DEVELOPMENT AND OPTIMIZATION OF SYNERGISTIC ANTIMICROBIAL COMBINATIONS AGAINST A VARIETY OF NON-TUBERCULOUS MYCOBACTERIA OF CLINICAL IMPORTANCE

by
Emma Carolyn Eisemann

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ABSTRACT

Non-tuberculous mycobacteria (NTM) are an increasing threat to the health of immunocompromised and immunocompetent individuals. *Mycobacterium abscessus* (MAB) and *M. avium* subspecies *paratuberculosis* (MAP) are two species of NTM that threaten respiratory and gut health, respectively. These two organisms have demonstrated both intrinsic and acquired resistance to many antibiotics. Of the two, MAB is associated with drug resistance due to genomic mutations, whereas MAP is known to adapt to an anaerobic state during which antibiotic targets are inactive. In this study, broth microdilution was used for both MIC and synergy-based assays; for MAP, the Wayne model was used for anaerobic susceptibility testing. Once MICs were established, a sub-inhibitory concentration of clofazimine was added to varying concentrations of 14 antibiotics to identify synergistic combinations. Aerobic synergy was defined as a ≥4-fold decrease in the MIC for an individual drug versus the paired MIC alone. For MAB, synergy was demonstrated between clofazimine and 12/14 antibiotics. The greatest synergy against MAB occurred with clofazimine and doxycycline, where the fold decrease in the latter was 211.20. For MAP, 4 strains were adapted to anaerobiosis and treated with 16 drugs or combinations. The threshold for successful inhibitory activity was defined as a 1-$\log_{10}$ reduction in viable counts. Success followed exposure to 4-drug combinations containing bedaquiline, clofazimine, and metronidazole with rifaximin or clarithromycin, or alternatively, the combination of gentamicin, rifaximin, clarithromycin, and metronidazole. No single, 2-, or 3-drug combinations achieved the targeted 1-$\log_{10}$ decrease. The most active drug combination against anaerobically-adapted MAP was bedaquiline, clofazimine, metronidazole, and rifaximin**.** These studies demonstrate that synergy

testing of drug resistant NTM species should be considered as an alternative to standard susceptibility testing using single drugs, particularly in settings in which few treatment options are available. In addition, adaptation of MAP and possibly other NTMs to anaerobiosis raises questions regarding the ability of aerobically active antibiotics to eradicate such organisms *in vivo*. Future studies are needed to help correlate *in vitro* susceptibility results with *in vivo* efficacy, especially given the rising tide of multidrug-resistant NTM infections, including those caused by MAB and MAP.

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CHAPTER 1: Introduction to non-tuberculous mycobacteria (NTM)

1.1 Mycobacteria

Mycobacteria are most infamous for the species *Mycobacterium tuberculosis* and *M. leprae*, the causative agents of tuberculosis and leprosy, respectively. However, the genus, *Mycobacterium,* hosts over 170 species that range in pathogenicity, host, environment, virulence, and disease (Forbes, 2017).

Despite the great diversity of mycobacteria, there are characteristics that define *Mycobacterium* as a genus. All *Mycobacterium* species are nonmotile, non-spore-forming, rod-shaped bacilli. The mycolic acid-rich cell wall of *Mycobacterium* species is the main genus-defining characteristic, resulting in an extremely high lipid content relative to other bacterial genera, comprising up to 60% of the dry weight of the cell. These mycolic acids, with their long, hydrophobic fatty-acid chains, are covalently bound to the peptidoglycan-arabinogalactan carbohydrate matrix in the plasma membrane. The mycolic acids also lie below a variety of additional hydrophobically-associated lipids that intercalate the ends of each mycolic acid molecule. These secondary lipids include glycopeptidolipids, trehalose-containing lipopolysaccharides, sulfolipids, phthiocerol dimycocerosates, and phenolic glycolipids (Chiaradia *et al*., 2017). While mycolic acids are the distinguishing characteristic of mycobacteria, other lipid components and proteins in the outer-layer of the cell wall are antigenic and function as virulence factors aiding intracellular survival.

This unique, hydrophobic, lipid-rich cell wall composition places *Mycobacterium* in the class of acid-fast bacilli (AFB), closely related to Gram-positive bacteria. During the Gram-staining

procedure, the mycolic acids prevent the primary stain from penetrating the cell wall. Therefore, unlike most bacterial genera, *Mycobacterium* species cannot be Gram-stained and must be classified using an acid-fast stain, such as the Ziehl-Neelson. The Ziehl-Neelson uses carbolfuchsin as the primary stain. Carbolfuchsin is extremely lipid-soluble and when used, in combination with heat, penetrates the mycolic acid layer staining the bacilli red. Subsequently, the slides are cooled to room temperature and a harsh decolorizer is used to remove the primary stain from non-AFB organisms, which appear blue when a counterstain is applied.

Genotypic and phenotypic characteristics divide mycobacteria into two main groups: the obligate pathogens, M. *leprae* and the *M. tuberculosis* complex (MTBC), and the non-tuberculous mycobacteria (NTM). *M. leprae* and members of the MTBC are well-studied human pathogens causing prevalent and catastrophic diseases while NTMs are environmental opportunistic pathogens residing in water, soil, wild and domesticated animals, and several animal products that humans consume. Further classification divides this group into rapid-growing and slow-growing NTMs, based on the rate of colony formation in culture. Rapid-growers develop colonies in ≤ 7 days, while slow-growers develop colonies in ≥ 7 days. Both rapid- and slow-growing *Mycobacterium* species are responsible for disease. Common rapid-growing NTMs include *M. abscessus* and *M. fortuitum* while the most noted slow-growing NTM is the *M. avium* complex (MAC).

Compared to MTBC and *M. leprae,* there is a paucity of clinical and research data for the NTM. However, NTMs pose an increasing threat to public health as incidence and burden of infections rise globally (Strollo *et al*., 2015; Lin *et al*., 2018; Ratnatunga *et al*., 2020). *Winthrop et al*. (2020) demonstrated that between 2008 and 2015, in the United States, the annual

prevalence of NTM increased from 6.78 to 11.70 per 100,000 persons. The greatest increase in prevalence and incidence were observed in women and elderly persons (Winthrop *et al*., 2020; Park *et al*., 2019). Additionally, deaths attributed to NTM-infections are increasing in the United States, especially in elderly women without comorbidities (Mirsaeidi *et al*., 2014). In developed countries including the United States, NTM-infections are on a trend to replace *M. tuberculosis* as the leading cause of mycobacteria-related deaths (Vinnard *et al*., 2016).

An explanation for the increase in NTM infection has not been clearly elucidated. However, several factors are thought to play a role. Of these, improved diagnostics, detection, and identification of mycobacteria have resulted in an increase in reporting of NTM infections. Culture and AFB staining have historically been the gold standard for mycobacterial detection. However, these methods cannot distinguish between mycobacterial species. The GeneXpert (Cepheid, Sunnyvale, CA) can distinguish MTBC from NTM as can several other methods including matrix-associated laser desorption/ionization-time of flight mass spectrometry (MALDI-Tof-MS), and other molecular sequencing-based technologies. The GenoType CM/AS (Hain Lifescience, Nehren, Germany) can distinguish between mycobacterial species, as it uses PCR, reverse hybridization, and a line probe to identify specific species (Richter *et al*., 2006). Additional PCR-based techniques are also common and are able to achieve species-level identification (Singh *et al*., 2020; Sarro *et al*., 2021).

Other contributors to the increase in NTM infections in developed countries include environmental factors impacting human contact with NTMs and decreased BCG vaccine administration to children. It is known that NTM, in part because some species are naturally resistant to antimicrobials, are common in water reservoirs, infrastructure, and bathroom

fixtures such as pipes, shower heads, and faucets. It is possible that the presence of NTMs in these environments is increasing contact between the organisms and humans with a concomitant increase in infections (Nishiuchi *et al*., 2017). However, this possibility has not been well studied or established. Better documented is the correlation between increased NTM incidence in young children and the decline of BCG vaccination in developed countries (Lacroix *et al*., 2018; Kontturi *et al*., 2018). Many developed nations where tuberculosis is well controlled do not require or offer BCG vaccination for children. It is hypothesized that cross-reactive immunity between BCG, a member of the MTBC, and NTMs may provide some protection against NTM infection in addition to tuberculosis. Thus, in settings in which BCG vaccinations decreased, NTM incidence may increase (Zimmerman *et al*., 2018).

1.2 NTM infection and virulence

NTM infections most commonly cause pulmonary disease, but also infect skin, intestinal, and lymphatic tissues. Several species of NTM, like *M. abscessus* and the *Mycobacterium avium* complex (MAC), have been previously acknowledged as a major respiratory threat to immunocompromised individuals, most commonly with chronic lung diseases or HIV. However, NTM infections have also gained attention as an increased threat in immunocompetent individuals with regard to hospital-acquired infections.

NTMs, like all other infectious organisms, utilize virulence factors to establish, maintain, and accelerate infections. Examples include the ability to switch between smooth and rough morphologies, the use of type-VII secretion systems to mitigate nutrient uptake, conjugation, and

phagolysosome escape, and the generation of biofilms on implanted medical devices (Howard *et al*., 2006; Faria *et al*., 2015; Lagune *et al*., 2021). Although virulence factors facilitate NTM infection and progression, the most clinically relevant and worrisome issue for treating infection is the prevalence of both intrinsic and acquired antibiotic resistance in NTM populations. Intrinsic and inducible antibiotic resistance mechanisms complicate the already difficult and individualized treatment regimens needed to suppress infection. NTM infections are usually treated with poorly-tolerated, often toxic, long-term combination-antibiotic therapies that vary depending on the species of NTM as well as the site of infection (Gopalaswamy *et al*., 2020). One possible solution which may decrease the emergence of antibiotic resistance while improve the toxicity and tolerability profile of various antibiotics is using combination drug therapies containing lower doses of synergistic drugs. Currently, there is a significant gap in knowledge regarding synergistic combinations targeting NTM species, both in vitro and in vivo, which must be addressed in order to gain key insights into best practices for treatment of NTM infections.

1.3 Antibiotic treatments for NTM infections

1.3.1 Antimycobacterial class

Antimycobacterial treatment regimens for NTM disease often include ethambutol, rifampin, and rifabutin. Although isoniazid and ethionamide are used in tuberculosis treatment regimens, most NTM are not susceptible to these drugs. Therefore, these drugs are rarely used in NTM treatments. Bedaquiline and clofazimine are second-line antibiotics used to treat tuberculosis, and likewise, are only used to treat NTM-infections when there is no alternative. Ethambutol kills mycobacteria by inactivating three arabinosyltransferases (EmbA, EmbB, and

EmbC) that translocate the mycobacterial cell membrane and catalyze the production of arabinogalactan, the component of the mycobacterial cell wall that connects the peptidoglycan layer to the outer mycolic acids (Alderwick *et al*., 2015). Ethambutol and rifampin are first-line drugs for tuberculosis treatment and, in the context of NTM infections, are more commonly used to inhibit slow-growing NTMs, like MAC.

The other antimycobacterial drugs work by shutting down a variety of cellular processes essential for growth, division, and replication. Rifampin and rifabutin, instead of inhibiting cell wall synthesis, inhibit DNA-dependent, RNA polymerase. Bedaquiline targets ATP synthase subunit c, without which the bacteria cannot produce enough ATP and energy for survival. Clofazimine, unlike the other antimycobacterials, is a prodrug, which interacts with NADH dehydrogenase inside the cell producing reactive oxygen species (ROS). In addition to the production of ROS, clofazimine also competes with the normal NADH dehydrogenase substrate, menaquinone, without which the mycobacterial electron transfer chain and ATP production become impaired (Lechartier & Cole, 2015).

1.3.2 Non-mycobacteria specific antibiotic classes

In addition to the previously mentioned antimycobacterial drugs, many nonmycobacterial-specific antibiotics may be used as components of the NTM treatment regimen.

Fluoroquinolones are an antibiotic drug class that inhibits DNA and RNA synthesis by interacting with DNA type II and IV topoisomerases that are essential for alleviating tension on

the DNA strands from supercoiling during replication or transcription. When a fluroquinolone drug interacts with the topoisomerase, the enzyme is unable to alleviate the tension caused by supercoiling resulting in DNA fragmentation, rendering the cell unable to replicate. Two fluoroquinolones that are often used in rapid- and slow-growing NTM treatment regimens are ciprofloxacin and moxifloxacin. Unfortunately, fluoroquinolones are commonly used to treat a broad range of not only mycobacterial but also other bacterial infections and, consequently, resistance to fluoroquinolones is common.

Other classes of antibiotics that are used to treat NTM-infections inhibit a component of translation in the bacterial cell. The aminoglycoside class of antibiotics bind to the A-site of the 30S ribosomal subunit. The drug-ribosome interaction disables the ribosome from binding to the incoming tRNA that is carrying the amino acid to be added to the growing peptide chain. Without translation, the bacterial cell is unable to produce the proteins it needs to function and survive. Common aminoglycosides found in NTM-treatment regimens include amikacin, streptomycin, gentamicin, and tobramycin. Like the aminoglycosides, the tetracycline antibiotics also shut down bacterial translation by binding to the A-site on the 30S ribosome, inhibiting tRNA binding and peptide elongation. Common tetracycline drugs in NTM-treatments include doxycycline, tigecycline, and minocycline.

Likewise, macrolide and oxazolidinone antibiotic classes also shut down bacterial translation. However, unlike the aminoglycosides and tetracyclines, the drugs in these classes do not act by inhibiting binding of the tRNA to the ribosome on the 30S subunit. Macrolides bind to the exit-site of the 50S ribosomal subunit and act as a plug, blocking the extension of the peptide chain. The most common macrolide in NTM treatments is azithromycin, which is used to treat

Gram-positive bacterial infections and mycoplasma species that lack a traditional cell wall (Alvarez-Elcoro & Enzler, 1999). The oxazolidinones bind to the 50S ribosomal subunit at the P-site where peptides are bound to the growing peptide chain. This interaction also disables the addition of subsequent amino acids. The oxazolidinone antibiotic of interest to NTM-infections is linezolid.

The beta-lactam antibiotic class is an extremely common class of drug that is used to treat both Gram-positive and Gram-negative bacterial infections. The beta-lactams inhibit cell wall synthesis, but unlike isoniazid, ethambutol, and ethionamide, beta-lactam antibiotics are not specific to mycobacterial cell-wall components. The beta-lactam antibiotics bind to transpeptidase enzymes that are responsible for crosslinking peptidoglycan molecules during cell wall synthesis. The beta-lactam class is further divided into subclasses, several of which contain antibiotics common in rapidly-growing NTM treatments: cephalosporins, carbapenems, and penicillin drugs. These antibiotics include cefoxitin, ceftriaxone, and cefepime in the cephalosporin subclass, imipenem in the carbapenem subclass, and amoxicillin/clavulanate in the penicillin subclass. Clavulanate is a beta-lactamase inhibitor, commonly paired with amoxicillin, which shuts down beta-lactamase enzymes that the bacteria may produce to degrade the antibiotic.

The last class of the non-mycobacterial-specific antibiotics are the sulfonamides. The "sulfa drugs" were the first class of antimicrobial to be discovered, and resistance has spread rapidly, so they are no longer commonly used to treat infections (Sköld, 2000). However, when bacteria are susceptible, sulfonamides disable bacterial production of folate, which is essential for bacterial survival. This inhibition results from the drug binding to the dihydropteroate

synthase enzyme and arresting folate biosynthesis. The sulfonamide used in NTM treatments is trimethoprim/sulfamethoxazole.

1.4 NTM mechanisms of antibiotic resistance

1.4.1 Resistance mechanisms in the NTM cell wall

Many antibiotics rely on diffusion across the cell wall and membrane into the cell where they can interact with their target molecules. However, mycolic acids in the mycobacterial cell wall contribute to an extremely hydrophobic environment through which only hydrophobic antibiotics can diffuse. This leaves more hydrophilic antibiotics, such as several macrolide and beta-lactam drugs, reliant on porin channels for cellular entry. However, compared to Gram-negative bacteria like *Escherichia coli*, mycobacteria can have 45-fold fewer membrane porins (Niederweis, 2003). Combined with the hydrophobicity of the cell wall, mycobacteria are only able to take up 1% of antibiotics, nutrients, and other compounds compared to *E. coli* (Jarlier and Nikaido, 1994). Taken together, these mycobacterial cell wall characteristics greatly inhibit entry of antibiotics into cells and, thus, complicate treatment options available to clinicians.

NTMs may also encode and express antibiotic efflux pumps, adding to the mycobacterial cellular defense. Unlike the lipid-rich cell wall, efflux pumps are not natural cell wall characteristics that act as a barrier to antibiotics. Rather, efflux pumps are encoded by genes and expression can be induced. Efflux pumps are ATP- or electrochemical gradient-powered transmembrane proteins that expel antibiotics from the cell cytoplasm into the extracellular space. This expulsion of antibiotic prevents intracellular drug concentrations from accumulating

to levels able to induce bacterial cell death. Further, some efflux pumps in NTMs have been well characterized and the specific mechanisms have been elucidated. *LfrA* was the first noted efflux pump in mycobacteria while *pstB*, *tetV*, and Tap are also NTM efflux pumps for rifampin, aminoglycosides, and tetracyclines (Takiff *et al*., 1996; Liu *et al*., 1996; Gupta *et al*., 2006; De Rossi *et al*., 1998; Aínsa *et al*., 1998; Ramón-García *et al*., 2006; Rindi, 2020). Other efflux pumps that have been identified in both rapid- and slow-growing NTMs include *mmpS-mmpL* efflux of bedaquiline and clofazimine in *M. abscessus* and MAV_3306, MAV_1406, and MAV_1695 efflux of macrolides in MAC species (Gutiérrez *et al*., 2019; Schmalstieg *et al*., 2012; Machado *et al*., 2015).

1.4.2 Resistance mechanisms in the NTM cytosol

Beyond the cell wall, the porins, and the efflux pumps, NTMs contain other intracellular resistance mechanisms. The main mechanism of resistance that occurs in the cytosol of mycobacteria is biotransformation. Biotransformation is the interaction between a drug and bacterial enzyme that inactivates that drug. Several specific mechanisms of biotransformation have been documented in mycobacteria. Acetylation and nitrosation of ethambutol, fluoroquinolones, and macrolides have been documented in several NTM species (Adjei *et al*., 2007; van Ingen *et al*., 2012). Additionally, NTM aminoglycoside O-phosphotransferases and ADP-ribosyltransferases inhibit streptomycin and rifampicin, respectively (Ramirez & Tolmasky, 2010; van Ingen *et al*., 2012; Baysarowich *et al*., 2008).

The beta-lactamases are a major group of enzymes that biotransform and disable various beta-lactam antibiotics across various genera of bacteria. In NTMs, examples of these enzymes include the major beta-lactamase, *blaS,* and the cephalosporin-specific beta-lactamase, *blaE* (Flores *et al*., 2005). One major reason that penicillin drugs are rarely used in mycobacterial treatments is the presence of beta-lactamases corresponding with penicillin resistance.

1.4.3 Mutation induced antibiotic resistance

Further inside the cell, resistance mutations naturally accumulate in DNA encoding the targets of gene products. When these mutations do not significantly impact the function of the gene product, but do inhibit the interaction of the antibiotic with the target molecule, the bacteria may be phenotypically resistant. It is important to note that mutant gene products, although often less efficient than their wild-type counterpart, are commonly selected for due to exposure to a given antibiotic. Oftentimes, antibiotic resistance resulting from mutation can be circumvented by combination antibiotic therapy.

There are several NTM genes that are subject to mutations resulting in acquired resistance to antibiotics. Broadly, mutations in the ribosomal RNA may interfere with the activity of a range of antibiotics which target translation. However, these mutations cannot completely inhibit ribosomal function, or the bacterial mutant will not be viable. Usually, these mutations occur in areas close to but not within the active part of the A-site, P-site, or E-site on ribosomal subunits. For example, mutations in the 16S ribosomal subunit gene, *rrs*, have conferred resistance to aminoglycosides, like amikacin, in *M. abscessus* and *M. chloenae* (Prammananan *et*

al., 1998). Documented mutations in the 23S ribosomal subunit gene confer resistance to macrolide antibiotics in MAC and *M. abscessus* (Bastian *et al*., 2011; Meier *et al*.,1994). The *rpoB* gene in *M. tuberculosis,* which typically functions during bacterial transcription, acquires mutations that confer rifampin resistance. Like many *M. tuberculosis* traits, these *rpoB* mutations are not specific to *M. tuberculosis* and can be found in NTMs as well, also conferring phenotypic resistance to rifampin.

1.4.4 Relevance

The increasing prevalence of NTM infections coupled with the large levels of intrinsic and emerging antibiotic resistance has spiked the need for new antibiotic treatment regimens. In efforts to better understand methods of NTM antibiotic evasion and persistence as well as develop better NTM treatment regimens, the experiments discussed hereafter focused on defining antibiotic synergy for the treatment of two, clinically relevant NTM species, *M. abscessus* and *M. avium* subspecies paratuberculosis. The data presented here can be applied to more successful NTM treatments and will contribute to an improved understanding of the role these opportunistic pathogens may play in associated chronic medical conditions.

CHAPTER 2: Antibiotic resistance and synergy against clinical *Mycobacterium abscessus* isolates

2.1 Abstract

M. abscessus is a NTM causing a significant threat to individuals with chronic respiratory disease and immunosuppressive disorders, like cystic fibrosis (CF). Antibiotic combination therapies, known for toxicity and poor patient tolerance, are the only treatment option for individuals with *M. abscessus* infections, and have led to high levels of antimicrobial resistance. Identifying combination therapeutic approaches that will result in drug synergy may enable lower doses of antibiotics, clinically, and potentially less toxicity. Previous research has demonstrated that clofazimine (CFZ), a second-line antimycobacterial, may act synergistically when combined with a variety of antibiotics (Huang *et al*., 2018; Obregón-Henao *et al*., 2015; Ruth *et al*., 2019; van Ingen *et al*., 2012). These data demonstrate that CFZ does demonstrate widespread synergy with 14 traditionally non-antimycobacterial drugs against 6 clinical multi-drug resistant *M. abscessus* isolates. CFZ, at sub-inhibitory concentrations, reduced the MIC of doxycycline (DOX), a tetracycline drug, consistently, by approximately 128-fold for five of the six isolates tested. The novel antibiotic synergy combinations demonstrated provides insight to potential optimization of clinical treatment regimens and suggest that clinical laboratories should add antibiotic synergy testing to the currently recommended single-drug susceptibility testing.

2.2 Introduction to *Mycobacterium abscessus* and association with antibiotic resistance and disease

M. abscessus is a rapid-growing, environmental NTM mostly found in water and soil. However, the organism is also increasingly notable for respiratory infections in immunocompromised individuals and those with underlying chronic diseases, such as cystic fibrosis (CF) (Degiacomi *et al*., 2019). *M. abscessus* infections pose a threat to these individuals because a patient mutation in the gene encoding the transmembrane conductance regulator protein results in the buildup of thick, sticky mucus, which prevents the body from expelling trapped bacteria. Without regular expulsion, *M. abscessus* can further multiply and progress to the lower respiratory tract where infection is most frequently established. Often, these infections lead to chronic pulmonary disease, despite antibiotic treatment. Although most recognized for causing respiratory infections, *M. abscessus* is also the causative agent of infections in the skin and mucosa throughout the body.

After exposure, *M. abscessus* establishes infection via evasion of phagocytosis and survives, intracellularly, within macrophages. One key virulence factor for this species is conversion between morphotypes where, after infection is established, the bacterium can switch between a rough and smooth cell wall. The rough morphotype is better able to evade the immune response because the lack of glycoproteins in the rough cell wall are less recognized by innate immune receptors (Johansen *et al*., 2020). These virulence factors, among others, complicate immune clearance of the infection.

Antibiotic resistance of *M. abscessus* is a major reason that treatment regimens require a year, minimum, of macrolide-combination therapy intravenously and orally. However, there is

no standardized broad range NTM-treatment regimen. Current combination regimens are poorly-tolerated, often have toxic side effects, and are at least a year in length. For this reason, new antibiotic combinations and therapies are desperately needed.

M. abscessus is commonly recognized as one of the most antibiotic-resistant mycobacteria, with resistance genes and mechanisms to several classes of antibiotics including fluoroquinolones, macrolides, beta-lactams, and aminoglycosides, and several antimycobacterials including bedaquilline, clofazimine (CFZ), ethambutol, and rifampicin (Johansen *et al*., 2020). In addition to intrinsic resistance and acquired mechanisms of resistance discussed in chapter one, *M. abscessus* encodes two transpeptidases, L,D- and D,D-transpeptidase, to build the cell wall. Because beta-lactam drugs will only target one form of the enzyme, if one transpeptidase form is inactivated by a drug, the other transpeptidase form is available for cell growth and division (Story-Roller *et al*., 2019). Assessments of *in vivo* synergy between two beta-lactam drugs collectively targeting both L,D- and D,D-transpeptidases have demonstrated the best synergy is between imipenem and cefoxitin, cefdinir, doripenem, or biapenem (Story-Roller *et al*., 2019). Likewise, studies have demonstrated synergy between limited combinations of antibiotics, but none have analyzed synergy between a broad range of drugs across several classes of antibiotics and species of NTM.

Previously, clofazimine combined with other antimycobacterials demonstrated synergy against *M. abscessus* and other NTMs (Huang *et al*., 2018; Obregón-Henao *et al*., 2015; Ruth *et al*., 2019; van Ingen *et al*., 2012). However, whether the synergy is maintained across a broader range of drug-combinations has not been studied. Our goal here was to assess the synergy of CFZ,

a drug previously used for the treatment of leprosy, with a broad variety of rapid-growing NTM antibiotics using a broth microdilution assay.

2.3 Materials and methods

All *M. abscessus* strains were maintained on Löwenstein–Jensen agar slants (Remel, Lenexa, KS) at 30ºC. CFZ powder (Sigma Aldrich, St. Louis, MO.) was stored at 4ºC as a powder and -20ºC as a 1 mg/ml solution in dimethyl sulfoxide (DMSO) (Sigma Aldrich). Sensititre™ RAPMYCO 96-well plates (Thermo Scientific, Waltham, MA) were stored at room temperature. Middlebrook 7H9 (M7H9) broth (Hardy Diagnostics, Santa Maria, CA) and Sensititre™ M7H9 with oleic acid, albumin, dextrose, and catalase (OADC, Thermo Scientific) were both stored at 4°C until use.

2.3.1 Drug preparation

CFZ stock solution was made in DMSO (Sigma Aldrich) so that the concentration was 1 mg/ml. On the day of use, the CFZ stock solution was diluted to 800 µg/ml in DMSO (Sigma Aldrich), from which an 84 µg/ml working stock was prepared in sterile deionized water (SDW). The CFZ working stock was diluted on a sterile, blank 96-well microtiter plate (Greiner bio-one, Monroe, NC), in DMSO (Sigma Aldrich), as illustrated in Figure 1.

2.3.2 Inoculum preparation

A 0.5 McFarland standard was prepared for each *M. abscessus* strain by inoculating a sterile bead tube filled with M7H9 broth (Hardy Diagnostics) from growth on solid media, transferring the supernatant to a second sterile tube, and adjusting the suspension using a nephelometer (Beckman Coulter, Brea, CA) for a final reading of 0.06 (Hardy Diagnostics). The

final inoculum was prepared by adding 50 µL of the adjusted suspension to 11 ml of M7H9 broth with OADC (ThermoFisher Scientific).

Viable counts were determined for each inoculum to ensure a target range of $5x10^4$ to $1x10^6$ CFU/ml. To do this, each final inoculum was diluted 1:50 in 490 µL NERL® Reagent Grade Water (Thermo Scientific). Subsequently, using a calibrated, sterile inoculating loop, 10 µL of the undiluted (1X) and diluted (1:50) inoculum was streaked onto one half of a corresponding M7H11 plate (Hardy Diagnostics). All plates were incubated for three days, inverted, at 30°C followed by determination of viable counts.

2.3.3 Sensititre™ minimum inhibitory concentration (MIC) assay

The Sensititre™ RAPMYCO plates (Thermo Scientific) contain a series of concentrations of 15 antibiotics commonly used to inhibit rapid-growing mycobacterial species. The antibiotics and concentrations are illustrated in Figure 2A. Plates were inoculated by adding 100 µl of each adjusted suspension in OADC to that strain's specified plate. Plates were then sealed with a sterile adhesive provided by Thermo Scientific, arranged no more than 3 plates per stack, and incubated at 30°C. After three days of incubation, plates were examined for visible growth using an inverted mirror box. The minimum inhibitory concentration (MIC) for each antibiotic was defined as the well containing the lowest concentration inhibiting visible growth for each antibiotic.

2.3.4 CFZ broth microdilution MIC assay

On a second blank 96-well microtiter plate (Grenier bio-one), 5 µl of the diluted CFZ was added to each well so that each row contained one concentration of CFZ, with row A containing the highest concentration and row H containing the lowest concentration of CFZ, illustrated in Figure 1. Two columns without drug were used as positive controls. After the CFZ was added to the wells, 100 µl of inoculum was added to each well. Two biological replicates were performed for each strain used in this assay. The plate configuration and set up are illustrated in Figure 1. Plates were then sealed with a sterile microplate adhesive (US Scientific, Ocala, FL) and incubated at 30ºC. After three days of incubation, plates were examined for visible growth using an inverted mirror box. The MIC for CFZ was defined as the well containing the lowest concentration inhibiting visible growth.

2.3.5 Clofazimine synergy assay

After the CFZ MICs for each strain were determined, the synergy assay was performed using Sensititre™ RAPMYCO plates (Thermo Scientific) and the same method of inoculum preparation. First, the CFZ working solution was prepared as previously described and diluted in DMSO (Sigma Aldrich) to the previously determined MIC, before diluting once more so that the final concentration was two-fold below the respective MIC for each strain to be tested. Once adjusted to the appropriate concentration, 5 µL of the CFZ was added to each well of a Sensititre™ RAPMYCO plate (Thermo Scientific), except for the marked positive control. Following this, 100 µl of inoculum was added to all wells and the plate sealed with the Thermo Scientific provided

adhesive. The CFZ synergy plate was read, and MICs determined as previously described in the Sensititre™ MIC assay method (Section *2.3.4*). Synergy between the sub-inhibitory concentration of CFZ and the paired antibiotic was noted if the MIC of the paired antibiotic was reduced by at least 4-fold. Two biological replicates were performed for all strains used in this assay.

2.4 Results

All positive controls grew without contamination and colony count plates indicated the inoculum of each isolate was between $5x10^4$ and $1x10^6$ CFU/ml. No DOX MIC was recorded for ABC 5 (Table 1) , so DOX/CFZ synergy was not included in analysis with this strain.

MIC results for all antibiotics alone and in combination with CFZ are displayed in Tables 1 - 3. The addition of CFZ at sub-inhibitory concentrations lowered the MICs of all antibiotics tested. However, variability was seen based on the isolate, drug combination, and original drug MIC. The two exceptions were demonstrated with the combination of CFZ (4 µg/ml) and SXT with isolate ABC 9 and the combination of CFZ (1 µg/ml) and IMI to isolate ABC 4 (Tables 2-3; Figure 3). All other isolates and synergy combinations (81 total) demonstrated at least a 4-fold decrease in MIC (Figure 3). The MIC fold-reduction resulting from the addition of a sub-inhibitory concentration of CFZ to MIN or AXO were constant for all isolates, 16-fold and 32-fold, respectively.

The largest decrease in MIC was observed with isolate ABC 5 where the combination of CFZ (2 µg/ml) and TGC resulted in a decrease in MIC from >4 µg/ml to 0.03 µg/ml (Table 1). All isolates with MICs to a given antibiotic consistent with resistance, defined as per the CLSI (Clinical Laboratory Standards Institute) recommendations, demonstrated susceptibility with the addition of a sub-inhibitory concentration of CFZ (CLSI Guideline M24 3rd Edition, 2018 & CLSI Guideline M62 1st Edition, 2018). This held true for all isolates and antibiotics where 100% of resistant strains (34/34) decreased their MICs to within the susceptible range when combined with a sub-inhibitory concentration of CFZ.

CFZ and DOX together demonstrated the greatest change in MIC across the six isolates (Figure 3). When combined with a sub-inhibitory concentration of CFZ, the MIC for DOX decreased from >16 μg/ml to 0.12 μg/ml for 4/6 isolates and 0.5 μg/ml for a single isolate (Table 1). No DOX MIC, and therefore no DOX MIC fold-reduction, was recorded for isolate ABC 5, however, the recorded DOX MIC after the addition of a sub-inhibitory concentration of CFZ was 0.12. In contrast, the least potent combination was observed with CFZ and FOX, where all the MICs for FOX alone were 32-64 μg/ml, which reduced to 4 μg/ml for all isolates with CFZ. By drug class, CFZ demonstrated the best synergy with the tetracycline antibiotics (Tables 1-3 & Figure 3).

MIC fold-reduction varied across isolates for several antibiotics, including SXT, CIP, AMI, TGC, LZD, IMI, and FEP after addition of sub-inhibitory CFZ (Figure 3). However, differences were noted between *M. abscessus* isolates. No synergy was observed with the addition of CFZ (4 μg/ml) to SXT with ABC 9, whereas the addition of CFZ (1 μg/ml) to SXT with ABC 4 resulted in a 64-fold reduction in the SXT MIC. With the exception of TGC, which demonstrated variable MICs in combination with CFZ, MICs for all other antibiotic-CFZ combinations resulted in the same MIC for all strains tested (Tables 1-3).

Due to the variability between biological replicates, there were no statistics performed during data analysis. However, overall trends were determined for each strain-antibiotic combination to evaluate if the same or different.

2.5 Discussion and future directions

This data supports the hypothesis that the combination of sub-inhibitory concentrations of CFZ to various other antibiotics *in vitro* would result in synergy against several clinical isolates of multi-drug resistant *M. abscessus*. These results are a compelling first step to provide a more comprehensive susceptibility-testing algorithm for multi-drug resistant *M. abscessus*. However, the small sample size used in this study warrants testing of a greater number of clinically-derived *M. abscessus* isolates with possible expansion to other NTM species, including both rapid- and slow-growers, such as *M. fortuitum*, *M. cholenae*, and MAC species. Should CFZ demonstrate synergy with a broad range of antibiotics against a wider number of NTM species, there may be basis for clinical trials with an eye towards preventing the development of drug resistance while shortening the course of treatment. For patients with multi-drug resistant NTM infections, few options exist for antimicrobial therapy. Multiple drugs, which are synergistic *in vivo* are needed to overcome the growing number of resistant infections.

This study has several limitations. The sample size was small and narrow with regards to the selection of NTM species. Thus, it remains unknown if exposure to decreased concentrations of antibiotics would add pressure, which would facilitate the development of antimicrobial resistance over time. Temporal genomics and transcriptomics data could partially help to answer these questions for individual antibiotics as well as combinations. However, this was beyond the scope of this project. In addition, CFZ-antibiotic combinations overall lowered the MICs of most antibiotics to the lowest concentration tested. This was regardless of the starting MIC for each antibiotic alone and the concentration of CFZ added. In all cases, the actual MIC for each antibiotic when combined with CFZ may have been below the minimum concentration tested.

Expanding the range of concentrations tested below the current minimum would better determine if the fold-decrease was proportional to the concentration of CFZ or paired drug MIC. This is especially important since most of the antibiotics utilized in this study have been well-characterized in terms of pharmacokinetic properties and we are only now beginning to understand the pharmacodynamics of individual drugs and combinations against MAP.

In addition to increased sample sizes and a broadened range of NTM species, more antimycobacterial drugs should be tested for synergy. Bedaquiline is a relatively new antibiotic developed for *M. tuberculosis* that may inhibit NTM growth synergistically with broader range aminoglycosides, fluoroquinolones, beta-lactams, tetracyclines, oxazolidinones, and sulfonamides. Other antibiotics, which could be tested in the synergy assay described in this study, include newer carbapenems, oxazolidinones, and cephalosporins.

This data demonstrates a novel approach to susceptibility testing of the NTM, which diverges from the traditional method currently in use which utilizes single drugs versus a synergy-based algorithm designed to identify inhibitory drug combinations against multi-drug resistant NTM. Overall, MICs for all 14 individual drugs were reduced when combined with a sub-inhibitory concentration of CFZ. However, significant strain-to-strain variability was noted between drugs with the exception of DOX, which had the most consistent inhibitory activity and demonstrated the greatest amount of synergy. Studies aimed at using 2, 3, and 4-drug combinations of antibiotics with different targets and mechanisms of action may help to elucidate the effectiveness of using such an approach to decrease the emergence of resistance while maximizing bactericidal activity.

This data supports further expansion of the number of clinical isolates and NTM species to be tested as well as increased ranges of drug concentrations. Additional studies could also examine development of drug resistance *in vitro* while following exposure to single versus combinations of antibiotics. This additional data would provide important considerations before initiation of clinical trials. Such clinical trials could be envisioned to provide for therapeutic NTM regimens, treatment, and determination of toxic side effects. For the NTM, the correlation between *in vitro* susceptibility and *in vivo* efficacy for the most part does not exist. Thus, this study is an important first step in trying to provide valuable information regarding an increasingly important issue.

CHAPTER 3: Adaptation of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) to anaerobic conditions and differential antimicrobial susceptibility

3.1 Abstract

Mycobacterium avium subspecies paratuberculosis (MAP), the causative agent of Johne's disease in cattle and ruminants, is controversially associated with Crohn's disease (CD), in humans. In addition to failing to meet Koch's Postulates, evidence that disassociates MAP from the etiological agent of CD includes unsuccessful curative treatment of CD patients with antimycobacterials. Previous investigators demonstrated that MAP can adapt to anaerobiosis using the Wayne Model of non-replicating persistence, and that many single antibiotics are not active under anaerobic conditions. In the current study, various combinations of antibiotics demonstrated synergy under aerobic and anaerobic conditions. Aerobic combinations, which showed marked inhibition in growth were those containing rifaximin (RFX) and gentamicin (GEN). Anaerobically adapted MAP was largely unaffected by most individual drugs at concentrations at or above the aerobic MIC. However, the combinations containing metronidazole (MET), an anaerobically active antibiotic, bedaquilline (BDQ), clofazimine (CFZ), and rifaximin (RFX) reduced viable counts by an average of 89.79%, which equated to 1 $\log_{10}$. An antibiotic that can reduce viable counts under anaerobic conditions by 1-$\log_{10}$ is considered very effective against mycobacteria. Taken together, the ability of MAP to survive under both aerobic and anaerobic conditions suggests the possibility that the differential susceptibility to antibiotics observed in this study may be reflected *in vivo* should the organism persist in both states in the gastrointestinal tract. As such, it is possible that previously unsuccessful treatment of CD with combination antibiotic therapy was hindered by not providing combinations of antimicrobials targeting both the aerobic and anaerobically-adapted states.

3.2 Introduction

3.2.1 Introduction to MAP

The *M. avium* complex (MAC) accounts for the majority of NTM infections. The complex is made up of two species, *M. avium* and *M. intracellulare*, both of which are slow-growing NTMs containing several subspecies. Like other opportunistic NTM, MAC species are most problematic for immunocompromised individuals like those living with HIV or chronic pulmonary disease. The most clinically notable MAC species is *M. avium*, an opportunistic pathogen of pulmonary, intestinal, and soft issues.

The *M.* avium species has four subspecies, one of which is *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease in ruminants and cattle (Taylor, 1953). It is understood that MAP can be transmitted via cow milk or fecal-orally to humans where it can penetrate mucosa and establish infection in the small intestine (Ssekitoleko *et al*., 2021). Controversially, MAP has been associated with CD, however, the exact role MAP plays in CD is unknown and ample evidence exists both for and against MAP as the causative agent of CD.

3.2.2 MAP association with Crohn's disease (CD)

Evidence both for and against MAP as CD's etiological agent centers around the clinically identical progression of Johne's disease and CD, the isolation and detection of MAP from CD patients, the ability of MAP to environmentally persist, and treatment of CD with antibiotics and immune dysregulating therapies. Overall, despite a strong association between MAP and CD,

MAP is not always recovered from CD patients and Koch's postulates have not been satisfied. Therefore, evidence is not strong enough to define MAP as the causative agent of CD.

Johne's disease and CD are both characterized by chronic inflammation of the gastrointestinal tract with development of diarrhea, weight loss, malaise, and abdominal pain. Further, a significant subset of CD patients develop epithelioid granulomas, which are also seen in Johne's disease in cattle (Hong *et al*., 2020). These granulomas distinguish a CD diagnosis from other inflammatory bowel disorders (Heresbach *et al*., 2005). It is not known if these granulomas are present in CD patients from which MAP is also isolated. These granulomas are also a defining characteristic of the immune response to other slow-growing mycobacteria, like members of the *Mycobacterium tuberculosis* complex (MTBC), so formation of granulomas after MAP infection would not be surprising.

Although Koch's postulates have not been met, MAP has been isolated from a significant portion of CD patients (Singh *et al*., 2016). Several studies have isolated significantly more MAP from CD patients than from control patients using culture and nucleic acid-based techniques from intestinal tissues, breast milk, and blood (Mishina *et al*., 1996; Bull *et al*., 2003; Naser *et al*., 2004; Ellingson *et al*., 2005; Seci *et al*., 2005; Abubakar *et al*., 2008; Bentley *et al*., 2008). In order to meet Koch's postulates, labs would need to consistently isolate MAP from CD patients, infect an animal model, and observe the development of CD.

MAP has also been demonstrated to persist in environmental niches that may be in frequent and close contact with humans. Alarmingly, Ellingson *et al*, 2005 demonstrated that MAP can survive pasteurization and be viable in commercial whole milk consumed by humans.

Likewise, Pickup *et al*, 2006 demonstrated the common presence of MAP in lake catchments in the United Kingdom and highlighted the route by which these organisms may enter the domestic water supply. Although these studies do not demonstrate causality between MAP and CD, they demonstrate that humans may be exposed to MAP more than previously thought, supporting the notion that MAP does play a role in common gastrointestinal inflammatory diseases like CD.

CD treatment options bring additional speculation to the role MAP plays in CD. Primarily, there is not a single treatment that cures CD and patients very often relapse. Common therapies include steroids, immune-modulating drugs, and anti-inflammatory drugs that subdue the overactive immune response causing chronic inflammation. However, if CD is promoted by MAP, then treatment with antibiotics should also be possible. Although some antibiotic therapy shows promise, CD is not consistently cured with antibiotics and relapse often occurs after initial success (Nitzan *et al*., 2016). A recent phase III clinical trial conducted by RedHill Biopharma (Tel-Aviv, Israel) in the United States tested a combination of the antimycobacterial drugs, clarithromycin, clofazimine, and rifabutin, in CD patients and, although remission was more common in those receiving drug, the regimen did not significantly initiate and maintain remission of CD patients (RedHill Biopharma, NCT01951326). In contrast, another combination of antibiotics (gentamicin, rifaximin, and metronidazole) used to treat an advanced CD patient, with a history of failed treatment and common relapse, was successful in clearance of all signs and symptoms without relapse to date (Goldberg *et al*., 2015). The conflicting data may suggest that the correct regimen has yet to be discovered. In addition to MAP, genetic factors and the gut microbiome are hypothesized to play a role in the development of CD, although additional research is also needed in these areas as well.

There are several hypotheses that may explain the contradicting evidence: the ability of MAP to enter an anaerobic, latent phase known as dormancy and persist at levels too low to detect, difficulty culturing and identifying anaerobically-adapted MAP leading to lower levels of detection, and mechanisms of antibiotic evasion or persistence related to anaerobiosis. The ability of MAP to adapt to a state of non-replicating persistence (NRP) in response to anaerobiosis, and the susceptibility to antibiotics under these versus aerobic conditions was the focus of this study.

3.2.3 MAP and the Wayne model of non-replicating persistence

It has been well established that *M. tuberculosis* can enter a state of NRP through the gradual depletion of oxygen, this is referred to as the Wayne model (Wayne & Hayes, 1996). More recently, it was shown that MAP is also able to enter a state of NRP via the same model (Parrish *et al*., 2017). The Wayne model is defined by two states, NRP1 and NRP2. The NRP1 state is microaerophilic, with low levels of oxygen accompanied by slowed growth and cellular processes which respond to adaptation to an increasingly anaerobic state. During NRP1, methylene blue begins to fade as oxygen is consumed, cellular division slows, while optical density temporarily increases slightly as cells enlarge and store nutrients for survival under anaerobic conditions. During NRP2, the methylene blue completely disappears, all cellular replication stops, and the optical density stabilizes, indicating adaptation to anaerobiosis.

Anaerobic adaptation enables MAP not only to survive in anoxic environments as would be expected in certain areas of the gastrointestinal tract, but also to be impervious to most

antibiotics because most enzymatic drug targets are inactive when the bacteria are not undergoing vegetative growth. For example, ethambutol specifically targets the mycobacterial cell wall, but when bacteria are not actively building a new cell wall during NRP2, the drug is ineffective. The MIC_{99}, defined as the concentration of drug that inhibited 99% of bacterial growth, of rifampin, rifaximin (RFX), amikacin (AMI), ciprofloxacin (CIP), clarithromycin (CLR), ethambutol (EMB), gentamicin (GEN), mesalamine, and salicilin all dramatically increased under anaerobic versus aerobic adaptation of five MAP strains (Parrish *et al*., 2017).

The goal of this study was to replicate previous work demonstrating the ability of MAP to adapt to anaerobiosis using the Wayne model and evaluate the activity of various antibiotics and combinations.

3.2.4 Cannabigerol and MAP

In addition to testing combinations of novel antibiotics on anaerobically adapted MAP, we also evaluated the inhibitory activity of cannabis derivates, termed cannabinoids. Tetrahydrocannabinol (THC) and Cannabidiol (CBD) are common cannabinoids that have recently become well-recognized pharmaceuticals, however, lesser known cannabigerol (CBG) also has promise for treating several diseases and infections, including CD.

IBD, and CD specifically, are characterized by a chronic, overactive inflammatory response in the gut. Because it has been demonstrated that cannabinoids contain anti-inflammatory properties, these compounds have increased interest in the treatment of inflammatory diseases, like CD. Although evidence for the successful treatment of CD with cannabinoids is minimal and

incomplete, approximately 15 percent of IBD patients use a cannabis derivative to alleviate inflammation and symptoms, with varying success (Nagarkatti *et al*., 2009; Naftali *et al*., 2017; Hoffenberg *et al*., 2017; Nso *et al*., 2021; Naftali *et al*., 2011; Naftali *et al*., 2013).

More relevant to MAP, several cannabinoids, including CBG, have demonstrated antimicrobial properties in addition to their anti-inflammatory properties (Farha *et al*., 2020; Karas *et al*., 2020). CBG has demonstrated antibacterial effects on several clinically problematic bacteria, most notably *Staphylococcus* species like *S. aureus*, *S. mutans*, and methicillin-resistant *S. aureus* (Aquawi *et al*., 2021; Appendino *et al*., 2018). Farha *et al*, 2020 demonstrates that CBG's mechanism of action, against *Staphylococcus*, targets the Gram-positive plasma membrane. Although MAP is not a Gram-positive organism, it closely resembles a Gram-positive bacteria.

Because of the association between MAP and CD, it is possible that successful treatment of CD symptoms with CBG is due to the combination of both anti-inflammatory and antimicrobial action. Use of CBG for treating CD raises the possibility to limit the steroids and immune modulators and limit development of resistance to crucial drugs. The variability in success of CD treatment with cannabinoids may be due to the switching of MAP between states of aerobic growth and anaerobic persistence, dosages, treatment lengths, and lack of data from large scale randomized controlled trials.

We tested the antimicrobial activity of CBG against MAP both aerobically and after adaptation to anaerobic persistence. We hypothesized that CBG would exhibit some antimicrobial effects on aerobically and anaerobically adapted MAP. However, we also

hypothesized that the degree of antimicrobial activity would decrease after the shift to anaerobic persistence.

3.3 Materials and methods

3.3.1 Strain maintenance & media

For all assays, five strains of MAP were used: the ATCC type strain 19698, Ben (ATCC 43544; human derived from intestinal tissue of Crohn's Patient), 44, 47, and Kay. All following assays were performed using each strain as one biological replicate and completed in two technical replicates. All MAP strains were maintained on Herrold's egg yolk agar slants with mycobactin J (VWR, Radnor, PA) at 37ºC and resubbed every 4 weeks.

A 2 mg/ml mycobactin J (Allied Monitor, Fayette, MO) stock solution was prepared in 95% ethanol. Mycobactin J was distributed into 1 ml aliquots and left at room temperature in the biosafety cabinet to dry. Each aliquot was again dissolved in 95% EtOH prior to use. All aliquots were stored at room temperature. Methylene blue (Sigma Aldrich) was prepared as a 10 mg/ml stock solution in reagent grade water and stored at room temperature. A 10 mg/ml CBG stock solution was prepared in DMSO.

All plating steps were completed using M7H11 plates (Hardy Diagnostics), brought to room temperature, and supplemented with 15 µl of mycobactin J stock directly prior to use. All MAP-inoculated plates were incubated at 37°C for 14 days.

Samples from all timepoints and dilutions in the following experiments were frozen and stored at -20ºC. Hungate anaerobic culture tubes with butyl rubber stoppers and screw caps (Fisher Scientific, Hampton, NH) were autoclaved within an hour of use and stored in the biosafety cabinet until needed.

3.3.2 Drug preparation

BDQ (Astatech Inc., Bristol, PA), CFZ (Sigma Aldrich), RFX (Sigma Aldrich), MET (Sigma Aldrich), CLR (Sigma Aldrich), and CBG (Curiowellness, Towson, Maryland) were all prepared as 1mg/ml stock solutions in DMSO (Sigma Aldrich). GEN (Sigma Aldrich) 1 mg/ml stock solution was prepared in sterile deionized water (SDW). All drugs were measured a tabletop Nimbus Precision Balance (Adam Equipment, Oxford, CT). CFZ working stock was prepared from the stock solution as described in section *2.3.1.* Each day of use, BDQ stock was thawed and diluted in SDW to 84 µg/ml as a working stock.

3.3.3 MAP inoculum preparation

Different methods were used to prepare the inoculum of each MAP strain for the aerobic and anaerobic assays. For all aerobic assays, inoculums were prepared using the same 0.5 McFarland standard protocol as described in section *2.3.2.* All methods were identical except that the growth added to the McFarland was taken from Herrold's egg yolk agar with mycobactin J (VWR), rather than Löwenstein–Jensen agar, and 100 µl, in lieu of the previously reported 50 µl, was inoculated into 11 ml M7H9/OADC broth.

For anaerobic assays, the 0.5 McFarland standard was prepared using an identical McFarland standard protocol as described for the aerobic assays above. However, unlike the protocol described above and in section *2.3.2*, 100 µl of the 0.5 McFarland standard was directly inoculated into Hungate culture tubes (Fisher Scientific) containing the prepared media. This

deviation from other inoculum protocols was due to the addition of OADC enrichment broth (BBL™ Becton Dickinson) to the master mix prepared as described below in *3.3.7*.

3.3.4 Sensititre™ MIC assay

This assay was identical to the assay described in section *2.3.3*, exchanging use of the Sensititre™ RAPMYCO plates (Thermo Scientific) for the Sensititre™ SLOMYCO plates (Thermo Scientific), illustrated in Figure 2B. Plates were inoculated with MAP prepared as previously described, and incubated for 14 days instead of three days.

3.3.5 Aerobic broth microdilution MIC assay – BDQ, CFZ, MET, and CBG

The MIC of BDQ, CFZ, MET and CBG was determined using the same method as described in section *2.3.4*. Preparation of initial and final concentrations are illustrated in Figure 1. MET was prepared identically to CBG. The MAP inoculum for aerobic assays was prepared as described in section *3.3.3* and 100 µl was added to corresponding wells of the broth microdilution MIC plate illustrated in Figure 1. The MIC plates incubated for 14 days at 37°C, stacked no more than 3 plates high inside the incubator. After incubation, MIC plates were read using an inverted mirror box. The MIC for each drug was defined as the well containing the lowest concentration inhibiting visible growth.

3.3.6 Aerobic BDQ and CFZ synergy assay

The CFZ synergy assay was completed using the same method as described in section 2.3.5, exchanging the Sensititre™ RAPMYCO plates (Thermo Scientific) for the Sensititre™ SLOMYCO plates (Thermo Scientific) and the *M. abscessus* strain inoculums for the MAP strain inoculums prepared as previously described in section *3.3.3*.

The BDQ synergy assay was completed with the same procedure as the CFZ synergy assay, substituting the addition of BDQ to each Sensititre™ SLOMYCO plate (Thermo Scientific) instead of CFZ. The amount of antibiotic added to each well was 5 µl, regardless of which antibiotic was tested for synergy. All synergy plates were incubated for 14 days at 37°C before being read on an inverted mirror box. Synergy was defined as a ≥4-fold MIC reduction after the addition of sub-inhibitory concentration of CFZ or BDQ.

3.3.7 Anaerobic Model

To set up the assay, vats of master mix were made by combining M7H9 broth (Hardy Diagnostics) and OADC (1:10, 10 ml/100 ml) (BBL™ Becton Dickinson), to which was added methylene blue (final concentration, 1.5 µg/ml) to visualize oxygen depletion, and mycobactin J stock (final concentration, 1.2 µg/ml). Each sterile, Hungate anaerobic culture tube (Fisher Scientific) received 6.8 ml of master mix and 100 µl of the inoculum prepared as previously described in section *3.3.3* for the anaerobic assay. After inoculation, all culture tubes were topped with a butyl rubber stopper, capped, and sealed with parafilm to ensure oxygen

deprivation. All tubes were placed in a 37°C shaking incubator (Thomas Scientific, Swedesboro, NJ), rotating at 250 rpm.

At 48-72 hour intervals, one tube per strain was removed from the incubator and the color and visual turbidity of the culture recorded. Subsequently, 2 mls of each broth culture was removed from the same tube and the OD_{A600} determined using a spectrophotometer (Spectronic 20D+, Thermo Scientific, Waltham, MA). Viable counts were determined by removing 1 ml of culture from the same tube and plating 10-fold dilutions onto 7H11 plates (Hardy diagnostics) as described in section *3.3.1*.

Antibiotics were added to respective tubes 48 hours after the complete disappearance of the methylene blue, indicating the absence of oxygen. Eighteen antibiotics were prepared in DMSO (Sigma Aldrich) with the exception of GEN, which was prepared in reagent grade water. The final concentration of each drug to be tested is listed in Table 6. Antibiotics were prepared such that the same volume of each antibiotic alone or in combination (100 µl) was injected per anaerobic culture using a tuberculin syringe (Becton Dickinson) which punctured the butyl rubber stopper so that no oxygen was introduced into the culture. To prepare the control, 100 µl of DMSO (Sigma Aldrich) was added to one culture without any antibiotics. Cultures were incubated in a shaking incubator (Thomas Scientific) with antibiotics at 37°C, rotating at 250 rpm for 72 hours before concentrating the bacterial pellet.

To wash off the antibiotics and CBG, cultures were centrifuged at room temperature for 15 minutes at 4000 rpm in a tabletop centrifuge (Model 5810, Eppendorf, Hamburg, Germany). After centrifuging, the supernatant was removed with a 10 ml pipette and discarded. Cells were

then washed twice with 1 ml reagent grade water and were centrifuged between washes on a benchtop microcentrifuge for 2 minutes at 4300 rpm. Serial ten-fold dilutions were subsequently prepared in reagent grade water out to 10^{-6} and 100 µl plated using sterile glass beads for determination of viable counts.

3.3.8 Statistics

Biological replicates, starting with different inocula, were done for all assays. Due to ongoing replicates for the aerobic MIC and synergy assays, no statistics could be applied at the time of this writing. For the MAP studies conducted to date, one strain, Kay, was not included in the analysis due to contamination, and only one replicate for strain 47 had been completed, the other having failed to grow *in vitro*. Thus, averages and standard deviations were calculated using data from the biological replicates for the remaining 3 strains, ATCC 19698, Ben, and 44.

3.4 Results

3.4.1 Aerobic MAP antibiotic susceptibility

Of the antibiotics tested on each Sensititre® SLOMYCO plate (Thermo Scientific), 8/13 single drugs had defined susceptible and resistant MICs based on CLSI recommendations (CLSI Guideline M24 3rd Edition, 2018 & CLSI Guideline M62 1st Edition, 2018): CIP, MXF, AMI, DOX, CLR, LZD, SXT, and RFB. Aerobic MICs for all MAP strains tested are reported in Table 4. Of the antibiotics tested, all strains [19698 (ATCC), Ben, 44, 47, and Kay] were susceptible to CLR, LZD, and SXT with MICs ranging from 0.500-4 µg/ml for CLR and 0.125/2.375-0.500/9.500 µg/ml for SXT. The LZD MIC for all strains was 1 µg/ml. As expected, MICs for INH (>8 µg/ml) and MET (>25 µg/ml) were at the top of their range for all strains. All MAP strains tested were phenotypically resistant to CIP, DOX, and RIF with MICs ranging from 8-16 µg/ml and 8->8 µg/ml for CIP and RIF, respectively. The DOX MIC was >16 µg/ml for all strains.

Differences were also noted between strains. While all MAP strains were resistant to MXF with MICs ≥ 2 µg/ml, 19698 (ATCC), 47, and Kay demonstrated higher MICs (4-8 µg/ml) versus Ben and 44 (2 µg/ml). Individual MICs for drugs without CLSI interpretation guidelines also varied between strains and [EMB, STR, ETH, CFZ, and BDQ] are reported in Table 4.

3.4.2 Aerobic BDQ and CFZ synergy

For all MAP strains, the addition of a sub-inhibitory concentration of BDQ was synergistic with CIP, MXF, AMI, DOX, and CLR. The only antibiotic that did not demonstrate synergy with BDQ, against any MAP strain, was LZD. CIP/BDQ demonstrated the most synergy, decreasing the

MIC from >4 µg/ml to 0.12 µg/ml for all strains. Accounting for all strain and antibiotic combinations with sub-inhibitory BDQ, 35/45 combinations demonstrated synergy.

For CFZ, the addition of a sub-inhibitory concentration with MXF, AMI, and CLR was synergistic for all MAP strains tested. CFZ/CIP demonstrated synergy against 3/5 MAP strains: 19698, 44, and Kay where the MIC decreased from 8-16 µg/ml to 2 µg/ml. CFZ/DOX synergy was only demonstrated against a single MAP strain (19698). However, although decreased, the MIC remained in the resistant range. Accounting for all strain and antibiotic combinations with sub-inhibitory CFZ, 29/45 demonstrated synergy.

Of the 34 strain and antibiotic combinations tested for which CLSI-based, interpretive guidelines exist for susceptibility/resistance, 71% (24/34) decreased the MIC from the resistant to the susceptible range using either a sub-inhibitory concentration of BDQ or CFZ. Further information regarding the MIC breakpoints for resistance versus susceptibility can be found in the following reference, CLSI Guideline M24 3rd Edition, 2018 & CLSI Guideline M62 1st Edition, 2018.

3.4.3 Antibiotic synergy against anaerobically adapted MAP

Kay was removed from the anaerobic experiments due to contamination, reducing the sample size to the remaining four MAP strains. The OD_{A600} and CFU/ml growth curves are represented in Figures 5-8. Strains 19698 (ATCC), Ben, 44, and 47 all adapted to anaerobic persistence using the Wayne model. For all strains considered together, NRP1 was entered an average of 20 days post inoculation (dpi), and NRP2, 23 dpi. However, variability was noted

between strains. For example, strains 19698 (ATCC) and Ben grew faster and adapted to anaerobiosis versus strains 44 and 47 (Figures 5-8).

The growth curves comparing aerobic versus anaerobic adaptation for strain 19698 are illustrated in Figure 11. As shown, aerobic cultures follow a standard bacterial curve climbing through log phase growth versus the leveling off seen in NRP2 with anaerobiosis which is characterized by a constant OD_{A600} and viable counts.

A 1-$\log_{10}$ decrease in viable counts, after the addition of an individual drug or drug combination was considered effective inhibition of MAP under anaerobic conditions.

After the addition of six antibiotics added at concentrations at or above the aerobically determined MIC, no single antibiotic, alone, significantly inhibited any anaerobically adapted culture. The inhibition that was noted was less than 1-$\log_{10}$ for all strains tested. The single drug with the greatest effect, alone, on any strain was RFX against Ben, which reduced the CFU/ml an average of 0.744 $\log_{10}$ (76.47%, range 61.36%-91.58%) (Figure 9A). BDQ, CFZ, and GEN had minimal activity against all strains of anaerobically adapted MAP. BDQ reduced the CFU/ml of all four MAP strains, on average, by 0.288 $\log_{10}$ (46.96%, range 27.36%-60.00%), CFZ by 0.297 $\log_{10}$ (46.73%, range 34.76%-67.43%), and GEN by 0.241 $\log_{10}$ (37.60%, range 2.42%-60.57%). Of the single drugs tested, RFX, MET, and CLR had the most individual activity against all strains of MAP when considered together, with RFX decreasing viable counts by 66.25% (0.513 $\log_{10}$, range 0.390-0.744), MET by 63.69% (0.467 $\log_{10}$, range 0.315-0.610), and CLR by 64.91% (0.484 $\log_{10}$, range of log reduction 0.306-0.671) (Figure 9A).

Strain to strain variability in susceptibility was observed after exposure to single drugs. For instance, 19698 (ATCC) and Ben were more easily inhibited with most antibiotics versus strain 44 (Figure 9). BDQ and CLR, individually, reduced 19698 (ATCC) CFU/ml by 0.320 $\log_{10}$ (52.05%, range 48.39%-55.71%) and 0.671 $\log_{10}$ (77.22%, range 69.29%-85.16%), respectively. In contrast, the same individual drugs, BDQ and CLR, reduced strain 44 by only 0.139 $\log_{10}$ (27.36%, range 24.37%-30.36%) and 0.423 $\log_{10}$ (61.29%, range 52.94%-69.64%). Similarly, GEN, RFX, and MET individually reduced Ben by 0.306 $\log_{10}$ (48.97%, range 36.36%-61.58%), 0.744 $\log_{10}$ (76.47%, range 61.36%-91.58%), and 0.510 $\log_{10}$ (66.74%, range 54.55%-78.95%), respectively, whereas for strain 44 the same drugs reduced CFU/ml by 0.012 $\log_{10}$ (2.42%, range -5.88%-10.71%), 0.390 $\log_{10}$ (58.67%, range 51.79%-65.55%), and 0.315 $\log_{10}$ (51.58%, range 49.58%-53.57%), respectively. The least potent drug for all strains considered together, and the drug with the greatest variability between strains was GEN, which inhibited growth an average of 0.241 $\log_{10}$ (37.60%, range 2.42%-60.57%) (Figure 9A).

The two-drug combinations tested against anaerobically-adapted MAP were BDQ/CFZ and GEN/RFX. BDQ/CFZ combined showed greater reduction in growth of all strains versus each antibiotic alone, defining BDQ/CFZ as a synergistic combination. Considering activity against all strains, BDQ and CFZ independently reduced the CFU/ml by 0.288 $\log_{10}$ (46.96%, range 27.36%-60.00%) and 0.297 $\log_{10}$ (46.73%, range 34.66%-67.43%), respectively, while BDQ/CFZ, together, reduced the average CFU/mL by 0.458 $\log_{10}$ (62.16%, range 50.21%-81.46%). For all strains considered together, GEN and RFX, alone, reduced CFU/ml on average by 0.241 $\log_{10}$ (37.60%, range 2.42%-60.57%) and 0.513 $\log_{10}$ (66.25%, range 58.67%-76.47%), respectively. In

comparison, when combined, GEN/RFX reduced CFU/mL by 0.591 $\log_{10}$ (57.439%, range 50.53%-67.70%).

Strain-to-strain variability was also noted with the two drug combinations. Of all the strains tested, 19698 (ATCC) was the most inhibited by both two-drug combinations. BDQ/CFZ reduced 19698 by 0.735 $\log_{10}$ (81.46%, range 79.36%-83.57%) and GEN/RFX reduced 19698 by 1.344 $\log_{10}$ (67.70%, range 35.71%-99.68%). In contrast, strain 44 was the least inhibited by both two-drug combinations: BDQ/CFZ and GEN/RFX decreased viable counts by 0.322 $\log_{10}$ (50.21%, range 35.71%-64.71%) and 0.307 $\log_{10}$ (50.53%, range 46.43 %-54.62%), respectively (Figure 9B).

Overall, the three-drug combinations improved the inhibitory activity against anaerobically-adapted MAP compared to that of the single or two-drug combinations. While two-drug combinations inhibited MAP, on average, by 0.525 $\log_{10}$ (59.80%, range 57.43%-62.16%), three-drug combinations inhibited MAP, on average, by 0.669 $\log_{10}$ (75.66%, range 74.69%-76.64%). The addition of MET or CLR to BDQ/CFZ improved the average reduction of all strains CFU/ml from 0.458 $\log_{10}$ (62.16%, range 50.21%-81.46%) to 0.655 $\log_{10}$ (76.39%, range 63.64%-84.50%) and 0.712 $\log_{10}$ (74.69%, range 61.13%-92.67%), respectively. Adding CLR to GEN/RFX also improved average reduction of CFU/ml from 0.591 $\log_{10}$ (57.439%, range 50.53%-67.70%) to 0.682 $\log_{10}$ (76.64%, range 67.23%-82.58%). The addition of MET to GEN/RFX improved inhibition of all MAP strains considered together: GEN/RFX reduced viable counts by an average of 0.591 $\log_{10}$ (57.439%, range 50.53%-67.70%) while GEN/RFX/MET reduced viable counts by 0.626 $\log_{10}$ (74.90%, range 71.38%-80.05%).

Once again, activity of each three-drug combination varied by strain (Figure 9C). 19698 (ATCC) and Ben were the strains most inhibited by each of the three-drug combinations, while the same drug combinations demonstrated significantly less activity against 47. While all three-drug combinations reduced 19698 (ATCC) an average of 0.865 $\log_{10}$ (84.37%, range 80.05%-92.67%), reduction of strain 47 was 0.492 $\log_{10}$ (67.26%, range 61.82%-76.36%). Considering all three-drug combinations against each strain, the drug-strain combination that exhibited the most inhibitory activity against any strain was BDQ/CFZ/CLR against 19698 (ATCC), which was reduced by 1.217 $\log_{10}$ (92.67%, range 88.57%-96.77%). However, the three-drug combination that exhibited the least activity against any strain was also BDQ/CFZ/CLR, which only inhibited 47 by 0.418 log10 (61.82%, no range).

The four-drug combinations resulted in the greatest inhibition for most of the strains tested compared to all other single antibiotics and combinations. All MAP strains considered together were reduced by four-drug combinations, on average, by 0.985 $\log_{10}$ (84.52%, range 74.15%-89.62%). The addition of MET to GEN/RFX/CLR improved the average reduction of CFU/ml from 0.682 $\log_{10}$ (76.64%, range 67.23%-82.58%) to 1.143 $\log_{10}$ (89.24%, range 78.99%-94.89%). However, it must be noted that strain 47 was excluded from GEN/RFX/CLR/MET exposure due to length of time it took to reach NRP2 and the consequential shortened supplies. The addition of RFX to BDQ/CFZ/MET improved the average reduction of CFU/ml from 0.655 $\log_{10}$ (76.39%, range 63.64%-84.50%) to 1.041 $\log_{10}$ (89.62%, range 87.27%-92.50%). BDQ/CFZ/MET/RFX also resulted in the most consistent inhibition between strains of MAP, despite GEN/RFX/CLR/MET resulting in the greatest average inhibition of all strains. The addition of MET to BDQ/CFZ/CLR improved the average reduction of CFU/ml for all strains, which was

0.712 $\log_{10}$ (74.69%, range 61.13%-92.67%) and 1.024 $\log_{10}$ (85.06%, range 73.53%-98.18%) for the three-drug and four-drug combination, respectively.

The one four-drug combination with a relatively small effect on inhibition was BDQ/CFZ/MET/GEN. The addition of GEN to BDQ/CFZ/MET did not significantly impact the average reductions in CFU/ml, which were 0.655 $\log_{10}$ (76.39%, range 63.64%-84.50%) and 0.732 $\log_{10}$ (74.15%, range 55.61%-94.66%) for the three-drug versus four-drug combinations, respectively.

After exposure to BDQ/CFZ/MET/RFX or BDQ/CFZ/MET/GEN, Ben was the strain most reduced. Ben, on average, was reduced by 91.89% (1.175 $\log_{10}$, range 0.910-1.371), and in contrast, 44 was reduced, on average, by 74.16% (0.685 $\log_{10}$, range 0.364-1.120). On average, four drug combinations inhibited 19698 (ATCC) by 87.45% (1.025 $\log_{10}$, range 0.839-1.381) and the strain 47 by 83.03% (1.025 $\log_{10}$, range 0.439-1.740).

Considering all single drugs and combinations, 19698 (ATCC) and Ben were more inhibited by the addition of antibiotics versus 44 and 47. Four antibiotic combinations reduced CFU/ml of 19698 by > 1 $\log_{10}$: GEN/RFX, BDQ/CFZ/CLR, BDQ/CFZ/MET/RFX, and GEN/RFX/CLR/MET (Figure 5). Three of the four-drug combinations, added to Ben, reduced the average CFU/ml by > 1 $\log_{10}$: BDQ/CFZ/MET/RFX, BDQ/CFZ/MET/GEN, and GEN/RFX/CLR/MET (Figure 6). Only BDQ/CFZ/MET/RFX reduced CFU/ml of MAP 44 by > 1 $\log_{10}$ and only BDQ/CFZ/CLR/MET reduced CFU/ml of MAP 47 by > 1 $\log_{10}$ (Figures 7-8).

3.4.4 CBG experiments

Aerobically, the CBG MIC was consistent for all MAP strains, 25 μg/ml, and growth of all MAP strains was reduced by approximately 50% at 12.5 μg/ml (Figure 10A). All positive controls grew without contamination. This inhibition was consistent despite differences in inoculum size where Ben had the smallest ($3x10^4$ CFU/ml) and 47 had the largest ($2.25x10^5$ CFU/ml). The three other MAP strains had inoculum sizes of $9x10^4$ CFU/ml for 19698 (ATCC), $1x10^5$ CFU/ml for 44, and $5x10^5$ CFU/ml for Kay.

After adaptation to anaerobiosis (NRP2), the addition of 25 μg/ml CBG had no inhibitory activity on the CFU/ml for any of the four strains of MAP tested (Figure 10B).

3.5 Discussion and future directions

In this study, all MAP strains tested were resistant to fluoroquinolones. However, since fluoroquinolones are commonly used for many bacterial infections and resistance is widespread, MAP resistance to CIP and MXF was not surprising. High MICs for INH and MET were also expected because MET is an anaerobic drug, hence is inactive under aerobic conditions and NTMs are rarely susceptible to INH.

Although there was some variability in MICs between MAP strains for most drugs, there was little variability in phenotypic susceptibility between strains. In general, if one strain was resistant to a specific drug, the other strains of MAP were resistant to the same drug, even if MICs varied. Where the MIC did vary between strains, so that two or more strains were split between resistant and susceptible MICs to the same drug, the varying MICs were within a two-fold dilution. This suggests that those strains were likely on the cusp between resistant and susceptible, as defined by current breakpoint ranges per CLSI (CLSI Guideline M24 3rd Edition, 2018 & CLSI Guideline M62 1st Edition, 2018).

One major abnormality observed was susceptibility to AMI, where all strains were susceptible for at least one replicate, but one replicate for both strains Ben and Kay had resistant MICs 8-fold higher. Particularly because replicate data was very consistent, this phenomenon is thought to be an issue of drug solubility on the Sensititre panels (Thermo Scientific).

Data from aerobic BDQ and CFZ synergy experiments also revealed several patterns. First, both SXT and LZD did not demonstrate synergy with BDQ or CFZ. However, it must be noted that the MICs for both SXT and LZD were the lowest concentrations on the Sensititre™ SLOMYCO

panel (Thermo Scientific). It is possible that synergy did exist between these drugs but remained unobserved because the lowest MIC already inhibited growth before the addition of SXT or LZD.

Synergy between BDQ and CIP or DOX was extremely consistent between most strains, all strains resulting in the same MIC-fold reduction or within one doubling dilution. Synergy between BDQ and MXF or CFZ and MXF was consistent for most strains, with small variations within 2-fold of other strains for MAP strains Ben and Kay.

In addition, significant synergy was observed between CLR and BDQ or CFZ, however, the level of synergy was more dependent on the specific MAP strain than were most other tested drugs. More synergy was observed between BDQ and CLR than between CFZ and CLR.

For all strains and antibiotics, BDQ was either just as synergistic or more synergistic compared to CFZ. BDQ acts on mycobacteria by deactivating a component of the ATP synthase proton pump. This mechanism slows production of ATP, or energy. BDQ may have been more synergistic with most drugs, compared to CFZ, because mechanisms to evade antibiotics often require a surplus of energy, and BDQ targets the production of that energy inside the cell, whereas CFZ does not.

The reported aerobic MICs were used to decide which drugs and combinations would be tested against anaerobically adapted cultures of MAP. All antibiotics tested were used at the aerobic MIC or higher; CFZ and BDQ synergies were conducted using a sub-inhibitory concentration of these two drugs. MET was used as a control under anaerobic conditions because, as previously discussed, it is known to be inactive aerobically. The data obtained in this study supports previous work demonstrating that MET is active against anaerobically adapted MAP, as

illustrated with the addition of MET to BDQ.CFZ and GEN/RFX/CLR where improved inhibition was observed (Parrish *et al*., 2017).

Parrish *et al*, 2017 previously demonstrated that MAP can shift into a state of anaerobic persistence via the Wayne Model. The data presented here further supports that claim, as all tested strains shifted through NRP1 and into NRP2. Further, we demonstrated how the Wayne Model of anaerobic persistence differs from anaerobic growth, using MAP strain 19698 (ATCC) as an example. On the growth curve where CFU/ml and OD_{A600} of the anaerobically-adapted culture stabilizes, the aerobic culture continued to multiply. This demonstrates that oxygen depletion is responsible for the stabilization of CFU/ml and OD_{A600}. What remains unknown is how long MAP can persist without oxygen before cells undergo cell death. Because of the debated association of MAP and CD, it would also be interesting to know if the time for which MAP can remain in NRP2 correlates with the amount of time between CD patient relapses. We demonstrate here that MAP strain 44 can persist for at least two weeks after entry into NRP2 (Figure 7A). This timeframe is likely much longer in reality but would require additional anaerobic cultures and months to conduct experiments.

Differences in growth rates and inoculums between strains of MAP impacted the growth curve for each strain and inconsistencies between replicates. This assay should be repeated for all strains in duplicate with strain inoculums prepared from the same starting inoculum. Starting anaerobic cultures from the same inoculum versus inoculums prepared separately and within a target range should make data more consistent as it will be removing a currently uncontrolled variable. Starting from the same inoculum, strains should enter NRP1 and NRP 2 on the same day and viable counts should be more consistent. This study aimed to normalize discrepancies

between inoculums by calculating $\log_{10}$ reduction of CFU/ml compared to a control prepared from the same inoculum, adapted to anaerobiosis, and treated with DMSO. Another potential contributor to error in this data was the freezing and thawing of some cultures after the removal of antibiotics for plating of dilutions. Freezing and unfreezing of cells may have resulted in some cell death that contributed to lower viable counts than accurate. Should cultures be frozen and thawed for additional viable count data, the control should be thawed as well, and the viable count compared only to the control plated at the same time.

Previously, no antibiotics were known to inhibit MAP adapted to anaerobiosis, even anaerobically active MET was not sufficient alone (Parrish *et al*., 2017). This data confirms that no antibiotics are sufficient alone but also demonstrates that a 1-$\log_{10}$ reduction in CFU/ml is possible when particular antibiotics are used in combination against anaerobically adapted MAP. Although not all strains exhibited the same degree of inhibition, it must be pointed out that even approaching a 1-$\log_{10}$ decrease in viable counts is a great improvement over each drug individually. The combination of BDQ/CLOF/MET/RFX was the most consistent combination against all strains considered together where the average CFU/ml reductions are near or above 1-$\log_{10}$ (Figure 9D). In general, the level of inhibition positively correlated with the number of antibiotics used (Figure 9). This finding is important considering that currently used antimicrobial treatment regimens for CD do not specifically target anaerobically adapted MAP.

There was an apparent divide in the effectiveness of some single antibiotics at reducing CFU/ml. Acting independently, BDQ, CFZ, and GEN were not as effective as RFX, MET, and CLR. There are several reasons that may be responsible for this divide. BDQ and CFZ, which are specifically antimycobacterial drugs can cross the mycobacterial cell well, therefore, the

relatively low inhibitory activity of these two drugs alone is likely due to MAPs halted growth when adapted to anaerobiosis. BDQ alone may have demonstrated lower inhibitory activity compared to RFX, MET, and CLR because BDQ acts on ATP synthase subunit c, slowing cellular energy production. However, although inhibiting energy production may slow or halt growth, anaerobically adapted organisms have already halted growth to conserve energy, so BDQ inhibition of energy production does not induce cell death. Inactivity of CFZ against anaerobically-adapted MAP may have been because CFZ is a prodrug that requires metabolism by a bacterial NADH dehydrogenase enzyme to produce ROS that are toxic to the cell. However, the halted growth of anaerobically adapted MAP may mean that the NADH dehydrogenase enzyme is inactive inside the cell, and CFZ is never metabolized and, therefore, able to release ROS. Perhaps future experiments could measure ROS in aerobic growth versus anaerobic persistent states after exposure to CFZ. Higher levels of ROS would be expected after the addition of CFZ to aerobically growing cultures. In contrast to BDQ and CFZ, the inactivity of GEN is likely due to the drugs inability to enter mycobacterial cells. Aminoglycosides, like GEN, are extremely water soluble and not lipid soluble. Due to mycobacterial cell wall high lipid content, it is likely that this drug cannot accumulate in high enough concentrations intracellularly to induce cell death via inhibition of protein translation. This is likely why GEN is not included on either the Senstitre™ RAPMYCO or SLOMYCO panels (Thermo Scientific), however, it was included in this study due to its success in the case study presented in Goldberg *et al*, 2015.

In contrast, RFX, MET, and CLR, which did inhibit MAP relative to BDQ, CFZ, and GEN, all inhibit protein translation. RFX and CLR directly bind to a subunit of the bacterial ribosome and halt amino acid chain elongation. MET causes bacterial DNA fragmentation so that the bacterial

cell cannot transcribe DNA into RNA or translate RNA into protein. Even during MAP adaptation to anaerobiosis, cells must maintain a very low level of metabolic stasis for survival. Therefore, there are likely some proteins that the cell must express which are essential to survival in anoxic environments. Drugs that inhibit the expression of these proteins may induce cell death to a greater degree than drugs that do not inhibit the expression of essential proteins. Comparing the transcriptome of MAP during aerobic log phase growth and anaerobic NRP2 would help elucidate which proteins play a role in anaerobic survival.

Also interesting, RFX is commonly prescribed to treat gastrointestinal infections, like traveler's diarrhea, since it is poorly absorbed and concentrates in the gut. This data paired with poor absorption makes RFX a promising candidate as a component of antibiotic regimens for the treatment of CD. Further studies should investigate the impact of RFX-containing combination antibiotic therapy on CD patients.

The ineffectiveness of CBG was not surprising under anaerobic conditions, given that no single drug was effective anaerobically. Additionally, although significant studies have demonstrated the inhibition of *Streptococcus* species by CBG, most cannabinoid literature focuses on the anti-inflammatory properties of CBG, rather than the antimicrobial properties. Therefore, perhaps CBG is a good addition to antibiotic combination therapies to calm inflammation. It would be interesting to test CBG in combination with antibiotics, aerobically and anaerobically, against MAP to determine if synergy exists between drug and cannabinoid. If CBG is synergistic with an antibiotic it may be beneficial to patients, and may slow the development of antibiotic resistance.

The anaerobic synergy demonstrated between BDQ and CFZ was interesting because these antibiotics are second-line drugs for treating mycobacterial infections. BDQ was developed as an anti-tubercular drug and CFZ as an anti-leprosy drug. Therefore, regimens containing this combination may be potential options for individuals with persistent NTM infections. The combination of BDQ and CFZ increased the activity against MAP compared to both BDQ and CFZ, independently, by approximately double. Although alone these drugs were less active, perhaps the two drugs together provided enough combined stress on anaerobically adapted MAP to reactivate cellular pathways, which these drugs target. However, the BDQ/CFZ combination remained less active than desired, with a 1-$\log_{10}$ reduction as the goal, indicating that the two-drug combination, although synergistic, should be considered as only a component of combination antibiotic therapy.

In contrast, GEN/RFX did not appear synergistic, as the combination of the two antibiotics resulted in approximately the same CFU/ml reduction as RFX alone. GEN did not demonstrate significant activity alone or improve the activity of combinations to which it was added. The lack of reported synergy between GEN and RFX supports the hypothesis that GEN, alone, was inactive against anaerobically adapted MAP because of the drugs lack of lipid solubility and consequential inability to enter mycobacterial cells. The combination of GEN/RFX was originally chosen based on their pairing in the Goldberg *et al*, 2015 case study in which the combination, with MET, was successful. Considering the success of that study with the data reported here, RFX/MET should be tested for synergy, without GEN.

CLR or MET, combined with BDQ/CFZ or GEN/RFX, further increased the activity against anaerobically-adapted MAP. Because RFX demonstrated comparable activity to CLR and MET,

individually, it would be beneficial to further test the activity of CLR/MET, CLR/MET/RFX, and BDQ/CFZ/RFX against anaerobic MAP cultures. While it is important to find a combination that sufficiently inhibits MAP, combining too many antibiotics may increase the likelihood of toxic side effects and make the regimen less tolerable for the patient.

Activity of drugs against MAP was not consistent across strains of MAP, demonstrating the variability of MAP and the importance of individual strain antibiotic susceptibility profiles to prescribed treatment regimens. In general, strains 19698 (ATCC) and Ben were more susceptible to antibiotics than were strains 44 and 47. This is likely because 19698 and Ben are both MAP ATCC strains that are lab-adapted and 44 and 47 are both clinically-derived strains. Therefore, although it would be clinically beneficial to have an antibiotic regimen that does not require the culture and MIC testing on a range of antibiotics, this protocol remains necessary. In fact, due to the strong association between MAP and CD, this data suggests that it may be beneficial to add the adaptation to anaerobiosis through the Wayne model before antibiotic susceptibility testing in CD patients with prior unsuccessful antibiotic treatments.

It must be noted that this study contained a very small sample size of anaerobically-adapted MAP, only four strains. In addition to the sample size, there were three instances where duplicates were performed, in which the antibiotic combination was added to two culture tubes started from the same inoculum (addition of GEN/RFX to Ben, addition of BDQ/CFZ/CLR/MET and GEN/RFX/CLR/MET to strain 44). The standard deviation for combinations conducted in duplicate were significantly smaller than combinations tested in replicate, indicating that the starting inoculum may impact drug activity. This problem may be alleviated by using synchronous cultures for the inoculum as previously discussed, and by duplicates rather than replicates.

The data reported here has generated further questions and directions for studies with MAP adapted to NRP2. Further experiments analyzing the shift in transcriptome between aerobic growth and anaerobic-persistence are necessary to begin understanding the molecular changes that MAP undergo to persist anaerobically. In addition to changes in gene expression between growth conditions, it is important to further analyze changes to the MAP cell wall as the cells shift into NRP2. As MAP adapts to anaerobic-persistence the cell wall may be lost, making cells appear as cell wall deficient mycobacteria, previously termed "spheroplasts", disguised as cocci with only a plasma membrane (Hines & Styer, 2003; Aitkens *et al*., 2021). Perhaps MAP's ability to downregulate the mycobacterial cell wall and shift into a state of anaerobic persistence are responsible for lacking success of antibiotic CD treatments and inability to detect MAP in all CD patients.

In addition to the addition of BDQ/CFZ/RFX, MET/CLR/RFX and BDQ/CFZ/MET/CLR/RFX drug combinations, more replicates, assays starting with synchronous cultures, gene expression studies, and the analysis of the cell wall, *in vivo* experiments are also necessary. Information remains unknown about MAP adaptation to an anaerobically-persistent state *in vivo*, raising questions about the possibility of MAP adaptation to NRP2, the duration of NRP2 before the organism is forced to find oxygen or undergo cell death, MAP population dynamics throughout the Wayne model, the kinetics in and out of NRP2, and whether oxygen must be depleted in the environment to induce the Wayne model or whether the bacteria can seek out a microaerophilic or anaerobic environment via a non-oxygen signal. In addition, the toxicity and adsorption of these drug combinations in the body will impact what drug regimens are feasible and effective for clinical treatment.

Many further experiments and studies are necessary to definitively conclude the role of MAP in CD and define treatments that are less toxic, better tolerated, and consistent between patients. Prior failure of antimicrobial treatments for CD remains a significant reason for claims dissociating MAP from the causative agent of CD. However, if MAP is a causative agent of CD and can adapt to anaerobiosis *in vivo*, then the ineffectiveness of many antibiotic combinations against anaerobically-adapted MAP, demonstrated in this study, may contribute to the prior lack of success using antibiotic therapy to treat CD patients.

Tables and Figures

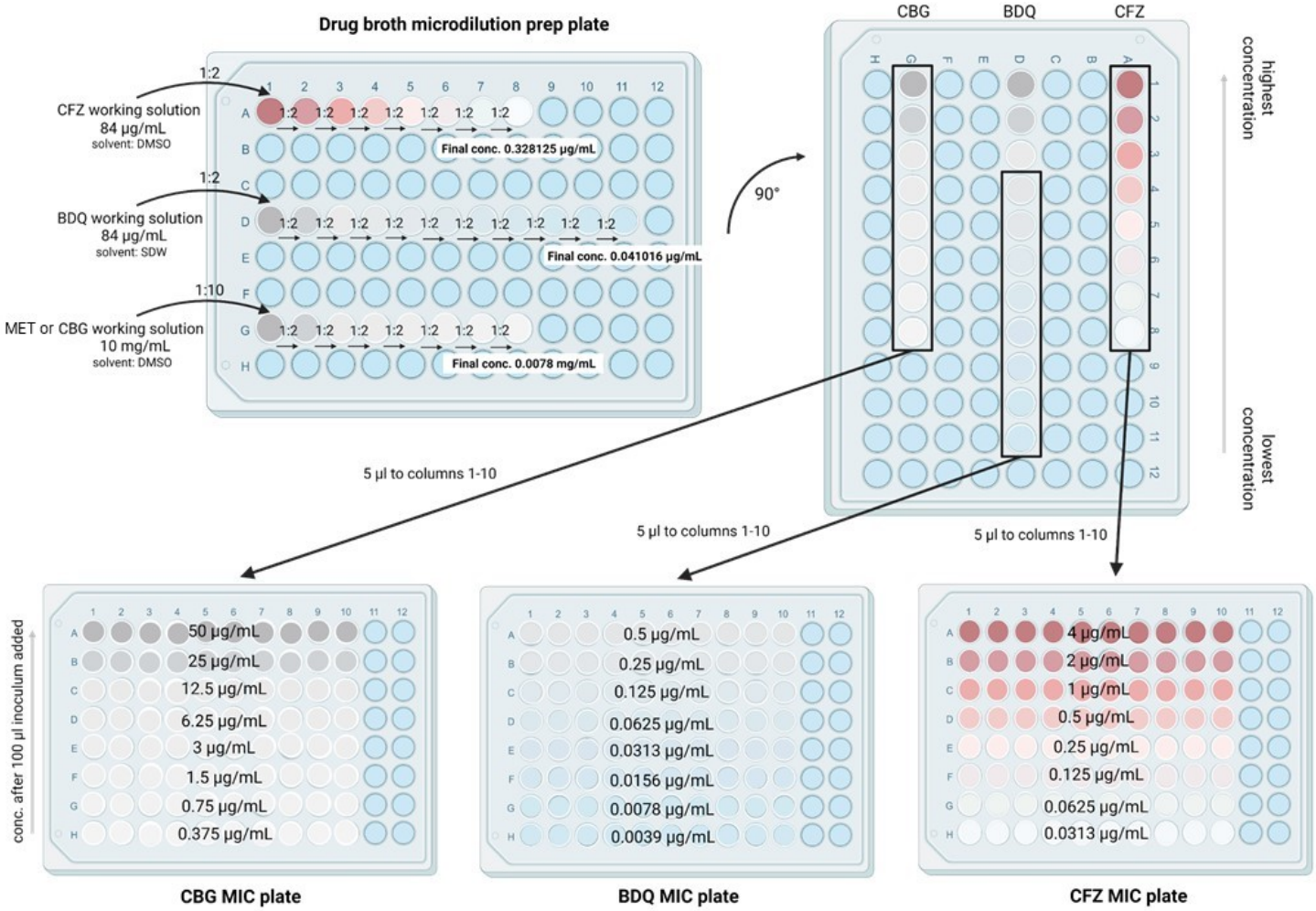


Figure 1. Illustration of BDQ, CFZ, and CBG microdilutions for minimum inhibitory concentration assays. * GEN and RFX were prepared as previously described (Parrish *et al*., 2107). Created with BioRender.com

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	SXT 0.25/4.75	SXT 0.5/9.5	SXT 1/19	SXT 2/38	SXT 4/76	SXT 8/152	LZD 1	LZD 2	LZD 4	LZD 8	LZD 16	LZD 32
B	CIP 0.12	CIP 0.25	CIP 0.5	CIP 1	CIP 2	CIP 4	IMI 2	IMI 4	IMI 8	IMI 16	IMI 32	IMI 64
C	MXF 0.25	MXF 0.5	MXF 1	MXF 2	MXF 4	MXF 8	FEP 1	FEP 2	FEP 4	FEP 8	FEP 16	FEP 32
D	FOX 4	FOX 8	FOX 16	FOX 32	FOX 64	FOX 128	AUG2 2/1	AUG2 4/2	AUG2 8/4	AUG2 16/8	AUG2 32/16	AUG2 64/32
E	AMI 1	AMI 2	AMI 4	AMI 8	AMI 16	AMI 32	AMI 64	AXO 4	AXO 8	AXO 16	AXO 32	AXO 64
F	DOX 0.12	DOX 0.25	DOX 0.5	DOX 1	DOX 2	DOX 4	DOX 8	DOX 16	MIN 1	MIN 2	MIN 4	MIN 8
G	TGC 0.015	TGC 0.03	TGC 0.06	TGC 0.12	TGC 0.25	TGC 0.5	TGC 1	TGC 2	TGC 4	TOB 1	TOB 2	TOB 4
H	CLR 0.06	CLR 0.12	CLR 0.25	CLR 0.5	CLR 1	CLR 2	CLR 4	CLR 8	CLR 16	TOB 8	TOB 16	POS

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	CLR 0.06	CLR 0.12	CLR 0.25	CLR 0.5	CLR 1	CLR 2	CLR 4	CLR 8	CIP 16	STR 64	DOX 16	ETH 20
B	CLR 16	CLR 32	CLR 64	MXF 8	RIF 8	SXT 8/152	AMI 64	LZD 64	CIP 8	STR 32	DOX 8	ETH 10
C	RFB 8	EMB 16	INH 8	MXF 4	RIF 4	SXT 4/76	AMI 32	LZD 32	CIP 4	STR 16	DOX 4	ETH 5
D	RFB 4	EMB 8	INH 4	MXF 2	RIF 2	SXT 2/38	AMI 16	LZD 16	CIP 2	STR 8	DOX 2	ETH 2.5
E	RFB 2	EMB 4	INH 2	MXF 1	RIF 1	SXT 1/19	AMI 8	LZD 8	CIP 1	STR 4	DOX 1	ETH 1.2
F	RFB 1	EMB 2	INH 1	MXF 0.5	RIF 0.5	SXT 0.5/9.5	AMI 4	LZD 4	CIP 0.5	STR 2	DOX 0.5	ETH 0.6
G	RFB 0.5	EMB 1	INH 0.5	MXF 0.25	RIF 0.25	SXT 0.25/4.75	AMI 2	LZD 2	CIP 0.25	STR 1	DOX 0.25	ETH 0.3
H	RFB 0.25	EMB 0.5	INH 0.25	MXF 0.12	RIF 0.12	SXT 0.12/2.38	AMI 1	LZD 1	CIP 0.12	STR 0.5	DOX 0.12	POS

Figure 2. Illustration of antibiotics and concentrations on the A) Sensititre™ RAPMYCO (Thermo Scientific) and B) Sensititre™ SLOMYCO (Thermo Scientific). The positive control was noted as POS and drugs included trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), moxifloxacin (MXF), cefoxitin (FOX), amikacin (AMI), doxycycline (DOX), tigecycline (TGC), clarithromycin (CLR), linezolid (LZD), imipenem (IMI), cefepime (FEP), amoxicillin/clavulanic acid (AUG2), ceftriaxone (AXO), minocycline (MIN), tobramycin (TOB), rifabutin (RFB), ethambutol (EMB), isoniazid (INH), rifampin (RIF), streptomycin (STR), ethionamide (ETH).

Table 1. Minimum inhibitory concentration (µg/ml) of aminoglycoside, tetracycline, and oxazolidinone antibiotics before and after the addition of sub-inhibitory levels of CFZ for six clinical *M. abscessus* isolates. [a] MIC greater than the highest concentration tested. MIC calculated be at least one doubling dilution above the highest concentration. [b] the replicates were within two doubling dilutions, higher MIC reported. [C] replicate not completed.

M. Abscessus isolate	CFZ MIC (µg/mL)	CFZ conc. Used (µg/mL)	CFZ Synergy MICs (µg/mL)											
			Aminoglycosides				Tetracyclines						Oxazolidinones	
			AMI	AMI+CFZ	TOB	TOB+CFZ	DOX	DOX+CFZ	TGC	TGC+CFZ	MIN	MIN+CFZ	LZD	LZD+CFZ
ABC 2	1	0.500	16	1	>16[a]	1	16	0.500[b]	0.500	0.125[c]	>8	1	32	1
ABC 4	2	1	16	1	>16[a]	1	>16[a]	0.125	0.500	0.015	>8	1	32	1
ABC 5	4	2	32	1	>16[a]	1	-	0.125	>4[d]	0.030	>8	1	>32[a]	1
ABC 8	>4[a]	4	4	1	16	1	>16[a]	0.125	1	0.015	>8	1	8	1
ABC 9	>4[a]	4	64	1	>16[a]	1	>16[a]	0.125	1	0.015	>8	1	32	1
ABC 11	>4[a]	4	>64[a]	1	>16[a]	1	>16[a]	0.125	0.500	0.030	>8	1	>32[a]	1

Table 2. Minimum inhibitory concentration (µg/ml) of carbapenem, cephalosporin, and penicillin beta-lactam antibiotics before and after the addition of sub-inhibitory levels of CFZ for six clinical *M. abscessus* isolates. [a] MIC greater than the highest concentration tested. MIC calculated be at least one doubling dilution above the highest concentration.

M. Abscessus isolate	CFZ MIC (µg/mL)	CFZ conc. Used (µg/mL)	Beta-lactam / CFZ Synergy MICs (µg/mL)									
			Carbapenems		Cephalosporins						Penicillins	
			IMI	IMI+CFZ	FOX	FOX+CFZ	FEP	FEP+CFZ	AXO	AXO+CFZ	AUG2	AUG2+CFZ
ABC 2	1	0.500	8	2	32	4	>32[a]	1	>64[a]	4	>64/32[a]	2/1
ABC 4	2	1	4	2	32	4	16	1	>64[a]	4	>64/32[a]	2/1
ABC 5	4	2	>64[a]	2	64	4[c]	>32[a]	1	>64[a]	4	>64/32[a]	2/1
ABC 8	>4[a]	4	8	2	32	4	16	1	>64[a]	4	64/32	2/1
ABC 9	>4[a]	4	>64[a]	2	64	4	32	1	>64[a]	4	64/32	2/1
ABC 11	>4[a]	4	16	2	32	4	>32[a]	1	>64[a]	4	>64/32[a]	2/1

Table 3. Minimum inhibitory concentration (µg/ml) of fluoroquinolone and sulfonamide antibiotics before and after the addition of sub-inhibitory levels of CFZ for six clinical *M. abscessus* isolates. [a] MIC greater than the highest concentration tested. MIC calculated be at least one doubling dilution above the highest concentration. [b] the replicates were within a doubling dilution, higher MIC reported.

M. Abscessus isolate	CFZ MIC (µg/mL)	CFZ conc. Used (µg/mL)	Fluoroquinolone / CFZ Synergy MICs (µg/mL)			
			CIP	CIP+CFZ	MXF	MXF+CFZ
ABC 2	1	0.500	4	0.125	4	0.250
ABC 4	2	1	>4[a]	0.125	>8[a]	0.250
ABC 5	4	2	>4[a]	0.125	>8[a]	0.250
ABC 8	>4[a]	4	2	0.125	4	0.250
ABC 9	>4[a]	4	>4[a]	0.125	>8[a]	0.250
ABC 11	>4[a]	4	>4[a]	0.250[b]	8	0.250

M. Abscessus isolate	CFZ MIC (µg/mL)	CFZ conc. Used (µg/mL)	Sulfonamide / CFZ Synergy MICs (µg/mL)	
			SXT	SXT+CFZ
ABC 2	1	0.500	8/152	0.250/4.750
ABC 4	2	1	>8/152[a]	0.250/4.750
ABC 5	4	2	>8/152[a]	0.250/4.750
ABC 8	>4[a]	4	1/19	0.250/4.750
ABC 9	>4[a]	4	0.250/4.750	0.250/4.750
ABC 11	>4[a]	4	2/38	0.250/4.750

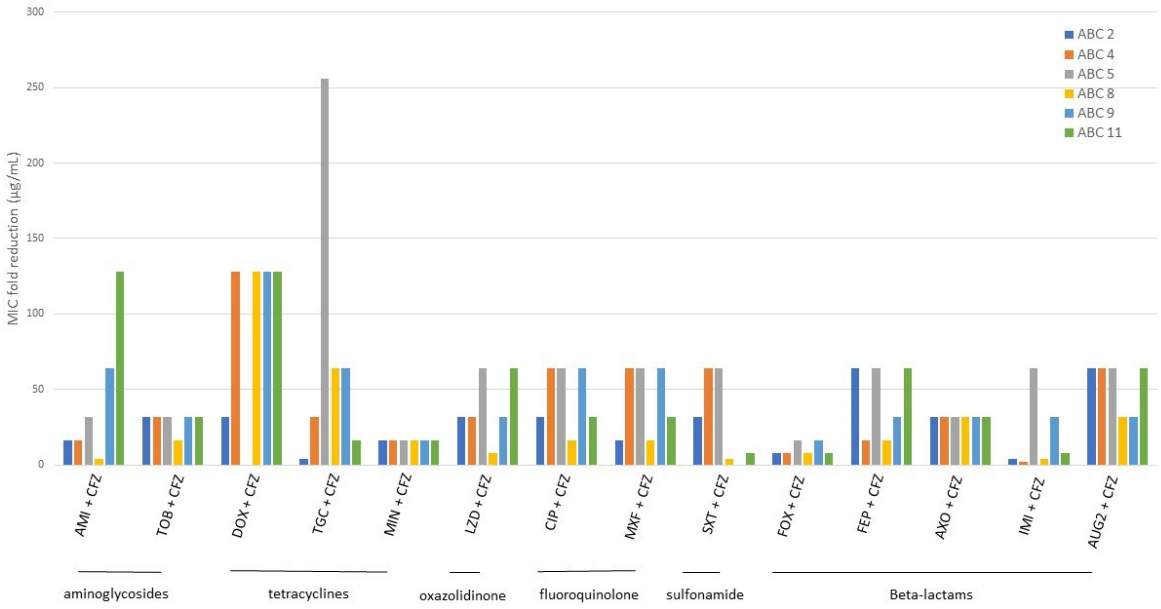


Figure 3. Fold reduction in MIC, for 14 antibiotics from six antibiotic classes, with and without a sub-inhibitory concentration of CFZ for six clinical isolates of *M. abscessus*. For all assays, 2 biological replicates were performed. Trends between replicates were the same. However, due to variability between biological replicates derived from differences in inocula, further statistical analysis was not performed.

Table 4. Aerobic MICs for antibiotics on the Sensititre™ SLOMYCO panel (Thermo Fisher) against five strains of MAP. The range of MICs tested is reported next to each antibiotic. [a] replicate within one doubling dilution, greater MIC reported. [b] replicate more than one doubling dilution apart, both MICs recorded

MAP strain	MIC (µg/ml)												
	SXT 0.125/2.375-8/152 µg/ml	**CIP** 0.125-16 µg/ml	**MXF** 0.125-8 µg/ml)	**AMI** 1-64 µg/ml	**DOX** 0.125-16 µg/ml	**CLR** 0.060-64 µg/ml	**LZD** 1-64 µg/ml	**EMB** 0.500-16 µg/ml	**STR** 0.500-64 µg/ml	**ETH** 0.300-20 µg/ml	**RIF** 0.125-8 µg/ml	**RFB** 0.250-8 µg/ml	**INH** 0.250-8 µg/ml
19698 (ATCC)	0.125/2.375	16[a]	4	8	>16	4[a]	1	16[a]	16[a]	20	>8	4[a]	>8
Ben	0.250/4.750	16	4[a]	64[b] 8	>16	4[b] 1	1	>16[b] 8	64[a]	>20[a]	8	1[a]	>8
44	0.250/4.750[a]	8	2	8[a]	>16	2[b] 0.500	1	8[a]	8	>20[b] 10	8	1[a]	>8
47	0.500/9.500[a]	16	4	8	>16	4[b] 1	1	8	32[b] 8	>20[a]	>8	1	>8
Kay	0.250/4.750[a]	16[a]	8[a]	64[b] 16	>16	4[a]	1	16	32	>20[b] 5	>8	2	>8

Table 5. Aerobic antibiotic synergy between sub-inhibitory concentrations of CFZ or BDQ with non antimycobacterials for five strains of MAP. [a]MIC greater than the highest concentration tested. MIC calculated be at least one doubling dilution above the highest concentration. [b] the replicates were within a doubling dilution, higher MIC reported. [c] the replicates were within two doubling dilutions, higher MIC reported. [d] replicate not completed

MAP strain	CFZ MIC (µg/ml)	CFZ conc. used (µg/ml)	CFZ Synergy MICs (µg/ml)													
			SXT	SXT/CFZ	CIP	CIP/CFZ	MXF	MXF/CFZ	AMI	AMI/CFZ	DOX	DOX/CFZ	CLR	CLR/CFZ	LZD	LZD/CFZ
19698	0.125[a]	0.031	0.250/4.750	0.250/4.750	16[b]	2	4	0.250	16	1	>16[a]	8	4	0.063	1	1
Ben	0.063	0.031	0.250/4.750	1/19	16	4	4	0.500	64	4	>16[a]	16	4	0.250	1	1
44	0.125[a]	0.031	0.250/4.750	0.250/4.750	8	2	4	0.250	8	1	>16[a]	16	1	0.063	1	1
47	0.250[b]	0.031	0.250/4.750	0.250/4.750	16	4	4	0.250	16	1	>16[a]	16	2	0.063	1	1
Kay	0.125[a]	0.031	0.250/4.750	0.250/4.750	16[b]	2	8	0.250	64	4	>16[a]	16	4	0.250	1	1

MAP strain	BDQ MIC (µg/ml)	BDQ conc. used(µg/ml)	BDQ Synergy MICs (µg/ml)													
			SXT	SXT/BDQ	CIP	CIP/BDQ	MXF	MXF/ BDQ	AMI	AMI/ BDQ	DOX	DOX/ BDQ	CLR	CLR/BDQ	LZD	LZD/BDQ
19698 (ATCC)	0.250	0.016	0.250/4.750	0.250/4.750	16[b]	0.125	4	0.250	16	1	>16[a]	0.125	4	0.063	1	1
Ben	0.250	0.016	0.250/4.750	0.250/4.750	16	0.125	4	0.250	64	1	>16[a]	0.125	4	0.063	1	1
44	0.250[b]	0.016	0.250/4.750	0.250/4.750	8	0.125	4	0.250	8	1	>16[a]	0.125	1	0.063	1	1
47	0.063	0.031	0.250/4.750	0.250/4.750	16	0.125	4	0.250	16	1	>16[a]	0.125	2	0.063	1	1
Kay	0.250[b]	0.016	0.250/4.750	0.250/4.750	16[b]	0.125	8	0.250	64	1	>16[a]	0.125	4	0.063	1	1

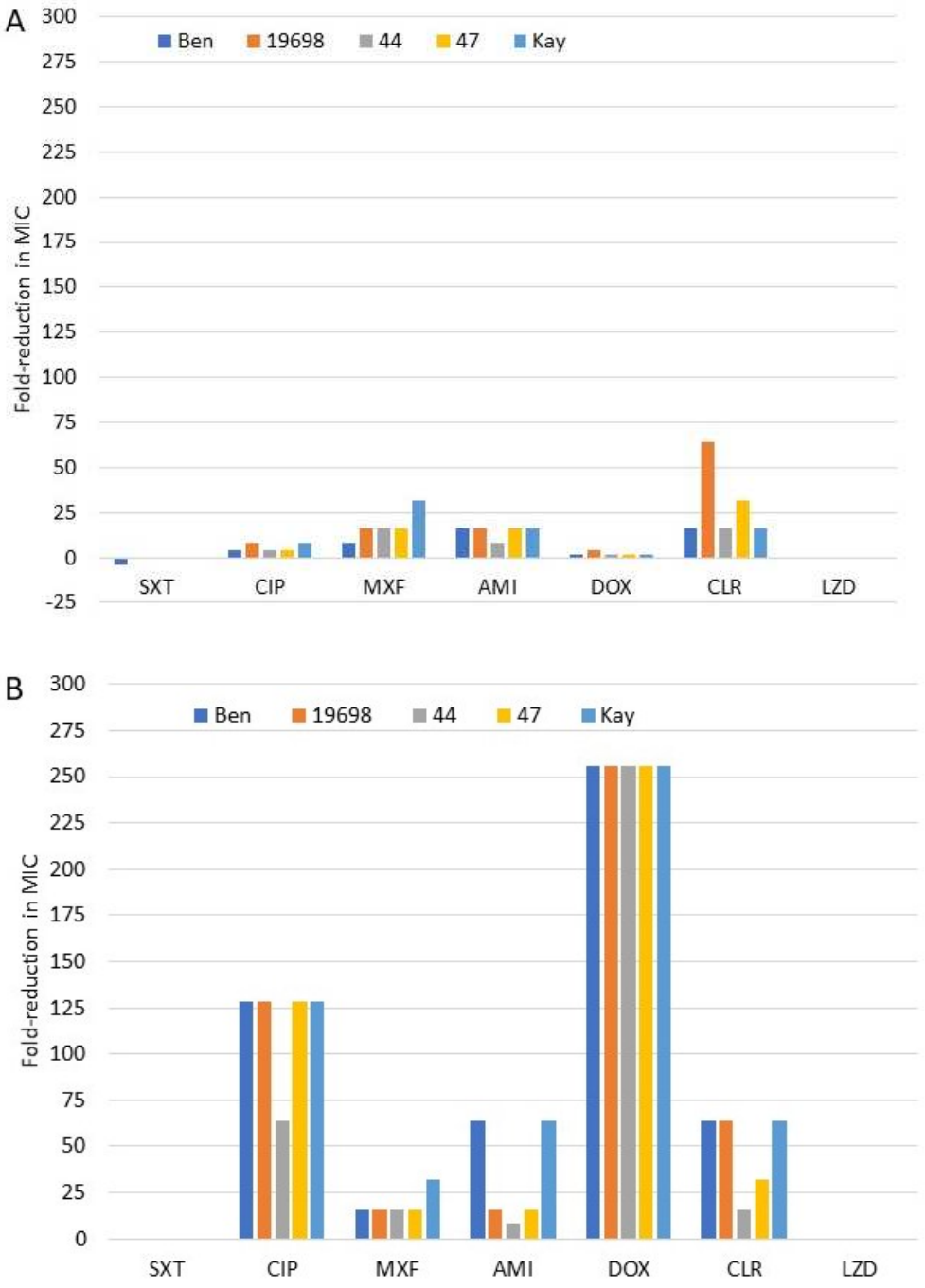
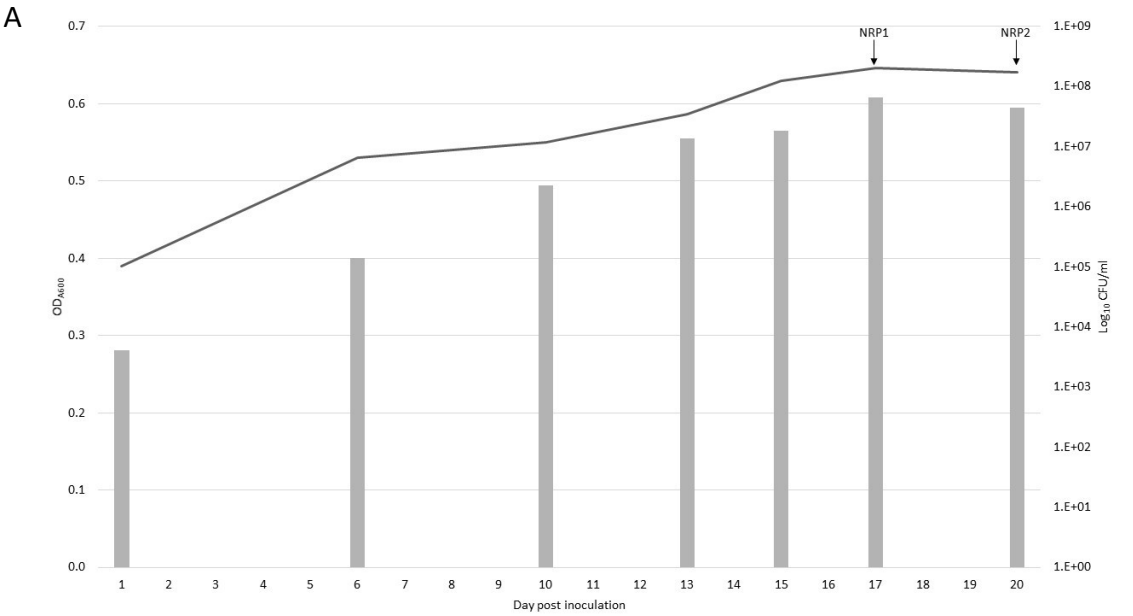


Figure 4. Aerobic synergy of A) CFZ and B) BDQ with antibiotics on the Sensititre™ SLOMYCO panel for five strains of MAP. For all assays, 2 biological replicates were performed. Trends between replicates were the same. However, due to variability between biological replicates derived from differences in inocula, further statistical analysis was not performed.

Table 6. MIC of BDQ, CFZ, GEN, RFX, MET, and CLR against aerobic MAP and the concentration of each antibiotic added to anaerobically-adapted cultures of MAP. The concentration of antibiotic added in anaerobic assays remained constant between single-drugs and drug combinations.

	BDQ	CFZ	GEN	RFX	MET	CLR
Solvent	DMSO	DMSO	SDW	DMSO	DMSO	DMSO
Concentration used in anaerobic assay (µg/mL)	**0.250**	**0.063**	**8**	**8**	**25**	**2**
MAP Strain	**MIC (µg/mL)**					
Ben	0.250 0.250	0.063 0.063	16 16	8 4	>25 >25	4 1
19698	0.250 0.250	0.125 0.063	8 16	8 8	>25 >25	4 2
44	0.250 0.063	0.125 0.063	8 4	0.125 0.063	>25 >25	2 0.500
47	0.063 0.063	0.063 0.250	0.250 0.500	0.063 0.125	>25 >25	4 1



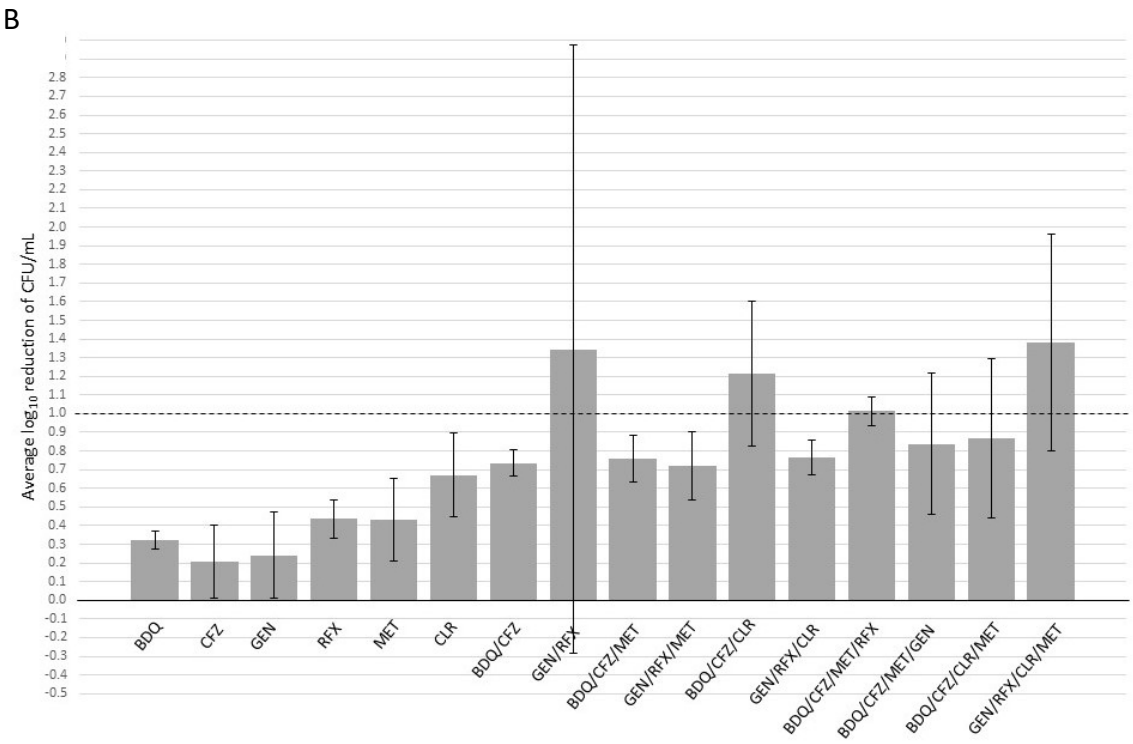


Figure 5. A) Growth and adaptation of MAP strain 19698 (ATCC) in the *in vitro* Wayne model of anaerobiosis. Lines indicate OD_{A600} and bars indicate CFU/ml. Methylene blue fading illustrated by the first arrow, indicating NRP1, and complete absence of color is illustrated by the second arrow, indicating NRP2. B) Log reduction, compared to a DMSO treated control, of CFU/ml after exposure to 16 antibiotic combinations against MAP strain 19698 (ATCC) after 48 hours in NRP2. Dotted line illustrates the desired 1-log_{10} decrease in viable counts which indicate adequate drug activity for anaerobic conditions.

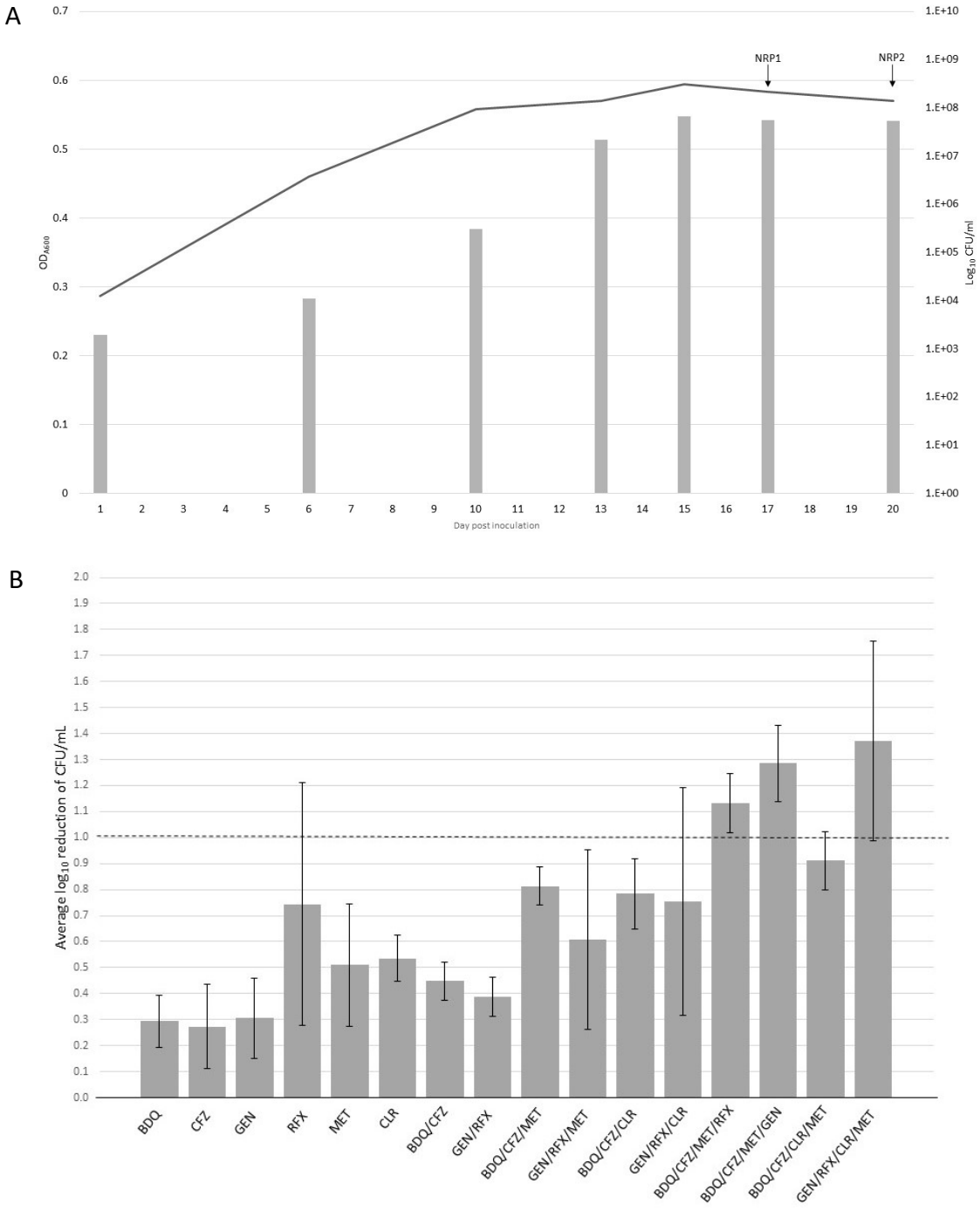
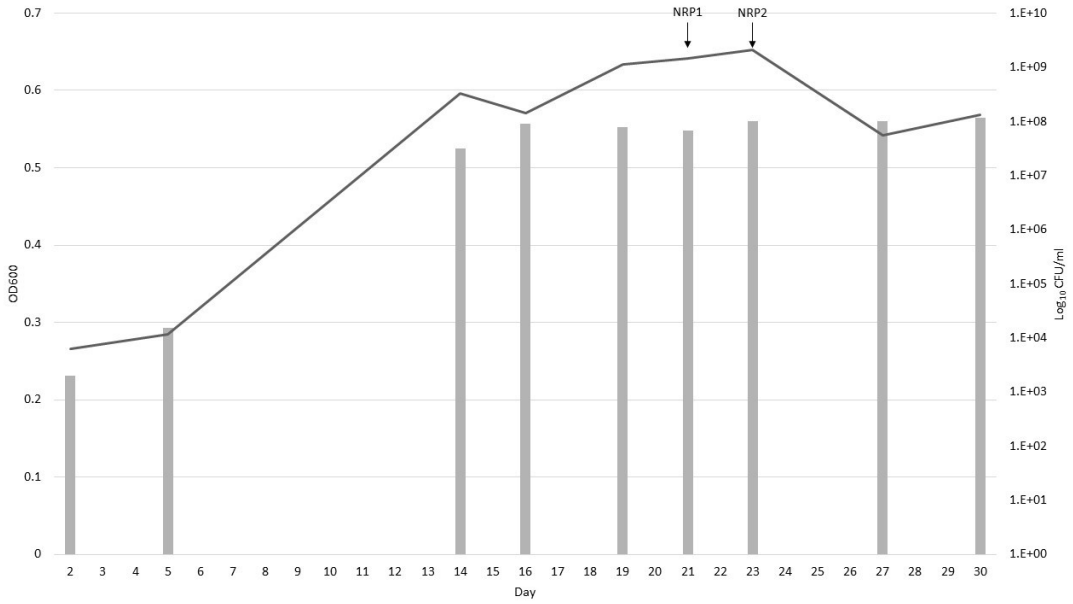


Figure 6. A) Growth and adaptation of MAP strain Ben in the *in vitro* Wayne model of anaerobiosis. Lines indicate OD_{A600} and bars indicate CFU/ml. Methylene blue fading illustrated by the first arrow, indicating NRP1, and complete absence of color is illustrated by the second arrow, indicating NRP2. B) Log reduction, compared to a DMSO treated control, of CFU/ml after exposure to 16 antibiotic combinations against MAP strain Ben after 48 hours in NRP2. Dotted line illustrates the desired 1-log_{10} decrease in viable counts which indicate adequate drug activity for anaerobic conditions.

A



B

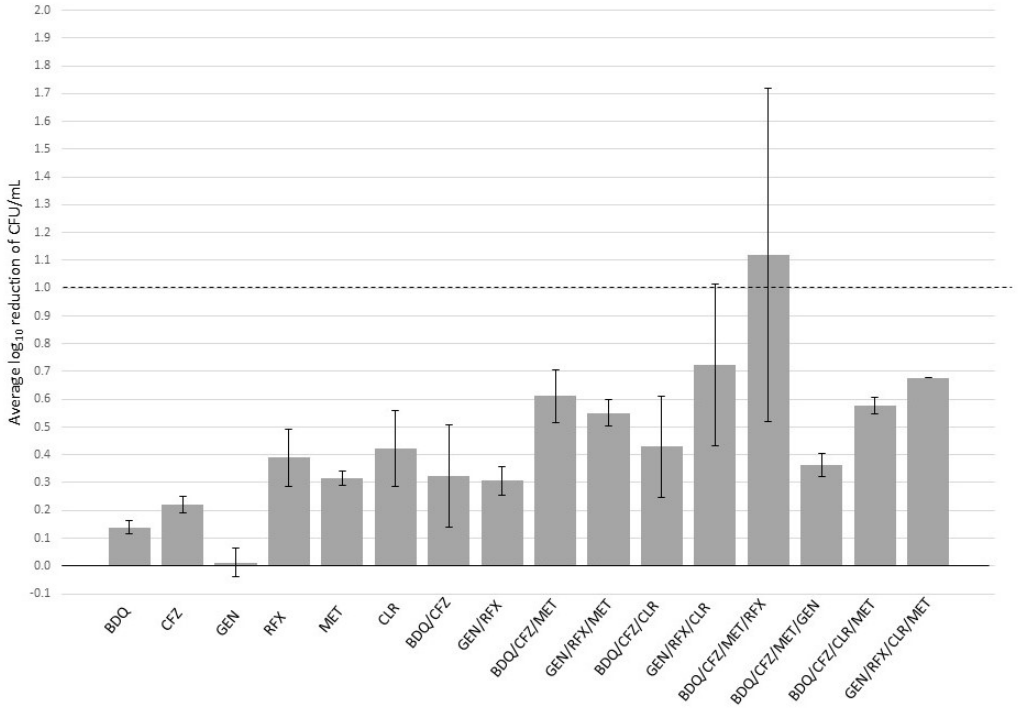


Figure 7. A) Growth and adaptation of MAP strain 44 in the *in vitro* Wayne model of anaerobiosis. Lines indicate OD_{A600} and bars indicate CFU/ml. Methylene blue fading illustrated by the first arrow, indicating NRP1, and complete absence of color is illustrated by the second arrow, indicating NRP2. B) Log reduction, compared to a DMSO treated control, of CFU/ml after exposure to 16 antibiotic combinations against MAP strain 44 after 48 hours in NRP2. Dotted line illustrates the desired 1-log_{10} decrease in viable counts which indicate adequate drug activity for anaerobic conditions.

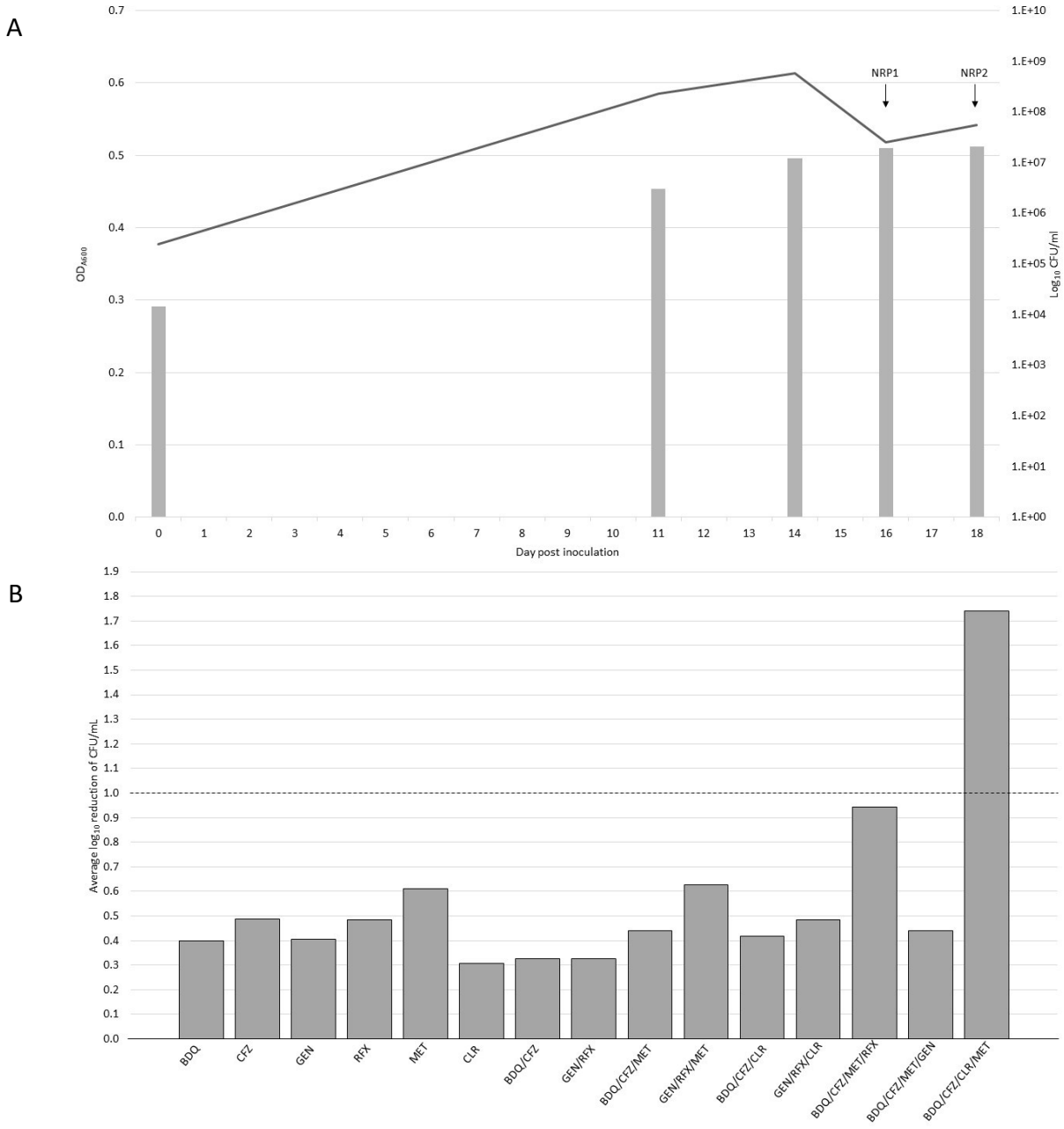
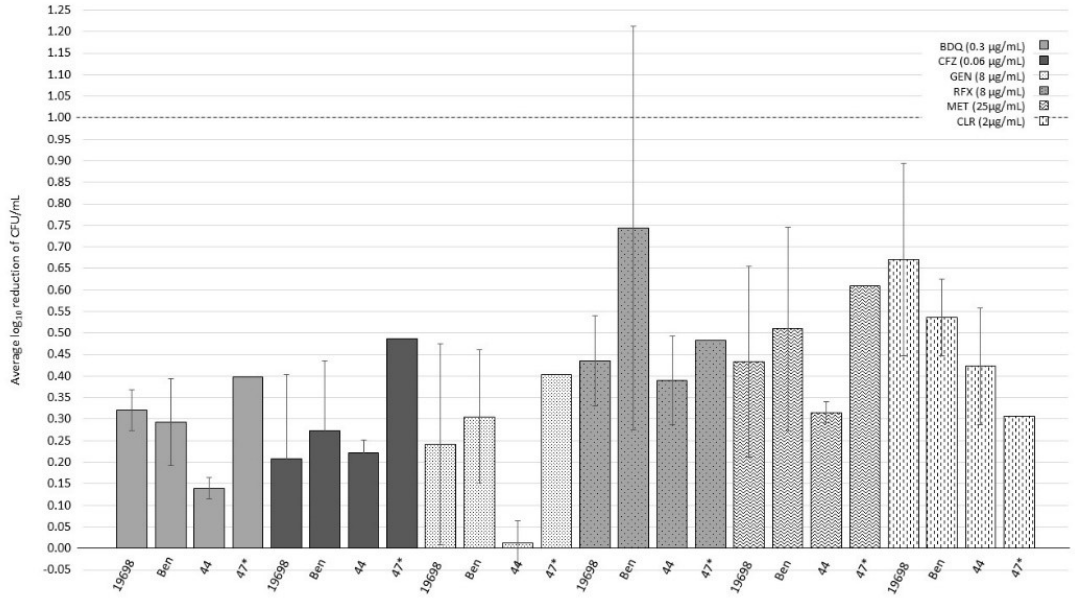


Figure 8. A) Growth and adaptation of MAP strain 47 in the *in vitro* Wayne model of anaerobiosis. Lines indicate OD_{A600} and bars indicate CFU/ml. Methylene blue fading illustrated by the first arrow, indicating NRP1, and complete absence of color is illustrated by the second arrow, indicating NRP2. B) Log reduction, compared to a DMSO treated control, in CFU/ml after exposure to 16 antibiotic combinations against MAP strain 47 after 48 hours in NRP2. Only data from one technical replicate of strain 47 is represented in figure. Dotted line illustrates the desired 1-log_{10} decrease in viable counts which indicate adequate drug activity for anaerobic conditions.

A



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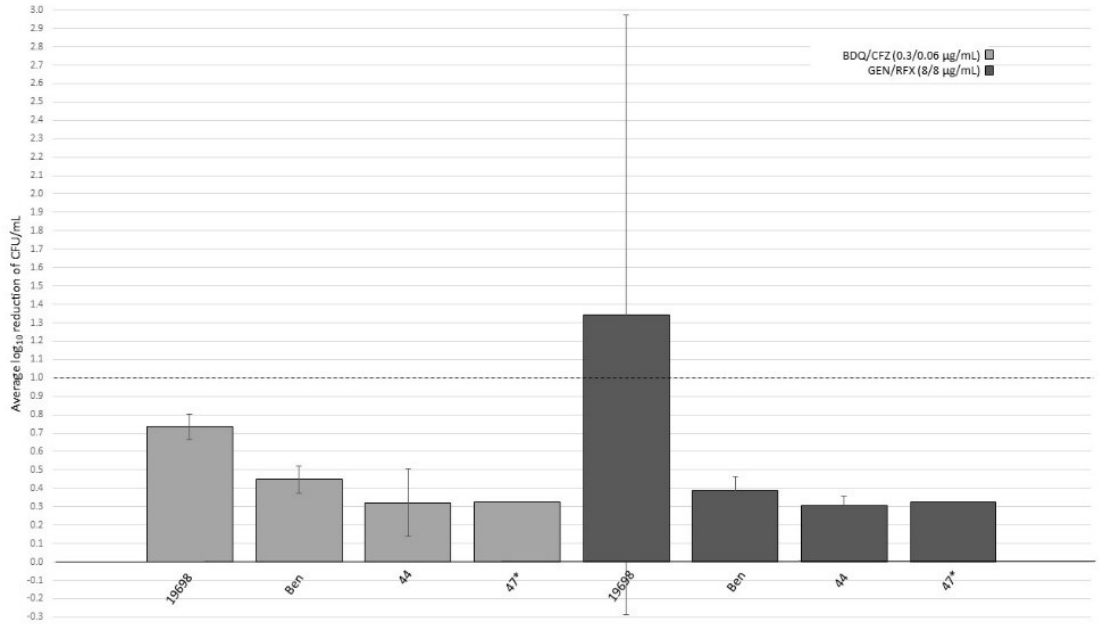
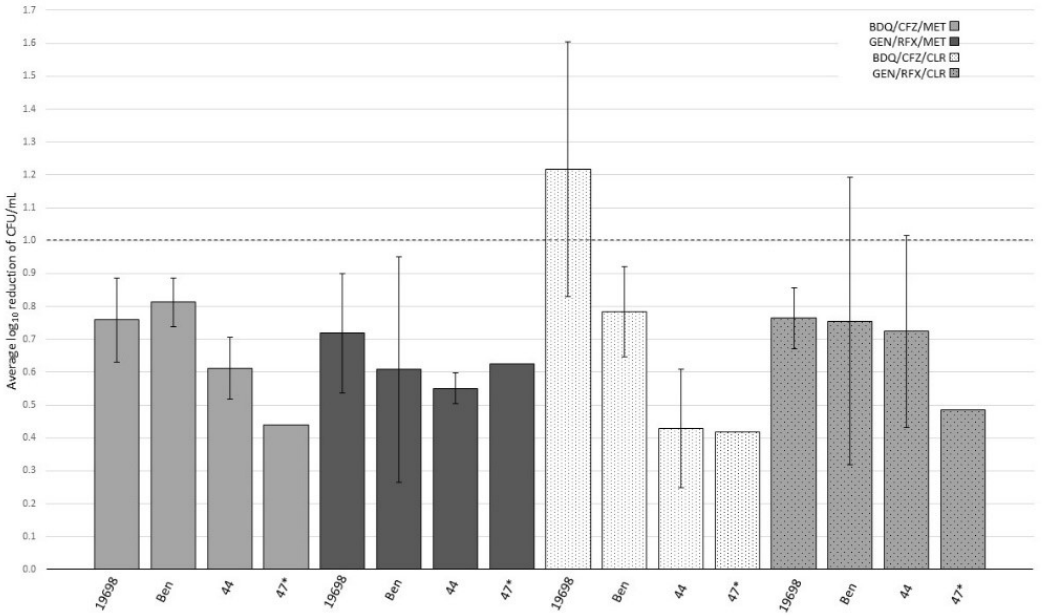


Figure 9A & 9B. Average log_{10} reduction in CFU/ml, compared to a DMSO treated control, of four biological replicates of MAP in NRP2 after exposure to A) single drugs BDQ (0.3 µg/ml), CFZ (0.06 µg/ml), GEN (8 µg/ml), RFX (8 µg/ml), MET (25 µg/ml), and CLR (2 µg/ml) and B) two-drug combinations of drugs [BDQ/CFZ & GEN/RFX]. Data collected from two technical replicates. *Only one biological replicate reported, the second failed to grow. Dotted line illustrates the desired 1-log_{10} decrease in viable counts which indicate adequate drug activity for anaerobic conditions.

C



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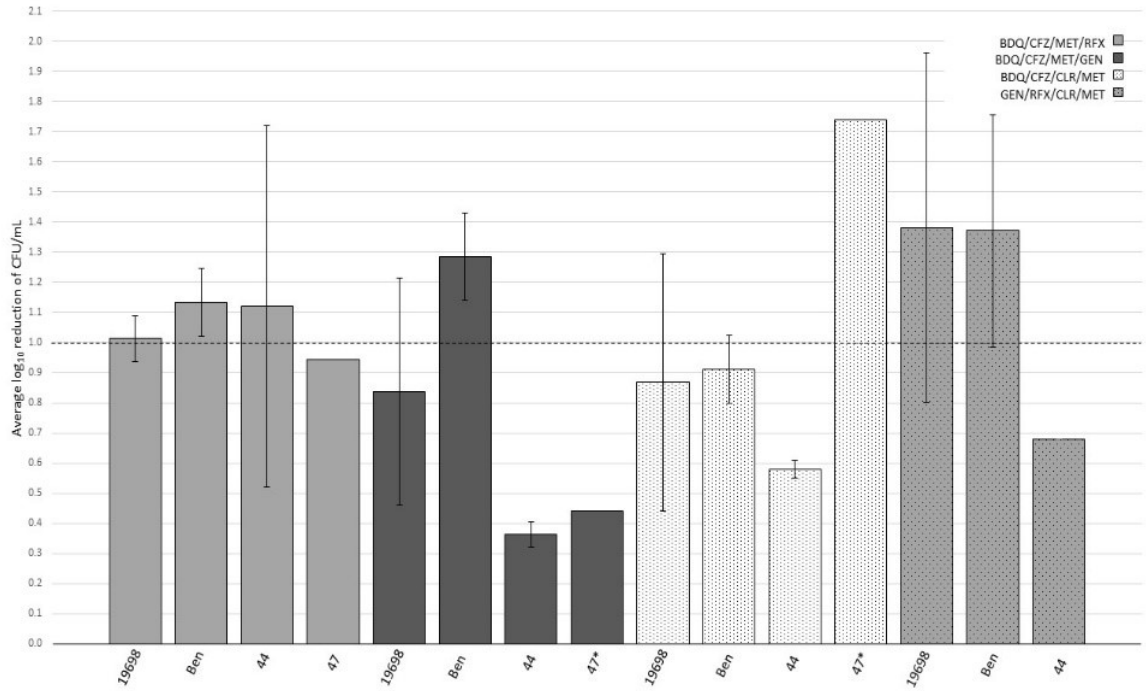

Figure 9C & 9D. Average log_{10} reduction in CFU/ml, compared to a DMSO treated control, of four strains of MAP in NRP2 after exposure to three-drug combinations [BDQ/CFZ/MET, GEN/RFX/MET, BDQ/CFZ/CLR, GEN/RFX/CLR], and four-drug combinations [BDQ/CFZ/MET/RFX, BDQ/CFZ/MET/GEN, BDQ/CFZ/CLR/MET, GEN/RFX/CLR/MET Data collected from two biological replicates per strain. *Only one biological replicate reported. Dotted line illustrates the desired 1-log_{10} decrease in viable counts which indicate adequate drug activity for anaerobic conditions.

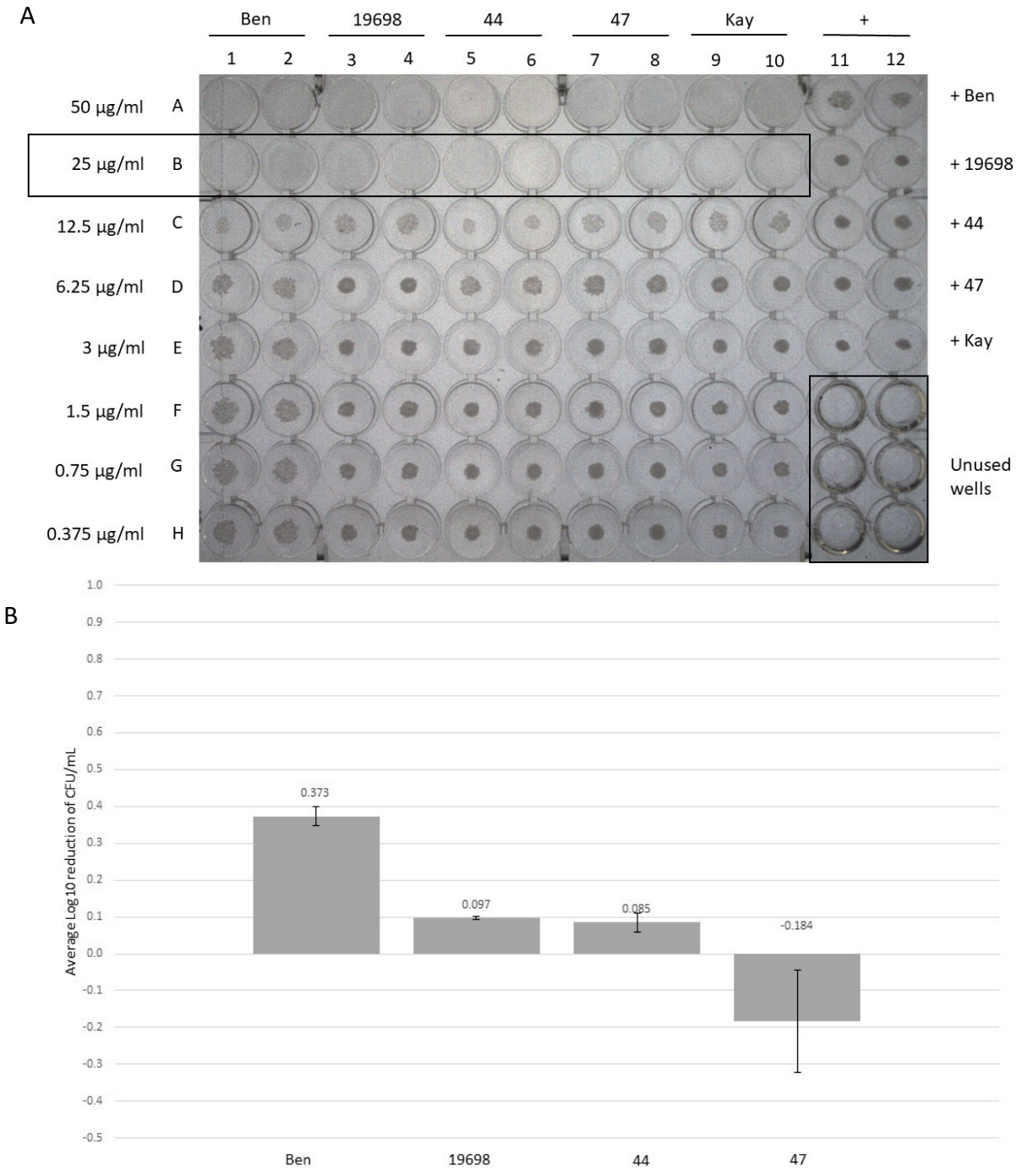

Figure 10. A) Aerobic MIC of CBG for two biological replicates of MAP. Ben positive control wells A11-12, 19698 (ATCC) pos. control wells B11-12, 44 pos. control wells C11-12, 47 pos. control wells D11-12, Kay pos. control wells E11-12. B) Log_{10} reduction, compared to a DMSO treated control, in CFU/ml after exposure to CBG for MAP strains Ben, 19698 (ATCC), 44, and 47 in NRP2. The average of two biological replicates for each strain is reported.

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