INVESTIGATION OF EFFECT OF CLOFAZIMINE ON TOXIN PRODUCTION OF CLOSTRIDIUM DIFFICILE STRAINS

By

Rashi Sultania

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Abstract

Clostridioles difficile is a ubiquitous anaerobic gram-positive bacterium that is responsible for severe gastrointestinal diseases. Lack of drugs for relapse of *C. difficile* infections (CDI) and emergence of strains that produce virulent toxins and spores establish an urgent need for an alternative treatment for CDI. KTI explored the potential of Clofazimine, an FDA approved drug for leprosy, for treatment of CDI due to its antimicrobial activity and low propensity to develop resistance. In the current study we study the effect of i) sub-inhibitory drug concentration of clofazimine on toxin levels of *C. Difficile, ii)* probiotics on *C. difficile,* and iii) combination of probiotics and clofazimine. Clofazimine is successful in reducing toxin levels of *C. difficile* at growth-inhibitory and bactericidal concentrations. We also see a direct correlation between toxin levels and the growth kinetics of the bacteria. Probiotic does not affect the growth of the bacteria as well as the toxin levels of the bacteria. CFZ is also successful in specific killing of C. difficile in presence of bacterial strains of probiotic. In conclusion, CFZ shows excellent in-vitro activity in reduction of toxin levels of *C. difficile*.

Keywords: toxins, probiotics, virulence, concentrations

Primary Reader and Advisor: Dr. Marc Ostermeier, Professor, Department of Chemical and Biomolecular Engineering, Johns Hopkins University.

Secondary Reader: Dr. Sharanjit VedBrat, President, KamTek Inc., Frederick, Maryland.

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1. Introduction

1.1. *Clostridium difficile*

Clostridum difficile, now called *Clostridioides difficile (C. difficile*), is a gram-positive, spore-forming anaerobic bacillus that has been isolated from patients worldwide. It is an obligate anaerobe that produces three toxins, toxin A (TcdA) and toxin B (TcdB) in most cases, and the binary toxin CDT in some strains. These toxins trigger host cellular responses that cause diarrhea, inflammation, and tissue necrosis. This bacterium spreads via the environment and is very common in hospitals and community-based setting such as retirement homes. Therefore, there is a need for new strategies to combat *C. difficile* infections in hospitals while maintaining patient safety at the same time [1].

The Centers for Disease Control and Prevention (CDC) estimates that the incidence of *Clostridioides difficile* infection (CDI) in the United States has increased to an estimated 462,000 cases in 2017 [2]. In 25% of cases, patients with an initial episode of CDI experience recurrence, and in 50% of cases that develop recurrent CDI, patients go on to suffer further episodes. The impact of CDI is not limited to the digestive tract; but can also lead to complications such as increased rates of subsequent sepsis, fulminant colitis and burdening the mental well-being of the patient eventually leading to a poor quality of life [3].

Clostridium difficile-associated disease (CDAD) is more common in elderly patients than younger patients, with a case rate of about 500 cases per 100,000 persons in those aged ≥ 65 years, compared to 90 cases per 100,000 persons among all adults in the United

States. Age 65 and above makes a patient more susceptible to CDAD and is one of the most important and high-risk factors. In fact, older patients have a 63% higher risk of recurrence than younger patients and each additional year of age, increases the risk. The elderly patients are more likely to develop complications such as sepsis, fulminant colitis, intensive care unit admission, morbidity, and mortality [4,5]. The incidence of *C. difficile* colonization in hospital populations is far greater than the number of patients who develop CDAD, and less than 10% of *C. difficile* colonized patients develop CDAD [6].

Early diagnosis and treatment of CDAD is important; if left untreated it has a high likelihood of leading to pseudomembranous colitis and spreading the infection to other patients. The presence of *C. difficile* toxins is only detected in 15 to 25% of patients with antibiotic-associated diarrhea, but in most cases the cause remains unidentified. To ensure accurate diagnostic detection with high sensitivity and specificity, the Society for Healthcare Epidemiology of America recommends two-step verification - culture and cytotoxin assay to be performed for detection of CDAD on the stool specimens submitted [6].

1.2 Epidemiology and economic burden

Occurrence of *Clostridium difficile* infections has increased 8-fold in the past two decades in patients over 65 years of age. This increase is likely due to following factors:

(a) Aging population, which is more susceptible to infection than younger people.

(b) The widespread use of antibiotics and other medications that can disrupt the natural microbiome making the gut more susceptible to *C. difficile* growth; and

(c) Inappropriate antibiotic treatment for patients with an underlying illness like diabetes or heart disease.

The increasing burden of the recurrent CDAD infections have remained constant over the last 7 years as reported by The Center for Disease Control (CDC) [7].

CDI produces a wide range of clinical problems, including prolonged hospital stays, increased risk of sepsis and the need for surgical mediation. Increase in CDI and its associated hospitalizations was first observed in the 2000s. The emergence of a new strain of the bacteria, ribotype 027, was largely responsible for the spike in cases. Prior to 2000, the strain was seen in fewer than 1% of U.S. *C. difficile* isolates. NAP1/ Ribotype 027 is now found in all provinces of Canada and more than 40 states in the United States. In vitro studies have shown that these strains produce significantly more toxin, and it was hypothesized that this virulence factor could be associated with increased severity and complications and is responsible for the 6.9% increase in mortality rate [2].

C. difficile is a microorganism commonly found in the environment, animals, and food; however, its presence is thought to be much higher in health care facilities. The infection is believed to occur shortly after the admission of patients to the facilities. The high incidence of CDI in health care facilities compared with the community can be attributed to the high number of susceptible individuals in hospitals, who are classically elderly patients with comorbid conditions. In recent years, however, cases of CDI acquired outside health care facilities are on the rise [2]. CDI not only causes morbidity and mortality but has a huge economic impact on the country's healthcare system. According to the studies performed, its reported that per patient costs range from $3427 to $33,055

[8]. Variability in costs occur due to readmission of patients due to resistant CDI and involvement of colectomy surgery as a part of the treatment. Patients infected with *C. difficile* have more comorbidities and complications with other illnesses compared to the ones without CDI and hence lead to prolonged hospital stays.

1.3 Pathogenesis

C. difficile is transmitted to humans by spores that are present in feces of infected patients. Being an anaerobic bacterium, it cannot survive in an oxygenated environment outside the host body and to allow for transmission between hosts and to survive, it produces spores called endospores which are metabolically inactive [3,9]. Spores are dormant cells and their resistance to environmental changes, disinfectants, microbials and gastric acid in stomach make them hard to eliminate [3]. Spores tend to grow, spread, and colonize in the intestine. Figure 1 shows the life cycle of *C. difficile* in human gastrointestinal tract. The microbiota present in the host greatly influences colonization. For example, increased ingestion of antibiotics causes a dysbiotic environment which helps in germination of the spores and results in *C. difficile* infections. The bacterium secretes enzymes such as cell protein Cwp84 that degrade the mucus of the colon. Vegetative *C. difficile* exists in two states- sessile and motile and switch between the two. In vitro studies have showed that *C. difficile* also forms robust biofilms consisting of extracellular polymer matrix that protects the bacteria against antibiotic activity [10]. The host produces antimicrobial agents such as lysozyme and cationic peptides as its first line of defense. [8].

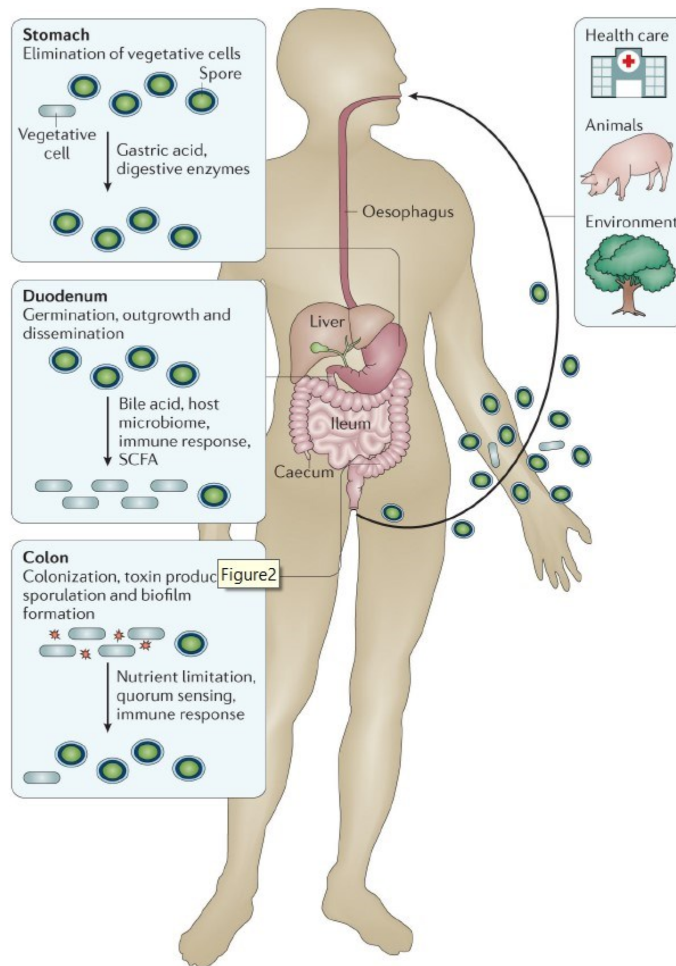

Figure 1: Stages of *C. difficile* life cycle in the human gut. Figure from Snell et al. [7]

The bacterium is non-intrusive and causes infection and diseases by the release of toxin A, 308-kd enterotoxin and toxin B, a 269-kd cytotoxin. These are present in the pathogenic locus (paLoc) in the bacterial genome, Along with TcdA and TcdB, the PaLoc encodes three proteins- TcdR, TcdE and TcdC that regulate toxin production and secretion. Toxins inactivate Rho GTPases and work together to open cellular tight junctions in the intestine. This increases vascular permeability and produces tumor necrosis factor and inflammatory cytokines. In addition, neutrophils secrete a chemical attractant that attracts other white blood cells to the site of an infection. This results in an

extensive inflammatory response and cellular necrosis, which, along with actin depolymerization induced by cytotoxin, cause the development of pseudomembranous colitis. Few of the strains have been found to produce an additional binary toxin which is directly related to high virulence, mortality rate and recurrence. As many as 65 different ribotypes of *C. difficile* have been identified including RT021, RT027 and RT078. Absence of *tcdC* gene in NAP1 causes high toxins in these strains as *tcdC* gene is responsible for regulating the decrease in the bacteria's toxin production.[8]

C. difficile transferase (CDT), also called the binary toxin, is produced by some strains, that include PCR ribotypes 027 and 028. CDT encloses cdtA that is present on the binary toxin locus (CdtLoc). CDTa disrupts the cytoskeleton, leading to cell death. Spo0A, the master regulator for sporulation in *C. difficile* also regulates toxin production in few of the strains.

The toxin productions vary between different isolates of *C. difficile*. Overall, the metabolic state of the bacteria and its environment play a huge role in the toxin synthesis. At high concentrations of TcdB, it produces reactive oxygen leading to cell death. Both mechanisms are important; cytopathic effects promote inflammation and disruption of the tight junctions, whereas TcdB induces necrosis which contributes to tissue damage observed in severe cases of CDI [3].

1.4 Diagnosis

Symptoms of *C. difficile* include watery diarrhea, fever, loss of appetite, nausea, abdominal pain, and fatigue. Stool tests for detection of *C. difficile* should be done on patients with recent hospitalization and/or those who develop diarrhea upon antibiotic use. The current testing options are (1) enzyme immunoassays (EIA) for toxin A/B, toxigenic *C. difficile* culture, nucleic acid amplification tests (NAAT), glutamate dehydrogenase (GDH) and cytotoxin neutralization assay. The clinical severity of *C. difficile* infection can range from mild, moderate, severe, and life-threatening. Patients experiencing mild CDI only show diarrhea as the only symptom. Moderate CDI includes diarrhea with additional symptoms. Patients that have a severe case have *hypoalbuminemia* with concentration lower than 3g/dl serum, WBC count greater than 15,000 cells/meter cube and abdominal tenderness without other signs of acute illness. In complicated cases, CDI presents with fever greater than 101°F, distended abdomen, mental stress, WBC count either greater than 35,000 cells/meter cube or lesser than 2,000 cells/meter cube, serum lactate level greater than 2.2 mmol/L, organ failure, need for admission to intensive care, requiring vasopressors or other pressor agents [11,12].

1.5 Current treatment and its setbacks

If the patient is still consuming antibiotics such as cephalosporins, clindamycin, and fluroquinolones, these are discontinued. Currently the three antibiotics which are used in the treatment are vancomycin, metronidazole and fidaxomicin. Until recently, treatment with mild and moderate CDI included oral medication of 500 mg metronidazole taken

thrice a day for a 10-day course. In severe cases, the patients were treated with oral medication of 125 mg vancomycin taken 4 times daily for a period of 10 days. For complicated cases, CDI should be injected with 500 mg metronidazole thrice a day, oral medication of 125 to 500 mg vancomycin 4 times a day and 500 mg vancomycin per 500 ml saline as enema 4 times a day. Treatments for severe and complicated cases include surgical intervention such as colectomy along with oral medication of vancomycin [11].

Metronidazole and vancomycin have shown increased failure due to increasing antibiotic resistance by *C. difficile*. In 2011, fidaxomicin was approved by the Food and Drug Administration for treatment of *Clostridium difficile* acquired diseases. Fidaxomicin does not alter the microbiome in the gut as much as vancomycin and shows similar efficacy for CDI treatments. However, treatment with fidaxomicin also experienced recurrent CDI cases with the hypervirulent strain. For complicated cases colectomy surgery is required and the mortality rate associated with it ranges from 30% to 80%. Risks associated with it are the need for vasopressors, increased lactate level beyond 5 mmol/L, organ and renal failure and the need for intubation [8].

10% to 25% of the patients with the initial infection go on to develop recurrent CDI within 3 months [8]. One of the main causes of recurrence is the alteration of the gut microbiome after the initial infection caused by drugs (vancomycin and metronidazole) given during the treatment which makes the gut more susceptible to growth of *C. difficile*. The treatment of the first recurrence is like the initial episode of the infection. However, after the third recurrence, the treatment includes fecal microbiota transplantation (FMT) that

aims to rehabilitate the balanced gut microbiome. FMT can take place by delivering the cultured stool in oral form where the stools from the donors are screened for toxins, antibiotics, and other diseases. FMT is not yet approved by the Food and Drug Administration. The procedure is not yet standardized and the long-term effects on the patient's gut microbiome is unknown, and we still require long-term data on the therapy [6,8]. Like FMT, probiotics could help restore the balanced microbiome. However, there is no strong evidence suggesting reduction in recurrence of CDI using probiotics [13, 14].

1.5 Clofazimine

The currently available drugs for treatment for CDI, metronidazole (MET), vancomycin (VAN), or fidaxomicin (FDX), shows high rates of recurrence. The last resort for recurrent CDI, Fecal Microbiota Transplant (FMT), has high risk of life-threatening infections as warned by FDA. The emergence of resistant *C. difficile* strains and lack of drugs with low CDI relapse establish an immediate need to develop a reliable product for treating the infections.

KamTek Inc. (KTI) has explored the possibility of repurposing clofazimine (CFZ), an FDA approved drug for multi-resistant tuberculosis, methicillin-resistant *Staphylococcus aureus* (MRSA) and leprosy, for treatment of CDI. Through multiple experiments, KTI observed high and specific activity of CFZ against CDI. Through previous studies, they also observed the drug's low propensity towards resistance development. Acquired resistance experiment after multiple passages (26) only increased the MIC of clofazimine by two-fold in contrast to MICs of vancomycin, metronidazole and fidaxomicin which

increased by 4, >8 and >16 fold respectively. [15,16]. In the current study, I focused my attention to the drug's effect on toxins produced by *C. difficile.*

1.5.1 Molecular structure

Clofazimine, a deep red colored crystalline drug, belongs to a class of tricyclic heterocycles called riminophenazines. It is a fat-soluble dye first found and developed for tuberculosis early in the 1950s but was discontinued due to its low activity against TB. CFZ,3-(p-chloroaniline)-10-(p-chlorophenyl)- 2,10- dihydro-2-iso-propyl amino phenazine (Fig 2) is an FDA approved drug for leprosy sold under the commercial name of lamprene [17] and continues to be used against multidrug resistant tuberculosis. The antibiotic is highly lipophilic and is orally bioavailable with a long pharmacokinetic half-life. The drug shows excellent antimicrobial and anti-inflammatory properties and accumulates in the epithelial cells of the gastrointestinal tract.

Clofazimine has been consistently effective against multibacillary leprosy for more than a decade and is declared as one of the key drugs and a recommended regimen for leprosy by the World Health Organization (WHO) [18].

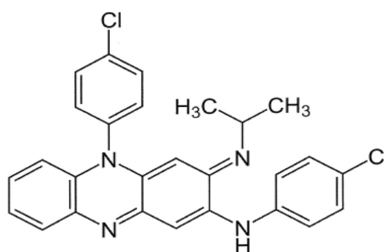

Figure 2: Molecular structure of clofazimine($C_{27}H_{22}Cl_2N_4$)

1.5.2 Mechanism of action

Mechanism of action of clofazimine is still unclear. It accumulates and precipitates within macrophages as crystals [10,12]. Because of its hydrophobic nature, it functions with membranes by inhibiting transport of potassium K+ ions as seen against *Mycobacterium tuberculosis* [11]. However, it is unclear whether the inhibition takes place due to a particular interaction or due to disruption of membranes. According to previous studies, CFZ binds to DNA as seen in its activity against leprosy [18]. The mechanism as observed in its activity against leprosy does not include disruption, however, more studies need to be performed to know about its mechanism.

The first study on CFZ reported the drug to be a redox active compound and its mechanism suggested the reduction and oxidation within mycobacterial species in association with respiratory activity [10,19]. Another suggested mechanism states that clofazimine kills bacteria by disruption of the bacteria membrane as seen against *Staphylococcus aureus* [19]. The combined mechanisms of selective accumulation in the membrane of bacteria, stimulation of reactive oxygen species, blocking K+ channels and release of lysophospholipids makes it an excellent drug against *C. difficile*. The drug also shows low plasma concentration and high concentration in tissues. It forms 3 metabolites in the liver and is mainly excreted out of the body through feces [15].

1.5.3 Adverse effects

Adverse effects after administration of CFZ in treatment with leprosy and tuberculosis includes skins conductions: red pigmentation of skin (>70% of cases), ichthyosis and dryness (10-25%), rash and gastrointestinal conditions including abdominal pain, nausea, committing, diarrhea [15]. In rare cases (around 1%), patients have experienced ocular conditions such as conjunctivital pigmentation, itchiness, and dryness in eyes. Overall, CFZ does not present with severe side effects except for skin pigmentation which is generally reversible after termination of treatment [20].

2. Materials and methods

2.1 Bacterial strains, antibiotics, and growth conditions

The strain VPI 10463(ATCC® 43255^{TM}) of *C. difficile* categorized as ribotype 003, NAP1 strains (NR-49278, NR-43522, NR- 49277) and the non-toxigenic strain P30 (NR-32904) were obtained from American Type Culture Collection (Manassas, VA, USA). Brain Heart Infusion broth, yeast extract, and 96 well microtiter plates were purchased from Becton, Dickinson and company (Franklin Lakes, NJ, USA). Resazurin was purchased from Beantown Chemical Corp. (Hudson, NH, USA). Brucella agar supplemented with Vitamin K and Hemin was purchased from Sigma Aldrich (St. Louis, MO, USA). The antibiotics used were acquired from authentic sources with their respective data, instruction sheets and MSDS sheets; clofazimine from Sangrose Laboratories Pvt Ltd, vancomycin from VWR International, metronidazole from Sigma Aldrich, and fidaxomicin from MedChemExpress. Five mg/ml stock solutions of clofazimine and metronidazole, one mg/ml stock solution of fidaxomicin were made in DMSO, whereas five mg/ml solution of vancomycin was made in sterile DI water. Except for clofazimine, stock solutions of all the other antibiotics were stored at -20°C in aliquots and were thawed whenever required. Clofazimine was made fresh a day before every experiment as our preliminary results showed reduced susceptibility of *C. difficile* on freeze-thawing the drug.

All experiments were performed in an anaerobic chamber (Sheldon Manufacturing Inc) that has an atmosphere composed of 85% N_2, 10% CO_2, and 5% H_2. Our previous studies showed that more than 5% DMSO in the growth media has an inhibitory effect on *C. difficile* and hence the DMSO concentration in the final medium never exceeded 3%.

Resazurin, a redox-sensitive dye, was used to monitor the anaerobicity of the chamber, media, and agar plates.

2.2 MIC and MBC of clofazimine on *C. difficile* strains through broth dilution method

The minimum inhibitory concentration (MIC) of selected antibiotics against various strains of *C. difficile* was determined by broth dilution method as described in Clinical and Laboratory Standards Institute (CLSI) guidelines M11-A8 [21], using BHIS broth (BHI broth supplemented with 5mg/ml yeast extract and 0.001 mg/ml resazurin). The antibiotics were serially diluted in 100 µl volumes in 96-well microtiter plates containing BHIS broth, pre-reduced overnight in the anerobic chamber. Subsequently, log-phase growth of the bacteria in BHIS was adjusted to 0.1 OD, diluted 1:10 in BHI and 100 µl volumes were dispensed in each well. A growth control (bacteria and media, no drug) row and a sterility control (only media, no bacteria, no drug) row were included in each 96-well microtiter plate. The plates were placed in the incubator of the anaerobic chamber at 37ºC. The lowest concentration of the drug showing no visible growth after 48 hours of the incubation compared to the growth of control wells was considered as the MIC of the respective drugs.

Minimum bactericidal concentrations (MBC) of the selected antibiotics were determined by broth microdilution method in BHIS subsequently plating on brucella agar. After reading the MIC, 50 µl of the broth from the last 4 wells showing no visible growth for each of the drugs, was spread on brucella agar plates and incubated in anaerobic chamber at 37ºC. The bacterial growth for each drug concentration was observed after

48 hours and the lowest concentration of the drug that showed no colonies on the plate was considered as MBC.

2.3 In-vitro microbiome study and toxin analysis

For studying the effect of clofazimine on *C. difficile* VPI 10463 in the presence of probiotic bacterial strains, commercially available formulation (Dr. Formula's Nexabiotic, Irvine, CA) was inoculated into BHIS media and incubated overnight in anaerobic chamber at 37ºC. Overnight grown probiotic culture and VPI cultures were adjusted to 0.1 OD and then diluted 1:10 in BHIS broth separately.. The samples for the study were as follows:

(a) VPI

(b) probiotic

(c) co-cultures of probiotic and VPI without the presence of drug

(d) VPI with 0.25 µg/ml clofazimine

(e) co-cultures of probiotic and VPI with 0.25 µg/ml

The experiment tubes (5ml) were taken after 72-hour incubation period and screened for total viable cell count and toxins.

For *C. difficile* total viable cell count, CHROMagar™ *C. difficile* agar plates (CHROMagar™, Paris, France) were used, which allow selective growth of *C. difficile* strains in the presence of co-cultures. Serial dilutions of the samples were prepared in pre-reduced BHIS broth and plated onto chromagar plates at 0 hour and 72-hours of incubation period.

For toxin analysis, an in vitro enzyme-linked immunosorbent assay (ELISA) for detection of toxin A and B produced by *C. difficile* was used. Samples were collected after 72-hour incubation period and centrifuged to allow sedimentation of bacterial pellet. The supernatant containing toxins was evaluated using ELISA kit. A total of 100 µl from each of the specimens were pipetted into wells coated with antibodies specific for *C. difficile* toxin A and B. This step was followed by adding 50 µl of conjugate anti-toxin A&B HRP. The plate was incubated at room temperature on a shaker at 700 rpm. This step was followed by addition of 100 µl substrate in wells and incubated at room temperature for 10 minutes. The color development was stopped by adding 50 µl to each well and the absorbance was read with a spectrophotometer (Spectracount, Packard) at 450nm with an air blank at 630nm.

3. Results and Discussion

There are multiple suggested mechanisms of CFZ to eliminate *C. difficile* but the effect of the drug on the toxin levels is unknown. Hence, I decided to determine the effect of i) sub-inhibitory drug concentration of clofazimine on toxin levels of *C. difficile,* ii) probiotics on *C. difficile,* and iii) combination of probiotics and clofazimine on *C. difficile.*

3.1. Toxin analysis at growth-inhibitory and sub-inhibitory concentrations of CFZ

As pointed out earlier, virulence of *C. difficile* is due to largely host cellular immune response to production of toxins: TcdA and TcdB, resulting in the inflammation that leads to clinical symptoms. The toxins modify the host's small GTPases that are responsible for actin polymerization and cytoskeleton assembly [22]. Previous studies have shown that drugs at sub-inhibitory concentration can have multiple effect on bacterial species such as stress and changes in metabolism and pathogenesis [23]. Vancomycin (VAN) targets killing of the bacteria by inhibiting cell wall synthesis and show an even higher level of toxins in sub-inhibitory concentrations of the drug as compared to that of untreated culture. The toxin levels only decreased at growth inhibitory level of vancomycin. One suggested mechanism for increase in toxin level at sub-inhibitory concentration is that the cell wall disruption may cause the release of intracellular toxin into its environment. Metronidazole (MET) showed the same trend as vancomycin showing increased toxin level as compared to untreated culture at sub-inhibitory concentrations of the drug and shows reduced levels of toxins only at growth-inhibitory concentration. The suggested

mechanism for this activity is that the drug causes disintegration of the bacterial cells by disrupting its plasma membrane. This phenomenon is known as sublethal lytic effect. In contrast to vancomycin and metronidazole, fidaxomicin (FDX) treated cultures showed reduced toxin levels as compared to untreated culture. Fidaxomicin targeting the bacteria by inhibiting synthesis of RNA, explains this activity [23]. On the same line, effect of growth-inhibitory and sub-inhibitory concentrations of CFZ on toxin levels of *C. difficile* was evaluated.

3.1.1 Growth-Inhibitory and bactericidal concentrations of antibiotics

To test what concentration of clofazimine influences toxin production and if there is any difference between response to inhibitory and subinhibitory concentrations, MIC, sub-MIC and MBC values of these antibiotics were established for 6 different strains of *C. difficile* by broth microdilution method (Table 1).

Table 1 : MIC and MBC values of CFZ, VAN, MET and FDX for different strains of C. difficile

Strains	MIC (µg/ml)				MBC (µg/ml)			
	CFZ	VAN	MET	FDX	CFZ	VAN	MET	FDX
VPI 10463	0.06-0.25	0.5	0.25	0.015	0.5	4	0.5	0.03
R20291	0.25	0.5	0.5	0.003	ND	ND	ND	ND
NR-49278	0.125	1	0.5	0.03	ND	ND	ND	ND
NR-43522	0.06	1	0.06	0.125	0.125	2	0.25	0.125
NR-49277	0.06	0.5	0.125	0.03	0.125	1	0.25	0.06
P30	0.125	0.5	0.25	0.015	0.125	0.5	0.5	0.015

[a]ND = not determined. Values stated in the table above are observed in two biological repeats

Variability in MIC values were observed for CFZ against VPI 10463 under same growth conditions and same strain which resulted in lack of clear reproducibility by the assay

system used. Variation in MIC values was also observed by Garrett et al [26] where they found *C. difficile* showing variations of rough and smooth colonies with different characteristics in the same clone. Due to phase variation, bacterial pathogens rapidly adapt to the change in environment pressures by balancing its need to move or adhere and avoiding immune recognition. This eventually results in variation in MIC values.

3.1.2 Toxin analysis

As a next step I decided to determine the effect of sub-inhibitory and growth-inhibitory concentrations of CFZ on toxin levels of VPI 10463 strain of *C. difficile* over a period of 72 hours. Given below are the toxin levels of VPI 10463 with treatment of CFZ in range 0.03- 0.5 µg/ml, monitored for a period of 72 hours. The toxins were measured at time intervals of 24 hours. using ELISA kit as described in Materials and Methods.

As seen in Figure 3, CFZ was successful at reducing toxin levels in comparison to the untreated culture (Control) at MIC (0.25 µg/ml) and MBC (0.5 µg /ml) values. The toxins were reduced below the cut-off value (as shown by red dashed line). At sub-inhibitory concentrations of CFZ, toxin levels were observed to be the same as compared to untreated culture. The toxin curve for 0.125 µg/ml CFZ overlapped with the toxin curve of 0.03 µg/ml CFZ. The toxin levels after CFZ treatment contrasted with results shown by VAN, MET and FDX where VAN and MET showed increased toxin levels at sub-inhibitory concentrations of the drug and FDX shows reduced levels of toxins at sub-inhibitory concentrations of the drug [23]. An increase in the level of toxins is seen with time for control and sub-inhibitory concentrations of the drug until it stabilized after 48 hours.

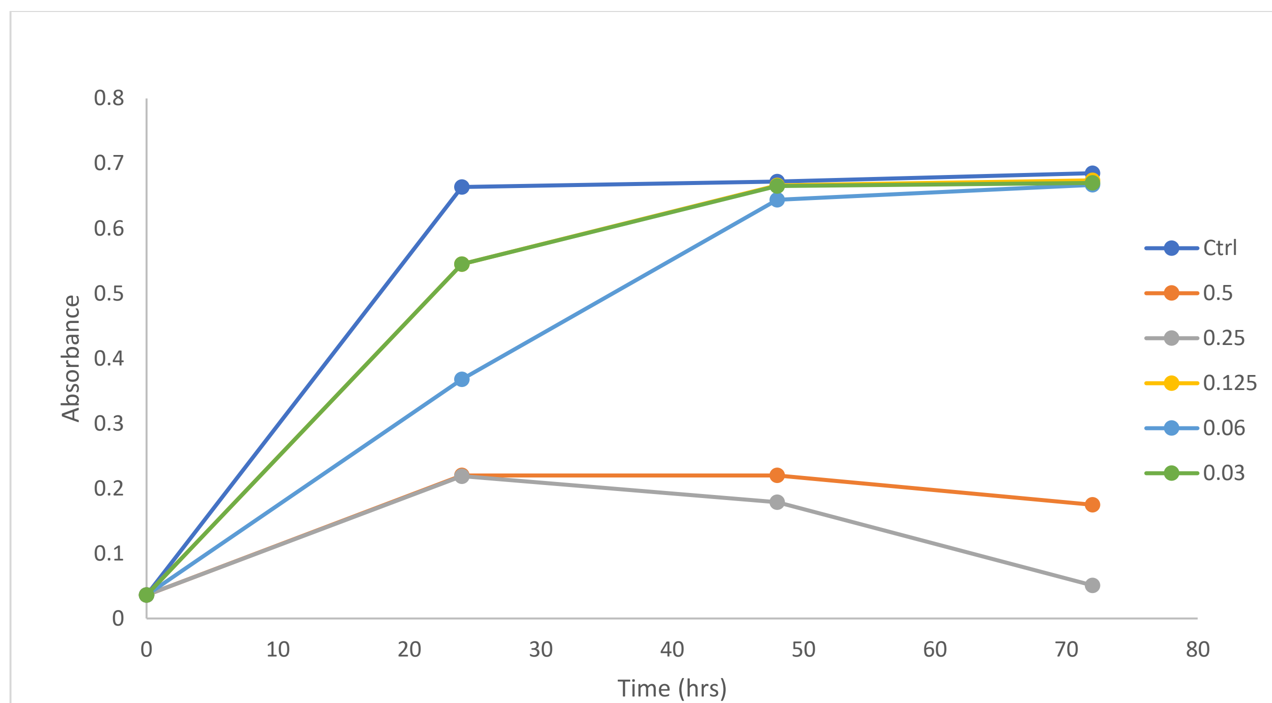

Figure 3: Toxin levels after treatment with clofazimine (CFZ) concentrations ranging 0.03ug/ml – 0.5ug/ml. VPI 10463 was grown in the presence of different CFZ concentrations and supernatants from the cultures were evaluated for presence of toxins A/B at 24-hour intervals using the ELISA kit.

3.1.3. Correlation of CFZ concentrations with growth kinetics and toxin level

Reduced level of toxins is observed at 0.25 µg/ml and 0.5 µg/ml CFZ and I wanted to further see the correlation of the toxin levels to the growth of the bacteria. It could be hypothesized that toxin levels are reduced because of elimination of *C. difficile* at MIC and MBC values and hence, the following study was done to study the effect of different concentrations of CFZ on growth kinetics of VPI 10463 over the period of 72 hours and its impact on the toxin levels. Both treated and untreated cultures were plated on brucella agar to quantify the growth and the cultures supernatant were analyzed for toxin levels

every 24 hours over a period of 72 hours. The cultures for control and drug concentrating were plated ranging from 0.03-0.5 μg/ml. To see countable colonies on the plate, multiple serial dilutions were made at 0, 24, 48 and 72 hours.

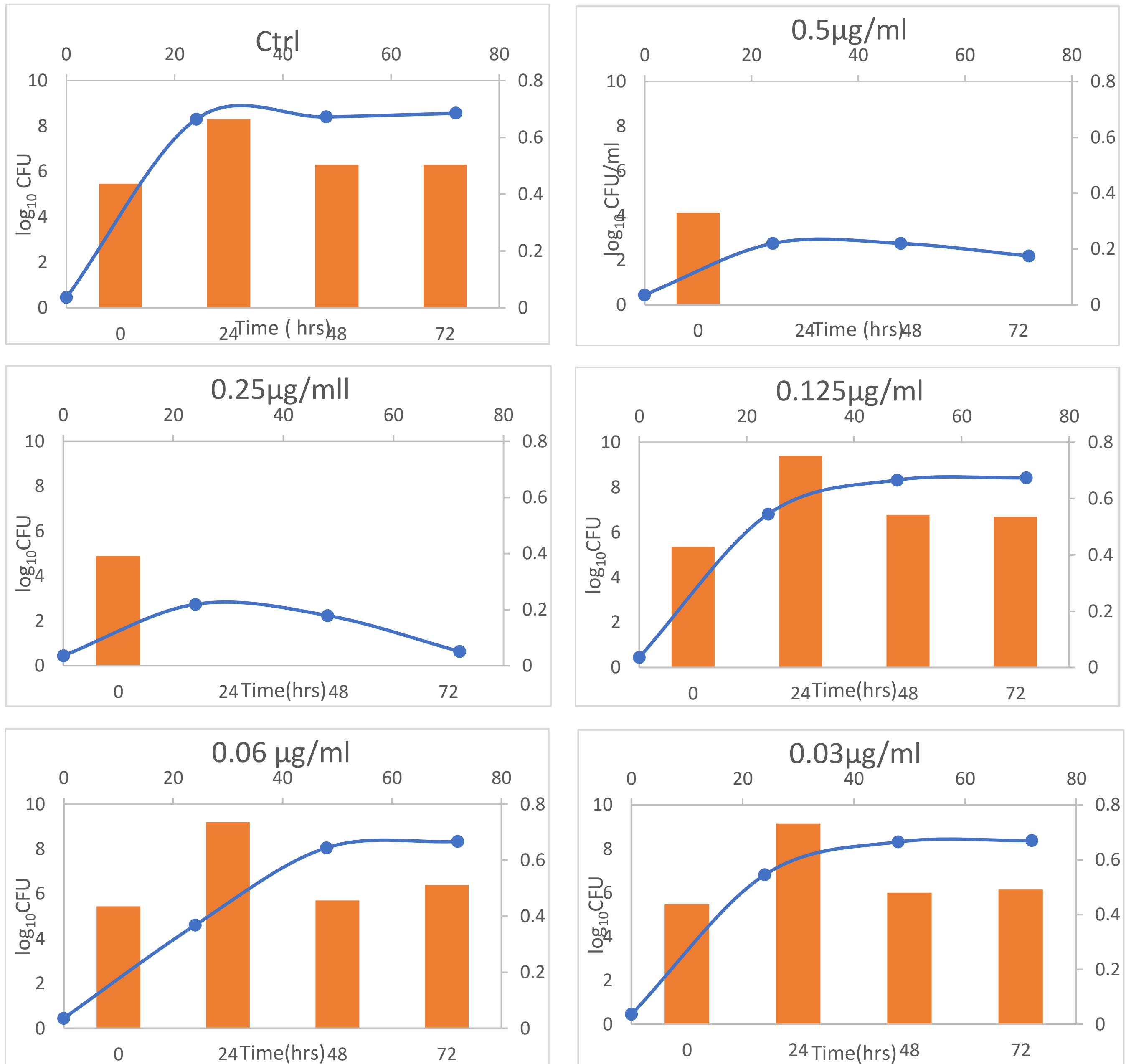

Figure 4: Toxin levels and Cell viability of *C. difficile* after treatment with clofazimine (CFZ) concentrations ranging 0.03ug/ml – 0.5ug/ml. Cell viability is measured in CFUs after observing the colonies on the next day after plating the cultures after serial dilutions. This is done in time periods of 24 hours.

Figure. 4 shows the CFU count of VPI 10463 against MIC and sub-MIC values of CFZ. For control and sub-MIC values, the growth of VPI 10463 reaches a peak at 24 hours at 10^9 CFUs and then decreases. This is consistent with previous studies which also showed growth of *C. difficile* peaking at 24 hours (growth phase) and then the CFUs decreasing because of stationary phase of the bacteria [24, 28].For drug concentrations at or above MIC, 0.25 μg/ml and 0.5 μg/ml, there is complete elimination of *C. difficile* within 24 hours and no colonies are observed on the agar plates. Due to elimination of the bacteria, toxins were not further produced. To conclude, toxin levels depend on the growth of the bacteria.

Although more research will be needed to determine if these findings have any relevance for patients, I hypothesize that reducing toxin levels with sub-growth-inhibitory concentrations of an antibiotic will be beneficial to alleviate symptoms.

3.2 Effect of CFZ on *C. difficile* in the presence of gut microbiome

Different bacterial strains in the gut could interfere with *C. difficile* by different mechanisms. Growth of *C. difficile* and its toxin production is influenced by its environment, drug type, drug concentration, presence of other bacterial strains, amino acids, butyric acid, carbon sources and biotin. It can be hypothesized that when more than one bacterial species is present, there is change in pattern of toxin production due to different factors one of them being competition for nutrients between the strains [25].

Gut microbiome greatly influences the growth of *C. difficile* and vice-versa, the presence of *C. difficile* influences the gut microbiome flora and diversity [25]. I wondered how the presence of probiotics would affect CFZ's ability to reduce *C. difficile* growth and toxin

levels and do so I set up the experiment with co-cultures of probiotic and VPI 10463 in presence of CFZ at concentration 0.25 µg/ml. On doing so, I observed the specific activity of clofazimine on *C. difficile* growth and its toxin levels in presence of probiotics comprising of 23 bacterial species representing the good microbiota of the gut microbiome.

3.2.1 Effect on *C. difficile* growth

I want to see the effect of growth-inhibitory concentration (0.25 µg/ml) of CFZ on growth of VPI 10463 in presence of probiotics. To do so the cultures for all samples were plated after three serial dilutions by a factor of 10 on brucella agar plates and chromagar plates. Brucella agar plates allows growth of all bacterial species plated and chromagar allows selective growth of *C. difficile.* This enabled me to see the selectivity of CFZ towards *C. difficile.* To validate the results, I compared it to growth of probiotic and VPI 10463 individually without any drug. The growth was also compared to co-cultures of VPI 10463 and probiotic. The fourth control for the experiment was to see the effect of 0.25 µg/ml CFZ on VPI 10463 culture. Given below is the graph showing growth of VPI 10463 with an initial concentration of 2×10^6 CFU/ ml in different conditions.

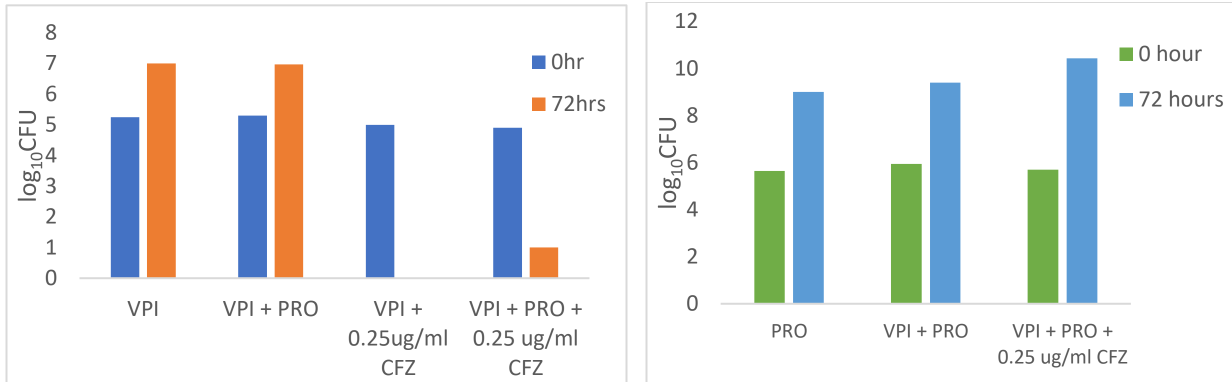

Figure 4: From left to right. (a)Growth of VPI 10463 as observed on chromagar plates in presence of CFZ and probiotics (b) Growth of probiotics as observed on brucella agar plates in presence of CFZ and VPI. Cell viability is measured in CFUs after observing the colonies on the next day after plating the cultures after serial dilutions. This is done in time periods of 24 hours

The colonies counted on the plates and total CFU calculated are represented on 10-log axis in the graph above. VPI 10463, probiotic and co-culture of VPI and probiotic grows to more than 10^7 CFUs after a period of 72 hours. In contrast, I see that after treatment with 0.25 µg/ml CFZ, there were no colonies, and the drug was successful in killing *C. difficile*. I also see around 10^1 CFUs of VPI 10463 after treatment with drug in presence of probiotic in comparison 10^7 CFUs without treatment of drug. The probiotic count was not affected as seen on brucella agar plates and this showed that CFZ is successful in specific killing of *C. difficile* and not the other bacterial species.

3.2.2 Effect on *C. difficile* toxin levels

Toxin analysis was also done on the above cultures after 72 hours to see the effect of microbiome on toxin levels of *C. difficile*. Given below is the graph showing toxin levels in the different conditions. Positive control comprises of pure Toxin A&B and negative control comprises of dilution buffer as given in the ELISA kit.

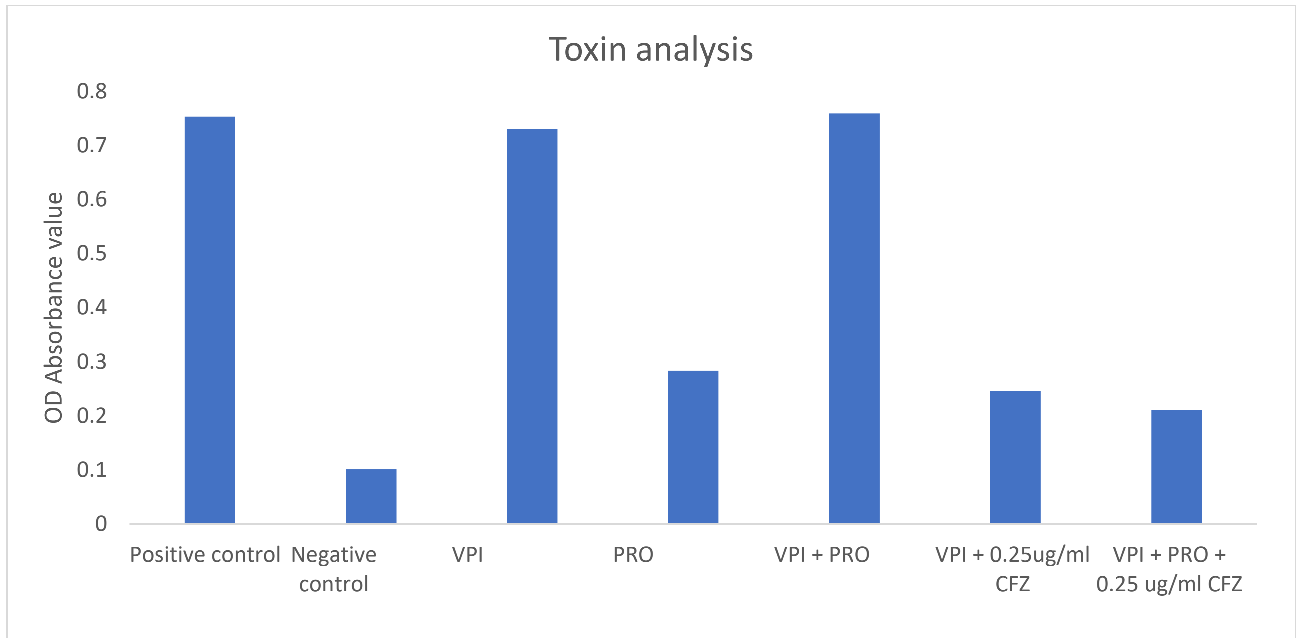

Figure 5: Toxin levels of *C. difficile* after treatment with clofazimine (CFZ) in presence of probiotics. VPI 10463 was grown in the presence of probiotics and 0.25 µg/ml CFZ and supernatants from the cultures were evaluated for presence of toxins A/B at 24-hour intervals using the ELISA kit

Toxin levels of untreated culture of VPI 10463 was observed close to pure toxins A&B. Probiotic culture shows toxins level closer to the negative control as expected. The untreated co-culture of VPI 10463 and probiotic shows toxin levels as that of positive control. This means that the probiotic culture does not influence toxin levels of VPI 10463. Treated culture of VPI with growth-inhibitory concentration of CFZ showed around 0.2

O.D. which is at cut-off value (negative control + 0.1). The same is also observed on co-cultures with growth-inhibitory concentration of CFZ. This helps us conclude that toxin levels were drastically reduced at growth-inhibitory concentrations of CFZ with or without the presence of probiotics.

4. Conclusion

Virulence of *C. difficile* is due to the host cellular immune response to production of toxins: TcdA and TcdB. Clofazimine is successful in reducing toxin levels at growth-inhibitory concentrations of the drug, i.e., 0.25μg/ml. At sub-inhibitory concentrations of the drug, the toxin levels are same as that of untreated culture. Our studies showed that probiotics does not have an influence on toxin levels of *C. difficile*, however, clofazimine at MIC value successfully reduced toxin levels of *C. difficile* in presence of probiotics. Toxin levels of *C. difficile* had a direct correlation to the growth kinetics of the bacteria. Clofazimine proves to be an effective treatment for *C. difficile* given its strong antimicrobial activity against the bacteria as well as the toxins produced.

References

1. Dawson, L. F., Valiente, E., Faulds-Pain, A., Donahue, E. H., & Wren, B. W. (2012). Characterisation of *clostridium difficile* biofilm formation, a role for SPO0A. *PLoS ONE*, *7*(12). https://doi.org/10.1371/journal.pone.0050527
2. Guh, A. Y., Mu, Y., Winston, L. G., Johnston, H., Olson, D., Farley, M. M., Wilson, L. E., Holzbauer, S. M., Phipps, E. C., Dumyati, G. K., Beldavs, Z. G., Kainer, M. A., Karlsson, M., Gerding, D. N., & McDonald, L. C. (2020). Trends in U.S. burden of *clostridioides difficile* infection and outcomes. *New England Journal of Medicine*, *382*(14), 1320–1330. https://doi.org/10.1056/nejmoa1910215
3. Smits, W. K., Lyras, D., Lacy, D. B., Wilcox, M. H., & Kuijper, E. J. (2016). *Clostridium difficile* infection. *Nature Reviews Disease Primers*, *2*(1). https://doi.org/10.1038/nrdp.2016.20
4. Feuerstadt, P., Nelson, W. W., Teigland, C., & Dahdal, D. N. (2022). Clinical burden of recurrent *clostridioides difficile* infection in the Medicare population: A real-world claims analysis. *Antimicrobial Stewardship and Healthcare Epidemiology*, *2*(1). https://doi.org/10.1017/ash.2022.2
5. Alfa, M. J., Kabani, A., Lyerly, D., Moncrief, S., Neville, L. M., Al-Barrak, A., Harding, G. K., Dyck, B., Olekson, K., & Embil, J. M. (2000). Characterization of a toxin A-negative, toxin B-positive strain of *clostridium difficile* responsible for a nosocomial outbreak of *clostridium difficile* -associated diarrhea. *Journal of Clinical Microbiology*, *38*(7), 2706–2714. https://doi.org/10.1128/jcm.38.7.2706-2714.2000
6. Yano, T., Kassovska-Bratinova, S., Teh, J. S., Winkler, J., Sullivan, K., Isaacs, A., Schechter, N. M., & Rubin, H. (2011). Reduction of clofazimine by mycobacterial

type 2 NADH:quinone oxidoreductase. *Journal of Biological Chemistry*, *286*(12), 10276–10287. https://doi.org/10.1074/jbc.m110.200501

7. Snell, H., Ramos, M., Longo, S., John, M., & Hussain, Z. (2004). Performance of the techlab *C. diff* Chek-60 enzyme immunoassay (EIA) in combination with the *C. difficile* Tox A/B II EIA kit, the triage *C. difficile* panel immunoassay, and a cytotoxin assay for diagnosis of *clostridium difficile* -associated diarrhea. *Journal of Clinical Microbiology*, *42*(10), 4863–4865. https://doi.org/10.1128/jcm.42.10.4863-4865.2004
8. Luciano, J. A., & Zuckerbraun, B. S. (2014). *Clostridium difficile* infection. *Surgical Clinics of North America*, *94*(6), 1335–1349. https://doi.org/10.1016/j.suc.2014.08.006
9. Nakamura, K., Inokuchi, R., Doi, K., Fukuda, T., Tokunaga, K., Nakajima, S., Noiri, E., & Yahagi, N. (2014). Septic ketoacidosis. *Internal Medicine*, *53*(10), 1071–1073. https://doi.org/10.2169/internalmedicine.53.1791
10. Vardanyan, R. S., & Hruby, V. J. (2006). Antimycobacterial Drugs. *Synthesis of Essential Drugs*, 525–534. https://doi.org/10.1016/b978-044452166-8/50034-0
11. Surawicz, C. M., Brandt, L. J., Binion, D. G., Ananthakrishnan, A. N., Curry, S. R., Gilligan, P. H., McFarland, L. V., Mellow, M., & Zuckerbraun, B. S. (2013). Guidelines for diagnosis, treatment, and prevention of *clostridium difficile* infections. *American Journal of Gastroenterology*, *108*(4), 478–498. https://doi.org/10.1038/ajg.2013.4

12. Gujja, D., & Friedenberg, F. K. (2009). T2059 predictors of serious complications due to *clostridium difficile* infection. *Gastroenterology*, *136*(5). https://doi.org/10.1016/s0016-5085(09)62905-x
13. McFarland, L. V. (1994). A randomized placebo-controlled trial of *saccharomyces boulardii* in combination with standard antibiotics for *clostridium difficile* disease. *JAMA: The Journal of the American Medical Association*, *271*(24), 1913–1918. https://doi.org/10.1001/jama.271.24.1913
14. Surawicz, C. M., McFarland, L. V., Greenberg, R. N., Rubin, M., Fekety, R., Mulligan, M. E., Garcia, R. J., Brandmarker, S., Bowen, K., Borjal, D., & Elmer, G. W. (2000). The search for a better treatment for recurrent *clostridium difficile* disease: Use of high-dose vancomycin combined with *saccharomyces boulardii*. *Clinical Infectious Diseases*, *31*(4), 1012–1017. https://doi.org/10.1086/318130
15. Reddy, V. M., Prensky, W., & VedBrat, S. (2020). Pharmaceutical compositions containing azaquinone for inhibiting *Clostridium difficile* activity (U.S. Patent No. 10,758,529 B2). U.S. Patent and Trademark Office. https://patents.google.com/patent/US10758529B2/en
16. Pasupuleti, C. (2021). Investigation of the added therapeutic potential of Clofazimine in combating antimicrobial resistance in *Clostridium difficile*. (Master's Thesis, Johns Hopkins University), Available from JHU ETD Database. http://jhir.library.jhu.edu/handle/1774.2/64235
17. National Center for Biotechnology Information (2022). *PubChem Compound Summary for CID 2794,* clofazimine. https://pubchem.ncbi.nlm.nih.gov/compound/clofazimine.

18. Cholo, M. C., Mothiba, M. T., Fourie, B., & Anderson, R. (2016). Mechanisms of action and therapeutic efficacies of the lipophilic antimycobacterial agents clofazimine and bedaquiline. *Journal of Antimicrobial Chemotherapy*, *72*(2), 338–353. https://doi.org/10.1093/jac/dkw426
19. Oliva, B. (2004). Anti-staphylococcal activity and mode of action of clofazimine. *Journal of Antimicrobial Chemotherapy, 53*(3), 435–440. https://doi.org/10.1093/jac/dkh114
20. Ujah O. F., Hough A. S (2020). Bioactive lead compounds effective against skin diseases,13, 211-220, https://doi.org/10.1016/B978-0-12-817890-4.00013-5.
21. Clinical and Laboratory Standards Institute (2012). Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria—Eighth Edition: Approved Standard M11-A8. CLSI.
22. Dapa, T., & Unnikrishnan, M. (2013). Biofilm formation by *clostridium difficile*. *Gut Microbes*, *4*(5), 397–402. https://doi.org/10.4161/gmic.25862
23. Sachdeva, M., & Leeds, J. A. (2015). Subinhibitory concentrations of LFF571 reduce toxin production by *clostridium difficile*. *Antimicrobial Agents and Chemotherapy*, *59*(2), 1252–1257. https://doi.org/10.1128/aac.04436-14
24. Locher, H. H., Seiler, P., Chen, X., Schroeder, S., Pfaff, P., Enderlin, M., Klenk, A., Fournier, E., Hubschwerlen, C., Ritz, D., Kelly, C. P., & Keck, W. (2014). *in vitro* and *in vivo* antibacterial evaluation of cadazolid, a new antibiotic for treatment of *clostridium* difficile infections. *Antimicrobial Agents and Chemotherapy*, *58*(2), 892–900. https://doi.org/10.1128/aac.01830-13

25. Ghimire, S., Roy, C., Wongkuna, S., Antony, L., Maji, A., Keena, M. C., Foley, A., & Scaria, J. (2020). Identification of *clostridioides difficile*-inhibiting gut commensals using culturomics, phenotyping, and Combinatorial Community Assembly. *MSystems*, *5*(1). https://doi.org/10.1128/msystems.00620-19
26. Garrett, E. M., Sekulovic, O., Wetzel, D., Jones, J. B., Edwards, A. N., Vargas-Cuebas, G., McBride, S. M., & Tamayo, R. (2019). Phase variation of a signal transduction system controls *clostridioides difficile* colony morphology, motility, and virulence. *PLOS Biology*, *17*(10). https://doi.org/10.1371/journal.pbio.3000379
27. Locher, H. H., Seiler, P., Chen, X., Schroeder, S., Pfaff, P., Enderlin, M., Klenk, A., Fournier, E., Hubschwerlen, C., Ritz, D., Kelly, C. P., & Keck, W. (2014). *in vitro* and *in vivo* antibacterial evaluation of cadazolid, a new antibiotic for treatment of *clostridium difficile* infections. *Antimicrobial Agents and Chemotherapy*, *58*(2), 892–900. https://doi.org/10.1128/aac.01830-13
28. Trejo, F. M., Pérez, P. F., & De Antoni, G. L. (2010). Co-culture with potentially probiotic microorganisms antagonises virulence factors of *clostridium difficile* in vitro. *Antonie Van Leeuwenhoek*, *98*(1), 19–29. https://doi.org/10.1007/s10482-010-9424-6