UNIVERSITY OF
LINCOLN

EVALUATING THE IMPACT OF ALTERNATIVE ANTIMICROBIALS ON BIOFILMS FORMED BY *Clostridium perfringens*

May Oyairibhor Omoigberale

Doctor of Philosophy

2021

EVALUATING THE IMPACT OF ALTERNATIVE ANTIMICROBIALS ON BIOFILMS FORMED BY *Clostridium perfringens*

May Oyairibhor Omoigberale

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

School of Life Sciences, College of Science,

University of Lincoln

December 2021

TABLE OF CONTENTS

Contents

DEDICATION

This thesis is dedicated to God almighty, my dear husband Jonathan Omoigberale and my lovely children Nita Omoigberale, Nannette Omoigberale and Netanel Omoigberale.

CERTIFICATE OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this thesis, that the original work is my own, except where specified in the acknowledgements and in references. Neither the thesis nor the original work contained therein has been previously submitted to any institution for a degree.

May Oyairibhor Omoigberale

ACKNOWLEDGEMENTS

I am most grateful to God Almighty for the gift of life and for His Grace that saw me through this research work.

I would like to appreciate my supervisor Dr Ronald Dixon for his motivation, advice, guidance, and numerous suggestions. This thesis would not have been completed without his support. Many thanks also to my co-supervisor, Dr. Lorna Lancaster.

My deepest gratitude goes to my lovely and supportive husband, Jonathan Omoigberale for his prayers, moral and financial support, for believing in me and for all the sacrifices made towards the completion of this research. My sincere appreciation to my daughters Nita and Nannette Omoigberale and my son Netanel Omoigberale for their understanding, courage, and perseverance throughout this PhD journey.

I would like to appreciate Dr. Joe Brown for his help in the lab, for providing the bacterial isolates used for this study and for providing the data on genome sequencing. I also would like to thank Philip Godon of Idonis Ltd for providing the antimicrobial solution (Acquorsol) used in this project. I am immensely grateful to PhD colleagues Joe Edward and Sammy Kay for the help and support they gave me in the laboratory.

Thanks to Julia Steele for training me on the SEM, Karen Staines, and Alex Aitken for supporting me with the confocal microscope, Nabilah Zunaidi for helping with the rotary evaporator, Nadia Andreani for assisting in the lab, Ratheesh Kallivalappil for assisting with the statistical analysis and to all staff and students at the School of Life Sciences who contributed to the success of this study.

Thanks to my parents, Mr Anthony Omionawele and Mrs Maria Omionawele for their provision, moral, physical, and spiritual support. I also want to appreciate my siblings; Anthonia Okoobo, Peter Omionawele, Rex Omionawele, Derek Omionawele and my in-laws; Dr. (Mrs.) Felicia Adeghe and Anita Adeghe for all the help rendered to my family whilst I was in the UK.

Finally, I would like to appreciate Tertiary Education Trust Fund (TETFUND) Nigeria for sponsoring this research and Ambrose Alli University Nigeria for approving my study leave.

LIST OF FIGURES

LIST OF TABLES

CONFERENCE PRESENTATIONS

Omoigberale, M.N.O. and Dixon, R. A. (2019.) *Clostridium perfringens:* Biofilm characterisation and antibiotics susceptibility profile. In: *American Society of Microbiology conference*, 20th-24th June, San Francisco.

Omoigberale, M. N. O. and Dixon R. A. (2019). Effect of biofilm on antibiotic resistance. In*: University of Lincoln, postgraduate research showcase Conference*, 20th, February, Lincoln UK.

Omoigberale, M. N. O. and Dixon, R.A. (2018). Biofilm formation and antibiotic susceptibility pattern of planktonic and biofilm grown *Clostridium perfringens in vitro.* In*: East Midland Doctoral Conference*, 19th September, Bishop Grosseteste University Lincoln, UK.

Omoigberale, M.N.O. and Dixon, R.A. (2018). Impact of biofilm on *Clostridium perfringens* antimicrobial tolerance and enhanced anti biofilm activity of gold nanoparticles. In: *National PhD Training in Antimicrobial Inaugural Conference*, 15th August, Bristol, UK.

Omoigberale, M. N. O. and Dixon, R.A. (2018). Impact of silver nanoparticles on *Clostridium perfringens* biofilm. In*: University of Lincoln, postgraduate research showcase Conference, 21st,* February, Lincoln UK.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BHI	Brain heart infusion
CBD	Calgary Biofilm Device
CDC	Centre for Disease Control and Prevention
CFU	Colony forming unit(s)
CLSI	Clinical and Laboratory Standards Institute
CLSM	confocal laser scanning microscope
DNA	deoxyribonucleic acid
EPS	Extracellular polymeric substance
g	gram
h	hour(s)
KPB	potassium phosphate buffer
MBCs	minimum bactericidal concentrations
MBECs	minimum biofilm eradication concentrations
MBIC	Minimum biofilm inhibitory concentration
Mg	milligram
MICs	minimum inhibitory concentrations
Min	minutes
ml	millilitre
NA	nutrient agar
NaCl	sodium chloride
NE	Necrotic enteritis
NEC	Necrotizing enterocolitis
NetB	Necrotic enteritis beta-like toxin
NICU	neonatal intensive care unit
NP	nanoparticles
OD	optical density
PBP	penicillin-binding protein
PBS	phosphate buffered saline.
PI	propidium iodide
PIA	polysaccharide intercellular adhesin
QM	Queen Mary's hospital
QS	quorum sensing
SEM	scanning electron microscopy
TSC	tryptose sulphite cycloserine agar
VLBW	very-low-birthweight
W/V	weight/volume
µg	microgram
µl	microliter
°C	degree Celsius

ABSTRACT

Clostridium perfringens is a Gram-positive, anaerobic bacterium that is a pathogen in humans and animals. *C. perfringens* has been associated with humans causing gas gangrene, food poisoning, necrotizing enterocolitis (NEC) in very premature neonates and necrotic enteritis (NE) in poultry. Although it is well established that pathogenic bacteria form biofilms extensively in nature to survive and resist adverse conditions by attaching to each other and to a substrate with extracellular polymeric substance (EPS). Biofilm formation in anaerobic organisms is under-researched.

One of the objectives of this study was to compare the biofilm-forming potential of *C. perfringens* isolates from broiler chickens, free-range poultry environments and neonatal humans using the traditional protocols of crystal violet staining assays in microtiter plates. In addition, the susceptibility of *C. perfringens* to a range of veterinary or human antimicrobial drugs in conventional or biofilm modes were tested using the broth microdilution method. All fifty-four (54) *C. perfringens* isolates tested from a variety of sources were shown to form biofilms. 7/54 (13%) were strong biofilm producers, 31/54 (57%) were moderate biofilm producers and 16/54 (30%) were weak biofilm producers according to current criteria. There was no significant difference observed in the density of biofilms formed by isolates from different sources but out of the 9 isolates tested previously for the presence of adhesin genes (virulence factors/appendages that facilitates bacteria attachment to host cell/surfaces), moderate biofilm producers harbored multiple adhesin genes whilst weak biofilm producers harbored only one adhesin gene each. As anticipated, the formation of biofilms significantly protected *C. perfringens* from the action of the antibiotics and resistance ranged between 83% to 100%.

Another objective of this study was to determine the *in vitro* activity of silver and gold nanoparticles against *C. perfringens* established biofilm. The antibiofilm activity of silver and

gold nanoparticles alone and the enhanced antibiotic effect of antibiotics in the presence of nanoparticles were determined by enumerating viable cells on agar plates after antimicrobial treatment. Treatment of *C. perfringens* with silver nanoparticles resulted in 19% to 58% (1.3log to 4.8log) biofilm reduction while treatment with gold nanoparticles recorded 11% to 39% (0.7log to 3.2log) biofilm reduction. Combination of antibiotic and nanoparticles improved biofilm reduction compared to antibiotic alone, but this was not generally significant at the concentration tested.

The third objective of this study was to test the antibiofilm potential of the ethanolic leaf extract of three medicinal plants (*Vernonia amygdalina, Ocimum gratissimum* and *Azadirachta indica*). The study showed that the plant's extracts inhibited or reduced biofilm differently for each tested *C. perfringens* isolate at the concentration tested. Using the viable count method to determine the effect of treatment showed minimal (2%/0.1 log) to good (28% /2.5 log) biofilm inhibition and reduction.

The final part of this study was aimed at testing the antibacterial and antibiofilm activity of a recently developed antimicrobial (QAC) solution (*Acquorso*l) on *C. perfringens*. The antibacterial and antibiofilm activity of *Acquorsol* was determined by enumerating viable cells using total viable count assay. 50% to 0.1% of A*cquorsol* solution completely inhibited the growth of four *C. perfringens* planktonic grown strains while 50% to 0.78% of *Acquorsol* solution completely prevented the growth of four *C. perfringens* biofilm grown strains. 50% to 6.25% of Acquorsol inhibited the growth of 100% of tested planktonic strains while 50% concentration of *Acquorsol* prevented the formation of *C. perfringens* biofilm in 91% (30 out of 33) of tested strains.

In summary, the results in this thesis show that *C. perfringens* grown in biofilm mode independent of the source of isolation could reduce the effective susceptibility of antimicrobials. Furthermore, it showed that alternative therapeutic strategies have the potential to control biofilm related contamination and infections in animals and people. These

alternative bacteriocides could be useful for decontaminating surfaces in hospital and may have a role in decontamination of skin surfaces and may be used in poultry environments as well as other public places.

CHAPTER: ONE

INTRODUCTION / LITERATURE REVIEW

1.1 Introduction

Clostridium perfringens is an encapsulated spore forming Gram-positive anaerobic pathogen that is widely distributed in nature. Although an anaerobe, this species can survive under low oxygen tension and is found in vegetation, marine sediment, soil, air, water and as part of the microbiome of the gastrointestinal tract of humans and animals (Piet, 2015). *C. perfringens* produces numerous exotoxins like alpha (α), beta (β), episilon (ε), iota (I) and enterotoxin (CPE) which are responsible for diseases in humans and animals caused by the bacterium (Pantanleon *et al*., 2014; Uzal *et al.,* 2014; Ferreira *et al*., 2016; Olkowski, *et al.*, 2008; Bannam *et al*., 2011). In humans it causes gas gangrene; a soft tissue disease causing necrosis (Stiles *et al.*, 2014; Hassen *et al.*, 2015; Garcia and Heredia, 2011; Stevens *et al*. 2010) and it has been suggested as the possible cause of necrotizing enterocolitis NEC); a severe inflammatory disease that affects the intestinal lining of very premature infants approximately 7% of the time with less than 1.5kg birth weights (Lin and Stoll, 2006, Neu *et al*., 2011; Shulhan *et al*., 2017; Agnoni *et al*., 2017). In poultry, *C. perfringens* is part of a complex aetiology of necrotic enteritis (NE) (Uzal *et al*., 2014; Timbermont *et al*., 2011; Keyburn *et al*., 2008). M'Sadeq *et al*., (2015); Skinner *et al*., (2010); Immerseel *et al*., (2004); Lovland and Kaldhusdal, (2001) have reported that NE is a significant disease of economic loss to the global poultry industry. Wade and Kayburn, (2015) reported that economic loss from NE increased from 2 billion US dollar as

estimated in the year 2000 to between 5-6 billion US dollar annually in prevention and control measures. The rise in the incident rate of the disease in some countries is due to the removal of antibiotic growth promoters (AGPs) especially when poor husbandry is identified (Shojadoost *et al.,* 2012; Stanley *et al*., 2012). Isolates of *C. perfringens* from animal origin can be transmitted to humans through the food chain especially in uncooked or poorly cooked meat (Garcia and Heredia, 2011; Brynestad and Granum, 2002).

There is a growing appreciation that the persistence of biofilms in the body contributes to the re-occurrence of diseases or chronic infections (Hall and Stoodley 2005). Generally, the ability of any bacterial species to form biofilms has been thought to significantly increase their persistence and enables them to adapt to environmental fluctuations (Davey and O Toole, 2000). The biofilm forming potential of *C. perfringens in vitro* was demonstrated for the first time by Vargal *et al*., (2008). Some reports have shown that formation of biofilms in *C. perfringens* is specifically associated with the pathogenicity of the microbe by increasing their resistance and tolerance to antimicrobial agents, environmental, physical, and chemical stress (Vargal *et al*., 2008; Charleboise *et al*., 2014; Charleboise *et al*., 2015). It is likely that pathogenic bacteria form biofilms extensively in nature to survive and resist adverse conditions by attaching to each other and to a substrate with the production of extracellular polymeric substance (EPS) but their formation by anaerobic organisms is under-researched and have been reported by Donelli *et al*.,(2012); Pantaleon, *et al*., (2014); Varga *et al*.,(2008), Obana *et al*., (2014), Vida *et al*.,(2015); Charleboise *et al*., (2014; 2016; 2017). Understanding of biofilm formed by *C. perfringens* is necessary for the development of novel and more efficient control methods since the organism is difficult to eradicate. The identification of *C. perfringens* isolates which readily form biofilm

from humans and animals will provide information on the potential of persistent infection in the health care, poultry industry and regulatory agencies for necessary mitigation strategies. Therefore, this study aims to characterize the biofilm forming potential of the anaerobic pathogen *C. perfringens* from diverse sources, to compare the susceptibilities of *C. perfringens* growing planktonically or in a biofilm to conventional antibiotics and to test the efficacy of different novel antimicrobial compounds on biofilm.

Literature review

1.2. The genus *Clostridium* / *Clostridium perfringens*

The genus *Clostridium* consist of over a hundred species, though most isolates fall within a few species. They are mainly anaerobic, spore-forming bacilli, some of which are medically important. Species of this genus have different biochemical properties, and some such as *C. perfringens* are aerotolerant anaerobe and can tolerate low oxygen tension. *Clostridium* species are ubiquitous, they are present in sewage, water, soil and as normal intestinal microbiota of humans and animals. Most *Clostridium* species are free living saprophytes while a few are opportunistic pathogens that have been associated with severe disease conditions with high mortality rate. Some of these species include *C. perfringens*, *C. difficile* (now known as *Clostridioides difficile* due to 16S rRNA gene sequence analysis), *C. histolyticum*, *C. novyi* and *C. septicum.* Diseases caused by the *Clostridium* genus include gas gangrene, tetanus, botulism, and antibiotic associated diarrhea (AAD) caused by *C. perfringens*, C*. tetani, C. botulinum* and *C. difficile,* respectively.

C. perfringens (formally *C. welchii*) was first described in 1892 by Welch and Nuttall when they isolated a Gram-positive anaerobe from a gangrenous wound (Hatheway, 1990). The organism is part of normal microbiome of the large intestine of humans

and animals. It is present in faeces and spores are frequently found in soil and vegetation. *C. perfringens* is listed as a 'Category B Bioterrorist agent' by CDC. It produces oval sub-terminal spores and Gram-stained smears appear as straight sided rods with blunt or truncated ends which are either single or paired.

1.3. Virulence / major toxins of *Clostridium perfringens*

In humans and animals, *C. perfringens* can cause different diseases most of which have an intestinal origin, from self-limiting food poisoning events to severe destruction of tissues. The pathogenicity of this organism is linked largely to its fast-doubling time, stress-resistant spores, and ability to produce numerous toxins and enzymes though no single strain can produce all toxins (Keyburn *et al*., 2008; Jihong *et al*., 2016). These lethal toxins are referred to as major or minor toxins and includes alpha (CPA), beta (CPB), episilon (ETX), Iota (ITX), enterotoxin (CPE), perfringolysin O (PFO)/theta, beta-2 (CPB2), TpeL, NetB, NetF, BecA, BecB, NanI, Nanj, Kappa, mu, Lambda, delta and α- clostripan (Ferreira *et al.,* 2016). They are classified into five toxin types designated A-E based on the production of four major exotoxins: α, β, ı and ε (see Table 1) (Songer 1996; Petit *et al*., 1999; Uzal *et al*., 2010). *C. perfringens* type A is a normal inhabitant of the gastrointestinal tract of humans. It is widely distributed in the environment and can be isolated from water, soil, sediments, dust, cooked and uncooked foods.

Most of the toxins are plasmid encoded and vary in size from approximately 45kb to approximately 140kb. Presence of these toxin genes in conjugative plasmids with insertion sequences enhances the mobility of these toxin genes which increases the virulence of *C. perfringens* diseases emanating from the intestine (Jihong *et al.*, 2013). Different diseases are caused by each group and infection is usually through a traumatic route into the body for the type A strains which are found in soil. Other routes

of infection are the fecal-oral route (ingesting food contaminated with spores) and dysbiosis (change in the gut microbiota) which can lead to excessive proliferation (or overgrowth) of *C. perfringens*. The second and last infection route are common to all toxin types.

Table 1.1: Classification of *Clostridium perfringens* based on the production of four major exotoxins

Toxin types	Exo-toxin			
	α	β	ε	ι
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

(+) indicates toxin production; (-) indicates no production of toxin.

C. perfringens produces various toxins which induce specific syndromes, and these toxins are produced during the exponential growth phase. The type of disease and the resulting symptoms is dependent on the strain of *C. perfringens* involved. Diseases caused by *C. perfringens* major toxins are shown in Table 2 below while the genetic location and mode of action of *C. perfringens* toxins are presented in Table 3.

Table 1.2: Diseases associated with *Clostridium perfringens* major toxins.
Source: (Stiles *et al*., 2013)

Toxin type (major toxins)	Associated diseases in humans	Associated diseases in animals
A (α)	Gangrene, food poisoning and antibiotic associated diarrhea	Myonecrosis in animals and necrotic enteritis in chicken and pigs
B (α, β and ε)		Dysentery in newborn lambs, hemorrhagic enteritis in neonatal calves and enterotoxaemia in sheep
C (α and β)	Necrotizing enterocolitis	Enterotoxaemia in sheep, necrotic enteritis in goat, calves, chicken and pigs
D (α and ε)		Enterotoxaemia in lamb, goat and calves.
E (α and ı)		Enterotoxaemia in calves and lamb

Table 1.3: Location of *Clostridium perfringens* toxins and their mode of action
Source: Kircanski, (2012)

Toxin/enzyme	Gene	Genetic location	Mode of action
Alpha	*Cpa*	Chromosome	Phospholipase C
Beta 1	*Cpb1*	Plasmid	Destruction of cell membrane
Beta 2	*Cpb2*	Plasmid	Destruction of cell membrane
Epsilon	*Etx*	Plasmid	Increases cell membrane permeability
Iota	*Iap*	Plasmid	Actin ADP – ribosylation
Iota	*Ibp*	Plasmid	Actin ADP – ribosylation
Enterotoxin	*Cpe*	Chromosome/plasmid	Pore forming ability
NetB	*NetB*	Plasmid	Pore forming
Theta	*pfoA*	Chromosome	Hemolysin specific to cholesterol

1.4. Major toxins of *Clostridium perfringens*

Alpha toxin (CPA)

C. perfringens alpha toxin is produced by all strains of *C. perfringens,* but the type A strains produce a very high amount of CPA compared to other toxin types. CPA consists of 370 amino acids (43kDa) and it has over 50% amino acid level similar to the phospholipases C from other species of *Clostridium* such as *C. novyi*, *C. baratii* and *C. bifermentans*. CPA is a zinc dependent phospholipase C that modifies cell membrane through degradation of phosphatidylcholine and sphingomyelin thereby causing cell damage. CPA enables *C. perfringens* to escape host phagocytes and promotes aggregation of *C. perfringens* which is responsible for blood vessel

blockage, anoxia and necrosis (Bryant *et al*., 2000). The genetic study has shown that the *Cpa* gene encodes the production of the alpha toxin and it is encoded by the chromosome unlike other toxins that are plasmid borne (Songer, 1996; Petit *et al*., 1999; Stiles *et al.*, 2013).

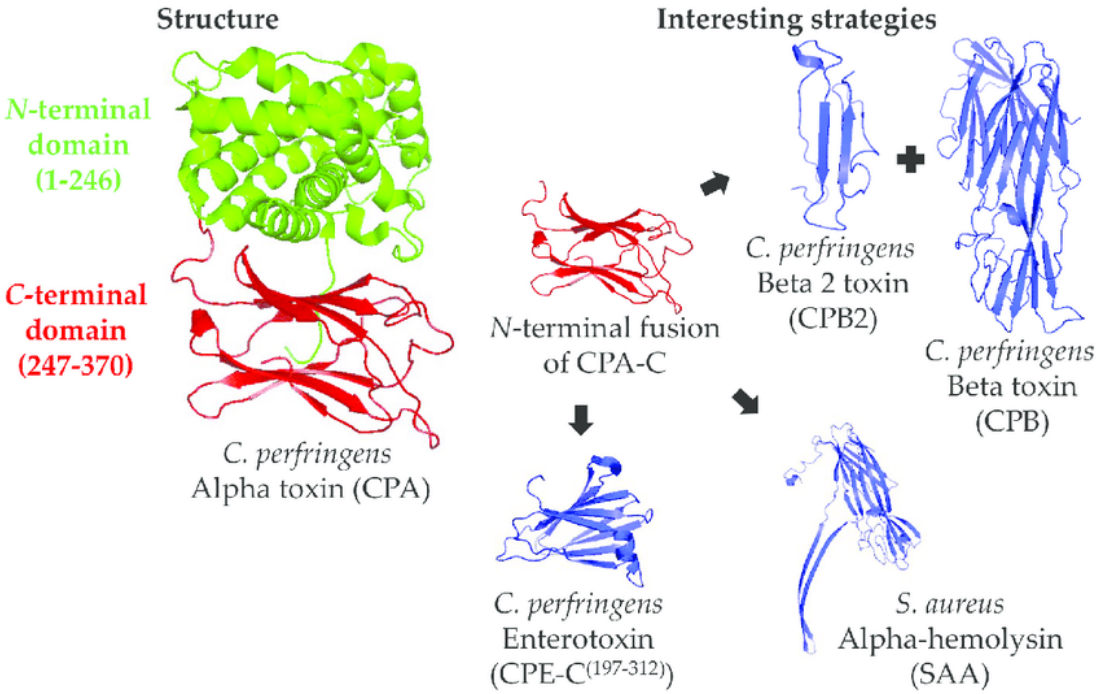


Figure 1.1: *Clostridium perfringens* alpha toxin structure

Source: Ferreira et al., (2016)

Beta toxin (CPB)

The *cpb* genes encodes the production of the CPB toxins - a thermolabile toxin. CPB toxin is a 35 kDa protein which shares similar sequence with alpha and gamma toxins of *Staphylococcus aureus* (Hunter *et al.*, 1993). The CPB toxin is responsible for 'pigbel' in humans - a disease that causes necrosis of the ileum and jejunum (Gui *et a*l., 2002). This disease is usually associated with the high consumption of meat by

people on a limited protein diet because of a low-basal level of pancreatic trypsin (Stiles *et al.,* 2013). Since high concentrations of protein in the intestinal tract encourages *C*. *perfringens* type B and C overgrowth, leading to lethal concentration of beta toxin which causes intestinal necrosis and bloody stools.

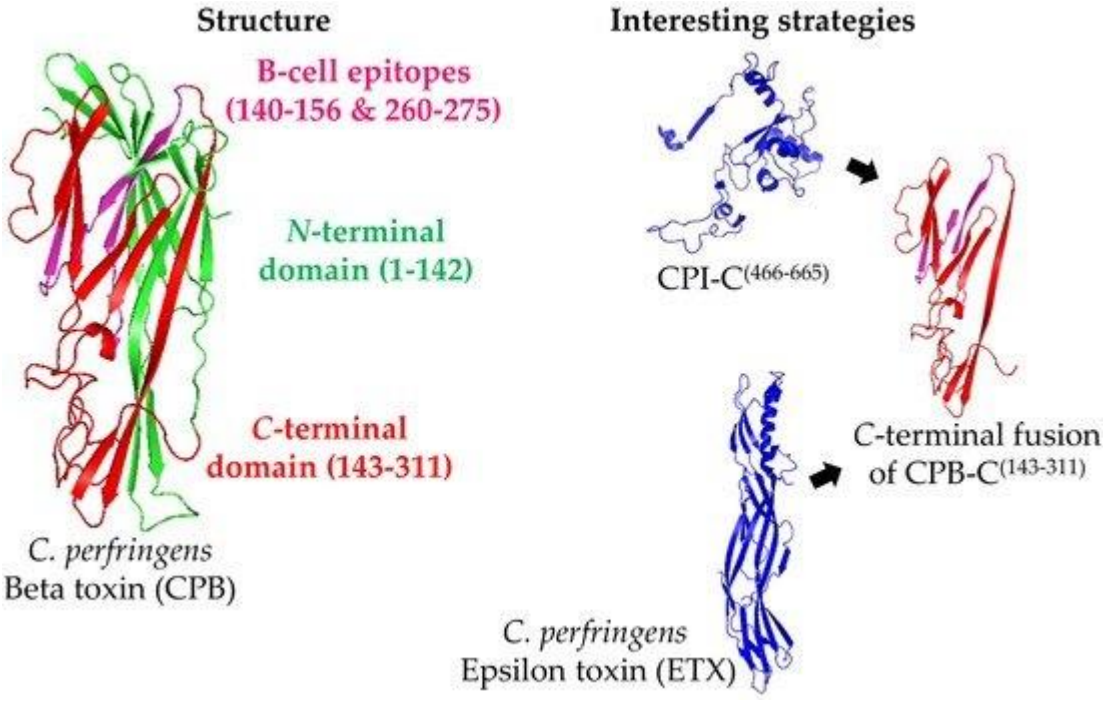


Figure 1.2: *Clostridium perfringens* beta toxin structure Source: (Ferreira *et al*., 2016)

Epsilon toxin (ETX)

C. perfringens ε-toxin is produced by type B and D strains (Petit *et al*., 1999). Type B causes dysentery in newborn lambs while Type D causes enterotoxaemia in sheep. It is considered the most potent clostridial toxin following tetanus and botulinum neurotoxins (Stiles *et al.*, 2013). ETX is located on a large plasmid and it is 32.7 kDa and matures when 13 or 14 basic amino acids are cleaved from its amino-terminal end (Rood 1998; Stiles *et al*., 2013). The precise role of the epsilon toxin like other toxins in the disease process is unclear. See Figure 1.2 for ETX structure.

Iota Toxin (ITX)

Iota toxin is a binary toxin found in *C. perfringens* type E. *C. perfringens* type E has been identified as the cause of diarrhoea in animals especially in domesticated livestock (Songer 1996; Petit *et al*., 1999). ITX is encoded by two genes (*Iap* and *Ibp)*. Iota toxin is composed of two independent polypeptides: Ia, is an ADP-ribosyltransferase and it has a molecular weight of approximately 47.5 kDa, and Ib, with a molecular weight of approximately 71.5 kDa which causes the binding and internalization of the toxin into the cell (Stiles *et al*., 2013). The light chain Ia is involved in ADP-ribosylation of globular skeletal muscle and non-muscle actin while the Ib heavy chain is needed for penetration of Ia into the cytosol (Sakurai *et al*., 2009).

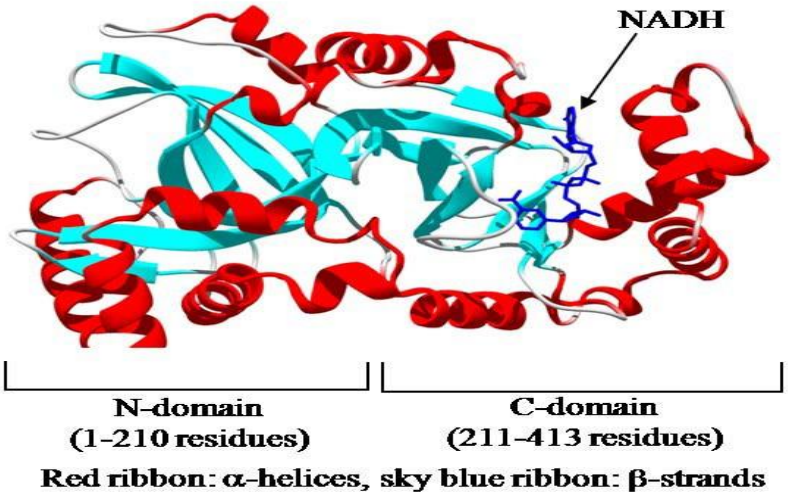


Figure 1.3: *C. perfringens* Iota toxin structure Source: (Sakurai *et al*., 2009)

Clostridium perfringens associated diseases (CPAD)

1.5. Gas gangrene in humans

Gas gangrene also known as clostridia myonecrosis and is an extensive and rapid necrosis and toxic infection of muscles/soft tissues accompanied by gas production caused by alpha and theta toxins produced by type A strains. Although several species of *Clostridium* (*C. novyi*, *C. sporogenes*, *C. septicum*, *C. tertium*, *C. histolyticum* and

C. bifermentans) can cause gas gangrene, *C. perfringens* is responsible for majority of gas gangrene cases (Verherstraeten *et al*., 2015). *C. perfringens* causes 80-95% of gas gangrene infections and is a serious traumatic and post-surgical wound infection that becomes fatal if it is not treated at the correct time (Buboltz 1999). Historically, *Clostridial myonecrosis* was a common wound infection during war with occurrence rate of 5% of wound infections. With improvement in wound care, use of antisepsis and antibiotics, the occurrence rate has reduced to 0.1% of war- wound infections since the Vietnam War era. Puncture wounds and surgical wounds, especially gastrointestinal tract surgeries done on the biliary tract or intestinal surgeries, remain causes of clostridial infections due to inadvertent inoculation of the surgical wound with gut bacteria including *C. perfringens*. The organism enters the human body through traumatized skin and the incubation period of this disease can be as low as 6h and it can progress to adjacent muscles several inches within an hour. The disease often leads to tissue death, shock and eventually death of host. Gas gangrene is usually characterized by absence of inflammatory cells in infection sites and accumulation of leukocytes between facial planes which results from vascular dysfunction and injury mediated by toxins. The perfringolysin O toxin causes leukocytes degeneration, cytolysis and polymorphonuclear cell destruction which leads to lack of inflammatory response during tissue necrosis and rapid spread of infection (Stevens *et al*., 2012; Steven and Bryant, 2017).

1.6. Necrotic enteritis (NE) in poultry

C. perfringens is a ubiquitous bacterium, and it is part of the normal gut microbiota of vertebrates (Songer, 1996; Porter, 1998). The organism is important in the development of necrotic enteritis along with other factors (Gholamiandehkordi *et al.,* 2007). The intestine of broilers suffering from necrotic enteritis contains up to 10^8-

10^9cfu/g of *C. perfringens* while healthy broilers, contains 0-10^5cfu/g of *C. perfringens* (Baba *et al*., 1997; Brown, 2018). Necrotic enteritis is caused by the toxins produced by types A and C strains of *C. perfringens*. The disease usually occurs in 4-week-old broiler chickens and affects poultry worldwide (Dahiya *et al.,* 2006). The plasmid-borne pore forming toxin NetB is an important virulence factor (Bannam *et al*., 2011; Prescott *et al*., 2016). The toxin causes necrotic lesions to develop in the intestines (ileum and jejunum) of poultry, makes holes in cell membranes and causes leakage of intestinal content. The intestine is covered with a yellow pseudomembrane, appears ballooned and contains a foul-smelling brown liquid (Van Immerseel *et al*., 2004).

Severe depression, ruffled feathers, sluggishness, drooping wings and head, diarrhea, drowsiness, anorexia, dehydration, and high mortality rate of flock are common signs of resulting enterotoxaemia. Craven, (2000); Nauerby *et al*., (2003) reported that high cell counts of *C. perfringens* in the intestine is not enough to cause NE disease. Pre-disposing factors are necessary for the disease to develop, and these include an intestinal environment that enhances the growth of the bacterium (*C. perfringens*) such as an intestinal infection by coccidial pathogen that damages the intestinal mucosa (Williams, 2005). Porter, (1998) reported that coccidiosis often occurs simultaneously or precedes outbreaks of necrotic enteritis. Collier *et al.,* (2008) in their article reports that Coccidiosis increases intestinal mucus production which then increases the availability of nutrient in the intestine which favors the proliferation *C. perfringens*. Furthermore, virulent strains of *C. perfringens* isolated from outbreaks of NE can produce bacteriocins (perfrin) (Timbermont *et al*., 2014). Bacteriocin production by these strains is capable of inhibiting growth of commensal strains thereby benefiting immensely from the high nutrient availability and then grow massively and release their toxins (Timbermont *et al.,* 2014). Studies have shown that *C. perfringens* and

Eimeria act synergistically to induce necrotic enteritis lesions (Baba *et a*l., 1997; Gholamiandehkordi *et al*., 2007). *Eimeria* parasites colonize the small intestine and damage the epithelial lining due to the intracellular stages of their life cycle. This creates gaps in the epithelial lining that allows the leakage of plasma proteins into the gut lumen. This is then utilized by *C. perfringens* as substrate for growth and subsequent disease (Van Immerseel *et al*., 2004). Olkowski *et al.,* (2006; 2008) pointed out the pivotal role of collagenolytic enzymes and collagenase produced by *C. perfringens* in the pathogenesis of NE. These enzymes are responsible for the morphological changes to the gut that appear during the early stages of the disease.

Another factor that influences the incidence of necrotic enteritis is diet. Diets high in undigestible, water-soluble non-starch polysaccharides increase the risk of necrotic enteritis in birds. Examples are wheat, rye, oat, and barley (Kaldhusdal and Skjerve, 1996; Craven, 2000; Jia *et al*., 2009). Also, high protein diets such as fishmeal, have been reported to favour the incidence of necrotic enteritis (Drew *et al*., 2004; Gholamiandehkordi *et al*., 2007). Generally, diets rich in protein contain high amounts of low digestible protein in the gastrointestinal tract which are used as substrates by the bacterium (Williams *et al*., 2001). The dietary fat source also affects *C. perfringens* growth. Animal fats promote *C. perfringens* counts compared to vegetable fat (Knarreborg *et al.,* 2002). Interestingly also, the physical form of the feed either mashed or pellet also determines the *C. perfringens* count in the intestine (Engberg *et al*., 2002). Pellet food reduce *C. perfringens* in intestine because there is high feed conversion ratio and little or no remains for *C. perfringens* to feed and proliferate. Another predisposing factor of birds to NE is stress because it could alter the intestinal environment and increase the risk of necrotic enteritis. Alterations in feeding pattern such as changing from starter diets to grower diets have been associated with necrotic

enteritis. Also, immunosuppressed birds that are suffering from diseases like chick anaemia virus, Gumboro disease or Marek's disease are more prone to gut infections (Engberg *et al*., 2002).

NE occurs as either acute clinical disease or sub-clinical disease. The acute clinical form of the disease is characterized by wet litter and a sudden high mortality rate. Death may occur in 1-2h and daily mortality may exceed 1% (Timbermont *et al.,* 2011). The chronic sub-clinical disease is mild, undetected and mortality is low but economically important since the birds fail to thrive and put on weight causing low productivity leading to high economic loss in the poultry industry (Wade and Kayburn, 2015).

Figure 1.4. Everted jejunal segment of broiler chicken showing gross lesion, obvious ulcers in the mucosa and necrotic tissues caused by *C. perfringens* infection. Source: (Keyburn *et al*., 2010)

1.7. Antibiotic use to control NE in poultry.

NE is an important problem in the poultry industry ~~due to~~ causing high economic losses due to high mortality rate, low weight gain, decreased feed conversion ratio, high cost of medication and risk of consumption of contaminated poultry product by humans (Timbermont *et al*., 2011). Control strategies for NE involves the use of antimicrobials

(McDevitt *et al*., 2006; Chan *et al*., 2015). For decades, antibiotics have been used to prevent NE and improve the yield of broilers. In general, antibiotics are used in animal feed, where they are either used for preventive or curative purpose. Several classes of antibiotics are used for control of *C. perfringens* infection in poultry and for growth promotion. Antibiotics used includes polypeptides (bacitracin), tetracyclines (chlortetracycline), β-lactams (penicillin), glycolipids (bambermycin), ionophores (salinomycin) and streptogramins (virginiamycin) (Diarra and Malouin 2014; Mehdi *et al*., 2018). Antibiotics are used greatly to improve poultry production, by (a) probably selectively changing and reducing the gut microbiome thereby increasing nutrient penetration through the intestinal wall (b) reducing bacterial fermentation of nutrient thus allowing for more nutrient absorption and (c) high feed conversion ratio through the reduction of NE subclinical infection. All these are deemed necessary to improve poultry health, nutrient availability, and overall poultry growth performance (M'Sadeq *et al*., 2015).

The use of antibiotics in the poultry industry raised concerns regarding the continued usage of antibiotics in antimicrobial growth promoters (AGPs) and how it has contributed to antimicrobial resistance in bacteria. This led to a re-evaluation on the use of antibiotics as a control strategy which eventually led to the ban of the use of in-feed antibiotics in poultry in European countries in 2006. With a ban of in-feed antibiotics, the incidence rate of NE has subsequently increased in European countries (Mcdevitt *et al*., 2006; Chan *et al*., 2015). Wade and Keyburn in 2015 reported that the ban of AGPs in Europe has increased the global economic loss in the poultry industry to range between 5 to 6 billion US dollar annually. With the ban of AGP, alternatives to antibiotics considered by poultry industries includes probiotics, prebiotics, enzymes, and organic acids (Dahiya *et al*., 2006).

1.8. Necrotizing enterocolitis (NEC) in humans

Necrotizing enterocolitis (NEC) is an inflammatory disease that causes extensive damage to intestinal tissues in neonates. This disease appears in premature infants and clinical NEC occurs when the lining of the intestinal walls dies off. The effect of this is inflammation of the intestine and if no proper intervention, it leads to perforation of the intestinal wall. In premature babies, this causes waste product and microorganisms in the intestine to pass through the wall of the intestine into the bloodstream or abdominal cavity progressing into a life-threatening disease. NEC is a disease that is almost exclusively confined to neonates during the first two weeks after birth. It is more common in very premature infants mostly those who are less than 32 weeks gestation period and those who have medical problems prior to birth (Heather *et al*. 2013).

The causes of NEC have not been clearly proven, but the overwhelming cause is immaturity. It is thought that a reduction in oxygen and blood flow to the intestine weakens the intestinal tissues, makes it lose its vigor and subjects it to damage by bacteria from food leading to perforation (Obladen, 2009). As a result, the intestine becomes highly immunoreactive which may lead to substantial increase in the inflammatory process causing systemic infections. If the central nervous system is affected, chances that such an infant will develop neurodevelopmental delays pertaining to the gastrointestinal tract are high. The chances that every infant who recovers from NEC will develop microcephaly and neurodevelopmental delays is 25% (Neu and Walker, 2011). The percentage mortality rate of NEC is about 20-30% and the occurrence of NEC based on NICU admission range from <1% to 5% and 5 in 1000 live births (Heather *et al*. 2013). The main factors involved in the complex

pathogenesis of NEC are ischemia, intestinal immaturity, intestinal microflora, enteral feeding, and inflammation.

1.9. Pathogenesis and clinical features of *Clostridium perfringens* food poisoning

C. perfringens food poisoning is a self-limiting, non-febrile disease. It is however one of the commonest causes of human gastrointestinal infection in the United States and Europe, costing millions of dollars in economic loss by staff absence each year. In the United States, between 1998 to 2002, *C. perfringens* was ranked as the third most frequently reported agent of human food-borne disease and the fifth leading cause of death from food poisoning (Lynch *et al*., 2006; Painter *et al*., 2006). *C. perfringens* type A have been associated with non-food-borne illness such as AAD (antibiotic associated diarrhoea) (Gorkiewicz, 2009; Joo *et al*., 2008; Modi and Wilcox, 2001). Further, it has been estimated that approximately 5% to 20% of all cases of AAD and 1non-food-borne sporadic diarrhoea are caused by *C. perfringens* type A.

Consumption of improperly cooked food contaminated with *C. perfringens* encourages the growth of spores in the nutrient rich gut environment which leads to the proliferation of large numbers of *C. perfringens* cells. Some of the cells ingested with the contaminated food are killed by the acidity of the stomach but some ingested cells can move to the small intestine where they reproduce and produce spores. The spores produced then release enterotoxins although some vegetative cells can also release enterotoxins. The enterotoxin is linked with the spore-coat though it is likely not to be an important structural component and it is released from the sporangium into the intestinal lumen when the sporangium is lysed. Symptoms of *C. perfringens* type A food poisoning usually starts 8 to 24hrs after consumption of contaminated food containing large number of *C. perfringens* vegetative cells. An estimated infective dose of 10^6-10^8 cfu/ml is required to cause illness by ingestion. The illness is usually

characterized by abdominal pain, diarrhea, and nausea. Vomiting is also a symptom though it is not frequent. Healthy individuals usually recover within 1-2 days without medical treatment (Uzal *et al*., 2014). However, it could be severe in the immunocompromised and immunosuppressed individuals such as the elderly. The disease is generally diagnosed by the delayed onset of symptoms compared to characteristic staphylococcal food poisoning and the detection of toxin in the faecal samples of patients if tested (Uzal *et al*., 2014).

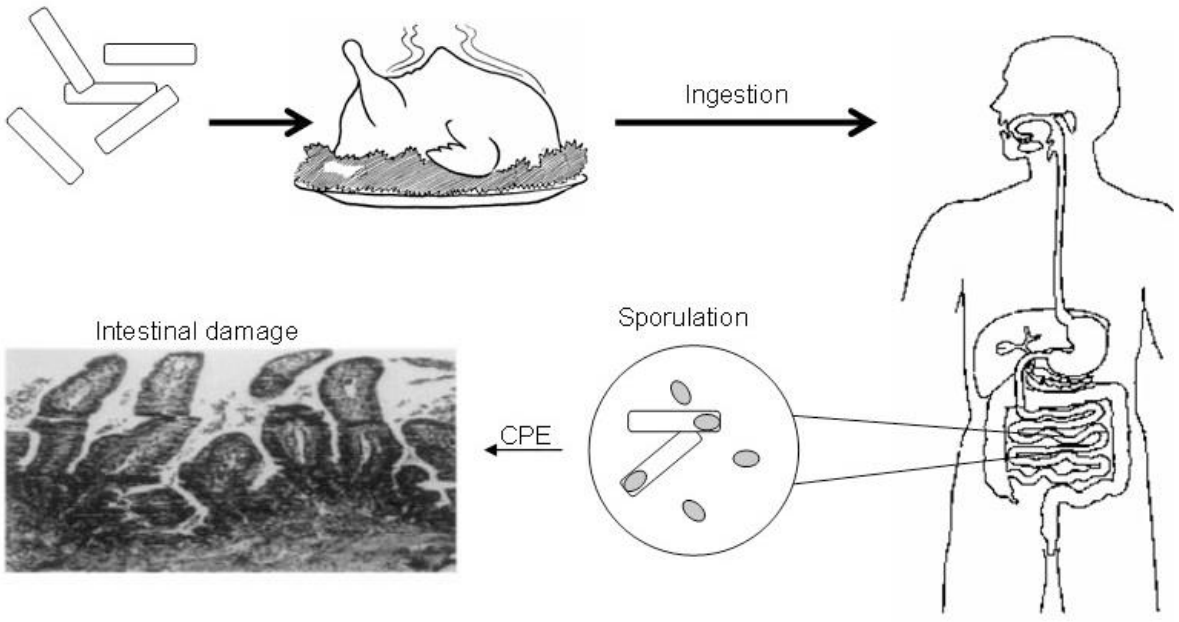


Figure 1.5: *C. perfringens* type A food poisoning. Source: (Huang, 2007)

Spores of *C. perfringens* that survive in improperly cooked food grow into vegetative cells and this results in ingestion of bacteria vegetative cells with food. Cells that survive the acidity of the stomach move to the intestine and sporulate. Spores release produce *Clostridium perfringens* enterotoxin (CPE) which is cytolytic causing diarrhoea.

.

1.10. Biofilms

Biofilms are sessile cells that are ubiquitous in nature. They can be found in industrial places, water channels, hospital settings and on living and inert surfaces (Jamal *et al.,* 2015; Donlan 2002). Biofilms were first observed by the Dutch researcher, Antonie

van Leeuwenhoek in the 17th century when he scraped his teeth surface and observed what he called animalculi with his primitive but compound microscope (Chandki *et al*., 2011, Costerton *et al.,* 1999).

Between 1980s and 1990s, it was observed that bacterial cells attached to surfaces did so in an organized pattern. A single bacterial cell can merge with several other cells which in turn aggregate with other cells leading to the formation of a dense microbial community that is encased in a self-produced extracellular polymeric substance (EPS) (Donlan and Costerton, 2002). Biofilm structure can either be simple or complex organization consisting of single or multiple species of organisms. The structure of biofilms is influenced by the type of organism in the biofilm and nutrient availability. Though this complex structure provided by biofilm results from communication and interaction among biofilm cells, it has conferred pathogenic properties to such cells (Hogan and Kolter 2002). Different biofilms display different physical and chemical properties which contribute to their unique characteristic making them difficult to destroy (Chandki *et al.,* 2011). The structural characteristics of biofilms and the physiological state of cells within the biofilms makes them highly recalcitrant to antimicrobial agents (Lewis 2001; Jamal *et al.,* 2018).

Components of the biofilm matrix are DNA (<1%), proteins (<1-2%), polysaccharide (1-2%), RNA (<1%) and water (97%) which makes up the largest component of the EPS (Charleboise *et al.,* 2014; Flemming *et al.,* 2007; Sutherland 2001). Water availability is key for the flow of nutrient within the matrix (Jamal *et al.,* 2015). The composition of the matrix makes biofilms resistant to antimicrobials and host immune cells (Flemming *et al.,* 2007). The chemical and physical composition of exopolysaccharides varies in different biofilms and is dependent on the biofilm growth stage (Jiao *et al.,* 2010). Some are made of neutral macromolecules while others are

made of polyanionic molecules (Donlan 2002). The extracellular matrix of biofilm cells is a glue-like substance that enables biofilm to adhere to surfaces and neighboring cells.

The protein component of the extracellular matrix includes adhesin, outer membrane vesicle proteins, secreted extracellular proteins and protein subunits of flagella and pili (Fong and Yildiz, 2015). Pili and flagella are necessary for the initial attachment of bacteria to surfaces and for movement across surfaces. The role of matrix protein in biofilm structure and stability has been confirmed from various mutational studies which has shown that the absence of matrix proteins reduces the stability, formation, and structure of biofilms (Fong and Yildiz, 2015)

The extracellular matrix helps biofilms to adhere to surfaces, protects them from antimicrobials, competing microbes, and desiccation, provides reservoir of nutrient and creates a suitable and distinct structure that enables microorganisms to persist in difficult situations (Vu *et al*., 2009; Oliviera *et al*., 2015). Biofilm extracellular polysaccharides can be protective, aggregative, and architectural/structural in function (Limoli *et al*., 2015). The aggregative polysaccharide is essential for the biofilm formation process. It plays a major role in attachment and microbial interaction that results in structure formation.

1.11. Formation of biofilms

The structure of a biofilm is dependent on the nature of species and environmental factors as there is usually interaction of bacterial subpopulations (Tolker-Nielsen *et al*., 2000). Biofilm formation starts when a specific environmental signal induces a genetic programe in free living planktonic cell to attach to nearby surface with the aid of flagella, pili and fimbriae. The attached cell coats the layer with organic monolayer of polysaccharide and glycoproteins to which more planktonic cells can attach. The initial

attachment of cells to surfaces is accomplished by a weak reversible bond known as Van der Waals forces - a temporary type of attraction of intermolecular forces. The van der waals bond is a relatively weak electric force that attract molecules to one another in liquefied and solidified gases which arise from temporary attractions between electron rich regions of one molecule and electron poor regions of another molecule. In a situation where the colonist is not detached immediately, they use cell surface proteins and molecules to adhere to other cells and bind themselves more permanently to the surface. The first bacterial colonist encourages the adhesion of pathogens and other cells that were unable to attach to surfaces on their own by providing many adhesion sites (Butt and Khan 2015). As cells go into biofilm mode, they no longer maintain their flagella but switch to twitching form of motility that involves the extension and retraction of pili and later stop moving and firmly attach to the surface. Adhered cells undergo cell division, forms microcolonies and start producing extracellular materials (Jamal *et al*., 2018). As more cells attach to the surface, they form microcolonies and begin to communicate with each other by sending and receiving chemical signals in a process called quorum sensing. When the population reaches a specific number analogus to an organizational quorum, the chemical signals reach a threshold or specific concentration that the cells can sense. This concentration triggers genetic regulated changes that makes cells bind tenaciously to the surface and to each other. Quorum sensing can regulate the amount of extracellular matrix produce by the cells. The biofilm progresses to its last stage called the development stage and at this level, the biofilm can only change in shape and size. Biofilms can move collectively across surfaces and detach clumps referred to as seeding. Biofilm formation can be terminated in response to growth conditions such as nutrient availability and this has been reported in *Pseudomonas putida* where

biofilms in a flow chamber dispersed completely due to stoppage of nutrient flow or a change in the nutrient content in the medium (that is from a medium with carbon to that with no carbon) (Gjermansen *et al*., 2005).

1.12. Process of biofilm formation by bacteria

1. Attachment of cells to surfaces

This phase is induced by environmental signals, and it includes nutrient concentration, pH, temperature, oxygen, iron and osmolarity. Microorganisms attach more easily to rough, hydrophilic, and coated environmental surfaces. Microbial attachment is also enhanced by presence of flagella, pili, fimbriae, and slime on bacterial surfaces (Donlan and Costerton, 2002). Cell appendages helps the microbial cells to overcome repulsive forces during the process of attachment thus enhancing the process of biofilm formation and contributing to virulence. Research by Koczan *et al*., (2011) demonstrated that bacterial cell appendages that play a role in the attachment process of biofilm formation contributes to their virulence. They reported that flagella have multifaceted effect on the process of biofilm attachment and virulence on *Erwinia amylovora*. They also showed that type 1 fimbriae and type iv pilus were utilized for early and late-stage biofilm attachment and lack of these appendages resulted in decrease in virulence of the pathogen. Also, Lazaro *et al*., (2009) reported the importance of FIC fimbriae in biofilm formation in *E. coli* commensal strain while Tan *et al.*, (2016) demonstrated the importance of flagella and fimbriae in the attachment process of *Salmonella* Typhimurium ATCC/14028 strain.

The reversible attachment occurs by Brownian motion and movement of motile cells. Irreversible attachment occurs when reversibly attached cell starts producing

extracellular polymeric substances that glues the cell to the substratum and provides an adhesion surface for other cells.

2 Formation of biofilm monolayer and microcolonies

This occurs after the successful attachment of bacteria cells to living or non-living surfaces. Microbial cells adhere to each other and secretes binding molecules such as adhesion proteins that enables it to bind irreversibly to the substrate. Also, cells start multiplying and emitting chemical signals that help the cells to communicate with each other. When the signal exceeds a certain threshold level, the genetic process that leads to the production of exopolysaccharides (EPS) is activated. Bacteria continue to proliferate and form cell aggregates within the exopolysaccharide matrix leading to the formation of micro- colonies (Toyofuku *et al.,* 2015). The bacterial cells are organized into communities with functional heterogenicity (Butt and Khan 2015, Stoodley *et al*., 2002).

3 Biofilm detachment

The ability of organisms to spread and colonize new habitats is necessary for the continuity of the organism, prevention of overcrowding and accumulation of biofilm cells that do not cooperate or participate in cell to cell signaling (quorum sensing mutants) (Popat *et al*., 2012). This has led to the dispersal phase in the biofilm life cycle and biofilms have a mechanism to release highly motile cells into the environment. Bacterial cells within the biofilm can disperse from the biofilm on a regular basis into the environment and attach to other surfaces. Cells that disperse from biofilm are either new cells detached from growing cells or voluntary dispersion of clumps of cells resulting from mechanical stress. Dispersed cells usually retain

certain properties of original biofilm cells (Vasudevan, 2014). Figure 1.6. shows a diagrammatic representation of the biofilm formation process.

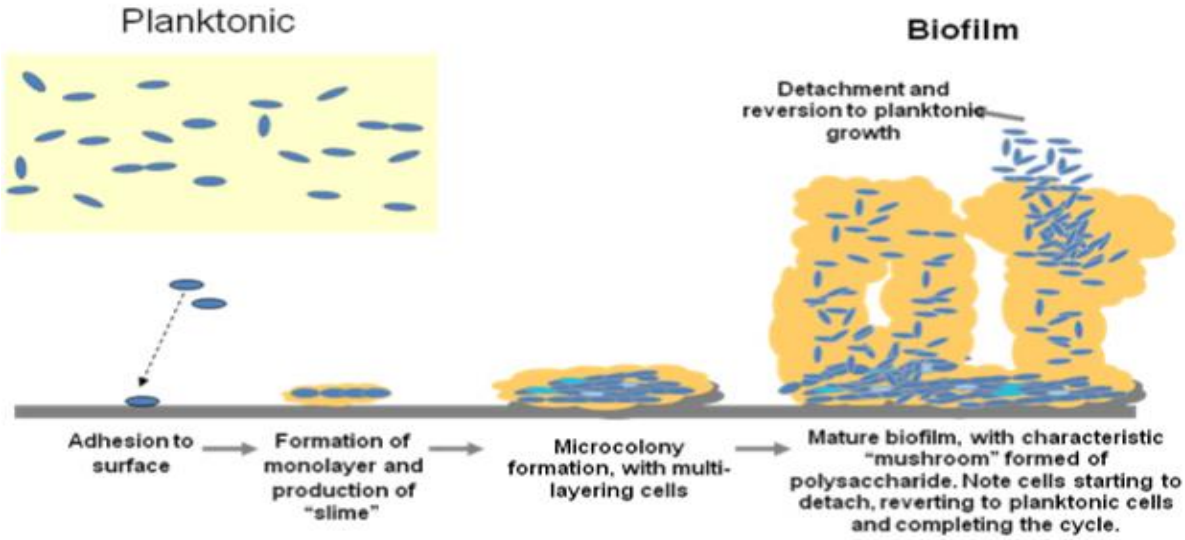


Figure 1.6.: Sequence of event leading to formation of biofilm. Source: (Vasudevan, 2014)

Planktonic cells attach to a suitable surface and proliferate. Cells adhere to each other to form monolayer which grows into a microcolony by forming a multilayer structure of bacterial. As the biofilm grows into maturity, more extracellular matrix and quorum sensing molecules are produced and bacterial cells at the centre grows slowing. At maturity, part of biofilm bacterial are dispersed and it goes back into planktonic mode.

1.13. Factors regulating biofilm formation (quorum sensing)

It is well known that organisms within a biofilm can communicate with each other through a process known as quorum sensing. This process involves the release, sensing and response to small diffusible signals. Quorum sensing (QS) enables bacterial cells sense the presence of cells in the environment and interact by cell-to-cell signals (Li and Tian, 2012). If a cell 'knows' that it is near a dense population of other cells, it will be inclined to contribute to biofilm formation. Microorganisms involved in quorum sensing communicate their presence in the environment by releasing chemical signals (e.g AHL in Gram-negative bacteria) that are recognized by co-inhabitants of the biofilm. If the signal increases and reaches a threshold, the organisms respond together and act as a group. Quorum sensing is observed either

in single bacterial species or in diverse species and can control different processes using various molecules as signals. Singh *et al*., (2000) demonstrated that *Pseudomonas aeruginosa* strains isolated from cystic fibrosis produced AHLs *in vitro.* This simple communication network by bacterial cells is activated by a low molecular weight auto-inducer present in the environment (Irie and Parsek, 2014). This auto inducer is activated when it gets to its threshold, and it regulates the transcription of certain genes, and it varies with different organisms. The autoinducers that trigger the QS mechanism are different among Gram-negative and Gram-positive bacteria. Gram-positive bacteria use peptides as signaling molecules while Gram negative bacteria makes use of N-acyl-homoserinelactone of varied length dependent on the extent of cell density (Vasudevan, 2014). Other types of autoinducers include amino acids and quinolones. Different phenotypic characters in bacteria such as biofilm formation, motility, and virulence factor expression (toxin production) could be controlled by QS (Parsek and Greenberg 2005).

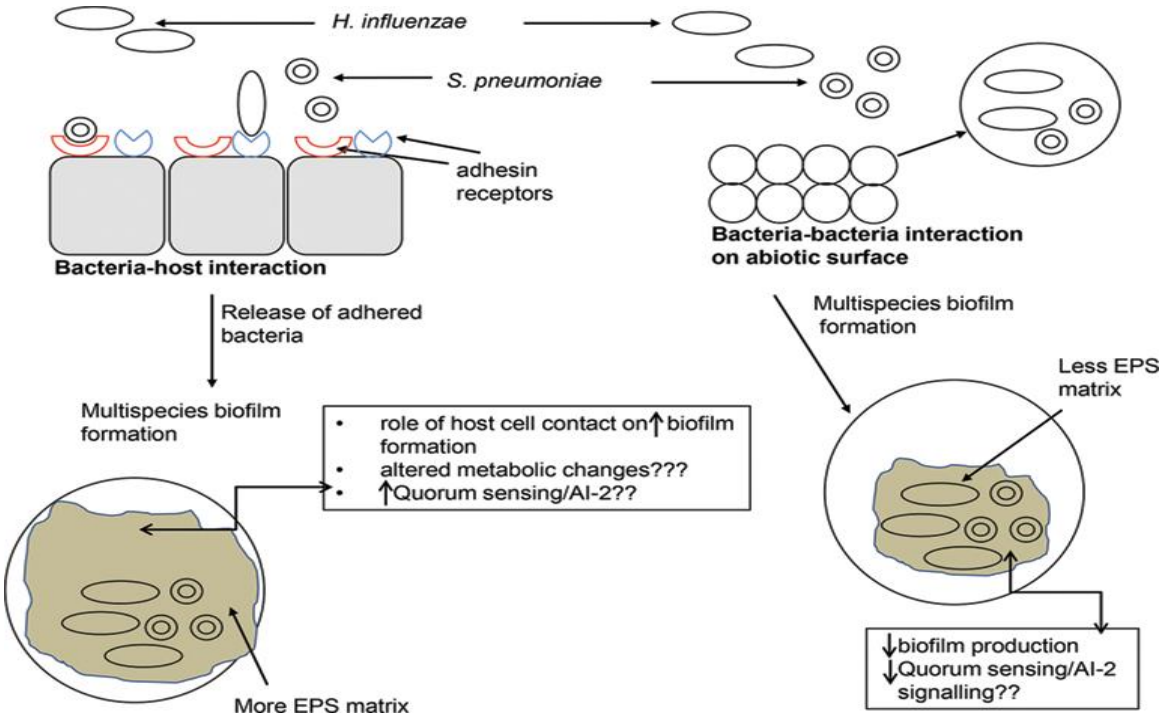


Figure 1.7.: Effect of contact surfaces and QS on biofilm. Source: (Kyd *et al*., 2016)

Bacteria produce biofilm differently upon contact with biotic (host epithelial cell) or abiotic surfaces (tissue culture polystyrene culture plate). The figure illustrates higher biofilm production (EPS matrix) in a dualspecies biofilm growing on biotic surface under increased QS autoinducer-2 (AI-2) (left) and low level of EPS matrix when growing on abiotic surface and low amount of AI-2 (right).

1.14. Mechanisms of antimicrobial resistance in biofilms

Microorganisms can either die or survive in the presence of antimicrobial agents. Microbes that survive in the presence of antimicrobial agents (resistant microbes) will replicate and spread continuously. Some non-resistant microbes can undergo mutation during replication. Mutation can make microbes resistant to treatment. Also, microbes can transfer genes to each other. This may lead to the transfer of antimicrobial resistance genes from resistant microbes to non-resistant microbes. Biofilms are often much more resistant to antibiotics than their planktonic counterparts. The reasons for biofilm loss of susceptibility or resistance are multifactorial depending on the type of antibiotic, the organism, and the nature of the biofilm. Temperature, pH, nutritional status of the growth medium, growth rate and prior exposure to antibiotics effects susceptibility of biofilms to antibiotics. Highly resistant bacteria have been

identified in biofilms which have multi-drug resistant pumps that expel antimicrobials from cells. Excellent reviews by Hall and Mah., (2017); Singh *et al*., (2017); Chadha (2014); Jamal *et al*., (2015); Bridier *et al*., (2011); Stewart, (2002) have identified specific mechanisms of biofilm resistance as follows.

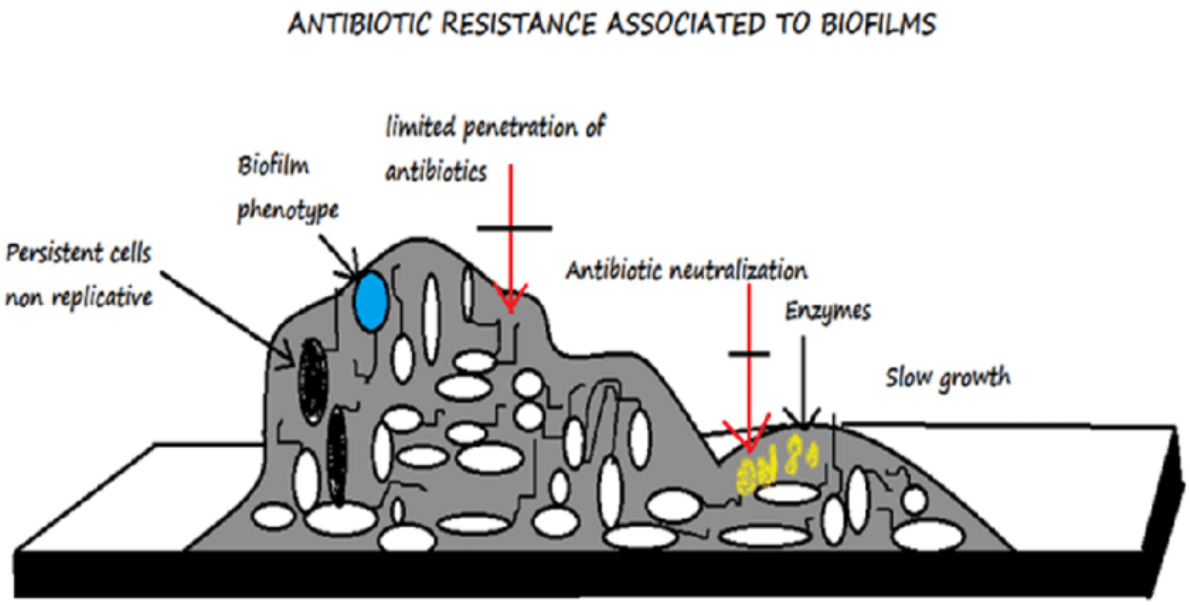


Figure 1.8.: Mechanisms involved in antibiotic resistance in biofilm. Source: (Jamal *et al*., 2015)

Mechanisms identified to be responsible for high resistance nature of biofilms includes (a) limited diffusion of antibiotics, (b) antibiotic neutralization by enzyme (c) heterogeneous nature of biofilm, (d) slow growth rate, (e) presence of non-dividing cells and (f) biofilm phenotype.

i. Limited penetration of antimicrobial through the biofilm matrix

The biofilm matrix limits movement of the antibiotics through the matrix and microorganisms are therefore probably exposed to sub-lethal concentrations of antibiotics. The matrix also serves as an adherent site for bacterial exoenzymes. These enzymes can use the antibiotics as substrates thereby degrading the antibiotics in the matrix which also slows down the penetration, movement, and activity of the

drug (Singh *et al*., 2011). Albayaty *et al*., (2019) studied the level of penetration of three different topical antimicrobial on *S. aureus* biofilm matrix and they reported that the biofilm matrix maturity as well as physicochemical properties of the antimicrobial agent influenced the penetrating ability of the tested antimicrobials through the biofilm matrix. Their report suggested that low antimicrobial penetration through the biofilm matrix results in failure in killing bacteria within the biofilm which leads to biofilm tolerance to antimicrobials.

ii Slow growth rate of bacterial in biofilm

Eng *et al*., (1991) examined the effect of different class of antibiotics on slow and optimal growing Gram-positive and Gram-negative bacteria. Their work showed that the activity of antibiotics was influenced by high growth rate of bacteria as the death of optimally growing bacteria was more compared to slow growing bacteria. Antibiotic treatment and bacterial susceptibility are influenced by the metabolic state of bacteria cell. This is because antibiotics target different cellular processes which can be grouped into four class- DNA replication, cell wall biosynthesis, protein synthesis and cell membrane (Stokes *et al*., 2019). In biofilm there is a reduced metabolic activity, reduction in energy production, cell division and protein synthesis. These changes increase the recalcitrance of biofilm to antimicrobial agents because they have reduced or no target for many antibiotics. The growth rate and metabolism of bacteria in a biofilm is affected by the nutrient and oxygen distribution within the structure. Also, bacteria within the biofilm have a lower supply of nutrient and experience depletion of oxygen compared to cells in the periphery region of biofilm, they grow slowly, and this makes such cells less susceptible to antibiotics (Hall and Mah 2017).

iii Development of persister bacteria

'Persisters' are part of the bacterial population that are tolerant to antibiotics. Persister cells in biofilm could be responsible for biofilm recalcitrance because these cells are not sensitive to antibiotics as the cells are not metabolically active thus antibiotics can not act on it leading to tolerance. These types of cells neither die nor proliferate in the presence of lethal doses of antibiotics and they are referred to as dormant variants of the wide type/metabolically inactive cells (Singh *et al*., 2011). Persister cells are about 1% of the biofilm and unlike resistant cells that grow in the presence of antibiotics, persister cells are unable to grow (Wood *et al*., 2013). Persisters results from the slow growth rate of bacteria within a biofilm which makes cells resistant to antibiotics. Some biofilm bacteria have developed into distinct phenotypes and such biofilm phenotypes shows specific adaptive response to antibiotics. This results from limited availability of nutrient leading to starvation, stress, and bacteria exposure to sub-lethal dose of antibiotics (Bridier *et al*., 2011). This phenotypic variant was first discovered in *S. aureus* and *S. pyogenes* when it was revealed that a small fraction of cells that was not killed by penicillin, did not undergo genetic changes hence such cells could not be called resistant cells rather phenotypic variants that are tolerant to antibiotics (persister cells).

iv Efflux pumps

The up regulation of genes coding for efflux pumps in biofilms compared to planktonic counterparts contributes to biofilm resistance (Alav *et al*., 2018). Efflux pumps are a system that enables cells to remove toxic substances including antimicrobials. Alav *et al*., (2018) studied the role of efflux pump in *E. coli*, *P. aeruginosa, P. mirabilis, A. baumanii* and in other bacteria biofilm formation process. Their study showed that

efflux pumps regulate biofilm formation in different ways which includes (1) Transport of extracellular polymeric substance and quorum sensing molecules to increase the formation of biofilm matrix. (2) Indirectly regulating genes that play a role in biofilm formation. (3) Efflux of toxic molecules (metabolites and antibiotics) and (4) influencing the aggregation of cells to surfaces. Also, Ikonomidis *et al*., (2008) reported that the efflux pump inhibitor - proton motive force (PMF) – carbonyl cyanide-m-chlorophenyhydrazone (CCCP) decreased biofilm formation in *Pseudomonas aeruginosa* significantly.

v Stress response genes

Oxidative stress plays a role in antibiotic susceptibility. Stewart *et al*., (2015) tested the effect of oxidative stress in biofilm resistance to antibiotics in *P. aeruginosa* biofilm. The development of stress response genes is an important feature of biofilms. Stress response is induced by stationary phase bacterial growth and nutrient deprivation. The *RpoS* is the regulator of stress response in *Pseudomonas aeruginosa* and this gene has been shown to be expressed three times more in 3-day old biofilms compared with stationary phase planktonic cells (Xu *et al*., 2001). Stewart *et al*., (2015) demonstrated that rpoS mutant strains treated with ciprofloxacin showed 2.4 – 2.9 log reduction in cell count compared to 0.9 log reduction observed in the wide type.

vi Gene transfer

Gene transfer is another characteristic associated with biofilms. The biofilm environment favours the exchange of genetic materials between bacteria (Molin and Tolker-Nielsen, 2003). Cells within a biofilm are so closely linked that there is easy exchange of virulence genes that can result in more pathogenic strains of microorganisms. There is also the possibility of transfer of plasmids carrying resistant

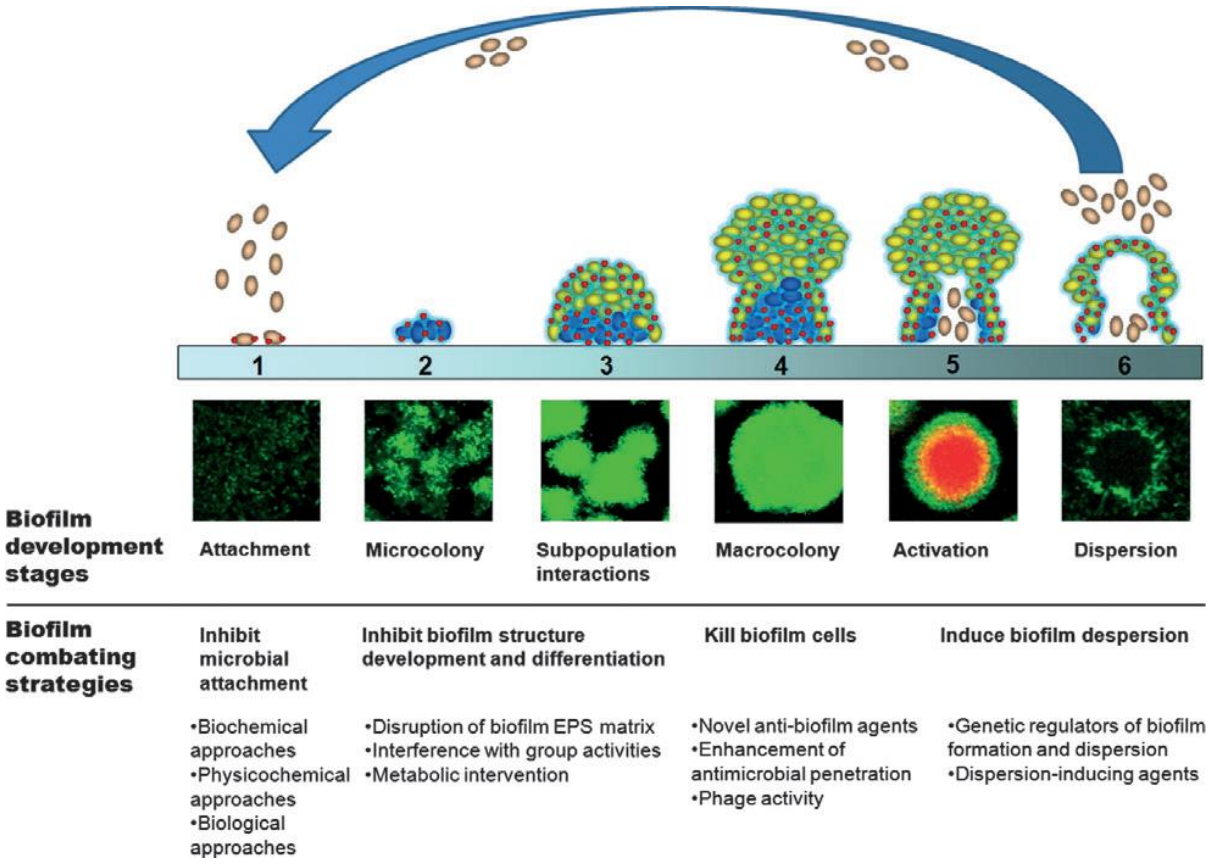
traits from one cell to another and genetic mutation. Horizontal gene transfer (HGT) enables bacteria resist antibiotics, adapt to changing environments (Stalder and Top, 2016). HGT in biofilm can occur through different mechanisms; conjugation, transformation, and transduction (bacteriophage mediated DNA transfer (Abe *et al.,* 2020). Conjugation occurs more in biofilm communities compared to when growing in a planktonic state because the transfer requires a direct contact between the donor and recipient cells. However, in biofilms, this is an advantage as cells are attached to a matrix and are located closely (Madsen *et al*., 2012). Li *et al*., (2018) studied the horizontal gene transfer of antibiotic resistant plasmid from *P. putida* biofilm using microfluidics to *E. coli* and activated sludge bacteria. Their study revealed that the composition and structure of the biofilm influences the route of gene transfer. Savage *et al.,* (2013) reported an increase in transfer rates of up to 16 000-fold compared to planktonic cells when a conjugative plasmid (pGO1) transferred trimethoprim and gentamicin resistance genes in *S. aureus* biofilms. This can be related to the fact that biofilms are dense communities that accelerates the spread of mobile genetic elements (MGEs). This spread is achieved because of the structural and spatial advantage of the biofilm growth mode whilst keeping the conjugative pili intact. Unlike conjugation, transformation does not require a physical contact between the donor and recipient cells. Free DNA released during cell lysis can serve as the donor for transformation. HGT by transformation occurs frequently in biofilms and this involves both small DNA fragments and big elements such as plasmids. Hendrickx, *et al*., (2003) demonstrated that *Acinetobacter sp.* BD413 biofilms formed in LB medium have been transformed with an exogenous plasmid (pGAR1) carrying a tetracycline resistance gene. Also, it has been reported that *Acinetobacter* sp. BD413 cells in river

biofilms were transformed with a mercury resistance plasmid pQM17 (Williams *et al*., 1996).

1.15. Traditional and non-traditional strategies for bacterial biofilm control

Treatment of infections caused by biofilms is a challenge as traditional antibiotic treatment alone is often insufficient to cure biofilm related infections. However, suitable alternative strategies to overcome biofilm resistance and associated infection by preventing cell attachment and induce biofilm disruption are in progress. Strategies include use of appropriate inhibitors that prevent the attachment of microbial cells, interference on the development of biofilm structure, killing of bacterial biofilm cells and induction of biofilm detachment (Yang *et al*., 2012).

The anti-biofilm potential of various natural antimicrobial compounds has been shown to control biofilm formation. Also, more effective anti-biofilm activity of antibiotics has been described when these antimicrobials are used in combination with traditional antibiotics (synergism) compared to their activity when each agent was used alone (Chhibber *et al*., 2017; Mu *et al*., 2014). The high antimicrobial activity is more likely since each agent acts on different targets. Figure 11 summarises biofilm control strategies.



Source: (Yang *et al*., 2012)
Figure 1.9: Strategies to control biofilm during different growth stages.

Currently employed experimental biofilm control strategies

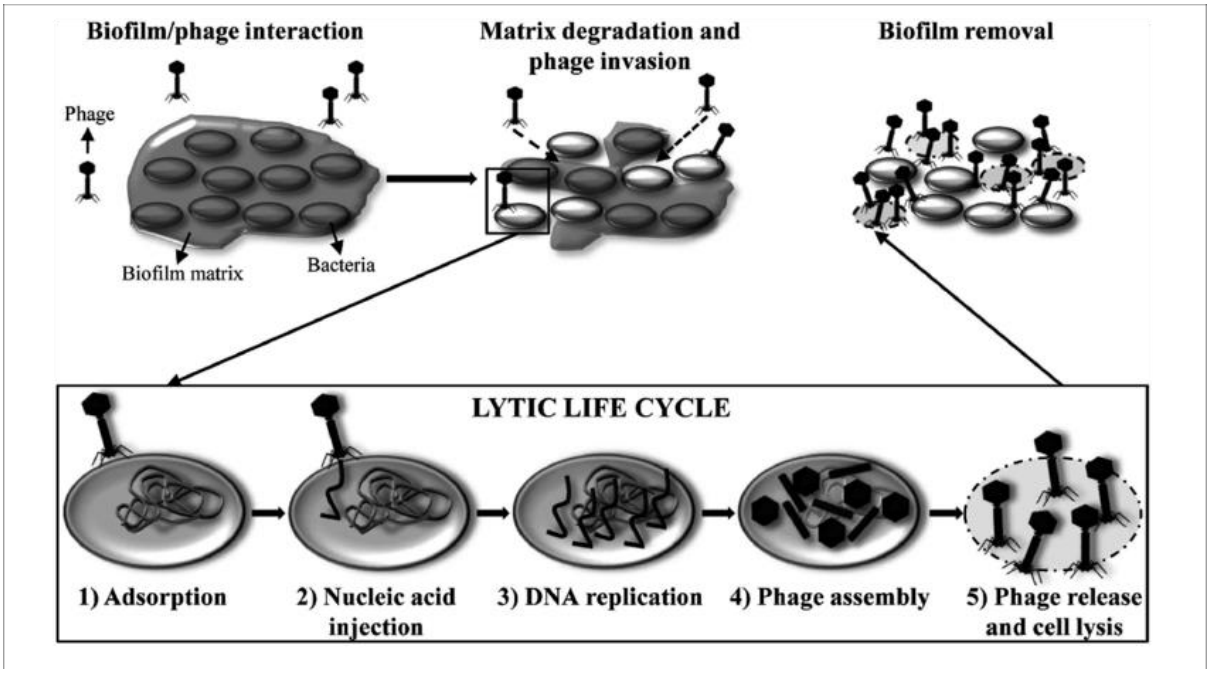
1.16. Bacteriophages (phages)

Phages are currently considered as alternative antimicrobial agents and are also used in combination with antibiotics to inhibit or disrupt biofilm. Phages are easy and quick to produce and since their antimicrobial activity is host specific, they do not affect the microbiota when used. Bacteriophages (also known as phages) are viruses that infect bacteria. Phages can either reside in the bacterial host genome (lysogenic phages) or they destroy/kill the bacteria (lytic phages). The lytic phages are most suitable as therapeutic agents. Currently phages are used as alternative therapeutic agent against bacterial infections. Phages are selective in action meaning they are active against a specific host or host range. Using the microtiter plate method and microscopic observation with the transmission electron microscope (TEM), phages have been

reported to show positive antibiofilm activity in many bacteria (Dickey and Perrot, 2019; Nair *et al*., 2016; Kim *et al*., 2019; Maszewska *et al*., 2018; Meng *et al*., 2011). However, in contrast there are reports in the literature that phages can enhance bacterial biofilm growth (Henriksen *et al*., 2019; Lucia *et al*. 2017; Tan *et al*. 2015; Zainab *et al*., 2013). Biofilm can confer resistance to phages because of the biofilm matrix barrier. Henriksen *et al*., (2019) reported that treatment of 72h *P. aeruginosa* biofilm with single virulent phages resulted to the growth of large microcolonies in flow cell system and they suggested this to be a defense mechanism against phage treatment. Tan *et al*., (2015) reported that strains of *Vibrio anguillarum* reacted differently to vibriophages. In their work, microscopic observation with the florescence microscope showed that there was an increase in biofilm formation (multilayered cell aggregate formed) when a strain of *V. anguillarum* (PF430-3) was treated with phage (KVP40) after 24h. Hosseinidousta *et al*., (2013) showed that pre- treatment of 48h *P. aeruginosa*, *S. aureus* and *S. enterica* Typhimurium biofilm with species-specific phages, resulted to 4 to 6 times greater biofilm formation than the control. Biofilm increase in growth after exposure to phages has been attributed to evolutionary and non-evolutionary mechanisms (Tan *et al*., 2015; Hosseinidousta *et al*., 2013; Lacqua *et al.,* 2006). Bacteria growing in biofilms have reduced metabolic activity and this affects the propagation of phages in biofilms. Abedon, (2012) reported that low number of phage and the abundance of phage resistance bacterial in biofilm is beneficial to the host bacteria as low phage number has been observed in situations where biofilm level increased above the level of the control during phage treatment (Fernandez *et al*., 2017).

Figure 1.10. shows the life cycle of phage in biofilm. Bacteriophages produce depolymerizing enzymes that aids in the degradation of the biofilm impermeable EPS

matrix. Phages attach to bacterial cell surface and penetrate through the cell by binding to specific surface receptors on bacterial cell wall. In the cell, phage replicates resulting in production of large number of bacteriophage (amplification). This releases numerous infectious phages into the biofilm capable of spreading through the biofilm and infecting other cells. This process can successfully eliminate the bacteria producing the EPS material and remove the biofilm. Phage can infect persister cells even though the phages are unable to replicate in such cells because they are inactive. Phage remain within the cell until such cells reactivate and then commence the lytic life cycle.



Source: (Gutierrez *et al*., 2016)

Figure 1.10: Biofilm destruction by phage (lytic life cycle of phage)

(1) phage attach to bacterial cell surface by binding its tail fibers to specific surface receptors on the cell surface of the host bacteria (2) nucleic acid of phage is injected into the cytoplasm of the bacterium. (3) Phage genome replication in bacteria cytoplasm. (4) virion maturation. New phage particles are formed by assembly of the phage heads and tails and packaging of the nucleic acid inside the heads. (5) Bacterial cell lysis and release of the phage progeny which then infect other cells within the biofilm and start a new cycle

1.17. Nanoparticles

The study of materials that range between 1 and 100 nanometers in size is known as nanotechnology. Nanoparticles are at the forefront of the rapidly growing field of nanotechnology. The use of nanomaterials has been beneficial to the industrial world. This technology has created materials with unique physico-chemical properties, high reactivity, conductivity, antimicrobial activity, and stability. NPs are usually classified as organic, inorganic and hybrid NPs based on the core materials they are made from (Liu *et al.,* 2007). Sond and Sondi, 2004 stated that the high stability of inorganic NPs (metal and metal oxides) results in more effective antibacterial activities. Novel and unique characteristic of nanomaterials makes it relevant in drug delivery includes shape, size, large surface area-to-volume ratio and surface charge (Allaker and Memarzadel 2014; Raghupathi *et al.,* 2011). The antibiofilm activity of different nanoparticles (NPs) have been reported against biofilm cells. This antibiofilm activity of NPs is associated with the generation of hydroxyl radicals by NPs, ATP-associated metabolism, and permeability of the outer membrane (Algburi *et al*., 2017).

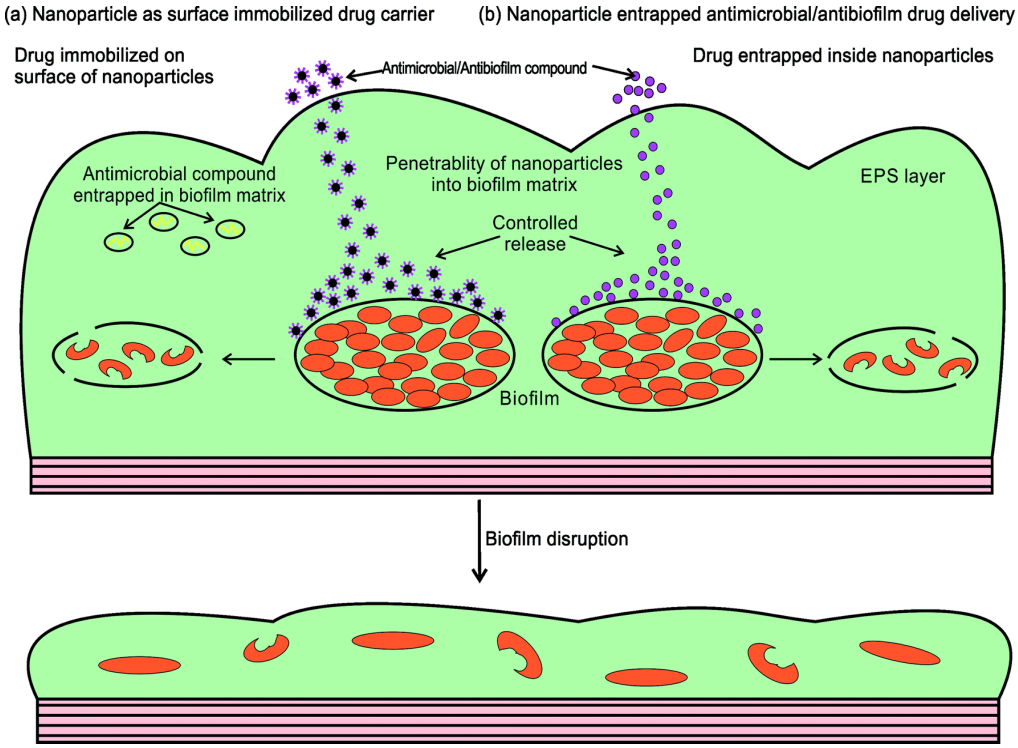
El-Gohary *et al*., (2020) reported the bactericidal activity of the combination of AgNPs and hydrogen peroxide on five multiple drug-resistant Gram-negative bacteria to range between 6.25µg/ml and 50µg/ml. Ramasamy *et al*., (2016) demonstrated that AuNPs and AgNPs inhibited biofilm formation in *P. aeruginosa*. *S. aureus*, *E. coli* and *E. faecalis* in both single specie and mixed culture biofilm in a concentration dependent manner. Gurunathan *et al*., (2014) reported that AgNPs showed effective antibiofilm effect on *S. aures*, *S. pnuemoniae*, *S. flexneri* and *P. aeruginosa* and could be used as an adjuvant to enhance antibiotic activity. Martinez-Gutierrez *et al*., (2013) reported biofilm reduction and inhibition activity of AgNPs on *P. aeruginosa*, *A. baumanii*. *Candida albican* and methicillin resistant *S. aureus.* They showed that 10mg/ml of AgNPs effectively prevented or reduced *P. aeruginosa* cells in established biofilm

resulting in 4 log reductions in colony forming unit. Ansari *et al*., (2014); showed that 50µg/ml of AgNps was effective in removing 24h old *K. pnuemoniae* and *E. coli* biofilm from glass slides.

Due to the increasing research aligned with multi-drug resistant bacteria, nanomaterials can also be functionalized/ manipulated by the addition of capping agent or coatings to their surface. This method of coating NPs with antibiotics, antimicrobial peptides, amino acids is used as an alternative method to treat resistant bacteria. Panacek *et al.,* (2016) investigated the effect of AgNPs alone and the combination of AgNPs with different antibiotics against *S. aureus*, *E. coli* and *P. aeuginosa*. Their results showed the AgNPs reduced the antibiotic MICs and made the bacteria susceptible to the tested antibiotics. Furthermore, their study revealed that antibacterial effect of the combined therapy was dependent on the antibiotic class. They were able to show an enhancing effect for AgNPs and antibiotics against either Gram-negative or Gram-positive bacteria by using different mechanisms. This suggests that combining NPs with antibiotics could be a possible alternative therapeutic strategy against biofilms. Singh *et al*., (2019) showed that alginate nanoparticles effectively penetrated *P. aeruginosa* biofilm. They also reported nanoparticles as a potential antibiofilm agent that will be useful in combination treatment against biofilm prevention and reduction.

The application of new technologies usually tends to raise public concerns on toxicity, health, and environmental risks. Studies have reported that silver in nanoform can cause adverse effects on humans and the environment. Tonnes of silver are released from industrial waste into the environment and exposure to soluble silver can cause toxic effects such as liver damage, eye, skin, respiratory and gastrointestinal tract irritations, and kidney damage. However, in the twenty-first century, the use of

nanosilver has gained much popularity; the same features that make NPs attractive, also raise important issues. There are reports that silver NPs are not selective as an antimicrobial agent and therefore can kill both harmful and beneficial microbes in the environment (Allsopp *et al.,* 2007). The application of nanomaterials is still controversial though most research have reported no or minimal risk to health and environment for most nanomaterials used. Hussain *et al*., 2005 tested the toxicity of silver in rat liver cells and reported that AgNPs was toxic to the cells even at low concentration which manifested as oxidative stress. It was reported that high doses of AgNPs causes cytotoxicity on human cells (Liu *et al.,* 2017). As more investigation has been carried out on the long- and short-term toxicity of NPs, there should be caution on the usage of nanomaterials.



Source: Qayyum and Khan, (2016)

Figure 1.11.: Disruption of preformed biofilm by NPs

Nanoparticles mechanism of action

Several properties of NPs such as the size, shape, surface area and surface charge confer numerous advantages to NPs over conventional antibiotics (Hu and Menco 2017). AgNPs are known to cause damage to cell membranes by inducing leakage when it infiltrates the membrane. AgNPs in cells can induce reactive oxygen species (ROS) generation, forming free radicals that cause strong oxidative stress in the cells. This activity leads to a powerful bactericidal action because it alters the DNA structure, inhibits protein synthesis and oxidation of lipids (Matsumura *et al*., 2006; Liu *et al.,* 2017). Also, released silver ions can react with enzymes, proteins, lipids, and DNA releasing ROS and free radical species such as hydrogen peroxide, singlet oxygen, superoxide ions and hydroxyl radicals. This can result in the denaturation of ribosomes and blockage of translation and transcription process by binding with the genetic material of the cell (Prabhu and Poulose 2012; Adhikari *et al*., 2013).

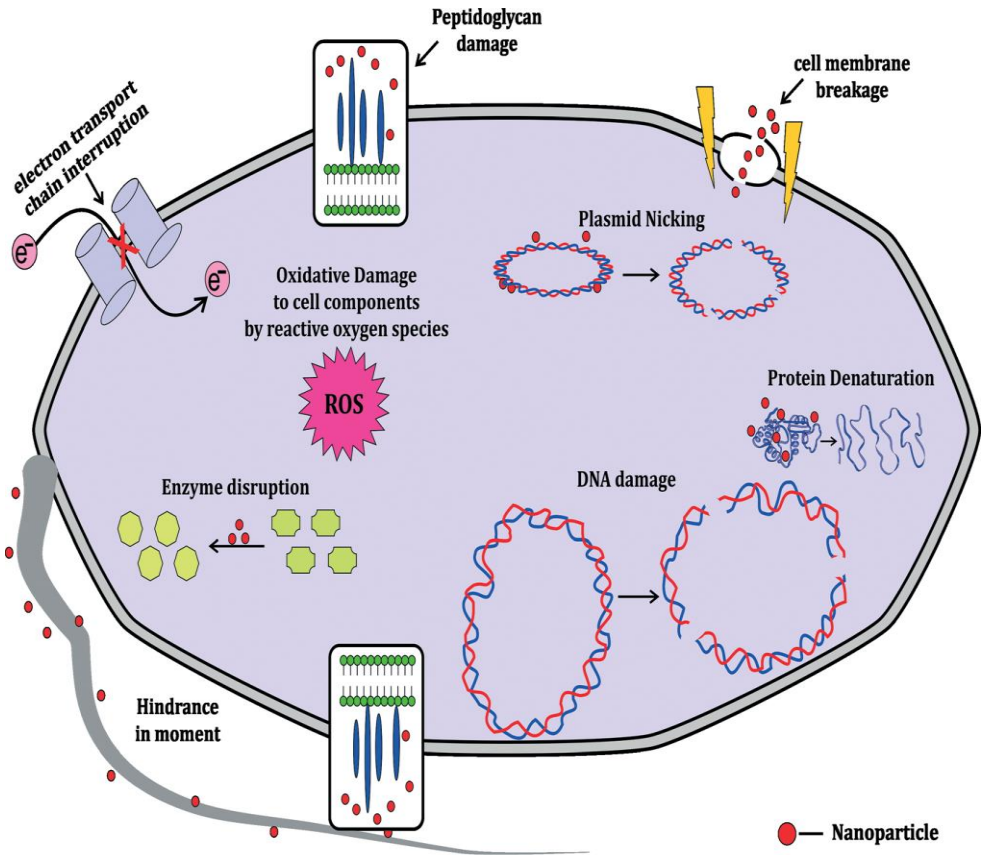


Figure 1.12: Mechanism of action of NPs on bacterial cell

Source (Qayyum and Khan, 2016)

The antibacterial activity of Nanoparticles is determined by the following.

1. Size of NPs

The extra-small sizes of NPs provide them with substantial advantages, which includes high penetrating ability, drug delivery and increased surface area which enhances its antimicrobial activities (Baker *et al.*, 2005). The large surface area of NPs enhances more intimate interaction between the NPs and bacterial membranes. This results in broad antimicrobial activity. For example, AgNPs has a greater surface area/volume ratio than the bulk material silver. This characteristic of NPs increases the number of interactions with the bacterial surfaces therefore facilitating higher antibacterial activity. Smaller size NPs release more radicals, which are the important factors needed for bacterial destruction. In metallic NPs, the smaller the nanomaterial

the higher the dissolution rate of the ions from the particles. The increased ion dissolution results in more ions available for attachment with bacterial surface leading to greater bacterial killing (Yeh, *et al*., 2020; Vassallo *et al*., 2020)

Makhluf *et al.,* (2005) reported that when *Escherichia coli* and *Staphylococcus aureus* where subjected to different sizes (23nm, 18nm,15nm, 11nm and 8nm) of MgO NPs, the 8nm which was the least NP size tested showed the greatest inhibition. The size, shape and concentration of NPs influences their ability to filter through the membrane (Zhang *et al.,* 2007). Nagarajan and Rajagopalan, (2008) demonstrated that smaller sizes of ZnO NPs increased their antimicrobial activity. This they attributed to the higher number of smaller sizes of ZnO NPs needed to cover a bacterial colony leading to the production of more reactive oxygen species which damages bacterial cells. Size of NPs and zeta potential are important parameters for bacterial growth inhibition and eradication (Azam *et al*., 2012; Franci *et al*., 2015).

2. Shapes.

There are different shapes of nanoparticles, and the type of shape influences their antimicrobial activity. Hong *et al*., (2016) reported the influence of shape of AgNPs on their antibacterial activity. The rod-shaped NPs penetrates bacterial wall faster as compared to spherical shape. They related this effect to the contact areas and reactive facets. Pal *et al.*, (2007) showed the antimicrobial activity of different shapes of NPs on *Escherichia coli*. Their work revealed that though all shapes tested showed antimicrobial activity, the triangular shape nanoparticles was more effective than the spherical shape nanoparticles and the rod-shaped NPs have (111) and (100) facets, compared to spherical nanoparticles having (100) facets. The greater antibacterial activity is linked to the higher facets which posses more atom density and exhibit less energy. Also, Slomberg *et al.*, (2013) reported that NPs that are rod like in shape are

more effective as anti-biofilm agent compared to the spherical shape NPs. Alshareef *et al*., (2017) reported bactericidal effect of truncated octahedral AgNPs which showed bacteriostatic effect.

3. Charges.

The surface charge of NPs is dependent on their zeta potential. The zeta potential is the electric charge on the surface of the NPs. The magnitude of the zeta potential determines the stability. A higher positive charge of NPs enhances its stability and the attachment of NPs to the negatively charge bacterial cell walls leading to cell damage through direct interaction or free radical production (Jiang *et al.*, 2009). Positively charged NPs shows strong affinity to negatively charged bacterial cell wall and tightly adhere and fuse with the bacterial cell wall (yeh *et al*., 2020. The positive surface charge in the NPs has led to bacterial cell damage which is a mode of antibacterial action of NPs.

1.18. Alternatives to antibiotics - Plant extracts

There has been a great interest in finding new plants for medicinal purposes. Medicinal plants are generally herbs which contain substances that are useful as therapeutic agents and are widely used in traditional medicine to treat diseases. The use of medicinal plants has a long history which was established long before the advent of pharmaceutical drug industry. Traditional (or herbal) medicine has existed worldwide in antiquity, and it depends on observation or past experiences. Early attempts to cure diseases made use of native plants and their extracts. In ethnomedicinal (traditional) treatment, a plant is either cooked, soaked in water or local wine, or eaten raw. Plant leaves, roots, bark, fruits, seeds, flowers, and stems can be used, and many have been validated with medicinal value which depends on substances in the plant known

as phytochemicals (Oyeyemi *et al*., 2018). Many antiseptics are phytochemicals. The increased use of antibiotics from specific filamentous bacteria or fungi in the treatment of bacterial infections has led to the emergence of multiple drug resistant (MDR) or AMR. This has become a major cause of failure in the treatment of infectious diseases and necessitates the discovery of new classes of compounds that inhibits or avoids bacteria resistance mechanisms (Gibbons 2005).

Ethnomedicinal treatments from plants presents as a promising alternative to combat MDR. Extracts of medicinal plants are known to show bacteriostatic or bactericidal effect and contain components which are useful for treatment or for drug production (Gibbons *et a*l., 2005). Some plants used in West Africa to control illnesses have been screened and shown to have medicinal value. Quave *et al*., (2008) revealed that ethnomedicinal plants used for the treatment of skin and soft tissue infections in Italy showed antibiofilm activity compared to plants that have no traditional application. Gislene *et al*., (2000) studied the antibacterial activity of some plant extracts and their findings suggest that they can be used in the treatment of diseases caused by resistant bacteria and potentially could be useful as antimicrobial agents. Plants such as *Vernonia amygdalina* commonly called bitter leaf, *Azadirachta indica* also known as neem and *Ocimum gratissimum* (Basil) have been used in the treatment of diverse conditions such as stomach discomfort, cough, and fever traditionally (Oyeyemi *et al*., 2018). The ethnomedicinal value of plants results from their bioactive compounds examples of which constituents are tannis, alkaloids, flavonoids, and phenolic compounds. In West African Countries *V. amygdalina* is a valuable medicinal plant. The leaves of *V. amygdalina* are green with characteristic bitter taste reflected in the local name bitter leaf and can be used as antimalarial, antibacterial and antiparastic agent (Okigbo and Mmeke, 2008; Alo *et al.,* 2012; Ghamba *et al*., 2014; Udochukwu

et al., 2015). Evbuomwan *et al.,* (2018) assessed the antibacterial activity of *V. amygdalina* leaf extract on selected pathogens. Their results showed that *V. amygdalina* has potential bioactive compounds that are responsible for its antibacterial activity. Therefore, investigating the antibiofilm activity of plants recognized for medicinal effect could show great potential in controlling biofilm infection.

1.19. Quartenary ammonium biocide

Quaternary ammonium compound (QACs) biocide also known as quats are cationic surfactants (positive charged surface-active agents). They reduce surface tension, form micelles, and allow dispersion in liquid (Gerba, 2015). QACs came into widespread use after World War II and their low toxicity and ability to be formulated for specific purpose and to target specific microorganisms account for their widespread application. QACs are added to numerous products for cleaning, disinfecting, and sanitizing surfaces both in food and health care industries (Gerba, 2015). Examples of QACs include cetrimonium, cethexonium bromoides, methylbenzethonium and benzalkonium and benzethonium. Generally, the activity of quartenary ammonium biocide varies significantly as it is based on the respective formulations of the compound.

The primary mode of action of QAC is the disruption of the structure and function of microbial cell membranes and QACs have shown bactericidal, fungicidal and virucidal activities. The antimicrobial activity of QACs can be affected by anionic surfactant and fat-containing substances. Some QACs are used at low concentrations as antiseptics examples includes biguanides- chlorhexidine (chlorhexidine gluconate, CHG) and polyhexamethylene biguanide (Vantocil) (Chauret 2014). Figure 1.13 shows the basic structure of a QAC.The basic QAC structure consist of the central nitrogen (cation portion) with four attached R groups, R_1, R_2, R_3, and R_4 representing a variety of alkyl

or aryl groups (Chauret, 2014). The anion portion (X) is negatively charged and is usually chlorine or bromine linked to the nitrogen to form the QAC salt.

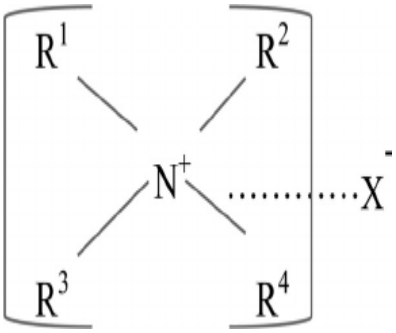


Figure 1.13.: Basic structure of QAC. Source: (Gerba, 2015)

QACs can be further classified based on the nature of the R groups. This variation includes branching of the carbon chain, number of nitrogen atoms and the presence of aromatic groups. These variations affect the antimicrobial activity of the QAC in terms of mode of action against different groups of organisms and dose. The length of the carbon chain in the R groups influences the quality of QACs as a disinfectant. Carbon chain between C_8 to C_{18} usually show the greatest antimicrobial activity. Several antimicrobial products contain a mixture of QACs and other adjuncts to enhance their efficacy and target a specific organism or group of organisms (Moore *et al.,* 2008). The different chemical structures available with QACs from first generation (Benzalkonium, alkyl chains, C12 to C18) to seventh generation (Bis-QACs with polymeric QACs) allows for evolution of their effectiveness and extension of their applications over the last century. This has led to continuous increase in efficacy while reducing toxicity and cost (Gerba, 2015).

The effective pH range for QACs is between pH 3 and pH10 with optimum activity around neutral pH. QACs have the advantage of being stable when diluted and are non-corrosive in nature. QACs are affected by organic soil, and they cling to surfaces

thus may be difficult to rinse off, resulting in taint problems. Chauret, (2014) reported that QACs are more effective against Gram – positive bacteria compared to Gram-negative bacteria and have limited activity against bacterial spores.

Mechanism of action of QACs.

QACs are positively charged thus they are attracted to the cell wall surface causing cell surface structure disruption and then penetrating the cell membrane. In the cell membrane, QACs interacts with phospholipids, causing disruption of membrane structure and function because of leakage of cytoplasmic components. These effects lead to cell death and loss of viability of microbial cells (Chauret, 2014). QACs are membrane-active agents and interact with the cytoplasmic membrane of bacteria and plasma membrane of yeast. The hydrophobic activity of QACs makes them effective against lipid-containing viruses. QACs can interact with intracellular organelles and bind to DNA (Gerba, 2015).

The mode of action of QACs against organisms follows a series of events; (1) Attachment and penetration of the cell wall; (2) Reaction with the cytoplasmic membrane (lipid or protein) (3) Leakage of intracellular material (4) Proteins and nucleic acids degradation and (5) Lysis of cell wall caused by autolytic enzymes. The nonspecific/multitarget mode of action of QACs makes it difficult for resistance to develop (Gilbert and McBain, 2003). This means that mutation in a single target is unlikely to result in treatment failure because there are other target site for action. Meyer and Cookson (2010) in their review analysed the resistance to biocides used in the health care industry and they concluded that the reasons for resistance to QACs was not clear. They suggested that resistance could be related to cross resistance, over dilutions of compound and incorrect handling of product. Also, efflux pumps can

cause biocide resistance. Efflux pumps can be induced by natural products, household chemicals, antibiotics, and biocides. Ortega, (2013); Weber and Rutala, (2006).

1.20. Research gap

C. perfringens strains can form biofilm-like structures either in their natural environment or in host tissues and this is considered as a major factor for its persistence in the host. There is a growing appreciation that the persistence of biofilm in the body contributes to the reoccurrence of diseases or chronic infections (Hall and Stoodley, 2005). The economic importance of biofilms has increased focus on the investigation of microbial biofilms and it's regulating factors (Jefferson, 2004). For several infections and opportunistic pathogens, the role of bacterial surface communities has been established and well-studied although not in *C. perfringens*. *C. perfringens* biofilms have not been studied extensively compared to other pathogenic bacterial species although initial attempts demonstrated *in vitro* biofilm formation (Vigal *et al*., 2008). Donelli *et al*., (2012) reported that *C. perfringens* forms mono species biofilms but the ability of *C. perfringens* to form mono, dual and mixed species biofilm was reported by Pantaleon *et al*., (2014). Investigations have revealed that strains of *C. perfringens* can display various biofilm phenotypes (Charleboise *et al.,* 2014) and it is very likely that persistence and strong biofilm phenotypes will be associated more frequently with certain isolates. Therefore, extensive study of *C. perfringens* biofilms could be key in understanding the ecological diversity existing amongst strains.

Research has revealed that microbial biofilms are naturally more tolerant to antibiotic administration than planktonic bacteria. Varga *et al*., (2008) reported that cells in biofilm showed 5 to 15-fold increase in survival rate compared to planktonic cells after exposure to penicillin G. Charlebois *et al.,* reported in 2017 that biofilm formed by *C. perfringens* were resistant to common disinfectants applied in farms and food

industries even as mono or dual species biofilms. *C. perfringens* biofilm formation has been shown to be influenced by temperature, growth media and type IV pili (Varga *et al.,* 2008). Other studies have characterized the biofilm of *C. perfringens* and have studied the structure of their exopolymeric components, namely polysaccharides and proteins (Type IV pilus) (Varga *et al*., 2008; Obana *et al.,* 2014; Charlebois *et al.,* 2014). Charleboise *et al*., (2014) revealed the presence of beta-1,4 linked polysaccharides and (extracellular) DNA in *C. perfringens* biofilm. Obana *et al.,* (2014) reported that temperature regulates biofilm morphology in *C. perfringens,* demonstrating that biofilm growth at 37^0C was more adherent (compared to biofilm formed at 25°C) which up-regulated *AbrB* and *pilA2* which encodes a subunit of the type IV pili. Recently, Vidal *et al.,* (2015) reported that CpAL regulates *C. perfringens* toxins (CPA and PFO) needed for the formation of biofilm structure. Furthermore, the transcription of the genes *cpa and pfoA* were up regulated in biofilm compared to planktonic cells. More recently, Charleboise *et al*., (2017) gave an insight on gene expression in *C. perfringens* biofilm. Their work showed the down regulation of virulence genes, energy production and quorum sensig genes whilst the up regulation of defense mechanism genes, EPS biosynthesis, sporulating genes and oxidative response genes occurred. Though studies have reported the persistence of *C. perfringens* biofilms following antimicrobial treatment, to our knowledge there has been no report on the influence or importance of isolate origin on biofilm formation or on antibiotic tolerance. Furthermore, to help prevent and control biofilm persistence, the need of the hour is to devise alternate therapeutic strategies.

1.21. Research Aim and objectives

The aim of this research was to assess for biofilm formation in the anaerobic pathogenic bacterium *C. perfringens* and to test the antibiofilm effect of alternative antimicrobial agents. The specific objectives of this research were as follows.

1. To evaluate and compare any difference in biofilm forming abilities of *C. perfringens* isolated from diverse sources - either preterm human neonate, free range poultry environment or broiler chickens.
2. To investigate the effect of growth temperature on *C. perfringens* biofilm formation in terms of density achieved.
3. To compare the differences in susceptibility to antibiotics of *C. perfringens* biofilm and planktonic cells *in vitro*
4. To investigate any antibiofilm activities of gold and silver nanoparticles on *C. perfringens* preformed biofilm.
5. To investigate the antibiofilm activities of medicinal plants extract on biofilm.
6. To determine the activity of quartenary ammonium compound - *Acquorsol* solution on planktonic and biofilm cells.

CHAPTER TWO:

GENERAL MATERIALS AND METHODS

2.1 Chemicals and reagents

List of growth media, chemicals, commercial kit, equipment, consumables, and their manufacturers are presented in Appendix A.

2.2 Preparation of growth media and solutions

Recipes for solutions and growth media used in the different experiments are provided in Appendix B. Manufacturers' specification was followed in preparation of all solutions and growth media.

2.3 Cleaning and sterilization of materials

Materials such as glassware and pipette tips were carefully wrapped and sterilized by autoclaving at 121˚C for 15min. Used glassware was washed with clean tap water and detergent prior to sterilization. Also, broth culture, used agar plates and all materials that were contaminated were placed in autoclave bags and autoclaved prior to disposal.

2.4 Bacterial isolates used in this study.

A total of fifty-four (54) isolates of *C. perfringens* were used in this study (Table 2.1). Nineteen (19) of the isolates were from faecal samples taken from preterm neonates diagnosed with Bell stage 3 of necrotizing enterocolitis (NEC) hospitalized in the neonatal intensive care unit in St Mary's Hospital, Paddington, London. Seventeen (17) of the isolates were recovered from various body parts from broiler chickens diagnosed with clinical or subclinical cases of necrotic enteritis (NE) and apparently

healthy chickens from a poultry farm in the UK. The final eighteen (18) isolates of *C. perfringens* were obtained from free range poultry farm environment in the UK (faeces, soil, litter beddings, wall). The isolates used in this study were isolated and identified as *C. perfringens* by Brown, (2018) using conventional cultural methods (growing in selective agar and gram staining) and molecular methods (PCR). *C. perfringens* ATCC 13124 strain was used as a positive control for biofilm formation in the laboratory. All isolates were stored at -80^{0}C in BHI with 30% glycerol stock until used. Before each experiment, the stock culture was streaked onto tryptose sulphite cycloserine agar (TSC) plates and incubated in anaerobic chamber at 37˚C for 24hrs.

Table 2.1: *Clostridium perfringens* isolates used in this study.

S/n	*C. perfringens isolates* ID	Source

1	N20.1	preterm neonate
2	N143.2	preterm neonate
3	N88.5	preterm neonate
4	N88.1	preterm neonate
5	N88.7	preterm neonate
6	N88.3	preterm neonate
7	N20.2	preterm neonate
8	N143.6	preterm neonate
9	N88.9	preterm neonate
10	N88.6	preterm neonate
11	N88.11	preterm neonate
12	N88.4	preterm neonate
13	N20.13	preterm neonate
14	N88.2	preterm neonate
15	N143.5	preterm neonate
16	N143.4	preterm neonate
17	N143.8	preterm neonate
18	N143.1	preterm neonate
19	N88.12	preterm neonate
20	E139	free range poultry
21	E25	free range poultry
22	E135	free range poultry
23	E61	free range poultry
24	E32	free range poultry
25	E2	free range poultry
26	E55	free range poultry

CONTINUATION

27	E38	free range poultry
28	E133	free range poultry
29	E15	free range poultry

30	E132	free range poultry
31	E60	free range poultry
32	E70	free range poultry
33	E14	free range poultry
34	E130	free range poultry
35	E11	free range poultry
36	E5	free range poultry
37	E6	free range poultry
38	C33	chicken clinical
39	CG36	chicken clinical
40	CI058	chicken clinical
41	CG43	chicken subclinical
42	CCR35	chicken subclinical
43	CG34	chicken subclinical
44	CG26	chicken subclinical
45	CG35	chicken subclinical
46	CG57	chicken subclinical
47	CG25	healthy chicken
48	CG27	healthy chicken
49	CCR27	healthy chicken
50	CJ29	healthy chicken
51	CC24	healthy chicken
52	CCR24	healthy chicken
53	CCR37	healthy chicken
54	CI056	healthy chicken

Antimicrobial agents

2.5. Antibiotics

Four antibiotics of different categories (drug class and mode of action) were used in this study. These antibiotics are administered to chickens either as antimicrobial growth promoter or to keep chickens healthy. Gentamicin (aminoglycoside /broad spectrum protein synthesis inhibitor), tetracycline (tetracyclines/broad spectrum protein synthesis inhibitor), penicillin (beta lactam/narrow spectrum inhibitor of bacterial cell wall synthesis) and bacitracin (polypeptide/ broad spectrum inhibitor of cell wall synthesis). Bacitracin is consistently used as an antibiotic growth promoter in the poultry industries.

Antimicrobial stock solutions

The stock solution for antibiotic used were prepared by dissolving antibiotic powder in deionised water in sterile universal bottles and then stored at 4˚C.

2.6 Gold and silver nanoparticles

Silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) have demonstrated broad spectrum antimicrobial effect against both Gram-positive and Gram-negative bacteria but there is no report in the literature of their antimicrobial activity on *Clostridium perfringens*. AgNPs were received as a gift from Dr Mark Baron, School of Chemistry, University of Lincoln. The average particle size (APS) was 31.2 nm diameter, zeta potential (mv) = 28.4 (minimal significant), uv-in was 390 (absorbance) and pH = 8.5 and the purity 99.9%. Gold nanoparticles were purchased from BBI solution UK (bbisolutions.com). Average particle size was 80nm (Product code EM GC80).

Calculation of Nanoparticles concentration

10mg of gold and silver nanoparticles was used. To calculate the concentration of nanoparticles in the stock solution, the following steps were performed.

Weight of empty Eppendorf = 0.49g

Weight of rack = 24.48g

Weight of Eppendorf + rack = 28.97g

Weight of Eppendorf containing 200µl of NPs solution + rack = 29.17g

Weight of NPs =0.2g

Weight of Eppendorf containing 200µl of NPs solution + rack after centrifuging = 28.98g

Weight of Eppendorf containing 200µl of NPs solution + rack after centrifuging - Weight of Eppendorf + rack = 28.98g-28.97g = 0.01g = 10mg

2.7. Leaf extract of medicinal plants

The antibiofilm activity of the ethanolic extract of three medicinal plants from the tropics were used. These are *Vernonia amygdalina* (common name - bitter leaf), *Ocimum gratissimum* (common name - scent leaf) and *Azadirachta indica* (Neem tree). These plants have been used traditionally for the treatment of various diseases and though there are scientific reports of their antimicrobial activity no report has been published on their specific antibiofilm effect.

To prepare the leaves for testing, they were washed thoroughly with distilled water and then air dried at room temperature before grinding into a fine powder in a sterile blender. The extract was prepared by weighing 12g of each ground sample and soaking in 100ml of 60% ethanol at room temperature for 72h with regular stirring. The plant extract was filtered using a Whatman no.1 filter paper. The filtrate was then

evaporated using a rotary evaporator to obtain the crude extract with a final concentration of 20mg/ml. The extract was stored at 4˚C until required.

2.8 Biocide

This work studied the antimicrobial properties of a novel compound acquorsol which is a QAC on C. perfringens to test for any antimicrobial activity. QAC is a common biocide primarily used as an intervention option against pathogenic bacteria on surfaces in healthcare facilities. Recontamination of surfaces, after disinfection, is a serious concern, therefore there is need to develop disinfectants that could prevent surface contamination and extend activity by exhibiting residual antimicrobial effect post disinfection.

2.9 General methods and procedures

Adequate care was taken during experiments to ensure all tests were carried out aseptically. The experiments were either carried out in a category II biohazard cabinet or on a working bench with a flaming bunsen burner. Work bench was wiped with alcohol and pipette was wiped with tissue soaked in alcohol. Glassware and pipette tips were sterilized by autoclaving before use to avoid any contamination. Disposable gloves were changed frequently to avoid contamination. Reagents were prepared from analytical laboratory grade chemicals. Deionized water was used as the solvent for most solutions. Weighing balance (Sartorius) was used to weigh dry chemicals. Appropriate pipettes were used for dispersing solutions with volumes ranging from 0.5-10µl, 2-20µl, 20-200 µl, 200-1000µl, 1-2 ml, 2-5ml and 5-25 ml. Measuring cylinders were used to measure volumes over 50ml. Media and reagents used were sterilized at 121°C for 15 min in a bench top autoclave after dissolving thoroughly in the solution.

Media and reagents were either stored at room temperature, in 55˚C incubator or in a 4°C refrigerator according to manufacturers' instructions.

2.10. Planktonic bacteria culture

Planktonic culture was prepared by taking three to four colonies of bacteria from an overnight bacterial culture on TSC agar plates. This was inoculated into 10ml of BHI broth and incubated at 37°C for 3 to 4h to obtain midlog phase culture. Growth curve for *C. perfringens* bacterial isolates is shown in appendix C.

Biofilm assay procedure

2.11. Biofilm growth in polystyrene well plates

C. perfringens biofilm were grown in 96 well microtiter plate which is a high through put method for biofilm assay and an effective method of growing anaerobic bacteria. Following an established method by Stepanovic *et al*., (2007); Donelli *et al*., (2012) with some modification. Isolates were grown on Tryptose sulphite cycloserine (TSC) agar by inoculating a loop (10µl) from *C. perfringens* stock broth recovered on agar plates. This was incubated at 37˚C for the required growth period in an anaerobic chamber. 3 to 4 colonies from the overnight culture plates were inoculated in 10ml of brain heart infusion (BHI) broth and incubated in an anaerobic cabinet without shaking at 37˚C for 3 to 4h. The optical density of the log phase broth culture was determined using a spectrophotometer (at 570nm) and when the optical density was 0.3, the inoculum was distributed. (The number of cells in the broth culture was confirmed as approximately 10^7CFU/ml by plating on sterile TSC agar plate for 24h at 37˚C both aerobically and anaerobically). Each well of the 96 well plate (83.3924 TC-plate 96 Well Standard.F SARSTEDT, Germany) was filled with 200µl of the broth culture. Three independent experiments were performed for each isolate and each experiment

was replicated (three replicates for each sample and three repeats). For each experiment, three wells of the 96 well plate was filled with 200µl of the sterile broth to serve as negative control (no bacteria). ATCC 13124 was used as a positive control for biofilm formation. The plates were sealed with 'parafilm' to minimize evaporation and incubated anaerobically at 37˚C in an anaerobic chamber connected to a compressed gas cylinder with a mixture of 95% nitrogen gas and 5% hydrogen gas. Because *C. perfringens* is an anaerobe and shaking were not necessary, growth was allowed to continue without shaking for the required number of days.

2.12. Biofilm quantification (Crystal violet staining)

To ascertain the level of biofilm produced, after the required growth period, the bacterial broth culture was carefully aspirated with pipette and the wells were rinsed three times with 200µl of sterile quarter strength Ringer's solution per well to remove non-adherent bacteria cells. Adherent cells in the wells were heat fixed at 55˚C for 1hr and 150µl of crystal violet (Pro-lab diagnostic) was added into each well for 10mins to stain biofilms. Afterwards, the crystal violet in the wells was discarded and the wells were rinsed with deionized water several times to remove excess stain. The tissue culture plates were tapped on paper towels to remove excess water in wells and then allowed to dry facing down. When wells were confirmed dry, 150µl of 33% acetic acid was added to biofilm in wells to solubilize the biofilm for 10mins. The absorbance value of each well was read with a microplate reader (BMG lab tech) at 570nm. This value determined the level/quantity of biofilm formed. Biofilm formation was determined by calculating the cut off OD (ODc) which is 3 standard deviations of the negative control added to the average optical density of the negative control. As described by Stepanovic *et al*. (2007), values less than ODc indicates no biofilm, values greater than ODc but less than 2 x ODc indicates weak biofilm. Values greater than 2 x ODc and less than 4 x ODc indicates moderate biofilm while values greater 4 x ODc

indicates strong biofilm. The average values obtained from three replicates of three repeats for each isolate were calculated and used for the determination of biofilm phenotype.

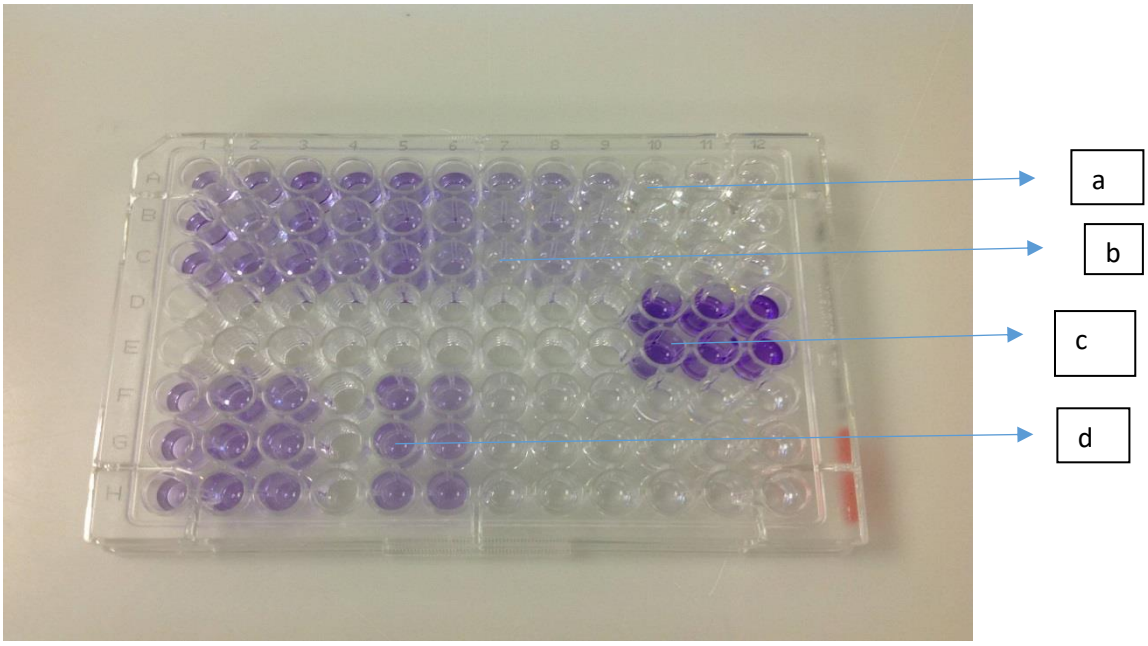


Figure 2.1: Microtitre plate for detecting biofilm formation showing solubilised adherent cells in wells.

The density of purple colour that appear after the addition of acetic acid indicates the extent of biofilm production. a; (negative control), b; (weak biofilm class), c; (strong biofilm class) and d; (moderate biofilm class).

2.13. Total viable cell counts

Biofilm was produced in wells of 96 well plates as described in section 2.10. Planktonic cells in the wells were removed and wells were washed gently with 200µl of sterile quarter strength Ringer's solution. Sterile pipettes were used to gently scrape adherent biofilms from wells and suspended in quarter strength Ringer's solution. A tenfold serial dilution was performed by pipetting 180µl of quarter strength Ringer's solution into wells in a new microtiter plate. 20µl from the bacterial suspension was transferred

into well A1, content was mixed by pipetting up and down before transferring 20µl to well A2 and this continued until the 8th dilution (10^{-1} to 10^{-8} dilution). After dilution, 10µl of each dilution was plated on TSC agar plates (spot plate) and plates were incubated overnight at 37˚C in an anaerobic chamber. To check that the cells were scrapped properly, crystal violet staining was performed on scrapped wells. Also, sterile broth culture was added onto wells and plates were incubated overnight to check for growth. and broth culturMicrobial loads of the adherent biofilm were reported in colony forming unit per ml. To calculate the number of biofilm cells per ml (CFU/ml) in the sample, the number of colonies was multiplied by the dilution factor and the result was divided by the volume of diluted cell suspension plated (inoculum).

CFU/ml = (Number of visible cell colonies X dilution factor)/ volume of inoculum plated

2.14. Coverslip biofilm assay

Colonies of bacteria from overnight culture on TSC agar plates were inoculated in BHI broth and incubated anaerobically at 37˚C for 3 to 4h to attain mid log stage and OD values of 0.3. 3ml of the broth culture was transferred into appropriate wells in 12 -well microtiter plates containing sterile broken pieces of microtiter plates. Plates were sealed with parafilm and incubated at 37˚C anaerobically for the required growth period. After the period of incubation, growth medium was aspirated from the wells and coverslips were washed three times with sterile quarter strength Ringer's solution.

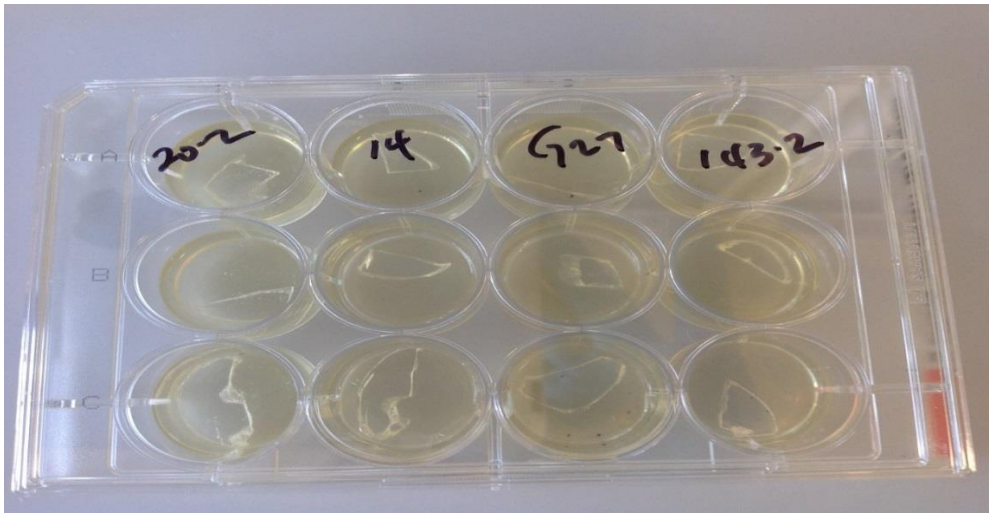


Figure 2.2: Broken pieces of microtiter plate in 12 well plate containing *C. perfringens* bacterial broth culture.

Microscopy

2.15. Light microscope

The ability of *C. perfringens* to adhere to surfaces was determined using a glass coverslip and imaging with a light microscope. *C. perfringens* broth culture (3 to 4h growth) were standardized to 0.3 optical density at 570nm and 3ml was transferred into 12 well plates containing coverslip. Following incubation at 37˚C for the required growth period, coverslips were gently washed in sterile quarter strength Rimger's solution, heat fixed and then stained with 2% crystal violet for 10mins. Adherent bacterial cells were imaged using 10X and 40X objectives giving overall magnification of 100X or 400X magnification.

2.16. Confocal laser scanning microscopy

Leica SP8 Confocal Microscope was used, and biofilm was imaged using the oil immersion objective 63X. Biofilms was grown on coverslips by transferring 3ml of *C. perfringens* mid log stage broth culture into 12 well microtitre plates containing coverslips glass. Coverslip placed in well with sterile BHI broth was used as control. This was incubated anaerobically for the required growth period and coverslips were

washed three times with 0.85% NaCl before staining. LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (Invitrogen, Molecular Probes Ltd) was used to determine the viability of the biofilm population and to estimate bacterial counts. *Bac*Light™ is composed of two nucleic acid-binding stains: SYTO9 and propidium iodide. SYTO9 is a membrane permeate dye that penetrates all bacterial membranes and stains the cells green. Propidium iodide (PI) can penetrate only dead cells or cells with compromised cell membranes and the combination of both stains in a cell produces a red fluorescing cell (Boulos *et al.* 1999). Dyes were stored in the freezer at -20˚C and thawed before use. 3µl of each dye was used and the dyed biofilms were incubated at 25˚C for 15min in the dark. A drop of *Bac*Light mounting oil (refractive index 25˚C= 1.517 +/- 0.003) was added to the biofilm and a coverslip glass (High precision coverslips Marienfold, Ref 0107032 was placed on the top of it and imaged with the CLSM. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide.

2.17. Scanning electron microscopy

Biofilms are established on glass cover slips or the bottom of polystyrene plates as described in section 3.2.2.4.2**.** After biofilm formation on microtiter plates or cover glass, surfaces were washed 2 times in quarter strength Ringer's solution and further washed in 0.1M potassium phosphate buffer (pH 7.2) for 3min at room temperature. Biofilm on plastic or glass surfaces were fixed by immersing the surface in 2.5% glutaraldehyde solution for 2h at room temperature. Thereafter, cover slips were washed 2 times in deionised water for 2mins each time. This was followed by dehydrating in graded series of ethanol 20%, 40%, 60%, 80% and 100% for 10mins each. After this, hexamethyldisilazane was used to freeze dry the sample for 10 minutes. Samples were mounted on aluminum stubs using an adhesive and coated with gold by sputtering prior to imaging (Eales *et al.,* 2018). Samples were inserted

into SEM chamber and beam of electron from an electron gun is used to produce a magnified image of the sample which is visualized in the computer.

2.18. Antimicrobial susceptibility testing

The minimum inhibitory concentration (MICs) of antimicrobials for planktonic cells in log phase were determined conventionally with the broth microdilution method as described by Clinical and Laboratory Standards Institute (CLSI, 2004). Briefly, bacterial culture at mid-log phase were diluted using sterile BHI broth to reach a bacterial cell density of about 1×10^6CFU/ml. 100µl of the diluted bacterial suspension were added to 100µl of two-fold serially diluted antimicrobial solutions in wells of 96 well microtiter plates and cultures were incubated in anaerobic chamber for 18- 24hr at 37°C. The MICs were determined by reading the absorbance in each well with the microplate reader. The lowest concentrations of antimicrobial agents that prevented turbidity after 18-24hr incubation were observed to have optical density of 0.1 or less.

2.19 Statistical analysis

Statistical analysis of the data was performed using R software version 4.0.3. Normality test of the data was checked by Shapiro-Wikinson normality test and Kruskal-Wallis test was performed to compare the means of multiple samples. Comparison of means of two samples was performed using t test or Wilcoxon test. All experiment results were expressed as mean ± standard deviation (SD) and each experiment was replicated at least two times. The test was considered statistically significant where *p* value was $p<0.05$

CHAPTER THREE:

BIOFILM FORMATION AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF PLANKTONIC AND BIOFILM GROWN *Clostridium perfringens IN VITRO*

3.1. Aims and objectives of study

The aim of this chapter is to evaluate the biofilm forming potential of *C. perfringens* recovered from a variety of sources. Specific objectives were,

(1) To determine the effect of temperature and time on *C. perfringens* biofilm formation

(2) To compare susceptibility of *C. perfringens* planktonic and biofilm cells to different antimicrobial classes.

(3) To compare biofilm susceptibility to antibiotics during their different growth stages in vitro.

3.2 Influence of growth temperature / time point on *C. perfringens* biofilm formation.

The difference in biofilm formation by *C. perfringens* when grown at different incubation temperatures was investigated on isolates from the different sources (n = 23). Tissue culture plates for growing biofilm were incubated in the anaerobic cabinet at 30˚C, 37˚C and 44˚C, respectively because *C. perfringens* is known to have a growth temperature range between 15˚C to 55˚C and an optimum temperature of between 43˚C to 47˚C. 3 to 4h BHI broth culture of *C. perfringens* was standardized to 0.3 optical density (10^7 CFU/ml). Biofilm was grown for the required period following the methods stated in sections 2.10 and 2.11. Biofilm formation was assessed after 24h growth period. The biofilm 'class' of each isolate was determined based on the method

by Stepanovic *et al.,* 2007. The experiment was conducted in triplicate and three separate experiments were performed. Subsequently analysis was performed to identify the best time for screening large number of *C. perfringens* isolates for their biofilm forming capability.

3.3. Determination of optimum period of incubation for biofilm formation

The optimum period of incubation to determine the growth of mature biofilm was by inoculating *C. perfringens* broth culture grown for 3 to 4h (OD_{570nm} 0.3) into wells of 96 well microtiter plates and incubating at 37˚C based on results from section 3.3. They were grown in an anaerobic chamber for 1 to 4 days, respectively. The extent of biofilm formed in the wells were determined each day by the crystal violet staining assay and viable cell counts as described in sections 3.11 and 2.12 Each experiment was replicated, and it was repeated on three separate occasions. For crystal violet staining, the average optical density of test and control wells was determined using a microplate reader and the cut off OD (ODc) was calculated as described by Stepanović *et al*. (2007).

3.4. Screening of *C. perfringens* isolates for biofilm formation

The biofilm forming potential of 54 *C. perfringens* isolates from a variety of sources and the positive reference control (ATCC 13124) was evaluated in BHI broth at 37˚C following 48h incubation based on results from preliminary experiments in this study. 3 to 4h broth culture was standardized to OD_{570nm} = 0.3 (~ 10^7 CFU/ ml) for biofilm assay. Each isolate was screened for their biofilm potential in three independent experiments and each experiment had three replicates. The cut off OD (ODc) was determined for each growth condition separately. The ODc is defined as 3 standard deviations (SD) of the negative control added to the average optical density of the negative control (uninoculated medium/ sterile broth) (Stepanovic *et al.,* 2007). Biofilm

formation of the isolates were assessed and classified as weak biofilm formers, moderate biofilm formers or strong biofilm formers based on the method published by Stepanović *et al*. (2007). In summary,

$OD \leq OD_C$ = no biofilm

$OD_C < OD \leq (2 \times OD_C)$ = weak biofilm

$2 \times OD_C < OD \leq 4 \times OD_C$ = moderate biofilm

$4 \times OD_C < OD$ = strong biofilm.

3.5. Comparison of antibiotic susceptibility profile of *C. perfringens* grown either planktonically or as a biofilm.

The minimum concentration of four different classes of antibiotics; gentamicin (aminoglycoside), tetracycline (protein synthesis inhibitor), penicillin (β-lactam antibiotic) and bacitracin (polypeptide) that inhibits the growth of planktonic and biofilm grown cells was determined as described by Balouiri, *et al.*, (2016). Broth microdilution assay for assessing minimum inhibitory concentration (MIC) of antibiotics was used. For planktonic grown cells, bacteria broth culture at midlog phase of growth was diluted using sterile broth to 0.01 optical density (10^6 CFU/ml). 100 µl of diluted broth culture was added to 100 µl of tested antibiotics. To determine the MICs for *C. perfringens* in biofilm, 100 µl of antibiotic was added to wells in microtiter plate holding pre-formed biofilm.

Minimum inhibitory concentration of planktonic cells

The antibiotic susceptibilities of selected isolates (n = 33) were determined using the broth microdilution method in microtiter plates. For planktonic grown cells, MIC was determined by inoculating single colonies from 24h TSC agar grown cultures in BHI broth, and this was incubated at 37°C in an anaerobic chamber for 3 to 4h. The antimicrobial challenge plates of tested antimicrobials (penicillin, gentamicin,

tetracycline, and bacitracin (Sigma) were prepared and were all tested at 2-fold concentrations between 0.13 µg/mL and 256 µg/mL. 100 µl of each concentration of the antimicrobial was transferred into wells of 96 well tissue culture plates (triplicate for each concentration). Broth culture was adjusted to 10^6CFU/ml and 100µl was added to each well in the challenge plate. Wells containing broth culture with no antibiotics served as negative control while sterility control wells contained sterile broth. Plates were incubated under anaerobic conditions overnight at 37˚C.The MIC of each antibiotic was determined by measuring the optical density in each well using the microplate reader at 600nm. The least concentration with optical density of 0.1 or less was considered the MIC.

Minimum inhibitory concentration of biofilm cells

The MICs for biofilm grown cells was determined by growing biofilms in 96 well flat bottom plates for 48h. Microtiter plates were inoculated with 3 to 4h broth culture of *C. perfringens* at OD_{570} (0.3). After 48h of biofilm growth in microtiter wells, broth containing planktonic cells was removed and wells washed three times with quarter strength Ringer's solution. Established biofilm in wells were exposed to 100µl of two-fold dilutions from 2µg/ml to 1024µg/ml of antibiotics; penicillin, gentamicin, tetracycline, and bacitracin (Sigma) and 100µl of sterile BHI broth was added to the test wells. Sterility control wells contained 200µl of sterile BHI broth with no preformed biofilm, growth control wells had preformed biofilm and 200µl of sterile BHI broth was added and all tests were performed in triplicate. Plates were incubated overnight at 37˚C anaerobically. The MICs of isolates in the biofilm mode was assessed by measuring the optical density in each well using the microplate reader at 600nm. Optical density of 0.1 or less was considered the MIC.

Results

3.6. Influence of different growth temperature on *C. perfringens* biofilm formation

The impact of different growth conditions on biofilm forming capability of *C. perfringens* including a reference strain (ATCC 13124) was investigated on selected isolates (n=23). Results are presented in Table 3.1. The strains produced different biofilm class under the tested growth conditions. For all the isolates, higher levels of biofilm mass were observed at 37˚C compared to 44˚C. The positive control strain ATCC 13124 displayed a weak biofilm phenotype. Interestingly, higher numbers of moderate biofilm phenotypes were observed during biofilm growth at 37˚C compared to biofilm growth at 44˚C. Biofilm growth at 44˚C showed higher numbers of weak phenotype or no biofilm formation.

The density of biofilm formation varied based on incubation temperature and tested isolates. Biofilms formed at 37˚C were significantly different ($P < 0.05$) than biofilm formed at 44˚C. Also, biofilm formed at 30˚C and 44˚C were significantly different ($P < 0.05$) showing higher biofilm mass at 30˚C. Biofilm growth at 30˚C and 37˚C was significantly different ($P < 0.05$). Since biofilm growth at 37˚C recorded the highest optical density, 37˚C was selected as the growth temperature for screening our isolates for their biofilm forming potential throughout the study.

A

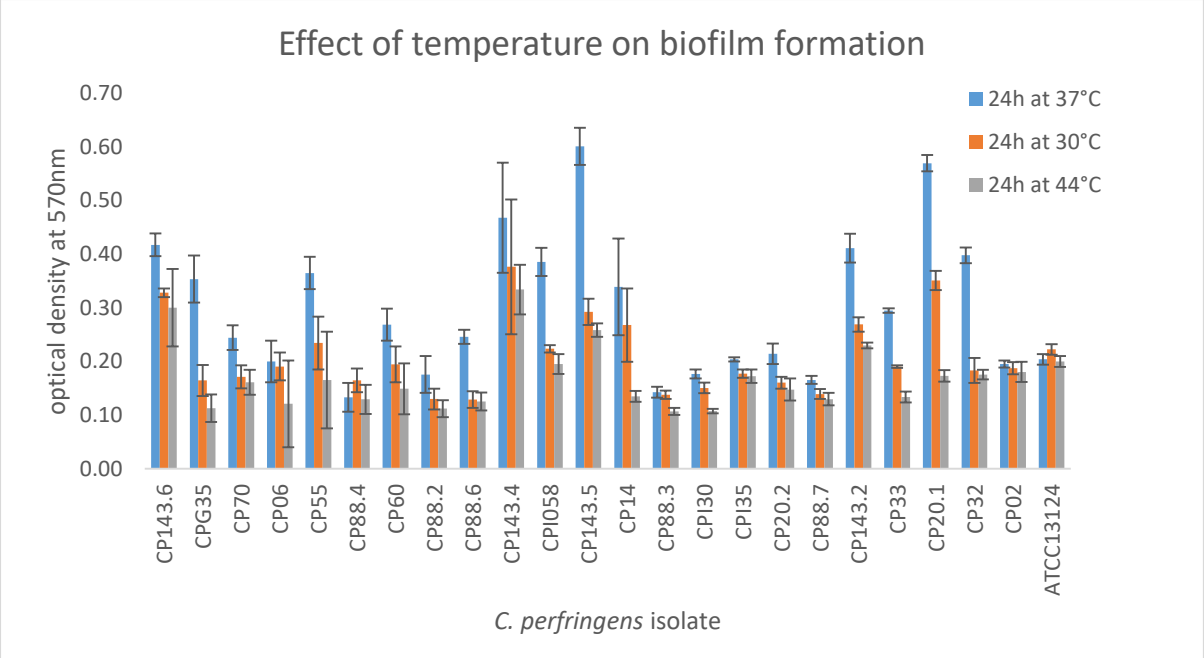
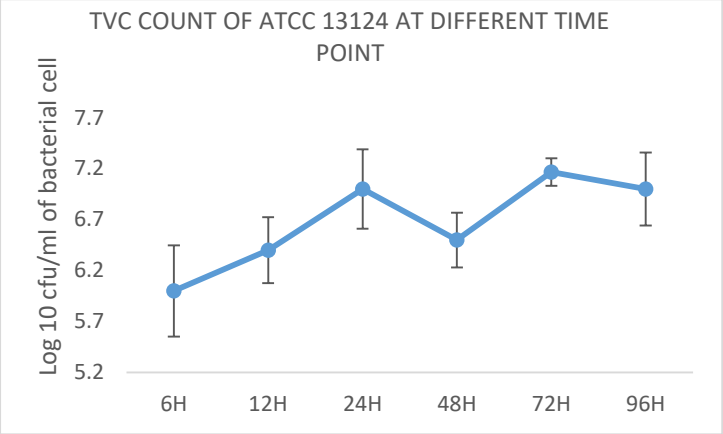


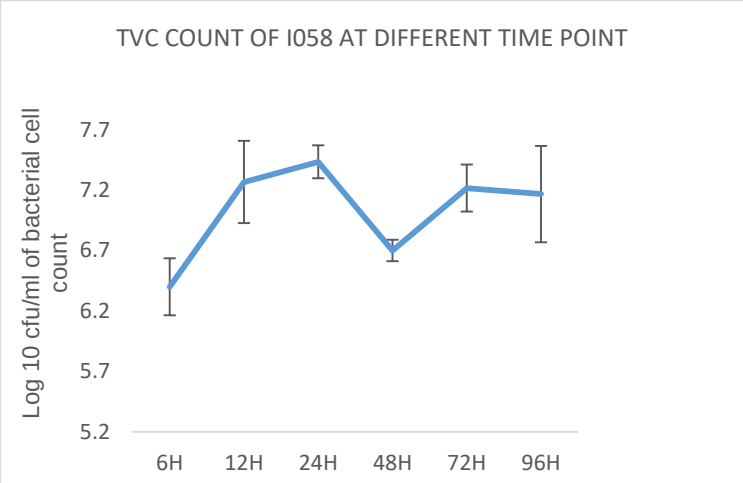
Figure 3.1 Comparison of biofilm formation by *C. perfringens* strains after 24hrs at different incubation temperature. Result is expressed as mean ± SD of triplicate assay.

3.7. Determination of optimum period of incubation for biofilm formation

Following the earlier work that showed higher biofilm mass when grown at 37˚C in BHI broth (section 3.7), biofilm production by *C. perfringens* at different incubation period in days was investigated using crystal violet assay and viable cell counts (Figure 3.2a and 3.2b). Biofilm mass obtained during the different incubation times were compared using ANOVA to determine an optimal incubation time for screening large number of *C. perfringens* isolates. Viable cell counts of biofilm cells and biofilm mass obtained from crystal violet assay both increased gradually from 6h biofilm growth to 24h biofilm growth. Fluctuation in biofilm cell count and biofilm mass was observed after 24h of biofilm growth thus biofilm growth after 24h were considered as matured biofilm. Though there was an initial rise in biofilm cell counts to 24h and then a decline, the difference was not statistically significant between all time points.



A



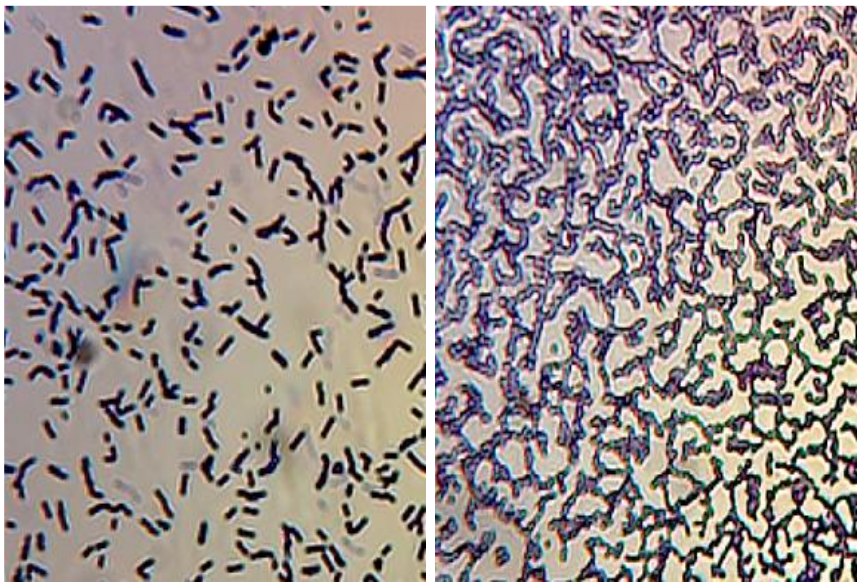
B

Figure 3.2: Optimum incubation period for biofilm growth using viable cell count. Result is expressed as mean ± SD of triplicate assay

3.8. Microscopic confirmation of biofilm formation

Light microscopy

Light microscopy was used to examine the morphology of adherent cells on coverslip glass. Adherent cells on glass surface stained with crystal violet were imaged using 100X magnification (Figures 3.2).



A B

Figure 3.3: Light microscope image of *C. perfringens* biofilm.

(A) 100X magnification of 1h old biofilm showing biofilm attachment (B) 100X magnification of 48h old biofilm showing clusters of bacterial cells considered as matured biofilm.

Scanning electron microscopy

As previously described in section 3.2.2.4.2, adherent *C. perfringens* cells on coverslip glass or microtiter plate coated with gold were mounted on an aluminum stub and imaged under different magnification (Figures 3.3.).

A

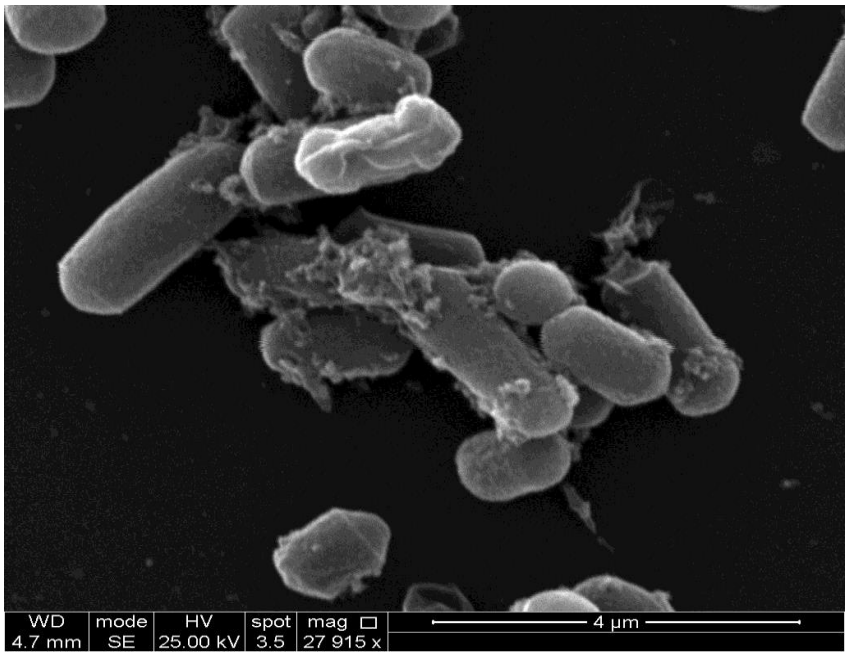


Figure 3.4: SEM image showing the formation of biofilm microcolony and EPS of 48h old *C. perfringens* biofilm grown anaerobcally at 37˚C on a cover glass.

3.9 Screening of *C. perfringens* isolates for biofilm formation

Table 3.1 shows the biofilm class of fifty-four *C. perfringens* tested isolates and the positive reference control (ATCC 13124) screened for their biofilm phenotypes by growing them individually in BHI broth at 37˚C anaerobically for 24h. OD values at 570nm obtained ranged from 0.13 ± 0.11 to 0.666 ± 0.003 (Table 3.2 and Table 3.3). Therefore, isolates with less than or equal to 0.109 were considered non-biofilm producers, OD_{570nm} values between 0.109 and 0.215 were considered as *weak* biofilm producers, OD_{570nm} values between 0.224 and 0.433 were considered *moderate* biofilm producers while OD_{570nm} values greater than 0.433 were considered *strong* biofilm producers. Of the 54 isolates tested, 7(13%) displayed strong biofilm phenotype, 31 (57%) produced moderate biofilm phenotypes, 16(30%) were weak biofilm producers and 0(0%) produced no biofilm under the conditions used in this study.

Table 3.1: Biofilm forming phenotypes of *C. perfringens*

***C. perfringens* isolates**	**OD 570nm**	**biofilm phenotypes**	***c. perfringens* isolates**	**OD 570nm**	**biofilm phenotypes**
CG57	0.51	Strong	N88.9	0.256	Moderate
CG27	0.43	Moderate	N143.4	0.254	Moderate
CG36	0.417	Moderate	N20.1	0.305	Moderate
CCR27	0.361	Moderate	N143.6	0.245	Moderate
CG35	0.352	Moderate	N88.4	0.27	Moderate
CJ29	0.325	Moderate	N88.3	0.132	Weak
CG34	0.319	Moderate	N88.7	0.13	Weak
CC24	0.298	Moderate	N143.5	0.283	Moderate
CG43	0.286	Moderate	E133	0.638	Strong
CG25	0.271	Moderate	E130	0.433	Moderate
CG26	0.271	Moderate	E15	0.377	Moderate
CC33	0.228	Moderate	E11	0.325	Moderate
CCR24	0.228	Moderate	E5	0.296	Moderate
CCR35	0.178	Weak	E139	0.293	Moderate
CCR37	0.177	Weak	E70	0.274	Moderate
CI056	0.17	Weak	E14	0.227	Moderate
CI058	0.152	Weak	E38	0.213	Weak
N88.5	0.666	Strong	E6	0.215	Weak
N88.2	0.623	Strong	E55	0.21	Weak
N20.2	0.6	Strong	E2	0.206	Weak
N88.1	0.573	Strong	E32	0.19	Weak
N143.2	0.443	Strong	E25	0.168	Weak
N20.13	0.422	Moderate	E60	0.264	Moderate
N143.8	0.371	Moderate	E61	0.151	Weak
N88.6	0.344	Moderate	E132	0.148	Weak
N143.1	0.305	Moderate	E135	0.224	Moderate
N88.11	0.185	Weak	ATCC 13124	0.134	Weak
N88.3	0.132	Weak			

Table 3.2: Classification of *C. perfringens* biofilm formation

Biofilm	Frequency	Percentage (%)	Absorbance (A_{570nm}) range
Strong producers	7	13	0.443-0.666
Moderate producers	31	57	0.224-0.433
Weak producers	16	30	0.13-0.215
Total	54	100	

Strains are classified as negative, when OD ≤ ODc; weak producers (ODc < OD ≤ 2 x ODc); moderate biofilm producers (2 x ODc < OD ≤ 4 x ODc) and strong biofilm producers (4 x ODc < OD). ODc = 3 x SD of negative control + average OD of negative control

3.10. Biofilm formation of *C. perfringens* isolated from different sources.

The biofilm phenotypes produced by *C. perfringens* isolates from three sources; human (n=19), broiler chicken (n=17) and free-range poultry environment (n=18) were compared (Figure 3.13). The highest OD values (0.666) was recorded from the preterm neonate group. All tested isolates were biofilm formers and isolates from the three groups displayed strong, moderate, and weak biofilm phenotypes. Figure 3.4. showed that out of the 19 isolates from neonates, 5(26%) displayed strong biofilm phenotype, 10(53%) displayed moderate biofilm phenotype, 4(21%) showed weak biofilm formation. Of the 18 samples from free range poultry environment investigated, 1(5.6%) isolate displayed strong biofilm phenotype, 9(50%) produced moderate biofilm while 8(44%) displayed weak biofilm phenotype. Of the 17 isolates from broiler chickens, 1(6%) produced strong biofilm, 12(71%) were moderate biofilm producers and 4(23%) were weak producers. Comparing biofilm formation (absorbance at

570nm) of strains based on the source of isolation using the single factor analysis of variance showed *p* values were > 0.05 and the difference was not significant.

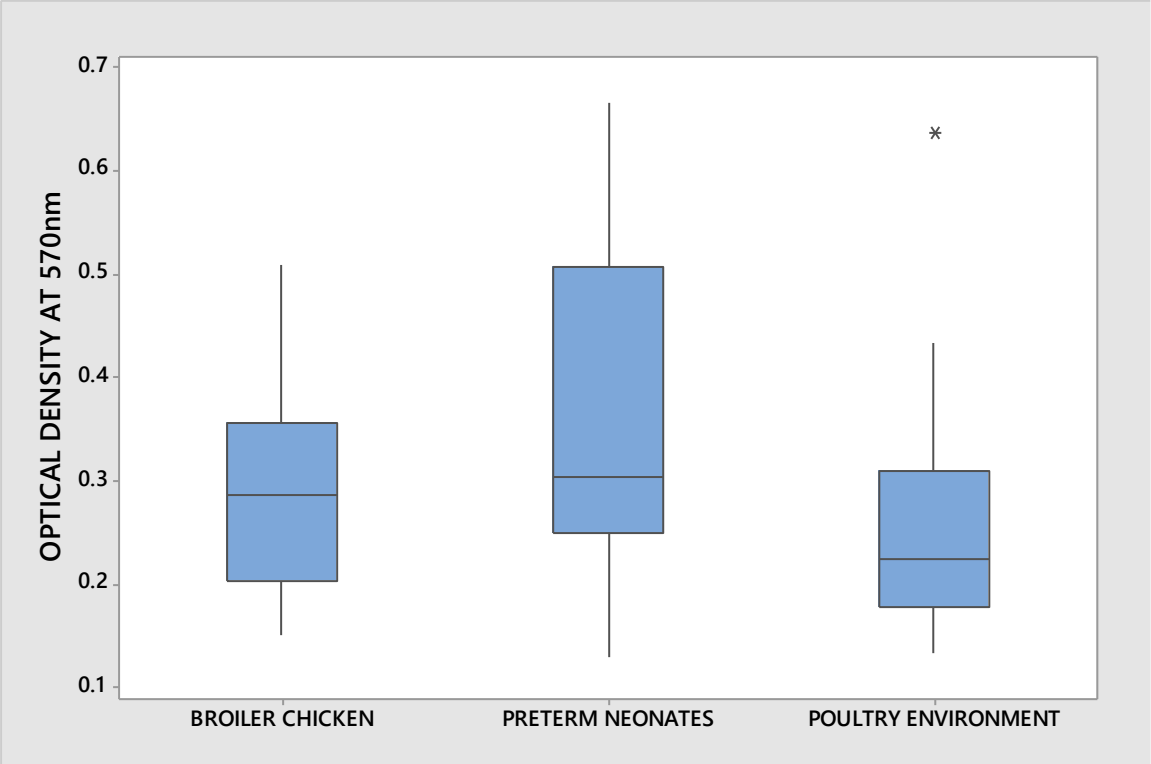


Figure 3.5: Biofilm formation by *C. perfringens* strains (n =54) grouped by origin of isolation.

Table 3.3 Density of biofilm formed by *C. perfringens* among source of isolates.

Isolation group	Strong Producers	Moderate producers	Weak Producers
Broiler chicken (n=17)	1(6%)	12(71%)	4(23%)
Free range poultry environment (n=18)	1(7%)	9(50%)	8(44%)
Preterm neonates (n=19)	5(26%)	10(53%)	4(21%)

3.11. Antibiotic susceptibility testing of biofilm and planktonic cells

The antibiotic susceptibility of planktonic compared with biofilm grown *C. perfringens* to four antibiotics (penicillin, gentamicin, tetracycline, and bacitracin) are presented in table 3.5 and table 3.6. Susceptibility to antibiotics was determined according to previous established clinical breakpoints on *C. perfringens* as reported by Charlebois *et al.,* (2014); Slavic *et al*., (2011) (penicillin: susceptible (S) ≤ 2µg/ml, resistant (R) > 2µg/ml; bacitracin: susceptible (S) ≤ 16µg/ml, resistant (R) > 16µg/ml; tetracycline: susceptible (S) ≤ 2µg/ml, resistant (R) > 2µg/ml; gentamicin: susceptible (S) ≤ 4µg/ml, resistant (R) ≥ 4µg/ml. Susceptibility pattern of isolates varied for different antibiotics. The MIC of four tested antibiotics on *C. perfringens* planktonic cells varied between 0.25µg/ml to >256 µg/ml whilst the MIC range of antibiotics on biofilm cell was considerably higher and different for each antibiotic. MIC of penicillin was between 2 µg/ml to 1024 µg/ml, MIC of bacitracin was between 64 µg/ml to >1024 µg/ml, MIC of gentamicin was between 8 µg/ml - >1024 µg/ml and tetracycline was between 2 µg/ml - >1024 µg/ml.

For the planktonic cells, 18 (53%) of tested isolates were resistant to penicillin and bacitracin while 26 (76%) and 30 (88%) of isolates were resistant to tetracycline and gentamicin, respectively. *C. perfringens* in the biofilm mode displayed 100% resistance to bacitracin and gentamicin while 28(82%) and 31(91%) were resistant to penicillin and tetracycline respectively (Figure 3.5). For bacitracin and gentamicin, 100% of the cells growing in biofilm became insensitive as opposed to 53% and 88% that were resistant in the planktonic growth mode.

The fold difference in MIC between planktonic and biofilm cells are shown in (Table 3.5). For penicillin, fold difference ranged between 2- 128, for bacitracin, fold difference was between 2 – 256, for gentamicin, it was between 2 – 128 while for tetracycline it was between 4 – 128.Though there was significant positive relationship between the MICs of antibiotics tested against planktonic and biofilm cells, ($P < 0.05$), some isolates that were susceptible in the planktonic mode of growth became insensitive when growing in biofilms.

Table 3.4: Susceptibility profile of *C. perfringens* planktonic and biofilm cells (n=34) tested in broth to antibiotics

C. perfringens	Penicillin MIC (µg/ml) MIC/MIC**	Fold diff	Bacitracin MIC (µg/ml) MIC/MIC**	Fold diff	Tetracycline MIC (µg/ml) MIC/MIC**	Fold diff	Gentamicin MIC (µg/ml) MIC/MIC**	Fold diff
N88.5	>256/512	2	>256/>1024	2	128/1024	8	>256/1024	4
N88.2	>256/1024	4	>256/>1024	2	64/1024	16	128/>1024	8
N88.9	0.25/2	8	4/128	32	2/256	128	64/>1024	16
N88.10	>256/1024	4	>256/>1024	2	64/1024	16	256/>1024	4
N88.6	0.5/8	16	2/64	32	0.5/4	8	128/>1024	8
N88.4	8/128	16	8/128	16	4/64	16	128/>1024	8
N143.4	0.25/2	8	4/128	256	0.25/2	8	0.25/8	32
N143.6	0.5/4	8	4/128	256	2/64	32	8/512	64
N20.13	0.5/64	128	8/256	32	1/8	8	4/512	128
N143.8	0.5/8	16	16/1024	64	16/128	8	32/1024	32
N143.2	0.25/2	8	0.25/64	256	0.25/2	8	0.25/8	32
N143.5	0.5/64	128	8/1024	128	8/64	8	128/512	4
N20.2	0.25/2	8	2/256	128	0.25/2	8	16/256	16
N88.11	0.25/256	4	4/>1024	256	8/512	32	32/256	8
N88.33	4/32	8	4/>1024	256	64/1024	16	32/256	8
CI058	128/1024	8	>256/>1024	2	256/>1024	4	32/>1024	32
CG35	4/64	16	>256/>1024	16	4/256	64	1/64	64
CG27	>256/512	2	64/>1024	2	64/1024	16	128/1024	8
CR37	2/64	32	64/512	8	32/1024	32	128/>1024	8
CG57	0.5/16	32	128/>1024	8	8/1024	128	256/>1024	4
C33	64/256	4	>256/>1024	2	64/>1024	16	64/>1024	16
CI056	64/512	8	>256/1024	2	64/>1024	16	128/512	4
CG43	128/1024	8	>256/>1024	2	2/64	32	128/>1024	8

Continuation of Table 1.

C. perfringens	Penicillin MIC (µg/ml) MIC/MIC**	Fold diff	Bacitracin MIC (µg/ml) MIC/MIC**	Fold diff	Tetracyclin MIC (µg/ml) MIC/MIC**	Fold diff	Gentamicin MIC (µg/ml) MIC/MIC**	Fold diff
E130	>256/1024	4	>256/>1024	2	128/1024	8	64/>1024	16
E5	4/64	16	64/1024	16	4/128	32	128/512	4
ATCC 13124	0.5/64	128	2/128	64	128/>1024	8	32/>1024	32
E15	>256/512	2	>256/>1024	2	256/1024	4	128/512	4
E14	0.25/2	8	4/256	64	16/1024	64	64/1024	16
E133	8/64	8	>256/>1024	2	128/>1024	8	128/>1024	8
E32	16/128	8	>256/>1024	2	8/512	64	64/256	4
E11	2/512	256	8/>1024	128	4/512	128	256/>1024	4
E02	8/32	4	>256/>1024	2	256/1024	4	256/1024	4
E55	16/512	32	>256/>1024	2	>256/1024	4	>256/512	2
E06	2/32	16	8/>1024	128	16/256	16	256/512	2

MIC: minimum inhibitory concentration for planktonic cells, MIC**: minimum inhibitory concentration of biofilm cells Statistical analysis of results revealed a significant positive relationship between the planktonic MICs and the biofilm MICs $P<0.05$

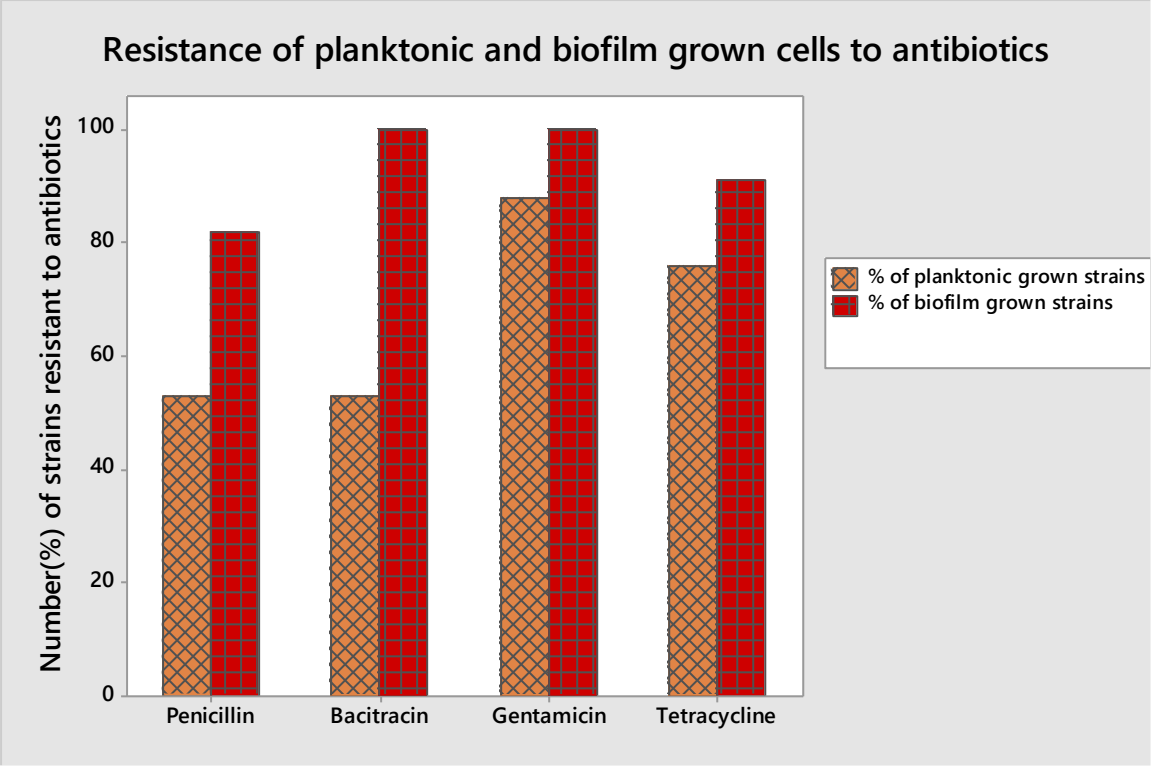


Figure 3.6: Antibiotic tolerance of *C. perfringens* planktonic and biofilm cells

Table 3.5: Minimum inhibitory concentration (MIC) of antibiotics µg/ml on *C. perfringens* planktonic and biofilm grown isolates.

	0.25		0.5		1		2		4		8		16		32		64		128		256		>256	
DRUGS	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*	MIC	MIC*
Penicillin	6	-	7	-	-	-	3	5	3	1	3	2	2	1	-	3	2	7	2	2	-	2	6	11
Bacitracin	1	-	-	-	-	-	3	-	6	-	5	-	1	-	-	-	3	2	1	5	-	3	14	24
Gentamicin	2	-	-	-	1	-	-	-	1	-	1	2	1	-	5	-	5	1	11	-	5	4	2	27
Tetracycline	3	-	1	-	1	-	3	3	4	1	4	1	3	-	1	-	6	4	4	2	3	4	1	19

Key: MIC – minimum inhibitory concentration for planktonic grown isolates; MIC* - minimum inhibitory concentration for biofilm grown isolates

3.12. Comparison of antimicrobial susceptibility of *C. perfringens* from different sources

The antibiotic susceptibility pattern of *C. perfringens* isolates from three sources growing in planktonic and biofilm mode to four antibiotics is shown in table 3.7. Antibiotics tested are commonly administered in the poultry industry is either as antimicrobial growth promoter or for the treatment of diarrhea. All 8 isolates tested from broiler chicken were resistant to all four antibiotics at the highest concentration tested (1024 µg/ml) when grown in biofilm. Only 13% and 27% of isolates from poultry environment and preterm neonates displayed any sensitivity to penicillin while 20% of isolates from preterm neonates displayed any sensitivity to tetracycline. Differences between susceptibilities of *C perfringens* from the various sources were strain and antibiotic dependent. Resistance to tested antibiotics observed could be attributed to overuse of these antibiotics which can make organisms develop tolerance.

Table 3.6: Antibiotic susceptibility of planktonic and biofilm cells from different sources

Source (N)	Penicillin	Bacitracin	Gentamicin	Tetracycline
	%S/%S*	%S/%S*	%S/%S*	%S/%S*
Broiler chicken (8)	25/0	0/0	13/0	13/0
Poultry environment (11)	36/13	36/0	0/0	0/0
Preterm neonates (15)	67/27	80/0	20/0	53/20

N: Number of isolates; %S: percentage susceptibility of planktonic cells; %S*: percentage susceptibility of biofilm cells

3.13. Comparison of antimicrobial susceptibility of *C. perfringens* grown in different modes.

The effect of bacterial growth stage on antibiotic susceptibility of 8 randomly selected *C. perfringens* isolates from broiler chicken and free-range poultry environment were tested. Differences in MICs of antibiotics on mature biofilms (48h old biofilm), early adherent cells (1h old biofilm) and planktonic cells was observed as presented in Table 3.8. For most isolates tested, the MICs of tested antibiotics were higher for the matured biofilm compared to the early adherent cells. Also, the bacterial counts of the matured biofilm were higher than the bacterial counts of the early adherent cells (Table 3.7). The higher bacterial count and MIC observed for the 48h old biofilm is inline with the the result of biofilm imaging observed with the light microscope as shown in Figure 3.7. were the 1h old biofilm showed fewer bacterial cells without clusters compared to the 48h old biofilm that formed clusters with more bacterial cells.

Table 3.7: Antimicrobial susceptibilities of biofilm producing isolates of *C. perfringens* grown in different modes.

Isolate growth mode	Bacterial count (CFU/Ml)	Penicillin (µg/ml)	Bacitracin (µg/ml)
		MICs	MICs
CI056			
Log planktonic cells	2×10^7	64	>256
Early adherent cells	2×10^6	64	>256
Mature biofilm	1.6×10^7	512	1024
CI058			
Log phase planktonic cells	2.5×10^7	128	>256
Early adherent cells	1×10^6	128	>256
Mature biofilm	1.3×10^7	1024	>1024
CC33			
Log phase planktonic cells	2.3×10^7	128	>256
Early adherent cells	1.5×10^6	64	>256
Mature biofilm	1.4×10^7	256	>1024
CG27			
Log phase planktonic cells	2.5×10^7	>256	>256
Early adherent cells	2×10^6	>256	>256
Mature biofilm	1.6×10^7	512	>1024
E32			
Log phase planktonic cells	2.5×10^7	64	>256
Early adherent cells	1.2×10^6	64	>256
Mature biofilm	1.6×10^7	128	>1024

Table 3.7: Continues

Isolate growth mode	Bacterial count (CFU/Ml)	Penicillin (µg/ml)	Bacitracin (µg/ml)
E55			
Log phase planktonic cells	2.5 X 10^6	32	>256
Early adherent cells	1.5 X 10^6	64	>256
Mature biofilm	1.6 x 10^7	512	>1024
E5			
Log phase planktonic cells	2.2 X 10^7	8	128
Early adherent cells	1 X 10^6	32	>256
Mature biofilm	1.3 x 10^7	64	>1024
CG35			
Log phase planktonic cells	2.4 X 10^7	16	>256
Early adherent cells	2 X 10^6	16	>256
Mature biofilm	1.3 x 10^7	64	>1024

3.14. Discussion

Biofilm formation is considered as a major factor in diseases (Mah and O'Toole, 2001). Antimicrobial tolerance, resistance to environmental stress and disinfectants by *C. perfringens* biofilms have been previously reported. Charleboise *et al*., (2017) demonstrated that *C. perfringens* biofilm showed tolerance to disinfectants such as sodium hypochorite, QAC, potassium monopensulphate, hydrogen peroxide and glutaraldehyde. Vargal *et al*., (2008) reported that *C. perfringens* can respond

carbohydrate limitation by producing biofilm. Considering the importance of biofilm in increasing drug resistance, there is a need for more efficient prevention and control strategies for infections, and more appropriate methods to control and prevent biofilm related infections. Studies by Charleboise *et al*., (2014) has characterized the biofilm forming potential of human and animal strains of *C. perfringens* and strains produced different biofilm class. This study agrees with the work of Charleboise *et al*., (2014) by showing biofilm formation of *C. perfringens* isolates from human and chicken and aims to extend the understanding of the biofilm forming capability of *C. perfringens* and how biofilms affect antibiotic tolerance.

Some studies have reported the influence of growth temperature on *C. perfringens* biofilm formation. *C. perfringens* is known to have a growth temperature range between 15˚C to 55˚C and an optimum temperature of between 43˚C to 47˚C (Li and McClane 2006). This present study compared biofilm growth at 30˚C, 37˚C and at 44˚C and in contrast with Charleboise *et al*., (2014) showed that biofilm grown at 30˚C and 37˚C produced more adherent cells than biofilm grown at 44˚C. This does not correlate with Charleboise *et al*., (2014) where 44˚C was reported as the optimal growth temperature for biofilm growth for *C. perfringens* strains. In contrast, Obana *et al.,* (2014) reported the influence of temperature on *C. perfringens* biofilm morphology. Their data showed that more adherent biofilm was formed at 37˚C compared to biofilm formed at 25˚C. Lee *et al*., (2013) discovered that *Listeria monocytogenes* formed biofilm faster at 44˚C compared to at 30˚C and 45˚C. Tezel *et al*., (2016) reported higher percentage of biofilm formation by *Salmonella* infantis strain at 28˚C compared to biofilm growth at 20˚C and 37˚C. The differences in results could be related to the different growth media used and bacteria tested.

In the present study, biofilm formation of two strains were analyzed for 4 days at 37˚C to obtain the growth pattern of biofilm at different biofilm growth stages. Measuring the biofilm formation at specific time intervals proved valuable. It showed that biomass increased from 6h to 24h. It is likely that matured biofilm is formed around 24h of biofilm growth and thereafter, disintegration of biofilm cells occurs which might be the likely cause of the fluctuating biofilm mass observed after 24h of biofilm growth as shown in figure 3.1. Results obtained from crystal violet assay were correlated with visual observation of biofilm morphology using the light microscope, confocal laser scanning microscope and scanning electron microscope. The ability of the established growth condition (24h at 37˚C) to support biofilm formation was confirmed microscopically using the positive control strain ATCC 13124 before the test isolates were screened.

Although growth conditions (media, temperature, and time) used for biofilm formation in this study were different from those described in earlier studies by Varga *et al*., (2008); Donelli *et al.,* (2012); Charlebois *et al*., (2014); Obana *et al*., 2014; Vidal *et al*., (2015), results from the present study agrees with data obtained by Charlebois *et al*., (2014). Charleboise *et al*., (2014) reported that clinical and commensal strains of *C. perfringens* grown at 44°C in Tryptic soy broth (TSB) for 6 days produced biofilm with different types of biofilm class (weak, moderate, and strong biofilm class).

Biofilm formation by microorganisms have been shown to contribute to the persistence of diseases as biofilm can adhere and grow on surfaces. Results from this present study suggest that isolates of *C. perfringens* can form biofilms and therefore may be associated with chronic infections. All *C. perfringens* isolates (from chicken, poultry environment and humans) (n=54) tested in this study were able to form biofilms under the growth conditions used in this study. Human clinical isolates formed more intense

biofilm than the chicken and environmental isolates with optical density (OD) values between 0.13 and 0.67 but the difference in OD was not significant (p>0.05). The chicken and environmental isolates displayed OD values between 0.15 and 0.51; 0.64 and 0.19, respectively. Donelli *et al*., (2012) obtained a higher OD value of 3.2 for *C. perfringens* strain (CpeBs31) and this strain was classified as a strong biofilm producer based on the growth conditions used in their study. There was no significant difference in biofilm formed by isolates from the three sources (chicken, human and environmental sources) or between clinical and commensal isolates. Similar results were reported by Reisner *et al.,* (2006). In their study, there was no significant difference in biofilm formed by *E. coli* isolates from diverse sources. Hussain and Oh, in 2018 reported that origin of strains influenced *Bacillus cereus* biofilm formation during early incubation period. Upadhyaha *et al*., (2011) also found that clinical isolates of *Enterococcus faecalis* produced significantly higher biofilm when compared to their commensal counterparts. In *Enterococcus faecium*, it was found that 67.5% of clinical isolates formed and this was significantly greater than biofilm formed by fecal isolates from community volunteers which was 32% (Almohamad *et al*., 2014). Barbosa *et al*., (2013) observed greater capability for biofilm formation from food isolates compared to clinical isolates from cases of listeriosis.

The application of antibiotics for the routine treatment of infections is more productive in treating planktonic cells compared to biofilm cells because the extracellular matrix of the biofilm protects bacteria from antimicrobial agents (Clayton and Thien-Fah Mah, 2017). The heterogeneous population of cells in biofilm has slow and fast-growing cells and this reduced metabolism and slow growth rate may lead to resistant to antibiotics which act on actively growing cells. Other factors that contribute to recalcitrance of biofilm cells is caused by the reduced growth rates of cells in the biofilm, little or no

penetration of antibiotics through the biofilm and exchange of genetic determinants of antibiotic tolerance. The viability and minimum inhibitory concentration of biofilm and planktonic cells of *C. perfringens* have rarely been studied compared to other biofilm producing bacteria. The ability of *C. perfringens* to produce biofilm could be an important virulence factor as biofilm can enhance antimicrobial tolerance, facilitate recurrent diseases, and hinder the immune system from eliminating infections. Understanding the biofilm producing potential of *C. perfringens* and their antibiotic tolerance will help to enhance the prevention and control strategies of *C. perfringens* infections.

This study showed that biofilm could protect *C. perfringens* from antibiotics. Vargal *et al*., (2008); Charlebois *et al.*, (2014) have previously reported increase in biofilm tolerance to 20µg/ml of penicillin G compared to their planktonic counterparts of same strain. In this study, *C. perfringens* isolates (n=34) displayed varied (MICs) value to tested antibiotics. In this study, 16 of the 34 planktonic strains were resistant to penicillin and bacitracin, 4 were resistant to gentamicin while 8 were resistant to tetracycline. A smaller number of biofilm cells were susceptible to the tested antibiotics; 6 were susceptible to penicillin, none of the biofilm cells were susceptible to bacitracin and gentamicin while 3 were susceptible to tetracycline. The presence of a biofilm increased the MICs up to 2 - 256-fold in biofilm grown cells and bacitracin and penicillin had the highest fold difference. Antibiotic susceptibility was not dependent on biofilm class, suggesting that resistance to antimicrobial was not dependent on class of biofilm but more on the existence of cells in biofilm mode. Charlebois *et al.,* (2014) compared the viability of planktonic and biofilm cells following exposure to antimicrobials and observed a significantly higher survival rate for the biofilm cells. Varga *et al*., (2008) observed that biofilm cells have 15-fold increase in

percentage survival rate over planktonic cells after exposure to 20µg/ml of penicillin G for 6h. Also, data from this study agrees with previous study that reported significantly higher viability rates of biofilm cells compared to those observed in planktonic cells. Charleboise *et al*., 2014 reported a rise in survival rate of 0.6 – 9-fold after 6h and 0.8 to 36-fold rise in survival rate after 24h of exposure to antibiotics. This suggests that biofilm formation may be an important survival mechanism of *C. perfringens* in infections such as necrotic enteritis and necrotizing enterocolitis.

This data on high tolerance of biofilm cells is consistent with the report of Sevanan *et al*., (2011) where 50% of the biofilm cells were resistant to gentamicin. Olson *et al*., (2002) found that *Streptococcus dysgalactiae* and *Streptococcus suis* biofilm and planktonic cells were sensitive to penicillin. They also reported sensitivity of *E. coli, Salmonella* spp*.* and *Pseudomonas aeruginosa* planktonic cells to gentamicin but gentamicin was only effective against *E. coli* biofilm cells. Tezel *et al.*, (2016) found that *Salmonella* infantis serovars biofilm were sensitive to gentamicin while nalidixic acid, spectinomycin, tetracycline and neomycin induced biofilm formation.

This study also investigated the effect of antibiotics on different stages of biofilm development. Planktonic cells at mid-log phase, early adherent monolayers (early biofilm) and mature biofilms (48h grown biofilm) were assessed for their susceptibility to two antibiotics. The aim was to test bacterial cultures at different growth stages with the same concentration of antibiotics. Results from this study showed significantly higher MICs for mature biofilm compared to when *C. perfringens* were in mid log planktonic growth stage and early adherent stage. In coagulase negative staphylococci, it was found that the MICs and MBECs of planktonic grown and early adherent monolayer cells were significantly lower than mature biofilm (Yu, 2017). This finding suggests that after initial bacterial cell attachment to a surface, *C perfringens*

may be susceptible to antimicrobials, but resistance may develop as the cells in the biofilm proliferate and develop into mature biofilm. As shown in Figure 3.2, early adherent biofilm displayed few bacterial cells that did not attach to each other unlike the mature biofilm that displayed more bacterial cells attached to each other. This therefore suggest that biofilm related infections could be controlled by early treatment.

Kiu *et al.,* (2019) analyzed the genome of 88 *C. perfringens* isolates from broiler chicken and poultry environment and these includes 9 of the isolates used in this present study. Isolates from this present study subjected to genome analysis includes four environmental isolates, four isolates from broiler chicken linked with necrotic enteritis and one isolate from healthy chicken. The toxin genes (*plc, ccp* and *colA* and at least 4 other toxin genes) out of the 12 toxin genes evaluated were expressed in all nine isolates tested. Collagen adhesin genes (*cna, cnaA, cnaC and cnaD*) were produced by seven out of the nine isolates. Four of the isolates that harbored the adhesin genes were moderate biofilm formers while three were weak biofilm formers. Also, the moderate biofilm producers harbored more than one adhesin genes while the weak biofilm producers harbored one adhesin gene each (Kiu *et al*., 2019). From these results, it is difficult to say if there is a correlation between biofilm class and adhesin gene production because of the low number of isolates tested.

3.15. Conclusion

In summary, the work in this chapter gives a general understanding of *C. perfringens* biofilm which has received little research attention. The role of biofilm formation in *C. perfringens* diseases and the possibility that biofilm could hinder the success of antimicrobial therapy has been highlighted here. The work reports that *C. perfringens* can produce biofilms which can protect the embedded bacterial cells from antibiotics.

Results shows no correlation between tolerance to antibiotics and source of isolates. Also, the ability of tested *C. perfringens* isolates to form biofilm and the biofilm class produced was not dependent on the source of the isolates. Furthermore, this study reports that the extent of biofilm formed by *C. perfringens* was dependent on growth temperature therefore, temperature should be considered while planning for the experimental control of *C. perfringens* biofilms in laboratory experiments. Future study will investigate the genetic components of *C. perfringens* to determine if there is any correlation between the presence of adhesin genes and biofilm density.

CHAPTER FOUR:

ANTIMICROBIAL EFFECTS OF SILVER AND GOLD NANOPARTICLES ON BIOFILMS OF *Clostridium perfringens*

4.1 Introduction

Many diverse bacteria growing as biofilms have been associated with AMR (Singh *et al*., 2017). AMR in pathogens is a growing problem and represents a serious public health concern. AMR arises from the development or acquisition of resistance genes by microorganisms promoted by the careless use of antibiotics in animals and people. AMR has been reported against all recent and 'old' discovered antibiotics thus encouraging the urgent need for new/alternative therapeutic strategies (El-Gohary *et al*., 2020; Chen *et al*., 2013; Liu *et al*., 2017; Khan and Khan 2016).

The use of nanomaterials as an alternative for antibiotics is intensively researched currently (Liu *et al.,* 2017; Karaman *et al.*, 2017). Nanotechnology is one of several potential strategies designed to control bacteria, fungi, viruses and other microorganisms (Qayyum and Khan 2016; Bakkiyaraj and Pandian 2014; Huh and Kwon 2011). Nanoparticles utilize the unique properties of metals when in nanoforms - the high surface to volume ratio of nanomaterials, high reactivity, small sizes in the nanometer range and shape of nanoparticles have been linked with various novel properties when compared with their bulk materials (Karaman *et al*., 2017; Franci *et al*., 2015). NPs act by either direct contact with the bacteria cell or through the release of silver ions. Although the precise nature of AgNPs antimicrobial action is unknown, they attach to the cell wall of target bacteria and penetrates it causing leakage through membrane damage. In the cell, AgNPs interact with cellular biomolecules like protein causing dysfunction leading to cell death. Also release of silver ions can react with

enzymes, lipids and DNA and release ROS and free radical species such as superoxide ions, hydrogen peroxide, singlet oxygen and hydroxyl radical which have antimicrobial action (Yeh, *et al.,* 2020; Qayyum and Khan, 2016).

The size of NPs influences their ability to penetrate inside the bacteria. Lu *et al*., (2013) reported that 5nm of AgNPs showed higher antibacterial activity compared to 12nm and 55nm AgNPs on *E. coli* and *S. mutans.* Moreover, the shapes of NPs have been reported to influence antibacterial activity. Copper nanocubes have been reported to show enhanced antibacterial activity on *E. coli* and *E. faecium* when compared to copper nanospheres (Alshareef *et al*., 2016). Pal *et al*. (2007) showed that only 1 µg of truncated triangular AgNPs was required to inhibit *E. coli* compared to 12.5 µg of spherical shaped NPs. Alshareef *et al*., (2017) demonstrated the antimicrobial activity of different concentrations (50, 100 and 1000µg/ml) of spherical and truncated octahedral shaped AgNPs against *E. coli* and *E. faecium* using crystal violet assay and viable counts. The truncated octahedral shaped AgNPs showed more antimicrobial activity when compared to spherical shaped AgNPs. The authors attributed the difference in efficacy to the higher surface area and more reactive facets of truncated shaped AgNPs compared to the spherical shaped AgNPs.

Research recently has demonstrated the broad spectrum antibacterial and antibiofilm activities shown with both silver nanoparticles (AgNPs) as well as gold nanoparticles (AuNPs) although they are chemically very different. El-Gohary *et al*., (2020) reported the bactericidal activity of the combination of AgNPs and hydrogen peroxide on five multiple drug-resistant Gram-negative bacteria to range between 6.25µg/ml and 50µg/ml. Panacek *et al.,* (2016) investigated the effect of AgNPs alone and the combination of AgNPs with different antibiotics against pathogenic bacteria. Their results showed the AgNPs reduced the antibiotic MICs and made the bacteria

susceptible to the tested antibiotics. Furthermore, their study revealed that the antibacterial effect of the combined therapy was dependent on the antibiotic class. They reported reduction in viability of cells from 85% to 71%, 73%, 72% and 73% when AgNPs was combined with ampicillin/sulbactam, meropenem, cefozolin and chloraphenical respectively. They were able to show an enhancing effect for AgNPs and antibiotics against either Gram-negative or Gram-positive bacteria by using different mechanisms. This suggests that combining NPs with antibiotics could be a possible alternative therapeutic strategy against biofilms. Singh *et al*., (2019) showed that alginate nanoparticles effectively penetrated *P. aeruginosa* biofilm. They also reported nanoparticles as a potential antibiofilm agent that will be useful in combination treatment against biofilm prevention and reduction. Reports by Cotton *et al*., (2012); Dixon *et al*., (2012); Guzman *et al*., (2012); Ramasamy *et al*., (2016); Gurunathan *et al*., (2014); Ansari *et al*., (2014); Martinez-Gutierrez *et al*., (2013) have shown biofilm reduction and inhibition when biofilms produced by Gram-positive and Gram-negative bacteria were treated with either silver or gold nanoparticles. These reports have shown the antibacterial activity of AgNPs and AuNPs on different bacteria but to my knowledge their activity on the Gram-positive anaerobe *C. perfringens* has not been reported.

4.2 Aim and objectives

The aim of this chapter was to determine the in vitro activity of silver and gold nanoparticles against bacterial biofilms formed by *C perfringens*. The specific objectives were.

(1)To determine the effect of AgNPs on *C. perfringens* pre-formed biofilm

(2)To determine the effect of AuNPs on *C. perfringens* pre-formed biofilm

(3)To determine the effect of biofilm growth stage on the anti-biofilm activity of AgNPs or AuNPs

(4)To assess the antibiofilm activity of antibiotics when combined with AgNPs or AuNPs

4.3. Reduction of preformed biofilm by metal nanoparticles

Biofilms of seventeen isolates representing different biofilm class and isolate origin of *C. perfringens* (see section 2.4 for strain identification) including the laboratory reference ATCC 13124 strain were grown for 6h. 12, 24h or 48h in 96 well microtitre plates and using the total viable counts assay to quantify bacteria as described in sections 2.10 and 2.12. Preformed biofilm in each well was then treated by adding 100µl of silver nanoparticles or gold nanoparticles (see section 2.6. for concentration and analysis). Plates were incubated at 37˚C anaerobically for the desired period. The viable cells remaining after treatment was counted as well as viable cells in untreated samples. The effect of silver or gold nanoparticles on 'mature' established biofilm was determined according to the formula:

Percentage reduction/kill = (A-B) X 100/A

Percentage survival = B/A X 100

A = Adherent cells in untreated wells

B = Adherent cells in treated wells

4.4. Evaluation of enhanced antibiofilm effect when combining metal Nanoparticles and antibiotics on *C. perfringens* biofilm.

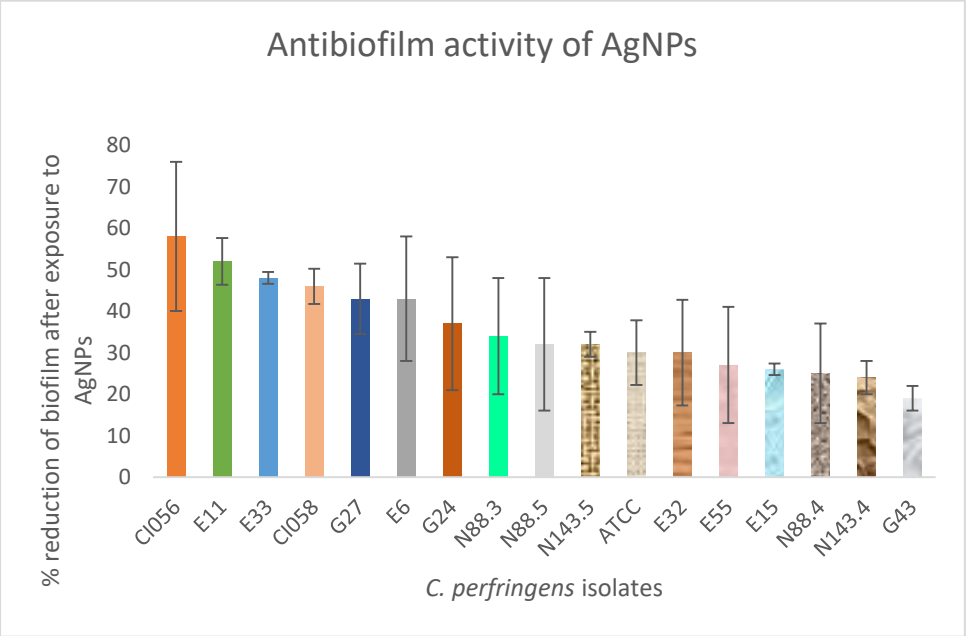
To evaluate the effect of combining metal nanoparticles with traditional antibiotics on *C. perfringens* biofilm, 48h established biofilm in wells of microtiter plate were treated with both antibiotics and metal nanoparticles. Biofilms were treated with 100µl of 10mg/ml solutions of either silver nanoparticles or gold nanoparticles alone and one of the following antibiotics at the MIC breakpoint. Penicillin (2µg/ml), gentamicin (8µg/ml), bacitracin (16µg/ml) or tetracycline (2µg/ml)) and a combination of antibiotic with nanoparticles. 100µl of the antimicrobial agent was added to each well and plates were incubated for 6h at 37˚C anaerobically. Following this, surviving bacteria in each well was determined as described in section 2.12. Percentages showing biofilm reduction were calculated as described in section 4.2.

Results

4.5. Antibiofilm activity of metal nanoparticles

The ability of silver nanoparticles and gold nanoparticles to reduce biofilms formed by the pathogen *C. perfringens* was determined under *in vitro* conditions. All 17 test isolates (for details of sources see section 2.4) were grown for 48h (to establish mature biofilm) in wells of microtiter plates and then treated with 10mg/ml concentration of nanoparticles for 24h. Though there are no reports in the literature on any antimicrobial effect of NPs on *C. perfringens*, researchers have reported the antibiofilm activity of varied concentrations of AgNPs and AuNPs on Gram-positive and Gram-negative bacteria. In this present study 10mg/ml of NPs was used to test for any antibiofilm activity on *C. perfringens* and the antibiofilm effect observed will determine if a higher or lower concentration will be tested in further study. The results in Figures 4.1A and 4.1B show the percentage of biofilm reduction for each of the tested isolates. Treatment of *C. perfringens* with silver nanoparticles resulted in 19% - 58% biofilm reduction (Figure 4.1A) whilst treatment with gold nanoparticles showed a lower

percentage reduction of biofilm ranging from 12% to 39% (Figure 4.1B). Although all test isolates were subjected to the same concentration of nanoparticles, different level of biofilm reduction for each strain were produced. The antibiofilm activity of nanoparticles appears strain dependent.



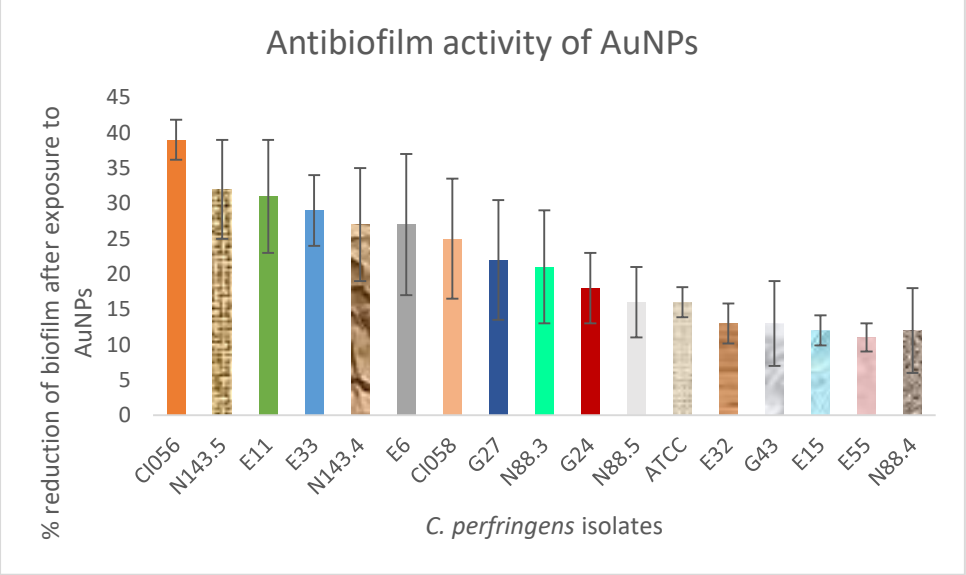
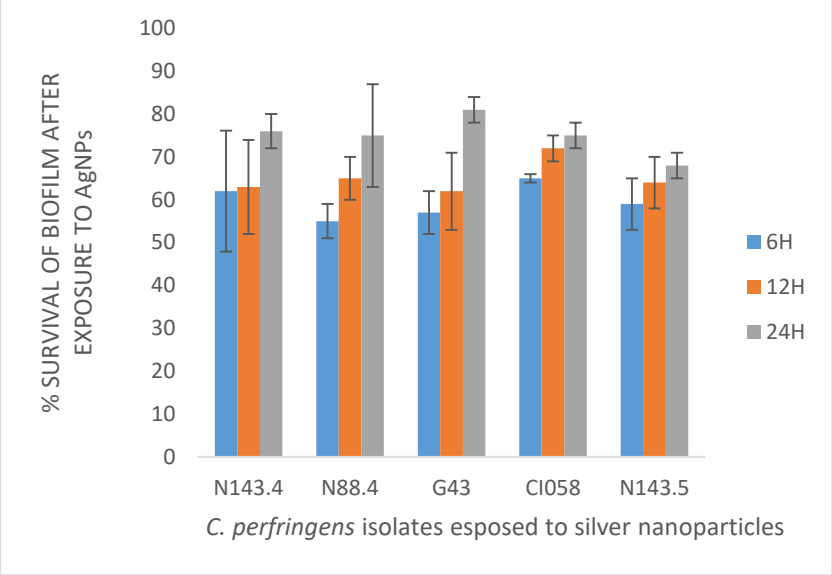


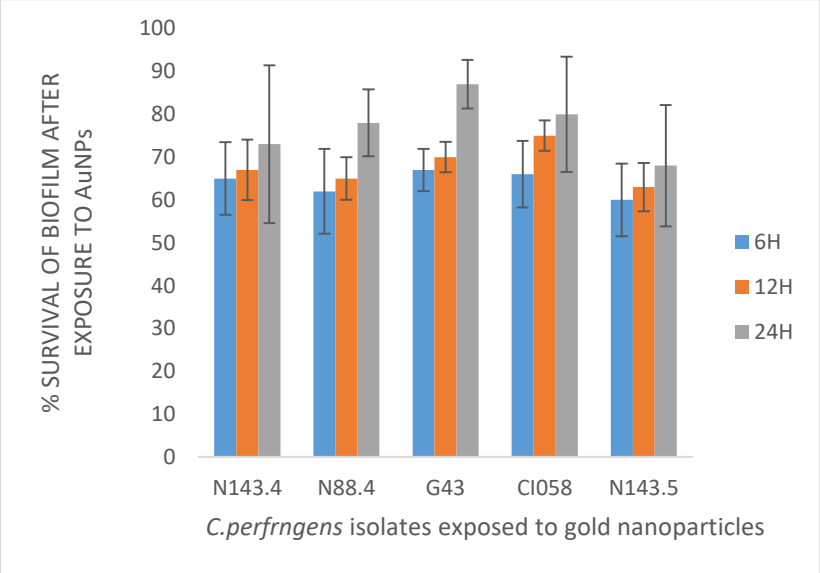
Figure 4.1A and 4.1B: Activity of silver nanoparticles and gold nanoparticles on the reduction of *C. perfringens* preformed biofilms. Results are expressed as mean ± SD of triplicate assay.

4.6. Activity of silver or gold nanoparticles on reduction of pre-formed biofilm at different growth stages

A test was taken to determine whether the reduction *C. perfringens* biofilms by silver or gold nanoparticles was dependent on the maturity (period of growth) of the established biofilm. *C. perfringens* biofilm were grown in 96-well plates to establish biofilms for either 6h, 12h or 24h before treating for 24h with nanoparticles. Effective reduction of biofilm by nanoparticles decreased with increases in the period of growth of established biofilm. Thus, biofilm reduction by tested nanoparticles on the 6h established biofilm was more than the 12h and 24h established biofilm growth.



A



B

Figure 4.2: Effect of AgNPs (A) and AuNPs (B) on reduction of pre-formed biofilm at different growth stage expressed as mean ±SD. P value < 0.05 for 6H and 24H biofilm growth stage

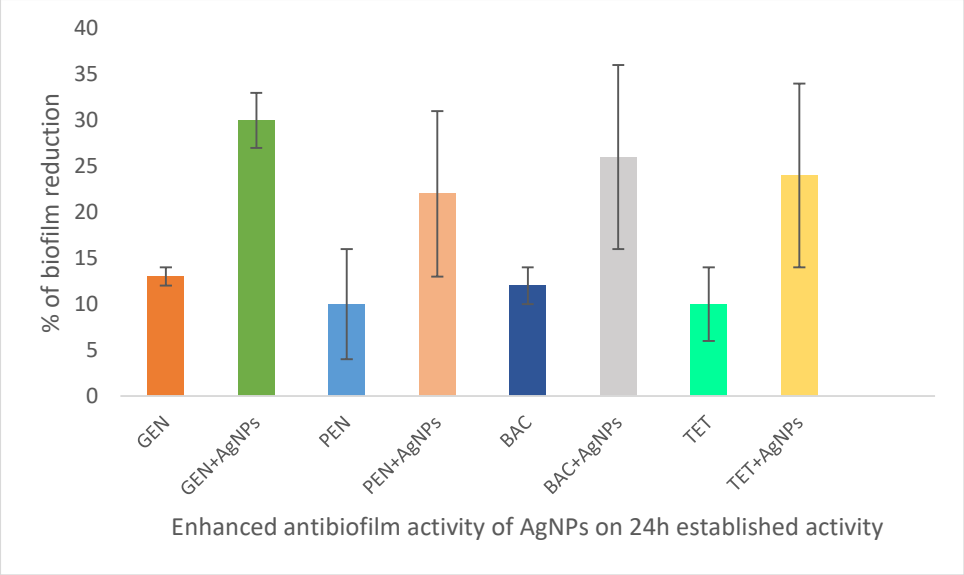
4.7. Enhanced antibiofilm effect of antibiotics in the presence of silver or gold nanoparticles

To investigate whether the combination of either silver or gold nanoparticles with antibiotics has synergistic effects, *C. perfringens* were grown to form biofilms and then

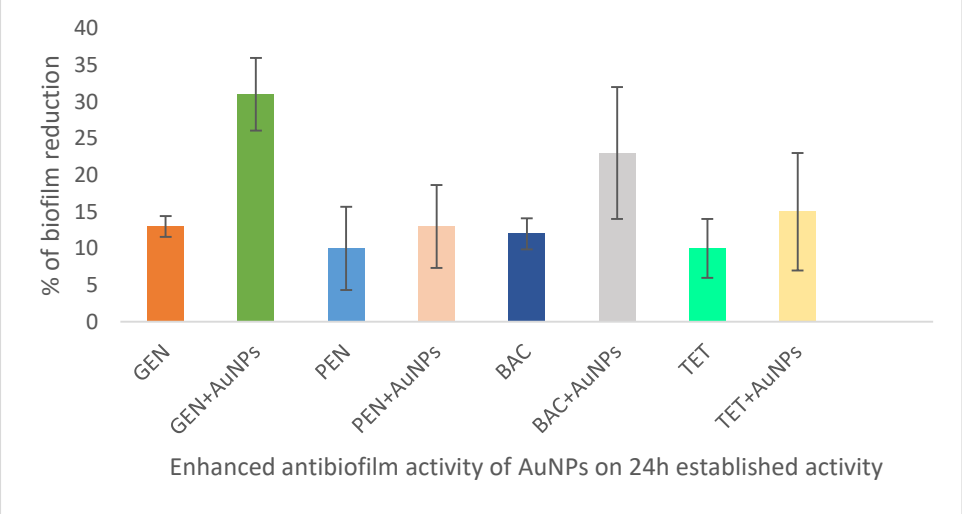
treated with antibiotic alone or nanoparticles in combination with antibiotics. The question asked was whether antibiotics tested together with either metal nanoparticles enhanced the reduction of established C*. perfringens* biofilms. As shown in figures 4.3A and 4.3B, the combination of nanoparticles and antibiotics showed an enhanced anti-biofilm effect than the antibiotics alone in 48h established biofilm treated for 6h. Specifically, the results demonstrated that gentamicin alone reduced biofilm activity by approximately 13% whereas combinations of gentamicin with either silver nanoparticles or gold nanoparticles reduced biofilm by 30% and 31% respectively. Similarly, penicillin alone reduced biofilm activity by 10% whilst combinations of penicillin with silver nanoparticles or gold nanoparticles reduced biofilm activity by 22% and 13% respectively. Biofilm reduction by bacitracin or tetracycline alone was 12% and 10% respectively whilst the combined effect of bacitracin with silver nanoparticles or gold nanoparticles was 26% and 23% respectively and the combined effect of tetracycline with silver nanoparticles or gold nanoparticles was 24% and 15% respectively.

The antibiofilm activity of the metal nanoparticles can be attributed to the release of reactive oxygen species (ROS) and other free radicals such as hydrogen peroxides, singlet oxygen, hydroxyl ions and superoxide ions. These released free radicals can cause oxidative stress in the cells, damage DNA, inhibit protein synthesis and block translation and transcription process. NPs can also cause leakage on plasma membrane by infiltrating the membrane. The enhanced antibiofilm effect recorded when nanoparticles were combined with antibiotics could be related to drug synergy were the different drugs acts on different pathways. Penicillin and bacitracin acts on bacteria cell wall (inhibiting peptidoglycan synthesis) while gentamicin and tetracycline hinder protein synthesis by inhibiting the 30S ribosomes subunit. Other possible

reason for enhanced antimicrobial effect is the improved solubility/penetration of antibiotics through the biofilm matrix by the NPs (pharmacokinetic) because of the small size, shape, high reactivity, and large surface area to volume ratio of NPs. Furthermore, the result showed that though NPs enhanced the antibiofilm activity of the tested antibiotics, NPs were more efficient when applied alone except when AuNPs and gentamicin were combined.



A



B

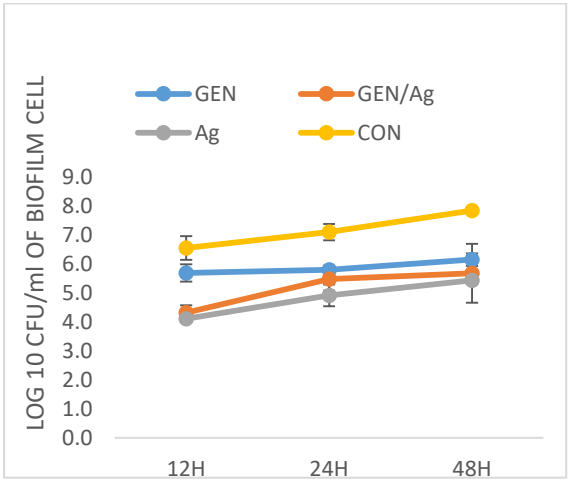
Figures 4.3 The enhanced antibiofilm activity of antibiotics in the presence of AgNPs (4.3A) and AuNPs (4.3B) on *C. perfringens* biofilms expressed as means ±SD. P value was < 0.05 for AgNPs and antibiotic combination whilst P value was < 0.05 for AuNPs/GEN and AuNPs/BAC combination.

Key: $AgNP_S$: silver nanoparticles; $AuNP_S$: gold nanoparticles; GEN: gentamicin; PEN: penicillin; BAC: bacitracin; TET: tetracycline

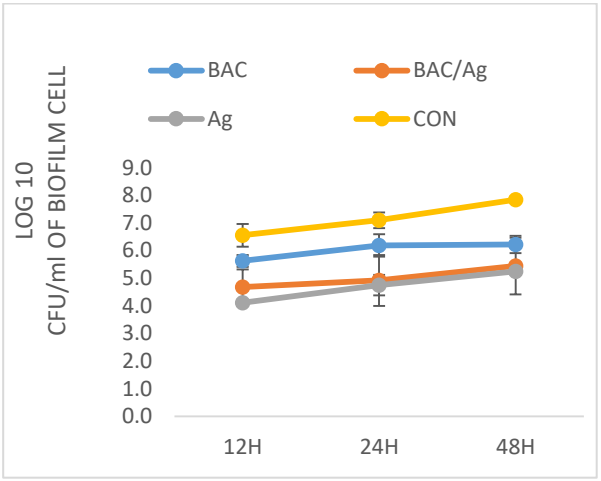
4.8. Antibiofilm activity of antibiotic combined with nanoparticles on different biofilm growth stage.

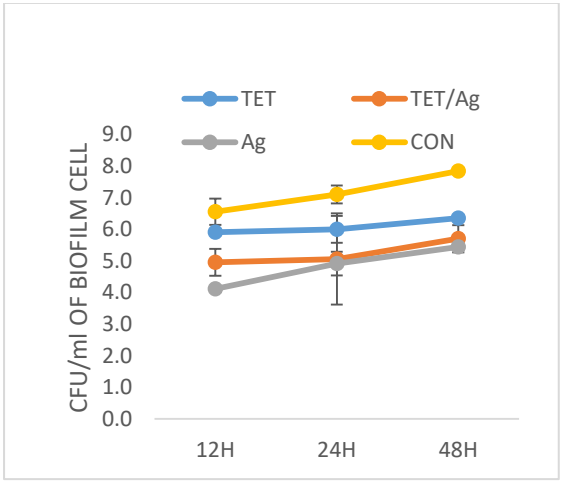
To test whether the anti-biofilm effect of antibiotics and nanoparticles combination shown in section 4.3.3 was dependent on the growth stage of biofilm, *C. perfringens* biofilm were grown in 96-well plates to establish biofilms for either 12h, 24h or 48h before treating for 6h with either nanoparticles or antibiotics or their combination. The antibiotics and nanoparticles combination displayed more effective anti-biofilm activity than antibiotic applied alone to pre-formed biofilms formed at all time-points tested (Figures 4.4A and 4.4B). Also, it was observed that nanoparticles applied alone were more effective at reducing preformed biofilm than the combination of antibiotic and nanoparticles except for the combination of gentamicin and gold nanoparticles. This result may suggest that antibiotics inhibit the effect of nanoparticles.

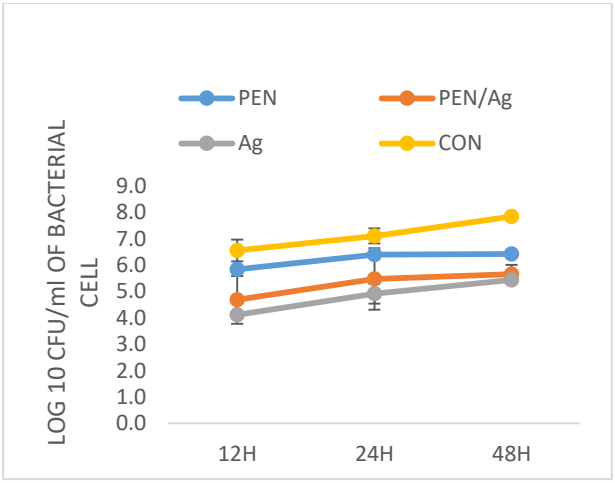
A



B





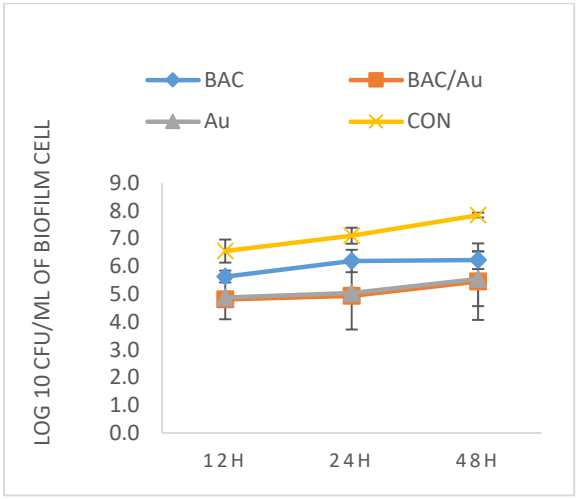


C D

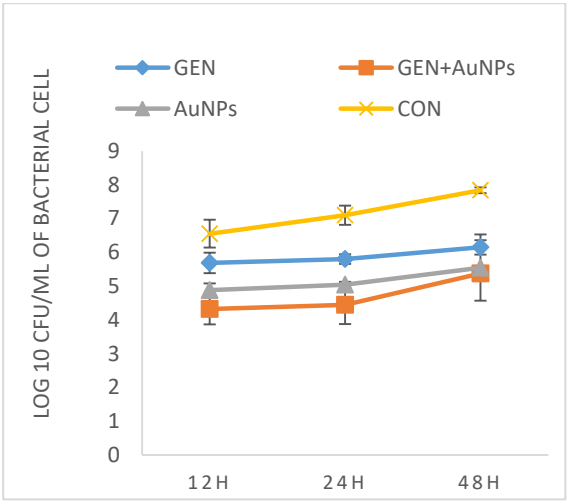
Figure 4.4A: Effects of antibiotics and AgNPs on different growth stages of *C. perfringens* biofilms expressed as means ±SD. 12h, 24h and 48h preformed biofilm treated with AgNPs and antibiotics showed that biofilm removal varied with the maturity of the biofilm.

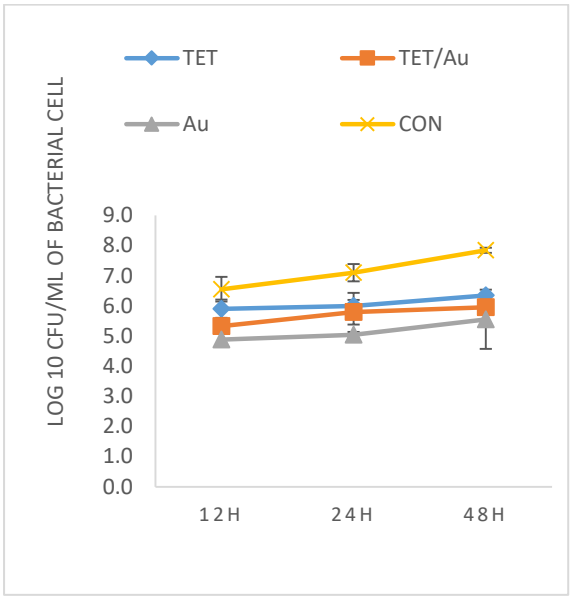
Key: AgNPs: silver nanoparticles; GEN: gentamicin; PEN: penicillin; BAC: bacitracin; TET: tetracycline

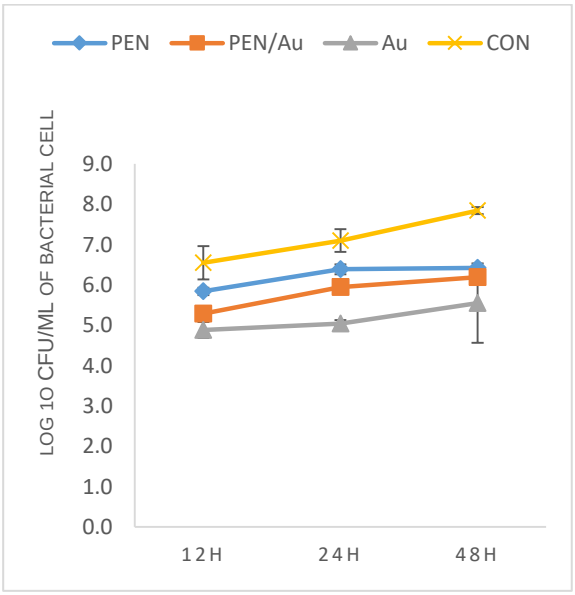
A



B







C D

Figure 4.4B: Effects of antibiotics and AuNPs on different growth stage of *C. perfringens* biofilms expressed as means ±SD. 12h, 24h and 48h preformed biofilm treated with AuNPs and antibiotics showed that biofilm removal varied with the maturity of the biofilm.

Key: AuNPs: gold nanoparticles; GEN: gentamicin; PEN: penicillin; BAC: bacitracin; TET: tetracycline

Data in this chapter showed that silver or gold nanoparticles has antibiofilm effect on *C. perfringens* pre-formed biofilm at all time points tested. Though the percentage of pre-formed biofilm cells at different growth stages that survived after 6h or 24h treatment with nanoparticles alone or nanoparticles + antibiotics combination increased with the level of maturity of the biofilm, this difference was statistically not significant (*P* value > 0.05). Also, pre-formed biofilm at all time-points tested, showed that the antibiotics and nanoparticles combination displayed a stronger anti-biofilm activity than antibiotics alone. Furthermore, it was observed that nanoparticles showed better antibiofilm activity when applied alone compared to combinations with antibiotics except for the combination of AuNPs and gentamicin.

4.9. Discussion

Nanoparticles are recognised as broad-spectrum antimicrobial agents that have shown successful application as antibacterial and antibiofilm agents (Karaman *et al*., 2017; *Ghotaslou et al.,* 2017; Han *et al*., 2017; Slomberg *et al.,* 2013; Ahmadi *et al.,* 2013; Guzman *et al*., 2012; Martínez-Gutierrez *et al*., 2010). In addition, nanoparticles have been used to inhibit bacteria from colonizing prostheses and other medical devices as well as a vehicle for the delivery of therapeutic agents (Qayyum and Khan, 2016; Huh and Kwon, 2011; Zhang *et al*., 2008).

Data in this chapter correlates with other studies that have shown that both silver and gold nanoparticles appear capable of reducing and 'dispersing' biofilms. Gurunathan *et al*., (2014) reported the anti-biofilm activity of biologically synthesized silver nanoparticles against two Gram- positive bacteria (*S. aureus* and *S. pneumoniae*) and recorded 90% biofilm reduction after 24h of treatment. They also found that silver

nanoparticles reduced biofilm formed by *Pseudomonas aeruginosa* and *Shigella flexneri* by more than 90%. Martinez- Gutierrez *et al.*, (2013) observed 4 log reductions in bacterial cell counts when *P. aeruginosa* was exposed to 100mg/ml of silver nanoparticles. Ansari *et al*., (2014) demonstrated that 50µl/ml of silver nanoparticles inhibited biofilm formation of *E*. *coli* and *Klbesiella* spp on the surface of agar plates. Honora *et al*., (2017) reported that silver nanoparticles removed pre-formed biofilm in frequently touched surfaces of surgical operating rooms in Suez Canal University Hospital Egypt.

Gold nanoparticles in this present study displayed less antibiofilm activity compared to silver nanoparticles. Though Castillo-Martínez *et al*., (2015) showed that 150µg/ml of gold nanoparticles completely inhibited oral biofilm *in vitro* whereas Gopinath *et al*., 2016 reported that gold nanoparticles did not inhibit biofilm formation by some Gram-positive and Gram- negative bacteria. To my knowledge, the present study is the first that has shown an antibiofilm effect of gold nanoparticles on biofilm reduction in *C. perfringens*. The antibiofilm effect of nanoparticles could be related to the ability of nanoparticles to adhere to the biofilm matrix and penetrate through the EPS and accumulate inside the bacterial cells thereby continuously causing destruction of cellular organelles. Mechanisms of bacterial killing by NPs include reactive oxygen species (ROS) production, membrane disruption, DNA synthesis inhibition and ATP depletion (see figure 1.12 in chapter 1) (Qayyum and Khan 2016; Yeh, *et al*., 2020).

The percentage of 6h, 12h and 24h preformed biofilm cells that survived treatment of nanoparticles after 24h of exposure increased though this difference was not statistically significant (*P* value > 0.05) (Figure 4.2A and 4.2B). This finding might imply that nanoparticles become less efficient in removing biofilm as the biofilm grows to maturity.

The investigation on whether metal nanoparticles in combination with antibiotics could improve the anti-biofilm activity of antibiotics normally tested alone showed that nanoparticles can enhance the activity of the tested antibiotics. This enhanced result is extended for pre-formed biofilm at different growth stages. Pre-formed biofilm formed at all time-points tested, showed that the antibiotics and nanoparticles combination displayed a stronger anti-biofilm activity than antibiotics alone. It is also observed that the combination of antibiotics and nanoparticles could enhance the antibiotic reduction of biofilms at different stages of growth. Therefore, antibiofilm activity of nanoparticles appears clearly not dependent on the growth stage of the biofilm. Furthermore, except for the combination of AuNPs and gentamicin, it was observed that nanoparticles showed better antibiofilm activity when applied alone compared to when combined with antibiotics. These findings agree with the report by Gurunathan *et al*., (2014) where they reported the enhanced anti-biofilm effect (40% to 60% reduction in biofilm) when sublethal concentration of ampicillin and vancomycin combined with AgNPs on *P. aeruginosa, S. flexneri, S. aureus* and *S. pueumoniae* using the crystal violet staining assay in tissue culture plates.

Results from this present study showed that combined treatments of silver nanoparticles or gold nanoparticles and antibiotics enhanced the number of cell deaths in biofilms although the precise mode of actions remains unknown. Notwithstanding this, combining nanoparticles with different antibiotics has the potential to become an effective anti-biofilm treatment and a method to increase antibiotic effectiveness.

4.10. Conclusion

Bacterial biofilms that are resistant to antibiotics are a public health challenge and has prompted a worldwide search for new therapeutic agents. Metal nanoparticles have been proposed as a new therapy for controlling bacterial infections. The present study

supports the claim that nanoparticles have antibiofilm therapeutic potential and suggests a potential use against *C. perfringens* biofilm as a possible disinfectant. However, since there has not been any previous study on nanoparticles activity with *C. perfringens*, it would be necessary to carry out larger studies to determine the antibiofilm reduction and enhanced activity of antibiotics when combined with nanoparticles on more strains. In addition, studies are required to determine the cytotoxicity of nanoparticles before considering their application.

CHAPTER FIVE:

ANTIBIOFILM ACTIVITY OF PLANT EXTRACTS AGAINST *Clostridium perfringens* BIOFILM

5.1 Introduction

Certain plants recognised as spices and for their medicinal effects, have not been investigated for their antibiofilm activity until recently. Despite some doubts, several effective natural compounds are attributed to different plant extracts (Sadekuzzaman *et al*., 2015). Plant extracts and their active compounds have shown antibiofilm activity and Coenye *et al.,* (2012) reported that the extracts of 5 plants (*Epimedium brevicornum*, *Malus pumila*, *Polygonum cuspidatum, Rhodiola crenulata,* and *Dolichos lablab)* out of 119 plant extracts investigated for antibiofilm activity on *Propionibacterium acnes (*reclassified as *Cutibacterium acnes)* effectively eradicated their biofilm. They also identified specifically that resveratrol, salidroside and icariin were the active compounds in those 5 plant extracts. Lee *et al.,* (2013) demonstrated that 16 out of the 498 plant extracts tested on enterohemorrhagic *Escherichia coli* (0157:H7) biofilm showed greater than 85% inhibition of biofilm formation. Extracts from green tea (*Camellia sinensis*) and dandasa (*Juglans regia*) successfully inhibited biofilm formation by *E. coli* isolated from a urinary catheter and *Streptococcus mutans* isolated from the oral cavity. They were active at a concentration of 12.5mg/ml and 6.5mg/ml respectively against *S. mutans* and 3.1mg/ml and 12.5mg/ml respectively against *E. coli* (Feraz *et al*., 2012). It was also reported that the aqueous extract of Caatinga plants (medicinal shrub from Brazil) prevented the growth of *Staphylococcus epidemidis* biofilm (Trentin, *et al*., 2011).

Medicinal plants with potential antimicrobial/ antibiofilm activity tested in this present study.

5.2. *Vernonia amygdalina* (Bitter leaf)

Vernonia amygdalina (*V. amygdalina)* belongs to the family Asteraceae. It is a perennial small shrub of about 2 to 5m in height found in tropical Africa. It is a soft wooded bitter plant with leaves of about 20cm long and it is commonly called bitter leaf in Nigeria because of the bitter taste of the plant. It is known by different local names in the different Nigerian languages, such as oriwo (Edo), onugbo (Igbo), Ewuro (Yoruba), etidot (Ibibio), Ityuna (Tiv), Chusar-doki (Hausa). In Uganda it is called Omubirizi, Ndoleh, in Cameroon and Ebichaa, in Ethiopia (Oyeyemi *et al.,* 2018). In Nigeria, the leaves are consumed as green leafy vegetables in soup, and it is used in the preparation of various delicacies following drying, boiling or several washing to reduce bitter taste. Studies that have analyzed the nutritional composition of *V. amygdalina* have shown that it is a rich source of protein, carbohydrate, fat, moisture, fibre, ash, and various minerals (Oboh and Masodje, 2009; Kadiri and Olawoye, 2016). *V. amygdalina* can thrive on all soil types but grows much better on humus-rich soils and humid environment though it can tolerate drought. It is easily propagated through stem cutting and is compatible with other plants as it is not a competitor for nutrient and moisture and yields high biomass and adapts easily to the environment. It favors the growth of other plants as it improves the soil fertility (Oyeyemi *et al.,* 2018).

V. amygdalina is a medicinal plant that serves a multi-purpose. It has been widely used for the treatment of numerous ailments in Africa. Different studies have reported the antimicrobial activities of ethanol, methanol, and aqueous extract of *V. amygdalina* on both Gram-positive and Gram-negative bacteria (Okigbo and Mmeke, 2008; Alo *et al.,* 2012; Ghamba *et al.,* 2014; Udochukwu *et al.,* 2015). *V. amygdalina* is used to treat malaria, dysentery and gastrointestinal disorder, constipation, cough, fever, worm

infestation and protozoal infection (amoebic dysentery). It is also reputedly used to treat hiccups, convulsions, diabetes, wounds, measles, jaundice, eczema, anaemia and to induce fertility in women (Farombi and Owoeye, 2011; Clement *et al*., 2014; Oyeyemi *et al*., 2018). Several phytochemicals responsible for *V. amygdalina* ethnobotanical use have been identified. Phytochemicals are natural bioactive compound that are beneficial to heath. Some established bioactive compounds in *V. amygdalina* includes terpenes, phenolic acids, flavonoids, steriods, liganans, saponins, xanthones, edotides, phytate, oxalate, cyanogenic glycoside and Anthraquinone. These compounds are responsible for the antimicrobial and antioxidant activity of *V. amygdalina*. Studies by Farombi and Owoeye *et al*., (2011); Foo *et al.,* (2014) revealed that the leaf extract of *V. amygdalina* is a significant source of dietary antioxidant. Evbuomwan *et al.,* (2018) assessed the antibacterial activity of *V. amygdalina* leaf extract on selected pathogens. Their results showed that *V. amygdalina* has potential bioactive compounds that are responsible for its antibacterial activity. Though several of the synthetic bioactive compounds have shown one or more of the activities exhibited by the plant extract, the activities are lower compared to when the extract is derived directly from the plant. This may imply that the medicinal effect of the plant results from a synergistic interaction of the different phytochemicals present in the plant (Oyeyemi *et al.,* 2018).

Figure 5.1: *Vernonia amygdalina* leaf

5.3. *Ocimum gratissimum* (Scent leaf)

Ocimum gratissimum (*O. gratissimum*) is a tropical herbaceous plant indigenous to Africa (West Africa) and Asia (India). The plant belongs to the family Labiatae. *O. gratissimum* is a home-grown shrub that measures up to 1.9m in height with branching stems. The leaves measure up to 10 x 5 cm and has a characteristic aromatic taste. The plant is known by different names in various parts of the world (Junaid *et al*., 2006). In India it is called Ram tulsi (by the Hindi) and Nimma tulasi (by the Kannada) (Prabhu *et al*., 2009). In the Eastern part of Nigeria (Igbos), the plant is called Nchanwu, Efinrin in the west and Daidoya in Hausa. *O. gratissimum* commonly called scent leaf in Nigeria serve both nutritional and medicinal purposes (Njoku *et al*., 2011). The plant has since been known for its ethnomedicinal value in many countries. The leaf of *O. gratissimum* is used as spices for cooking various delicacies like soup, stew, and pasta. The leaves and flowers are rich in essential oils. The essential oil contains geraniol, linalol, citral, eugenol and thymol (Dubbey *et al.,* 2000; Prabhu *et al*., 2009).

The oil has wide spectrum of antifungi activity, and it is used as an insect repellent and in preparation of teas. In Nigeria, *O. gratissimum* is used to treat cough, catarrh, gastroenteritis, severe diarrhoea and other stomach disorder, malaria, vomiting, high fever, convulsion, skin diseases such as ring worm, urinary infections, epilepsy, mental illness, and cleaning of the baby cord to keep the surface sterile. People of India use the plant as a diaphoretic and antipyretic treatment of headache and influenza (Ijeh *et al*., 2005; Adebolu and Salau, 2005; Akinmoladun *et al*., 2007; Prabhu *et al*., 2009).

Researchers have shown that *O. gratissimum* medicinal values depends on its bioactive compound that exhibit antibacterial (*S. aureus*, *S. faecalis*, *P. aeruginosa* and *E. coli* and antifungal *(Aspergillus niger*, *A. flavus*, *Fusarium oxsporium*, *Rhizopus stolonifer*, *Botryodiplodia theobromae* and *Penicillium chrysogenum*) activities that is of health benefit. Phytochemicals that have been identified include tannins, steroids, oligosaccharides, saponins, carbohydrates, flavonoids, and alkaloids (Junaid *et al*., 2006; Akinmoladun *et al*., 2007; Prabhu *et al*., 2009). The antibacterial activity of cold water, hot water, and steam distillation oil extract from the leave of *O. gratissimum* was tested against pathogenic bacteria that causes diarrhoea (S*taphylococcus aureus, Escherichia coli, Salmonella typhi and Salmonella typhimurium)*. Results showed that only the plant oil extracted via steam distillation had inhibitory effect on the tested bacteria with MICs ranging from 0.1% for *S. aureus*, 0.01% for *E. coli* and *S. typhimurium* and 0.001% for *S. typhi* (Adebolu and salau, 2005). The authors suggested that the likely course of this could be because the steam distillation technique extracted the oil better than the other methods used in the study. Junaid *et al*., (2006) supported the ethnomedicinal application of *O. gratissiumum* as they demonstrated that leaf extract of the plant has potential for the control of gastroenteritis.

Investigation by Offiale and Chikwendu, (1999) on the antidiarrhoeal effect of aqueous leaf extract of *O. gratissimum* on castor oil induced diarrhoea in rats revealed that the extract could hinder the production of watery faeces and propulsive movement of the gastrointestinal tract. Nweze and Eze, (2009) also confirmed the folkloric application of *O. gratissimum* use in treating wound infection. In their study, they tested the effect of ethanolic leaf extract of *O. gratissimum* on some bacterial commonly associated with wound infection. They revealed that the plant extract could inhibit the growth of the bacteria and reported positive synergistic interaction between plant extract and antibiotics.

Figure 5.2: *Ocimum gratissimum* plant

5.4 *Azadirachta indica* (Neem)

Azadirachta indica (A. indica) is commonly called the neem plant and it belongs to the family Meliaceae. It is used traditionally as a therapeutic agent. The leaf, seed, oil and

bark of *A. indica* are known to contain antibacterial and antifungal substances effective against pathogenic microorganisms. There has also been report on their antiviral activity against measles and vaccinia viruses (Raja *et al*., 2013; Biswas *et al.,* 2002). Different parts of neem (leaf, bark seed pulp and oil) have shown various medicinal use which includes antiulcer, antibacterial, antifungal, antiviral, antimalarial, antioxidant, anticarcinogenic, antifertility, anti-inflammatory, antipyretic and analgesic, antioxidant, antimutagenic and anti-diabetic activities (Biswas *et al*., 2002). Neem is also used to treat leprosy, intestinal helminthiasis, heumatism, constipation, ringworm, eczema, scabies and as a general health promoter. Bioactive compounds in neem such as nimbidin, azadarachtin, nimbin, gedunin, polysaccharides and nimbolide are responsible for the biological activities in different plant parts. Raj *et al*., (2013) investigated the antimicrobial activity of the leaf, seed, and bark extract of *A. indica* and they revealed that the leaf and bark extract had high antimicrobial properties on *S. aureus*, *P. aeruginosa*, *P. mirabilis*, *E. faecalis*, *A. fumigatus* and *C. albicans*. Natarajan *et al*., (2003) studied the effect of the leaves and seeds extract of neem plant on dermatophyte growth. They reported that the seed extract of neem had antidermatophytic properties as it could hinder the growth of dermatophytes. Their study supports the claim that *A. indica* seed oil can be used to treat ringworm and other dermatological diseases. Also, Coventry and Allan, (2001) showed that the neem seed extract had antimicrobial properties.

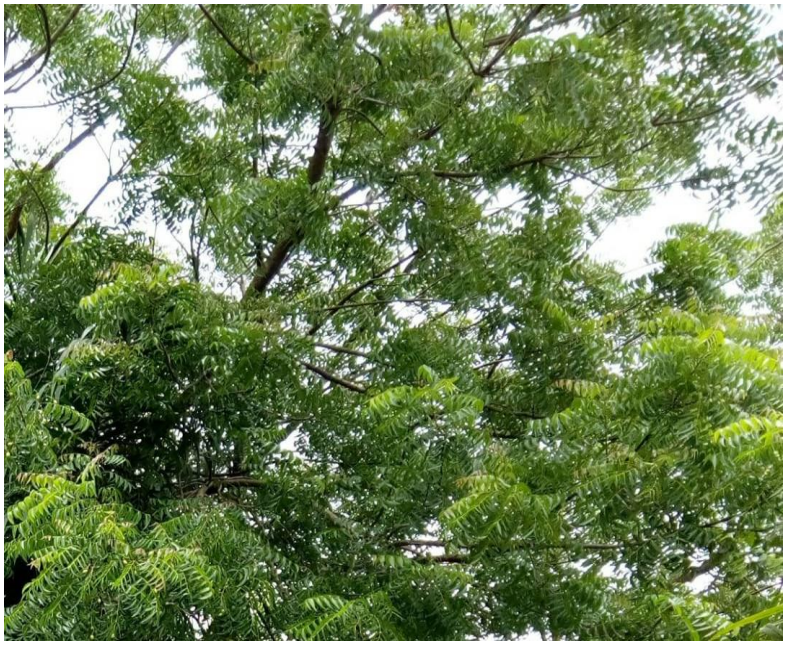


Figure 5.3: *Azadirachta indica* plant

Although several studies have reported good but varying antimicrobial activity of ethnomedicinal herbs, only limited research has been published on their antibiofilm potentials.

5.5 Aims and Objectives

The aim of this chapter was to test the antibiofilm potential of the ethanolic leaf extracts of three medicinal plants (*V. amygdalina, O. gratissimum* and *A. indica*) against *C. perfringens* bacteria. The specific objectives were.

(1)To test the effect of different concentrations of ethanolic leaf extract on *C. perfringens* bacteria

(2)To evaluate the biofilm inhibitory effect of ethanolic leaf extract on *C. perfringens* biofilm

(3)To evaluate the biofilm removal/reduction effect of ethanolic leaf extract on *C. perfringens* biofilm

5.6. Preparation of crude extract

Fresh leaf samples of three plants; *V. amygdalina*, *O. gratissimum* and *A. indica* were collected from a garden at Irrua, Esan Central LGA Edo state, Nigeria. Leaf extract was prepared following the methods described by (Okigbo and Mmeke 2008; Alo *et al*., 2012). Leaves were collected from the same plant and at a particular time. Leaves were washed thoroughly with distilled water and then air dried at room temperature before grinding into a fine powder in a sterile blender. The extract was prepared by weighing twelve grams (12g) of each ground leaf sample and soaking in 100ml of 60%w/w ethanol at room temperature for 72h with regular stirring. The plant extract was filtered using a Whatman number1 filter paper. The filtrate was then evaporated using a rotary evaporator to obtain the crude extract. This was stored at 4^0C until required.

Figure 5.4: Ground dried leaves of plant extract. From left to right; *Vernonia amygdalina, Ocimum gratissimum, Azadirachta indica*.

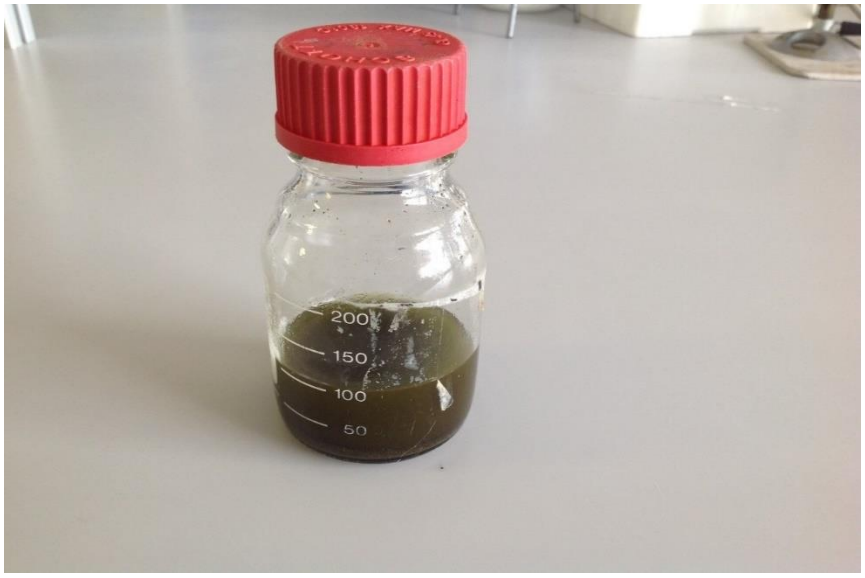


Figure 5.5: Blended leaf of plant extract dissolved in 60%w/w of ethanol.

5.7 Preparation of concentration of plant extract:

This present study tested the antibiofilm activity of 20mg/ml of the plant extract. Varied MICs of tested plant leaf extract on different bacteria have been reported in the literature and since there is no previous report on *C. perfringens*, 20mg/ml concentration of tested plant extract was used to establish if there is any antibiofilm activity on *C. perfringens*. Due to the oily nature of the ethanol extract, it failed to dissolve directly in deionized water. To overcome this, 0.2g of the extract was first dissolved in 1ml of 99.9% ethanol before 10ml of distilled water was added to produce a concentration of 20mg/ml. 1ml of ethanol dissolve in 10ml of deionized water was used as a negative control to ensure ethanol was not contributing to the antimicrobial effect. Other concentrations of 10, 5, 2.5 and 1.25mg/ml were prepared by the serial double dilution method.

5.8. Prevention of biofilm formation

To assess the potential of plant extract to prevent the attachment of *C. perfringens* biofilm cells from the different biofilm class in 96 well plates, 100µl of each plant extract

was pipetted into wells in 96 well plates containing 100µl of BHI broth culture (10^7CFU/ml) of *C. perfringens* making a total volume of 200µl in each well. 200µl of sterile BHI broth was added into empty wells as sterility control while 200µl of broth culture was pipetted in wells as negative control (untreated biofilm). Plates were wrapped with parafilm and then incubated anaerobically at 37°C for 24h without shaking to allow for cell attachment. Percentages showing biofilm inhibition were calculated as described in section 4.2.

5.9. Reduction/removal of preformed biofilm

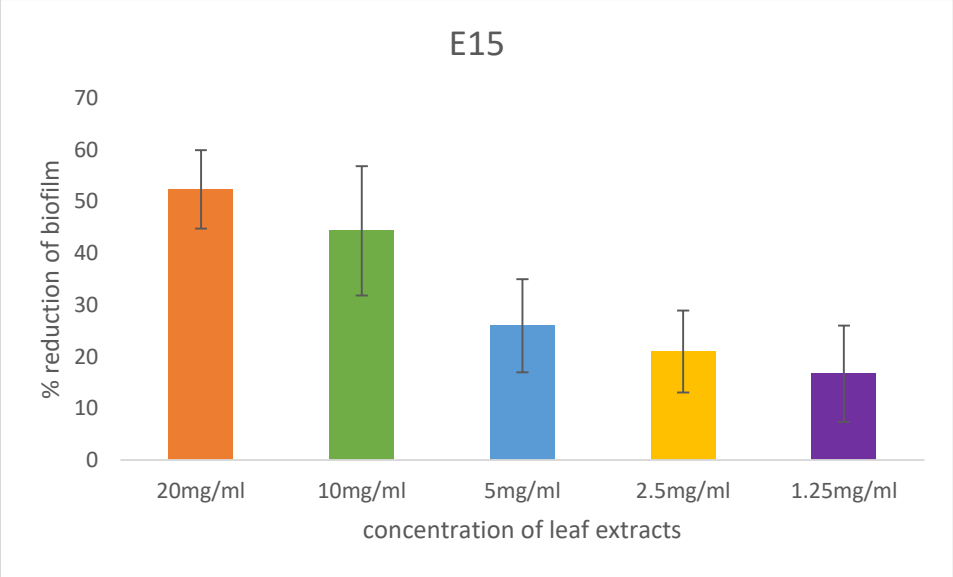
Firstly, biofilm was preformed for 48h before treatment with the plant extract. 200µl of *C. perfringens* bacterial broth culture was added to each well in 96 well microtiter plate and incubated at 37°C for 48h to allow for cell attachment. Following incubation and cell attachment, wells with pre-formed biofilm were washed three times with 200µl of sterile quarter strength Ringer's solution to remove unattached cells and then treated with 100µl of each plant extract. For the negative control, preformed biofilm in wells were treated with 100µl of sterile quarter strength Ringer's solution. Plates were again incubated at 37°C anaerobically for 24h. After the period of incubation, the level of biofilm growth reduction was compared against untreated biofilm cells and biofilm growth was quantified by total viable count method (TVC) as described in section 2.12. Percentages showing biofilm reduction were calculated as described in section 4.2.

RESULTS

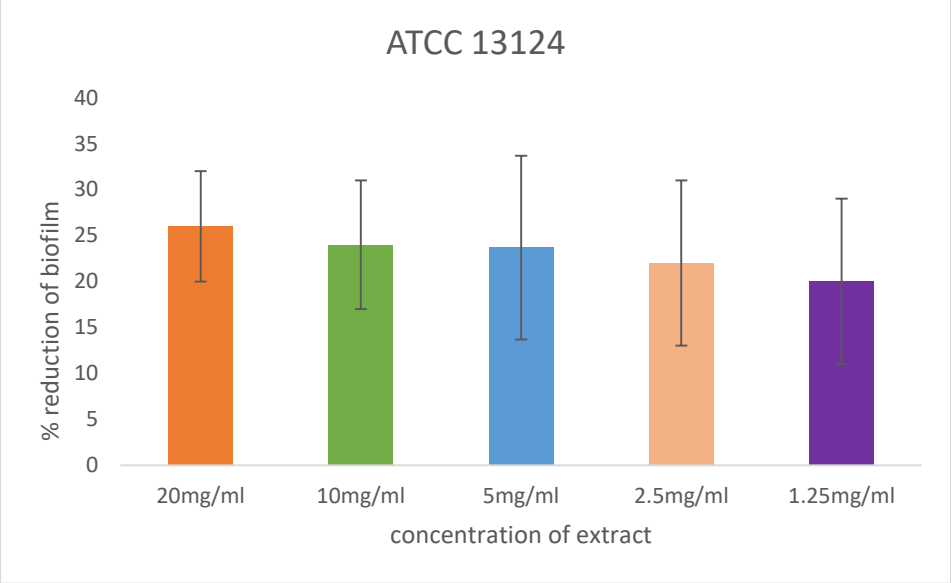
5.10. Antibiofilm activity of *V. amygdalina* extract

Antibiofilm activity revealed that the ethanolic extract of *V. amygdalina* leaf (20mg/ml, 10mg/ml, 5mg/ml, 2.5mg/ml and 1.25mg/ml) tested on two strains of *C. perfringens* reduced pre-formed biofilm as shown in Figures 1a and 1b. It was observed that the

antibiofilm activity of different concentrations of *V. amygdalina* leaf extract was concentration dependent as there was a reduction in bacterial cell count as the concentration decreased. Also, the antibiofilm activity was strain dependent as the percentage of biofilm reduced varied between the two strains tested. The ATCC strain was more susceptible to reduction and there was no significant difference ($P > 0.05$). In the environmental isolate (E15), significant difference was seen only between 20mg/ml and 1.25mg/ml and 10mg/mg and 1.25mg/ml.



a



b

Figure 5.6: Antibiofilm activity of different concentrations of *V. amygdalina* leaf extract on *C. perfringens* biofilm expressed as means ±SD of triplicate assays. The higher the concentration tested the increase in the removal of bacterial cell.

5.11. Activity of *V. amygdalina*, *O. gratissimium*, and *A. indica* leaf extracts in prevention of *C. perfringens* biofilm formation and reduction of established biofilm

The ability of the tested plant extract to prevent the attachment of biofilm cells as well as reduce already formed biofilm was investigated. Figures 5.7 and 5.8 shows the

percentage inhibition of biofilm formation and the reduction of pre-formed biofilm compared against untreated *C. perfringens* biofilm (negative control) by a concentration of 20mg/ml of leaf extracts on six isolates of *C. perfringens* that represented the different source of our isolates. This study revealed that the three-plant extracts did inhibit or reduced biofilm for each tested isolate although the dynamics were different. From our results, the ethanolic leaf extract of these plants showed varied level of reduction of biofilm formation or prevention of biofilm formation. The highest percentage of biofilm reduced by *V. amygdalina* was 28% and the least was 15%. For *O. gratissimium*, the highest percentage reduced was 27% and the least was 12%. While for *A. indica*, the highest percentage of reduction was 28% and least was 10% (figure 5.7). For prevention of biofilm formation, the highest percentage of inhibition recorded by *V. amygdalina* was 11% and the least was 2%. *O. gratissimium* had 15% as the highest percentage of inhibition and 5% as the least. While *A. indica* showed 20% as the greatest percentage of inhibition and 4% as the least (figure 5.8).

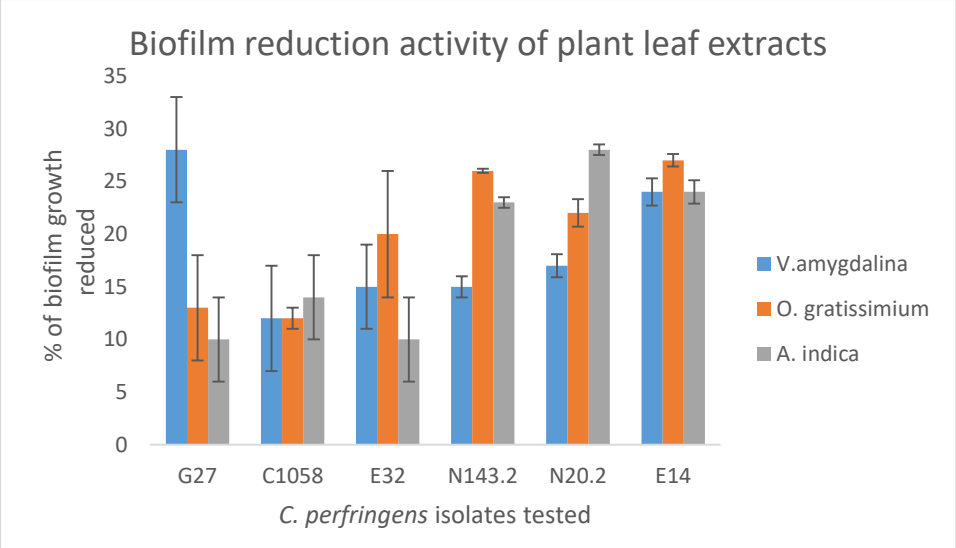


Figure 5.7: Percentage reduction/kill of 48h established biofilm by plant leaf extract expressed as means ± SD of triplicate assays. After treatment of preformed biofilm for 24h, leaf extract reduced biofilm in all tested isolates.

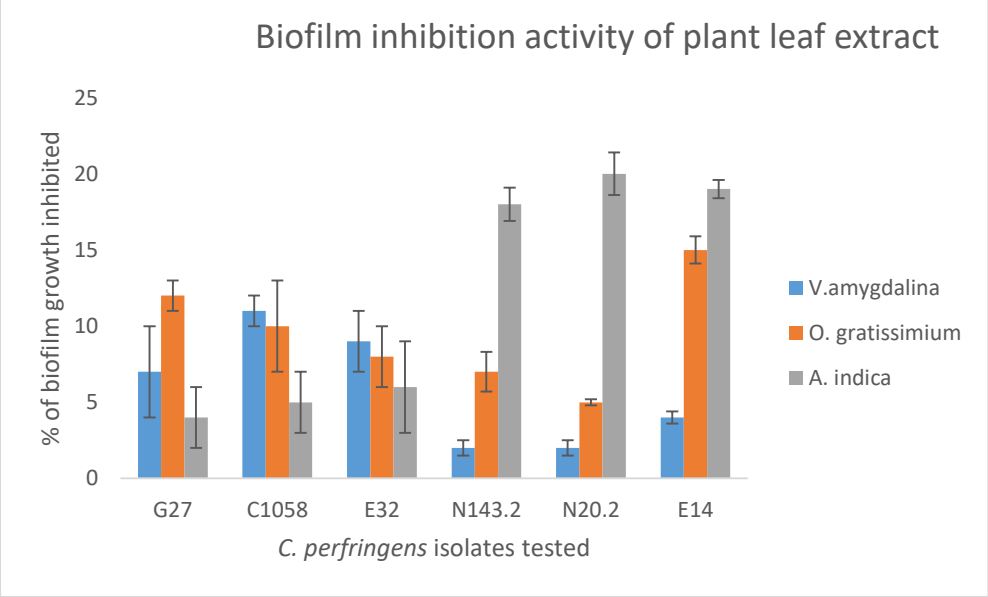


Figure 5.8: Percentage inhibition of biofilm growth by plant leaf extract after treatment for 24h expressed as means ±SD of triplicate assays. Plant leaf extract inhibited biofilm formation in all tested isolates.

Data from this chapter showed that the studied plant leaf extracts showed modest biofilm inhibitory activity in the range of 20% to 2% and reduction effect in the reange of 28% to 10% at the concentrations tested. The dynamics for biofilm reduction and

inhibition was different for each plant extract tested and varied for the different *C. perfringens* isolates.

5.12. DISCUSSION

The antibiofilm activity of the plant extracts tested in this study are probably as a result from the presence of a variety of phytochemicals which have previously been identified from the leaf extracts. Such compounds include tannins, flavonoid, steroids, alkaloids, terpenoids, saponins and glycosides (Biu *et al*., 2009; Adeniyi *et al.,* 2010; Akinmoladun *et al.,* 2007; Ayoola *et al.,* 2008). Since our samples were crude ethanolic extracts, these compounds could have acted individually or in synergy to produce the effects observed at the concentrations tested.

Although there has not been any published work on the antimicrobial activities of any of these plant extracts (*V. amygdalina, O. gratissimium* and *A. indica*) specifically on C. perfringens, several studies have been carried out on the antimicrobial activities of leaf extracts of the plants on other bacteria and particularly ethanolic leaf extract *of V. amygdalina*, *O. gratissimium* and A. indica on other microorganisms. Romero *et al.,* (2016) evaluated the antibiofilm activity of native plant extract from northwestern Argentina. They reported that extract concentration significantly affected biofilm inhibition and reduction activity and that the biofilm inhibition effect of all tested plant extract (*Lycium chilense, Schinus fasciculatus, T. minuta, T. absinthioides*, and *L. divaricate*) were higher than biofilm reduction effect on Bacillus sp Mcn4 and *S. aureus* Mcr1 with 30% and 39% difference respectively. Sanchez *et al.,* (2016) studied the biofilm inhibitory effect of different concentrations (75, 50, and 25%) of methanolic plant extracts from Prosopis laevigata, Opuntia ficus-indica, and Gutierrezia microcephala on *E. coli* and reported a dose dependent activity. The highest specific biofilm formation (SBF) recorded was 0.4, 1.5 and 3 after treatment with 75%, 50% and 25% of the plant extract. Famuyide *et al.,* (2019) studied the antibiofilm activity of acetone plant extracts from the Myrcateae family and reported that the tested plant extract showed varied degree of biofilm inhibition and reduction activity. Plant extracts

showed good biofilm inhibition activity with all tested isolates showing greater than 50% biofilm inhibition. Their work showed that removal of preformed biofilm was more difficult for the tested plant extract compared to inhibition of biofilm formation or establishment. This shows clearly that pathogens are more resistant to antimicrobials when in the biofilm mode of growth. Laird *et al.,* (2011) reported citrus EO vapour reduced vancomycin resistant Enterococcus spp. (VRE) and MRSA biofilm formed on stainless steel surfaces by 1.5 – 3 log10 and 72% reduction in metabolic activity after exposure for 24h. The EO vapour showed both biofilm inhibition and biofilm reduction effect on *S. aureus* biofilm but showed only significant biofilm reduction effect on enterococcal biofilm.

Zeuko'o et al., (2016) reported that the MIC of ethanolic leaf extract of *V. amygdalina* on the fungi - *Candida albicans* and *Candida glabrata* was greater than 40mg/ml. Okigbo and Mmeka (2008) reported that the 20mg/ml of the ethanolic leaf extract of V. amygdalina inhibited the growth of *E. coli*, *S. aureus* and *C. albicans* planktonic cells. A study by Evbuomwan *et al.,* (2012) observed that the MIC of *V. amygdalina* on some Gram positive and Gram-negative bacteria varied between 25mg/ml to 50mg/ml. Alo *et al*., (2012) observed that the MIC of the ethanolic leaf extract of *V. amygdalina* and *O. gratissimium* on *E. coli* was 500mg/ml and 200mg/ml, respectively. Another study by Nwinyi *et al.,* (2009) recorded MIC of 10mg/ml and 2.3mg/ml for *O. gratissimium* extract on *E. coli* and *S. aureus*, respectively. Udochukwu *et al.,* (2015) showed that the MIC of *V. amygdalina* was 50µl/ml for *E. coli* and *S. aureus* while the MIC for *P. aeruginosa* was 25µl/ml. They also observed that *O. gratissimium* had MIC of 50µl/ml for the three tested bacteria (*E. coli, P. aeruginosa* and *S. aureus*) respectively. Rajasekaran *et al*., (2008) demonstrated that the ethanolic leaf extract of *A. indica* in a concentration of 5mg/ml inhibited the growth of some Gram positive and

Gram-negative bacteria. Aslam *et al.,* (2012) also reported the activity of 50mg/ml and 75mg/ml of *A. indica* extract on *S. aureus*, *Corynebacterium bovis* and *E. coli*. To our knowledge this present study is the first to assess their antibiofilm activity. No study has so far been carried out using these extracts on *C. perfringens* or any other bacterial biofilm.

5.13. CONCLUSION

Results of this study showed that the studied plant extracts demonstrated modest ability to reduce biofilm formation at the concentrations tested when added before biofilm was formed or removed biofilms once formed. This suggest that the bioactive compounds of these plant leaf extracts are responsible for these activities. We recommend further investigation on the antibiofilm activity at higher concentrations in *C. perfringens* and other bacteria biofilms. It will also be necessary to apply both quantitative and qualitative phytochemical screening following the methods of Gul *et al.,* (2017); Ezeonu and Ejikeme (2016) to analyse and determine the different classes of natural compounds as well as the percentage of phytochemical constituents in the extract responsible for the antibiofilm activity observed by separating and identifying the components. These compounds would be tested individually and when effective, could be used to develop antibiofilm agents and then tested against biofilm produced by other pathogenic microorganisms.

CHAPTER SIX:

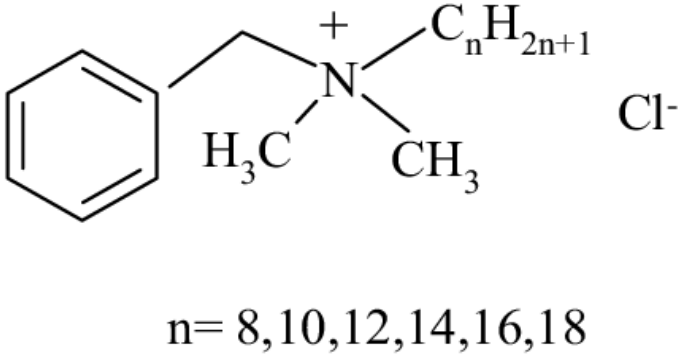
ANTI-BACTERIAL AND ANTI-BIOFILM ACTIVITY OF A NEW ANTIMICROBIAL COMPOUND (*ACQUORSOL*) ON *Clostridium perfringens* PLANKTONIC CELLS AND BIOFILM.

6.1 Introduction

Biocides are disinfectants that are used in homes, hospitals, food industries and farms to prevent and control the spread of pathogens. There are various studies in the literature that have reported the antibiofilm activity of biocides on pathogenic bacteria. Zineb *et al*., (2014) demonstrated that sodium hypochlorite, hydrogen peroxide and ethanol showed good to excellent biofilm inhibition effect on *Staphylococcus werneri* and *Staphylococcus sciuri* biofilm. Skowon et al., (2019) tested the susceptibility of Listeria monocytogenes biofilm to four disinfectants (Jodat, Peroxat, Chlorox S and Quotosept) on *Listeria monocytogene*s biofilm. They reported that though the disinfectant tested reduced biofilm, biofilm susceptibility to disinfectant was strain dependent and was influenced by environmental factors such as temperature, pH, and nutrient availability. Also, Trevisan *et al*., (2018) recorded 5 log and 5.12 log reduction in bacterial count respectively when *Salmonella* Typhimurium preformed biofilm on stainless steel and polypropylene was treated with 4 x MIC of carvacrol. Charleboise *et al.,* (2017) tested the susceptibility of *C. perfringens* in biofilm to five different disinfectants: hydrogen peroxide (10%), potassium monopersulfate (1%), glutaraldehyde (2%), quaternary ammonium chloride (1%) and sodium hypochlorite solution (0.27%). Their investigation showed that not surprisingly, sodium hypochlorite solution (0.27%) was very efficient at reducing *C. perfringens* in biofilm.

When considering the healthcare significance of biofilm and its role in increasing drug resistance, there is an urgent need for appropriate and alternative approaches to inhibit biofilm growth and treat biofilm related infections. This chapter aims at testing the antibiofilm activity of a recently developed QAC compound (*acquorsol*) on *C. perfringens* biofilms. Acquorsol is a very similar compound to Bacoban a common bactericide composed of the active component Benzalkonium chloride. Benzalkonium chlorides (BACs) has broad-spectrum antimicrobial properties against bacteria, fungi, and viruses and therefore has widespread application. They are categorized as biocidal agents with long durations of action (Pereira and Tagkopoulos, 2019). Benzalkonium chloride are bacteriostatic or bactericidal in action based on their concentration. Generally, their activity is not affected by pH, but such activity increases with prolonged exposure time and at higher temperatures.

The mode of action of benzalkonium chloride is due to the disruption of intermolecular interactions in the microbial cell. This disruption can cause the separation of membrane lipid bilayers of bacteria, resulting in uncontrolled cellular permeability, leakage of important cellular contents and deactivation of enzymes involve in different metabolic activities (Pereira and Tagkopoulos, 2019).



Structure of benzalkonium chloride Source: (Blazheyevskiy and Kovalska, 2017).

When considering the healthcare significance of biofilm and its role in increasing drug resistance, there is an urgent need for appropriate and alternative approaches to inhibit biofilm growth and treat biofilm related infections.

6.2 Aims and objectives

This chapter aims at testing the antimicrobial activity of a recently developed research compound (acquorsol) on *C. perfringens* biofilms. The specific objectives are.

(1)To determine the antimicrobial activity of acquorsol on *C. perfringens* planktonic cells

(2)To determine the biofilm inhibitory activity of acquorsol on *C. perfringens* biofilm cells

(3)To evaluate the effect of acquorsol on *C. perfringens* pre-formed biofilm

(4)To determine the effect of time of exposure on the antibacterial activity of acquorsol on *C. perfringens* planktonic and biofilms

Antimicrobial assay

6.3. Activity of *Acquorsol* on planktonic cell

The bactericidal effect of different concentrations of *Acquorsol* on *C. perfringens* planktonic culture was determined on isolates from human, chicken and poultry environment representing different biofilm class. In a sterile 96-well microtiter plate, 200µl of stock *Acquorsol* solution was transferred to wells in column 1 of the plate. 100µl of deionized water was transferred to other wells. Two-fold serial dilutions of *Acquorsol* solution were prepared by transferring 100µl of *Acquorsol* solution from column 1 into column 2 and then mix thoroughly by gently pipetting up and down. Then

100µl of the mixture in column 2 was transferred with a pipette and added to the next set of wells in column 3. This twofold serial dilution continued up to the 12th dilution. 100µl of the *C. perfringens* broth culture was transferred into each well containing the antimicrobials and producing a final cell density of 5x10^5 CFU/ml. The negative control wells were inoculated with *C. perfringens* test isolates with no *Acquorsol* solution, while the positive control wells contained sterile BHI broth and *Acquorsol* solution with no bacterial suspension. The microtiter plates were incubated anaerobically overnight at 37˚C. The minimum bactericidal concentration (MBC) of *Acquorsol* solutions were reported as the lowest concentration that inhibited bacterial growth completely which was determined by spread plating. Briefly, TSC agar plates were prepared and 100µl from each well after incubation was inoculated onto plates and incubated anaerobically at 37˚C for 24h.

6.4. Prevention of biofilm formation by *Acquorsol* solution

Different concentrations of A*cquorso*l solution; (1) 50%, (2) 25%, (3) 12.5%, (4) 6.25%, (5) 3.13%, (6) 1.56%, (7) 0.78%, (8) 0.39%, (9) 0.195%, (10) 0.097%, (11) 0.05%, (12) 0.02%, (13) 0.01% were prepared and tested on *C. perfringens* biofilm isolates representing different biofilm class using 96 well microtiter plates. For each isolate, 100µl of antimicrobial solution was transferred to three wells in microtiter plate and 100µl of *C. perfringens* midlog phase broth culture was inoculated into each of the wells. Microtiter plates were then incubated anaerobically at 37˚C for 24h. The effect of the treatment was determined by TVC assay as described in section 2.12. The percentage of biofilm reduction or kill was determined as shown in section 4.2.

6.5. Biofilm removal/reduction effect of Aquorsol

The effect of *Acquorsol* solution on the removal of established biofilm was evaluated by counting the number of viable bacterial cells in treated and untreated wells after exposure of pre-formed biofilm to acquorsol. Preformed biofilm in wells of microtiter plates were treated with 100µl of acquorsol and incubated for 6h to 24h. The number of adherent cells left after treatment was quantified following the procedure described in section 2.12.

Results

6.6. Antibacterial activity of A*cquorsol* on planktonic cells

The antibacterial activity of 13 concentrations (1) 50%, (2) 25%, (3) 12.5%, (4) 6.25%, (5) 3.13%, (6) 1.56%, (7) 0.78%, (8) 0.39%, (9) 0.20%, (10) 0.097%, (11) 0.05%, (12) 0.02%, (13) 0.01% of *Acquorsol* solution was determined on four *C. perfringens* isolates. *Acquorsol* solution affected the bacterial cell survival of tested *C. perfringens* isolates compared to the negative control (bacterial culture with no antimicrobial solution). Bacterial cell viability in planktonic culture was increased as the concentration of the antimicrobial solution decreased. It was observed that 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.02% and 0.097% concentrations inhibited 100% of planktonic cell growth in all isolates tested whilst 0.05% concentration inhibited 100% of planktonic cell in only one of the test isolates. Figure 6.1 shows the effect of 0.097% to 0.01% of *Acquorsol* on planktonic cells of four *C. perfringens* isolates. Table 6.1 shows the viability of thirty-three isolates of *C. perfringens* planktonic cells following exposure to 50%, 25%, 12.5%, 6.25%, 3.13% and 1.56%, concentrations of *Acquorsol* solution. Exposure to 50%, 25%, 12.5%, 6.25% caused a complete inhibition of planktonic bacterial growth in the thirty-three

isolates tested whilst 12.5% and 6.25% concentrations inhibited 85% and 64% respectively of *C. perfringens* planktonic cell density.

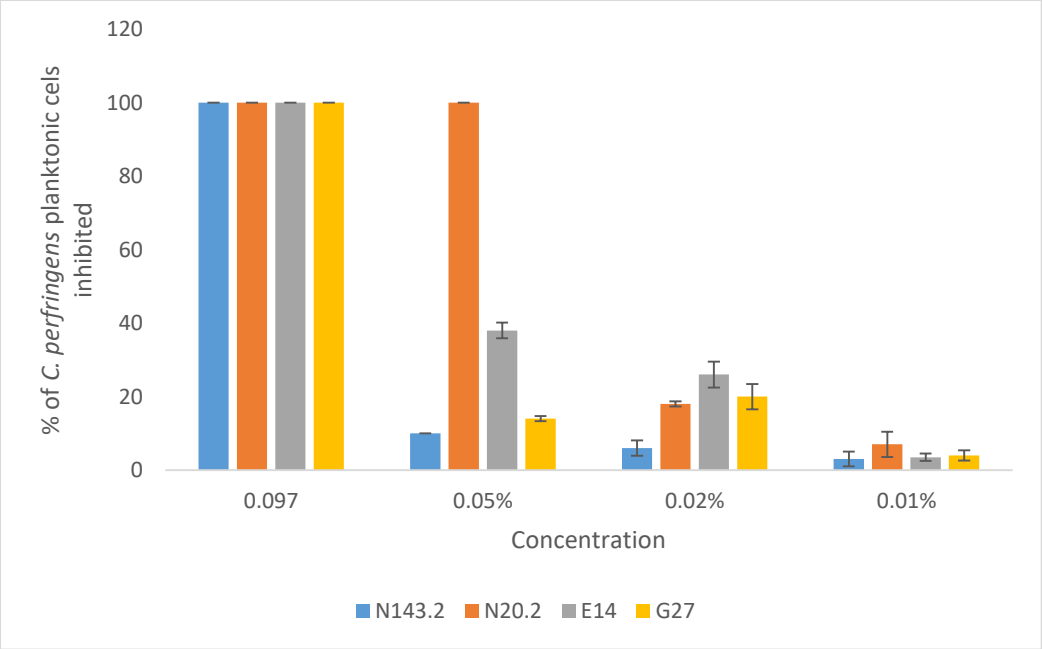


Figure 6.1: Effect of different concentrations of *Acquorsol* on C. *perfringens* planktonic cell expressed as means±SD of triplicate assays

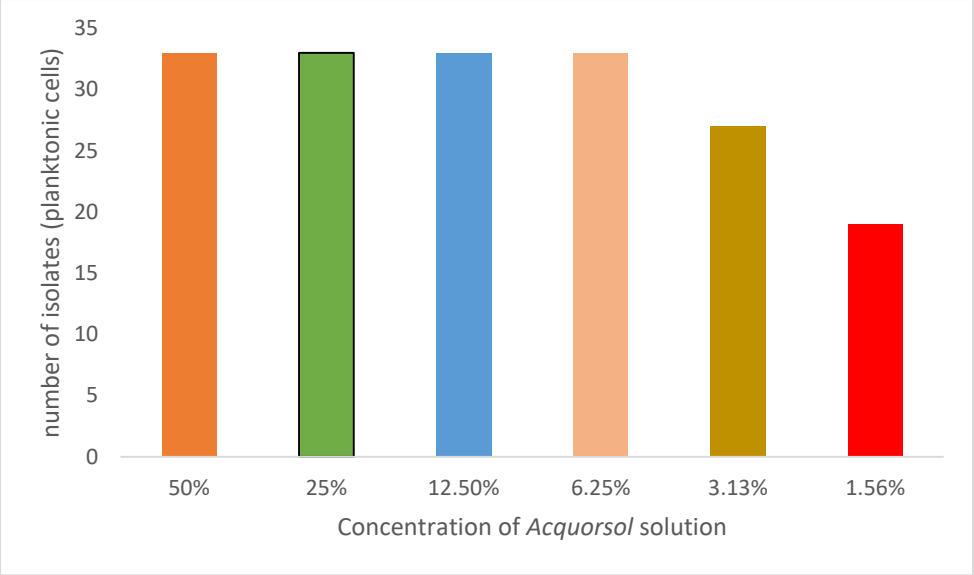


Figure 6.2: Antibacterial activity of Acquorsol solution on *C. perfringens* planktonic cells expressed as mean of triplicate assay.

6.7. Antibiofilm activity of *Acquorsol* solution

The antibiofilm activity of different concentrations (1) 50%, (2) 25%, (3) 12.5%, (4) 6.25%, (5) 3.13%, (6) 1.56%, (7) 0.78%, (8) 0.39%, (9) 0.20%, (10) 0.097%, (11) 0.05%, (12) 0.02% (13) 0.01 of *Acquorsol* solution was determined on four *C. perfringens* isolates. 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78% concentrations of *Acquorsol* solution inhibited 100% of biofilm growth in all test isolates. Whilst 0.39%, 0.20% and 0.097%, of *Acquorsol* solution inhibited 100% of biofilm growth in three of the four tested isolates. Figure 6.2. shows the effect of 0.78% to 0.01% of *Acquorsol* on biofilm cells of four *C. perfringens* isolates. Table 6.2 shows the antibiofilm effect of 50%, 25%, 12.5% concentrations of A*cquorsol* solution on thirty-three *C. perfringens* isolates. A dose dependent antibiofilm activity was observed as the biofilm cell inhibited from attachment decreased as the concentration of the solution decreased. 50% concentration of *Acquorsol* solution inhibited the formation of biofilms in 30 out of 33 isolates tested whilst 25% and 12.5% concentrations of the solution

inhibited the formation of biofilms in 26 and 9 respectively out of the 33 *C. perfringens* bacteria tested.

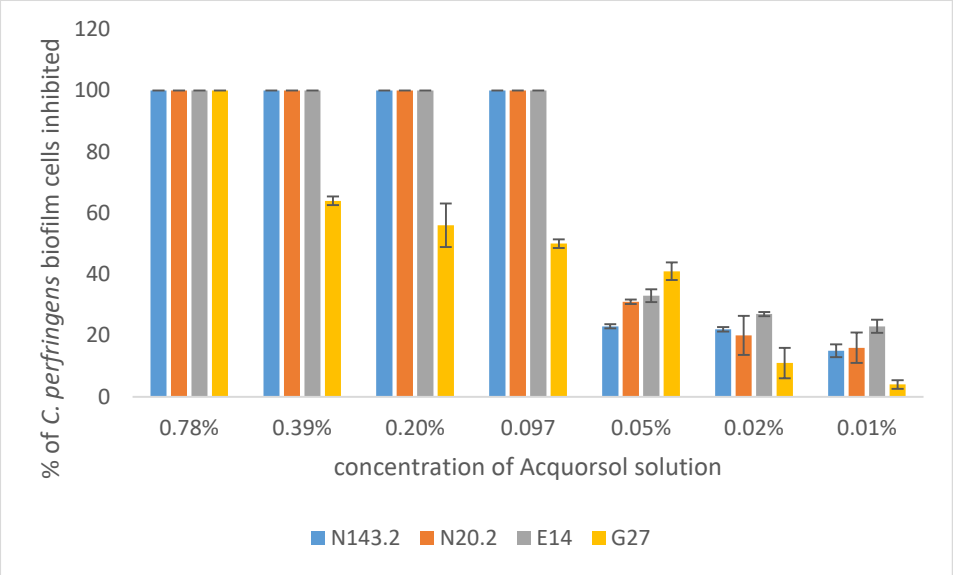
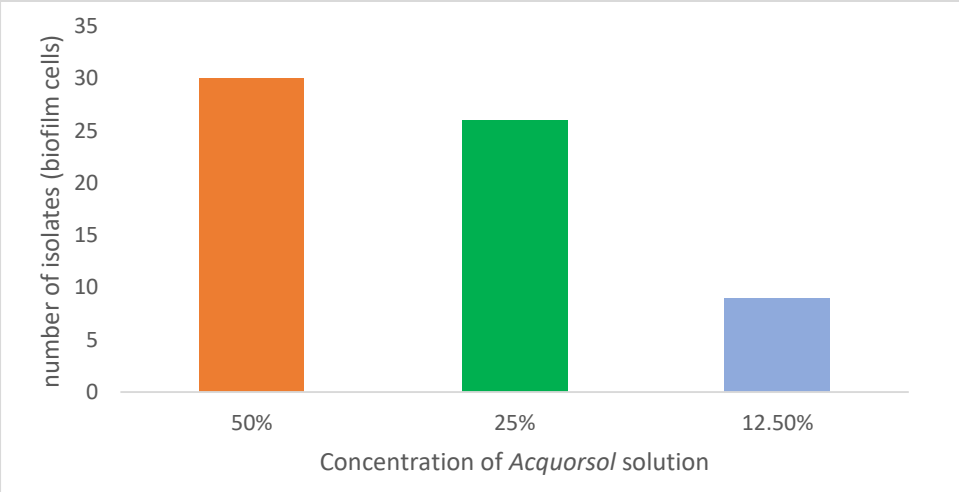


Figure 6.3: Effect of *Acquorsol* solution on biofilm inhibition expressed as mean ± SD of triplicate assay.



6.4: Antibiofilm activity of Acquorsol solution on *C. perfringens* biofilm inhibition/prevention expressed as mean of triplicate assay.

6.8. Effect of time on antibacterial activity of *Acquorsol* in planktonic growth

Following results on the dose dependent antibacterial effect of *Acquorsol* solution on planktonic cell survival, the influence of exposure time of *Acquorsol* solution on bacterial cells in planktonic growth was investigated. This test was performed on three of our isolates to determine if the antimicrobial activity was related to exposure time. 100µl of planktonic culture of *C. perfringens* was exposed to 100µl of 0.02% concentration of *Acquorsol* solution for 6h and 24h respectively. The viable planktonic cells of *C. perfringens* after treatment remained the same after 6h and 24h for one isolate whilst a 1% reduction in bacterial cell count was observed with increase in incubation time from 6h to 24h in two of the tested isolates although this difference was not significant as the *p* value was > 0.05 (Figure 6.3).

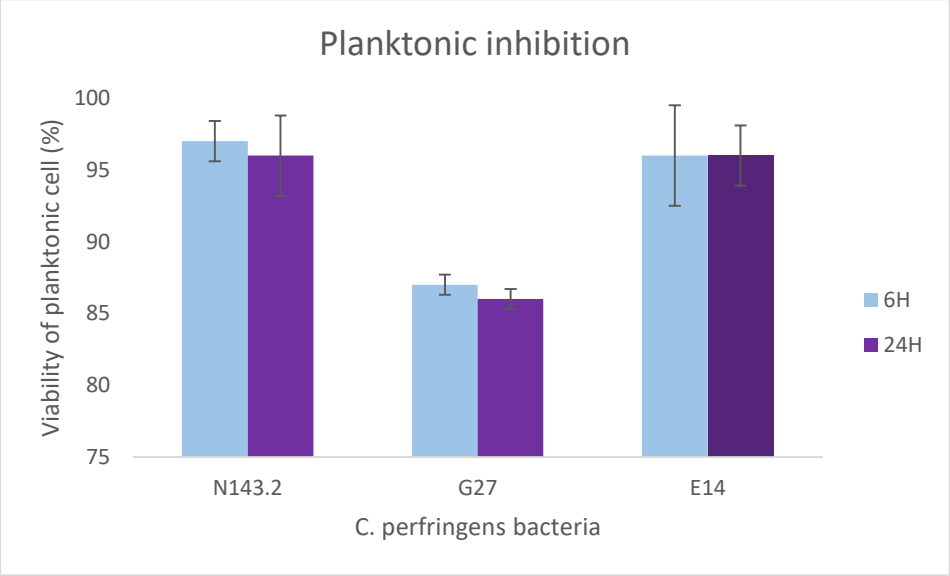


Figure 6.3: Viability of planktonic cells following exposure to *Acquorsol* antimicrobial solution at different time points expressed as means ±SD of triplicate assays

6.9. Effect of time on biofilm inhibition activity of A*cquorsol* solution

The time dependent antibiofilm activity of *Acquorsol* solution was investigated on three isolates to determine if the antibiofilm activity observed was influenced by the exposure time to the agent. Planktonic culture of *C. perfringens* was exposed to 0.05% concentration of *Acquorsol* solution for 6h and 24h respectively. After the desired period of biofilm growth, planktonic culture was discarded, and the adherent cells in each well were enumerated by careful scrapping and plating on agar plate. Our result as represented in figure 6.4 showed similar viability of biofilm cells after treatment with 0.05% of *Acquorsol* for 6h and 24h for one isolate, 4% reduction in *C. perfringens* biofilm cell count for a second isolate and 1% reduction in biofilm cell survival in the third isolate with increase in incubation time from 6h to 24h. The difference in biofilm cell viability was not significant as *p* value was > 0.05 for (Figure 6.4).

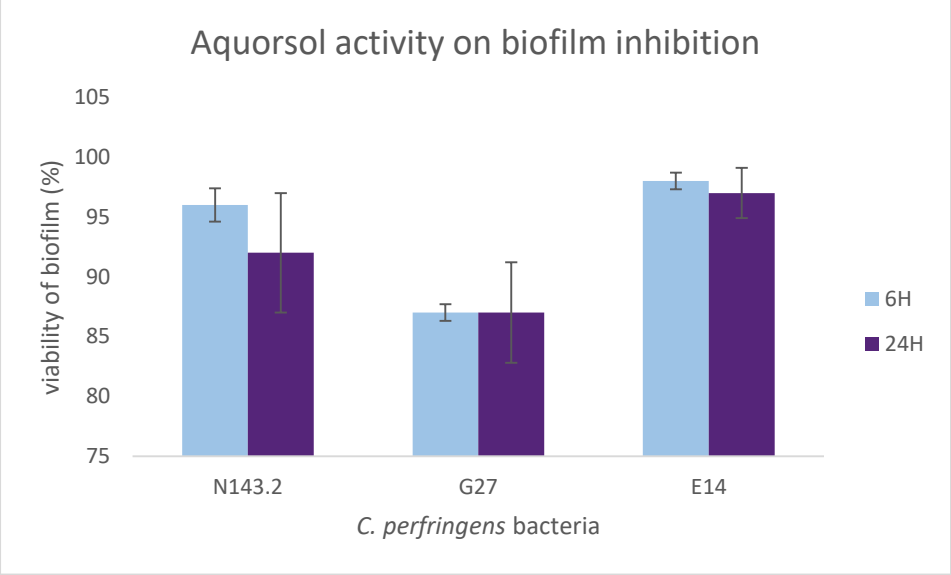


Figure 6.4: Effect of *Acquorsol* solution on biofilm inhibition on *C. perfringens* at different time points expressed as means ±SD of triplicate assays

6.10. Effects of *Acquorsol* on biofilm inhibition at 37˚C or 44˚C

C. perfringens broth culture in wells of microtiter plates were treated with 100µl of antimicrobial solution for 24h and incubated anaerobically at 37˚C or 44˚C to investigate the extent of biofilm formation. Results of viable cell count after the period of biofilm formation showed that although there were differences in bacterial count between the two temperatures this difference was not significant.

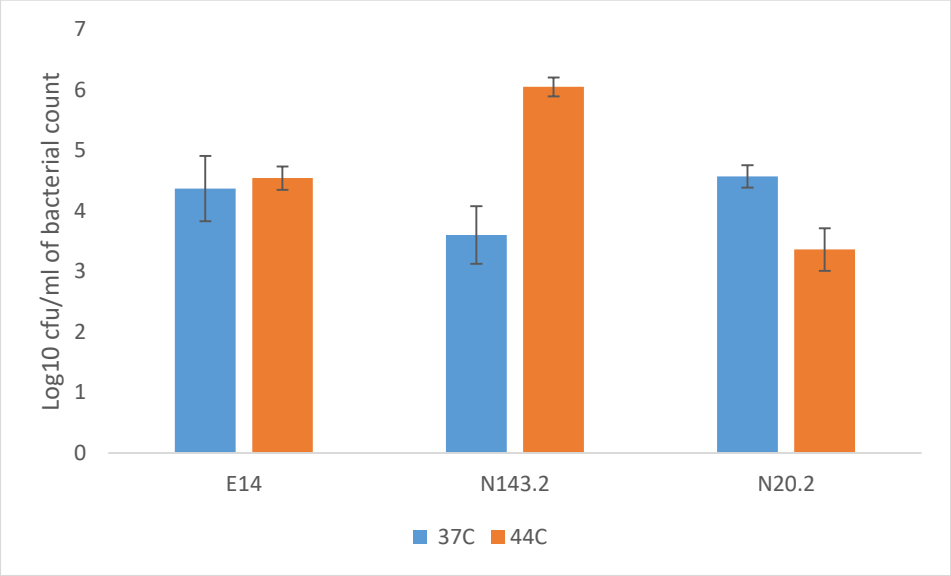


Figure 6.5: Effects of temperature on Acquorsol biofilm inhibition expressed as means ±SD of triplicate assays

.

6.11. Discussion

This present study tested the effect of a new research compound on *C. perfringens* planktonic and biofilm cells. The study revealed that *Acquorsol* solution has high bactericidal effect on *C. perfringens* planktonic cells. The minimum bactericidal concentration was determined to evaluate antibacterial activity and was observed to be 6.25% concentration as shown in figure 6.2. Antibiofilm activity was evaluated by quantifying total biofilm biomass using total viable count assay. The antibiofilm

and antibacterial activity of *Acquorsol* was not dependent on temperature and time. The results shown in our study agree generally with the studies of Charleboise *et al*., (2017). Their work showed that *C. perfringens* in planktonic growth mode were significantly more susceptible to disinfectants (hydrogen peroxide, quaternary ammonium chloride, potassium monopersulphate and glutaraldehyde solution) compared to when grown in biofilm. However, our present study revealed that *Acquorsol* solution was efficient against *C. perfringens* biofilm either in preventing biofilm formation or removing pre-formed/established biofilm. Acquorsol solution at 0.02% prevented biofilm growth in the range of 2 to 13% after 6h and 24h whilst 0.05% of acquorsol solution completely removed preformed biofilm. The difference in the biofilm inhibition and removal activity of acquorsol is because of the difference in the concentration used. The concentration tested in the biofilm removal assay was twice the concentration used in the biofilm inhibition test. Figure 6.4 shows the biofilm prevention activity of acquorsol at different times. 50% concentration of *Acquorsol* inhibited the formation of *C. perfringens* biofilm in 91% of tested bacteria. The work by Charleboise *et al*., (2017) agrees with the findings of this study when they investigated the antibiofilm activity of sodium hypochlorite (0.27%) on *C. perfringens* in established biofilms and reported high reduced biofilm cell viability to less than 20% after biofilm exposure to treatment.

Though there are only a few reports on disinfectant-like activity on *C. perfringens*, different articles have reported the antibiofilm activity of disinfectant on other bacteria. Skowron *et al*., (2019) reported that 100% and 50% of the disinfectant - Quatosept was efficient in removing *Listeria monocytogenes* biofilm. Lineback *et al*., (2018) reported significantly higher antibiofilm activity for Sodium hypochlorite and hydrogen peroxide compared to QAC on *S. aureus* and *P. aeruginosa* biofilm.

Their work showed that Sodium hypochlorite produced a log reduction of 8.73 and 8.75 while hydrogen peroxide produced a log reduction of 8.73 and 8.51 for *S. aureus* and *P. aeruginosa* respectively. The antibiofilm effect for QAC produced a log reduction of 4.37 and 0.82 for *S. aureus* and *P. aeruginosa*

Silva *et al.,* (2018) reported that peracetic acid and sodium hypochlorite disinfectant were not effective in removing *B. cereus* biofilm formed on stainless steel surfaces in contact with milk. They reported no significant difference in the cell count of *B. cereus* in coupons cleaned with water, peracetic acid, and sodium hypochlorite. The cell counts ranged from 1.5×10^5 to 8.7×10^6, 2.5×10^5 to 8.5×10^6 and 3.0×10^4 to 4.6×10^6 for water, peracetic acid and sodium hypochlorite respectively. Murray *et al.* (2017) evaluated the antibiofilm properties of a novel organosilane biocide (Goldshield). They reported that Goldshield had long term residual antibiofilm effect and recorded bactericidal effect of up to 6days when applied on *S. aureus* biofilm formed on stainless steel surfaces. They also showed that Goldshield had no significant effect on *P. aeruginosa* biofilm inhibition.

Although this present study was limited in terms of the number of isolates tested, this result showed that *Acquorsol* solution exhibits both antibacterial and particularly antibiofilm activity on *C. perfringens* observed by the reduction in planktonic and biofilm cell count.

6.12. Conclusion

Acquorsol solution at reasonably low concentrations effectively prevents the formation of *C. perfringens* biofilms and was remarkably effective against *C. perfringens* bacteria in established biofilms and planktonic cells. Results also showed that biofilm inhibition or reduction did not depend on the period of incubation, or the temperature used to

grow the biofilm. Although this present study was limited in terms of number of isolates tested, the proof of principle is confirmed and A*cquorsol* can be considered as a potentially useful cleaning agent in livestock management as well as a possible topical treatment for the control of *C. perfringens* and other commonly encountered wound pathogens. Future study would determine the possible mode of action of acquorsol and to assess the influence of environmental factors such as growth temperature. Also, the residual antimicrobial properties of acquorsol would be investigated.

CHAPTER SEVEN:

GENERAL DISCUSSION, CONCLUSION AND FUTURE WORK

7.1 Biofilm as unique structures

Biofilms are structures composed of bacteria that are protected from antimicrobial agents, environmental stresses, and the host immune response because of the production of extracellular polymeric substance (EPS matrix) associated with them (Jamal *et al*., 2015; Vasudavan, 2014). Biofilms have been defined as a virulence factor since biofilm embedded bacteria are more tolerant to antimicrobial treatment than their planktonic counterparts and are therefore responsible for chronic infections such as wound infections, (diabetic foot), surgical implants and probably infections affecting the intestinal lining (Jamal *et al*., 2018; Kirketerp-Moller *et al.,* 2008; Darouiche, 2004). Although it is well established that pathogenic bacteria form biofilms extensively in nature to survive and resist adverse conditions by attaching to each other and to a substrate via the extracellular polymeric substance (EPS), little data exists on the biofilm formation and antimicrobial resistance from a large sector of the natural world – the anaerobic bacteria (Charleboise *et al*., 2017; Vargal *et al*., 2008). Information regarding biofilm formation by Clostridia particularly *C. perfringens* is extremely limited and there are (as far as we know) currently no documented reports on the biofilm forming ability and antimicrobial susceptibility of *C. perfringens* isolated from humans.

This is the first study to show that *C. perfringens* isolates from humans (neonates), can form biofilms in the laboratory *in vitro*. This study shows that although some variability in the abundance of biofilm production from either chicken, the environment or humans was noted *in vitro,* this was clearly not dependent on the original source of the isolates. The ability of *C. perfringens* to form biofilm is probably an important virulence factor since this would enhance the establishment of infections following colonization of a site and help the bacteria to evade the host immune system and antimicrobials (Singh *et al*., 2017). The present work confirms the findings of others that both time and temperature affect biofilm formation in most organisms studied. With *C. perfringens*, it was confirmed that growth time and temperature affected the extent of the biofilm and could be an important but neglected factor when considering the prevention and control of *C. perfringens* biofilm related infections. Previous studies with *C. perfringens* biofilms have reported higher antimicrobial values (minimum inhibitory concentrations - MICs) for biofilm-embedded bacteria compared with testing against planktonic bacteria alone (Charleboise *et al*., 2014). This effect is in common with many other studies using a diverse range of bacteria and agree with results recorded in the present study. Studies by Hoffman *et al*., (2005) have shown that there is the possibility that antimicrobial treatment could enhance biofilm formation and although this paradoxical effect was not investigated in the present study, it could be playing a role in the higher MICs recorded with biofilms.

In human medicine, the ability of *C. perfringens* to form biofilm may play an important role in the pathogenesis of diseases and particularly in our ability to treat recurrent and refractory infections. Results from this present study also indicates the limitations in the application of routine laboratory culture and sensitivity results to a guide for infection control and treatment. Routine laboratory testing with planktonic bacteria is

likely not a true representation of the response to antibiotic treatment when biofilms are involved.

7.2 Genomic analysis of *C. perfringens*

Although this present study did not consider the genetic factors that play a role in biofilm formation, Kiu *et al.,* (2019) analyzed the genome of 88 *C. perfringens* isolates from broiler chicken and poultry environment and these include 9 of the isolates used in this present study. Their work showed that the 88 tested isolates expressed different virulence genes (toxin genes, collagen adhesin genes and antimicrobial resistance genes). They also observed the presence of more toxin genes and adhesin genes in *C. perfringens* associated with necrotic enteritis compared to healthy chickens. Isolates from this present study subjected to genome analysis includes 4 environmental isolates, 4 isolates from broiler chicken linked with necrotic enteritis and 1 isolate from healthy chicken. All 9 isolates harbored the toxin genes *plc, ccp* and *colA* and at least 4 other toxin genes out of the 12 toxin genes evaluated. Collagen adhesin genes (*cna, cnaA, cnaC and cnaD*) were absent in only two of the 9 isolates but was seen in 7 of the isolates. 4 of the 7 isolates that harboured the adhesin genes were moderate biofilm formers and they all harboured more than one adhesin gene. The other 3 isolates were weak biofilm formers and they harboured one adhesin gene each (Kiu *et al*., 2019). It is difficult to say if there is a correlation between biofilm class and adhesin gene production because of the low number of isolates tested. Though the evidence supports the model, future studies would investigate the genetic components of *C. perfringens* from human, broiler chicken and environmental isolates that may contribute to biofilm formation.

Bacterial adhesive structures enhance the attachment of bacterial to surfaces and aids biofilm formation. Although all our isolates formed biofilms, they did not all harbour the collagen adhesin genes, suggesting other genes may be involved in biofilm formation. Works by different researchers have revealed the involvement of different adhesin genes in biofilm formation of other bacteria. A study by Pereyra *et al*., (2016) detected the *cna* genes in 20% of *S. aureus* isolated from bovine mastitis. Chen *et al*., (2019) showed that *cna* genes were significantly higher in biofilm producing strains of *S. aureus* compared to non-biofilm producers while Khoramian *et al*., (2015) reported a higher prevalence of *cna* genes in *S. aureus* isolated from humans than in bovine. Ghasemian *et al.,* (2018) reported that adhesin genes *fimA, mrkA, matB* and *pilQ* were associated with biofilm formation in *Klebsiella oxytoca*. Saba *et al.*, (2018) demonstrated the presence of *icaA* and ica*D* genes in biofilm formation of clinical isolates of *S. aureus* but also showed that these genes were not associated in all cases of biofilm formation in the strains tested. Azmi *et al.,* (2019) reported that the presence of *icaA* and *icaD* genes in MRSA strains did not correlate with their biofilm forming capacity. Qin *et al*., (2007) reported the absence of *ica* genes in two biofilms producing strains of *S. epidermidis.* Charlebois *et al*., (2017) has shown the expression of genes in biofilm formed by *C. perfringens* isolated from chicken. Their work revealed the up regulation of genes responsible for EPS biosynthesis, oxidative stress resistance and general stress response genes, haemolysin encoding genes, iron acquisition genes and genes involved in sporulation.

7.3 Novel treatment (Nanoparticles)

Another aim of this present study was to investigate whether silver nanoparticles or gold nanoparticles have the potential of directly reducing the biofilm formed by *C. perfringens* and/or enhancing the susceptibility of antimicrobials against *C. perfringens* when embedded in biofilm. The ability of tested nanoparticles to reduce bacteria in established biofilms suggest that nanoparticles could be a useful adjuvant (Vazquez-Munoz *et al*., 2019; Singh *et al.,* 2019). The exact mechanism of action of nanoparticles against biofilms remains unclear (Grudniak and Wolska, 2013) but it may relate to the unique characteristics of nanoparticles which includes their highly reactive surface, small size and large surface to volume ratio that enhances their quality as good drug delivery agents (Huh and Kwon, 2011). Nanoparticles have been used as carriers of antibiotics to enhance antibiotic effectiveness thus enhancing antibiotic penetration through the biofilm matrix. Alabresm *et al*., (2020) demonstrated that NPs functioned as antibiotic delivery vehicles and enhanced the antimicrobial activities of penicillin G against MRSA and other multidrug resistant bacteria (*E. coli, P. aeruginosa, P. vulgaris*). PenG-NPs complex was formed by mixing 2µl/ml carboxylic acid functionalized NPs with 1mg of Pen G. Using the broth microdilution assays to assess for antimicrobial activity, they reported significantly ($P < 0.05$) enhanced antimicrobial activity of PenG-NPs against Gram-negative and Gram-positive bacteria. The increased bactericidal abilities of PenG-NPs were attributed to increase in concentration of antibiotic delivered by each NPs to the bacterial cell compared with when the antibiotic was applied alone, and this overwhelms the bacterial resistance mechanisms. The inhibition by NP-Pen G complex suggests that NPs delivery of this antibiotic was able to overcome bacteria membrane barriers and penicillin-binding protein defences.

In this study, we reported that although the percentage of bacterial biofilm reduction recorded when the antibiotic was applied alone and when combined with nanoparticles, was not significantly different, it could be related to the size and concentration of the nanoparticles used. As far as we know there has been no study focusing on *C. perfringens* previously on the anti-biofilm activity of silver or gold nanoparticles in the literature. This present study supports the claim that nanoparticles have antibiofilm therapeutic potential and suggest that nanoparticles could be used against *C. perfringens* biofilm probably as a specialist disinfectant. However, since there has not been any previous study of nanoparticles activity on *C. perfringens*, it is therefore necessary to carryout larger studies (more strains and higher concentrations) to determine the anti-biofilm and enhanced activity of antibiotics in combination with nanoparticles on anaerobic pathogenic bacteria.

7.4 Novel treatment (Plant extracts)

Results of the third section of these studies shows that medicinal plant extracts could be identified and developed as a possible antibiofilm agents. Medicinal plants have been applied for decades to treat various illness and there are reports of their useful antibacterial activity (Quave *et al*., 2008; Gibbons *et al*., 2005). However, detailed studies on the antibiofilm activities of medicinal plant extracts on *C. perfringens* are limited. Generally, plant materials are extracted in various solvents such as ethanol, acetone, distilled water, and methanol. Alan *et al*., (2020) reported that the antibiofilm activity of extracts varied depending on the solvent used for extraction. They studied the biofilm inhibition activity of three medicinal plant extract *(Berginia ciliata*, *Clematis grata* and *Clematis viticella)* on *P aeruginosa* PAO1 biofilm using different solvents (methanol, ethanol, ethyl acetate, acetone, hexane, chloroform, and distilled water). Biofilm of PAO1 was grown

at 30°C and crystal violet assay was used to assess the biofilm inhibiting activity of plant extracts. Inhibition of biofilm formation ranged from -100% recorded in the chloroform extract to 80% recorded in the methanol and ethanol extract.

Plant extracts has expressed antibiofilm activity against a range of bacteria. Bazargani and Rohloff, (2016) investigated the invitro antibiofilm activities of essential oil (EO) and plant extract of coriander (*Coriandrum sativum* L.), anise (*Pimpinella anisum* L.) and peppermint (*Mentha piperita* L) on *S. aureus* and *E coli* bacteria. They used the crystal violet and XTT reduction assays to assess biofilm growth and development. Though all tested plant extract exhibited antibiofilm activity against both bacterial strains, a more potent activity was demonstrated against *S. aureus*. Their work showed that EO and plant extract inhibited bacteria attachment by at least 50%. Also, EO from coriander showed the highest antibiofilm activity against biofilm formed by the tested bacteria (*S. aureus* and *E. coli*) with 0.8ml/ml and 1.6ml/ml as the least MIC values respectively. Similar range of biofilm inhibition by EO has been reported in the literature. EO from oregano, carvacrol and thymol showed biofilm inhibitory MIC at 1.25mg/ml, 0.31mg/ml and 1.25mg/ml respectively (Nostrol *et al*., 2007). Also, Adukwu *et al.,* (2012) and Oral *et al*., (2010) reported MIC value of 1.25mg/ml and 0.5mg/ml when EO from lemon grass and EO from oregano were tested on *S. aureus* biofilm respectively.

The biofilm inhibition activity of Canadian medicinal plants on *S. pyogenes* biofilm was reported by Wijesundara and Rupasinghe (2019). The MTT assay was performed to study biofilm formation of fourteen ethanolic plant extract and their study showed that only five (Licorice root, purple coneflower flower, purple coneflower stem, sage leaves, and slippery elm inner bark) of the fourteen plants tested had biofilm inhibition effect with the minimum biofilm inhibitory concentration (MBIC) of extracts ranging from 31.5

– 250 µg/ml. Also, study by Al Shahwany *et al*., (2016) showed that phenolic plant extract from three medicinal plant (*Zingiber officinal* Roscoe, *Thymus vulgaris* L. and *Cinnamomum zeylanicum)* using the crystal violet assay on *S. aureus* and *K. pnuemoniae* biofilm. Their result showed that the tested bacteria behaved differently in their sensitivity to the different extracts. *Z. officinale* showed the greatest antibiofilm activity against *K. pneumoniae* at 0.106µg/ml and *S. aureus* at 0.150µg/ml. This was followed by *T. vulgaris,* and the least was *C. zeylanicum* extracts.

Though the plant extract investigated in this present study demonstrated limited biofilm inhibition and biofilm removal potential at the concentration tested, the effect was sufficient to reduce bacterial counts from treated biofilm compared with the negative control. The extract has clear anti-biofilm potential if applied at an appropriate concentration but there is need for further investigation of the anti-biofilm activity of tested plant extract at a range of concentrations with *C. perfringens* to determine the lowest concentration where activity can be achieved. It will be interesting to further determine what bioactive compounds in the plant extract are responsible for the antibiofilm activities observed and investigate the antibiofilm activity at various higher concentrations in *C. perfringens* and other bacteria biofilms. The bioactive compounds would be identified and analysed using both quantitative and qualitative analysis. These methods will determine the different classes of natural compounds in the extract responsible for the antibiofilm activity observed.

7.5 Novel treatment (*Acquorsol* solution)

The final part of this study investigated the activity of an entirely new antimicrobial biocide called *Acquorsol* against *C. perfringens* planktonic and biofilm cells. The study reported here reveals that *Acquorsol* solution at low concentrations has highly effective

antibacterial effects and interestingly reduces *C. perfringens* biofilm. The activity of disinfectant could be influenced by microorganism and time of exposure. Iñiguez-Moreno *et al*., (2017) determined the effectiveness and antimicrobial activity of 15 disinfectants commonly used in food industry in Mexico against *E. coli* ATCC 11229, *S. Choleraesuis* ATCC 10708, *P. aeruginosa* ATCC 15442, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538 and *L. monocytogenes* ATCC 19111. Their work showed that the MIC was dependent on the bacteria tested as well as the exposure time. They reported 99.999% (5 log 10) reduction in bacterial count after 30 seconds of exposure for most bacteria. In addition, for all disinfectants tested, the MIC after 5 min of exposure was two to four times greater than the MIC with the same effect after 10min of treatment. The MIC was not different after 10min and 15 min of exposure in most cases.

Although there is little published data on regular disinfectant activity against *C. perfringens*, 0.27% hypochlorite solution was reported by Charlebois *et al*., (2017) to be more effective at reducing *C. perfringens* in established biofilm compared to planktonic cells. Ferreira *et al.,* (2017) reported that though sodium hypochlorite showed antibiofilm activity against *Candida* species mature biofilm, the disinfectant chloramine T was significantly more effective than sodium hypochlorite in reducing mature biofilm after 1min of exposure with MIC range from 0.69 – 5.54 and 4.19 to 16.79 for chloramine T and sodium hypochlorite respectively.

This present study shows that *Acquorsol* solution a QAC has considerable potential as an antimicrobial biocide especially against biofilms and could be a possible novel control measure for *C. perfringens.* Appropriate concentration of *Acquorsol* solution can be used as a useful effective disinfectant to clean surfaces in homes, hospitals, schools, poultry environment and food processing industries. QAC are surface-active

agents and are widely used as disinfectants. Gerber, (2015) reported that QACs are multitarget in nature and development of resistance by organisms is rare as there are alternative mechanisms of action when mutation occurs. Ramzi *et al*., (2020) assessed the antibacterial activity of three synthetic disinfectants classified as QAC (DDN9® (0.5%), spray (0.4%), and Phagosurf ND® (0.4%)) on different bacterial strains (*E coli*, *K pneumoniae, Enterobacter cloacae, P. aeruginosa, A. baumannii*, and *S. aureus*) isolated from the hospital environment. They used both disk diffusion and broth dilution methods to determine their activity and reported that QAC (NDD9) only inhibited the growth of *S. aureus and S. aureus* ATCC 29213 with an MIC of 0.25 mg/ml. The second disinfectant (spray) had effect on *E. coli, S. aureus, E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 with an MIC of 4 mg/ml and 2mg/ml for *S. aureus* ATCC 29213. The third disinfectant, Phagosurf ND® at an MIC of 4mg/ml inhibited only the growth of *S. aureus* ATCC 29213. Furthermore, it is important to use disinfectant with caution as excessive domestic and industrial usage of QACs has been reported to enhance bacteria resistance to antibiotics and led to accumulation of QACs in the environment and abundance of QACs in the environment is toxic to aquatic and terrestrial animals. Therefore, it is recommended that QACs are effectively removed in wastewater treatment plants before releasing into the environment (Zhang *et al*., 2015).

CONCLUSION

This present study shows that biofilm formation is common among *C. perfringens* isolates and revealed that *C. perfringens* isolated from different sources (human neonates, broiler chicken and free-range poultry environment) can form different classes of biofilm (weak, moderate, and strong categories).

Biofilm formation by *C. perfringens* was not dependent on the source of isolation of the bacteria but was shown to be dependent on growth temperature and time-period of growth. As expected, the minimum inhibitory concentration (MIC) of tested antibiotics was higher for biofilm-embedded bacteria than when compared to their planktonic counterparts.

This study investigated the anti-biofilm activity of silver and gold nanoparticles and found that silver and gold nanoparticles were effective in removing *C. perfringens* established biofilm in microtiter plates and further enhanced the activity of penicillin, gentamicin, tetracycline, and bacitracin on *C. perfringens* biofilm removal.

The antibiofilm potential of the ethanolic leaf extract of three medicinal plants (*V. amygdalina*, *O. gratissimum* and *A. indica*) on *C. perfringens* was examined. This was the first study to investigate the activity of medicinal plants on *C. perfringens* and it showed that the tested plant extracts could be potential anti-biofilm agent for *C. perfringens.* Further work is needed to improve on these findings by testing various higher concentrations of the extract on *C. perfringens* biofilm and identify the phytochemicals present in the extract using qualitative approach.

C. perfringens harboured various toxin genes as well as collagen adhesin genes. Although a correlation between the production of collagen adhesin genes and development of biofilm class formed by *C. perfringens* could not be established because of the low number of isolates tested.

Finally, this present study demonstrated that a novel antimicrobial research solution (*Acquorsol* - a QAC product) has good antimicrobial and excellent antibiofilm effects against *C. perfringens* and could be considered as a disinfectant or for the control of *C. perfringens* related infection.

Limitations of study

In this study, the 96 well plate biofilm assay method was explored. Biofilm formation using biofilm reactor was not considered.

Only the biofilm reduction activity of NPs was assessed. It would be necessary to determine both biofilm inhibition and biofilm reduction effect of tested NPs at similar concentrations to compare results. Also, the checkerboard method was not considered to determine the impact of the combination of NPs and antibiotics in comparison to their individual activities to give the fractional inhibitory concentration (FIC) index.

This study did not analyse the component of the plant leaf extract. It would be necessary to conduct GC-MS analysis on the extract to understand the different component and consistency within the product.

Future work

To test for biofilm formation in the anaerobe if possible, using biofilm reactor and to investigate the genetic components of *C. perfringens* to determine if there is any correlation between the presence of adhesin genes and biofilm density.

Future work will perform larger study on the antibiofilm activity of a range of concentrations of metal nanoparticles as well as antibiotics and measure synergy of the enhanced antibiofilm effect when nanoparticles are combined with antibiotics on anaerobic bacteria using the checkerboard testing.

To extend the study on the antibacterial and anti-biofilm activity of tested plant extract. Leaf extract of plants will be tested at a range of concentrations on pathogenic bacteria to determine the MIC for planktonic and biofilm grown strains. Also, the phytochemicals present in the extract will be characterised using quanlitative assay such as Gas chromatography - Mass spectrometry method.

To determine the possible mode of action of acquorsol and to assess the influence of environmental factors such as growth temperature and contaminating organics (fats) on the antimicrobial activity. Also, it will be necessary to study acquorsol for residual antimicrobial activity when applied on surfaces.

REFERENCES

Aaron, S. D., Ferris, W., Ramotar, K., Vandemheen, K., Chan, F. and Saginur, R. (2002). Single and combination antibiotic susceptibilities of planktonic, adherent, and biofilm-grown *Pseudomonas aeruginosa* isolates cultured from sputa of adults with cystic fibrosis*. Journal Clinical Microbiology* 40, 4172-4179.

Abe. K., Nomura. N. and Suzuki, S. (2020) Biofilms: hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. *FEMS Microbiology Ecology*, 96:1-11.

Abedon, S.T. (2012). Spatial Vulnerability: Bacterial Arrangement, Microcolonies, and Biofilms as Responses to Low Rather than High Phage Densities. *Viruses,* 4,663-687.

Adebolu, T.T. and Oladimeji, S. A. (2005). Antimicrobial activity of leaf extracts of *Ocimum gratissimum* on selected diarrhoea causing bacteria in southwestern Nigeria. *African Journal of Biotechnology,* 4 (7) 682-684.

Adhikari, M.D., Goswami, S., Panda, B.R., Chattopadhyay, A., Ramesh, A. (2013). Membrane-Directed High Bactericidal Activity of (Gold Nanoparticle)–Polythiophene Composite for Niche Applications against Pathogenic Bacteria*. Advanced Healthcare Materials*. 2:599–606.

Adukwu, E. C., Allen, S. C. H., and Phillips, C. A. (2012). The anti-biofilm activity of lemon grass (Cymbopogonflexuosus) and grapefruit (Citrus paradisi) essential oils against five strains of *Staphylococcus aureus*. *Journal of Applied Microbiology*,113 (5):1217-1227.

Agnoni, A., PA-C, L.A., Christine, P. (2017). Necrotizing enterocolitis: Current concepts in practice *Journal of American Academy of Physician,* 30(8) 16-21.

Ahangari, A., Salouti, M., Heidari, Z., Kazemizadeh, A.R. and Safari, A.A. (2013). Development of gentamicin-gold nanospheres for antimicrobial drug delivery to Staphylococcal infected foci. *Journal of Drug Delivery*, 20(1): 34–39.

Ahmadi, F., Abolghasemi, S., Parhizgari, N., Moradpour, F. (2013). Effect of silver nanoparticles on common bacteria in hospital surfaces. *Jundishapur Journal of Microbiology* 6(3) 209-214.

Akinmoladun, A.C., Ibukun, E.O., Afor, E., Obuotor, E.M. and Farombi, E.O. (2007). Phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum. Scientific Research and Essay,* 2(5)163-166.

Alabresm, A., Cen, Y. P., Witcher-Chandler, S., Lead, J., Benicewienz, B. C., Decho, A. W. (2020). Nanoparticles as antibiotic-delivery vehicles (ADVs) overcomeresistance by MRSA and other MDR bacterial pathogens: The grenade hypothesis, *Journal of Global Antimicrobial Resistance*, 22:811-817.

Alama, K., Al Farraj, D. A., Mah-e-Fatima, S., Yameen, M. A., Elshikhb, M. S., Alkufeidy, R. M., Abd El-Zaher M.A. Bhasme, M. P., Alshammari, M. K., Alkubaisi, N. A., Arshad Abbasig, M., Naqvia, T. A. (2020). Anti-biofilm activity of plant derived extracts against infectious pathogen-*Pseudomonas aeruginosa* PAO1.*Journal of Infection Public Health.* 13:1734-1741.

Alav, I., Sutton, M.J. and Rahman, M.K. (2018). Role of bacterial efflux pumps in biofilm formation. *Journal of Antimicrobial Chemotherapy,* 73: 2003-2020.

Algburi, A., Comito, A.A., Kashtanov, D, N., Dicks, L.M.T., Chikindas, M.L. (2017). Control of biofilm formation: antibiotics and beyond. *Applied Environmental Microbiology* 83:3-83.

Alkhulaifi, M.M. (2017). Using phages to exterminate biofilms. *Journal of Medical Microbiology Diagnosis* 6:(3) 1-5.

Allaker, R.P, Memarzadeh, K. (2014). Nanoparticles and the control of oral infections. *International Journal of Antimicrobial Agents*, 43:95–104.

Almohamad, S., Somarajan, S.R., Kavindra, K.V., Nallapareddy, S.R., Murray, B.E. (2014). Influence of isolate origin and presence of varous genes on biofilm formation by *Enterococcus faecium. FEMS Microbiology Letters,* 353: 151–156.

Alo, M. N., Anyim, C., Igwe, J. C., Elom, M. and Uchenna, D. S. (2012). Antibacterial activity of water, ethanol, and methanol extracts of *Ocimum gratissimum*, *Vernonia amygdalina* and *Aframomum melegueta. Advances in Applied Science Research* 3 (2): 844-848.

Alshareef, A., Laird, K. and Cross, R. (2016). "Chemical synthesis of copper nanospheres and nanocubes and their antibacterial activity against *Escherichia coli* and *Enterococcus* sp." *Acta Metallurgica Sinica* (*English letters),* 1-7.

Al Shahwany A.W., Tawfeeq H. K., Hamed, S.E. (2016). Antibacterial and Anti-biofilm Activity of Three Phenolic Plant Extracts and Silver Nanoparticles on *Staphylococcus aureus* and *Klebsiella pneumoniae*. *Biomedicine and Biotechnology*, 4: 12-18.

Alshareef, A., Laird, K. and Cross, R. (2017). Shape-dependent antibacterial activity of silver nanoparticles on Escherichia.coli and Enterococcus.faecium bacterium, Applied Surface Science, 424:310-315.

Ankri, S. and Mirelman D. (1999). Antimicrobial properties of allicin from garlic. *Microbes and Infection,* 2: 125-129.

Ansari, A., Khan, H.M., Khan, A.A., Sultan, A., Azam, A. (2012). Synthesis and characterization of the antibacterial potential of ZnO nanoparticles against extended-spectrum β-lactamases-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from a tertiary care hospital of North India Mohammad. *Applied Microbiology and Biotechnology,* 94:467–477.

Ansari, M.A., Khan, H.M., Khan, A.A., Cameotra, S.S., Pal, R. (2014). Antibiofilm efficacy of silver nanoparticles against biofilm of extended spectrum b-lactamase isolates of *Escherichia coli* and *Klebsiella pneumoniae. Applied Nanoscience,* 4:859–868.

Aparna, M.S. and Yadav, S. (2008). Biofilms: microbes and diseases. *Brazilian Journal of Infectious Diseases*, 12(6): 1-7.

Aslam, F., Rehman, K., Asghar, M. and Sarwar, M. (2009). Antibacterial activity of various phytoconstituents of neem. *Pakistan Journal of Agriculture Science,* 46(3): 209-213.

Ayoola, G.A., Coker, H.A.B., Adesegun, S.A., Adepoju-Bello, A.A., Obaweya, K., Ezennia, E.C., Atangbayila, T.O. (2008). Phytochemical Screening and Antioxidant Activities of Some Selected Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. *Tropical Journal of Pharmaceutical Research*, 7(3): 1019-1024.

Azam, A., Ahmed, A. S., Oves, M., Khan, M.S. and Memic, A. (2012). Size-dependent antimicrobial properties of CuO nanoparticles against Gram-positive and Gram-negative bacterial strains. *International Journal of Nanomedicine,* 7: 3527-3535.

Azmi, K., Qrei, W. and Abdeen, Z. (2019). Screening of genes encoding adhesion factors and biofilm production in methicillin resistant strains of *Staphylococcus aureus* isolated from Palestinian patients. *BMC Genomics,* 20(578):1-12

Baba, E., Ikemoto, T., Fukata, T., Sasai, K., Arakawa, A. and McDougald, L.R. (1997). Clostridial population and the intestinal lesions in chickens infected with *Clostridium perfringens* and *Eimeria necatrix*. *Veterinary Microbiology*, *54*: 301-308.

Baker, C., Pradhan, A., Pakstis, L., Pochan, D.J., Shah, S.I. (2005). Synthesis and antibacterial properties of silver nanoparticles. *Journal of Nanoscience and Nanotechnol*ogy, 5:244-249.

Balouiri, M., Sadiki, M., Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis,* 6 (2): 71-79.

Bandiola, T.M.B. (2018). Extraction and Qualitative Phytochemical Screening of Medicinal Plants: A Brief Summary. *International Journal of Pharmacy*, 8(1): 137-143

Barbosa, J., Borges, S., Camilo, R., Magalhaes, R., Ferreira, V. Santos, I. (2013). Biofilm formation among clinical and food isolates of *Listeria monocytogenes*. *International Journal of Microbiology, Biochemistry (Moscow),* 70(2): 267-274.

Barzargani, M. M. and Rohloff, J. (2016*). Antibiofilm activity of essential oils and plant extracts against Staphylococcus aureus and Escherichia coli biofilms. Food Control,* 61: 156-164.

Biswas, K., Chattopadhyay, I., Banerjee R.K. and Bandyopadhyay, U. (2002). Biological activities and medicinal properties of neem (*Azadirachta indica*). *International Journal of Current science*, 82(11): 1336-1345.

Bjerkan, G., Witsø, E. and Bergh, K. (2009). Sonication is superior to scraping for retrieval of bacteria in biofilm on titanium and steel surfaces in vitro. *Acta Orthopaedica*, 80 (2): 245-250.

Blazheyevskiy, M .and Koval'ska, O. (2017). A novel colorimetric biosensor for determination of cationic surfactants. *Physics Mathematics and Chemistry*,1-7.

Boisver, A., Cheng, M.P., Sheppard, D.C. and Nguyen, D. (2016). Microbial Biofilms in Pulmonary and Critical Care Diseases, *Annals of American Thoracic Society,* 13(9): 1615-1623.

Bonaventura G.D., Piccolomini, R., Paludi, D., Orio, V.D., Vergara, A., Conter, M. and Lanieri, A. (2007). Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food contact surfaces: relationship with motility and cell surface hydrophobicity. *Journal of Applied Microbiology,* 104(6):1552-1561.

Branda, S. S., S. Vik, L. Friedman, and R. Kolter. (2005). Biofilms: the matrix revisited. *Trends in Microbiology,* 13(1): 20-26.

Bridier, R. Briandet, V. Thomas & F. Dubois-Brissonnet (2011). Resistance of bacterial biofilms to disinfectants: a review, *Biofouling,* 27(9): 1017-1032.

Brockhurst, M.A., Buckling, A. and Gardner, A. (2007). Cooperation peaks at intermediate disturbance. *Current Biology,* 17: 761-765.

Brown, J.C.S. (2018). The characterisation of *Clostridium perfringens* from human and animal origins with varied health status. PhD University of Lincoln (unpublished).

Bryant, A.E, Chen, R.Y, Nagata, Y. (2000). Clostridial gas gangrene. Phospholipase C-induced activation of platelet gpIIbIIIa mediates vascular occlusion and myonecrosis in *Clostridium perfringens* gas gangrene. *Journal of Infectious Diseases* 182 (3):808–815.

Bryers, J.D. (2008). Medical Biofilms. *Biotechnology and Bioeng*ineering, 100(1): 1–18.

Burmolle, M., Ren, D., Bjarnsholt, T., and Sorensen S.J. (2014). Interaction in multispecies biofilms: do they actually matter? *Trends in Microbiology* 22(2)84-91.

Burmolle, M., Thomsen, T.R., Fazli, M., Dige, I., Christensen, L., Homoe, P., Tvede, M., Nyvad, B., Tolker-Nielsen, T. Givskov, M., Moser, C., Kirketerp-Moller, K., Johansen, H. K., Hoiby, N., Jensen, P. O., Sorensen, S.J., and Bjarnsholt, T. (2010). Biofilms in chronic infections - a matter of opportunity - monospecies biofilm in multispecies infections. *Immunology and Medical Microbiology,* 59:324-336.

Butt, A. and Khan, A. (2015). Antibiotics Resistance of bacterial Biofilms. *Middle East Journal of Business* 10(4) 38-45.

Caiazza, N.C. and O'Toole, G.A. (2004). SadB Is Required for the Transition from Reversible to Irreversible Attachment during Biofilm Formation by *Pseudomonas aeruginosa* PA14. *Journal of Bacteriology*, 186(14) 4476–4485.

Castillo-Martínez, J. C., Martínez-Castañón, G. A., Martínez-Gutierrez, F., Zavala-Alonso, N.V., Patiño-Marín, N., Niño-Martinez, N., Zaragoza-Magaña, V. and Cabral-Romero, C. (2015). Antibacterial and Antibiofilm Activities of the Photothermal

Therapy Using Gold Nanorods against Seven Different Bacterial Strains. *Journal of Nanomaterials* 1-7.

Centers for Disease control and prevention (2016). Antibiotic use and resistance. *Available from: antibiotic use.org/antibiotic-resistance faqs.html*

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D. and Buret, A. (1999). The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology* 37, 1771-1776.

Chan, G., Guthrie, A., Sivaramalingam, T., Wilson, J., Vancraeynest, D., Moody, R. and Steven Clark, S. (2015). A framework for assessing the efficacy of antimicrobials in the control of necrotic enteritis in broiler chickens. *Journal of Applied Poultry Research*, 24:246–256.

Chandki, R. banthia P. and Banthia R. (2011). Biofilms: A microbial home. *Journal of Indian Society of Periondontology* 15(2) 111-114.

Charlebois, A., Jacques, M. and Archambault M. (2017). Comparative transcriptomic analysis *of Clostridium perfringens* biofilms and Planktonic cells. *Avian pathology,* 45(5) 593–601.

Charlebois, A., Jacques, M. and Archambault M. (2017). Tolerance of *Clostridium perfringens* biofilms to disinfectants commonly used in the food industry*. Food Microbiology,* 62*,* 32-38.

Charlebois, A., Jacques, M. and Archambault, M. (2014). Biofilm formation of *Clostridium perfringens* and its exposure to low- dose antimicrobials. *Frontiers in Microbiology*, 5(183)1-11.

Chauret, C. P., (2014). *Encyclopedia of Food Microbiology*, 2nd edition. Indiana University Kokomo, Kokomo, IN, USA: 360-364.

Chen, M., Yu, Q. and Sun, H. (2013). Novel strategies for the prevention and treatment of biofilm related infections. *International Journal of Molecular Sciences, 14*, 18488-18501.

Chen, F., Gao, Y., Chen, X., Zhimin, Y. and Li, X. (2013). Quorum quenching enzymes and their application in degrading signal molecules to block quorum sensing-dependent infection. *International Journal of Molecular Science, 14*, 17477-17500.

Chen, Q., Xie, S., Lou, X., Cheng, S., Liu, X., Zheng, W., Zheng, Z., Wang, H. (2019). Biofilm formation and prevalence of adhesion genes among *Staphylococcus aureus* isolates from different food sources. *Microbiology*, 1-11.

Chhibber, S., Gondil, V.S., Sharma, S., Kumar, M., Wangoo, N. and Sharma, R.K. (2017). A novel approach for combating *Klebsiella pneumoniae* biofilm using Histidine functionalized silver nanoparticles. *Frontiers in Microbiology,* 8(1104)1-10.

Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Melton, D.M. And Beachey, E.H. (1985). Adherence of Coagulase-Negative Staphylococci to Plastic Tissue Culture Plates: A Quantitative Model for the Adherence of Staphylococci to Medical Devices. *Journal of Clinical Microbiology*, 22(6) 996-1006.

Clement, E., Erharuyi, O., Vincent, I., Joy, A., Christopher, A., Anthony, A., Udu-Cosi, O., Theophilus, O., James, I., and Abiodun, F. (2014). Significance of bitter leaf (*Vernonia amagdalina*). In Tropical Diseases and Beyond: A Review. *Malaria Chemotherapy, Control and Elimination*, 3(1)1-10.

Cochran, W.L., McFeters, G.A. and Stewart, P.S. (2000). Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. *Journal of Applied Microbiology*, 88, 22–30.

Coenye, T., Brackman, G., Rigole, P., DeWitte, E., Honraet, K., Rossel, B., Nelis, H.J. (2012). Eradication of *Propionibacterium acnes* biofilms by plant extracts and putative identification of icariin, resveratrol and salidroside as active compounds. *Phytomedicine* 19:409–12.

Coenye, T., De Prijck, K., De Wever, B. and Nelis, H.J. (2008). Use of the modified Robbins device to study the *in vitro* biofilm removal efficacy of NitrAdine TM, a novel disinfecting formula for the maintenance of oral medical devices, *Journal of Applied Microbiology,* 105, 733–740.

Cornelissen, A., Ceyssens, P.J., T'Syen, J., Van Praet, H., Noben, J.P., Olga, V., Shaburova, O.V., Krylov, V.N., Guido Volckaert, G., and Lavigne, R. (2011). The T7-related *Pseudomonas putida* Phage Q15 Displays Virion-Associated biofilm degradation properties. *PLoS ONE* 6(4):1-11.

Costerton, J. W, Stewart, P. S., Greenberg, E. P. (1999). Bacterial biofilms: A common cause of persistent infections. *Science,* 284 (5418): 1318–1322.

Cotton, C., Baron, M., and Dixon, R.A. (2012). Silver nanoparticles: Antibacterial potential and applications. *Interscience Conference on Antimicrobial Agents and Chemotherapy* (ICCAC), San Francisco.

Coventry, E., and Allan, E.J. (2001). Microbiological and chemical Analysis of Neem (*Azadarachta indica*) extracts: New Data on antimicrobial activity *Phytoparasitica,* 29(5) 441-450.

Craven, S.E. (2000). Colonization of the intestinal tract by *Clostridium perfringens* and fecal shedding in diet-stressed and unstressed broiler chickens. *Poultry Science*, 79: 843-849.

Dahiya, J.P., Wilkie, D.C., Van Kessel, A.G. and Drew, M.D. (2006). Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. *Animal Feed Science and Technology,* 129: 60–88.

Davey M. E. and O'Toole G. A. (2000). Microbial Biofilms: From ecology to molecular genetics. *Microbiology and Molecular Biology reviews*. 64(4) 847–867.

Dawson, L. F, Valiente, E., Faulds-Pain, A., Donahue, E. H., Wren, B. W. (2012). Characterisation of *Clostridium difficile* Biofilm Formation, a Role for Spo0A. *PLOS ONE* 7(12).

Dean, S., Bishop, B., van Hoek, M. (2011). Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against *Staphylococcus aureus*. *BMC Microbiology, 11:114.*

De Silva, G.O., Abeysundara, A. T. and Aponso, M.M.W. (2017). Extraction methods, qualitative and quantitative techniques for screening of phytochemicals from plants. *American Journal of essential oils and natural products*, 5(2): 29-32.

Dickey, J., Perrot, V. (2019). Adjunct phage treatment enhances the effectiveness of low antibiotic concentration against *Staphylococcus aureus* biofilms *in vitro*. *PLOS ONE* 14(1).

Darouiche, R.O. (2004). Treatment of Infections Associated with Surgical Implants. *The New England Journal of Medicine* 350: 1422-1429.

Diarra, M.S., Malouin, F. (2014). Antibiotics in Canadian poultry productions and anticipated alternatives. *Frontiers in Microbiology*, 5:282

Dixon, R.A., Gillett, A., Cotton, C. and Baron, M. (2012). Activity of silver nanoparticles against MRSA tested in a *Galleria mellonella* model of infection. *International Conference on Antimicrobial Research*, Lisbon, Portugal.

Donelli, G., Voutto, C., Cardines, R. and Mastrantonio, P. (2012). Biofilm growing intestinal anaerobic bacteria. *Immunology and Medical Microbiology*, 65(2): 318-325.

Donlan, R.M. (2001). Biofilms and device-associated infections. *Emerging Infectious Diseases*, *7*, 277–281.

Donlan, R.M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, 8(9):881-890.

Donlan, R.M. and Costerton, W. (2002). Biofilms: Survival Mechanisms of clinically relevant Microorgaisms. *Clinical Microbiology Reviews,* 15(2): 167-193.

Dragoš, A. and Kovács, A.T. (2017). The peculiar functions of the bacterial extracellular matrix. *Trends in Microbiology*, 25(4):257-266.

Drew, M.D., Syed, N.A., Goldade, B.G., Laarveld, B. and Van Kessel, A.G. (2004). Effects of dietary protein source and level on intestinal populations of *Clostridium perfringens* in broiler chickens. *Poultry Science*, *83*: 414-420.

Dubeya, N.K., Tiwaria, T.N., Mandinb, D., Andriamboavonjyc, H., Chaumont, J. (2000). Antifungal properties of *Ocimum gratissimum* essential oil (ethyl cinnamate Chemotype). *Fitoterapia* 71: 567 -569.

Dunne, W.M. (2002). Bacterial Adhesion: Seen any good biofilm lately? *Clinical Microbiology Review,* 15(2): 155-166.

Eales, M.G., Ferrari, E., Goddard, A.D., Lancaster, L., Sanderson, P., Miller, C. (2018). Mechanistic and phenotypic studies of bicarinalin BP100 and colistin action on *Acenetobacter baumannii*. *Research in Microbiology*, 169: 296 -302.

Eckhart, L., Fischer, H., Barken, K.B., Tolker-Nielsen, T. and Tschachler, E. (2007). DNase1L2 suppresses biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *British Journal of Dermatology,* 156: 1342–1345.

Economou, N. J., Cocklin, S. and Loll, P. J. (2013). High-resolution crystal structure reveals molecular details of target recognition by bacitracin. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(35): 14207–14212.

El-Gohary, A.F., Abdel-Hafez, L.J.M., Zakaria, A.I., Shata, R.R., Tohoun, A., El-mleeh, A., Elfadl, E.A.A. and El- Mahallawy, E.K. (2020). Enhanced antibacterial activity of silver nanoparticles combined with hydrogen peroxide against multipledrug-resistant pathogens isolated from dairy farms and beef slaughterhouses in Eygpt. *Infection and Drug Resistance*, 13:3485-3499.

Eng, R.H.K., Padberg, F.T., Smith, S.M., Tan, E.N. and Cherubin, C. (1991). Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrobial agents and Chemotherapy*, 35(9):1824-1828.

Engberg, R.M., Hedemann, M. S., and Jensen, B.B. (2002). The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. *British Poultry Science*, *43*: 569-579.

European society of clinical microbiology and infectious diseases (ESCMID) (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection*, 9(8):9-15.

Evbuomwan, l, Chukwuka, E.P., Obazenu, E.I., Ilevbare, l. (2018). Antibacterial activity of *Vernonia amygdalina* leaf extracts against multidrug resistant bacterial isolates. *Journal of Applied Science and Environmental Management,* 22 (1): 17-21.

Ezeonu, C.S., and Ejikeme, C. M. (2016). Qualitative and quantitative determination of phytochemicalcContents of indigenous Nigerian softwoods. *New Journal of Science*, 1-9.

Famuyide, I. M., Aro, A. O., Fasina, F. O., Eloff, J. N. and McGaw, L. J. (2019). Antibacterial and antibiofilm activity of acetone leaf extracts of nine underinvestigated south African Eugenia and Syzygium (Myrtaceae) species and their selectivity indices. *BMC Complementary and Alternative Medicine*, 19:141.

Faraz, N., Islam, Z.U., Rehman, R., Sehrish. (2012). Antibiofilm forming activity of naturally occurring compound*. Biomedical,* 28:171–5.

Farombi, E.O. and Owoeye, O. (2011). Antioxidative and Chemopreventive properties of *Vernonia amygdalina* and *Garcinia biflavonoid. International Journal of Environmental Research and Public Health 8*, 2533-2555.

Fernandez, L., Gonzalez, S., Campelo, A., Martinez, B., Rodriguez, A. and Pilar Garcia, P. (2017). Low-Level predation by lytic phage Phiipla-RODI promotes biofilm

formation and triggers the stringent response in *Staphylococcus aureus. Nature,* 7:40965.

Ferreira, M.A., Moreira, G.S.G., Da Cunha Marcelo Mendonça, C.P., Salvarani, F.M., Moreira, A.N. and Conceição, F.R. (2016). Recombinant Alpha, Beta, and Epsilon Toxins of *Clostridium perfringens*: Production Strategies and applications as veterinary vaccines. *Toxins*, 8(11): 340-364.

Flemming, H., Neu, T.R. and Wozniak, D.J. (2007). The EPS Matrix: The "House of Biofilm Cells". *Journal of Bacteriology*, 189(22): 7945–7947.

Fong, J.N.C. and Yildiz, F.H. (2015). Biofilm matrix protein. *Microbiology Spectrum,* 3(2):1-27.

Foo, R.Q., Manogaran, E. and Gabriel, A.A. (2014). Antimicrobial and antioxidant studies of *Vernonia amygdalina. Journal of Applied Pharmacology* 6(4): 360-371.

Franci, G., Falanga, A., Galdiero, S., Palomba, L., Rai, M., Morelli, G. and Galdiero, M. (2015). Silver nanoparticles as potential antibacterial agents. *Molecules, 20*: 8856-8874.

Freeman, D.J., Falkiner, F.R., Keane, C.T. (1989). New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology 42:872-874.*

Fuqua, W.C., Winans, S. C., Greenberg, E.P. (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology,* 176:269-275.

Fux, C.A., Costerton, J.W., Stewart, P.S., Stoodley, P. (2005). Survival strategies of infectious biofilms. *Trends in Microbiology, 13*: 34-40.

García, S. and Heredia, N. (2011). *Clostridium perfringens*: A dynamic foodborne pathogen*. Food and Bioprocess Technology,* 4(4) 624-630.

Gerba, C.P. (2015). Quaternary ammonium biocides: efficacy in application. Applied *Environmental Microbioiogy*, 81: 464-469.

Ghamba, P.E., Balla, H., Goje, L.J., Halidu, A. and Dauda, M.D. (2014). *In vitro* antimicrobial activities of *Vernonia amygdalina* on selected clinical isolates. *International Journal of Current Microbiology and Applied Sciences,* 3(4): 1103-1113.

Ghasemian, A., Mobarez, A.M., Peerayeh, S.N. and Bezmin Abadi, A.T. (2018). The association of surface adhesin genes and the biofilm formation among *Klebsiella oxytoca* clinical isolates. *New Microbes and New Infections,* 27:36-39.

Gholamiandehkordi, A.R., Timbermont, L., Lanckriet, A., Van den Broeck, W., Pedersen, K., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., Van Immerseel, F. (2007). Quantification of gut lesions in a subclinical necrotic enteritis model. *Avian Pathology*, 36: 375- 382.

Ghotaslou, R., Bahari, Z., Aliloo, A., Gholizadeh, P., Eshlaghi, S. B. (2017). The in vitro effects of silver nanoparticles on bacterial biofilms. *Journal of Microbiology, Biotechnology and Food Science,* 6(4): 1077-1080.

Gibbons, S. (2005). Plants as source of bacterial resistance modulators and anti-infective agents. *Photochemistry Reviews,* 4:63-74.

Gislene, G.F., Juliana, L., Paulo, C.F. and Giuliana, L.S. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. *Brazilian Journal of Microbiology,* 31:247-256.

Gjermansen, M., Ragas, P., Sternberg, C., Molin, S. and Tolker-Nielsen, T. (2005). Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environmental Microbiology,* 7(6), 894-904.

Goeres, D.M, Loetterle, L.R., Hamilton, M.A., Murga, R., Kirby, D.W. and Donlan, R.M. (2005). Statistical assessment of a laboratory method for growing biofilms. *Microbiology,* 151(3):757-62

Gopinath, K., Kumaraguru, S., Bhakyaraj, K., Mohan, S., Venkatesh, K. S., Esakkirajan, M., Kaleeswarran, P., Naiyf, S. A., Kadaikunnan, S., Govindarajan, M., Benelli, G., Arumugam, A., (2016). Green synthesis of silver, gold and silver/gold bimetallic nanoparticles using the Gloriosa superba leaf extract and their antibacterial and antibiofilm activities*. Microbial Pathogenesis,* 101: 1-11.

Gorkiewicz, G. (2009). Nosocomial and antibiotic-associated diarrhoea caused by organisms other than *Clostridium difficile. International Journal of Antimicrobial Agents,* 33(1): 37 -41.

Gui, L., Subramony C., Fratkin, J., Hughson, M.D. (2002). Fatal Enteritis Necroticans (Pigbel) in a Diabetic Adult*. Modern Pathology*, 15(1): 66-70.

Gul, R., Jan, S. U., Faridullah, S., Sherani, S. and Jahan, N. (2017). Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extracts from *Ephedra intermedia* indigenous to Balochistan. *The Scientific World Journal*, 1-7.

Gurunathan, S., Han, J.W., Kwon, D. and Kim. J. (2014). Enhanced antibacterial and antibiofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. *Nanoscale Research Letters,* 9(373):1-17.

Gutierrez, D., Rodriguez-Rubio, L., Martinez, B., Rodrigez, A. and Garcia, P. (2016). Bacteriophages as weapons against bacterial biofilms in the food industry. *Frontiers in Microbiology,* 7:825.

Guzman, M., Jean, D., and Stephne, G. (2012). "Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria. *Nanomedicine*: Nanotechnology, *Biology and Medicine,* 8: 37-45.

Gutiérrez, D., Rodríguez-Rubio, L., Martínez, B., Rodríguez, A. and García, P. (2016). Bacteriophages as weapons against bacterial biofilms in the food Industry. *Frontiers in Microbiology*, 7:8.

Hall, C.W and Mah, T. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Review*, 41(3): 276–301.

Hall-Stoodley, L., and Stoodley P. (2005). Biofilm formation and dispersal and the transmission of human pathogens. *Trends in Microbiology,* 13(1) 7–10.

Han, C., Romero, N., Fischer, S., Dookran, J., Berger, A. and Doiron, A. L. (2017). Recent developments in the use of nanoparticles for treatment of biofilms *Nanotechnology Reviews,* 6(5): 383–404

Harper, D.R., Parracho, H.R.T., Walker, J., Sharp, R., Hughes, G., Werthén, M., Lehman, S. and Sandra Morales, S. (2014). Bacteriophages and Biofilms. *Antibiotics, 3*, 270-284

Harrison, J.J., Turner, R.J. and Ceri, H. (2005). Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa. Environmental Microbiology*, 7(7): 981–994.

Hatheway, C. L. (1990). Toxigenic clostridia. *Clinical Microbiology* Revision, 3:66-98.

Heilmann, S., Sneppen, K., Krishna, S. (2012). Coexistence of phage and bacteria on the boundary of self-organized refuges. *Proceedings of The National Academy of Science USA*, 109:12828-12833.

Hendrickx, L., Hausner, M., Wuertz, S. (2003). Natural genetic transformation in monoculture Acinetobacter sp. strain BD413 biofilms. *Applied Environmental Microbiology,* 69:1721–7.

Henriksen, K., Rørbo, N., Rybtke, M. L., Martinet, M.G., Tolker-Nielsen, T., Høiby, N., Middelboe, M. and Ciofu, O. (2019). *P. aeruginosa* flow-cell biofilms are enhanced by repeated phage treatments but can be eradicated by phage–ciprofloxacin combination. *Pathogens and Disease*, 77 (2): 1-16

Hentzer, M., Wu, H., Andersen, J.B., Rieldel, K., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z., Kritoffersen, P., Manefield, M., Eberl, L., Steinberg, P., Kjellberg, S., Hoiby, N., and Givskov, M. (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *The EMBO Journal* 22 (15): 3803-3815.

Herten, M., Bisdas T., Knaack D., Becker, K., Osada, N., Torsello, G.B. and Idelevich, E. A (2017). Rapid *in vitro* quantification of *S. aureus* biofilms on vascular graft surfaces. *Frontiers in Microbiology*, 8:2333 1-8

Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang, Z., Jones, R.A., Miller, S.I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature,* 436: 1171-1175.

Høiby, N., Bjarnsholt, T., Moser, C., Bassi, G. L., Coenye, T., Donelli, G., and Lebeaux, D. (2015). ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. *Clinical Microbiology and Infection*, *21*, 1-25.

Hong, X., Wen, J., Xiong, X., Hu, Y. (2016). Shape effect on the antibacterial activity of silver nanoparticles synthesized via a microwave-assisted method. *Environmental Science and Pollution Research,* 23 (5) 4489-4497.

Hosseinidousta, Z., Tufenkjia, N. Zeinab H, Nathalie, T. and Theo G.M. (2013). Formation of biofilms under phage predation: Considerations concerning a biofilm increase. *Biofouling*, 29:4, 457-468.

Hoyle, B.D, Jass, J, Costerton, J.W. (1990). The biofilm glycocalyx as a resistance factor. *Journal of Antimicrobial Chemotherapy,* 26(1): 1-5.

Hu, L., and Menco B. I.M. (2017). The Use of nanoparticles to prevent and eliminate bacterial biofilms. *Antimicrobial research,* 344-350.

Huang, I.H. (2007). *Clostridium perfringens* Sporulation and Gliding Motility.PhD Thesis (unpublished).

Huh, A.J., Kwon, Y.J. (2011). "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotic resistant era. *Journal of Controlled Release,* 156:128–45.

Hunter, S.E.C., Brown, J.E., Oyston, P.C.F., Sakurai, J., Titball, R.W. (1993). Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence

homology with alpha-toxin, gamma-toxin and leukocidin of *Staphylococcus aureus*. *Infection and Immunity, 61*:3958–3965.

Hussain M., Oh D. H. (2018). Impact of the Isolation source on the biofilm formation characteristics of *Bacillus cereus. Journal of Microbiology and Biotechnology,* 28(1): 77-86.

Hussain, S.M, Hess, K.L, Gearhart, J.M, Geiss, K.T, Schlager, J.J. (2005). *In vitro* toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicology in vitro* 19: 975–983.

Ijeh, I.I., Omodamiro, O.D. and Nwanna, I.J (2005). Antimicrobial effects of aqueous and ethanolic fractions of two spices, *Ocimum gratissimum* and *Xylopia aethiopica. African Journal of Biotechnology,* 4 (9): 953-956.

Ikonomidis, A., Tsakris A, Kanellopoulou M., Maniatis, A.N. and Pournaras, S. (2008). Effect of the proton motive force inhibitor carbonyl cyanide-m-chlorophenylhydrazone (CCCP) on *Pseudomonas aeruginosa* biofilm development. *Letter of Applied Microbiology*, 47: 298–302.

Iñiguez-Moreno, M., Avila-Novoa, M. G., Iñiguez-Moreno, E., Javier Guerrero-Medina, P., Gutiérrez-Lomelí, M. (2017). Antimicrobial activity of disinfectants commonly used in the food industry in Mexico. *Journal of Global Antimicrobial Resistance*, 10:143 - 147.

Irie, Y. and Parsek, M.R. (2008). Quorum sensing and microbial biofilms bacterial biofilms*. Current Topics in Microbiology and Immunology,* 322: 67-84*.*

Izano E. A, Wang H, Ragunath C, Ramasubbu N, Kaplan J.B. (2007). Detachment and killing of *Aggregatibacter actinomycetemcomitans* biofilms by dispersin B and SDS. *Journal of Dental Research,* 86:618–622.

Jamal, M., Tasneem, U., Hussain T., and Andleeb S., (2015). Bacterial biofilm: Its composition, formation, and role in human infections. *Journal of Microbiology and Biotechnology*, 4(3) 1-14.

Jamal, M., Wisal A., Saadia A., Fazal J., Muhammad I., Muhammad, A.N., Tahir H., Muhammad A., Muhammad R., and Muhammad A. K. (2018). Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association,* 81: 7 – 11.

Jefferson, K. K. (2004). What drives bacteria to produce a biofilm? *FEMS Microbiology Letters*, 236 (2)163–173.

Jensen, L.K., Johansen, A.S.B. and Jensen, H.E. (2017). Porcine Models of Biofilm Infections with Focus on Pathomorphology. *Frontiers in Microbiology*, 8 (1961) 1-16.

Jesaitis A. J, Franklin M. J, Berglund D., Sasaki M., Lord C. I, Bleazard J. B, Duffy J. E, Beyenal H., Lewandowski, Z. (2003). Compromised host defense on *Pseudomonas aeruginosa* biofilms: characterization of neutrophil and biofilm interactions. *Journal of Immunology,* 171(8):4329-39.

Jia, W., Slominski, B.A., Bruce, H.L., Blank, G., Crow, G. and Jones, O. (2009). Effect of diet type and enzyme addition on growth-performance and gut health of broiler chickens during subclinical *Clostridium perfringens* challenge. *Poultry Science*, *88*, 132-140.

Jiang, J., Oberdörster, G., Biswas, P. (2009a) Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *Journal of Nanoparticle Research,* 11:77–89.

Jiao, Y., Cody, G.D., Harding, A. K., Wilmes, P., Schrenk, M., Wheeler, K.E. Banfield, J.F. and Thelen, M. P. (2010). Characterization of extracellular polymeric substances

from acidophilic microbial biofilms. *Applied and Environmental Microbiology,* 76(9). 2916–2922.

Jorgensen, J. H. and Ferraro, M. (2009). Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Journal of Medical Microbiology,* 49, 1749-1755.

Kadiri, O. and Olawoye, B. (2016). *Vernonia amygdalina*: An underutilized vegetable with nutraceutical potentials – A Review Turkish. *Journal of Agriculture - Food Science and Technology*, 4(9): 763-768.

Kaldhusdal, M. and Skjerve, E. (1996). Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway. *Preventive Veterinary Medicine*, *28*, 1-16.

Karaman, D.S., Manner, S., Fallarero, A., and Rosenholm, J.M. (2017). Current Approaches for Exploration of Nanoparticles as Antibacterial Agents. *Web of Science,* 61-86.

Keyburn, A.L., Bannam, T.L., Moore, R. J., and Rood, J.L. (2010). NetB, a pore-forming toxin from necrotic enteritis strains of *Clostridium perfringens. Toxins*, *2*, 1913-1927.

Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E. (2008). Netb, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. *Plos Pathogens,* 4(2):1-11.

Khan, S. N., and Khan, A.U. (2016). Breaking the Spell: Combating Multidrug Resistant ‘Superbugs’. *Frontiers in Microbiology,* 7(174): 1-11.

Khoramian, B., Jabalameli, F., Niasari-Naslaji, A., Taherikalani, M., and Emaneini, M. (2015). Comparison of virulence factors and biofilm formation among Staphylococcus aureus strains isolated from human and bovine infections. Microbial Pathogenesis, 88: 73-77.

Kim, J.S., Kuk, E., Yu, K., Kim, J.H., Park, S.J., Lee, H.J., Kim, S.H., Park, Y.K., Park, Y.H., Hwang, C.Y., Kim, Y.K., Lee, Y.S., Jeong, D.H., Cho, M.H. (2007). Antimicrobial effects of silver nanoparticles. *Nanomedicine,* 3: 95-101.

Kim, S. G., Jun, J. W., Giri, S.S., Yun, S., Kim, H. J., Kim, S. W., Kang, J.W., Han, S.J., Jeong, D. and Park, S.C. (2019). Isolation and characterisation of pVa-21, a giant bacteriophage with anti-biofilm potential against *Vibrio alginolyticus. Nature, 9:6284.*

Kim, S. and Yoo, K. (2008). Antibiotic-Associated Diarrhea: Candidate Organisms other than *Clostridium Difficile*. *The Korean Journal of Internal Medicine*, 23:9-15.

Kircanski, J. (2012). *Clostridium perfringens* and the beta2 (CPB2) toxin: Development of a diagnostic ELISA for neonatal piglet enteritis, and distribution of the gene in isolates from selected animal species. MSc. University of Guelph.

Kirketerp-Moller K, Jensen P.O, Fazli M., Madsen, K.G., Pedersen, J., Moser, C., Tolker-Nielsen, T., Hoiby, N., Givskov, M. and Bjarnsholt, T. (2008). Distribution, organization, and ecology of Bacteria in chronic wounds. *Journal of Clinical Microbiology,* 46: 2717-2722.

Kiu, R., Brown, J., Bedwell, H., Leclaire, C., Caim, S., Pickard, D., Dougan, G., Dixon, R.A. and Hall, L.J. (21019). Genomic analysis on broiler-associated *Clostridium*

perfringens strains and exploratory caecal microbiome investigation reveals key factors linked to poultry necrotic enteritis. *Animal Microbiome*, 1(12):1-14.

Knarreborg, A., Simon, M.A., Engberg, R.M., Jensen, B.B. and Tannock, G.W. (2002). Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Applied and Environmental Microbiology*, *68*: 5918-5924.

Kobayashi, H., Tuohy, M.J. and Bauer, T. W. (2009). Improved detection of biofilm-formative bacteria by vortexing and sonication. *Clinical Orthopaedics and Related Research*, 467:1360–1364.

Koczan, J.M., Lenneman, B.R., McGrath, M.J. and Sundin, G.W. (2011). Cell surface attachment structures contribute to biofilm formation and xylem colonization by *Erwinia amylovora. Applied and Environmental Microbiology*, 77(19): 7031-7039.

Kostakioti, M., Hadjifrangiskou, M., and Hultgren, S. J. (2013). Bacterial Biofilms: Development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harbour Perspectives in Medicine,* 3(4):1-22.

Kyd, J.M., Krishnamurthy, A. and Kidd, S. (2016). Interactions and mechanisms of respiratory tract biofilms involving *Streptococcus pneuniae* and non-typeable *Haemophilus influenzae*. INTECH http://dx.doi.org/10.5772/63500

Labbate, M., Queck, S.Y., Koh, K.S., Rice, S.A., Givskov, M. and Kjelleberg, S. (2004). Quorum sensing – controlled biofilm development in *Serratia Liquefaciens* MG1.*Journal of Bacteriology,* 186: 692-698.

Lacqua, A., Wanner, O., Colangelo, T., Martinotti, M.G., Landini, P. (2006). Emergence of biofilm-forming subpopulations upon exposure of *Escherichia coli* to environmental bacteriophages. *Applied Environmental Microbiology,* 72:956–959.

Laird, K., Armitage, D. and Phillips, C. (2012). Reduction of surface contamination and biofilms of Enterococcus sp. and Staphylococcus aureus using a citrus-based vapour. *Journal of Hospital Infections,* 80:61-66.

Lasaro, M.A., Salinger, N., Zhang, J., Wang, Y., Zhong, Z., Goulian, M. and Zhu, J. (2009). F1C fimbriae play an important role in biofilm formation and intestinal colonization by the *Escherichia coli* commensal strain Nissle. *Applied and Environmental Microbiology*, 75(1): 246–251.

Lee, H.Y., Chai, L.C., Pui, C.F., Mustafa, S., Cheah, Y.K., Nishibuchi, M., Radu, S. (2013). Formation of biofilm by *Listeria monocytogenes* ATCC 19112 at different incubation temperature and concentrations of sodium chloride. *Brazilian Journal of Microbiology*, 44(1) 51-55.

Lebeaux, D., Chauhan, A., Rendueles, O., Beloin, C. (2013). From *in vitro* to *in vivo* models of bacterial biofilm-related infections. *Pathogens,* 2:288-356.

Lee, J.H., Cho, H.S., Joo, S.W., Regmi, C.S., Kim, J.A., Ryu, S.H., Yong, R.C., Cho, M.H., Lee, J. (2013). Diverse plant extracts and trans resveratrol inhibit biofilm formation and swarming of *Escherichia coli* O157:H7. *Biofouling,* 29:1189–203.

Le, K.Y. and Otto, M. (2015). Quorum-sensing regulation in staphylococci-an overview. *Frontiers in Microbiology,* 6:1174.

Leid, J. G., Willson, C. J, Shirtliff, M. E, Hassett D.J, Parsek, M. R, Jeffers, A. K. (2005). Exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-gamma-mediated macrophage killing. *Journal of Immunology*, 175 (11):7512-8.

Leid, J. G., Costerton, J.W, Shirtliff, M. E, Gilmore, M. S, Engelbert, M. (2002). Immunology of Staphylococcal biofilm infections in the eye: new tools to study biofilm endophthalmitis. *DNA and Cell Biology.* 21(5-6):405-13.

Leiman P.G., Chipman, P. R., Kostyuchenko, V.A., Mesyanzhinov, VV., Rossmann, M.G. (2004). Three-dimensional rearrangement of proteins in the tail of bacteriophage T4 on infection of its host. *Cell,* 118: 419-429.

Lewis, K. and Spoering, A. L. (2001). Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *Journal of Bacteriology,* 183(23):1-6.

Lewis, K., (2005). Persister cells and the riddle of biofilm survival. *Biochemistry,* 70(2): 267-274.

Li J. and McClane B.A. (2006). Further Comparison of Temperature Effects on Growth and Survival of *Clostridium perfringens* Type A Isolates Carrying a Chromosomal or Plasmid-Borne Enterotoxin Gene. Applied and Environmental Microbiology 72(7):4561-4568.

Li, B., Qiu, Y., Zhang, J., Huang, X., Shi, H. andYin, H. (2018). Real – Time study of rapid spread of antibiotic resistance plasmid in biofilm using microfluidics. *Environmental Science and Technoligy,* 52:11132-11141

Li, Y., and Tian, X. (2012). Quorum Sensing and Bacterial Social Interactions in Biofilms. *Sensors,* 12:2519-2538.

Limoli, D.H., Jones, C.J. and Wozniak, D.J. (2015). Bacterial extracellular polysaccharides in biofilm formation and function. *Microbiology Spectrum*, 3(3):1-30.

Lin, P.W. and Stoll, B.J. (2006). Necrotising enterocolitis. *Lancet,* 368: 1271–83.

Lineback, C. B., Nkemngong, C. A., Wu, S. T., Li, X., Teska., P. J. and Oliver. H., F. (2018). Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms than quaternary ammonium compounds. *Antimicrobial Resistance and Infection Control*, 7:154.

Linton, C.J., Sherriff, A., and Millar, M.R. (1999). Use of a Modified Robbins Device to directly compare the adhesion of *Staphylococcus epidermidis* RP62A to surfaces. *Journal of Applied Microbiology,* 86, 194-202.

Little, B., Wagner, P., Ray, R. and Pop, R. (1991) Biofilms: An ESEM evaluation of artifacts introduced during SEM preparation. *Journal of Industrial Microbiology and Biotechnology,* 8(4):213-221.

Liu, C., Guo, J., Yan, X., Tang, Y., Mazumder, A., Wu, S., and Liang, Y. (2017). Antimicrobial nanomaterials against biofilms: an alternative strategy. *Environmental Reviews,* 25: 225–244.

Liu, Y., Miyoshi, H. & Nakamura, M. (2007). Nanomedicine for drug delivery and imaging: a promising avenue for cancer therapy and diagnosis using targeted functional nanoparticles. *International Journal of Cancer,* 120: 2527-2537.

Lovland, A., Kaldhusdal, M. (2001). Severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated hepatitis. *Avian Pathology,* 30(1): 73-81.

Lu, Z., Rong, K., Li, J., Yang, H. and Chen, R. (2013). "Size-dependent antibacterial activities of silver nanoparticles against oral anaerobic pathogenic bacteria. *Journal of Materials Science: Materials in medicine*, 24(6),1465-1471.

Lynch, M., J. Painter, R. Woodruff, and C. Braden (2006). Surveillance for foodborne-disease outbreaks--United States, 1998-2002. Morbility and Mortality Weekly Report Surveillance Summary, 55:1-42.

Madsen, J.S., Burmolle, M., Hansen, L.H., Sorenson, S.J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology and Medical Microbiology*, 65:183–95.

Mah, T. F, O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology,* 9(1):34-9.

Mahmoud M. B., Samar, M. S., Ahmed I. El-Batal, A., Hanora, M. (2017). Evaluation of silver nanoparticles as disinfectant in surgical operating rooms *Der Pharmacia Lettre,* 9(10): 1-23.

Makhluf, S., Dror, R., Nitzan, Y., Abramovich, Y., Jelinek, R., Gedanken, A. (2005). Microwave-assisted synthesis of nanocrystalline MgO and its use as a bacteriocide. *Advanced Functional Materials,* 15:1708-15.

Marrie, T.J. and Costerton, J. W. (1984). Morphology of bacterial attachment to cardiac pacemaker Leads and power packs. *Journal of Clinical Microbiology*, 19(6): 911-914.

Martinez, J.L.; Baquero, F. (2002). Interactions among strategies associated with bacterial infection: Pathogenicity, epidemicity, and antibiotic resistance. *Clinical Microbiology Review, 15*: 647–679.

Martinez-Gutierrez, F., Boegli, L., Agostinho, A., Elpidio Sánchez, E.M., Bach, H., Ruiz, F. and James, G. (2013). Anti-biofilm activity of silver nanoparticles against different microorganisms, *Biofouling*, 29 (6): 651-660.

Maszewska, A., Zygmunt, M., Grzejdziak, I., and Zalski, A.R. (2018). Use of polyvalent bacteriophages to combat biofilm of *Proteus mirabilis* causing catheter associated urinary tract infections. *Journal of Applied Microbiology,* 125: 1253-1265

Matsumura, Y., Yoshikata, K., Kunisaki, S., Tsuchido, T. (2003). Mode of bacterial action of silver zeolite and its comparison with that of silver nitrate. *Applied and Environmental Microbiology,* 69: 4278-4281.

Mcdevitt, R., Brooker, J., Acamovic, T. and Sparks, N.H.C. (2006). Necrotic enteritis; a continuing challenge for the poultry industry. *World Poultry Science Journal,* 62:221-248.

Mehdi, Y., Letourneau-Montminy, M., Gaucher, M., Chorfi, Y., Suresh, G., Rouissi, T., Kaur Brar, S., C^ot_e, C., Avalos Ramirez, A., Godbout, S. (2018). Use of antibiotics in broiler production: Global impacts and alternatives. *Animal Nutrition*, 4:170-178.

Melo, P.D., Ferreira, L.M., Filho, A.N., Zafalon, L.F., Vicente, H.I.G., De Souza, V. (2013). Comparison of methods for the detection of biofilm formation by

Staphylococcus aureus isolated from bovine subclinical mastitis. *Brazilian Journal of Microbiology,* 44(1):119-124.

Meng, X., Shi, Y., Ji, W., Meng, X., Zhang, J., Wang, H., Lu, C., Sun, J. and Yan, Y. (2011). Application of a Bacteriophage Lysin to disrupt biofilms formed by the Animal Pathogen *Streptococcus suis. Applied and Environmental Microbiology*, 77(23): 8272-8279.

Merritt, J.H., Kadouri, D.E. and O'Toole, G.A. (2005). Growing and analyzing static biofilms. *Current Protocols in Microbiol*ogy, 1: 1-17.

Meyer B, Cookson B. (2010). Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control? *Journal of Hospital Infection*, 76:200 –205

Miyake, Y., Fujiwara, S., Usui, T., Suginaka, H. (1992). Simple method for measuring the antibiotic concentration required to kill adherent bacteria. *Chemotherap*y, 38(5): 286-90.

Mizunaga, S., Kamiyama, T., Fukuda, Y., Takahata, M. & Mitsuyama, J. (2005). Influence of inoculum size of *Staphylococcus aureus* and *Pseudomonas aeruginosa* on in vitro activities and in vivo efficacy of fluoroquinolones and carbapenems. *Journal of Antimicrobial Chemotherapy,* 56: 91-96.

Modi, N., and Wilcox, M.H. (2001). Evidence for antibiotic induced *Clostridium perfringens* diarrhoea. *Journal of Clinical Pathology,* 54:748–751.

Molin, S. and Tolker-Nielsen, T. (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Current Opinion in Biotechnology,* 14(3): 255-261.

Moore, L.E., Ledder, R.G., Gilbert. P., McBain, A.J. (2008). *In vitro* study of the effect of cationic biocides on bacterial population dynamics and susceptibility. *Applied Environmental Microbiology,* 74:4825–4834.

M'Sadeq, S.A., Wu, S., Swick, R. A. and Choct, M. (2015). Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. *Animal nutrition,* 1(1) 1-11.

Mu, H., Guo, F., Niu, H., Liu, Q., Wang, S. and Duan, J. (2014). Chitosan improves anti-biofilm efficacy of gentamicin through facilitating antibiotic penetration. *International Journal of Molecular Science*, 15: 22296-22308.

Mu, H., Zhang, A., Zhang, L., Niu, H. and Duan J. (2014). Inhibitory effects of chitosan in combination with antibiotics on *Listeria monocytogenes* biofilm. *Food Control,* 38: 215-220.

Murray, J., Muruko, T., Gill, C. I. R., Kearney, M. P., Farren, D., Scott, M. G., McMullan, G. and Ternan, N. G. (2017). Evaluation of bactericidal and anti-biofilm properties of a novel surface-active organosilane biocide against healthcare associated pathogens and Pseudomonas aeruginosa biolfilm. PLoS ONE, 12(8):1-13.

Murugan, S., Devi, P. U. and John, P. N. (2011). Antimicrobial susceptibility pattern of biofilm producing *Escherichia coli* of urinary tract infections. *Current Research in Bacteriology,* 4(2): 73-80.

Nadell, C.D., Drescher, K. and Kevin R. (2016) Foster Spatial structure, cooperation, and competition in biofilms. *Nature Reviews*, 14: 589-600.

Nair, S., Desai, S., Poonacha, N., Vipra, A., Sharma, U. (2016). Antibiofilm activity and synergistic inhibition of *Staphylococcus aureus* biofilms by bactericidal protein P128 in combination with antibiotics. *Antimicrobial Agents Chemotherapy,* 60:7280 –7289.

Natarajan, V., Venugopal, P.V., Menon, T. (2003). Effect of *Azadirachta indica* (neem) on the growth pattern of dermatophytes. *Indian Journal of Medical Microbiologists,* 21(2): 98-101.

Neu, J., and Walker, W.A., (2011) Necrotizing enterocolitis. New *England Journal of Medicine,* 364(3): 255–264.

Njoku, O. U., Joshua, P. E., Agu, C. V., and DIM, N. C. (2011). Antioxidant properties of *Ocimum gratissimum* (Scent Leaf). *New York Science Journal*, 4(5):98-103.

Nogueira, T., Rankin, D. J., Touchon, M., Tadde, F., Brown, S. P., and Rocha, E.P.C. (2009). Horizontal gene transfer of the secretome drives the evolution of bacterial cooperation and virulence. *Current biology* 19: 1683-1691.

Nostro, A., Roccaro, A. S., Bisignano, G., Marino, A., Cannatelli, M. A., Pizzimenti, F. C., Cioni, P. L., Procopio, F. and Blanco, A, R. (2007). Effects of oregano, carvacrol and thymol on *Staphylococcus aureus and Staphylococcus epidermidis* biofilms. *Journal of Medical Microbiology*, 56(4), 519-523.

Nweze, E.I. and Eze, E.E. (2009) Justification for the use of *Ocimum gratissimum* leaves in herbal medicine and its interaction with disc antibiotics. *BMC Complementary and Alternative Medicine* 9:37 1-6.

Nwinyi, O. C., Nwodo, C. S., Olayinka, A. O., Ikpo C.O and Ogunniran, K. O. (2009). Antibacterial effects of extracts of *Ocimum gratissimum* and *piper guineense* on

Escherichia coli and *Staphylococcus aureus. African Journal of Food Science,* 3(1): 022-025.

Obana, N., Nakamura, K. and Nomura, N. (2014). A sporulation factor is involved in the morphological change of *Clostridium perfringens* biofilms in response to temperature. *Journal of Bacteriology*, 196(8: 1540-1550.

Obladen, M. (2009) Necrotizing enterocolitis-150 years of fruitless search for the cause. *Neonatology,* 96(4): 203-210.

Oboh, F.O.J. and Masodje, H.I. (2009). Nutritional and Antimicrobial Properties of *Vernonia amygdalina* Leaves. *International Journal of Biomedical and Health Sciences,* 5(2): 51-56.

Offiah, V.N., and Chikwendu, U.A. (1999). Antidiarrhoeal effects of *Ocimum gratissimum* leaf extract in experimental animals. *Journal of Ethnopharmacology* 68: 327–330.

Okigbo, R.N. and Mmeka E.C. (2008). Antimicrobial effects of three tropical plant extracts on *Staphylococcus aureus, Escherichia coli and Candida albicans*. *African Journal of Traditional Complementary and Alternative Medicine,* 5 (3): 226 – 229.

Oliveira, N.M., Martinez-Garcia, E., Xavier J., Durham, W.M., Kolter, R., Kim, W. (2015). Biofilm formation as a response to ecological competition. *PLOS Biology*, 13(7): 1-23.

Olkowski, A.A., Wojnarowicz, C., Chirino-Trejo, M., Laarveld, B. and Sawicki, G. (2008). Sub-clinical necrotic enteritis in broiler chickens: Novel Etiological consideration based on ultra-structural and molecular changes in the intestinal tissue. *Research in Veterinary Science,* 85(3): 543–553.

Olson, M.E., Ceri, H., Morck, D.W., Buret, A.G., and Read, R.R. (2002). Biofilm bacteria: formation and comparative susceptibility to antibiotics. *The Canadian Journal of veterinary Research,* 66:86-92.

Olszowska-Zaremba, N., Borysowski, J., Dabrowska, J., Górski, A. (2012). Phage translocation, safety, and immunomodulation. In Bacteriophages in Health and Disease; Hyman P, Abedon S.T. (eds); CABI Press: Wallingford, UK. pp. 168-184.

Oral, N. B., Vatansever, L., Aydin, B. D., Sezer, C., Güven, A., Gülmez, M., Baser, C. K. H., and Kürkçüoğlu, M. (2010). Effect of oregano essential oil on biofilms formed by Staphylococci and *Escherichia coli*. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 16(Suppl-A), S23-S29.

Ortega, M. E., Fernández-Fuentes, M.A., Grande, M.J., Abriouel, H., Pérez Pulido, R., Gálvez, A. (2013). Biocide tolerance in bacteria. *International Journal of Food Microbiology*, 162:13–25.

Oyeyemi, I.T., Akinlabi, A.A., Adewumi, A. and Aleshinloye, A. O. (2018). *Vernonia amygdalina*: A folkloric herb with anthelminthic properties. *Journal of Basic and Applied Science, 7: 43-49.*

Padmavathy, N. and Vijayaraghavan, R. (2008). Enhanced bioactivity of ZnO nanoparticles-an antimicrobial study. *Science and Technology of Advanced Materials,* 9 (3):1-7.

Paiva, D. and McElroy, A. (2014). Necrotic enteritis: Applications for the poultry industry. *The Journal of Applied Poultry Research, 23(3):* 557-566.

Pal, S., Tak, Y.K., Song, J.M. (2007). Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium *Escherichia coli*. *Applied and Environmental Microbiology, 73*: 1712–1720.

Pan, M., Zhu, L., Qiu, Y. and Wang, J. (2016). Detection techniques for extracellular polymeric substances in biofilms: A Review*. BioResources,* 11(3): 8092-8115.

Paná˘cek, A., Smékalová, M., Kilianová, M., Prucek, R., Bogdanová, k., Ve˘ce˘rová, V., Kolá˘r, M., Havrdová, M., Płaza, G.A., Chojniak, J., Zbo˘ril, R. and Kvítek, L. (2016). Strong and nonspecific synergistic antibacterial efficiency of antibiotics combined with silver nanoparticles at very low concentrations showing no cytotoxic effect. *Molecules*, 21(26): 1-17.

Pantaleon, V., Bouttier, S. Soavelomandroso, A.P., Janoir, C., Candela, T. (2014). Biofilms of *Clostridium* species. *Anaerobes,* 30:193-198.

Parish, W.E. (1961). Necrotic enteritis in fowl (*Gallus Gallus domesticus*). Histopathology of the disease and isolation of a strain of *Clostridium welchii*. *Journal of Comparative Pathology*, *71*: 337-393.

Parsek, M. R. and Greenberg, E.P. (2005). Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends in Microbiology*, 13 (1): 27-33.

Patel, R. (2005). Biofilms and antimicrobial resistance. *Clinical Orthopaedics and Related Research*, 437: 41–47.

Paula-Ramos, L., Da Rocha Santos, C., Reis Mello, D.C., Theodoro, L., Eduardo De Oliveira, F., Graziella N. Brito, B., Junqueira, J. C., Cardoso Jorge, A.O., and Dias de Oliveira, L. (2016). *Klebsiella pneumoniae* planktonic and biofilm reduction by different plant extracts: *In Vitro* Study, *The Scientific World Journal,* 1- 5.

Pereira B. M. P. and Tagkopoulos I. (2019). Benzalkonium chlorides uses, regulatory status, and microbial resistance. *Applied Environmental Microbiology*, 85(13): 1-13.

Pereyra, E. A., Picech, F., Renna, M. S., Baravalle, C., Andreotti, C. S., Russi, R., Dallard, B. E. (2016). Detection of *Staphylococcus aureus* adhesion and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells. *Veterinary Microbiology*, 183: 69-77.

Petit, L., Gilbert, M., Popoff, M. (1999). *Clostridium perfringens*: Toxinotype and genotype. *Trends in Microbiology*, 7: 04-110.

Pettit, R.K., Weber, C.A., Kean, M.J., Hoffmann, H., Pettit, G.R., Tan, R., Franks, K.S. and Horton, M.L. (2005). Microplate Alamar Blue Assay for *Staphylococcus epidermidis* Biofilm Susceptibility Testing. *Antimicrobial Agents and Chemotherapy,* 49(7): 2612-2617.

Popat, R., Crusz, S. A., Messina, M., Williams, P., West, S. A. and Diggle, S. P. (2012). Quorum-sensing and cheating in bacterial biofilms. *Proceedings of the Royal Society of Biology*, 279: 4765-4771.

Porter, R.E. (1998). Bacterial enteritides of poultry. *Poultry Science, 77*: 1159-1165.

Prabhu, K.S., Lobo, R., Shirwaikar, A.A and Shirwaikar, A. (2009). *Ocimum gratissimum*: A Review of its chemical, pharmacological and ethnomedicinal properties. *The Open Complementary Medicine Journal, 1,* 1-15.

Prabhu, S. and Poulose, E.K. (2012). Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. *International Journal of Nano Letters,* 2:32-42.

Qin, Z., Yang, X., Yang, L., Jiang, J., Ou, Y., Molin, S. and Qu, D. (2007). Formation and properties of *in vitro* biofilms of *ica* negative *Staphylococcus epidermidis* clinical isolates. *Journal of Medical Microbiology*, 56(1):83-93.

Quavea, C.L., Lisa R.W., Planob, Traci Pantusoa, T., Bradley C. B. (2008). Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus. Journal of Ethnopharmacology,* 118, 418-428.

Raad, I., Chatzinikolaou, I., Chaiban, G., Hanna, H., Hachem, R., Dvorak, T., Cook, G. and Costerton, W. (2003). In vitro and ex vivo activities of minocycline and EDTA against microorganisms embedded in Biofilm on Catheter Surfaces. *Antimicrobial Agents and Chemotherapy*, 47(11): 3580-3585.

Raghupathi, K.R, Koodali, R.T, Manna, A.C. (2011). Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles. *Langmuir,* 27:4020-4028.

Raja, R.R., Krishna, Y., Kumari C., Lokanatha O., Mamatha, S., Damodar, Reddy, C. (2013) Antimicrobial activity of *Azadirachta Indica* (neem) leaf, bark, and seed extracts. *International Journal of Research in Phytochemistry and Pharmacology,* 3(1): 1-4.

Rajasekaran, C., Meignanam, E., Vijayakumar, V., Kalaivani, T., Ramya, S., Premkumar, N., Siva, R. and Jayakumararaj, R. (2008) Investigations on antibacterial activity of leaf extracts of *Azadirachta indica* A. Juss (Meliaceae): A Traditional medicinal plant of India. *Ethnobotanical Leaflets,* 12: 1213-1217.

Ramasamy, M., Lee, J. and Lee, J. (2016). Potent antimicrobial and antibiofilm activities of bacteriogenically synthesized gold–silver nanoparticles against pathogenic bacteria and their physiochemical characterizations. *Journal of Biomaterials Applications,* 31(3): 366-378.

Ramzi, A., Oumokhtar, B., Ez zoubi, Y., Mouatassem, T.F., Benboubker, M., and El Ouali Lalami, A. (2020). Evaluation of antibacterial activity of three quaternary ammonium disinfectants on different germs isolated from the hospital environment. *BioMed Research International,* 1-6.

Ratner, B. D. and Bryant, S. J. (2004). Biomaterials: where we have been and where we are going. *Annual Review Biomedical Engineering*, 6:41-75.

Reisner, A., Krogfelt, K.A., Klein, B.M., Zechner, E.L., and Molin, S. (2006) In vitro biofilm formation of commensal and pathogenic *Escherichia coli* strains: impact of environmental and genetic factors. *Journal of Bacteriology,* 188(10): 3572-3581.

Rendueles O, Ghigo J.M. (2015). Mechanisms of competition in biofilm communities. *Microbiology Spectrum* 3(3): 1-18.

Romero, C. M., Vivacqua, C. G., Abdulhamid, M., Baigori, M. D., Slanis, A.C., Gaudioso de Allori, M. C. and Tereschuk, M. L. (2016). Biofilm inhibition activity of traditional medicinal plants from Northwestern Argentina against native pathogen and environmental microorganisms. *Revista da Sociedade Brasileira Medicinal Tropical* 49(6):703-712.

Rood, J. I. (1998). Virulence genes of *Clostridium perfringens*, *Annual Review of Microbiology,* 52:333-360.

Rossi, L.M., Rangasamy, P., Zhang J, Qiu, X-Q., Wu, G.Y. (2008). Research advances in the development of peptide antibiotics. *Journal of Pharmaceutical Science,* 97: 1060-1070.

Sadeghi, B., Garmaroudi, F.S., Hashemi, M., Nezhad, H., Nasrollahi, A. Ardalan, S. and Ardalan, S. (2012). "Comparison of the anti-bacterial activity on the nanosilver shapes: Nanoparticles, nanorods and nanoplates. *Advanced Powder Technology*, 23(1): 22-26.

Sakuragi, Y. and Kolter, R. (2007). Quorum sensing regulation of the biofilm matrix genes (*pel*) of *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 189: 5383-5386.

Sakurai, J., Nagahama, M., Oda, M., Tsuge, H. and Keiko Kobayashi (2009). *Clostridium perfringens* Iota-Toxin: Structure and Function. *Toxins 1*: 208-228.

Sánchez, E., Morales, C. R., Castillo, S., Leos-Rivas, C., García-Becerra, L and Ortiz Martínez, D.M. (2016). Antibacterial and antibiofilm activity of methanolic plant extracts against nosocomial microorganisms. *Evidence-Based Complementary and Alternative Medicine,* 1-8.

Sandasi, M., Leonard, C. M. and Viljoen, A.M. (2010). The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes, Letters in Applied Microbiology,* 50:30-35.

Sangiliyandi Gurunathan, S., Jae Woong Han, J.W., Deug-Nam Kwon, D. N. and Jin-Hoi Kim, J. (2014). Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. *Nanoscale Research Letters,* 9:373.

Savage, V.J., Chopra, I., O'Neill, A.J. (2013). Staphylococcus aureus biofilms promote horizontal transfer of antibiotic resistance. *Antimicrobial Agents Chemotherapy*, 57:1968–70

Shojadoost, B., Vince, A.R. and Prescott, J.F. (2012). The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. *Veterinary Research*, 43(1): 74-86.

Shulhan, J., Dicken, B., Hartling, L. and Larsen, B.M (2017). Current knowledge of necrotizing enterocolitis in preterm infants and the impact of different types of enteral nutrition products. *American Society for Nutrition*. 8:80-91.

Silva, H. O., Santos Lima,J.A., Gamero Aguilar, C. E., Marques Rossi,G. A., Mathias L. and Centola Vidal, A. (2018). Efficiency of different disinfectants on *Bacillus cereus* Sensu Stricto biofilms on stainless-steel surfaces in contact with milk. *Frontiers in Microbiology*, 9(2934):1-11.

Silva Ferreira, G. L., Rosalen, P., Peixoto, L., de Lima Pérez, A., de Carvalho Carlo, F., Cançado Castellano, L., de Lima, J. M., Almeida Freires, I., de Oliveira Lima, E., and de Castro, R. (2017). Antibiofilm activity and mechanism of action of the disinfectant Chloramine T on *Candida* spp., and Its toxicity against human cells. *Molecules*, 22, 1527-1542.

Singh, N., Romero, M., Travanut, A., Monteiro, P. F., Jordana-Lluch, E., Hardie, K. R., Williams, P., Alexander, M. R. and Alexander, C. (2019). Dual bioresponsive antibiotic and quorum sensing inhibitor combination nanoparticles for treatment of *Pseudomonas aeruginosa* biofilms *in vitro* and *ex vivo*. *Biomaterials Science*, **7**, 4099-4111.

Singh, P.K., Schaefer, A. L., Parsek, M.R., Moninger T.O., Welsh, M.J. and Greenberg, E.P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407: 762-764.

Singh, S., Singh, S.K., Chowdhury, I. and Singh, R. (2017). Understanding the mechanism of bacterial biofilms resistance to antimicrobial agents. *The Open Microbiology Journal*, 28(11): 53-62.

Skinner, J.T., Bauer, S., Young, V., Pauling, G., Wilson, J. (2010). An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. *Avian Diseases*, 54(4): 1237-1240.

Skowron, K., Wałecka-Zacharska, E., Grudlewska, K., Gajewski, P., Wiktorczyk, N., Wietlicka-Piszcz, M., Dudek, A., Skowron, K.J. and Gospodarek-Komkowska, E. (2019). Disinfectant susceptibility of biofilm formed by *Listeria monocytogenes* under selected environmental conditions. *Microrganisms,* 7(280):1-16.

Slomberg, D.L, Lu, Y., Broadnax, A.D, Hunter, R.A, Carpenter, A.W, Schoenfisch, M.H. (2013). Role of size and shape on biofilm eradication for nitric oxide-releasing silica nanoparticles. *ACS Applied Materials and Interfaces*. 5:9322-9329.

Smith, J. (2001). The social evolution of bacterial pathogenesis. *Proceedings of Royal Society London B.* 268: 61-69.

Son, J.S., Lee, S.J., Jun, S.Y., Yoon, S.J., Kang, S.H., Paik, H.R., Kang, J.O., Choi, Y.J. (2010). Antibacterial and biofilm removal activity of a podoviridae *Staphylococcus aureus* bacteriophage SAP-2 and a derived recombinant cell-wall-degrading enzyme. *Applied Microbiology Biotechnology. 86*, 1439-1449.

Sondi, I. and Sondi, S.B. (2004). Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram negative bacteria. *Journal of Colloid and Interface Science,* 275:177- 82.

Song, H.J., Shim, K., Jung, S., Choi, H.J., Lee, M.A., Ryu, K.H., Songer J. G. (1996). Clostridial enteric diseases of domestic animals. *Clinical Microbiology Review*. 9(2): 216-234.

Stalder, T. and Top, E. (2016). Plasmid transfer in biofilms: a perspective on limitations and opportunities. *Biofilms and Microbiomes,* 2 (16022):1-5.

Stanley, D., Wu, S-B., Rodgers, N., Swick, R. A., Moore, R. J. (2014). Differential responses of cecal microbiota to fishmeal, Eimeria and *Clostridium perfringens* in a necrotic enteritis challenge model in chickens. *PLOS ONE* 9(8):1-10.

Stepanovic, S., Cirkovic, I., Majac, V., and Svabic-Vlahovic, M. (2003). Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. *Food Microbiology*, 20(3): 339-343.

Stepanovic´ S, Vukovic´ D, Hola V, Di Bonaventura G, Djukic´ S, C´ irkovic´ I, Ruzicka F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS*, 115 (8): 891-899.

Sternberg, C., Bjarnsholt, T. and Shirtliff, M. (2014). Methods for dynamic investigations of surface-attached In vitro bacterial and fungal biofilms. *Methods in Molecular Biology,* 1147: 3–22.

Sternberg, C., Christensen, B.B., Johansen, T., Nielsen, A.T., Andersen, J.B., Givskov, M. and Molin, S. (1999). Distribution of bacterial growth activity in flow-chamber biofilms. *Applied and Environmental Microbiology*, 65(9): 4108-4117.

Stevens, D. L., Aldape, M.J., and Bryant, A. E. (2012) Life threatening clostridial infection. *Anaerobes,* 18(2) 254-259.

Stevens, D.L. and Bryant, A.E. (2017). Necrotizing Soft-Tissue Infections. *The New England Journal of Medicine,* 377: 2253-2265.

Stewart, P.S. and Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet, 358* (9276):135-138.

Stewart, P.S., Franklin, M.J., Williamson, K.S., Folsom, J.P., Boegli, l. ad James, G.A. (2015). Contribution of stress responses to antibiotic tolerance in *Pseudomonas aeruginosa* biofilms. *Antimicrobial agents Chemotherapy*, 59:3838-3847.

Stiles, B. G., Barth, G., Barth, H. and Popoff, M.R. (2013). *Clostridium perfringens* Epsilon Toxin: A Malevolent Molecule for Animals and Man? *Toxins, 5:* 2138-2160.

Stokes, J. M., Lopatkin, A.J., Lobritz, M.A. and Collins, J.J. (2019). Bacterial metabolism and antibiotic efficacy. *Cell Metabolism*, 30: 1-9.

Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W. (2002). Biofilms as complex differentiated communities. *Annual Review of Microbiology, 56*: 187-209.

Sutherland, I.W. (2001) Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology,* 147: 3-9.

Tan, M.S.F., White, A.P, Rahman, S., Dykes, G.A. (2016). Role of fimbriae, flagella and cellulose on the attachment of *Salmonella* Typhimurium ATCC 14028 to plant cell wall models. *PLOS ONE,* 11(6):1-13.

Tan, D., Dahl, A., Middelboe, M. (2015). Vibriophages differentially influence biofilm formation by *Vibrio anguillarum* strains. *Applied and Environmental Microbiology,* 81:4489 - 4497.

Tezel, B.U., Akçelik, N., Yüksel, F.N., Taşkale, N., Karatuğ and Akçelik, M. (2016). Effects of sub-MIC antibiotic concentrations on biofilm production of *Salmonella* Infantis, *Biotechnology and Biotechnological Equipment*, 30(6), 1184-1191.

Timbermont, L., Haesebrouck, F., Ducatelle, R. & Van Immerseel, F. (2011). Necrotic enteritis in Broilers: An Updated Review on the Pathogenesis. *Avian Pathology,* 40(4) 341-347.

Timbermont, L., Smet, L.D., Nieuwerburgh, F.V., Parreira, V.R., Driessche, G. V., Freddy Haesebrouck, F., Ducatelle, R., Prescott, J., Deforce, D., Devreese, B. and Immerseel, F.V. (2014). Perfrin, a novel bacteriocin associated with *netB* positive *Clostridium perfringens* strains from broilers with necrotic enteritis. *Veterinary Research,* 45(1): 40.

Tejpal, J., Cross, R., Owen, L., Jenkins, R., Armtage, D., Laird, K. (2018). A multi-faceted approach to determining the efficacy of metal and metal oxide nanoparticles against bacterial biofilms. *Journal of Bionanoscience,* 12(5):705-714.

Tolker-Nielsen, T. and Molin, S. (2000). Spatial organization of microbial biofilm communities. *Microbial Ecology,* 40(2):75-84.

Toyofuku, M., Inaba, T., Kiyokawa, T., Obana, N., Yawat, Y. and Nomura, N. (2015). Environmental factors that shape biofilm formation. *Bioscience, Biotechnology and Biochemistry,* 1-6.

Trentin, D. D. S., Giordani, R.B., Zimmer, K.R., da Silva, A.G., da Silva, M.V., Correia, M.T., Baumvol, I.J., Macedo, A.J. (2011). Potential of medicinal plants from the Brazilian semi-arid region (Caatinga) against *Staphylococcus epidermidis* planktonic and biofilm lifestyles. *Journal of Ethnopharmacology*, 137:327-35.

Trevisan, D.A.C., Silva, A. F., Negri, M., Abreu Filho, B. A., Junior, M. M., Patussi, E.V., Campanerut-Sá, P. A. Z., Mikcha, J. M. G. (2018). Antibacterial and antibiofilm activity of carvacrol against *Salmonella* enterica serotype Typhimurium. *Brazilian Journal of Pharmaceutical Sciences,* 54(1):1-8.

Turkey, A.M., Barzani, K.K., Suleiman, A.A.J., Abed, J.J. (2018). Molecular assessment of accessory gene regulator (*agr*) quorum sensing system in biofilm forming *Staphylococcus aureus* and study of the effect of silver nanoparticles on *agr* system. *Iran Journal of Microbiology,* 10 (1): 14-21.

Udochukwu, U., Omeje, F. I., Uloma, I.S. and Oseiwe, F. D. (2015). Phytochemical analysis OF *Vernonia amygdalina* and *Ocimum gratissimum* extracts and their antibacterial activity on some drug resistant bacteria, *American Journal of Research Communication,* 3(5):225-235.

Upadhyaya, G.P.M., Lingadevaru, U.B., Lingegowda, R.K. (2011) Comparative study among clinical and commensal isolates of *Enterococcus faecalis* for presence of *esp* gene and biofilm production. *Journal of Infection in Developing countries,* 5(5): 365-369.

Uzal, F. A., Freedman, J. C., Shrestha, A., Theoret, J. R., Garcia, J., Awad, M. M., Adams, V., Moore, R. J., Rood, J. I. and Mcclane, B. A. (2014). Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. *Future Microbiology,* 9(3): 361-377.

Van Immerseel, F., De Buck, J., Pasmans, F., Huyghebaert, G, Haesebrouck, F., Ducatelle, R. (2004). *Clostridium perfringens* in poultry: an emerging threat for animal and public health. *Avian Pathology*, 33 (6): 537-549.

Varga, J. I., Therit, B. And Melville, S. B. (2008). Type IV Pili and the Ccpa protein are needed for maximal biofilm formation by the Gram positive anaerobic pathogenic *Clostridium perfringens. Infection and Immunity*, 76 (11): 4944-4951.

Vassallo, A., Silletti, M. F., Faraone, I. and Milella, L. (2020). Nanoparticulate antibiotic systems as antibacterial agents and antibiotic delivery platforms to fight infections. *Journal of Nanomaterials*, 1-31.

Vasudevan, R. (2014). Biofilms: Microbial Cities of Scientific Significance. *Journal of Microbiology and Experimentation,* 1(3) 2-16.

Vazquez-Munoz, R., Meza-Villezcas, A., Fournier, P.G.J., Soria-Castro, E., Juarez-Moreno, K., Gallego-Hernandez, A.L., Bogdanchikova, N., Vazquez-Duhalt, R. and Huerta-Saquero, A. (2019). Enhancement of antibiotics antimicrobial activity due to the silver nanoparticles impact on the cell membrane. *Plos One*, 14(11).

Verherstraeten, S., Goossens, E., Valgaeren, B., Pardon, B., Timbermont, L., Haesebrouck, F., Ducatelle, R., Deprez, P., Wade, K.R. and Tweten, R. (2015). Perfringolysin O: The underrated *Clostridium perfringens* toxin? *Toxins*, 7(5): 1702-1721.

Vida, J.E., Shak, J.R., and Canizalez-Roman, A. (2015). The Cpal quorum sensing system regulates production of hemolysins CPA and PFO to build *Clostridium perfringens* biofilms. *Infection and Immunity*, 83 (6): 2430-2442.

Vu, B., Chen, M., Crawford, R.J. and Ivanova, E.P. (2009). Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules*, 14: 2535-2554.

Vuong, C., Saenz, H. L., Gotz, F. and Otto, M. (2000). Impact of the *agr* quorum – sensing system on adherence to polystyrene in Staphylococcus aureus. *Journal of Infectious Diseases,* 182: 1688-1693.

Wade, B. and Keyburn, A. (2015). The true cost of necrotic enteritis. *World Poultry,* 31(7):16-17.

Walker, T.S., Tomlin, K.L., Worthen, G.S., Poch, K. R., Lieber, J. G., Saavedra, M.T., Fessler, M.B., Malcolm, K. C., Vasil, M. L., and Nick, J. A. (2005). Enhanced *Pseudomonas aeruginosa* Biofilm Development Mediated by Human Neutrophils*. Infection and Immunity,* 73(6): 3693-3701.

Weber, D.J., Rutala, W.A. (2006). Use of germicides in the home and healthcare setting: is there a relationship between germicide use and antibiotic resistance? *Infection Control Hospital Epidemiology*, 27:1107–1119

Wenzel, R. P. (2007). Health care-associated infections: major issues in the early years of the 21st century. *Clinical Infectious Diseases,* 15(45): 85-8.

Wijesundara, N. M. and Rupasinghe, H. P. V. (2019). Anti-biofilm activity of plant derived extracts against infectious pathogen-*Pseudomonas aeruginosa* PAO1*. Molecule,* 24:1-19

Williams, H.G., Day, M.J., Fry, J.C., and Stewart, G.J. (1996). Natural transformation in river epilithon. *Applied Environmental Microbiology*, 62:2994–8

Williams, R.B. (2005). Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. *Avian Pathology, 34:* 159- 180.

Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., Okuom, M., Huber, C., Brooks, T.D., Wilson, E., Clement, B., Wentworth, C.D. Holmes A.E. (2017). Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini review. *Research and Review: Journal of Engineering and Technology,* 6(4):1-42.

Wilson, M. (2001). Bacterial biofilms and human Disease. *Science Progress,* 84 (3): 235-254.

Wimley, W., Hristova, K. (2011). Antimicrobial peptides: successes, challenges, and unanswered questions. *Journal of Membrane Biology,* 239:27-34.

Wood, T.K., Knabel, S.J. and Kwan, B.W. (2013). Bacterial persister cell formation and dormancy. *Applied and Environmental Microbiology*, 79(23):7116-7121.

Xu K.D, Franklin, M. J, Park, C. H, McFeters, G. A, Stewart, P.S. (2001). Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously fed *Pseudomonas aeruginosa* biofilms. *FEMS Microbiology Letters* 199:67-71.

Yan, J., Mao, J. and Xie, J. (2014). Bacteriophage Polysaccharide depolymerases and Biomedical Applications, *BioDrugs*, 28:265-274.

Yang, L., Liu, Y., Wu, H., Song, Z., Høiby, N., Molin, S. and Givskov, M. (2012). Combating biofilms. *FEMS Immunology and Medical Microbiology,* 65: 146-157.

Yarwood, J.M., Bartels, D. J., Volper, E.M. and Greenberg, E.P. (2004). Quorum sensing in *S. aureus* biofilms. *Journal of Bacteriology,* 186:1838-1850.

Yeh, Y., Huang, T., Yang,S., Chen C.and Fang, J. (2020). Nano-Based Drug Delivery or Targeting to Eradicate Bacteria for Infection Mitigation: A Review of Recent Advances. Frontiers in Chemistry, *https://doi.org/10.3389/fchem.2020.00286*

Yue, Q. (2010). Biofilm formation and antimicrobial resistance of coagulase-negative staphylococci isolated from neonatal intensive care units. PhD. RMIT University.

Zeuko´o, M. E., Virginio, C. L., Sara, M. S. and Fekam, B. (2016). Anti-*candida* biofilm properties of Cameroonian plant extracts. *Journal of Medicinal Plants Research,* 10(35): 603-611.

Zhang, C., Cui, F., Zeng, G., Jiang, M., Yang, Z., Yu, Z., Zhu, M., Shen, L. (2015). Quaternary ammonium compounds (QACs), a review on occurrence, fate and toxicity in the environment. *Science of the Total Environment,* 518-519: (353-362).

Zhang, L., Jiang, Y., Ding, Y., Povey, M., York, D. (2007) Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). *Journal of Nanoparticle Research,* 9:479-489.

Zhu, X., Long, F., Chen, T., Knochel, S., She, Q. and Shi X. (2008). A putative ABC transporter is involved in negative regulation of biofilm formation by *Listeria monocytogens*. *Applied and Environmental Microbiology*, 74(24):7675-7683.

Zineb, G., Mostafa, M.E., Youssef, G., Abdellatif, K., Salwa, O., Abdellah, H., Saad, I. K., Naima, E. and Mohammed, T. (2014). Anti-Adhesion and Anti-Biofilm Effectiveness of Disinfectants used in Hemodialysis against both *Staphylococcus warneri* and *Staphylococcus sciuri* Biofilms. *International Journal of Engineering Research and Applications*, 4 (7):86-92.

APPENDIX A

LIST OF CHEMICALS, MEDIA, COMMERCIAL KITS, CONSUMABLES AND EQUIPMENTS/MANUFACTURERS

Chemicals	Manufacturers
Ethanol absolute (99.9%)	Fischer scientific chemical UK
Tetracycline hydrochloride	Sigma-Aldrich, UK
Crystal violet	Pro-lab diagnostic UK
Iodine	Pro-lab diagnostic UK
Safranin	Pro-lab diagnostic UK
Gentamicin sulfate	Sigma-Aldrich, UK
Bacitracin	Sigma- Aldrich, UK
Industrial methylated spirit	Fischer scientific chemical
Glycerol ≥99%	Fischer scientific chemical
Penicillin G sodium salt	Sigma- Aldrich, UK
Sodium chloride	Fischer scientific chemical, UK
Ringer solution tablet	Oxoid BR0052G
Acetic acid glacier	Fischer scientific chemical, UK
Glutaraldehyde solution	Sigma- Aldrich, UK
Gold nanoparticles	BBI solution, UK
Silver nanoparticles	Metnano UK
Hexamethyldisilazane	Sigma- Aldrich, UK
Dipotassium phosphate	ACROS Organic, UK
Monopotassium phosphate	Sigma-Aldrich, UK

Growth media	Manufacturers
Brain Heart Infusion	OXOID Ltd., UK
Nutrient agar	OXOID Ltd., UK
Tryptose Sulphite cycloserine agar	OXOID Ltd., UK

Commercial Kits	Manufacturers
Live/Dead BacLight bacterial viability kit Invitro probe	ThermoFischer scientific UK

Equipments and Consumables	Manufacturers
Weighing balance	Sartorius
Cuvettes	Sarstedt Germany
Parafilm	Bemis USA
Stirrer	Bio cote
Biological safety cabinet class II	Thermo Scientific
Autoclave	Astell
Bench Top autoclave	Classic prestige Medical
Nitrile gloves	VWR International bvba malaysia
Fume hood	Zurich
Pipette holder	Integra pipetboy
25ml universal	Sarstedt
Cover glass	VWR
Ultralow temperature freezer (-80˚C)	New Brunswick eppendorf
-20˚C freezer	Medline LIEBHERR
4˚C fridge	Medline LIEBHERR
37˚C aerobic incubator	Thermo scientific
Anaerobic incubator	don Whitley scientific
Spectrophotometer	Thermo scientific
Micro plate reader	FLUOstar Optima
Eppendorf tubes (1.5ml)	SARSTEDT
Eppendorf tubes (25ml)	SARSTEDT
Eppendorf tubes (50ml)	SARSTEDT
2ml/5ml/25ml Sarstedt serological pipette	HERAEUS
Finnpipette single channel digital pipettes	NICHIRYO/NICHIPET EXII Japan
Finnpipettes multichannel digital pipette (20-300 µl)	GILSON
Pippette tips (100-1000µl)	SARSTEDT
Pippette tips (20-200 µl)	SARSTEDT
Pippette tips (2-20 µl)	SARSTEDT
Pippette tips (0.5-10 µl)	SARSTEDT
Gold sputter coater	EMITECH
Eppendorf centrifuge (1.5ml, 25ml, 50ml)	Bio-labs
Microplates,	SARSTEDT Germany
Microscope slide	Fischer Brand UK
Confocal Laser Scanning Microscope	Leica
Petri dishes (90 mm)	SARSTEDT
Scanning electron microscope	FEI INSPECT
Vortex	Bio cote UK
Light binocular microscope	Primo star
50ml/25ml Eppendorf centrifuge	Bio-Labs
pH meter	HANNAH instruments

APPENDIX B

PREPARATION OF GROWTH MEDIA AND SOLUTIONS

Tryptose Sulphite cycloserine agar (TSC agar)

28g of TSC agar powder was added into 500ml deionised water in a 1000ml bottle. The solution was mixed with a magnetic stirrer and then autoclaved for 15 min at 121˚C. The medium was poured into petri dishes and agar was allowed to set at room temperature and then stored in a 4˚C until use.

Brain Heart infusion broth (BHI)

37g of BHI powder was added to 1 litre of distilled water in a 1000ml glass bottle. The medium was mixed properly with a magnetic stirrer before autoclaving for 15mins at 121˚C. The media was stored at room temperature until used.

Nutrient Agar Plates

28g of nutrient agar powder was added to 1 litre of deionised water. This was mixed properly with a magnetic stirrer and autoclaved for 15 min at 121˚C. The medium was then poured into Petri dishes and allowed to set in the fume hood or on the work bench. Nutrient agar plates were stored at 4˚C until use.

Pottassium phosphate buffer (pH 7.2)

12.36g of K_2HPO4 (Dipotassium phosphate) was dissolved in 71.7ml of deionised water. 3.85g of KH_2PO4 (Monopotassium phosphate) was dissolved in 28.3ml of deionised water. The final volume was adjusted to 1000ml with deionised water. Final

pH was 7.2. The solution was autoclaved at 121˚C for 15min and stored at room temperature.

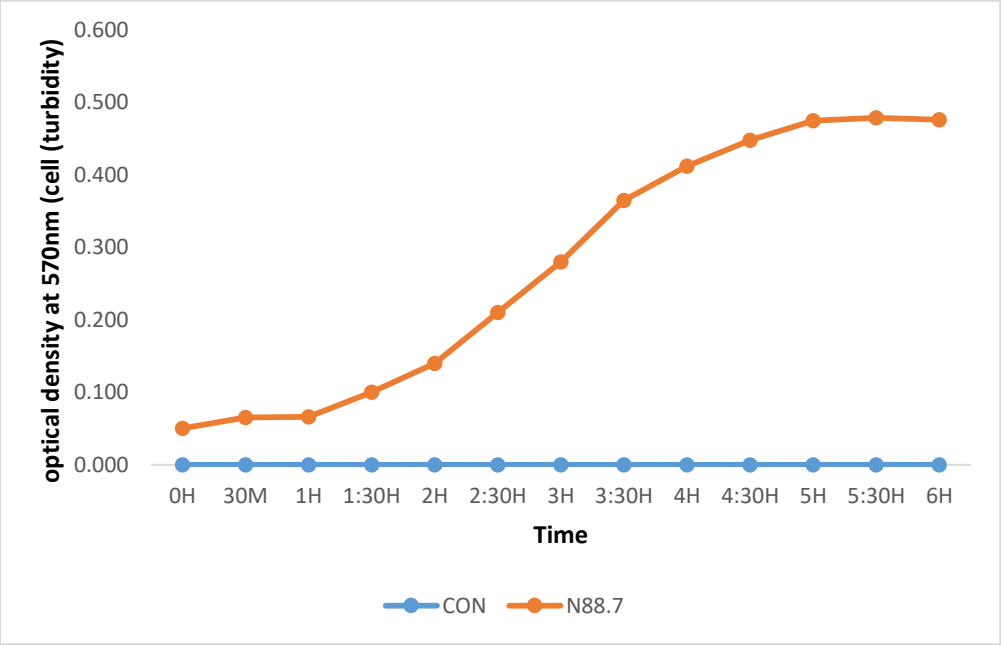
Ringer® solution

One Ringer solution tablet was dissolved in 500ml of deionised water. The solution was then autoclaved at 121˚C for 15min and stored at room temperature.

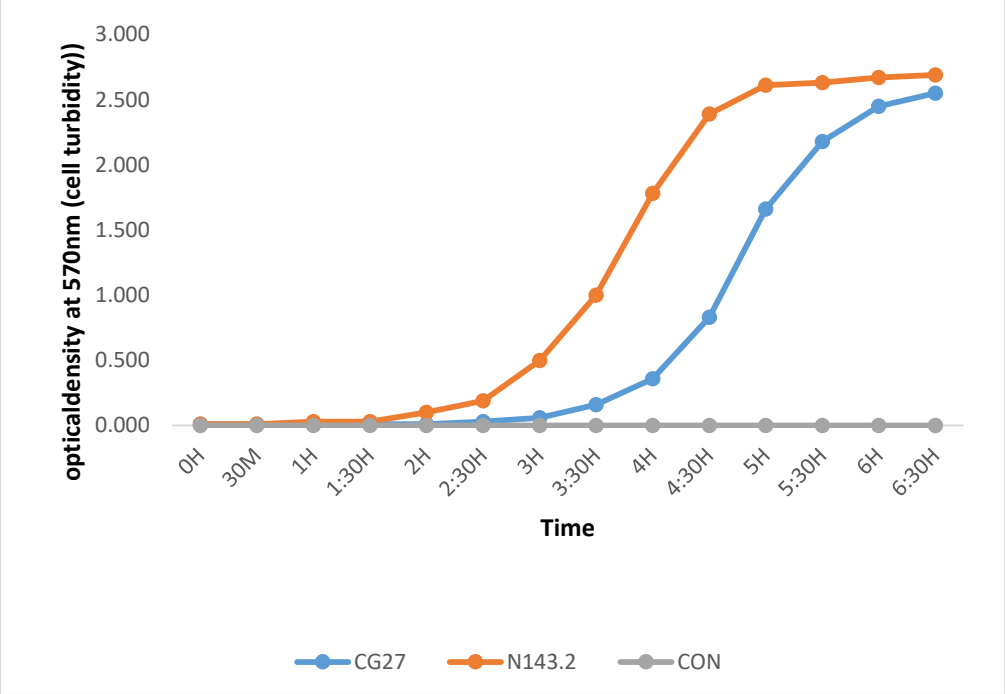
0.85% Sodium Chloride

8.5g of sodium chloride pellet was weighed and added to 1000ml of deionised water in a 1 litre glass bottle. The solution was allowed to mix properly using a magnetic stirrer. The reagent was then autoclaved at 121˚C for 15min and stored at room temperature until use.

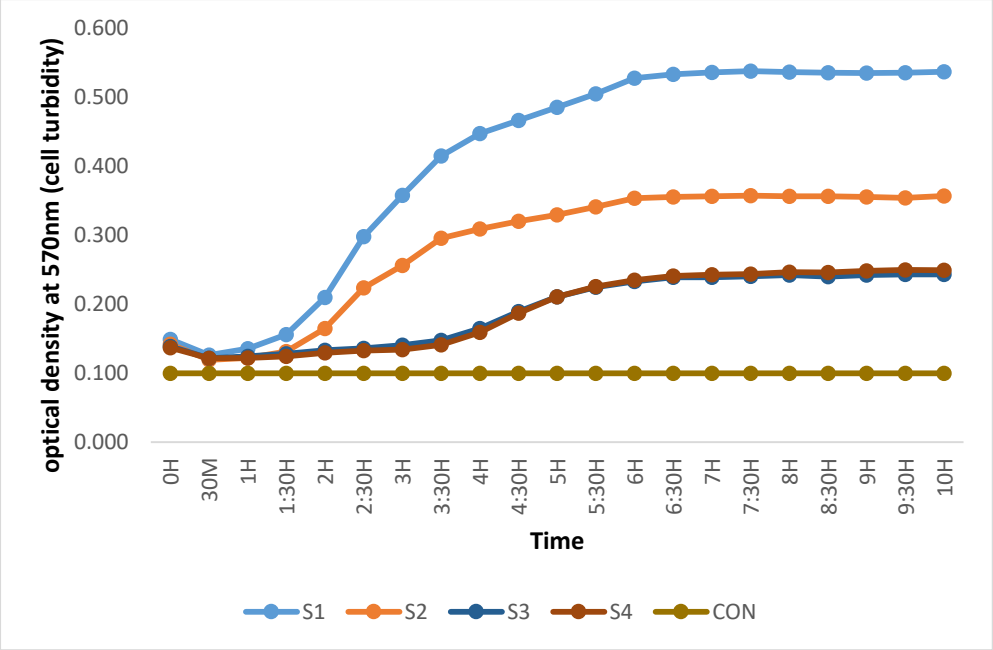
APPENDIX C
FIGURES



A



B



C

Figure 1: Growth curve for *Clostridium perfringens* isolates

C. perfringens isolates were grown under static condition at 37˚C in an anaerobic chamber to determine the mid-log phase of growth.

Key: S1=E32, S2=E14, S3=ATCC 13124, S4=CG43, CON= sterile BHI broth

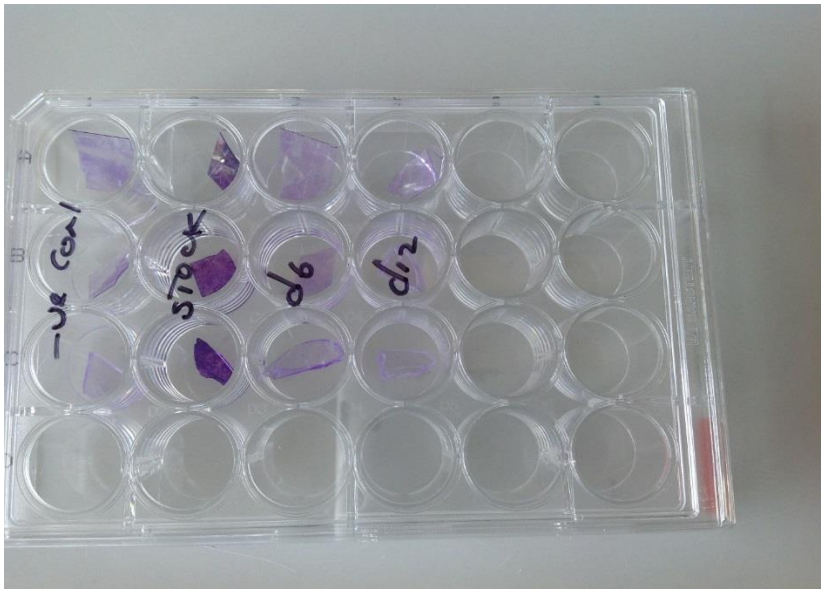


Figure 2: Biofilm grown on cover slips/ piece of microtitre plate and stained with crystal violet.