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CHARACTERIZING THE ACTIVITY OF ANTIMICROBIAL PEPTIDES AGAINST THE PATHOGENIC BACTERIUM *CLOSTRIDIUM DIFFICILE* IN AN ANAEROBIC ENVIRONMENT

by

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

MASTER OF SCIENCE

CHEMISTRY

OLD DOMINION UNIVERSITY
August 2018

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ABSTRACT

CHARACTERIZING THE ACTIVITY OF ANTIMICROBIAL PEPTIDES AGAINST THE PATHOGENIC BACTERIUM *CLOSTRIDIUM DIFFICILE* IN ANAEROBIC ENVIRONMENT

Adenrele Mojeed Oludiran
Old Dominion University, 2018
Director: Dr. Purcell Erin

Clostridium difficile is an anaerobic Gram-positive pathogen with high treatment costs and mortality and very high antibiotic tolerance. Antimicrobial host-defense peptides (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.

Piscidins, fish-derived HDPs that can also form complexes with copper (Cu) to enhance their activities, are very active against multiple bacterial species in an aerobic environment. We examined their activity against *C. difficile* and other species in an anaerobic environment and found that the interaction of piscidins and copper is different in different oxygen environments. Piscidins are highly active against *C. difficile* and could be a good candidate for drug development.

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This thesis is dedicated first to Almighty God because that’s where all my strength come from, it is dedicated to my parents they both really sacrificed a lot for me and my siblings for us to have education. The dedication also extends to my brothers, my son and my queen. Finally, to all the children struggling to get education, now on the street of Mushin Lagos state Nigeria. Keep on fighting one day you shall overcome.

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My first appreciation to my parents for a lot I cannot completely account for here, I would like to thank my brother for his strong support and the rest of my brothers. I would also like to appreciate the department of chemistry and biochemistry Old Dominion University. Special appreciation to Dr. Erin Purcell and Dr. David Courson for the positive contribution they both have made to the success of my education in United States of America. I really appreciate my committee who have helped in my research thank you all. I would like to also appreciate my colleagues in the lab for their constant helping hands may God reward you all accordingly. Thank you to my peers at Old Dominion University and my family back home for your undying support. I really learn a lot in all the classes in chemistry and biochemistry department thank you all to my Professors. To all my students in Lagos and children on the street of Mushin Lagos state do not stop dreaming the day you stop dreaming is the day your success goes to the lands of the silence and thank you all for the prayers. To my son and my queen thanks for the prayers and patient.

Table of Contents

Page

LIST OF TABLES viii

LIST OF FIGURES ix

1. INTRODUCTION 1

2. LITERATURE REVIEW 6

2.1 INNATE IMMUNE SYSTEM AND OXIDATIVE STRESS 6

2.2 METAL ION INDUCED OXIDATIVE STRESS 7

2.3 HOST-DEFENSE PEPTIDES (HDPs) INDUCED OXIDATIVE STRESS 8

2.4 PISCIDINS AND THEIR METAL COMPLEXES 9

2.5 OXIDATIVE STRESS RESPONSE IN BACTERIA 10

2.6 BIOFILM AS STRESS RESPONSE 11

2.7 OXIDATIVE STRESS RESPONSE IN BACTERIA 11

2.8 OXIDATIVE STRESS RESPONSE IN AEROBIC BACTERIA 12

2.9 OBLIGATE ANAEROBE 14

2.10 *C. DIFFICILE* VEGETATIVE CELLS AND PATHOGENESIS 15

2.11 *C. DIFFICILE* AND ANTIMICROBIAL AGENTS 17

2. 12 OXIDATIVE STRESS RESPONSE IN *C. DIFFICILE* 18

3. RESEACH OBJECTIVES 20

4. EXPERIMENNTAL PROCEDURE 21

4.1 MATERIAL AND SAMPLES 21

4.2 INSTRUMENTATIONS 21

4.3 MINIMAL INHIBITORY CONCENTRATIONS (MIC) 22

4.4 MIC FOR P1 AND P3 22

4.5 MIC FOR Cu^{2+} CONCENTRATIONS 22

4.6 BIOFILM ASSAY 23

4.7 P1 AND P3 BIOFILM ASSAY 23

4.8 PISCIDINS AND THEIR COMPLEXES BIOFILM ASSAY 23
4.9 Cu^{2+} BIOFILM ASSAY 24
4.10 TIME KILL ASSAY 24

5. RESULTS AND DISCUSSIONS 25
5.1 MINIMAL INHIBITORY CONCENTRATIONS (MIC) 25
5.2 MIC FOR 630Δerm AND R20291 25
5.3 MIC FOR R20291 USING DIFFERENT COPPER SALTS 27
5.4 TIME KILL ASSAY 28
5.5 BIOFILM INHIBITORY ASSAY 37
5.6 BIOFILM METALLIC STIMULATORY EFFECT 40

6. CONCLUSIONS 47

REFERENCES 49

VITA 58

LIST OF TABLES

Table Page

1. Distribution of antimicrobials tested 110 toxigenic *C. difficile* isolates……..…

2. The data analysis of p1 and p1+Cu two ways ANOVA…..…………………………30

3. The p3 and p3+Cu time kill data analysis by two ways ANOVA..…………………31

4. The data comparison of lab mixed peptides complex (p1+Cu) and pre-complex peptides (p1Cu)…………………………………...…………………33

5. The data comparison of lab mixed peptides complex (p3+Cu) and pre-complex peptides (p3Cu)…………………………………………………...35

6. The data analyses of peptides and their complexes with copper salt alone……….....39

7. Copper salt alone biofilm data formation analyses…………………………………..42

8. The data analyses of gradient concentrations of copper(II) salts on RT527 *C. difficile* strain………………………………..………………………………44

9. The data analyses of different copper salt biofilm formation…………..……………46

LIST OF FIGURES

Figure Page

1. The longitudinal section of Clostridium difficile bacterium……………..…….……...1

2. The amino acid residues conserved in p1 & p3………………………...…..................3

3. PDB CODE: 2MCX. The α-Helix structure of piscidin-3 found by solid-state NMR..........3

4. The illustration of the biological roles of HDPs by innate immune modulation peptides direct killings and intracellular target killings .………………...9

5. The life cycle of *Clostridium difficile*…………………………………………….…14

6. Pathogenesis of *Clostridium difficile* Infection……………………………………...16

7. The MICs for p1 & p3 against both strains of *C. difficile 630Δerm & R20291* through measure of cell density after 24 hours....................………….…….26

8. The copper concentration gradient for three copper(II) salts inhibitory effect against the virulent strain of *C. difficile*…..……………………...………..27

9. The time kill curve for p1 and its copper complex lab mixture………….…………....29

10. The time kill curve for p3 and its copper complex for lab mixture………..……..…30

11. Comparative data curve for p1+Cu and p1Cu complexes………………...………....33

12. Comparative data plot for p3+Cu & p3Cu complexes……………..……………..….34

13. The time kill curve Cu^{2+} at 2uM & 4uM………..…………………………….……36

14. Copper concentration/µM curve in media as determined by FAAS in three different media ……………………...………………………………….…36

15. The amount of biofilm treated and the untreated control……………………………38

16. The copper salt (Cu^{2+}) alone concentration gradient for biofilm quantitative analysis of *R20291 C. difficile* strain ...………………………………..41

17. The copper(II) salt (Cu^{2+}) biofilm quantitative for *RT527 C. difficile* strain…..…..43

18. The different Cu^{2+} biofilm quantitative effect curve……………..…………………45

CHAPTER 1

INTRODUCTION

Combating harmful bacterial infection is a complex process that requires a sophisticated and diligent approach, especially when it involves *Clostridium difficile* bacteria (Figure 1). *Clostridium difficile* is a Gram-positive anaerobic spore producing bacterium, found in the intestine of many animals and humans. Imbalance to the commensal microbiota due to antibiotic treatment, is the major risk factor in *Clostridium difficile* infection (CDI) after exposure to *C. difficile* spores (1). The longitudinal section of *C. difficile* bacterium is shown in Figure 1

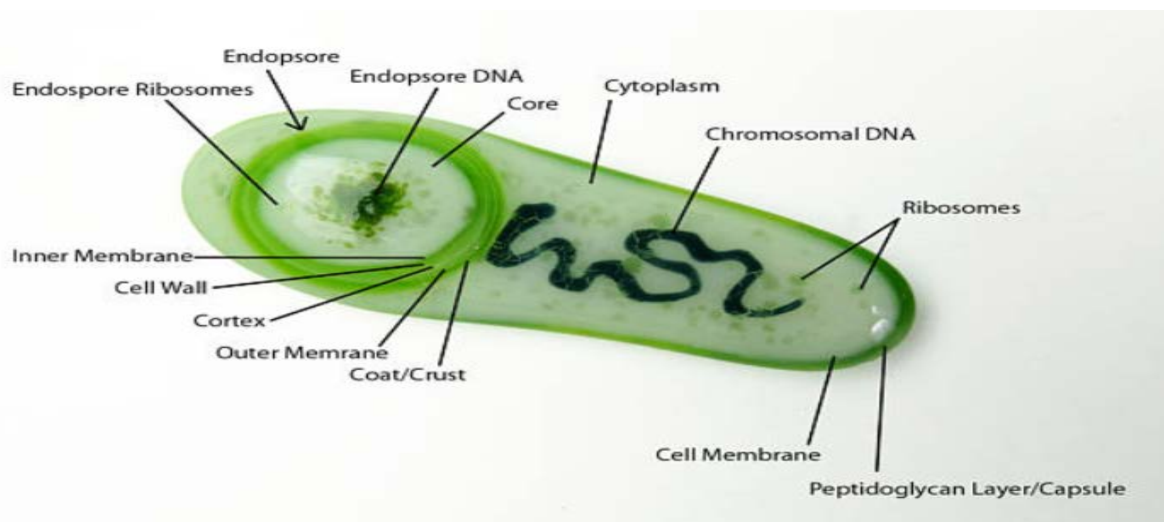


Figure 1: The longitudinal section of Clostridium difficile bacterium. Shown are the vegetative cell, the peptidoglycan capsule which absorbs Gram stain, and daughter which has differentiated into spore. Image adapted from Jane Hartman (trilobiteglasswork) Etsy.com

The *Clostridium difficile* life cycle has two main stages. Metabolically dormant *C. difficile* spores persist in the environment and germinate if and only if they are ingested by a susceptible host (36). *Clostridium difficile infection* (CDI) is a consequence of exposure to *C. difficile* spores which germinate in the digestive tract to form the vegetative cells. Vegetative cells are the replicative form that produced the toxin, causes disease symptoms and can germinate into new spores to spread the infection into new host (33). *C. difficile* toxins disrupt host cell-cell junctions in the epithelium, which results in CDI symptoms, including fever, diarrhea, stomach pain, nausea, enlargement of colon and death in humans (2). CDI is associated with more than 20% of hospital-acquired diarrhea cases in United State (3). It costs more than $5billion yearly to treat and up to 45,000 lives are lost annually in United States alone (3).

CDI is resistant to multiple classes of antibiotics and has a 15 – 26% recurrence rate after treatment (4-6). When humans are exposed to pathogens the first line of defense is the innate immune system, which releases reactive oxygen species (ROS) and cationic antimicrobial host defense peptides (HDPs) to kill foreign cells. These antimicrobial peptides can kill bacterial cells through a variety of mechanisms, attacking the cell membrane and/or intracellular targets (37-39). More than 2700 of these peptides has been discovered in different families but, this research is focused on the specific family called piscidins (18). Piscidins are derived from fish (hybrid striped bass) mast cells and play very significant role in antimicrobial defense in the fish innate immune system and are occasionally part of adaptive immune responses as well (25), piscidin-1 (p1) and piscidin-3 (p3) are the two antimicrobial peptides which play important role in this study.

These two peptides are intrinsically disordered in solution and contain a high number of hydrophobic amino acids (26). They differ in their antimicrobial mechanisms with p1 targeting the bacterial membrane and p3 showing a strong intracellular nuclease activity (26 & 27).

The piscidins each contain 22 amino acids residues, of which about 60% are hydrophobic in nature, and conserved amino terminal regions. Figure 2 showed conserved region and conserved amino acid residue in these peptides p1 (PDB ID 2OJM) and p3 (PDB ID 2MCX) respectively.

```
2MCX:A|PDBID|CHAIN|SEQUENCE        FIHHIFRGIVHAGRSIGRFLTGX
2OJM:A|PDBID|CHAIN|SEQUENCE        FFHHIFRGIVHVGKTIHRLVTG-
                                   *:*********.*.:*  *::**
```

Figure 2: The amino acid residues conserved in p1 & p3. The three underlined N-terminal amino acid residues represent the ATCUN region for binding of Cu^{2+} and Ni^{2+} respectively.

This conserved region can bind some transition metals (like copper(II) and nickel(II)) and is called the amino terminal copper and nickel binding (ATCUN). The ATCUN regions for p1 and p3 are FHH and FIH amino acid residues respectively. The two piscidins fold into alpha helical secondary structure but are inherently stable (28). The piscidin-3 peptide structure is shown in the Figure 3 below.

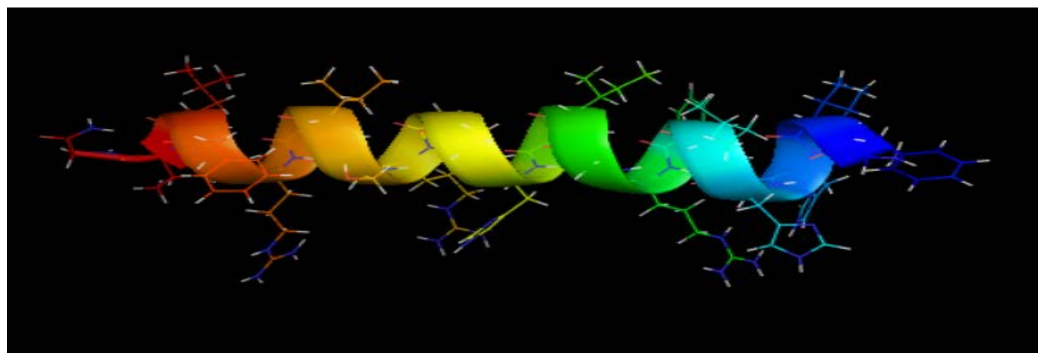


Figure 3: PDB CODE: 2MCX. The α-Helix structure of piscidin-3 found by solid-state NMR. Figure adapted from pymol protein structure software.

The transition metals copper has well-known antimicrobial metallic properties. It has bactericidal effect in its ground state (Cu) and in multiple ionic state, most commonly Cu(I) and Cu(II) (29 & 30). The capability of this transition metal to act as an antimicrobial agent alone or coordinate to other ligands to form different complexes are exploited by many scientific studies (29). Recently, Cu and its complexes are used in water purification and hospital waste management.

Today, much scientific research support copper been algaecide, fungicide, nematicide, antimicrobial and antifouling agent respectively (29 & 31).

In the +2 oxidation states (Cu(II) [Ar] $4s^0$ $3d^9$), copper can coordinate up to six bonds resulting into an octahedral shape, which is very useful in the formation of many complexes. The p1 and p3 ATCUN motifs have been shown to bind Cu^{2+} in a 1:1 stoichiometric measurement with Nano molar affinity (31). At the ATCUN amino acid residues, Cu^{2+} binds directly to the two subsequent backbone amide nitrogen and the terminal nitrogen on the conserved histidine of the peptides (32). These two disordered peptides show high antimicrobial activity against a variety of pathogenic bacteria (31).

Both peptides inhibit bacterial growth, kill actively growing bacterial, and can disperse bacterial biofilm (28). The efficacy of both peptides is enhanced by Cu^{2+} binding (31 & 43). The production of reactive oxygen species (ROS) in the presence of these peptides when planktonic bacteria are exposed to them create an imbalance between ROS and the bacterial defense, leading to high oxidative stress experienced, either at the membrane (leading to membrane disruption) or covalent DNA damage (nuclease activity) (40, 41 & 42). The peptides performance in an anaerobic environment have yet to be tested, but HDPs have shown to have antimicrobial activities in all parties of the body tissues including anaerobic ones.

So, this work examines the effect of HDPs (piscidins and their complexes) on *Clostridium difficile* in an anaerobic environment. Piscidin activity is assessed against *C. difficile,* an obligate anaerobic bacterium, and *Escherichia coli* and *Pseudomonas aeruginosa* facultative anaerobes capable of living in aerobic and anaerobic environments. The methods applied for combating these bacteria started from the measurement of minimal inhibitory concentration (MIC), the peptide concentration required to inhibit the bacterial growth called minimal inhibitory concentration (MIC) peptide concentration required to inhibit the bacterial growth (44). Time kill assays, determined the rate of peptides killing of *C. difficile* vegetative cells and the amount of biofilm formed in the presence of peptides, their copper complexes and transition metals alone are measured (31). The method helps to elucidate the antibiofilm properties of piscidins on *C. difficile* and oxidative response of the bacteria to transition metals.

CHAPTER 2

LITERATURE REVIEW

2.1 INNATE IMMUNE SYSTEM AND OXIDATIVE STRESS

The innate immune system, also known as the first line of immunological defense in advanced organisms, is fast and has having multiple mechanisms to attack pathogens. This protects host organisms (higher animals) from foreign pathogens, before the more comprehensive adaptive immune system is activated (51). Infection recognition is a complex process initiated by the pathogen's production of specific molecules called pathogen-associated molecular patterns (PAMPs) which are sensed by host pathogen recognition receptors (PPRs). This triggers the innate immune system to release a series of anti-pathogens substances like host defense peptides (HDPs), cytokines, chemokines and sometimes type1 interferons (52). Some of the innate immune system molecules generate reactive oxygen species (ROS) and /or reactive nitrogen species (RNS) to help combat the harmful microbial cells (54).

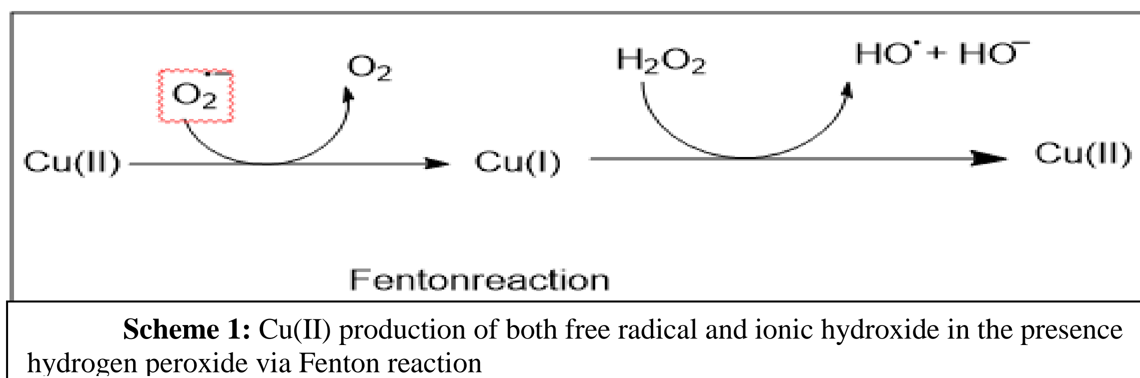
Oxidative stress is the disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses in organisms' system. Oxidative stress is known to be "the imbalance that exist between oxidation and antioxidation in a system, in which oxidation exceed the counter effect of the system" (46). Oxidative stress in the system is a very detrimental process because it results in the damage including DNA mutation, lipid peroxidation, and oxidation of membrane layers. It is also employed by the body's innate immune system sometimes to combat foreign bodies (46). Oxidative stress is well documented in many organisms. It can occur in different parts of many systems and they are implicated in many heavy metal ions' toxicities because transition metals can change their oxidation state, serving as electron donors (47).

2.2 METAL ION INDUCED OXIDATIVE STRESS

Transition metals can induce oxidative stress due to the ability to change their oxidation states (50). Gallego *et al* conducted a study which involved the treatment of plant leaves with 0.5m of Fe(II), Cu(II) or Cd(II) ions. Their study showed that after incubation, chlorophyll and glutathione (GSH), activity decreased but peroxidation and lipoxygenase activity increased. This result concluded that excess ions of these metals created oxidative stress for the plant leaves' cells (47).

Copper(II), or cupric ion, is the form of copper most likely to induce oxidative stress via its reduction to Cu(I) (cuprous ion) in a biological system, when in the presence of a strong reductant. Scheme 1 shows that this process results in the decomposition of hydrogen peroxide through the Fenton reaction process. Cupric ion does not cause oxidative stress directly. Instead, the (free radicals and reactive hydroxide ions generated in these reactions, cause the oxidative damage to biological molecules) (48).

More evidence demonstrated how metallic-induced oxidative stress through a multifunctional mechanistic process decomposed hydrogen peroxide (49). The result of that study also identified an indirect mechanism, sulfhydryl reserves which was hypothesized to be by redox-inactive metals (50). Many transition metals induce their form of oxidative stress because they can easily change their oxidation states (50).



Scheme 1: Cu(II) production of both free radical and ionic hydroxide in the presence hydrogen peroxide via Fenton reaction

2.3 HOST-DEFENSE PEPTIDES (HDPs) INDUCED OXIDATIVE STRESS

The elevation of reactive oxygen species (ROS) in the host cell's physiology can either aid pathological processes by bacteria or kill the bacteria by host deference systems (53). Many host-defense peptides combat bacteria by introducing oxidative stress on bacterial cell surfaces or in the bacterial cytoplasm (54). The innate immune systems produce these host-defense peptides to act as broad-spectrum antimicrobial agents in the body (18 & 31).

HDPs in the innate immune system have immunomodulatory activities, apart from acting directly as antimicrobial peptides (18). That is, they can also recruit leukocytes to the site of infection, suppress the potentially harmful inflammatory responses, stimulate angiogenesis and wound healing (55), because of all these functions include upregulation of chemokines (a group very small proteins that attract T-cells to site of injury) production. HDPs are ubiquitous in all complex organisms' innate and sometime adaptive immune system. As such they are strong candidate for new antimicrobial drugs. Figure 4 shows how these peptides can directly attack the membranes of bacteria (like membrane targeting piscidin p1) or target the intracellular DNA of the bacteria (piscidin p3) with nuclease activities (55, 56 & 57).

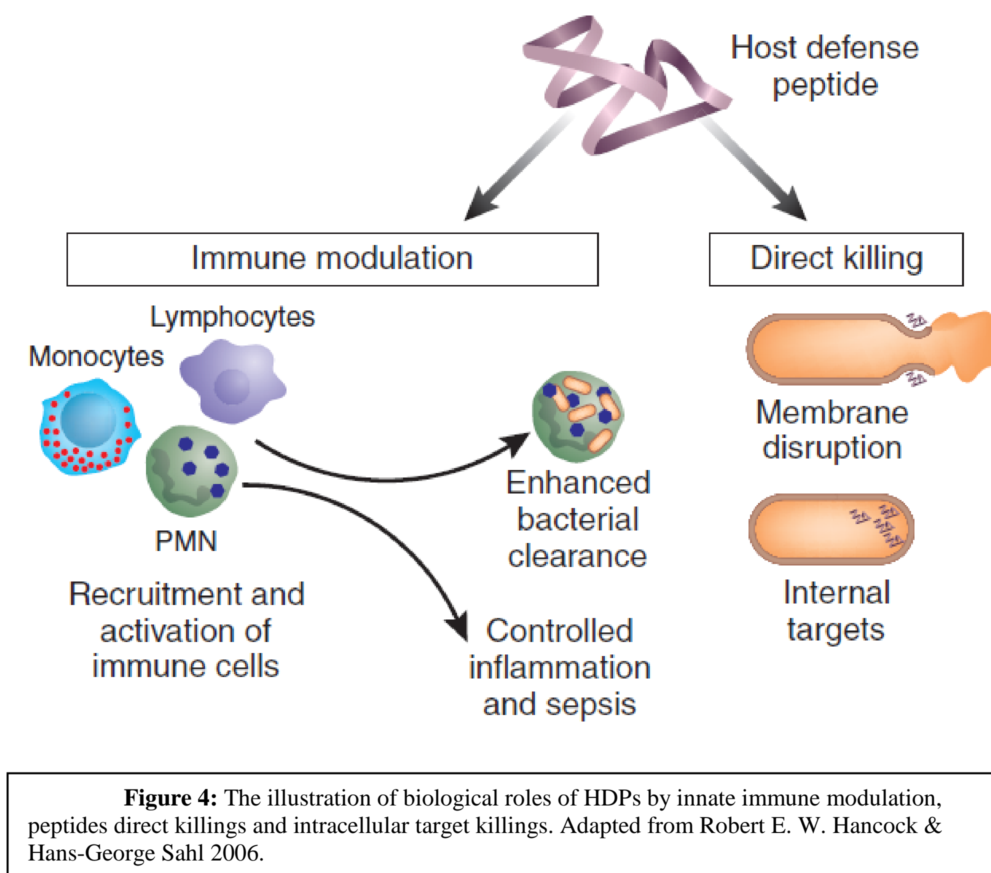


Figure 4: The illustration of biological roles of HDPs by innate immune modulation, peptides direct killings and intracellular target killings. Adapted from Robert E. W. Hancock & Hans-George Sahl 2006.

2.4 PISCIDINS AND THEIR METAL COMPLEXES

Piscidin are a prominent family of HDPs first discovered in the mast cells of vertebrates (31). These broad-spectrum antimicrobial peptides were isolated in hybrid bass striped fish (*Morone saxatilis*) and NMR studies suggest that they are intrinsically disordered in solution but adopt an amphipathic α-helix structural conformation when bound to model membranes (31 & 58). Piscidins can be isolated from other fish species and their ubiquity can be justified in fish because, fish are constantly exposed to pathogens (59 & 60).

The piscidin focused on in this investigation are p1 and p3 respectively. They are bactericidal in nature, but their mechanisms of action are different (31).

These two peptides (p1: **FFHHIFRGIVHVGKTIHRLVTG and** p3: **FIHHIFRGIVHAGRSIGRFLTG),** are intrinsically disordered in solution. This is likely due to the high numbers of hydrophobic amino acid residues in these peptides, compared to the hydrophilic residues. p1 & p3 are isoforms of one another with similarities and variations in their biological activities, specifically varying where they attack bacteria (i.e., p1 attacks the cell membrane and p3 the DNA) (26-31).

The overall positive charge in the piscidin peptides' amino acid residues helps them to interact with the phospholipids of the cell membrane and/or the acidic backbone of DNA, causing reactive oxygen production that leads to oxidative stress within the cell bilayer membrane (26). Piscidins also employ the same method in the intracellular targets, and they can be self-promoting, inserting themselves into the bilayer cell membrane and interrupting the membrane integrity (55).

2.5 OXIDATIVE STRESS RESPONSE IN BACTERIA

Bacteria use different methods to respond to oxidative stressors encountered in their environment from various sources like drugs, metals, nutrients, host defense system and sometimes UV-radiation (62 &63). The nutrient in their surroundings are both helpful and harmful, particularly those that create imbalance in their oxidative environment (62). In some cases, the oxidative stress experienced by bacteria are because of ROS, and RNS (63). Some well-known oxidative stress responses in bacteria during anomalous growth process are of two main types' gene regulation and/or repair responses (62).

The oxidative stress gene regulation response is the production or increased activity rate of antioxidant enzymes or proteins (like the OxyR system for hydrogen peroxides and the SoxRS system for superoxide) in bacteria (65). The OxyR and SoxRS system for regulation of oxidative stress created by ROS and others are said to be conserved in both Gram-positive and Gram-negative bacteria (66).

The repair response is focused primarily on DNA either by exertion or methylation repair methods of the bacteria genes (64). Some bacteria have other response to oxidative stress or extracellular stress in general, including the production of biofilm.

2.6 BIOFILM AS STRESS RESPONSE

Biofilms are structured multicellular communities of microorganisms that adhere to surfaces (67). Bacteria biofilm are associated with more than 65% of infections and recurrent infections in hospitalized patient in the United States of America alone and lead to high rate of chronic diseases in humans' health around the globe (67 &68). Biofilm containing multiple or single bacteria taxa, are mostly composed of a secreted exopolysaccharide matrix which protect the cells within the biofilm from extracellular stress, including antibiotics (76) Cells in a biofilm can be up to 1000-fold more resistant to antibiotics than cells of the same bacterial species outside of a biofilm (69). Growth in a biofilm also leads to changes in bacterial metabolism and gene expression (66).

Hundreds of genes are responsible for stress associated biofilm formation in bacteria. One example is the MqsA/MqsR pair of genes in *E. coli* that induce biofilm formation in response to toxin stress has antitoxin stress (70). Biofilm production has been reported as a direct response to antibiotic stress (71). In May 2014, Cesar and colleagues implicated biofilm formation as part of stress response to antimicrobial peptides (68).

2.7 OXIDATIVE STRESS RESPONSE IN BACTERIA

The oxidative stress experienced in both aerobic and anaerobic bacteria differ because of the availability of oxygen. While aerobes make use of oxygen to produce energy, ROS are also produced as by-products that can be harmful to their cells (72). Anaerobes also experience oxidative stress. Since this stress is not only external or extracellular based only, ROS can also be generated intracellularly (62).

2.8 OXIDATIVE STRESS RESPONSE IN AEROBIC BACTERIA

Aerobic bacteria that use oxygen in respiration can also create "ROS" and RNS, which leads to oxidative stress (62). The response of bacteria to this type of stress is the production of antioxidant enzymes (62). The innate immune system also adopts this strategy against foreign bodies through the production of different molecules or peptides that initiate or induce a process called the "oxidative burst" (51-54 & 62). Some of the antioxidant enzymes used by the bacteria are superoxide dismutase (SOD), catalase, alkylhydroperoxidaes, superoxide reductase and peroxidase. The co-factor for most of those antioxidant enzymes is iron. Iron can absorb the electrons that make ROS reactive, but excess iron can also result in metallic ion oxidative stress for the bacteria (62).

Pseudomonas aeruginosa and *Escherichia coli* are facultative anaerobes that can live in the presence of absence of oxygen. *Pseudomonas aeruginosa* is a Gram-negative bacterium that can be a life-threatening with strong resistance to antibiotics (8). The model *P. aeruginosa* strain, PA01, was isolated from a wound, while other strains were found in from burns and cystic fibrosis (9 & 10).

P. aeruginosa, is an opportunistic, infectious bacterium that causes chronic infection in patients who already have cystic fibrosis, increasing their mortality rates (12 & 13). This opportunistic pathogen, well-known for its large biofilm formation also causes chronic biofilm infection and has ability to form three exopolysaccharides types of biofilm (14 &15). The high rate of infection by *P. aeruginosa*, has been traced back to the bacteria ability to grow and flourish easily in sputum, found in large volume along the human airways. So, in the case of cystic fibrosis of the lungs (CF Lungs) they can be easily determined in the sputum after 50 minutes of infection (16 & 17). Unlike *E. coli, Pseudomonas aeruginosa (PA01)* makes use of many genes and complex

way of responding to oxidative stress, including formation of large amounts of biofilm. *P. aeruginosa PA01* exhibits high rates of biofilm formation and copper-piscidin complexes have been previously demonstrated to disperse *PA01* biofilms (31).

PA01 genes that combat oxidative stress are katB-ankB, ahpB and ahpC-ahpF (73). In-vitro the katB-lacZ reporter expression level increased 250-folds when cells were exposed to oxidative generating compounds (H_2O_2-generating compounds) (73). Both anhB-lacZ and ahp-lacZ expression level increased by 90 and 3 folds respectively when the bacteria are induced by paraquat (an oxidative generating compound), within 5 minutes (73).

The mutant PA01 (ΔoxyR) does not express those genes mentioned before when exposed to either of the oxidative generating compounds above and any other form of oxidative stress because exposure to the same compounds kill the mutant before gene expression can change. They are highly susceptible to oxidative stress but, none of the oxidative stress genes get expressed. What was inferred from this was that oxyR is responsible for the control of oxidative stress response genes and those genes are used to counter the effect of oxidative stress in *P. aeruginosa (*62, 70 – 73).

Escherichia coli is a known Gram-negative bacterium and widely spread in food, water and air contamination (19). *E. coli* can live aerobically or anaerobically and responds to oxidative stress by two well documented strategies of antioxidant enzyme formation and DNA-repair protein formation, as well as biofilm formation as a result of oxidative stress (68, 70 & 71).

2.9 OBLIGATE ANAEROBE

Clostridium difficile is the only obligate anaerobic microbe principally investigated in this research.

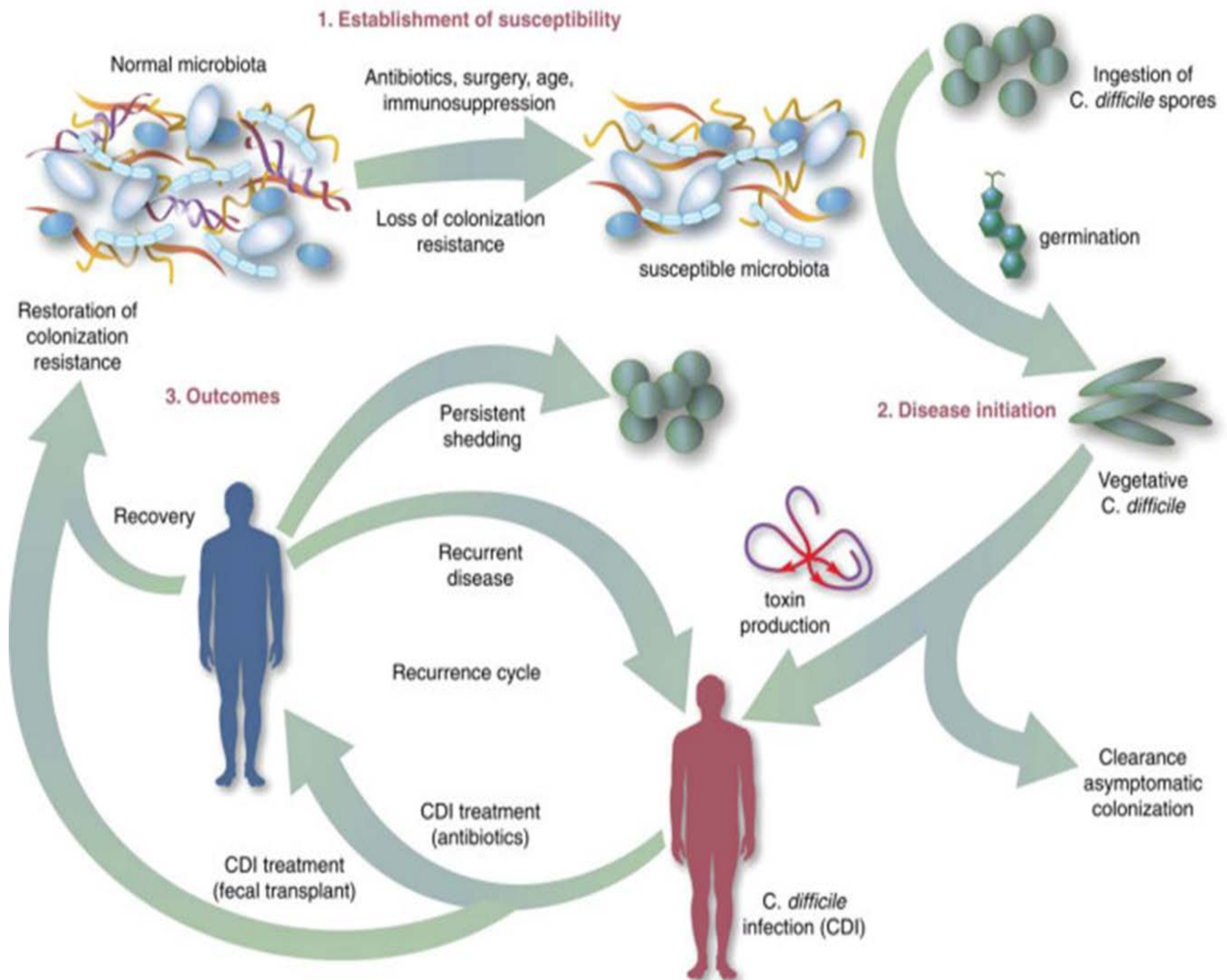


Figure 5: The life cycle of *Clostridium difficile.* Adapted from Karissa Culbreath *et al.* Journal of clinical microbiology, Oct. 2010, p. 3738–3741

This bacterium is a flagellated, Gram-positive rod-like (bacillus), and has two states in its life cycle (1). Dormant non-metabolizing spore are resistant to oxygen, alcohol, and other stresses. Spores let the bacteria spread through the oxygenated environment to new hosts (34). The actively germinating phase that can produce spores and cause infection through the production of exotoxin

in *C. difficile* life cycle the vegetative cells (3 & 75). This destructive phase is the main target of this investigation. The vegetative cells highly antibiotic resistant, and they form biofilm. Their biofilm in the hypervirulent strains aid in the bacteria protection and infection processes (1-7 &33). All the life cycle stages are described in Figure 5 (1, 2 & 75).

2.10 *C. DIFFICILE* VEGETATIVE CELLS AND PATHOGENESIS

C. difficile is the causative agent of hospital related antibiotic-associated diarrhea and pseudomembranous colitis (1 & 2). The bacteria produce two main toxins in the intestine called toxin A (Ted A) and toxin B (Ted B), some strains of *C. difficile* also produce a third type of toxin called binary toxin (CDT) (74).

The pathogenesis of this bacterium starts from the vegetative cell production of toxin after colonization of the animal gut (33). When the innate immune response of the host cells fails, bacteria colonization increases, and disease symptoms set in, shown in the figure 6 flow chart. Unless the antitoxin treatment works, or vegetative cells are killed off before toxin production, the symptoms rapidly surface in the infected individual (75). The major risk factor which increases susceptibility to CDI is the disruption of microbiota intestinal community is antibiotics and others are secondary to antibiotics (33 &75).

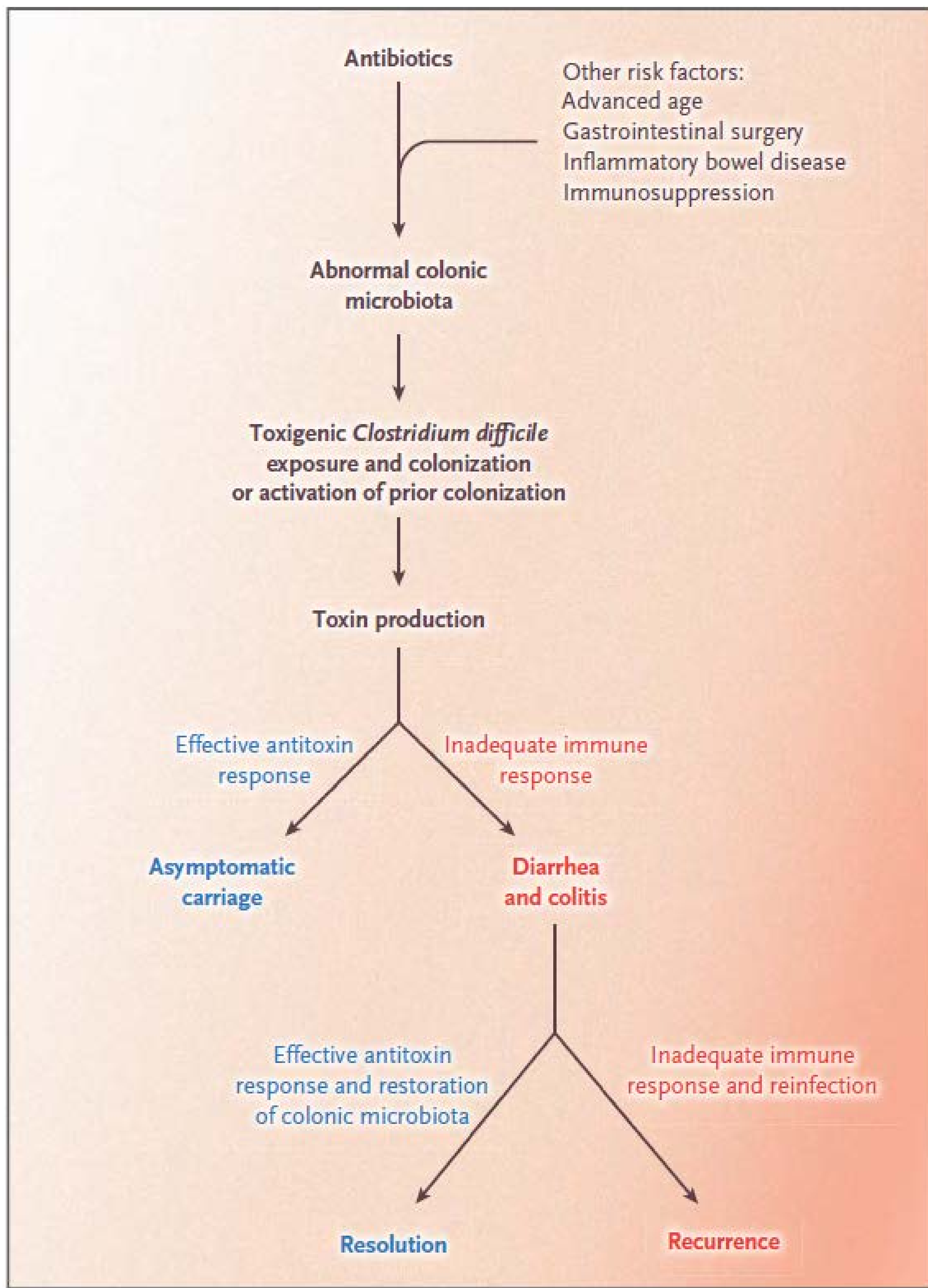


Figure 6: Pathogenesis of *Clostridium difficile* Infection. Chart adapted from Daniel A. Leffler and J. Thomas Lamont N Engl J Med 2015;372: 1539-48

2.11 *C. DIFFICILE* AND ANTIMICROBIAL AGENTS

Treatment with fluoroquinolone antibiotics results in the disruption of microbiota communities that normally out-compete *C. difficile* for nutrients and allows *C. difficile* colonization and CDI associated diarrhea with roughly 33% recurrence after treatment (34). The antibiotic was meant to fight microorganisms but in doing so, it created an environment susceptible to the *C. difficile* infection, Jacques Pepin *et al* have found similar results with the antibiotic metronidazole used to treat recurrent CDI; 33.3% of patients had a second recurrence after metronidazole treatment (34). Both studies not only point out that antibiotics have not been effective against *C. difficile,* but also indicate promotion of adaptive *C. difficile* strains in the toxins production by this pathogen [5 &34]. According to the medical community of the early 1980s the use of metronidazole and vancomycin were effective antibiotics against *C. difficile,* but in recent years, the high rate of recurrence, despite the usage of those antibiotics, is not just anomaly it is a serious and an ongoing problem (34 & 35).

The Center for Disease Control and Prevention (CDC) warned against the usage of those two antibiotics in treatment of *Clostridium difficile-associated disease* (CDAD) to avoid vancomycin resistant enterococci and staphylococci bacterial infections. This reduced the confidence level in these antibiotics and set stage for a new and urgent need for antimicrobial drugs [35]. Another study by Hetch D W. and Galang M. A *et al,* used 15 well-known antibiotics against, 110 toxigenic clinical strains of *C. difficile* to check effectiveness. This study came about as a result of the decrease in confidence in the use of antibiotics against this bacterium. Yet, none of the 15 antibiotics were able to kill the strains of *Clostridium difficile in-vitro.* Table 1 shows the distribution test of all the antimicrobial agents tested on isolated clinical *C. difficile* strains [35].

Table 1: Distribution of antimicrobials tested against 110 toxigenic clinical C. difficile isolates

Antimicrobial agent	No. of strains for which the antimicrobial agent MIC (μg/ml) was:															
	≤0.0019	0.0039	0.0078	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64[a]
Rifalazil	14	20	45	27	1											3*
Rifaximin	12	1	73	20	1											3*
OPT-80				9	11	29	50	11								
Tizoxanide				1	7	83	18		1							
Nitazoxanide					4	71	33		2							
Metronidazole					2	3	70	32	3							
Tinidazole					1	2	66	35	5	1						
Ramoplanin						1	19	45	45							
Tigecycline						2	89	17	1	1						
Vancomycin						2			34	68	5	1				
Doripenem										78	31	1				
Meropenem										16	89	5				
Gatifloxacin									3	73	17		1	9	6	1†
Moxifloxacin										57	38		1	8	6	
Levofloxacin											9	82	1		5	13‡

[a] *, Three strains had MICs of >256 μg/ml for rifalazil and rifaximin; †, one strain had an MIC of >32 μg/ml for gatifloxacin; ‡, thirteen strains had an MIC of >32 μg/ml for levofloxacin.

In view of this looming antimicrobial failure, we turn to naturally occurring antimicrobial peptides, HDPs. These peptides, as already discussed, channel oxidative stress that they create against bacteria cells to kill them (30 &31). The peptides alone, their complexes and sometime copper alone can generate diverse oxidative stress, which can quickly overwhelm the bacteria cells, resistance will not easily be develop against them. Moreover, it is possible that as the piscidin are naturally produced in organisms' immune systems, health challenges posed by them may be low.

2. 12 OXIDATIVE STRESS RESPONSE IN *C. DIFFICILE*

C. difficile an anaerobic bacterium but, can survive oxygen level up to around 2%. So, *C. difficile* must have mechanisms of defense to tolerate oxygen molecule and ROS generated as a result of responding to oxidative stress from these oxygen molecules as well as antimicrobial

peptide peptides and metal ions (77). Aside the endogenous ROS that might be produced during metabolic reactions of the bacteria. Antimicrobial peptides, metal ions and oxygen molecules can aggravate the situation (49 & 56).

In *C. difficile* the oxidative stress response has yet to be well established, although ROS scavenging enzymes like catalase, peroxidase and superoxide dismutase (SOD) have been identified being identify on their spores. The vegetative cells' defense against oxidative stress require more intensive investigation because reports on this aspect of bacterial physiology are not yet sufficient. A conserved putative superoxide reductase (SOR) is also encoded in the *C. difficile* genome and expressed in transcriptomic and proteomic studies of vegetative cells (80). The sensitive of *C. difficile* to piscidin peptides and complexes with the oxidative stress response of this bacterium to metal ions are reported in these investigations.

CHAPTER 3

RESEACH OBJECTIVES

The advent of failure in many strong antibiotics against the *Clostridium difficile* and the fast adaptation of the bacteria to them, there is urgent need for antimicrobial agents that can kill and minimize the adaptation of the bacteria to them. Oxidative stress adaptation in bacteria are difficult because it can come from diverse sources, like transition metal ions and antimicrobial peptides produced by animal immune systems. The objective of this research is to:

1. Characterize the oxidative stress response of *C. difficile* to antimicrobial peptides and transition metals
2. Determine the effect of antimicrobial peptides in the aerobic and anaerobic environments

CHAPTER 4

EXPERIMENNTAL PROCEDURE

4.1 MATERIAL AND SAMPLES

LB-broth, Agar, Bacto yeast extract, BHI (Brain-Heart Infusion), Glucose, drugs are Host defense peptides (HDPs) piscidin-1 (p1), piscidin-3 (p3), $CuSO_4$, $CuCl_2$ and $Cu(NO_3)_2$ (Cu^{2+}) and piscidin complexes. Piscidins and complexes were provided by the Cotton lab (William and Mary). Na_2PO_4, Nuclease free water, TY-media, dry ice, 630 Δerm *Clostridium difficile* strain (lab strain), R20291 *Clostridium difficile* strain (virulent strain), *Escherichia coli (DH5 alpha* Lab strain from New England Biolabs, *PDJ1* and *MWF1* two gift strains from Liberdo lab) and *Pseudomonas aeruginosa (PA01* strain*)*. 1×PBS, 1% crystal violet, variable percentage of Ethanol Antibiotics (Ampicillin and Kanamycin). Unless otherwise specified, reagents were purchased from Fisher scientific.

4.2 INSTRUMENTATIONS

Centrifuge (1) Accuspin micro 17 (Fisher Scientific) (2) Legend micro 21R (Thermoscientific), Imaging is through syngene image (U); Genius 3. Optical density (OD); (1) Genesys ws UV-Vis spectrophotometer (Thermoscientific) (2) Smart Bio drop (Dr. Pascal's lab) (3) Ultraspec 10 (Bio-Rad), Anaerobic chamber (Coy laboratories), Incubator and sample shaker Max Q 6000 by (Thermoscientific), Dry and wet bath (Thermoscientific and Corning/LSE). Fluorescence UV-Vis spectrophotometer plate reader (Bio-Tek) and smart pH-meter (SevenCompact).

4.3 MINIMAL INHIBITORY CONCENTRATIONS (MIC)

4.4 MIC FOR P1 AND P3

The minimal inhibitory concentrations MIC for p1 and p3 was verified for both Δ*erm 630* strain and virulent strain of *R20291* respectively. Four biologicals of both *C. difficile*. Strains were cultured in TY-media for 24hrs at 37^0C in the anaerobic chamber's incubator using glass test tubes. After 24hrs these cultures were used to inoculate fresh culture in 96 well plates, leaving the first and last columns in the plate full of non-inoculated medium as blanks. Each experimental well contained peptide and /or copper at the indicated concentration and the total volume /well was constant at 200µl, with volume adjusted as needed with nuclease free water. Inoculated plates were incubated at 37^0C for 16 hours.

After 16 hours plates were taken out of the chamber, plate exteriors were sterilized with bleach, and plates were read at 630/nm excitation wavelength in Bio-Tek plate reader. The 96 well plates used for these experiments has been deoxygenated in the anaerobic chamber for more than 72hours before usage.

4.5 MIC FOR Cu^{2+} CONCENTRATIONS

Four fresh cultures of the two *C. difficile*. Strains (R20291 and 630Δerm) were inoculated from overnight in 96 well plates with TY medium containing copper ion in a gradient concentration from 64uM to 0.5uM incubated and measured as described above. We employed three different strains of *E, coli* (*DH5α, PDJ1,* and *MWF1* respectively) to establish the piscidin MICs for them in the presence and absent of oxygen. Anaerobic cultures were grown in the incubator in the anaerobic chamber and aerobic cultures were grown in a laboratory incubator. We maintained the same MICs growth assay steps and setup already discussed above and after 16 hours growth data was obtained with the plate reader.

4.6 BIOFILM ASSAY

4.7 P1 AND P3 BIOFILM ASSAY

We grew four biologically independent cultures of *R20291* and inoculated 2 mL/well of BHIS media supplemented in 1% glucose and 50 mM Na_2PO_3 pH **7.**4 with 20µL of saturated culture in 24 well plates. Culture were incubated for 2 hours before treatment and left in incubator at 37^0C for 24 hours. Plates were degassed in the anaerobic chamber for 72 hours before using. Biofilm cultures contained piscidin and/or copper at the indicated concentrations. After 24 hours of growth, cell density measured at 630 nm. Afterwards, non-adhered cells were removed so biofilm could be measured.

Carefully slant the 24 well plates to remove the media without allowing the pipette tips to touch the bottom part of the wells. Wash the left over in each well with 1×PBS, 1ml for each well, pouring the wash buffer gently and in slant orientation. Stain each well with 0.1% crystal violet and allowed to stand for 30 minutes or more. To the resulting setup after 30 minutes wash twice with 1×PBS 1ml each time again carefully. Then, suspend the left over in 70% ethanol and take the picture. Scan the plate with 570/nm wavelength, using the plate reader.

4.8 PISCIDINS AND THEIR COMPLEXES BIOFILM ASSAY

Well reduced 24 well plates, each well was filled with 2ml BHIS (glucose 1% and NaPO4 p H= 7.4). Except for the first row, inoculate with 20ul of starter culture of R20391 of biological A, B, C, D, E & F respectively. No drug to the first column, 4uM of Cu^{2+} added to the second column only, third column contain 2uM of P1 only while fourth contain mixture of 2uM p1 and 2uM Cu^{2+} fifth column was p3 alone and sixth well column contain the mixture of 4uM of Cu^{2+} and p3 each. The experimental setup was kept in incubator at 37^OC in anaerobic environment for 24hrs. After 24hrs, washing procedure, discussed earlier for other biofilm and piscidin 1 and 3 was carefully followed and plate reader used to scan the 24 well plate at 570/nm wavelength.

4.9 Cu^{2+} BIOFILM ASSAY

P. aeruginosa and *E. coli* strains biofilm were grown in LB medium containing the indicated concentrations of copper ions rather than supplemented BHIS. The protocols for biofilm inoculation, growth, staining, and measuring were the same as those listed above. Duplicate plates were set up for *P. aeruginosa* and *E. coli* strains. To account for slower growth in anaerobic conditions, one plate was allowed to grow for 48 hours at 37^0C in the anaerobic chamber and one was allowed to grow 24 hours at 37^0C aerobically.

4.10 TIME KILL ASSAY

Four biologically independent cultures of *R20291* were grown overnight in TY medium and then used to use to inoculate fresh TY medium. Culture were grown to mid-exponential phase. The culture density was measured by monitoring optical density at 600 nm (OD600). At OD600 of 0.5-0.7, piscidin and/or copper were added at the indicated concentrations. At the indicated time points, 20 µL aliquots of treated culture were added removed and serially diluted 10-folds in fresh TY medium. Each sample was plated on BHIS plates at a $1{:}10^6$ dilution and incubated anaerobically at 37^0C for 24 hours. Colonies were enumerated

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 MINIMAL INHIBITORY CONCENTRATIONS (MIC)

The following are the various results obtain for MIC of 630Δerm and R20291.

5.2 MIC FOR 630Δerm AND R20291

The minimum inhibitory concentration (MIC) is the lowest concentration sufficient to stop the growths of microbes' in-vitro. This is determined by inoculating media containing a concentration gradient of p1 and p3 peptides (from 0.063uM to 64uM) in a 96-well plate, with 20 µL of saturated starter culture of R20291 and 630Δerm *C. difficile* to a final volume of 200µL per well. Uninoculated TY-medium was used as a blank. After 24 hours incubation, the plate reader, using 630nm wavelength, measured the cell density in each well. Figure 7 showed the growth curve for the two strains on three separate days. For the p1 the MIC was estimated on the curve to be 4uM, although growth decline started at 2uM. The MIC for p3 was 8uM and growth decline started at 4uM. The MIC determination for the two strains were carried out in the absence of oxygen and at 37^0 C incubation.

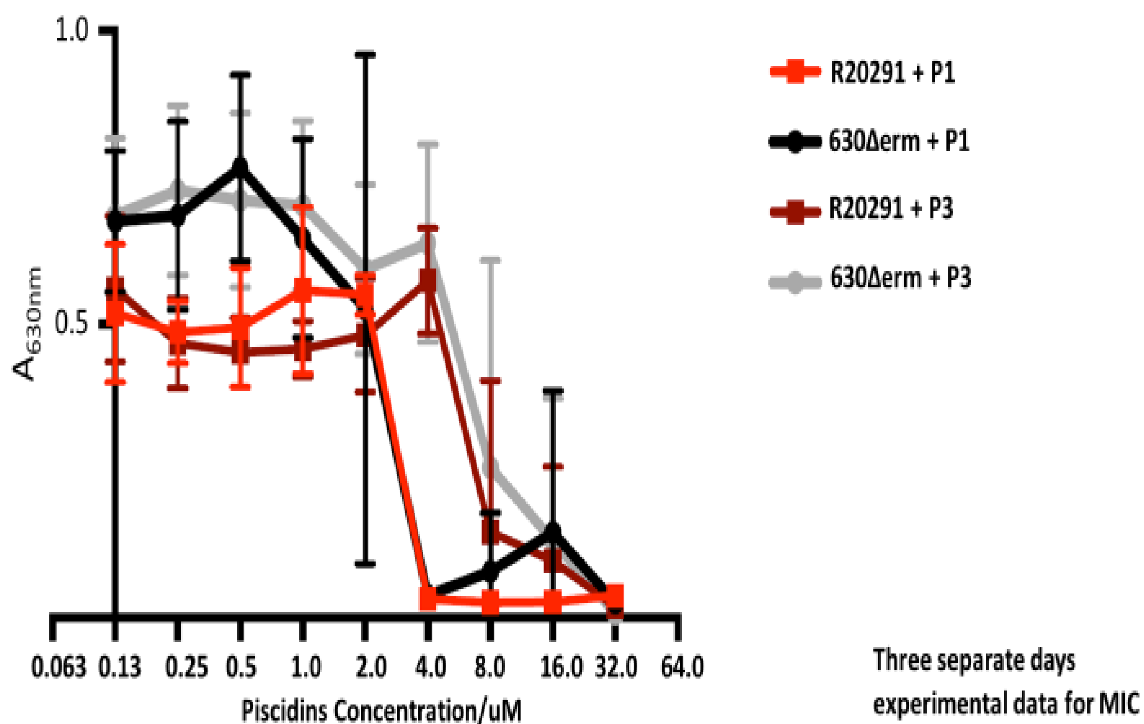


Figure 7: The MICs for p1 & p3 against both strains of *C. difficile 630Δerm & R20291* through measure of cell density after 24 hours. The figure above represents average three days data acquisition plots

5.3 MIC FOR R20291 USING DIFFERENT COPPER SALTS

It has previously been reported that piscidin peptides have stronger antibiotic activity when complexed to copper ions (31). To investigate the effect of copper ions alone on *C. difficile* growth, three different copper salts were tested against the virulent *R20291 C. difficile* strain. The concentrations of the three copper salts employed ranges from 0 to 8uM**.** This is to test how those copper concentrations affect the growth of C. difficile virulent strain. Figure 8 below, as at 8uM, toxicity of copper(II) salts, no significant growth inhibitory effect and the bacteria growth was stable above 1 of absorbance measured at 630/nm.

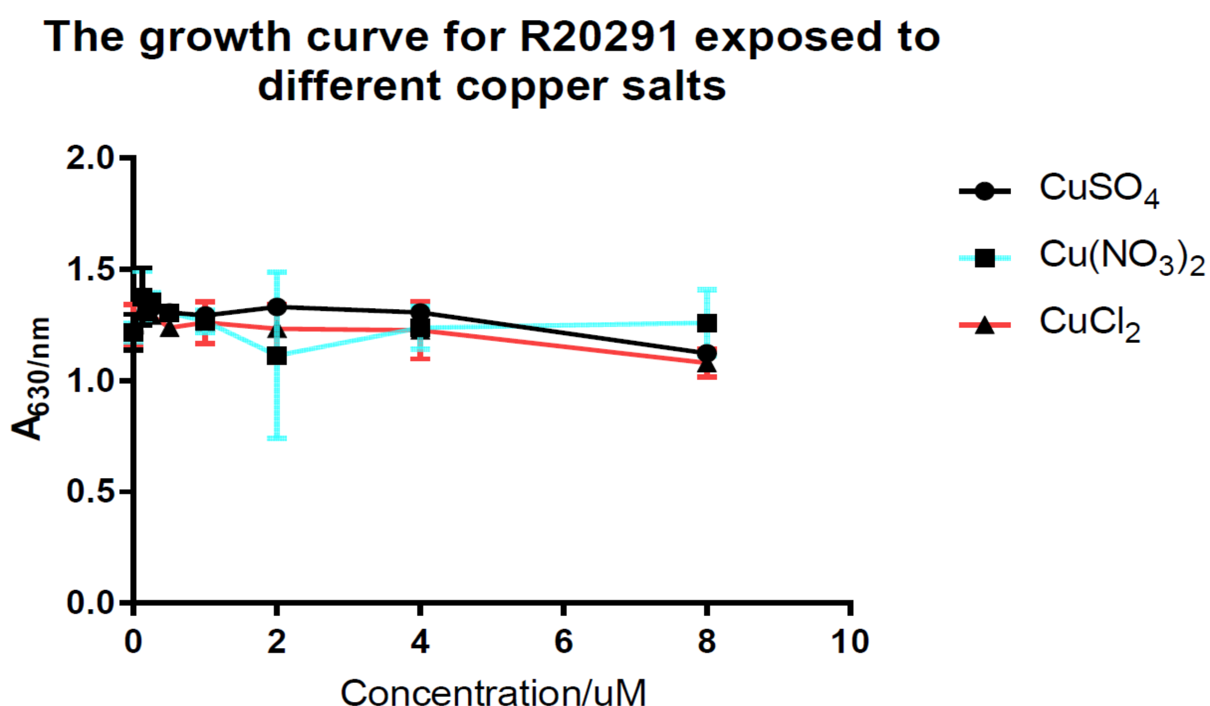


Figure 8: The copper concentration gradient for three copper(II) salts inhibitory effect against the virulent strain of *C. difficile.*

5.4 TIME KILL ASSAY

5.4.1 Time kill assay for p1, p3 and their copper complexes

The time kill assay was used to determine the killing rate of the peptides and their complexes in both presence and absence of oxygen. Unlike MIC assays, time kill assays measure the effects the peptides have on the *C. difficile* culture after they have been removed from the bacterial cells by dilution. The time kill made use of sub-MIC concentrations of p1 and p3. The concentrations used for p1 was 2µM and p3 was 4µM. To investigate potential synergy between piscidins and copper, piscidins were tested in the presence and absence of equimolar $CuSO_4$. The method involved enumeration of colony forming unite per mL of culture (CFU/mL) of the treated and non-treated cultures after plating for 24 hours at 37^0C in the incubator.

Enumeration of colonies revealed p1 alone had not significantly reduced CFU/mL at t=0 but the p1-Cu complex immediately reduced CFU mL by approximately 40% (Figure 9) in comparison to NT. After 30 minutes, there was significant killing in comparison to untreated CFU. Specifically, p1 vs p1+Cu only had low significant different at t=0 (p=0.0063) (Table 2) but as time progressed no other time point showed any significant difference between the peptide alone and its complex. The p3 and p3+Cu had no immediate impact after treatment because there was less than 5% difference between NT, p3 and p3+Cu and the comparison showed no significant difference at t=0 (Figure 10) (Table 3). Subsequently there were significant differences between the p3 and p3+Cu to NT at all other time points. More than 50% reduction in CFU had occur in 30 minutes between the p3, p3+Cu to NT but, the peptide and its complex showed no significant difference in them. At the four-hour point, no significant difference occurred between the p3 and p3+Cu.

The data analyses (Table 2) showed there was initially no significant difference between the samples treated with piscidin-1 (p1) and untreated (NT) samples. The samples treated with p3+Cu complex had significantly lower CFU counts than the untreated samples ($p < 0.001$) and samples treated with piscidin alone ($p < 0.001$) at the zero time point. After 30 minutes of treatment, both p1 and p1+Cu were significantly different from the untreated samples ($p < 0.001$) but showed no significant different from each other, meaning copper initially accelerated p1 killing of the bacterial cells, but, the peptide alone quickly 'caught up' to the peptide-copper complex. This same killing rate was observed at 2 and 4 hours' time points after treatment.

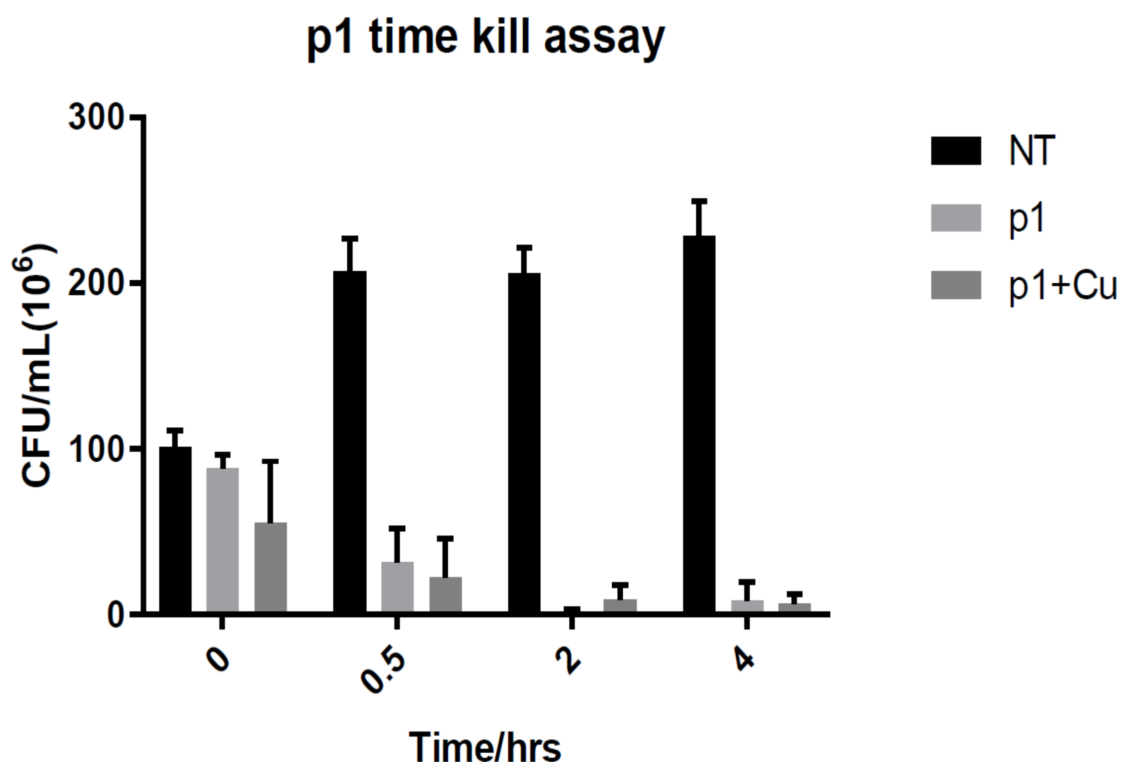


Figure 9: The time kill curve for p1 and its copper complex lab mixture. The time kill assay data which resulted into the above Figure 9 was an average of the peptides antimicrobial activity carried out months apart

Table 2: The data analysis of p1 and p1+Cu two ways ANOVA

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
NT vs. p1	12.5	-12.25 to 37.25	No	ns	0.4496
NT vs. p1+Cu	45.33	20.59 to 70.08	Yes	***	0.0001
p1 vs. p1+Cu	32.83	8.088 to 57.58	Yes	**	0.0063
0.5					
NT vs. p1	175.3	150.6 to 200.1	Yes	****	<0.0001
NT vs. p1+Cu	184.2	159.4 to 208.9	Yes	****	<0.0001
p1 vs. p1+Cu	8.833	-15.91 to 33.58	No	ns	0.6687
2					
NT vs. p1	204.5	179.8 to 229.2	Yes	****	<0.0001
NT vs. p1+Cu	197	172.3 to 221.7	Yes	****	<0.0001
p1 vs. p1+Cu	-7.5	-32.25 to 17.25	No	ns	0.7477
4					
NT vs. p1	220.2	195.4 to 244.9	Yes	****	<0.0001
NT vs. p1+Cu	221.7	196.9 to 246.4	Yes	****	<0.0001
p1 vs. p1+Cu	1.5	-23.25 to 26.25	No	ns	0.9884

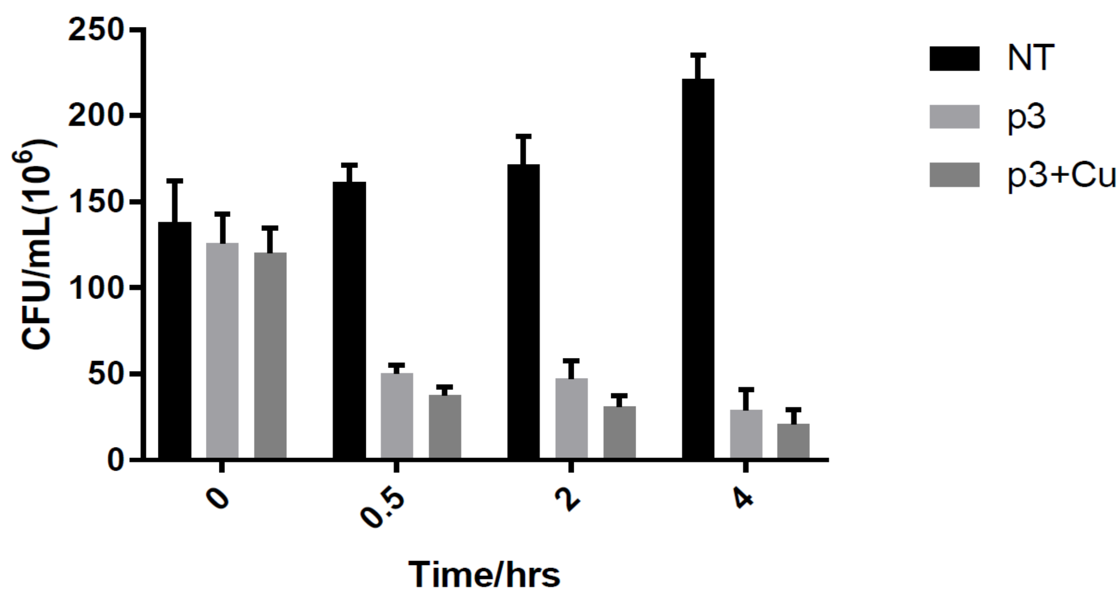


Figure 10: The time kill curve for p3 and its copper complex for lab mixture

Table 3: The p3 and p3+Cu time kill data analysis by two ways ANOVA

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
NT vs. p3	12.33	-5.851 to 30.52	No	ns	0.2411
NT vs. p3+Cu	17.67	-0.5173 to 35.85	No	ns	0.0586
p3 vs. p3+Cu	5.333	-12.85 to 23.52	No	ns	0.7616
0.5					
NT vs. p3	110.8	92.65 to 129	Yes	****	<0.0001
NT vs. p3+Cu	123.5	105.3 to 141.7	Yes	****	<0.0001
p3 vs. p3+Cu	12.67	-5.517 to 30.85	No	ns	0.2235
2					
NT vs. p3	124.2	106 to 142.4	Yes	****	<0.0001
NT vs. p3+Cu	140.5	122.3 to 158.7	Yes	****	<0.0001
p3 vs. p3+Cu	16.33	-1.851 to 34.52	No	ns	0.0868
4					
NT vs. p3	192.5	174.3 to 210.7	Yes	****	<0.0001
NT vs. p3+Cu	200.3	182.1 to 218.5	Yes	****	<0.0001
p3 vs. p3+Cu	7.833	-10.35 to 26.02	No	ns	0.5577

Table 3 above showed that at zeroth hour there was no significant difference in the three conditions NT, p3 and p3 complex. As time progress the trends noticed in p1 and its complex activities was the same at rest time points from half an hour to the fourth hour time point.

What was noticeable during data analysis, neither p1 nor p3 showed significant changes to the killing rate when copper was added. This shows that adding copper in +2 oxidation states do not increase the peptides' activities "rate of vegetative cells killing". This is directly contrary to what was previously measured against *E. coli* in aerobic conditions (31). The time kill assay was carried out several times over the course of four months.

The copper concentration in the TY medium is 1.24 µM (Figure 14). This is low compared to the amount of copper added in the assays. To account for the possibility that the extra copper was being chelated by something else in the media and not forming complexes with piscidins, we repeated the assays using pre-formed piscidin-copper complexes. Figure 11 and 12 show the activities of the pre-formed peptides-copper'-complexes. In peptide p1+Cu and p1Cu complexes. Where, p1+Cu represent the lab mixture and p1Cu is for pre-copper complex, respectively. For any form of p1-copper complexes data analyses showed no significant difference in their rate of killing (Figure 11). The p3 and its two different copper complexes showed significant difference at starting point of t=0. This is likely due to slow chelation process between the peptide and copper compound, in this case $CuSO_4$. As time progressed, particularly at the 4-hour time point, there was no significant difference in the killing rate of the two forms of peptide complexes. Figure 12 properly shows the curve for the two forms of p3 and its complexes.

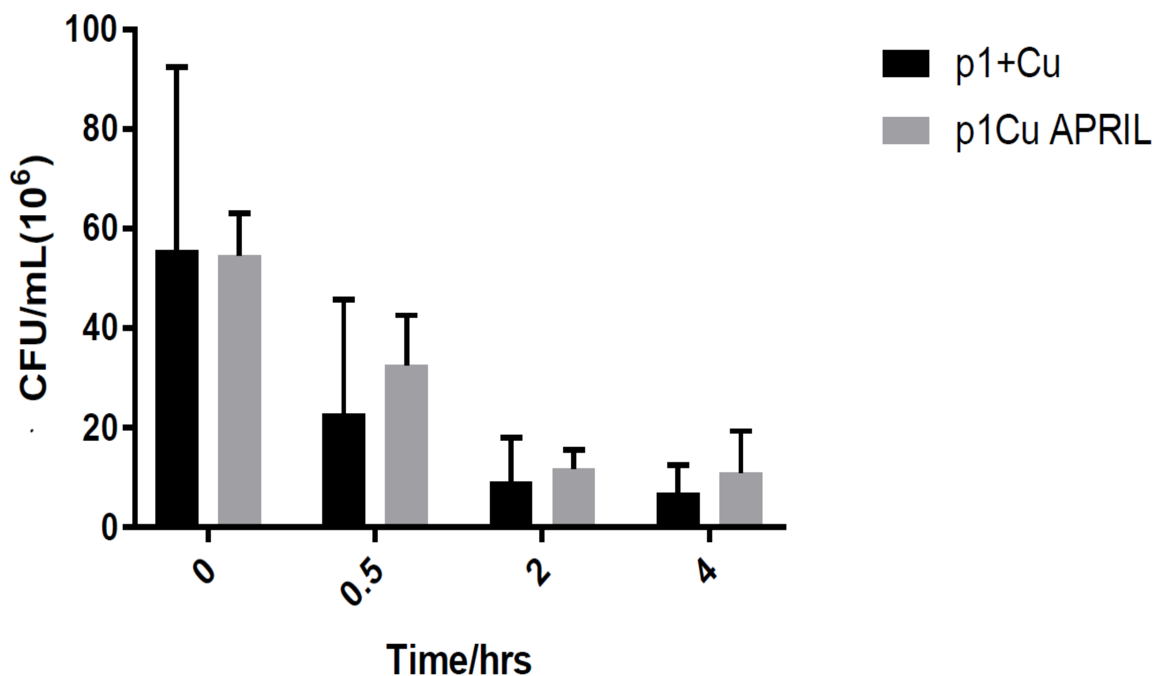


Figure 11: Comparative data curve for p1+Cu and p1Cu complexes. Where p1+Cu and p1Cu represent the peptide copper mixed in the lab and pre-formed peptide-copper complexes.

Table 4: The data comparison of lab mixed peptides complex (p1+Cu) and pre-complex peptides (p1Cu)

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
p1+Cu JAN - p1Cu APRIL								
0	1	-24.31 to 26.31	No	ns	>0.9999			
0.5	-9.667	-34.98 to 15.65	No	ns	0.7927			
2	-2.667	-27.98 to 22.65	No	ns	0.9979			
4	-4.167	-29.48 to 21.15	No	ns	0.9881			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	t	DF
p1+Cu JAN - p1Cu APRIL								
0	55.67	54.67	1	9.706	6	6	0.103	40
0.5	22.83	32.5	-9.667	9.706	6	6	0.9959	40
2	9.167	11.83	-2.667	9.706	6	6	0.2747	40
4	6.833	11	-4.167	9.706	6	6	0.4293	40

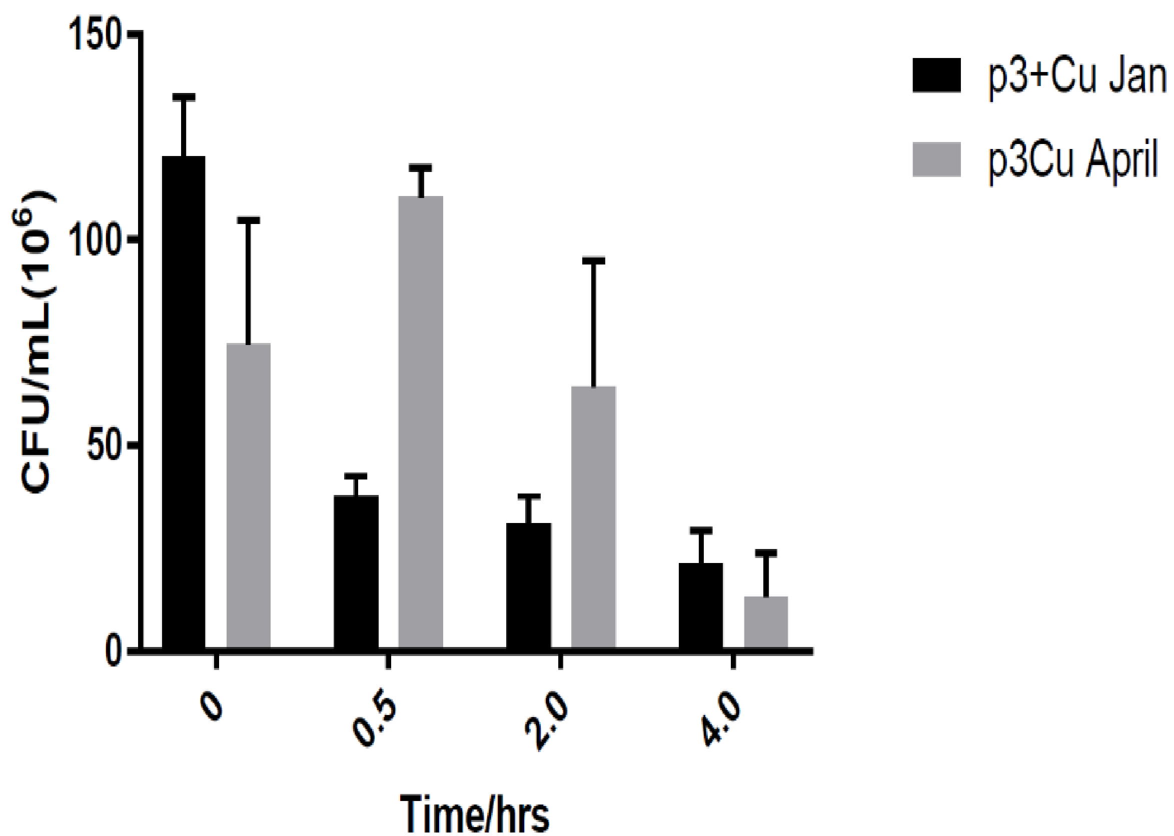


Figure 12: Comparative data plot for p3+Cu & p3Cu complexes. P3+Cu and p3Cu are lab mixed and pre-formed peptide-copper complexes

Table 5: The data comparison of lab mixed peptides complex (p3+Cu) and pre-complex peptides (p3Cu)

Compare each cell mean with the other cell mean in that row								
Number of families	1							
Number of comparisons per family	4							
Alpha	0.05							
Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
p3+Cu Jan - p3Cu April								
0	45.67	19.82 to 71.51	Yes	***	0.0002			
0.5	-72.5	-98.35 to -46.65	Yes	****	<0.0001			
2.0	-33.17	-59.01 to -7.321	Yes	**	0.0071			
4.0	7.667	-18.18 to 33.51	No	ns	0.9042			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	t	DF
p3+Cu Jan - p3Cu April								
0	120.3	74.67	45.67	9.91	6	6	4.608	40
0.5	37.83	110.3	-72.5	9.91	6	6	7.316	40
2.0	31	64.17	-33.17	9.91	6	6	3.347	40
4.0	21	13.33	7.667	9.91	6	6	0.7736	40

The time kill assay extended to the "sub-MICs" of p1 and p3 of copper(II) salts of 2uM and 4uM respectively, in copper(II) alone samples treatment. At t=0 to t=4, time point as previously done for the peptides and their complexes. The data curve (Figure 13) simply showed that at those copper concentrations, there were no significant killing of the R20291 *C. difficile* vegetative cells. The curve resulted from CFU/mL enumeration after 24 hours on each of the plates. The curve showed that copper(II) at 2uM and 4uM had an initial impact, but, the vegetative cells were able to recover as time went on.

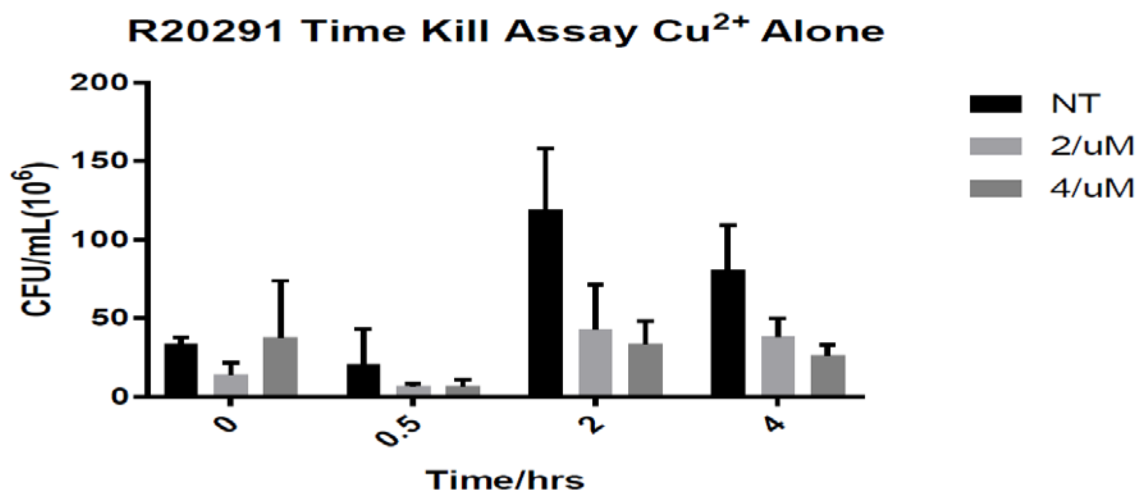


Figure 13: The time kill curve Cu^{2+} at 2uM & 4uM. This help to monitor what happed to *C. difficile* vegetative cells' growth at each time point because of the copper concentration they are exposed to

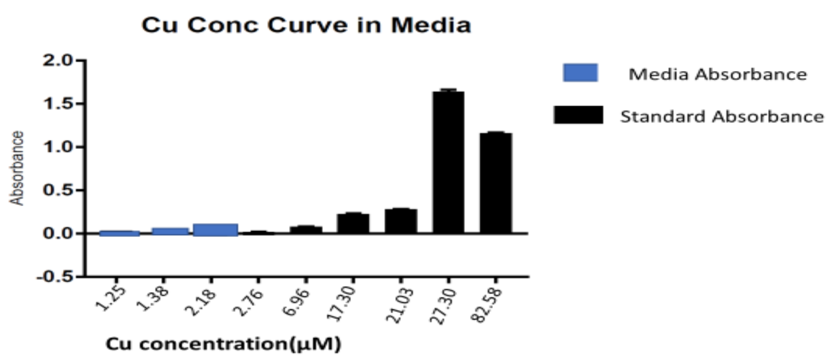


Figure 14: Copper concentration/µM curve as determined by FAAS in three different media. Concentration of Cu TY-medium is 1.24/µM, BHIS is 1.38/µM and LB 2.18/µM respectively

5.5 BIOFILM INHIBITORY ASSAY

5.5.1 BIOFILM ASSAY FOR PEPTIDES, COMPLEXES AND COPPER(II)

We measured the effect of piscidin peptides in the presence and absence of copper on *C. difficile* biofilm formation. Biofilm were grown anaerobically at 37° C for 24 hours and then quantitated by crystal violet staining. Figure 15 showed the amount of biofilm formation in the treated and untreated 24-well plates, to a final volume of 2mL in each well. In contrast to previous reports that piscidins disperse biofilm formation in anaerobic conditions, and that copper enhances this effect, our results showed. Stimulatory effect in the treated wells, compared to the untreated control. Figure 12 shows the highest biofilm formation in Cu^{2+} treated wells, to be higher than the rest. This appear to be due to the copper, as the peptides did not further increase biofilm formation.

Table 6 showed that the amount of untreated (NT) biofilm formed had no significant difference to the two peptides alone. More biofilm formation in the p1+Cu complex treated wells resulted into significant difference ($p < 0.001$) when compared to the NT and p3+Cu were not significant. However, any data comparison to copper(II) salt ($CuSO_4$) alone biofilm formation, showed significant difference ($p < 0.001$), consistently and convincingly. Other comparison not against $CuSO_4$ treated ones are not affirmative, but, when compared with the copper salt alone its straight forward significant difference.

This result prompted a lot of questions rather than answers. We continued to investigate the metallic oxidative stress response in *C. difficile* virulent strain *(R20291)* and high biofilm forming mutant strain *(RT527).* The comparison of p1+Cu to p3+Cu showed significant difference ($p = 0.0059$), this may be because of p1 and its complex having more of its bactericidal activities to be membrane specific rather than nuclease.

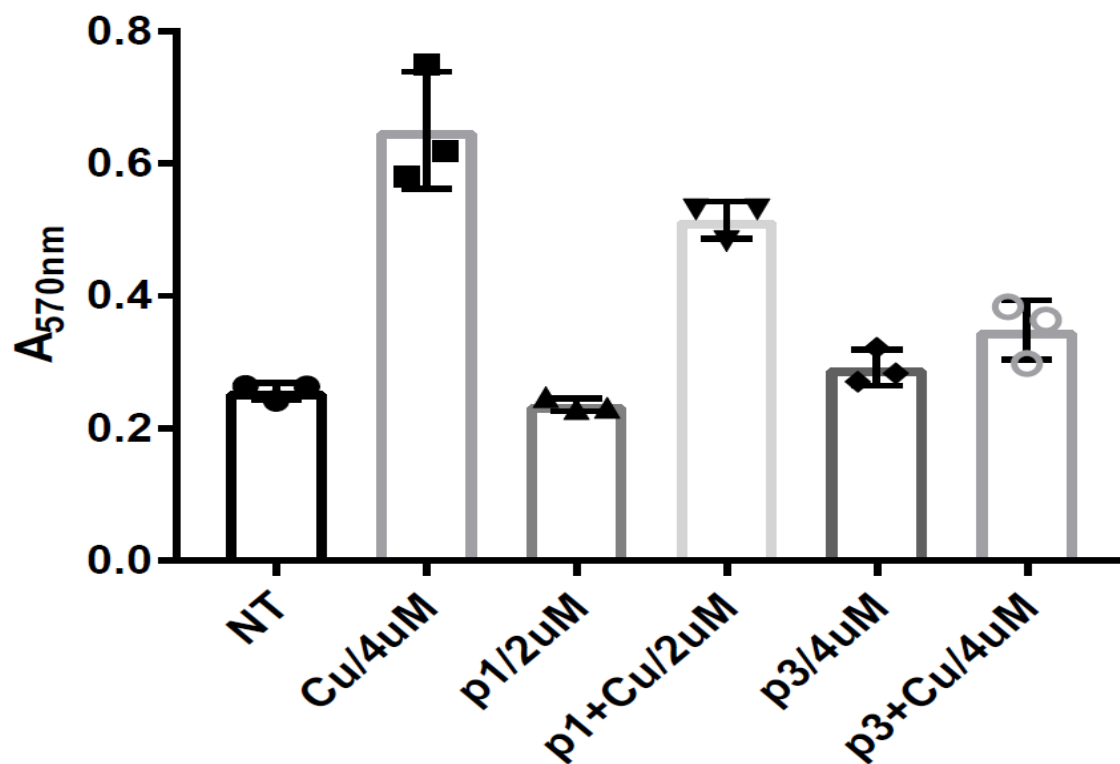


Figure 15: The amount of biofilm treated and the untreated control. Treated ones are peptides, peptide-copper complexes and copper(II) alone. NT in this figure represents the untreated control

Table 6: The data analyses of peptides and their complexes with copper salt alone

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
NT vs. Cu/4uM	-0.394	-0.5146 to -0.2734	Yes	****	<0.0001	A-B
NT vs. p1/2uM	0.02067	-0.09996 to 0.1413	No	ns	0.9909	A-C
NT vs. p1+Cu/2uM	-0.2587	-0.3793 to -0.138	Yes	***	0.0001	A-D
NT vs. p3/4uM	-0.03533	-0.156 to 0.0853	No	ns	0.9146	A-E
NT vs. p3+Cu/4uM	-0.09233	-0.213 to 0.0283	No	ns	0.1783	A-F
Cu/4uM vs. p1/2uM	0.4147	0.294 to 0.5353	Yes	****	<0.0001	B-C
Cu/4uM vs. p1+Cu/2uM	0.1353	0.0147 to 0.256	Yes	*	0.0251	B-D
Cu/4uM vs. p3/4uM	0.3587	0.238 to 0.4793	Yes	****	<0.0001	B-E
Cu/4uM vs. p3+Cu/4uM	0.3017	0.181 to 0.4223	Yes	****	<0.0001	B-F
p1/2uM vs. p1+Cu/2uM	-0.2793	-0.4 to -0.1587	Yes	****	<0.0001	C-D
p1/2uM vs. p3/4uM	-0.056	-0.1766 to 0.06463	No	ns	0.6368	C-E
p1/2uM vs. p3+Cu/4uM	-0.113	-0.2336 to 0.007629	No	ns	0.0712	C-F
p1+Cu/2uM vs. p3/4uM	0.2233	0.1027 to 0.344	Yes	***	0.0005	D-E
p1+Cu/2uM vs. p3+Cu/4uM	0.1663	0.0457 to 0.287	Yes	**	0.0059	D-F
p3/4uM vs. p3+Cu/4uM	-0.057	-0.1776 to 0.06363	No	ns	0.6208	E-F

5.6 BIOFILM METALLIC STIMULATORY EFFECT

We measured the effect various copper(II) salts on biofilm formation in *C. difficile R20291* and *RT527,* or *R20291* pDccA, a strain with elevated c-di-GMP levels and high biofilm formation (33). The *C. difficile* strains were exposed to gradient concentrations of copper(II) salts. These concentrations appear to stimulate more biofilm formation, in direct relation to increased concentration. The biofilm production almost showed linear proportionality (Figure 16 and 17) the curve of the two strains in the increase Copper(II) concentration.

Table 7 and 8 respectively showed no statistically significant differences due to copper treatment. The figures however, showed trend towards increase biofilm formation response to increases in copper(II) salt concentrations when compared to untreated well. In RT527, known for its large biofilm formation capability, the copper(II) salt gradient concentration was from 0.25/µM to 2/µM. However, in further investigations the concentration level may be raised, but at these concentrations the figure showed the trend, but differences were not statistically significant. The trend did concur with that seen in the initial piscidin with or without copper experiments (Figure 12).

Investigations continued with the usage of other copper(II) salts, $CuSO_4$, $CuCl_2$ and $Cu(NO_3)_2$ respectively. The stimulatory effect appeared to be the same in *R20291* and *RT527*. Figure 18 shows the different copper(II) salts curves. The figure showed a mild stimulatory effect, but, according to the data analyses (Table 9), there were no significant differences in the amount of biofilm formed. Notably, addition of any type of copper(II) salts do not inhibit biofilm formation in *C. difficile* rather it appears to stimulate more biofilm formation in all experimental results as seen in figure 18. So, the anaerobic compared to aerobic antimicrobial activities of copper and other transition metal merit further investigations.

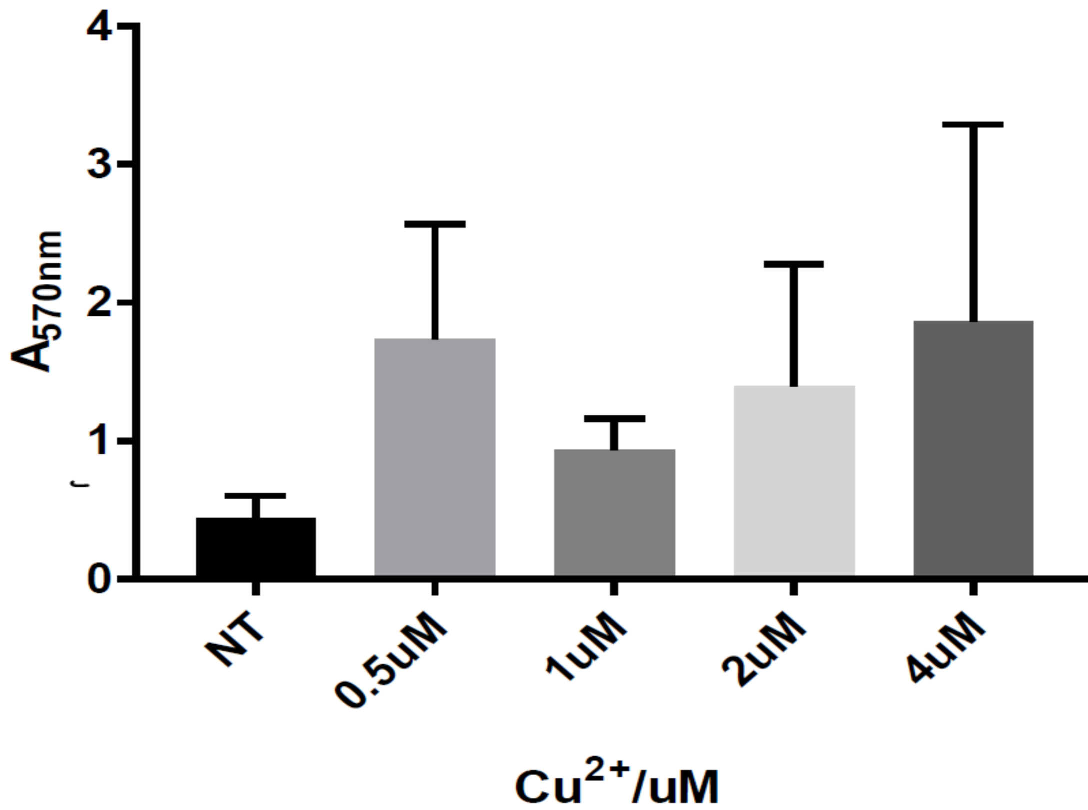


Figure 16: The copper salt (Cu^{2+}) alone concentration gradient for biofilm quantitative analysis of *R20291 C. difficile* strain

Table 7: Copper salt alone biofilm data formation analyses

NT vs. 0.5uM	-1.294	-3.14 to 0.5533	No	ns	0.2457	A-B		
NT vs. 1uM	-0.4965	-2.343 to 1.35	No	ns	0.9171	A-C		
NT vs. 2uM	-0.9545	-2.801 to 0.8923	No	ns	0.5217	A-D		
NT vs. 4uM	-1.419	-3.265 to 0.4283	No	ns	0.1767	A-E		
0.5uM vs. 1uM	0.797	-1.05 to 2.644	No	ns	0.6764	B-C		
0.5uM vs. 2uM	0.339	-1.508 to 2.186	No	ns	0.9780	B-D		
0.5uM vs. 4uM	-0.125	-1.972 to 1.722	No	ns	0.9995	B-E		
1uM vs. 2uM	-0.458	-2.305 to 1.389	No	ns	0.9366	C-D		
1uM vs. 4uM	-0.922	-2.769 to 0.9248	No	ns	0.5533	C-E		
2uM vs. 4uM	-0.464	-2.311 to 1.383	No	ns	0.9337	D-E		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
NT vs. 0.5uM	0.4423	1.736	-1.294	0.5981	4	4	3.059	15
NT vs. 1uM	0.4423	0.9388	-0.4965	0.5981	4	4	1.174	15

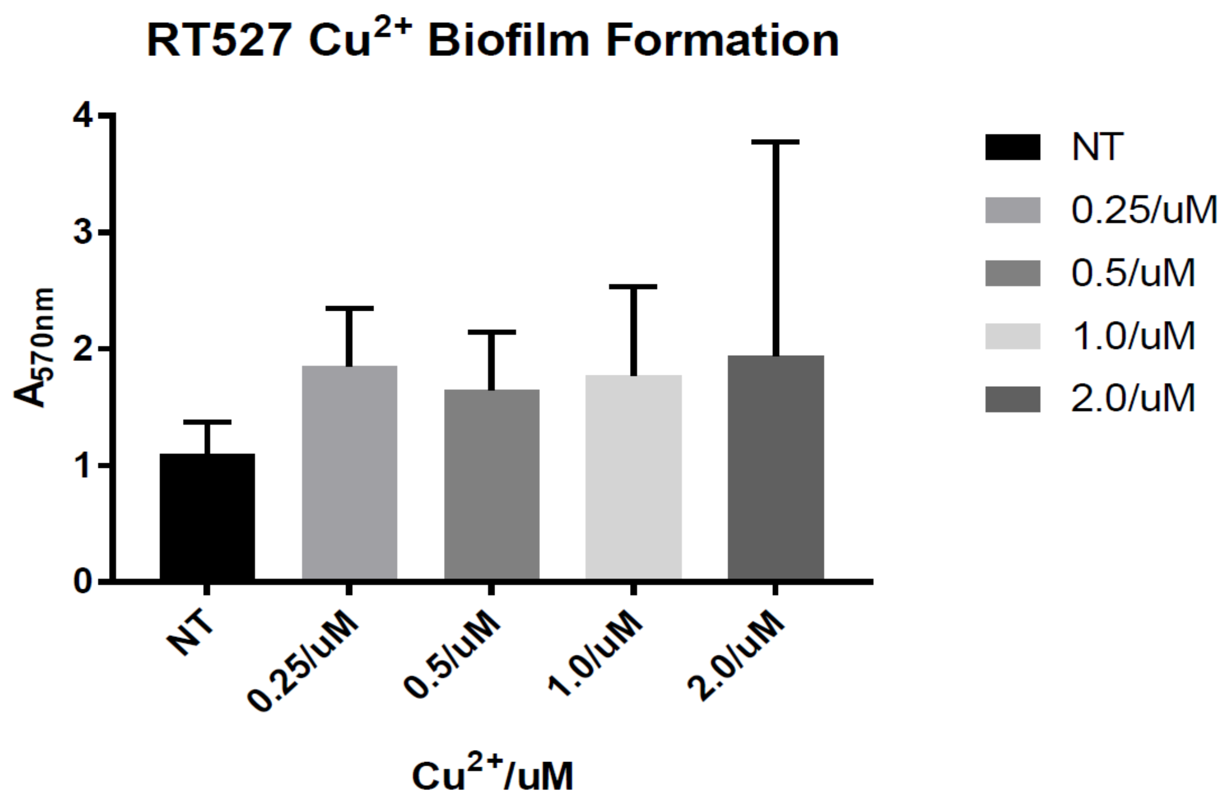


Figure 17: The copper(II) salt (Cu^{2+}) biofilm quantitative for *RT527 C. difficile* strain

Table 8: The data analyses of gradient concentrations of copper(II) salts on RT527 *C. difficile* strain

Table Analyzed	Data 1				
Data sets analyzed	A : NT	B : 0.25/uM	C : 0.5/uM	D : 1.0/uM	E : 2.0/uM
ANOVA summary					
F	0.2467				
P value	0.9001				
P value summary	ns				
Significant diff. among means (P < 0.05)?	No				
R square	0.1649				
Brown-Forsythe test					
F (DFn, DFd)	2.371e+031 (4, 5)				
P value	<0.0001				
P value summary	****				
Are SDs significantly different (P < 0.05)?	Yes				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.8862	4	0.2216	F (4, 5) = 0.2467	P=0.9001
Residual (within columns)	4.49	5	0.8979		
Total	5.376	9			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	10				

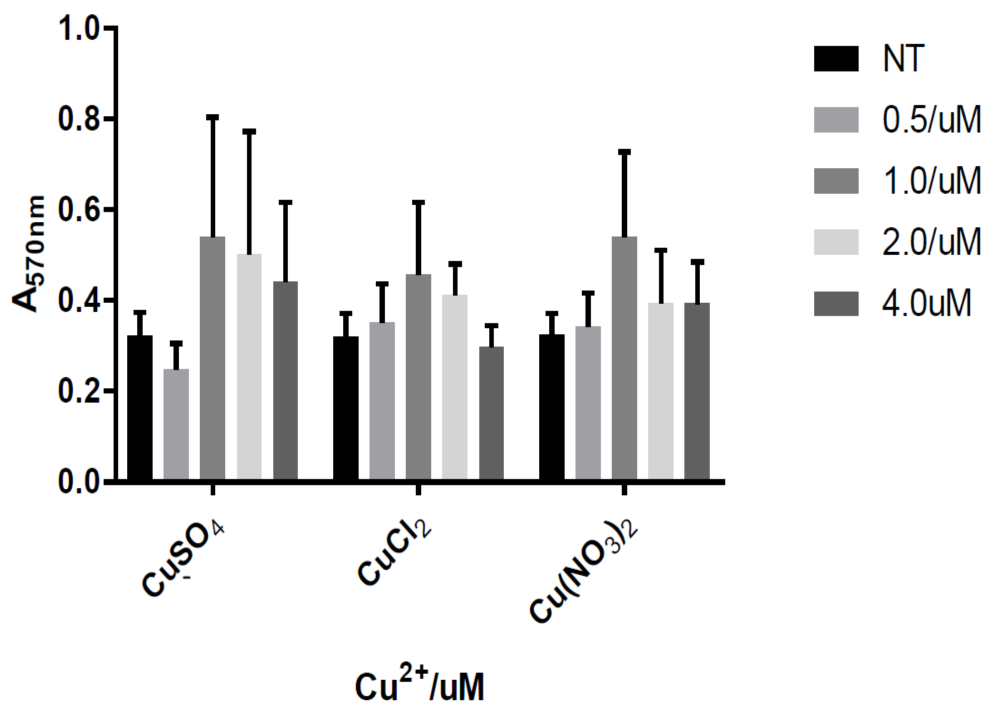


Figure 18: The different Cu^{2+} biofilm quantitative effect curve. Each bar from NT to 4uM represent $CuSO_4$, $CuCl_2$ & $Cu(NO_3)_2$ respectively and NT represent the untreated control

Table 9: The data analyses of different copper salt biofilm formation

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CuSO4					
NT vs. 0.5/uM	0.07325	-0.2042 to 0.3507	No	ns	0.9433
NT vs. 1.0/uM	-0.217	-0.4945 to 0.06049	No	ns	0.1903
NT vs. 2.0/uM	-0.1805	-0.458 to 0.09699	No	ns	0.3596
NT vs. 4.0uM	-0.1185	-0.396 to 0.159	No	ns	0.7437
0.5/uM vs. 1.0/uM	-0.2903	-0.5677 to -0.01276	Yes	*	0.0363
0.5/uM vs. 2.0/uM	-0.2538	-0.5312 to 0.02374	No	ns	0.0877
0.5/uM vs. 4.0uM	-0.1918	-0.4692 to 0.08574	No	ns	0.3001
1.0/uM vs. 2.0/uM	0.0365	-0.241 to 0.314	No	ns	0.9957
1.0/uM vs. 4.0uM	0.0985	-0.179 to 0.376	No	ns	0.8501
2.0/uM vs. 4.0uM	0.062	-0.2155 to 0.3395	No	ns	0.9685
CuCl2					
NT vs. 0.5/uM	-0.031	-0.3085 to 0.2465	No	ns	0.9977
NT vs. 1.0/uM	-0.1375	-0.415 to 0.14	No	ns	0.6259
NT vs. 2.0/uM	-0.0925	-0.37 to 0.185	No	ns	0.8768
NT vs. 4.0uM	0.022	-0.2555 to 0.2995	No	ns	0.9994
0.5/uM vs. 1.0/uM	-0.1065	-0.384 to 0.171	No	ns	0.8104
0.5/uM vs. 2.0/uM	-0.0615	-0.339 to 0.216	No	ns	0.9695
0.5/uM vs. 4.0uM	0.053	-0.2245 to 0.3305	No	ns	0.9823
1.0/uM vs. 2.0/uM	0.045	-0.2325 to 0.3225	No	ns	0.9904
1.0/uM vs. 4.0uM	0.1595	-0.118 to 0.437	No	ns	0.4847
2.0/uM vs. 4.0uM	0.1145	-0.163 to 0.392	No	ns	0.7668
Cu(NO3)2					
NT vs. 0.5/uM	-0.018	-0.2955 to 0.2595	No	ns	0.9997
NT vs. 1.0/uM	-0.2158	-0.4932 to 0.06174	No	ns	0.1950
NT vs. 2.0/uM	-0.06925	-0.3467 to 0.2082	No	ns	0.9534
NT vs. 4.0uM	-0.06825	-0.3457 to 0.2092	No	ns	0.9557
0.5/uM vs. 1.0/uM	-0.1978	-0.4752 to 0.07974	No	ns	0.2710
0.5/uM vs. 2.0/uM	-0.05125	-0.3287 to 0.2262	No	ns	0.9844
0.5/uM vs. 4.0uM	-0.05025	-0.3277 to 0.2272	No	ns	0.9855
1.0/uM vs. 2.0/uM	0.1465	-0.131 to 0.424	No	ns	0.5678
1.0/uM vs. 4.0uM	0.1475	-0.13 to 0.425	No	ns	0.5613
2.0/uM vs. 4.0uM	0.001	-0.2765 to 0.2785	No	ns	>0.9999

CHAPTER 6

CONCLUSIONS

The characterization of the *Clostridium difficile* oxidative stress response and antimicrobial effect of piscidin peptides and complexes on these bacteria was determined in anaerobic conditions. The p1 has a lower MIC (4µM) than p3 (8µM) but both the piscidins are active antimicrobial peptides in anaerobic conditions against *Clostridium difficile*. Their antimicrobial activities include growth inhibition and permanent bacterial killing. The killing rate in the absence of oxygen *invitro* gave these confirmatory results. In the time kill assay, p1 and its copper complex had started killing immediately after treatment while it takes 30 minutes for p3 and its copper complex to show any bactericidal activities. The estimation of number of colony forming unite (CFU) at each time points till the fourth hour, was that the total number of CFU in p1 and its complex compared to p3 and its complex. As a result of these data analyses p1 appear to be a slightly better antimicrobial agent than p3 in an anaerobic environment. This is consistent with what was previously observed aerobically but the lack of synergy between piscidins and copper is novel to this work.

In the absence of piscidin, copper(II) alone at the sub-MICs of both peptides' p1 and p3 which were 2uM and 4uM sub-MICs respectively. At these concentrations there was no significant killing of the bacteria. Within half hour exposure of the bacteria, the metallic oxidative stress inferred from the CFU plot at this time point, showed lower number of viable cells. However, the bacteria cells were able to overcome the stress within two hours and the CFU showed they had recovered from the oxidative stress.

The peptides in comparison to their complexes show no significant difference in their bacteria killing rates. So, the bactericidal activities of both peptides are not enhanced by the

presence of copper(II) ion as the time progressed in the anaerobic environment. This is a direct contrast to what was observed in Gram-positive and Gram-negative bacteria in aerobic environment by Daben J. Libardo et al in 2017.

The lab mixed and pre-complex "piscidin complexes" had approximately the same end results in their killing rates, confirming that the peptides are not failing to form the complexes in those conditions tested. This result confirmed that the mixed copper(II) salts are not used up by any other *C. difficile* metabolic activities and they got to the targets in the media. This means that while the piscidin peptides and copper ions can both induce stress in *C. difficile*, the peptides and copper ions do not exhibit the same synergy in an anaerobic environment that they do aerobically.

The biofilm prevention assay appears to have different result in anaerobic environment to the bacteria biofilm dispersal assay in aerobic environment as reported. It was previously report that modest inhibitory activities occur when peptides alone are used for biofilm inhibitory assay. The same assay in the presence of peptide complex, showed stronger biofilm clearance was reported in aerobic condition. In anaerobic conditions peptides and their complexes do not show any biofilm inhibitory effect, but, the peptide complexes and metal alone at sub-MICs tend to show stimulatory effect. So, *C. difficile* appears to respond to oxidative stress through formation of more biofilm to protect themselves from the reactive oxygen species generated in the oxidative stress process. The investigations up to this point support the conclusion that the peptides' antimicrobial mechanisms and metallic ion interactions appear to be different based on their environment and their targets. So, drugs design based of these peptides, the designer must keep in mind the environment and the physiological target of the peptides. The aerobic and anaerobic biofilm formation in *E. coli* strains are really promising and this among others will be forming the bases of my PhD research, in fall.

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The word processor for the thesis was Microsoft word 2016. Graphs and tables were made using Prism 2016. Chemical scheme was created by ChemDraw Pro 13.0