Summer 8-2022

Clostridioides Difficile Biofilm and Spore Production in Response to Antibiotics and Immune Stress

Adenrele M. Oludiran

Old Dominion University, adenreleoludiran@gmail.com

CLOSTRIDIOIDES DIFFICILE BIOFILM AND SPORE PRODUCTION IN RESPONSE TO

ANTIBIOTICS AND IMMUNE STRESS

by

Adenrele M. Oludiran
BSc. December 2011, Olabisi Onabanjo University, Nigeria
M.S. August 2018, Old Dominion University

A Dissertation Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

CHEMISTRY

OLD DOMINION UNIVERSITY
August 2022

Approved by:

Erin B. Purcell (Director)

Piotr J Kraj (Member)

Lesley Greene (Member)

Steven M. Pascal (Member)

ABSTRACT

CLOSTRIDIOIDES DIFFICILE BIOFILM AND SPORE PRODUCTION IN RESPONSE TO
ANTIBIOTICS AND IMMUNE STRESS

Adenrele M. Oludiran
Old Dominion University, 2022
Director: Dr. Erin B. Purcell

The development of new therapeutic options against *Clostridioides difficile (C. difficile)*
infection is a critical public health concern, as the causative bacterium is highly resistant to
multiple classes of antibiotics. *C. difficile*, an anaerobic spore-forming Gram-positive pathogenic
bacterium, is a major cause of hospital-acquired infections. *C. difficile* persists in the
environment and spreads the infection to new hosts in the form of dormant spores and can persist
within hosts as surface-attached biofilms. These studies investigate bacterial vegetative cell
survival, biofilm formation, and sporulation in response to stress. Antimicrobial host-defense
peptides (HDPs) are highly effective at simultaneously modulating immune system function and
directly killing bacteria through membrane disruption and oxidative damage. HDPs produced
naturally by animal immune systems are promising candidates to develop novel therapies for
bacterial infection because they cause oxidative stress that damages multiple targets in bacterial
cells, so it is difficult for bacteria to evolve resistance to these attacks. We investigate the *C.
difficile* response to HDPs applied alone or in combination with antibiotics and to oxidative
stresses similar to those caused by the human immune system. In our investigation of fish-
derived copper-binding HDPs known as piscidins applied to *C. difficile* in an anaerobic
environment, we found that the interaction of piscidin and copper depends on environmental
oxygen. While copper-binding increases piscidin potency in an aerobic environment, copper

does not synergize with these peptides anaerobically. Piscidins suppress the proliferation of *C. difficile* by killing bacterial cells and strongly increase the efficacy of multiple classes of antibiotics when applied in combination. Piscidins disrupt the bacterial cell membrane and increase the uptake of exogenous substances. We find that piscidins are effective against epidemic *C. difficile* strains that are highly resistant to other stresses. While extracellular stress can induce *C. difficile* to sporulate or form biofilm for protection, piscidins alone kill vegetative *C. difficile* cells without triggering spore formation and repress antibiotic-induced sporulation after combined treatments. Piscidins may stimulate more *C. difficile* biofilm formation at sub-inhibitory doses, so dosage will need to be carefully considered in any potential infection treatments using these peptides.

This dissertation is dedicated to my family for their unreserved love and support; especially to my mother and father, Ms. Oludiran Mistura, and Capt. Oludiran Gbadegesin (rtd).

ACKNOWLEDGEMENTS

I appreciate all the helping hands who supported me to be able to complete this dissertation. My heartfelt appreciation to my advisor, Dr. Erin B. Purcell for her dedicated guidance, encouragement, and tolerance throughout my Ph.D. program at Old Dominion University. I would like to extend my sincere thanks to the members of my dissertation committee, Drs. Steven M. Pascal, Lesley Greene, and Piotr J. Kraj for their helpful knowledge and support through my graduate study. In addition, I would like to acknowledge current and earlier Purcell lab group members for their contribution and support in this work. I extend my sincere gratitude to Dr. David Courson for his support and guidance in my research. I would like to thank all the faculties and staff at the Department of Chemistry and Biochemistry at ODU for their support during my graduate study. I am thankful to Dr. Alvin Holder for all the advice and encouragement. I would like to reaffirm my admiration and special thanks to all my colleagues who supported me. Furthermore, I would like to acknowledge Dr. Myriam L Cotten (College of Williams and Mary), and Dr. Muratori (Frank Reidy Research Center For Bioelectrics ODU).

NOMENCLATURE

CDI	*Clostridioides difficile* infection
FMT	Fecal microbiota transplantation
FDA	Food and Drug Administration
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
°C	Degrees Celsius
BHIS	Brain heart infusion supplemented
TY	Tryptone yeast
LB	Luria-Bertani
×g	Times gravity
DNA	Deoxyribonucleotide
PBS	Phosphate buffered saline
DIC	Differential interface contrast
PI	Propidium iodide
CFU	Colony forming unit
ECM	Extracellular matrix
VAN	Vancomycin
MET	Metronidazole
FID	Fidaxomicin
p1	Piscidin 1
p3	Piscidin 3

p1Cu	Piscidin 1 Copper complex
p3Cu	Piscidin 3 Copper complex
SEM	Scanning Electron Microscope
SLP	Surface Layer Protein
EPS	Extracellular Polymeric Substances
GI	Gastrointestinal Tract
HDP	Host Defense Peptides
AMP	Antimicrobial Peptides
MRSA	Methicillin-Resistant *Staphylococcus aureus*
TAMRA	5-Carboxytetramethylrhodamine
ATCUN	Amino-Terminal Copper and Nickel-binding

TABLE OF CONTENTS

xi

LIST OF TABLES

LIST OF FIGURES

Figure																					Page

CHAPTER 1

INTRODUCTION

OVERVIEW

Bacterial Phenotypic Stress Responses

The prokaryotic organisms called bacteria are categorized by their relationship to environmental oxygen [3]. Obligate aerobic bacteria require oxygen as the terminal electron acceptor for energy generation and cannot live in its absence [4]. Facultative anaerobes can either perform aerobic respiration in oxygen-rich environments or anaerobically generate energy through fermentation. All anaerobes encode strong antioxidant defense mechanisms against reactive oxygen species (ROS) generated by aerobic metabolism and respiration [5]. Obligate anaerobic bacteria utilize carbon dioxide, sulfur, fumarate, or ferric acid rather than oxygen as terminal electron acceptors and are quickly killed by oxygen exposure as they have limited antioxidant capacity to detoxify ROS [3, 4]. Anaerobic pathogens infect oxygen-poor or anoxic environments within hosts, such as the large intestine in mammals. Most anaerobic pathogens are pathobionts and originate from commensal gut relationships to become infectious pathogens that are major health concerns [6]. Bacteria experience a variety of stresses in their natural environments, including stress from the host immune system in the case of pathogens [7]. Bacterial stress can lead to several adaptive and protective responses, altering gene expression patterns and cell physiology [8, 9]. This can include the formation of resilient, metabolically dormant spores or adherence to surfaces and secretion of a protective extracellular matrix to form biofilm as a direct phenotypic expression in response to environmental stresses [10].

Stressors in the pathogenic bacterial niche include but are not limited to antibiotics, innate immune system effects such as the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), gastrointestinal (GI) metal homeostasis, chemicals (such as sanitizers in the food industry or in medical settings), physical stresses (temperature from fever in a host or from industrial or healthcare sterilization processes), and competition for nutrients from other bacteria; however, some highly resilient pathogens survive and thrive in these stressed environments [11, 12]. Bacterial pathogenesis is an urgent health crisis around the world because microbes have developed both genetic and phenotypic processes of evading stresses and are increasingly antibiotic-resistant [13]. Studies already exist focusing on the regulation of resistance genes with little attention to the phenotypes that result from gene transcription and translation. It is the phenotypic changes that allow the infection to persist and cause symptoms in the host [14]. This study focuses on phenotypic stress responses, namely spore production and biofilm formation of the pathogenic anaerobic bacteria *Clostridioides difficile*.

Clostridioides difficile

Clostridioides (formerly *Clostridium*) *difficile* was first isolated and identified in 1935 by Ivan Hall and Elizabeth O'Toole [15]. The pathogen was isolated during a study investigating the bacterial flora in the stool samples of healthy infants [16]. *C. difficile* is the cause of *Clostridioides difficile* infection (CDI). Symptoms of CDI can include inflammation of intestinal walls, profuse diarrhea, and pseudomembranous colitis. CDI has been recognized as an urgent world health crisis by the U.S. Center for Disease Control [17, 18]. Since its discovery, *Clostridioides difficile* in the 21st century became the leading nosocomial infection around the world. This opportunistic pathogen is responsible for over 462,100 cases resulting in 20,500 deaths annually in the United States alone [19]. The main risk factor for contracting CDI is the

use of broad spectrum antibiotics, resulting in disruption of the gut microbiota [20]. This creates

an opportunity for *C. difficile* colonization, as the pathogen has less competition for nutrients.

Old age is an additional risk factor, as people over 65 years old are 8.65 times more likely to

contract CDI than those younger than the age [16].

Epidemiology data for the past two decades show that CDI is not only found in

developed countries, but also present in the African continent. Data show that on the African

continent, unlike in Europe and North America, younger people get CDI more than the elderly.

This is probably due to the high prevalence of co-morbid conditions such as tuberculosis,

particularly in west Africa [21]. *C. difficile* has primarily been a western hospital-acquired

disease, but the number of community-acquired infections has increased in recent years as have

cases in infants in developing nations [22, 23]. The severity of CDI has also increased during the

21st century with the emergence of so-called "hypervirulent" epidemic strains including the

R20291 strain responsible for the infamous 2003-2005 Stokes Mandeville hospital outbreak in

Great Britain. This hypervirulent strains are associated with higher levels of disease recurrence

and death in infected patients [24, 25]. *C. difficile* is resistant to several families of antibiotics

and it is becoming increasingly resistant to next-generation therapeutics including fidaxomicin,

metronidazole, and vancomycin [26]. Recently, the most clinically effective treatment for CDI is

the replenishment of the protective gut microbiota through fecal transplants. The procedures

have a high risk of introducing other pathogens, making them not recommended for

immunocompromised patients, so there is great interest in the development of new strategies for

prevention and treatment of CDI [27]. Few labs study *C. difficile* because it is a strict anaerobe

that requires an artificially created anaerobic atmosphere with in an anaerobic chamber (Figure

1) [28]. Anaerobic experiments commonly occur in an atmosphere of 85% nitrogen, 10% CO_2, 5% H_2.

Figure 1. Representative image of an anaerobic chamber. The sealed glove box is accessed by a vacuum airlock to allow the transfer of materials in and out of the chamber without filling it with environmental oxygen. Shown is the Purcell lab anaerobic chamber.

Spores

    C. difficile persists in the environment and infects new hosts in the form of metabolically dormant spores, which are highly resilient to chemical and physical stresses and remain viable for months [29]. Spore formation and germination are important for the spread of CDI but are still not well characterized in *C. difficile* [30]. Spores germinate into vegetative cells which are

the metabolically active and toxin-producing stage of the bacterial life cycle. Most known regulatory information was adapted from *Bacillus subtilis* but many of the sporulation regulation pathways are not conserved between the two species [31, 32]. Spores germinate into vegetative cells and proliferate in the gut following exposure to germinants including primary bile acids such as taurocholate and the amino acids alanine and glycine [33]. Antibiotic treatments disrupt the intestinal microbiota, allowing *C. difficile* spores or vegetative cells to either germinate or replicate easily because other competing microbes in the same niche die off, allowing *C. difficile* greater access to bioavailable nutrients [34].

Spores are extremely difficult to eradicate from contaminated environments. The resistance of bacterial spores results from a protective multilayered structure (Figure 2a) and from the unique composition of the spore core containing highly dehydrated bacterial DNA, ribosomes, dipicolinic acid (for maintenance of spore dormancy) and inorganic minerals (Figure 2b) [35, 36].
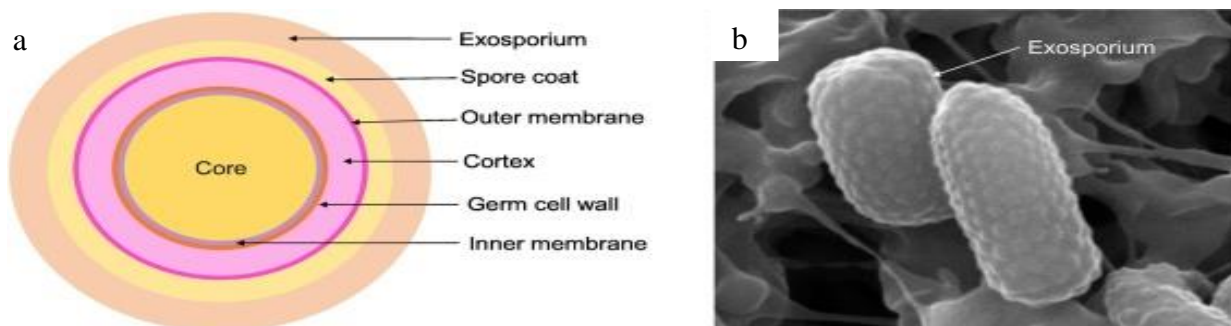
Figure 2. Structure of *C. difficile* spores. (**a**) Schematic drawing of longitudinal spore structure and the multilayered spore coat. (**b**) Scanning Electron Microscope (SEM) image of aggregated spores in the extracellular matrix of a biofilm. Adapted from Lawler, A. J., *et al* 2020

The spore core is dehydrated which keeps DNA, tRNA, ribosomes, and metabolic enzymes dormant or inactive until bile salt activation of spore germination [37]. The inner membrane is the bridge between the core of the spore and the germ cell wall. The spore core membrane is made up of derivative forms of cellulose which protect against desiccation and prevents inlet of DNA damaging reagents [38]. The next layer is the cortex which is made of peptidoglycans that are degraded by lytic enzymes during spore germination [39]. The outer membrane only creates space between the rest of the layers. The spore coat consists of more than 30% water insoluble proteins creating an impermeable barrier to most biological substances [40]. Though not present in many other sporulating bacteria, the exosporium contributes to *C. difficile* spore resiliency against well characterized stresses due to its high number of cysteine-rich proteins necessary for resistance to lysozyme, ethanol, and heat treatment as well as surface layer protein (SLP), which is involved in sensing bile salt germinants [41].

Primary bile salts, including cholic acid and chenodeoxycholic acid (Figure 3a), are produced by liver hepatocytes and stored in the gallbladder before diffusing into the small intestine, where they solubilize dietary lipids [42]. Intestinal bacteria metabolize primary bile salts into secondary bile salts which accumulate in the large intestine (Figure 3b) before being trafficked back to the liver and recycled into primary bile salts (Figure 3c) [43]. Primary bile salts induce *C. difficile* spore germination while secondary bile salts inhibit *C. difficile* vegetative growth [44]. Disruption of the gut microbiota reduces the conversion of primary bile salts to secondary bile salts, creating conditions hospitable for *C. difficile* colonization [45].
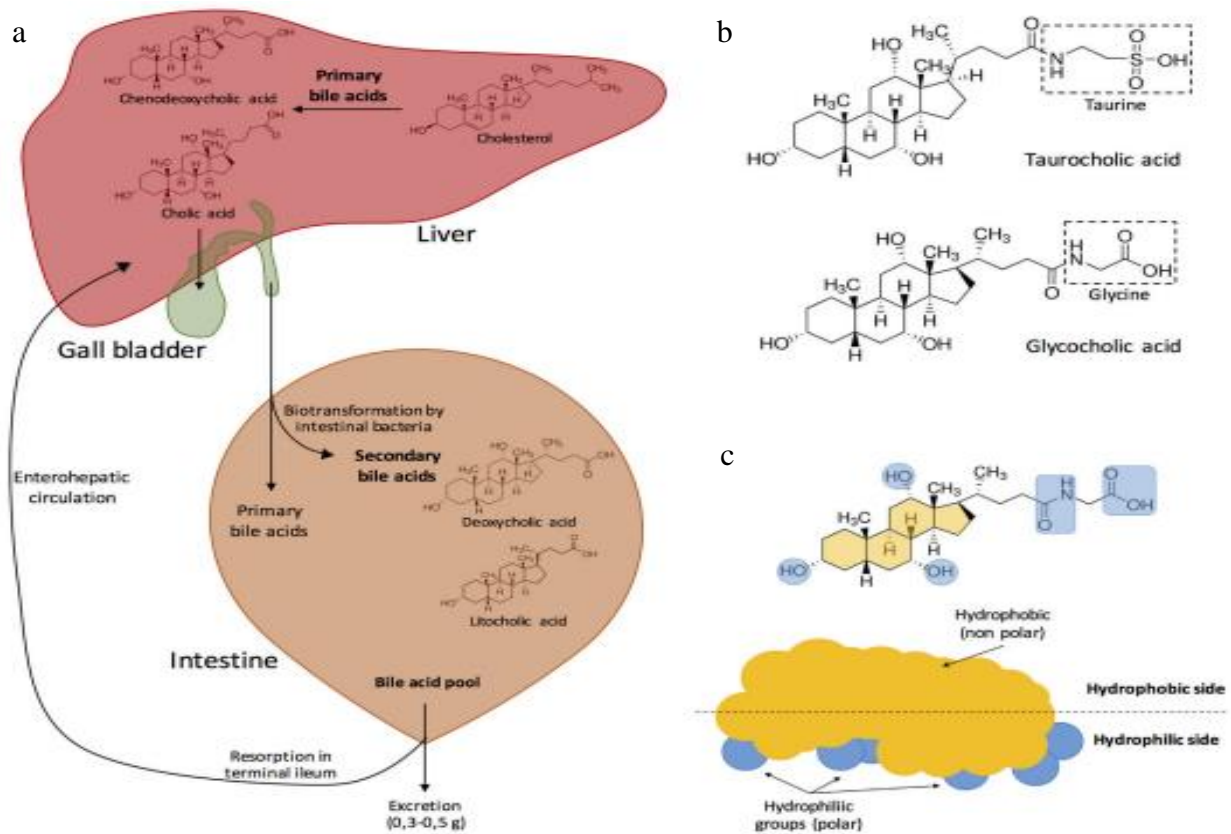
Figure 3. Synthesis of the major bile acids of human bile and circulation in the hepatic, biliary, and digestive systems. (**a**) Bile acids are converted into bile salts by the association with metal cations. Produced bile salts are stored in the gall bladder. After food ingestion, bile is released into the small intestine. Bile acids are modified into secondary bile acids in the small intestine by commensal microbiota. Primary and secondary bile acids are reabsorbed in the terminal ileum. Modified from Fontana *et al*. (**b**) Bile acids can be conjugated with amino acids. In this example, cholic acid becomes either taurocholic acid or glycocholic acid after conjugation with taurine or glycine, respectively. (**c**) Bile acids are amphipathic; the hydrophobic side is associated with the surface of lipid droplets while the polar groups interact with water creating a stable emulsion of small, mixed micelles. Modified from Hofmann and Vander *et al*.

Spores appear to have affinity for the extracellular matrix of biofilms, which may act as a reservoir for them *in vivo* [46]. They can persist on surfaces or in patients' intestines in hospital and healthcare facilities for extended periods, leading to new or recurrent infections [47, 48]. Recurring episodes affect about 15–35% of patient and present a particular risk for the elderly [34]. Spore germinate in the intestine is activated by cholate and deoxycholate stimulants [49]. Ingested *C. difficile* spores germinate into vegetative cells, which either reproduce and produce toxins or differentiate into spores to continue the bacterial life cycle (Figure 4) [50].
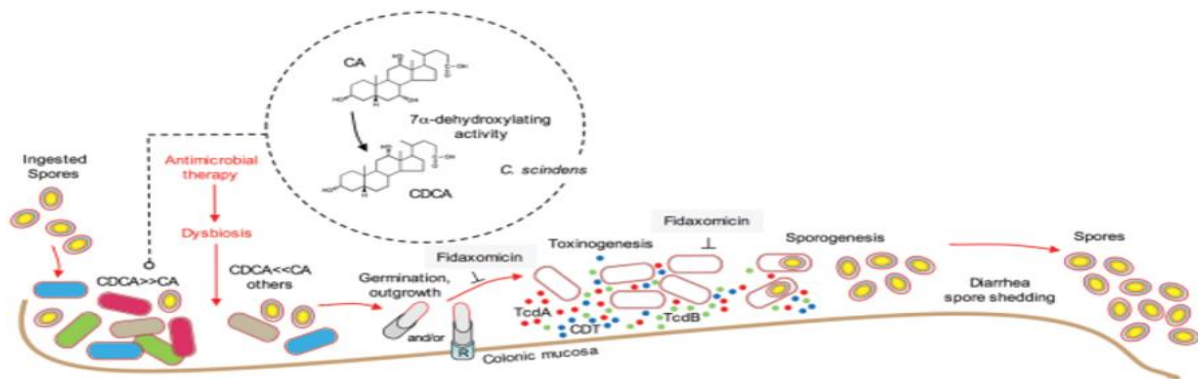
Figure 4. Schematic representation of the *C. difficile* infection cycle. Spores are ingested and can pass the gastric barrier to reach the intestine. In the intestine, when the normal gut microbiota is disturbed, for example by antibiotic treatment, the concentration of the secondary bile salts cholate (CA) and chenodeoxycholate (CDCA) increases and spores will germinate. Spore germination produces a population of vegetative, actively growing cells, which will initiate toxin production and sporulation. Shedding of the highly resistant spores will allow their accumulation in the environment and the infection of new hosts, and spores retained in the host can cause recurrence. Reproduced from Isidro Mendes *et al* 2017.

Bacterial Biofilms

The mammalian gastrointestinal tract (GI) is home to a complex community of microbes, composed of prokaryotes and eukaryotes, collectively referred to as the GI microbiota, that are present mostly as biofilms [51]. Biofilm can protect vegetative bacterial cells and harbor spores [46]. Formation attached of biofilms involves secretion of biofilm components like matrix and adhesins at high cell density as shown in Figure 5 [52]. The biofilm matrix includes polysaccharides, proteins, and nucleic acids, together called extra polymeric substances (EPS) [53, 54]. Some extracellular conditions, such as carbohydrate availability or antibiotic exposure, can stimulate increased biofilm formation [55]. After maturation, the biofilm can disperse vegetative cells and spores [56]. We explore sporulation and biofilm formation of planktonic *C. difficile* cells in response to antibiotics, antimicrobial metals, antimicrobial peptides, and oxidants that mimic stressful environments within a mammalian host.

Planktonic   Biofilm

(5) Detachment and reversion to planktonic growth, starting a new cycle

(1) Attachment to surface

(2) Formation of monolayer and production of matrix

(3) Microcolony formation, multi-layer

(4) Mature biofilm, with characteristic "mushroom" formed of polysaccharide
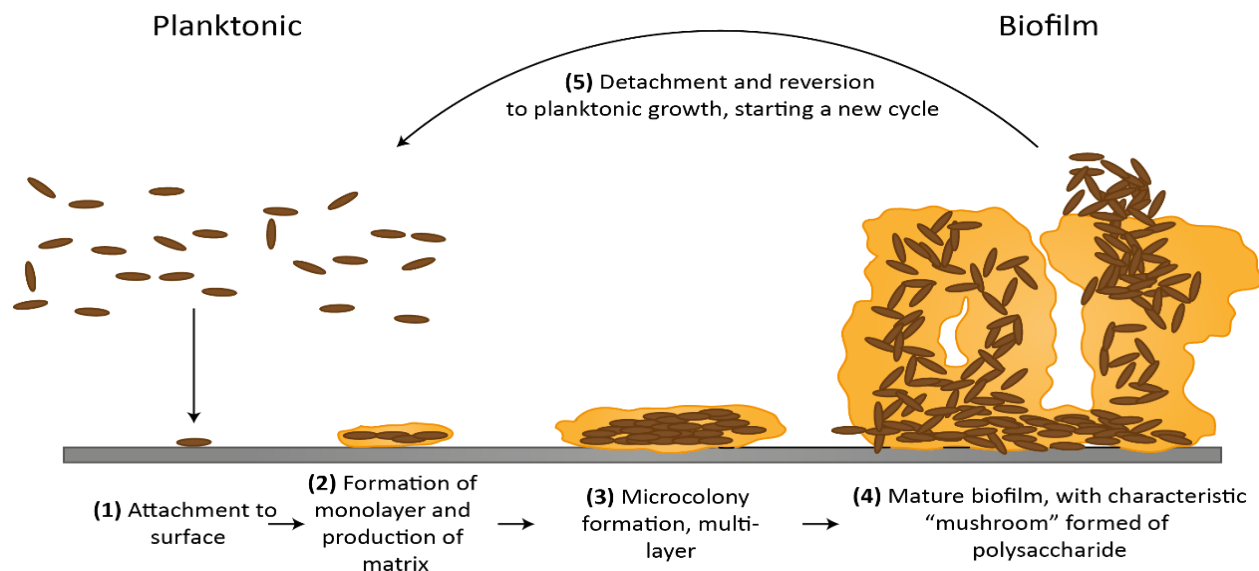
Figure 5. Schematic representation of biofilm formation. Formation begins with a reversible attachment of planktonic cells (brown ovals) followed by the adhesion to the surface (grey) (1). The bacteria then form a monolayer and irreversibly attach by producing an extracellular matrix (yellow) (2). Next, a microcolony containing multiple layers is formed (3). During later stages, the biofilm is mature, forming characteristic "mushroom" structures (4). Finally, some cells start to detach, and the biofilm (shown in yellow) will disperse (5). Adapted from Vasudevan, 2014.

Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs), also known as host defense peptides (HDPs), are a common endogenous defense mechanism against pathogens, which occur in all classes of life. AMPs are relatively small peptides consisting of 4−50 amino acid residues and are highly cationic [57]. Piscidins are antimicrobial HDPs found in fish which have previously shown

broad-spectrum antimicrobial activity against aerobic bacteria [58]. They can induce immunomodulatory effects by enhancing the immune system's ability to attract leukocytes to the infection and directly kill bacteria by attacking plasma membranes or targeting intracellular DNA with nuclease activity [59-61]. These antimicrobial peptides are promising therapies, because they are active against highly drug resistant bacteria [58].

The peptides employed in these investigations are piscidin 1 (p1) **(FFHHIFRGIVHVGKTIHRLVTG)** and piscidin (p3) (**FIHHIFRGIVHAGRSIGRFLTG**). The peptides fold into alpha helical secondary protein structures (Figure 6) and are derived from the mast cells of hybrid striped sea bass, which exhibit broad spectrum antibacterial activities with dual mechanisms [62, 63]. Both piscidins have conserved amino terminal copper and nickel binding (ATCUN) motifs that can bind +2 transition metals, most notably copper (II) and nickel (II). The ATCUN regions for p1 and p3 are FHH and FIH, respectively [61, 64]. p1 primarily disrupts the bacterial cell membrane and exhibits weak DNA nuclease activity while p3 causes less membrane disruption and displays strong DNA nuclease activity [58]. There are two proposed models for piscidin-mediated membrane disruption. The first is that piscidins enter the membrane perpendicular to the membrane lipids and disrupt contacts between the lipids through a combination of hydrophobic and hydrophilic interactions. [63]. Additionally, their bound copper ions induce oxidative stress by inducing the formation of ROS, which cause oxidative damage to the lipid headgroups [63]. These interactions have been studied in the presence of atmospheric oxygen levels but had not previously been studied in the absence of molecular oxygen. In this work, we investigate the efficacy and response of these peptides against *Clostridioides difficile* in an anaerobic environment, focusing on the bacterial cells' proliferation, viability, sporulation, and biofilm production.

Figure 6: Structure of piscidin 1. Piscidins form amphipathic alpha-helices that interact with the plasma membranes. The structure of piscidin 1 was determined by solid-state NMR. (PDB CODE: 6PF0). Adapted from Cotten M. L *et al* 2019.

Common Antibiotics

C. difficile has supplanted methicillin-resistant *Staphylococcus aureus* as the most common cause of nosocomial infection [65]. Antibiotics commonly used in the treatment of CDI now include fidaxomicin (FID), vancomycin (VAN) and metronidazole (MET), but resistance to all these drugs is rising over time [66]. These antibiotics have different cellular targets. Fidaxomicin binds to a four amino acid sequence on the *C. difficile* RNA polymerase, which is not broadly conserved among the commensal gut bacteria, to inhibit the initiation of RNA transcription with high specificity (Figure 7) [1, 67]. Metronidazole denatures genomic DNA and causes strand breakage by intercalating into the helical fold and also induces oxidative damage (Figure 7) [1, 68]. Vancomycin inhibits the biosynthesis of the major structural cell wall polymer, peptidoglycan, interfering with cell growth and replication (Figure 7) [1, 69]. Many of

other classes of commonly used antibiotics, including fluroquinolones and tetracycline, are ineffective against *C. difficile;* their interactions with *C. difficile* are summarized in Table 1 [70].

Currently, MET is rarely used in developed countries because many strains of bacteria are now resistant to it; the same issue is beginning to affect VAN, which is a non-ideal treatment for CDI because it is not narrow spectrum. A narrow spectrum antimicrobial agent are genus or species specific in action because they target or are more suited to inhibit species specific process [71]. FID is the most recent and specific treatment for CDI but has a very high cost and there are already some clinical isolates of *C. difficile* with low susceptibility to this antibiotic [66]. There is an urgent need for new and highly specific therapeutic methods or combination of methods for treatment of CDI that bacteria will not easily develop resistance against.
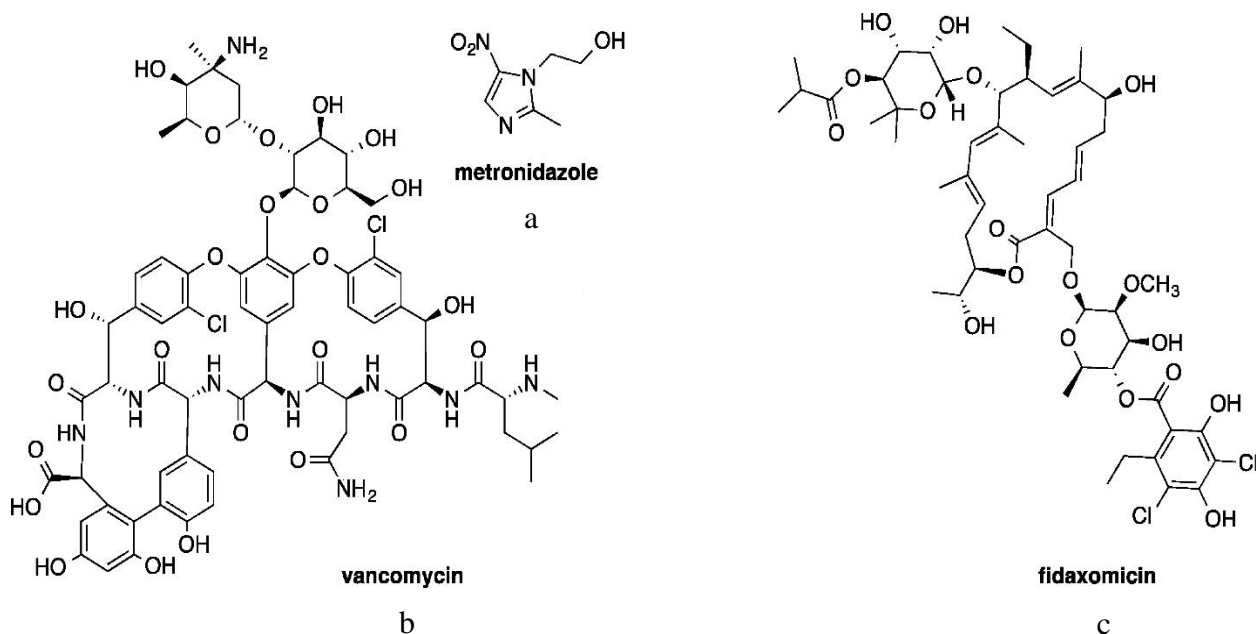
Figure 7. Current antibiotic treatments primarily used to treat *C. difficile* infection: (**a**) metronidazole, (**b**) vancomycin, and (**c**) fidaxomicin. Adapted from Jarrad *et al* 2015 [1].

Table 1. Summary of *Clostridioides difficile* resistance to antibiotics and associated

mechanisms. Adapted and modified from Isidro and Mendes et al 2017.

Antibiotic	Mechanism	Relevance in CDI	Resistance frequency	Mechanism of resistance
Metronidazole	DNA damage after reduction of metronidazole inside the bacterial cell	Treatment of mild/moderate CDI	High	Multifactorial 5-nitroimidazole reductase and modifications in multiple proteins involved in DNA repair, iron uptake and metronidazole reduction
Vancomycin	Inhibition of cell wall synthesis by binding to the dipeptide D-Ala-D-Ala of peptidoglycan	Treatment of severe and recurrent CDI	Moderate	Mutations in *murG*
Fidaxomicin	Inhibition of RNA synthesis by binding to RNA polymerase (in a site distinct from rifamycin)	Treatment of severe and recurrent CDI	Rare	Mutations in *rpoB, rpoC* and *rarR*
Fluoroquinolones	Inhibition of DNA synthesis by binding to DNA gyrase and topoisomerase IV	Associated with elevated risk of CDI; resistance contributed to spread of the epidemic ribotype 027	High	Target modification by mutations in *gyrA* and *gyrB*
Rifamycin	Inhibition of RNA synthesis by binding to RpoB	Used adjunctively for the treatment of recurrent CDI	Common	Target modification by mutations in *rpoB*
Tetracyclines	Inhibition of protein synthesis by binding to 30S ribosomal subunit	Resistance found in multi-resistant isolates; resistance shared between human and swine isolates	Common	Target protection by *tetM*, carried by the elements Tn5398 or Tn916-like
Chloramphenicol	Inhibition of protein synthesis binding to the 50S ribosomal subunit	Resistance associated with prevalent ribotypes	Uncommon	Inactivation of the antibiotic by *catD*, carried by Tn4453 elements

The Human Gastrointestinal Tract

The gastrointestinal (GI) tract consists of the esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (colon the ascending colon and cecum) and the rectum, which include a continuous tube; each part of the GI tract comes together to act in concert for the digestion of food. The GI tract is home to millions of microbes (Figure 10) [72]. In recent years, studies have confirmed that the human GI microbial community, or microbiota, play a significant role not only in food digestion but also in immune system function [73]. Alterations in GI microbial composition are strongly associated with many intestinal diseases, including colorectal cancer and inflammatory bowel disease as well as CDI (Figure 8). The small intestine is the major site of digested food absorption and is the location where *C. difficile* spores encounter their bile acid germinants [74]. Peristalsis, the contractile movement of the digestive tract, moves the majority of these germinated spores to the large intestine, which is an anaerobic environment with $< 2\%$ oxygen [75]. If the commensal gut microbiota have not consumed all of the food sources for *C. difficile*, the germinated spores can proliferate in the proximal colon where the *C.difficile* permeate through the mucosa, appear to adhere to the epithelial tissue (Figure 8), and secrete toxins that cause intestinal inflammation (Figure 9) [2, 76, 77].
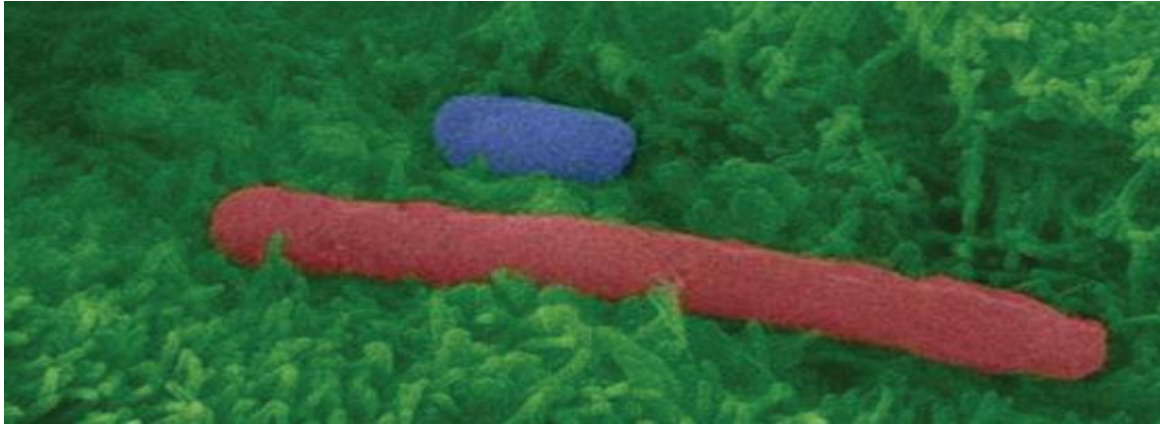
Figure 8. *C. difficile* spores and vegetative cells adhere to the host intestinal epithelium. False-color, high resolution, scanning electron micrograph of a *C. difficile* spore (blue) and vegetative cell (red) adhering to human intestinal epithelial cells (green) grown in culture. Microvilli are clearly visible (feather-like green protrusions). Micrograph collection, Vedantam laboratory [2]
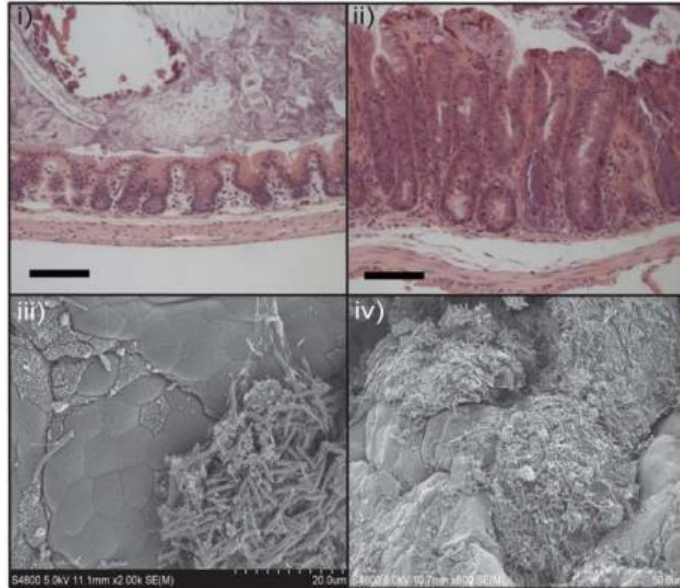
Figure 9. *C. difficile* adheres to sites of intestinal damage in infected mice. (**i–ii**) Hematoxylin (a basic dye) and eosin (an acidic dye) double edge staining of cecal biopsies where hematoxylin stains the nuclear components and eosin stains the cytoplasm components from (**i**) healthy, clindamycin-treated mice and (**ii**) mice infected with *C. difficile* 027/BI-7-day 49 post-infection. Scale bars represent 100 mm. (**iii-iv**) Scanning electron micrographs illustrating the presence of *C. difficile* microcolonies (**iii**) and biofilm-like structures (**iv**) on the intestinal mucosal surface (or epithelial cells of the same mice). Adapted from Lawley *et al* 2012
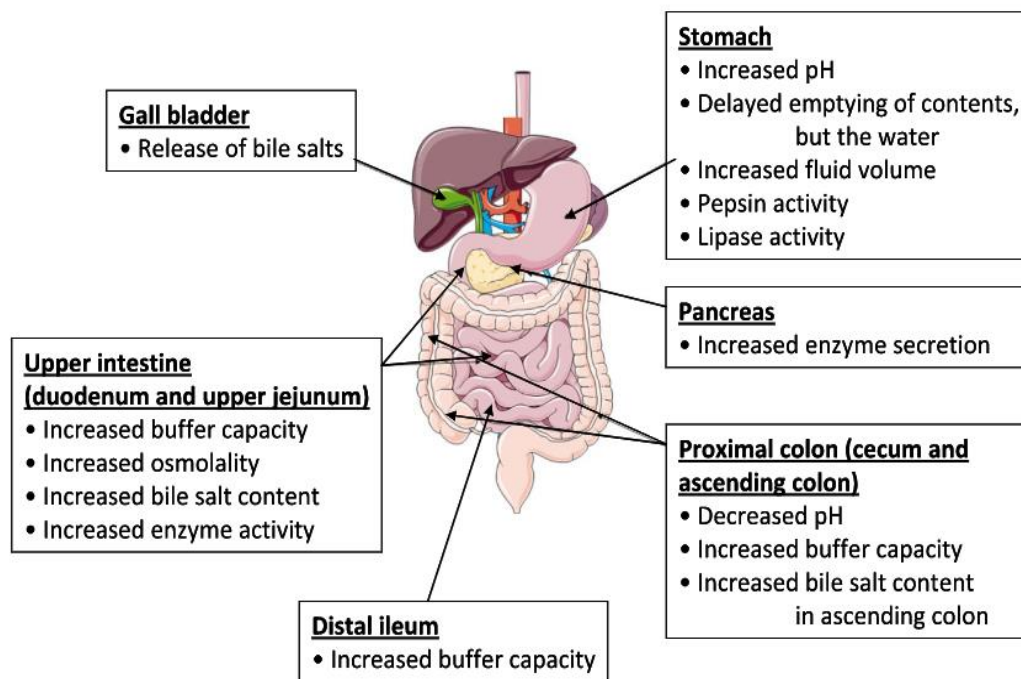
Figure 10. The human gastrointestinal tract. Shown are the organs of the human gastrointestinal tract and a summary of their biological roles. Adapted from Koziolek *et al* 2019

RESEARCH AIMS

The main objectives of the work presented in this dissertation are to:

1. Determine the efficacy of piscidins and their copper complexes against *Clostridioides difficile* in anaerobic conditions using growth inhibition assays, where peptides are applied before cells are inoculated into medium and time kill assays, where peptides are added to growing cells and viability is measured for each time (Chapter II).

2. Investigate synergistic or antagonistic effects between piscidins and different classes of antibiotics used to treat *Clostridioides difficile* using growth inhibition assays (Chapter III).

3. Determine the effect of piscidins and other environmental stresses on *Clostridioides difficile* phenotypic stress responses by quantitating sporulation and biofilm production (Chapters III and IV).

CHAPTER II

EFFICACY OF ANTIMICROBIAL PEPTIDES PISCIDINS AND THEIR COPPER COMPLEXES AGAINST *C. DIFFICILE* IN ANAEROBIC CONDITONS

PREFACE

The content in this chapter is reprinted with the permission from "Oludiran, A.; Courson, D.S.; Stuart, M.D.; Radwan, A.R.; Poutsma, J.C.; Cotten, M.L.; Purcell, E.B. How Oxygen Availability Affects the Antimicrobial Efficacy of Host Defense Peptides: Lessons Learned from Studying the Copper Binding Peptides Piscidins 1 and 3. Int. J. Mol. Sci. 2019, 20, 5289. https://doi.org/10.3390/ijms20215289". Permission is provided in Appendix G. Reported is a modified version of the published manuscript.

OVERVIEW

*Clostridioides (*formerly *Clostridium) difficile* infection (CDI), whose symptoms can include inflammation, profuse diarrhea, and pseudomembranous colitis, has been recognized as an urgent public health threat in the United States and other industrialized nations [17]. CDI is primarily a hospital-acquired disease, as disruption of the native gut microbiota by prior antibiotic usage is the major risk factor for *C. difficile* colonization, although the number of community-acquired infections has increased in recent years [22]. The severity of CDI has also increased during the 21st century with the emergence of so-called "hypervirulent" epidemic ribotypes of the bacterium, most notably ribotype 027, that are associated with higher levels of disease recurrence and death in infected patients [24]. *C. difficile* is resistant to several families of antibiotics, including penicillin-family beta lactams and fluoroquinolones, and is increasingly resistant to next-generation therapeutics including fidaxomicin and vancomycin [26, 78]. Currently, the most clinically effective treatment for CDI is replenishment of the protective gut microbiota through

fecal transplants. As these procedures have a high inherent risk of introducing uncharacterized pathogens and are not recommended for immunocompromised patients, there is great interest in the development of new strategies for prevention and treatment [79]. *C. difficile* persists in the environment in the form of metabolically dormant spores, which are highly resilient to chemical and physical stresses and remain viable for months [29]. If mammals ingest these spores, amino acids and bile salts in the digestive system trigger their germination into metabolically active vegetative cells [80].

Vegetative *C. difficile* often cannot integrate well into the diverse, metabolically efficient microbial ecosystem of a healthy intestinal microbiome but can take advantage of the loss of bacterial species diversity and rise in nutrient availability induced by antibiotic exposure to establish colonization [81]. *C. difficile* colonization triggers the innate immune response, including the release of reactive oxygen species (ROS) and cationic host defense peptides (HDPs) [82]. These antimicrobial peptides can kill bacterial cells directly through several mechanisms, attacking the cell membrane and/or intracellular targets, and indirectly by activating the host's innate immune response [83]. As these peptides have multiple cellular targets, bacteria cannot quickly develop or transmit genetically encoded resistance to them, and they are a promising precursor for the development of stand-alone antibiotics or adjuvants designed to work synergistically with existing antibiotics [84, 85].

The antimicrobial peptides piscidins are a family of HDPs found in teleost (bony) fish species with demonstrated efficacy against a wide range of bacteria and viruses. The piscidins p1 and p3, which are derived from the mast cells of hybrid striped sea bass, exhibit broad spectrum antibacterial activity although their mechanisms of action differ [86]. Both peptides localize to bacterial cell membranes and are internalized at sub-lethal concentrations. While p1 is more

damaging to membrane integrity than p3, the latter is more disruptive to DNA [64]. Furthermore, studies done on live bacteria and model membranes indicate that the peptides, especially p1, take advantage of lipid heterogeneity to deploy their mechanism of membrane disruption [64, 87]. Recently, it was demonstrated that under aerobic conditions both peptides use their amino-terminal copper and nickel-binding (ATCUN) motifs to coordinate Cu^{2+} with picomolar affinity [58, 88]. Piscidin-copper complexes form ROS and exhibit nuclease activity against double stranded DNA, resulting in increased lethality against multiple bacterial species [58]. Such copper-ATCUN complexes can serve as sources of oxidative stress, increasing peptide lethality against bacteria in an aerobic environment [89].

Oxidative stress can be harmful or lethal to organisms, depending on their oxidative stress tolerance. Obligate anaerobes such as Clostridia are considered completely intolerant to oxygen, although they can employ scavenger and reductase enzymes to survive transient exposure to environmental oxygen or immune-mediated oxidative bursts [29, 81, 90]. Application of antimicrobial peptides sensitizes *C. difficile* to antibiotics, although epidemic strains from ribotype 027 are less sensitive than other strains [85, 91]. Importantly, as HDPs, piscidins have immunomodulatory effects. Our investigations have demonstrated that both p1 and p3 induce chemotaxis in neutrophils [88]. These effects are exclusively mediated by formyl peptide receptors 1 and 2 (FPR1 and FPR2), both of which are G-protein coupled receptors (GPCRs) that play crucial functions in the immune system [92-96]. Interestingly, Cu^{2+}-coordination decreases the chemotactic effects of p1 and p3, suggesting a regulatory effect of copper between the direct and indirect antimicrobial effects of the peptides [88]. Given the role of FPR2 for the resolution of inflammation, it has become an important drug target [92]. p1 has also been shown to decrease

the inflammatory response through a process that may involve binding lipopolysaccharides and decreasing toll-like receptor (TLR)-mediated inflammatory pathways [97, 98].

The immunomodulatory properties of HDPs such as piscidins have emerged as an important topic of research given that these effects are indirect, and thus unlikely to activate mechanisms of drug resistance observed with traditional antibiotics that directly attack bacteria [59]. In addition, HDP modulation of the inflammatory immune response can mitigate infection symptoms and has been shown to reduce toxin-dependent inflammation in mouse models of *C. difficile* infection [99]. As indicated above, the antimicrobial effects of piscidin have previously been measured in aerobic environments. However, there is a ten-fold range of partial oxygen pressure among the tissues of the human body, with organs such as the large intestine providing a habitat for anaerobic microbes, both commensal and pathogenic [100].

Here, we report that the antimicrobial activity of p1 and p3 differ in aerobic and anaerobic environments. In an anaerobic environment, both p1 and p3 are incorporated into *C. difficile* cells, inhibit bacterial proliferation, and are highly toxic against actively dividing *C. difficile*. Both peptides associate extensively with bacterial cell membranes, exhibiting preferential localization at sites of high curvature such as cell poles and septa. In contrast to previously observed aerobic data, anaerobic piscidin antibacterial activity does not appear to be enhanced by metal complex formation. Our findings suggest that the mechanism by which these peptides induce bacterial cell death is influenced by the availability of environmental oxygen. Future mechanistic investigations of HDPs focused on potential medical applications must account for oxygen levels at the desired site of action to accurately model antimicrobial activity.

MATERIALS AND METHODS

Materials, Chemicals, Bacterial Strains and Growth Conditions

Materials and chemicals were purchased from Fisher Scientific (Hampton, NH, USA) unless otherwise indicated. The bacterial strains used in this study are listed in Appendix D. *C. difficile* 630Δerm and R20291 were maintained on brain-heart infusion supplemented with 5% yeast extract (BHIS) agar plates and liquid cultures were grown in TY medium [101, 102]. All anaerobic bacterial culture took place at 37 ∘C in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 85% N2, 10% CO2, 5% H2. All plastic consumables were allowed to equilibrate in the anaerobic chamber for a minimum of 72 h.

Growth Inhibition Assays

C. difficile was grown overnight and re-inoculated in fresh TY medium to log phase in a Coy anaerobic chamber in an 85% nitrogen, 10% CO_2, 5% H_2 environment. Two-fold dilution series of TY medium containing the indicated concentrations of peptide and/or copper salts were prepared in sterile 96-well plates as detailed in Wiegand et al [103]. Wells containing 200 µL of medium were inoculated with 20 µL of saturated overnight culture of *C. difficile* 630Δ*erm* or R20291 containing approximately 10^8 CFU/mL and incubated anaerobically for 16 h at 37 ^{0}C. Closed microplates were removed from the anaerobic chamber and the outsides of the plates were disinfected with 10% bleach before examination to determine the minimum concentration of each peptide and/or metal ion sufficient to completely inhibit visible growth. Culture density at 630 nm was measured in a BioTek (Winooski, VT, USA) microplate reader. As removal from the anaerobic chamber killed the anaerobic *C. difficile* bacteria, we were not able to plate samples to determine CFU/mL after spectroscopic measurements. Inhibitory concentrations were reported as the peptide concentration necessary to reduce the overnight OD_{630} by at least 50%

from that of untreated samples. Data reported are the means and standard deviations of four biologically independent samples in duplicates.

Time-Kill Assays

3mL of TY media were inoculated with single colonies of *C. difficile* R20291 and allowed to grow at 37 ^{0}C to an optical density at 600 nm (OD_{600}) of 0.5–0.7. At the onset of the experiment 20 µL aliquots were removed from the exponentially growing culture and inoculated into fresh TY medium containing the indicated concentration of peptide and/or copper sulfate ($CuSO_4$). The final volume was adjusted to 1 mL with fresh TY medium. After 0, 0.5, 2, and 4 h of incubation at 37 ^{0}C, 10 µL aliquots were removed for serial 10-fold dilution in TY. 10^6 dilutions were plated in duplicate on BHIS agar plates for colony enumeration. Colony forming units (CFU) were counted after 24 h. Data reported are the averages of three biologically independent samples measured in duplicate. Treated samples were compared to untreated samples and to each other by two-way ANOVA using Tukey's multiple comparison test with Prism (GraphPad Software, San Diego, CA, USA).

Microscopy

Live-cell, time-lapse, wide-field fluorescence, and differential interference contrast (DC) microscopy of the interaction between TAMRA-labeled piscidin peptides and *C. difficile R20291* bacteria was performed on a Nikon Ti-E inverted microscope equipped with apochromatic TIRF 60X oil immersion objective lens (N.A. 1.49), pco.edge 4.2 LT sCMOS camera, and SOLA SE II 365 Light Engine as well as complementary DIC components (Nikon Instruments Inc, Melville, NY, USA). Mid-logarithmic phase cells and peptides at the indicated concentration were mixed inside the anaerobic chamber and injected into home-built anaerobic rose-type imaging chambers as previously described [104]. Imaging chambers were removed from the anaerobic chamber and

placed on the microscope after sterilization. The microscope was maintained at 37 ◦C using a home-built enclosure and a Nevtek Air Stream microscope stage warmer (Nevtek, Williamsville, VA, USA). Nikon Perfect Focus system (Nikon Instruments Inc, Melville, NY, USA) was employed to eliminate focal drift during recordings. Movies consisting of a fluorescence and DIC image each minute for 60 min were then recorded for each condition. Movies started 6–8 min after the bacteria and peptide were mixed. Data analysis was performed using the Nikon Elements imaging suite. During the recordings, the amount of fluorescence background increased with time, presumably as peptide was deposited on the coverslip surface. During analysis, this background change was corrected using background-leveling tools, then a second rolling-ball type background correction was used to remove imaging artifacts. A threshold binary mask was then applied to the fluorescence images to isolate and count each fluorescent object (peptide-labeled bacteria) in the movie. Fluorescence levels of objects were monitored as a function of time.

Flame Atomic Absorption Spectroscopy

Flame atomic absorbance spectroscopy (FAAS), the copper concentration in TY medium was measured using an AA-7000 atomic absorption spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA) with a hollow cathode lamp using an acetylene flame [105]. Copper from TY medium was detected at 324.8 nm and quantified using a standard curve of copper chloride ($CuCl_2$) diluted in water.

RESULTS

Piscidins Prevent *C. difficile* Proliferation

To measure the inhibitory effects of piscidin peptides on *C. difficile* growth we inoculated *C. difficile* strains 630Δ*erm* and R20291 into a medium containing the peptides. *C. difficile* 630Δ*erm* is an erythromycin-sensitive derivative of the reference strain *C. difficile* 630, while R20291 is an epidemic strain isolated from a 2003–2005 hospital outbreak of *C. difficile* infection in the United Kingdom [106, 107]. R20291 is a so-called "hypervirulent" strain of ribotype 027 and is more resistant than 630 to multiple classes of antibiotics including clindamycin and ciprofloxacin [108]. The presence of piscidin peptides prevented *C. difficile* proliferation. Notably, R20291 was as susceptible as 630Δ*erm* to growth inhibition by piscidins. p1 inhibited proliferation of both strains at concentrations at or above 4.00 µM and p3 inhibited growth at or above 8.00 µM (Figure 11).
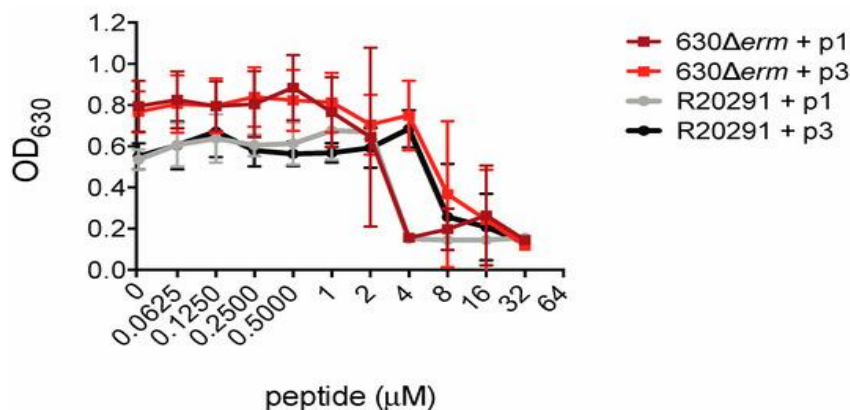
Figure 11. Piscidins inhibit *C. difficile* growth. Optical densities of overnight *C. difficile* 630Δerm and R20291 cultures grown in the presence of the indicated concentrations of piscidins. Data shown are the means and standard deviations of four biologically independent samples triplicates. The figure is reproduced from Oludiran *et al*. 2019.

Piscidins Are Incorporated into *C. difficile* and Appear to Localize to Sites of Membrane Curvature

Confocal microscopy of fixed bacterial cells exposed to fluorescently labeled p1 and p3 has previously shown that they enter both Gram-negative and Gram-positive bacterial cells and appear to be concentrated at bacterial nucleoids and cell septa [64, 87]. We exposed live *C. difficile* R20291 cells to 0.75 µM 5-carboxytetramethylrhodamine (TAMRA)-labeled p1 and p3 and observed peptide uptake and localization in unfixed live cells. As *C. difficile* exhibits green autofluorescence, the red TAMRA labeling was distinct from any intrinsic signal produced by the cells [109]. Exponential-phase cells and peptides were mixed and sealed within microscopy chambers in an anaerobic chamber and then transported to the microscope, resulting in a 6-min delay between the onset of peptide exposure and the first image [104]. Mean fluorescence

intensity within cells was stable over the course of 1 h of monitoring, indicating that peptide

incorporation into cells occurs within the first few min of exposure (Figure 12a,b). Peptide

integration appeared to be complete within 6 min even at lower peptide concentrations of 0.25

and 0.075 µM (data not shown). The addition of additional unlabeled peptide or unlabeled

peptide complexed with Cu^{2+} did not increase fluorescence intensity, and thus there was no

evidence of potential cooperativity in peptide uptake. There were distinct fluorescent puncta at

the septa of provisional cells (Figure 12 c,e,f), consistent with prior observations in *Escherichia*

coli (E. coli) and *Bacillus megaterium (B. megaterium)* [64]. In addition, there were fluorescent

puncta at cell poles, suggesting that piscidin generally localize to sites of high curvature (Figure

12d). While unlabeled cells were motile and maintained rod-like shapes, many of the

fluorescently labeled cells exhibited curvature or surface irregularities suggestive of cell

envelope damage (Figure 12e). Performing these experiments on live cells allowed real-time

observation of cellular response to peptide intoxication. We observed a motile chain of

provisional rod-shaped cells over the course of 10 min (Figure 12g). During this time, the chain

of cells took up labeled p1 at one pole and subsequently developed progressively severe

curvature at cell septa and separated into smaller fragments (Figure 12g). The resulting pieces

were asymmetrically curved and non-motile, indicating that lysis rather than healthy cell division

had occurred.
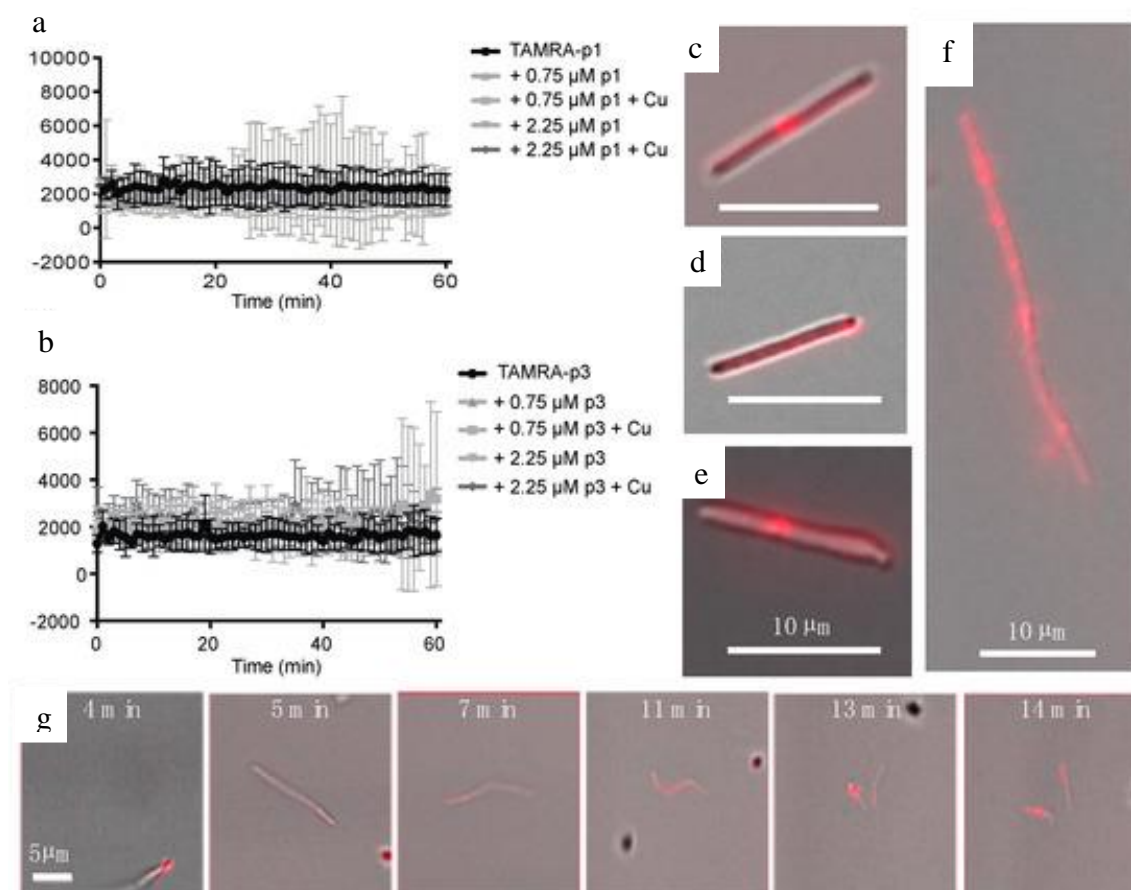
Figure 12. Incorporation of TAMRA-piscidin into live *C. difficile.* (**a, b**) Fluorescent signal per cell of 0.75 μM TAMRA-labeled p1 (**a**) and p3 (**b**) mixed with live *C. difficile* R20291. Cells had already reached maximum peptide incorporation by the time recording began, roughly 6 min after peptides and cells were mixed. The addition of unlabeled peptide, in the presence or absence of equimolar amounts of copper sulfate, did not cooperatively increase peptide incorporation. (**c-f**) Representative images of *C. difficile* labeled with: (**c**) 0.75 μM TAMRA-labeled p1 plus 0.75 μM unlabeled p1; (**d**) 0.75 μM TAMRA-labeled p3; (**e**) 0.75 μM TAMRA-labeled p1 plus 2.25 μM unlabeled p1; (**f**) 0.75 μM TAMRA-labeled p1 plus 0.75 μM unlabeled p1. (**g**) Time course showing the rupture of a divisional cell labeled with 0.75 μM TAMRA-labeled p1 plus 0.75 μM unlabeled p1. Scale bars in the panels (**c-f**) represent 10 microns. The scale bar in panel (**g**) represents 5 microns and this figure is reproduced from Oludiran *et al* 2019.

Piscidins Reduce Established *C. difficile* Populations

Growth inhibition assays do not distinguish between substances that kill cells and bacteriostatic substances that inhibit growth only if compounds are present in sufficient quantities prior to bacterial proliferation. To confirm that piscidins can reduce the number of viable cells in established bacterial populations, we performed time-kill assays to confirm that the number of viable *C. difficile* R20291 cells in exponentially growing culture decreases with exposure to p1 and p3 at sub-inhibitory concentrations. As shown in Figure 13, both p1 and p3 reduce *C. difficile* viability at half of the concentration needed to inhibit bacterial growth. The addition of 2.00 μM p1 significantly reduces the number of viable cells in the culture within 30 min, with continued loss of colony forming units over the course of 4h (Figure 13a). Similarly, incubation with 4.00 μM p3 significantly reduced the number of viable cells within 30 min (Figure 13b). The presence of copper did not enhance bacterial killing by piscidins, in contrast to what had previously been reported against *E. coli* and *Pseudomonas aeruginosa* (*P. aeruginosa*) in aerobic condition [58]. To define the effects of copper alone against *C. difficile*, we determined that the copper concentration of the TY growth medium used to culture *C. difficile* under anaerobic conditions is 1.46 μM, which is lower than that of the Mueller-Hinton broth previously used to culture *E. coli* and *P. aeruginosa* under aerobic conditions [58, 110]. We found that copper salts are capable of inhibiting *C. difficile* growth at the same concentrations used for both p1 and p3 (Figure 13c). This means that copper alone at the indicated concentration can still inhibit exponentially growing *C. difficile* anaerobically but inhibition and killing of *C. difficile* were not enhanced by the addition of Cu^{2+} to either of the peptides in anaerobic conditions. By contrast, in aerobic environments the bacterial response to piscidins with Cu^{2+} is of higher killing than either the peptides or Cu^{2+} alone [57]
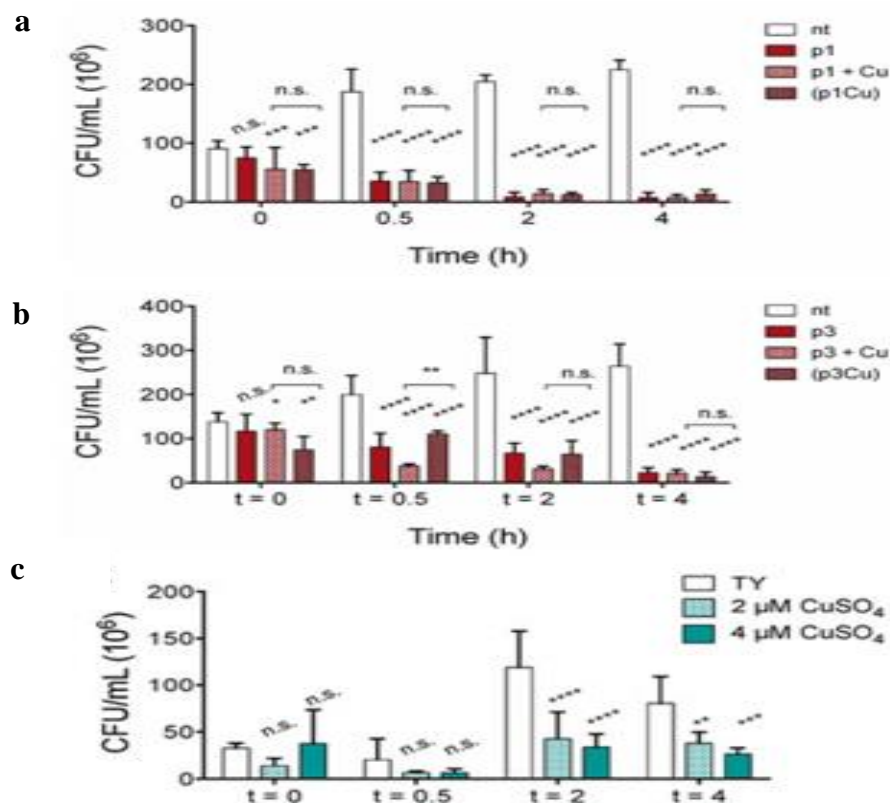
Figure 13. Copper does not accelerate anaerobic *C. difficile* killing by piscidins. Time-kill assays compared viable colony forming units per milliliter (CFU/mL) of bacterial culture before exposure to p1 (**a**) and p3 (**b**) the CFU/mL 30 min, 2, and 4 h post-exposure. Cells were exposed to peptides (p1 and p3), peptides, and equimolar copper sulfate added simultaneously (p1 + Cu and p3 + Cu), peptides were allowed to form piscidin-copper complexes in aerobic prior to addition to the anaerobic bacterial cultures ((p1Cu) and (p3Cu)). (**c**) Time-kill assays comparing viable colony forming units per milliliter (CFU/mL) of bacterial culture before exposure to the indicated concentrations of copper sulfate with the CFU/mL 30 min, 2, and 4 h post-exposure. CFU/mL in treated samples were compared to those in untreated samples and to each other using two-way ANOVA with Tukey's post-test comparison. nt, not treated; n.s., not significant; $* p < 0.05$; $** p < 0.01$; $*** p < 0.001$; $**** p < 0.0001$. This figure is reproduced from Oludiran *et al* 2019.

a

b
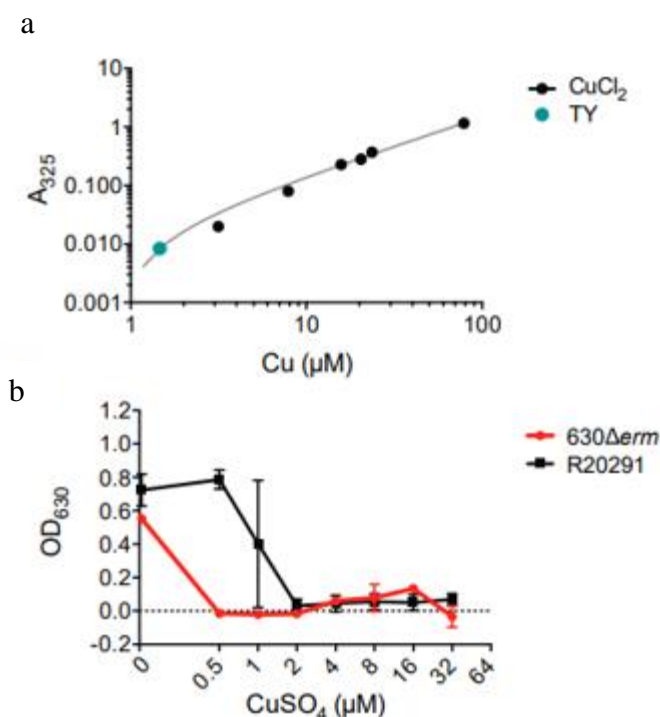
Figure 14. Copper is still antimicrobial in anaerobic environments. (**a**) The copper concentration

of TY medium is 1.46µM as determined by comparison to a standard curve. (**b**) Optical densities

of overnight *C. difficile* 630Δ*erm* and R20291 cultures grown in the presence of the indicated

concentrations of copper sulfate. Data shown are the means and standard deviations of four

biologically independent samples and this figure is reproduced from Oludiran *et al* 2019.

DISCUSSION

The development of new therapies to combat antibiotic-resistant infections, including *C.*

difficile infection, is an urgent public health priority. Antimicrobial peptides capable of

simultaneously killing bacterial pathogens and stimulating the host innate system are a promising

avenue for the development of new antimicrobial therapies. Piscidins previously showed efficacy

against Gram-negative and -positive bacteria in aerobic environments [58]. Here, we investigated their effect on anaerobic bacteria. While piscidins are still highly lethal against *C. difficile* growing anaerobically, we found that piscidin growth inhibition and killing against *C. difficile* were not enhanced by the addition of Cu^{2+}. This is true even though copper alone is capable of inhibiting *C. difficile* growth and killing actively growing *C. difficile* cells. While in aerobic environments, the bacterial response to piscidins with Cu^{2+} is greater than that to either piscidins or copper alone, it appears that the anaerobic response to piscidin with copper is less than the sum of the parts, and thus does not feature the synergistic effects observed aerobically.

Bacteria employ general stress response pathways which can be activated by multiple diverse extracellular stresses. Bacterial cells that have previously been exposed to an extracellular stress such as antibiotic exposure may respond through phenotypic expression like spore formation to go into dormant state [111, 112]. The fact that the *C. difficile* strains 630*erm* and R20291 exhibit identical inhibition in response to p1 and p3, but differential inhibition to copper alone, suggests that piscidins and copper inhibit bacterial growth and viability by different mechanisms. This makes sense because Cu^{2+} has no specificity while piscidins have specific targets. It should be noted that free Cu^{2+} *in vivo* is highly toxic to mammalian as well as bacterial cells, and complexation by HDPs such as p1 and p3 can provide the critical specificity of targeting the metal ions to bacterial rather than host cells. HDPs are a viable treatment option against both aerobic and anaerobic bacteria, and in the case of *C. difficile*, it is extremely encouraging that the epidemic strain R20291 is just as susceptible to HDP inhibition and killing as the less robust 630Δ*erm* strain. As the symptoms of *C. difficile* infection are inflammatory, and treatments based on piscidins could potentially reduce inflammation while killing the causative pathogen, this is a very promising strategy to pursue. However, while clinical

antibiotics derived from HDPs would benefit from the inclusion of copper in tissues with elevated levels of oxygen, such as the lungs, antibiotics targeted to less aerobic tissues, such as the kidneys or large intestine, may not. Future work to investigate the interaction of piscidins with *C. difficile* in animal models of infection will be necessary to determine whether copper could or should be included with the peptides. More broadly, it appears that clinical treatments developed from HDPs should be designed in a tissue-specific manner, as metal ion adjuvants may be beneficial or necessary in some organs and unneeded in others, based on the oxygen levels at the site of activity

Summary

We have established that the antimicrobial peptides piscidin 1 and 3 and their copper-complexes have strong efficacy in anaerobic conditions. They significantly reduced the proliferation of *C. difficile* and reduced previously established *C. difficile* populations. We discovered that in anaerobic environments, coordinated or complexed copper does not increase piscidin efficacy, even though copper alone is toxic to the bacterial cells at high concentrations. The FAAS result showed that the amount of copper in the media is not enough to inhibit *C. difficile* growth or viability without supplementation. The microscopy images indicate the localization points of the fluorescently labeled peptides on the bacterial cells.

CHAPTER III

PISCDINS AND THEIR COPPER COMPLEXES SENSITIZE *C. DIFFICILE* TO

ANTIBIOTICS AND SUPPRESS SPORULATION IN BACTERIAL CELLS

OVERVIEW

Bacteria need to survive stresses in their natural environments [7]. Environmental stress can lead to several adaptive and protective responses, which can induce gene regulation and cell physiological changes [8, 9]. These can include increased replication, formation of biofilm on surfaces, altered metabolism, or dormancy (which in some Gram-positive Firmicutes bacteria takes the form of sporulation) [113]. *C. difficile* is an anaerobic Gram-positive spore and biofilm-forming pathogen that causes serious disease in humans [114]. *C. difficile* infection (CDI) is now established as a growing health challenge in both developed and developing countries of the world [115]. CDI predominantly affects long-term hospital inpatients, the elderly, immune-compromised patients, and people undergoing prolonged antibiotic treatments or exposure. The symptoms of CDI include but are not limited to the inflammation of intestinal walls, profuse diarrhea, and pseudomembranous colitis in human [116, 117]. This bacterium remains a leading nosocomial pathogen, which is strenuous to healthcare systems.

C. difficile can form spores, highly resistant to environmental assaults, with a very serious role in both the persistence and transmission of infection [35]. Any therapeutic agents that can reduce *C. difficile* spore production could significantly minimize the transmission and relapse of the *C. difficile* infection. In 2014, epidemiological data showed that *C. difficile* has surpassed methicillin-resistant *Staphylococcus aureus* (MRSA) as the most common hospital-acquired infection with high rates of recurrence and readmission. In an analysis of nationwide hospital readmission data 2013 to 2017, 48.5% of patients had MRSA and 22% were readmitted to the

health care center for the same infection, while 50% of patients had hospital-acquired CDI and 27% were readmitted with the same infection [118]. *C. difficile* spores are dormant and do not require active ATP consumption for any metabolic processes [31]. In the presence of germinants, including bile salts such taurocholic acid, and amino acids, and especially if antibiotic treatments have killed off other bacteria, spores can develop into free-living vegetative cells which are the toxin-producing stage of the bacterium's life cycle [33]

Dormant spores can survive for months in an hostile environment with the help of their protective multilayered structure [35]. Spores are extremely robust and resistant to both chemical and physical attacks enabling *C. difficile* to survive exposure to heat, low levels of oxygen, alcohol, and many disinfectants and spread to new patients in households and health care facilities. Reports on *C. difficile* spores have implicated genes including *csiA*, which is involved in the control of sporulation initiation in *C. difficile* and the master regulator *spo0A* which is the main regulatory protein controlling sporulation, as factors which increase the spore formation in this bacterium [119]. *C. difficile* spores are the cell form that spread infection, and mutants with defective spore formation have a low infection rate [35, 76].

The antibiotic metronidazole (or nitroimidazole in its reduced form) inhibits nucleic acid synthesis by disrupting the DNA of the cells [120]. The antibiotic vancomycin inhibits cell wall synthesis [33]. Fidaxomicin is the most recent and well-known antibiotic treatment for CDI, yet it comes at a high cost and there are still reports of recurrencies after treatment [121]. Some newly isolated clinical strains of *C. difficile* harboring the *rpoB*V1143D allele have shown higher resistance to fidaxomicin [122]. Thus, there is a need for better treatment strategies.

Piscidins are short antimicrobial host-defense peptides (HDPs) produced by the innate immune systems of fish. These helical structured peptides have broad-spectrum antibacterial

activities against most aerobic bacteria [58]. They also show strong antibacterial efficacy in anaerobic conditions [123]. They can potentiate immune cells including lymphocytes and monocytes by sensitizing the surface layer proteins (SLPs) of bacteria [59]. Due to their efficacy against muti-drug resistance pathogens, novel therapies could be developed from HDPs. Piscidins p1 and p3, derived from hybrid striped sea bass, can cause cell membrane disruption and have DNA nuclease activity [62, 63]. These peptides have a very short, conserved N-terminal motif for metal binding called ATCUN, which binds some transition metals like copper (Cu^{2+}) and nickel (Ni^{2+}) leading to increased efficacy in aerobic condition [124], but Cu-binding does not enhance efficacy of either piscidins in anaerobic conditions [61].

The peptides accumulate in *C. difficile* at sites of membrane curvature and later cause cell death [123]. Both piscidins are effective against the historical strain 630Δ*erm* and the epidemic R20291 strain that is highly resistant to antibiotics and other stressors [123]. Piscidins attack different cellular targets than medical antibiotics, meaning that simultaneous treatments are not likely to be redundant. We investigated the effect of piscidins on vegetative *C. difficile* survival after antibiotic treatment and its effect on clostridial spore formation, as well as synergy between the peptides and common antibiotics used for CDI treatment. We discovered that piscidin treatment enhances antibiotic efficacy and prevents antibiotic-induced sporulation. Piscidins reduced bacterial membrane integrity and increased uptake of exogenous substances, which could increase uptake into bacterial cells.

MATERIALS AND METHODS

Materials, Chemicals, Bacterial Strains and Growth Conditions

Materials and chemicals were purchased from Fisher Scientific (Hampton, NH, USA) unless otherwise indicated. The bacterial strains used in this study are listed in Appendix D. *C. difficile* 630Δ*erm* and R20291 were maintained on brain-heart infusion supplemented with 5% yeast extract (BHIS), agar plates [101, 102]. Where indicated, cells were treated with ciprofloxacin, fidaxomicin, (Cayman Chemicals), erythromycin (Acros Organic), metronidazole (BTC) or vancomycin (VWR). For spore enumeration, vegetative cells were killed by aerobic incubation at room temperature with 50% ethanol for one hour and spores were germinated anaerobically with 0.1% taurocholic acid (TCA from Sigma Life Science). Host defense peptides (HDPs) piscidin 1 (p1), piscidin 3 (p3), and piscidin copper complexes were provided by Myriam Cotten (College of William & Mary, Williamsburg, VA). All anaerobic bacterial culture took place at 37^0C in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 85% N_2, 10% CO_2, 5% H_2. All plastic consumables were equilibrated in the anaerobic chamber for a minimum of 72 h.

Growth Inhibition Measurements

Saturated overnight cultures of *C. difficile* were inoculated dilution factor of the first innoculation into fresh BHIS media and grown to log phase (OD600 + 0.4-0.6) and diluted 1:10 into fresh BHIS media in 200 µL sterile 96-well plates (Thermo Fisher Scientific) containing the indicated concentration of peptides. Piscidins were either equilibrated for 3h in the anaerobic chamber before adding or fresh peptides used immediately after import into the chamber. p1 and p3 synergy were first tested with gradient concentrations from 0 to 32 µM and three independent biologicals. The plates were maintained in the anaerobic incubator at 37^0C for 16h. After 16h,

plate exteriors were removed from the anaerobic chamber and the exterior was sterilized before the optical density was read at 630nm with a microplate reader (BioTek). Each experiment was repeated three more times, mean and standard deviation reported.

Antibiotic Synergy Assay

The antibiotic synergy with piscidins and their copper complexes was measured at half of the published inhibitory peptide concentrations [123]. Antibiotic gradients were designed to end at 0.5x of the previously published inhibitory concentration each of the antibiotics [125-127]. The well-established antibiotics ciprofloxacin, erythromycin, fidaxomicin, metronidazole and vancomycin were used at the indicated concentrations. Data represent the means and standard deviations of three biologically independent samples.

Combinations of piscidins and drugs that restricted overnight *C. difficile* R20291 growth to OD 600 ≤ 0.05 were assessed for viable cells by plating of the treated samples on BHIS. Cells were exposed to synergistic combinations of antibiotics and piscidins as described above and incubated for 16h before plating 100µL on BHIS plates with 20 µg/mL erythromycin. Plates were incubated at 37ºC for 48h before enumeration of colonies. Treated samples were compared to untreated samples and to each other by two-way ANOVA using Tukey's multiple comparison test with Prism (GraphPad Software, San Diego, CA, USA). Data represent the means and standard deviations of three biologically independent samples.

Live Cell Microcopy

Time-lapse, differential interference contrast (DIC) microscopic imaging of live *C. difficile* R20291was bacteria performed on a Nikon Ti-E inverted microscope equipped with apochromatic TIRF 60X oil immersion objective lens 4.2 LT sCMOS camera, and SOLA SE II 365 Light Engine as well as complementary DIC components (Nikon Instruments Inc, Melville,

NY, USA). Exponentially growing (OD_{600} = 0.5 - 0.6) cells were mixed with peptides (p1 and p3 at 0.5x the inhibitory concentration), peptide-metal complexes (p1Cu and p3Cu at 0.5x the inhibitory concentration) or 4.00 µM $CuSO_4$, $FeSO_4$, $MgSO_4$ and $AgNO_3$. and phosphate-buffer saline (PBS) was added inside the anaerobic chamber to a final volume of 1.5 mL.

The samples were injected into mobile anaerobic rose-type imaging chambers as previously described by Courson *et al* 2019 [104]**.** The airtight imaging chamber was removed from the anaerobic chamber, the exterior sterilized, and placed on the microscope with an average time lapse of ten minutes. The microscope was maintained at 37 ^{0}C using a home-built enclosure and a Nevtek Air Stream microscope stage warmer (Nevtek, Williamsville, VA, USA). Nikon Perfect Focus system was employed to eliminate focal drift during recordings. Cells were recorded for 24h, with three randomly selected fields of view image every 30 minutes. Data analyses were performed using the Nikon Elements imaging suite ImageJ image tools. During imaging analysis spores, pre-spores (or mother cells), and vegetative cells were quantitated.

Piscidin Uptake Assays

4 mL samples of *C. difficile* cultures in late exponential growth (OD_{600} 0.7-0.8) were co-incubated anaerobically at 37°C with 20 µg/mL propidium iodide (PI) (Biotium), and inhibitory concentrations of piscidins or piscidin-copper complexes (4 µM for p1 and p1Cu, 8 µM for p3 and p3Cu) [123]. Samples containing apo-peptides were incubated for 30 minutes and samples containing metalated peptides were incubated for 10 minutes. After incubation, cells were centrifuged for 5 minutes and resuspended in 1.5 mL fresh BHIS. Negative controls were incubated with PI for 10 or 30 minutes, with no peptide and then centrifuged at 3000rpm for 5 minutes and resuspended. Positive control samples were exposed to oxygen for 1h and incubated aerobically at room temperature with propidium iodide and 50% ethanol. Samples were injected

into rose chambers for microscopic imaging as detailed in Courson *et al* 2019 [104]. The rose chambers were removed from the anaerobic chamber and the outsides were sterilized with 70% ethanol and 10% bleach. Nikon Perfect Focus system was employed to eliminate focal drift during recordings.[128]. The PI fluorescence was measured using 553/650 nm excitation/emission filters.

Spore Enumeration Assay

BHIS liquid media and BHIS agar plates containing 20µg/mL erythromycin alone or 20µg/mL erythromycin + 0.1% taurocholic acid (TCA) (Cayman Chemicals) were equilibrated in the anaerobic chamber overnight. 4mL of fresh liquid media was inoculated 1:10 with a saturated overnight culture. The inoculated culture was allowed to grow anaerobically for 4h at 37 ^{0}C until late log-phase (OD_{600} 0.8 to 0.9) [129], and then, treated with 0.5x inhibitory concentration of piscidins, peptide-metal complexes, $CuSO_4$ or antibiotics respectively [123, 125, 126]. 0.5 mL each of the samples were removed from the anaerobic chamber at the indicated time points, with one untreated control removed to aerobic conditions and one left anaerobic. Samples was exposed to oxygen for 1h and ethanol were added to a final concentration of 50%. All samples were serially diluted and plated in duplicate on BHIS containing 20µg/mL erythromycin and 0 or 0.1% TCA [130]. Plates were incubated anaerobically for 48h at 37^{0}C before colony enumeration. Treatments were repeated on a minimum of three biologically independent samples. For antibiotic-piscidin synergy experiments, samples were exposed to the combined treatments for 16h before dilution and plating.

RESULTS

Piscidins Are Most Effective When Equilibrated to Their Oxygen Environment

We determined that the piscidins must be equilibrated to the anaerobic environment to yield consistent results. The efficacy of 2.00μM p1 and p3 4.00μM p3 at killing *C. difficile* was measured using peptides either equilibrated in the anaerobic chamber for 3h before use or immediately upon bringing them into the chamber, still contaminated with environmental oxygen. The equilibrated peptides were more effective at inhibiting anaerobic *C. difficile* growth, keeping the OD_{630} after 16h at 0.0 at 2.00 μM (p1) or 4.00 μM (p3) compared to 4.00 μM (p1) and 8.00 μM (p3), for the unequilibrated peptides. The unequilibrated numbers were consistent with those reported in Oludiran *et al* 2019 after 16h incubation (Figure 15a). The equilibrated piscidins act additively, as both peptides inhibit the *C. difficile* growth at lower concentration compared to either piscidins alone (Figure 15b).
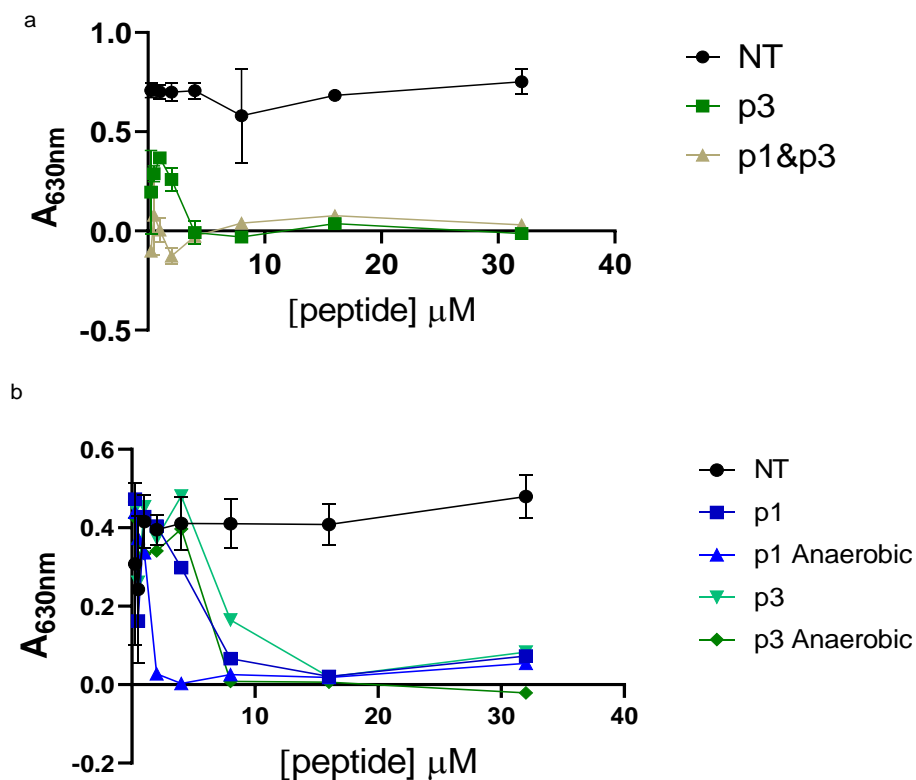
Figure 15: Piscidins strongly inhibit the *C. difficile* growth anaerobically and they act additively (**a**) When p1 and p3 are combined at 0.25x their respective MICs the combined treatment (gold) is more effective than p3 alone (green) at 0.5x MIC. Similar results were obtained with p1 (data not shown) (**b**) p1 equilibrated to the anaerobic environment for 3h before application (blue triangles) inhibited *C. difficile* growth at a lower concentration than p1 applied immediately after import into the anaerobic chamber while it was still contaminated with oxygen (blue squares). The same result was found for anaerobic p3 (green diamonds) and oxygen-contaminated p3 (green triangles). The data shown are the means and standard deviation of six biologically independent samples.

Piscidins Synergize With Antibiotics to Strongly Inhibit Proliferating *C. difficile* Cells

The ability of piscidins to synergize with antibiotics was tested with three clinically relevant antibiotics commonly used for the treatment of CDI [34]; fidaxomicin (FID), metronidazole (MET) and vancomycin (VAN). Ciprofloxacin (Cipro), to which *C. difficile* is resistant, was used as a control [131]. Varying concentration of antibiotics were combined with constant concentration of apo or metalated peptides at 0.5x inhibitory concentrations. Both FID and MET inhibited growth at lower concentrations in the presence of piscidins (Figure 16-19). The MICs of FID, MET, and VAN alone were 0.5 µg/mL, 4 µg/mL, and 4 µg/mL, respectively. The presence of p1, p1Cu, p3, or p3Cu at 0.5x inhibitory concentrations increases the efficacy of all the functioning antibiotics far below their published *in vitro* minimum inhibitory concentrations (MICs) as summarized in Table 2 and Figures 16-19 ([125, 126].

Table 2. Co-treatment with antibiotics-piscidins combinations reduces *C. difficile* growth.

Antibiotic	No peptide	2µM p1	2µM p1Cu	4µM p3	4µM p3Cu	2 µM p1 4 µM p3
Metronidazole	>4	1	1	2	0.5	0.25
Vancomycin	4	2	0.5	0.5	0.25	0.25
Fidaxomicin	0.25	0.1	0.05	0.05	>0.1	0.0125
Ciprofloxacin	>128	n/a	n/a	n/a	n/a	n/a
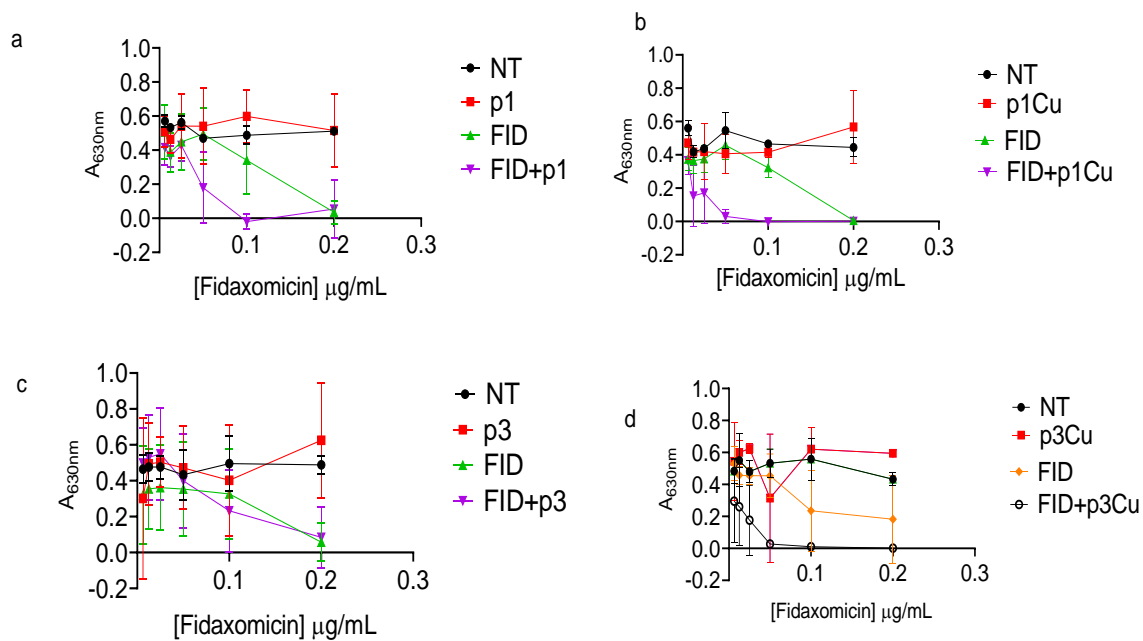
Figures 16. *C. difficile* growth in the presence of FID and 0.5x inhibitory levels of piscidins. Overnight growth was monitored at 630nm in the presence of the indicated concentration of fidaxomicin and subinhibitory levels of p1 alone (**a**), p1 with copper (**b**), p3 alone (**c**), or p3 with copper (**d**). Shown are the means and standard errors of at least three biologically independent samples.
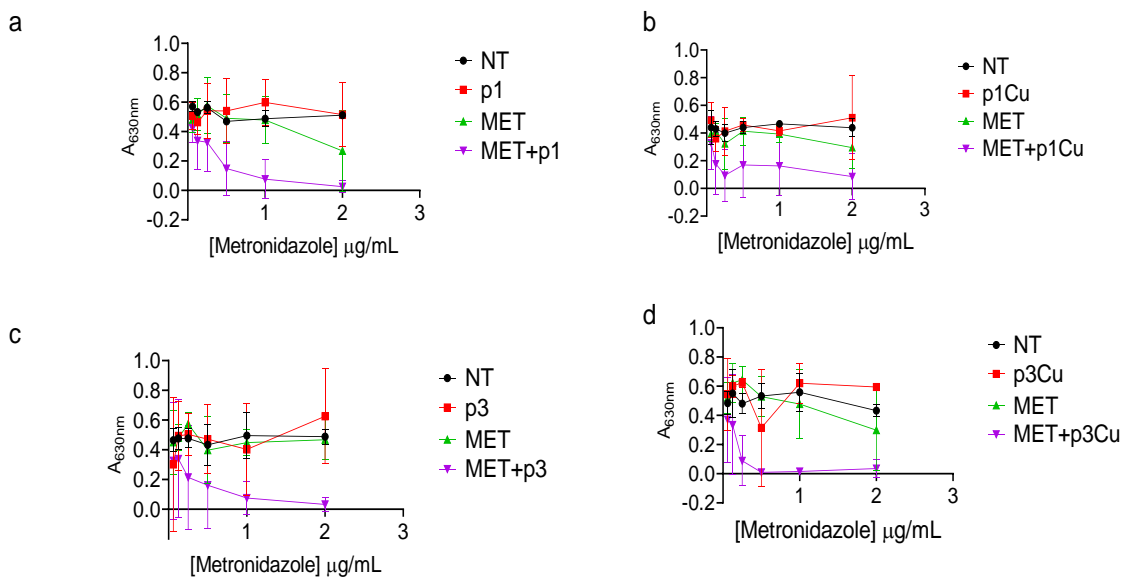
Figures 17. *C. difficile* growth in the presence of MET and 0.5x inhibitory levels of piscidins.

Overnight growth was monitored at 630nm in the presence of the indicated concentration of

fidaxomicin and subinhibitory levels of p1 alone (**a**), p1 with copper (**b**), p3 alone (**c**), or p3 with

copper (**d**). Shown are the means and standard errors of at least three biologically independent

samples.
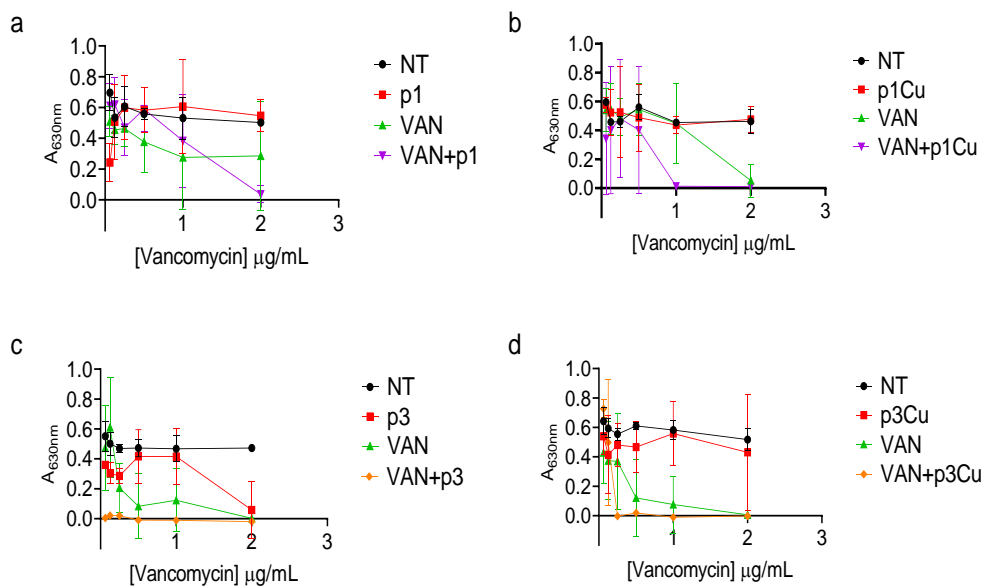
Figures 18. *C. difficile* growth in the presence of VAN and 0.5x inhibitory levels of piscidins.

Overnight growth was monitored at 630nm in the presence of the indicated concentration of

fidaxomicin and subinhibitory levels of p1 alone (**a**), p1 with copper (**b**), p3 alone (**c**), or p3 with

copper (**d**). Shown are the means and standard errors of at least three biologically independent

samples.

a

b

c

d
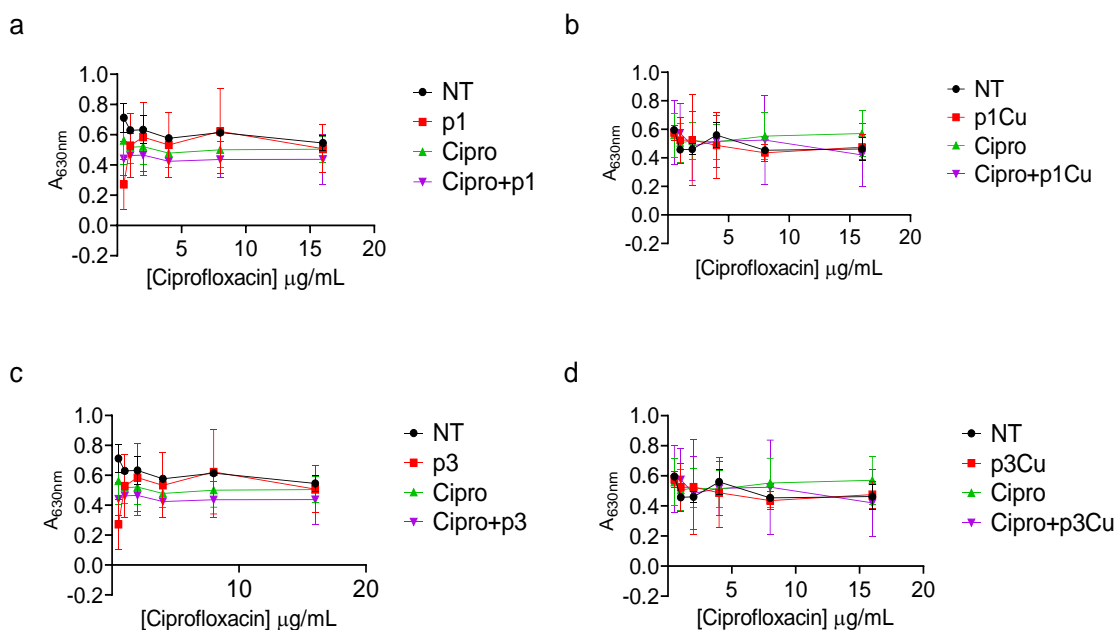
Figures 19. *C. difficile* growth in the presence of Cipro and 0.5x inhibitory levels of piscidins. Overnight growth was monitored at 630nm in the presence of the indicated concentration of fidaxomicin and subinhibitory levels of p1 alone (**a**), p1 with copper (**b**), p3 alone (**c**), or p3 with copper (**d**). Shown are the means and standard errors of at least three biologically independent samples.

Peptide-Antibiotic Synergy Against Viable Cells

We needed to determine whether the piscidin-antibiotic combinations that kept bacterial culture density below $OD_{630} \leq 0.05$ were killing bacteria or merely suppressing bacterial growth. We diluted samples treated with these combinations and plated them to count viable colonies. We found that co-treated samples containing FID or MET had at least 90% fewer live cells than untreated controls (Figure 20a, b). Combined treatments containing VAN reduced the number of

cells by 50-90% (Figure 20c). The combination of piscidin and the ineffective antibiotic Cipro did not kill bacteria, showing that the piscidins were enhancing the antibiotics but were not sufficient to kill the bacteria in the absence of an effective antibiotic at the tested concentrations (Figure 20d). Copper appeared to increase the p1 and p3 synergy with FID (Figure 20a) and p1 synergy with MET (Figure 20b) but had no effect on the synergy with VAN (Figure 20c) or Cipro (Figure 20d). The cell counts of samples treated with FID or MET in combination with piscidin-metal complexes were at least 90% fewer than untreated controls.
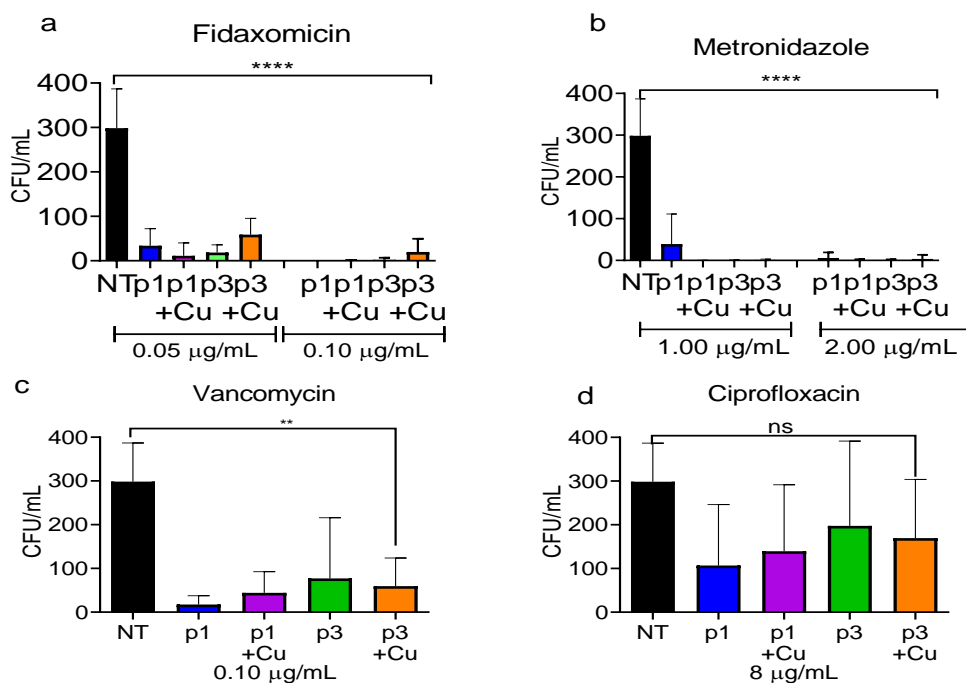
Figure 20. Piscidins lower the effective MICs of antibiotics and reduce viable cell numbers. Colony-forming units per mL of *C. difficile* cells after treatment with piscidins and fidaxomicin (**a**), metronidazole (**b**), vancomycin (**c**), or ciprofloxacin (**d**). CFU counts were compared to those in untreated controls by two-way ANOVA with Tukey's post-test comparison and using at least three independent biologicals. nt, not treated; n.s., not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Shown are the means and standard errors of at least three biologically independent samples.

We used microscopy to investigate a possible mechanism for the increased efficacy of antibiotics in the presence of piscidin. The peptides are known for directly killing bacteria aerobically by either causing DNA damage or membrane disruption, so we speculate that membrane disruption could allow antibiotics to penetrate the cytoplasm. We employed

propidium iodide as a fluorescent trace to verify the idea that membrane disruption allowed

external substances to move freely into the cells. PI is excluded from cells with intact

membranes. The number of cells with red staining is very sparse in the untreated, undamaged

negative control (Figure 21). Oxygen and ethanol triggered extensive PI uptake, as expected

(Figure 21). PI staining was extremely high in cells treated with both piscidins and their copper

complexes (Figure 21). This confirmed the ability of the piscidins and their copper complexes to

cause membrane disruption that could enhance antibiotic uptake and efficacy.
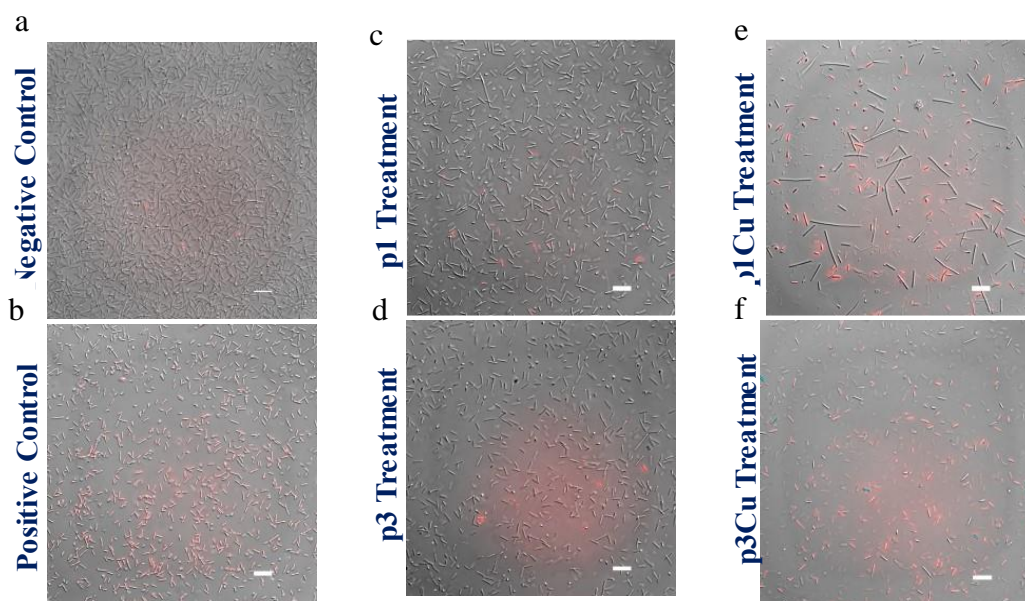
Figure 21. Piscidins allow increased propidium iodide uptake (**a**) Negative control with PI but no

oxygen and no other treatment. (**b**) Positive control with PI, oxygen, and 50% ethanol treatment.

(**c**) PI and p1 alone were co-treated, with no oxygen. (**d**) PI and p3 alone were co-treated with no

oxygen. (**e**) PI and p1Cu treatment but no oxygen. (**f**) PI and p3Cu co-treatment without oxygen.

Exposure to Antimicrobial Peptides and Metals Affects *C. difficile* Morphology

      C. difficile R20291 was imaged on an inverted microscope at 60x magnification at 30-minute intervals after treatment with piscidins or with transition metals known to induce oxidative stress. The bacterial morphology varies according to the source of the induced stress. The images show that sub-inhibitory 4.00 μM concentration of non-antimicrobial metals of +2 oxidation states Fe, Mn, and Mg, enabled robust continuous cell growth and stimulated only moderate spore formation (Figure 22), while piscidins and their piscidins copper complexes appeared to have both fewer numbers of *C. difficile* vegetative cells and spores (Figure 23) . However, the known antimicrobial metals Ag and Cu increased the number of spores visible by bright-field microscopy (Figure 24).
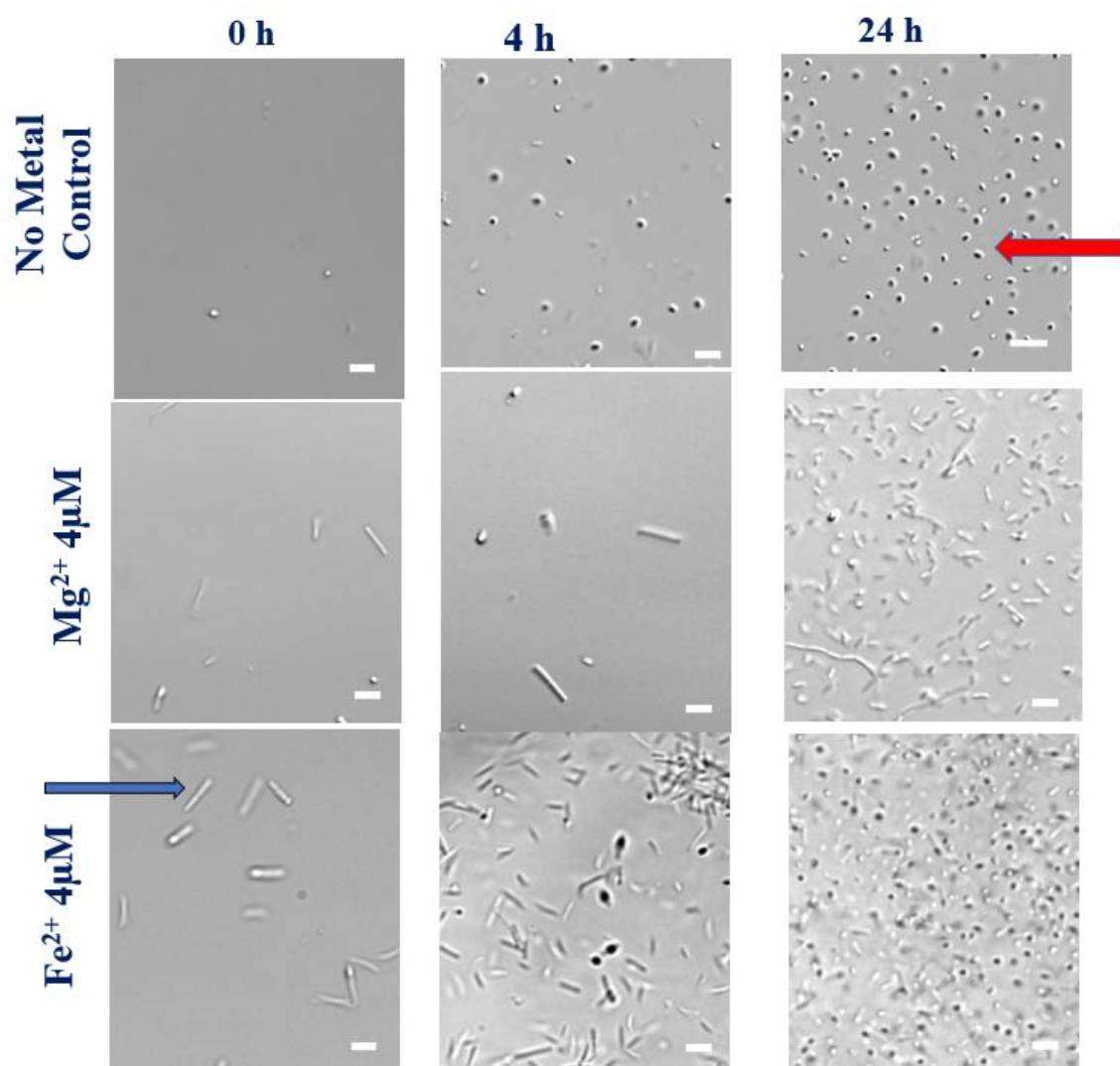
Figure 22. Non-antimicrobial metal treatment does not affect *C. difficile* morphology. Cells treated with 4 µM of Cu^{2+}, Fe^{2+}, Mg^{2+}, and an untreated control are shown 0, 4h, and 24h after exposure. Scale bars in all images represent 10 microns. Selected vegetative cells are marked with blue arrows and selected spores are marked with red arrows.
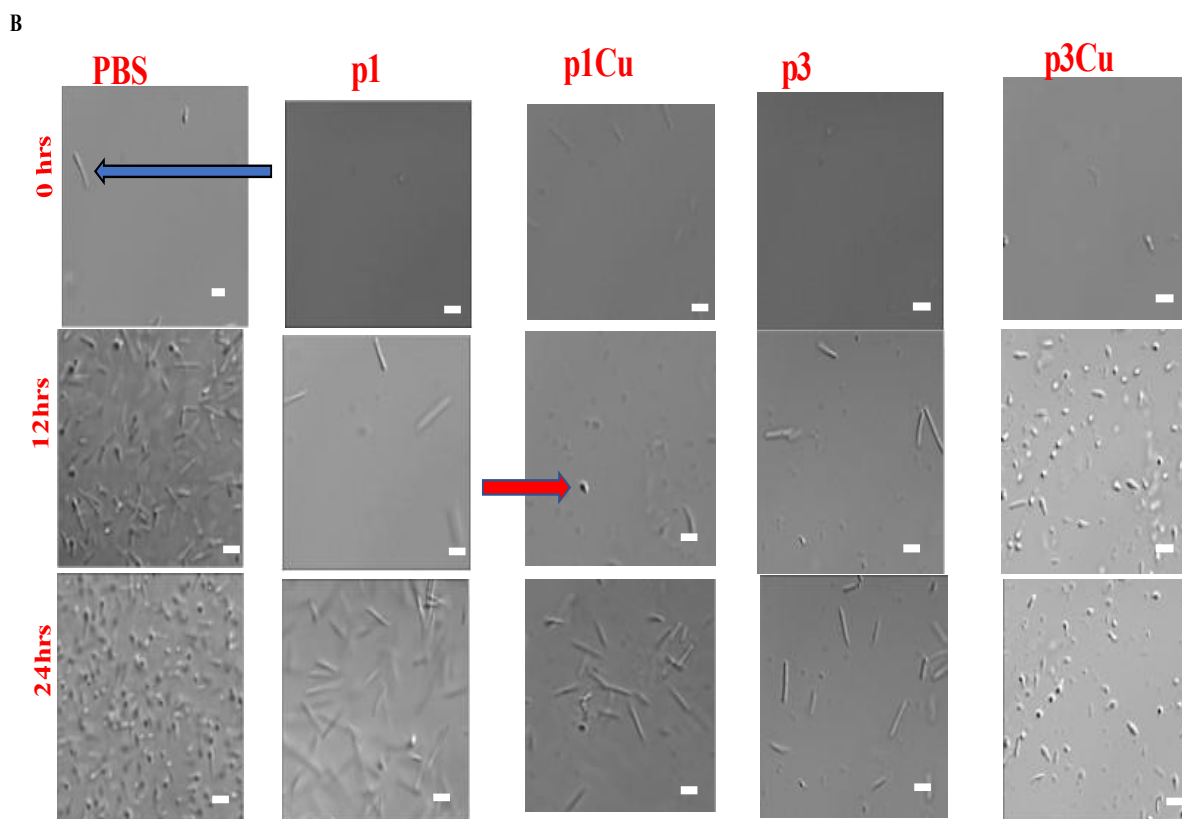
Figure 23. Piscidin treatment appeared to reduce *C. difficile* cells. Cells treated with 0.5x of p,

p1Cu, p3, p3Cu, and a 1XPBS treated control are shown at 0, 12h, and 24h. Scale bars in all

images represent 10 microns. Selected vegetative cells are marked with blue arrows and

selected spores are marked with red arrows.
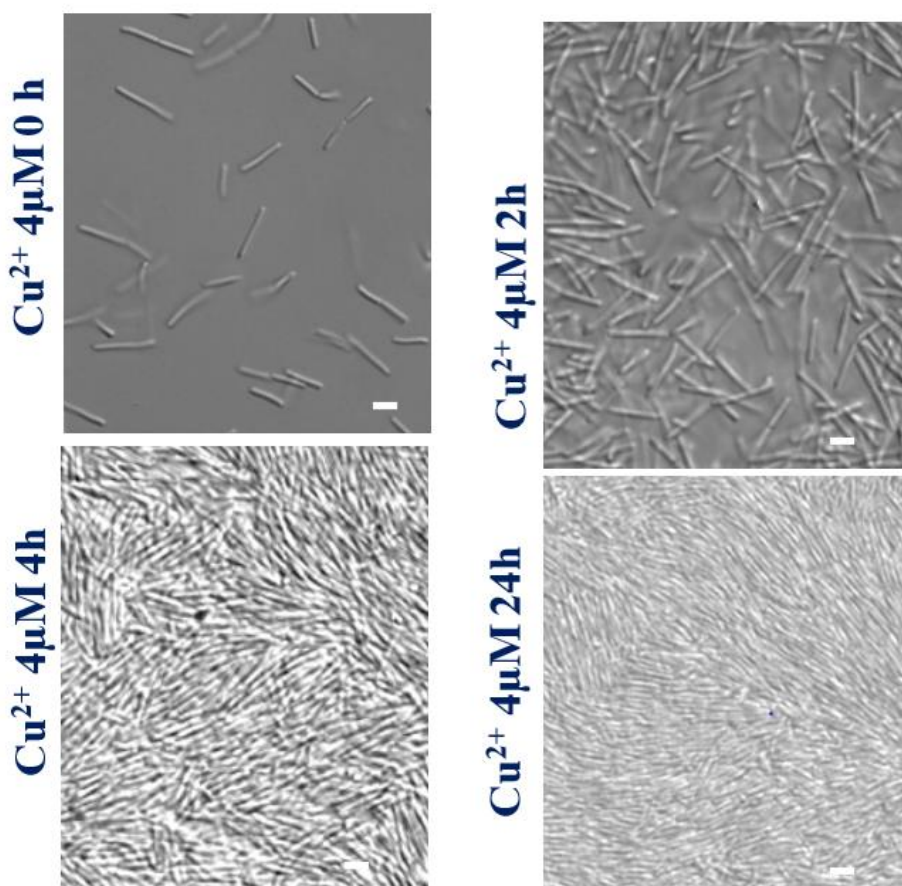
Figure 24. Metal treatment affects *C. difficile* morphology. Treatment with 4 µM Cu^{2+}, cause cells to sediment on the bottom surface of the imaging chamber in a nest-like morphology after 2h continuing through 24h. The same morphological changes were seen when treated with an Ag^+ (image not shown). Scale bars in all images represent 10 microns

Piscidins and Piscidin-Copper Complexes Do Not Induce Sporulation in *C. difficile*

To confirm the visual observation that samples treated with piscidin had fewer spores we subjected treated samples to oxygen and ethanol to kill off any surviving vegetative cells and then germinated the spores to count them. Both piscidin and piscidin-copper complexes caused a

significant reduction in the numbers of spores over time at their 0.5x inhibitory concentrations of 2.00µM (p1&p1Cu) and 4.00µM (p3&p3Cu). The cells exposed to 0.5x inhibitory concentrations of both peptides and their complexes had no significant differences from the untreated controls at the beginning of the experiment. After 3h, the number of spores in the untreated control increased significantly and the number of spores in the treated samples was constant or diminished. p1 and p1Cu induced a threefold reduction in the number of spores compared to the untreated negative control (Figure 25a). In p3 and p3Cu there was twofold reduction in the number of spores (Figure 25b). Copper ions made no appreciable difference in the effect of p1 on sporulation, and copper-bound p3 appeared to suppress sporulation even more than p3 alone.

Copper Alone Induces Sporulation in *C. difficile*

We have previously reported that copper alone is inhibitory against *C. difficile* presumably by causing oxidative stress. Exposure to sub-inhibitory copper sulfate increases spore formation after 3h (Figure 25 c), even though copper complexed to p1 and p3 did not have this effect (Figure 25 a, b).
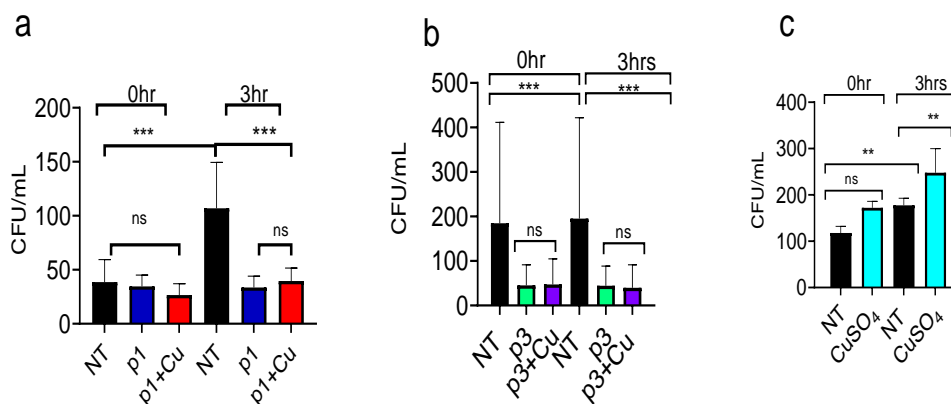
Figure 25. Piscidins do not stimulate sporulation, but copper does. (**a**) p1 and p1Cu decrease the formation of viable spores after 3h of treatment compared to non-treated controls (**b**) p3 and p3Cu reduce the number of germinating spores immediately and after 3h of treatment plots (**c**) Treatment with copper sulfate stimulate the formation of more spores in 3h than in the nontreated control. Using two-way ANOVA with Tukey's post-test comparison. nt, not treated; n.s., not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Shown are the means and standard errors of at least three biologically independent samples.

Piscidin-Antibiotic Combinations Do Not Trigger Sporulation

Samples treated with piscidins, and piscidin-copper complexes showed reduced sporulation even though copper alone increased spores (Figure 25c). While fidaxomicin reduced expression of *C. difficile* sporulation gene and inhibits sporulation *in vitro*, metronidazole and vancomycin have been reported to not affect sporulation or to mildly stimulate sporulation in some straw and clindamycin [48, 132-134].. We evaluated the effect of 0.5x of inhibitory concentrations of p1 and p3 administered in the presence of fidaxomicin, metronidazole or

vancomycin. *C. difficile* sporulation was reduced in the presence of these combinations (Figure 26 a, b, and c). p1 alone and p3Cu appeared to suppress spores in the presence of the ineffective antibiotic Cipro, but the result was not statistically significant (Figure 26d).
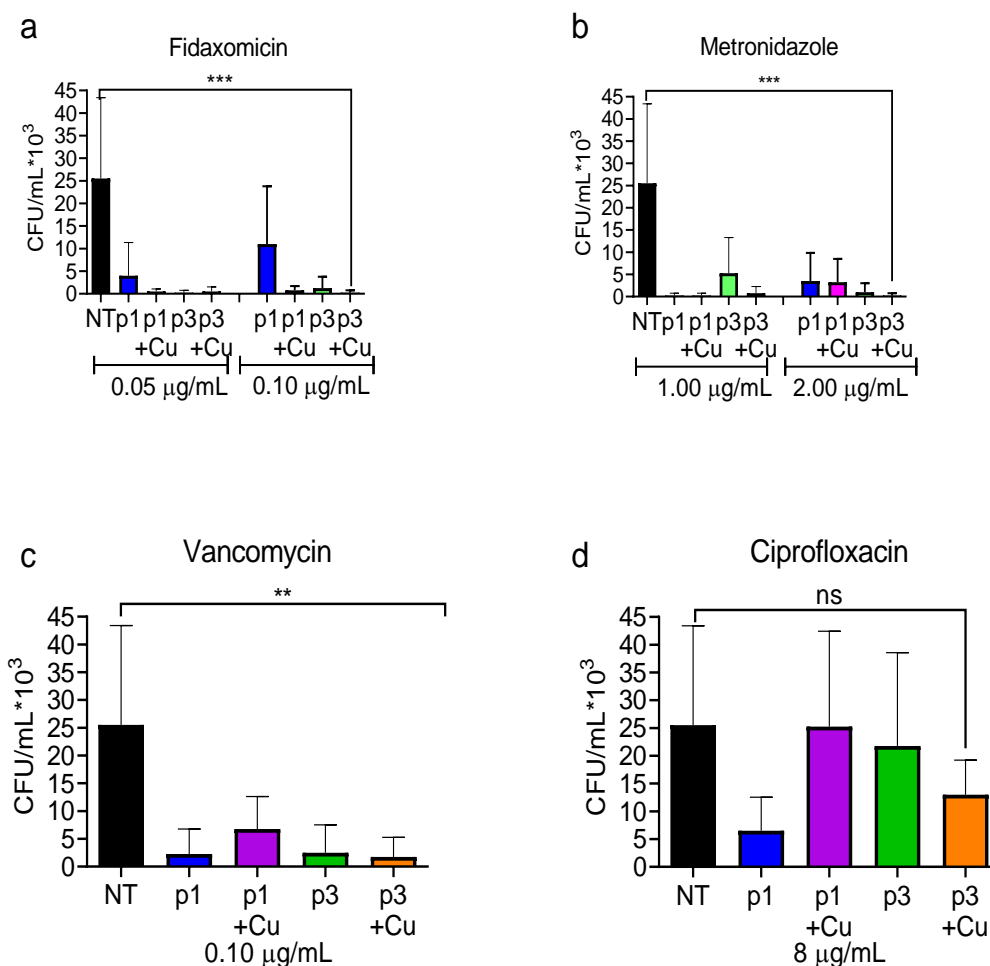
Figure 26. Spore enumeration after treatment with piscidins and antibiotics (**a**) Fidaxomicin in combination with piscidins and their copper complexes reduced *C. difficile* sporulation (**b**) Metronidazole in combination with piscidins and their copper complexes reduced *C. difficile* sporulation (**c**) Vancomycin in combination with piscidins and their copper complexes slightly reduced sporulation in the case of p1 and p3Cu (**d**) Ciprofloxacin in combination with piscidins and their copper complexes sporulation not reduced. Data were analyzed using two-way ANOVA with Tukey's post-test comparison. nt, not treated; n.s., not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Shown are the means and standard errors of at least three biologically independent samples.

DISCUSSION

p1 and p3 act additively in an anaerobic environment against *C. difficile*. The two peptides were able to inhibit bacterial proliferation at a lower concentration of both in comparison to each peptide acting alone. Oxygen-contaminated peptides applied immediately after being brought into the anaerobic chamber supported the level of efficacy established by Oludiran *et al* 2019, but peptides equilibrated for 3h in anaerobic chamber before usage are more efficient. We speculate that trace oxygen activates the bacterial oxidative response pathways, which incidentally increases the resistance to piscidin. In addition to synergizing with each other, sub-inhibitory amounts of piscidins increase the efficacy of antibiotics used in the treatment of CDI.

The ability of the peptides to increase uptake of exogenous propidium iodide in living cells suggested that the peptides may permeabilize the cell membrane, increasing uptake of antibiotics and making it easier for antibiotics to access their intracellular targets. While copper does not enhance the anaerobic antimicrobial activity of p1 or p3 alone, metalated peptides were more effective than peptides alone at stimulating PI uptake or synergizing with antibiotics. This may be because copper stabilizes peptide interactions with the membrane to increase permeability has been suggested by studies monitoring fluorescent dye entry into non-living lipid vesicles [135]. DIC microscopy suggested an increase in sporulation in the presence of antimicrobial metals that cause oxidative stress, which was confirmed by spore germination and quantification assays. Interestingly, treatment with 4µM Cu (II) salts also affect vegetative cell morphology. The treated cell showed a nest-like new arrangement not seen before they settled out of the solution. This aggregation could be a precursor to biofilm formation to try to collectively reduce the effect of oxidative stress.

The discovery that oxidative stress from metal ions induces sporulation in *C. difficile* suggested that the loss of vegetative growth observed after piscidin treatment could be partly due to cells differentiating into spores instead of reproducing. However, all the metalated and non-metalated peptides reduced spore formation in direct comparison with the untreated samples, showing that piscidins kill vegetative *C. difficile* without triggering sporulation. Interestingly, copper alone stimulates sporulation but not if the piscidins are present, suggesting that the peptides suppress sporulation. However, piscidins can be used to boost antibiotic treatments without triggering increased *C. difficile* sporulation, and no cell will be able evade antibiotic treatment.

Summary

These antimicrobial peptides and their copper complexes give a helping hand to the effective antibiotics fidaxomicin, metronidazole, and vancomycin by lowering their needed dosage and increasing their efficacy against vegetative *C. difficile*. This effect does not extend to non-functioning antibiotics like ciprofloxacin, showing that both partners need to be functional to synergize. Peptides alone or with their copper complexes can suppress sporulation in *C. difficile,* even in the presence of antibiotics that induce stress needed to stop bacterial cell proliferation.

CHAPTER IV

BIOFILM PRODUCTION AS PHENOTYPIC RESPONSE TO ENVIRONMENTAL AND IMMUNE STRESS IN *C. DIFFICILE*

OVERVIEW

Biofilms are the aggregation of microbes of one or many species embedded within a matrix in surface-attached communities that secrete biofilm-specific extracellular products. While anaerobic biofilms impact health, industry, and the environment, biofilms are mostly studied *in vitro* in the presence of oxygen because of the difficulty of growing and analyzing them anaerobically [10, 135]. *C. difficile* can form biofilms as a single species or with other anaerobic intestinal bacteria on different biotic and abiotic surfaces, which helps them survive antibiotics and other stresses [136]. *C. difficile* strains that form strong biofilms *in vitro* are more likely to cause recurrent CDI [137]. In recent years, the potential contribution of biofilms to *C. difficile* pathogenesis has been a strong subject of interest especially their part in CDI recurrence. The role of stresses that induce or disrupt biofilm in the intestinal microbiota is not well understood and there is little information on *C. difficile* colonization of the gastrointestinal tract.

Environmental and physiological stresses can stimulate biofilm formation response in other bacterial species [138]. Some harsh environmental conditions can lead microorganisms to accumulate reactive oxygen species (ROS), which produce or mimic intracellular oxidative stress. Oxidative stress acts as a cue, triggering bacteria to activate effective oxygen scavenging mechanisms or to shift metabolic pathways. Oxidative stress responses which are shared among most bacteria include gene expression induced by conserved regulators, production of extracellular polymeric substances, and biofilm formation. Mitigation of *C. difficile* biofilm

production could be helpful to control the virulence and infection recurrence of this organism [138].

The biofilm matrix can include polysaccharides, proteins, and nucleic acids, together called extracellular polymeric substances (EPS); the exact nature of the biofilm matrix depends on the identity of the bacteria within it [53, 54]. The biofilm structure and /or architecture dictate the ability to attach either to biotic or abiotic surfaces and survive stress. Biofilm composition, compactness and amount also vary according to the available nutrients in the environment [139]. The three-dimensional structure of biofilms give insight into their attachment ability to different surfaces and antibiotics resistance capacity of cells in the biofilm. Biofilms with more polysaccharide composition with moderate extracellular nucleic materials and proteins tend to form clumped aggregates and are less resistant to external stresses. Biofilms with higher proportions of nuclear material and protein form three-dimensional carpet-like aggregates which protect the component cells and improve stress survival [56]. Previous studies have shown that deoxycholate or pyruvate induced *C. difficile* to form surface adherent biofilms in 48h. We investigate environmental stressors like antibiotics, antimicrobial metals, antimicrobial peptides, and oxidants (diamide and tert-butyl hydroperoxide tBHP) applied *in vitro* to mimic the *in vivo* environment of the bacteria during infection and quantify stress-induced *C. difficile* biofilm formation.

MATERIALS AND METHODS

Materials, Chemicals, Bacterial Strains and Growth Conditions

Materials and chemicals were purchased from Fisher Scientific (Hampton, NH, USA) unless otherwise indicated. The bacterial strains used in this study are listed in Appendix D. *C. difficile* 630Δ*erm* and R20291 were maintained on brain-heart infusion supplemented with 5% yeast extract (BHIS) agar plates [101, 102]. Biofilms were grown in untreated 24 well plates in BHIS media supplemented with 1% glucose and 50mM Na_2PO_3 (pH = 7.4). Where indicated, cells were treated with $CuSO_4$, $AgNO_3$, $MgSO_4$, diamide, (MP Biomedicals) or tert-Butyl hydroperoxide (tBHP) to induce oxidative stress. Host defense peptides (HDPs) piscidin-1 (p1), piscidin-3 (p3), and piscidin copper complexes were provided by Myriam Cotten's lab (College of William & Mary, Williamsburg, VA). All anaerobic bacterial culture took place at 37^0C in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 85% N_2, 10% CO_2, 5% H_2. All plastic consumables were equilibrated in the anaerobic chamber for a minimum of 72 h [140, 141].

C. difficile Biofilm Growth and Quantification

Four biologically independent cultures of R20291 were used to inoculate 2.00mL/well of BHIS media with supplemented 1% glucose and 50 mM Na_2PO_3 (Fisher Scientific) at pH 7.4 (Mettler-Toledo), using 200μL of log phase culture in sterile equilibrated 24-well plates. Culture was incubated for 2 hours before treatment with the indicated concentrations of respective treatments at sub-lethal concentrations and incubated anaerobically at 37^0C for 48h. After the 48h of growth, cell density was measured at 630/nm. Non-adhered planktonic cells were removed by pipetting and wells were washed with 1 mL 1x phosphate buffered saline solution (1xPBS), stained for 30 minutes with 0.1% crystal violet, and then washed twice with 1 mL each

of 1xPBS. The adhered stain was suspended in 70% ethanol before scanning the plate at 570/nm, using the Bio-Tek plate reader [61].

RESULTS

Piscidins and/or their copper complexes showed significant stimulation of *C. difficile* biofilm production. Gradient concentrations of piscidin and piscidin-copper complexes from 0.0 to 2.0 µM, showed that apo- and metalated peptides increase the amount of biofilm produced. At 1µ M p1 there is a two-fold increase in the amount of biofilm produced and this continued up to 2µM concentration point. The same response was seen with p1Cu (Figure 17a). Biofilm production was also increased by both p3 and the p3Cu complex, with the p3Cu complex having a more pronounced effect (Figure 17b).

Transition metals are known to be antimicrobial, with a presumed mechanism of action involving serving as a source of oxidative stress antimicrobial metals. We have shown that copper kills *C. difficile* and inhibit its growth (Chapter II Figure 6) [123]. Here we have shown that silver is also antimicrobial at millimolar concentrations (Figure 26). A similar level of magnesium is not antimicrobial, showing that the observed effect of the transition metals is not osmotic (Figure 26). The antimicrobial metals of have a dose-dependent effect on biofilm. $AgNO_3$ showed significant increase in biofilm production at 4µM (Figure 25d), and the same response is seen in $CuSO_4$ at 32µM (Figure 25c). We compared antimicrobial metals (Ag & Cu) and non-antimicrobial metal (Mg). We found a significant difference in biofilm formation for both concentration gradient of Ag (2 µM and 4 µM) and a strong trend but no significant differences for Cu for the tested concentrations while Mg does not cause any observable changes (Figure 25). In the case of oxidative stress inducers (diamide and tert-Butyl hydroperoxide

tBHP) which mimic environmental conditions *in vitro,* we show that biofilm production response by the bacteria is selective. There was a twofold increase for biofilm produced in the presence of diamide but no significant difference in the biofilm produced by tBHP at gradient concentrations of both oxidants in treated media and cell culture (Figure 25). There was a twofold increase in the amount of biofilm produced in the presence of diamide but no significant difference in biofilm produced after treatment with tBHP.
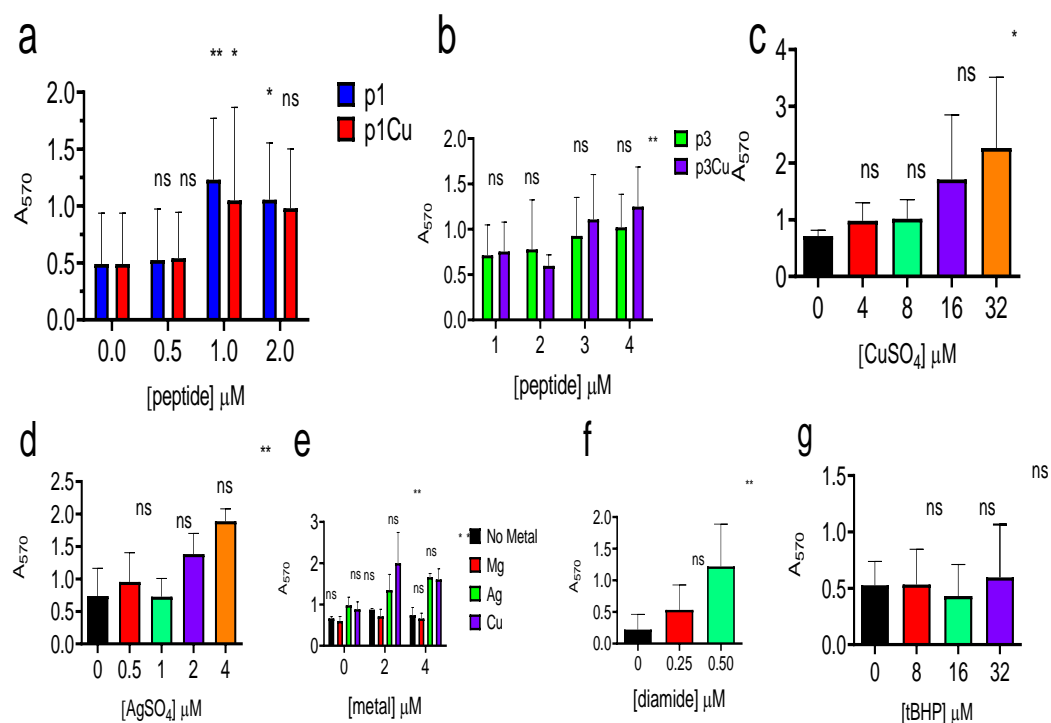
Figure 27. *C. difficile* biofilm production in response to different inducers **(a)** Biofilm produced after treatment with p1 and p1Cu **(b)** Biofilm produced after treatment with p3 and p3Cu **(c and d)** Biofilm produced with the treatment of antimicrobial metals **(e)** Biofilm produced after treatment with antimicrobial metals (Ag and Cu) and a non-antimicrobial metal (Mg)**. (f)** Graphical representation of biofilm produced after treatment with the oxidative stressor diamide **(g)** Biofilm produced after treatment with the oxidative stressor tBHP. Treated conditions were compared to untreated conditions by using two-way ANOVA with Tukey's post-test comparison. nt, not treated; n.s., not significant; $*$ $p < 0.05$; $**$ $p < 0.01$; $***$ $p < 0.001$; $****$ $p < 0.0001$. Shown are the means and standard errors of at least three biologically independent samples.
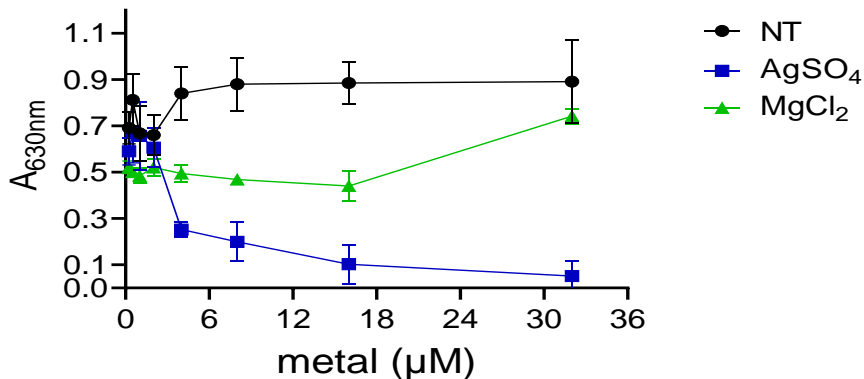
Figure 28. *C. difficile* growth in the presence of metal. Optical density at 600 nm after overnight growth in BHIS supplemented with the indicated concentration of silver nitrate ($AgNO_3$) and magnesium (II) sulfate ($MgSO_4$). Shown are the means and standard deviation of two samples.

DISCUSSION

C. difficile biofilm formation in response to oxidative stress varies depending on the source of the stress. p1 and p1Cu are major biofilm inducers at their published 0.5x inhibitory concentrations and showed dose dependance as the concentrations increased. The effect was less pronounced with p3 and was only significant in the presence of copper. P1, p3 and their copper complexes cause membrane disruption which might sensitize the activation of surface defense mechanism of the bacteria to quickly secrete extracellular matrix so as to protect themselves from extracellular attack. Metal bioavailability also influences the bacterial cells. Disturbance to metal homeostasis in the GI tract can lead to diverse types of response by different bacteria in that habitat. Many vertebrates maintain a consistent amount of metals in their GI tract to not only

promote enzymatic activities (by acting as cofactors), but to also prevent proliferation of bacterial cells, for which metals can be growth-limiting nutrients but which can suffer redox damage in the presence of excessive metal [142]. Ag and Cu used in medical tools are believed to prevent bacterial growth, but they have never been evaluated for the prevention of biofilm formation [143].

Supplementation with non-transition metal salts did not stimulate biofilm formation, indicating that biofilm is formed as a result of oxidative stress induced by the antimicrobial metals at the indicated concentrations. Antimicrobial peptides and metals are not the only sources of oxidative stress but the fact that they have the same effect on biofilm formation suggests that it is a general response to oxidative stress. We employ diamide and tBHP as both are organic oxidant; they are used as sources of oxidative stress because they do not break down as easily as hydrogen peroxide in an anaerobic condition. These organic oxidants use two different mechanisms in exacting oxidative stress. Diamide induce disulfide bond breakage, while tBHP accelerate the oxidation of glutathione, and both are subcategories of oxidative stress. Only diamide at sub-inhibitory concentrations stimulated more biofilm formation *in vitro* against *C. difficile* while tBHP does not, which suggests that biofilm induction in response to oxidants could be source dependent. So, in all indication, exposure to sub-lethal oxidative stress induces biofilm formation as a survival mechanism.

Summary

The production of biofilms shield bacterial communities from extracellular stress and increases bacterial survival. While piscidins are effective killers of the *C. difficile*, sublethal concentrations do stimulate biofilm formation. This could complicate the use of piscidins as treatments for CDI *in vivo*.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Stress survival by the pathogen *C. difficile* contributes to the infection's spreading to new host via spore formation as well as vegetative cell survival within one host. In this dissertation we investigate the phenotypic stress responses of *C. difficile*. Sporulation and biofilm formation are both important morphological responses to different stresses. More information about these processes is very necessary to design new treatments to combat the pathogenicity of this multi-drug resistant bacterium, wreaking havoc in both industrialized nations and developing countries. Since the beginning of the 21st century CDI is not slowing down in infection spreading. So strong alternative treatments for the pathogen are urgently needed.

We propose a new strategy, the usage of AMPs (specifically piscidins). In chapter II of this dissertation, we applied piscidins to an anaerobic pathogen for the first time and found that they are a viable strategy to attack anaerobic bacteria. We investigated the response of the pathogen to environmental and immune stresses in terms of the vegetative cell viability, spore production and biofilm formation. The importance of the phenotypic expression of spore and biofilm for this bacterium cannot be over-stressed because of their contribution to infection spread and resistance to most classes of antibiotics. Piscidins and their copper complexes inhibit the growth of *C. difficile* in anaerobic conditions. The peptides and their metalated complexes were able to significantly reduce the population of already existing *C. difficile* cells in the same anaerobic condition. Aerobically, it was reported that addition of copper metal to the piscidin significantly increases their efficacy, but this is not the same in anaerobic condition. Copper on its own can inhibit bacterial proliferation but does not enhance piscidin lethality anaerobically.

We established that piscidins can associate with the membranes of the bacterial cells, which positions them to impair membrane integrity [123].

In chapter III of this dissertation, we showed that the piscidins' antimicrobial activities are stronger when residual oxygen has been removed and prevented from activating antioxidant stress responses, suggesting that piscidins induce oxidative stress in this organism. We also showed that the piscidins evaluated can synergize with each other and with clinically relevant antibiotics. Piscidins strengthen antibiotics by lowering their dosage and increasing their efficacy against the anaerobic pathogen. This complimentary effect could help improve the potency of CDI treatment antibiotics that are already lost or are losing their efficacy. The cooperation effect between antibiotics and sublethal amount of piscidins are not extended to non-functioning antibiotics, proving that the peptides boost the antibiotics even when they are not present at high enough level to be lethal themselves, the peptide at the supplied concentrations will not be able to provide effective killing unless they synergize with functioning antibiotics. The propidium iodide uptake experiments suggest that the piscidins boost the antibiotic efficacy by increasing antibiotic uptake into the cells.

The most exciting finding is that the synergy between piscidins and antibiotics leads to sporulation suppression in *C. difficile*. *C. difficile* sporulation stage was reported to be the major bacteria life cycles stage that many of the antibiotics do not have direct inhibitory or suppression effect on except fidaxomicin. Presence of piscidins, with those functioning antibiotics at their sub-inhibitory concentrations, simultaneously carryout their antibacterial activities and sporulation suppressions. So, functioning antibiotics worked in synergy with the peptides and peptides-metal complex. Earlier investigation already established that piscidins can stand alone in killing off the vegetative cells of *C. difficile* and now our new findings confirmed that they can

work in cooperation with other antibiotics to conduct the antimicrobial effect. Treatment that attacks vegetative *C. difficile* without triggering sporulation are highly sought after to combat CDI and piscidin show exciting potential for this.

Our investigation in chapter IV of this dissertation reveals that biofilm formation is a stress survival strategy activated separately from sporulation in *C. difficile*. Biofilm formation is another phenotypic expression to survive extracellular stress. Piscidins, reported to induce unique form of stress by causing both cell membrane disruption and DNA damage, can also induce the bacterial cells to produce biofilm at sub-inhibitory concentration. Other environmental stressors, including antimicrobial metals, can also stimulate biofilm formation. The non-oxidative stress agent diamide, which breaks disulfide bonds, also induces biofilm formation, while tBHP, an organic oxidant that induces oxidative stress by causing an increase in ROS, does not influence the amount of biofilm produced by *C. difficile*. This shows that stress induced biofilm formation in this organism is specific to different stress sources. This could limit piscidins' usefulness as a treatment against *C. difficile* because sub-inhibitory concentration of these peptides might encourage biofilm formation and bacterial resilience. Future work will evaluate whether combinations of peptides and antibiotics exhibit cooperation against *C. difficile* biofilm and whether they disrupt already formed biofilm in anaerobic conditions as these have been reported to do aerobically. Bacteria live up to the saying "what does not kill you makes you stronger." So, when these peptides are coupled into therapies it is not only the oxygen level of the target sites that should be considered, but the lethality dosage threshold is also particularly important to avoid triggering biofilm formation.

REFERENCES

1.   Jarrad, A. M.; Karoli, T.; Blaskovich, M. A.; Lyras, D.; Cooper, M. A., *Clostridium difficile* drug pipeline: challenges in discovery and development of new agents. *Journal of Medicinal Chemistry* **2015,** 58, (13), 5164-5185.

2.   Vedantam, G.; Clark, A.; Chu, M.; McQuade, R.; Mallozzi, M.; Viswanathan, V., *Clostridium difficile* infection: toxins and non-toxin virulence factors, and their contributions to disease establishment and host response. *Gut Microbes* **2012,** 3, (2), 121-134.

3.   Cyprowski, M.; Stobnicka-Kupiec, A.; Ławniczek-Wałczyk, A.; Bakal-Kijek, A.; Gołofit-Szymczak, M.; Górny, R. L., Anaerobic bacteria in wastewater treatment plant. *International Archives of Occupational and Environmental Health* **2018,** 91, (5), 571-579.

4.   Johnson, L. A.; Hug, L. A., Distribution of reactive oxygen species defense mechanisms across domain bacteria. *Free Radical Biology and Medicine* **2019,** 140, 93-102.

5.   Ma, X.; Wu, M.; Wang, C.; Li, H.; Fan, A.; Wang, Y.; Han, C.; Xue, F., The pathogenesis of prevalent aerobic bacteria in aerobic vaginitis and adverse pregnancy outcomes: a narrative review. *Reproductive Health* **2022,** 19, (1), 1-11.

6.   Wallace, M.; Fishbein, S.; Dantas, G., Antimicrobial resistance in enteric bacteria: current state and next-generation solutions. *Gut Microbes* **2020,** 12, (1), 1799654.

7.   Poole, K., *Pseudomonas aeruginosa:* resistance to the max. *Frontiers in Microbiology* **2011,** 2, 65.

8.   Proctor, R. A.; Von Eiff, C.; Kahl, B. C.; Becker, K.; McNamara, P.; Herrmann, M.; Peters, G., Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Reviews Microbiology* **2006,** 4, (4), 295-305.

9.   Lewis, K., Persister cells. *Annual Review of Microbiology* **2010,** 64, 357-372.

10.   Béchon, N.; Ghigo, J.-M., Gut biofilms: Bacteroides as model symbionts to study biofilm formation by intestinal anaerobes. *FEMS Microbiology Reviews* **2022,** 46, (2), fuab054.

11.   Wetzel, D.; McBride, S. M., The impact of pH on *Clostridioides difficile* sporulation and physiology. *Applied and Environmental Microbiology* **2020,** 86, (4), e02706-19.

12.   Marmion, M.; Macori, G.; Ferone, M.; Whyte, P.; Scannell, A., Survive and thrive: Control mechanisms that facilitate bacterial adaptation to survive manufacturing-related stress. *International Journal of Food Microbiology* **2022**, 109612.

13.   Fjalstad, J. W.; Esaiassen, E.; Juvet, L. K.; van den Anker, J. N.; Klingenberg, C., Antibiotic therapy in neonates and impact on gut microbiota and antibiotic resistance development: a systematic review. *Journal of Antimicrobial Chemotherapy* **2018,** 73, (3), 569-580.

14.   Shawky, M.; Suleiman, W. B.; Farrag, A. A., Antibacterial resistance pattern in clinical and non-clinical bacteria by phenotypic and genotypic assessment. *Journal of Pure and Applied Microbiology* **2021,** 15, (4), 7220-7229.

15.   Hall, I. C.; O'toole, E., Intestinal flora in new-born infants: with a description of a new pathogenic anaerobe, Bacillus difficilis. *American journal of diseases of children* **1935,** 49, (2), 390-402.

16.   Tan, D., Germination and Sporulation of *Clostridioides difficile*. **2020**.

17.   Miller, M., Fidaxomicin (OPT-80) for the treatment of *Clostridium difficile* infection. *Expert Opinion on Pharmacotherapy* **2010,** 11, (9), 1569-1578.

18.   Newsroom, C., Nearly half a million Americans suffered from *Clostridium difficile* infections in a single year. In 2019.

19.   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., Trends in US burden of *Clostridioides difficile* infection and outcomes. *New England Journal of Medicine* **2020,** 382, (14), 1320-1330.

20.   Giordano, N.; Hastie, J. L.; Carlson, P. E., Transcriptomic profiling of *Clostridium difficile* grown under microaerophillic conditions. *Pathogens and Disease* **2018,** 76, (2), fty010.

21.   Kullin, B.; Abratt, V. R.; Reid, S. J.; Riley, T. V., *Clostridioides difficile* infection in Africa: A narrative review. *Anaerobe* **2022**, 102549.

22.   Gupta, A.; Khanna, S., Community-acquired *Clostridium difficile* infection: an increasing public health threat. *Infection and Drug Resistance* **2014,** 7, 63.

23.   Brennhofer, S. A.; McQuade, E. T. R.; Liu, J.; Guerrant, R. L.; Platts-Mills, J. A.; Warren, C. A., *Clostridioides difficile* colonization among very young children in resource-limited settings. *Clinical Microbiology and Infection* **2022**.

24.   Smits, W. K., Hype or hypervirulence: a reflection on problematic *C. difficile* strains. *Virulence* **2013,** 4, (7), 592-596.

25.   Collins, J.; Robinson, C.; Danhof, H.; Knetsch, C.; Van Leeuwen, H.; Lawley, T.; Auchtung, J.; Britton, R., Dietary trehalose enhances virulence of epidemic *Clostridium difficile*. *Nature* **2018,** 553, (7688), 291-294.

26.   Drekonja, D. M.; Butler, M.; MacDonald, R.; Bliss, D.; Filice, G. A.; Rector, T. S.; Wilt, T. J., Comparative effectiveness of *Clostridium difficile* treatments: a systematic review. *Annals of Internal Medicine* **2011,** 155, (12), 839-847.

27.   Boyle, M. L.; Ruth-Sahd, L. A.; Zhou, Z., Fecal microbiota transplant to treat recurrent *Clostridium difficile* infections. *Critical Care Nurse* **2015,** 35, (2), 51-64.

28.   Dharmasena, M.; Jiang, X., Improving culture media for the isolation of *Clostridium difficile* from compost. *Anaerobe* **2018,** 51, 1-7.

29.   Edwards, A. N.; Karim, S. T.; Pascual, R. A.; Jowhar, L. M.; Anderson, S. E.; McBride, S. M., Chemical and stress resistances of *Clostridium difficile* spores and vegetative cells. *Frontiers in Microbiology* **2016,** 7, 1698.

30.   Shen, A., *Clostridioides difficile* spore formation and germination: New insights and opportunities for intervention. *Annual Review of Microbiology* **2020,** 74, 545-566.

31.   Kumar, N.; Browne, H. P.; Viciani, E.; Forster, S. C.; Clare, S.; Harcourt, K.; Stares, M. D.; Dougan, G.; Fairley, D. J.; Roberts, P., Adaptation of host transmission cycle during *Clostridium difficile* speciation. *Nature Genetics* **2019,** 51, (9), 1315-1320.

32.   DiCandia, M. A.; Edwards, A. N.; Jones, J. B.; Swaim, G. L.; Mills, B. D.; McBride, S. M., Identification of functional Spo0A residues critical for sporulation in *Clostridioides difficile*. *Journal of Molecular Biology* **2022**, 167641.

33.   Kraus, C. N.; Lyerly, M. W.; Carman, R. J., Ambush of *Clostridium difficile* spores by ramoplanin: activity in an *in vitro* model. *Antimicrobial Agents and Chemotherapy* **2015,** 59, (5), 2525-2530.

34.   Asempa, T. E.; Nicolau, D. P., *Clostridium difficile* infection in the elderly: an update on management. *Clinical Interventions in Aging* **2017,** 12, 1799.

35.   Dembek, M.; Kelly, A.; Barwinska-Sendra, A.; Tarrant, E.; Stanley, W. A.; Vollmer, D.; Biboy, J.; Gray, J.; Vollmer, W.; Salgado, P. S., Peptidoglycan degradation machinery in

Clostridium difficile forespore engulfment. *Molecular Microbiology* **2018,** 110, (3), 390-410.

36. Lawler, A. J.; Lambert, P. A.; Worthington, T., A revised understanding of *Clostridioides difficile* spore germination. *Trends in Microbiology* **2020,** 28, (9), 744-752.

37. Setlow, P., Spores of *Bacillus subtilis:* their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology* **2006,** 101, (3), 514-525.

38. Nicholson, W. L.; Munakata, N.; Horneck, G.; Melosh, H. J.; Setlow, P., Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews* **2000,** 64, (3), 548-572.

39. Paredes-Sabja, D.; Shen, A.; Sorg, J. A., *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends in Microbiology* **2014,** 22, (7), 406-416.

40. Leggett, M. J.; McDonnell, G.; Denyer, S. P.; Setlow, P.; Maillard, J. Y., Bacterial spore structures and their protective role in biocide resistance. *Journal of Applied Microbiology* **2012,** 113, (3), 485-498.

41. Pizarro-Guajardo, M.; Calderón-Romero, P.; Castro-Córdova, P.; Mora-Uribe, P.; Paredes-Sabja, D., Ultrastructural variability of the exosporium layer of *Clostridium difficile* spores. *Applied and Environmental Microbiology* **2016,** 82, (7), 2202-2209.

42. Di Ciaula, A.; Garruti, G.; Baccetto, R. L.; Molina-Molina, E.; Bonfrate, L.; Portincasa, P.; Wang, D. Q., Bile acid physiology. *Annals of Hepatology* **2018,** 16, (1), 4-14.

43. Urdaneta, V.; Casadesús, J., Interactions between bacteria and bile salts in the gastrointestinal and hepatobiliary tracts. *Frontiers in Medicine* **2017,** 4, 163.

44. Winston, J. A.; Theriot, C. M., Impact of microbial derived secondary bile acids on colonization resistance against *Clostridium difficile* in the gastrointestinal tract. *Anaerobe* **2016,** 41, 44-50.

45. Abbas, A.; Zackular, J. P., Microbe–microbe interactions during *Clostridioides difficile* infection. *Current Opinion in Microbiology* **2020,** 53, 19-25.

46. Rashid, T.; Haghighi, F.; Hasan, I.; Bassères, E.; Alam, M. J.; Sharma, S. V.; Lai, D.; DuPont, H. L.; Garey, K. W., Activity of hospital disinfectants against vegetative cells and spores of *Clostridioides difficile* embedded in biofilms. *Antimicrobial Agents and Chemotherapy* **2019,** 64, (1), e01031-19.

47. Paredes-Sabja, D.; Setlow, P.; Sarker, M. R., Germination of spores of Bacillales and Clostridiales species: mechanisms and proteins involved. *Trends in Microbiology* **2011,** 19, (2), 85-94.

48. Babakhani, F.; Bouillaut, L.; Gomez, A.; Sears, P.; Nguyen, L.; Sonenshein, A. L., Fidaxomicin inhibits spore production in *Clostridium difficile*. *Clinical Infectious Diseases* **2012,** 55, (suppl_2), S162-S169.

49. Zhu, D.; Sorg, J. A.; Sun, X., *Clostridioides difficile* biology: sporulation, germination, and corresponding therapies for C. difficile infection. *Frontiers in Cellular and Infection Microbiology* **2018,** 8, 29.

50. Isidro, J.; Mendes, A. L.; Serrano, M.; Henriques, A. O.; Oleastro, M., *Overview of Clostridium difficile infection: life cycle, epidemiology, antimicrobial resistance and treatment*. 2017.

51. Sender, R.; Fuchs, S.; Milo, R., Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. *Cell* **2016,** 164, (3), 337-340.

52.   Blunk, B.; Perkins, M.; Walsh, D.; Chauhan, V.; Camara, M.; Williams, P.; Aylott, J.; Hardie, K., Use of nanosensor technology to investigate biofilm formation and resulting malodour in washing machines. *Access Microbiology* **2019,** 1, (1A), 238.

53.   Anwar, H.; Dasgupta, M.; Costerton, J., Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrobial Agents and Chemotherapy* **1990,** 34, (11), 2043-2046.

54.   Matz, C.; Bergfeld, T.; Rice, S. A.; Kjelleberg, S., Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. *Environmental Microbiology* **2004,** 6, (3), 218-226.

55.   Wu, S.; Li, X.; Gunawardana, M.; Maguire, K.; Guerrero-Given, D.; Schaudinn, C.; Wang, C.; Baum, M. M.; Webster, P., Beta-lactam antibiotics stimulate biofilm formation in non-typeable Haemophilus influenzae by up-regulating carbohydrate metabolism. *PLoS One* **2014,** 9, (7), e99204.

56.   Poquet, I.; Saujet, L.; Canette, A.; Monot, M.; Mihajlovic, J.; Ghigo, J.-M.; Soutourina, O.; Briandet, R.; Martin-Verstraete, I.; Dupuy, B., *Clostridium difficile* biofilm: remodeling metabolism and cell surface to build a sparse and heterogeneously aggregated architecture. *Frontiers in Microbiology* **2018**, 2084.

57.   Meurer, M.; O'Neil, D. A.; Lovie, E.; Simpson, L.; Torres, M. D.; de la Fuente-Nunez, C.; Angeles-Boza, A. M.; Kleinsorgen, C.; Mercer, D. K.; von Köckritz-Blickwede, M., Antimicrobial Susceptibility Testing of Antimicrobial Peptides Requires New and Standardized Testing Structures. *ACS Infectious Diseases* **2021**.

58.   Libardo, M. D. J.; Bahar, A. A.; Ma, B.; Fu, R.; McCormick, L. E.; Zhao, J.; McCallum, S. A.; Nussinov, R.; Ren, D.; Angeles-Boza, A. M., Nuclease activity gives an edge to host-defense peptide piscidin 3 over piscidin 1, rendering it more effective against persisters and biofilms. *The FEBS Journal* **2017,** 284, (21), 3662-3683.

59.   Mansour, S. C.; de la Fuente-Núñez, C.; Hancock, R. E., Peptide IDR-1018: modulating the immune system and targeting bacterial biofilms to treat antibiotic-resistant bacterial infections. *Journal of Peptide Science* **2015,** 21, (5), 323-329.

60.   Silva, O. N.; Torres, M. D.; Cao, J.; Alves, E. S.; Rodrigues, L. V.; Resende, J. M.; Lião, L. M.; Porto, W. F.; Fensterseifer, I. C.; Lu, T. K., Repurposing a peptide toxin from wasp venom into antiinfectives with dual antimicrobial and immunomodulatory properties. *Proceedings of the National Academy of Sciences* **2020,** 117, (43), 26936-26945.

61.   Oludiran, A. M., *Characterizing the Activity of Antimicrobial Peptides Against the Pathogenic Bacterium Clostridium difficile in an Anaerobic Environment. Old Dominion University*: 2018.

62.   Salger, S. A.; Cassady, K. R.; Reading, B. J.; Noga, E. J., A diverse family of host-defense peptides (piscidins) exhibit specialized anti-bacterial and anti-protozoal activities in fishes. *PLoS One* **2016,** 11, (8), e0159423.

63.   Portelinha, J.; Duay, S. S.; Yu, S. I.; Heilemann, K.; Libardo, M. D. J.; Juliano, S. A.; Klassen, J. L.; Angeles-Boza, A. M., Antimicrobial Peptides and Copper (II) Ions: Novel Therapeutic Opportunities. *Chemical Reviews* **2021,** 121, (4), 2648-2712.

64.   Hayden, R. M.; Goldberg, G. K.; Ferguson, B. M.; Schoeneck, M. W.; Libardo, M. D. J.; Mayeux, S. E.; Shrestha, A.; Bogardus, K. A.; Hammer, J.; Pryshchep, S., Complementary effects of host defense peptides piscidin 1 and piscidin 3 on DNA and

lipid membranes: biophysical insights into contrasting biological activities. *The journal of physical chemistry B* **2015,** 119, (49), 15235-15246.

65. Mullane, K. M.; Gorbach, S., Fidaxomicin: first-in-class macrocyclic antibiotic. *Expert Review of Anti-Infective Therapy* **2011,** 9, (7), 767-777.

66. Brauer, M.; Herrmann, J.; Zühlke, D.; Müller, R.; Riedel, K.; Sievers, S., Myxopyronin B inhibits growth of a Fidaxomicin-resistant *Clostridioides difficile* isolate and interferes with toxin synthesis. *Gut Pathogens* **2022,** 14, (1), 1-11.

67. Cao, X.; Boyaci, H.; Chen, J.; Bao, Y.; Landick, R.; Campbell, E. A., Basis of narrow-spectrum activity of fidaxomicin on *Clostridioides difficile*. *Nature* **2022,** 604, (7906), 541-545.

68. Patel, O. P.; Jesumoroti, O. J.; Legoabe, L. J.; Beteck, R. M., Metronidazole-conjugates: A comprehensive review of recent developments towards synthesis and medicinal perspective. *European Journal of Medicinal Chemistry* **2021,** 210, 112994.

69. Guan, D.; Chen, F.; Xiong, L.; Tang, F.; Qiu, Y.; Zhang, N.; Gong, L.; Li, J.; Lan, L.; Huang, W., Extra sugar on vancomycin: new analogues for combating multidrug-resistant Staphylococcus aureus and vancomycin-resistant Enterococci. *Journal of Medicinal Chemistry* **2018,** 61, (1), 286-304.

70. Nelson, R. L.; Suda, K. J.; Evans, C. T., Antibiotic treatment for *Clostridium difficile*-associated diarrhea in adults. *Cochrane Database of Systematic Reviews* **2017,** (3).

71. Raza, T.; Ullah, S. R.; Mehmood, K.; Andleeb, S., Vancomycin resistant *Enterococci:* A brief review. *J Pak Med Assoc* **2018,** 68, (5), 768-772.

72. Vertzoni, M.; Augustijns, P.; Grimm, M.; Koziolek, M.; Lemmens, G.; Parrott, N.; Pentafragka, C.; Reppas, C.; Rubbens, J.; Van Den Abeele, J., Impact of regional differences along the gastrointestinal tract of healthy adults on oral drug absorption: An UNGAP review. *European Journal of Pharmaceutical Sciences* **2019,** 134, 153-175.

73. Kelly, J.; Daly, K.; Moran, A. W.; Ryan, S.; Bravo, D.; Shirazi-Beechey, S. P., Composition and diversity of mucosa-associated microbiota along the entire length of the pig gastrointestinal tract; dietary influences. *Environmental Microbiology* **2017,** 19, (4), 1425-1438.

74. Wexler, A. G.; Guiberson, E. R.; Beavers, W. N.; Shupe, J. A.; Washington, M. K.; Lacy, D. B.; Caprioli, R. M.; Spraggins, J. M.; Skaar, E. P., *Clostridioides difficile* infection induces a rapid influx of bile acids into the gut during colonization of the host. *Cell Reports* **2021,** 36, (10), 109683.

75. Zeitouni, N. E.; Chotikatum, S.; von Köckritz-Blickwede, M.; Naim, H. Y., The impact of hypoxia on intestinal epithelial cell functions: consequences for invasion by bacterial pathogens. *Molecular and Cellular Pediatrics* **2016,** 3, (1), 1-9.

76. Lawley, T. D.; Clare, S.; Walker, A. W.; Stares, M. D.; Connor, T. R.; Raisen, C.; Goulding, D.; Rad, R.; Schreiber, F.; Brandt, C., Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. *PLoS Pathogens* **2012,** 8, (10), e1002995.

77. Koziolek, M.; Alcaro, S.; Augustijns, P.; Basit, A. W.; Grimm, M.; Hens, B.; Hoad, C. L.; Jedamzik, P.; Madla, C. M.; Maliepaard, M., The mechanisms of pharmacokinetic food-drug interactions–A perspective from the UNGAP group. *European Journal of Pharmaceutical Sciences* **2019,** 134, 31-59.

78. Louie, T. J.; Miller, M. A.; Mullane, K. M.; Weiss, K.; Lentnek, A.; Golan, Y.; Gorbach, S.; Sears, P.; Shue, Y.-K., Fidaxomicin versus vancomycin for *Clostridium difficile* infection. *New England Journal of Medicine* **2011,** 364, (5), 422-431.

79. Rodriguez-Palacios, A.; LeJeune, J. T., Moist-heat resistance, spore aging, and superdormancy in *Clostridium difficile*. *Applied and Environmental Microbiology* **2011,** 77, (9), 3085-3091.

80. Sarker, M. R.; Paredes-Sabja, D., Molecular basis of early stages of *Clostridium difficile* infection: germination and colonization. *Future Microbiology* **2012,** 7, (8), 933-943.

81. Theriot, C. M.; Koenigsknecht, M. J.; Carlson, P. E.; Hatton, G. E.; Nelson, A. M.; Li, B.; Huffnagle, G. B.; Z Li, J.; Young, V. B., Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection. *Nature Communications* **2014,** 5, (1), 1-10.

82. Frädrich, C.; Beer, L.-A.; Gerhard, R., Reactive oxygen species as additional determinants for cytotoxicity of *Clostridium difficile* Toxins A and B. *Toxins* **2016,** 8, (1), 25.

83. Bahar, A. A.; Ren, D., Antimicrobial peptides. *Pharmaceuticals* **2013,** 6, (12), 1543-1575.

84. CM Wong, C.; Zhang, L.; X Ren, S.; Shen, J.; LY Chan, R.; H Cho, C., Antibacterial peptides and gastrointestinal diseases. *Current Pharmaceutical Design* **2011,** 17, (16), 1583-1586.

85. Nuding, S.; Frasch, T.; Schaller, M.; Stange, E. F.; Zabel, L. T., Synergistic effects of antimicrobial peptides and antibiotics against *Clostridium difficile*. *Antimicrobial Agents and Chemotherapy* **2014,** 58, (10), 5719-5725.

86. Chen, W.; Cotten, M. L., Expression, purification, and micelle reconstitution of antimicrobial piscidin 1 and piscidin 3 for NMR studies. *Protein Expression and Purification* **2014,** 102, 63-68.

87. Comert, F.; Greenwood, A.; Maramba, J.; Acevedo, R.; Lucas, L.; Kulasinghe, T.; Cairns, L. S.; Wen, Y.; Fu, R.; Hammer, J., The host-defense peptide piscidin P1 reorganizes lipid domains in membranes and decreases activation energies in mechanosensitive ion channels. *Journal of Biological Chemistry* **2019,** 294, (49), 18557-18570.

88. Kim, S. Y.; Zhang, F.; Gong, W.; Chen, K.; Xia, K.; Liu, F.; Gross, R.; Wang, J. M.; Linhardt, R. J.; Cotten, M. L., Copper regulates the interactions of antimicrobial piscidin peptides from fish mast cells with formyl peptide receptors and heparin. *Journal of Biological Chemistry* **2018,** 293, (40), 15381-15396.

89. Libardo, M. D. J.; Nagella, S.; Lugo, A.; Pierce, S.; Angeles-Boza, A. M., Copper-binding tripeptide motif increases potency of the antimicrobial peptide Anoplin via Reactive Oxygen Species generation. *Biochemical and Biophysical Research Communications* **2015,** 456, (1), 446-451.

90. Zhang, L.; Nie, X.; Ravcheev, D. A.; Rodionov, D. A.; Sheng, J.; Gu, Y.; Yang, S.; Jiang, W.; Yang, C., Redox-responsive repressor Rex modulates alcohol production and oxidative stress tolerance in *Clostridium acetobutylicum*. *Journal of Bacteriology* **2014,** 196, (22), 3949-3963.

91. McQuade, R.; Roxas, B.; Viswanathan, V.; Vedantam, G., *Clostridium difficile* clinical isolates exhibit variable susceptibility and proteome alterations upon exposure to mammalian cationic antimicrobial peptides. *Anaerobe* **2012,** 18, (6), 614-620.

92.   Corminboeuf, O.; Leroy, X., FPR2/ALXR agonists and the resolution of inflammation. *Journal of Medicinal Chemistry* **2015,** 58, (2), 537-559.

93.   Le, Y.; Murphy, P. M.; Wang, J. M., Formyl-peptide receptors revisited. *Trends in Immunology* **2002,** 23, (11), 541-548.

94.   Migeotte, I.; Communi, D.; Parmentier, M., Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. *Cytokine & Growth Factor Reviews* **2006,** 17, (6), 501-519.

95.   Pundir, P.; Catalli, A.; Leggiadro, C.; Douglas, S.; Kulka, M., Pleurocidin, a novel antimicrobial peptide, induces human mast cell activation through the FPRL1 receptor. *Mucosal Immunology* **2014,** 7, (1), 177-187.

96.   Park, Y. J.; Lee, S. K.; Jung, Y. S.; Lee, M.; Lee, H. Y.; Lee, H. Y.; Park, J. S.; Koo, J.; Koo, J.; Bae, Y.-S., Promotion of formyl peptide receptor 1-mediated neutrophil chemotactic migration by antimicrobial peptides isolated from the *centipede Scolopendra subspinipes mutilans*. *BMB Reports* **2016,** 49, (9), 520.

97.   Chen, W.-F.; Huang, S.-Y.; Liao, C.-Y.; Sung, C.-S.; Chen, J.-Y.; Wen, Z.-H., The use of the antimicrobial peptide piscidin (PCD)-1 as a novel anti-nociceptive agent. *Biomaterials* **2015,** 53, 1-11.

98.   Lee, E.; Shin, A.; Jeong, K.-W.; Jin, B.; Jnawali, H. N.; Shin, S.; Shin, S. Y.; Kim, Y., Role of phenylalanine and valine10 residues in the antimicrobial activity and cytotoxicity of piscidin-1. *PLoS One* **2014,** 9, (12), e114453.

99.   Hing, T. C.; Ho, S.; Shih, D. Q.; Ichikawa, R.; Cheng, M.; Chen, J.; Chen, X.; Law, I.; Najarian, R.; Kelly, C. P., The antimicrobial peptide cathelicidin modulates *Clostridium difficile*-associated colitis and toxin A-mediated enteritis in mice. *Gut* **2013,** 62, (9), 1295-1305.

100.   Carreau, A.; Hafny-Rahbi, B. E.; Matejuk, A.; Grillon, C.; Kieda, C., Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *Journal of Cellular and Molecular Medicine* **2011,** 15, (6), 1239-1253.

101.   Sorg, J. A.; Sonenshein, A. L., Bile salts and glycine as cogerminants for *Clostridium difficile* spores. *Journal of Bacteriology* **2008,** 190, (7), 2505-2512.

102.   Purcell, E. B.; McKee, R. W.; McBride, S. M.; Waters, C. M.; Tamayo, R., Cyclic diguanylate inversely regulates motility and aggregation in *Clostridium difficile*. *Journal of Bacteriology* **2012,** 194, (13), 3307-3316.

103.   Wiegand, I.; Hilpert, K.; Hancock, R. E., Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols* **2008,** 3, (2), 163-175.

104.   Courson, D. S.; Pokhrel, A.; Scott, C.; Madrill, M.; Rinehold, A. J.; Tamayo, R.; Cheney, R. E.; Purcell, E. B., Single cell analysis of nutrient regulation of Clostridioides (Clostridium) difficile motility. *Anaerobe* **2019,** 59, 205-211.

105.   Ghaedi, M.; Ahmadi, F.; Shokrollahi, A., Simultaneous preconcentration and determination of copper, nickel, cobalt and lead ions content by flame atomic absorption spectrometry. *Journal of Hazardous Materials* **2007,** 142, (1-2), 272-278.

106.   Stabler, R. A.; He, M.; Dawson, L.; Martin, M.; Valiente, E.; Corton, C.; Lawley, T. D.; Sebaihia, M.; Quail, M. A.; Rose, G., Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome Biology* **2009,** 10, (9), 1-15.

107. Lachowicz, D.; Pituch, H.; Obuch-Woszczatyński, P., Antimicrobial susceptibility patterns of *Clostridium difficile* strains belonging to different polymerase chain reaction ribotypes isolated in Poland in 2012. *Anaerobe* **2015,** 31, 37-41.

108. Wang, B.; Lv, Z.; Zhang, P.; Su, J., Molecular epidemiology and antimicrobial susceptibility of human *Clostridium difficile* isolates from a single institution in Northern China. *Medicine* **2018,** 97, (25).

109. Buckley, A. M.; Jukes, C.; Candlish, D.; Irvine, J. J.; Spencer, J.; Fagan, R. P.; Roe, A. J.; Christie, J. M.; Fairweather, N. F.; Douce, G. R., Lighting up *Clostridium difficile*: reporting gene expression using fluorescent Lov domains. *Scientific Reports* **2016,** 6, (1), 1-11.

110. Fernández-Mazarrasa, C.; Mazarrasa, O.; Calvo, J.; del Arco, A.; Martínez-Martínez, L., High concentrations of manganese in Mueller-Hinton agar increase MICs of tigecycline determined by Etest. *Journal of Clinical Microbiology* **2009,** 47, (3), 827-829.

111. Manteca, A.; Alvarez, R.; Salazar, N.; Yagüe, P.; Sanchez, J., Mycelium differentiation and antibiotic production in submerged cultures of Streptomyces coelicolor. *Applied and Environmental Microbiology* **2008,** 74, (12), 3877-3886.

112. Poole, K., Bacterial stress responses as determinants of antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* **2012,** 67, (9), 2069-2089.

113. Miller, C.; Thomsen, L. E.; Gaggero, C.; Mosseri, R.; Ingmer, H.; Cohen, S. N., SOS response induction by ß-lactams and bacterial defense against antibiotic lethality. *Science* **2004,** 305, (5690), 1629-1631.

114. Mooyottu, S.; Kollanoor-Johny, A.; Flock, G.; Bouillaut, L.; Upadhyay, A.; Sonenshein, A. L.; Venkitanarayanan, K., Carvacrol and trans-Cinnamaldehyde Reduce *Clostridium difficile* Toxin Production and Cytotoxicity *in vitro*. *International Journal of Molecular Sciences* **2014,** 15, (3), 4415-4430.

115. Doughari, J.; Kachalla, N.; Jaafaru, M., Prevalence of *Clostridium difficile* in Hospital Environment within Yola Adamawa State Nigeria. *Microbiol Infect Dis* **2021,** 5, (2), 1-5.

116. Varela-Aramburu, S.; Ghosh, C.; Goerdeler, F.; Priegue, P.; Moscovitz, O.; Seeberger, P. H., Targeting and inhibiting Plasmodium falciparum using ultra-small gold nanoparticles. *ACS applied materials & interfaces* **2020,** 12, (39), 43380-43387.

117. Bressuire-Isoard, C.; Broussolle, V.; Carlin, F., Sporulation environment influences spore properties in Bacillus: evidence and insights on underlying molecular and physiological mechanisms. *FEMS Microbiology Reviews* **2018,** 42, (5), 614-626.

118. Reeves, A. B. G.; Trogdon, J. G.; Stearns, S. C.; Lewis, J. W.; Weber, D. J.; Weinberger, M., Are Rates of Methicillin-Resistant *Staphylococcus aureus* and *Clostridioides difficile* Associated With Quality and Clinical Outcomes in US Acute Care Hospitals? *American Journal of Medical Quality* **2021,** 36, (2), 90-98.

119. Martins, D.; Mendes, A. L.; Antunes, J.; Henriques, A. O.; Serrano, M., A regulatory protein that represses sporulation in *Clostridioides difficile*. *bioRxiv* **2020**.

120. Pelaez, T.; Alcala, L.; Alonso, R.; Rodriguez-Creixems, M.; Garcia-Lechuz, J.; Bouza, E., Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. *Antimicrobial agents and chemotherapy* **2002,** 46, (6), 1647-1650.

121. Khanna, S.; Pardi, D. S.; Kelly, C. R.; Kraft, C. S.; Dhere, T.; Henn, M. R.; Lombardo, M.-J.; Vulic, M.; Ohsumi, T.; Winkler, J., A novel microbiome therapeutic increases gut microbial diversity and prevents recurrent *Clostridium difficile* infection. *The Journal of infectious diseases* **2016,** 214, (2), 173-181.

122. Schwanbeck, J.; Riedel, T.; Laukien, F.; Schober, I.; Oehmig, I.; Zimmermann, O.; Overmann, J.; Groß, U.; Zautner, A. E.; Bohne, W., Characterization of a clinical *Clostridioides difficile* isolate with markedly reduced fidaxomicin susceptibility and a V1143D mutation in rpoB. *Journal of Antimicrobial Chemotherapy* **2019,** 74, (1), 6-10.

123. Oludiran, A.; Courson, D. S.; Stuart, M. D.; Radwan, A. R.; Poutsma, J. C.; Cotten, M. L.; Purcell, E. B., How oxygen availability affects the antimicrobial efficacy of host defense peptides: lessons learned from studying the copper-binding peptides piscidins 1 and 3. *International Journal of Molecular Sciences* **2019,** 20, (21), 5289.

124. Maiti, B. K., Potential role of peptide-based antiviral therapy against SARS-CoV-2 infection. *ACS pharmacology & translational science* **2020,** 3, (4), 783-785.

125. Pokhrel, A.; Poudel, A.; Castro, K. B.; Celestine, M. J.; Oludiran, A.; Rinehold, A. J.; Resek, A. M.; Mhanna, M. A.; Purcell, E. B., The (p) ppGpp synthetase RSH mediates stationary-phase onset and antibiotic stress survival in *Clostridioides difficile*. *Journal of Bacteriology* **2020,** 202, (19), e00377-20.

126. Poudel, A.; Pokhrel, A.; Oludiran, A.; Coronado, E. J.; Alleyne, K.; Gilfus, M. M.; Gurung, R. K.; Adhikari, S. B.; Purcell, E. B., Unique features of magic spot metabolism in *Clostridioides difficile*. *bioRxiv* **2021**.

127. Poudel, A.; Pokhrel, A.; Oludiran, A.; Coronado, E. J.; Alleyne, K.; Gilfus, M. M.; Gurung, R. K.; Adhikari, S. B.; Purcell, E. B., Unique Features of Alarmone Metabolism in *Clostridioides difficile*. *Journal of Bacteriology* **2022,** 204, (4), e00575-21.

128. Oludiran, A. C., D.S.; Stuart, M.D.; Radwan, A.R.; Poutsma, J.C.; Cotten, M.L.; Purcell, E.B., How Oxygen Availability Affects the Antimicrobial Efficacy of Host Defense Peptides: Lessons Learned from Studying the Copper-Binding Peptides Piscidins 1 and 3. *Int. J. Mol. Sci.* **2019,** 20, (20), 5289.

129. Mooyottu, S.; Flock, G.; Venkitanarayanan, K., Carvacrol reduces *Clostridium difficile* sporulation and spore outgrowth *in vitro*. *Journal of Medical Microbiology* **2017,** 66, (8), 1229-1234.

130. Poudel, A.; Oludiran, A.; Sözer, E. B.; Casciola, M.; Purcell, E. B.; Muratori, C., Growth in a biofilm sensitizes *Cutibacterium acnes* to nanosecond pulsed electric fields. *Bioelectrochemistry* **2021,** 140, 107797.

131. Hindi, N. K. K.; Alsaadi, Z. H.; Abbas, A. F.; Al-Saadi, A. G. M., The emergence of multidrug-resistant and hypervirulent *Clostridium difficile* clinical isolates. *Meta Gene* **2020,** 24, 100644.

132. Garneau, J. R.; Valiquette, L.; Fortier, L.-C., Prevention of *Clostridium difficile*spore formation by sub-inhibitory concentrations of tigecycline and piperacillin/tazobactam. *BMC Infectious Diseases* **2014,** 14, (1), 1-10.

133. Ochsner, U. A.; Bell, S. J.; O'Leary, A. L.; Hoang, T.; Stone, K. C.; Young, C. L.; Critchley, I. A.; Janjic, N., Inhibitory effect of REP3123 on toxin and spore formation in *Clostridium difficile*, and in vivo efficacy in a hamster gastrointestinal infection model. *Journal of Antimicrobial Chemotherapy* **2009,** 63, (5), 964-971.

134. Lawley, T. D.; Clare, S.; Walker, A. W.; Goulding, D.; Stabler, R. A.; Croucher, N.; Mastroeni, P.; Scott, P.; Raisen, C.; Mottram, L., Antibiotic treatment of *Clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infection and Immunity* **2009,** 77, (9), 3661-3669.

135. Paredes, S. D.; Kim, S.; Rooney, M. T.; Greenwood, A. I.; Hristova, K.; Cotten, M. L., Enhancing the membrane activity of piscidin 1 through peptide metallation and the presence of oxidized lipid species: implications for the unification of host defense mechanisms at lipid membranes. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **2020,** 1862, (7), 183236.

136. Dubois, T.; Tremblay, Y. D.; Hamiot, A.; Martin-Verstraete, I.; Deschamps, J.; Monot, M.; Briandet, R.; Dupuy, B., A microbiota-generated bile salt induces biofilm formation in *Clostridium difficile*. *NPJ Biofilms and Microbiomes* **2019,** 5, (1), 1-12.

137. Meza-Torres, J.; Auria, E.; Dupuy, B.; Tremblay, Y. D., Wolf in Sheep's Clothing: *Clostridioides difficile* Biofilm as a Reservoir for Recurrent Infections. *Microorganisms* **2021,** 9, (9), 1922.

138. Gambino, M.; Cappitelli, F., Mini-review: Biofilm responses to oxidative stress. *Biofouling* **2016,** 32, (2), 167-178.

139. Pantaléon, V.; Soavelomandroso, A. P.; Bouttier, S.; Briandet, R.; Roxas, B.; Chu, M.; Collignon, A.; Janoir, C.; Vedantam, G.; Candela, T., The *Clostridium difficile* protease Cwp84 modulates both biofilm formation and cell-surface properties. *PloS One* **2015,** 10, (4), e0124971.

140. Ewald, C. Y.; Hourihan, J. M.; Blackwell, T. K., Oxidative Stress Assays (arsenite and tBHP) in Caenorhabditis elegans. *Bio-Protocol* **2017,** 7, (13).

141. Beier, N.; Kucklick, M.; Fuchs, S.; Mustafayeva, A.; Behringer, M.; Härtig, E.; Jahn, D.; Engelmann, S., Adaptation of Dinoroseobacter shibae to oxidative stress and the specific role of RirA. *PloS One* **2021,** 16, (3), e0248865.

142. Murdoch, C. C.; Skaar, E. P., Nutritional immunity: the battle for nutrient metals at the host–pathogen interface. *Nature Reviews Microbiology* **2022**, 1-14.

143. Rtimi, S.; Dionysiou, D. D.; Pillai, S. C.; Kiwi, J., Advances in catalytic/photocatalytic bacterial inactivation by nano Ag and Cu coated surfaces and medical devices. *Applied Catalysis B: Environmental* **2019,** 240, 291-318.

APPENDIX A

## INHIBITORY CONCENTRATION DETERMINATION

a

b

c
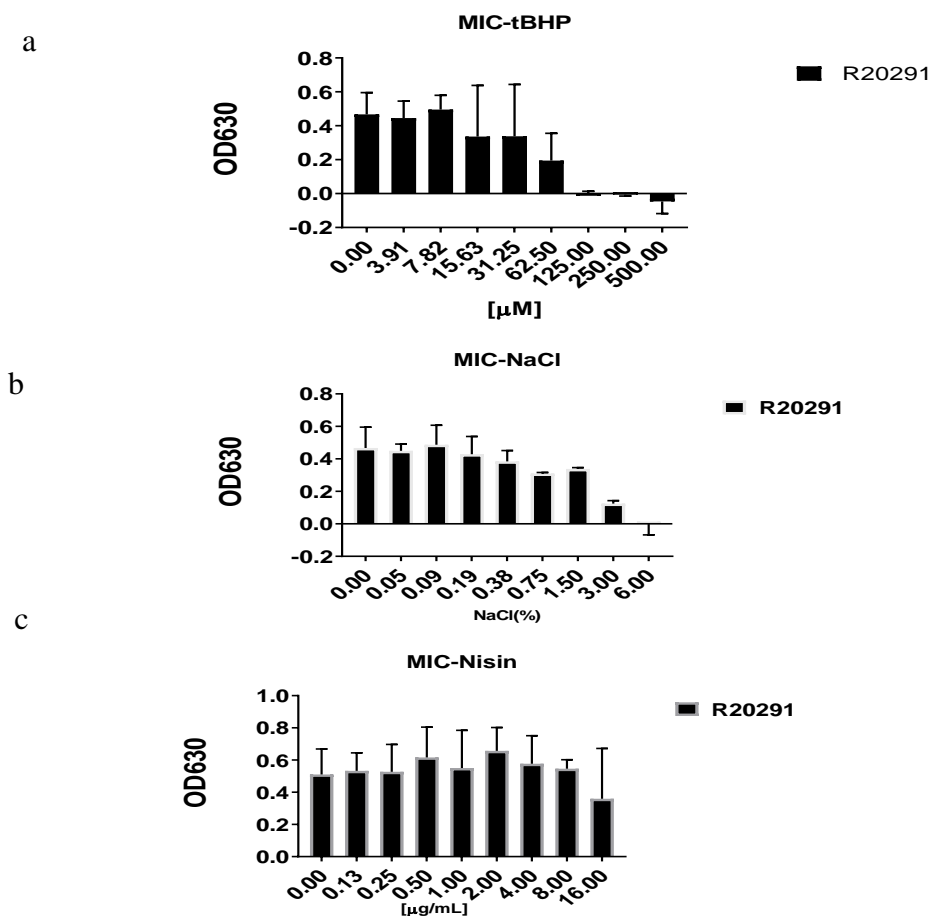
Inhibitory concentration determination. *C. difficile* R20291 cell density after overnight growth in BHIS medium having the indicated concentrations of the treatments. Log phase cells of R20291 were inoculated at 1:10 into fresh BHIS media treated with gradient concentrations. Cells were incubated anaerobically at 37°C to check cells' turbidity at 630nm. (**a**) tBHP inhibitory concentrations (**b**) NaCl inhibition concentrations and (**c**) Nisin inhibitory concentration determination. Shown are the means and standard deviations of at least three biologically independent samples.

APPENDIX B

DIAMIDE INHIBITORY CONCENTRATION DETERMINATION
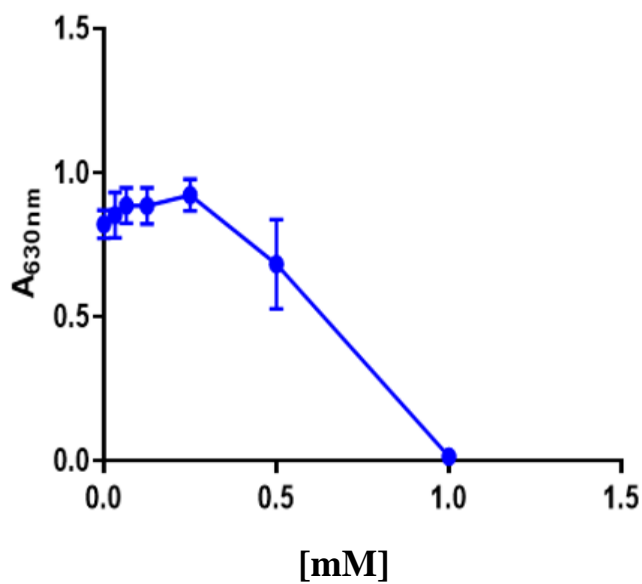
Diamide inhibitory concentration determination. Log phase cells of R20291 were inoculated at 1:10 into fresh BHIS media treated with gradient concentrations of diamide and samples were incubated anaerobically at 37°C to check turbidity at 630nm. Shown are the means and standard deviations of at least three biologically independent samples.

APPENDIX C

C. DIFFICILE **SPORES AT 0.5x ANTIBIOTIC CONCENTRATIONS**

a

b
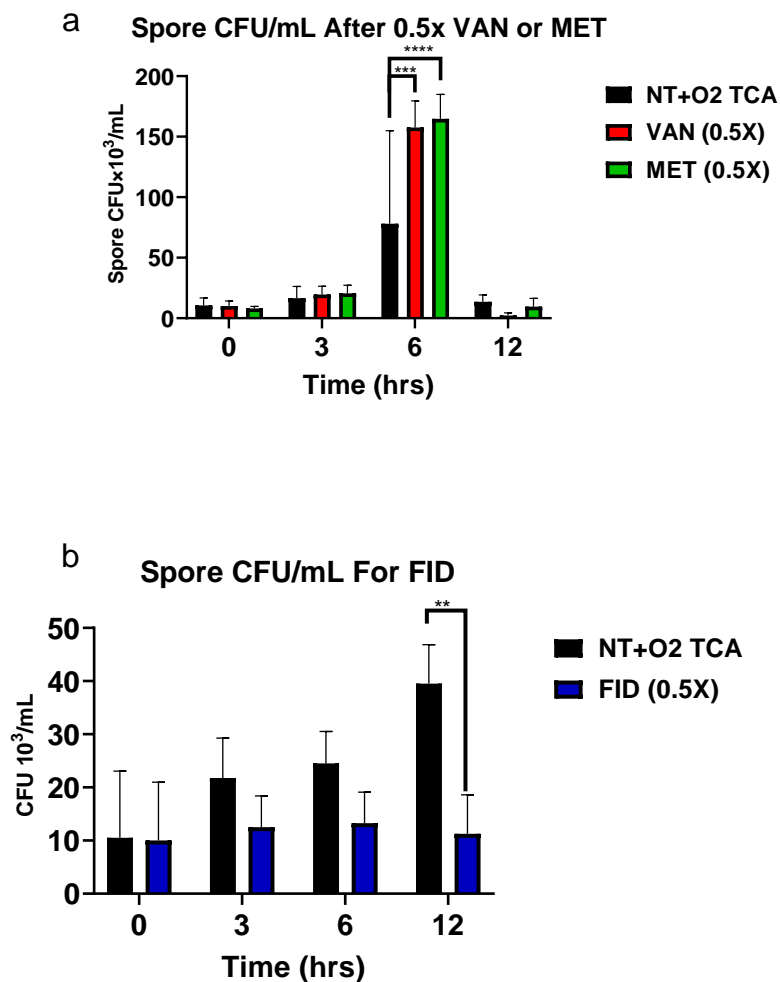
C. difficile spores at 0.5x antibiotic concentrations. Late log phase cells of R20291 were inoculated with antibiotics at one-half lethal dosages. (a) shows the plot of MET and VAN spore count against time (b) FID spore count against time. Samples were treated aerobically at room temperature with ethanol to make 50% the sample volume and left for 1h with occasional mixing in order to kill all vegetative *C. difficile* before plating on 0.1%TCA+ 20µg/mL erythromycin plates and incubated anaerobically at 37°C.

APPENDIX D

C. *DIFFICILE* STRAINS USED IN THIS WORK

| *Clostridioides difficile*	
630Δ*erm*	Derivative of strain 630 lacking the erythromycin resistance gene *ermB*
R20291	Epidemic strain

APPENDIX E

C. DIFFICILE GROWTH IN THE PRESENCE OF METAL
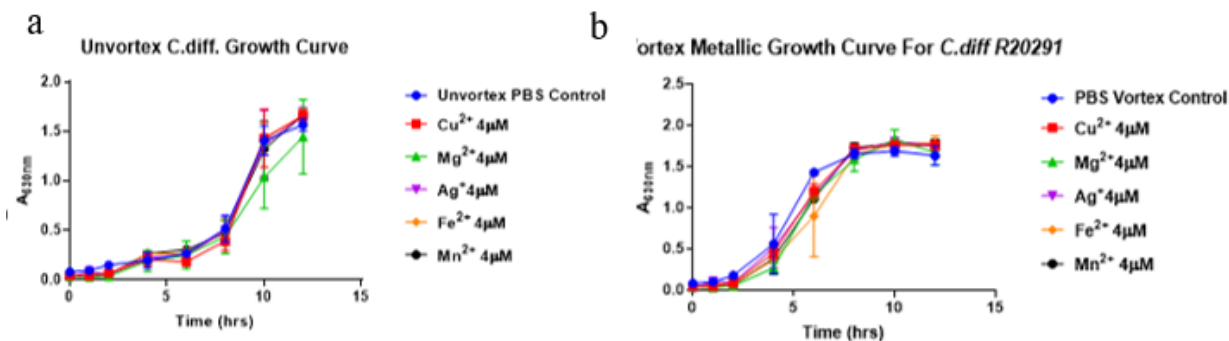
C. difficile growth in the presence of metals. R20291 growth for in the presence of the indicated concentrations of metal. Log phase cells of R20291 were inoculated at 1:10 into fresh BHIS media treated with indicted concentration metals. Cells were incubated anaerobically at 37°C to check cells' turbidity every 1hr before and after mixing. (**a**) OD at 600nm unvortex samples and (**b**) is the OD 600nm vortex samples. Shown are the means and standard deviations of at least three biologically independent samples [45].

APPENDIX F

MODIFICATION OF PRIMARY BILE ACIDS
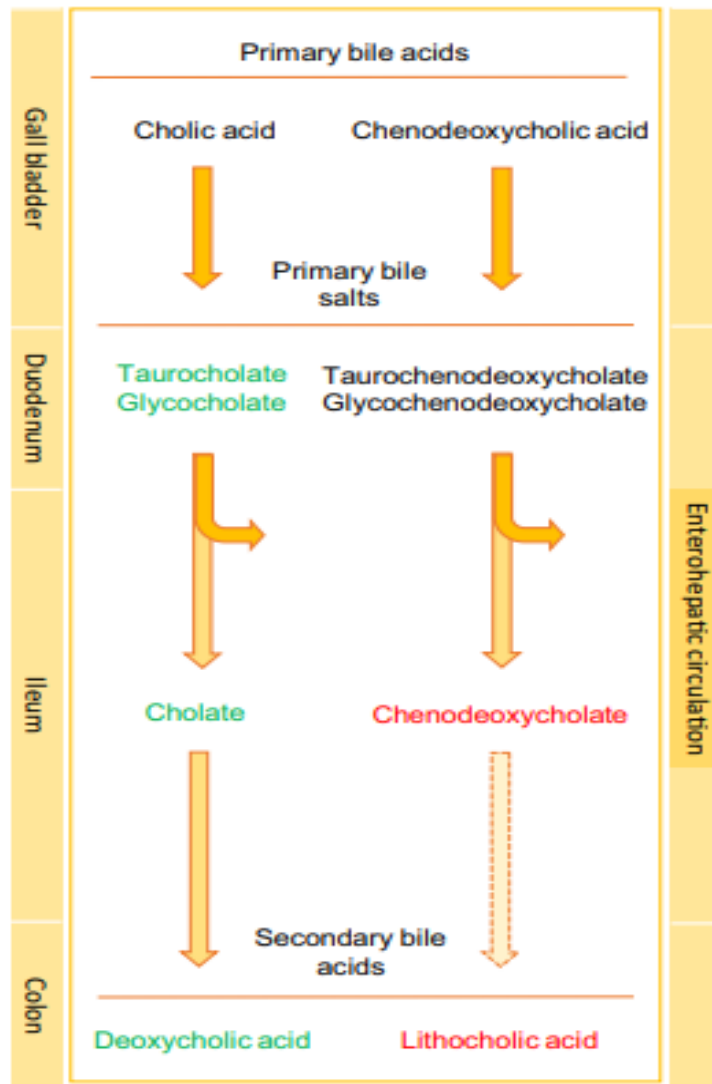
Modification of primary bile acids. The schematic diagram illustrate the conversion of bile acids from the primary state to secondary form in the GI tract making use of enterohepatic circulation [45].

APPENDIX G

RIGHTS AND PERMISSION

International Journal of
Molecular Sciences

MDPI

To whom it may concern,

This is to inform that the article titled «**How Oxygen Availability Affects the Antimicrobial Efficacy of Host Defense Peptides: Lessons Learned from Studying the Copper-Binding Peptides Piscidins 1 and 3**» in the *International Journal of Molecular Sciences* and that can be found under Int. J. Mol. Sci. 2019, 20(21), 5289; https://doi.org/10.3390/ijms20215289 was published under a Creative Commons License CC-BY which grants permission to any person to reproduce, share or reprint this work totally or partially should it be cited properly as follows:

MDPI and ACS Style

Oludiran, A.; Courson, D.S.; Stuart, M.D.; Radwan, A.R.; Poutsma, J.C.; Cotten, M.L.; Purcell, E.B. How Oxygen Availability Affects the Antimicrobial Efficacy of Host Defense Peptides: Lessons Learned from Studying the Copper-Binding Peptides Piscidins 1 and 3. *Int. J. Mol. Sci.* **2019**, *20*, 5289. https://doi.org/10.3390/ijms20215289

AMA Style

Oludiran A, Courson DS, Stuart MD, Radwan AR, Poutsma JC, Cotten ML, Purcell EB. How Oxygen Availability Affects the Antimicrobial Efficacy of Host Defense Peptides: Lessons Learned from Studying the Copper-Binding Peptides Piscidins 1 and 3. *International Journal of Molecular Sciences*. 2019; 20(21):5289. https://doi.org/10.3390/ijms20215289

Chicago/Turabian Style

Oludiran, Adenrele, David S. Courson, Malia D. Stuart, Anwar R. Radwan, John C. Poutsma, Myriam L. Cotten, and Erin B. Purcell. 2019. "How Oxygen Availability Affects the Antimicrobial Efficacy of Host Defense Peptides: Lessons Learned from Studying the Copper-Binding Peptides Piscidins 1 and 3" *International Journal of Molecular Sciences* 20, no. 21: 5289. https://doi.org/10.3390/ijms20215289

————————Jesús García Cano, PHD.
Publishing Manager

VITA
Adenrele M. Oludiran
Department of Chemistry and Biochemistry
Old Dominion University
Norfolk, VA, 23529

Education

BSc. (December 2011) in Biochemistry, Olabisi Onabanjo University, Nigeria

M.S. (August 2018) in Chemistry and Biochemistry, Old Dominion University, Norfolk, VA

Ph.D. (Expected August 2022) in Chemistry and Biochemistry, Old Dominion University,

Norfolk, VA

Selected Presentations

A Oludiran, & EB Purcell. Using Antimicrobial Peptides Against *Clostridium difficile*
Pathogen. EVMS Graduate Research Conference 2018, EVMS, Norfolk VA

A Oludiran, M. Cotten, & EB Purcell. Piscidins and Copper Complexes Against Anaerobic
Clostridioides difficile Bacteria. Graduate Symposium College of Williams & Mary VA 2020

A Oludiran, M. Cotten, & EB Purcell. *Clostridioides difficile* Biofilm and Spore Production in
Response to Antibiotic and Immune Stress Mid Atlantic Microbial Pathogenesis Meeting,
Wintergreen, VA February 2022, and Biotech (Pfizer) April 2022.

Selected Publications

Oludiran, A. M. (2018). Characterizing the Activity of Antimicrobial Peptides Against the
Pathogenic Bacterium *Clostridium difficile* in an Anaerobic Environment. Old Dominion
University.

Oludiran, A., Courson, D. S., Stuart, M. D., Radwan, A. R., Poutsma, J. C., Cotten, M. L., &
Purcell, E. B. (2019). How oxygen availability affects the antimicrobial efficacy of host defense
peptides: lessons learned from studying the copper-binding peptides piscidins 1 and 3.
International journal of molecular sciences, 20(21), 5289.

Pokhrel, A., Poudel, A., Castro, K. B., Celestine, M. J., **Oludiran, A**., Rinehold, A. J., ... &
Purcell, E. B. (2020). The (p) ppGpp synthetase RSH mediates stationary-phase onset and
antibiotic stress survival in *Clostridioides difficile*. *Journal of Bacteriology*, 202(19), e00377-20.

Poudel, A., **Oludiran, A.,** Sözer, E. B., Casciola, M., Purcell, E. B., & Muratori, C. (2021).
Growth in a biofilm sensitizes *Cutibacterium acnes* to nanosecond pulsed electric fields.
Bioelectrochemistry, 140, 107797.