A lower MIC of 0.029 g/mL was observed for MGO40 (Figure 2.14 A). Interestingly, MGO55, MGO70 and MGO83 also appeared to inhibit *M. abscessus* at a concentration of 0.029 g/mL until 72 h, after which growth was observed by optical density. A reduction in growth was observed for MGO40 at 0.014 g/mL and 0.007 g/mL compared to the control of *M. abscessus* only. End point data, taken at 96 h, had normal distribution and was analysed using a one-way ANOVA. This identified a statistically significant difference between treatments, P=<0.0001. To further analyse where the differences were, a Dunnett's comparison was conducted, comparing the growth of all treatments to the control of *M. abscessus* only. The Dunnett's comparison identified a significant difference between all concentrations compared to *M. abscessus* only (P=<0.0001 for all), apart from for 0.014 g/mL and 0.007 g/mL, P=0.0051 and P=0.1290, respectively. MGO55 was inhibitory against *M. abscessus* at 0.0595 g/mL, with a reduction in growth observed for 0.029 g/mL and no inhibition for 0.014 g/mL and 0.007 g/mL compared to *M. abscessus* only (Figure 2.14 B). A one-way ANOVA identified a statistically significant difference between the treatments, P=<0.0001. A Dunnett's comparison was performed, comparing all treatments to *M. abscessus* only. This identified a significant difference between all honey treatments and *M. abscessus* only, P=<0.0001, apart from for 0.014 g/mL and 0.007 g/mL, P=0.3771 and P=0.3064 respectively. Similar observations were made for MGO70. The MIC was 0.0595 g/mL with a reduction in growth for 0.029 g/mL and no inhibition for 0.014 g/mL and 0.007 g/mL. The end point data was analysed for normal distribution before further analysis. A one-way ANOVA identified a statistically significant difference between the treatments, P=<0.0001. A Dunnett's comparison identified a significant difference between the growth of *M. abscessus* only and the 4 highest concentrations of honey tested, P=<0.0001. For 0.029 g/mL, a significant difference was still identified but the P value was P=0.0046. The two lowest concentrations of 0.014 g/mL and

0.007 g/mL had no significant difference compared to *M. abscessus* only, P=0.9976 and P=0.9075 respectively. MGO83 also had an MIC of 0.0595 g/mL, but a reduction in growth was observed for 0.029 g/mL, 0.014 g/mL and 0.007 g/mL compared to *M. abscessus* only. The end point data had normal distribution and a one-way ANOVA was conducted. The one-way ANOVA identified a statistically significant difference between all treatments, P=<0.0001. A Dunnett's comparison identified a statistically significant difference for all concentrations of honey compared to *M. abscessus*, P=<0.0001 for all, apart from 0.014 g/mL and 0.007 g/mL which had a P value of P=0.0003 for both.

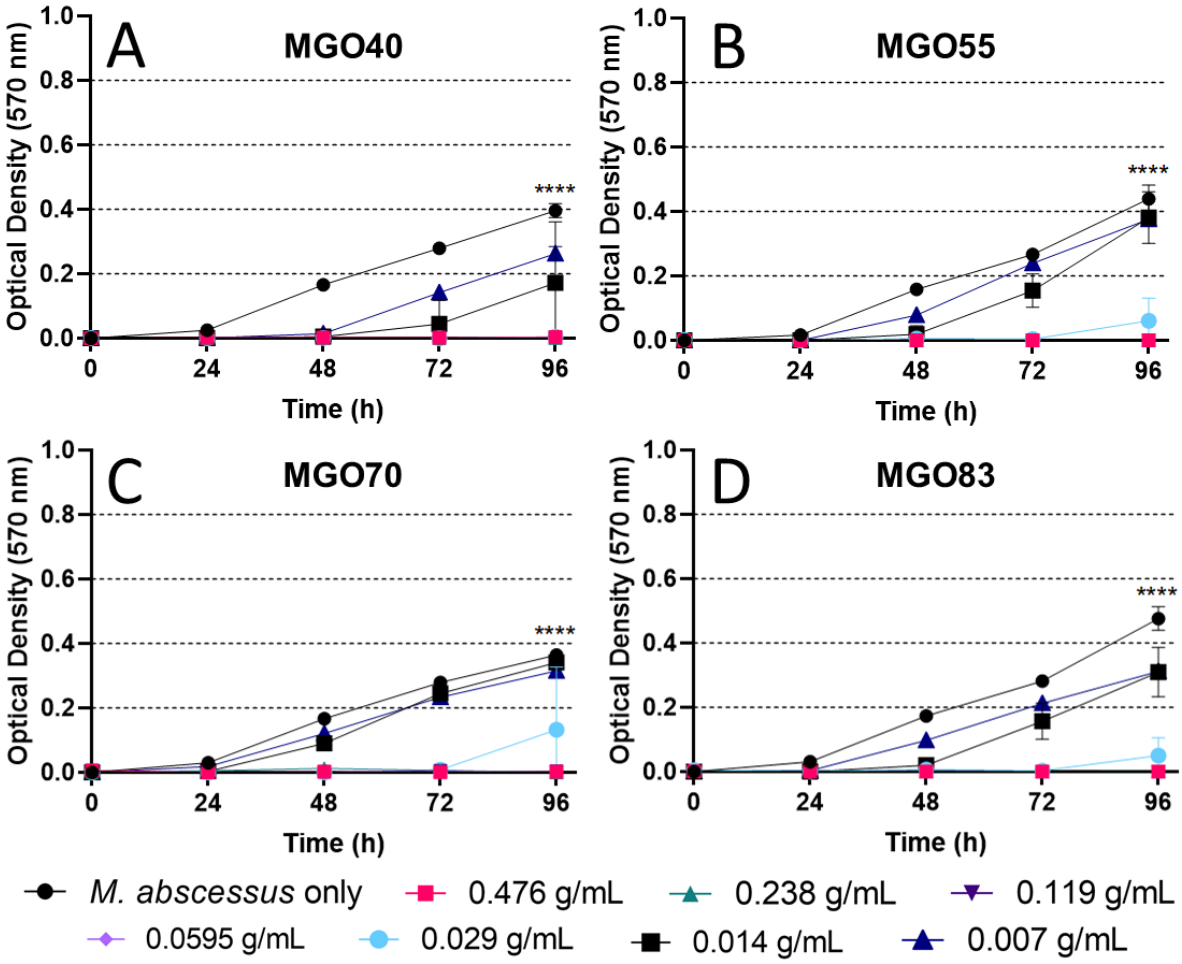

Figure 2.14 Growth Curves of *M. abscessus* NCTC 13031, cultured at 37 °C treated with 4 manuka honeys stored at 4 °C for 112 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 of the manuka honeys inhibited *M. abscessus* at a concentration of 0.0595 g/mL or less End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) MGO40 growth curve identifying an MIC of 0.029 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve identifying an MIC of 0.0595 g/mL. A significant difference was observed for all treatments, one-way ANOVA P=<0.0001. C) MGO70 growth curve showing an MIC of 0.0595 g/mL. A significant difference was identified between all treatments, one-way ANOVA P=<0.0001. D) MGO83 growth curve showing an MIC of 0.0595 g/mL. A one-way ANOVA identified a significant difference between all honey treatments, P=<0.0001.

The MBCs observed for manuka honey stored at 4 °C for 112 days prior to testing varied depending on the honey tested. MGO40 had an MBC of 0.029 g/mL, which was consistent with the MIC. This was also the same MBC observed for the same honey stored at 4 °C for 30 days prior to testing. MGO55 had the highest MBC of the 4 manuka honey's tested, which was 0.119 g/mL. This was largely increased from the lower MBC of 0.029 g/mL after 30 days of storage. However, this is still an improved MBC compared to an MBC of 0.476 g/mL for honey used immediately or no MBC for honey stored for up 16 days prior to testing. Both MGO70 and MGO83 had an MBC of 0.0595 g/mL, which was consistent with the MIC observed. This was slightly higher than the MBC observed after 30 days. However, this is still an improved MBC compared to the previous MBCs of 0.476 g/mL for no prior storage.

Manuka honey stored at 20 °C for 112 days prior to testing exhibited higher MICs than those observed for the same duration of storage at 4 °C. For all 4 manuka honey samples tested, the MIC was 0.238 g/mL, and a range of inhibition was observed for other concentrations tested (Figure 2.15). MGO40 exhibited a range of activity against *M. abscessus*. The higher concentrations of 0.476 g/mL and 0.238 g/mL were inhibitory, and some lower concentrations reduced the growth of *M. abscessus*. However, 0.007 g/mL honey appeared to improve the growth of *M. abscessus* in comparison to the control with no honey (Figure 2.15 A). The end point data was checked for normal distribution and a one-way ANOVA was conducted. This identified a significant difference between all treatments, one-way ANOVA P=<0.0001. A Dunnett's comparison was conducted comparing all honey concentrations to *M. abscessus* only. This identified a statistically significant difference between both 0.476 g/mL and 0.238 g/mL compared to *M. abscessus*, P=<0.0001 for both. A significant difference was also seen for 0.119 g/mL, P=0.0008. No significance was identified for 0.0595 g/mL, 0.029 g/mL and 0.014 g/mL, P=0.2326, P=0.9999 and P=0.3288, respectively. Interestingly, a significant difference was observed for 0.007 g/mL and *M. abscessus*, P=0.0056, suggesting that this concentration significantly improved the growth, rather than having no effect. MGO55 also had an MIC of 0.238 g/mL, but there was less variation in inhibition for the lower concentrations (Figure 2.15 B). A one-way ANOVA showed a significant difference between all treatments, P=<0.0001. A Dunnett's comparison was conducted comparing all honey concentrations to *M. abscessus* only. A significant difference was identified between both 0.476 g/mL and 0.238 g/mL compared to *M. abscessus* only, P=<0.0001 for both. No significance was identified for 0.119 g/mL, 0.0595 g/mL and 0.029 g/mL, P=0.9994, P=0.9058 and P=0.1883 respectively. Similar to observations for MGO40, the lower concentrations of 0.014 g/mL and 0.007 g/mL for MGO55 had a significant difference compared to *M. abscessus* only, P=0.0171 and P=0.0213 respectively. This suggests that at these concentrations the growth of *M. abscessus* was improved in the presence of manuka honey. MGO70 also exhibited an MIC of 0.238 g/mL with all other concentrations having minimal impact on the growth of *M. abscessus* compared to the

growth of *M. abscessus* only (Figure 2.15 C). A one-way ANOVA identified a significant difference between the treatments, P=<0.0001. A Dunnett's comparison, comparing all honey concentrations to *M. abscessus* was conducted and identified a significant difference between 0.476 g/mL and 0.238 g/mL against *M. abscessus* only, P=<0.0001 for both. No significant difference was observed for 0.119 g/mL, 0.014 g/mL and 0.007 g/mL, P=0.9995, P=0.4756 and P=0.8730 respectively. However, there was a significant difference between both 0.0595 g/mL and 0.029 g/mL against *M. abscessus*, P=0.0166 and P=0.0155 respectively. This was not due to the inhibition of *M. abscessus* because the OD reads for these concentrations were higher than that of *M. abscessus* only, suggesting the growth of *M. abscessus* at these concentrations was improved compared to the control (Figure 2.15 C). The same occurrence noted for MGO70 was also observed for MGO83. The MIC remained at 0.238 g/mL with some variation in the growth of lower concentrations. A one-way ANOVA showed a significant difference between all treatments, P=<0.0001. A Dunnett's comparison showed a significant difference for 0.476 g/mL and 0.238 g/mL against *M. abscessus* only, P=<0.0001 for both. Furthermore, no significant difference was observed for 0.119 g/mL, 0.014 g/mL and 0.007 g/mL, P=0.3553, P=0.88365 and P=0.4794 respectively. Additionally, a significant difference was identified for 0.0595 g/mL and 0.029 g/mL against *M. abscessus*, P=0.0018 and P=0.0002 respectively. This was also due to higher ODs for these concentrations of honey compared to the control of *M. abscessus* only, not due to inhibition.

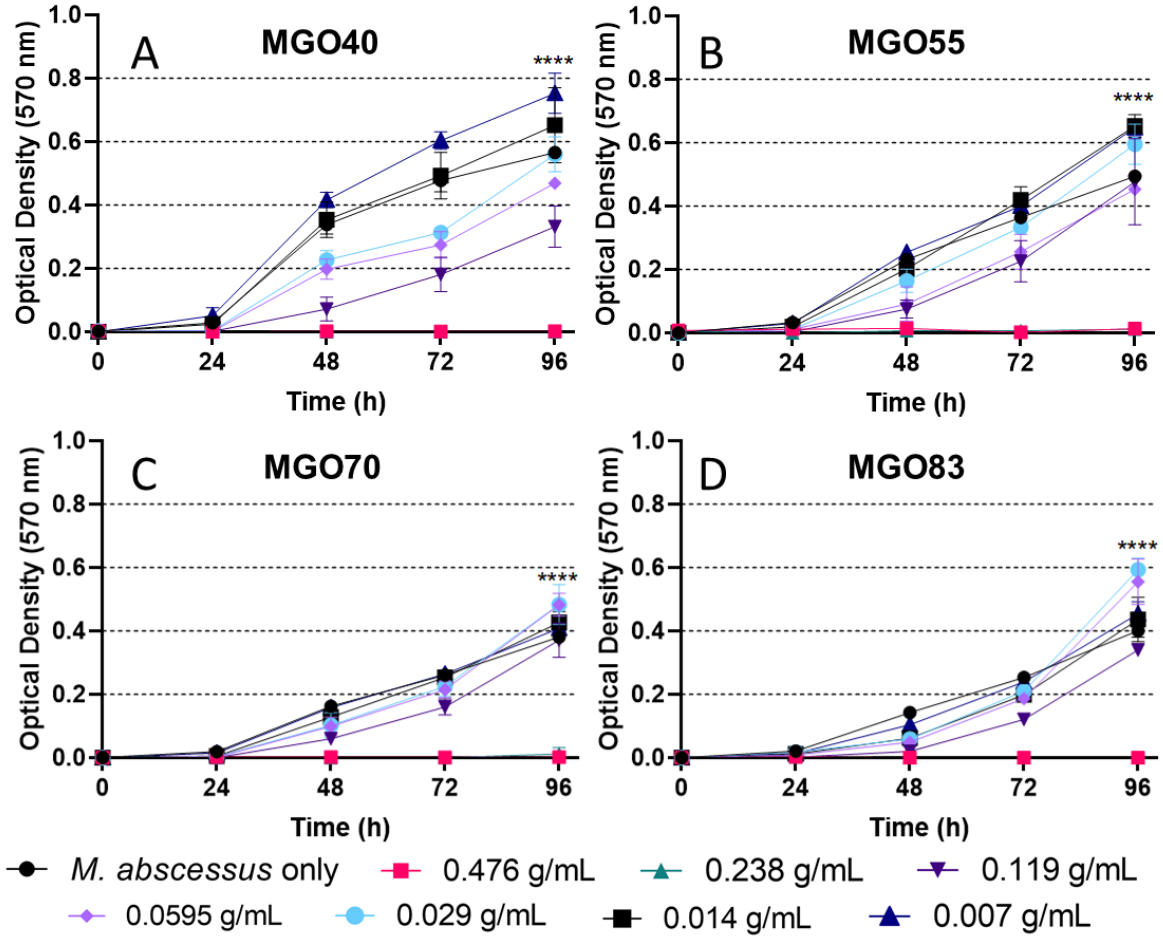

Figure 2.15 Growth Curves of *M. abscessus* NCTC 13031, cultured at 37 °C treated with 4 manuka honeys stored at 20 °C for 112 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 of the manuka honeys inhibited *M. abscessus* at a concentration of 0.238 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) MGO40 growth curve showing and MIC of 0.238 g/mL. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. B) MGO55 growth curve showing an MIC of 0.238 g/mL. A one-way ANOVA showed a significant difference for all treatments, P=<0.0001. C) MGO70 growth curve showing an MIC of 0.238 g/mL. A significant difference was identified between all treatments, one-way ANOVA P=<0.0001 D) MGO83 growth curve with an MIC of 0.238 g/mL. One-way ANOVA identified a significant difference between all treatments, P=<0.0001.

The MBCs for all 4 manuka honey's stored at 20 °C for 112 days were 0.476 g/mL. This was similar to the observations of the honey stored at 20 °C and tested for 16 days (Section 2.3.4). For all of the manuka honey's an MBC of 0.476 g/mL was observed after 16 days, with variations of no MBC and of 0.476 g/mL depending on day of testing. This suggests that manuka honey can maintain bactericidal activity with long term storage prior to use.

Manuka honey stored at 37 °C for 112 days prior to testing exhibited an MIC of 0.238 g/mL or less, with reductions in growth of *M. abscessus* for subsequent concentrations of honey (Figure 2.16). MGO40 had the lowest MIC of the honey's tested, which was 0.119 g/mL (Figure 2.16 A). A reduction in growth was observed for all concentrations of honey. A one-way ANOVA was conducted and a significant difference was identified for all treatments, P=<0.0001. A Dunnett's comparison was conducted comparing all honey concentrations to the control of *M. abscessus* only and there was a significant difference between all treatments, P=<0.0001 for all. This was not observed for MGO55. The MIC for MGO55 was 0.238 g/mL and a larger variation in inhibition was seen for lower concentrations of honey (Figure 2.16 B). A one-way ANOVA identified a significant difference between the treatments, P=<0.0001. A Dunnett's comparison, comparing all honey concentrations to *M. abscessus* only, showed a significant difference between all honey concentrations and *M. abscessus*, P=<0.0001 for all apart from 0.014 g/mL (P=0.0002) and 0.007 g/mL (P=0.0491). This suggests that all concentrations of honey had an impact on the growth of *M. abscessus*, even if not fully inhibitory. MGO70 also exhibited an MIC of 0.238 g/mL, with a reduction in growth for 0.119 g/mL and 0.0595 g/mL (Figure 2.16 C). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's comparison, comparing all honey treatments to *M. abscessus* only, identified a significant difference between 0.476 g/mL, 0.238 g/mL, 0.119 g/mL and 0.0595 g/mL compared to *M. abscessus*, P=<0.0001 for all. No significant difference was identified between 0.029 g/mL (P=0.9761), 0.014 g/mL (P=0.1081) and 0.007 g/mL (P=0.1599). Suggesting that these lower concentrations did not impact the growth of *M. abscessus*. A similar occurrence was observed for MGO83, with an MIC of 0.238 g/mL and a reduction in growth for 0.119 g/mL (Figure 2.16 D). A one-way ANOVA identified a significant difference between the treatments, P=<0.0001. A Dunnett's comparison was conducted comparing all honey concentrations to *M. abscessus* only. A significant difference was identified for 0.476 g/mL, 0.238 g/mL and 0.119 g/mL compared to *M. abscessus* only, P=<0.0001 for all. A significant difference was also identified for 0.0595 g/mL (P=0.0015) and 0.029 g/mL (P=0.0398). No significant difference was observed for 0.014 g/mL (P=0.8943) and 0.007 g/mL (P=0.6511), suggesting these concentrations did not impact the growth of *M. abscessus*.

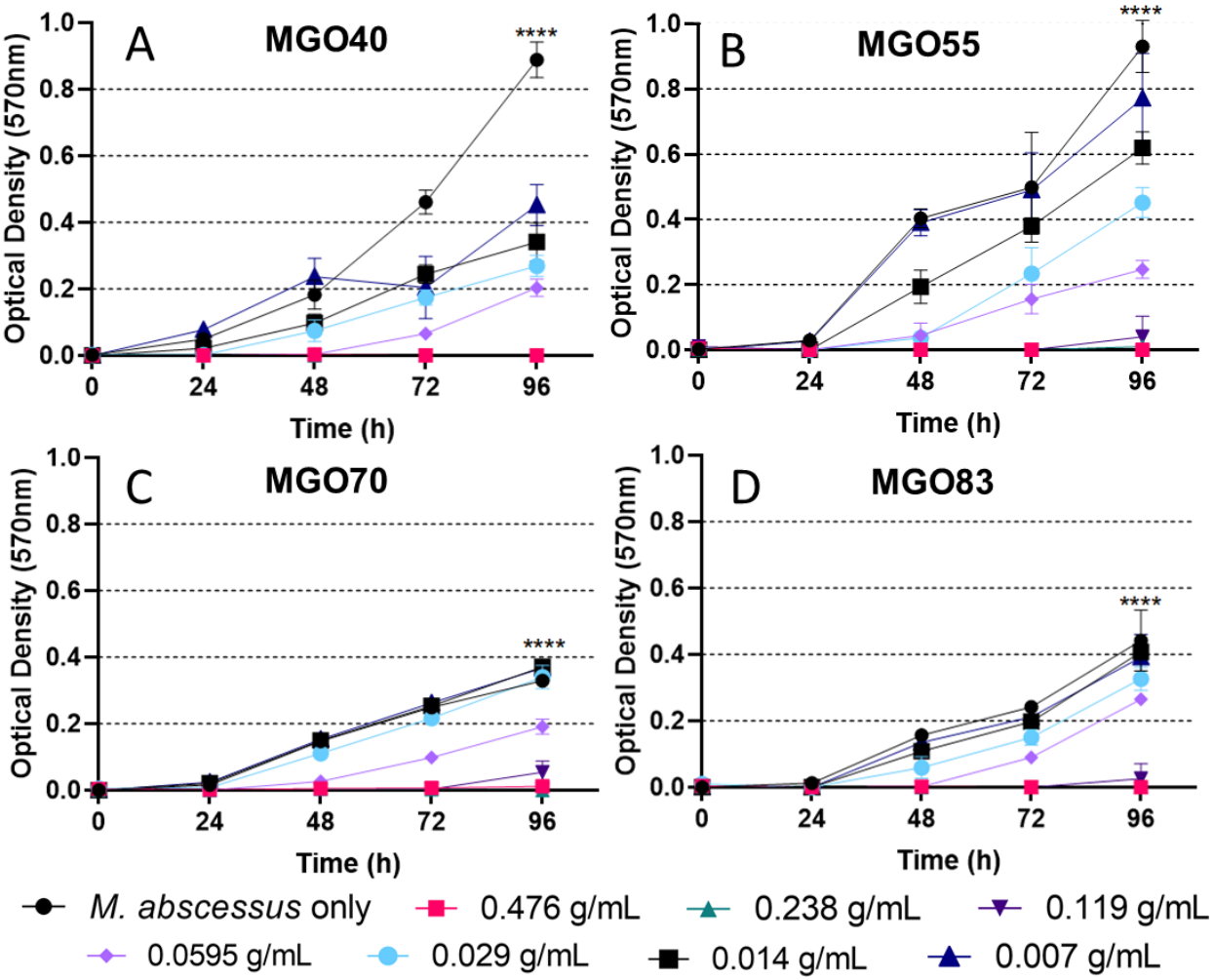

Figure 2.16 Growth Curves of *M. abscessus* NCTC 13031, cultured at 37 °C treated with 4 manuka honeys stored at 37 °C for 112 days prior to testing. Data shown are mean + SD for n=3 technical replicates. The manuka honeys inhibited *M. abscessus* at a concentration of 0.238 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus*. A) MGO40 growth curve, showing an MIC of 0.119 g/mL. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. B) MGO55 growth curve, identifying an MIC of 0.238 g/mL. One-way ANOVA showed a significant difference between all treatments, P=<0.0001. C) MGO70 growth curve showing an MIC of 0.238 g/mL. One-way ANOVA identified a significant difference between the treatments, P=<0.0001. D) MGO83 growth curve identifying an MIC of 0.238 g/mL. One-way ANOVA identified a significant difference between the treatments, P=<0.0001.

There was some variation in MBC for the manuka honey's stored at 37 °C for 112 days prior to testing. MGO40, MGO70 and MGO83 all had an MBC of 0.238 g/mL, which was consistent with the MICs observed for MGO70 and MGO83, but not for MGO40 which exhibited the lowest MIC. MGO55 had a higher MBC of 0.476 g/mL, regardless of maintaining the MIC of 0.238 g/mL. However, this was consistent with MGO55 stored at 37 °C for 16 days. Suggesting that the antimicrobial activity was not lost by longer term storage.

Table 2.27 MICs and MBCs for manuka honey stored for 112 days at 4 °C, 20 °C and 37 °C tested against *M. abscessus* NCTC 13031

Honey stored for 112 days, culture at 37 °C	Minimum Inhibitory Concentration (g/mL)			
Storage Temperature	**MGO40**	**MGO55**	**MGO70**	**MGO83**
4 °C	0.029	0.0595	0.0595	0.0595
20 °C	0.238	0.238	0.238	0.238
37 °C	0.119	0.238	0.238	0.238
	Minimum Bactericidal Concentration (g/mL)			
4 °C	0.029	0.119	0.0595	0.0595
20 °C	0.476	0.476	0.476	0.476
37 °C	0.238	0.476	0.238	0.238

2.4 Discussion

M. abscessus infections, both pulmonary and skin and soft tissue, have become increasingly prevalent and complicated to treat. With the antibiotics available for treatment dwindling due to increased resistance, alternatives are urgently required. In this chapter, it has been demonstrated that manuka honey can successfully inhibit *M. abscessus* and a panel of clinical isolates, all with differing drug resistance susceptibilities (Appendix 8.1.1). Not only can manuka honey act as a bacteriostatic agent, but bactericidal activity was also observed, something that has not yet been reported. Furthermore, the culture temperature of *M. abscessus* can impact its susceptibility to antimicrobial treatment, a consideration for future *M. abscessus* susceptibility testing. These were not the only important findings. Storage conditions of manuka honey can also impact the activity, with freshly made manuka honey being the least effective. Additionally, storage at 4 °C for 30 days results in drastically improved activity. These findings are of importance when considering manuka honey as a possible treatment for *M. abscessus* infections, both practically within a medical setting and for ensuring the optimum treatment outcomes for patients.

The initial assessment of manuka honey against *M. abscessus* was conducted with honey made fresh on the day of testing and *M. abscessus* cultured at 30 °C. Due to the nature of honey's antimicrobial activity, it is often suggested that honey should be made fresh at the point of use to ensure that no activity is lost. This is attributed to the presence and generation of hydrogen peroxide within the honey, which can be activated rapidly upon the addition of H_2O and also expended in a short time (Bang, Buntting and Molan, 2003; Brudzynski *et al.*, 2017; Brudzynski, 2020). Therefore, all initial screening was conducted with fresh manuka honey. Additionally, the culture conditions used to grow *M. abscessus* can be varied, due it being an environmental pathogen. When *M. abscessus* is isolated from patients, the culture temperature selected is typically 30 °C (Preece *et al.*, 2016; Brown-Elliott *et al.*, 2019). Therefore, this was the temperature initially selected. Under these conditions, the manuka honey successfully inhibited *M. abscessus* NCTC 13031 at a concentration of 0.476 g/mL. This was also observed for the *M. abscessus* clinical isolates, where the MIC was either 0.476 g/mL or less, regardless of the MGO strength of the honey (Table 2.2, Appendix 8.1.2). Interestingly, MGO55 exhibited an MIC of 0.238 g/mL for the majority of clinical isolates (Table 2.2). Although an MIC was observed for all strains of *M. abscessus* tested, this was not the same for MBCs (Table 2.3). The only honey to exhibit an MBC for all isolates tested was MGO55. This increased activity observed for MGO55 could be due to either higher concentrations of hydrogen peroxide in this honey or inaccurate classification of MGO. Due to how manuka honey is classified, the content of the precursor DHA is not always considered at the time of testing and therefore, over time a higher concentration of MGO could be present. It is also possible that there was a loss of MGO in the stronger manuka honey's tested, but this is unlikely. Scientific literature on the loss of MGO in manuka honey

is limited but New Zealand Honey Co suggest that with proper storage the MGO should last long after the expiration date, indicating that this should not have happened for the manuka honey used here, since they were purchased and used inside the expiration date. Furthermore, a study exploring the conversion of DHA to MGO over time identified in an artificial honey doped with a known concentration of MGO in an aqueous solution that MGO loss did not occur over 479 days (Grainger, Manley-harris, *et al.*, 2016b). Additionally, a loss of MGO was only observed in artificial honey doped with either propylamine or diethylamine. The artificial honey used only simulated the high sugar content and osmolarity without consideration for the multiple other components that make honey unique, resulting in demonstrating that the propylamine or diethylamine were the main causes for MGO loss and not the artificial honey itself. It was also stated that experiments were conducted in aqueous solutions which would not be indicative of occurrences in undiluted honey (Grainger, Manley-harris, *et al.*, 2016a; Grainger, Manley-Harris, *et al.*, 2016b). This suggests that loss of MGO within the undiluted manuka honey would not have occurred. Unfortunately, the MGO concentrations were not independently determined and therefore the concentrations of MGO present could be different to those stated by the manuka honey rating on the jar.

Considering that *M. abscessus* is a human opportunistic pathogen, the initial screening was repeated at the clinically relevant culture temperature of 37 °C. The MIC for *M. abscessus* NCTC 13031 remained at 0.476 g/mL, regardless of the manuka honey used. This was also the case for the majority of the clinical isolates. Although the MGO rating of MGO83 is double that of MGO40, the difference in MGO concentration is not significant enough to cause a noticeable difference in MIC or MBC, moreover manuka honey can be in excess of MGO925, highlighting the minimal difference between MGO40 and MGO83. Arguably, the difference in antimicrobial activity from MGO83 should be double that of MGO40 but considering the scale of MGO content in other available manuka honey it can be seen that this would not necessarily be the case. Notably, there was an increased MIC of 0.476 g/mL for the majority of clinical isolates tested against MGO55 compared to the MIC of 0.238 g/mL when cultured at 30 °C. More importantly, the biggest difference between culturing at 30 °C and 37 °C was in bactericidal activity. When cultured at 30 °C, only 7 of the clinical isolates had an MBC for MGO40, whereas culturing at 37 °C resulted in 12 isolates now having an MBC. Similar was also observed for MGO83, where 7 clinical isolates exhibited an MBC at 30 °C, but 13 clinical isolates had an MBC when cultured at 37 °C. However, the opposite was observed for MGO55 and MGO70 and there was a loss of MBC when culturing at 37 °C compared to 30 °C. These differences in MIC and MBC could be due to multiple factors. One of the main contributing factors could be due to a morphological switch in *M. abscessus* (Section 1.1.4). This change in morphology from the environmental smooth variant to the rough variant has been associated with increased virulence and the ability to evade host immune defences (Kim *et al.*, 2019). This switch has been suggested to occur due, in part, to changes in

temperature (Ryan and Byrd, 2018). Thus, the higher culture temperature of 37 °C could be resulting in phenotypic changes in *M. abscessus*, resulting in an altered response to manuka honey treatment. Another contributing factor that could be influencing this change in susceptibility could be due to the temperature affecting the manuka honey. The presence of MGO in manuka honey is due to the precursor of DHA that is present in the flower nectar (Section 1.2.3.4). The conversion of DHA to MGO happens nonenzymatically over time and is referred to as manuka honey ripening (Cokcetin *et al.*, 2016). However, by heating to 37 °C this process can be accelerated, and this temperature has been highlighted as the optimum temperature for DHA to MGO conversion (Adams, Manley-Harris and Molan, 2009). Furthermore, it is standard in honey research to make honey stocks fresh at the time of testing and not allow them to age due to the possible degradation of antimicrobial components, most commonly the hydrogen peroxide which exhausts rather rapidly upon dilution (Brudzynski, 2020). However, the antimicrobial activity of manuka honey is based on MGO, not hydrogen peroxide, therefore the antimicrobial activity should not be impacted by preparing dilutions in advance. Additionally, the MGO rating attributed to a given honey is due to the amount of MGO present at the time of testing, therefore this is the minimum amount of MGO present and could increase over time. Suggesting that the manuka honey could potentially have more MGO present, than the MGO grade indicates. Therefore, changing the culture temperature to 37 °C could have increased the amount of MGO present within the honey and impacted the antimicrobial activity. To further investigate the cause of change in MIC and MBC, the broth microdilution assay was repeated with some alterations (Section 2.2.7 and Section 2.3.3).

Since it was unknown whether it was the culture temperature or the heating of the manuka honey that influenced the change in MIC and MBC, both of these factors were altered and the broth microdilution assay repeated. To determine if heating of the manuka honey influenced the activity, the honey stocks were prepared and heated to 37 °C for 1 h prior to testing. After the initial 1 h, subsequent testing was conducted every hour, up to 6 h. Simultaneously, to see if culture temperature was the driving force for MIC and MBC changes, *M. abscessus* cultures were grown at 30 °C and 37 °C, with testing conducted at both temperatures. This would allow for accelerated conversion of DHA to MGO, whilst also being able to determine if the culture temperature impacted the activity. Overall, there was no variation in MIC for all manuka honey's and time points tested against *M. abscessus* when cultured at 30 °C, remaining at 0.476 g/mL (Section 2.3.3). However, variation in response to the lower concentrations was observed. A variation in MIC was observed for *M. abscessus* cultured at 37 °C depending on honey and preheating time, with the majority of time points exhibiting an MIC of 0.476 g/mL but some having an MIC of 0.238 g/mL (Section 2.3.3). Interestingly, there was no MBC observed for *M. abscessus* cultured at 30 °C. There was also inconsistent MBCs for *M. abscessus* cultured at 37 °C, with only some MBCs identified

for each manuka honey. Additionally, MBCs were identified for *M. abscessus* cultured at 37 °C treated with MGO40, MGO55 and MGO70 when preheated for 3 h before testing. There was only one MBC identified in response to MGO83, this was for *M. abscessus* cultured at 37 °C with honey preheated for 6 h prior to testing. These findings suggest that preheating the honey can improve the efficacy when culturing at 37 °C, which is an important consideration for therapeutic uses. However, within a clinical setting it may not be possible to heat honey before administration, but this does suggest that making a honey solution ahead of time could result in improved antimicrobial activity, a finding that has not yet been explored for honey therapy. This is also beneficial for clinical use, as honey treatments can be premade before administering without compromising the activity. Furthermore, *M. abscessus* cultured at 30 °C resulted in a loss of bactericidal activity when the manuka honey was preheated. Indicating that the culture temperature is impacting the response to antimicrobial treatment, for *M. abscessus*. Considering that *M. abscessus* is a pathogen that causes pulmonary infections that are difficult to treat, it is arguably more important to focus on the internal physiological temperature of 37 °C for the purpose of this study, rather than lower temperatures of 30 °C which are more associated with external physiological temperatures of skin. Therefore, further exploration into the storage conditions, both length of time and temperature, of manuka honey were investigated, with the focus on *M. abscessus* cultured at 37 °C.

The storage temperatures selected to assess the influence upon manuka honey's antimicrobial activity were 4 °C, 20 °C and 37 °C. Both 4 °C and 20 °C were selected due to the likelihood of storage conditions within a clinical setting and those realistically achievable, such as fridge or cupboard storage. The continuation of storage at 37 °C was also included, to observe if the accelerated conversion of DHA to MGO would impact the antimicrobial activity. Interestingly, there was limited variation in MIC and MBC in response to honey storage. Manuka honey stored at 4 °C and 37 °C largely remained the same with the majority of MICs and MBCs staying at 0.476 g/mL and only day 4 of honey storage having a loss of MBC. Storage at 20 °C showed more variation in MIC, with all of the manuka honey's having an MIC of 0.238 g/mL for at least one of the time points. However, this was not the same for MBC, and MBCs either remained at 0.476 g/mL or did not have an MBC. This overall maintenance of MIC and MBC in response to honey storage is an important finding, suggesting potential conversion of DHA to MGO occurring more rapidly upon dilution. The occurrence of 1,2-dicarbonyls in honey, such as MGO, glyoxalase and 3-deoxyglucosone were originally considered a consequence of sugar degradation or caramelisation (Weigel, Opitz and Henle, 2004). Exploration into a wide range of honey types identified between 80 mg/kg and 1270 mg/kg of 3-deoxyglucosone but relatively low levels of MGO or glyoxalase, roughly 5 mg/kg, apart from the honey derived from manuka which contained up to 760 mg/kg (Weigel, Opitz and Henle, 2004; Mavric *et al.*, 2008; Marceau and Yaylayan, 2009).

The occurrence of MGO within manuka honey was later determined to be due to the presence of DHA within the flower nectar of manuka plants (Adams, Manley-Harris and Molan, 2009) (Section 1.2.3.4). Further exploration into this identified that DHA within honey exists in the dimer form but for MGO conversion the monomer form of DHA is required (Grainger, Manley-Harris, *et al.*, 2016a). The conversion of DHA dimer to monomer happens readily in an aqueous solution (Davis, 1973). The irreversible generation of MGO from DHA is then achieved non-enzymatically through a dehydration reaction (Rückriemen *et al.*, 2015). Therefore, it is possible that with the addition of H_2O to generate the honey stocks the conversion of DHA dimer to DHA monomer occurs and allows for greater acceleration of DHA to MGO conversion. This conversion from DHA to MGO can also be accelerated by heating to 37 °C (Grainger, Manley-Harris, *et al.*, 2016a). This could result in improved antimicrobial activity due to an increase of MGO, the main component giving rise to the antimicrobial activity. Therefore, it is possible that the antimycobacterial activity observed here is due to increased MGO, and not hydrogen peroxide. Additionally, any loss of hydrogen peroxide could be contributing to the changes in bactericidal activity, due to the possible synergistic activity of the two components in honey. This shows that storage of premade manuka honey does not negatively impact the antimicrobial activity and it can in some cases improve the inhibitory activity.

It has been suggested that the conversion of DHA to MGO is accelerated at 37 °C but does not occur at 4 °C (Grainger, Manley-Harris, *et al.*, 2016a). However, over the 16 days of honey storage a change in activity was observed for manuka honey stored at 4 °C. For MGO40 there was no bactericidal activity until after 7 days of storage and MGO83 exhibited the lower MIC of 0.238 g/mL for days 7 and 11. This suggests that the DHA to MGO conversion could be occurring, since the hydrogen peroxide is likely to be expended. If storage of premade honey stocks remain stable or improve at 4 °C it could be an important and beneficial step in the treatment of *M. abscessus* infections. Meaning that treatment could be made ahead of time and used over multiple days, rather than being limited to only one day. To further explore if storage at 4 °C would impact the antimicrobial activity, manuka honey was prepared and stored at 4 °C for 30 days prior to testing. The repeat testing also included the clinical isolates to gain further insight into how the honey would impact the growth of *M. abscessus*, and thus be impacted in a clinical setting. All testing was conducted with *M. abscessus* cultured at 37 °C. Interestingly, the storage of manuka honey at 4 °C for 30 days greatly improved the activity of the manuka honey and MICs were drastically reduced, from 0.476 g/mL to either 0.029 g/mL for MGO70 or 0.014 g/mL for the other manuka honey's (Figure 2.13) (Table 2.5 and Table 2.25). This was also the same for MBCs, with all of the clinical isolates now having an MBC and almost all of the clinical isolates having a lower MBC than for fresh manuka honey (Table 2.6 and Table 2.26). This change in MIC and MBC could be due to the DHA to MGO conversion, not previously observed

occurring at 4 °C. However, this could not be determined because there was no access to either a high performance liquid chromatography or mass spectrometer to measure the MGO concentration. Nevertheless, the improved antimycobacterial activity is significant and requires further exploration into why this occurred.

After identifying that storage of manuka honey for 30 days improved the antimicrobial activity significantly, longer term storage was explored. Honey stocks were stored at 4 °C, 20°C and 37 °C for 112 days before testing. All of the MICs after 112 days were lower than the initial MICs observed for fresh manuka honey, regardless of the storage temperature (Table 2.5) (Figure 2.14, Figure 2.15 and Figure 2.16). Interestingly, the manuka honey stored at 4 °C exhibited the lowest MICs and MBCs, regardless of the manuka honey tested. The MICs were higher than those observed for the 30 day storage, typically 0.0595 g/mL compared to the previous 0.014 g/mL or 0.029 g/mL, however the MBCs remained similar being 0.0595 g/mL. It is possible that a saturation point of DHA to MGO conversion occurred and resulted in the degradation of MGO between 30 days and 112 days of storage. Thus, impacting the MIC due to a reduction in MGO, however still maintaining higher levels of MGO than initially and therefore maintaining the MBC. Storage at 20 °C was arguably the least effective, having the highest MICs and MBCs, though these were still lower than in the initial testing with fresh manuka honey. The activity of manuka honey stored at 37 °C was similar to that of 20 °C but with improved MBCs of 0.238 g/mL rather than 0.476 g/mL. Ultimately, the storage of manuka honey for 112 days improved the activity compared to no storage and the storage temperature determined to what extent, with the lower temperature exhibiting the most improved activity. This is something that has not yet been explored in honey research and outlines that there are more complex interactions occurring within honey than originally considered. Limiting antimicrobial susceptibility testing to only freshly made honey is drastically limiting the potential of this antimicrobial agent and further exploration into what is happening is required to utilize the full potential of manuka honey and manuka honey therapy.

More importantly, it has been shown here that manuka honey is inhibitory against *M. abscessus* and a range of clinical isolates. The MGO strength of the manuka honey used in this study were also typically low, with significantly higher grades available on the market, however all 4 exhibited inhibitory activity. This shows potential to further this work and explore the possibility of higher grade manuka honey and some of the more potent medical grade honey's as a solution for *M. abscessus* infections. Furthermore, under the right conditions, the manuka honey was also bactericidal, highlighting that manuka honey is an effective antimycobacterial agent. Future work around the efficacy of manuka honey against *M. abscessus* and other mycobacterial species should be considered, exploring higher strength manuka honey and other opportunistic mycobacteria. A focus on the impact of storage conditions on the antimicrobial efficacy of the honey should also be conducted, with

an emphasis on the changes in chemical composition upon dilution and subsequent storage. Repeating the long term storage conditions with mass spectrometer analysis at the beginning of the experiment, during the experiment and at the end of the experiment would help to elucidate not only what is happening to the manuka honey over time but also highlight the components causing the antimycobacterial activity. If the antimicrobial components were to be identified, this could be developed into a targeted treatment for mycobacterial infections, lessening the burden on the antimicrobial resistance crisis whilst also helping to combat otherwise difficult to treat infections.

Chapter 3: Nebulised manuka honey and antibiotic combinations for *M. abscessus* treatment using an *in vitro* assay

3.1 Introduction

3.1.1 Current inhalation therapies

Inhalation therapy for *M. abscessus* pulmonary infections is often used alongside an I.V. administered drug regimen as an effective strategy to combat pulmonary infections. It allows for direct administration of antibiotic to the site of infection, delivering a high dose and reducing side effects associated with I.V. antibiotics (Tiddens *et al.*, 2014). When considering antibiotics for inhalation treatments, antibiotic absorption rates are important. Slower absorbed antibiotics allow for higher local concentrations and reduced systemic side effects (Zhou *et al.*, 2015). This is due to the dead end of the alveolar space, where antibiotic elimination is achieved through clearance mechanisms within the lung or through absorption, unlike oral antibiotics that are eliminated through absorption, distribution, metabolism and excretion (Hastedt *et al.*, 2016). The antibiotics tobramycin, colistin and aztreonam have been identified as ones with low absorbance rates, thus resulting in higher concentrations within the epithelial lining fluid (Marchand *et al.*, 2010, 2015, 2016). Whereas, inhaled moxifloxacin and ciprofloxacin have the opposite effect, with high permeability resulting in low concentrations maintained within the lung and therefore achieving similar concentrations to intravenous administration (Gontijo *et al.*, 2014). These findings have provided a basis for further development of inhalation therapies based on amikacin, tobramycin, aztreonam and colistin (Brillault and Tewes, 2020).

One of the first approved inhalation therapies for NTM pulmonary infections was amikacin (Banaschewski and Hofmann, 2019). Now it is routinely used and is a major component of treatment regimes, often administered in the continuation phase of treatment (Table 1.1). Although administering nebulised amikacin for NTM pulmonary infection can result in sputum conversion, it unfortunately has negative side effects associated, with up to 50% of patients developing toxicity and 35% of patients reporting adverse events (Olivier *et al.*, 2014; Yagi *et al.*, 2017). This led to further research into improving nebulised amikacin, resulting in the development of liposomal amikacin which has been shown to be as effective as free amikacin, with improved alveolar macrophage uptake (Rose *et al.*, 2014; Malinin *et al.*, 2016; Zhang *et al.*, 2018). It gained FDA approval in 2018 and appears a promising alternative to free amikacin. However, side effects of cough, dyspnoea and ototoxicity are still present, causing some patients to discontinue the treatment (Henriette Zweijpfenning *et al.*, 2022). Further studies and clinical trials into the clinical use of liposomal amikacin are required to fully assess its potential role in treatment of pulmonary infection.

Another potential inhalation treatment for pulmonary infections is powdered tobramycin. The use of inhaled tobramycin was first introduced in the 1990s, where it was administered to cystic fibrosis patients with *Pseudomonas aeruginosa* infections (Quon, Goss and Ramsey, 2014). The benefit of powdered tobramycin over liposomal amikacin is due to the administration of the antibiotic formulation. By administering a dry powder using a specifically

designed inhaler, as opposed to a solution, the lengthy treatment time is drastically reduced and the deep cleaning of nebulising equipment is removed, making it a favourable alternative (Geller *et al.*, 2007). However, inhaled tobramycin is not routinely used for *M. abscessus* infections, and only one case study in a paediatric patient has shown its potential application for NTM pulmonary infections (Anisowicz, Welsh and Gross, 2016). The slower absorption rate of tobramycin make it a potential candidate for *M. abscessus* pulmonary infections.

Azithromycin is routinely prescribed as part of the drug regimen to treat *M. abscessus* infections (Table 1.1). Although it is often administered orally, it is a good candidate for inhalation therapy due to high concentrations being achieved in lung epithelial lining fluid and alveolar macrophages (Capitano *et al.*, 2004). The side effects associated with azithromycin are often intolerable, such as gastrointestinal issues and hearing loss, which could be mitigated by inhalation, rather than oral doses (Hickey *et al.*, 2006). Nebulised azithromycin has also been shown to be effective at reducing *P. aeruginosa* infections in cystic fibrosis patients, with improved patient quality of life (Maneshi *et al.*, 2019). The subspeciation of *M. abscessus* could impact the efficacy of azithromycin therapy, but it could also provide an effective treatment (Section 1.1.3).

These inhalation therapies present a possible solution for *M. abscessus* pulmonary infection but could be improved. After demonstrating the efficacy of manuka honey against *M. abscessus* clinical isolates (Chapter 2), the next step would be to improve on established current therapies, such as combining manuka honey with nebulisation therapy to improve efficacy. Previously it has been shown that the use of nebulised honey can reduce the symptoms of asthma in rabbits (Kamaruzaman *et al.*, 2014). It has also been proposed that manuka honey in combination with antibiotics could be a useful strategy to combat *P. aeruginosa* cystic fibrosis infections (Jenkins *et al.*, 2015; Roberts *et al.*, 2019). Therefore, this chapter will aim to explore the activity of 3 antibiotics, amikacin, tobramycin and azithromycin, in combination with manuka honey against *M. abscessus* and subspecies. Followed by exploration into nebulising the combination of antibiotic with manuka honey against *M. abscessus*.

3.1.3 Aims and Objectives

Firstly, the MICs and MBCs of 3 antibiotics of interest will be determined, these are amikacin, tobramycin and azithromycin. Then, using a checkerboard assay, each antibiotic will be tested with manuka honey at varying concentrations to assess the combinatorial effect. Lastly, an *in vitro* nebuliser assay will be developed using both antibiotic and honey in combination, as a potential new treatment for *M. abscessus* pulmonary infections.

3.2 Materials and Methods

3.2.1 Media Preparation

All chemicals and reagents were purchased from Sigma-Aldrich or Melford, unless otherwise stated. For growth of *Mycobacterium abscessus* cultures, Middlebrook 7H9 broth and Middlebrook 7H11 agar were selected. The 7H9 broth was made by weighing 2.35 g of broth added to 450 mL of distilled H_2O and supplemented with 4 mL of 50% (w/v) glycerol before autoclaving (121 °C for 15 min). Once cooled, 1.25 mL of 20% filter sterile Tween80 was added (0.055% w/v). The 7H11 agar was made by weighing 10.25 g of agar added to 450 mL distilled H_2O and supplemented with 5 mL 50% (w/v) glycerol before autoclaving (121 °C for 15 min). The agar was subsequently poured into petri dishes.

3.2.2 M. abscessus strains and culture

The *M. abscessus* strains used were the type strain NCTC 13031 (also called ATCC 19977) and a panel of 16 clinical isolates obtained from Brighton and Sussex Medical School, isolated from patients with *M. abscessus* pulmonary infection. All organisms were grown for 72 h in 7H9 broth before being stored in 25% (w/v) glycerol stock solutions and stored at -80 °C. Prior to testing, *M. abscessus* isolates were grown in 10 mL 7H9 broth, prepared as described in 3.2.1, for 72 h at either 30 °C or 37 °C, depending on the experiment, with orbital shaking at 180 rpm.

3.2.3 Honey sample storage and preparation

The 4 manuka honey samples selected for this study were all of differing MGO concentrations. The lowest grade selected was MGO40 (Manuka Doctor, UK), followed by MGO55 (ManukaPharm, UK), MGO70 (Manuka Doctor, UK) and the highest grade selected was MGO83 (Comvita, UK). The MGO rating determines that each manuka honey will have certain levels of MGO, for example the MGO40 will contain 40 mg/kg MGO. All honey jars were stored in the dark at room temperature prior to testing and not used beyond any use by dates. For each experiment honey stocks were made up to 1 g/mL in distilled H_2O (w/v) and filter sterilised in a two-step filtration process using 0.8 µm filter and 0.22 µm filter (Sartorius), unless otherwise stated. No further sterility testing was conducted, such as testing for *Bacillus* spores.

3.2.4 Broth microdilution assay for antibiotic susceptibility testing

To determine the MIC and MBC of amikacin against *M. abscessus* NCTC 13031 and 3 clinical isolates of known subspecies (subsp. *abscessus* 159544, subsp. *bolletii* DC088A and subsp. *massiliense* DC088D), the broth microdilution assay was used. In brief, a 12.8 mg/mL stock of amikacin was made in sterile distilled H_2O. A master plate was set up by adding 100 µL of sterile distilled H_2O to wells A2-A11 and 200 µL of amikacin stock to well A1. The amikacin was serially diluted down the plate from well A1 to A10 by mixing and removing 100 µL and adding to the next row. On well A10 the excess 100 µL was removed and discarded,

leaving row A11 as a control row of sterile distilled H_2O. From the master plate, 1 µL was removed from each well of the plate and added to the corresponding wells in the experimental plate. Then, 94 µL of 7H9 broth, prepared as described in 3.2.1, was added to all wells of the 96 well plate. The plate was then inoculated along rows A-C and E-G with 5 µL of $OD_{600\ nm}$=0.1 *M. abscessus* cells, prepared as described in 3.2.2. The control rows of D and H had an additional 5 µL of 7H9 broth. This resulted in the final concentrations of: 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL and 0.25 µg/mL amikacin (Figure 3.1). Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C for a total of 96 h, with $OD_{570\ nm}$ reads every 24 h. After 96 h, the final $OD_{570\ nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using 10 µL aliquots and spotting onto 7H11 agar plates, prepared as described in 3.2.1. The agar plates were incubated for a further 72 h at 37 °C and the minimum bactericidal concentration (MBC) was determined as the minimum concentration where no bacterial growth was visually observed.

	1	2	3	4	5	6	7	8	9	10	11	12
A	128 µg/mL *M. abscessus*	64 µg/mL *M. abscessus*	32 µg/mL *M. abscessus*	16 µg/mL *M. abscessus*	8 µg/mL *M. abscessus*	4 µg/mL *M. abscessus*	2 µg/mL *M. abscessus*	1 µg/mL *M. abscessus*	0.5 µg/mL *M. abscessus*	0.25 µg/mL *M. abscessus*	0 µg/mL *M. abscessus*	*M. abscessus*
B	128 µg/mL *M. abscessus*	64 µg/mL *M. abscessus*	32 µg/mL *M. abscessus*	16 µg/mL *M. abscessus*	8 µg/mL *M. abscessus*	4 µg/mL *M. abscessus*	2 µg/mL *M. abscessus*	1 µg/mL *M. abscessus*	0.5 µg/mL *M. abscessus*	0.25 µg/mL *M. abscessus*	0 µg/mL *M. abscessus*	*M. abscessus*
C	128 µg/mL *M. abscessus*	64 µg/mL *M. abscessus*	32 µg/mL *M. abscessus*	16 µg/mL *M. abscessus*	8 µg/mL *M. abscessus*	4 µg/mL *M. abscessus*	2 µg/mL *M. abscessus*	1 µg/mL *M. abscessus*	0.5 µg/mL *M. abscessus*	0.25 µg/mL *M. abscessus*	0 µg/mL *M. abscessus*	*M. abscessus*
D	128 µg/mL	64 µg/mL	32 µg/mL	16 µg/mL	8 µg/mL	4 µg/mL	2 µg/mL	1 µg/mL	0.5 µg/mL	0.25 µg/mL	0 µg/mL	Broth only
E	128 µg/mL *M. abscessus*	64 µg/mL *M. abscessus*	32 µg/mL *M. abscessus*	16 µg/mL *M. abscessus*	8 µg/mL *M. abscessus*	4 µg/mL *M. abscessus*	2 µg/mL *M. abscessus*	1 µg/mL *M. abscessus*	0.5 µg/mL *M. abscessus*	0.25 µg/mL *M. abscessus*	0 µg/mL *M. abscessus*	*M. abscessus*
F	128 µg/mL *M. abscessus*	64 µg/mL *M. abscessus*	32 µg/mL *M. abscessus*	16 µg/mL *M. abscessus*	8 µg/mL *M. abscessus*	4 µg/mL *M. abscessus*	2 µg/mL *M. abscessus*	1 µg/mL *M. abscessus*	0.5 µg/mL *M. abscessus*	0.25 µg/mL *M. abscessus*	0 µg/mL *M. abscessus*	*M. abscessus*
G	128 µg/mL *M. abscessus*	64 µg/mL *M. abscessus*	32 µg/mL *M. abscessus*	16 µg/mL *M. abscessus*	8 µg/mL *M. abscessus*	4 µg/mL *M. abscessus*	2 µg/mL *M. abscessus*	1 µg/mL *M. abscessus*	0.5 µg/mL *M. abscessus*	0.25 µg/mL *M. abscessus*	0 µg/mL *M. abscessus*	*M. abscessus*
H	128 µg/mL	64 µg/mL	32 µg/mL	16 µg/mL	8 µg/mL	4 µg/mL	2 µg/mL	1 µg/mL	0.5 µg/mL	0.25 µg/mL	0 µg/mL	Broth only

Figure 3.1 Plate map of amikacin broth microdilution at 37 °C. The broth microdilution was prepared by adding 94 µL of 7H9 broth to all 96 wells followed by 1 µL of amikacin (w/v) taken from a master plate. Rows A, B, C, E, F and G were all inoculated with OD adjusted culture of *M. abscessus*. Rows D and H were used as control rows and therefore not inoculated. The concentrations of amikacin are indicated in blue.

3.2.5 Antibiotic and honey checkerboard assay

To assess if there was a combinatorial effect between manuka honey and the 3 antibiotics of interest against *M. abscessus* a checkerboard assay was used. The *M. abscessus* isolates selected for testing were *M. abscessus* NCTC 13031, clinical isolate 159544 (subsp. *abscessus*), DC088A (subsp. *bolletii*) and DC088D (subsp. *massiliense*). For amikacin checkerboards plates, a 10 mg/mL stock of amikacin was made in sterile distilled H_2O. A master plate was set up by adding 100 µL of sterile distilled H_2O to wells A2-8 and 20 µL of amikacin stock to well A1 with 180 µL of sterile distilled H_2O. The amikacin was serially diluted along the plate by mixing and removing 100 µL and adding to the next well. On well A7 the excess 100 µL was removed and discarded, leaving well A8 as sterile distilled H_2O only. For tobramycin checkerboard plates a 16 mg/mL stock was made in sterile distilled H_2O. A master plate was set up by adding 100 µL of sterile distilled H_2O to wells A2-8 and 20 µL of stock to well A1 with 180 µL of sterile distilled H_2O. For azithromycin checkerboard plates a 16 mg/mL stock was made in DMSO. A master plate was set up by adding 100 µL of DMSO to wells A2-8 and 20 µL of stock to well A1 with 180 µL of DMSO. From the master plates, 1 µL was removed from each well of row A and added to rows A-H of a 96 well plate. The final concentrations of amikacin were: 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.125 µg/mL and 0 µg/mL along the x axis. The final concentrations of tobramycin and azithromycin were: 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL and 0 µg/mL along the x axis.

The honey stocks were made as previously described in section 3.2.3. For each concentration of honey to be tested, the honey stock was aliquoted individually. The final concentrations of honey were: 0.277 g/mL, 0.237 g/mL, 0.197 g/mL, 0.157 g/mL, 0.117 g/mL, 0.077 g/mL, 0.037 g/mL and 0 g/mL along the y axis. The plates were inoculated with 5 µL of $OD_{600\ nm}$=0.1 *M. abscessus* cells, prepared as described in 3.2.2, into columns A-H and rows 1-8. Control rows of *M. abscessus* only and 7H9 broth only were on columns 9 and 10. These had 100 µL of 7H9 broth with the addition of 5 µL $OD_{600\ nm}$=0.1 *M. abscessus* for column 9 and 5 µL of 7H9 broth for column 10 (Figure 3.2 and 3.3). Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C for a total of 96 h, with $OD_{570\ nm}$ reads every 24 h. After 96 h, the final $OD_{570\ nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using 5 µL aliquots and spotting onto 7H11 agar plates, prepared as described in 3.2.1. The agar plates were incubated for a further 72 h at 37 °C and the MBC was determined as the minimum concentration where no bacterial growth was visually observed.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.277 g/mL 8 µg/mL	0.277 g/mL 4 µg/mL	0.277 g/mL 2 µg/mL A	0.277 g/mL 1 µg/mL	0.277 g/mL 0.5 µg/mL	0.277 g/mL 0.25 µg/mL	0.277 g/mL 0.125 µg/mL	0.277 g/mL 0 µg/mL	*M. abscessus*		Broth	0.452 g/mL
B	0.237 g/mL 8 µg/mL	0.237 g/mL 4 µg/mL	0.237 g/mL 2 µg/mL	0.237 g/mL 1 µg/mL	0.237 g/mL 0.5 µg/mL	0.237 g/mL 0.25 µg/mL	0.237 g/mL 0.125 µg/mL	0.237 g/mL 0 µg/mL	*M. abscessus*		Broth	0.2375 g/mL
C	0.197 g/mL 8 µg/mL	0.197 g/mL 4 µg/mL	0.197 g/mL 2 µg/mL	0.197 g/mL 1 µg/mL	0.197 g/mL 0.5 µg/mL	0.197 g/mL 0.25 µg/mL	0.197 g/mL 0.125 µg/mL	0.197 g/mL 0 µg/mL	*M. abscessus*		Broth	0.118 g/mL
D	0.157 g/mL 8 µg/mL	0.157 g/mL 4 µg/mL	0.157 g/mL 2 µg/mL	0.157 g/mL 1 µg/mL	0.157 g/mL 0.5 µg/mL	0.157 g/mL 0.25 µg/mL	0.157 g/mL 0.125 µg/mL	0.157 g/mL 0 µg/mL	*M. abscessus*		Broth	0.0593 g/mL
E	0.117 g/mL 8 µg/mL	0.117 g/mL 4 µg/mL	0.117 g/mL 2 µg/mL	0.117 g/mL 1 µg/mL	0.117 g/mL 0.5 µg/mL	0.117 g/mL 0.25 µg/mL	0.117 g/mL 0.125 µg/mL	0.117 g/mL 0 µg/mL	*M. abscessus*		Broth	0.0296 g/mL
F	0.077 g/mL 8 µg/mL	0.077 g/mL 4 µg/mL	0.077 g/mL 2 µg/mL	0.077 g/mL 1 µg/mL	0.077 g/mL 0.5 µg/mL	0.077 g/mL 0.25 µg/mL	0.077 g/mL 0.125 µg/mL	0.077 g/mL 0 µg/mL	*M. abscessus*		Broth	0.0148 g/mL
G	0.037 g/mL 8 µg/mL	0.037 g/mL 4 µg/mL	0.037 g/mL 2 µg/mL	0.037 g/mL 1 µg/mL	0.037 g/mL 0.5 µg/mL	0.037 g/mL 0.25 µg/mL	0.037 g/mL 0.125 µg/mL	0.037 g/mL 0 µg/mL	*M. abscessus*		Broth	0.0074 g/mL
H	0 g/mL 8 µg/mL	0 g/mL 4 µg/mL	0 g/mL 2 µg/mL	0 g/mL 1 µg/mL	0 g/mL 0.5 µg/mL	0 g/mL 0.25 µg/mL	0 g/mL 0.125 µg/mL	0 g/mL 0 µg/mL	*M. abscessus*		Broth	

Figure 3.2 Plate map of manuka honey and amikacin checkerboard. The checkerboard broth microdilution was prepared by adding 1 µL of amikacin (concentration indicated in blue) from a master plate and the desired concentration of manuka honey (indicated in red) along with 7H9 broth to bring the final volume to 100 µL. Columns 1 to 9 were all inoculated with OD adjusted culture of *M. abscessus*. Columns 11 and 12 were used as controls and therefore not inoculated.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.277 g/mL 16 µg/mL	0.277 g/mL 8 µg/mL	0.277 g/mL 4 µg/mL **A**	0.277 g/mL 2 µg/mL	0.277 g/mL 1 µg/mL	0.277 g/mL 0.5 µg/mL	0.277 g/mL 0.25 µg/mL	0.277 g/mL 0 µg/mL	*M. abscessus*		Broth	0.452 g/mL
B	0.237 g/mL 16 µg/mL	0.237 g/mL 8 µg/mL	0.237 g/mL 4 µg/mL	0.237 g/mL 2 µg/mL	0.237 g/mL 1 µg/mL	0.237 g/mL 0.5 µg/mL	0.237 g/mL 0.25 µg/mL	0.237 g/mL 0 µg/mL	*M. abscessus*		Broth	0.2375 g/mL
C	0.197 g/mL 16 µg/mL	0.197 g/mL 8 µg/mL	0.197 g/mL 4 µg/mL	0.197 g/mL 2 µg/mL	0.197 g/mL 1 µg/mL	0.197 g/mL 0.5 µg/mL	0.197 g/mL 0.25 µg/mL	0.197 g/mL 0 µg/mL	*M. abscessus*		Broth	0.118 g/mL
D	0.157 g/mL 16 µg/mL	0.157 g/mL 8 µg/mL	0.157 g/mL 4 µg/mL	0.157 g/mL 2 µg/mL	0.157 g/mL 1 µg/mL	0.157 g/mL 0.5 µg/mL	0.157 g/mL 0.25 µg/mL	0.157 g/mL 0 µg/mL	*M. abscessus*		Broth	0.0593 g/mL
E	0.117 g/mL 16 µg/mL	0.117 g/mL 8 µg/mL	0.117 g/mL 4 µg/mL	0.117 g/mL 2 µg/mL	0.117 g/mL 1 µg/mL	0.117 g/mL 0.5 µg/mL	0.117 g/mL 0.25 µg/mL	0.117 g/mL 0 µg/mL	*M. abscessus*		Broth	0.0296 g/mL
F	0.077 g/mL 16 µg/mL	0.077 g/mL 8 µg/mL	0.077 g/mL 4 µg/mL	0.077 g/mL 2 µg/mL	0.077 g/mL 1 µg/mL	0.077 g/mL 0.5 µg/mL	0.077 g/mL 0.25 µg/mL	0.077 g/mL 0 µg/mL	*M. abscessus*		Broth	0.0148 g/mL
G	0.037 g/mL 16 µg/mL	0.037 g/mL 8 µg/mL	0.037 g/mL 4 µg/mL	0.037 g/mL 2 µg/mL	0.037 g/mL 1 µg/mL	0.037 g/mL 0.5 µg/mL	0.037 g/mL 0.25 µg/mL	0.037 g/mL 0 µg/mL	*M. abscessus*		Broth	0.0074 g/mL
H	0 g/mL 16 µg/mL	0 g/mL 8 µg/mL	0 g/mL 4 µg/mL	0 g/mL 2 µg/mL	0 g/mL 1 µg/mL	0 g/mL 0.5 µg/mL	0 g/mL 0.25 µg/mL	0 g/mL 0 µg/mL	*M. abscessus*		Broth	

Figure 3.3 Checkerboard plate map of manuka honey and either tobramycin or azithromycin. The checkerboard broth microdilution was prepared by adding 1 µL of either tobramycin or azithromycin (concentration indicated in blue) from a master plate and the desired concentration of manuka honey (indicated in red) along with 7H9 broth to bring the final volume to 100 µL. Columns 1 to 9 were all inoculated with OD adjusted culture of *M. abscessus*. Columns 11 and 12 were used as controls and therefore not inoculated.

3.2.6 *In vitro* nebulisation assay of manuka honey and amikacin

To explore the possibility of using nebulised honey in combination with amikacin to inhibit *M. abscessus* and the subsp., a nebuliser assay was developed. Initially, 7H11 agar plates, prepared as described in 3.2.1, were inoculated with log phase *M. abscessus* cultures, grown at 37 °C as described in 3.2.2, using a 10 µL loop and confluent streak. To prepare the setup of the nebuliser assay (Figure 3.4), the agar plates were transferred to the biosafety cabinet along with the metal bar support with attached plate holders made from 3 mm thick laser cut acrylic. Inoculated agar plates were placed onto the plate holders and this was transferred into an autoclave biohazard bag (Fisherbrand, 40 µm thickness, 410 mm x 630 mm). The autoclave bag, containing the inoculated agar plates was removed from the biosafety cabinet and heat sealed (VWR bag sealer with cutter) for 4 seconds of heating. A small gap at the top of the bag was not sealed, to allow attachment of the medicine cup. Treatments to be nebulised, either manuka honey, amikacin or sterile distilled H_2O, were placed into the medicine cup and transferred to the fume hood along with the heat sealed bag containing the inoculated agar plates. The metal bar was attached to two retort stands and the medicine cup was taped to the top corner of the autoclave bag. The lids for the agar plates were then removed and the tubing to the nebuliser (OMRON C28P) was attached. Nebulisation was started and allowed to run until the medicine cup was empty or sputtering occurred (~15 min). After the nebuliser was finished, the tubing was removed and the autoclave bag was transferred to the biosafety cabinet where it was cut open and the agar plates retrieved. The agar plates had the lids replaced and were incubated at 37 °C for 72 h. Inhibition was determined by the visual lack of bacterial growth.

The treatments that were nebulised included sterile distilled H_2O, MGO55 manuka honey, amikacin and combination of MGO55 manuka honey and amikacin. To prepare the honey sample for nebulising, a 0.74 g/mL stock was prepared in distilled H_2O and filter sterilised in a two-step filtration process using 0.8 µm filter and 0.22 µm filter (Sartorius). To achieve the concentration of 0.37 g/mL required for nebulising, 2.5 mL of MGO55 manuka honey stock was mixed with 2.5 mL sterile distilled H_2O. To prepare the amikacin for nebulising, a stock of 20 mg/mL amikacin was made with sterile distilled H_2O. From this the stock was then made to the following concentrations for nebulising: 0.16 mg/mL, 1.6 mg/mL and 16 mg/mL. For nebulising of both MGO55 manuka honey and amikacin, these were prepared as described above to the following concentrations: MGO55 manuka honey 0.37 g/mL and 1.6 mg/mL amikacin.

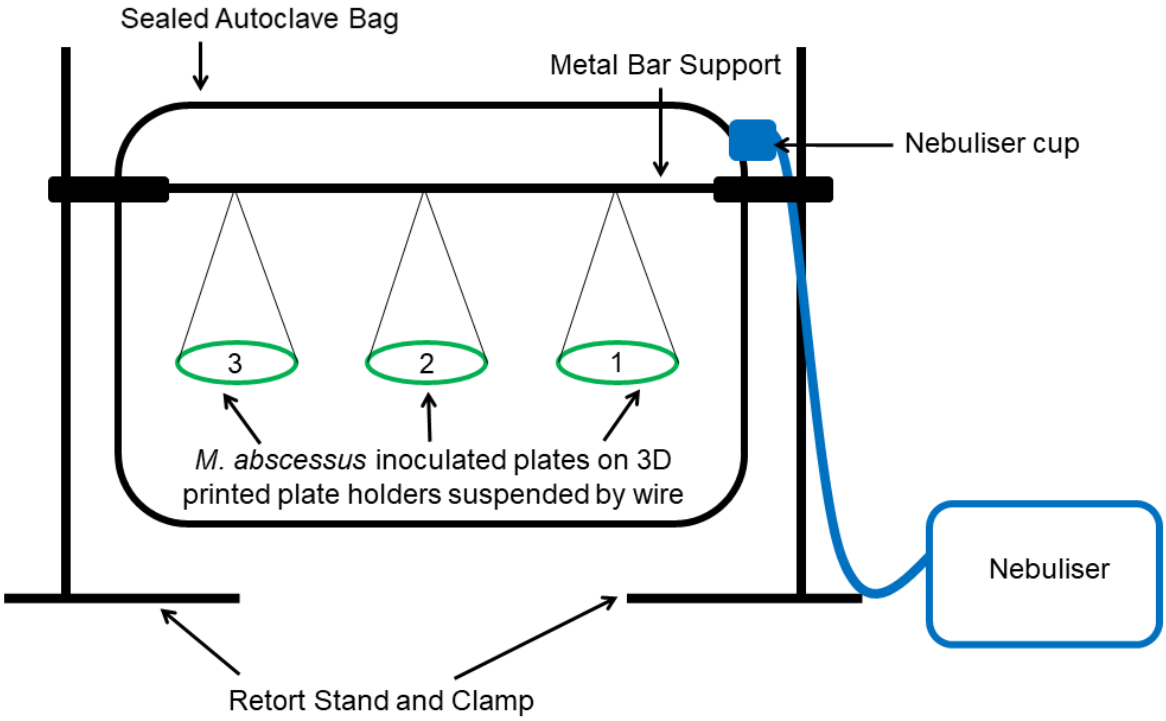

Figure 3.4 Nebuliser assay experimental design. Initially, 7H11 agar was inoculated with *M. abscessus* culture and place onto a plate holder before being sealed inside a bag, leaving the top corner open for the nebuliser medicine cup to be attached. The desired treatment to be nebulised was added to the nebuliser cup and attached to the nebuliser unit (OMRON C28P). The sealed bag containing the inoculated agar plates was attached to a retort stand. The medicine cup was then attached to the top right corner of the sealed bag and secured in place. The nebuliser was then started and allowed to run until the medicine cup was empty. The positions of the plates labelled 1, 2 and 3 identify the position of the agar plates in relation to the proximity of the nebuliser medicine cup.

3.2.7 Quantifying the *in vitro* nebulisation assay by using CFU/mL

To quantify the nebuliser assay colony forming units per mL (CFU/mL) were used. Cultures of *M. abscessus* NCTC 13031 were grown as described in section 3.2.2 and OD adjusted to 0.2 before serial dilution. The serial dilution was done by taking 1 mL of OD adjusted culture and adding it to 9 mL of 7H9 broth and mixed before 1 mL was removed and added to another 9 mL of 7H9 broth. This was repeated until a 10^{-4} dilution was obtained. From the 10^{-4} dilution, 50 µL was inoculated onto 7H11 agar and spread using an L-shaped spreader, to allow for full coverage of the agar plate. Inoculated plates were allowed to dry for 30 minutes prior to nebulised treatment. The nebuliser treatment was conducted as described in section 3.2.6.

3.2.8 Quantifying the *in vitro* nebulisation assay by using inoculated spots

Another method utilised to quantify the nebuliser assay was by inoculating agar plates with spots of known *M. abscessus* inoculum. Briefly, *M. abscessus* cultures were grown as described in 3.2.2 and OD adjusted to 0.1. Then a total of 8, 10 µL spots of culture were inoculated onto 7H11 agar and allowed to dry before nebulised treatment (Lin *et al.*, 2020). The nebulised treatment was conducted as described in section 3.2.6.

3.2.9 Data processing and statistical analysis

All data collected were n=3 technical replicates and 2 biological replicates, the broth microdilution assays and checkerboard assays were processed in Microsoft Excel 2016 and subsequently analysed using GraphPad Prism 8. Prior to data analysis, the OD value of honey only was subtracted from corresponding experimental honey values, containing *M. abscessus* culture, to account for the OD of the honey samples. The data was then analysed for normal distribution using Shapiro-Wilk test and subsequently analysed using a One-Way ANOVA. A Dunnett's multiple comparisons analysis was also conducted. For each experiment conducted, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The MIC was defined as the lowest concentration required to inhibit *M. abscessus* determined by an OD value of 0. The MBC was defined as the lowest concentration required for no visible growth after transfer onto solid media.

For the checkerboard assays the fractional inhibition concentration index (FICI) and fractional bactericidal concentration index (FBCI) were calculated. The equation for these were:

$$FICI = (MIC_A^{combi} / MIC_A^{alone}) + (MIC_B^{combi} / MIC_B^{alone})$$

$$FBCI = (MBC_A^{combi} / MBC_A^{alone}) + (MBC_B^{combi} / MBC_B^{alone})$$

The MIC^{alone} was defined as the minimum concentration of one antimicrobial compound required to inhibit *M. abscessus* alone. The MIC^{combi} was defined as the minimum concentration of one antimicrobial compound required to inhibit *M. abscessus* in combination with the second antimicrobial compound. The MBC^{alone} was defined as the minimum concentration required for one antimicrobial compound to kill *M. abscessus* alone. The MBC^{combi} was defined as the minimum concentration of one antimicrobial compound required to kill *M. abscessus* in combination with the second antimicrobial compound. The interpretation of the FICI or FBCI values were: an FICI or FBCI of <0.5 was defined as synergy, an FICI or FBCI of >0.5 to 4 was defined as no interaction and an FICI or FBCI of >4 was defined as antagonism (Odds, 2003).

3.3 Results

3.3.1 Amikacin

The MICs and MBCs of amikacin against *M. abscessus* NCTC 13031 and the 3 subspecies were established, and growth curves were generated (Figure 3.5). The MIC of amikacin against *M. abscessus* NCTC 13031 was 4 µg/mL (Figure 3.5 A). The MBC was determined to be 16 µg/mL. The 3 subspecies also had a similar response to amikacin, with all of them having an MIC of 4 µg/mL (Figure 3.5 B, C and D). This was also the same for MBCs, with all 3 subspecies having an MBC of 8 µg/mL.

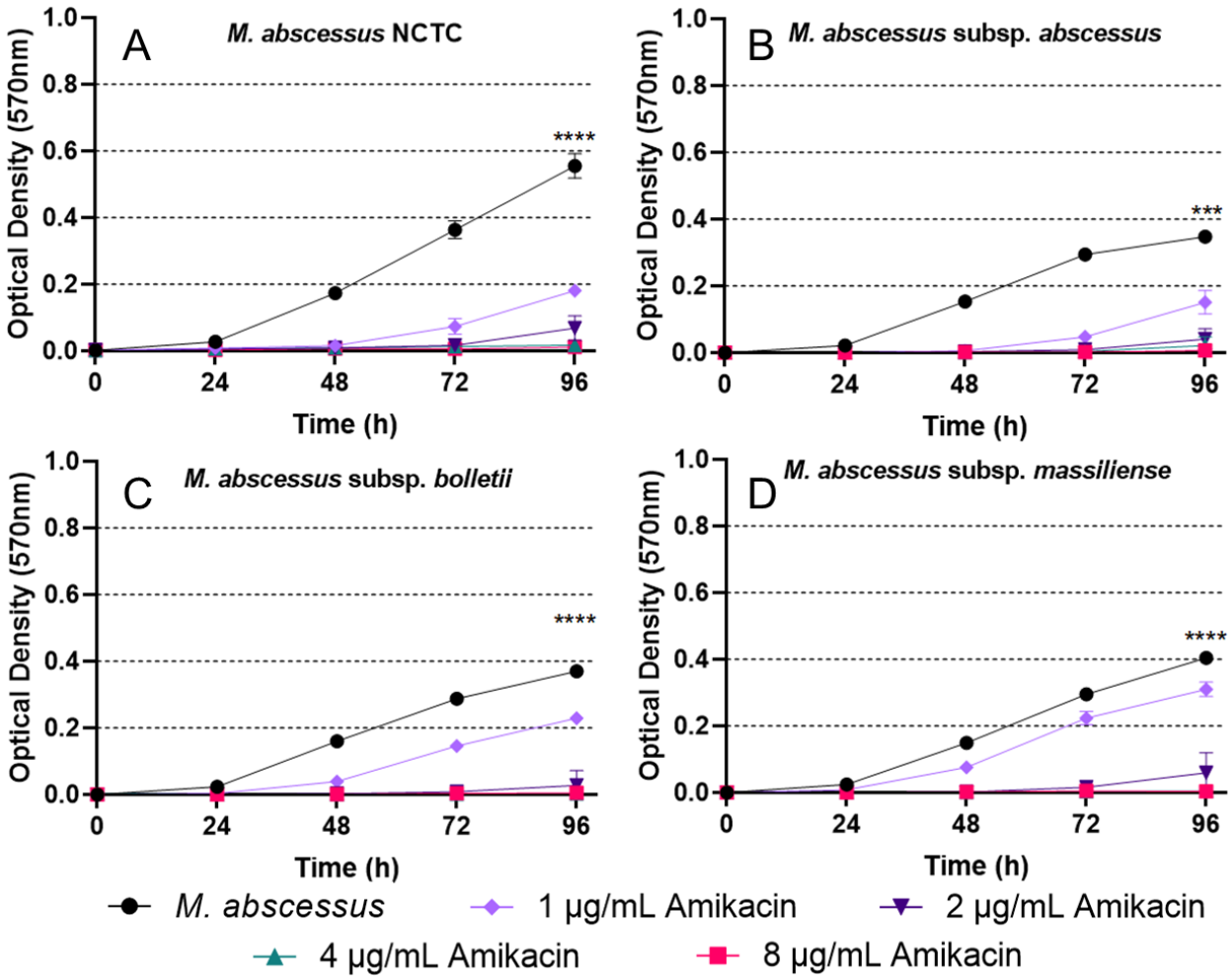

Figure 3.5. Growth curves of *M. abscessus* treated with amikacin. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of amikacin concentration on the growth of *M. abscessus*. A) *M. abscessus* NCTC 13031, with an MIC of 4 µg/mL amikacin. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. B) *M. abscessus* subsp. *abscessus* with an MIC of 4 µg/mL amikacin. Kruskal-Wallis shows a significant difference between all treatments, P=0.0003. C) *M. abscessus* subsp. *bolletii* showing an MIC of 4 µg/mL amikacin. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. D) *M. abscessus* subsp. *massiliense* with an MIC of 4 µg/mL amikacin. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001.

3.3.2 Combinatorial effect of manuka honey and amikacin against *M. abscessus*

After establishing the MIC and MBC of amikacin, the checkerboard assay was explored to determine the efficacy of manuka honey in combination with amikacin against *M. abscessus*. A combinatorial effect of manuka honey and amikacin was observed for all 4 of the manuka honey samples tested against *M. abscessus*. The concentrations required for enhanced activity varied depending on both the manuka honey tested and the *M. abscessus* isolate subspecies. Typically, the amount of manuka honey required for increased antimicrobial activity was 0.037 g/mL. This is considerably lower than the concentration required for inhibition without amikacin, which was 0.476 g/mL of manuka honey (Section 2.3.2, Table 2.5). The concentration of amikacin required for improved activity was more varied but was still reduced with the addition of manuka honey.

3.3.2.1 MGO40 in combination with amikacin against *M. abscessus*

The combination of MGO40 manuka honey and amikacin resulted in increased activity by using subinhibitory concentrations of both antimicrobial compounds against all 4 *M. abscessus* isolates tested. For *M. abscessus* NCTC 13031, the concentrations required for the improved activity were 0.037 g/mL of MGO40 and 1 µg/mL of amikacin. This is reduced from 0.476 g/mL of MGO40 and 4 µg/mL of amikacin required alone to inhibit *M. abscessus*, giving an FICI value of 0.327, indicating synergy (Table 3.1). The growth curve for these concentrations show that both MGO40 and amikacin alone did not inhibit the growth of *M. abscessus* NCTC 13031, but together inhibition was observed (Figure 3.6 A). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's comparison, comparing all treatments to *M. abscessus* NCTC 13031 only, showed a significant difference between the growth of *M. abscessus* NCTC 13031 treated with the combination and amikacin only, P=<0.0001 for both, and no difference between *M. abscessus* treated with MGO40 and *M. abscessus* NCTC 13031 only, P=0.9993. To see if there was a statistical difference between the combination and the treatments alone, a Dunnett's comparison was conducted comparing all treatments to the combinations. This identified a significant difference between all treatments, with *M. abscessus* NCTC 13031 only and MGO40 alone both having a P value of P=<0.0001, and amikacin only having a P value of P=0.0477. Both the FICI value and the Dunnett's comparison show a synergistic interaction between MGO40 and amikacin at these concentrations. However, this improved inhibition was not bactericidal at these concentrations, and 2 µg/mL amikacin with 0.037 g/mL MGO40 were required for cidality (Figure 3.6 B). The MBC of amikacin alone was higher than the MIC, being 16 µg/mL rather than 4 µg/mL, resulting in an FBCI of 0.202, indicating synergy (Table 3.2).

Table 3.1. FICI values for MGO40 manuka honey and amikacin against *M. abscessus*.

M. abscessus isolate	Honey alone MIC (g/mL)	Honey combination MIC (g/mL)	Amikacin alone MIC (µg/mL)	Amikacin combination MIC (µg/mL)	FICI value
NCTC 13031	0.476	0.037	4	1	0.327
subsp. *abscessus*	0.476	0.077	4	2	0.661
subsp. *bolletii*	0.476	0.237	4	0.5	0.622
subsp. *massiliense*	0.476	0.277	4	2	1.081

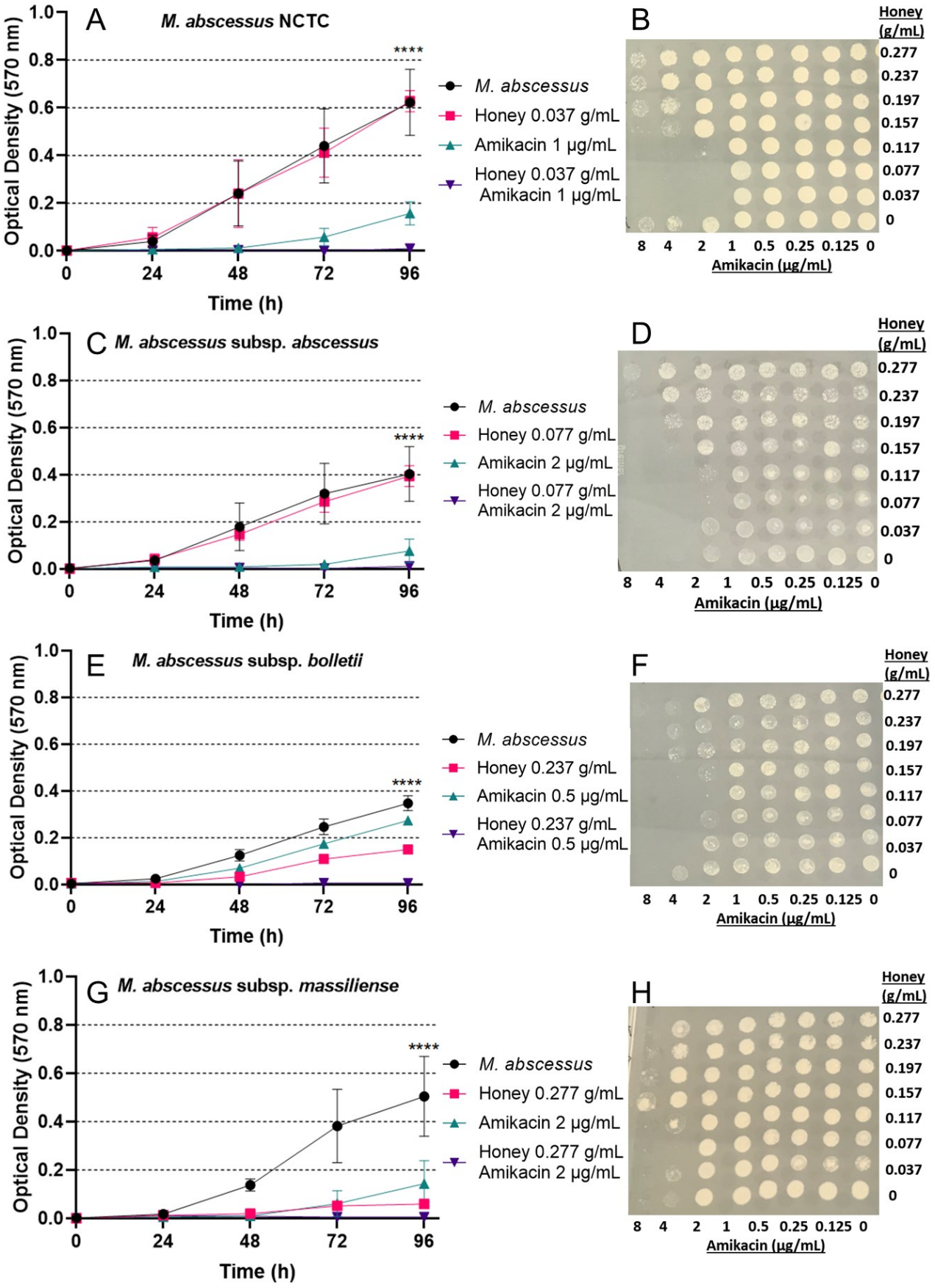

Figure 3.6. Combination of MGO40 manuka honey and amikacin results in increased inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in combination with

amikacin on the growth of *M. abscessus*. A) Growth curve of *M. abscessus* NCTC 13031 by combining 0.037 g/mL MGO40 and 1 µg/mL amikacin. One-way ANOVA shows a significant difference between all treatments, P=<0.0001. B) *M. abscessus* NCTC 13031 growth on solid media showed increased bactericidal activity by combining 0.037 g/mL MGO40 and 2 µg/mL amikacin. C) The growth curve showed enhanced inhibition of *M. abscessus* subsp. *abscessus* when exposed to 0.077 g/mL MGO40 and 2 µg/mL amikacin. One-way ANOVA shows significant difference between all treatments, P=<0.0001. D) *M. abscessus* subsp. *abscessus* on solid media had improved bactericidal activity with both 0.077 g/mL MGO40 and 2 µg/mL amikacin. E) Growth curve combination of 0.237 g/mL MGO40 and 0.5 µg/mL amikacin results in improved inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=<0.0001. F) *M. abscessus* subsp. *bolletii* increased bactericidal activity after transfer to solid media with the combination of 0.117 g/mL MGO40 and 2 µg/mL amikacin. G) Growth curve showing inhibition of *M. abscessus* subsp. *massiliense* by combining 0.277 g/mL MGO40 and 2 µg/mL amikacin, one-way ANOVA P=<0.0001. H) Bactericidal activity of 0.077 g/mL MGO40 and 4 µg/mL amikacin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

Table 3.2 FBCI values for MGO40 manuka honey and amikacin against *M. abscessus*.

***M. abscessus* isolate**	**Honey alone MBC (g/mL)**	**Honey combination MBC (g/mL)**	**Amikacin alone MBC (µg/mL)**	**Amikacin combination MBC (µg/mL)**	**FBCI value**
NCTC 13031	0.476	0.037	16	2	0.202
subsp. *abscessus*	0.476	0.077	4	2	0.661
subsp. *bolletii*	0.476	0.117	8	2	0.495
subsp. *massiliense*	0.476	0.077	8	4	0.661

For *M. abscessus* subsp. *abscessus* there was a difference in concentrations required for improved activity compared to *M. abscessus* NCTC 13031. The concentrations required in combination to inhibit *M. abscessus* subsp. *abscessus* were 0.077 g/mL of MGO40 and 2 µg/mL of amikacin (Table 3.1). This was a reduction from 0.476 g/mL of MGO40 and 4 µg/mL of amikacin when used alone, giving an FICI value of 0.661 (Table 3.1). This suggests there is no interaction between the two antimicrobial compounds. The growth curve of *M. abscessus* subsp. *abscessus* shows no inhibition of growth when treated with 0.077 g/mL MGO40, but there is a reduction of growth for amikacin alone, although the growth is not fully inhibited (Figure 3.6 C). A one-way ANOVA identified a significant difference between the treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *M. abscessus* subsp. *abscessus* only showed a significant difference between the combination of MGO40 and amikacin and amikacin only, P=<0.0001. No significant difference was observed between *M. abscessus* subsp. *abscessus* only and MGO40, P=0.9941. A Dunnett's multiple comparison was also conducted comparing all treatments to the growth of *M.*

abscessus subsp. *abscessus* treated with both MGO40 and amikacin. This showed a significant difference between the combination and either no treatment or treatment with MGO40, P=<0.0001 for both. No significant difference was identified between the combination and amikacin only, P=0.4092. This further suggests there was no interaction between MGO40 and amikacin at these concentrations. However, considering the bactericidal activity of MGO40 and amikacin at the same concentrations, there was growth for both amikacin alone and MGO40 alone, but no growth for the combination (Figure 3.6 D).

The combined activity for MGO40 and amikacin against *M. abscessus* subsp. *bolletii* required higher concentrations of MGO40 and lower concentrations of amikacin compared to the two previous isolates (Table 3.1). These were 0.237 g/mL MGO40 and 0.5 µg/mL, which are still reduced compared to each antimicrobial compound alone. The FICI value was calculated using these values and it suggests there is no interaction between MGO40 and amikacin, with an FICI value 0.622 (Table 3.1). The growth curve for *M. abscessus* subsp. *bolletii* shows a reduction in growth for treatment with amikacin or MGO40 alone and no growth for amikacin and MGO40 together, at these concentrations (Figure 3.6 E). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001, and a Dunnett's multiple comparison identified a significant difference between all treatments compared to *M. abscessus* subsp. *bolletii*, P=<0.0001 for MGO40 alone and in combination and P=0.0005 for amikacin alone. A Dunnett's multiple comparison was then conducted comparing the growth of all treatments of *M. abscessus* subsp. *bolletii* to the growth of *M. abscessus* subsp. *bolletii* exposed to the combination of MGO40 and amikacin. This showed a significant difference between all treatments compared to the combination, with all having a P value of P=<0.0001. This suggests that the difference in growth of *M. abscessus* subsp. *bolletii* when exposed to the combination was significantly reduced compared to either MGO40 alone or amikacin alone. Suggesting that the combination was synergistic, when considering inhibition. However, these concentrations were not bactericidal (Figure 3.6 F). Furthermore, at the concentrations of 0.117 g/mL MGO40 and 2 µg/mL amikacin a synergistic relationship could be observed, FICI value 0.495 (Table 3.1).

The least effective combination was observed for *M. abscessus* subsp. *massiliense*. The combination required for inhibition was 0.277 g/mL MGO40 and 2 µg/mL amikacin (Table 3.1). These concentrations have an FICI value of 1.081, which show no interaction between the two antimicrobial compounds. The growth curve shows that at these concentrations the growth of *M. abscessus* subsp. *massiliense* was reduced for MGO40 and amikacin alone compared to the control and no growth for both MGO40 and amikacin together (Figure 3.6 G). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the growth of *M. abscessus* subsp. *massiliense* showed a significant difference for all, P=<0.0001 for MGO40 alone and in combination with amikacin, and P=0.005 for amikacin alone. Another Dunnett's multiple

comparison was also conducted, comparing the growth for *M. abscessus* subsp. *massiliense* treated with the combination of MGO40 and amikacin to the other treatments. This identified a significant difference between the growth for the combination and the control, P=<0.0001 but no significant difference for either treatments alone compared to the control, MGO40 alone P=0.7667 and amikacin alone P=0.1495. This suggests there was no difference between the treatments alone and in combination on the growth of *M. abscessus* subsp. *massiliense*. Furthermore, these concentrations of 0.277 g/mL MGO and 2 µg/mL amikacin were not bactericidal. However, at 0.077 g/mL MGO40 and 4 µg/mL amikacin, bactericidal activity could be observed that is not seen when these concentrations are used alone (Figure 3.6 H). The FBCI value for these concentrations is 0.661 (Table 3.2). This suggests there is not a synergistic relationship between the two antimicrobial compounds.

3.3.2.2 MGO55 in combination with amikacin against *M. abscessus*

The combination of MGO55 manuka honey and amikacin resulted in improved inhibition and bactericidal activity for *M. abscessus* NCTC 13031 and the 3 subspecies tested. The most improved activity was observed for *M. abscessus* NCTC 13031, which required 0.037 g/mL MGO55 and 2 µg/mL amikacin to inhibit *M. abscessus* (Table 3.3). This is a reduction from 0.476 g/mL MGO55 and 4 µg/mL amikacin when used alone. However, the FICI value suggests there is no interaction between the two antimicrobials, FICI value 0.577. The growth curve at these concentrations shows similar growth for *M. abscessus* NCTC 13031 alone and treated with MGO55. A reduction in growth can be seen for *M. abscessus* treated with 2 µg/mL and no growth observed for the combination of MGO55 and amikacin (Figure 3.7A). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing the growth of all treatments to the growth of *M. abscessus* alone identified no difference between the control and MGO55, P=0.9996, and a significant difference between both amikacin and the combination, P=<0.0001 for both. Another Dunnett's comparison was conducted comparing all treatments to the combination. A significant difference between the combination and MGO55 alone and the control was observed, P=<0.0001 for both. No significant difference was observed between amikacin alone and the combination, P=0.9565. This suggests that the addition of MGO55 did not have a significant impact on the growth of *M. abscessus* compared to amikacin alone. However, when looking at the bactericidal activity, growth can be observed for 0.037 g/mL MGO55 and 2 µg/mL amikacin alone but no growth was observed for these concentrations when used together (Figure 3.7 B). This is further supported by the FBCI value of 0.327, indicating synergy between the two (Table 3.4). This shows that by using both of these subinhibitory concentrations together increased inhibition and bactericidal activity can be observed for *M. abscessus* NCTC 13031.

Table 3.3 FICI values for MGO55 manuka honey and amikacin against *M. abscessus*.

M. abscessus isolate	Honey alone MIC (g/mL)	Honey combination MIC (g/mL)	Amikacin alone MIC (µg/mL)	Amikacin combination MIC (µg/mL)	FICI
NCTC 13031	0.476	0.037	4	2	0.577
subsp. *abscessus*	0.476	0.277	4	0.25	0.644
subsp. *bolletii*	0.476	0.277	4	1	0.831
subsp. *massiliense*	0.476	0.277	4	1	0.831

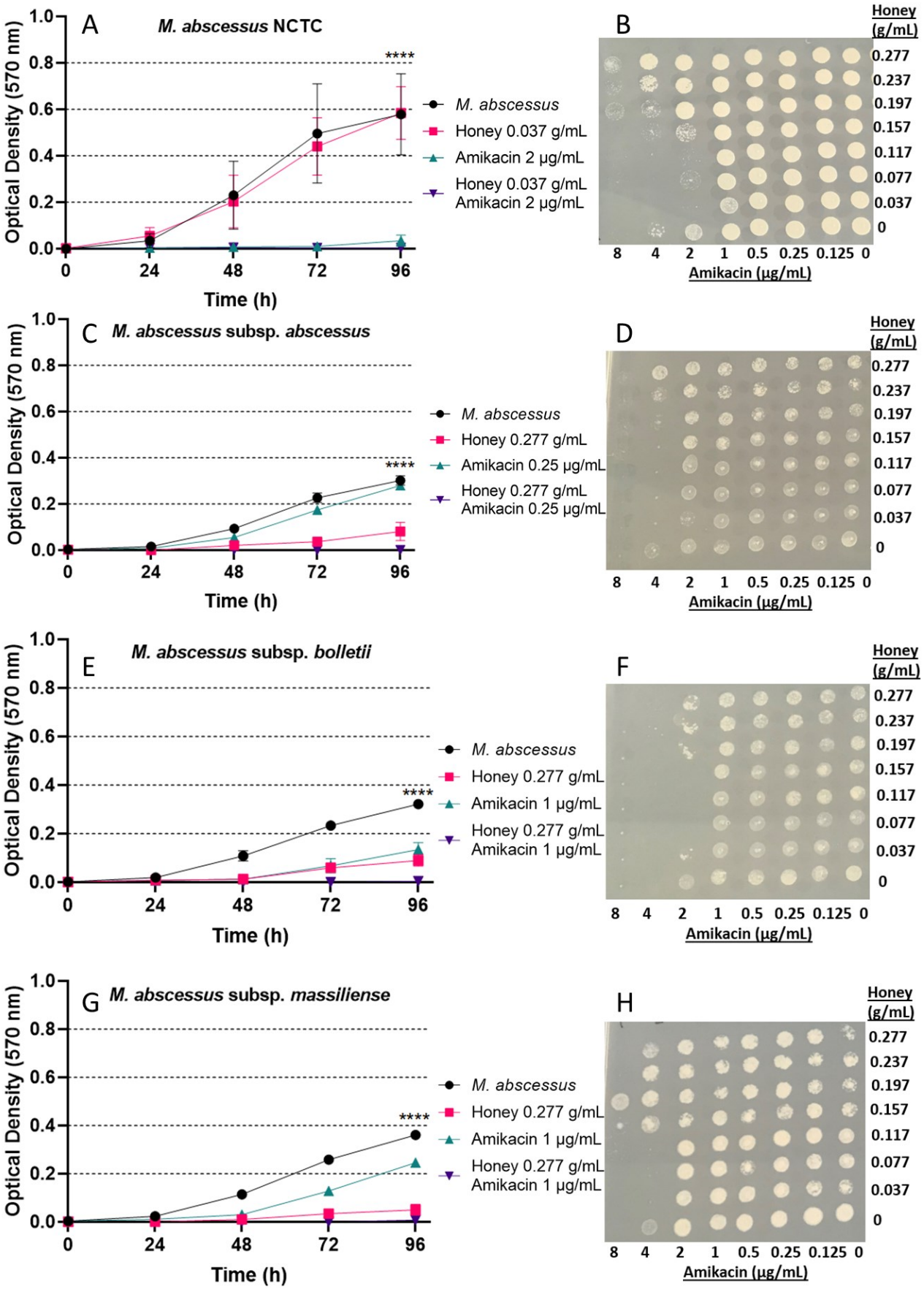

Figure 3.7. Combination of MGO55 manuka honey and amikacin results in increased inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in combination with

amikacin on the growth of *M. abscessus*. A) The growth curve showed improved inhibition of *M. abscessus* NCTC 13031 by combining 0.037 g/mL MGO55 and 2 µg/mL amikacin. One-way ANOVA shows a significant difference between all treatments, P=<0.0001. B) *M. abscessus* NCTC 13031 had increased bactericidal activity after transfer onto solid media by combining 0.037 g/mL MGO55 and 2 µg/mL amikacin. C) Growth curve showing enhanced inhibition of *M. abscessus* subsp. *abscessus* when exposed to 0.277 g/mL MGO55 and 0.25 µg/mL amikacin. One-way ANOVA shows significant difference between all treatments, P=<0.0001. D) On solid media *M. abscessus* subsp. *abscessus* had improved bactericidal activity with both 0.037 g/mL MGO55 and 4 µg/mL amikacin. E) Growth curve indicating the combination of 0.277 g/mL MGO55 and 1 µg/mL amikacin results in improved inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=<0.0001. F) *M. abscessus* subsp. *bolletii* had increased bactericidal activity with the combination of 0.117 g/mL MGO55 and 2 µg/mL amikacin when transferred to solid media. G) Growth curve of *M. abscessus* subsp. *massiliense* with improved inhibition by combining 0.277 g/mL MGO55 and 1 µg/mL amikacin, one-way ANOVA P=<0.0001. H) Bactericidal activity of 0.037 g/mL MGO55 and 4 µg/mL amikacin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

Table 3.4 FBCI values for MGO55 manuka honey and amikacin against *M. abscessus*.

***M. abscessus* isolate**	**Honey alone MBC (g/mL)**	**Honey combination MBC (g/mL)**	**Amikacin alone MBC (µg/mL)**	**Amikacin combination MBC (µg/mL)**	**FBCI**
NCTC 13031	0.476	0.037	8	2	0.327
subsp. *abscessus*	0.476	0.037	8	4	0.577
subsp. *bolletii*	0.476	0.117	4	2	0.661
subsp. *massiliense*	0.476	0.037	8	4	0.577

An improvement in inhibition was also observed for *M. abscessus* subsp. *abscessus* when using both MGO55 and amikacin in combination (Figure 3.7 C). The concentrations required for inhibition were 0.277 g/mL MGO55 with 0.25 µg/mL amikacin, which is a reduction from 0.476 g/mL and 4 µg/mL, respectively (Table 3.3). This resulted in an FICI value of 0.644, suggesting that there is no synergistic interaction. The growth curve shows a reduction in growth for *M. abscessus* subsp. *abscessus* treated with amikacin alone and MGO55 alone, compared to the control, and no growth observed for the combination (Figure 3.7 C). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the growth of the control of *M. abscessus* subsp. *abscessus* showed a significant difference between MGO55 alone and the combination, P=<0.0001 for both, but no significant difference between the control and amikacin only, P=0.4619. Another Dunnett's multiple comparison was conducted comparing

all treatments to the combination. This identified a significant difference between all of the treatments and the combination, P=<0.0001 for amikacin only and *M. abscessus* subsp. *abscessus*, and P=0.0016 for the combination and MGO55 alone. This suggests that there is a statistically significant difference between the treatments and the combination. However, there was no bactericidal activity observed for the combination at these concentrations (Figure 3.7 D). Furthermore, there was bactericidal activity observed at higher concentrations of the combination, which appear as inhibition when considering the growth curves. The combination of 0.037 g/mL MGO55 and 4 µg/mL amikacin resulted in no growth of *M. abscessus* subsp. *abscessus*, but growth can be seen for these concentrations alone (Figure 3.7 D). The FBCI value indicated there was no relationship between the antimicrobial compounds, FBCI value 0.577.

The concentrations of MGO55 and amikacin required for improved inhibition of *M. abscessus* subsp. *bolletii* were 0.277 g/mL MGO55 and 1 µg/mL amikacin (Table 3.3). At these concentrations, the FICI value shows no interaction, FICI value 0.831. The growth curve shows that there was a reduction in growth of *M. abscessus* subsp. *bolletii* exposed to either MGO55 alone or amikacin alone, compared to the control, and no growth for the combination (Figure 3.7 E). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001, and a Dunnett's multiple comparison showed a significant difference for all treatments compared to the control of *M. abscessus* subsp. *bolletii* only, P=<0.0001 for all. Another Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This showed a significant difference between all treatments and the combination, P=<0.0001 for *M. abscessus* subsp. *bolletii* only and amikacin only, P=0.0003 for MGO55 only. This suggests there is a difference between MGO55 and amikacin alone compared to being used in combination. At these concentrations, no bactericidal activity was observed, but there was bactericidal activity observed for 0.117 g/mL MGO55 and 2 µg/mL amikacin together that was not seen for these concentrations alone (Figure 3.7 F). This gave an FBCI value of 0.661, which still suggests no interaction (Table 3.4).

M. abscessus subsp. *massiliense* also required 0.277 g/mL MGO55 and 1 µg/mL amikacin together for inhibition of growth (Table 3.3). This was also determined as no interaction by the FICI value of 0.831. The growth curve shows a reduction in growth for *M. abscessus* subsp. *massiliense* treated with amikacin compared to the control, and a greater reduction in growth for MGO55 (Figure 3.7 G). No growth was observed for the combination of MGO55 and amikacin together. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001, and a Dunnett's multiple comparison showed a significant difference between all treatments compared to the growth of *M. abscessus* subsp. *massiliense* only, P=<0.0001 for all. Another Dunnett's multiple comparison was conducted, comparing all treatments to the growth of *M. abscessus* subsp. *massiliense* with the combination of MGO55 and amikacin together. This showed a significant difference between all treatments,

P=<0.0001 for *M. abscessus* subsp. *massiliense* only and amikacin only, and P=0.0109 for MGO55 alone. This suggests that there is a significant difference between the combination of MGO55 and amikacin compared to them alone. Again, this combination of MGO55 and amikacin were not bactericidal. However, bactericidal activity could be seen for 0.037 g/mL MGO55 and 4 µg/mL amikacin (Figure 3.7 H). This gives an FBCI value of 0.577, which still suggests no interaction (Table 3.4).

3.3.2.3 MGO70 in combination with amikacin against *M. abscessus*

The combination of MGO70 manuka honey and amikacin had improved inhibition and bactericidal activity when used together rather than alone against *M. abscessus* NCTC 13031 and the 3 subspecies. Inhibition was observed against *M. abscessus* NCTC 13031 with 0.037 g/mL MGO70 and 2 µg/mL amikacin together, compared to 0.476 g/mL MGO70 and 4 µg/mL amikacin when used alone (Table 3.5). These concentrations have an FICI value of 0.577, which suggests there is no interaction between the two antimicrobial compounds (Table 3.5). The growth for *M. abscessus* NCTC 13031 was similar for *M. abscessus* alone and *M. abscessus* with 0.037 g/mL MGO70. A reduction in growth was observed for treatment with amikacin and no growth was observed for both MGO70 and amikacin together (Figure 3.8 A). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the control of *M. abscessus* alone showed no significant difference between the control and MGO70, P=0.8451. A significant difference was shown between amikacin alone and the combination compared to *M. abscessus* only, P=<0.0001. A second Dunnett's multiple comparison was conducted comparing all treatments to the response of the combination. This identified a significant difference between *M. abscessus* treated with MGO70 and the control, P=<0.0001. No significant difference was observed between amikacin only and the combination, P=0.1384. This suggests that there was no difference between the combination and amikacin only. However, the bactericidal activity observed at these concentrations show no growth for the combination and growth for both amikacin and MGO70 alone (Figure 3.8 B). In this instance the MBC of amikacin against *M. abscessus* NCTC 13031 was 16 µg/mL, not 8 µg/mL which was observed previously (Table 3.4). Using these concentrations, the FBCI was calculated and identified a synergistic response for MGO70 and amikacin together against *M. abscessus* NCTC 13031, FBCI value 0.202 (Table 3.6). This suggests that these concentrations do act synergistically together against *M. abscessus* NCTC 13031 at these concentrations.

Table 3.5 FICI values for MGO70 manuka honey and amikacin against *M. abscessus*.

M. abscessus isolate	Honey alone MIC (g/mL)	Honey combination MIC (g/mL)	Amikacin alone MIC (µg/mL)	Amikacin combination MIC (µg/mL)	FICI
NCTC 13031	0.476	0.037	4	2	0.577
subsp. *abscessus*	0.476	0.037	8	4	0.577
subsp. *bolletii*	0.476	0.197	4	2	0.913
subsp. *massiliense*	0.476	0.037	8	8	1.077

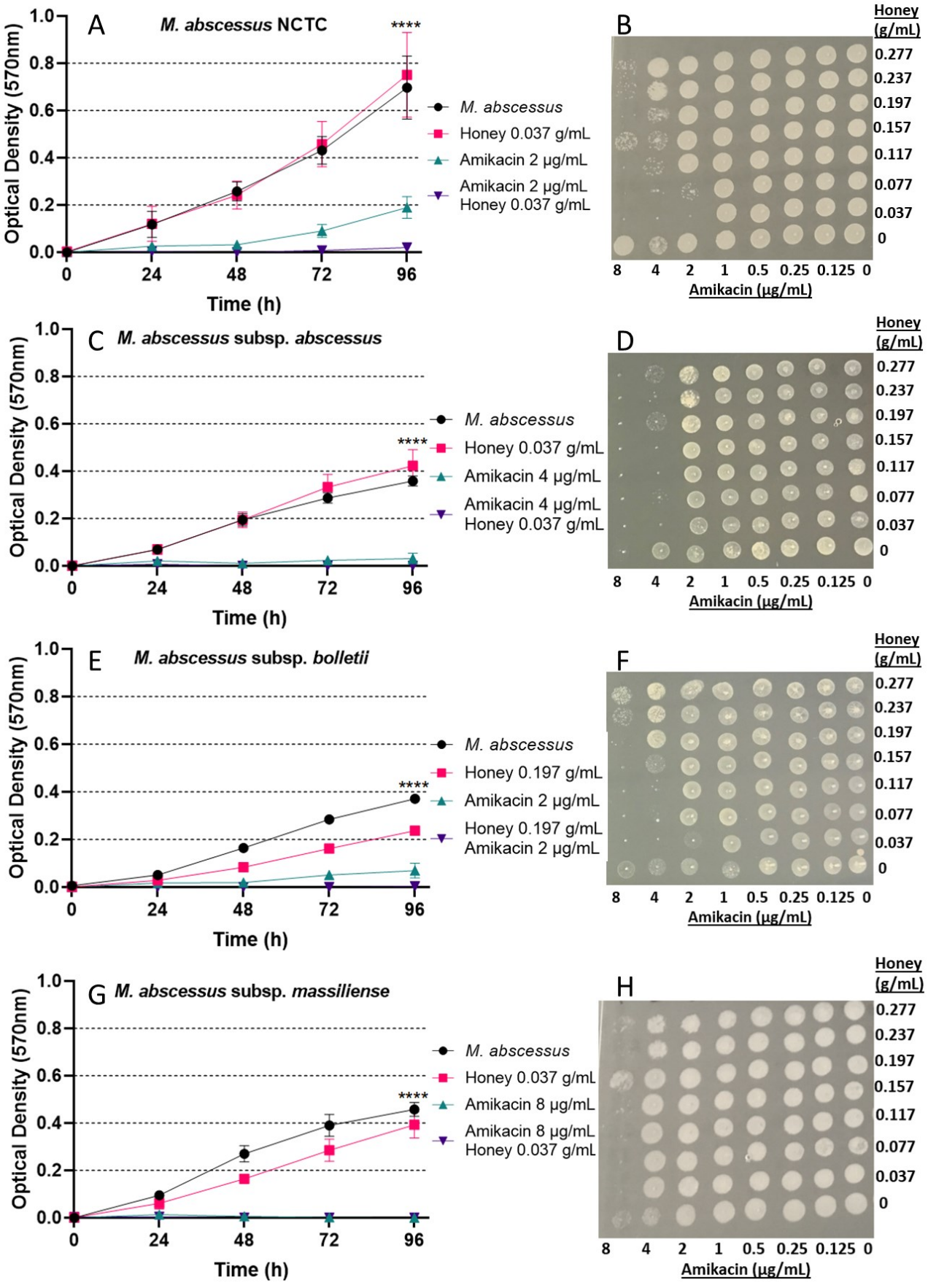

Figure 3.8 Combination of MGO70 manuka honey and amikacin results in increased inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in combination with

amikacin on the growth of *M. abscessus*. A) Growth curve showing improved inhibition of *M. abscessus* NCTC 13031 by combining 0.037 g/mL MGO70 and 2 µg/mL amikacin. One-way ANOVA shows a significant difference between all treatments, P=<0.0001. B) *M. abscessus* NCTC 13031 showing increased bactericidal activity on solid media by combining 0.037 g/mL MGO70 and 2 µg/mL amikacin. C) Growth curve indicating enhanced inhibition of *M. abscessus* subsp. *abscessus* when exposed to 0.037 g/mL MGO70 and 4 µg/mL amikacin. One-way ANOVA shows significant difference between all treatments, P=<0.0001. D) *M. abscessus* subsp. *abscessus* improved bactericidal activity when transferred to solid media with both 0.037 g/mL MGO70 and 4 µg/mL amikacin. E) The growth curve shows the combination of 0.197 g/mL MGO70 and 2 µg/mL amikacin results in improved inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=<0.0001. F) On solid media *M. abscessus* subsp. *bolletii* had increased bactericidal activity with the combination of 0.037 g/mL MGO70 and 4 µg/mL amikacin. G) Growth curve showing inhibition of *M. abscessus* subsp. *massiliense* by combining 0.037 g/mL MGO70 and 8 µg/mL amikacin, one-way ANOVA P=<0.0001. H) Bactericidal activity of 0.037 g/mL MGO70 and 8 µg/mL amikacin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

Table 3.6 FBCI values for MGO70 manuka honey and amikacin against *M. abscessus*.

***M. abscessus* isolate**	**Honey alone MBC (g/mL)**	**Honey combination MBC (g/mL)**	**Amikacin alone MBC (µg/mL)**	**Amikacin combination MBC (µg/mL)**	**FBCI**
NCTC 13031	0.476	0.037	16	2	0.202
subsp. *abscessus*	0.476	0.037	8	4	0.577
subsp. *bolletii*	0.476	0.037	16	4	0.327
subsp. *massiliense*	0.476	0.037	16	8	0.577

For *M. abscessus* subsp. *abscessus*, the combination of 0.037 g/mL MGO70 and 4 µg/mL were required improved activity (Table 3.5). The growth curve shows similar growth for *M. abscessus* subsp. *abscessus* treated with MGO70 and *M. abscessus* alone (Figure 3.8 C). This is also similar for amikacin alone and the combination, where the growth for amikacin only is very minimal and there is no growth observed for the combination. A one-way ANOVA identified a significant difference for all treatments, P=<0.0001. A Dunnett's multiple comparison showed no significant difference between the growth of *M. abscessus* subsp. *abscessus* compared to *M. abscessus* subsp. *abscessus* exposed to MGO70, P=0.1232. No significant difference was observed for amikacin only or the combination compared to the control, P=<0.0001 for both. Another Dunnett's multiple comparison was conducted

comparing all treatments to the response of the combination. This identified a significant difference for *M. abscessus* subsp. *abscessus* only and MGO70, P=<0.0001, but no difference between the combination and amikacin only, P=0.5705. This suggests there was no difference between amikacin alone and the combination. The FICI value shows no interaction at these concentrations, FICI value 0.577 (Table 3.5). However, the bactericidal activity at these concentrations is more interesting, showing growth of *M. abscessus* subsp. *abscessus* exposed to 4 µg/ml amikacin and 0.037 g/mL MGO70, but no growth for the combination of these (Figure 3.8 D). So, although the growth for amikacin alone was very minimal it was not fully inhibited and was still able to grow. The FBCI value suggests no interaction between the compounds, FBCI value 0.577.

The inhibition of *M. abscessus* subsp. *bolletii* with both MGO70 and amikacin was observed at a higher concentration of 0.197 g/mL MGO70 and 2 µg/mL amikacin compared to observations of the other isolates (Table 3.5). The growth curve for these concentrations show a reduction in growth of *M. abscessus* subsp. *bolletii* for MGO70 alone and amikacin alone compared to the control, and no growth for the combination (Figure 3.8 E). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001, and a Dunnett's multiple comparison showed a significant difference for all treatments when compared to the control of *M. abscessus* subsp. *bolletii* only, P=<0.0001 for all. A second Dunnett's multiple comparison was conducted comparing all treatments to the combination. This showed a significant difference for all treatments, P=<0.0001 for *M. abscessus* subsp. *bolletii* alone and MGO70, P=0.0014 for amikacin only. This suggests there is a difference between the combination and MGO70 and amikacin alone. However, the FICI value for these concentrations indicate there is no interaction, FICI value 0.913 (Table 3.5). Furthermore, these concentrations were not bactericidal. Although bactericidal activity could be observed for 0.037 g/mL MGO70 and 2 µg/mL (Figure 3.8 F). As observed for *M. abscessus* NCTC 13031, the bactericidal activity of amikacin alone was 16 µg/mL. Using these concentrations, the FBCI value is 0.327, suggesting these concentrations are synergistic (Table 3.6).

The inhibition of *M. abscessus* subsp. *massiliense* was based on the bactericidal activity. The concentrations focused on were 0.037 g/mL MGO70 and 8 µg/mL amikacin (Table 3.5). At these concentrations, there appears to be no growth of *M. abscessus* subsp. *massiliense* for 8 µg/mL amikacin alone, as well as for the combination (Figure 3.8 G). The growth for *M. abscessus* subsp. *massiliense* alone and exposed to 0.037 g/mL MGO70 showed very little difference. A one-way ANOVA did identify a significant difference for all treatments, P=<0.0001, and a Dunnett's multiple comparison showed a significant difference for amikacin alone and the combination compared to the control, P=<0.0001. A significant difference was also shown between MGO70 and the control P=0.0349. A Dunnett's multiple comparison, comparing all treatments to the combination showed a significant difference for *M. abscessus* subsp. *massiliense* only and MGO70, but no difference was observed between

the combination and amikacin only, P=>0.9999. However, the bactericidal activity shows that at these concentrations bactericidal activity was achieved when used together (Figure 3.8 H). Similar to *M. abscessus* NCTC 13031 and *M. abscessus* subsp. *bolletii*, the MBC of amikacin alone was higher than observed previously (Table 3.2 and 3.4), being 16 µg/mL rather than 8 µg/mL. Using these concentrations, the FBCI was 0.577. This suggests there is no interaction at these concentrations.

3.3.2.4 MGO83 in combination with amikacin against *M. abscessus*

The combination of MGO83 manuka honey and amikacin against *M. abscessus* NCTC 13031 and the 3 subspecies resulted in improved inhibition and bactericidal activity. The improved inhibition of *M. abscessus* NCTC 13031 was achieved by combining 0.037 g/mL MGO83 and 2 µg/mL amikacin, which is reduced from 0.476 g/mL MGO83 and 4 µg/mL amikacin when used alone (Table 3.7). These concentrations result in an FICI value of 0.577, suggesting there is no interaction between the two antimicrobial compounds. The growth curve shows similar growth for *M. abscessus* NCTC 13031 only and MGO83 alone, and a reduction in growth for amikacin alone with no growth observed for the combination of MGO83 and amikacin together (Figure 3.9 A). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001, and a Dunnett's multiple comparison showed a significant difference for the combination and amikacin only compared to *M. abscessus* NCTC 13031 only, P=<0.0001 for both. No difference was identified between MGO83 and *M. abscessus* NCTC 13031 only, P=9583. Another Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This identified a significant difference for *M. abscessus* NCTC 13031 and MGO83 alone, P=<0.0001, but no difference was observed for the combination and amikacin only, P=0.4879. This suggests there was a similar response to amikacin only and the combination. However, the bactericidal activity at these concentrations shows growth for 0.037 g/mL MGO83 and 2 µg/mL amikacin but when used in combination there was no visible growth (Figure 3.9 B). Moreover, the FBCI value, 0.202, further indicates that there is a synergistic interaction between the two compounds Table 3.8).

Table 3.7 FICI values for MGO83 manuka honey and amikacin against *M. abscessus*.

M. abscessus isolate	Honey alone MIC (g/mL)	Honey combination MIC (g/mL)	Amikacin alone MIC (µg/mL)	Amikacin combination MIC (µg/mL)	FICI
NCTC 13031	0.476	0.037	4	2	0.577
subsp. *abscessus*	0.476	0.277	4	0.25	0.644
subsp. *bolletii*	0.476	0.037	8	2	0.327
subsp. *massiliense*	0.476	0.277	4	2	1.081

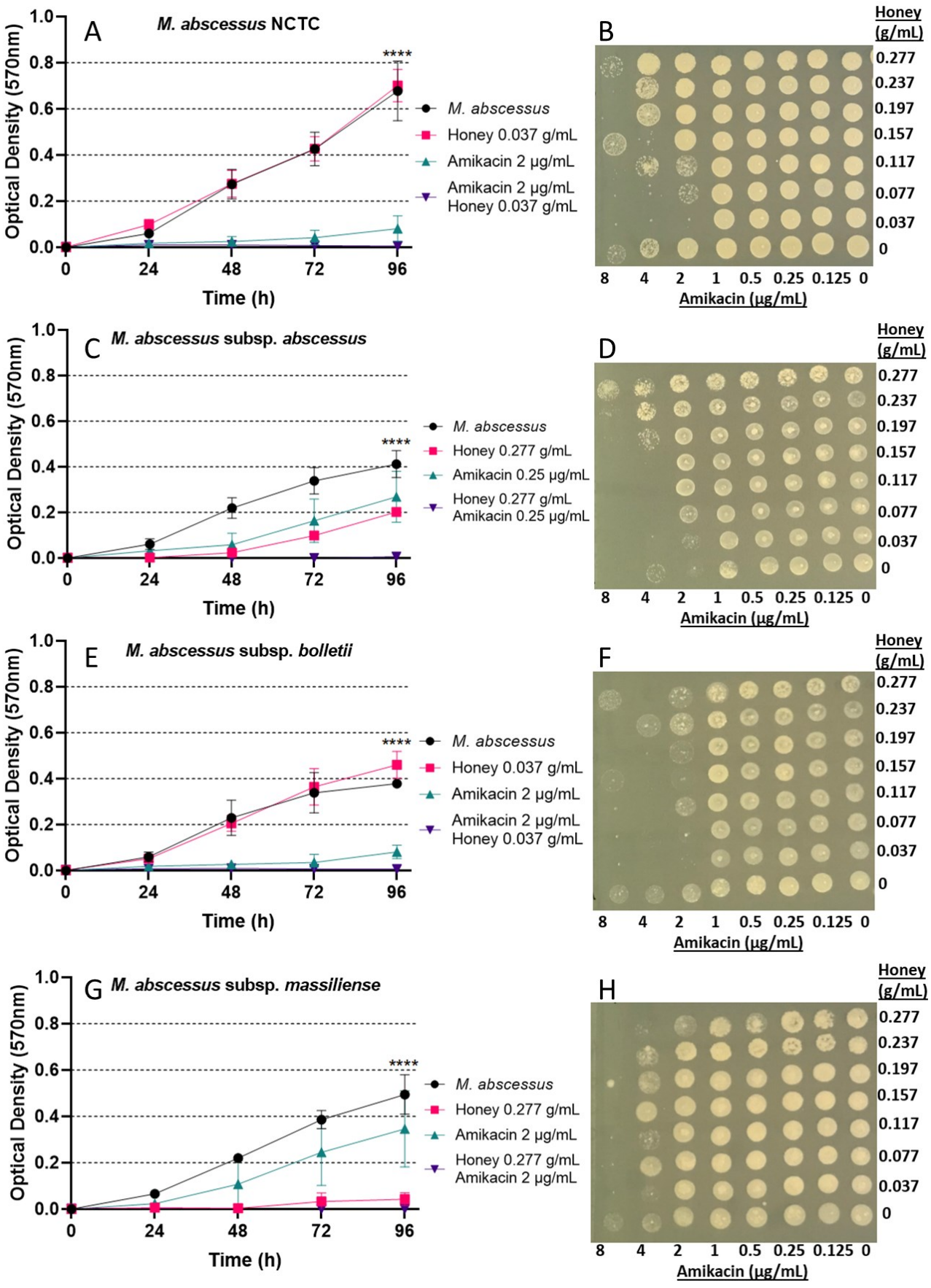

Figure 3.9 Combination of MGO83 manuka honey and amikacin results in increased inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for

statistical analysis to determine the impact of manuka honey concentration in combination with amikacin on the growth of *M. abscessus*. A) Growth curve showing improved inhibition of *M. abscessus* NCTC 13031 by combining 0.037 g/mL MGO83 and 2 µg/mL amikacin. One-way ANOVA shows a significant difference between all treatments, P=<0.0001. B) *M. abscessus* NCTC 13031 increased bactericidal activity on solid media by combining 0.037 g/mL MGO83 and 2 µg/mL amikacin. C) Growth curve showing enhanced inhibition of *M. abscessus* subsp. *abscessus* when exposed to 0.277 g/mL MGO83 and 0.25 µg/mL amikacin. One-way ANOVA shows significant difference between all treatments, P=<0.0001. D) *M. abscessus* subsp. *abscessus* improved bactericidal activity when transferred to solid media with both 0.037 g/mL MGO83 and 4 µg/mL amikacin. E) Growth curve indicating the combination of 0.037 g/mL MGO83 and 2 µg/mL amikacin results in improved inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=<0.0001. F) *M. abscessus* subsp. *bolletii* increased bactericidal activity on solid media with the combination of 0.037 g/mL MGO83 and 2 µg/mL amikacin. G) Growth curve showing inhibition of *M. abscessus* subsp. *massiliense* by combining 0.277 g/mL MGO83 and 2 µg/mL amikacin, one-way ANOVA P=<0.0001. H) Bactericidal activity of 0.037 g/mL MGO83 and 8 µg/mL amikacin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

Table 3.8 FBCI values for MGO83 manuka honey and amikacin against *M. abscessus*.

***M. abscessus* isolate**	**Honey alone MBC (g/mL)**	**Honey combination MBC (g/mL)**	**Amikacin alone MBC (µg/mL)**	**Amikacin combination MBC (µg/mL)**	**FBCI**
NCTC 13031	0.476	0.037	16	2	0.202
subsp. *abscessus*	0.476	0.037	8	4	0.577
subsp. *bolletii*	0.476	0.037	16	2	0.202
subsp. *massiliense*	0.476	0.037	16	8	0.577

A different response was observed for *M. abscessus* subsp. *abscessus*, which required 0.277 g/mL MGO83 with 0.25 µg/mL amikacin for improved inhibition (Table 3.7). This is a decrease from 0.476 g/mL MGO83 and 4 µg/mL amikacin when used alone. Based on these values, the FICI value of 0.577 suggests these concentrations were not synergistic and there was no interaction. The growth curve shows that there was a reduction in growth for MGO83 and amikacin alone compared to the control of *M. abscessus* subsp. *abscessus* only, and no growth was observed for the combination (Figure 3.9 C). A one-way ANOVA indicated there was a significant difference for all treatments, P=<0.0001, and a Dunnett's multiple comparison showed there was a difference for all treatments compared to the control of *M. abscessus* subsp. *abscessus* only, P=<0.0001 for the combination, P=0.0026 for MGO83 alone and P=0.0287 for amikacin alone. Another Dunnett's comparison was conducted,

comparing all treatments to the combination. This identified a significant difference between all treatments compared to the combination, P=<0.0001 for *M. abscessus* subsp. *abscessus*, P=0.0008 for amikacin and P=0.0069 for MGO83. This suggests there was a difference between the combination and MGO83 and amikacin used alone. Although these concentrations were inhibitory when used together, they were not bactericidal. The concentrations required for bactericidal activity were 0.037 g/mL MGO83 and 4 µg/mL amikacin (Figure 3.9 D). The FBCI value for these concentrations indicate there was no interaction between these concentrations, FBCI value 0.577 (Table 3.8).

The combination of MGO83 and amikacin required for improved inhibition against *M. abscessus* subsp. *bolletii* was the same as for *M. abscessus* NCTC 13031 (Table 3.7). These were 0.037 g/mL MGO83 and 2 µg/mL amikacin, which is reduced from 0.476 g/mL and 8 µg/mL. The FICI value based on these concentrations was 0.327, suggesting a synergistic interaction. The growth curve shows similar growth for MGO83 compared to *M. abscessus* subsp. *abscessus* only and a reduction in growth for amikacin, with no growth for the combination (Figure 3.9 E). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001, a Dunnett's multiple comparison showed a significant difference between all treatments compared to *M. abscessus* subsp. *bolletii* only, P=<0.0001 for the combination and amikacin only, P=0.0142 for MGO83 only. Another Dunnett's multiple comparison was also conducted, comparing all treatments to the combination, this also showed a significant difference for all treatments, P=<0.0001 for *M. abscessus* subsp. *abscessus* and MGO83 alone, P=0.0226 for amikacin. This suggests there was a difference in response to the combination compared to either amikacin or MGO83 alone. Furthermore, this was also reflected in the bactericidal activity, with visible growth for 0.037 g/mL MGO83 and 2 µg/mL amikacin alone and no growth visible for the combination (Figure 3.9 F). The FBCI value for these concentrations also suggests they are synergistic, FBCI value 0.202 (Table 3.8).

The concentrations needed to inhibit *M. abscessus* subsp. *massiliense* were higher than those for the other isolates, requiring 0.277 g/mL MGO83 and 2 µg/mL amikacin (Table 3.7). This was still less than the concentrations used alone for inhibition, which were 0.476 g/mL MGO83 and 4 µg/mL amikacin, giving an FICI value of 1.081, showing there was no interaction between the two compounds. The growth curve shows a reduction in growth for amikacin alone, compared to *M. abscessus* subsp. *massiliense*, with a further reduction for MGO83 alone and no growth for the combination (Figure 3.9 G). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001, and a Dunnett's multiple comparison, comparing all treatments to *M. abscessus* subsp. *massiliense* only, showed a significant difference for the combination and MGO83 alone, P=<0.0001, and no significance between amikacin alone and *M. abscessus* subsp. *massiliense*, P=0.1088. Another Dunnett's multiple comparison, comparing all treatments to the combination, showed a significant

difference between *M. abscessus* subsp. *massiliense* only, P=<0.0001 and amikacin only, P=0.0006 compared to the control, and no difference between MGO83 alone and the combination, P=0.8560. This suggests that there was no difference in response to MGO83 and the combination. This is further supported by the bactericidal activity, which shows visible growth for MGO83 alone, amikacin alone and the combination at these concentrations (Figure 3.9 H). However, looking at 0.037 g/mL MGO83 and 8 µg/mL amikacin, growth was visible for these concentrations alone but no visible growth can be seen for these in combination. Using these values to calculate the FBCI, there was still no interaction, FBCI value 0.577 (Table 3.8).

3.3.3 Combinatorial effect of manuka honey and tobramycin against *M. abscessus*

Since both tobramycin and amikacin are aminoglycoside antibiotics with similar chemical structures and for a direct comparison between the 2, the same concentrations that were explored for synergistic interactions with manuka honey and amikacin were chosen for tobramycin. This was unless a relationship between tobramycin and manuka honey were noticed at other concentrations. In most instances there were no interactions between tobramycin and manuka honey, but in some instances, antagonism was observed.

3.3.3.1 MGO40 in combination with tobramycin against *M. abscessus*

An interaction between tobramycin and MGO40 against *M. abscessus* was not observed for all isolates tested. There was no relationship observed for *M. abscessus* NCTC 13031 and *M. abscessus* subsp. *abscessus*, but there was an interaction observed for *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*. For *M. abscessus* NCTC 13031, the same concentrations explored for amikacin were focused on for tobramycin (Section 3.3.2.1), this was 0.037 g/mL MGO40 and 1 µg/mL (Figure 3.10 A). No inhibition was observed for the combination of tobramycin and MGO40. There was also no bactericidal activity observed for any concentration tested (Figure 3.10 B). This was also the same for *M. abscessus* subsp. *abscessus*. The same concentrations observed for amikacin were focused on for tobramycin (Section 3.3.2.1), these were 0.077 g/mL MGO40 and 2 µg/mL tobramycin. No interaction was observed and growth was seen for all concentrations, including the combination (Figure 3.10 C). There was also no bactericidal activity observed (Figure 3.10 D).

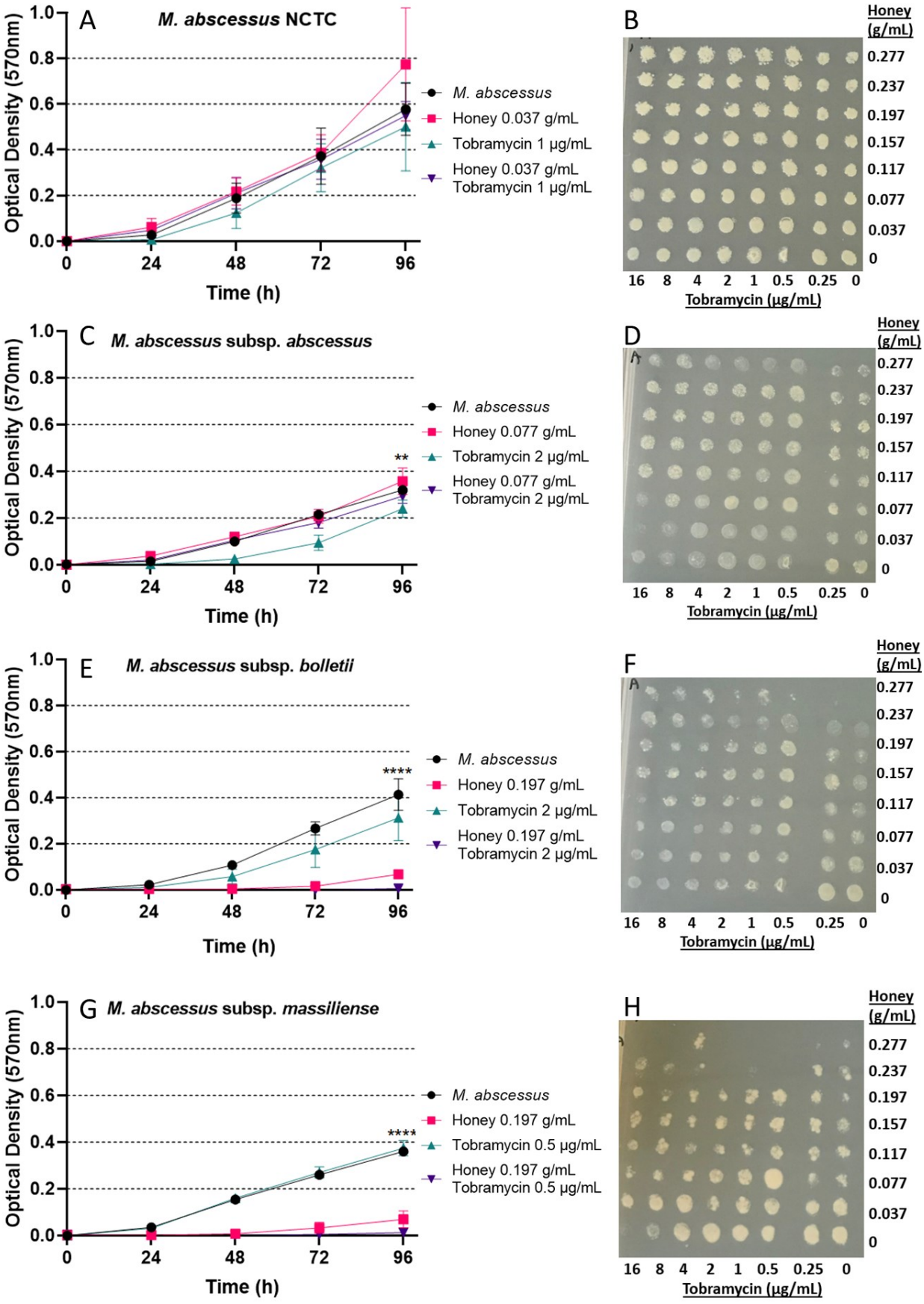

Figure 3.10 Combination of MGO40 manuka honey and tobramycin results in variable inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in combination with

tobramycin on the growth of *M. abscessus*. A) Growth curve showing no interaction between tobramycin and MGO40 against *M. abscessus* NCTC 13031. B) *M. abscessus* NCTC 13031 exhibiting no bactericidal activity on solid media of tobramycin and MGO40 for any concentration tested. C) Growth curve indicating no interaction for MGO40 and tobramycin against *M. abscessus* subsp. *abscessus.* D) *M. abscessus* subsp. *abscessus* showing no bactericidal activity for MGO40 and tobramycin on solid media for any concentration tested. E) Growth curve showing combination of 0.197 g/mL MGO40 and 2 µg/mL tobramycin results in improved inhibition of *M. abscessus* subsp. *bolletii*, Kruskal-Wallis P=<0.0001. F) *M. abscessus* subsp. *bolletii* showing no improved bactericidal activity on solid media at any concentration tested. G) Growth curve identifying inhibition of *M. abscessus* subsp. *massiliense* by combining 0.197 g/mL MGO40 and 0.5 µg/mL tobramycin, one-way ANOVA P=<0.0001. H) Bactericidal activity of 0.237 g/mL MGO40 and 0.5 µg/mL tobramycin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

For *M. abscessus* subsp. *bolletii* an interaction between tobramycin and MGO40 was observed at 0.197 g/mL MGO40 and 2 µg/mL tobramycin (Figure 3.10 E). Growth was observed for MGO40 and tobramycin alone at these concentrations but no growth was observed for both MGO40 and tobramycin together at the same concentrations. A Kruskal-Wallis identified a significant difference between all treatments, P=<0.0001, and a Dunn's multiple comparison identified a significant difference between *M. abscessus* subsp. *bolletii* and the combination, P=0.0048 and no difference between MGO40 alone and tobramycin alone compared to *M. abscessus* subsp. *bolletii* only, P=0.0642 and P=>0.9999, respectively. A further analysis, comparing all treatments to the combination identified a significant difference for *M. abscessus* subsp. *bolletii* only and tobramycin only, P=0.0048 and P=0.0460, respectively. No difference was identified between MGO40 alone compared to the combination, P=0.9113. This suggests there was no difference between MGO40 alone and the combination. The FICI value based on these concentrations was calculated, FICI value 0.663, which suggested there was no interaction between the two antimicrobial compounds at these concentrations. Furthermore, there was no bactericidal activity observed for *M. abscessus* subsp. *bolletii* at any combination of MGO40 and tobramycin tested (Figure 3.10 F).

An interaction between MGO40 and tobramycin was also identified for *M. abscessus* subsp. *massiliense* (Figure 3.10 G). The concentrations of 0.197 g/mL MGO40 and 0.5 µg/mL tobramycin showed growth for these concentrations alone but no growth for the combination together. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001, and a Dunnett's multiple comparison identified a significant difference between MGO40 and the combination compared to the control of *M. abscessus* subsp. *massiliense* alone, P=<0.0001 for both, and no difference for tobramycin alone, P=0.7842. Another Dunnett's multiple comparison was also conducted, comparing all treatments to the combination. This showed a significant difference for all treatments compared to the

combination, *M. abscessus* subsp. *massiliense* P=<0.0001, tobramycin P=<0.0001 and MGO40 P=0.0215. This suggests the combination was more effective than each antimicrobial compound alone. The FICI value was also calculated using these concentrations which identified a synergistic interaction, FICI value 0.476. This further indicates that these concentrations were synergistic. However, this was not reflected in the bactericidal activity (Figure 3.10 H). Bactericidal activity was observed for 0.237 g/mL MGO40 and 0.5 µg/mL tobramycin, giving an FBCI value of 0.560, suggesting this was not synergistic.

3.3.3.2 MGO55 in combination with tobramycin against *M. abscessus*

The activity of tobramycin and MGO55 was varied, with only one subspecies, *M. abscessus* subsp. *bolletii*, exhibiting an interaction (Figure 3.11). The MIC of MGO55 also altered and was lower than observed previously (Section 2.3.2, Table 2.5), being 0.277 g/mL rather than 0.476 g/mL, which could have impacted the interactions between MGO55 and tobramycin. There was no interaction observed for MGO55 and tobramycin against *M. abscessus* NCTC 13031. Therefore, the concentrations focused on were 0.037 g/mL MGO55 and 2 µg/mL tobramycin because these were beneficial for amikacin and MGO55. No interaction was observed and growth was seen for all treatments (Figure 3.11 A). There was also no bactericidal activity observed for any concentration tested, either alone or in combination (Figure 3.11 B). There were also no interactions observed for MGO55 and tobramycin against *M. abscessus* subsp. *abscessus*, so the same concentrations for amikacin were focused on. These were 0.277 g/mL MGO55 and 0.25 µg/mL tobramycin (Figure 3.11 C). At these concentrations MGO55 appeared to inhibit *M. abscessus* at 0.277 g/mL, not 0.476 g/mL previously observed (Section 2.3.2, Table 2.5). This meant that there was no interaction observed between MGO55 and tobramycin at these concentrations. However, the bactericidal activity showed 2 possible combinations of MGO55 and tobramycin that resulted in bactericidal activity (Figure 3.11 D). These were 0.077 g/mL MGO55 with 16 µg/mL tobramycin, or 0.037 g/mL MGO55 with 8 µg/mL, although these were not MBCs.

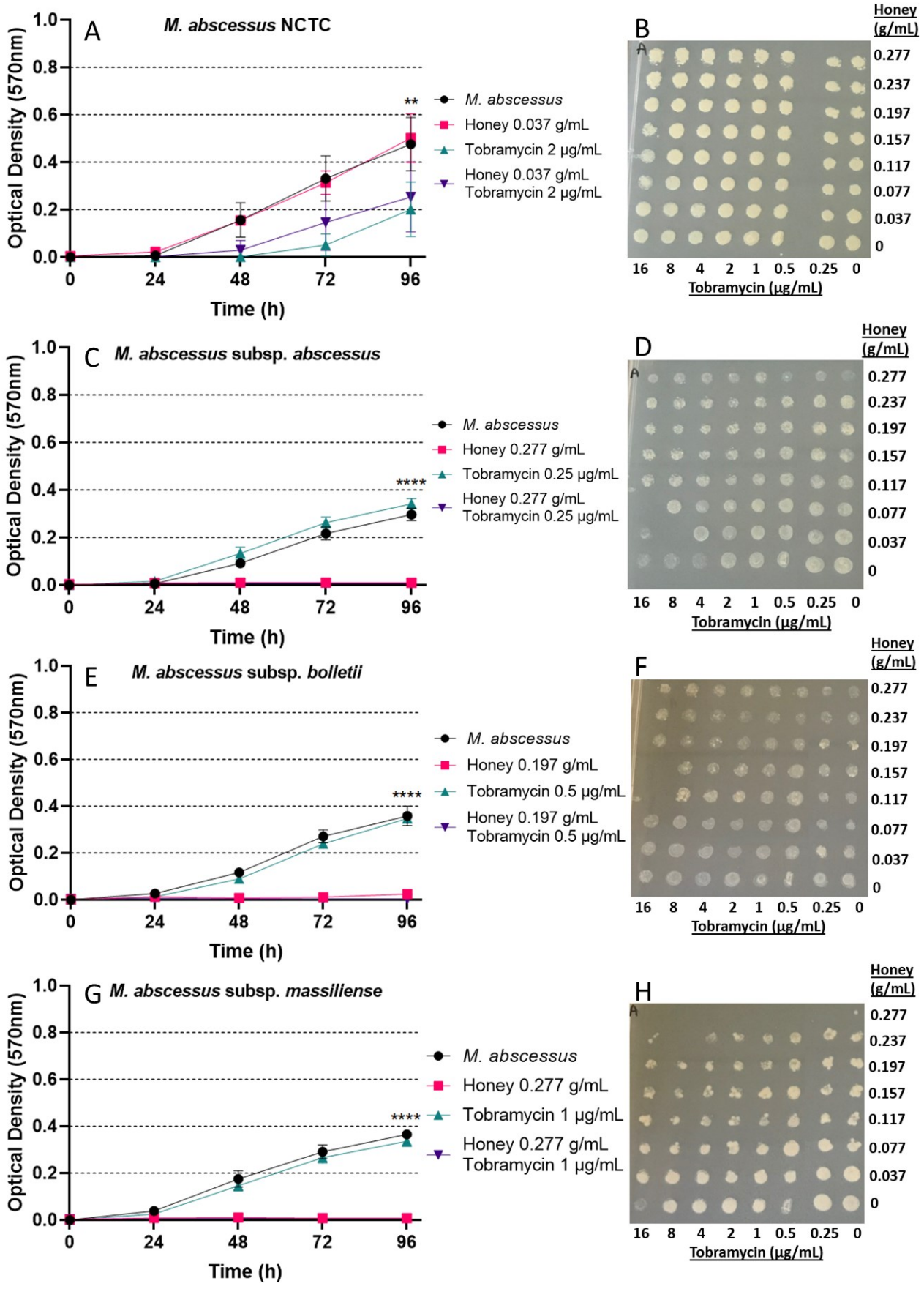

Figure 3.11 Combination of MGO55 manuka honey and tobramycin results in variable inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in combination with

tobramycin on the growth of *M. abscessus*. A) Growth curve showing no interaction between tobramycin and MGO55 against *M. abscessus* NCTC 13031. B) *M. abscessus* NCTC 13031 exhibiting no bactericidal activity of tobramycin and MGO55 on solid media for any concentration tested. C) Growth curve identifying no interaction for MGO55 and tobramycin against *M. abscessus* subsp. *abscessus.* D) *M. abscessus* subsp. *abscessus* showing bactericidal activity for MGO55 and tobramycin for 2 concentrations on solid media. These were 0.077 g/mL MGO55 and 16 µg/mL tobramycin or 0.037 g/mL MGO55 and 8 µg/mL tobramycin. E) Growth curve showing combination of 0.197 g/mL MGO55 and 0.5 µg/mL tobramycin results in inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=<0.0001. F) *M. abscessus* subsp. *bolletii* showing improved bactericidal activity at 2 concentrations when transferred to solid media. These were 16 µg/mL tobramycin with either 0.157 g/mL MGO55 or 0.117 g/mL MGO55. G) Growth curve identifying no improved inhibition of *M. abscessus* subsp. *massiliense* by MGO55 and tobramycin. H) Bactericidal activity of 0.277 g/mL MGO55 and all concentrations of tobramycin tested against *M. abscessus* subsp. *massiliense* after transfer to solid media.

For *M. abscessus* subsp. *bolletii* a possible interaction was observed between 0.197 g/mL MGO55 and 0.5 µg/mL (Figure 3.11 E). The growth for MGO55 alone was drastically reduced compared to the control of *M. abscessus* subsp. *bolletii* only, and the growth for 0.5 µg/mL tobramycin was similar to that of the control, and the combination showed no growth. A one-way ANOVA identified a significant difference between all treatments P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the control, show a significant difference between MGO55 alone and the combination, P=<0.0001, and no difference between tobramycin alone P=0.9029. A second Dunnett's multiple comparison was also conducted, comparing all treatments to the combination. This identified a significant difference for *M. abscessus* subsp. *bolletii* alone and tobramycin alone, P=<0.0001, and no difference between MGO55 alone and the combination P=0.6058, suggesting there was no difference between the treatments. Furthermore, the FICI value at these concentrations suggested there was no interaction, FICI value 0.956. However, considering the bactericidal activity, no growth was observed for 2 concentration combinations, 0.157 g/mL MGO with 16 µg/mL tobramycin and 0.117 g/mL with 16 µg/mL tobramycin, but these were not MBCs (Figure 3.11 F). This further suggests there was no interaction between MGO55 and tobramycin.

There was no interaction detected for MGO55 and tobramycin against *M. abscessus* subsp. *massiliense*, so the same concentrations observed for amikacin were focused on. These were 0.277 g/mL MGO55 and 1 µg/mL tobramycin. The MIC of MGO55 appeared to be lower again and no growth was observed for 0.277 g/mL MGO55 alone. Growth for 1 µg/mL tobramycin was similar to that of *M. abscessus* subsp. *massiliense* only and no growth was observed for the combination (Figure 3.11 G). The bactericidal activity showed no growth for MGO55 at 0.277 g/mL with any concentration of tobramycin (Figure 3.11 H). The FBCI value for the lowest concentration of tobramycin, 0.25 µg/mL, with 0.277 g/mL MGO55 is 0.589 which suggests that this is not a synergistic interaction.

3.3.3.3 MGO70 in combination with tobramycin against *M. abscessus*

There was no interaction observed between MGO70 and tobramycin against *M. abscessus* for all isolates tested (Figure 3.12). The same concentrations explored for amikacin and MGO70 were focused on, and no inhibition was identified for the combination of both manuka honey and antibiotic. *M. abscessus* NCTC 13031 showed improved growth for the combination of MGO70 and tobramycin compared to tobramycin only, but this was less inhibition than that for MGO70 or *M. abscessus* only (Figure 3.12 A). The bactericidal activity showed no growth for the combination of 0.157 g/mL MGO70 and 16 µg/mL, giving an FBCI value of 0.829, which suggests this concentration was not synergistic (Figure 3.12 B). A similar interaction was observed for *M. abscessus* subsp. *abscessus*, with a reduction in growth for tobramycin compared to the combination of MGO70 and tobramycin. This was still reduced compared to the growth of *M. abscessus* subsp. *abscessus* only and MGO70 (Figure 3.12 C). The bactericidal activity indicated no growth for 2 combinations, 0.117 g/mL MGO70 with 16 µg/mL and 0.077 g/mL MGO70 with 16 µg/mL (Figure 3.12 D).

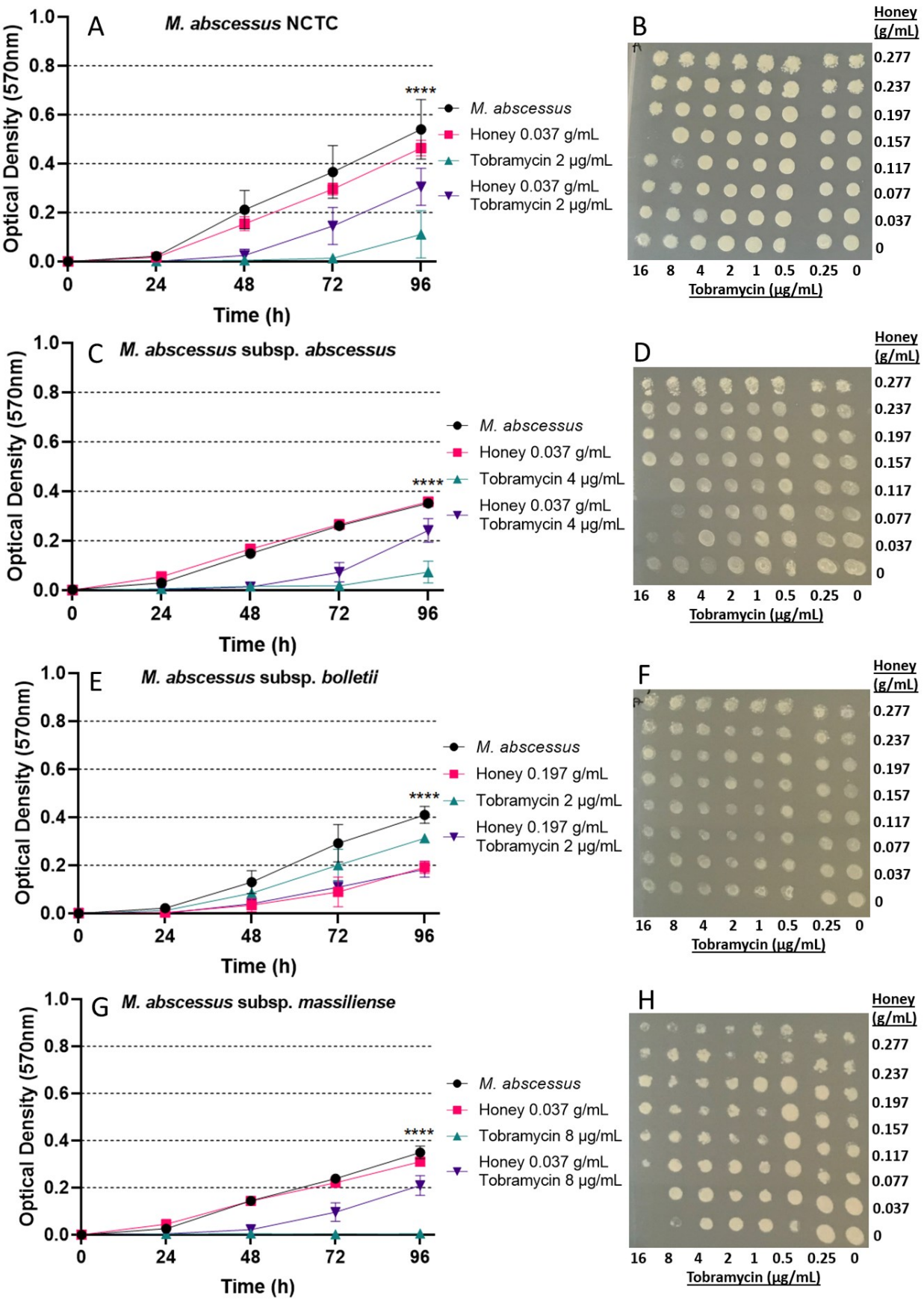

Figure 3.12 Combination of MGO70 manuka honey and tobramycin results in variable inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in combination with

tobramycin on the growth of *M. abscessus*. A) Growth curve showing no interaction between tobramycin and MGO70 against *M. abscessus* NCTC 13031. B) *M. abscessus* NCTC 13031 exhibiting no bactericidal activity of tobramycin and MGO70 for any concentration tested on solid media. C) Growth curve indicating no interaction for MGO70 and tobramycin against *M. abscessus* subsp. *abscessus.* D) *M. abscessus* subsp. *abscessus* showing bactericidal activity for MGO70 and tobramycin for 2 concentrations after transfer to solid media. These are 0.177 g/mL MGO70 and 16 µg/mL tobramycin or 0.077 g/mL MGO70 and 16 µg/mL tobramycin. E) The growth curve identified no interaction for 0.197 g/mL MGO70 and 2 µg/mL tobramycin against *M. abscessus* subsp. *bolletii*. F) *M. abscessus* subsp. *bolletii* showing no bactericidal activity on solid media after treatment with MGO70 and tobramycin. G) Growth curve showing no interaction of *M. abscessus* subsp. *massiliense* by MGO70 and tobramycin. H) No bactericidal activity of MGO70 and tobramycin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

There was no interaction observed for MGO70 and tobramycin against *M. abscessus* subsp. *bolletii* (Figure 3.12 E). The growth observed in response to both MGO70 and the combination of MGO70 with tobramycin had very similar growth, suggesting that the addition of the tobramycin did not impact the growth. The bactericidal activity also showed no growth for any combination (Figure 3.12 F). This suggests that MGO70 and tobramycin had no interaction.

The interaction between MGO70 and tobramycin resulted in improved growth of *M. abscessus* subsp. *massiliense* for the combination compared to MGO70 and tobramycin alone (Figure 3.12 G). The growth for MGO70 was similar to that of *M. abscessus* subsp. *massiliense* only, and no growth was observed for tobramycin alone. However, the combination together resulted in growth of *M. abscessus* subsp. *massiliense*. This was further reflected in the bactericidal activity, with no growth observed for 16 µg/mL tobramycin alone or with 0.037 g/mL, but growth can be seen for all other concentrations of MGO70 tested (Figure 3.12 H).

3.3.3.4 MGO83 in combination with tobramycin against *M. abscessus*

No synergistic interactions were observed between MGO83 and tobramycin against *M. abscessus*. This resulted in the same concentrations observed for amikacin being focused on for tobramycin. Moreover, the bactericidal activity did show some interactions between MGO83 and tobramycin.

For *M. abscessus* NCTC 13031 the concentrations focused on were 0.037 g/mL MGO83 and 2 µg/mL tobramycin (Figure 3.13 A). The previous data sets showed growth for *M. abscessus* NCTC 13031 with 2 µg/mL tobramycin, however, no growth was observed. The growth for *M. abscessus* NCTC 13031 alone and with 0.037 g/mL MGO83 were very similar and the combination of both tobramycin and MGO83 showed no growth. Since there was no growth observed for tobramycin only at this concentration, it resulted in no interaction being observed. The bactericidal activity did show an interaction between 0.117 g/mL MGO83 and 16 µg/mL tobramycin (Figure 3.13 B). These concentrations give an FBCI of 0.745, suggesting this was not synergistic.

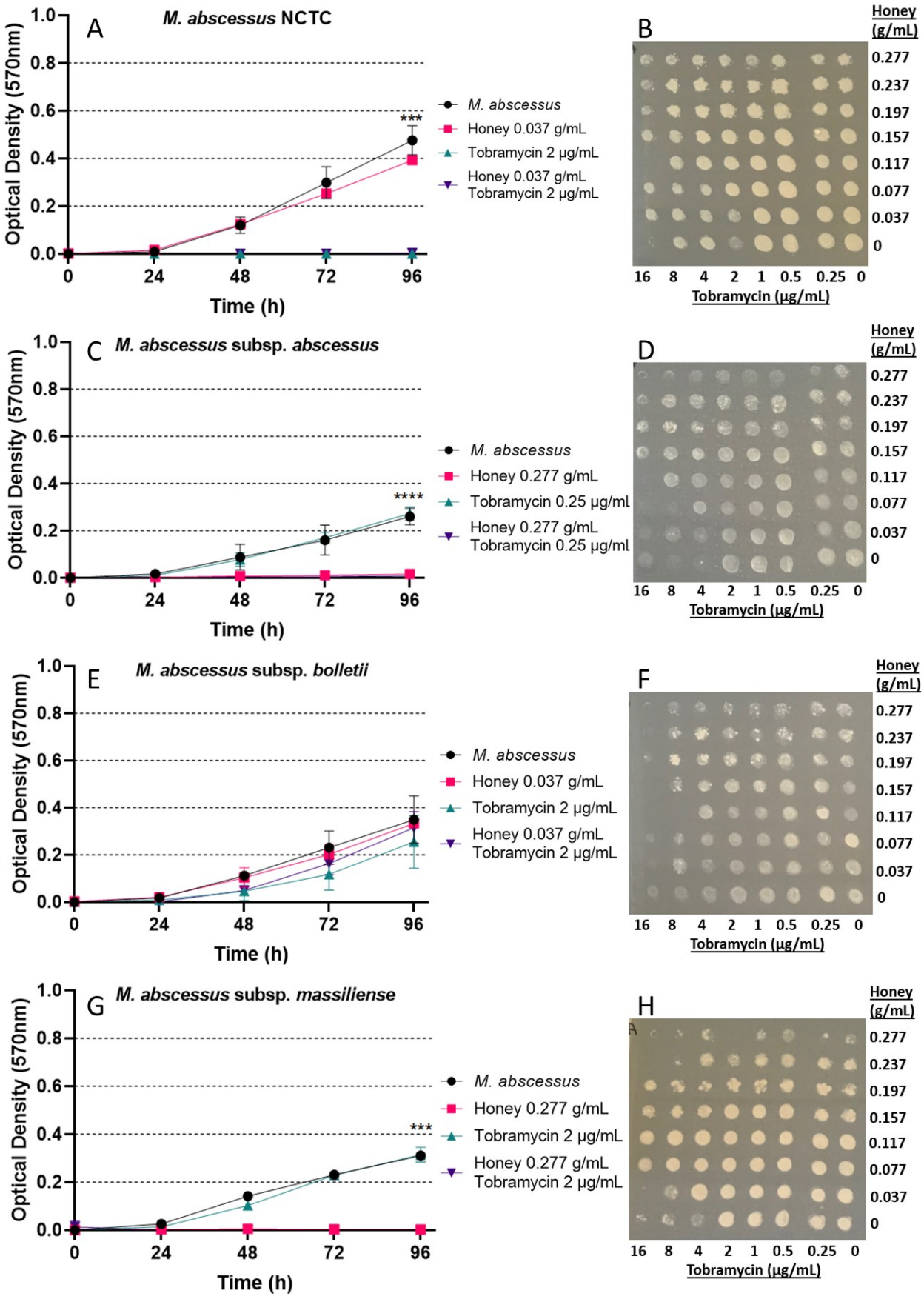

Figure 3.13 Combination of MGO83 manuka honey and tobramycin results in variable inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in combination with

tobramycin on the growth of *M. abscessus*. A) Growth curve showing no interaction between tobramycin and MGO83 against *M. abscessus* NCTC 13031. B) *M. abscessus* NCTC 13031 exhibiting an interaction on solid media between 0.117 g/mL MGO83 and 16 µg/mL tobramycin. C) Growth curve indicating no interaction for MGO83 and tobramycin against *M. abscessus* subsp. *abscessus.* D) *M. abscessus* subsp. *abscessus* showing bactericidal activity for MGO83 and tobramycin for 0.177 g/mL MGO70 and 16 µg/mL tobramycin on solid media. E) The growth curve showed no interaction for MGO83 and tobramycin against *M. abscessus* subsp. *bolletii*. F) *M. abscessus* subsp. *bolletii* showing bactericidal activity on solid media with 0.117 g/mL MGO83 and 8 µg/mL tobramycin. G) Growth curve showing no interaction of *M. abscessus* subsp. *massiliense* by MGO83 and tobramycin. H) Interaction of 3 concentrations of MGO83 with tobramycin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

A similar occurrence was observed for *M. abscessus* subsp. *abscessus*, however in this instance no growth was observed for MGO83 (Figure 3.13 C). The concentrations examined were 0.277 g/mL MGO83 and 0.25 µg/mL tobramycin. The growth for *M. abscessus* subsp. *abscessus* alone and with 0.25 µg/mL tobramycin were very similar. No growth was observed for MGO83 alone or in combination with tobramycin. This resulted in no interaction being observed between MGO83 and tobramycin against *M. abscessus* subsp. *abscessus*. However, the bactericidal activity showed that 0.117 g/mL MGO83 and 16 µg/mL tobramycin resulted in no growth (Figure 3.13 D). The FBCI showed no interaction, FBCI value of 0.745.

There was no synergistic interaction observed for MGO83 and tobramycin against *M. abscessus* subsp. *bolletii*, therefore the same concentrations observed for amikacin were focused on (Figure 3.13 E). For all treatment's growth was observed, and no interaction observed. The assessment of bactericidal activity did show that 0.117 g/mL MGO83 and 8 µg/mL tobramycin had growth for each concentration alone but no growth was observed for these concentrations together (Figure 3.13 F). This results in an FBCI of 0.621, indicating no interaction.

For MGO83 and tobramycin against *M. abscessus* subsp. *massiliense* the MIC concentrations observed were the same as those for amikacin, 0.277 g/mL MGO83 and 2 µg/mL tobramycin (Figure 3.13 G). In this instance there was no growth observed for 0.277 g/mL MGO83 alone, and the growth of tobramycin only and *M. abscessus* subsp. *massiliense* alone were very similar. No growth was observed for the combination. Due to no growth being observed for MGO83, no interaction was identified. The bactericidal activity showed 3 possible combinations of interest, 0.277 g/mL MGO83 with 2 µg/mL tobramycin, 0.237 g/mL MGO83 with 16 µg/mL tobramycin and 0.037 g/mL with 16 µg/mL tobramycin (Figure 3.13 H). Interestingly, one of these combinations was the same as the one explored for inhibitory synergistic interactions, which results in an FBCI of 0.644. The combination of 0.237 g/mL MGO83 with 16 µg/mL tobramycin gives an FBCI of 0.997 and 0.037 g/mL MGO83 with 16 µg/mL gives an FBCI of 0.577. This suggests that none of these combinations are synergistic.

3.3.4 Combinatorial effect of manuka honey and azithromycin against *M. abscessus*

Increased bacteriostatic activity was observed for all manuka honey samples tested in combination with azithromycin against *M. abscessus* isolates. Typically, only 0.037 g/mL manuka honey was required for enhanced activity, depending on manuka honey strength and isolate tested. Bactericidal activity had more variation in effective concentrations and there was limited bactericidal activity observed for *M. abscessus* subsp. *bolletii*.

3.3.4.1 MGO40 in combination with azithromycin against *M. abscessus*

The combination of MGO40 and azithromycin resulted in improved antimicrobial activity for *M. abscessus* NCTC 13031 and the subspecies tested. The concentrations required for improved inhibition against *M. abscessus* NCTC 13031 were 0.037 g/mL MGO40 with 1 µg/mL azithromycin. This is a reduction from 0.476 g/mL MGO40 and 4 µg/mL azithromycin when used alone, giving an FICI value of 0.327, suggesting a synergistic interaction (Table 3.9). The growth curve shows similar growth for *M. abscessus* NCTC 13031 alone and treated with 0.037 g/mL MGO40, with a reduction in growth for 1 µg/mL azithromycin and no growth for the combination of MGO40 and azithromycin together (Figure 3.14 A). A Kruskal-Wallis identified a significant difference for all treatments, P=<0.0001, and the Dunn's multiple comparison identified no significant difference between MGO40 alone and azithromycin alone compared to *M. abscessus* NCTC 13031 only, P=>0.9999 and 0.5512 respectively. However, this improved inhibition was not bactericidal at these concentrations, and bactericidal activity was only observed at 0.117 g/mL MGO40 with 16 µg/mL azithromycin (Figure 3.14 B). At these concentrations the FBCI value was 0.745, suggesting there was no interaction at these concentrations (Table 3.10).

Table 3.9 FICI values for MGO40 manuka honey and azithromycin against *M. abscessus*

M. abscessus isolate	Honey alone MIC (g/mL)	Honey combination MIC (g/mL)	Azithromycin alone MIC (µg/mL)	Azithromycin combination MIC (µg/mL)	FICI
NCTC 13031	0.476	0.037	4	1	0.327
subsp. *abscessus*	0.476	0.077	2	0.5	0.411
subsp. *bolletii*	0.476	0.117	32	4	0.370
subsp. *massiliense*	0.476	0.037	1	0.5	0.577

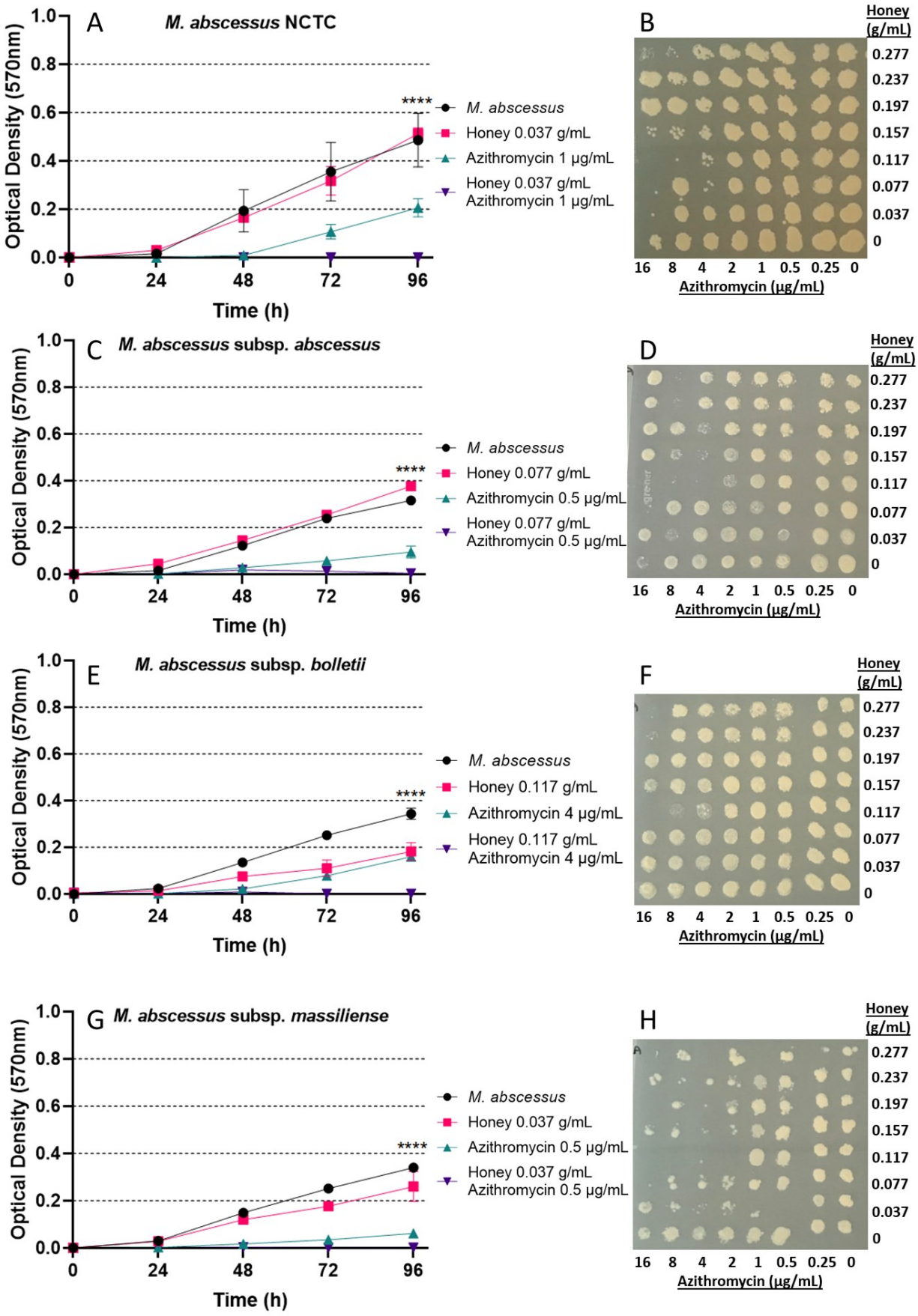

Figure 3.14 Combination of MGO40 manuka honey and azithromycin results in increased inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in

combination with azithromycin on the growth of *M. abscessus*. A) Growth curve showing improved inhibition of *M. abscessus* NCTC 13031 by combining 0.037 g/mL MGO40 and 1 µg/mL azithromycin. Kruskal-Wallis identifies a significant difference between all treatments, P=<0.0001. B) *M. abscessus* NCTC 13031 has increased bactericidal activity on solid media by combining 0.117 g/mL MGO40 and 16 µg/mL azithromycin. C) The growth curve showed enhanced inhibition of *M. abscessus* subsp. *abscessus* when exposed to 0.077 g/mL MGO40 and 0.5 µg/mL azithromycin. One-way ANOVA shows significant difference between all treatments, P=<0.0001. D) *M. abscessus* subsp. *abscessus* improved bactericidal activity with both 0.117 g/mL MGO40 and 4 µg/mL azithromycin after transfer to solid media. E) Growth curve indicates combination of 0.117 g/mL MGO40 and 4 µg/mL azithromycin results in improved inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=<0.0001. F) *M. abscessus* subsp. *bolletii* had increased bactericidal activity on solid media with the combination of 0.117 g/mL MGO40 and 16 µg/mL azithromycin. G) Growth curve showing inhibition of *M. abscessus* subsp. *massiliense* by combining 0.037 g/mL MGO40 and 0.5 µg/mL azithromycin, Kruskal-Wallis P=<0.0001. H) Bactericidal activity of 0.037 g/mL MGO40 and 0.5 µg/mL azithromycin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

Table 3.10 FBCI values for MGO40 manuka honey and azithromycin against *M. abscessus*

***M. abscessus* isolate**	**Honey alone MBC (g/mL)**	**Honey combination MBC (g/mL)**	**Azithromycin alone MBC (µg/mL)**	**Azithromycin combination MBC (µg/mL)**	**FBCI**
NCTC 13031	0.476	0.117	32	16	0.745
subsp. *abscessus*	0.476	0.117	32	4	0.370
subsp. *bolletii*	0.476	0.117	32	16	0.871
subsp. *massiliense*	0.476	0.037	32	0.5	0.093

For *M. abscessus* subsp. *abscessus* the combination required for improved inhibition was 0.077 g/mL MGO40 with 0.5 µg/mL azithromycin compared to 0.476 g/mL manuka honey alone and 2 µg/mL alone. These concentrations give an FICI value of 0.411, suggesting synergy between antimicrobials at these concentrations (Table 3.9). The growth curve for these concentrations show similar growth for *M. abscessus* subsp. *abscessus* alone and MGO40 alone, with reduced growth for azithromycin and no growth for the combination (Figure 3.14 C). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the growth of *M. abscessus* subsp. *abscessus* showed a significant difference for all treatments, P=<0.0001

for azithromycin only and the combination, P=0.0012 for MGO40 alone. A second Dunnett's multiple comparison was conducted, comparing all treatments to the combination, this identified a significant difference for all treatments compared, P=<0.0001 for all. However, these concentrations were not bactericidal, but bactericidal activity was observed for 0.117 g/mL MGO40 and 4 µg/mL azithromycin (Figure 3.14 D). The FBCI value for these concentrations also suggests synergy, FBCI value 0.370 (Table 3.10).

The combination for improved inhibition against *M. abscessus* subsp. *bolletii* was 0.117 g/mL MGO40 and 4 µg/mL azithromycin, which is reduced from 0.476 g/mL MGO40 only and 32 µg/mL azithromycin only (Table 3.9). These concentrations give an FICI value of 0.370, suggesting these concentrations are synergistic. The growth curve for these concentrations shows a reduction in growth for MGO40 alone and azithromycin alone compared to *M. abscessus* subsp. *bolletii* alone, and no growth for the combination (Figure 3.14 E). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001, and a Dunnett's multiple comparison, comparing all treatments to the control of *M. abscessus* subsp. *bolletii* only showed a significant difference for all, P=<0.0001 for all. A second Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This identified a significant difference for all treatments, P=<0.0001 for *M. abscessus* subsp. *bolletii* only and MGO40 alone compared to the combination, and P=0.0001 for azithromycin only compared to the combination. These concentrations were not bactericidal, but bactericidal activity was observed for 0.117 g/mL MGO40 and 16 µg/mL azithromycin (Figure 3.14 F). These concentrations give an FBCI value of 0.871 suggesting no interaction (Table 3.10).

The combination of 0.037 g/mL MGO40 and 0.5 µg/mL azithromycin resulted in improved activity against *M. abscessus* subsp. *massiliense*, which is reduced from 0.476 g/mL MGO40 alone and 1 µg/mL azithromycin alone (Table 3.9). This results in an FICI value of 0.577, suggesting there is no interaction. The growth curve shows similar growth for both *M. abscessus* subsp. *massiliense* only and MGO40, with a reduction in growth for azithromycin and no growth for the combination (Figure 3.14 G). A Kruskal-Wallis identified a significant difference for all treatments, P=<0.0001. A Dunn's multiple comparison, comparing all treatments to *M. abscessus* subsp. *massiliense* only showed no significant difference for MGO40 alone or azithromycin alone, P=>0.9999 and P=0.1119, respectively. A significant difference was observed between the combination and *M. abscessus* subsp. *massiliense* only, P=0.0032. These concentrations of 0.037 g/mL MGO40 and 0.5 µg/mL azithromycin were also bactericidal (Figure 3.14 H). The FBCI value for these concentrations further identifies that these concentrations are synergistic, 0.093 (Table 3.10).

3.3.4.2 MGO55 in combination with azithromycin against *M. abscessus*

A variation in response to the combination of MGO55 and azithromycin was observed, with all subspecies tested exhibiting increased inhibition to the combination of antibiotic and manuka honey, as opposed to each antimicrobial alone. For every isolate tested, only 0.037 g/mL MGO55 was required for the increased inhibitory activity, with a variation in amount of azithromycin required. Bactericidal activity was also observed for the combination of manuka honey and azithromycin at varying concentrations for all subspecies tested.

For *M. abscessus* NCTC 13031, increased inhibition was observed with 0.037 g/mL MGO70 and 1 µg/mL azithromycin, a reduction from 0.476 g/mL MGO55 alone and 4 µg/mL azithromycin alone (Table 3.11). These concentrations result in an FICI value of 0.327, suggesting these concentrations act synergistically. The growth curve for these concentrations show similar growth for *M. abscessus* NCTC 13031 only and MGO55 alone, with a reduction in growth observed for azithromycin alone and no growth for the combination (Figure 3.15 A). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001 and a Dunnett's multiple comparison showed a significant difference between azithromycin alone and the combination compared to *M. abscessus* NCTC 13031 alone, P=<0.0001 for both. No difference was observed between MGO55 alone and *M. abscessus* NCTC 13031 only, P=0.5640. Another Dunnett's multiple comparison was conducted, comparing all treatments to the combination, this identified all treatment were significantly different, P=<0.0001 for all. However, these concentrations were not bactericidal (Figure 3.15 B). One combination of MGO55 and azithromycin was bactericidal, this was 0.117 g/mL MGO55 with 16 µg/mL azithromycin. These concentrations give an FBCI value of 0.745, suggesting no interaction (Table 3.12).

Table 3.11 FICI values for MGO55 manuka honey and azithromycin against *M. abscessus*

M. abscessus isolate	Honey alone MIC (g/mL)	Honey combination MIC (g/mL)	Azithromycin alone MIC (µg/mL)	Azithromycin combination MIC (µg/mL)	FICI
NCTC 13031	0.476	0.037	4	1	0.327
subsp. *abscessus*	0.476	0.037	2	1	0.577
subsp. *bolletii*	0.476	0.037	32	8	0.327
subsp. *massiliense*	0.476	0.037	1	0.5	0.577

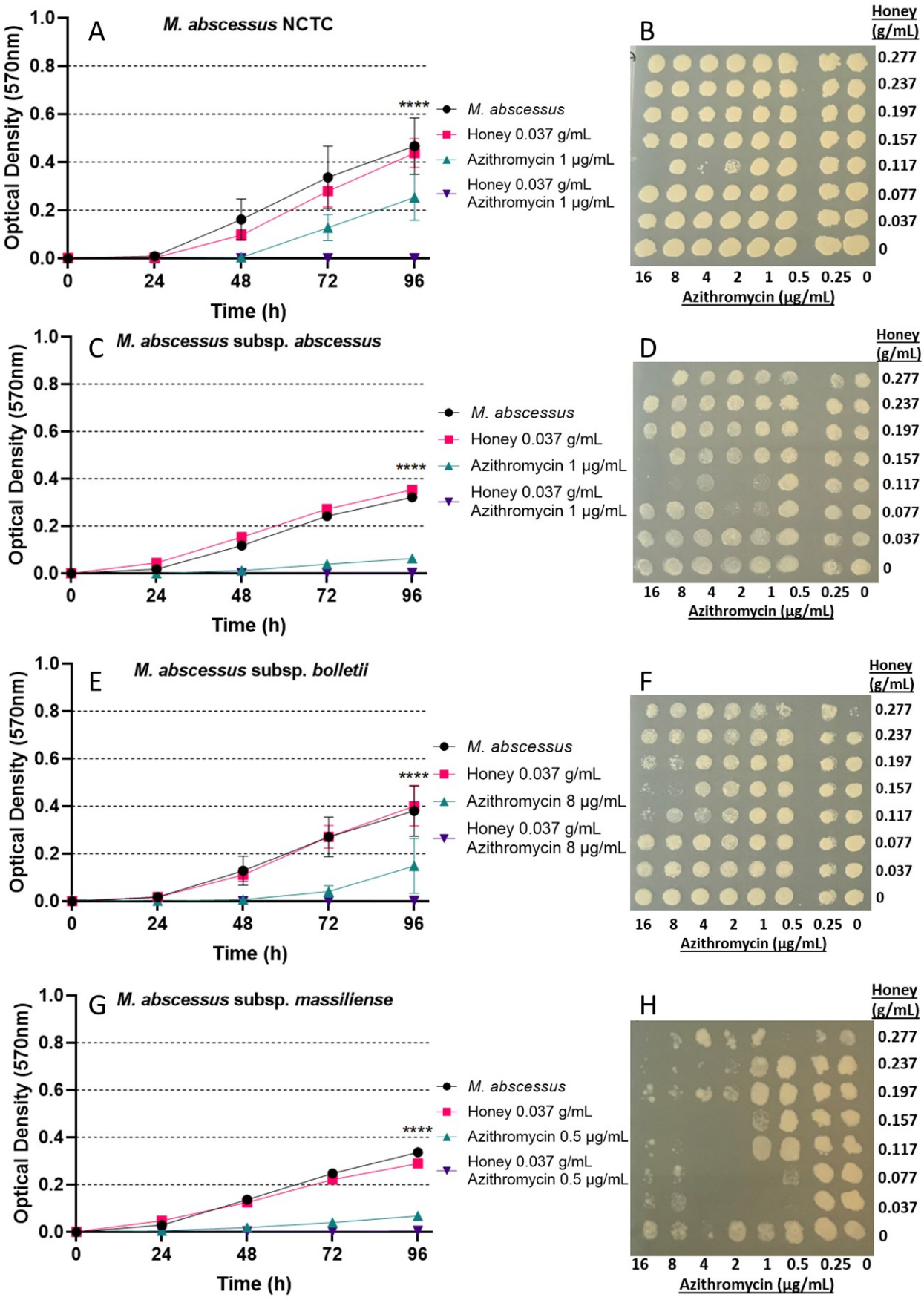

Figure 3.15 Combination of MGO55 manuka honey and azithromycin results in increased inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in

combination with azithromycin on the growth of *M. abscessus*. A) Growth curve showing improved inhibition of *M. abscessus* NCTC 13031 by combining 0.037 g/mL MGO55 and 1 µg/mL azithromycin. One-way ANOVA shows a significant difference between all treatments, P=<0.0001. B) *M. abscessus* NCTC 13031 increased bactericidal activity on solid media by combining 0.117 g/mL MGO55 and 16 µg/mL azithromycin. C) The growth curve shows enhanced inhibition of *M. abscessus* subsp. *abscessus* when exposed to 0.037 g/mL MGO55 and 1 µg/mL azithromycin. One-way ANOVA shows significant difference between all treatments, P=<0.0001. D) *M. abscessus* subsp. *abscessus* improved bactericidal activity with both 0.117 g/mL MGO55 and 2 µg/mL azithromycin when transferred to solid media. E) Growth curve identifying the combination of 0.037 g/mL MGO55 and 8 µg/mL azithromycin results in improved inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=<0.0001. F) *M. abscessus* subsp. *bolletii* has no bactericidal activity on solid media for any concentration tested. G) Growth curve showing inhibition of *M. abscessus* subsp. *massiliense* by combining 0.037 g/mL MGO55 and 0.5 µg/mL azithromycin, one-way ANOVA P=<0.0001. H) Bactericidal activity of 0.037 g/mL MGO55 and 0.5 µg/mL azithromycin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

Table 3.12 FBCI values for MGO55 manuka honey and azithromycin against *M. abscessus*

***M. abscessus* isolate**	**Honey alone MBC (g/mL)**	**Honey combination MBC (g/mL)**	**Azithromycin alone MBC (µg/mL)**	**Azithromycin combination MBC (µg/mL)**	**FBCI**
NCTC 13031	0.476	0.117	32	16	0.745
subsp. *abscessus*	0.476	0.117	32	2	0.308
subsp. *bolletii*	0.476	No interaction	32	No interaction	No interaction
subsp. *massiliense*	0.476	0.037	32	0.5	0.093

Improved inhibition was observed for *M. abscessus* subsp. *abscessus* treated with 0.037 g/mL MGO55 (Table 3.11). This was reduced from 0.476 g/mL MGO55 alone and 2 µg/mL azithromycin alone, resulting in an FICI of 0.577, suggesting no interaction. The growth curves shows improved growth for *M. abscessus* subsp. *abscessus* exposed to MGO55 alone at 0.037 g/mL, compared to the growth of *M. abscessus* subsp. *abscessus* only, and a reduction in growth for azithromycin, with no growth for the combination (Figure 3.15 C). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001, and a Dunnett's multiple comparison identified a difference between all treatments compared to the control of *M. abscessus* subsp. *abscessus* only, P=<0.0001 for azithromycin alone and the combination, P=0.0154 for MGO55 alone. A second Dunnett's multiple comparison was

conducted, comparing all treatments to the response of the combination. This identified a significant difference between all treatments, P=<0.0001 for all. This suggests that the response to the combination was significantly different to either MGO55 or azithromycin alone. These concentrations were not bactericidal, but bactericidal activity was observed for 5 different combinations (Figure 3.15 D). The MBC was 0.117 g/mL MGO55 with 2 µg/mL azithromycin, resulting in an FBCI value of 0.308 (Table 3.12). This suggests that these concentrations are synergistic.

The increased bacteriostatic activity observed for *M. abscessus* subsp. *bolletii* was achieved with 0.037 g/mL MGO55 with 8 µg/mL azithromycin, which is improved from 0.476 g/mL and 32 µg/mL azithromycin (Table 3.11). These concentrations give an FICI value of 0.327, which suggests a synergistic interaction. The growth curve shows similar growth for *M. abscessus* subsp. *bolletii* only and MGO55 alone (Figure 3.1 E). The growth for azithromycin alone is reduced and there is no growth observed for the combination. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the control of *M. abscessus* subsp. *bolletii* only, showed a significant difference for the combination and azithromycin only, P=0.0004 and P=0.0242 respectively. No significant difference was observed between MGO55 and *M. abscessus* subsp. *bolletii* only, P=0.4469. Another Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This showed a significant difference for all treatments, P=<0.0001 for MGO55, P=0.0004 for *M. abscessus* subsp. *bolletii* only and P=0.0458. This further supports these concentrations being synergistic. No bactericidal activity was observed for any combination tested (Figure 3.15 F).

The concentrations required for improved inhibition of *M. abscessus* subsp. *massiliense* were 0.037 g/mL MGO55 with 0.5 µg/mL azithromycin (Table 3.11). This is a reduction from 0.476 g/mL MGO55 alone and 1 µg/mL azithromycin alone, giving an FICI value of 0.577, suggesting no interaction. The growth curve shows similar growth for *M. abscessus* subsp. *massiliense* and MGO55 alone (Figure 3.15 G). A reduction in growth could be seen for azithromycin alone and no growth for the combination. A one-way ANOVA identified a significant difference for all treatments, P=<0.0001, and a Dunnett's multiple comparison showed a significant difference for all treatments compared to *M. abscessus* subsp. *massiliense* only, P=<0.0001 for azithromycin and the combination, P=0.0002 for MGO55. A second Dunnett's multiple comparison was conducted, comparing all treatments to the combination, this identified a significant difference for all treatments, P=<0.0001 for all. This suggests that there was a significant difference between the response to the combination and MGO55 and azithromycin alone. Furthermore, this concentration was bactericidal (Figure 3.15 H). This results in an FBCI of 0.093, which shows that these concentrations are synergistic.

3.3.4.3 MGO70 in combination with azithromycin against *M. abscessus*

The combination of MGO70 and azithromycin resulted in increased inhibition against all *M. abscessus* isolates tested. For increased inhibition only 0.037 g/mL MGO70 was required and the concentration of azithromycin was between 4 µg/mL and 0.5 µg/mL, depending on the subspecies. A variation in bactericidal activity was observed, with all isolates apart from *M. abscessus* subsp. *bolletii* exhibiting increased activity when in combination.

Increased inhibition of *M. abscessus* NCTC 13031 was observed by combining 0.037 g/mL MGO70 and 0.5 µg/mL azithromycin (Table 3.13). This was a reduction from 0.476 g/mL MGO70 and 4 µg/mL azithromycin alone, resulting in an FICI value of 0.202. This indicates synergy between the two antimicrobials. The growth curve shows similar growth for *M. abscessus* NCTC 13031 only, MGO70 alone and azithromycin alone (Figure 3.16 A). The combination of MGO70 with azithromycin shows no growth. A Kruskal-Wallis identified a significant difference between all treatments, P=0.0014 and a Dunn's multiple comparison identified no significance between MGO70 alone and azithromycin alone compared to the control of *M. abscessus* NCTC 13031 only, P=0.7917 and P=0.3534 respectively. A significant difference was identified between the combination and *M. abscessus* NCTC 13031 only, P=0.0031. These concentrations together were not bactericidal, but bactericidal activity was observed for 0.037 g/mL MGO70 with 4 µg/mL azithromycin (Figure 3.16 B). These concentrations give an FBCI value of 0.202, which further suggests that MGO70 and azithromycin are synergistic (Table 3.14).

Table 3.13 FICI values for MGO70 manuka honey and azithromycin against *M. abscessus*

***M. abscessus* isolate**	**Honey alone MIC (g/mL)**	**Honey combination MIC (g/mL)**	**Azithromycin alone MIC (µg/mL)**	**Azithromycin combination MIC (µg/mL)**	**FICI**
NCTC 13031	0.476	0.037	4	0.5	0.202
subsp. *abscessus*	0.476	0.037	2	1	0.577
subsp. *bolletii*	0.476	0.037	32	4	0.202
subsp. *massiliense*	0.476	0.037	2	0.5	0.327

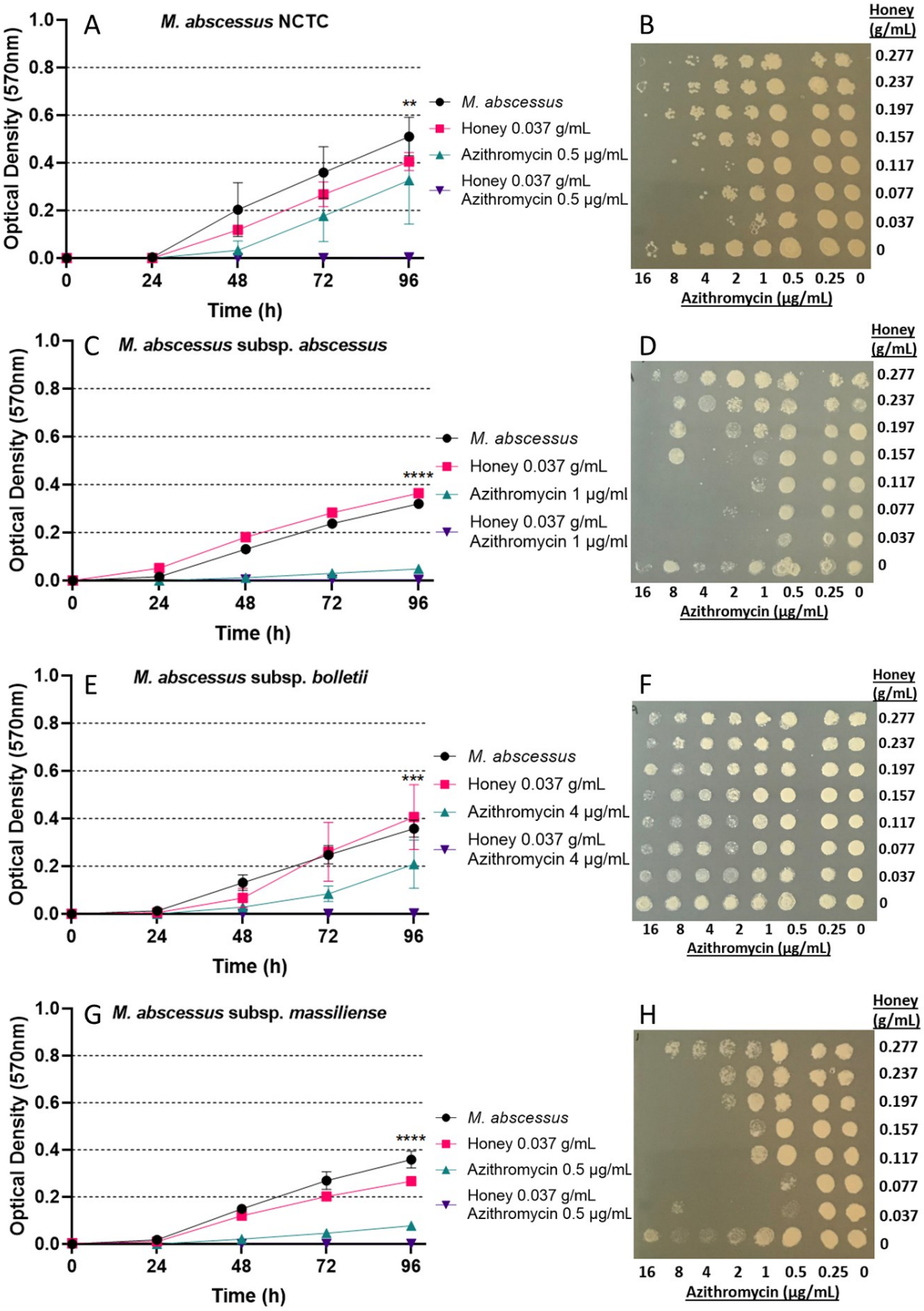

Figure 3.16 Combination of MGO70 manuka honey and azithromycin results in increased inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in

combination with azithromycin on the growth of *M. abscessus*. A) Growth curve showing improved inhibition of *M. abscessus* NCTC 13031 by combining 0.037 g/mL MGO70 and 0.5 µg/mL azithromycin. Kruskal-Wallis shows a significant difference between all treatments, P=0.0014. B) *M. abscessus* NCTC 13031 increased bactericidal activity on solid media by combining 0.037 g/mL MGO70 and 4 µg/mL azithromycin. C) Growth curve indicating enhanced inhibition of *M. abscessus* subsp. *abscessus* when exposed to 0.037 g/mL MGO70 and 1 µg/mL azithromycin. One-way ANOVA shows significant difference between all treatments, P=<0.0001. D) *M. abscessus* subsp. *abscessus* improved bactericidal activity with both 0.037 g/mL MGO70 and 1 µg/mL azithromycin on solid media. E) Growth curve identifying combination of 0.037 g/mL MGO70 and 4 µg/mL azithromycin results in improved inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=0.0001. F) *M. abscessus* subsp. *bolletii* has no bactericidal activity for any concentration tested on solid media. G) Growth curve showing inhibition of *M. abscessus* subsp. *massiliense* by combining 0.037 g/mL MGO70 and 0.5 µg/mL azithromycin, one-way ANOVA P=<0.0001. H) Bactericidal activity of 0.037 g/mL MGO70 and 1 µg/mL azithromycin against *M. abscessus* subsp. *massiliense* when transferred to solid media.

Table 3.14 FBCI values for MGO70 manuka honey and azithromycin against *M. abscessus*

***M. abscessus* isolate**	**Honey alone MBC (g/mL)**	**Honey combination MBC (g/mL)**	**Azithromycin alone MBC (µg/mL)**	**Azithromycin combination MBC (µg/mL)**	**FBCI**
NCTC 13031	0.476	0.037	32	4	0.202
subsp. *abscessus*	0.476	0.037	32	1	0.108
subsp. *bolletii*	0.476	No interaction	32	No interaction	No interaction
subsp. *massiliense*	0.476	0.037	32	1	0.108

The concentrations required to achieve increased inhibition against *M. abscessus* subsp. *abscessus* were 0.037 g/mL MGO70 with 1 µg/mL azithromycin. These concentrations result in an FICI value of 0.577, suggesting no interaction (Table 3.13). The growth curve for these concentrations show similar growth for *M. abscessus* subsp. *abscessus* only and MGO70 alone, with a reduction in growth for azithromycin and no growth for the combination (Figure 3.16 C). A one-way ANOVA showed a significant difference between the treatments, P=<0.0001, and a Dunnett's multiple comparison showed a significant difference between all treatments compared to the control of *M. abscessus* subsp. *abscessus* only, P=<0.0001 for all. A second Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This showed a significant difference between all treatments and the combination, P=<0.0001 for all, suggesting that there was a difference between the response to MGO70 and azithromycin alone compared to both combined. This suggests they could be synergistic. Furthermore, these concentrations were also bactericidal (Figure 3.16 D). The FBCI value for these concentrations was 0.108, which suggests that these concentrations are synergistic against *M. abscessus* subsp. *abscessus* (Table 3.14).

Improved inhibition was observed with 0.037 g/mL MGO70 and 4 µg/mL azithromycin against *M. abscessus* subsp. *bolletii* (Table 3.13). This was reduced from 0.476 g/mL MGO70 and 32 µg/mL azithromycin, giving an FICI value of 0.202. The growth curve shows similar growth for *M. abscessus* subsp. *bolletii* only and MGO70 alone, with a reduction in growth for azithromycin and no growth observed for the combination (Figure 3.16 E). A one-way ANOVA identified a significant difference between all treatments, P=0.0001. A Dunnett's multiple comparison identified a significant difference between the combination and *M. abscessus* subsp. *bolletii* only, P=0.003. No significant difference was identified between MGO70 alone and azithromycin alone compared to *M. abscessus* subsp. *bolletii* only, P=0.7792 and P=0.0775 respectively. A second Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This identified a significant difference for all of them, P=<0.0001 for MGO70, P=0.0003 for *M. abscessus* subsp. *bolletii* and P=0.0144 for azithromycin. This further supports that the concentrations used in combination are synergistic. However, no bactericidal activity was observed, for any concentration or combination tested (Figure 3.16 F).

For *M. abscessus* subsp. *massiliense*, increased inhibition was observed with the combination of 0.037 g/mL MGO70 and 0.5 µg/mL, a reduction from 0.476 g/mL MGO70 and 2 µg/mL azithromycin (Table 3.13). This results in an FICI of 0.327, suggesting synergy between the two antimicrobials. The growth curve shows similar growth for *M. abscessus* subsp. *massiliense* only and MGO70 alone, with a reduction in growth for azithromycin and no growth for the combination (Figure 3.16 G). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison identified a significant difference between all treatments compared to *M. abscessus* subsp. *massiliense*

only, P=0.0001 for MGO70, and P=<0.0001 for azithromycin alone and the combination. Another Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This identified a significant difference between all treatments, P=<0.0001 for *M. abscessus* subsp. *massiliense* only and MGO70 alone, P=0.0001 for azithromycin. This further supports that there was a significant difference between the response to MGO70 and azithromycin alone compared to the combination of the two, further indicating a synergistic response. This was not the same for the bactericidal activity, and growth could be seen for these concentrations (Figure 3.16 H). However, the combination of 0.037 g/mL MGO70 with 1 µg/mL azithromycin resulted in bactericidal activity. This gives an FBCI value of 0.108, which suggests that these concentrations are synergistic.

3.3.4.4 MGO83 in combination with azithromycin against *M. abscessus*

Improved bacteriostatic activity was observed for against all *M. abscessus* isolates tested when exposed to both MGO83 and azithromycin. The concentration of MGO83 required for increased activity was 0.037 g/mL for all isolates, which is reduced from 0.476 g/mL. The concentration of azithromycin required for the improved activity was typically 0.5 µg/mL or lower, apart from for *M. abscessus* subsp. *bolletii*. Bactericidal activity was varied, depending on the *M. abscessus* isolate.

To achieve improved inhibition of *M. abscessus* NCTC 13031, 0.037 g/mL MGO83 and 0.5 µg/mL azithromycin were required. These were reduced from 0.476 g/mL MGO83 and 2 µg/mL azithromycin (Table 3.15). This gives an FICI value of 0.327, suggesting they are acting synergistically. The growth curve for these concentrations shows similar growth in response to both MGO83 and azithromycin compared to the control of *M. abscessus* NCTC 13031 only (Figure 3.17 A). No growth was observed for the combination. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001, and a Dunnett's multiple comparison found no difference between MGO83 and *M. abscessus* NCTC 13031 only, P=0.9456, but a difference was observed between azithromycin and *M. abscessus* NCTC 13031 and the combination and *M. abscessus* NCTC 13031, P=0.0457 and P=<0.0001 respectively. A second Dunnett's comparison was conducted, comparing all treatments to the combination. This identified a significant difference between all treatments, P=<0.0001 for *M. abscessus* NCTC 13031 only and MGO83 alone, P=0.0006 for azithromycin alone. This further supports that these concentrations are acting synergistically. However, these concentrations did not have bactericidal activity, but bactericidal activity was observed for 0.037 g/mL MGO83 with 1 µg/mL azithromycin (Figure 3.17 B). These concentrations are still synergistic, with an FBCI value 0.108 (Table 3.16).

Table 3.15 FICI values for MGO83 manuka honey and azithromycin against *M. abscessus*

M. abscessus isolate	Honey alone MIC (g/mL)	Honey combination MIC (g/mL)	Azithromycin alone MIC (µg/mL)	Azithromycin combination MIC (µg/mL)	FICI
NCTC 13031	0.476	0.037	2	0.5	0.327
subsp. *abscessus*	0.476	0.037	1	0.5	0.577
subsp. *bolletii*	0.476	0.037	32	8	0.327
subsp. *massiliense*	0.476	0.037	1	0.25	0.577

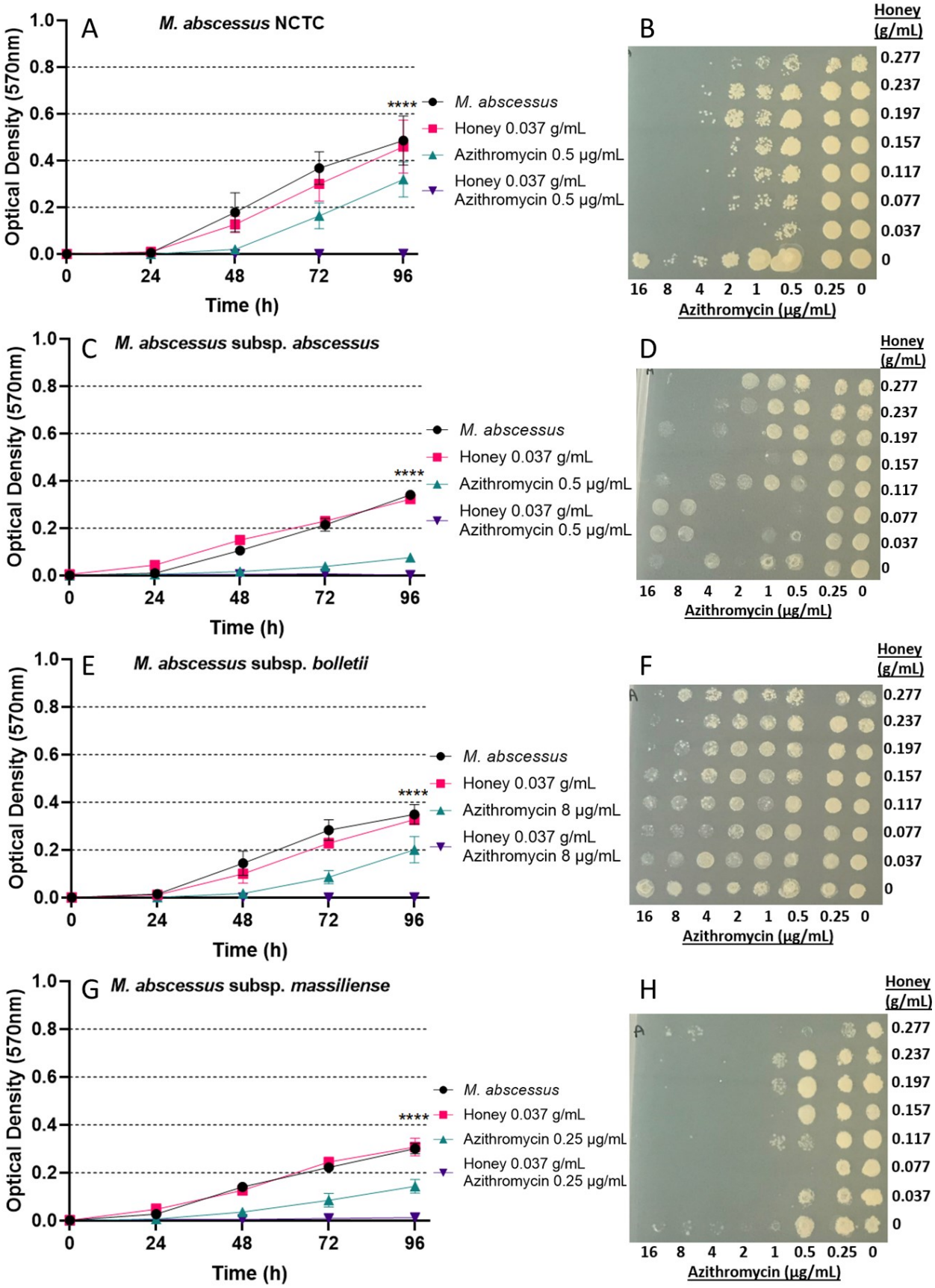

Figure 3.17 Combination of MGO83 manuka honey and azithromycin results in increased inhibition and bactericidal activity against *M. abscessus* NCTC 13031 and *M. abscessus* subspecies. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration in

combination with azithromycin on the growth of *M. abscessus*. A) Growth curve showing improved inhibition of *M. abscessus* NCTC 13031 by combining 0.037 g/mL MGO83 and 0.5 µg/mL azithromycin. A one-way ANOVA shows a significant difference between all treatments, P=<0.0001. B) *M. abscessus* NCTC 13031 increased bactericidal activity on solid media by combining 0.037 g/mL MGO83 and 1 µg/mL azithromycin. C) Growth curve indicating enhanced inhibition of *M. abscessus* subsp. *abscessus* when exposed to 0.037 g/mL MGO83 and 0.5 µg/mL azithromycin. One-way ANOVA shows significant difference between all treatments, P=<0.0001. D) *M. abscessus* subsp. *abscessus* had improved bactericidal activity with both 0.037 g/mL MGO83 and 2 µg/mL azithromycin when transferred onto solid media. E) The growth curve identified combinations of 0.037 g/mL MGO83 and 8 µg/mL azithromycin results in improved inhibition of *M. abscessus* subsp. *bolletii*, one-way ANOVA P=0.0001. F) *M. abscessus* subsp. *bolletii* has no bactericidal activity for any concentration tested on solid media. G) The growth curve showed inhibition of *M. abscessus* subsp. *massiliense* by combining 0.037 g/mL MGO83 and 0.25 µg/mL azithromycin, one-way ANOVA P=<0.0001. H) Bactericidal activity of 0.037 g/mL MGO83 and 1 µg/mL azithromycin against *M. abscessus* subsp. *massiliense* after transfer to solid media.

Table 3.16 FBCI values for MGO83 manuka honey and azithromycin against *M. abscessus*

***M. abscessus* isolate**	**Honey alone MBC (g/mL)**	**Honey combination MBC (g/mL)**	**Azithromycin alone MBC (µg/mL)**	**Azithromycin combination MBC (µg/mL)**	**FBCI**
NCTC 13031	0.476	0.037	32	1	0.108
subsp. *abscessus*	0.476	0.037	32	2	0.140
subsp. *bolletii*	0.476	No interaction	32	No interaction	No interaction
subsp. *massiliense*	0.476	0.037	32	1	0.108

Increased inhibition of *M. abscessus* subsp. *abscessus* was seen with 0.037 g/mL MGO83 with 0.5 µg/mL azithromycin. These concentrations result in an FICI value of 0.557, suggesting no interaction (Table 3.15). The growth curve shows very similar growth for *M. abscessus* subsp. *abscessus* only and MGO83 alone. A reduction in growth was observed for treatment with azithromycin alone and no growth was seen for the combination (Figure 3.17 C). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the growth of *M. abscessus* subsp. *abscessus* only identified no significant difference for MGO83, P=0.1862

and a significant difference for both azithromycin alone and the combination, P=<0.0001 for both. Another Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This showed a significant difference for all treatments, P=<0.0001 for *M. abscessus* subsp. *abscessus* only and MGO83, P=0.0001 for azithromycin. This suggests there was a significant difference in response to MGO83 alone and azithromycin alone compared to being used in combination. The bactericidal activity for *M. abscessus* subsp. *abscessus* was largely varied, but an MBC was observed for 0.037 g/mL MGO83 with 2 µg/mL azithromycin (Figure 3.17 D). These concentrations produce an FBCI value of 0.140, suggesting synergy.

M. abscessus subsp. *bolletii* was inhibited by a combination of 0.037 g/mL MGO83 with 8 µg/mL azithromycin (Table 3.15). This was a decrease from 0.476 g/mL MGO83 and 32 µg/mL azithromycin, giving an FICI value of 0.327. The growth curve for these concentrations show similar growth for *M. abscessus* subsp. *bolletii* only and MGO83 with a reduction in growth for azithromycin and no growth for the combination (Figure 3.17 E). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001, and a Dunnett's multiple comparison showed no difference for MGO83 compared to *M. abscessus* subsp. *bolletii*, P=0.7335, but a significant difference was found for azithromycin alone and the combination compared to *M. abscessus* subsp. *bolletii*, P=0.0003 and P=<0.0001 respectively. Another Dunnett's multiple comparison was conducted, comparing all treatments to the combination, this identified a significant difference for all treatments, P=<0.0001, further supporting a synergistic interaction between MGO83 and azithromycin against *M. abscessus* subsp. *bolletii*. However, no bactericidal activity was observed for any concentration tested (Figure 3.17 F).

For *M. abscessus* subsp. *massiliense* 0.037 g/mL MGO83 with 0.25 µg/mL azithromycin were required for improved inhibition (Table 3.15). This was a reduction from 0.476 g/mL MGO83 and 1 µg/mL azithromycin, giving an FICI value of 0.577, suggesting no interaction. The growth curve shows very similar growth for *M. abscessus* subsp. *massiliense* only and MGO83 alone (Figure 3.17 G). A reduction in growth was seen for azithromycin alone and no growth was seen for the combination. A one-way ANOVA identified a significant difference for all treatments, P=<0.0001. A Dunnett's multiple comparison showed no significant difference between MGO83 alone and the control of *M. abscessus* subsp. *massiliense* only, P=0.9695. A Significant difference was identified between both azithromycin alone and the combination compared to the control of *M. abscessus* subsp. *massiliense* only, P=<0.0001 for both. A second Dunnett's multiple comparison was conducted, comparing all treatments to the combination. This showed a significant difference for all treatments, P=<0.0001. This suggests there was an interaction between MGO83 and azithromycin due to a significant difference in response to the combination as opposed to each one alone. There was no bactericidal activity observed for these concentrations. However, bactericidal activity could be observed for 0.037 g/mL MGO83 with 1 µg/mL azithromycin (Figure 3.17 H). The results in an FBCI value of 0.108, indicating synergy.

3.3.5 Nebulised Honey and amikacin

Due to the established therapeutic use of nebulised amikacin, this was the antibiotic selected for exploration into a nebulised treatment with the addition of manuka honey. Only one manuka honey was selected, MGO55, for exploration into nebulising due to its improved activity with all the subspecies in combination with amikacin (Section 3.2.3.2). As a preliminary investigation, the type strain *M. abscessus* NCTC 13031 was used to establish the assay. Initially, amikacin was nebulised alone at 3 concentrations to determine the minimum required concentrations for use in the assay. The concentrations selected were based on the bactericidal activity of amikacin, which was determined to be 16 µg/mL against *M. abscessus* NCTC 13031. Therefore, it was decided a 10 fold increase, 100 fold increase and 1000 fold increase in concentration would be tested, resulting in 0.16 mg/mL amikacin, 1.6 mg/mL amikacin and 16 mg/mL amikacin being nebulised. These concentrations are lower than the currently utilised therapeutic concentrations that are currently administered to patients, which are 125 mg/mL or higher. No growth was observed for *M. abscessus* after nebulised treatment with 16 mg/mL amikacin (Figure 3.18 A), but growth of *M. abscessus* was observed for both 1.6 mg/mL amikacin (Figure 3.19 B) and 0.16 mg/mL amikacin (Figure 3.18 C). It was decided that 1.6 mg/mL amikacin would be used as the concentration for pairing with MGO55 manuka honey.

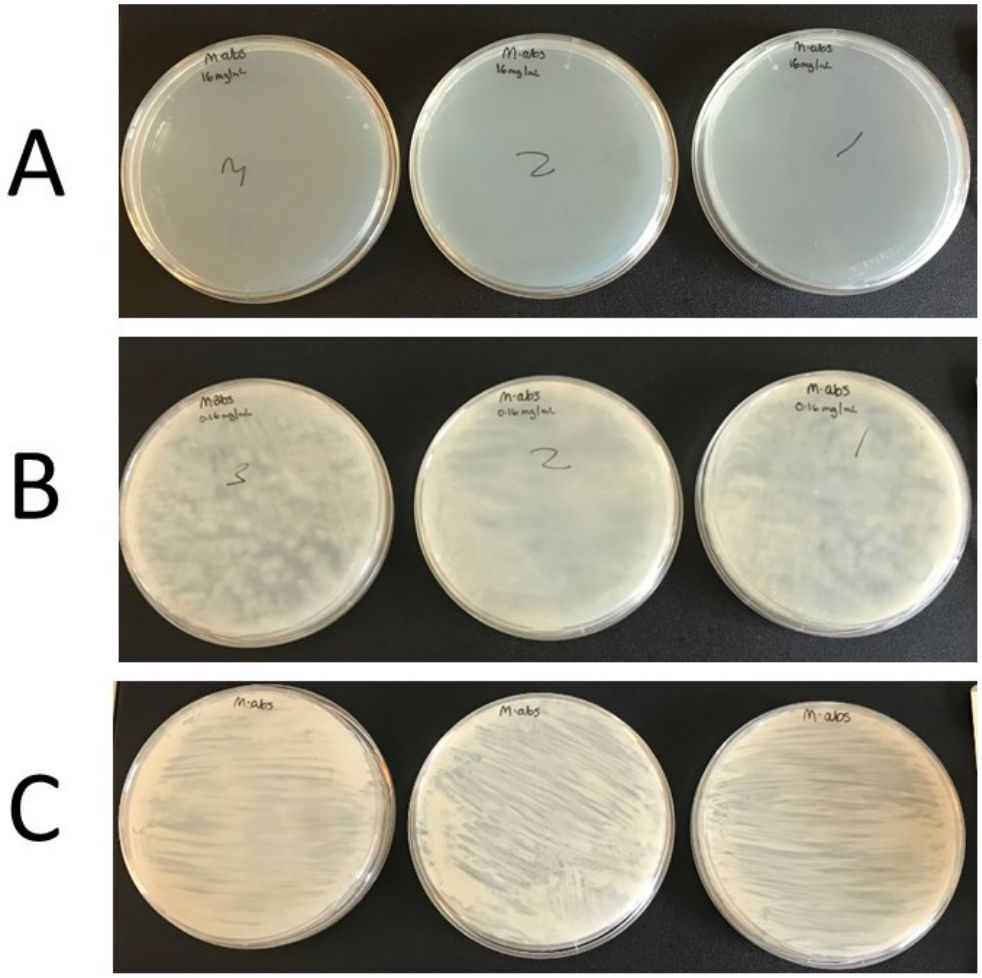

Figure 3.18 Growth on agar plates of *M. abscessus* NCTC 13031 treated with nebulised amikacin and control of nebulised distilled H_2O. A) No visible growth of *M. abscessus* NCTC 13031 after nebulised treatment of 16 mg/mL amikacin. B) Visible growth of *M. abscessus* NCTC 13031 after nebulised treatment of 1.6 mg/mL amikacin. C) Visible growth of *M. abscessus* NCTC 13031 after nebulised treatment with distilled H_2O.

The same approach was considered for MGO55, however there were limitations to manuka honey concentration due to the viscosity. Therefore, the fixed concentration of 0.37 g/mL was used. This was a 10 fold increase in concentration, and an achievable concentration, at which a combinatorial affect could be seen between manuka honey and amikacin. The nebulising of MGO55 alone resulted in similar growth of *M. abscessus* compared to the control of nebulised sterile distilled H_2O (Figure 3.18 C and Figure 3.19 C).

After determining that growth could be seen for both 1.6 mg/mL amikacin and 0.37 g/mL MGO55, they were both nebulised together. This resulted in almost no visible growth after incubation (Figure 3.19 A). Interestingly, the position of the agar plate within the bag had an impact on the reduction of growth (Figure 3.4), with the agar plate in the middle showing the most reduction of growth compared to the agar plates either closest to or furthest away from the nebuliser medicine cup. Furthermore, no growth could be seen in the middle of the agar plates but growth was visible at the edges of the petri dishes.

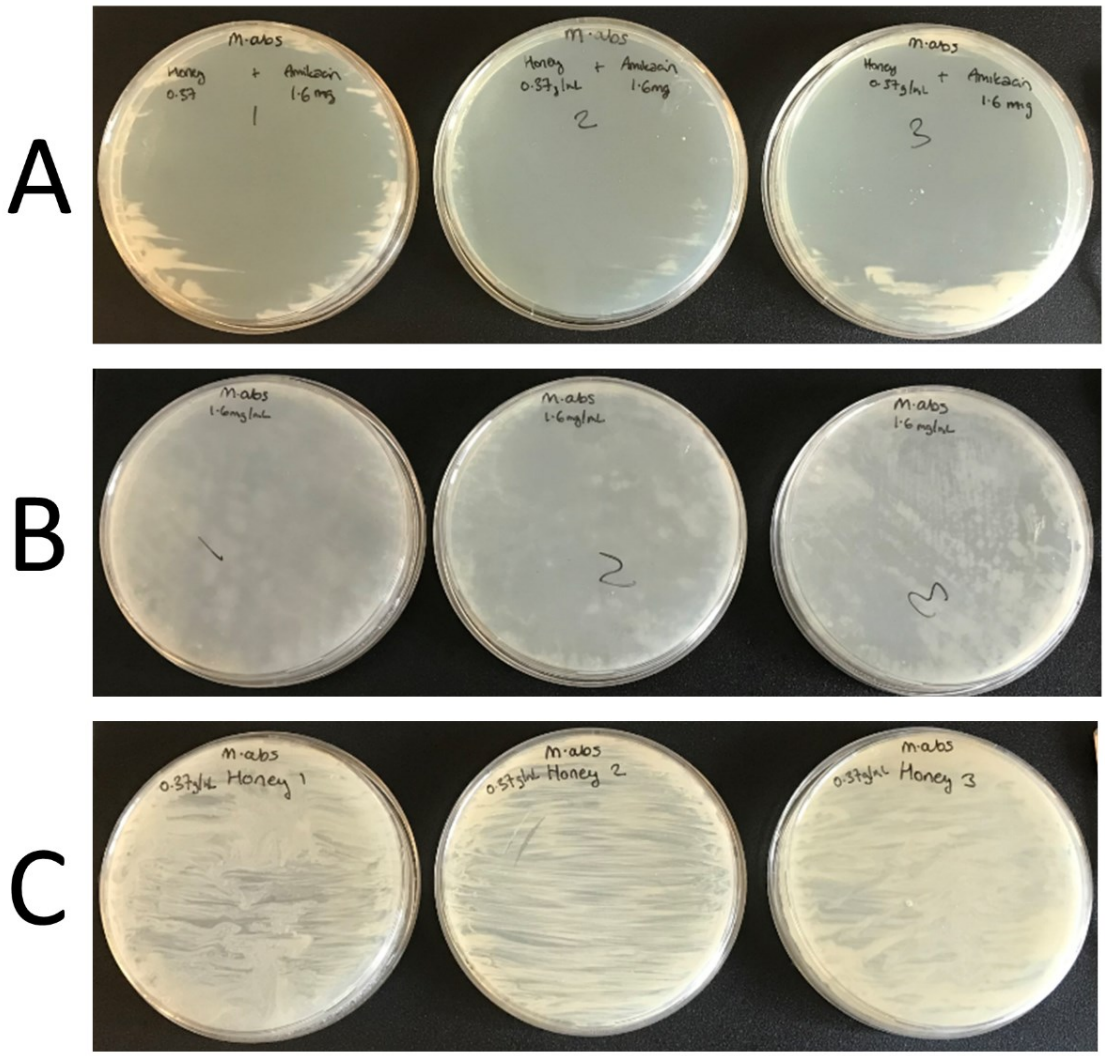

Figure 3.19 Growth on agar plates of *M. abscessus* treatment with nebulised amikacin and MGO55 honey alone and in combination. A) Combination treatment of *M. abscessus* NCTC 13031 with 1.6 mg/mL amikacin and 0.37 g/mL MGO55 manuka honey. B) Treatment of *M. abscessus* NCTC 13031 with 1.6 mg/mL amikacin. C) Treatment of *M. abscessus* NCTC 13031 with 0.37 g/mL MGO55 manuka honey.

3.3.6 Quantification of the nebuliser assay

After establishing that the combination of nebulised MGO55 and amikacin enhanced the inhibition of growth of *M. abscessus* NCTC 13031, a quantifiable method was pursued. This initially used the colony forming units per mL (CFU/mL) method to determine loss of viable cells after treatment. However, after nebulising treatment with sterile distilled H_2O, no growth was observed (Figure 3.21 A). There was also no growth observed for treatment with amikacin or the combination of MGO55 with amikacin (Figure 3.21 C and D). However, growth was observed for MGO55 alone (Figure 3.21 B). Due to the requirement the inoculum on the plate being in a countable range, it was not possible to use a higher dilution. Therefore, this method was considered not viable and another approach was considered.

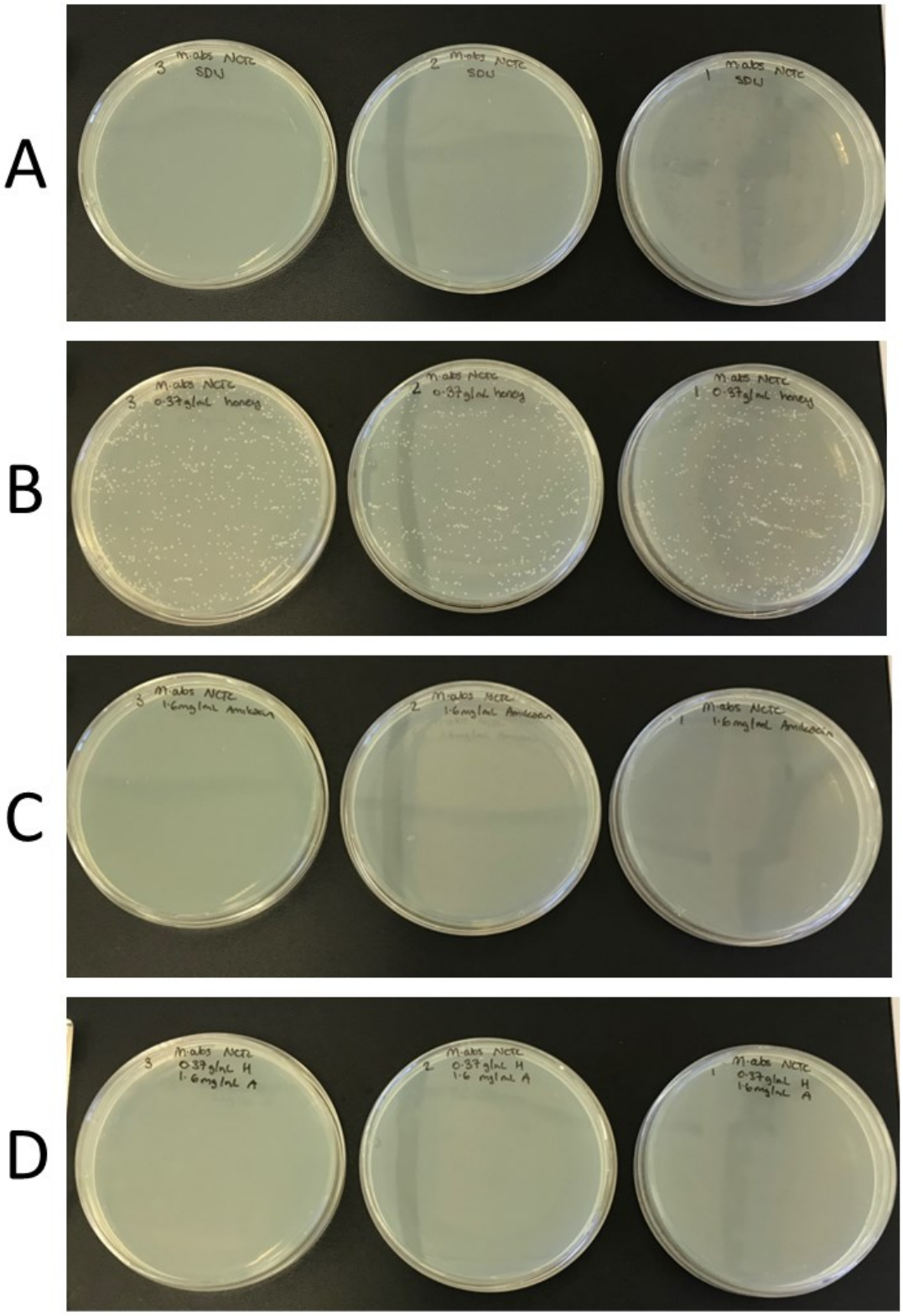

Figure 3.21 Growth on agar plates of *M. abscessus* CFU/mL with nebulised amikacin and MGO55 honey alone and in combination. *M. abscessus* cultures were adjusted to an OD_{600nm} of 0.2 and then serially diluted to 10^{-4} before inoculating 50 µL onto solid agar. After inoculation nebulised treatment was administered. A) No growth observed for *M. abscessus* after exposure to nebulised sterile distilled H_2O. B) Visible growth of countable colonies after 0.37 g/mL nebulised MGO55 was administered. C) No growth of *M. abscessus* observed after 1.6 mg/mL nebulised amikacin. D) No growth observed of *M. abscessus* after 0.37 g/mL MGO55 and 1.6 mg/mL amikacin nebulised treatment.

Another method that was used in the quantification of the nebuliser assay was applying 8 equally distanced spots of 10 µL 0.1 OD_{600nm} adjusted *M. abscessus* culture to the agar prior to nebulising. This allowed a known cell inoculum to be administered onto the agar and the impact of nebulised treatment to be measured (Lin *et al.*, 2020). The control of sterile distilled H_2O and the treatment of 0.37 g/mL MGO55 showed growth of *M. abscessus* (Figure 3.22 A and B). After treatment with 1.6 mg/mL amikacin, the growth of *M. abscessus* was visible but reduced in comparison to the control of sterile distilled H_2O (Figure 3.22 C). The combination of 0.37 g/mL MGO55 and 1.6 mg/mL amikacin showed a further reduction in growth compared to amikacin only (Figure 3.22 D). However, growth was still visible. Interestingly, the position of the agar plate within the autoclave bag appeared to impact the growth. The agar plate closest to the nebuliser cup (Figure 3.4, plate 1) had the most growth and the agar plate furthest away (Figure 3.4, plate 3) had the least amount of growth. Suggesting that there was not uniform circulation of nebulised treatment within the bag.

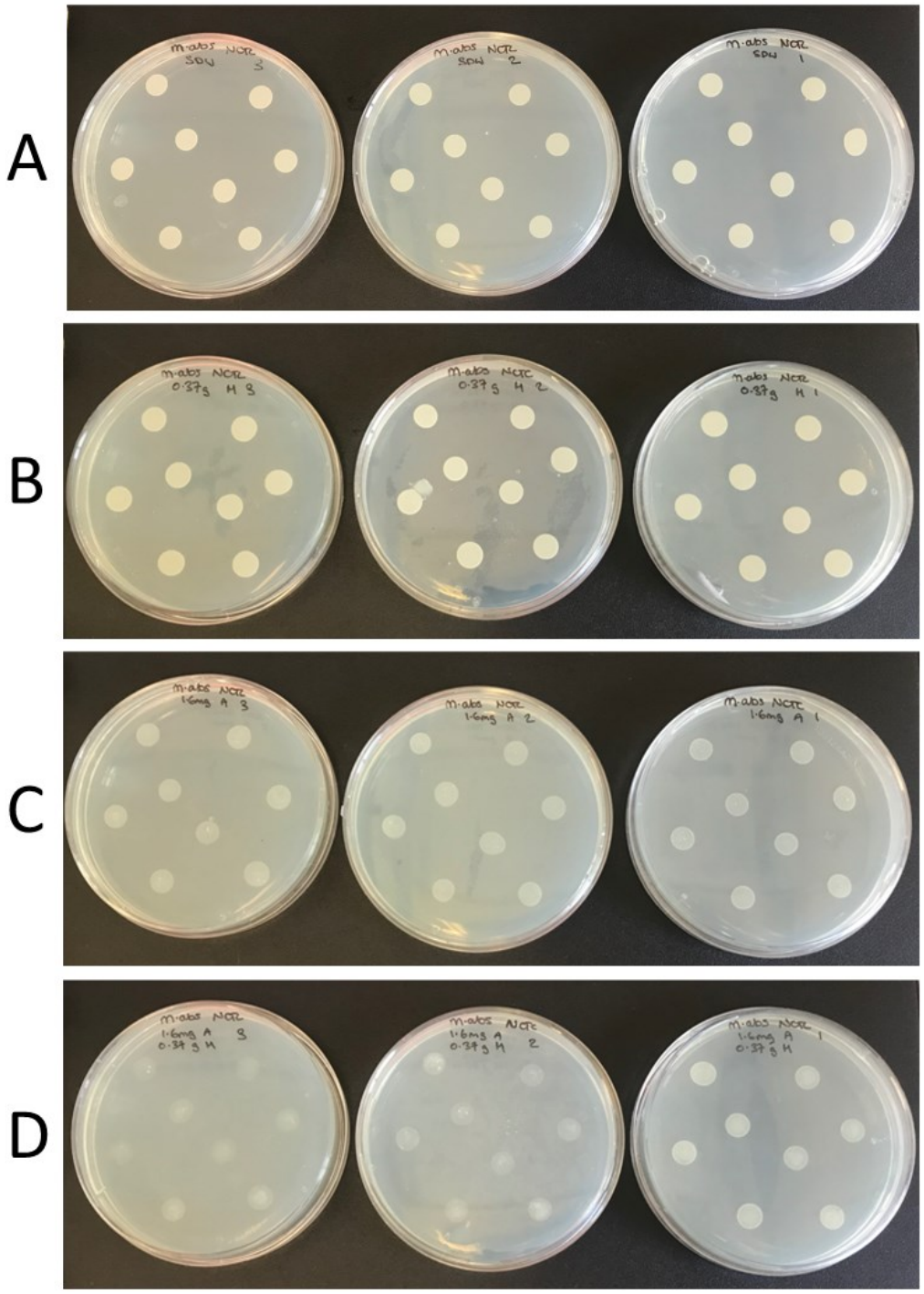

Figure 3.22 Growth on agar plate of *M. abscessus* NCTC 13031 spots with nebulised amikacin and MGO55 honey alone and in combination. *M. abscessus* cultures were OD_{600nm} adjusted to 0.1 and 10 µL spots were inoculated onto solid media before nebulised treatment. The agar plates on the right were those that were the closest to the medicine cup and those on the left were furthest away (Figure 3.4, plates 1-3). A) The growth of *M. abscessus* spots after nebulised sterile distilled H_2O. B) Growth of *M. abscessus* after 0.37 g/mL nebulised MGO55. C) A reduction in growth of *M. abscessus* after 1.6 mg/mL nebulised amikacin compared to the control of sterile distilled H_2O. D) A further reduction in *M. abscessus* growth after treatment with 0.37 g/mL MGO55 with 1.6 mg/mL amikacin compared to amikacin alone.

3.4 Discussion

One of the frontline treatments for pulmonary *M. abscessus* infections is initially I.V. amikacin, followed by inhaled amikacin (Haworth *et al.*, 2017). Side effects associated with amikacin include; nausea, vomiting, abdominal pain, ototoxicity and nephrotoxicity (Novosad *et al.*, 2016; Weng *et al.*, 2020). The use of nebulised amikacin can reduce these systemic side effects by administering the antibiotic directly to the site of infection which improves efficacy and reduces toxicity (Tiddens *et al.*, 2014). Although the toxicity of the drug may be reduced, other side effects become present such as dyspnoea, bronchospasms and nausea (Ailiyaer *et al.*, 2018). This is due to the concentration of the dosage of inhaled amikacin. It is exponentially higher than that of the I.V. amikacin, with initial nebulised dosage starting at 250 mg/mL twice daily, but this can be increased to 500 mg/mL twice daily if tolerated by the patient (Olivier *et al.*, 2014). Therefore, if the dosage of inhaled amikacin could be lowered but its efficacy maintained, it would be beneficial to the patient as it would help to reduce these side effects, allowing patients to complete the course of treatment and improve patient outcomes. Some developments have been made to improve aminoglycoside inhalation therapy, such as liposomal amikacin and powdered tobramycin, but these are limited due to patient eligibility owing to the novel treatments only recently gaining FDA and EMA approval and therefore not being currently widespread. As well as the intended bacterial isolate often being *P. aeruginosa* and not *M. abscessus* which increases the likelihood of not being prescribed for mycobacterial infections. Recent advances in asthma treatments however, have identified nebulised honey is a safe and effective strategy to reduce symptoms, showing that natural remedies may be implemented as an alternative (Kamaruzaman *et al.*, 2014). After demonstrating the efficacy of manuka honey against *M. abscessus* and a panel of clinical isolates, exploration into synergistic interactions with some key antibiotics used in the treatment of *M. abscessus* pulmonary infections would provide a basis for a possible new treatment (Section 2.3.2) (Nolan, Harrison and Cox, 2022a). In this study, it has been shown that manuka honey and either amikacin or azithromycin can have a synergistic affect resulting in improved inhibition or bactericidal activity against *M. abscessus* and the distinct subspecies.

To determine any interactions between the chosen antibiotics and manuka honey against *M. abscessus* and the 3 subspecies, the checkerboard assay was selected. This allowed for multiple concentrations to be examined in a high throughput screening. The initial antibiotic of interest was amikacin, due it its already established use in the treatment of *M. abscessus* pulmonary infections regardless of subspecies. There was a varied response for *M. abscessus* and the 3 subspecies to manuka honey used in combination with amikacin. The concentrations required for improved inhibition often depended on the grade of manuka honey and the subspecies. This also differed between bacteriostatic activity and bactericidal activity, with the bactericidal activity often requiring lower concentrations of manuka honey.

The two highest grade manuka honey's, MGO70 and MGO83, required different concentrations ranging from 0.037 g/mL to 0.277 g/mL for increased inhibition, depending on *M. abscessus* isolate. However, for improved bactericidal activity only 0.037 g/mL was required for all isolates. This was not the case for the lower grades, MGO40 and MGO55, which required 0.037 g/mL to 0.117 g/mL for increased bactericidal activity, depending on the isolate. Interestingly, it was *M. abscessus* subsp. *bolletii* that required the higher concentrations of manuka honey. The concentration of amikacin required for improved bactericidal activity in combination with manuka honey was between 2 µg/mL and 4 µg/mL, which is a reduction from typically 8 µg/mL, depending on the isolate. The improved combined activity could be attributed to MGO within the honey exerting pressure on the mycobacterial cell membrane, allowing for easier penetration of amikacin into the mycobacterial cell (Combarros-Fuertes *et al.*, 2019). Indicating why the higher grade manuka honey's required lower concentrations to achieve improved bactericidal activity.

The combination of manuka honey and tobramycin did not have the same interactions as amikacin. Both tobramycin and amikacin are aminoglycoside antibiotics, with similar chemical structures and mechanisms of action (Figure 3.23). It was hypothesised that similar interactions would have been observed for both tobramycin and amikacin combined with manuka honey because of this. However, no interactions were observed, bacteriostatic or bactericidal, for any concentration tested or any isolate. If the MGO within the manuka honey was exerting pressure on the mycobacterial cell membrane, allowing for easier penetration of antibiotic, similar observations would have been made. Previously, it has been identified that manuka honey and tobramycin work synergistically against *P. aeruginosa* isolates obtained from cystic fibrosis patients (Roberts *et al.*, 2019). It was suggested in *P. aeruginosa*, that subinhibitory concentrations resulted in antagonism in some cases, whereby the antimicrobial activity of the honey was ineffective and the additional sugar from the honey provided an alternative carbon source, thus allowing the bacterial isolates to overcome the antibiotic pressure. Therefore, higher concentrations of manuka honey may be required for a synergistic interaction to be observed.

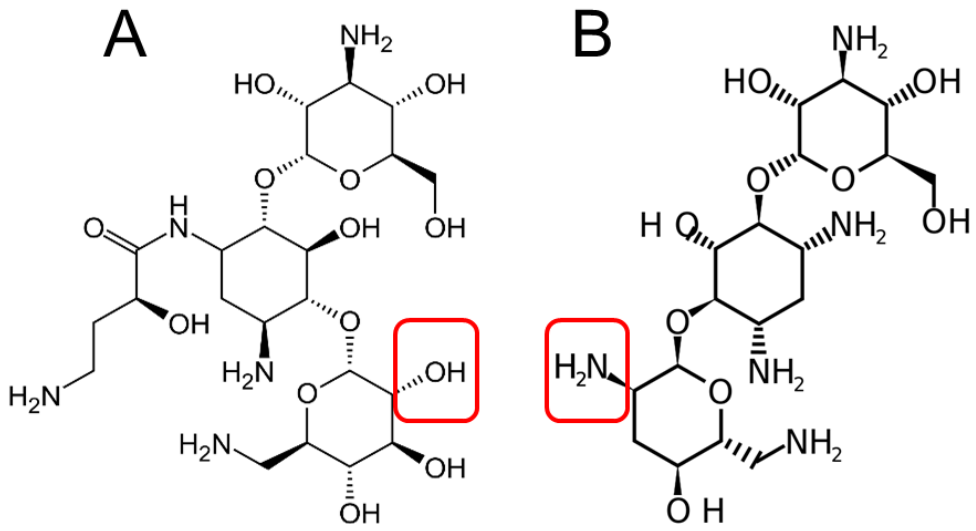

Figure 3.23 Chemical structure of amikacin and tobramycin antibiotics. A) The chemical structure of amikacin. The red box indicates the 2'-hydroxyl group, one of the main differences between amikacin and tobramycin. B) The chemical structure of tobramycin. The red box indicates the 2'amino group not present in amikacin.

Another possibility for the difference in activity between amikacin and tobramycin could be due to their chemical structures. One of the key differences between amikacin and tobramycin is the presence of either a 2'-hydroxyl group or a 2'-amino group, respectively (Figure 3.23). It has previously been identified that aminoglycoside antibiotics with a 2'-amino group are less effective at inhibiting *M. abscessus* than those with the 2'-hydroxyl group. Interestingly, this was only observed for *M. abscessus* and both modifications were effective against *Mycobacterium smegmatis* (Maurer *et al.*, 2014). This has been attributed to the presence of aminoglycoside acetyltransferases, more specifically aminoglycoside 2'-N-acetyltransferase (AAC(2')), which have only been identified in Gram negative bacteria and mycobacterial species (Ramirez and Tolmasky, 2010). The AAC(2') utilises acetyl-CoA to detoxify aminoglycoside antibiotics through acetylation of the 2'-amino group (Bacot-Davis, Bassenden and Berghuis, 2016). The lack of the amino group, replaced with a hydroxyl group in amikacin prevents the AAC(2') from being effective. Furthermore, amikacin contains an n (S)-4-amino-2-hydroxybutyrate (HABA) group at the N-1 position, which prevents hydrogen bonding with the enzyme and forces the enzyme to accommodate this group in a confirmation away from the active site (Bassenden *et al.*, 2021). These differences in chemical structure could be the reason why tobramycin was not as effective as amikacin when combined with manuka honey. It is possible however, to improve the efficacy of tobramycin by the addition of other antibiotics, the most commonly suggested being β-lactams (Sanz-García *et al.*, 2019). Another possibility to improve its activity would be through the use of AAC inhibitors, which prevent the modification of the antibiotic, thus allowing it to remain effective (Jana and Deb, 2005; Labby and Garneau-Tsodikova, 2013).

Improved activity was also observed for manuka honey in combination with azithromycin, a macrolide antibiotic. The concentration of manuka honey required for improved inhibition was typically 0.037 g/mL, apart from for MGO40 which required up to 0.117 g/mL depending on the subspecies. This was typically lower than the concentrations required for improved amikacin activity. Similar concentrations were required for bactericidal activity, with 0.037 g/mL manuka honey resulting in increased activity. Importantly, bactericidal activity was only observed for one of the manuka honey samples, MGO40, against *M. abscessus* subsp. *bolletii*. Due to one of the main defining characteristics between the subspecies, inducible macrolide resistance, it was not surprising that bactericidal activity was not observed for *M. abscessus* subsp. *bolletii*. However, it is interesting to note that improved bacteriostatic activity was observed for all manuka honey samples tested, suggesting there could be a potential interaction between azithromycin and manuka honey. Other than MGO concentration, it is unknown what specific differences were present between the manuka honey samples. Therefore, it is hard to suggest why MGO40 in combination with azithromycin resulted in bactericidal activity compared to the other manuka honeys. This could be attributed to multiple variables such as polyphenolic compounds present, hydrogen peroxide, bee defensin-1 or currently unknown compounds (Section 1.2.3).

For interactions between manuka honey with amikacin or manuka honey with azithromycin, the FICI or FBCI values would often indicate there was no relationship between the two compounds. However, there are other factors to consider. One of those is the defined FICI or FBCI values, where <0.5 is synergistic, between 0.5 and 4 is no interaction and above 4 is antagonism. Although this is the standard, it has been suggested that the value to show synergy should be <0.75 and not <0.5 (Pryjma, Burian and Thompson, 2018). Considering this, some of the suggested no interactions between manuka honey and amikacin could be considered synergistic. Additionally, although not considered synergistic by the FBCI values, bactericidal activity was observed between several concentrations, suggesting an additive effect was observed, and should therefore be considered beneficial.

After establishing that amikacin can act synergistically with manuka honey, a nebuliser assay was developed to explore the possibility of an inhalation treatment combining both antimicrobial compounds. One of the main concerns with nebulised antibiotics is the side effects associated with it due to the administering of significantly high doses. Therefore, if it is possible to lower the dosage of amikacin, with the addition of manuka honey, it could provide a more tolerable, yet still effective treatment. Initially, concentrations of amikacin were nebulised at 10 times, 100 times and 1000 times the MBC observed in the broth microdilutions. This provided a base for determining concentrations that resulted in the growth of *M. abscessus* and concentrations at which growth inhibition was observed. To proceed with combining amikacin and manuka honey, 1.6 mg/mL amikacin was selected and 0.37 g/mL manuka honey. Growth was observed for manuka honey alone and amikacin

alone at these concentrations but combined there was almost no growth. This provided a preliminary basis for furthering the nebuliser assay to quantify the reduction of *M. abscessus* growth. Considering the use of CFU/mL, to allow a countable range to be inoculated onto solid media required a diluted culture resulting in less than 300 CFU/mL on the agar. The use of this dilution appeared to impact the growth of *M. abscessus* in two different ways. One way suggests that the individual cells of mycobacteria were washed off the agar in the nebulising process, evident by the only growth observed being for the manuka honey. The viscous nature of manuka honey could have prevented the wash off affect observed for those with distilled H_2O. The second possibility is due to how mycobacteria grow with the use of quorum sensing. With such a low inoculum on the plate, it is possible that quorum sensing could not be achieved, therefore preventing the growth of *M. abscessus*. When considering the spots of culture onto the agar plate, a small impact on the growth of *M. abscessus* was noted between amikacin and manuka honey alone compared to in combination. However, this did not result in complete eradication of mycobacterial growth. In this instance, it is possible that a pedestal effect is taking place, whereby the bacterial cells are layered on top of each other, and bacteria on the surface of the agar are being protected by bacteria on top exposed to the treatment. This could result in the death or inhibition of growth to the mycobacteria exposed to the treatment but allow for those below to grow and be unaffected by the treatment.

The nebuliser assay used here is a promising preliminary exploration into an *in vitro* assay. However, there are some other limitations that need to be addressed. The first consideration here is the lack of determination of particle size produced by the nebuliser and therefore the dosage that it can administer. This was something that could not be achieved within the scope of this study, but for any further work conducted utilising this assay these factors need to be determined. Without knowing the dosage of antibiotic or honey reaching the agar plate, concentrations that are beneficial are somewhat unknown. Further to this, an occurrence that was observed throughout the experiments was growth of *M. abscessus* at the edge of the petri dish, regardless of what was nebulised, inhibitory or not. This suggested one of two things. One being there is an interaction that results in the lack of distribution of nebulised particulate around the whole agar plate. This either being that the edge of the petri dish acted as a shield preventing nebulised particles from descending onto the plate, or some unknown interaction between the plastic of the petri dish and the nebulised particles. To further explore the cause of this there are two possibilities, either pour agar plates that are flush with the edges of the plastic or use glass petri dishes. The second possibility for growth at the edge of the petri dish is that the circulation within the autoclave bag was not consistent. Another occurrence that was observed throughout these experiments was inconsistent inhibition between the agar plates. The positioning within the autoclave bag was noted on the petri dishes allowing for identification of location to the medicine cup after incubation. In some

instances, more inhibition of growth was observed on agar plates closest to the medicine cup and in other instances it was those furthest away. This suggests that circulation was varied at every round of nebulising and for future experiments this needs to be controlled. One such way would be with the addition of a mechanical ventilator, which would allow for improved distribution of nebulised particles and a more determined dosage to be administered (Forde *et al.*, 2019).

Other considerations for future developments of this model include the type of nebuliser used. There are a large variety of nebuliser types and inhalation models that could be used for direct delivery of antibiotic to the lung, including jet nebuliser, mesh nebuliser, ultrasonic nebuliser, dry powdered inhalers and pressurized metered dose inhalers (Newman, 2005; Tiddens *et al.*, 2014). During the development of a drug for nebulised treatment a specific nebuliser type is used. This results in the requirement for a specific unit to be used for optimum delivery of that specific antibiotic (Elphick *et al.*, 2015). It is possible that the nebuliser type required for amikacin differs to that for manuka honey, resulting in suboptimal nebuliser treatment for one of the components. Therefore, development of these two antimicrobials together with a specific nebuliser unit is essential. Further to this, the use of liposomal amikacin should also be explored. By delivering the antibiotic in a liposome, improved cellular uptake could occur due to the hydrophobic nature of the mycobacterial cell wall (Section 1.1.1). Combining this with manuka honey could further improve the efficacy of this combination. Additionally, it could be possible to generate liposomal manuka honey to aid with delivery into the mycobacterial cell, which could further improve the activity of this combination. Other considerations could explore combining the assay with infected alveolar macrophages, by utilising a liposomal drug delivery system this should improve drug permeability.

A final limitation that requires consideration is the lack of a cell cytotoxicity study of manuka honey and the combination treatment. The efficacy of the manuka honey against *M. abscessus* is suggested to be due to MGO, which is known to be toxic to human cells. Further exploration into this combined treatment requires the examination of toxicity to eukaryotic cells.

Future work developing the use of antibiotics in combination with manuka honey against *M. abscessus* and other species of mycobacteria should be explored. The effective combinations of amikacin with manuka honey and azithromycin with manuka honey provide a strong basis for the possibility of other improved treatments by combining antibiotics with manuka honey. Other antibiotics currently used in a nebulised form to treat *M. abscessus* pulmonary infections include aztreonam and colistin, therefore combinations of these antibiotics alongside manuka honey should be explored. Furthermore, it is important to note that *M. abscessus* does not only cause pulmonary infections but infections of the skin and

soft tissue. As well as the highly drug resistant nature of *M. abscessus*, these infections can also be difficult to treat. With the availability of medical grade honey and various wound dressings, along with the discovery that antibiotic resistance does not impede the efficacy of honey, the use of honey in treatment of these infections alongside antibiotic chemotherapy should be implemented (Nolan *et al.*, 2020). The combination of manuka honey and phage therapy should also be considered. Recent advances in *M. abscessus* pulmonary infection utilised phage therapy as a method of mycobacterial eradication (Dedrick *et al.*, 2019). It has also been demonstrated that honey combined with phage therapy resulted in a synergistic interaction against *E. coli* biofilms (Oliveira *et al.*, 2017). Identifying another exciting possibility for the future use of manuka honey in combination with other strategies for fighting these infections.

Recent advances into the development of 3D printing and hydrogels should also be explored as an intermediary step to move this model from *in vitro* towards *in vivo*. By utilising these, it is possible to develop an environment similar to lung tissue which would provide a more accurate representation of a lung infection. Combined with a more advanced setup containing a ventilator and a different nebuliser, this could improve the results and provide more information about how effective this treatment would be. This would also lessen the requirement for the use of animals in scientific testing.

A final note on the future direction of this work is the consideration of exploring the ability of manuka honey to penetrate *M. abscessus* biofilms as well as the sputum within which the infections develop. Manuka honey has been shown to disrupt microbial biofilms (Lu *et al.*, 2014, 2019). Therefore, it is important to determine if *M. abscessus* biofilms would also be impacted by manuka honey. Currently, there is no information regarding manuka honey and its impact on sputum, thus this should be explored. If manuka honey is able to penetrate and disrupt sputum and biofilms this would be a very important finding in combating these infections.

Chapter 4: Analysis into the antimicrobial components of honey and their role in inhibiting *Mycobacterium abscessus*

4.1 Introduction

The antimicrobial activity of honey has been previously attributed to 3 main components, namely hydrogen peroxide, bee defensin-1 and in the case of manuka honey, MGO (Mieles *et al.*, 2022). The occurrence of these vary among honey types and impact the efficacy of the honey against a variety of microorganisms (Nolan, Harrison and Cox, 2019). The contribution of each component to the antimicrobial activity of honey is largely understudied, with the majority of reports focusing on the variety of microorganisms inhibited. Some studies have explored the origin of these components, such as identifying DHA as the precursor for MGO, or glucose oxidase being the driving force behind hydrogen peroxide production (Section 1.2.3) (White, Subers and Schepartz, 1963; Adams, Manley-Harris and Molan, 2009). However, their direct impact on the antimicrobial activity is not thoroughly understood. Investigations into the mechanism of action of MGO identified that concentrations of up to 2 mM MGO were required to cause the loss of fimbriae and flagella in *B. subtilis* and *E. coli* as well as cause shrinking and rounding of the cell (Rabie *et al.*, 2016). The mechanism behind hydrogen peroxide is considered to be due to DNA degradation by generation of hydroxyl radicals (Brudzynski *et al.*, 2012). The action of bee defensin-1 has not yet been reported. Therefore, the focus should be on the role these components play in inhibiting microorganisms, not just the variety they inhibit. It has been suggested that these components act synergistically together as a combination therapy to inhibit bacterial growth, and that physiochemical properties of honey contribute to its activity (Alvarez-Suarez *et al.*, 2014). However, there have been other reports that MGO in manuka honey can have a negative effect on hydrogen peroxide accumulation and also degrade bee defensin-1 (Majtan *et al.*, 2012, 2014). Suggesting that in the case of manuka honey, MGO is the driving force behind its antimicrobial activity.

The occurrence of MGO in manuka honey is due to the production of DHA in the flower nectar of *Leptospermum* flowers (Section 1.2.3.4). The conversion of DHA to MGO occurs nonenzymatically over time. However, it has been suggested that within honey the DHA exists as a dimer, whereas the monomer form is required for MGO conversion. The highly viscous nature of honey and largely dehydrating environment result in very slow conversion of the DHA dimer to the DHA monomer and therefore MGO (Grainger, Manley-Harris, *et al.*, 2016). The accumulation of MGO within the manuka are directly related to the MGO rating of a given manuka honey. Therefore, this continual conversion of DHA dimer, to DHA monomer and ultimately MGO is a very important step in the manufacturing of a manuka honey and the attributed antimicrobial activity.

These observations have not gone unnoticed and the development of various medical grade honeys are reportedly based on at least one of these components. Examples of these are Medihoney based on MGO content, Surgihoney based on reactive oxygen species and Revamil which utilises bee defensin-1 (Kwakman *et al.*, 2008; Dryden *et al.*, 2014; Tirado,

Hudson and Maldonado, 2014). However, due to intellectual property associated with these products, the exact mechanisms behind their activity are not full elucidated. Additionally, studies exploring the conversion of DHA to MGO have not reported how this impacts the antimicrobial activity of a honey, just that it occurs (Grainger, Manley-harris, *et al.*, 2016; Grainger, Manley-Harris, *et al.*, 2016). Consequently, this remains a largely underexplored area of research. Therefore, the exploration into the 3 main components of manuka honey and their impact on *M. abscessus* would be beneficial.

4.1.1 Aims and Objectives

Initially, each of the 3 main components in manuka honey will be identified. This will be achieved through the development of several assays, including an N-acetyl cysteine assay to detect MGO, hydrogen peroxide detection assay and the use of protein dialysis to determine bee defensin-1. Then, the antimicrobial activity of the manuka honey samples will be reassessed after removal of the major components. This will allow an exploration into the importance of their role in antimicrobial activity against *M. abscessus*. Additionally, each component will be examined alone against *M. abscessus*. These will be achieved through the broth microdilution assay. And finally, exploration into modification of a vegan honea (lacking the natural antimicrobial products found in manuka honey) to generate antimicrobial activity will be conducted. This will be achieved through the addition of precursors to generate MGO and hydrogen peroxide.

4.2 Materials and Methods

4.2.1 Media Preparation

All chemicals and reagents were purchased from Sigma-Aldrich or Melford Laboratories, UK, unless otherwise stated. For growth of *Mycobacterium abscessus* cultures, Middlebrook 7H9 broth and Middlebrook 7H11 agar were selected. The 7H9 broth was made by adding 2.35 g of 7H9 to 450 mL of distilled H_2O and supplemented with 4 mL of 50% (w/v) glycerol before autoclaving (121 °C for 15 min). Once cooled, 1.25 mL of 20% (w/v) filter sterile Tween80 was added (final concentration of 0.055%). The 7H11 agar was made by adding 10.25 g of 7H11 to 450 mL distilled H_2O and supplemented with 5 mL 50% (w/v) glycerol before autoclaving (121 °C for 15 min). The agar was subsequently poured into petri dishes.

4.2.2 *M. abscessus* strains and culture

The *M. abscessus* strain used was the type strain NCTC 13031 (also called ATCC 19977). All organisms were grown for 72 h in 7H9 broth before being stored in 25% (w/v) glycerol stock solutions and stored at -80 °C. Prior to testing, *M. abscessus* isolates were grown in 10 mL 7H9 broth, prepared as described in 2.2.1, for 72 h at 37 °C with orbital shaking at 180 rpm.

4.2.3 Honey sample storage and preparation

The 4 manuka honey samples selected for this study were all of differing MGO concentrations. The lowest grade selected was MGO40 (Manuka Doctor, UK), followed by MGO55 (ManukaPharm, UK), MGO70 (Manuka Doctor, UK) and the highest grade selected was MGO83 (Comvita, UK). The MGO rating determines that each manuka honey will have certain levels of MGO, for example the MGO40 will contain at least 40 mg/kg MGO. A vegan honea (Plant Based Artisan) was also used. All honey jars were stored in the dark at room temperature prior to testing and not used beyond any use by dates. For each experiment honey stocks were made up to 1 g/mL in distilled H_2O (w/v) and filter sterilised in a two-step filtration process using 0.8 µm filter and 0.22 µm filter (Sartorius), unless otherwise stated. No further sterility testing was conducted, such as testing for *Bacillus* spores.

4.2.4 Antimicrobial susceptibility testing of honey

To assess the antimicrobial activity of various honey samples and modified honey, a broth microdilution was used. Initially, 100 µL of 7H9 broth, prepared as described in 4.2.1, was added to all wells of the 96 well plate. Then 100 µL of honey, prepared as described in 4.2.3, was added to wells A1-A6 and a different honey was added to wells A7-A12, these were then mixed and serially diluted down the plate (Figure 4.1). On row G, the excess 100 µL was removed and discarded. The plates were inoculated with 5 µL of $OD_{600\ nm}$=0.1 *M. abscessus* culture, prepared as described in 4.2.2, down columns 1-3 and 7-9. This included control wells of H1-3 and H7-9 for *M. abscessus* only and H4-6 and H10-12 for 7H9 broth only (Figure 4.1). Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C for a total of 96 h, with $OD_{570\ nm}$ reads every 24 h. After 96 h, the final $OD_{570\ nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using 5 µL aliquots and spotting onto 7H11 agar plates, prepared as described in 4.2.1. The agar plates were incubated for a further 72 h at 37 °C and the minimum bactericidal concentration (MBC) was determined as the minimum concentration where no bacterial growth was visually observed.

	MGO40						MGO55					
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL	0.476 g/mL	0.476 g/mL	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL *M. abscessus*	0.476 g/mL	0.476 g/mL	0.476 g/mL
B	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL	0.238 g/mL	0.238 g/mL	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL *M. abscessus*	0.238 g/mL	0.238 g/mL	0.238 g/mL
C	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL	0.119 g/mL	0.119 g/mL	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL *M. abscessus*	0.119 g/mL	0.119 g/mL	0.119 g/mL
D	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL	0.0595 g/mL	0.0595 g/mL	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL *M. abscessus*	0.0595 g/mL	0.0595 g/mL	0.0595 g/mL
E	0.0297 g/mL *M. abscessus*	0.0297 g/mL *M. abscessus*	0.0297 g/mL *M. abscessus*	0.0297 g/mL	0.0297 g/mL	0.0297 g/mL	0.0297 g/mL *M. abscessus*	0.0297 g/mL *M. abscessus*	0.0297 g/mL *M. abscessus*	0.0297 g/mL	0.0297 g/mL	0.0297 g/mL
F	0.014 g/mL *M. abscessus*	0.014 g/mL *M. abscessus*	0.014 g/mL *M. abscessus*	0.014 g/mL	0.014 g/mL	0.014 g/mL	0.014 g/mL *M. abscessus*	0.014 g/mL *M. abscessus*	0.014 g/mL *M. abscessus*	0.014 g/mL	0.014 g/mL	0.014 g/mL
G	0.007 g/mL *M. abscessus*	0.007 g/mL *M. abscessus*	0.007 g/mL *M. abscessus*	0.007 g/mL	0.007 g/mL	0.007 g/mL	0.007 g/mL *M. abscessus*	0.007 g/mL *M. abscessus*	0.007 g/mL *M. abscessus*	0.007 g/mL	0.007 g/mL	0.007 g/mL
H	*M. abscessus*	*M. abscessus*	*M. abscessus*	Broth	Broth	Broth	*M. abscessus*	*M. abscessus*	*M. abscessus*	Broth	Broth	Broth

Figure 4.1 Plate map of honey broth microdilution. The broth microdilution was prepared by adding 100 µL of 7H9 broth to all 96 wells followed by 100 µL manuka honey from a stock concentration of 1 g//mL (w/v) to wells A1 to A12, with MGO40 being added to wells A1 to A6 and MGO55 being added to well A7 to A12. This was serially diluted from row A to row F. Columns 1, 2, 3, 7, 8 and 9 were inoculated with OD adjusted culture of *M. abscessus*. Columns 4, 5, 6, 10, 11 and 12 were used as controls and therefore not inoculated.

4.2.5 Identification of antimicrobial components in manuka honey

4.2.5.1 Methylglyoxal identification using N-acetyl cysteine

To determine the concentration of MGO, the N-acetyl cysteine assay was used (Wild *et al.*, 2012). Initially, a 0.5 M stock solution of N-acetyl cysteine in distilled H_2O was prepared. From this, the subsequent concentrations were made: 5 mM, 2.5 mM, 1.25 mM, 0.625 mM and 0.3125 mM to the volume of 2.4 mL using distilled H_2O. The manuka honey stock was made by weighing 0.2 g honey and adding 2 mL distilled H_2O to generate a 0.1 g/mL stock. Then 100 µL of 0.1 g/mL manuka honey was added to 2.3 mL phosphate buffered saline. To start the reaction 600 µL 500 mM N-acetyl cysteine was added and incubated at 37 °C for 30 min. After incubation, each reaction was placed into a quartz cuvette and read in a bench top spectrophotometer (Jenway Genova plus spectrophotometer) at 288 nm. The concentrations of MGO were also calculated based on the MGO rating.

To calculate the concentrations of MGO, the MGO rating was used and the concentration determined in mg/kg. First, the rating needed to be converted from mg/kg to mg/L. This was done by multiplying the MGO rating by the density of MGO, which is 1.046. Once converted, the following equation was used to determine molar concentration:

Mass (g) = concentration (M) x volume (L) x molecular weight (g/mol).

4.2.5.2 Hydrogen peroxide identification using a colourimetric assay

A colourimetric hydrogen peroxide assay was conducted, as described by Lehmann et al., 2019. Initially, a 10 mM sodium phosphate buffer was made by preparing a 1 M sodium phosphate buffer of 6.9 g of NaH_2PO_4 dissolved in 50 mL of distilled H_2O and a 1 M disodium phosphate buffer of 7.1 g Na_2HPO_4 dissolved in 50 mL distilled H_2O. Then, 3.15 mL of disodium phosphate buffer and 6.85 mL sodium phosphate buffer was added to 990 mL of sterile distilled H_2O. To prepare a working stock solution, a 5 mg/mL (w/v) o-dianisidine was prepared by weighing out 20 mg of o-dianisidine and adding 4 mL of 95% ethanol. This was vortexed until dissolved. From this, 250 µL was added to 1 mL 10 mM sodium phosphate buffer, making a 1 mg/mL working solution.

Then, a 10 mg/mL stock of horseradish peroxidase type II (HRP) (w/v) was prepared in 10 mM sodium phosphate buffer by weighing out 10 mg HRP and adding 1 mL sodium phosphate buffer. This was vortexed until dissolved. The HRP reagent mixture was then prepared by combining 1 mL of the working solution (1 mg/mL o-dianisidine) with 40 µL of HRP stock and adding it to 18.96 mL sodium phosphate buffer.

A 2 mg/mL (w/v) stock of catalase was prepared by weighing out 10 mg catalase and adding 5 mL 10 mM sodium phosphate buffer which was vortexed until dissolved. The sulphuric acid stock solution was prepared by slowly adding 67 mL of 18 M H_2SO_4 to 50 mL distilled H_2O. The final volume was adjusted to 200 mL with distilled H_2O.

The Manuka honey used for testing was made up to a 1 g/mL stock in sterile distilled H_2O by weighing 8 g of manuka honey and adding 8 mL distilled H_2O that was preheated to 37 °C. The honey was kept at 35 °C for 20 min on an orbital shaker at 180 rpm. The honey was then filter sterilised in a two-step filtration process using a 0.45 µm and 0.22 µm filters. These were then further diluted by adding 2.5 mL honey to either 2.5 mL sterile distilled H_2O, 2.5 mL catalase solution or 2.5 mL 10 mM sodium phosphate (blank). These were further incubated for 2 h at 35 °C with orbital shaking at 180 rpm. Hydrogen peroxide (H_2O_2) standards were then prepared in sodium phosphate buffer to the final concentrations of 2200 µM, 1100 µM, 550 µM, 275 µM, 137.5 µM, 68.8 µM, 34.4 µM, 17.2 µM, 8.6 µM, 4.3 µM, 2.1 µM and 0 µM.

To prepare the assay a flat bottom 96 well plate (Greiner Bio-One) was used. The experimental honey sample was prepared by adding 135 µL of HRP stock solution and 40 µL honey sample in distilled H_2O to wells A1-C1, A3-C3, A5-C5 and A7-C7 (Figure 4.2). The blank was prepared by adding 135 µL sodium phosphate buffer and 40 µL honey sample in distilled H_2O to wells D1-F1, D3-F3, D5-F5 and D7-F7 (Figure 4.2). Honey samples with catalase were prepared by adding 40 µL honey made in catalase solution with 135 µL HRP stock in wells A2-C2, A4-C4, A6-C6 and A8-C8 (Figure 4.2). Honey catalase blanks were prepared by adding 40 µL honey made in 10 mM sodium phosphate buffer with 135 µL HRP

stock solution in wells D2-F2, D4-F4, D6-F6 and D8-F8 (Figure 4.2). For the H_2O_2 standard, 40 µL of the corresponding H_2O_2 concentration was added to 135 µL HRP stock in rows G and H. The catalase negative control was prepared by adding 20 µL of 550 µM H_2O_2 and 20 µL catalase solution to 135 µL HRP stock in wells A11, B11 and C11. The catalase negative control blank was prepared by adding 20 µL of 550 µM H_2O_2 and 20 µL catalase blank to 135 µL HRP stock in wells D11, E11 and F11 (Figure 4.2). Once the samples were fully loaded into the 96 well plate it was protected from light by covering with foil and incubated at room temp for 5 min. To stop the reaction, 120 µL of 6 M sulphuric acid was added to all wells. The plate was then read using a BMG FLUOstar omega plate reader at 560 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	MGO40 with HRP	MGO40 catalase HRP	MGO55 with HRP	MGO55 catalase HRP	MGO70 with HRP	MGO70 catalase HRP	MGO83 with HRP	MGO83 catalase HRP			Catalase negative	
B	MGO40 with HRP	MGO40 catalase HRP	MGO55 with HRP	MGO55 catalase HRP	MGO70 with HRP	MGO70 catalase HRP	MGO83 with HRP	MGO83 catalase HRP			Catalase negative	
C	MGO40 with HRP	MGO40 catalase HRP	MGO55 with HRP	MGO55 catalase HRP	MGO70 with HRP	MGO70 catalase HRP	MGO83 with HRP	MGO83 catalase HRP			Catalase negative	
D	MGO40 blank	MGO40 catalase blank	MGO55 blank	MGO55 catalase blank	MGO70 blank	MGO70 catalase blank	MGO83 blank	MGO83 catalase blank			Catalase positive	
E	MGO40 blank	MGO40 catalase blank	MGO55 blank	MGO55 catalase blank	MGO70 blank	MGO70 catalase blank	MG083 blank	MGO83 catalase blank			Catalase positive	
F	MGO40 blank	MGO40 catalase blank	MGO55 blank	MGO55 catalase blank	MGO70 blank	MGO70 catalase blank	MGO83 blank	MGO83 catalase blank			Catalase positive	
G	2,200 µM H2O2	1,100 µM H2O2	550 µM H2O2	275 µM H2O2	138.5 µM H2O2	67.8 µM H2O2	34.4 µM H2O2	17.8 µM H2O2	8.6 µM H2O2	4.3 µM H2O2	2.1 µM H2O2	0 µM H2O2
H	2,200 µM H2O2	1,100 µM H2O2	550 µM H2O2	275 µM H2O2	138.5 µM H2O2	67.8 µM H2O2	34.4 µM H2O2	17.8 µM H2O2	8.6 µM H2O2	4.3 µM H2O2	2.1 µM H2O2	0 µM H2O2

Figure 4.2. Plate map for hydrogen peroxide assay. The plate map identifies experimental wells containing manuka honey and HRP solution in dark red. The control wells containing manuka honey with catalase and HRP solution are indicated in light red. The controls of manuka honey with sodium phosphate buffer are indicated in cream and the catalase blank is white. The hydrogen peroxide standard curve is shown on rows G and H.

4.2.5.3 Bee defensin-1 identification using dialysis

To determine if bee defensin-1 was present in the honey samples, an isolation method utilising protein dialysis was conducted (Bíliková, Wu and Šimúth, 2001). Initially, 10 g of each manuka honey was weighed out and diluted in 30 mL phosphate buffered saline. Once dissolved the honey was filter sterilised in a two-step filtration process using a 0.45 µm filter and then 0.22 µm filter. The honey was then transferred to a 3.5K molecular weight cut off tubing (Thermo Scientific SnakeSkin) and placed into dialysis buffer made of distilled H_2O with acetic acid at pH 2.7. This was then placed on a stirrer plate at 20 °C for 72 h and the dialysis buffer changed every 24 h. After dialysis, the contents of the tubing were transferred to 10 kDa spin columns in 10 mL aliquots and centrifuged at 4000 rpm for 15 mins at 4 °C and the flow through retained. This removed all particulates bigger than 10 kDa, leaving anything below this in the flow through. The flow through was then placed into 5 kDa spin columns and centrifuged at 4000 rpm for 15 mins at 4 °C until concentrated to roughly 2 mL. The samples were kept at 4 °C until determination of protein content.

To determine if protein was present in the samples sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was used. Tricine gels were prepared to a concentration of 10.5 % running gel and 2.9 % stacking gel. To ensure the running gel was flat, 100 µL isopropanol was added on top and the gel allowed to set. Once set, the isopropanol was removed with blotting paper. The stacking gel was then pipetted on top of the running gel, until level with the glass plates before adding the comb and leaving to set.

Once the gel had set, the comb was removed and the gel was mounted into the cassette and placed inside the electrophoresis tank. Two buffers were used to run the gel, a cathode buffer and an anode buffer. The cathode buffer was prepared to a final concentration of 0.1 M Tris, 0.1 M Tricine and 0.1 % SDS. This was added inside the cassette. The anode buffer was prepared to a final concentration of 0.2 M Tris-HCl, pH 8.9. This was added to the gel tank outside of the cassette.

To prepare the samples, 10 µL 5x SDS loading dye was added to an Eppendorf tube with 30 µL sample. These were then placed in a heat block at 100 °C for 10 min and allowed to cool before loading onto the gel. To load the gel, 20 µL of low molecular weight marker (Thermo Fisher PageRuler) was added in the first well and 20 µL of the samples were loaded in the subsequent wells. The gel was then run at 100 V, 55 mA for 90 min. The gel was removed from the tank and stained with InstantBlue Coomassie protein stain (Abcam) for 12 h before rinsing with distilled H_2O and viewed on a light box for protein bands.

4.2.6 Removal of the antimicrobial components of manuka honey and re-evaluation of antimicrobial activity

4.2.6.1 Removal of methylglyoxal using N-acetyl cysteine

To assess the impact of manuka honey after removal of MGO, the N-acetyl cysteine assay was used due to irreversible conversion of MGO to imidazolone, thus eradicating the MGO (Lo *et al.*, 1994). A stock solution of N-acetyl cysteine was prepared as described in section 4.2.5.1. Manuka honey samples were prepared by weighing 3 g honey and adding 1.2 mL N-acetyl cysteine with 1.8 mL phosphate buffered saline. A control of N-acetyl cysteine was also prepared by adding 1.2 mL N-acetyl cysteine and 4.8 mL phosphate buffered saline. The samples were mixed and heated at 37 °C for 30 min prior to testing. The broth microdilution assay was used and prepared as described in section 4.2.4.

4.2.6.2 Removal of hydrogen peroxide using catalase

To assess the antimicrobial activity of manuka honey after the removal of hydrogen peroxide, catalase was used. A catalase stock was made by weighing 100 mg catalase and adding 10 mL distilled H_2O. Then, 5 g of each manuka honey was weighed and 4 mL distilled H_2O was added with 1 mL catalase stock. The manuka honey was mixed until dissolved before filter sterilising in a two-step filtration process using 0.8 µm filter and 0.22 µm filter. A control of catalase only, hydrogen peroxide only and hydrogen peroxide with catalase were also tested. The catalase only was prepared by adding 1 mL catalase stock to 9 mL sterile distilled H_2O, giving a final concentration in the experimental plate of 0.476 mg/mL. To prepare the hydrogen peroxide only a 1 M stock of hydrogen peroxide was made by first adding 1.02 mL hydrogen peroxide (9.8 M) to 8.98 mL distilled H_2O. Then, 1 mL of this was added to 9 mL distilled H_2O to generate a 100 mM stock. From this 0.01 mL was added to 9.99 mL distilled H_2O to have a working stock of 100 µM hydrogen peroxide. For the hydrogen peroxide with catalase, 1 mL of the 100 µM hydrogen peroxide stock was removed and added to 2 mg catalase. Once all the samples were prepared, the broth microdilution was then used as described in section 4.2.4.

4.2.7 Antimicrobial components of honey tested alone and in combination against *M. abscessus*

4.2.7.1 Broth microdilution assay of MGO against *M. abscessus*

To determine the efficacy of MGO against *M. abscessus*, a broth microdilution assay was used. Initially, a stock of 500 mM MGO was prepared by adding 38.28 µL MGO (6.53 M) to 461.72 µL sterile distilled H_2O and mixed. A master plate was then prepared by using a 96-well plate and adding 100 µL sterile distilled H_2O from column B1 down to H1. Then 200 µL of 500 mM MGO stock was added to well A1 and 100 µL of this was removed and added to well B1 and mixed. This was repeated down the plate until well G1, where the excess 100 µL was discarded. Then, 1 µL from each well of the master plate was removed and added to the corresponding wells of a new 96 well plate in rows 1-4. Then 94 µL of 7H9 broth, prepared as described in section 4.2.1, was added to all experimental wells. The plate was inoculated with 5 µL of optical density $(OD)_{600\ nm}$=0.1 *M. abscessus* culture, prepared as described in 4.2.2, down rows 1-3. This included control wells of H1-3 for *M. abscessus* only and row 4 with 7H9 broth only. The final concentrations for testing were 5 mM, 2.5 mM, 1.25 mM, 0.625 mM, 0.3125 mM, 0.156 mM, 0.078 mM and 0 mM MGO. Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C for a total of 96 h, with $OD_{570\ nm}$ reads every 24 h. After 96 h, the final $OD_{570\ nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using 5 µL aliquots and spotting onto 7H11 agar plates, prepared as described in 4.2.1. The agar plates were incubated for a further 72 h at 37 °C and the minimum bactericidal concentration (MBC) was determined as the minimum concentration where no bacterial growth was visually observed.

4.2.7.2 Broth microdilution assay of hydrogen peroxide against *M. abscessus*

An initial screen of hydrogen peroxide against *M. abscessus* was conducted at concentrations that would be observed by the hydrogen peroxide assay described in section 4.2.5.2. A stock of 0.05 M hydrogen peroxide was prepared by first adding 1 mL hydrogen peroxide (9.8 M) to 9 mL sterile distilled H_2O to generate a 0.98 M stock. Then, 25.5 µL hydrogen peroxide stock (0.98 M) was added to 474.5 µL sterile distilled H_2O to achieve the 0.05 M working stock. From this a master plate was prepared by using a 96-well plate and adding 100 µL sterile distilled H_2O from row B1 down to H1. Then 200 µL of 0.05 M hydrogen peroxide stock was added to well A1 and 100 µL of this was removed and added to well B1 and mixed. This was repeated down the plate until well G1, where the excess 100 µL was discarded. Then, 1 µL from each well of the master plate was removed and added to the corresponding wells of a new 96 well plate in rows 1-4. Then 94 µL of 7H9 broth, prepared as described in section 4.2.1, was added to all experimental wells. The plate was inoculated with 5 µL of optical density $(OD)_{600\ nm}$=0.1 *M. abscessus* culture, prepared as described in 4.2.2, down rows 1-3. This included control wells of H1-3 for *M. abscessus* only and row 4

with 7H9 broth only. The final concentrations for testing were 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.6 µM, 7.8 µM and 0 µM hydrogen peroxide. Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C for a total of 96 h, with $OD_{570\,nm}$ reads every 24 h. After 96 h, the final $OD_{570\,nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using 5 µL aliquots and spotting onto 7H11 agar plates, prepared as described in 4.2.1. The agar plates were incubated for a further 72 h at 37 °C and the minimum bactericidal concentration (MBC) was determined as the minimum concentration where no bacterial growth was visually observed.

A second screen of hydrogen peroxide against *M. abscessus* was also conducted but at higher concentrations. The concentrations tested were 20 mM, 10 mM, 5 mM, 2.5 mM, 1.25 mM, 0.625 mM, 0.3125 mM and 0 mM hydrogen peroxide. For this, a 2 M stock of hydrogen peroxide was prepared by adding 2.04 mL hydrogen peroxide (9.8 M) to 7.96 mL sterile distilled H_2O. Then, a master plate was prepared as described above and the experiment conducted as described above.

4.2.7.3 Broth microdilution assay of bee defensin-1 against *M. abscessus*

The bee defensin-1 protein (Kingfisher Biotech) was prepared by adding 25 µL of sterile phosphate buffered saline to 25 µg of defensin-1 protein to make a 1 mg/mL stock. Then a master plate was prepared by adding 10 µL from the stock to well A1 of a v-bottom 96 well plate. Wells A2 to A8 had 5 µL of sterile phosphate buffered saline added and 5 µL from well A1 was removed and added to A2 and mixed. This was repeated up to well A7, leaving well A8 as a control of phosphate buffered saline only. Then 1 µL from each well was added to corresponding wells on a flat-bottom 96 well plate in rows 1-4 and 94 µL 7H9 broth was added to all wells. The plates were inoculated with 5 µL of optical density $(OD)_{600\,nm}$=0.1 *M. abscessus* culture, prepared as described in 4.2.2, down rows 1-3, leaving row 4 as a control of only 7H9 broth. Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C for a total of 96 h, with $OD_{570\,nm}$ reads every 24 h. After 96 h, the final $OD_{570\,nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using 5 µL aliquots and spotting onto 7H11 agar plates, prepared as described in 4.2.1. The agar plates were incubated for a further 72 h at 37 °C and the minimum bactericidal concentration (MBC) was determined as the minimum concentration where no bacterial growth was visually observed.

4.2.7.4 Checkerboard assay of MGO and hydrogen peroxide against *M. abscessus*

To determine if there was a synergistic relationship between MGO and hydrogen peroxide, a checkerboard assay was used. First, a master plate was prepared using a 96 well plate. A stock of 0.2 M MGO was prepared by adding 15.31 µL to 484.96 µL sterile distilled H_2O. A stock of 0.02 M hydrogen peroxide was prepared by first making a 0.98 M stock of 10 µL hydrogen peroxide (9.8 M) in 90 µL sterile distilled H_2O. Then, from this 10.2 µL hydrogen peroxide was added to 489.8 µL sterile distilled H_2O. In the master plate down row 11, a serial dilution of MGO was prepared by adding 100 µL sterile distilled H_2O in wells B11 to H11. Then, 200 µL of 0.2 M MGO was added to well A11 and 100 µL was removed and added to well B11 and mixed. This was repeated down all wells until well G11 where the excess 100 µL was discarded. This was repeated for hydrogen peroxide down row 12. Next, using a multichannel pipette 10 µL was removed from all wells in row 11 and added to rows 1 to 8, to generate the MGO concentrations required. This was repeated for hydrogen peroxide in row 12, however these were added across the plate from columns A to H. From the master plate the experimental plates could be prepared, the was done by adding 1 µL of each well in the master plate to the subsequent wells in the experimental plate. Then 94 µL 7H9 broth was added to all wells, including control rows of 7H9 broth only and *M. abscessus* only. The plates were inoculated with 5 µL of $OD_{600\ nm}$=0.1 *M. abscessus* culture, prepared as described in 4.2.2. The final concentrations for testing were 2 mM, 1 mM, 0.5 mM, 0.125 mM, 0.0625 mM, 0.03 mM and 0 mM MGO and 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM and 0 µM hydrogen peroxide. Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C for a total of 96 h, with $OD_{570\ nm}$ reads every 24 h. After 96 h, the final $OD_{570\ nm}$ read was taken and each well was plated out on to solid media to observe bactericidal activity, using 5 µL aliquots and spotting onto 7H11 agar plates, prepared as described in 4.2.1. The agar plates were incubated for a further 72 h at 37 °C and the MBC was determined as the minimum concentration where no bacterial growth was visually observed.

4.2.8 Modification of vegan honea to generate antimicrobial activity

To determine if an artificial honey could be engineered to have antimicrobial activity, a series of experiments was developed using vegan honea (Plant Based Artisan). The vegan honea was manufactured without honeybees, containing: sugar, inulin, apple juice, natural flowers, lemon juice, natural flavours and molasses. Therefore, does not contain any of the precursors that provide the antimicrobial activity of honey, these include DHA which converts to MGO, glucose oxidase which converts into hydrogen peroxide and bee defensin-1. To determine if the vegan honea had antimicrobial activity against *M. abscessus*, it was initially tested using the broth microdilution method described in section 4.2.4.

4.2.8.1 Generation of methylglyoxal in vegan honea tested against *M. abscessus*

To generate methylglyoxal in the vegan honea to promote antimicrobial activity, the precursor dihydroxyacetone (DHA) was used. A stock of 10 mg/mL DHA was prepared by weighing 200 mg DHA and adding 20 mL distilled H_2O with mixing until dissolved. Then, 10 g vegan honea was weighed and 8 mL of the DHA stock was added with 2 mL distilled H_2O and vortexed until dissolved. A stock of DHA only was prepared by adding 8 mL DHA stock to 12 mL distilled H_2O. Both the vegan honea with DHA and DHA only were filter sterilised in a two-step filtration process using a 0.8 µM filter and 0.22 µM filter. These were prepared in duplicate and stored at either 37 °C or 4 °C prior to testing. The first broth microdilutions were set up, as described in 4.2.4, after 2 h of incubation at either 37 °C or 4 °C and subsequent broth microdilutions were set up every 14 days for a total of 112 days to monitor antimicrobial activity. The final concentrations tested in each broth microdilution were 0.476 g/mL vegan honea with 1.9 mg/mL DHA, 1.9 mg/mL DHA only, 0.238 g/mL vegan honea with 0.9 mg/mL DHA, 0.9 mg/mL DHA only, 0.119 g/mL vegan honea with 0.49 mg/mL DHA and 0.49 mg/mL DHA only. Each set of broth microdilutions was plated out onto solid media after 96 h to observe bactericidal activity, as described in 4.2.4.

4.2.8.2 Generation of hydrogen peroxide in vegan honea tested against *M. abscessus*

To generate hydrogen peroxide in the vegan honea, the enzymes invertase and glucose oxidase were used. Initially, a 10 mg/mL stock of glucose oxidase was prepared by weighing 10 mg glucose oxidase and adding 1 mL sterile phosphate buffered saline. A 5 mg/mL stock of invertase was prepared by weighing 5 mg invertase and adding 1 mL sterile phosphate buffered saline. Then, 5 g vegan honea was weighed into 3 different 50 mL conical tubes to assess quantity of enzyme required for hydrogen peroxide production. For 10 U/mL, 14.7 µL glucose oxidase and 15 µL invertase was added, the 100 U/mL had 147 µL glucose oxidase and 150 µL invertase added and 500 U/mL had 735 µL glucose oxidase and 150 µL invertase added. The remaining volume was made up to 5 mL with sterile distilled H_2O, mixed and filter sterilised in a two-step filtration process using a 0.8 µm filter and then a 0.22 µm filter. This was then incubated at 37 °C for 2 h and the hydrogen peroxide detection assay was also conducted, as described in section 4.2.5.2.

For the broth microdilution assay, 5 g vegan honea was weighed and 1.47 µL glucose oxidase and 1.5 µL invertase were added to generate 1 U/mL of both enzymes in the vegan honea. The final volume was adjusted to 5 mL and mixed until dissolved before filter sterilising in a two-step filtration process using a 0.8 µm filter and then a 0.22 µm filter. This was then incubated at 37 °C for 2 h prior to testing. The broth microdilution assay was then conducted as described in section 4.2.4.

4.2.9 Data processing and statistical analysis

All data collected were n=3 technical replicates and 2 biological replicates, the broth microdilution assays were processed in Microsoft Excel 2016 and subsequently analysed using GraphPad Prism 8. Prior to data analysis, the OD value of honey only was subtracted from corresponding experimental honey values, containing *M. abscessus* culture, to account for the OD of the honey samples. The data was then analysed for normal distribution using Shapiro-Wilk test and subsequently analysed using a One-Way ANOVA. A Dunnett's multiple comparisons analysis was also conducted. For each experiment conducted, the MIC and MBC were determined. The MIC was defined as the lowest concentration required to inhibit *M. abscessus* determined by an OD value of 0. The MBC was defined as the lowest concentration required for no visible growth after plating out onto solid media.

4.3 Results

4.3.1 Identification of the 3 main components identified in manuka honey

To determine if the major components attributed to the antimicrobial activity of honey were present within the manuka honey samples, several assays were used. The main components focused on were MGO, hydrogen peroxide and bee defensin-1.

4.3.1.1 Analysis of MGO content

To identify and quantify concentrations of MGO within the manuka honey samples the N-acetyl cysteine method was used (Section 4.2.5.1) (Wild *et al.*, 2012). The standard curve of MGO shows concentrations of up to 5 mM MGO. Unfortunately, no variation in MGO concentration was observed for the manuka honey samples and all samples indicated 0.5 mM MGO (Figure 4.3). Some variation was expected and should have been observed due to the differing strengths of manuka honey tested. Unfortunately, additional experiments adding known concentrations of MGO to the manuka honey and testing again were not conducted and therefore it cannot be determined why all 4 manuka honey samples indicated the same concentration. Consequently, the MGO concentrations were calculated based on the MGO rating. The molar concentration ranged from 0.58 mM to 1.2 mM, depending on manuka honey (Table 4.1).

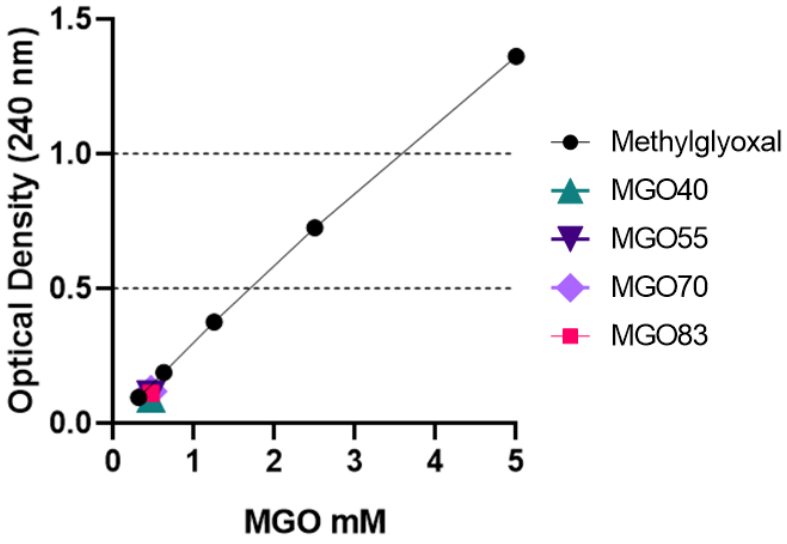

Figure 4.3. MGO detection of manuka honey using the N-acetyl cysteine assay. Data shown are mean for n=3 technical replicates. The standard curve shows detection of MGO up to 5 mM. No variation in MGO concentration was detected for the manuka honey samples tested compared to the standard curve.

Table 4.1 The calculated MGO concentration in the manuka honey samples compared to the concentrations observed using the N-acetyl cysteine assay

Manuka honey	Calculated MGO concentration	Observed MGO concentration
MGO40	0.58 mM	0.5 mM
MGO55	0.798 mM	0.5 mM
MGO70	1.016 mM	0.5 mM
MGO83	1.2 mM	0.5 mM

4.3.1.2 Analysis of hydrogen peroxide content

To identify hydrogen peroxide levels in the manuka honey samples, the hydrogen peroxide assay was used (Section 4.2.5.2) (Lehmann *et al.*, 2019). All 4 of the manuka honey samples had detectable concentrations of hydrogen peroxide present (Figure 4.4). The highest amount of hydrogen peroxide detected was 68 µM observed for MGO55. Both MGO40 and MGO70 had 40 µM hydrogen peroxide, and MGO83 had the lowest amount of hydrogen peroxide at 18 µM. This suggests there is no correlation of hydrogen peroxide to MGO concentration.

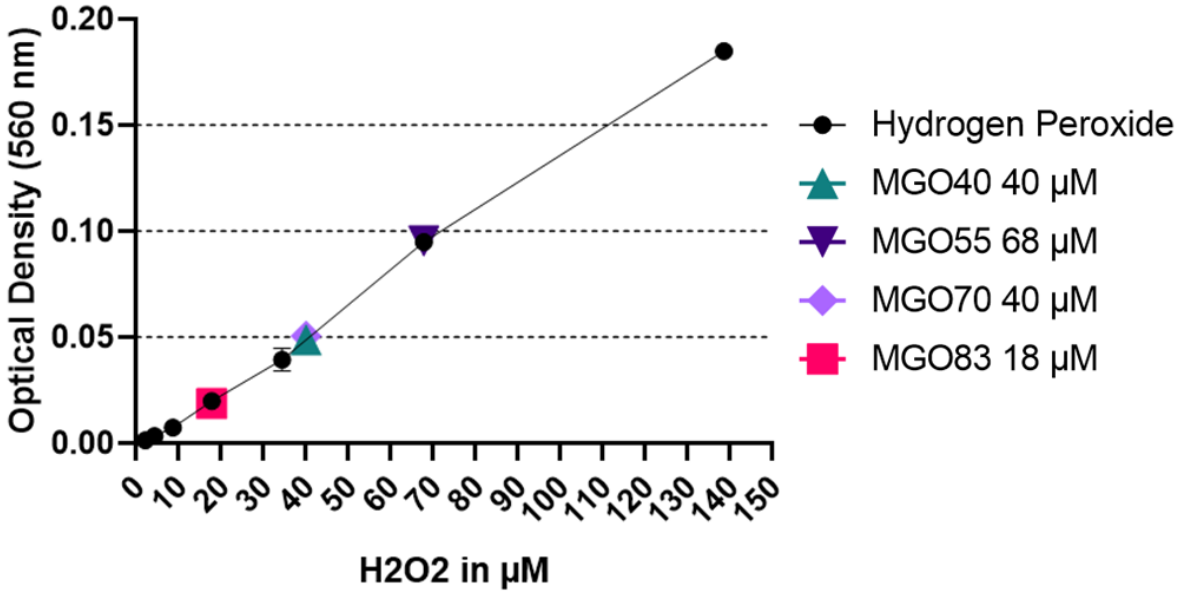

Figure 4.4 Detection of hydrogen peroxide within the manuka honey samples. **Data shown are mean for n=3 technical replicates.** All 4 manuka honeys had detectable levels of hydrogen peroxide present. MGO40 and MGO70 both had 40 µM, whereas MGO83 had the lowest at 18 µM and MGO55 had the highest at 68 µM.

4.3.1.3 Identification of defensin-1

To identify if bee defensin-1 was present in any of the manuka honey samples, protein dialysis was used and the samples obtained were subsequently analysis using SDS PAGE (Section 4.2.5.3). The defensin-1 protein is 5.2 kDa and would be visible at the bottom of the gel inline with the 5 kDa marker of the PageRuler (Figure 4.5). The SDS PAGE showed no protein was present in any of the samples, concentrates or flow through.

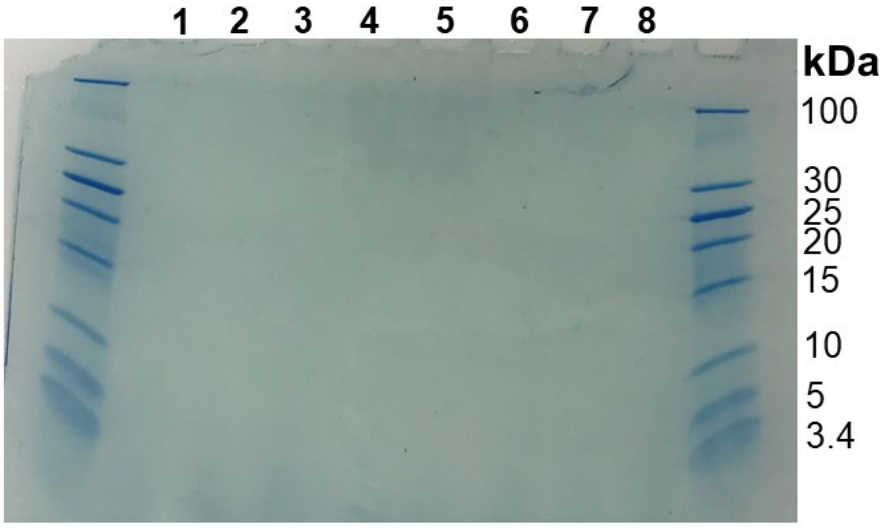

Figure 4.5. Tricine gel stained with Coomassie blue after manuka honey dialysis to identify bee defensin-1. No defensin-1 was identified in any of the samples. 1) MGO40 concentrate showing no sample. 2) MGO55 concentrate with no sample. 3) MGO70 concentrate with no sample. 4) MGO83 concentrate with no sample. 5) MGO40 flow through. 6) MGO55 flow through. 7) MGO70 flow through. 8) MGO83 flow through.

4.3.2 Efficacy of manuka honey after removal of the major components attributed to antimicrobial activity

4.3.2.1 Efficacy of manuka honey after removal of MGO

To determine if manuka honey would maintain antimicrobial activity against *M. abscessus* after the removal of MGO, the N-acetyl cysteine assay was used and broth microdilution assay repeated (Section 4.2.6.1). Unfortunately, the concentration of N-acetyl cysteine used was inhibitory to *M. abscessus* at the highest concentration tested and any reduction in activity could not observed because of this (Figure 4.6). Another assay based on the conversion of MGO into D-lactate by reduced glutathione and glyoxalase was attempted but was unsuccessful and therefore not shown here.

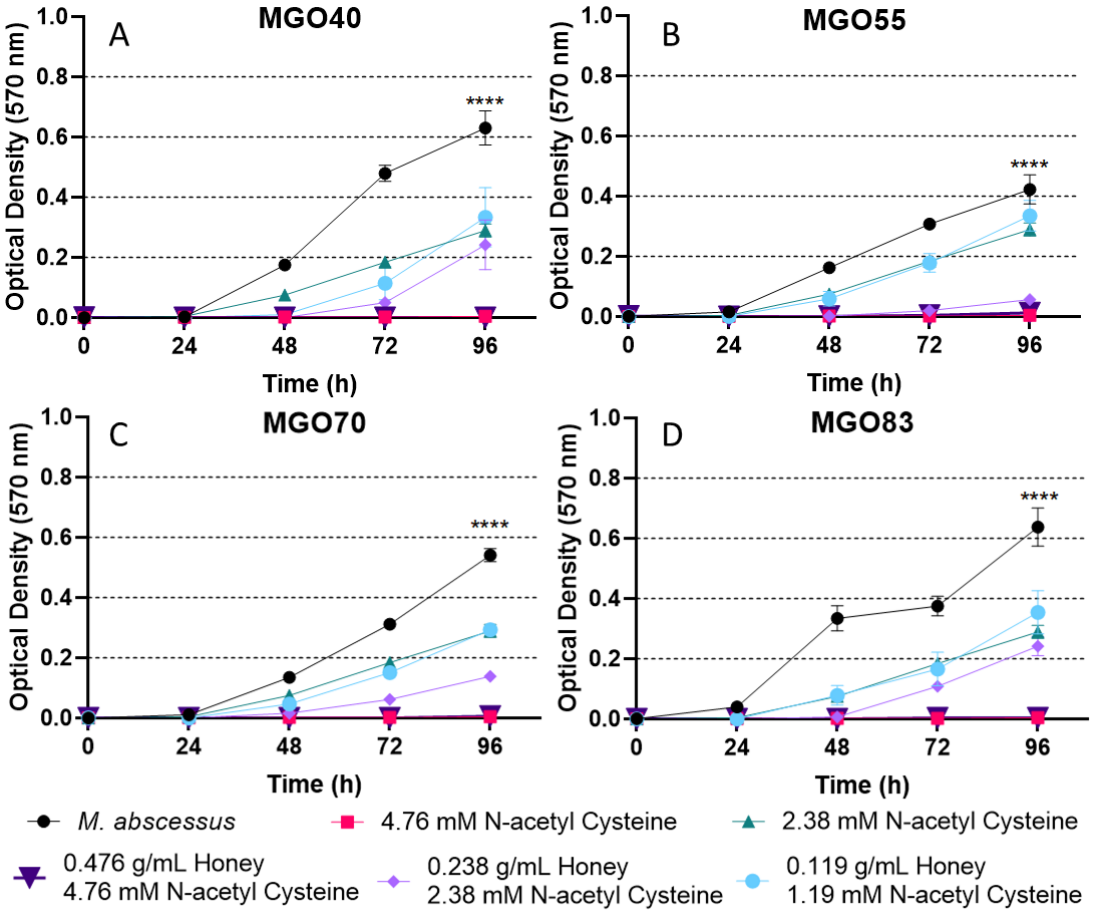

Figure 4.6. Growth curves of *M. abscessus* NCTC 13031 after removal of MGO by N-acetyl cysteine. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus* after MGO removal with N-acetyl cysteine. The N-acetyl cysteine alone appeared to be inhibitory to *M. abscessus* and no change in activity could be observed. A) MGO40 manuka honey and N-acetyl cysteine inhibiting *M. abscessus* at the highest concentrations tested. B) MGO55 manuka honey with N-acetyl cysteine inhibiting the growth of *M. abscessus* at the highest concentrations tested. C) MGO70 showing no growth of *M. abscessus* for the highest concentrations of manuka honey with N-acetyl cysteine and N-acetyl cysteine alone. D) MGO83 manuka honey showing inhibition against *M. abscessus* for N-acetyl cysteine alone and with manuka honey.

4.3.2.2 Efficacy of manuka honey after removal of hydrogen peroxide

To assess the antimicrobial activity of manuka honey after removal of hydrogen peroxide, manuka honey samples were treated with catalase before retesting for antimicrobial activity. All 4 of the manuka honey samples exhibited similar activity to previous observations despite catalase treatment and therefore hydrogen peroxide removal (Section 2.3.2, Figure 2.4 and Figure 4.7). All of the manuka honey samples maintained an MIC of 0.476 g/mL with a reduction in growth for 0.238 g/mL and 0.119 g/mL (Figure 4.7). Importantly, catalase only had no impact on the growth of *M. abscessus*. Interestingly, no bactericidal activity was observed for any of the manuka honey samples tested after catalase treatment, where bactericidal activity was observed previously with no catalase treatment (Section 2.3.2, Table 4.2).

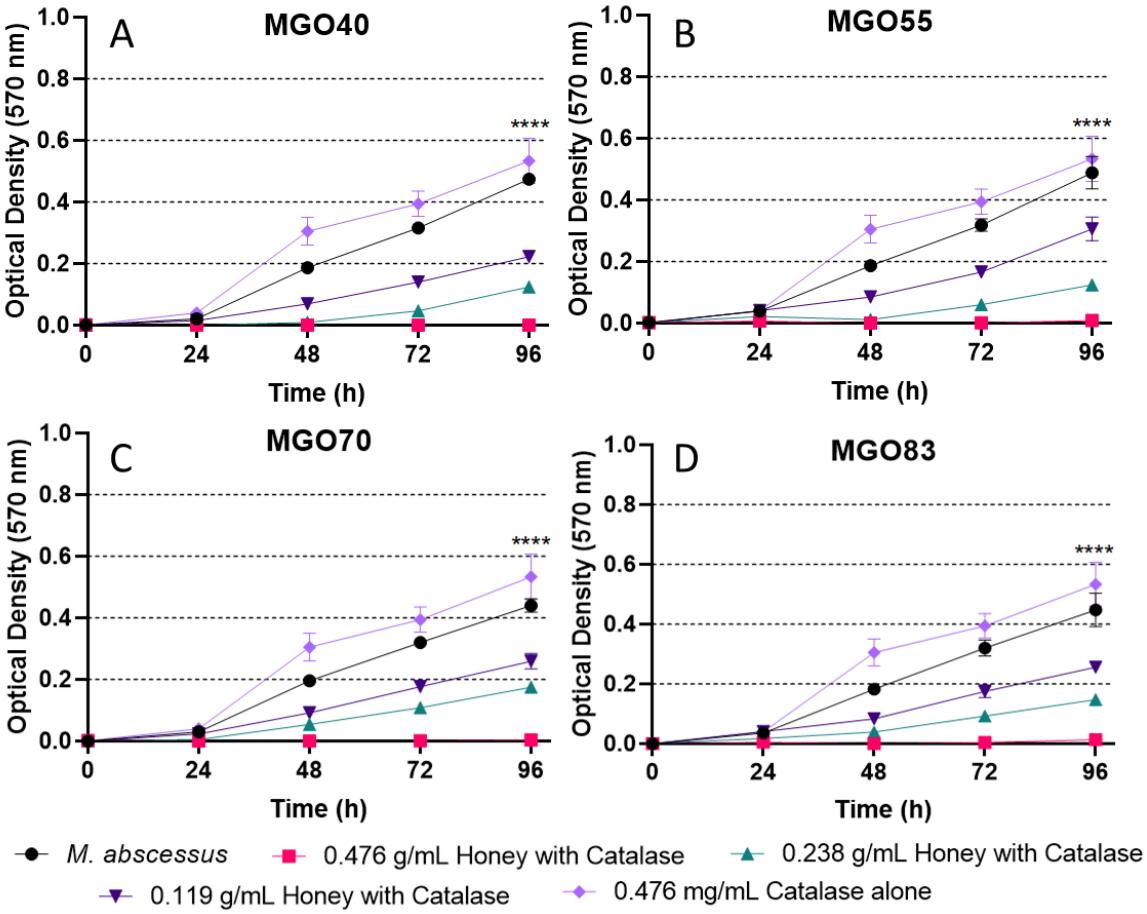

Figure 4.7 Growth curve of *M. abscessus* NCTC 13031 after exposure to manuka honey treated with catalase to remove hydrogen peroxide. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus* after hydrogen peroxide removal with catalase. All 4 of the manuka honeys tested maintained an MIC of 0.476 g/mL. The catalase only control shows catalase had no impact on the growth of *M. abscessus*. A) MGO40 exhibited an MIC of 0.476 g/mL against *M. abscessus*. One-way ANOVA identified a significant difference for all treatments, P=<0.0001. B) MGO55 had an MIC of 0.476 g/mL against *M. abscessus* and a one-way ANOVA showed a significant difference between all treatments, P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70 against *M. abscessus*. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. D) MGO83 also had an MIC of 0.476 g/mL against *M. abscessus* and a one-way ANOVA identified a significant difference between all treatments.

Table 4.2 MBCs of manuka honey against *M. abscessus* with and without catalase treatment

Manuka honey	MBC with catalase treatment	MBC with no catalase treatment
MGO40	>0.476 g/mL	0.476 g/mL
MGO55	>0.476 g/mL	0.476 g/mL
MGO70	>0.476 g/mL	0.476 g/mL
MGO83	>0.476 g/mL	0.476 g/mL

4.3.3 The efficacy of each antimicrobial component tested alone and in combination against *M. abscessus*

To observe the impact of the main antimicrobial components in manuka honey against *M. abscessus* NCTC 13031, each one was tested in a broth microdilution assay. For MGO and hydrogen peroxide, a checkerboard assay was also conducted to observe any synergistic interaction.

4.3.3.1 The efficacy of MGO alone against *M. abscessus*

Although the concentrations of MGO could not be determined within the honey samples, the minimum concentration within the manuka honey was calculated based on MGO rating (Section 4.2.5.1).

Therefore, the highest concentration of MGO tested was 5 mM, with subsequent dilutions also tested. The highest concentrations of 5 mM MGO and 2.5 mM were inhibitory to *M. abscessus*, with a reduction in growth observed for exposure to 1.25 mM MGO (Figure 4.8). There was a small reduction in growth for 0.625 mM MGO compared to the control of *M. abscessus* only. A one-way ANOVA identified a significant difference for all treatments, P=<0.0001. A Dunnett's multiple comparison identified a significant difference between 5 mM MGO, 2.5 mM MGO and 1.25 mM MGO compared to the control of *M. abscessus* only, P=<0.0001, P=<0.0001 and P=0.0001 respectively. No significant difference was observed for 0.625 mM MGO compared to *M. abscessus*, P=0.1035. Bactericidal activity was observed for 5 mM and 2.5 mM MGO against *M. abscessus*, with growth from 1.25 mM MGO.

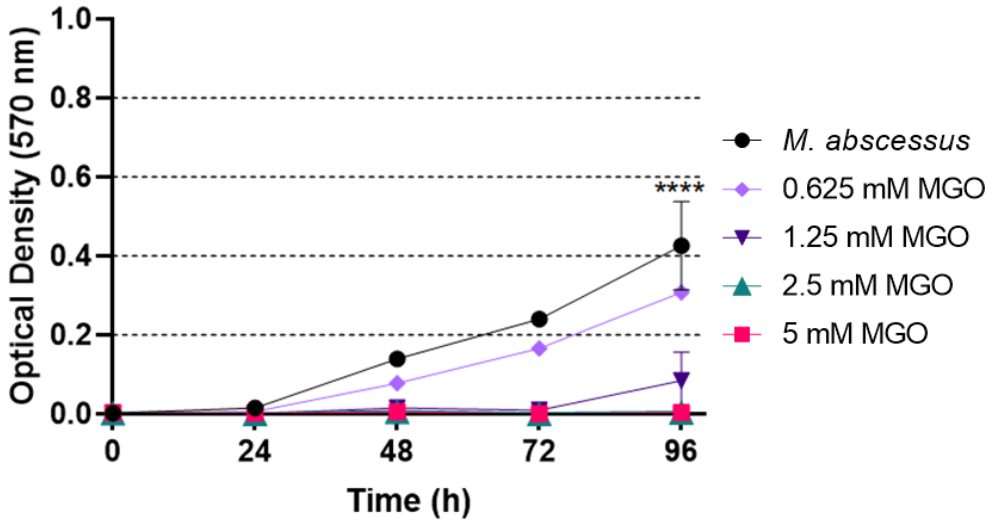

Figure 4.8 Growth curve of M. abscessus NCTC 13031 treated with MGO. Data shown are mean + SD for n=3 technical replicates. The highest concentrations of 5 mM MGO and 2.5 mM MGO inhibited the growth of *M. abscessus*. A reduction in growth was observed for exposure to 1.25 mM MGO.

4.3.3.2 The efficacy of hydrogen peroxide against *M. abscessus*

To observe the impact of hydrogen peroxide on the growth of *M. abscessus*, the broth microdilution assay was used (Section 4.2.7.2). The initial experiment focused on concentrations that were similar to those observed within the manuka honey samples (Section 4.3.2, Figure 4.4). The highest concentration tested was 500 µM hydrogen peroxide which was not inhibitory to *M. abscessus* (Figure 4.9). Therefore, higher concentrations of hydrogen peroxide were also tested.

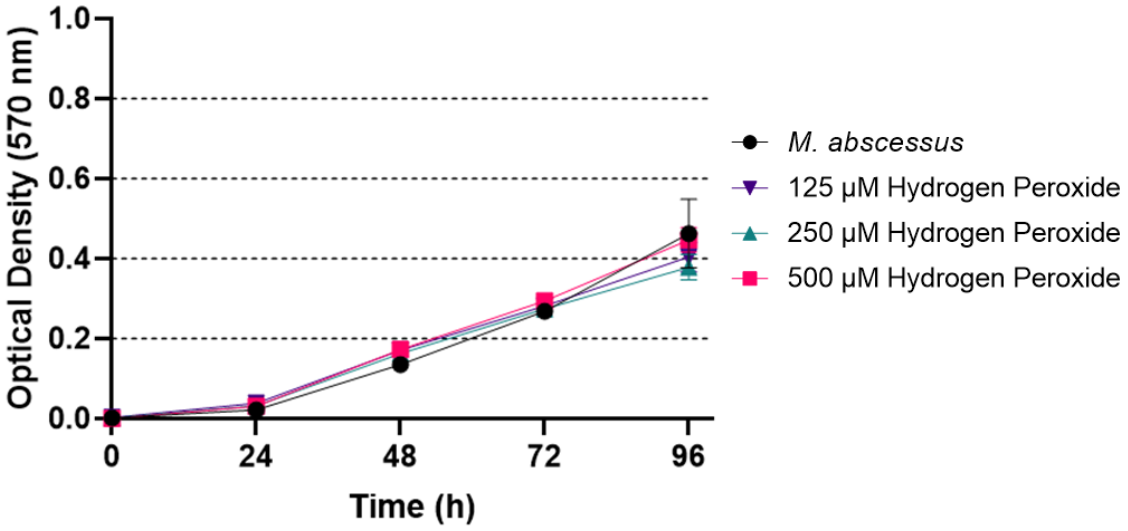

Figure 4.9 Growth curve of *M. abscessus* NCTC 13031 exposed to hydrogen peroxide. Data shown are mean + SD for n=3 technical replicates. The concentrations tested were similar to those found within honey samples. None of the concentrations tested were inhibitory to *M. abscessus*.

The concentrations selected for further testing started at 20 mM hydrogen peroxide to ensure a large range could be observed. The highest concentration tested of 20 mM and the next concentration of 10 mM hydrogen peroxide were both inhibitory to *M. abscessus* (Figure 4.10). The 5 mM hydrogen peroxide appeared to have no impact on the growth of *M. abscessus* and a one-way ANOVA identified a significant difference between the treatments, P=<0.0001. The bactericidal activity reflected the MICs, with no growth observed for 20 mM or 10 mM hydrogen peroxide, but growth was observed for 5 mM hydrogen peroxide.

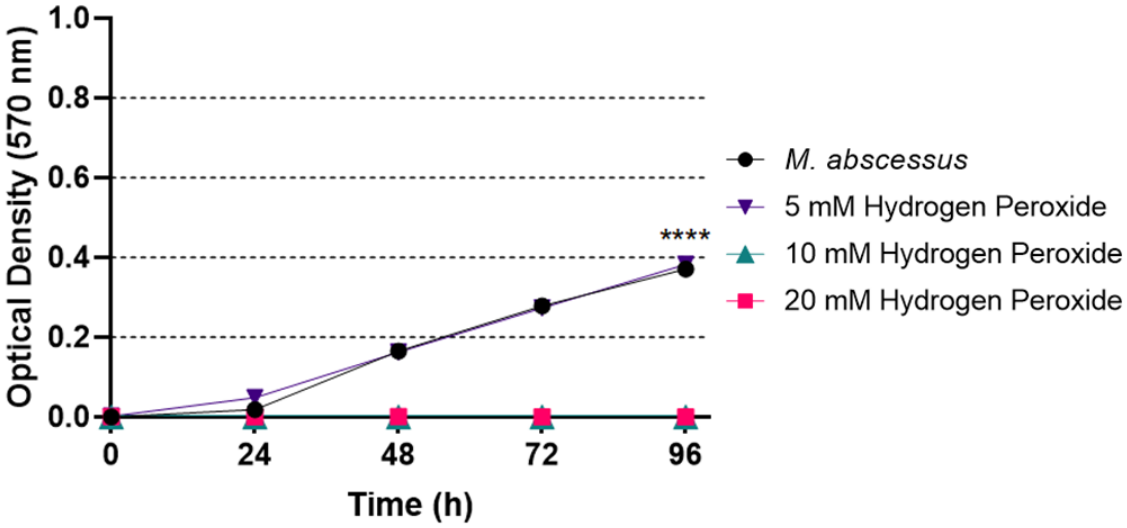

Figure 4.10 Growth curve of *M. abscessus* NCTC 13031 exposed to higher concentrations of hydrogen peroxide. Data shown are mean + SD for n=3 technical replicates. The concentrations of 20 mM and 10 mM hydrogen peroxide were inhibitory to *M. abscessus*. Growth was observed for *M. abscessus* exposed to 5 mM hydrogen peroxide.

4.3.3.3 The efficacy of bee defensin-1 against *M. abscessus*

Due to no bee defensin-1 detected within the honey samples, and therefore no quantifiable concentration, the highest concentration of protein that could be achieved from the purchased stock was tested against *M. abscessus*. This was a starting concentration of 10 µg/mL bee defensin-1. No impact on the growth of *M. abscessus* could be observed for defensin-1 (Figure 4.11).

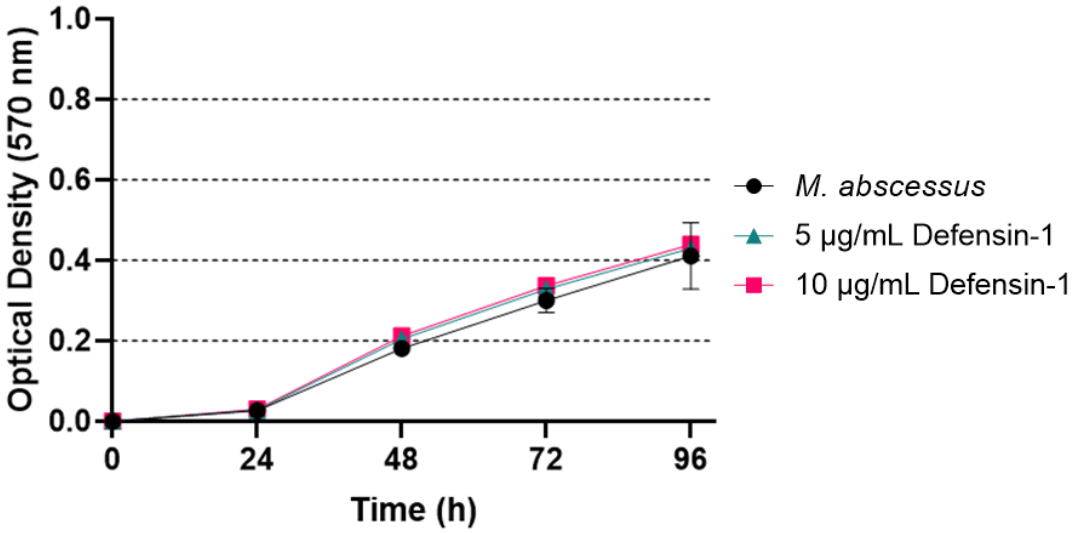

Figure 4.11 Growth curve of M. abscessus NCTC 13031 treated with bee defensin-1. Data shown are mean + SD for n=3 technical replicates. The bee defensin-1 protein had no impact on the growth of *M. abscessus*.

4.3.3.4 The efficacy of MGO and hydrogen peroxide in combination against *M. abscessus*

To observe if MGO and hydrogen peroxide act synergistically together a checkerboard assay was conducted. The concentrations selected for testing were similar to those that would be observed within honey. At the higher concentrations of MGO, 2 mM and 1 mM, no growth was observed for any combination. Growth was observed for all subsequent concentrations, both alone and in combination. No growth inhibition for any subsequent combination suggests there was no synergistic relationship between MGO and hydrogen peroxide against *M. abscessus*. This was also reflected in the bactericidal activity, where no growth was observed for any combination containing either 2 mM or 1 mM MGO, and all subsequent concentrations exhibited growth.

4.3.4 Vegan honea modified for antimicrobial activity against *M. abscessus*

To explore if a vegan honea could be modified to replicate manuka honey, several experiments were developed. Initially the vegan honea was assessed for antimicrobial activity against *M. abscessus* NCTC 13031 before modification. The vegan honea was then prepared with different concentrations of DHA, a precursor to MGO, and monitored for antimicrobial activity over 112 days. Another experiment utilised the enzymes glucose oxidase and invertase to generate hydrogen peroxide within the vegan honea and antimicrobial activity observed.

4.3.4.1 Efficacy of vegan honea against *M. abscessus*

To assess any antimicrobial activity of a vegan honea, lacking the bee-derived components, a broth microdilution was used. No MIC was observed for any concentration of the vegan honea, although the growth of *M. abscessus* was reduced by the honea when compared to the *M. abscessus* only control (Figure 4.12). A one-way ANOVA identified a statistically significant difference between the treatments, P=0.0011. A Dunnett's multiple comparisons analysis identified a significant difference between 0.476 g/mL and *M. abscessus* only, P=0.0006, as well as for 0.238 g/mL and *M. abscessus* only, P=0.0358. No significant difference was observed for 0.119 g/mL and *M. abscessus* only, P=0.5519. This suggests that although no MIC was observed, the vegan honea did impact the growth of *M. abscessus*. Furthermore, no bactericidal activity was observed for any concentration tested.

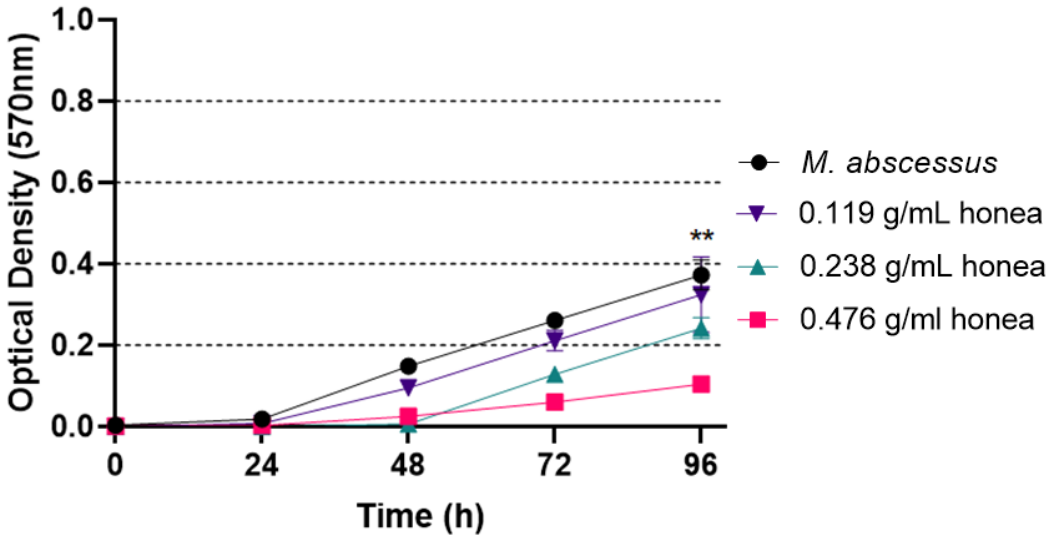

Figure 4.12 Growth curve of *M. abscessus* NCTC 13031 treated with vegan honea. Data shown are mean + SD for n=3 technical replicates. No MIC was observed for vegan honea but growth inhibition of *M. abscessus* was observed for the concentration tested compared to *M. abscessus* alone.

4.3.4.2 The efficacy of vegan honea against *M. abscessus* after modification with DHA to generate MGO against

To assess the generation of MGO within the vegan honea, DHA was added to the vegan honea and antimicrobial activity observed over time (Section 4.2.8.1). Variations in MIC and MBC were observed, with improved antimicrobial activity observed for vegan honea containing DHA stored at 37 °C over time.

For vegan honea with DHA stored at 37 °C, there was no initial change in antimicrobial activity and no inhibition was observed (Figure 4.13 A). The growth of *M. abscessus* was reduced when treated with vegan honea, but this was not an MIC. The DHA alone also did not impact the growth of *M. abscessus* and the growth for the highest concentration of DHA was similar to that of *M. abscessus* only. The growth for the lower concentrations of DHA only also showed improved growth compared to *M. abscessus* only, further showing the DHA alone did not impact *M. abscessus*. This indicates any observations of improved antimycobacterial activity are not attributed to DHA, but the conversion to MGO. No bactericidal activity was observed for any concentration. Similar observations were made for vegan honea with DHA after 14 days of storage (Figure 4.13 B). No MIC or MBC was observed and the DHA only continued to not impact the growth of *M. abscessus*. After 28 days of storage at 37 °C MICs were observed for 0.476 g/mL vegan honea with 1.9 mg/mL DHA (Figure 4.13 C). Interestingly, the same concentration of 1.9 mg/mL DHA alone did not impact the growth of *M. abscessus*. All other concentrations of vegan honea with DHA and DHA only showed a reduction in growth compared to *M. abscessus* only, but these were not considered inhibitory. No MBCs were observed for any concentration.

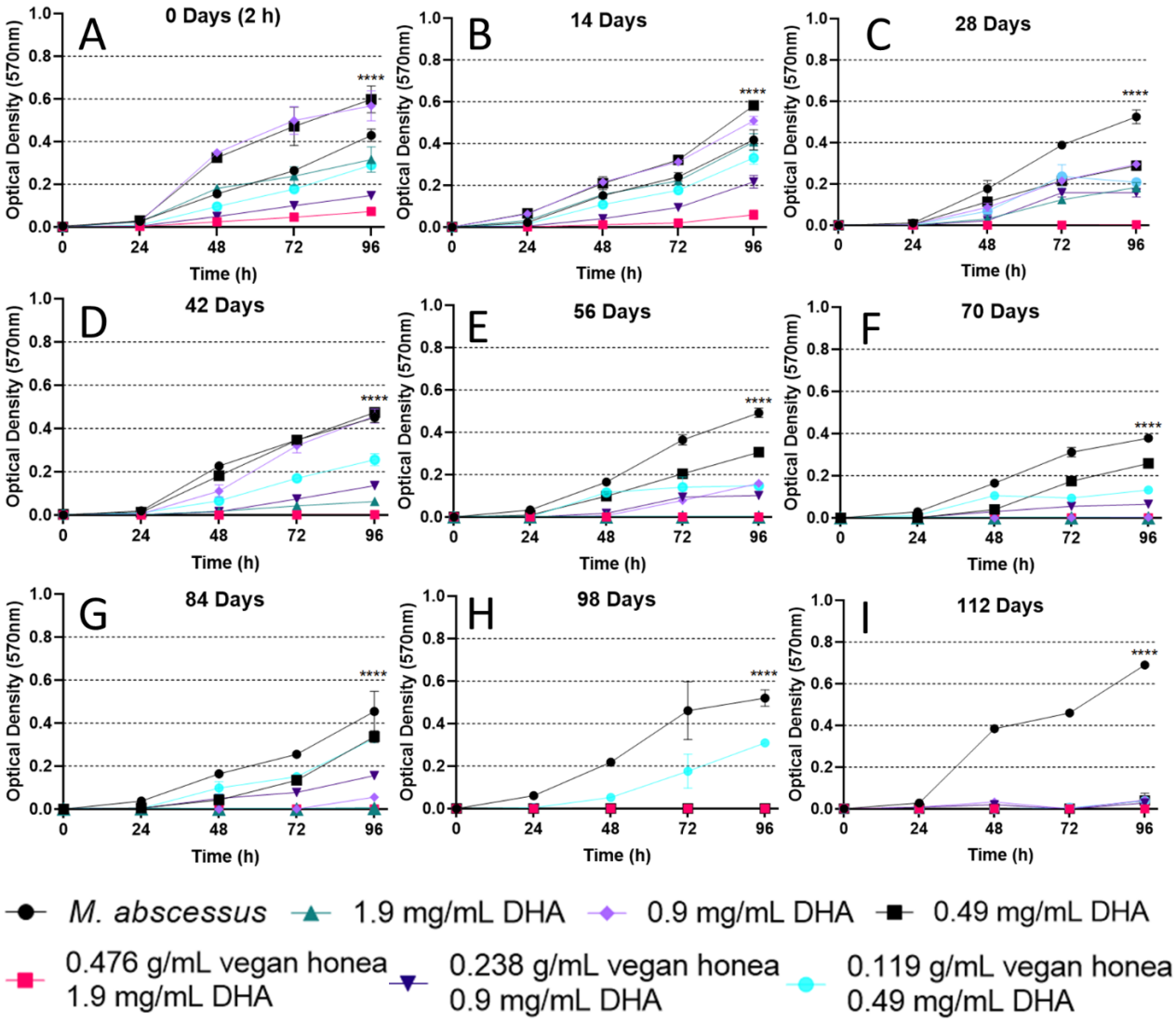

Figure 4.13 Growth curves of *M. abscessus* NCTC 13031 treated with vegan honea containing DHA stored at 37 °C for 112 days. Data shown are mean + SD for n=3 technical replicates. An increase in antimicrobial activity of vegan honea containing DHA can be observed over time. End point data, taken at 96 h, was used for statistical analysis to determine the impact of vegan honea with DHA on the growth of *M. abscessus*. A) No inhibitory activity of vegan honea with DHA at any concentration tested against M. abscessus after 2 h incubation. B) Vegan honea with DHA stored for 14 days showing no inhibition of M. abscessus at any concentration. C) Inhibition of M. abscessus by 0.476 g/mL vegan honea with 1.9 mg/mL DHA stored for 28 days prior to testing. No inhibition was observed for any other concentration tested. D) Vegan honea with DHA after 42 days of storage exhibiting inhibition by 0.476 g/mL vegan honea with 1.9 mg/mL DHA. No inhibition was observed for any other concentration tested. E) Vegan honea with DHA stored for 56 days prior to testing inhibiting M. abscessus with 0.476 g/mL vegan honea with 1.9 mg/mL DHA and 1.9 mg/mL DHA only. F) After 70 days of storage, 0.476 g/mL vegan honea with 1.9 mg/mL DHA, 1.9 mg/mL DHA and 0.9 mg/mL DHA alone were inhibitory to M. abscessus. G) The storage of vegan honea with DHA for 84 days prior to testing showing M. abscessus growth inhibition for 0.476 g/mL vegan honea with 1.9 mg/mL DHA and 1.9 mg/mL only. H) All concentrations tested inhibited the growth of M. abscessus apart from 0.119

g/mL vegan honea with 0.49 g/mL DHA after 98 days of storage. I) After 112 days of storage, all concentrations of vegan honea with DHA and DHA alone inhibit M. abscessus apart from 0.119 g/mL vegan honea with 0.49 mg/mL DHA and 0.49 mg/mL DHA only.

For vegan honea with DHA stored at 37 °C for 42 days the MICs were maintained, with 0.476 g/mL vegan honea with 1.9 mg/mL DHA remaining the only inhibitory concentration (Figure 4.13 D). However, *M. abscessus* exposed to 1.9 mg/mL DHA exhibited a significant reduction in growth compared to the growth of *M. abscessus* only, Dunnett's multiple comparison P=<0.0001. After 56 days both 0.476 g/mL vegan honea with 1.9 mg/mL DHA and 1.9 mg/mL DHA only were inhibitory (Figure 4.13 E). All other concentrations showed a reduction in growth compared to *M. abscessus* only, but these were not inhibitory. No MBC was identified for any concentration after storage at either 42 days or 56 days.

After 70 days of storage, 3 concentrations were inhibitory to *M. abscessus* (Figure 4.13 F). These were 0.476 g/mL vegan honea with 1.9 mg/mL DHA, 1.9 mg/mL DHA only and 0.9 mg/mL DHA alone. All other concentrations maintained a reduction in growth compared to *M. abscessus*. Interestingly, there was no MBC for 0.476 g/mL vegan honea with 1.9 mg/mL DHA but an MBC was observed for 1.9 mg/mL DHA only.

The antimicrobial activity observed after 84 days of storage maintained inhibition against *M. abscessus* at 0.476 g/mL vegan honea with 1.9 mg/mL DHA and 1.9 mg/mL DHA only (Figure 4.13 G). However, a loss of inhibition was observed for 0.9 mg/mL DHA only. All other concentrations were not inhibitory to *M. abscessus* but a reduction in growth was observed. A change in MBC was also observed, with an MBC identified for 0.476 g/mL vegan honea with 1.9 mg/mL DHA and 1.9 mg/mL DHA alone.

A significant difference in activity was observed after 98 days, with almost all concentrations being inhibitory towards *M. abscessus* (Figure 4.13 H). The only concentration that did not inhibit *M. abscessus* were 0.119 g/mL vegan honea with 0.49 mg/mL DHA. The MBCs remained the same with only 0.476 g/mL vegan honea with 1.9 mg/mL DHA and 1.9 mg/mL DHA only being bactericidal.

After 112 days of storage at 37 °C, almost all concentrations remained inhibitory towards *M. abscessus* (Figure 4.13 I). The concentration of 0.119 g/mL vegan honea with 0.49 mg/mL DHA remained not inhibitory and a loss of inhibition was observed for 0.49 mg/mL DHA only. The MBCs were also maintained, with 0.476 g/mL vegan honea with 1.9 mg/mL DHA and 1.9 mg/mL DHA only having bactericidal activity.

The antimicrobial activity of vegan honea with DHA stored at 4 °C against *M. abscessus* differed to that stored at 37 °C and did not have the same inhibitory affect (Figure 4.14). There was no MIC observed for any concentration tested until day 98 of storage, which was 0.476 g/mL vegan honea with 1.9 mg/mL DHA (Figure 4.14 H), and after that the MIC was lost. The vegan honea with DHA had a greater inhibitory effect against *M. abscessus* than compared to DHA only for all days tested. No bactericidal activity was observed for any day of testing at any concentration.

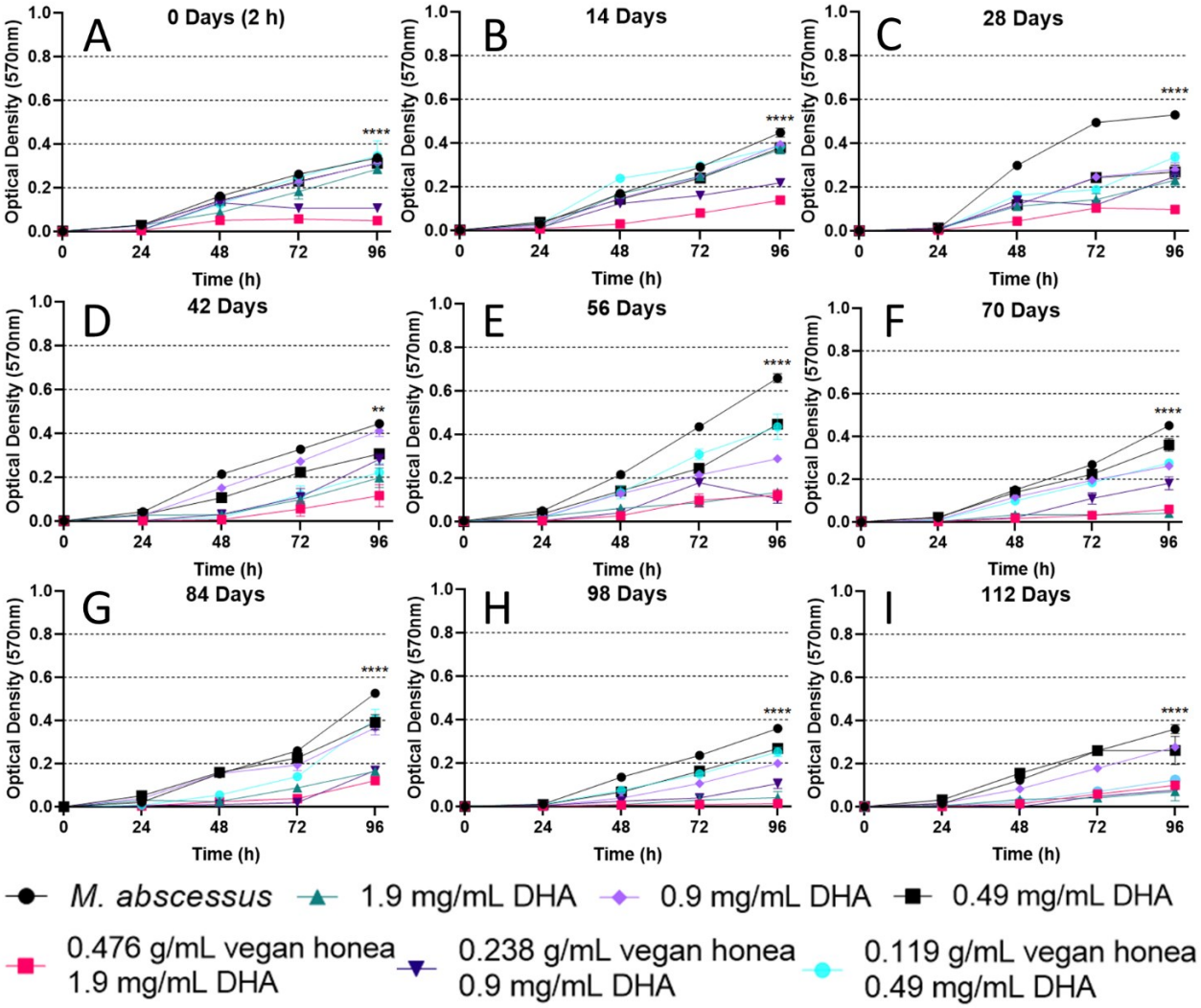

Figure 4.14 Growth curves of *M. abscessus* NCTC 13031 treated with vegan honea containing DHA stored at 4 °C for 112 days. Data shown are mean + SD for n=3 technical replicates. No increased antimicrobial activity of vegan honea containing DHA was observed, regardless of storage time. End point data, taken at 96 h, was used for statistical analysis to determine the impact of vegan honea with DHA on the growth of *M. abscessus*. A) Vegan honea with DHA stored for 2 h showed a reduction in growth for M. abscessus but no MIC. B) Vegan honea with DHA stored for 14 days prior to testing maintaining the reduction in growth for higher concentrations of vegan honea and DHA, but no MIC was observed. C) Storage of vegan honea with DHA for 28 days showing no MIC against M. abscessus. D) No MIC observed for vegan honea with DHA against M. abscessus after 42 days of storage. E) Storage of vegan honea for 56 days maintaining no MIC but a reduction in growth of M. abscessus was observed. F) A reduction in growth of M. abscessus for 0.476 g/mL vegan honea with

1.9 mg/mL DHA and 1.9 mg/mL DHA alone stored for 70 days but no MIC. G) No MIC observed for M. abscessus exposed to vegan honea with DHA stored for 84 days. H) Vegan honea with DHA stored for 98 days inhibited M. abscessus at 0.476 g/mL vegan honea with 1.9 mg/mL DHA. No other concentration impacted the growth of M. abscessus. I) Loss of inhibition of M. abscessus by vegan honea with DHA after 112 days of storage.

4.3.4.3 The efficacy of vegan honea against *M. abscessus* after modification with glucose oxidase and invertase to generate hydrogen peroxide

To determine that hydrogen peroxide was generated within the vegan honea samples containing glucose oxidase and invertase, the hydrogen peroxide detection assay was used (Section 4.2.5.2). To observe if the concentration of enzymes would impact the amount of hydrogen peroxide generated, 3 concentrations were selected. These were 10 U/mL, 100 U/mL and 500 U/mL of both glucose oxidase and invertase. All concentrations of enzyme produced hydrogen peroxide that was detected by the assay. Regardless of amount of enzyme, the same concentration of hydrogen peroxide was detected (Figure 4.15).

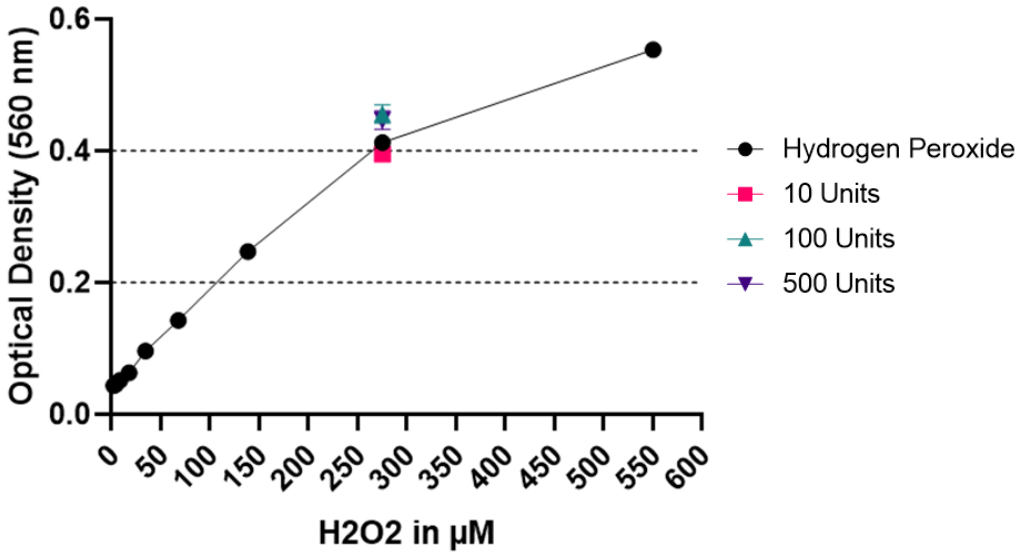

Figure 4.15 Detection of hydrogen peroxide in vegan honea with glucose oxidase and invertase. Data shown are mean + SD for n=3 technical replicates. The glucose oxidase and invertase enzymes were added to the concentrations of 10 units, 100 units and 500 units. No difference in hydrogen peroxide concentration was observed regardless of unit of enzyme added.

After determining that the amount of enzyme used resulted in the same amount of hydrogen peroxide conversion, the broth microdilution assay was conducted using vegan honea containing 1 U/mL of both enzymes in the stock solution. The vegan honea containing glucose oxidase and invertase had an MIC of 0.014 g/mL against *M. abscessus* NCTC 13031 (Figure 4.16). Growth could be seen for 0.007 g/mL vegan honea with glucose oxidase and invertase. The control of 1 U/mL glucose oxidase and 1 U/mL invertase did not impact the growth of *M. abscessus*. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison was conducted comparing all treatments to *M. abscessus* only. This identified no significant difference for glucose oxidase and invertase combined, P=0.2064, confirming that this did not impact the growth of *M. abscessus*. A significant difference was observed for all other concentrations, P=<0.0001 for all apart from 0.007 g/mL, P=0.0002. The MICs were also reflected in the MBCs with no growth observed for 0.014 g/mL vegan honea with glucose oxidase and invertase. Growth of *M. abscessus* was observed for 0.007 g/mL vegan honea with glucose oxidase and invertase as well as the control of glucose oxidase and invertase only.

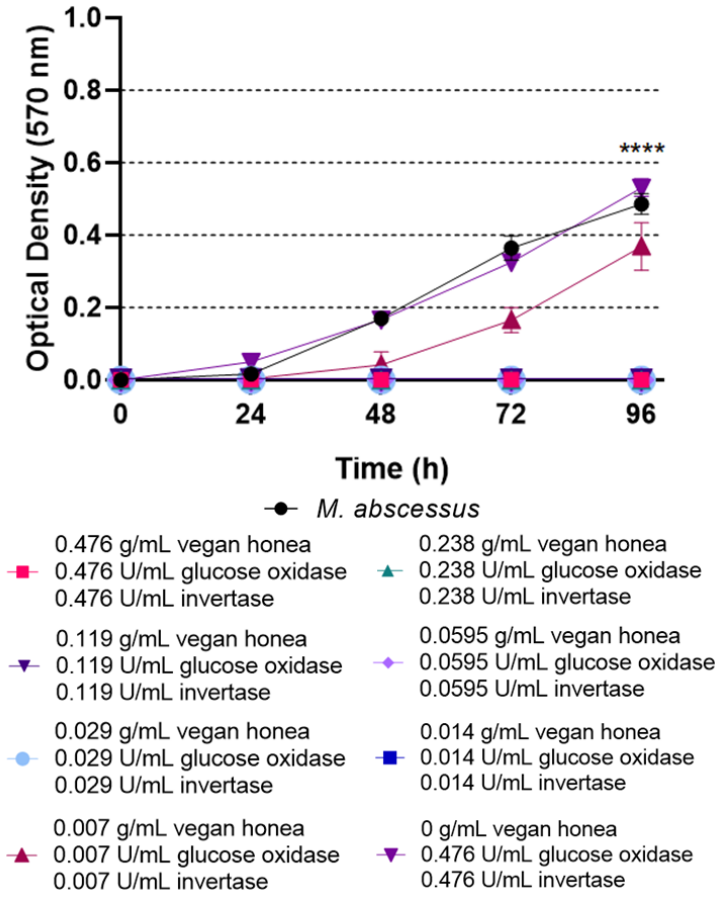

Figure 4.16 Antimicrobial activity of vegan honea with glucose oxidase and invertase against *M. abscessus* NCTC 13031. Data shown are mean + SD for n=3 technical replicates. The MIC of vegan honea with glucose oxidase and invertase was 0.014 g/mL. Growth of *M. abscessus* was observed for 0.007 g/mL vegan honea with glucose oxidase and invertase. The enzymes glucose oxidase and invertase alone had no impact on the growth of *M. abscessus*.

4.4 Discussion

The 3 main antimicrobial components of honey are present at varying levels and can influence the antimicrobial activity of a given honey. This variation is attributed to the geographical location, botanical origin and bee colony health (Section 1.1.2) (Nolan, Harrison and Cox, 2019). Other factors can also influence the antimicrobial activity of honey, including the high sugar content, low pH and osmotic pressure. After establishing that manuka honey has antimicrobial activity against *M. abscessus* (Section 2.3.2), the focus was to determine to what extent the components influence this. Initially, the determination and quantification of the 3 major components, MGO, hydrogen peroxide and bee defensin-1 was conducted followed by assessment of their activity and role in the inhibition of *M. abscessus*.

The MGO grades of manuka honey used in this study indicate the minimum concentration of MGO (mg/kg) present within the honey. However, due to the conversion of DHA to MGO over time, it is possible that at the time of MGO determination not all DHA had been converted and therefore the MGO concentration could be higher. The N-acetyl cysteine assay was selected for determination of MGO (Wild *et al.*, 2012). This assay relies on the formation of the Na-acetylcysteine-methylglyoxal hemithioacetal, Na-acetyl-S-(1-hydroxy-2-oxoprop-1-yl)cysteine, which can be detected spectrophotometrically at 288 nm. Unfortunately, there was no variation detected between the manuka honey samples and all were indicated to be 0.5 mM MGO, which would be incorrect due to their MGO ratings. It has been identified that the presence of amino acids can result in deterioration of MGO over time within manuka honey (Adams, Manley-Harris and Molan, 2009; Grainger, Manley-harris, *et al.*, 2016). However, this would likely cause a loss in antimicrobial activity, which was not observed. Furthermore, the assay used was adapted and not originally for honey samples. Therefore, it is possible that other compounds within the honey interfered with the reaction and this resulted in inaccurate results. To determine if this was the case, an additional experiment should have been conducted by adding known concentrations of MGO to a honey sample and testing again. This would allow for comparison of the known concentration to the standard curve and indicate if the adapted experiment was suitable to detect MGO within manuka honey. Moreover, the industry standard for detection of MGO in manuka honey samples is through high performance liquid chromatography, which was unachievable for this research. However, MGO will be present within the samples due to the classification of MGO grades.

The determination of hydrogen peroxide within the manuka honey was successful. The hydrogen peroxide assay was based on a colourimetric assay that utilised HRP to catalyse the oxidation of o-dianisidine by hydrogen peroxide (Lehmann *et al.*, 2019). HRP has an iron rich haem group that contains iron and protoporphyrin, which acts as the binding site for a variety of substrates (Hamid and Khalil-ur-Rehman, 2009). This provides a site of attachment for hydrogen peroxide during enzyme activation, forming a complex (Veitch, 2004). The

HRP-hydrogen peroxide complex can oxidise a variety of hydrogen donors, such as o-dianisidine. This results in a brown colour change that is unstable, requiring sulfuric acid to stop the reaction through the denaturing of all enzymes. Upon the addition of sulfuric acid, the brown oxidised o-dianisidine turns pink, which can be detected spectrophotometrically at 560 nm. The concentrations of hydrogen peroxide within the 4 manuka honey samples were determined to be 68 µM for MGO40, 40 µM for MGO55, 40 µM for MGO70 and 18 µM for MGO83 (Section 4.3.1.2). These concentrations are within the typical range observed in other honey samples of 10 µM to 92 µM (Alygizou *et al.*, 2021). Interestingly, these concentrations are subinhibitory for a wide range of microorganisms and are significantly lower than concentrations required for disinfection (Brudzynski *et al.*, 2011). These low concentrations of hydrogen peroxide in the manuka honey suggest its impact on the inhibition of *M. abscessus* is minimal.

The last component to be identified within the manuka honey samples was bee defensin-1, an antimicrobial peptide produced by the honeybee. This was conducted by protein dialysis of the manuka honey samples and subsequent detection using SDS PAGE (Section 4.3.1.3). No protein was identified within the samples. This was not unsurprising as several reports have suggested that MGO could have negative impacts on defensin-1 over time, resulting in the inactivation or degradation of the protein (Kwakman *et al.*, 2011; Majtan *et al.*, 2012). It is possible that the selection of a Coomassie blue stain impacted the detection. However, in previous reports identifying the bee defensin-1 protein a Coomassie blue stain was used successfully (Kwakman *et al.*, 2011; Bucekova *et al.*, 2017). Further to this, the detection sensitivity of Coomassie blue is impacted by it interacting with only hydrophobic amino acids and the bee defensin-1 protein contains 46 hydrophobic amino acids out of the 95 amino acid sequence, suggesting that detection sensitivity might not be an issue. To determine if no bee defensin-1 was identified or the Coomassie blue did not detect the protein on the SDS PAGE gel, a control of bee defensin-1 should have been used. The suggestion that bee defensin-1 might not be present as well as the lack of identification in the manuka honey samples used here, indicates that the bee defensin-1 protein is not having an impact on the antimicrobial activity of the manuka honey against *M. abscessus*. This coupled with the low levels of hydrogen peroxide identified in the manuka honey samples suggest that MGO is the main component attributed to the activity observed against *M. abscessus*.

After determination of the presence of MGO and hydrogen peroxide within the manuka honey, removal of the components was conducted and the antimicrobial activity re-evaluated. For the removal of MGO, the N-acetyl cysteine assay was utilised. Unfortunately, the N-acetyl cysteine was inhibitory to *M. abscessus* and therefore any changes in activity due to the loss of MGO could not be determined. In a previous study that used reduced glutathione and the glyoxalase enzyme to convert MGO to S-D-lactoglutathione, they determined that after the conversion of MGO some inhibitory activity still remained

(Kwakman *et al.*, 2011). This depended on the microorganism tested, with methicillin resistant *S. aureus*, *B. subtilis* and *P. aeruginosa* still being inhibited but often requiring higher concentrations of honey and *E. coli* was no longer inhibited by the honey at any concentration tested. This suggests that the activity of manuka honey against *M. abscessus* would be altered after the removal of MGO, although growth inhibition might still be observed.

The removal of hydrogen peroxide was achieved by utilising catalase, which breaks down hydrogen peroxide into H_2O and oxygen. The antimicrobial activity of the manuka honey was slightly impacted by hydrogen peroxide removal, as the MICs remained the same but bactericidal activity was lost. Importantly, the addition of catalase also did not have any impact on the growth of *M. abscessus*. This suggests that hydrogen peroxide does not have an important role in the inhibition of *M. abscessus*. Furthermore, it has previously been suggested that the presence of MGO within a honey can cause degradation of glucose oxidase, the enzyme responsible for hydrogen peroxide generation (Kwakman *et al.*, 2011). However, concentrations of hydrogen peroxide were detected within these manuka honey samples. The assay used to detect the hydrogen peroxide had previously been developed to optimise its detection within a honey sample (Lehmann *et al.*, 2019). It is possible that previous attempts to detect hydrogen peroxide within a manuka honey were unsuccessful due to several factors overlooked, such as honey dilution and dilution time before performing the assay as well as aeration during incubation prior to performing the assay. This indicates that hydrogen peroxide is a constituent of manuka honey and may have some role in its antimicrobial activity. This is further supported by the study conducted by Kwakman *et al.,* 2011, outlining that antimicrobial activity was still observed after MGO removal. Furthermore, other studies have also identified hydrogen peroxide within manuka honey, at concentrations of 0.72 mM and 1.04 mM (Brudzynski *et al.*, 2011). Overall, the concentrations of hydrogen peroxide detected within the manuka honey samples were typically low and considered subinhibitory, suggesting that MGO was more important for *M. abscessus* inhibition than hydrogen peroxide.

To further explore the role each component had on the inhibition of *M. abscessus*, each one was tested alone using the broth microdilution assay. Further exploration into the relationship between MGO and hydrogen peroxide was also conducted to see if they acted synergistically against *M. abscessus*. It was identified that 2.5 mM MGO was required for inhibition of *M. abscessus*, which is higher than the concentrations of MGO calculated within the manuka honey, that ranged from 0.58 mM to 1.2 mM MGO. This suggests that MGO alone does not inhibit *M. abscessus* and other components may also impact the activity of manuka honey. Previously it has been shown that concentrations of 0.8 mM and 1.2 mM MGO are required for growth inhibition of *B. subtilis* and *S. aureus*, respectively. Moreover, the mechanism of action of MGO is due to its ability to alter the structure of bacterial fimbriae and flagella,

where 2 mM MGO resulted in the complete loss of these structures, as well as inducing damage to cell membranes and causing shrinking and rounding of bacterial cells (Rabie *et al.*, 2016). Although *M. abscessus* does not have fimbriae or flagella, it is likely that MGO causes damage to the mycobacterial cell membrane. If MGO causes damage to the outer mycobacterial membrane, accumulation could occur within the periplasmic space (Figure 1.1), This accumulation would result in damage to the cytoplasmic membrane and could result in cell death. Furthermore, it has been identified that MGO is not recognised by drug efflux pumps in other bacterial isolates (Hayashi *et al.*, 2014). This suggests that after entry into the cell it is very unlikely MGO would be exported out, improving its antimicrobial action and preventing one of the main resistance mechanisms employed by mycobacteria (Section 1.1.5). Further exploration into the mechanisms behind the activity of MGO and manuka honey should be conducted, with a focus on microscopy to determine if MGO impacts the mycobacterial cell and does cause membrane disruption. Additionally, the pressure exerted by MGO, coupled with other factors such as high sugar content, osmotic pressure and low pH of manuka honey could influence the antimicrobial activity being observed. This could explain why subinhibitory concentrations of MGO within manuka honey result in growth inhibition of *M. abscessus*.

A different occurrence was observed with hydrogen peroxide, where 10 mM was required to inhibit the growth of *M. abscessus*. This is significantly higher than the concentrations observed within the manuka honey, which were 68 µM, 40 µM, 40 µM and 18 µM, indicating that hydrogen peroxide alone would not be sufficient to inhibit *M. abscessus*. Typically, the concentrations of hydrogen peroxide alone required to inhibit *E. coli* and *B. subtilis* are 1.25 mM and 2.5 mM, respectively (Brudzynski *et al.*, 2011). This is still considerably lower than the concentrations required to inhibit *M. abscessus*. The mechanism behind hydrogen peroxides activity is DNA degradation through oxidation (Finnegan *et al.*, 2010; Brudzynski, Abubaker and Wang, 2012). It has been shown that hydrogen peroxide alone requires 2.5 mM for DNA damage to occur, whereas similar DNA damage can be observed for honey's containing less than 2.5 mM hydrogen peroxide (Brudzynski *et al.*, 2011). It is possible that significantly higher concentrations of hydrogen peroxide are required for *M. abscessus* due to the impermeable cell wall. This further supports that hydrogen peroxide alone is not the driving force behind the inhibition of manuka honey but could be a contributing factor.

To determine if MGO and hydrogen peroxide act synergistically at subinhibitory concentrations, a checkerboard assay was conducted. The concentrations tested were similar to those identified within the honey, but no synergy was observed for any concentration tested. This suggests that MGO and hydrogen peroxide together are not sufficient to inhibit *M. abscessus* and other factors are impacting the activity, such as osmotic pressure, sugar content and low pH. Future experiments exploring this relationship should be considered to determine if these indirect factors are enough to influence the activity of MGO

and hydrogen peroxide at such low concentrations. Additionally, the bee defensin-1 protein was also assessed for antimicrobial activity and no activity was observed. Considering no defensin-1 was observed in the manuka honey it is unlikely that this protein impacted the growth inhibition of *M. abscessus*.

Considering indirect mechanisms of honey, specifically the sugar content, osmolarity and pH, exploration into a vegan honea against *M. abscessus* was conducted. The vegan honea selected did not contain any bee derived components, such as glucose oxidase and bee defensin-1, and was not of botanical origin so was therefore void of DHA and other compounds such as polyphenolics. Additionally, the sugar content was similar to that of manuka honey, thus allowing investigation into the impact of the indirect mechanisms. The broth microdilution assay was used to assess activity of the vegan honea and no MIC or MBC was observed for *M. abscessus*. However, a reduction in growth was observed for *M. abscessus* treated with vegan honea compared to *M. abscessus* only. This was most likely due to the high sugar content and osmotic pressure providing an unfavourable environment for bacterial growth. The presence of apple juice in the vegan honea would have also created a low pH environment, suggesting this could have impacted the growth of *M. abscessus*. The typical pH of manuka honey is between 3.1 and 4.5, which is comparable to that of apple juice which is between 3.35 and 4, further helping to mimic the conditions of manuka honey (Safii, Tompkins and Duncan, 2017). After determining that vegan honea was not inhibitory to *M. abscessus*, exploration into developing an antimicrobial vegan honea was conducted by addition of precursors to MGO and hydrogen peroxide.

To generate MGO in the vegan honea, DHA was used. The conversion of DHA to MGO occurs over time and can be influenced by temperature (Adams, Manley-Harris and Molan, 2009). Therefore, 2 different temperatures were selected, these were 37 °C and 4 °C. It has been suggested that conversion of DHA to MGO does not occur at 4 °C, however, the observations of the time dependent study indicate that this might not be the case (Section 2.3.6). The vegan honea with DHA stored at 37 °C was not inhibitory initially and an MIC was not observed until after 28 days of incubation. The MIC was also only observed for vegan honea containing DHA and not for DHA alone. This suggested that the combination of MGO generation and indirect mechanisms of the vegan honea were causing this inhibition. Interestingly though, there was no MBC observed, suggesting that concentrations of MGO were not sufficient to cause bactericidal activity. The next change in activity was observed after 56 days of storage, where the highest concentration of DHA both with or without vegan honea were inhibitory to *M. abscessus* This suggested that an inhibitory concentration of MGO had been converted, however this was still not bactericidal. A further increase in antimicrobial activity was observed after 70 days of storage at 37 °C, with 0.476 g/mL vegan honea with 1.9 mg/mL DHA, 1.9 mg/mL DHA only and 0.9 mg/mL DHA only causing inhibition of *M. abscessus*. The concentration of 0.238 g/mL vegan honea with 0.9 mg/mL

DHA was not inhibitory to *M. abscessus*. This could be due to the amount of MGO converted having some inhibitory pressure on *M. abscessus* when tested alone but the added sugar of the vegan honea with the same concentration of DHA could be allowing it to overcome this pressure, as 0.238 g/mL vegan honea is subinhibitory on its own. This inhibition observed by 0.9 mg/mL DHA only was not observed after 84 days of storage, suggesting the concentration achieved was either similar to the MIC of MGO required or after a further 14 days of storage the converted MGO has dissipated. Furthermore, bactericidal activity was observed for 1.9 mg/mL DHA only after 70 days of storage but bactericidal activity was observed for both 0.476 g/mL vegan honea and 1.9 mg/mL DHA by day 84. This also suggests that the sugar in the honea might be influencing the activity observed, by providing an additional sugar source.

The biggest change observed was after 98 days of storage, with almost all concentrations being inhibitory to *M. abscessus*. Again, the only concentration not inhibitory was one containing vegan honea, which continues to suggest the subinhibitory concentration of sugar is allowing *M. abscessus* to overcome the inhibitory pressures of DHA to MGO conversion. The bactericidal activity also remained the same with 0.476 g/mL vegan honea with 1.9 mg/mL DHA and 1.9 mg/mL DHA alone being bactericidal, indicating inhibitory concentrations of MGO were reached.

After 112 days of incubation a slight change in inhibition was observed. The lowest concentration of vegan honea with DHA and DHA alone were not inhibitory to *M. abscessus*. This could be due to a saturation point in DHA to MGO conversion being reached where the loss of MGO starts to occur, resulting in the loss of activity. Importantly though, the bactericidal activity was maintained for the highest concentrations, suggesting that if MGO loss was occurring within the honea, this was minimal and not enough to impact the higher concentrations tested.

On the other hand, the vegan honea with DHA stored at 4 °C exhibited no inhibitory activity until 98 days of incubation, where only 0.476 g/mL vegan honea with 1.9 mg/mL DHA was inhibitory to *M. abscessus*. There was also no bactericidal activity observed for any concentration tested. This highlights that slower conversion of DHA to MGO occurs at lower temperatures. This also supports the previous results observing increased antimicrobial activity over time when stored at 4 °C (Section 2.3.6). It also highlights that the indirect mechanisms of honey might be impacting the antimicrobial activity since DHA alone was not inhibitory. The accelerated conversion of DHA to MGO at 37 °C meant that this was not observed at the higher incubation temperature. However, at 4 °C the concentrations were not bactericidal, alluding to other influences on the antimicrobial activity than DHA to MGO conversion and indirect mechanisms alone. Furthermore, it has been suggested that other factors might influence the conversion of DHA to MGO that were not addressed here, such

as proton donors/acceptors that influence the change of DHA from a dimer to a monomer, a necessary step for MGO conversion (Grainger, Manley-Harris, *et al.*, 2016). A limiting factor of this study was also the absence of MGO detection throughout. The concentrations of MGO achieved could not be determined so the full extent of DHA to MGO conversion and therefore the impact on the antimicrobial activity cannot be fully elucidated. However, the increase in antimicrobial activity over time is significant and should not be overlooked. This provides a promising basis for further developing honey products and understanding important mechanisms behind their action.

The second approach for altering the vegan honea with a view to generating antimicrobial activity was to generate hydrogen peroxide by using glucose oxidase and invertase. These enzymes are responsible for the conversion of glucose into hydrogen peroxide within the honey (Section 1.2.3.2). Initially, the amount of enzyme required for this conversion was unknown, therefore different enzyme concentrations were used and hydrogen peroxide content determined using the hydrogen peroxide assay. Interestingly, the amount of enzyme required for conversion did not impact the amount of hydrogen peroxide converted, with all 3 concentrations of enzyme resulting in the same amount of hydrogen peroxide conversion. Therefore, for the broth microdilution assay with vegan honea containing both enzymes, 1 U/mL of enzyme was used. This resulted in an MIC and MBC of 0.014 g/mL vegan honea with 0.014 U/mL invertase and glucose oxidase against *M. abscessus*, which was significantly reduced compared to vegan honea alone, where no MIC or MBC was noted. The concentration of hydrogen peroxide generated was indicated to be 275 µM. However, considering the activity of hydrogen peroxide alone against *M. abscessus*, this seems inaccurate. The assay used can quantify hydrogen peroxide up to 550 µM, suggesting that concentrations higher than this might not be detected accurately (Lehmann *et al.*, 2019). It is possible that the amount of hydrogen peroxide produced by the enzymes is significantly higher, indicated by the significant increase in antimicrobial activity. Overall, the antimicrobial activity achieved through the addition of 1 unit of enzyme to the stock solution of the vegan honea is highly significant and of great importance.

Infections caused by *M. abscessus* are notoriously difficult to treat due to many factors, such as innate drug resistance mechanisms and an impermeable cell wall (Section 1.1.5). Therefore, new treatments are imperative for combating these infections. Here, it has been demonstrated that manuka honey can successfully inhibit *M. abscessus* and the likely driving force behind this activity is the presence of MGO. However, it is likely that MGO alone is not the only driving force and that hydrogen peroxide along with the indirect mechanisms of honey impact the activity. Furthermore, the development of an antimicrobial vegan honea with the addition of either DHA or glucose oxidase with invertase, shows a very promising future for the development of specific and targeted therapies based on manuka honey. Overall, manuka honey is effective against *M. abscessus* and medical grade honey should be considered for the treatment of *M. abscessus* infections.

Future work should be focused on full determination of the antimicrobial impact of each component against *M. abscessus* both alone and in combination. This would include mass spectrometer analysis of the manuka honey samples and laboratory based replication of the honey with differing of the main antimicrobial component. This would provide a better determination of the antimicrobial impact through a more controlled experiment, it would also allow different combinations to be tested such as the addition of all components or 2 at a time and how this impacts the antimicrobial activity. Further exploration into modifying a vegan honea should also be conducted. The conversion of DHA to MGO and the increase of MGO over time within the samples was never determined. Therefore, this experiment should be repeated with mass spectrometer analysis on the selected days of testing to identify the changes in chemical composition over time, helping to fully elucidate if there is an increase in MGO and if that is the driving force for the improved antimicrobial activity, or if other currently unknown changes are occurring. The vegan honea experiments both with the addition of DHA or the glucose oxidase and invertase enzymes should also be further explored with a focus on eukaryotic cell cytotoxicity, as this would be a limiting factor in further developing this as a potential wound treatment. The vegan honea should also be explored for more microorganisms than *M. abscessus* due to the potential of reducing the reliance on antibiotics and the possibility of developing a targeted treatment for different microorganisms.

Chapter 5: Antimicrobial activity of 4 ruthenium complexes against a variety of pathogenic bacteria

5.1 Introduction

The ever present and evolving threat of antimicrobial resistance (AMR) has drastically diminished the amount of antibiotics that are available to treat microbial infections, and is estimated that AMR will cause 10 million deaths a year by 2050 (O' Neil, 2016; Pulingam *et al.*, 2022). As of 2020, there were only 42 new antimicrobial compounds in the clinical development pipeline. Out of these, 11 were of new structural classes, meaning the other 31 were either modified or derivatives of currently approved antibiotics (Frei, 2020). Therefore, the likelihood that bacteria will gain resistance to the majority of these compounds rather rapidly is highly likely, providing only a short term solution to the growing problem. A limitation and commonality shared between these new compounds is that they are organic molecules, based around carbon atoms, which limits the chemical variability (Frei *et al.*, 2020). This largely overlooks the possible potential of inorganic compounds, such as metal based compounds, that can achieve chemical structures that are impossible for organic molecules, due to the difference in valence between metal ions and carbon ions. Metal compounds and metallofragments can utilise 3 dimensional structural space that is impossible for organic based compounds, increasing the diversity of molecules that can be produced and their biologically relevant capabilities (Lovering, Bikker and Humblet, 2009; Morrison *et al.*, 2020). Furthermore, since these compounds would be of new chemical structure, not explored before, existing bacterial resistance mechanisms are unlikely to overcome the new antimicrobials, potentially extending the lifespan of these compounds. Additionally, some metals have been noted for their antimicrobial potential, highlighting further that metal based compounds provide a potential avenue for new antimicrobials.

5.1.1 Metal compounds and medicinal uses

Within the medical industry certain metals have been utilised for many years, such as copper and silver. Copper has historically been used to treat a plethora of infectious diseases, including syphilis caused by *Treponema pallidum*, impetigo caused by *Streptococcus pyogenes* and tubercular infections caused by *M. tuberculosis* (Grass, Rensing and Solioz, 2011). In more recent years it has been shown to be an effective antimicrobial against *Staphylococcus aureus* and *Escherichia coli* (Noyce, Michels and Keevil, 2006; Santo *et al.*, 2011). The use of cooper coated surfaces in high infectious areas, such as bathroom fixtures, door handles or bed rails has been implemented in hospital trials to help curb nosocomial infections. The change to copper surfaces in 3 separate hospitals showed a reduction in microbial contamination compared to non-copper surfaces (Casey *et al.*, 2010; Marais, Mehtar and Chalkley, 2010; Mikolay *et al.*, 2010). These studies resulted in the exploration of how copper can effectively inhibit microorganisms, with suggestions that dissolved copper ions are involved in the bactericidal activity (Grass, Rensing and Solioz, 2011). Furthermore, the mechanisms behind copper's antimicrobial activity have been attributed to causing cell membrane disruption, interfering with protein synthesis and damaging microbial DNA (Reyes-Jara *et al.*, 2016). However, the majority of applications are limited to surface killing of bacteria and not medical treatment, unlike silver.

Silver has been utilised within the medical industry for a variety of ailments, including wound antisepsis and burn wounds to prevent infection (Urnukhsaikhan *et al.*, 2021). Historically, silver filings were used as a blood purifier, to treat bad breath and for heart palpitations (Frei, 2020). However, the discovery of antibiotics slowed the use of silver as an antimicrobial, but some silver based compounds are still used, such as silver laced wound dressings, silver coated catheters and silver nitrate to treat abscesses and cysts (Mjos and Orvig, 2014). The mechanism of action behind silver's antimicrobial activity is due to the disruption of cellular bacterial processes, including metabolism, disulphide bond formation and iron homeostasis. These result in increased membrane permeability and the production of reactive oxygen species (ROS) (Morones-Ramirez *et al.*, 2013). However, it has been suggested that silver is not the most effective treatment option, and often non-silver based treatments are more effective and less expensive (Frei, 2020).

The antimicrobial activity of metals is not limited to copper and silver. Another metalloid compound, arsenic, was developed into the first treatment for syphilis called Arsphenamine and is now known as Salvarsan (Gasser, 2015). Other metal based compounds have also been further developed for use within medicine, such as mercury into an antiseptic and thiomersal as a vaccine preservative (Frei *et al.*, 2020). Gold has also been used for treating rheumatoid arthritis and various other metals have been developed for cancer therapies, including iron, gallium, palladium, bismuth and ruthenium due to their high toxicity to eukaryotic cells (Kean and Kean, 2008; Wu, Yang and Yan, 2019).

The most recent advances in metal based antimicrobial compounds has been through the repurposing of existing drugs. Ruthenium based complexes have been of particular interest for their anticancer properties, however the antimicrobial potential of ruthenium, first reported in the 1950s, has since become of interest once again (Dwyer *et al.*, 1952; Frei *et al.*, 2020). This is due to ruthenium being able to bind to proteins and nucleic acids either through intercalation or ligand exchange reactions (Flamme *et al.*, 2018). Ruthenium complexes can also be modified to improve or change their function, such as through the addition of an arene ring which alters the DNA binding, facilitating hydrogen bonding to guanine residues or hydrophobic interactions (Li, Collins and Keene, 2015). Modifications adding alkyl chains to dinuclear ruthenium complexes resulted in antibacterial activity when the chain reached a certain length (Li *et al.*, 2011). Interestingly, the longer alkyl chain resulted in improved cellular uptake due to an increase in lipophilic interactions (Li *et al.*, 2012). The antibacterial activity of the ruthenium complex was achieved through preferential binding to RNA, ultimately inhibiting protein synthesis (Li *et al.*, 2014). Other modifications to ruthenium complexes include the addition of the β-lactam antibiotic 6-aminopenicilinic acid to a ruthenocene complex. The resulting complex exhibited activity against *Staphylococcus epidermidis* and *Enterococcus faecalis*, but no activity was observed for methicillin resistant *S. aureus*, suggesting the ruthenocene was acting as a carrier for the β-lactam (Lewandowski *et al.*, 2018). Showing the variability that can be achieved with ruthenium based compounds.

Further to this, ruthenium based complexes can act as photosensitisers and have shown improved activity by utilising antimicrobial photodynamic therapy to generate ROS (Heinemann, Karges and Gasser, 2017). The generation of ROS, especially in close proximity to DNA, RNA, proteins or enzymes can cause damage, furthering the antimicrobial potential (Frei, 2020). This is of particular interest for topical application, where improved activity can be activated on site. Recent advances in endoscopes and fibre optics widen the scope of this, allowing for photodynamic therapy to be administered to most areas of the body, including the nasal cavity, urogenital tract and the lungs (Wainwright *et al.*, 2017). Initial developments into improving the antimicrobial activity of ruthenium based compounds utilised white light to improve the MICs against *S. aureus*, *P. aeruginosa* and *Candida albicans* (Donnelly *et al.*, 2007). More recent advances utilised photodynamic therapy to release the antibiotic isoniazid from a ruthenium complex. Upon the addition of light irradiation at 465 nm, the isoniazid antibiotic is released from the ruthenium complex, allowing for targeted antibiotic treatment (Smith *et al.*, 2016). The isoniazid-ruthenium complex was shown to be effective against *Mycobacterium smegmatis*, which shows a promising application for photodynamic therapy and a potential therapy for mycobacterial pulmonary infections.

Advances in the field have led to the development of a new range of molecules based around ruthenium, originally developed for anticancer therapy. The lack of toxicity to eukaryotic cells has facilitated exploration of the antimicrobial potential of these compounds, which will be examined across a range of bacterial pathogens.

5.1.2 Aims and Objectives

A total of 4 ruthenium based complexes have been developed and will be explored for antimicrobial activity. The 4 ruthenium complexes will be tested for antimicrobial activity against a variety of bacterial pathogens. This will be achieved by using the broth microdilution assay. Bactericidal activity will also be determined by regrowth on compound-free media.

5.2 Materials and Methods

5.2.1 Media Preparation

All chemicals and reagents were purchased from Sigma-Aldrich or Melford Laboratories UK, unless otherwise stated. For growth of *Mycobacterium abscessus* cultures, Middlebrook 7H9 broth and Middlebrook 7H11 agar were selected. The 7H9 broth was made by adding 2.35 g of 7H9 to 450 mL of distilled H_2O and supplemented with 4 mL of 50% glycerol before autoclaving (121 °C for 15 min). Once cooled, 1.25 mL of 20% (w/v) filter sterile Tween80 was added (final concentration of 0.055% (w/v)). The 7H11 agar was made by adding 10.25 g of 7H11 to 450 mL distilled H_2O and supplemented with 5 mL 50% glycerol before autoclaving (121 °C for 15 min).

The media selected for all other organisms was nutrient broth and nutrient agar. The nutrient broth was prepared by adding 25 g nutrient broth to 1 L distilled H_2O and autoclaved (121 °C for 15 min). The nutrient agar was prepared by adding 28 g nutrient agar to 1 L distilled H_2O and autoclaved (121 °C for 15 min).

5.2.2 Bacterial strains and culture

The *M. abscessus* strain used was the type strain NCTC 13031 (also called ATCC 19977) and was grown for 72 h in 7H9 broth before being stored in 25% (w/v) glycerol stock solutions and stored at -80 °C. Prior to testing, *M. abscessus* isolates were grown in 10 mL 7H9 broth, prepared as described in 5.2.1, for 72 h at 37 °C, depending on the experiment, with orbital shaking at 180 rpm.

The other strains used were *Escherichia coli* ATCC 11775, *E. coli* I469 ESBL, *E. coli* J53 2138E, *E.* coli J53 2140E, *Klebsiella pneumoniae* H467 KPC, *Proteus mirabilis* NCTC 8309, *Pseudomonas aeruginosa* ATCC 10145, *Salmonella enterica* serovar *Typhi and Staphylococcus aureus* ATCC 29213. All organisms were grown for 24 h in nutrient broth at 37 °C before being stored in 25 % (w/v) glycerol stock solutions and stored at -80 °C. Prior to testing, all strains were grown in 10 mL nutrient broth for 24 h at 37 °C with orbital shaking at 180 rpm.

5.2.3 Experimental metal compounds

The 4 metal compounds used within this study were developed at University of Bradford. The compounds were based around ruthenium, with some alterations (Figure 5.1).

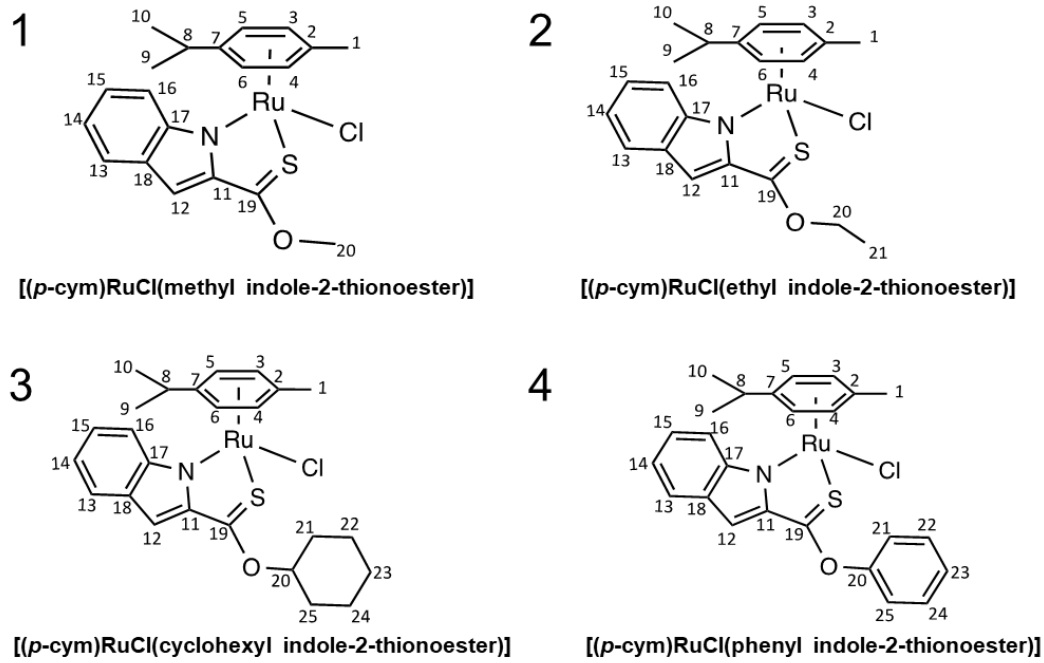

Figure 5.1 Ruthenium based metal compounds. 1) Complex 1 (HS08). 2) Complex 2 (HS07). 3) Complex 3 (HS09). 4) Complex 4 (HS06) (Nolan *et al.*, 2022b).

5.2.5 Broth microdilution assay

To determine the MIC and MBC of the metal compounds the broth microdilution assay was used. Initially, 10 mg/mL stocks of compound were made in dimethyl sulfoxide (DMSO). A master plate was set up by adding 100 µL DMSO to columns B to H and 1 to 4, leaving wells A1 to A4 empty. In column A, 200 µL of the corresponding compound was added. The compounds were serially diluted down the plate by mixing and removing 100 µL compound and adding to the next column. At column G, the excess 100 µL was discarded, leaving column H as a control row of DMSO only. From the master plate, 1 µL was removed from each well of the plate and added to the corresponding wells in the experimental plate (Figure 5.2). Experimental wells were conducted in triplicate and due to the highly coloured nature of the compounds, controls containing no bacteria were also completed in triplicate. All wells had 94 µL of either 7H9 broth for *M. abscessus* or nutrient broth for all other organisms. The plate was then inoculated down rows 1-3 and 7-9 with 5 µL of $OD_{600\ nm}$=0.1 bacterial culture. The control rows of 4-6 and 10-12 had an additional 5 µL of 7H9 broth. This resulted in the final concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.56 µg/mL and 0 µg/mL for each compound. Once the plates were prepared, OD reads at 570 nm using a spectrophotometric plate reader (Biotek EL808) were taken and plates incubated at 37 °C. For *M. abscessus* the plates were incubated for a total of 96 h,

with $OD_{570\ nm}$ reads every 24 h. For all other bacteria the plates were incubated for 24 h, with an initial plate read and final plate read. After the final plate read for all bacteria, each well was plated out on to solid media to observe bactericidal activity, using 10 µL aliquots and spotting onto 7H11 agar plates for *M. abscessus* or nutrient agar for all other bacteria. The 7H11 agar plates were incubated for a further 72 h at 37 °C and the nutrient agar plates were incubated for a further 24 h at 37 °C. The MBC was determined as the minimum concentration where no bacterial growth was visually observed (n=3).

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Complex 1** 100 µg/mL	**Complex 1** 100 µg/mL	**Complex 1** 100 µg/mL	**Complex 1** 100 µg/mL	**Complex 1** 100 µg/mL	**Complex 1** 100 µg/mL	**Complex 2** 100 µg/mL	**Complex 2** 100 µg/mL	**Complex 2** 100 µg/mL	**Complex 2** 100 µg/mL	**Complex 2** 100 µg/mL	**Complex 2** 100 µg/mL
B	**Complex 1** 50 µg/mL	**Complex 1** 50 µg/mL	**Complex 1** 50 µg/mL	**Complex 1** 50 µg/mL	**Complex 1** 50 µg/mL	**Complex 1** 50 µg/mL	**Complex 2** 50 µg/mL	**Complex 2** 50 µg/mL	**Complex 2** 50 µg/mL	**Complex 2** 50 µg/mL	**Complex 2** 50 µg/mL	**Complex 2** 50 µg/mL
C	**Complex 1** 25 µg/mL	**Complex 1** 25 µg/mL	**Complex 1** 25 µg/mL	**Complex 1** 25 µg/mL	**Complex 1** 25 µg/mL	**Complex 1** 25 µg/mL	**Complex 2** 25 µg/mL	**Complex 2** 25 µg/mL	**Complex 2** 25 µg/mL	**Complex 2** 25 µg/mL	**Complex 2** 25 µg/mL	**Complex 2** 25 µg/mL
D	**Complex 1** 12.5 µg/mL	**Complex 1** 12.5 µg/mL	**Complex 1** 12.5 µg/mL	**Complex 1** 12.5 µg/mL	**Complex 1** 12.5 µg/mL	**Complex 1** 12.5 µg/mL	**Complex 2** 12.5 µg/mL	**Complex 2** 12.5 µg/mL	**Complex 2** 12.5 µg/mL	**Complex 2** 12.5 µg/mL	**Complex 2** 12.5 µg/mL	**Complex 2** 12.5 µg/mL
E	**Complex 1** 6.25 µg/mL	**Complex 1** 6.25 µg/mL	**Complex 1** 6.25 µg/mL	**Complex 1** 6.25 µg/mL	**Complex 1** 6.25 µg/mL	**Complex 1** 6.25 µg/mL	**Complex 2** 6.25 µg/mL	**Complex 2** 6.25 µg/mL	**Complex 2** 6.25 µg/mL	**Complex 2** 6.25 µg/mL	**Complex 2** 6.25 µg/mL	**Complex 2** 6.25 µg/mL
F	**Complex 1** 3.125 µg/mL	**Complex 1** 3.125 µg/mL	**Complex 1** 3.125 µg/mL	**Complex 1** 3.125 µg/mL	**Complex 1** 3.125 µg/mL	**Complex 1** 3.125 µg/mL	**Complex 2** 3.125 µg/mL	**Complex 2** 3.125 µg/mL	**Complex 2** 3.125 µg/mL	**Complex 2** 3.125 µg/mL	**Complex 2** 3.125 µg/mL	**Complex 2** 3.125 µg/mL
G	**Complex 1** 1.56 µg/mL	**Complex 1** 1.56 µg/mL	**Complex 1** 1.56 µg/mL	**Complex 1** 1.56 µg/mL	**Complex 1** 1.56 µg/mL	**Complex 1** 1.56 µg/mL	**Complex 2** 1.56 µg/mL	**Complex 2** 1.56 µg/mL	**Complex 2** 1.56 µg/mL	**Complex 2** 1.56 µg/mL	**Complex 2** 1.56 µg/mL	**Complex 2** 1.56 µg/mL
H	**Bacteria Only**	**Bacteria Only**	**Bacteria Only**	**Broth Only**	**Broth Only**	**Broth Only**	**Bacteria Only**	**Bacteria Only**	**Bacteria Only**	**Broth Only**	**Broth Only**	**Broth Only**

Figure 5.2 Plate map of experimental compounds. The broth microdilution was prepared by adding 1 µL (w/v) of the chosen complex taken from a master plate and 94 µL of broth (7H9 for *M. abscessus* or nutrient broth for all other organisms). The plate was then inoculated with 5 µL OD adjusted culture in columns 1, 2, 3, 7, 8 and 9. Columns 4, 5, 6, 10, 11 and 12 were used as controls and therefore not inoculated.

5.2.8 Data processing and statistical analysis

All data collected were n=3 technical replicates and 2 biological replicates, the broth microdilution assays were processed in Microsoft Excel 2016 and subsequently analysed using GraphPad Prism 8. Prior to data analysis, blank control values were deducted from experimental values that were inoculated with bacteria. The data was then analysed for normal distribution using Shapiro-Wilk test and subsequently analysed using a One-Way ANOVA. A Dunnett's multiple comparisons analysis was also conducted. For each experiment conducted, the MIC and MBC were determined. The MIC was defined as the lowest concentration required to inhibit *M. abscessus* determined by an OD value of 0. The MBC was defined as the lowest concentration required for no visible growth after plating out onto solid media.

5.3 Results

Varying levels of antimicrobial activity were observed for all 4 complexes tested. The complex with the best antimicrobial activity was complex 3, having MICs and MBCs for almost all microorganisms tested. The complex that was least effective was complex 4, exhibiting activity against only one of the microorganisms tested.

5.3.1 Antimicrobial activity of complex 1

Complex 1 exhibited antimicrobial activity against 3 of the microorganisms tested, with MICs observed for *M. abscessus*, *S. enterica* ser. Typhi and *S. aureus* at 50 µg/mL or less (Table 5.1). All other microorganisms tested showed no inhibition of growth for the highest concentration tested of 100 µg/mL complex 1.

Table 5.1 MICs and MBCs for complex 1 against all microorganisms tested.

Organism	MIC (µg/mL)	MBC (µg/mL)
Acinetobacter baumannii NCTC 12156	>100	>100
Escherichia coli ATCC 11775	>100	>100
Escherichia coli I469 ESBL	>100	>100
Escherichia coli J53 2138E	>100	>100
Escherichia coli J53 2140E	>100	>100
Klebsiella pneumoniae H467 KPC	>100	>100
Mycobacterium abscessus NCTC 13031	50	>100
Proteus mirabilis NCTC 8309	>100	>100
Pseudomonas aueruginosa ATCC 10145	>100	>100
Salmonella enterica serovar Typhi	50	50
Staphylococcus aureus ATCC 29213	6.25	12.5

M. abscessus was inhibited by 50 µg/mL complex 1, and a reduction in growth could be observed for subsequent concentrations (Figure 5.3 A). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the control of *M. abscessus* only, identified a significant difference between all treatments, P=<0.0001 for all concentrations apart from 3.125 µg/mL, P=0.0064. This indicates that although complete inhibition of growth was not achieved at the lower concentrations, the growth of *M. abscessus* was still impacted by complex 1.

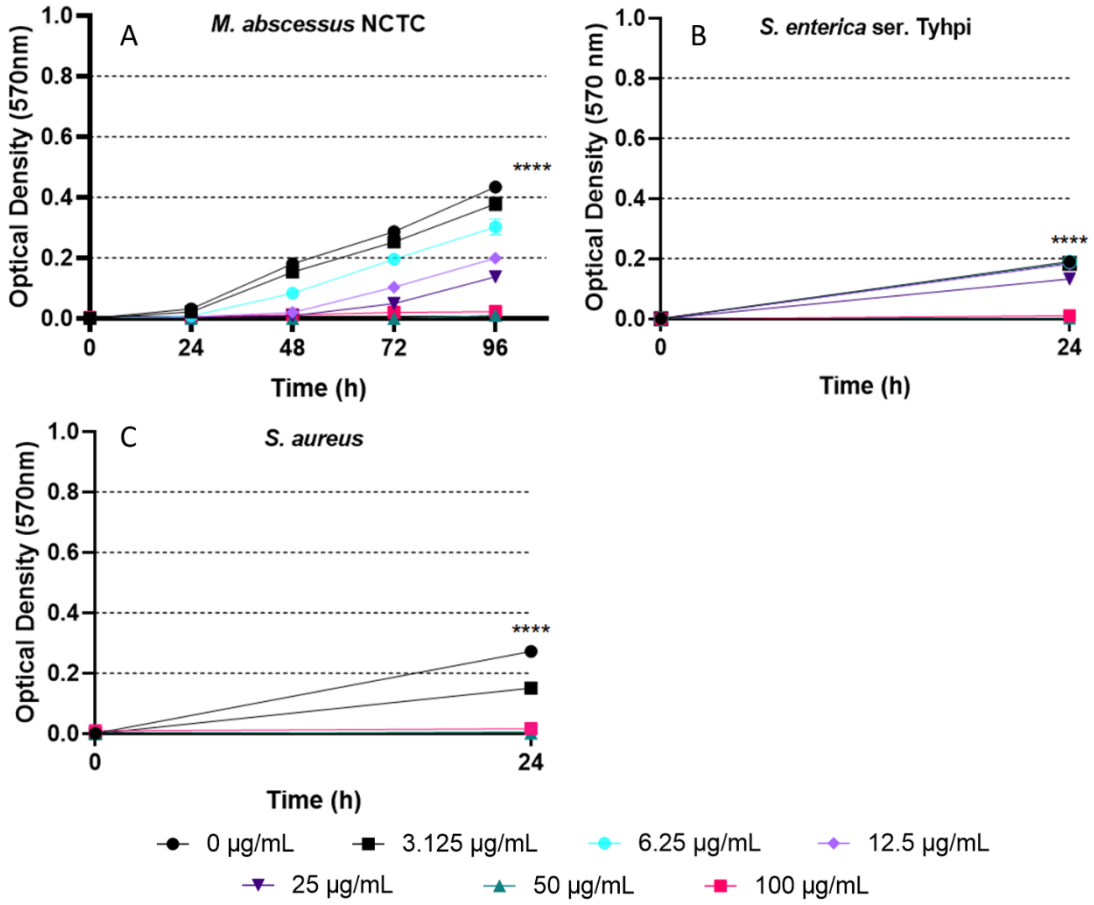

Figure 5.3 Growth curves of microorganisms inhibited by complex 1. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of complex 1 on the growth of *M. abscessus, S. enterica* ser. Typhi and *S. aureus*. A) Growth inhibition of *M. abscessus* was observed with 50 µg/mL complex 1. One-way ANOVA identified a significant difference between all treatments, P=<0.0001. B) Inhibition of *S. enterica* ser. Typhi was achieved with 50 µg/mL complex 1. One-way ANOVA showed a significant difference between all treatments, P=<0.0001. C) The growth of *S. aureus* was inhibited by 6.25 µg/mL complex 1. One-way ANOVA identified a significant difference between all treatments, P=<0.0001.

The MIC for *S. enterica* ser. Typhi was also 50 µg/mL, but subsequent concentrations had less impact on the growth (Figure 5.3 B). A one-way ANOVA identified a significant difference between the treatments, P=<0.0001. A Dunnett's multiple comparison was conducted, comparing all treatments to the growth of *S. enterica* ser. Typhi only. This identified a significant difference between 100 µg/mL, 50 µg/mL and 25 µg/mL, P=<0.0001. No significant difference was observed for the lower concentrations of 12.5 µg/mL, 6.25 µg/mL and 3.125 µg/mL, P=0.7956, P=9997 and P=0.9147 respectively. This indicates the lower concentrations had no impact on the growth of *S. enterica* ser. Typhi.

The lowest MIC of 6.25 µg/mL for complex 1 was observed for *S. aureus* (Figure 5.3 C). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001. A Dunnett's multiple comparison was conducted, comparing all treatments to *S. aureus* only, this identified a significant difference between all treatments, P=<0.0001 for all. This suggests that the growth of *S. aureus* exposed to complex 1 was significantly impacted, and although the concentration of 3.125 µg/mL did not cause inhibition of *S. aureus* it did impact the growth.

The bactericidal activity of complex 1 was varied between the 3 microorganisms that exhibited an MIC (Table 5.1). No bactericidal activity was observed for *M. abscessus* at the concentrations tested. Bactericidal activity was observed for *S. enterica* ser. Typhi at 50 µg/mL, which was consistent with the MIC. The MBC for *S. aureus* was higher than the MIC, but this was still considerably low compared to *S. enterica* ser. Typhi, being 12.5 µg/mL.

5.3.2 Antimicrobial activity of complex 2

Similar antimicrobial activity was observed for complex 2, with the majority of microorganisms tested remaining unaffected. MICs were observed for *E. coli* J53 2138E, *E. coli* J53 2140E, *M. abscessus*, *S. enterica* ser. Typhi and *S. aureus* (Table 5.2). A range of MIC concentration was also observed, with the highest MIC of 100 µg/mL and the lowest of 12.5 µg/mL.

Table 5.2 MICs and MBCs for complex 2 against all microorganisms tested.

Organism	MIC (µg/mL)	MBC (µg/mL)
Acinetobacter baumannii NCTC 12156	>100	>100
Escherichia coli ATCC 11775	>100	>100
Escherichia coli I469 ESBL	>100	>100
Escherichia coli J53 2138E	100	>100
Escherichia coli J53 2140E	100	>100
Klebsiella pneumoniae H467 KPC	>100	>100
Mycobacterium abscessus NCTC 13031	25	>100
Proteus mirabilis NCTC 8309	>100	>100
Pseudomonas aueruginosa ATCC 10145	>100	>100
Salmonella enterica serovar *Typhi*	25	25
Staphylococcus aureus ATCC 29213	12.5	12.5

The two strains of *E. coli* inhibited by complex 2 had the same MIC of 100 µg/mL but variations in response to the lower concentrations varied (Figure 5.4 A and B). *E. coli* J53 2138E showed a reduction in growth for 50 µg/mL compared to the control of no complex 2, and dose dependent variation in the subsequent concentrations was observed. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison was conducted, comparing all treatments to *E. coli* only. A significant difference was observed for 100 µg/mL and 50 µg/mL with a P value of P=<0.0001. A significant difference was also observed for 25 µg/mL and 12.5 µg/mL, P=0.0003 and P=0.0039 respectively. No difference was observed for 6.25 µg/mL and 3.125 µg/mL, P=0.2043 and P=0.1201 respectively. This suggests that some of the non-inhibitory concentrations of complex 2 still impacted the growth of the *E. coli* J53 2138E. Whereas, *E. coli* J53 2140E had less variation between the non-inhibitory concentrations of complex 2. A one-way ANOVA identified a significant difference between the treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *E. coli* only, showed a significant difference for 100 µg/mL only, P=0.0006. All other concentrations were not significant, suggesting they did not impact the growth of *E. coli* J53 2140E. This shows there was a difference in response between the two different strains of *E. coli*. In addition, no MBC was observed for any strain of *E. coli* for any concentration tested.

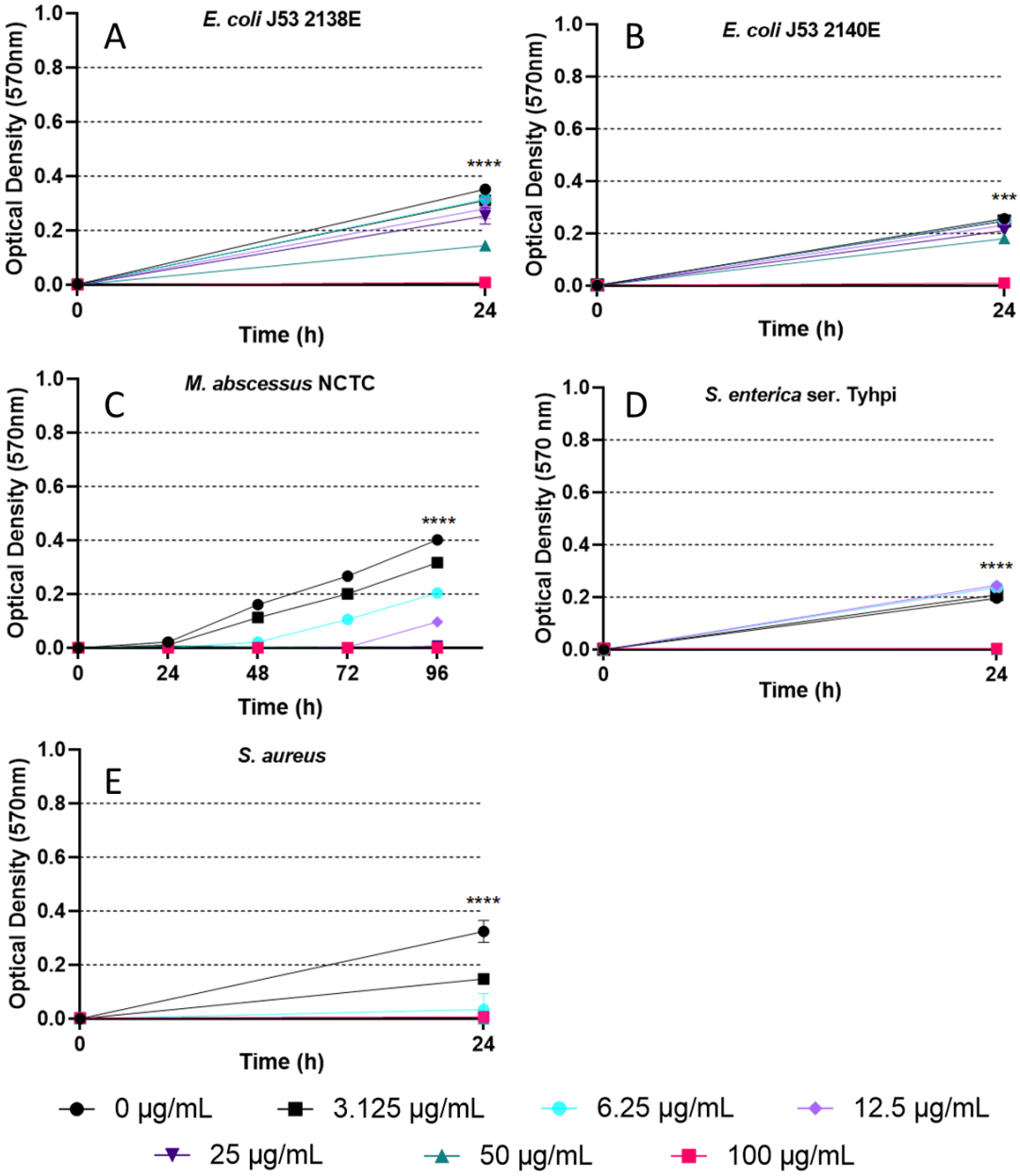

Figure 5.4 Growth curves of microorganisms inhibited by complex 2. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of complex 2 on the growth of *E. coli* J53 2138E, *E. coli* J53 2140E, *M. abscessus, S. enterica* ser. Typi and *S. aureus*. A) Growth curve of *E. coli* J53 2138E showing inhibition for 100 µg/mL complex 2. One-way ANOVA identified a significant difference between all treatments, P=<0.0001. B) The growth *E. coli* J53 2140E after exposure to complex 2, growth inhibition was observed at 100 µg/mL. One-way ANOVA identified a significant difference between all treatments, P=0.0001 C) The growth inhibition of *M. abscessus* by 25 µg/mL complex 2. A one-way ANOVA showed a significant difference between all treatments, P=<0.0001. D) *S. enterica* ser. Typhi growth inhibition by 25 µg/mL complex 2. One-way ANOVA identified a significant difference between the treatments, P=<0.0001. E) Inhibition of *S. aureus* by 12.5 µg/mL complex 2. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001.

M. abscessus had an MIC of 25 µg/mL for complex 2, and a dose dependent response for the subsequent concentrations tested (Figure 5.4 C). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *M. abscessus* only, showed a significant difference for all, P=<0.0001 for all. This shows that the non-inhibitory concentrations still impacted the growth of *M. abscessus*. Additionally, no MBC was observed for any concentration tested.

Complex 2 exhibited an MIC of 25 µg/mL against *S. enterica* ser. Typhi and the growth curve shows that lower concentrations of complex 2 had no impact on *S. enterica* ser. Typhi (Figure 5.4 D). A one-way ANOVA identified a significant difference between all concentrations, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *S. enterica* ser. Typhi only, showed a significant difference for all treatments, P=<0.0001, apart from 3.125 ug/mL, P=0.0777. However, the OD values for 12.5 µg/mL and 6.25 µg/mL were higher than that for *S. enterica* ser. Typhi only, so this difference was not due to any inhibition of growth. An MBC was observed at 25 µg/mL, which was consistent with the MIC.

The lowest MIC observed of 12.5 µg/mL in response to complex 2 was observed against *S. aureus* (Figure 5.4 E). A reduction in growth was observed for the lower concentrations tested of 6.25 µg/mL and 3.125 µg/mL compared to *S. aureus* only. A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *S. aureus* only, showed a significant difference for all treatments, P=<0.0001 for all. This suggests that the concentrations below the MIC had an impact on the growth of *S. aureus* but were not enough to completely inhibit growth. An MBC was also identified at 12.5 µg/mL, which was consistent with the MIC.

5.3.3 Antimicrobial activity of complex 3

Complex 3 had the best antimicrobial activity of all the complexes tested, having MICs for 8 out of the 11 microorganisms tested (Table 5.3). The microorganisms that were not inhibited were *K. pneumoniae*, *P. mirabilis* and *P. aeruginosa*. All other microorganisms tested were inhibited by 50 µg/mL or less. Complex 3 also had the lowest MIC observed for any of the complexes tested, inhibiting *S. aureus* with 3.125 µg/mL.

Table 5.3 MICs and MBCs for complex 3 against all microorganisms tested.

Organism	MIC (µg/mL)	MBC (µg/mL)
Acinetobacter baumannii NCTC 12156	25	50
Escherichia coli ATCC 11775	50	50
Escherichia coli I469 ESBL	50	50
Escherichia coli J53 2138E	25	25
Escherichia coli J53 2140E	25	25
Klebsiella pneumoniae H467 KPC	>100	>100
Mycobacterium abscessus NCTC 13031	12.5	>100
Proteus mirabilis NCTC 8309	>100	>100
Pseudomonas aueruginosa ATCC 10145	>100	>100
Salmonella enterica serovar *Typhi*	6.25	6.25
Staphylococcus aureus ATCC 29213	3.125	3.125

The MIC observed for *A. baumannii* was 25 µg/mL and the growth curve indicates concentrations below this had no impact on the growth of *A. baumannii* (Figure 5.5 A). A one-way ANOVA identified a significant difference for all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the growth of *A. baumannii* only, showed a significant difference for 100 µg/mL, 50 µg/mL and 25 µg/mL, P=<0.0001 for all. No difference was observed for 12.5 µg/mL, 6.25 µg/mL and 3.125 µg/mL, P=0.0915, P=0.6522 and P=0.3917, respectively. An MBC of 50 µg/mL was also observed.

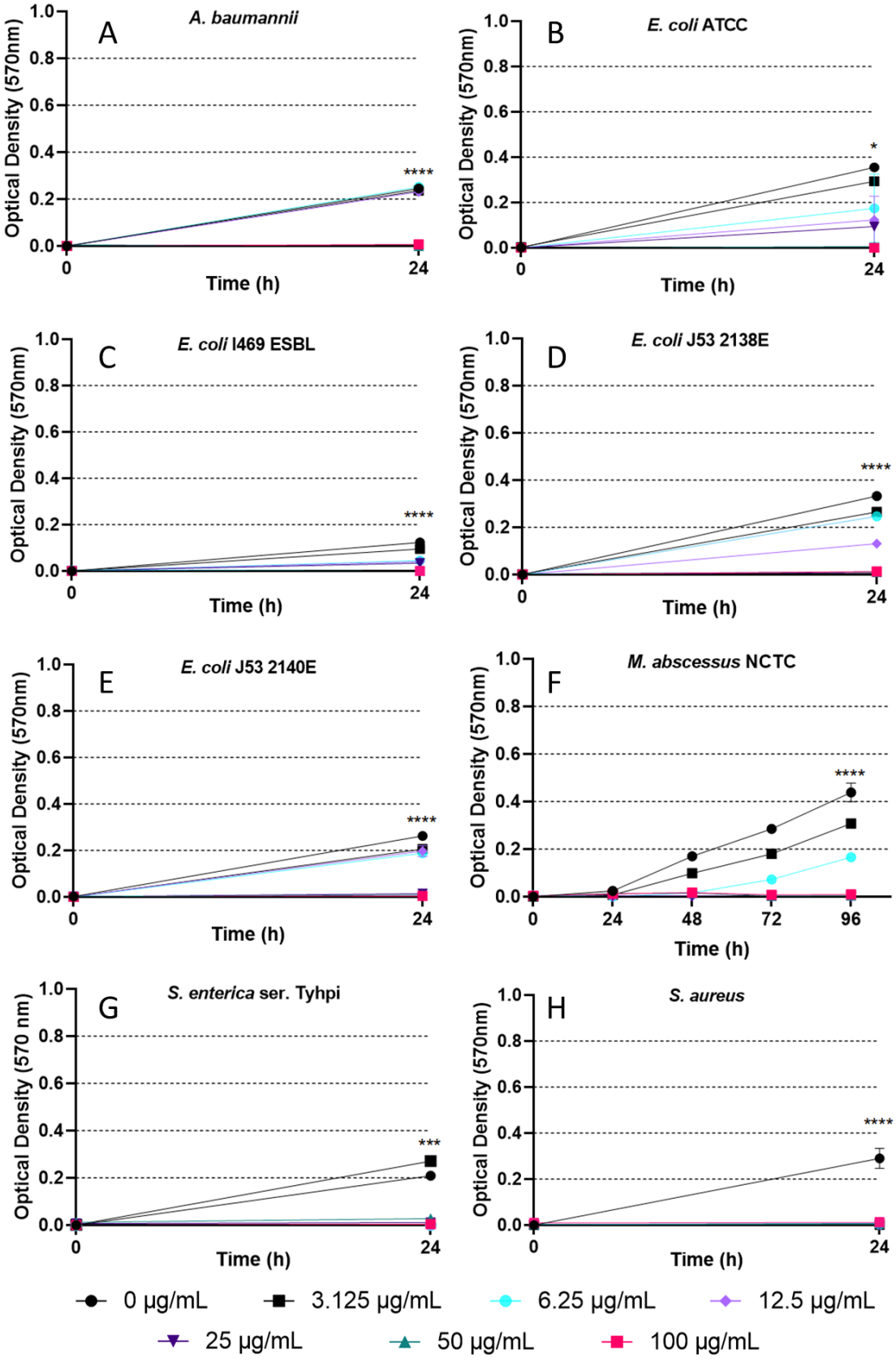

Figure 5.5 Growth curves of microorganisms inhibited by complex 3. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of complex 3 on the growth of *A. baumannii, E. coli* ATCC, *E. coli* I469 ESBL, *E. coli* J53 2138E, *E. coli* J53 2140E, *M. abscessus, S. enterica* ser. Typi and *S. aureus*. A) Growth curve of *A. baumannii* identifying an MIC of 25 µg/mL. A one-way ANOVA showed a significant difference

between all treatments, P=<0.0001. B) *E. coli* ATCC growth curve showing inhibition by 50 µg/mL complex 3. A Kruskal-Wallis identified a significant difference between the treatments, P=0.0118. C) Growth of *E. coli* I469 ESBL inhibited by 50 µg/mL. A one-way ANOVA showed a significant difference for all treatments, P=<0.0001. D) *E. coli* J53 2138E growth curve with an MIC of 25 µg/mL. One-way ANOVA identified a significant difference between the treatments, P=<0.0001. E) Growth inhibition of *E. coli* J53 2140E with 25 µg/mL complex 3. One-way ANOVA showed a significant difference between all treatments, P=<0.0001. F) Growth curve of *M. abscessus* showing an MIC of 12.5 µg/mL. One-way ANOVA identified a significant difference between all treatments, P=<0.0001. G) The growth of *S. enterica* ser. Typhi inhibited by 6.25 µg/mL complex 3. Kruskal-Wallis showed a significant difference between the treatments, P=0.0005. H) *S. aureus* showing inhibition for all concentrations of complex 3. A one-way ANOVA showed a significant difference for all treatments, P=<0.0001.

All 4 *E. coli* tested were inhibited by complex 3, but the MIC varied depending on strain. *E. coli* ATCC was inhibited by 50 µg/mL and a reduction in growth was observed for subsequent concentrations (Figure 5.5 B). A Kruskal-Wallis identified a significant difference between the treatments, P=0.0118. A Dunn's multiple comparison was conducted, comparing all treatments to *E. coli* ATCC only, and a significant difference was identified for 100 µg/mL and 50 µg/mL, P=0.0044 and P=0.0325, respectively. No difference was identified for any other concentrations. This suggests that concentrations below 50 µg/mL did not impact the growth of *E. coli* ATCC. For *E. coli* I469 ESBL the MIC was 50 µg/mL, with a reduction in growth observed for subsequent concentrations (Figure 5.5 C). A one-way ANOVA identified a significant difference between the concentrations, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *E. coli* I469 ESBL only, showed a significant difference for all treatments, P=<0.0001 for all apart from 3.125 µg/mL which was P=0.0010. This suggests that all concentrations of complex 3 impacted the growth. The MIC observed for *E. coli* J53 2138E was 25 µg/mL. The growth curve showed a reduction in growth for 12.5 µg/mL compared to the control and the 2 lowest concentrations showed a small reduction in growth compared to the control (Figure 5.5 D). A one-way ANOVA showed a significant difference for all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the growth of *E. coli* J53 2138E only, identified a significant difference for all treatments, P=<0.0001. This shows that non-inhibitory concentrations of complex 3 had a significant impact on this strain of *E. coli*. A similar occurrence was also observed for *E. coli* J53 2140E, with an MIC of 25 µg/mL and a reduction in growth for lower concentrations (Figure 5.5 E). A one-way ANOVA showed a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *E. coli* J53 2140E only, also showed a significant difference between all treatments, P=<0.0001 for all apart from 3.125 µg/mL P=0.0001. This also suggests that all concentrations of complex 3 tested impacted the growth of *E. coli*. Furthermore, all 4 of the *E. coli* strains had an MBC of 50 µg/mL in response to complex 3.

The MIC for complex 3 against *M. abscessus* was 12.5 µg/mL. The growth curve shows a reduction in growth was observed for exposure to 6.25 µg/mL and 3.125 µg/mL complex 3 when compared to the control (Figure 5.5 F). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *M. abscessus* only, showed a significant difference for all concentrations, P=<0.0001 for all. This shows that complex 3 impacted the growth of *M. abscessus* at subinhibitory concentrations. However, no MBC was observed at the concentrations tested.

The MIC observed for *S. enterica* ser. Typhi was 6.25 µg/mL complex 3 (Figure 5.5 G). A Kruskal-Wallis identified a significant difference between the treatments, P=0.0005. A Dunn's multiple comparison, comparing all treatments to *S. enterica* ser. Typhi only, showed significance for only 100 µg/mL and 6.25 µg/mL, P=0.0412 and P=0.0128 respectively. All other concentrations were identified as not significant. However, no growth was observed for any concentration above 3.125 µg/mL. The OD observed for some of the concentrations was not 0, this is due to the highly coloured nature of the compounds which could impact the Dunn's multiple comparison. Looking at the growth curve, all concentrations above 3.125 µg/mL had an impact on the growth of *S. enterica* ser. Typhi. An MBC of 6.25 µg/mL was observed for treatment with complex 3, which is consistent with the MIC.

The MIC observed for complex 3 against *S. aureus* was 3.125 µg/mL (Figure 5.5 H). A one-way ANOVA identified a significant difference between the treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to *S. aureus*, showed a significant difference for all concentrations of complex 3, P=<0.0001 for all. The MBC observed was the same as the MIC of 3.125 µg/mL complex 3.

5.3.4 Antimicrobial activity of complex 4

Only one microorganism was inhibited by complex 4, this was *S. aureus* (Table 5.4). The MIC observed for *S. aureus* was 12.5 µg/mL complex 4. The growth curve shows no growth for 12.5 µg/mL or higher of complex 4 and a reduction in growth for the lower concentrations of 6.25 µg/mL and 3.125 µg/mL (Figure 5.6). A one-way ANOVA identified a significant difference between all treatments, P=<0.0001. A Dunnett's multiple comparison, comparing all treatments to the control of *S. aureus* only, showed a significant difference between all treatments, P=<0.0001 for all. An MBC of 50 µg/mL was observed for complex 4 against *S. aureus*.

Table 5.4 MICs and MBCs for complex 4 against all microorganisms tested.

Organism	MIC (µg/mL)	MBC (µg/mL)
Acinetobacter baumannii NCTC 12156	>100	>100
Escherichia coli ATCC 11775	>100	>100
Escherichia coli I469 ESBL	>100	>100
Escherichia coli J53 2138E	>100	>100
Escherichia coli J53 2140E	>100	>100
Klebsiella pneumoniae H467 KPC	>100	>100
Mycobacterium abscessus NCTC 13031	>100	>100
Proteus mirabilis NCTC 8309	>100	>100
Pseudomonas aueruginosa ATCC 10145	>100	>100
Salmonella enterica serovar *Typhi*	>100	>100
Staphylococcus aureus ATCC 29213	12.5	50

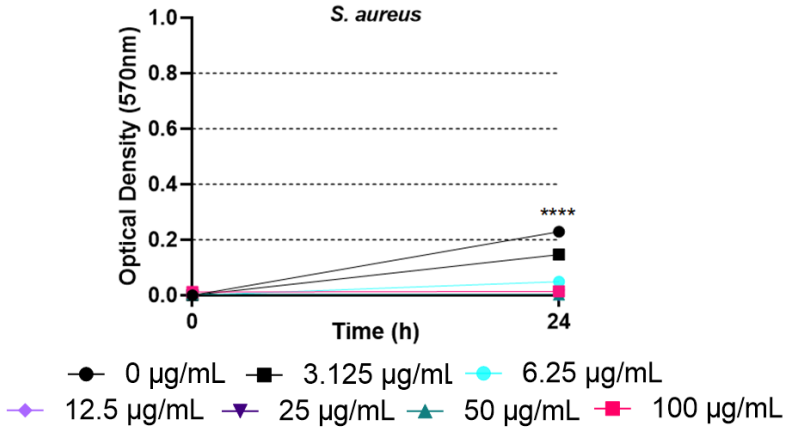

Figure 5.6 Growth curve for *S. aureus* treated with complex 4. Data shown are mean + SD for n=3 technical replicates. End point data, taken at 96 h, was used for statistical analysis to determine the impact of complex 4 on the growth of *S. aureus*. *S. aureus* was inhibited by 12.5 µg/mL complex 4. A one-way ANOVA identified a significant difference between the treatments, P=<0.0001.

5.4 Discussion

The 4 complexes explored had variation in antimicrobial activity depending on the microorganism tested. All 4 complexes were active against *S. aureus* and 3 of the complexes were active against *M. abscessus* and *S. enterica* ser. Typhi. Complex 3 (*p*-cym)RuCl(cyclohexyl indole-2-thionoester) exhibited the best antimicrobial activity, inhibiting the majority of microorganisms tested. The least effective complex was complex 4 (*p*-cym)RuCl(phenyl indole-2-thionoester), with activity only against *S. aureus*. Interestingly, no complex had bactericidal activity against *M. abscessus*, although growth inhibition was observed, indicating they were bacteriostatic for this microorganism. In addition, no activity was observed for *K. pneumoniae, P. mirabilis* or *P. aeruginosa* for any of the complexes tested.

The continued search for new antimicrobial compounds has resulted in the exploration of natural sources and the repurposing of other drugs. Initially, the ruthenium complexes used in this study were developed as anticancer drugs. However, low cytotoxicity to eukaryotic cells made them poor candidates for anticancer therapy, but good candidates for antimicrobial drug discovery. This is not uncommon for ruthenium complexes, most of which have been largely explored for anticancer treatments, and only recently regained interest for their antimicrobial potential. A large scale screen of multiple metal based complexes that were initially developed for other applications was conducted to explore the possibility of identifying new antimicrobials (Frei *et al.*, 2020). Among the potential candidates were 4 ruthenium based compounds that previously exhibited activity against cancer cells after photodynamic therapy. Antimicrobial activity was assessed and the compounds were found to be active against *A. baumannii, S. aureus* and 2 fungal species, *Candida albicans* and *Cryptococcus neoformans*. Interestingly, no activity was observed against *E. coli, K. pneumoniae* or *P. aeruginosa*, which is consistent with the findings of the ruthenium complexes used in this study. Another study exploring the activity of ruthenium based intercalators identified that the complexes were active against *B. subtilis* and *S. aureus* but no activity was found against *E. coli* (Bolhuis *et al.*, 2011). However, the compounds were shown to be effective against *S. aureus in vivo* using infected *Caenorhabditis elegans* nematode worms, showing promise for ruthenium based antimicrobial chemotherapy. Other ruthenium based complexes have exhibited antimicrobial activity against *S. aureus, E. faecalis, A. baumanni, E. coli* and *P. aeruginosa* (Smitten *et al.*, 2020). Although activity was observed for all microorganisms reported, the concentrations of some complexes required to inhibit the bacteria were significantly higher than the concentrations tested here. Typically, this was between 300 μM and 688 μM compared to the highest concentration of 216 μM (100 μg/mL) tested here, suggesting that the complexes used here might have exhibited antimicrobial activity against *K. pneumoniae* and *P. aeruginosa* if higher concentrations were explored. However, within a clinical setting, this would be unrealistic and unachievable,

therefore the concentrations explored were limited to 216 μM (100 μg/mL). Furthermore, due to the solubility of the complexes, higher concentrations would be difficult to achieve. Therefore, higher concentrations were not explored.

Another potential benefit of a ruthenium based complex is the lack of antimicrobial resistance gained towards them. After determining MICs of a ruthenium complex against *Streptococcus pyogenes*, the rate at which resistance was gained was explored. It was identified that after 25 passages only a minimal increase in MIC was observed, compared to a 10,000 fold increase for the same exposure with penicillin (Li, Collins and Keene, 2015). Another ruthenium complex tested against *S. aureus* showed only an 8 fold increase in MIC after 20 passages (Wang *et al.*, 2021). Demonstrating the potential of these compounds, likely due to the novel chemical structure.

The mechanism of action behind the ruthenium based complexes used in this work is an important area for future study. However, the ability of ruthenium based complexes to intercalate with DNA and RNA indicate that this disruption is a likely candidate (Flamme *et al.*, 2018). The generation of ROS after photodynamic therapy of ruthenium complexes also supports that DNA damage, along with damage to RNA, proteins and enzymes could be occurring (Frei, 2020). Furthermore, exploration into the antimicrobial action of other ruthenium complexes has suggested that due to the lipophilicity of ruthenium based complexes, cell membrane incorporation resulting in morphological changes could be the likely cause (Smitten *et al.*, 2019; Sur *et al.*, 2020). Further exploration will elucidate the exact mechanism of action behind these ruthenium complexes and should be included in further studies. Additionally, photodynamic therapy to enhance these complexes should also be conducted. Furthermore, the bacterial pathogens tested here are not exhaustive and these complexes should be examined against more microorganisms of growing AMR concern. Alongside this, further clinical testing should be conducted and exploration into animal model testing should be considered if these complexes are to be considered for future clinical use. Importantly, the broad spectrum of activity of these ruthenium complexes against a range of microorganisms, and low toxicity to eukaryotic cells, puts them in an encouraging position for future clinical use.

Chapter 6: General Discussion

6.1 Conclusions and Future Directions

The prevalence of NTM infections is increasing globally, with reported cases rising from 3.3% to 22.6% in the last 2 decades (Degiacomi *et al.*, 2019). One of the NTMs of concern is *M. abscessus,* which has recently been reported to spread from person-to-person, further exacerbating the situation (Bryant *et al.*, 2016). The highly drug resistant nature of mycobacteria drastically limits the treatment options available, and the current drug regimen prescribed is largely ineffective (Section 1.1.5 and 1.1.6). The lengthy course of multiple antibiotics results in many patients choosing not to complete the course of treatment, with severe side effects being listed as a main contributing factor. Ultimately, this means treatment outcomes are poor. Those most at risk of infection are immunocompromised individuals, such as those with cystic fibrosis or bronchiectasis, who are more susceptible to pulmonary infections. It is critical that new antimicrobials or novel treatment strategies are developed to combat these infections. Therefore, this thesis aimed to explore the antimycobacterial potential of manuka honey, a well-established antimicrobial, that has largely been overlooked for mycobacteria.

The antimicrobial potential of manuka honey has been well documented and explored for a variety of bacterial pathogens, but it was considered ineffective against mycobacterial species (Efem, 1988; Dunford *et al.*, 2000). However, in Chapter 2 it was demonstrated that manuka honey can be an effective antimicrobial against *M. abscessus* and clinical isolates, both inhibiting growth and exerting bactericidal action. Further investigations into both culture temperature and storage conditions identified that these factors can largely impact the efficacy and changes in these can actually improve both the MIC and MBC. The improved activity was observed when culturing *M. abscessus* at 37 °C, which is a physiologically relevant temperature and more beneficial in regard to clinical use of manuka honey treatment. Previously, it has been highlighted that honey dilutions should be made fresh and used rapidly for testing (Sherlock *et al.*, 2010; Kwakman *et al.*, 2011). However, in this study it has been shown that this is not the case. Improved activity was observed over time, with storage for 30 days at 4 °C being the most effective. This is also an optimum and achievable temperature for a clinical setting, meaning dilutions can be prepared and stored in refrigeration prior to administering. Furthermore, a recent systematic review has outlined antimicrobial resistance of bacterial isolates does not impact the efficacy of manuka honey (Nolan *et al.*, 2020). This also means that manuka honey can be administered prior to lengthy susceptibility testing that would need to be undertaken before prescribing patients traditional antibiotics. This conclusion is further supported by the clinical isolates used within this thesis, all of which were a variety of the subspecies, giving rise to a mixture of drug susceptibility phenotypes, and ultimately all of them were susceptible to manuka honey. Considering *M. abscessus* causes infections of skin and soft tissue, an effective treatment option would be the use of medical grade honey, specifically topical ointments, and is

something that should be explored. The use of a non-antibiotic based treatment can help to lessen the burden of antimicrobial resistance, whilst also improving wound healing by preventing infection, reducing inflammation and promoting re-epithelisation (Tashkandi, 2021).

Chapter 3 explored the possible synergistic effect of combining manuka honey with existing antibiotics as a therapy against mycobacteria. There have been several reports of synergistic relationships between honey and antibiotics against different bacterial pathogens, therefore the potential for combining antibiotics with manuka honey was promising (Jenkins and Cooper, 2012; Müller *et al.*, 2013). Up until now, this had not been demonstrated in mycobacteria. The 3 antibiotics selected were amikacin, tobramycin and azithromycin. These were selected because of their established use in the drug regimen for *M. abscessus*, as well as all 3 being approved and utilised in a nebulised form. Interestingly, amikacin was found to be synergistic with manuka honey, but the chemically similar tobramycin was not. Azithromycin also showed potential for synergy and exhibited a bacteriostatic effect against *M. abscessus* subsp. *bolletii*, which possess the inducible macrolide resistance gene. The potential for these combinations to be utilised in clinical practice is very promising and paves the way for improving antibiotic therapy. Considering that *M. abscessus* causes infections of skin and soft tissue, shows that medical grade honey can be administered alongside antibiotic treatment to vastly improve wound healing. This opens up the potential of manuka honey for the treatment of *M. abscessus* infections and more honey-antibiotic combinations should be examined as any advances improving current therapies are urgently required. Additionally, the concentrations of manuka honey required for improved activity were distinctly lower than the MIC, and typically 0.037 g/mL of fresh honey. The findings that premade manuka honey stored at 4 °C had improved activity, suggest that both the activity could be improved and the concentration lowered if the honey dilutions were stored in the optimal conditions prior to testing. This requires further exploration into the impacts this has on antimicrobial potential, by re-examining the synergistic relationship after manuka honey storage, and continuing this *in vitro* model into an *in vivo* study using mouse models. With the successful continuation *in vivo*, this could result in clinical use.

Given the efficacy of manuka honey against *M. abscessus* and the synergy it shows when used with antibiotics, an in depth exploration into the 3 main components of manuka honey was conducted in Chapter 4, to understand the mechanistic action behind the antimycobacterial activity of the honey. Importantly, at higher concentrations than observed in manuka honey, both MGO and hydrogen peroxide were inhibitory to *M. abscessus*. However, when tested in combination at lower concentrations, similar to those found in honey, a synergistic effect was not observed, indicating other factors influence the activity. The combination of MGO and hydrogen peroxide also did not account for the indirect mechanisms of honey, such as high sugar content, which could be the additional component

required for activity to be seen. The bee defensin-1 protein was also shown to have no activity alone against *M. abscessus* and was also not isolated from any manuka honey sample. It has been suggested that the presence of MGO deteriorates the bee defensin-1 protein, which could explain why none was identified (Majtan *et al.*, 2012). Furthermore, modified honey based solely around bee defensin-1 has shown higher concentrations are required for bacterial growth inhibition compared to manuka honey or other modified honey, suggesting its antimicrobial potential is enhanced by the other components (Nolan *et al.*, 2020). It is possible that bee defensin-1 could be active against *M. abscessus* if combined with the high sugar solution or the other 2 main components.

To further explore the combinatorial impact of the main components within manuka honey and determine if the indirect mechanisms alone were enough to cause inhibition of *M. abscessus*, a vegan honea alternative was explored. The vegan honea selected was void of any bee derived components, such as bee defensin-1, glucose oxidase and invertase, and also lacked the DHA as no manuka flower nectar was present. The precursors for MGO and hydrogen peroxide were therefore added and explored for any change in antimicrobial activity, to generate an antimicrobial synthetic honea. The conversion of DHA to MGO occurred over time and was observed by the improved antimicrobial activity. Interestingly, the storage temperature impacted the conversion, with 37 °C showing improved activity and 4 °C remaining similar to no conversion. Previously it has been suggested that additional components, such as the presence of specific amino acids can impact the antimicrobial activity, with arginine increasing DHA to MGO conversion and proline, alanine and serine increasing DHA consumption but not conversion to MGO (Grainger, Manley-harris, *et al.*, 2016). Additionally, inhibitory activity was originally observed for vegan honea with DHA, but over time the DHA alone also exhibited inhibition of *M. abscessus*. Bactericidal activity was also observed for the highest concentrations of vegan honea with DHA and DHA alone, indicating that a threshold bactericidal concentration of MGO had been converted, regardless of sugar content. This further supports that MGO within manuka honey is of significant importance for the inhibition of *M. abscessus*.

The generation of hydrogen peroxide within the vegan honea using glucose oxidase and invertase resulted in vastly improved antimicrobial activity, requiring 0.014 g/mL vegan honea for *M. abscessus* inhibition, compared to 0.476 g/mL of unmodified manuka honey (Section 2.3.2). This was achieved with only 0.014 U/mL of glucose oxidase and invertase with 0.014 g/mL vegan honea. Furthermore, the enzymes alone had no inhibitory activity against *M. abscessus*. This improved activity was comparable to that of Surgihoney, which was engineered to generate ROS (Dryden *et al.*, 2014). The concentration of hydrogen peroxide generated could not be quantified with the assay used but was considered to be higher than concentrations typically found in honey due to the increase in activity. This was further supported by concentrations of hydrogen peroxide tested alone, showing a concentration of

10 mM was required for inhibition of *M. abscessus*, indicating *M. abscessus* has increased tolerance for hydrogen peroxide. Upon further exploration, this tolerance of hydrogen peroxide is most likely due to the presence of a KatG gene, encoding a catalase-peroxidase enzyme, initially identified in *M. tuberculosis*. The KatG gene infers resistance to isoniazid in *M. tuberculosis* and is a main detoxifier of hydrogen peroxide (Manca *et al.*, 1999). A comparison of $KatG^{Mtb}$ and $KatG^{Mabs}$ shows a 72% similarity in gene sequence, suggesting that this is likely the reason for requiring such high concentrations of hydrogen peroxide for an inhibitory affect to be observed (Gagliardi *et al.*, 2020). Further indicating MGO being the driving force behind manuka honey's inhibitory activity against *M. abscessus*. Although cytotoxicity studies were not conducted, the generation of antimicrobial activity upon the addition of either DHA or the combination of glucose oxidase and invertase is very exciting and shows the potential for further developing antimicrobial therapies based on honey. Furthermore, the addition of these components is relatively inexpensive, and would be a cheaper alternative to manuka honey. There is also potential to further develop this into a targeted honey with improved antimicrobial activity for specific bacterial pathogens, as well as synergistic interactions with antibiotics, making this modified vegan honea a very exciting future therapeutic.

A different aspect of determining new antimicrobials was explored in Chapter 5, by testing 4 ruthenium based complexes against a variety of bacterial pathogens. The exploration into metal based compounds has gained interest in recent years due to the chemical variability that can be achieved. The 4 complexes tested showed varying antimicrobial activity with complex 3 exhibiting the best antimicrobial action, inhibiting 8 out of 11 bacterial strains tested, with typically low MICs, ranging from 3.125 µg/mL to 50 µg/mL. Interestingly, not all bacterial strains tested were inhibited by the complexes and *K. pneumoniae, P. aeruginosa* and *P. mirabilis* were unaffected by any of the complexes tested. Additionally, the mechanism of action of these complexes was not determined in this study but should be included in any further research. Additionally, photodynamic therapy should also be explored due to the increased potential observed by other ruthenium based complexes. Further testing should also be extended to more bacterial pathogens of increasing concern, to better understand the spectrum of activity of these complexes. Regardless, these complexes show great potential by inhibiting a variety of bacterial pathogens.

The current treatments for *M. abscessus* pulmonary infections are inadequate, requiring lengthy antibiotic regimens that result in severe side effects for patients. The highly drug resistant nature of *M. abscessus* and lack of new antibiotics means alternative treatment strategies are urgently required. The lack of exploration into manuka honey for these infections has overlooked a large area of antimicrobial potential. By exploring the antimycobacterial activity of manuka honey, and its synergistic activity with amikacin, a new treatment strategy has been identified. Nebulised honey has previously been used to alleviate the symptoms of asthma and is therefore a realistic clinical option. By combining manuka honey and amikacin in a nebulised form, the concentration of amikacin required can be lowered, lessening the associated side effects and improving the efficacy of treatment. Furthermore, the potential for developing a more potent and cheaper modified honea could further improve the efficacy of this therapy. This could also be utilised for skin and soft tissue infections caused by *M. abscessus*. Ultimately, treatment options could be largely improved with the implementation of manuka honey treatment and further development of modified honea.

7.1 Reference List

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Appendices

8.1 Appendices

8.1.1 Appendices for clinical isolate susceptibility data

Data for clinical isolate antibiotic susceptibility, obtained from Thesis (Rose Lopeman, 2021). All data was collected by Rose Lopeman and conducted at 30 °C culture temperature.

Table 8.1 Antibiotic susceptibility of the clinical isolates used within this thesis, taken from Lopeman, 2021.

	Antibiotics (µg/mL)							
Isolate ID	IMI	AMI	TIG	LIN	CLOF	CFX	MIN	MOX
NCTC	2	16	1	64	>4	32	16	8
DC088 A	4	64	>4	>64	>4	64	32	32
DC088 B	4	>64	2	>64	>4	32	32	2
DC088 C	8	>64	>4	>64	>4	32	>32	8
DC088 D	4	16	1	32	>4	64	8	4
DC088 E	2	4	0.5	64	>4	32	32	4
DC088 Ref	4	64	0.5	>64	>4	32	32	2
211666	4	4	>4	>64	>4	16	8	8
137071	2	2	0.25	>64	>4	16	16	16
199277	4	>128	4	>64	>4	16	>16	>16
194891	4	>128	1	>64	>4	16	8	16
159544	4	16	>4	>64	>4	16	16	>16
186433	2	8	2	64	>4	16	16	16
186144	8	16	0.5	ND	>4	16	ND	ND
186154	16	4	0.25	>64	>4	32	8	16
147028	4	16	>4	>64	>4	16	>16	16
GOSH1BAL2	32	>128	>4	64	>4	>64	>16	>16
GOSH2	32	4	>4	>64	>4	>64	8	8
GOSH3	8	>128	>4	>64	>4	8	16	16
GOSH4	16	16	4	>64	>4	>64	>16	16
GOSH5	4	2	4	16	>4	>64	2	2
GOSH6	8	32	4	>64	>4	>64	8	8
GOSH7	8	8	>4	>64	>4	>64	16	16
GOSH8	>32	2	4	>64	>4	>64	16	8
BCH1	4	>128	4	>64	>4	>64	8	8

8.1.2 Graphical data for all clinical isolates for the efficacy of manuka honey cultured at 30 °C

All graphical data for the *M. abscessus* clinical isolates tested against the 4 manuka honey samples and cultured at 30 °C.

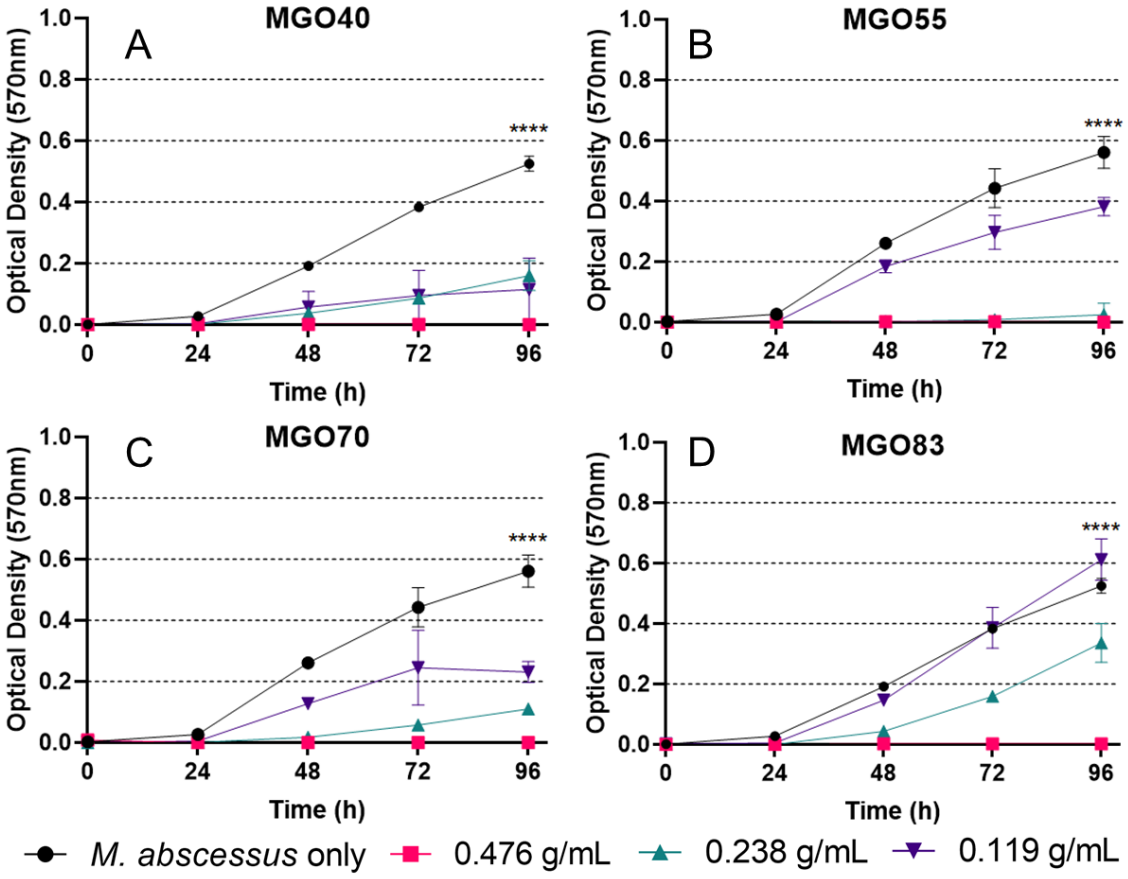

Figure 8.1 Growth Curves of *M. abscessus* 137071, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 137071 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

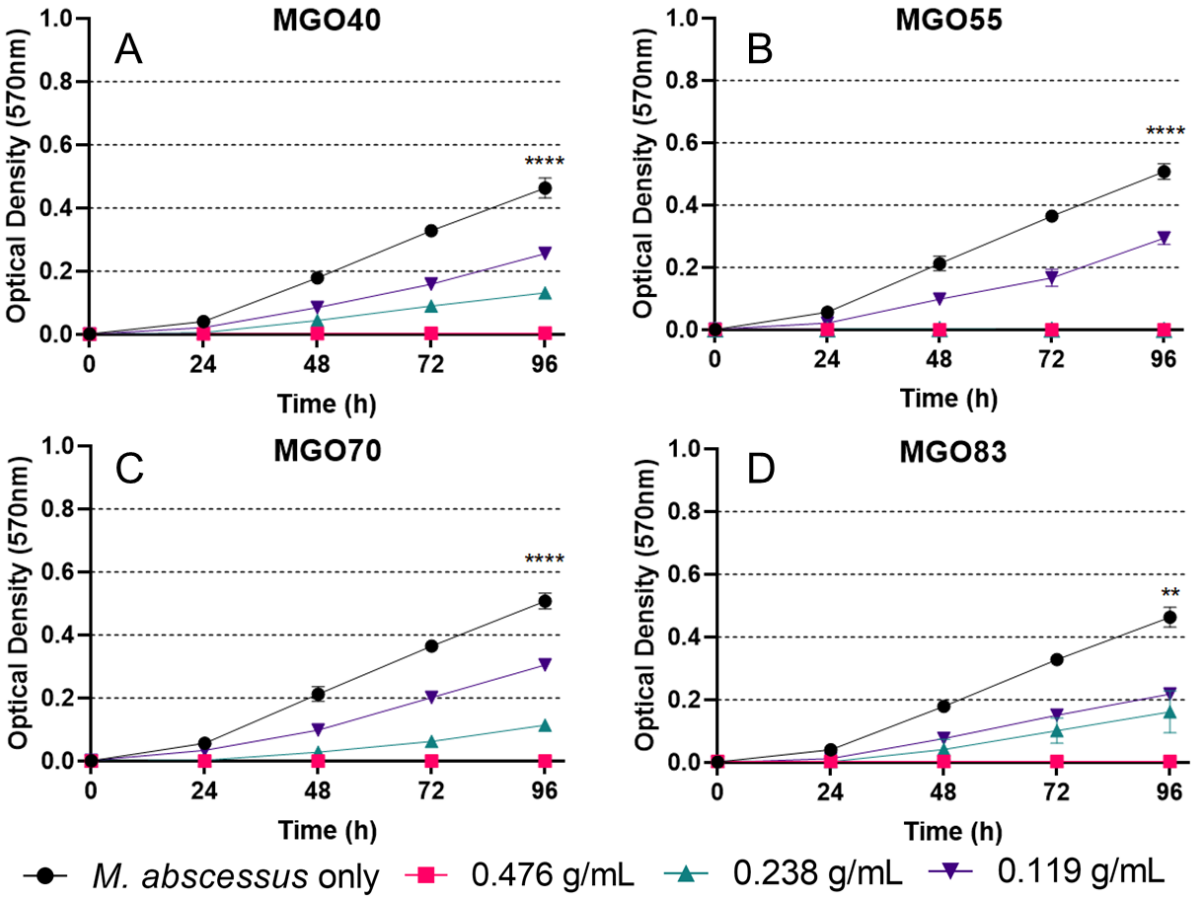

Figure 8.2 Growth Curves of *M. abscessus* 147028, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 147028 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, Kruskal-Wallis P=0.0014.

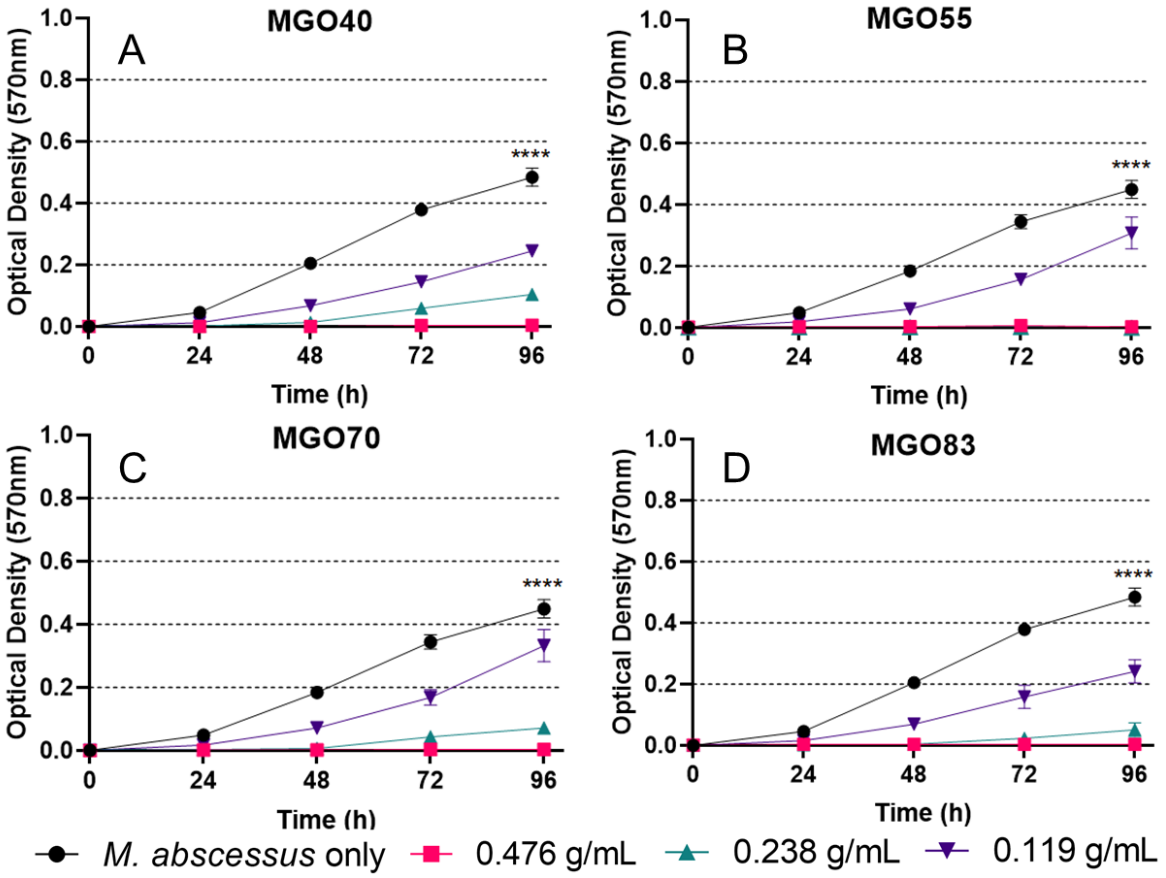

Figure 8.3 Growth Curves of *M. abscessus* 159544, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 159544 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

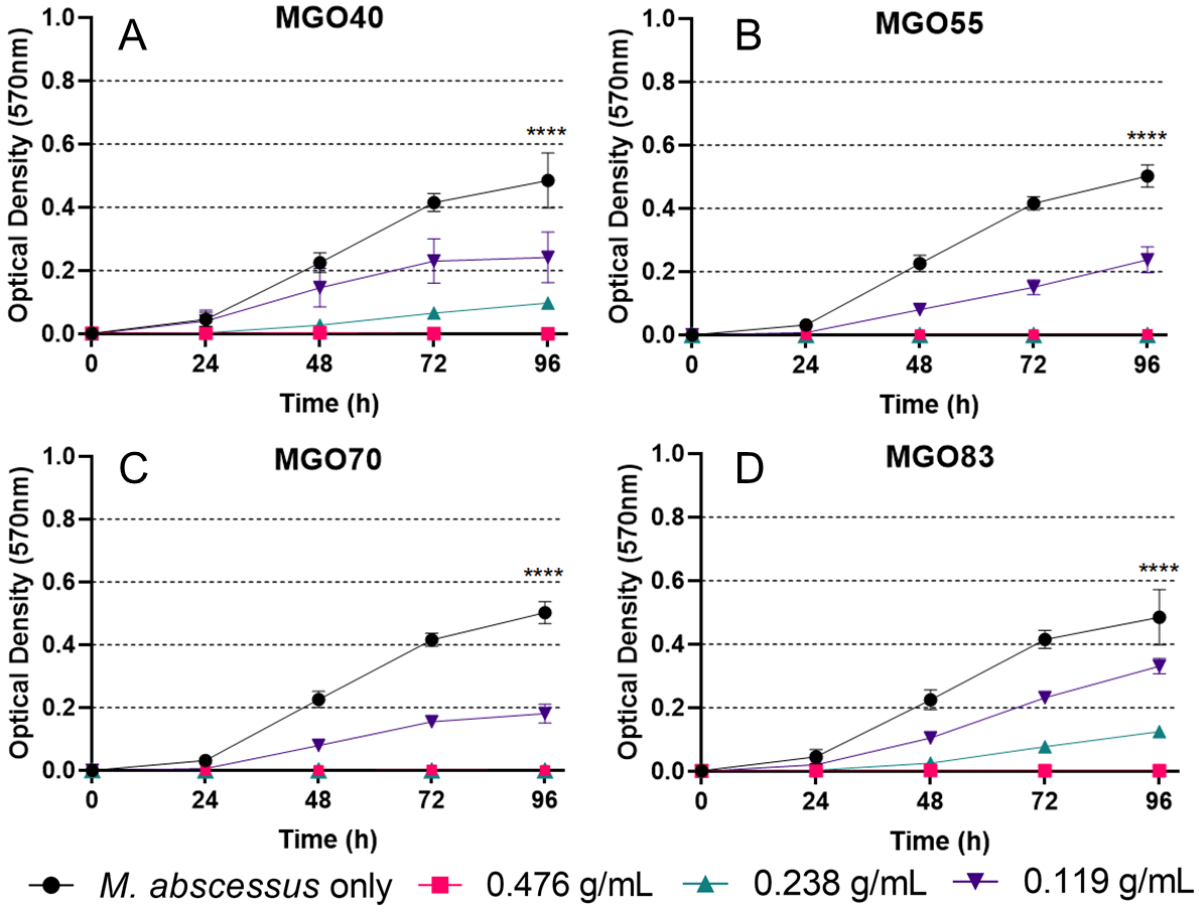

Figure 8.4 Growth Curves of *M. abscessus* 186144, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186144 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.238 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

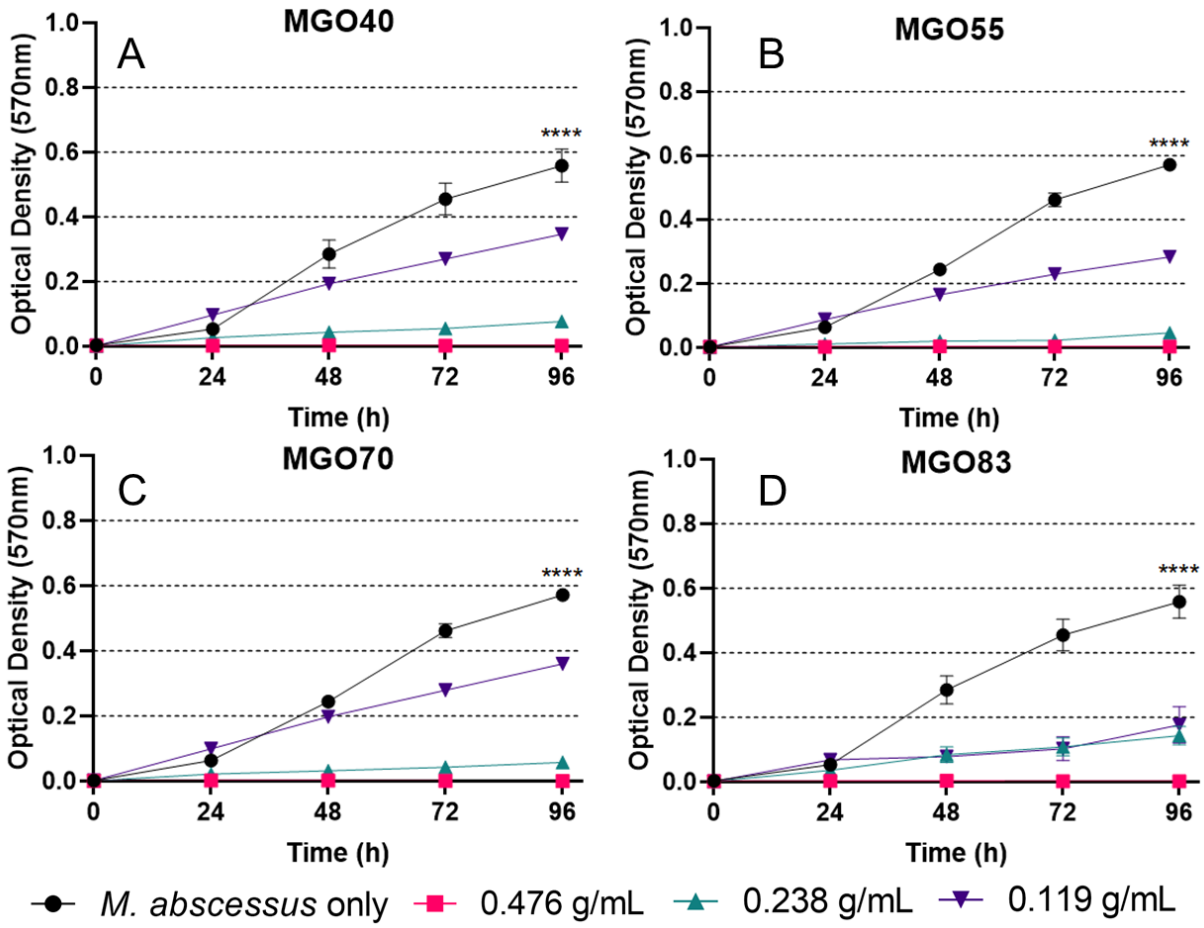

Figure 8.5 Growth Curves of *M. abscessus* 186154, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186154 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

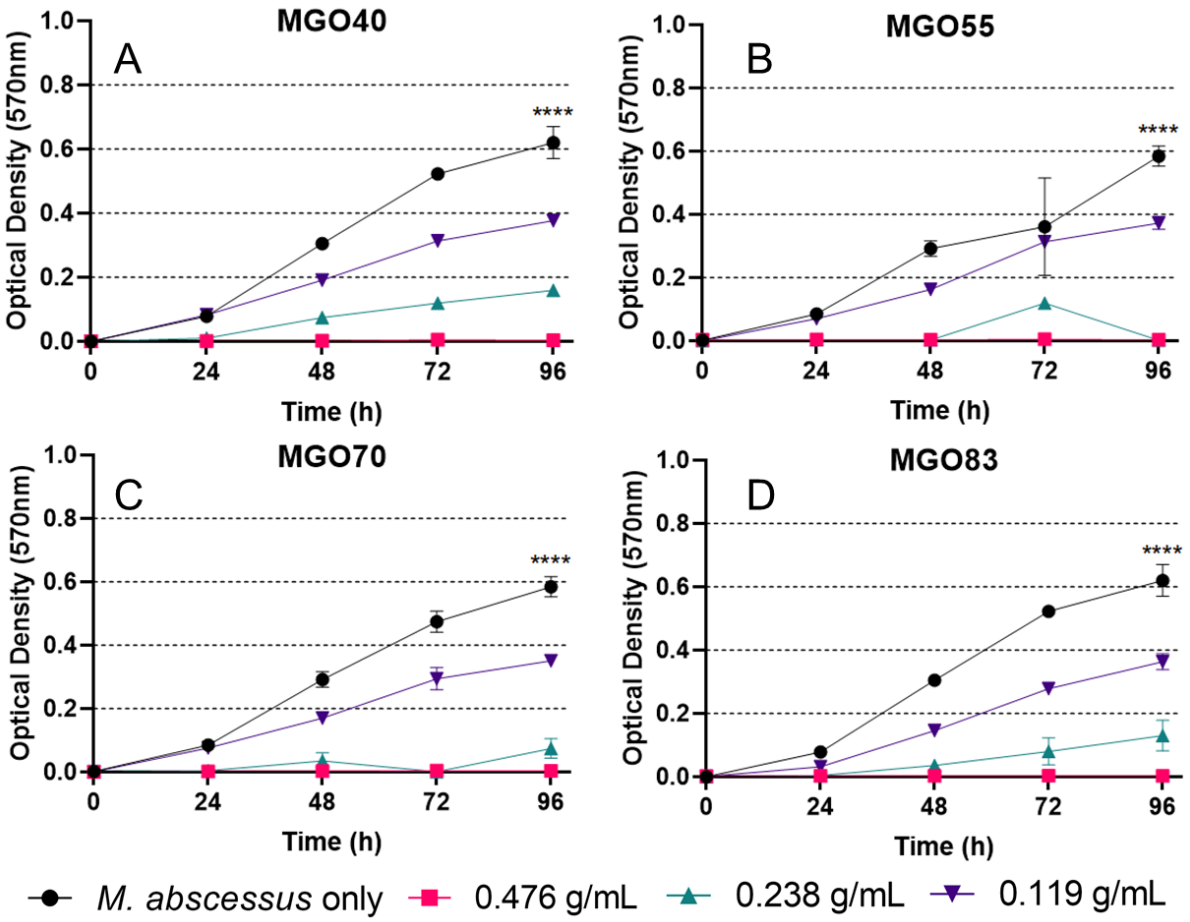

Figure 8.6 Growth Curves of *M. abscessus* 186433, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186433 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

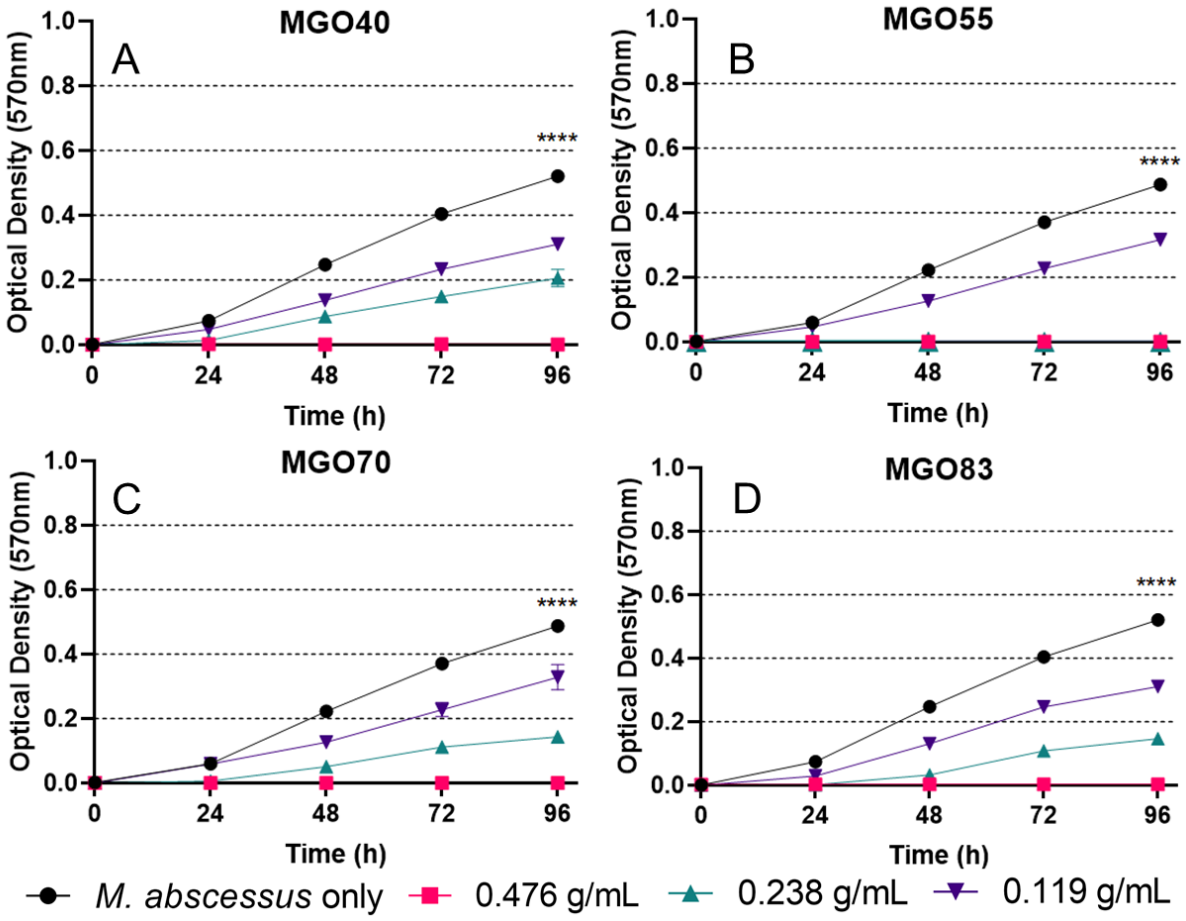

Figure 8.7 Growth Curves of *M. abscessus* 189961, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 189961 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

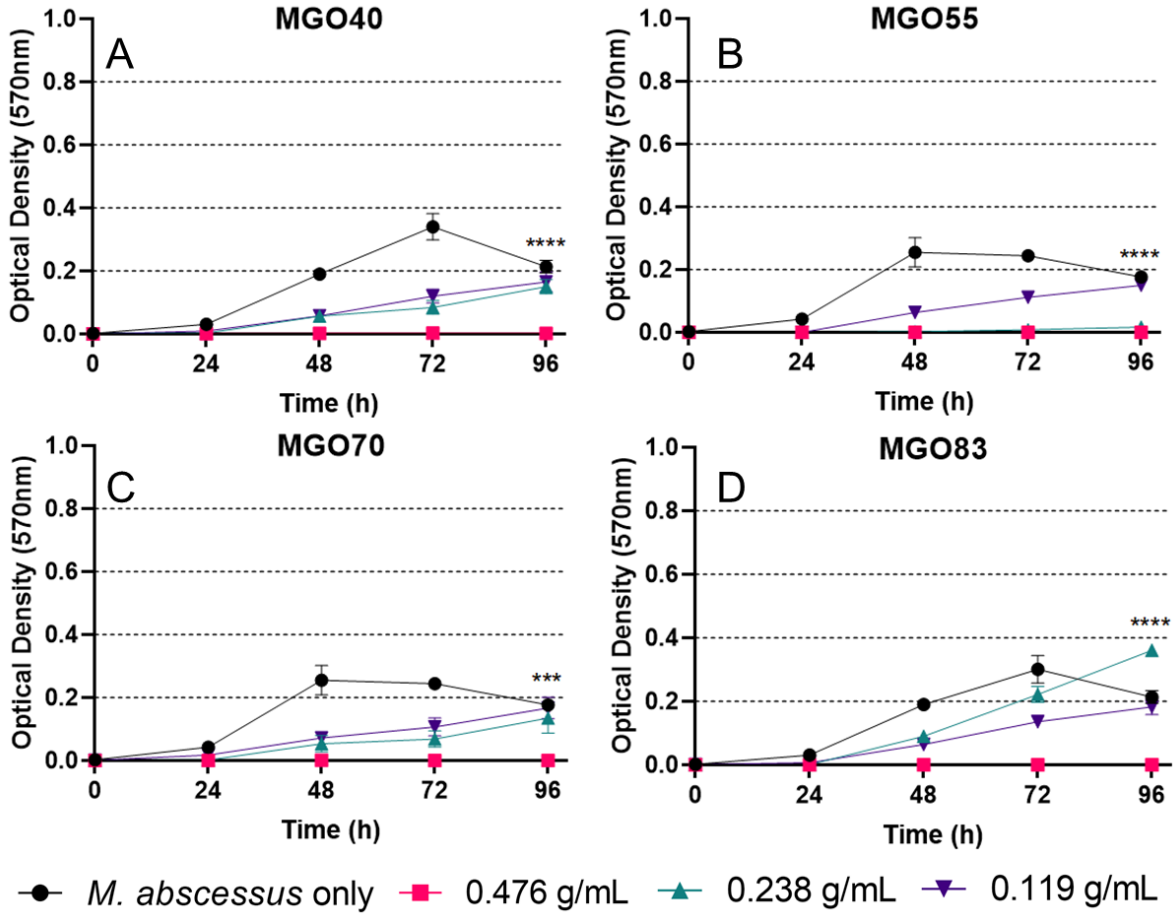

Figure 8.8 Growth Curves of *M. abscessus* 194891, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 194891 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=0.0003. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

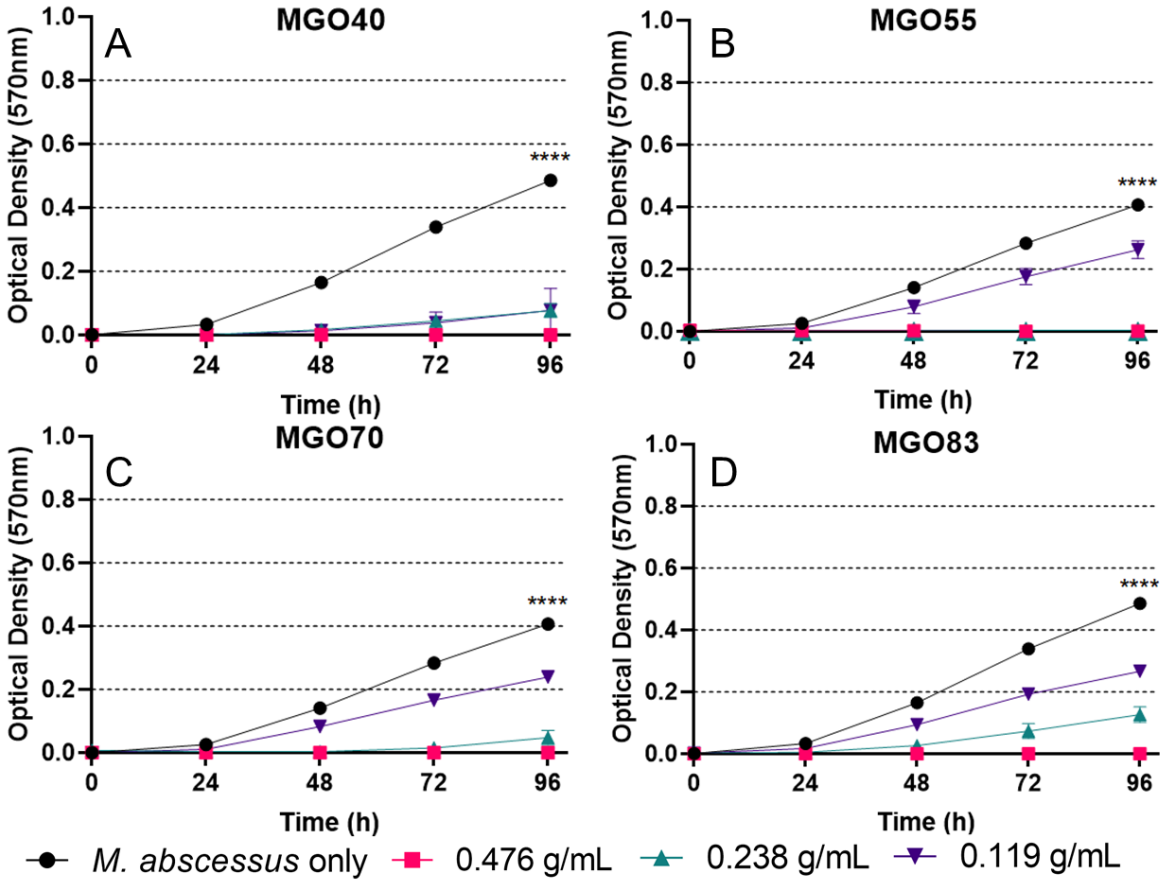

Figure 8.9 Growth Curves of *M. abscessus* 199277, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 199277 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

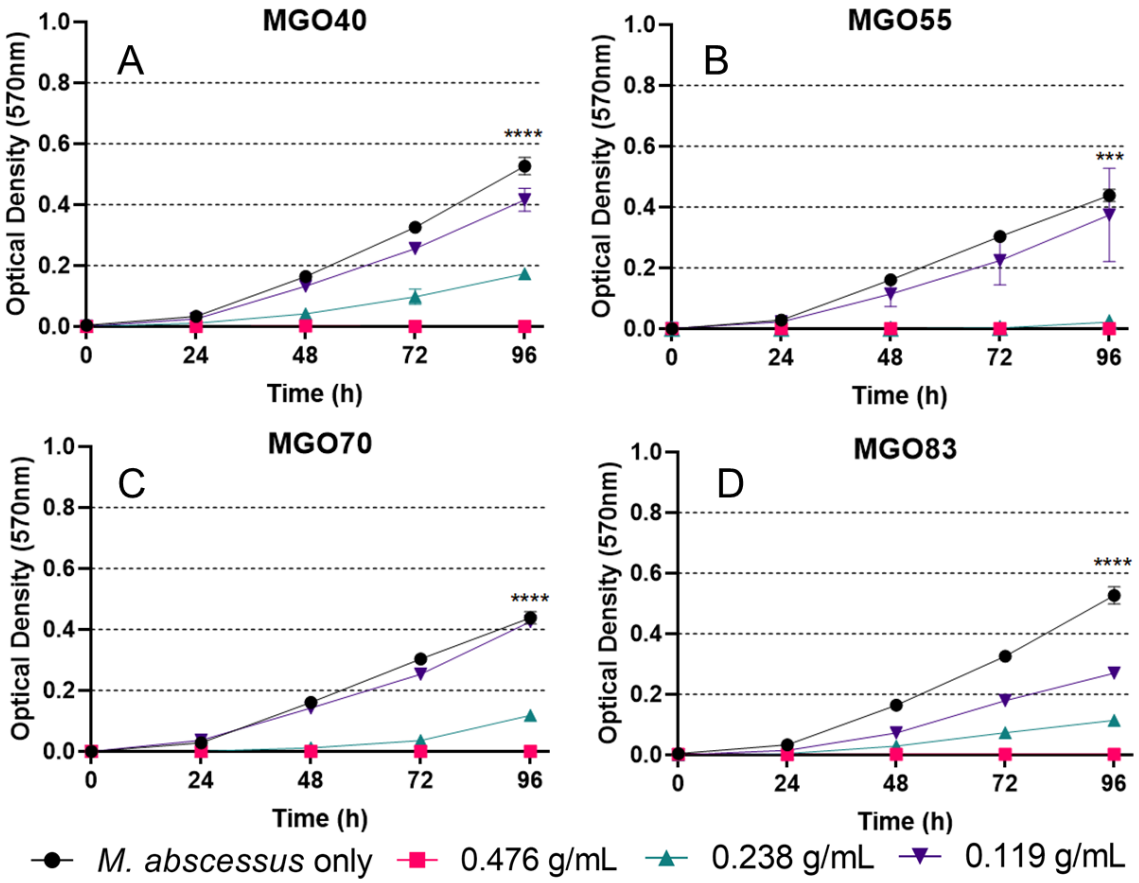

Figure 8.10 Growth Curves of *M. abscessus* 211666, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 211666 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=0.0002. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

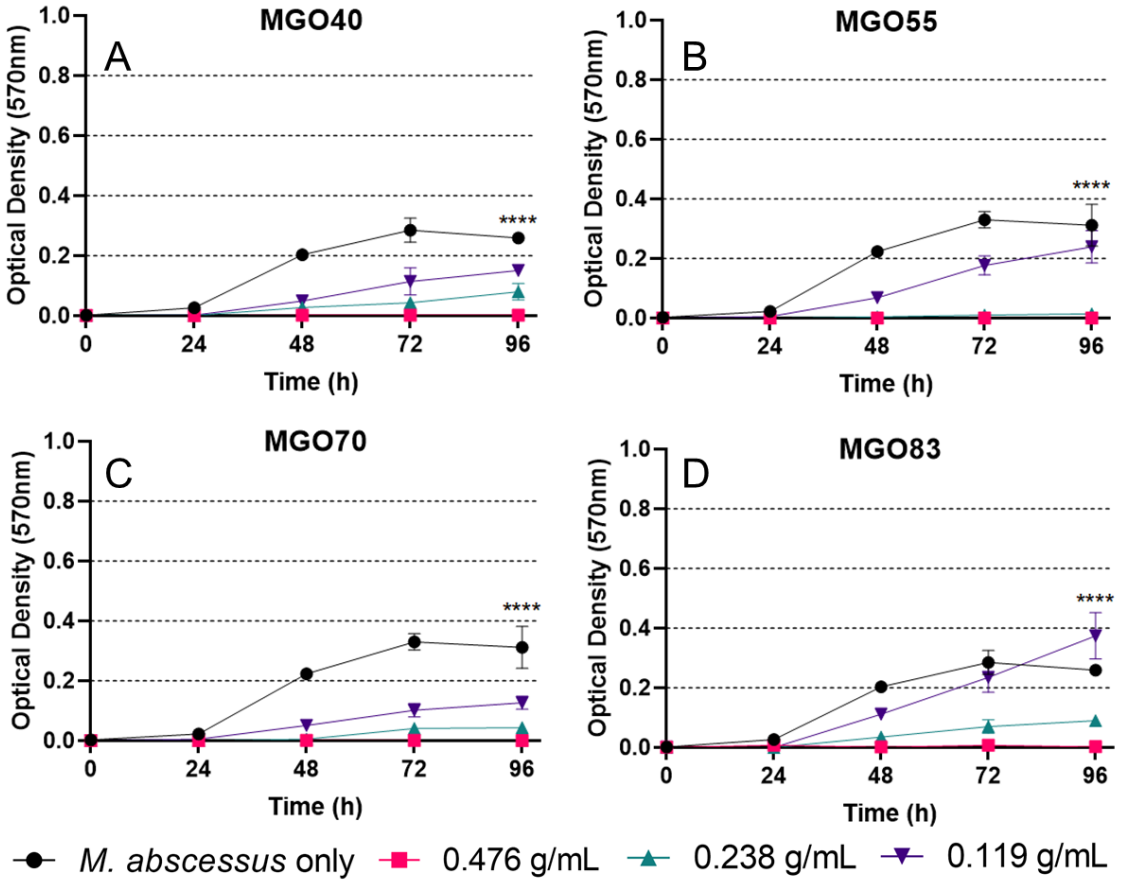

Figure 8.11 Growth Curves of *M. abscessus* DC088A, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088A at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

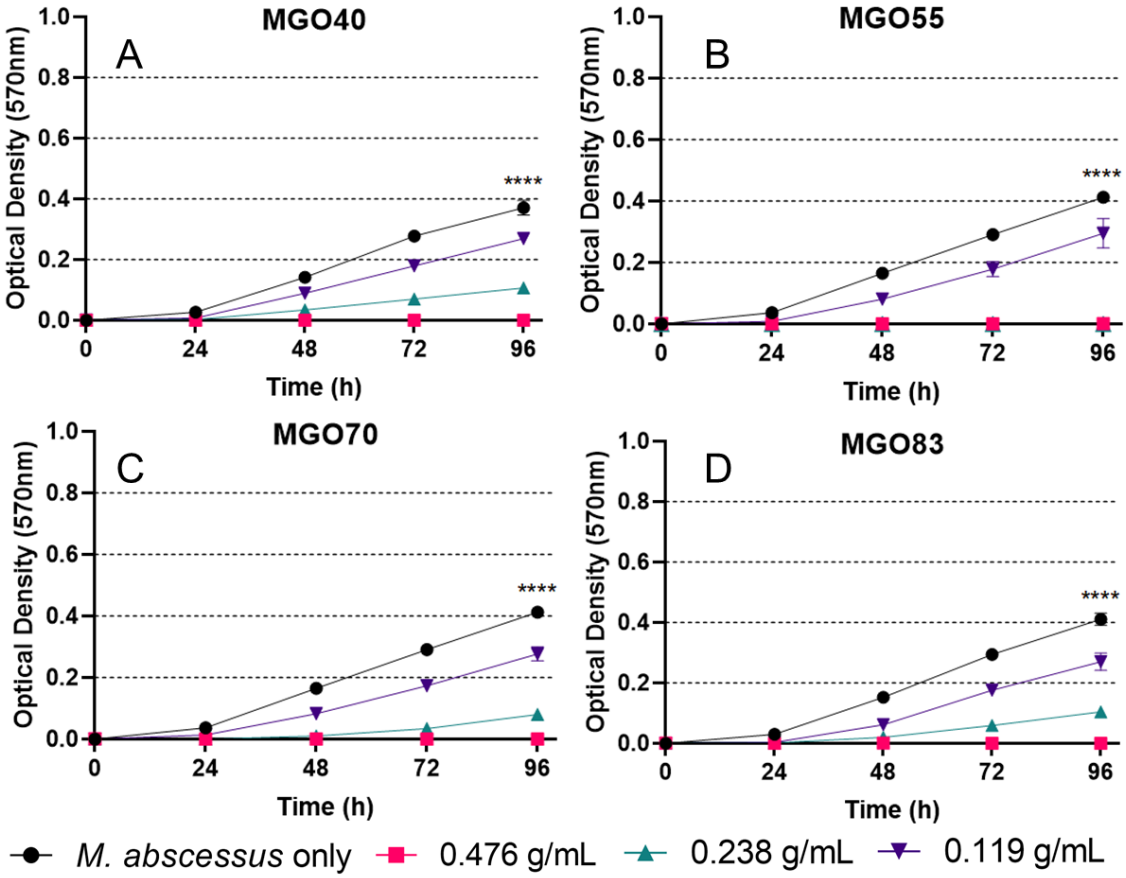

Figure 8.12 Growth Curves of *M. abscessus* DC088B, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088B at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

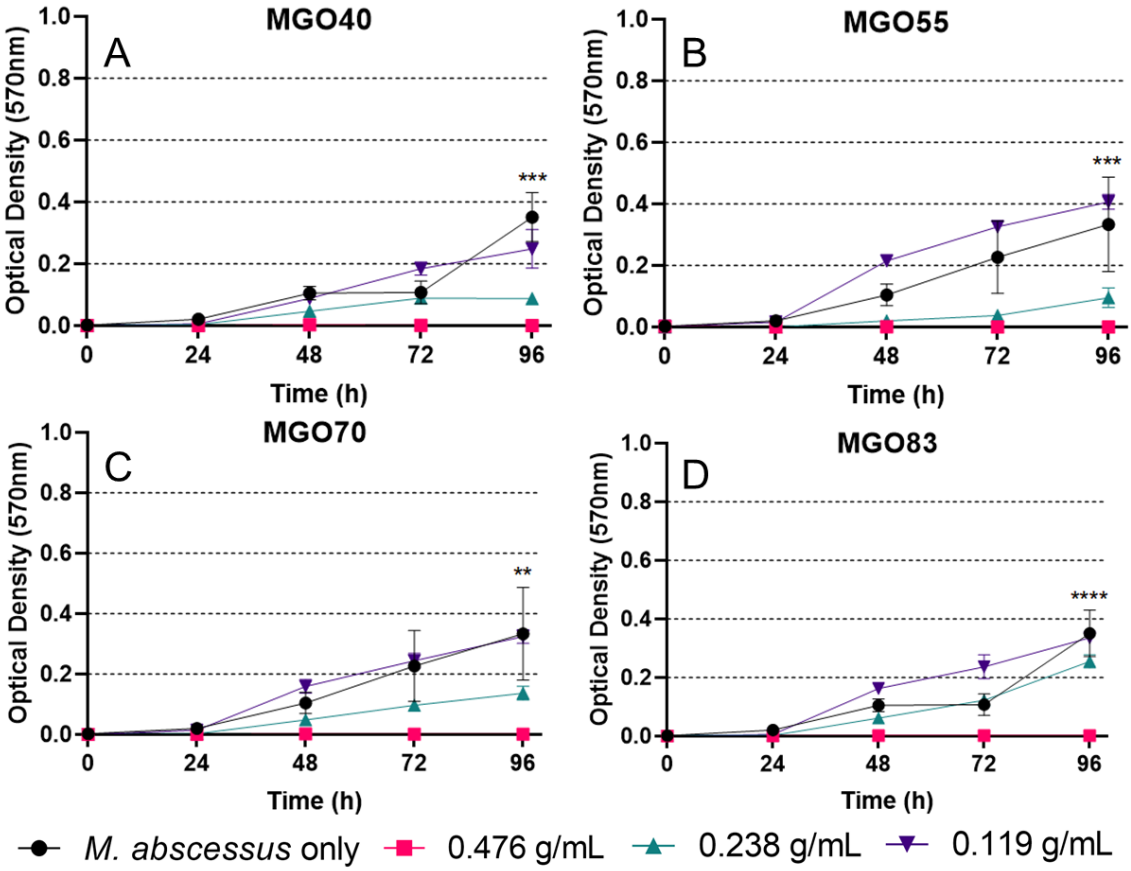

Figure 8.13 Growth Curves of *M. abscessus* DC088C, cultured at 30 °C treated with 4 different manuka honey sampless. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088C at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=0.0007. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=0.0021. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

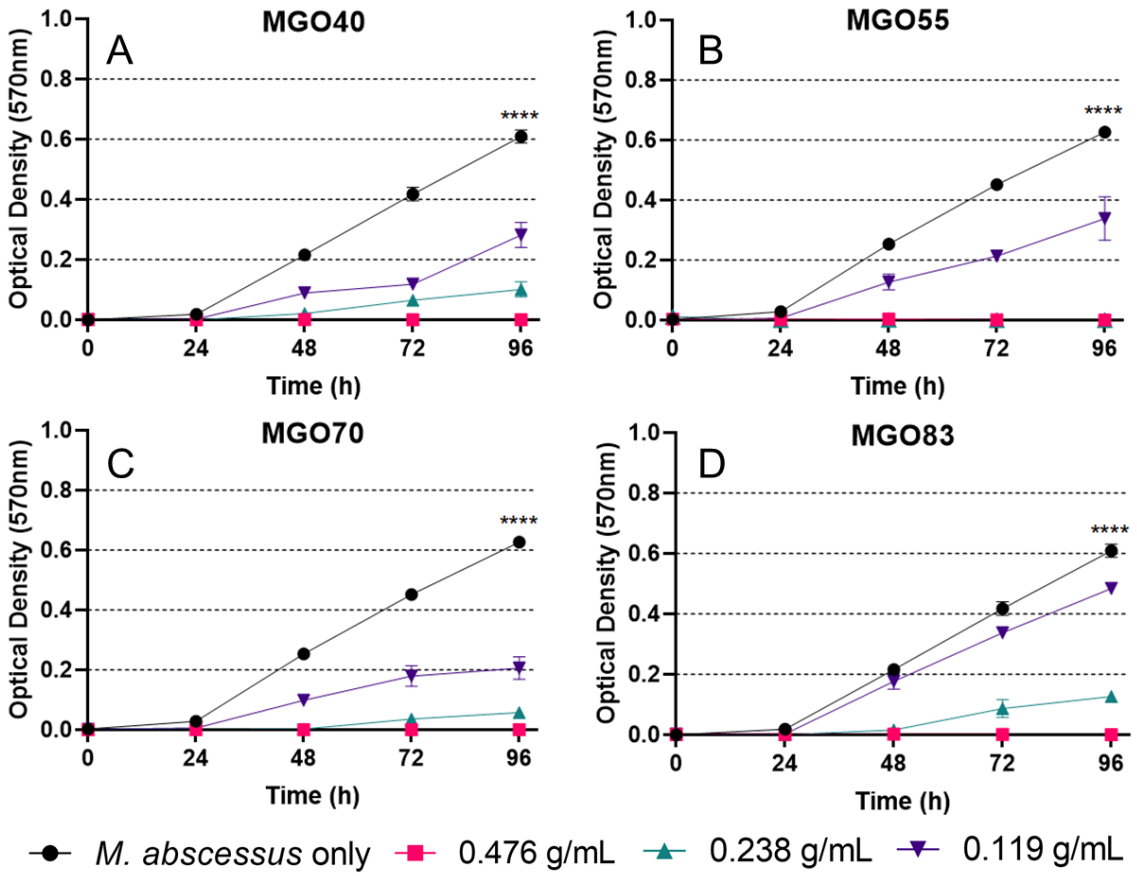

Figure 8.14 Growth Curves of *M. abscessus* DC088D, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088D at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, Kruskal-Wallis P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

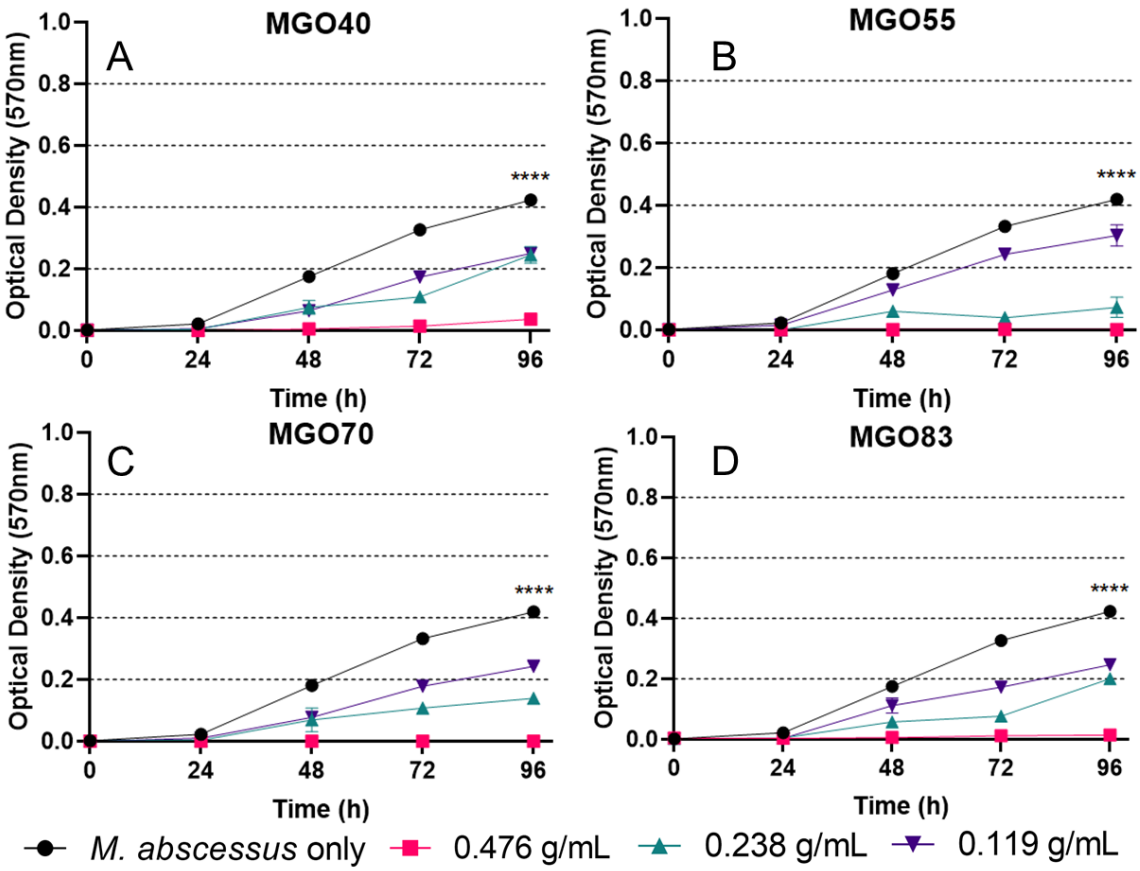

Figure 8.15 Growth Curves of *M. abscessus* DC088E, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088E at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

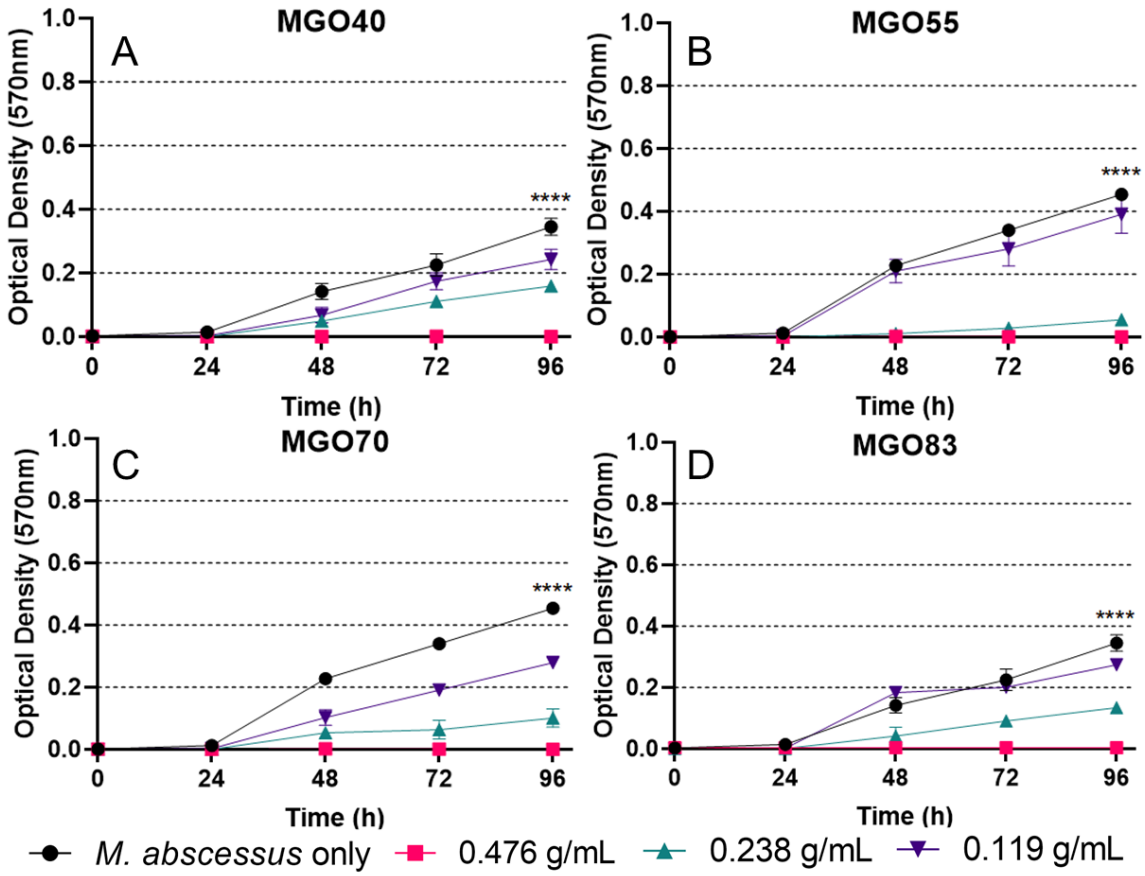

Figure 8.16 Growth Curves of *M. abscessus* DC088ref, cultured at 30 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088ref at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

8.1.3 Graphical data for all clinical isolates for the efficacy of manuka honey cultured at 37 °C

All graphical data for the *M. abscessus* clinical isolates tested against the 4 manuka honey samples and cultured at 37 °C.

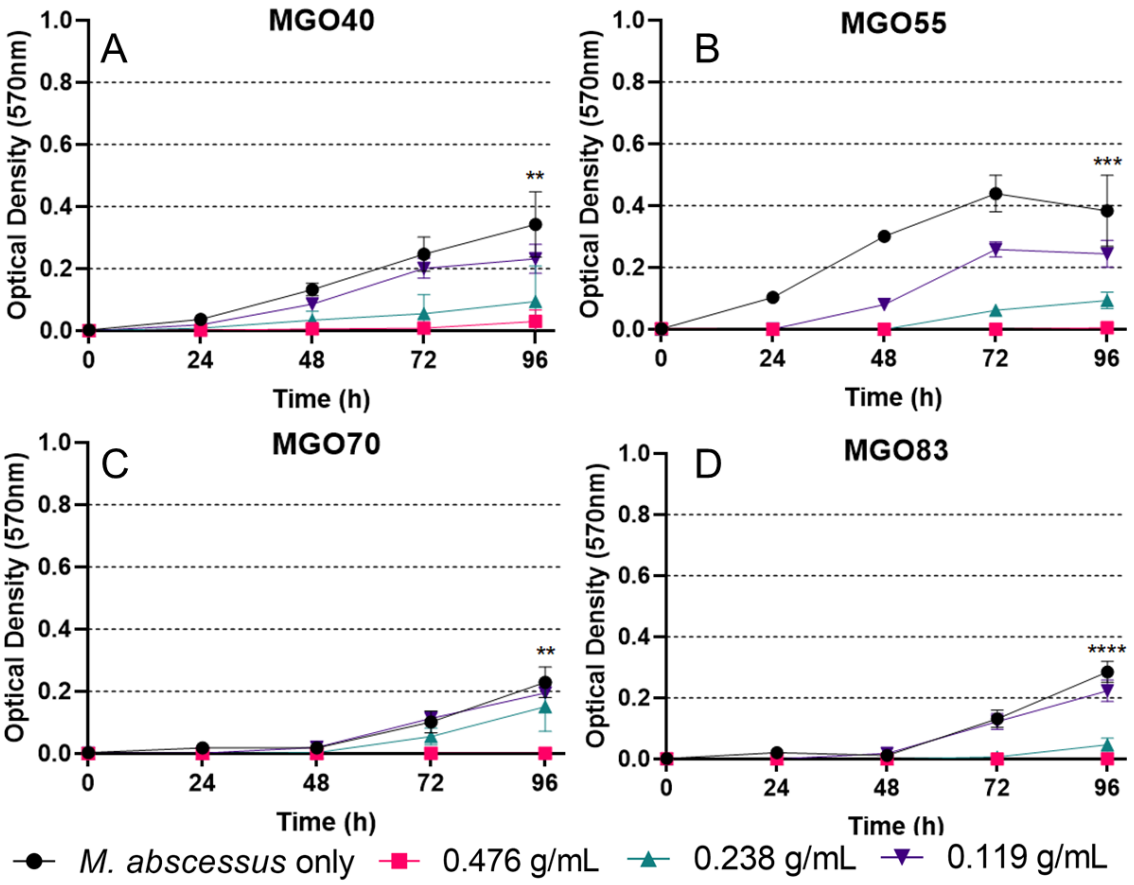

Figure 8.17 Growth Curves of *M. abscessus* 137071, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 137071 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=0.0072. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=0.0004. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=0.0015. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

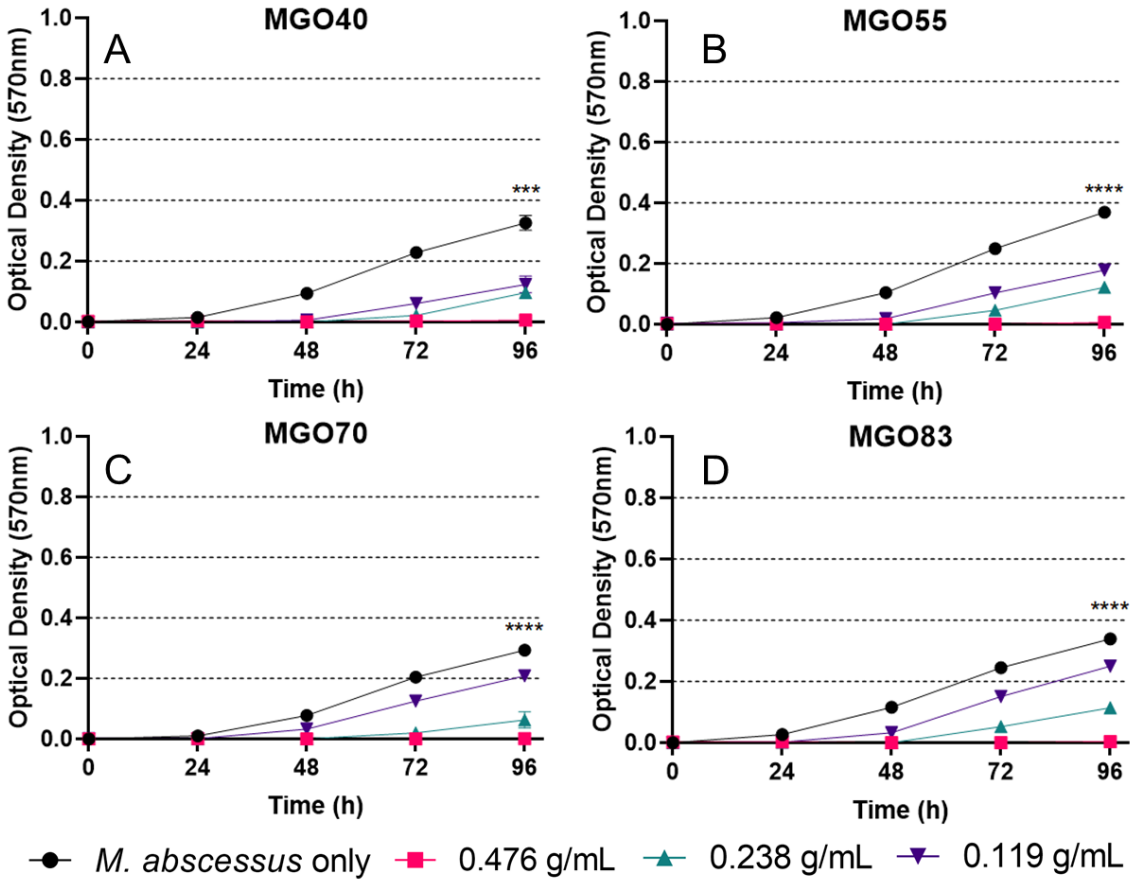

Figure 8.18 Growth Curves of *M. abscessus* 147028, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 147028 at 0.476 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A Kruskal-Wallis identified a significant difference between all honey treatments and no treatment, P=0.0006. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

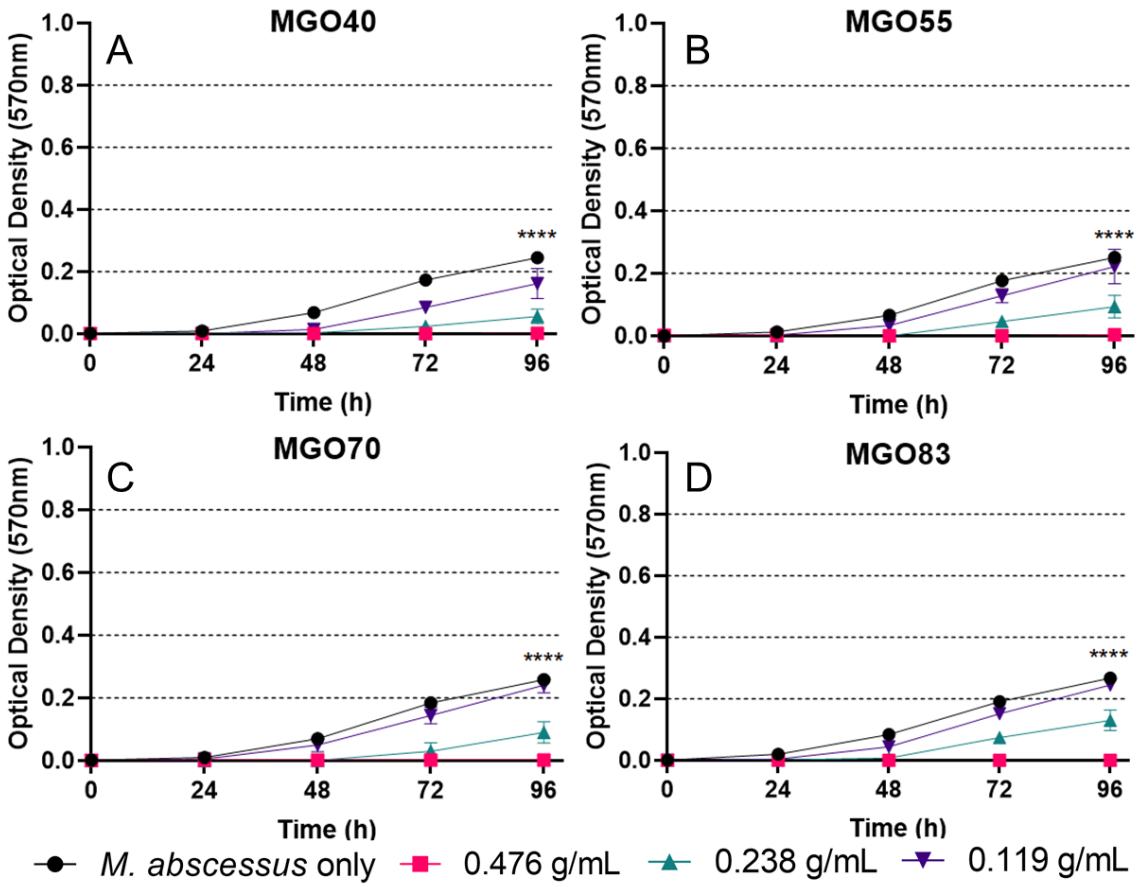

Figure 8.19 Growth Curves of *M. abscessus* 159544, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 159544 at 0.476 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A Kruskal-Wallis identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

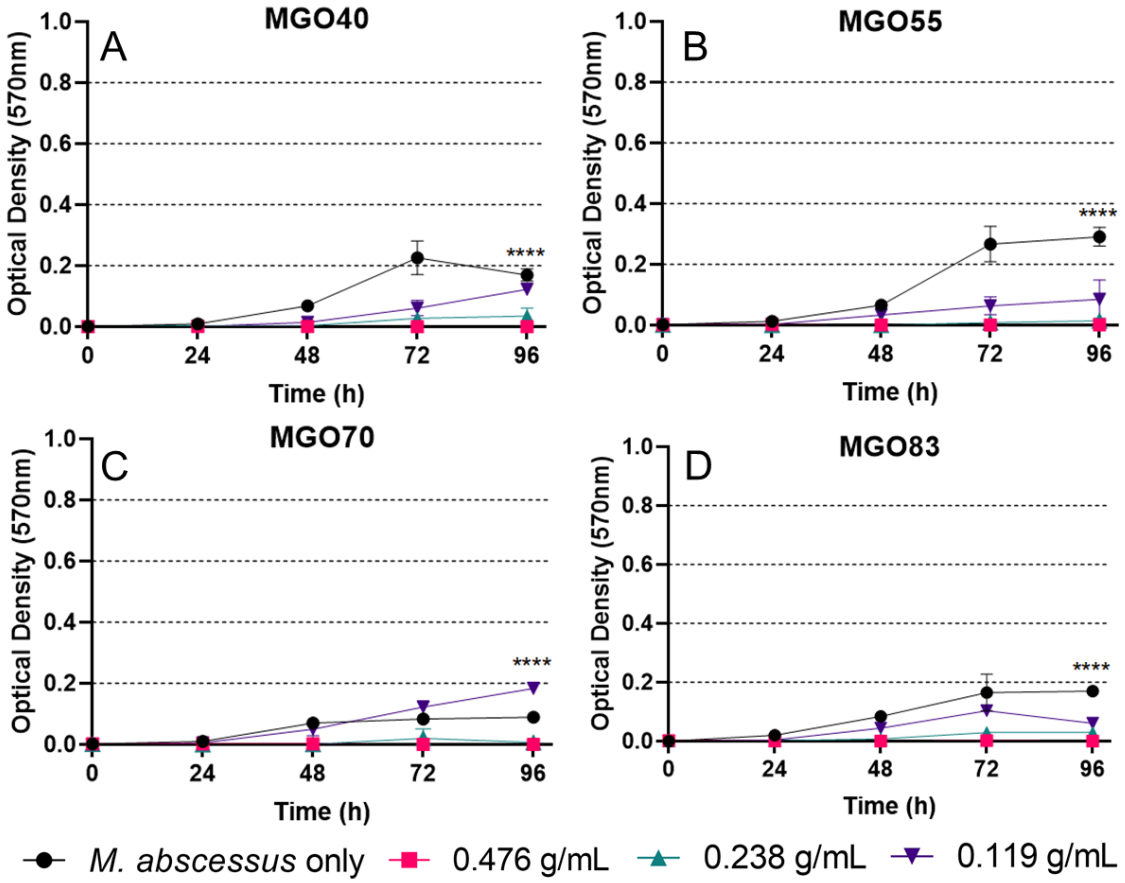

Figure 8.20 Growth Curves of *M. abscessus* 186144, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186144 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.238 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

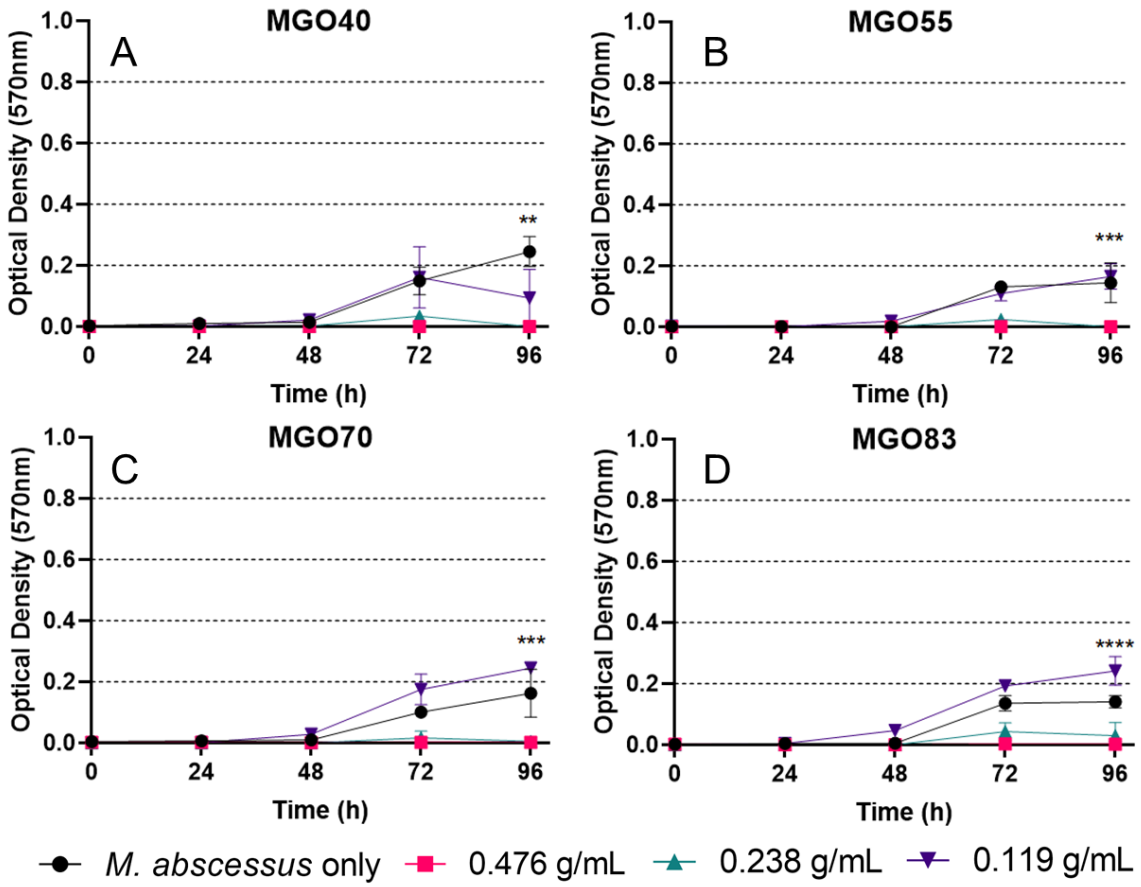

Figure 8.21 Growth Curves of *M. abscessus* 186154, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186154 at 0.238 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.238 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=0.0014. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=0.0009. C) An MIC of 0.238 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=0.0002. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

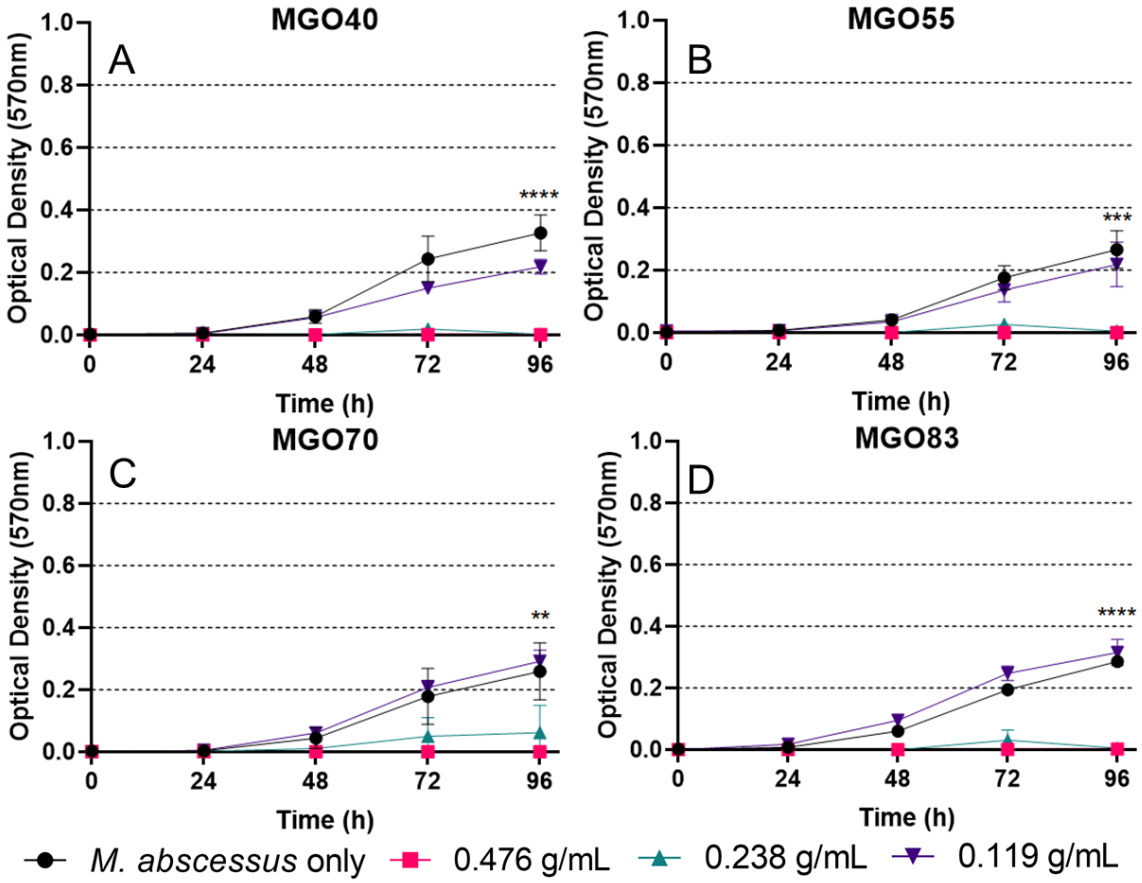

Figure 8.22 Growth Curves of *M. abscessus* 186433, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186433 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.238 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=0.0002. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, Kruskal-Wallis P=0.0014. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

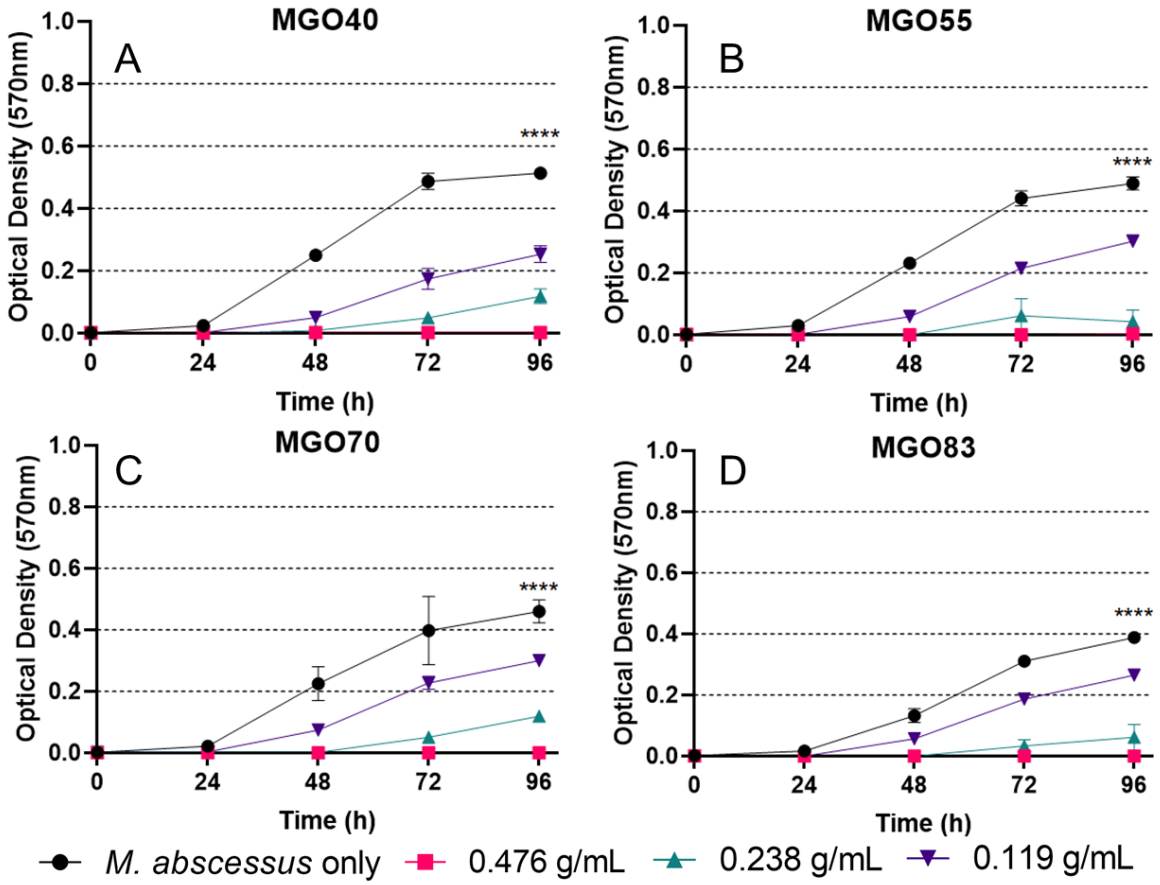

Figure 8.23 Growth Curves of *M. abscessus* 189961, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 189961 at 0.476 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

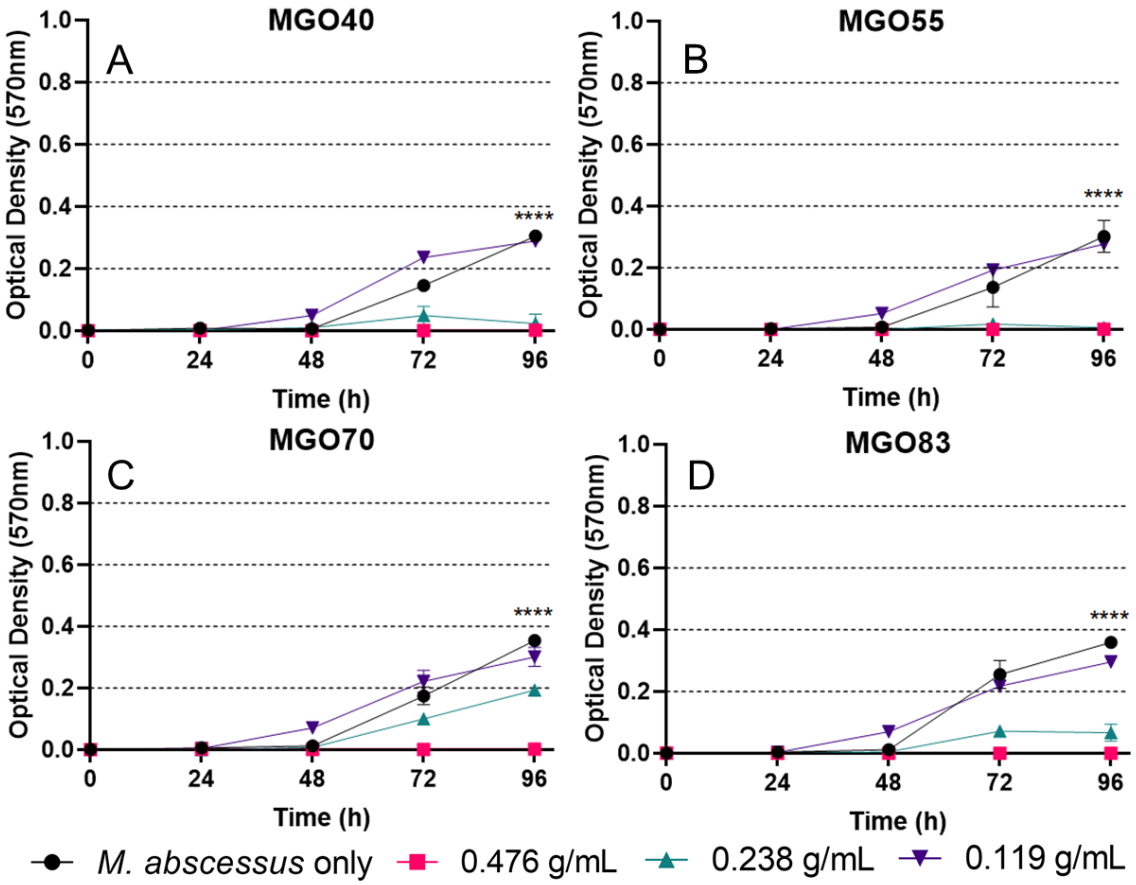

Figure 8.24 Growth Curves of *M. abscessus* 194891, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 194891 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.238 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

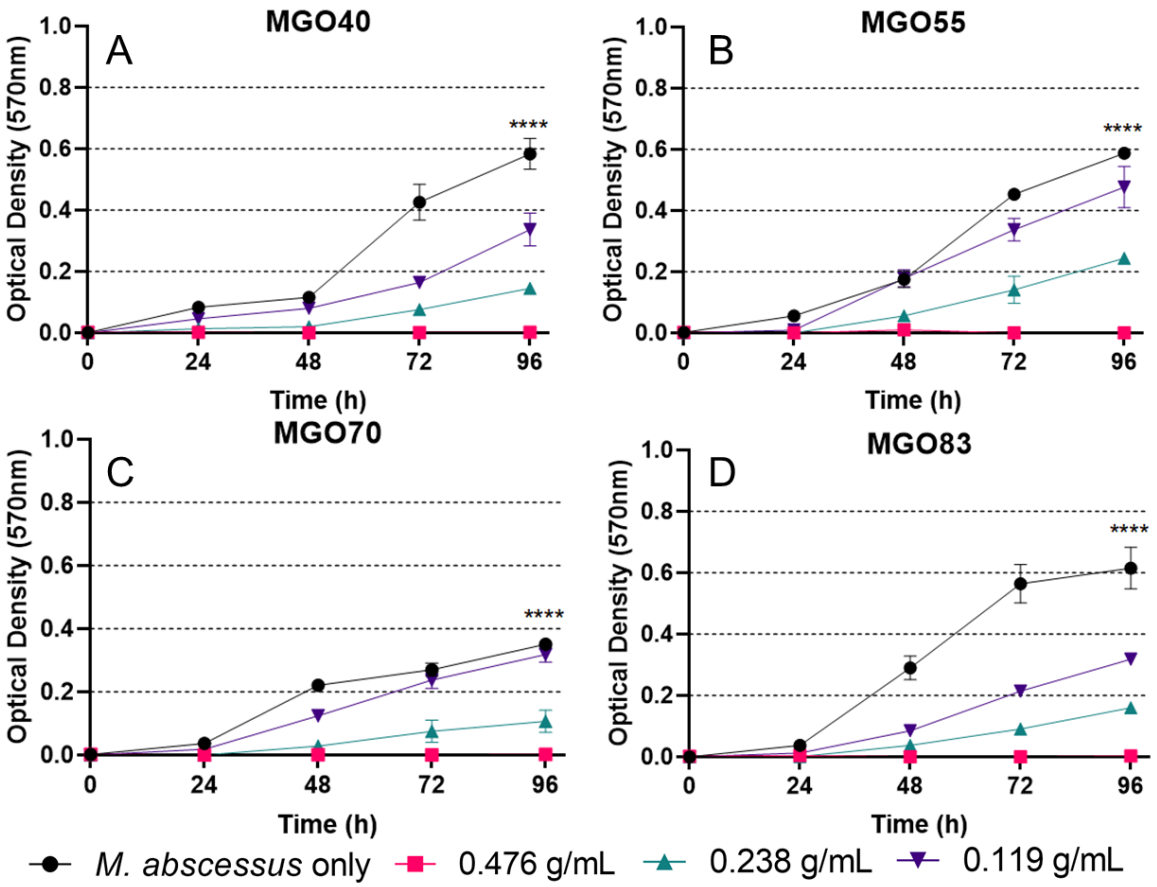

Figure 8.25 Growth Curves of *M. abscessus* 199277, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 199277 at 0.476 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

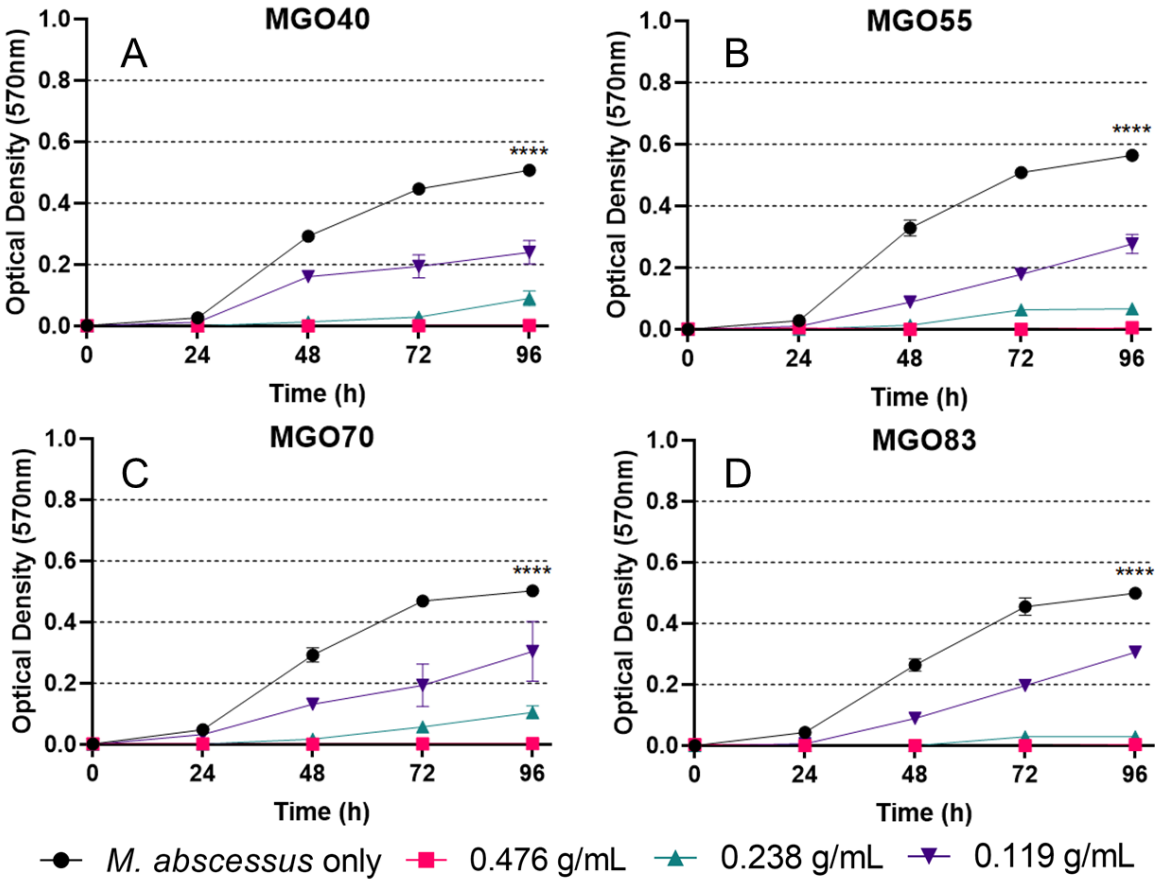

Figure 8.26 Growth Curves of *M. abscessus* 211666, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 211666 at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, Kruskal-Wallis P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

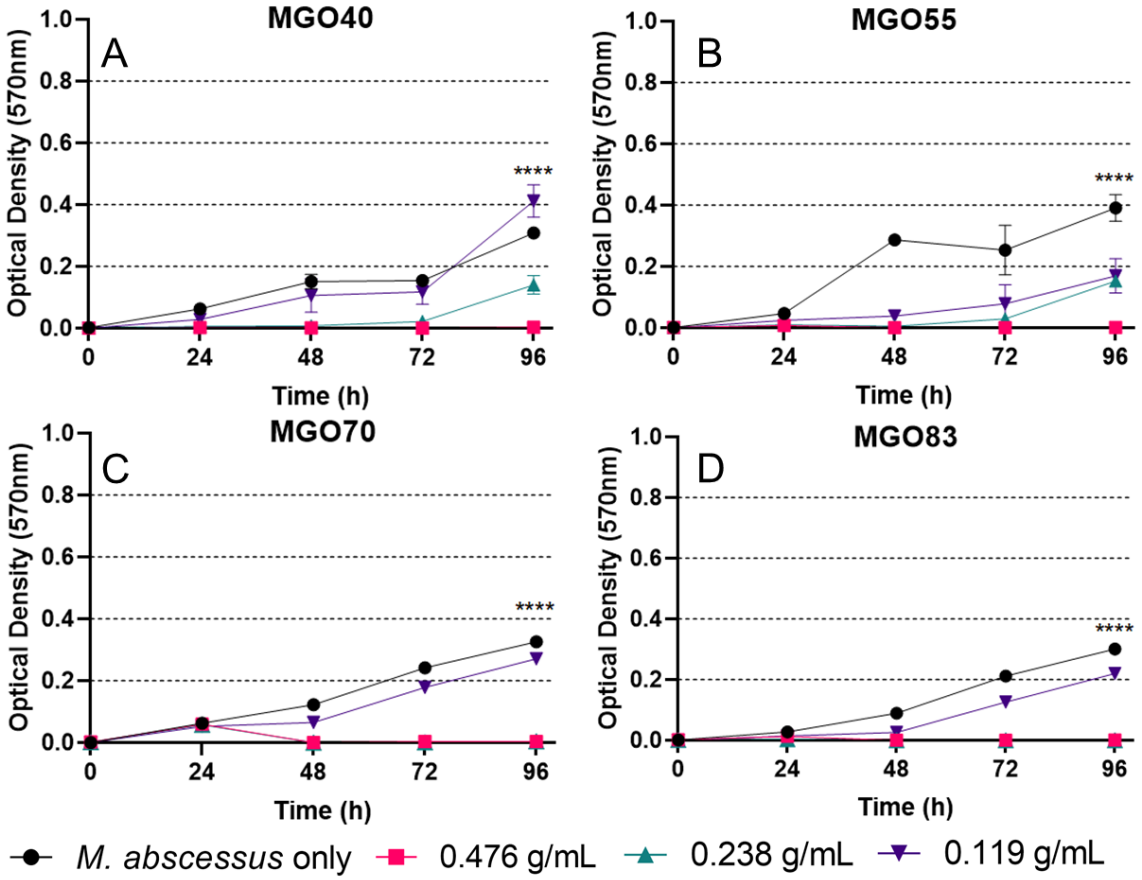

Figure 8.27 Growth Curves of *M. abscessus* DC088A, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088A at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.238 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

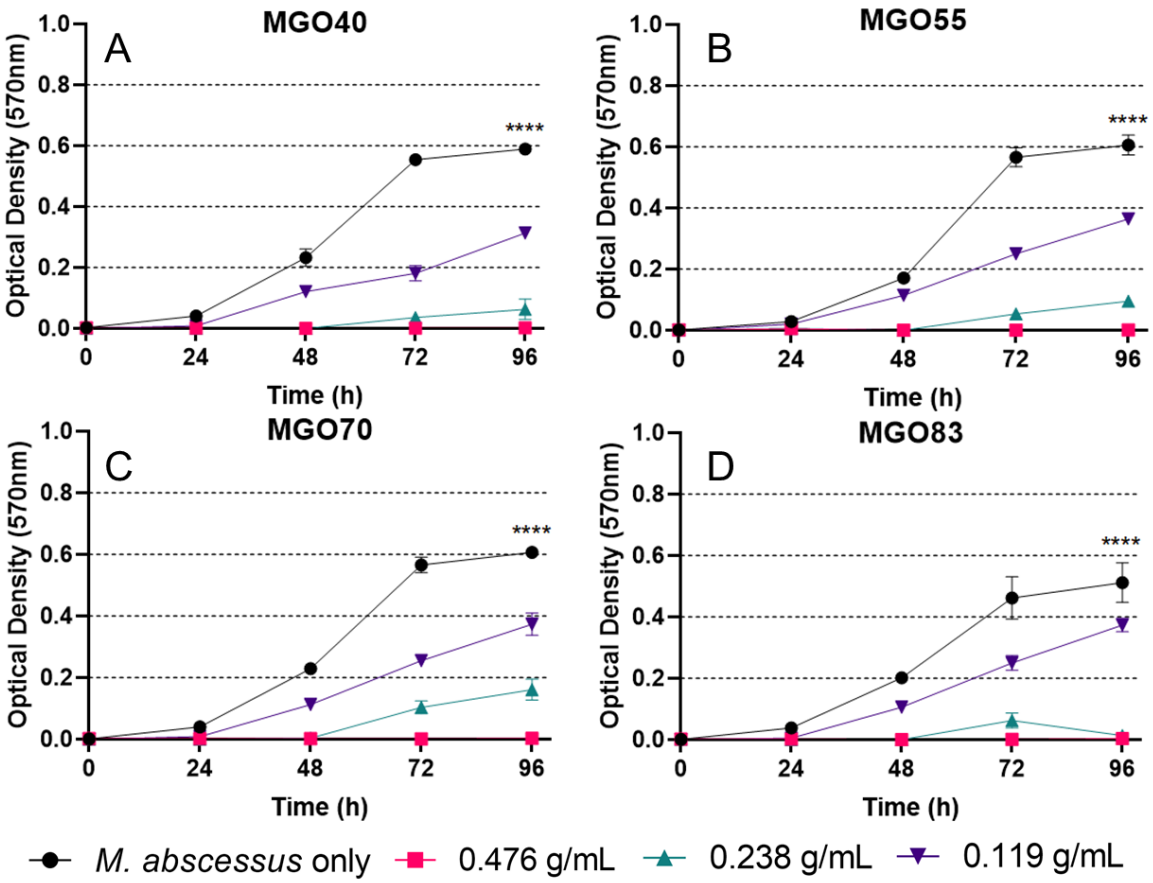

Figure 8.28 Growth Curves of *M. abscessus* DC088D, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088D at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

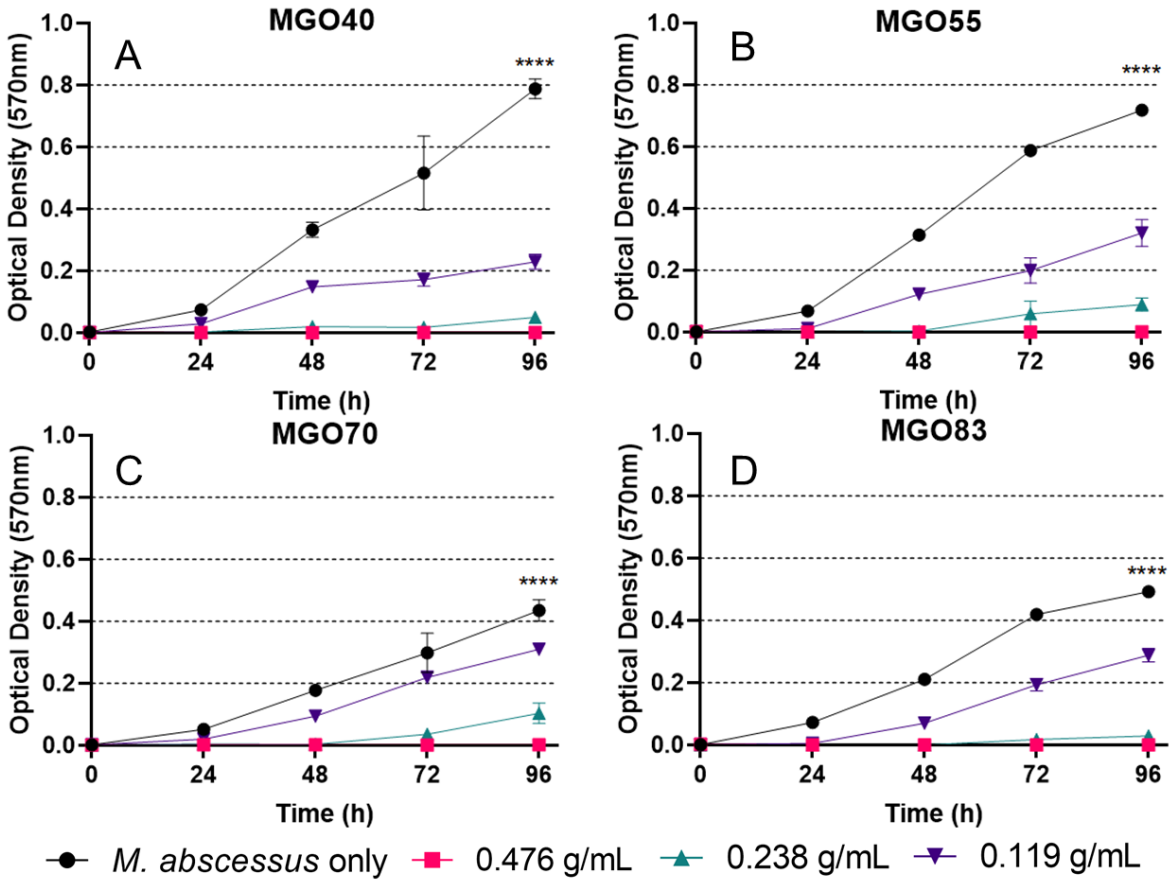

Figure 8.29 Growth Curves of *M. abscessus* DC088C, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088C at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

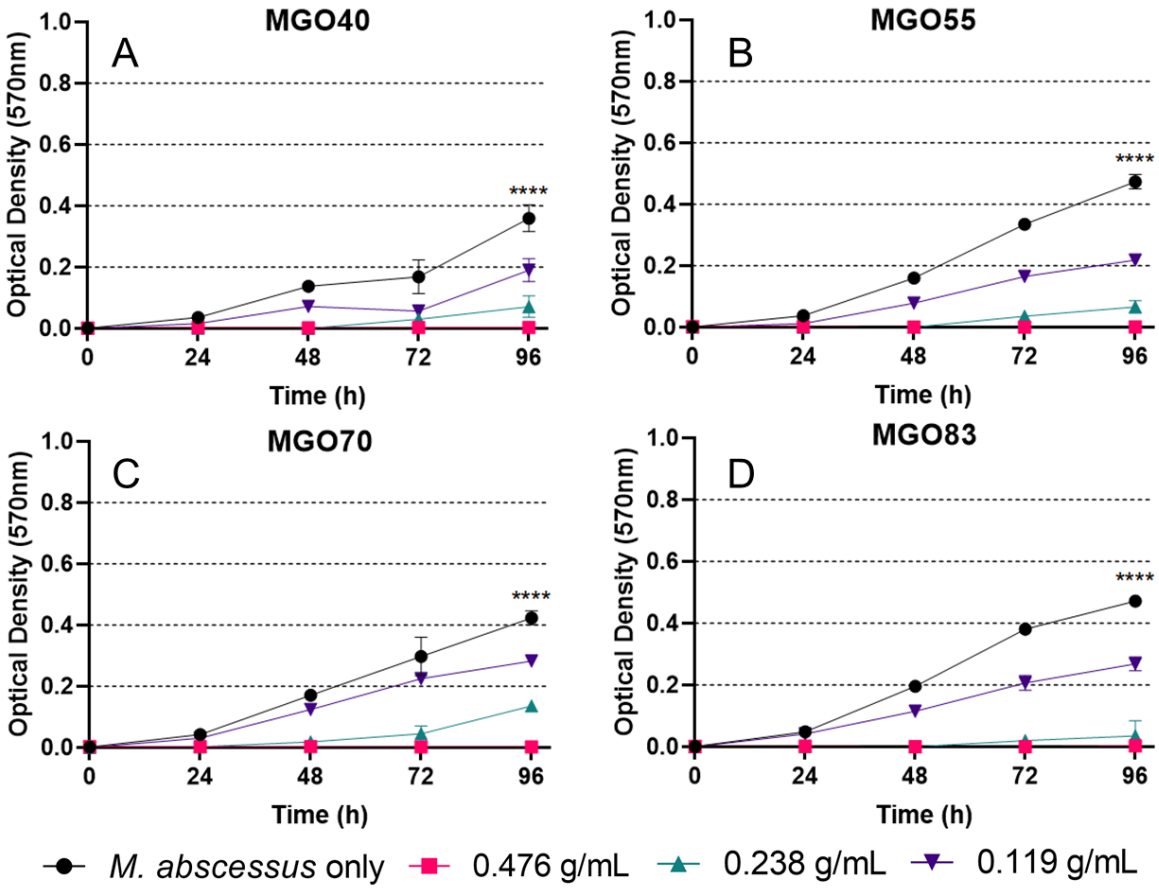

Figure 8.30 Growth Curves of *M. abscessus* DC088D, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088D at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

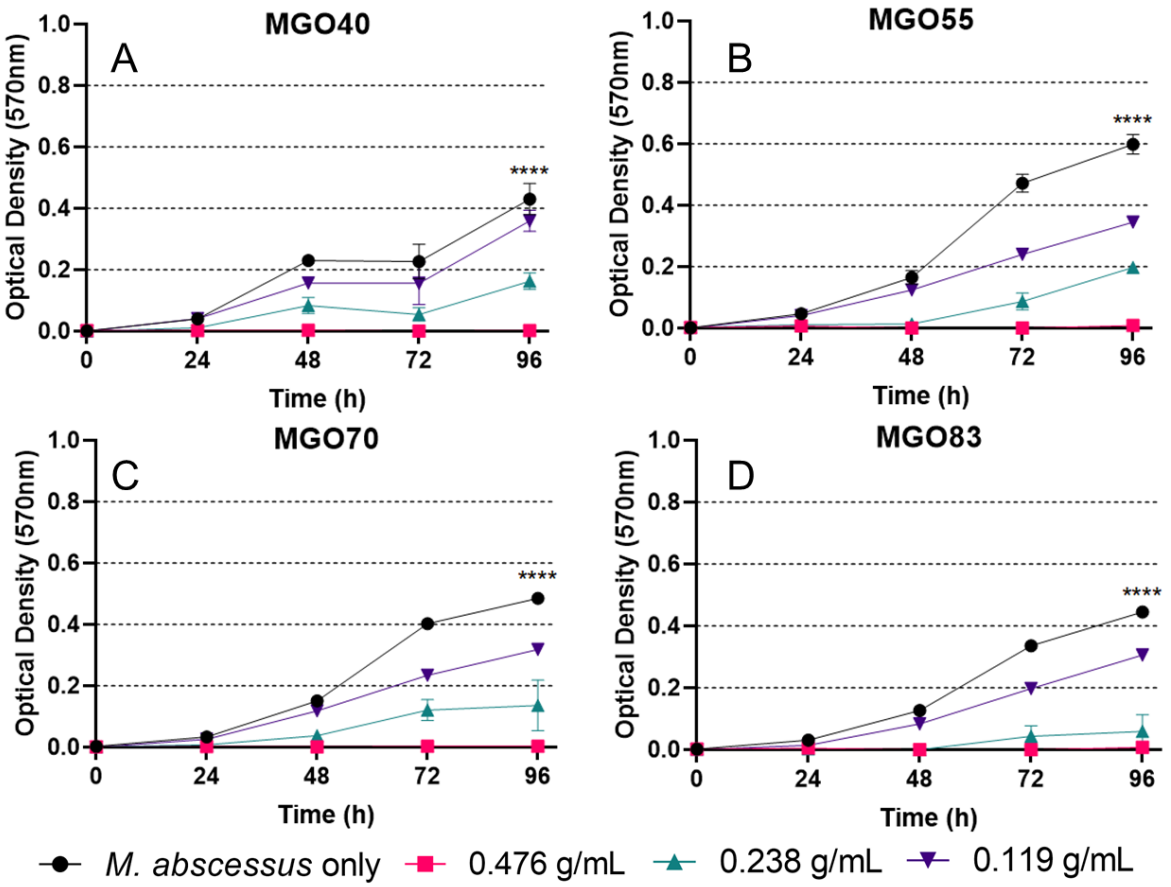

Figure 8.31 Growth Curves of *M. abscessus* DC088E, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088E at 0.476 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.476 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

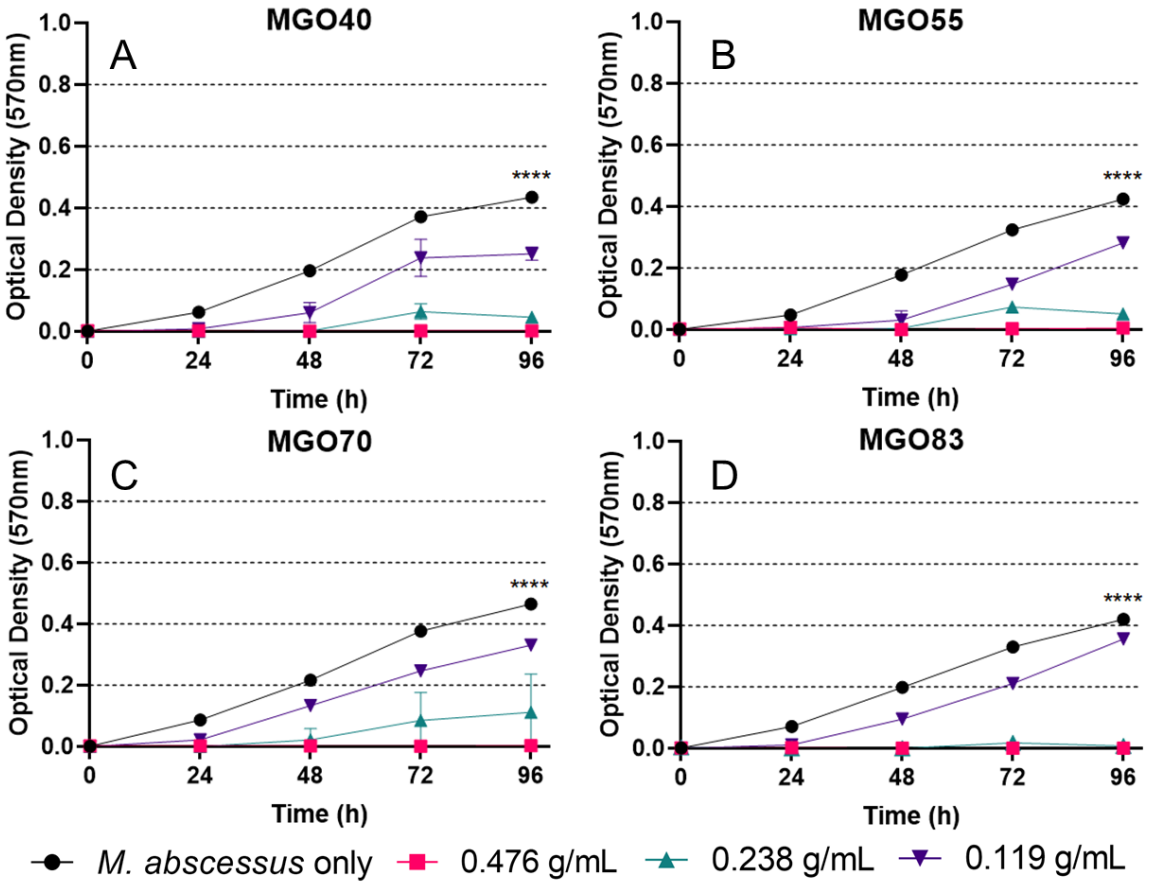

Figure 8.32 Growth Curves of *M. abscessus* DC088ref, cultured at 37 °C treated with 4 different manuka honey samples. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088ref at 0.476 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.476 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.476 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.476 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, Kruskal-Wallis P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

8.1.4 Graphical data for all clinical isolates for the efficacy of manuka honey cultured at 37 °C and stored at 4 °C for 30 days

All graphical data for the *M. abscessus* clinical isolates tested against the 4 manuka honey samples and cultured at 37 °C and stored at 4 °C for 30 days.

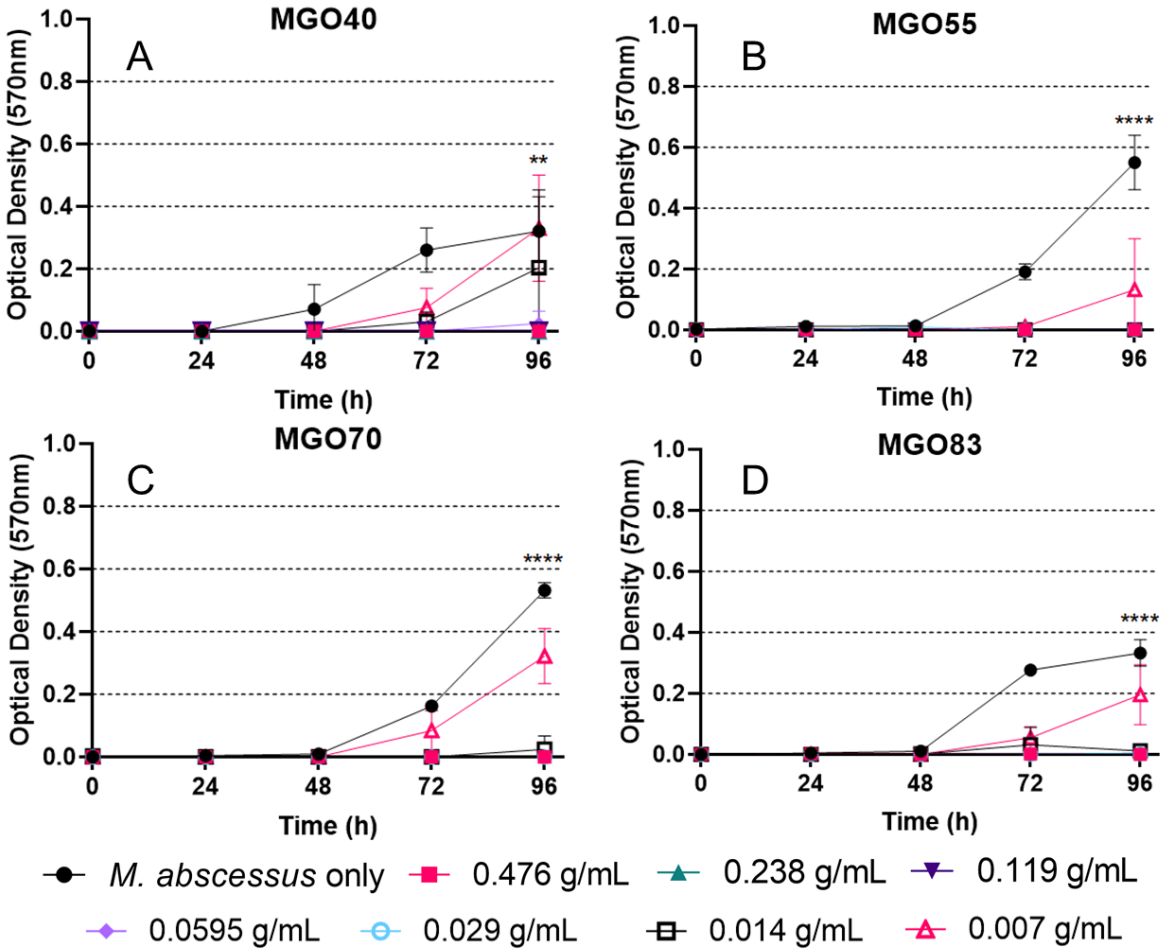

Figure 8.33 Growth Curves of *M. abscessus* 137071, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 137071 at 0.029 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.029 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=0.0025. B) MGO55 growth curve with an MIC of 0.014 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.014 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.014 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

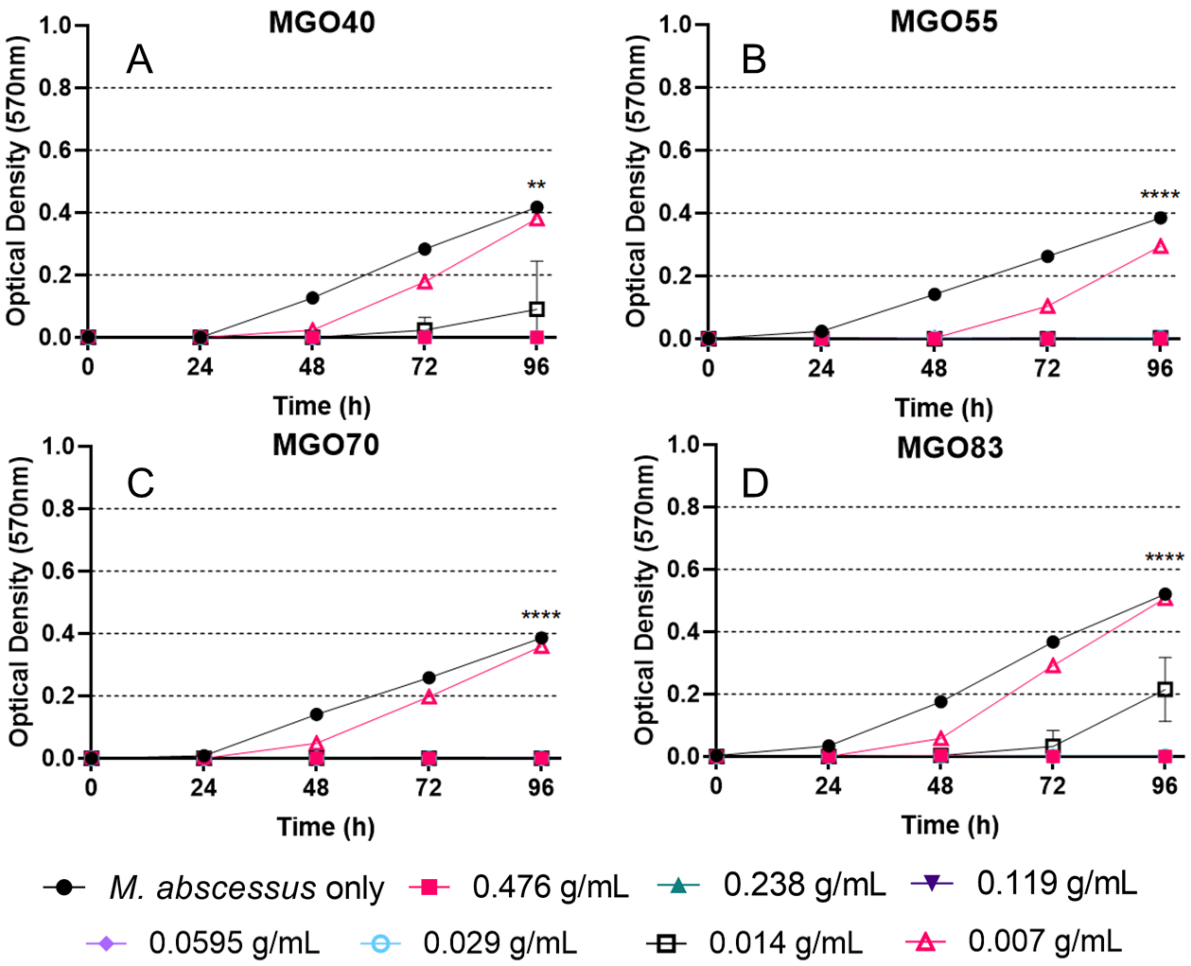

Figure 8.34 Growth Curves of *M. abscessus* 147028, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 147028 at 0.029 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.029 g/mL. A Kruskal-Wallis identified a significant difference between all honey treatments and no treatment, P=0.0033. B) MGO55 growth curve with an MIC of 0.014 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.014 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.029 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

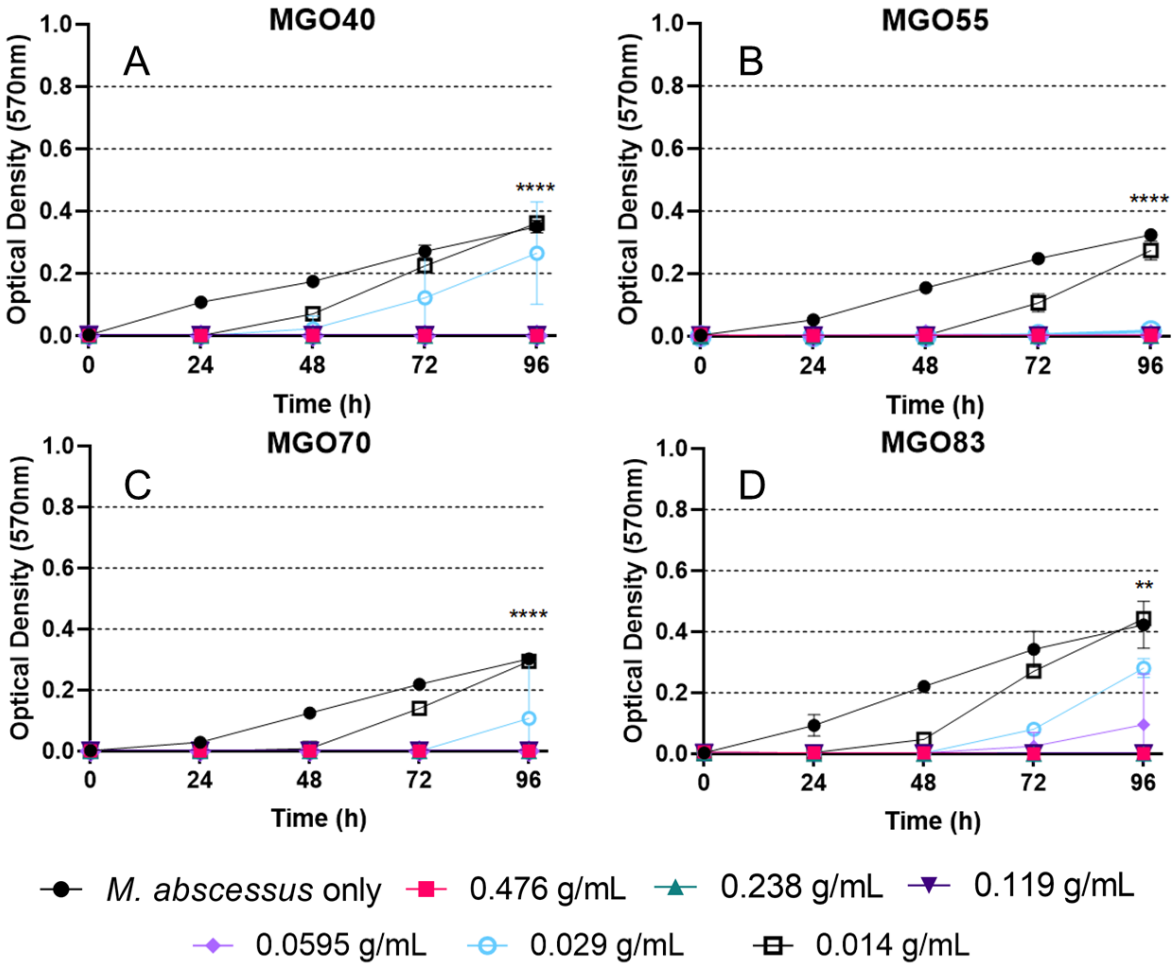

Figure 8.35 Growth Curves of *M. abscessus* 159544, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 159544 at 0.0595 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.0595 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001 B) MGO55 growth curve with an MIC of 0.029 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.029 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.0595 g/mL. A significant difference was observed for honey treatments, Kruskal-Wallis P=0.0099.

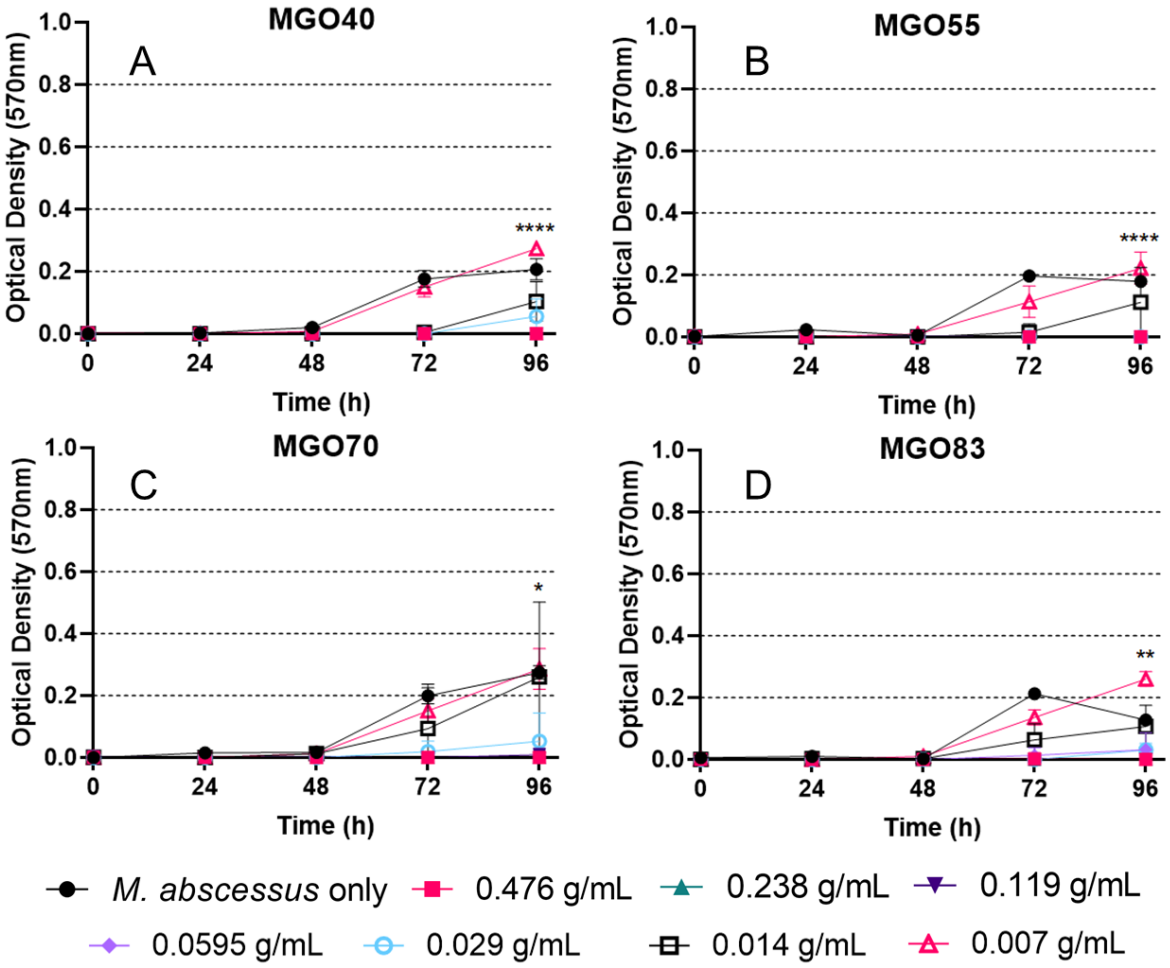

Figure 8.36 Growth Curves of *M. abscessus* 86144, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186144 at 0.0595 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.029 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.029 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.0595 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, Kruskal-Wallis P=0.0125. D) MGO83 exhibiting an MIC of 0.029 g/mL. A significant difference was observed for honey treatments, Kruskal-Wallis P=0.0054.

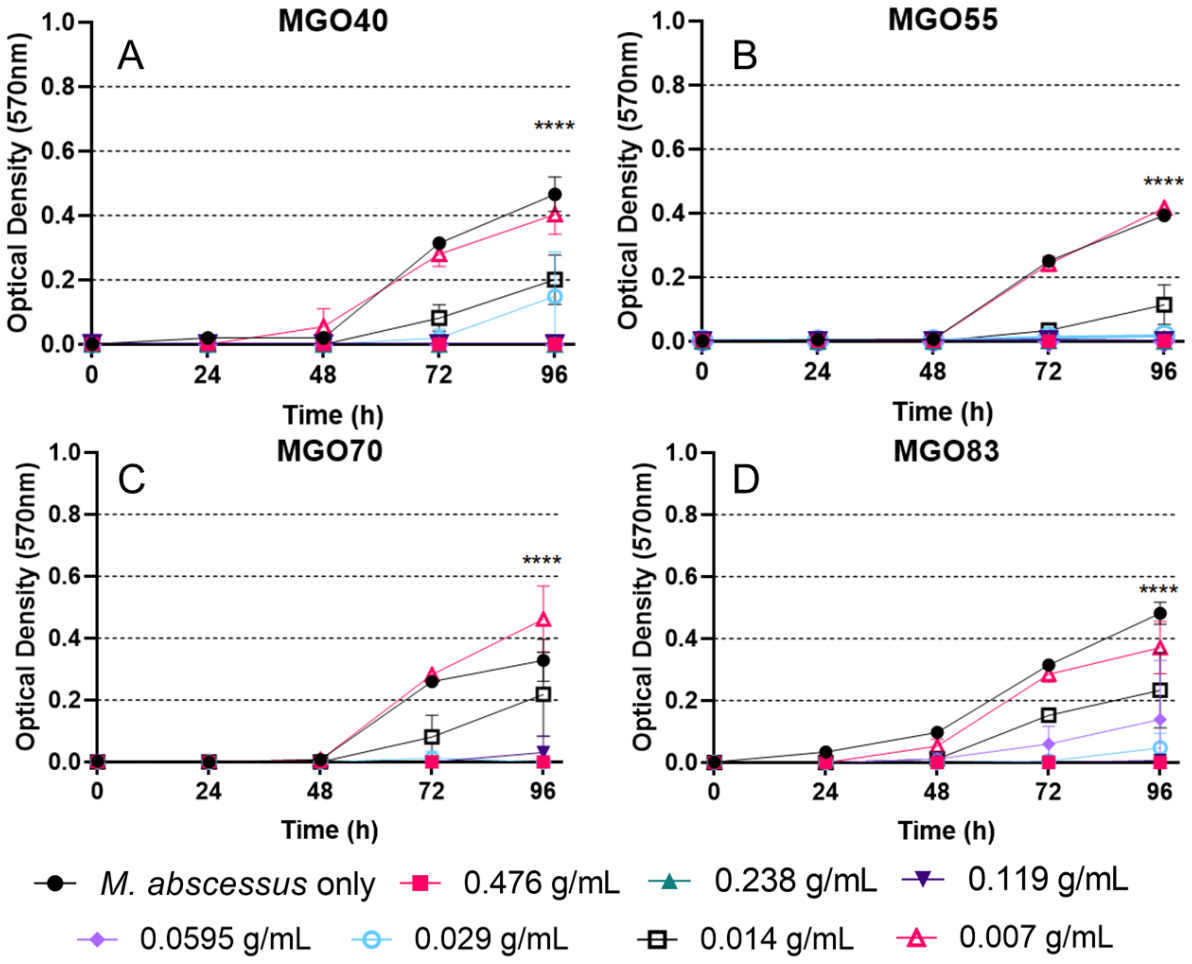

Figure 8.37 Growth Curves of *M. abscessus* 186154, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186154 at 0.0595 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.0595 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.029 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.029 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.029 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

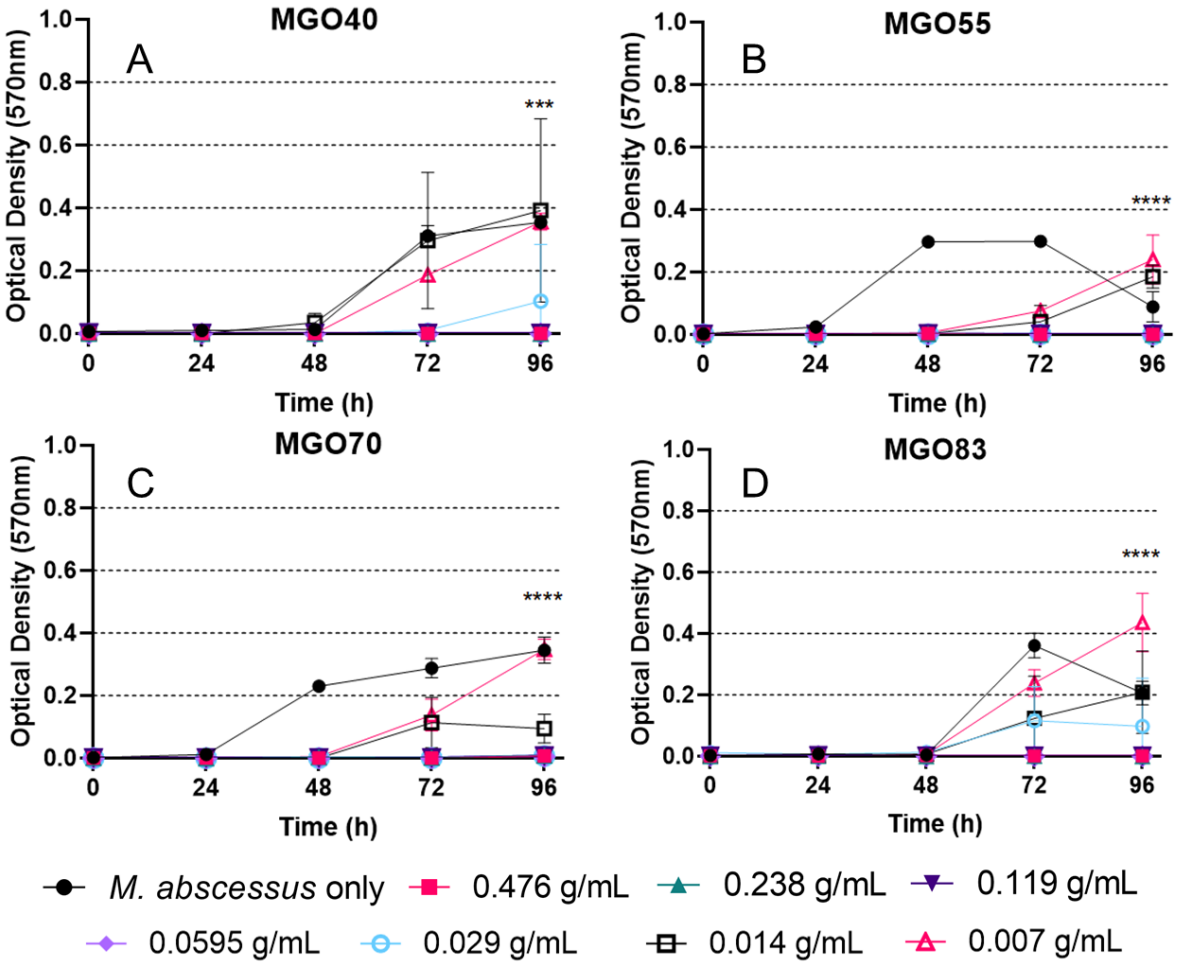

Figure 8.38 Growth Curves of *M. abscessus* 186433, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 186433 at 0.0595 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.029 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=0.0008. B) MGO55 growth curve with an MIC of 0.029 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.029 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.0595 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

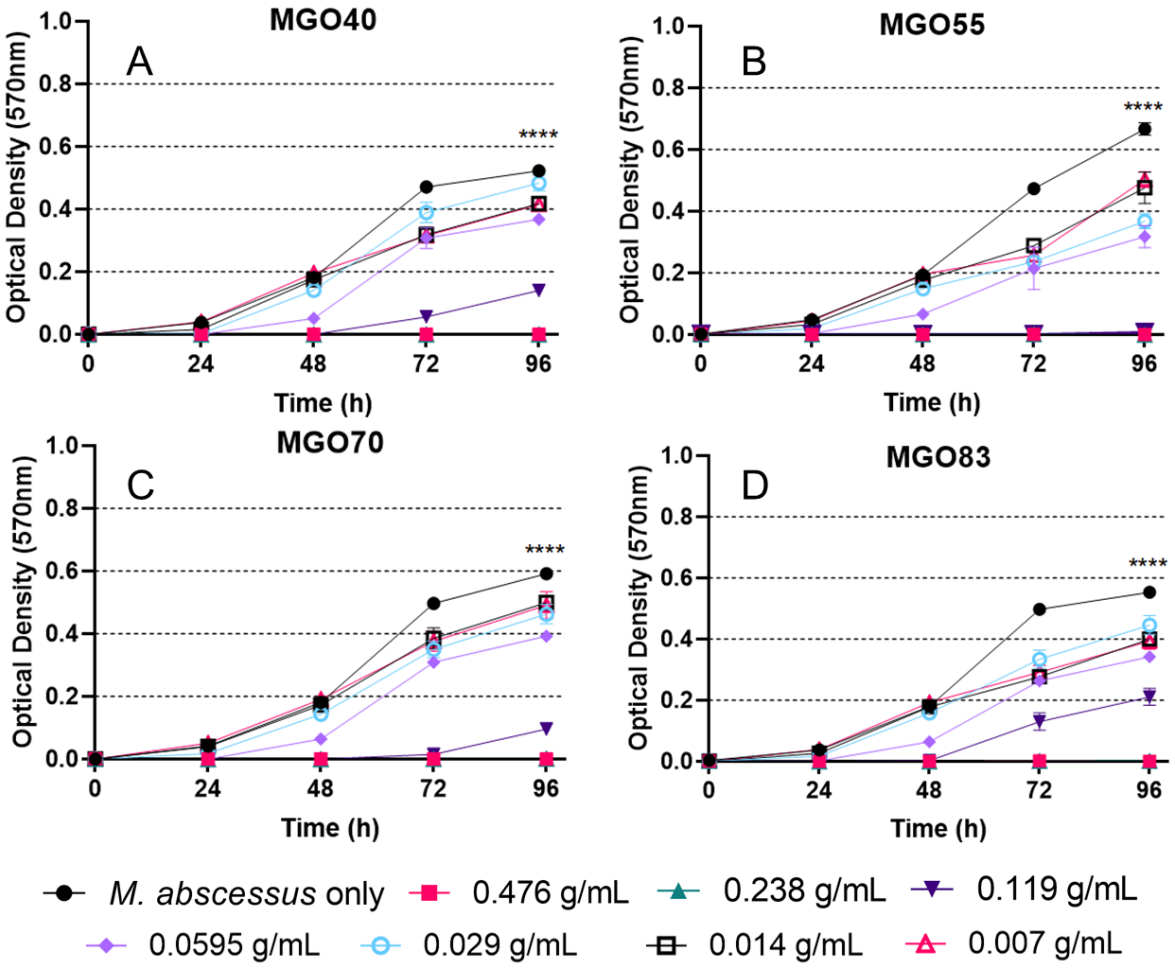

Figure 8.39 Growth Curves of *M. abscessus* 189961, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 189961 at 0.238 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.238 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.119 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.119 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

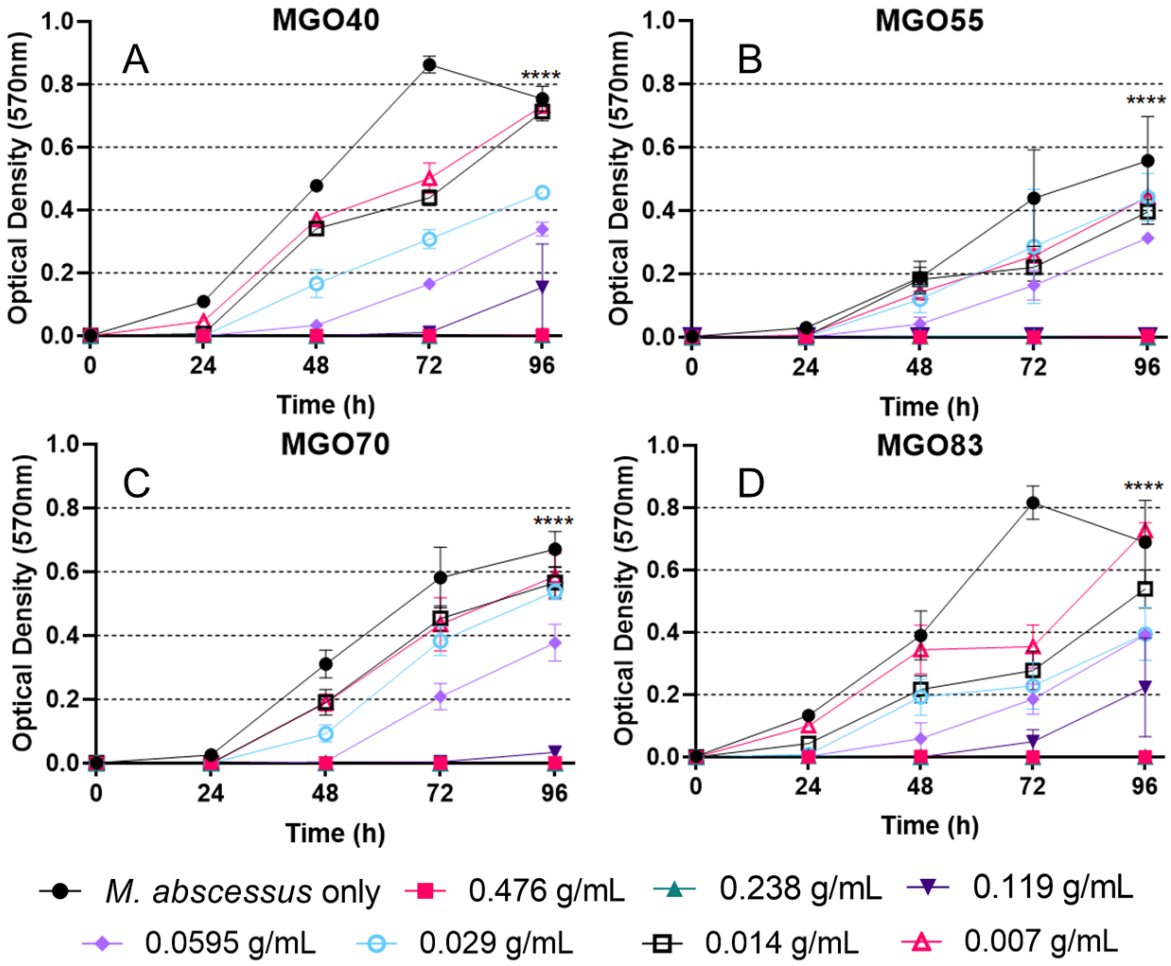

Figure 8.40 Growth Curves of *M. abscessus* 194891, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 194891 at 0.238 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.119 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.119 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.119 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

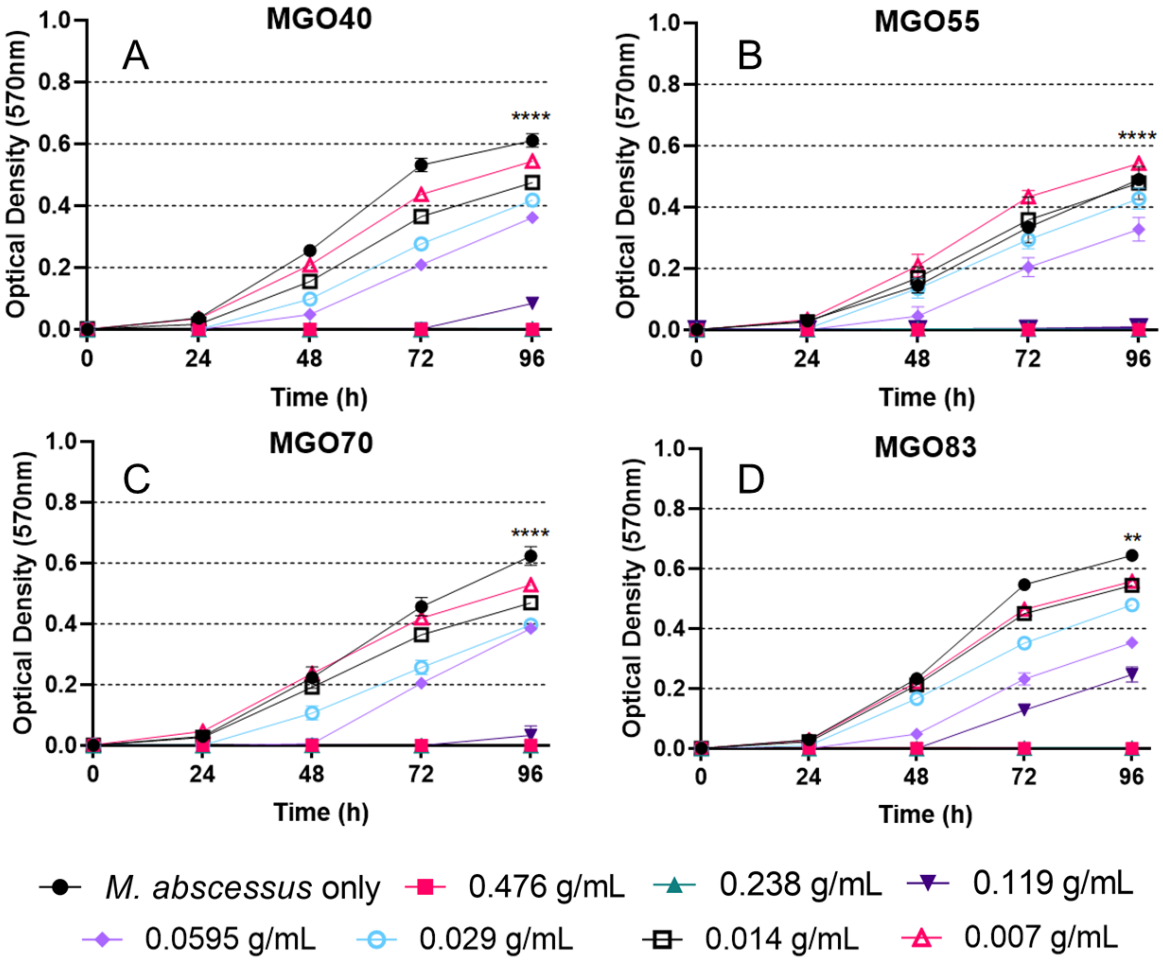

Figure 8.41 Growth Curves of *M. abscessus* 199277, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 199277 at 0.238 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.238 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.119 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.119 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, Kruskal-Wallis P=0.0020.

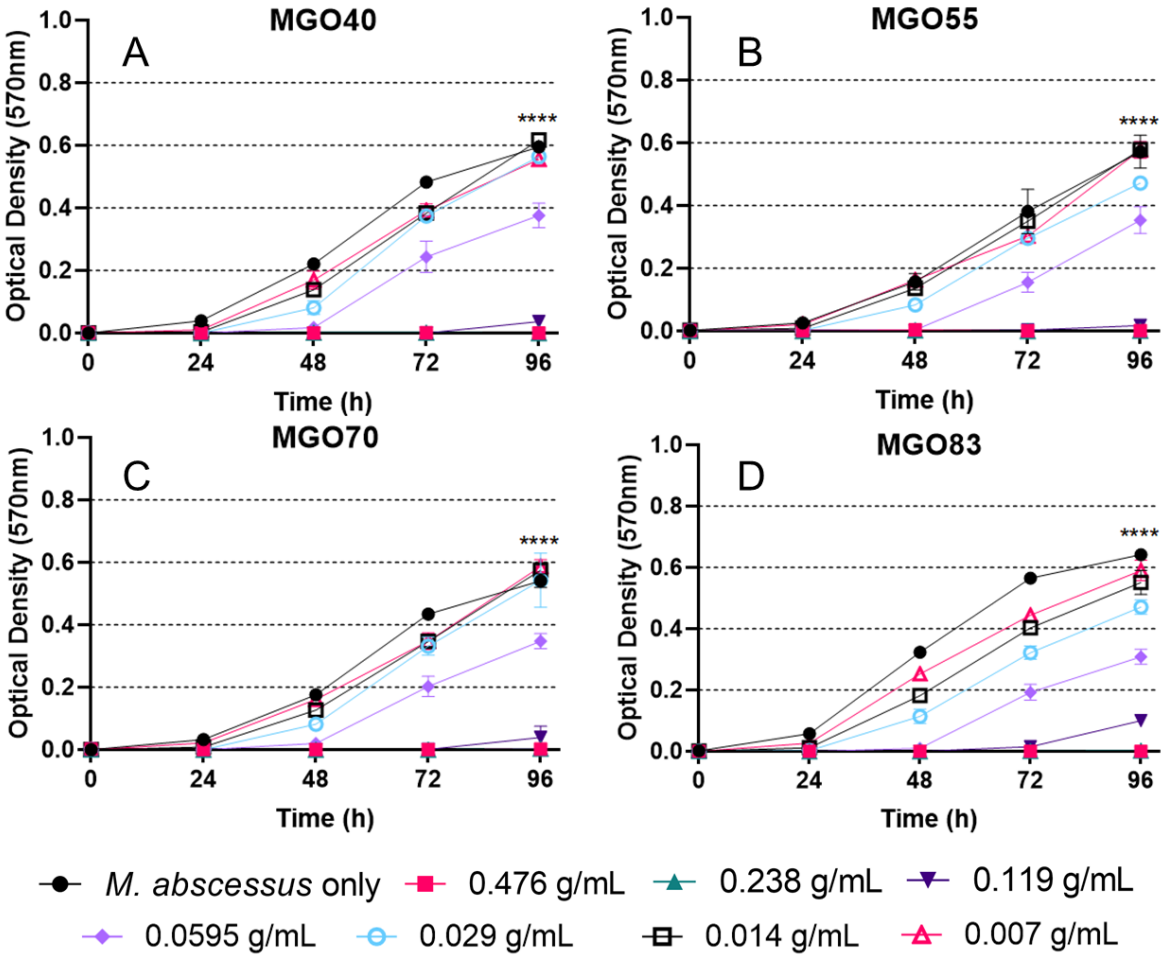

Figure 8.42 Growth Curves of *M. abscessus* 211666, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* 211666 at 0.238 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.238 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.238 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.238 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

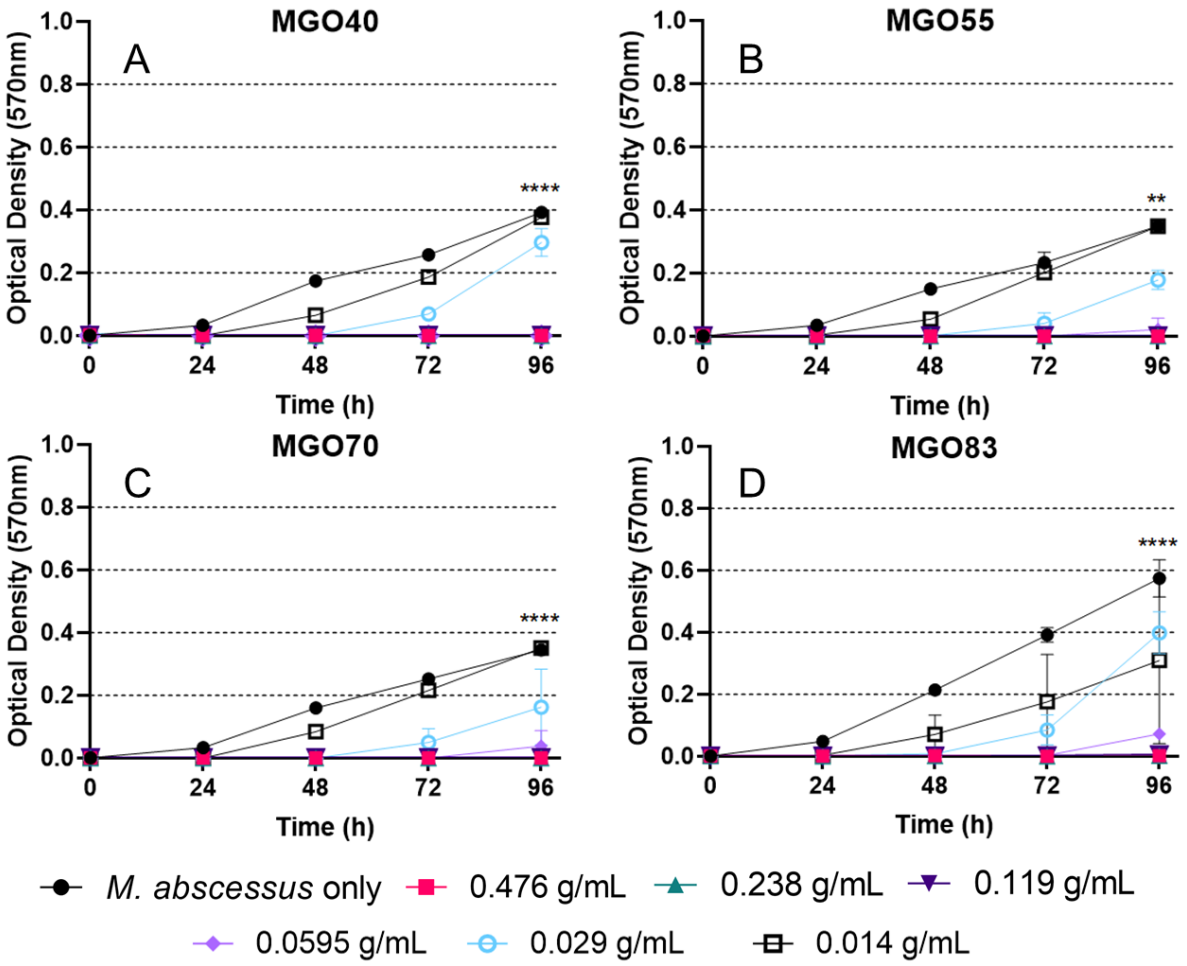

Figure 8.43 Growth Curves of *M. abscessus* DC088A, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088A at 0.0595 g/mL. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.0595 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.0595 g/mL, a Kruskal-Wallis identified a significant difference for all honey treatments P=0.0051. C) An MIC of 0.0595 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.0595 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

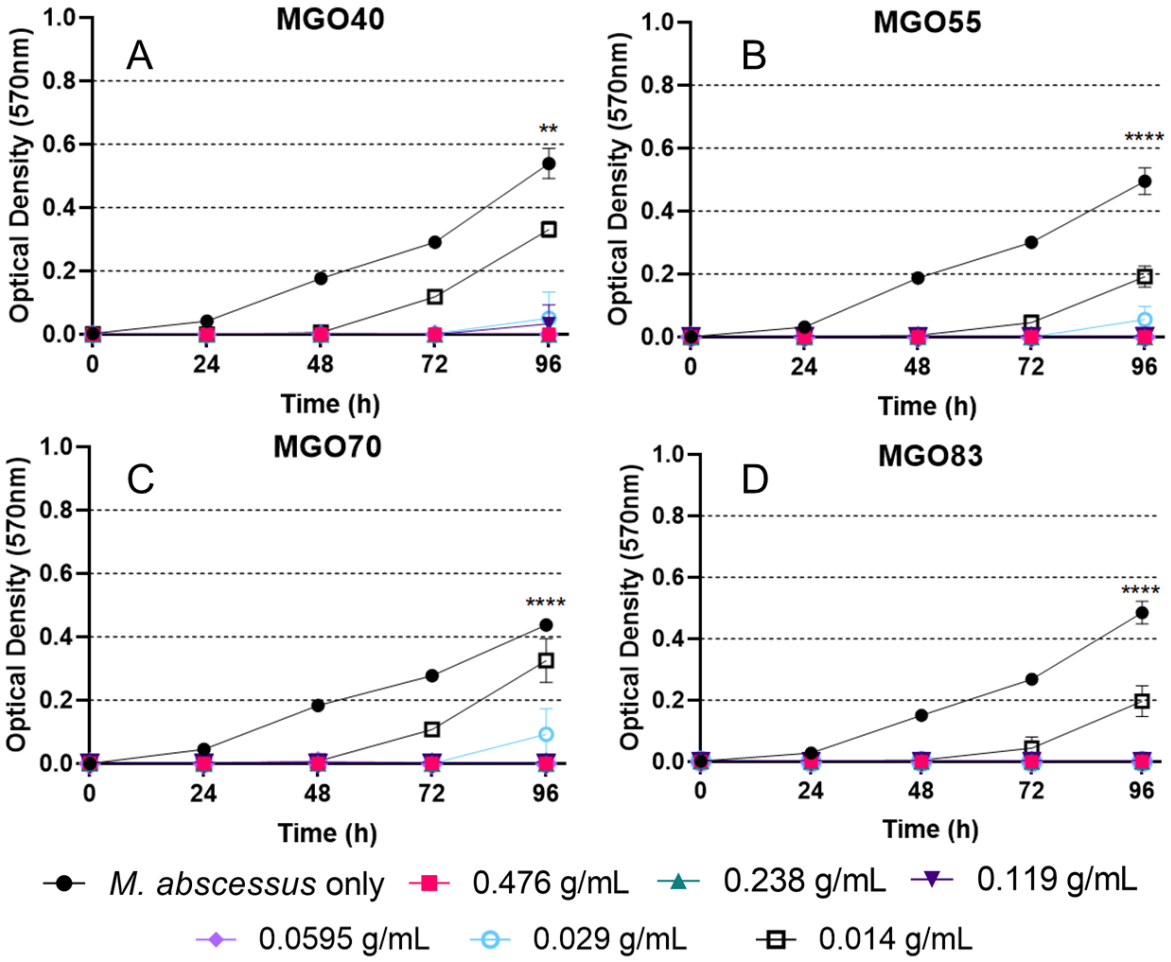

Figure 8.44 Growth Curves of *M. abscessus* DC088B, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088B at 0.0595 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.029 g/mL. A Kruskal-Wallis identified a significant difference between all honey treatments and no treatment, P=0.0079. B) MGO55 growth curve with an MIC of 0.029 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.0595 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.029 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

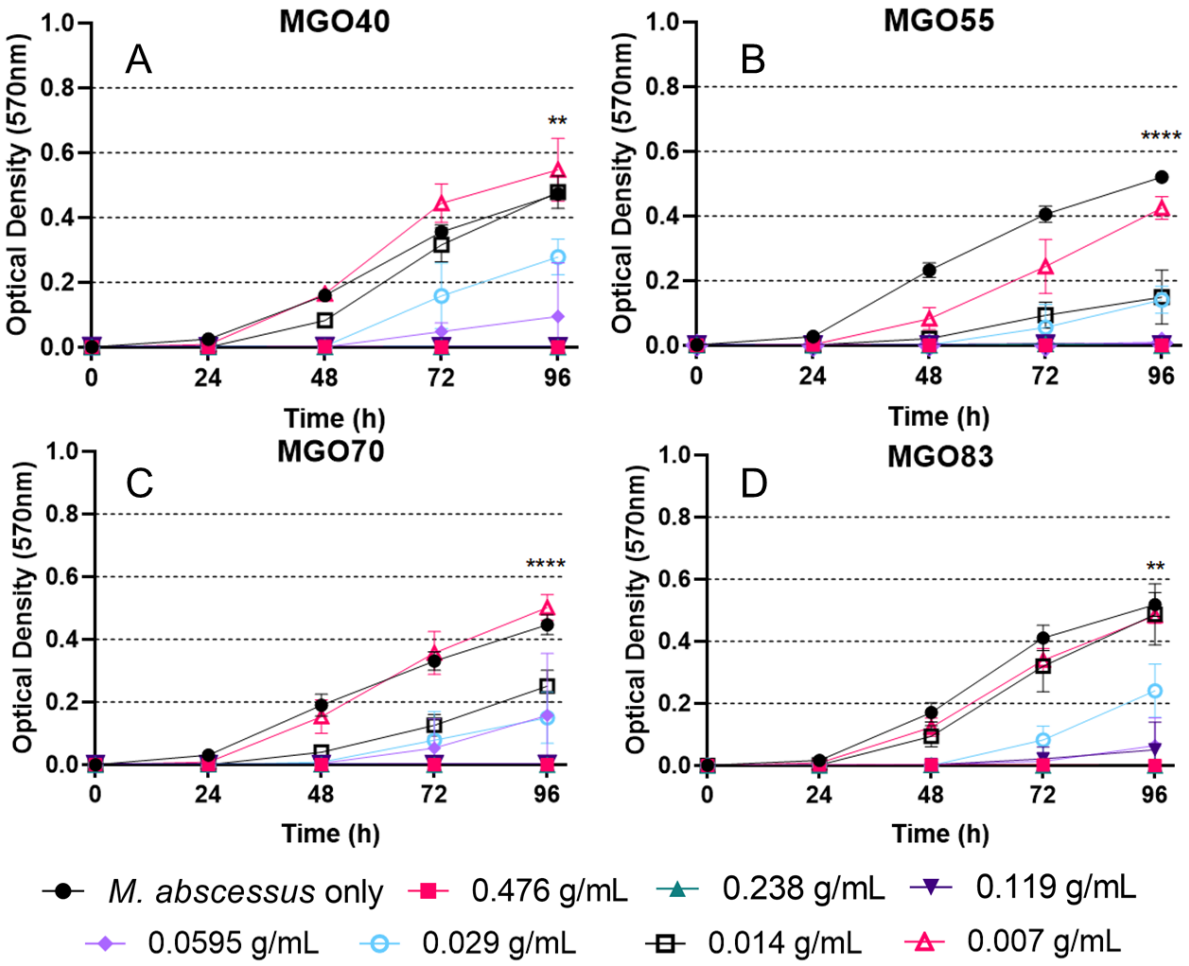

Figure 8.45 Growth Curves of *M. abscessus* DC088C, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088C at 0.119 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.0595 g/mL. A Kruskal-Wallis identified a significant difference between all honey treatments and no treatment, P=0.0056. B) MGO55 growth curve with an MIC of 0.0595 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.119 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, Kruskal-Wallis P=0.0068.

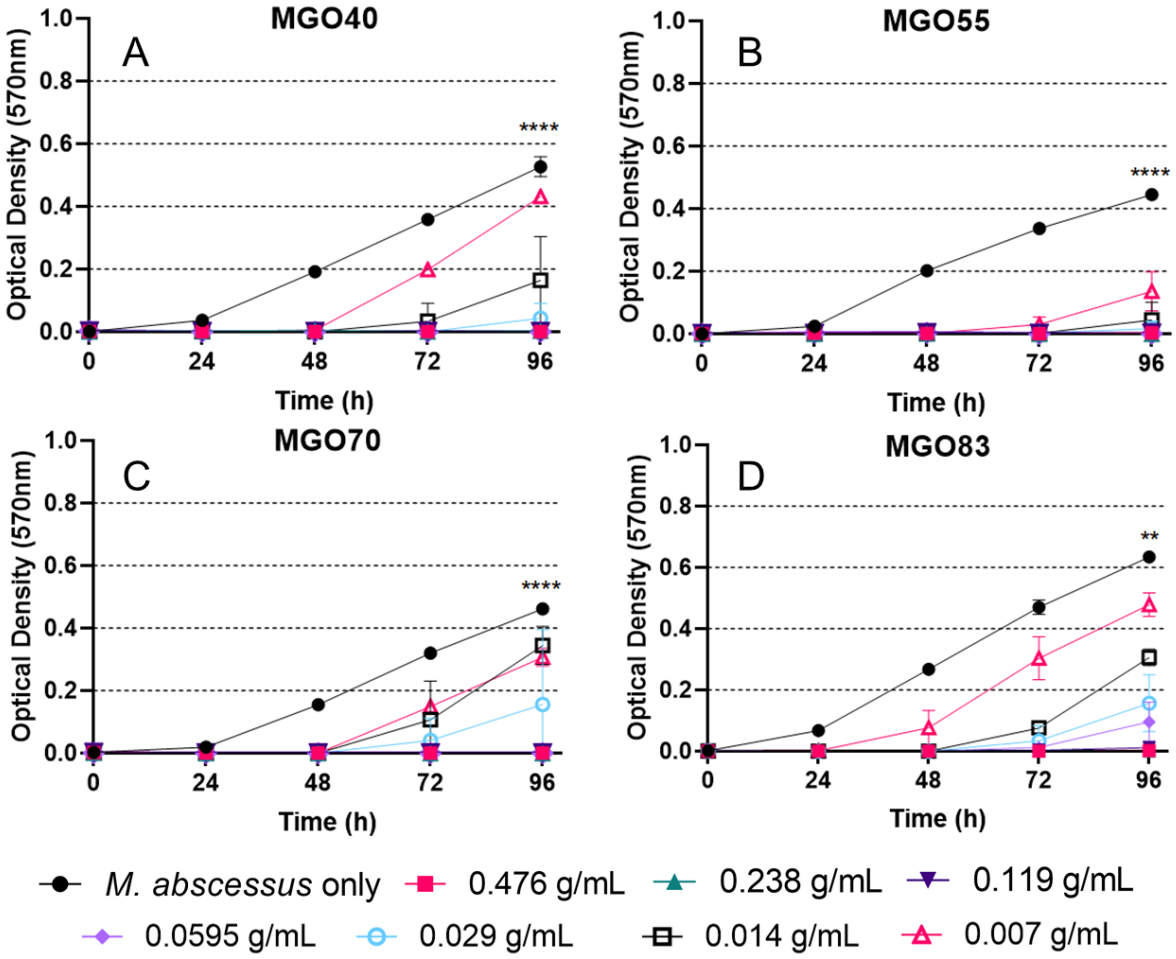

Figure 8.46 Growth Curves of *M. abscessus* DC088D, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088D at 0.0595 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.029 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.014 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.0595 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.119 g/mL. A significant difference was observed for honey treatments, Kruskal-Wallis P=0.0030.

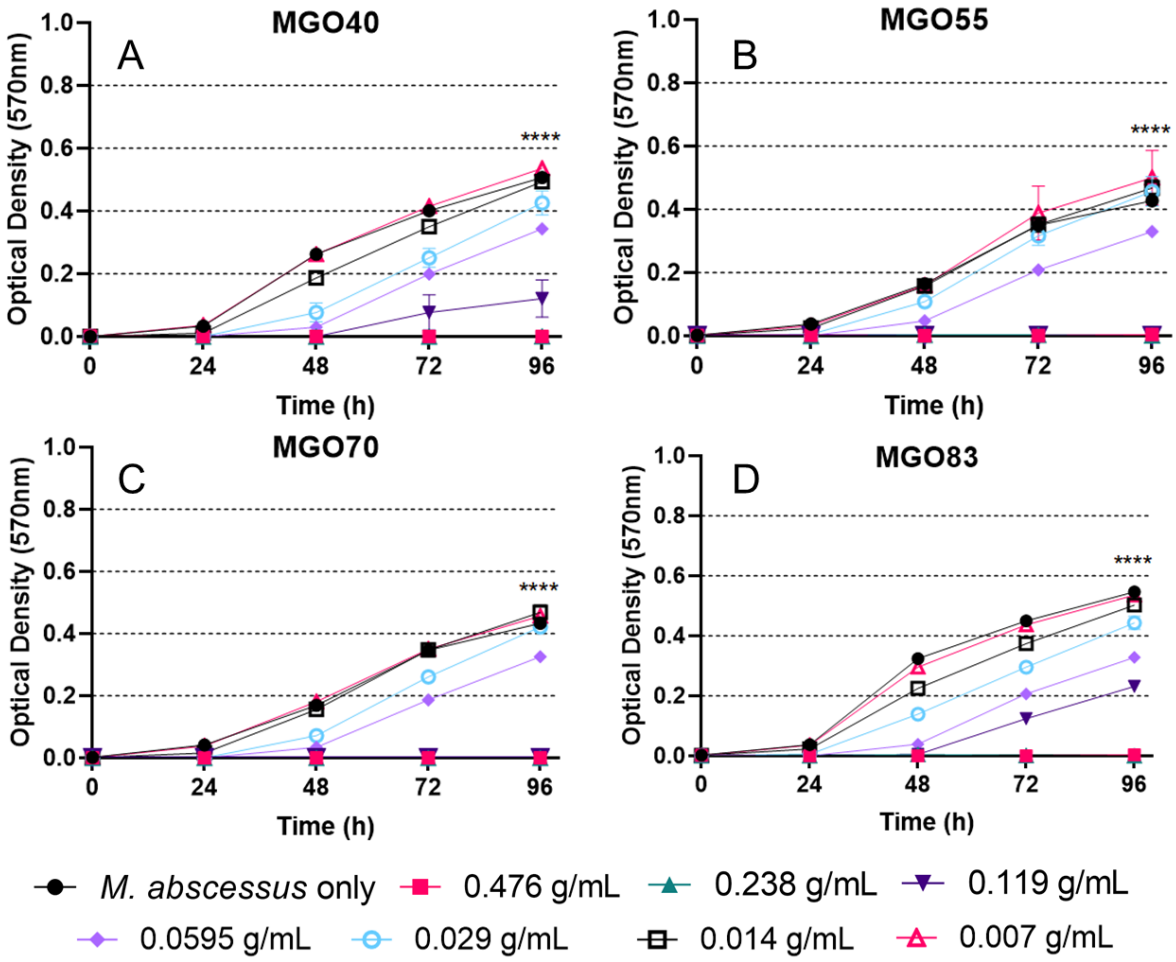

Figure 8.47 Growth Curves of *M. abscessus* DC088E, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088E at 0.238 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.238 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.119 g/mL, a one-way ANOVA identified a significant difference for all honey treatments P=<0.0001. C) An MIC of 0.119 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.238 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.

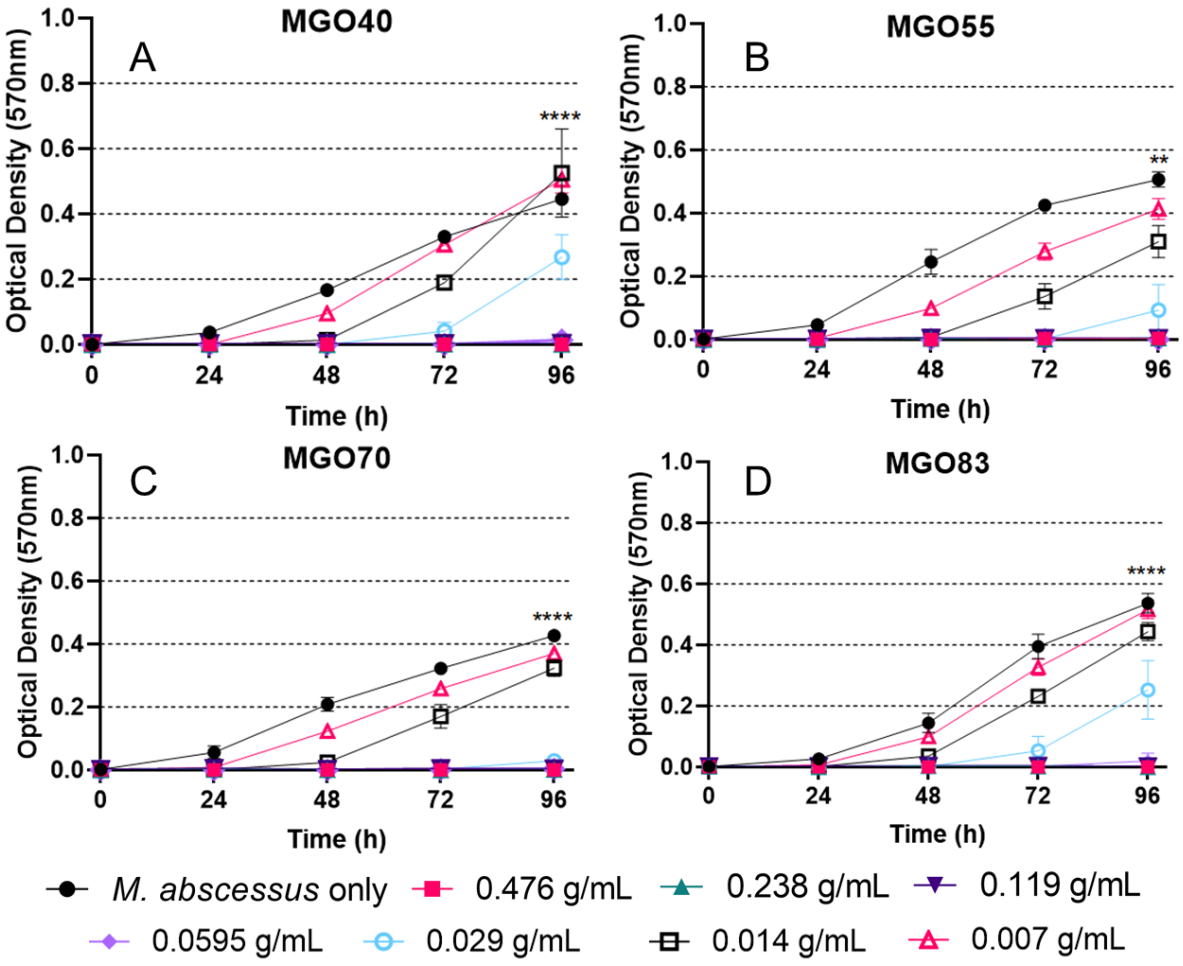

Figure 8.48 Growth Curves of *M. abscessus* DC088ref, cultured at 37 °C treated with 4 different manuka honey samples that were stored at 4 °C for 30 days prior to testing. Data shown are mean + SD for n=3 technical replicates. All 4 manuka honeys inhibited *M. abscessus* DC088ref at 0.0595 g/mL or less. End point data, taken at 96 h, was used for statistical analysis to determine the impact of manuka honey concentration on the growth of *M. abscessus.* A) MGO40 growth curve with an MIC of 0.0595 g/mL. A one-way ANOVA identified a significant difference between all honey treatments and no treatment, P=<0.0001. B) MGO55 growth curve with an MIC of 0.029 g/mL, a Kruskal-Wallis identified a significant difference for all honey treatments P=0.0086. C) An MIC of 0.029 g/mL was observed for MGO70. A significant difference was identified between all honey treatments, one-way ANOVA P=<0.0001. D) MGO83 exhibiting an MIC of 0.0595 g/mL. A significant difference was observed for honey treatments, one-way ANOVA P=<0.0001.