THE EFFECT OF PECTINEX ULTRA SP-L ON BACTERIAL BIOFILMS AND HUMAN CELL CULTURES *IN VITRO*

IAN PAUL OLWOCH

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Supervisor: Prof V. Steenkamp

Co-Supervisor: Prof O.B.W. Greeff

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DECLARATION

I: Ian Paul Olwoch

Student number: 29677051

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ABSTRACT

Biofilms are surface-bound bacterial colonies that are held together by a self-produced extracellular polymeric matrix. They are highly resistant to antibiotics and host defence mechanisms, and are known to be the cause of persistent infections. Biofilm-degrading enzymes have been shown to prevent biofilm formation, remove mature biofilm, and enhance the efficacy of antibiotics. This study investigated the antibacterial and antibiofilm actions of the commercial enzyme Pectinex Ultra SP-L (Pectinex), alone and in combination with antibiotics, on standard and clinical cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The cytotoxicity of Pectinex was determined on human cell cultures *in vitro*.

Pectinex (7.42 – 950 PGU/ml) was not bactericidal, and had no effect on the antibacterial efficacy of amoxicillin-clavulanate and ciprofloxacin in cultures of *S. aureus* (ATCC 12600) and *P. aeruginosa* (ATCC 9027), respectively. However, in clinical cultures of *P. aeruginosa*, Pectinex caused an 89.0% (from 1.0 to 1.89 µg/ml) and 92.8% (from 1.67 to 3.22 µg/ml) increase in the MIC and MBC of ciprofloxacin, respectively. In clinical cultures of *S. aureus*, both bactericidal indices of amoxicillin-clavulanate were increased by 28.0% (from 2.0 to 2.56 µg/ml). In all bacterial cultures, low concentrations of Pectinex (≤ 118.75 PGU/ml) and prolonged incubation periods (≥ 6 h) were both associated with increased viability and biofilm biomass. Over a short incubation period (≤ 6 h), higher concentrations of Pectinex (237.5 – 950 PGU/ml) effectively inhibited biofilm formation in *P. aeruginosa* ATCC (237.5 – 950 PGU/ml) and clinical (950 PGU/ml) strains but not in *S. aureus* cultures.

Pectinex (237.5 – 950 PGU/ml) was cytotoxic to HeLa cells, lymphocytes and neutrophils, and induced morphological features that included shrunken rounded cells, blebs, apoptotic

bodies, cytoplasmic vacuoles and cell debris. The effects at 475 and 950 PGU/ml were comparable to mitomycin C 10 µg/ml and staurosporine 1 µg/ml.

Pectinex was shown to either enhance or reduce biofilm biomass and cell viability in cultures of *S. aureus* and *P. aeruginosa*. The manifested effects depended on the concentration of the enzyme, the specific bacterial species and strain, and the maturity of the biofilms. Further studies are still needed in order to determine the actions of Pectinex on other clinical pathogens.

KEYWORDS

antibiotic, amoxicillin-clavulanate, biofilm, ciprofloxacin, cytotoxicity, enzyme, Pectinex, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

DEDICATION

This work is dedicated

to

my wife, Jane,

and to

my daughters, Irene and Margaret,

in recognition of

their

patience, understanding, and encouragement.

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LIST OF ABBREVIATIONS

agr	Accessory gene regulator
AHL	Acylhomoserine lactone
AI	Autoinducer
AIP	Autoinducing peptide
alg	Alginate biosynthetic gene
ATCC	American type culture collection
ATP	Adenosine triphosphate
Bap	Biofilm-associated proteins
*bdl*A	Biofilm dispersion locus A
CCN	Cut-off colony number
c-di-GMP	Cyclic diguanosine monophosphate
CF	Cystic fibrosis
CFU	Colony-forming unit
CLSM	Confocal laser scanning microscopy
CTC	5-cyano-2, 3-ditolyl tetrazolium chloride
CV	Crystal violet
DAPI	4', 6-diamidino-2-phenylindole
DLVO	Derjaguin-Landau-Verwey-Overbeek
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's minimal essential medium
EMEM+	Eagle's minimal essential medium supplemented with 10% foetal bovine serum and 1% penicillin and streptomycin
EPS	Exopolymeric substance
FBS	Foetal bovine serum
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
FDA	Food and Drug Administration (United States of America)
GRAS	Generally recognized as safe
HCl	Hydrochloric acid

LIST OF ABBREVIATIONS

HIV	Human immunodeficiency virus
ica	Intercellular adhesin
IC_{50}	50% inhibitory concentration
las	Elastase structural gene
LPS	Lipopolysaccharides
Lux	Luciferase
MBC	Minimum bactericidal concentration
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules family
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NO^{\bullet}$	Nitric oxide free-radical
nuc	Nuclease gene
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBP	Penicillin-binding proteins
Pel	Pellicle; pellicle polysaccharide
PGU	Polygalacturonase unit
PIA	Polysaccharide intercellular adhesin
*pil*A	Pilus gene A
p-INT	*p*-Iodo-nitrotetrazolium violet (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
PlasDIC	Polarization-optical transmitted light differential interference contrast microscopy
PNAG	Poly-*N*-acetylglucosamine polysaccharide
PNSG	*β*-1-6-linked N-succinylglucosamine polysaccharide
PQS	Pseudomonas quinolone signal
Psl	Polysaccharide synthesis locus; polysaccharide synthesis locus-dependent polysaccharide
QS	Quorum-sensing
rhl	Rhamnolipid
RPMI	Roswell Park Memorial Institute medium
sarA	Staphylococcal accessory regulator A

LIST OF ABBREVIATIONS

SCV	Small colony variant
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
S.E.M.	Standard error of the mean
SERAM	Secretable expanded repertoire adhesive molecules family
UV	Ultraviolet
VCC	Viable colony count

CHAPTER 1

INTRODUCTION

Since the discovery of penicillin, antibiotics have been one of the most important classes of drugs prescribed in everyday clinical practice. Their central role in the management of critically ill patients, and as a complement to virtually every surgical procedure, can hardly be overstated[1]. They have also been extensively used for the treatment of disease and prevention of bacterial growth in plant agriculture, animal husbandry, veterinary medicine and the food processing industry. The ongoing use of currently available antibiotics and biocides, whether judicious or otherwise, has repeatedly exposed bacteria to hostile environmental challenges to which they have invariably adapted[2]. The broad consequence of this adaptation has been the worldwide emergence, rapid evolution and spread of multidrug-resistant pathogens[1]. Furthermore, the slow pace at which new antibiotics have been developed has lagged behind the rate of emergence and spread of antibiotic-resistant bacteria, and may have inadvertently aggravated the problem of drug resistance[3].

The alarming worldwide increase in the incidence of bacterial resistance to antimicrobial agents has prompted the urgent need for the development of new drugs and treatment strategies to counter the occurrences of infections caused by resistant pathogens[1,3,4]. The scientific approach to the problem of antimicrobial resistance is further compounded by the realization that bacteria naturally occur as integrated surface bound communities, the biofilm, and that the biofilm-state confers additional unique antimicrobial resistance properties upon the bacteria[5,6]. A biofilm* is defined as an interacting, organized, three-dimensional, structured community of single or multiple species of microorganisms encapsulated within a complex self-developed, hydrated, polyionic, predominantly exopolysaccharide matrix, and adherent to a living or inert solid surface (substratum) and to each other[7].

Biofilm-mediated bacterial resistance to antimicrobial agents, disinfectants and host immune factors depends upon protective mechanisms that are made possible through their strategy of communal co-existence[5]. One logical treatment strategy that has received attention, is to overcome biofilm-dependant antimicrobial resistance by causing the dispersal of the biofilm[7].

*By definition the term “biofilm” includes structures formed by bacteria, fungi and viruses; unless otherwise stated, the term biofilm as used in this study will describe a collection of surface-bound and/or mutually adherent bacterial microcolonies embedded in an exopolymeric substance.

This approach gained some credibility from reported evidence in a study which suggested that, when bacteria were dispersed from a biofilm they reverted to the independent planktonic phenotype and rapid restoration of antibiotic sensitivity occured[8].

A variety of bacterial biofilm eradication strategies have been employed with varying degrees of success, primarily in industrial and laboratory settings[9]. From amongst published industrial and experimental methods of biofilm dispersal, commercial biofilm-removing enzymes that act by degrading the exopolymeric substance (EPS) of the biofilm, appear to offer a potential solution to the clinical management of biofilm-related infections[10–12]. In addition, a growing body of *in vitro* laboratory-based studies lends further support to the notion that enzymes may offer a novel solution to the problem of persistent microbial infections when they are used either as the sole antimicrobial agents or as adjuncts to conventional antibiotic therapy[13–15]. The use of EPS degrading enzymes in the management of biofilm-related infections is not established in therapeutic medicine, and research into their role as antimicrobial agents is still in its infancy. The study of enzyme-based antibacterial and antibiofilm therapy is important as this mode of treatment appears to hold potential benefits to both clinical medicine and veterinary science; therefore, research into the future usefulness of enzyme therapy still deserves further attention[7].

Bacterial biofilms are heterogeneous and vary widely in structure and composition, ranging from single to multiple cell layers which may comprise of single or mixed bacterial species with a diverse and dynamic composition in EPS[16]. Because of this diversity, it is reasonable to assume that bacterial biofilms are more likely to undergo extensive degradation by the action of enzymes or enzyme mixtures which provide a wide range of substrate activity[16,17]. One such enzyme preparation is Pectinex Ultra SP-L (Pectinex®; Novozymes, South Africa), a widely used commercial enzyme complex that is derived from the fungus *Aspergillus aculeatus* and has both protease and carbohydrase activity[10,17]. Pectinex appears to have the potential to be of therapeutic benefit, as to date there are no publications that document any serious adverse effects that have occurred in either animals or humans. Furthermore, there are no documented *in vitro* cytotoxicity studies on the effect of Pectinex on animal or human tissues. Notwithstanding that there are no reports of toxicity; there is no literature where safety has been proven scientifically.

In this study the existing body of scientific literature on the management of bacterial biofilms is reviewed. Also, the safety and usefulness of Pectinex as a possible replacement and/or supplement to standard antibacterial therapy in the treatment of biofilms formed by

Staphylococcus aureus and *Pseudomonas aeruginosa* is assessed. In addition this study examines the cytotoxicity of Pectinex with regard to human epithelial cells (HeLa), lymphocytes and neutrophils. The motivational factor behind this study is that enzyme induced biofilm dispersal, as a therapeutic strategy, is still a virtually unexplored area of research. The potential outcome(s) of this study will contribute to the existing body of knowledge with the potential to make an impact on the future management of biofilm-related bacterial infections in clinical medicine and encourage further research.

CHAPTER 2

LITERATURE REVIEW

2.1 Early studies on surface-bound (benthic) bacteria

Towards the beginning of the 20th century, scientists became aware that bacteria, in their natural environment, preferentially adhered to solid surfaces. Their findings and subsequent publications were based on observations made from bacteria found in aqueous marine and industrial environments. A few of these studies are most frequently cited, and describe with clarity what we now accept to be a bacterial biofilm[18–21].

In 1933 Henrici observed the growth of a film of bacteria on the surface of sterile, glass microscope slides that were immersed into fresh water[18]. He noted that the bacteria were surrounded by an acellular matrix (*slime*) which developed within a few days and progressively thickened up to a point where individual bacterial cells could not easily be identified by light microscopy. The *slime* served as an adhesive ("*holdfast*") that held the cells together and attached them to the glass slide, such that they were not removed by running tap water. In a follow-up study Henrici and Johnson[19] described discrete bacterial colonies that grew as filamentous stalks enveloped by the adhesive acellular matrix and suggested that the matrix was produced by the bacteria. They regarded the production of this matrix as being unique only to certain species of bacteria and not a universal bacterial phenomenon. Furthermore, they hypothesised that newly formed cells from actively dividing bacteria in outer layers of the colonies either remained within the expanding colony or became detached and floated (or swam) away until they encountered a suitable surface to which they may attach and form a new colony.

Butterfield[21] examined flocculent matter in sludge samples from industrial sewage treatment plants and observed that they comprised of bacteria and a gelatinous matrix[21]. The bacteria isolated from the flocculants were of the same "type" which, when cultured, were eventually surrounded by an extracellular matrix (ECM). Butterfield[21] described it as: *"These bacteria were packed together usually in solid masses, sometimes with finger-like projections extending from the mass. These bacterial masses (flocs) were held together in a gelatinous matrix which appears to be a gummy exudate of the bacterial cell and may be likened to huge bacterial colonies suspended in a liquid medium."*

In 1943 Zobell[20] studied bacterial cultures in nutrient-enriched sea water and observed that the bacteria which grew in discrete micro-colonies were surrounded by an extracellular substance, and also firmly adhered to glass. He reinforced the suggestion that this extracellular substance was either part of, or a product of, the bacterial cell. In addition he observed that the rate of bacterial growth was exponential and not directly related to the concentration of nutrients. This led to the conclusion that not only did the bacteria multiply on solid surfaces but the surface attachment of bacteria also promoted further attachment of bacteria[20].

2.2 The biofilm

The period of relative quiescence into research on natural bacterial films (*slime*) ended during the 1970's when a renewed interest developed[22]. This interest was facilitated to a great extent by advanced developments in microscopy and microelectrode technology namely light microscopy, electron microscopy, microelectrode technology and confocal laser scanning microscopy (CLSM). Detailed visualization of bacteria has revealed that they adhere tenaciously to virtually all solid surfaces by means of complex hydrated polysaccharide and protein molecules that appear to extend from their cell surface. It is also appreciated that bacteria collect into colonies, some of which contain several different species, and amongst the bacteria within the colonies there is a high degree of functional differentiation and complex spatial organization[22,23]. As a result of these observations a biofilm has been defined as an interacting, organized, three-dimensional, structured community of single or multiple species of microorganisms (e.g. bacteria, archaea, fungi and algae) encapsulated within a self-developed, complex, polyionic, hydrated exopolymeric substance (EPS), that are adherent to a living or inert solid surface (substratum) and to each other, and exhibit an altered phenotype with regard to growth, gene expression and protein production[7,23,24].

Bacterial biofilms are ubiquitous in nature and form on the surface of virtually all living or dead tissue as well as inert material. They are hailed as being "*the most successful life form on earth*"[7,25]. The current consensus is that bacteria preferentially live as surface bound biofilms which affords the advantage of protection and survival in hostile environments, by providing a haven in which bacteria can thrive, multiply and disperse[6]. Over 99% of natural bacterial populations are found within biofilms whereas planktonic forms exist only transiently as a comparatively minor proportion of the overall population[26,27]. Biofilms also

occur at air-water interfaces and within water by way of their adhesion to microscopic debris[21].

The hardiness of bacterial biofilms is partly attributed to the physicochemical properties and metabolic functions of the EPS[25,28].Within EPS, the environment is characterized by different ecological niches which occur as a result of local differences in factors such as oxygen tension, nutrient availability and pH. EPS composition is not static but changes continuously in response to internal and external signals in order to maintain the well being of the biofilm[29]. Spatial and temporal orientation of bacterial cells in a biofilm depends on cell proliferation and cellular migration in response to external cues such as nutrient availability, as well as on self-generated intercellular signal molecules and the production of the EPS matrix[30]. Bacteria within a biofilm must constantly adapt to their dynamic local microenvironments. As a result of the latter they develop and maintain diverse and physiologically distinct subpopulations which vary phenotypically and genotypically. This heterogeneity increases the collective ability of biofilm bacteria to survive adverse conditions[31]. When compared to their free floating planktonic counterparts, biofilm bacteria show an increased resistance to physical shearing forces, ultraviolet (UV) radiation, dehydration, predation, viral infection, chemical biocides and antibiotics, which contribute to a decreased susceptibility to immune defense mechanisms during chronic infectious diseases[26,31].

2.3 The biofilm theory of disease

Over 150 years ago, Koch's postulates provided the basis for the acceptance of "*the germ-theory of disease*" which demonstrated that infectious diseases like anthrax, tuberculosis and dysentery were caused by microbiological agents[26]. The *"Germ Theory"*, which still remains the paradigm for the management of infectious disease, is based entirely on the identification of pathogenic species of planktonic bacteria (free-floating) cultured in nutrient-rich media. The theory declares that a specific clinical illness is caused by a single species of microorganism and that the appearance and behaviour of the pathogenic microorganism does not vary. An assumption is then made that effective antibiotic treatment restores health by eradicating the microorganism. However, scientists have encountered circumstances where the germ theory fails to offer a reasonable explanation. For example, microbiologists have described cases in which clinical specimens were "culture negative" although the bacteria were alive and visible under a microscope[32]. Equally perplexing, are cases in which infections

remain resistant to appropriate antibiotic therapy despite the proven antibiotic sensitivity of the isolated pathogenic microorganism(s)[5,32].

Recognition of the biofilm as the natural mode of bacterial life has led to the formulation of the *"biofilm theory of disease"* and a paradigm shift away from the *"Germ Theory"*. The *"Biofilm Theory"* acknowledges biofilm formation as a bacterial virulence strategy that enhances their ability to establish an infection, resist the onslaught of antibiotic treatment and evade host immune responses[33]. The inability of many biofilm bacteria to be cultivated *in vitro* is explained in part by their transformation into a dormant state[6]. The majority of bacterial infections are caused by normal commensals and species that are commonly found in the environment. Unlike the *"Germ Theory"* which advocates the presence of a single species, biofilm infections are understood to be caused either by a single species or by a mixture of species of bacteria[23].

It is now recognized that biofilms are involved in 65% to 80% of clinical infections and an appreciation of their importance has led to a multidisciplinary approach to the diagnosis and management of biofilm-related infections[26,34]. Disease causing biofilms may form on apparently healthy living tissues but preferentially develop on fragments of dead tissue (e.g. bone sequestra) and on inert surfaces such as medical implant devices[23,35]. Upon gaining a foothold at a suitable site, a complex interaction takes place between the invading bacteria and the host immune response. The result is a modified host environment and altered bacterial behaviour towards the biofilms' mode of growth[32]. Biofilm disease may remain clinically asymptomatic or manifest as mild, persistent, recurrent or chronic, with or without acute exacerbations. Treatment with antibiotics is usually instituted when symptoms occur in response to release of antigenic substances and planktonic cells from the biofilm. Unfortunately, the available arsenal of antibiotics is directed at the planktonic form and although therapy may reverse the symptoms it could fail to disinfect the biofilm. Infection caused by surviving microorganisms may recur at a later stage or immediately exacerbate when treatment is withdrawn[23,26,32]. Biofilm infections may also progress to a point where surgical intervention is indicated in addition to medical treatment. At surgery, infected tissues and/or contaminated medical implants are removed[32].

2.4 Biofilm life cycle and structure

Biofilm formation, growth and cell behaviour is a highly regulated process, from early attachment of planktonic bacteria, to growth of the biofilm and subsequent dissemination to

colonize new territories. An understanding of biofilm life cycle (formation, maturation and dispersal) and structure is required to facilitate the identification of potential antibiofilm targets which may help to formulate new strategies for the development of novel antibiofilm therapies. The stages in the life cycle of a biofilm are a dynamic overlapping series of processes influenced by numerous factors[29]. Species of bacteria continuously interact with each other and with bacteria of other species. This bacterial cell-to-cell communication is mediated through the secretion and detection of chemical signal molecules which accumulate in proportion to the bacterial population density; the process is known as *"quorum sensing"*. Through quorum sensing, bacteria are able to regulate biofilm growth and metabolic behaviour to the overall benefit of the biofilm and coordinate their response to local and external influences[36]. Although several studies have investigated the phenomenon of, and offered some explanation to, the surface binding by bacteria, the precise mechanisms involved are not fully understood and are still a subject of ongoing research[37]. The mechanisms that mediate adhesion between bacteria and solid surfaces are broadly classified into physicochemical, molecular and cellular interactions[38].

Stages of biofilm formation

The biofilm life cycle is generally described as occurring in three to five discrete stages with slight variations as to the exact definition of each stage (Figure 1)[39]. The stages of biofilm formation include surface conditioning, reversible bacterial attachment, irreversible bacterial attachment, maturation and dispersal[37,39,40]

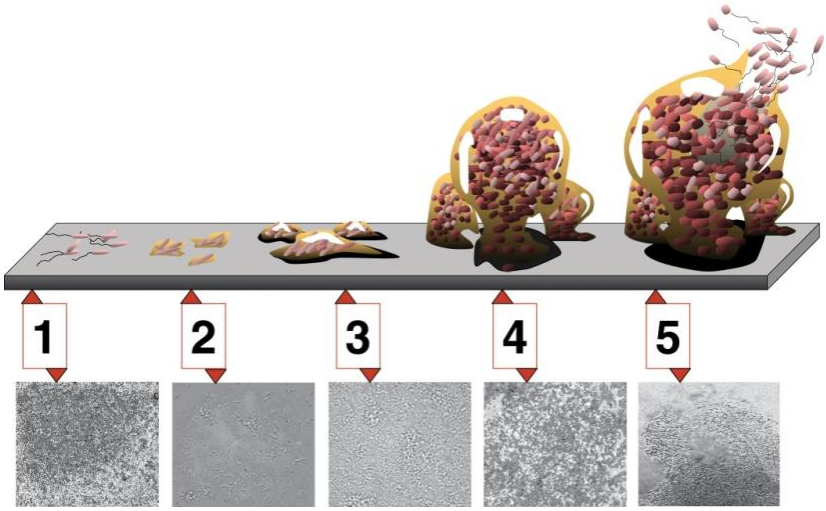

Figure 1: Five stages of biofilm formation paired with a photomicrograph of a developing *P. aeruginosa* biofilm (Stage 1, initial attachment; stage 2, irreversible attachment; stage 3, maturation I; stage 4, maturation II; stage 5, dispersion)[39].

(i) Surface conditioning

Surface conditioning is important with respect to inert surfaces, as a precursor to biofilm formation; biotic surfaces already exist in a state that is suitable for bacterial colonization[37,41]. During this process the substratum surface is hydrated and, organic and inorganic molecules that are present in the liquid phase are adsorbed[38]. Surface conditioning of inert substrata also occurs when bacterial cells from a pre-existing biofilm shear off and leave behind layers of surface-bound bacterial proteins. Conditioning alters the physicochemical properties (e.g. surface free energy, hydrophobicity and electrostatic charges) of the substratum which usually promotes the initial bacterial attachment although in some instances inhibition of bacterial attachment may occur[37,42,43].

(ii) Reversible bacterial attachment (physicochemical interaction)

The process of bacterial adhesion to a solid surface is initially reversible. This is thought to be due to weak physicochemical forces and is most often explained by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloid stability[44]. The theory is based on the assumption that bacteria in a liquid medium behave like perfectly spherical inert colloidal particles and attach to smooth surfaces according to physicochemical defined predictions. Initial contact between bacteria and the substratum is considered to be a random event during which planktonic bacteria are brought close to the substratum by non-specific forces that include Brownian motion, Van der Waals forces, bulk hydrodynamic forces and gravity. When the bacteria-substratum distance is less than 3 nm, specific short-range interactions become effective. The latter include chemical bonds (e.g. hydrogen bonding), ionic, dipole interactions and hydrophobic interactions[37,38]. The DLVO theory thus explains bacterial attachment as the net result of attractive Lifshitz-van der Waals forces and Lewis acid-base reactions, and repulsive electrostatic interactions between the microorganisms and the substratum. Two other theoretical models, namely the extended DLVO and microbial thermodynamic theory are further developments from the DLVO and take into consideration factors such as surface charge and hydrophobicity[44].

In some species (e.g. *Pseudomonas aeruginosa*) innate bacterial motility, chemotaxis, haptotaxis and temperature gradients also contribute in the movement of bacteria towards the substratum. However, none of the theoretic models offers an explanation for the biological, chemical and hydrodynamic factors that influence microbial transport and attachment[44,45]. Initial attachment to the substratum makes the following molecular-cellular phase of irreversible adhesion possible through the production of bio-polymers which 'glue' the microorganisms and their daughter cells onto the surface and to each other.

(iii) Irreversible bacterial attachment (molecular and cellular interactions)

The second process of bacterial adhesion is irreversible attachment to either an abiotic or biotic surface. This is a vital step towards the establishment of an infection by pathogenic bacteria. During biofilm formation, the reversible attachment of bacteria progressively transitions into an irreversible state in which there is a firmer adhesion of bacteria to the substratum. Bacteria-to-surface adhesion matures by way of an increase in the number and complexity of molecular-mediated bonds between specific bacterial adhesins and receptors of the extracellular matrix of the conditioning film or biotic tissue. Surface attachment triggers up-regulation of genes leading to transformation of bacteria from the planktonic to the sessile biofilm phenotype[46,47]. Intricate internal and environmental signals direct processes that consolidate surface adhesion through the production of EPS which then complex with receptor sites on the substratum. There is an increase in the production and secretion of EPS which results in selective bridging between the bacterial surface structures, which include the capsules, pili, flagella, and the exopolymeric substances.

The main contribution to irreversible adhesion is from exopolymeric substances that are secreted by attached microorganisms, which become incorporated into the conditioning film (or biotic tissue) and strengthen its adhesive properties. In addition to physicochemical forces encountered during the earlier phase, adhesion during this phase is also influenced by surface conditioning, the time period of bacterial exposure, bacterial concentration, the physical nature and chemistry the substratum, extracellular deoxyribonucleic acid (DNA), electrostatic interaction, hydrophobic interactions, steric hindrance, pH, CO_2, electrolytes and the presence of bactericidal agents[37,38].

a) Irreversible attachment of *S. aureus*

Species-specific differences exist between *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the manner in which the pioneer planktonic bacteria of each species gains an initial foothold onto the substratum. Since *S. aureus* is non-motile, physical forces are largely responsible for delivering the microorganisms to the surface of the substratum. Initial surface attachment of *S. aureus* to an abiotic surface is facilitated by a hydrophobic surface chemistry and by surface pre-conditioning[41,42]. Specific bacterial surface components known as adhesins mediate adhesion to conditioned inert surfaces, host tissues and to each other by participating in complex interactions with receptors in the extracellular matrix (ECM) and on adjacent cell surfaces. *S. aureus* possesses a large complement of both proteinaceous and non-proteinaceous adhesins that are responsible for both surface attachment and aggregation of bacteria[48]. *In vitro* and *in vivo*, many proteins are believed to serve as active and redundant sites to which *S. aureus* can bind, however, the full range of bacterial components, and the precise mechanisms involved, still remains unknown. Although a comprehensive discussion of adhesins is beyond the scope of this study, a few examples are mentioned to highlight the complexity of the involved processes.

The majority of *S. aureus* adhesins are proteinaceous adhesins that include covalently surface-anchored proteins of the microbial surface components recognizing adhesive matrix molecules family (MSCRAMM) and proteins that are surface-associated by different means, such as ionic or hydrophobic interactions[49]. In the pathogenesis of disease, MSCRAMM's are thought to play a major role in the initiation of cell attachment of *S. aureus*, by binding with specific receptors on fibronectin, fibrinogen, collagen and heparin-related polysaccharides in the ECM[41,42,49]. Other non-covalently surface-associated proteins include the autolysin-adhesins, proteins of the secretable expanded repertoire adhesive molecules family (SERAM) and membrane-spanning proteins[50,51]. Non-proteinaceous adhesins include the polysaccharide intercellular adhesin/poly-N-acetylglucosamine polysaccharide (PIA/PNAG), wall teichoic and lipoteichoic acids[52,53].

b) Irreversible attachment of *P. aeruginosa*

Pseudomonas aeruginosa bacilli are actively motile by means of a polar flagellum and numerous pili that protrude throughout their cell wall (Figure 2). They use their flagella to mediate near-surface swimming and surface-bound spinning movements, whilst surface-

bound horizontal crawling, vertical walking and *"slingshot"* actions are performed using pili[54,55].

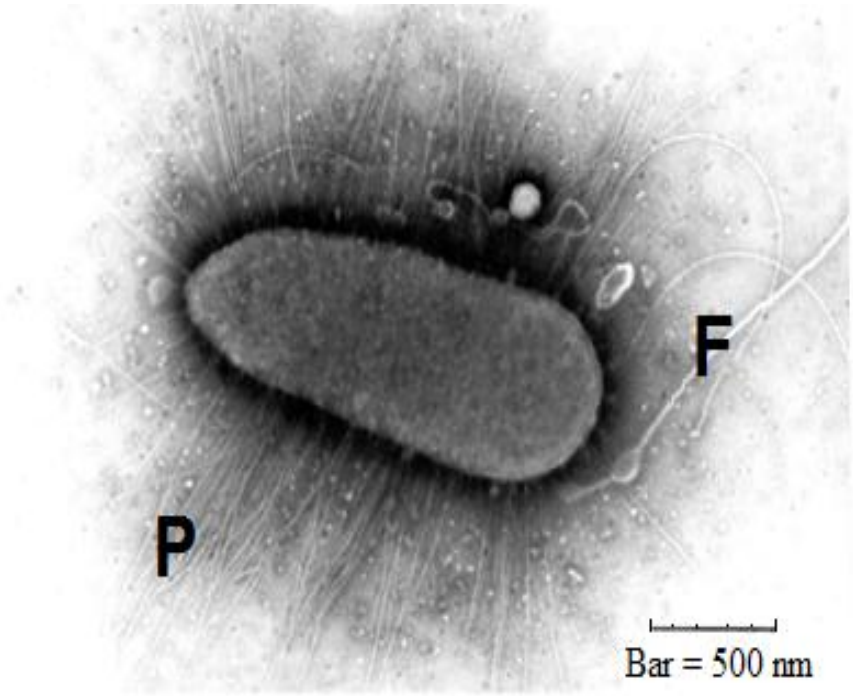

Figure 2: Negative stain electron micrograph of *Pseudomonas aeruginosa* showing flagellum (F) and pili (P)[56].

At the substratum surface, the bacilli interchange between the various modes of motility and actively traverse the substratum in a semi-attached condition which is independent of the direction of fluid bulk flow[55,57]. However, in some cases, irreversible adhesion has been shown to occur after only seconds of surface contact[44]. The presence of a conditioning film favours the attachment of *P. aeruginosa* by inducing specific responses such as chemotaxis towards the substratum and attachment to specific surface receptors[58]. After a suitable attachment site is selected, attachment subsequent seems to be effected through mechanisms that involve the secretion of polysaccharides along with interactions with adhesins located on the cell wall, flagella and pili. Type IV pili and pilus-associated adhesins have been shown to enable *P. aeruginosa* to bind readily to surfaces including abiotic surfaces devoid of a conditioning film[59]. Other studies highlight the importance of the flagella by demonstrating that mutant strains defective in flagella-mediated motility are not able to form biofilms[57].

In response to stimuli at the substratum surface and to cell-cell interactions, *P. aeruginosa* produces and secretes several different types of extracellular polysaccharides, of which three namely alginate, polysaccharide synthesis locus (Psl)-dependent polysaccharide and pellicle

polysaccharide (Pel), play a crucial role in facilitating surface attachment[60–62]. Psl and Pel polysaccharides are also expressed on non-biofilm, planktonic *P. aeruginosa* cells[63]. Most environmental and clinical isolates of *P. aeruginosa* secrete either Pel or Psl, whereas alginate is only produced in the lungs of cystic fibrosis patients[64,65]. Psl polysaccharide is essential during the early stages of biofilm formation. It is anchored on the cell surface in a helical pattern where it promotes cell-surface interactions, cell-cell interactions and the assembly of a Psl matrix which binds the bacteria within the biofilm and onto the substratum[61]. The function of Psl in attachment is independent of both alginate and Pel[66]. Pel promotes cell-cell interactions which enable *P. aeruginosa* to form thin biofilm layers (pellicles) at air–liquid interfaces. Alginate synthesis is not essential for biofilm development; both alginate rich and deficient *P. aeruginosa* form morphologically similar biofilms[63].

(iv) Biofilm maturation

The phase of biofilm maturation is characterized by the generation of a complex architecture of channels and pores, the formation of microcolonies and a redistribution of bacteria away from the substratum[29]. The microorganisms enter an "accumulative phase" of biofilm formation which involves cell-to-cell interactions and the formation of cell aggregates on the substratum. Continued EPS production along with cell multiplication of adherent bacteria, further recruitment of planktonic bacteria and dynamic spatial orientation leads to the formation of a monolayer and subsequently to more complex multilayered structures. The initial microcolonies are formed mainly by growth of surface attached cells with a lesser contribution by continued planktonic cell aggregation[67,68]. CSLM examination of biofilms revealed that the typical ultrastructure of a mature biofilm is a heteromorphic, three-dimensional, hydrated structure which consists of clusters, pillars and mushroom-like projections of heterogeneous EPS. The latter contain bacterial microcolonies which are permeated by an elaborate network of water-filled channels[69,70]. Other structural forms have been known to develop as a consequence of environmental conditions (e.g. hydrodynamic forces)[29]. CSLM also revealed that within the microcolonies, the bacteria are arranged such that there is a central core of viable bacteria with non-viable bacteria occupying the outer layer with projections into the core[71]. The microcolonies were found to be separated by acellular voids which serve as channels through which water and nutrients flow. These water channels are formed by coalescence of the overhanging canopies of the bacterial stalks and possibly by controlled cell death and enzymatic EPS degradation from within cell dense areas of the biofilm[72].

a) The role of quorum-sensing (QS)

In addition to the influence of local environmental factors and cellular responses, the development and behaviour of a mature multicellular biofilm is highly regulated by a range of specific cell-to-cell communication activities that operate under conditions of high cell density[73]. A cell-to-cell communication mechanism known as quorum-sensing has been found to play an important role in biofilm formation. Quorum-sensing is defined as a process in which gene expression is regulated, in response to fluctuations in cell-population density, and mediated through the release of self-produced chemical signal molecules called autoinducers[74]. The concentration of the autoinducer increases concomitantly with bacterial cell density, and upon reaching a critical threshold, when a sufficient number of cells are present, the bacteria respond to the signal by altering the expression of target genes. As a result of this change, a wide range of bacterial cell functions are affected including biofilm formation, biofilm cell density, cell activity (e.g. EPS production, motility and viability), symbiosis and virulence[74,75]. However, because quorum-sensing requires a sufficient density of bacteria, these signals may be involved in biofilm differentiation of formed biofilm and not in the initial stages of bacterial attachment and proliferation[73].

The full spectrum of autoinducer (AI) molecules associated with quorum-sensing is diverse and still expanding; however, the majority of known AI's are classified into four main signalling systems namely; acylhomoserine lactone (AHL) systems, oligopeptides, LuxS/AI-2 and LuxS/AI-3-epinephrine-norepinephrine[75–77].

i) Acylhomoserine lactone (AHL) systems

The regulation of AHL quorum-sensing in bacteria is most commonly mediated by two proteins belonging to the family of LuxR and LuxI homologs[78]. LuxI-type proteins are cytoplasmic synthases responsible for the production of a range of AHL signalling molecules, and LuxR proteins are transcription factors which bind with AHL's to effect the transcription of a variety of target genes[75]. AHL systems are associated with intraspecies signalling by several Gram-negative bacteria including *P. aeruginosa*[77].

ii) Oligopeptides

Gram-positive bacteria primarily use peptide signalling systems[75,76]. The signalling molecules are small peptides derived from oligopolysaccharides, which are detected by

receptor kinases at threshold concentrations and which in turn catalyse the activation of specific response regulators. This two-component signalling system ultimately leads to gene transcription[75].

iii) LuxS/AI-2

The LuxS/autoinducer-2 (AI-2) quorum-sensing system is shared by both Gram-positive and Gram-negative bacteria and involves the production of AI-2 by LuxS (AI-2 synthase). LuxS is widely distributed among bacteria, which may suggest that AI-2 is used for interspecies communication.

iv) LuxS/AI-3-epinephrine-norepinephrine

In the LuxS/AI-3 system, the autoinducer-3 (AI-3) activates the transcription of genes involved in the production of adhesins and modulation of flagella function; both of these functions have the potential to enhance biofilm development. AI-3 is involved in interspecies signalling among intestinal bacteria and may also participate in bacteria-host communication by way of agonistic cross-stimulation of receptors between AI-3 and the mammalian hormones, epinephrine and norepinephrine[79].

In general, Gram-negative bacteria use the LuxR/I signalling system to communicate, Gram-positive bacteria use oligopeptides and the LuxS/AI-2 system is common to both. The ability to communicate with one another allows bacteria to coordinate gene expression thereby regulating biofilm growth and the behaviour of the entire community within and between bacterial species[74,75].

b) Nanotube cell-to-cell communication

Knowledge of the complex nature of cell-to-cell interaction has advanced further with the revelation, by electron microscopy, of a previously undescribed type of bacterial communication that may involve the transfer of cytoplasmic molecules through a network of variously sized nanotubes that interlink adjacent cells. Researchers have suggested that, by providing a conduit for exchange of cellular molecules within and between species, these nanotubes may represent an important form of bacterial communication[80].

c) Maturation of *S. aureus* biofilm

S. aureus produces a multilayered biofilm embedded within a chemically heterogeneous EPS, that it produces in a phase-variable manner (Figure 3). The growth and maturation in *S. aureus* biofilm is largely dependent upon adhesins that promote adhesive interactions between the bacterial cells. Although a number of polysaccharide and protein intercellular adhesins (*ica*) have been described, there is a general consensus that the main determinant of the accumulation phase of staphylococcal biofilm formation is PIA/PNAG. Evidence in support of this notion is derived from *in vitro* studies which have demonstrated that *S. aureus*, possesses the *ica* gene locus, which encodes functions required for PIA/PNAG synthesis and, deletion of the *ica* locus results in a loss of the ability to form biofilms, to produce PIA/PNAG, or mediate *N*-acetylglucosaminyltransferase adhesin activity[46,52]. Despite their importance, both the *ica* gene locus and PIA/PNAG are not absolute requirements for the process of *S. aureus* biofilm formation as *ica*-independent biofilms are also formed in the absence of *ica* expression[48,81]. A recent study found that the *ica* gene was not expressed by all strains of *S. aureus* and was present in about 51% of clinical isolates. Furthermore, the study found that some isolates carried the *ica* gene but were unable to form a biofilm[82].

Biofilm associated proteins (Bap) are a group of surface active proteins which are important during the development of a *S. aureus* biofilm[81,83]. The protein is regulated by the *bap* gene locus and its transcription induces strong biofilm formation by an alternative PIA/PNAG-independent mechanism even in the absence of the *ica* locus[84]. Recently, evidence has emerged that suggests that Bap is not found in all *S. aureus*, strains and that the *bap* gene is expressed in less than 50% of clinical isolates[82].

Extracellular DNA (eDNA) is another important constituent of *S. aureus* biofilm. It accumulates in the EPS, as a result of controlled bacterial cell lysis, where it contributes towards the structural support and adhesive properties of the biofilm matrix[72,85,86]. In addition, a number of other genetic regulators (e.g. staphylococcal accessory regulator, accessory gene regulator, staphylococcal respiratory response regulator and a nuclease gene) have also been implicated in *ica*-dependent and non-*ica* dependent biofilm formation; however, their specific roles are not fully understood[24,46,52,87].

The accessory gene regulator (*agr*) is a quorum-sensing cluster of genes that regulates biofilm formation and virulence in *S. aureus*. *S. aureus* strains are known to have four types of *agr*

systems, *agr*-I through to *agr*-IV, each of which recognizes a unique corresponding autoinducer (AIP-I to AIP-IV)[88].

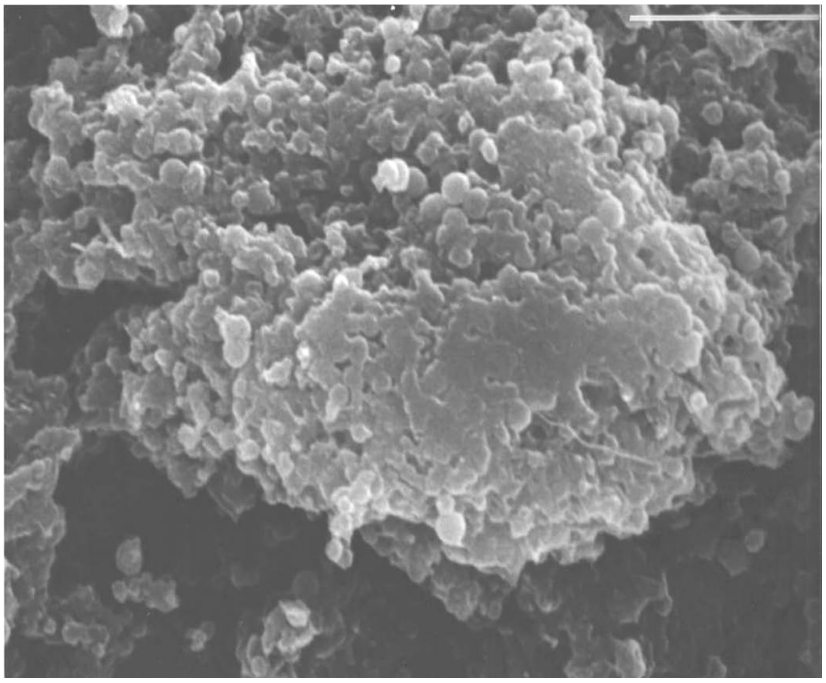

Figure 3: Scanning electron microscopy (SEM) image from an implant recovered at five days showing massive numbers of cocci partially occluded by dehydrated material. Bar = 5 μm[24].

The *agr* system is activated in response suprathreshold concentrations of an extracellular peptide called autoinducing peptide (AIP). *Agr* activation up-regulates secretion of pathogenic virulence factors (e.g. haemolysins, super-antigens and tissue degrading enzymes) while down regulating production of surface adhesins[89]. Alpha-haemolysin is one of the downstream products regulated by the *agr* system that is known to promote biofilm formation. Mutant strains defective in alpha-haemolysin production do not form biofilms and exhibit an apparent defect in cell-to-cell interactions[90]. Also involved in the development of *S. aureus* biofilm is a regulatory gene locus known as staphylococcal accessory regulator (*sarA*), which controls *agr*-regulated pathways and the *ica* operon. *SarA* and *agr* have opposing roles in that the induction of *sarA* or inhibition of *agr* results in increased biofilm formation[91]. However, the effects of the *agr* system on *S. aureus* biofilm development are not definitive and vary under the influence of environmental factors. Studies have demonstrated that, repression of *agr* may have no discernible influence on biofilm formation in some instances, while under other conditions it could either enhance or inhibit biofilm formation[76,88].

d) Maturation of *P. aeruginosa* biofilm

P. aeruginosa initially adheres to a substratum as a monolayer; this is followed thereafter by the rapid formation of microcolonies consisting of between 30 and 100 cells. Microcolony formation occurs largely as a consequence of cell division by non-motile bacteria with only a minor contribution from recruitment of planktonic bacteria[30,92]. At the same time, motile *P. aeruginosa* bacteria spread over the substratum by means of twitching motility and eventually settle at other sites[57]. Depending on conditions, *P. aeruginosa* can form biofilms that are either flat multilayer structures or microcolonies that have defined 3-dimensional arrangements[61,93]. The formation of mushroom-shaped microcolonies is achieved through colonization of initially pillar-shaped microcolonies with bacteria from the motile subpopulation that subsequently form a mushroom 'cap' at the top of the pillars (Figure 4)[30]. Voids ("hollowed-out" cavities) are created inside the large (>80 µm diameter) microcolonies during biofilm differentiation as consequence of endogenous phage-mediated cell death[94,95]. As the biofilm matures further, the bacilli differentiate into two distinct phenotypes; highly motile cells within the voids and non-motile cells which constitute the outer wall of the colony[95]. The microcolonies are typically separated by acellular fluid-filled channels and a matrix envelope composed of polysaccharides, eDNA and proteins[29,96].

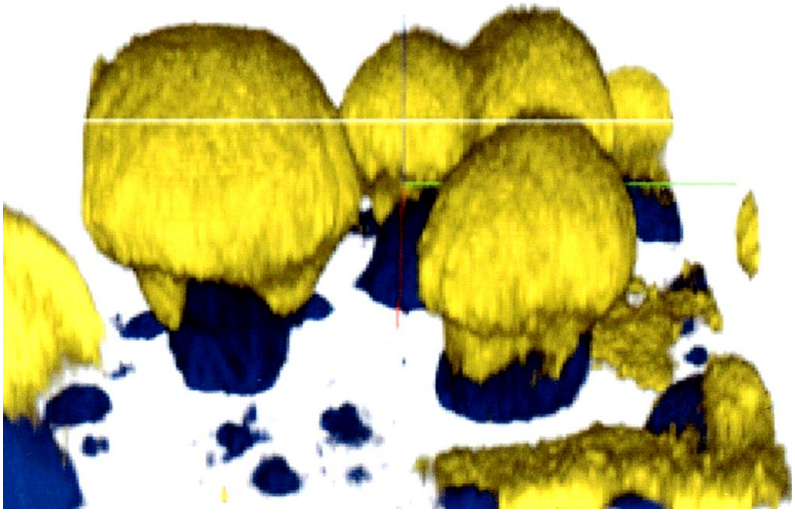

Figure 4: Three-dimensional reconstructions of *P. aeruginosa* biofilms formed in a flow cell reactor after an initial 1:1 inoculation of the system with yellow fluorescent protein-tagged wild-type strain PAO1 and a cyan fluorescent protein-tagged *pil*A mutant defective in type IV-mediated twitching motility. The stalks of the biofilm mushroom structures were formed by clonal growth of the non-motile *pil*A mutant. The caps of biofilm mushroom structures were formed by the wild-type strain, which migrated up the stalks of the *pil*A mutant (Magnification, ×400)[96].

The various exopolysaccharides and eDNA interactively influence the architecture of *P. aeruginosa* biofilms (Figure 5). Continuous production of the Psl polysaccharide has been shown to be essential for both the increase in biofilm biomass and the maintenance of an existing biofilm structure. Loss of Psl expression has been reported to result in cessation of biofilm growth and degradation of the existing biofilm structure[61,97]. Secreted Psl polysaccharide matrix acts by promoting further attachment of planktonic bacteria onto the substratum and by recruitment of bacteria into the microcolonies. Formation of characteristic mushroom-shaped microcolonies requires both Psl and alginate. In addition, Psl serves to maintain the 3-dimensional structure of the microcolony as well as the surface adherence of the biofilm. During biofilm maturation, Psl accumulates, mainly at the outer periphery of the microcolonies, resulting in a Psl matrix-free cavity at the centre of the microcolony in which planktonic cells are found along with dead cells and extracellular DNA (eDNA)[61,66]. Pel is a glucose-rich polysaccharide adhesin that is required for cell aggregation and it provides the primary structural support in some biofilms. It affects the biofilm cell population and intercellular proximity within the biofilm. Unlike Psl, continuous Pel production is not essential for either surface attachment or the structural maintenance of a mature biofilm. However, inhibition of Pel expression has been found to result in a biofilm that fails to increase in size without altering the existing architecture[63,97].

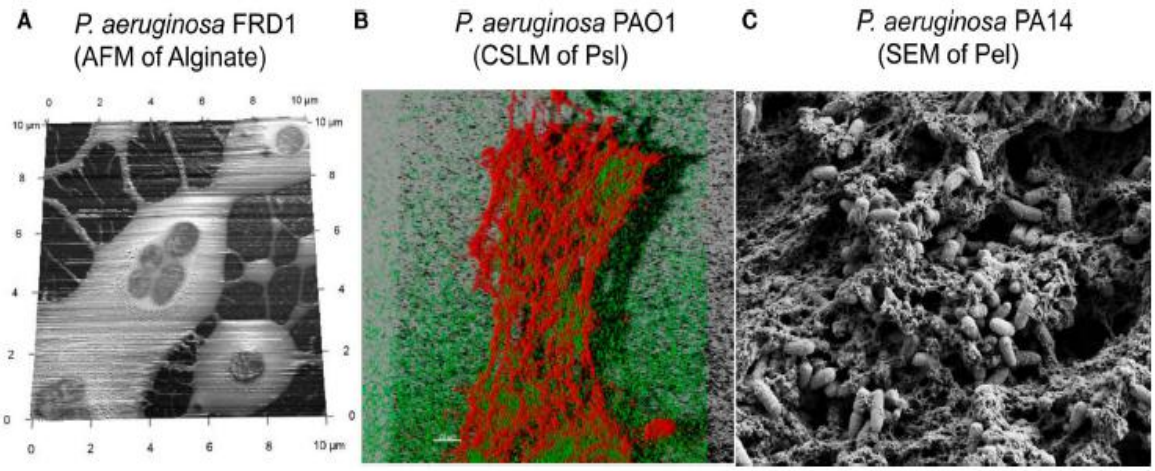

Figure 5: Extracellular polysaccharides of *P. aeruginosa* visualized using three approaches: (A) atomic force microscopy, showing alginate as a soft loosely adherent polymer that surrounds the cells, (B) confocal laser scanning microscopy (CLSM) image of hydrated *P. aeruginosa*, cultured as a pellicle, with the *P. aeruginosa* cells expression the green fluorescent protein and the Psl polysaccharide counterstained with CellMask Orange, (C) SEM image of *P. aeruginosa* PA14, cultured as a pellicle. The image shows the extracellular matrix which includes Pel and a fabric-like matrix that surrounds and connects the cells that form a microbial mat at the air-water interface[64].

P. aeruginosa has at least two AHL-based quorum-sensing systems, namely *las*R-*las*I and *rhl*R-*rhl*I, that function through the autoinducers N-(3-oxododecanoyl)-l-homoserine lactone and N-butyryl-l-homoserine, respectively[73,77]. In the former system, *las*I synthesizes the autoinducer and *las*R acts as the transcriptional regulator. In the latter, *rhl*I and *rhl*R are the synthase and transcriptional regulators, respectively. *Las*R-*las*I quorum-sensing is involved in the development of *P. aeruginosa* biofilms. Secretion of the respective AHLs from a biofilm enhances the proliferation of neighbouring cells that are in contact with surfaces and promotes their transformation into biofilm communities[98]. Strains deficient in N-(3-oxododecanoyl)-l-homoserine lactone are not able to initiate normal biofilms but form thin, flat, undifferentiated biofilms[73]. In addition to the two AHL's, P. *aeruginosa* also produces a third signalling molecule, 2-heptyl-3-hydroxy-4-(1H)-quinolone, known as the Pseudomonas Quinolone Signal (PQS). This signal appears to be involved in the later stages of biofilm maturation. PQS deficient strains have been found to make mature biofilms but with an altered structure that lacks normal water channels[92]. Although the contribution of the *rhl*R-*rhl*I quorum-sensing system in biofilm formation has not been clearly established, it has been observed that the *las*R-*las*I system exerts both an excitatory and inhibitory control over the *rhl* quorum-sensing system, whilst the alginate biosynthetic gene, *alg*R, modulates suppression[99,100]. The conclusion drawn form these observations is that specific repression of *rhl* during biofilm growth may be a requirement for normal biofilm formation[99].

(v) Biofilm dispersal

Bacteria live in the environment as sessile surface-bound biofilms. As a composite part of their propagation and survival, they are obliged to disperse from established biofilms and colonise new locations. The process of bacterial biofilm dispersal is divided into three phases namely detachment of cells from the biofilm, translocation of the cells to a new location and attachment of the cells to a substrate at the new location[7].

Many authors, however, limit their descriptions of dispersal to the process of cell detachment [39]. Detachment* is a generalized term used to describe the release of cells (either individually or in groups) from a biofilm or substratum[101]. In the process of dispersal (or detachment), single cells and variable-sized clusters of bacteria detach either directly from the surface, leaving bare spots, or detach from other bacteria and leave behind a layer of cells and

*In this review, the term "*dispersal*" is regarded as being synonymous with "*detachment*" the two terms are used interchangeably.

EPS[102,103]. The size of a detached bacterial cluster affects the phenotypic characteristics of the organisms within the cell aggregate. Relatively large clusters tend to retain their biofilm characteristics e.g. antimicrobial resistance, whereas single cells that have been shed will quickly revert to the planktonic phenotype[102,104].

Five distinct modes of biofilm dispersal have been described: erosion, sloughing, abrasion, swarming and seeding:

- Erosion refers to the continuous release of single cells and/or small clusters of cells from a biofilm at low levels over the course of biofilm formation[7,103].
- Sloughing is characterized by sudden detachment of large portions of mature biofilm[7].
- Abrasion is a process in which cells from a biofilm are dispersed after collision between particles in the bulk fluid and the biofilm (i.e. hydrodynamic shearing)[104].
- In swarming, the bacteria spread, in a surface-based creeping fashion, over solid and semi-solid substrata, leading to rapid colonization of the surfaces of suitable environments[55,67].
- Seeding occurs from cavities in the interior of the biofilm where bacterial cells are actively freed from cohesion between each other, and from adhesion to the substratum, to be released in large numbers of single cells or small clusters of cells[95].

Erosion and sloughing may have both active (i.e. metabolic) and passive (i.e. physical, non-metabolic) components, whereas abrasion is an entirely passive processes, with swarming and seeding being active[7]. During swarming and seeding, bacteria undergo phenotypic transformation from the sessile surface-bound state to the free-swimming planktonic form[95].

Although the mechanisms that underlie or facilitate the processes of bacterial dispersal are not well understood, they are considered to be genetically controlled and to involve enzymatic degradation of biofilm EPS, increased cell motility and programmed cell death[7,55,72,105]. A wide range of internal biofilm signals and external environmental factors are known to trigger and/or mediate the dispersal of *S. aureus* and *P. aeruginosa* biofilm. Some of the biofilm dispersing agents that have been studied include antibiotics[106], detergents and disinfectants[107], chelating agents[108,109], signalling molecules[76,91,110], surfactants[17], enzymes (hydrolases, proteases and DNase)[10,11,111], nutrient availability[112], nutrient deprivation[88], nitric oxide[113], and ultrasound[114].

a) Dispersal of *S. aureus* biofilm

Mature *S. aureus* biofilm spreads to new locations by detachment and re-attachment of variable size biofilm clusters (small: 2 to 10 cells, medium: 11 to 100 cells and large: 101 to 1000 cells), detachment and remote site re-attachment of single cells, continuous outward growth of bacterial cell clusters and rolling motility (velocity ≈ 1 μm/min) along the surface by cell clumps of various diameters[102].

The relative contribution of each mode of biofilm cell detachment has been studied *in vitro*, where 18 to 44% of the detached particles were found to be single cells, whilst 23 to 45% were from fragments containing more than 10 cells[102]. The single cells and small clusters were found to detach with an equivalent frequency to medium and large emboli, whereas the larger aggregates were observes as containing a higher fraction of the total detached biomass[103].

Naturally occurring dispersal is regulated through quorum-sensing[36]. Activation of the *agr* quorum-sensing system, along with its associated increase in extracellular protease activity, is considered to be central to the detachment of established *S. aureus* biofilms. This is supported by the *in vitro* finding that activation of all *agr* systems (*agr*I to *agr*IV) by exogenous autoinducing peptides, glucose depletion or by inhibition of the *sar*A gene locus, results in biofilm detachment in laboratory strains and clinical isolates[88,91]. Several other staphylococcal regulatory genes that influence the *agr* regulatory pathway, have been identified. However, their contribution towards biofilm dispersal is not clearly established[115,116].

In response to cell density-induced signals, a *nuc* (nuclease) gene that is highly specific for *S. aureus* encodes a thermostable nuclease, called Staphylococcal thermonuclease. The substrate for this enzyme is eDNA, which is an important structural component of the Staphylococcal biofilm[86]. Furthermore, eDNA contributes several key functions that include cell-surface adhesion, cell aggregation, cell-cell cohesion and horizontal gene transfer between biofilm cells[25,117]. The degradation of eDNA by thermonuclease could be one of the mechanisms involved in the disintegration and dispersal of *S. aureus* biofilm[86,87].

b) Dispersal of *P. aeruginosa* biofilm

Mature biofilms of *P. aeruginosa* exhibit a distinct and particularly dramatic form of seeding dispersal, accounting for about 50 to 80% of the detached biofilm[95,102]. The process is

characterized by evacuation of numerous motile single cells from clusters contained in voids at the interior of microcolonies. The cells at the periphery of microcolonies appear to become motile and highly agitated as a prelude to dispersal, after which, they escape through local 'breakout' points at random locations in the outer walls of the colonies[94]. After seeding, the emptied voids do not replenish themselves with new cells nor do they exhibit further growth in size[95]. Only a small proportion of detached cell clusters, about 1 to 3%, consist of aggregates with over 10 cells[102]. Detachment from the substratum is mediated by flagella and pili which interact in a well-coordinated sequence to change the orientation from horizontal to vertical and then detach; vertical orientation appears to facilitate surface detachment[55]. *P. aeruginosa* also disperses by migrating over the substratum. Surface translocation of bacilli to new sites, away from their inoculation point, is achieved through twitching, swimming and swarming motility[54,55,67].

The innate mechanisms that regulate dispersal in *P. aeruginosa* biofilm are still unclear and the study of these mechanisms is ongoing. Research has unveiled numerous interrelated factors that are associated with biofilm dispersal which include EPS matrix degrading enzymes, changes in the intracellular levels of cyclic diguanosine monophosphate (c-di-GMP), activation of genes responsible for cell motility, lytic bacteriophages, changes in nutrient levels and changes in microbial growth status[7].

In vitro studies suggest that the matrix-degrading enzymes are involved in active biofilm dispersal[118]. Mucoid strains of *P. aeruginosa* produce alginate polysaccharide and alginate lyase, an enzyme that degrades alginate[64,118]. Alginate is important in that it promotes surface attachment, contributes to the structure of the biofilm EPS matrix and is required to efficiently retain the bacteria with the biofilm[58]. When expressed by the alginate lyase gene (*alg*L), the enzyme causes enzymatic degradation of alginate which disrupts the EPS structure and induces biofilm dispersal through sloughing of cells[118].

The intracellular nucleotide, c-di-GMP, is a ubiquitous second messenger found in many bacterial species, where it is known to influence a wide range of physiological processes[119,120]. Intracellular c-di-GMP levels may be increased or decreased by the antagonistic activities of diguanylyl cyclases and phosphodiesterases, respectively[121]. It has been shown that these changes in c-di-GMP levels influence bacterial choice of lifestyle in *P. aeruginosa*; accumulation promotes biofilm formation and sessile mode, while a decrease favours a planktonic existence and biofilm dispersal[119,120]. Much attention has been drawn to the involvement of secreted quorum-sensing signals and a central biofilm-regulating role has

been ascribed to c-di-GMP[95,120,122]. Elevated levels of c-di-GMP stimulate production of EPS and adhesins, reduce cell motility, up-regulate virulence factors and induce biofilm formation. Low levels of c-di-GMP have the opposite effect and trigger biofilm dispersal (Figure 6)[120,121].

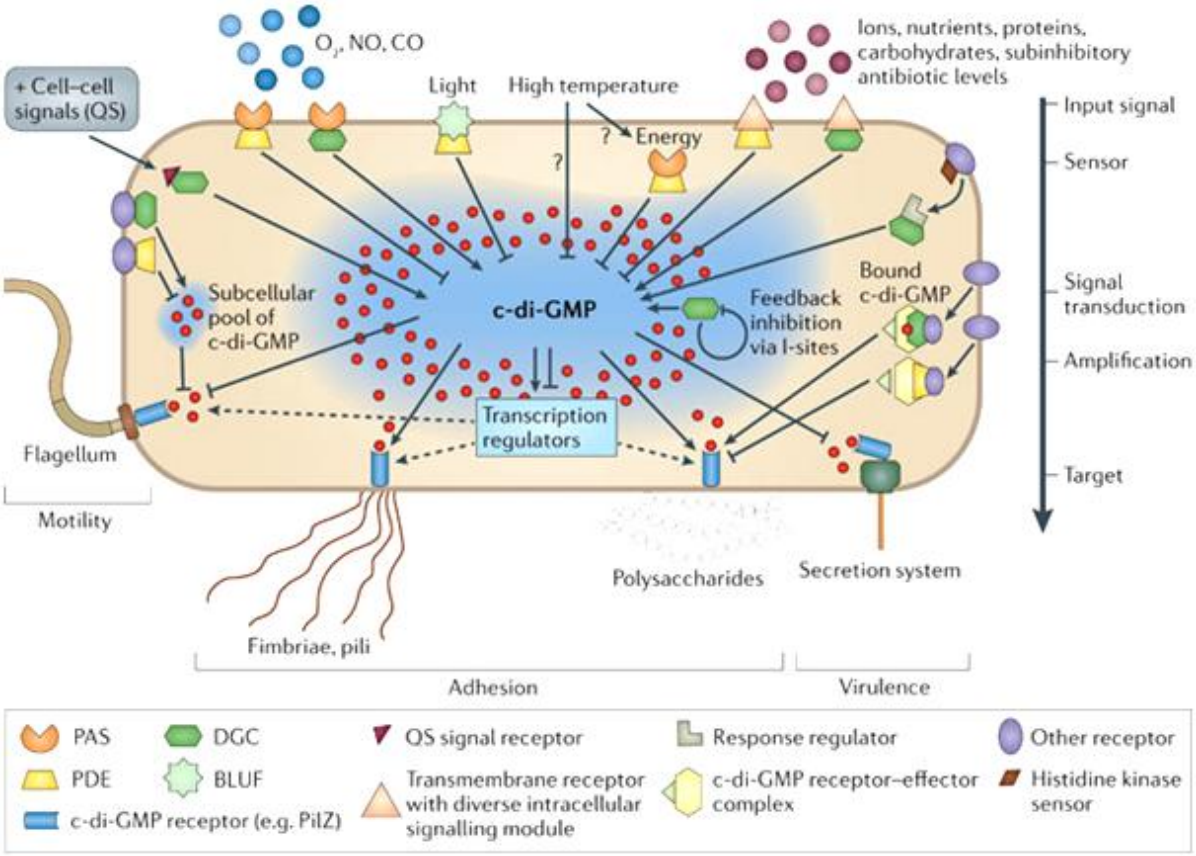

Figure 6: Cyclic di-GMP and the regulation of biofilm dispersal[121].

P. aeruginosa is a facultative anaerobe which, during anaerobic metabolism, utilizes nitrate, nitrite and nitrous oxide as terminal electron acceptors through the activity of nitrite reductase enzyme. Nitric oxide free-radicals ($NO^{\bullet}$) are generated as either a by-product or product of the anaerobic metabolism[123]. Recent studies have demonstrated that $NO^{\bullet}$ is able to induce biofilm dispersal at concentrations that are non-toxic to *P. aeruginosa*, by stimulating phosphodiesterase activity which catalyses the degradation of c-di-GMP thereby lowering its levels and activity and leading to biofilm dispersal[95,113,122]. A study by Morgan *et al.* identified a gene locus, named *bdl*A (biofilm dispersion locus), that encodes a protein; *bdl*A-protein[110]. The study demonstrated that *bdl*A deficient biofilms possessed increased intracellular levels of c-di-GMP, exhibited increased adherent properties and lacked the ability to disperse. An assumption was made that sufficient levels of *bdl*A were likely to have the opposite effect and result in biofilm dispersal associated with low c-di-GMP levels. It was

also noted that *bdl*A protein possessed several domains which are essential for responding to environmental signals. Also biocide-induced dispersion in *bdl*A deficient mutants was found to be independent of cell motility. These observations prompted the authors to suggest that *bdl*A may act as a link between environmental stimuli and c-di-GMP levels in bringing about cell detachment.

Rhamnolipids are a class of glycolipid biosurfactants, produced and secreted by *P. aeruginosa*, that are essential for the formation of water channels and hollow cavities within the biofilm and for dispersal of mature biofilms[7,124,125]. Some authors report that, while rhamnolipids may be involved in the maintenance of biofilm architecture in *P. aeruginosa*, they do not appear to be required for seeding dispersal[95]. The biosynthesis of rhamnolipids, in response to genetic and environmental signals, is under the control of the *rhl*R-*rhl*I quorum-sensing system and is catalysed by genes (e.g. *rhl*AB and *rbd*A) that encode a rhamnosyltransferase[95,124,126]. The *rbd*A locus of genes in particular has also been found to cause c-di-GMP degradation, up-regulate swarming and swimming motility and to down-regulate EPS production[124]. In addition to quorum-sensing, *in vitro* studies have demonstrated that the production of rhamnolipids is induced by the deprivation of a selection of nutrients including nitrogen, phosphate, magnesium, calcium, potassium, sodium, iron and trace elements[127]. Seeding dispersal in *P. aeruginosa* biofilms is facilitated by rhamnolipids through a mechanism that involves the formation of hollow cavities filled with motile planktonic cells and the rapid evacuation of these cavities[124,125]. In addition, rhamnolipids lower the surface tension and alter the surface charge of both the substratum and the cell surface, through their biosurfactant properties[127]. It is postulated that the induced change in the physical properties of the surfaces promotes swarming motility which may serve to foster detachment. Furthermore, it is suggested that rhamnolipids could facilitate dispersal by acting directly to solubilise components on the biofilm matrix and to disrupt cell surface structures that act as adhesins[7,125].

An extracellular unsaturated fatty acid that is produced and secreted by *P. aeruginosa*, that was recently identified is cis-2-decenoic acid. It accumulates in a cell-density dependent manner and induces dispersion and inhibition of growth in *P. aeruginosa* biofilm and other Gram-negative and Gram-positive bacteria[128]. It is thought that this agent serves as an extracellular cell-to-cell signal whose actions are mediated by c-di-GMP[121,128].

Both nutrient excess and nutrient deficiency can induce dispersal of *P. aeruginosa* biofilm. The genetic locus of control that is essential for nutrient-induced dispersion is the *bdl*A gene.

The bdlA gene is considered to be a chemotaxis gene that regulates the dispersion response by sensing chemical gradients or changes in the presence of nutrients[110]. High nutrient levels (increase in the availability of succinate, glutamate or glucose) were found to cause an estimated 80% reduction in biofilm biomass[112]. Deficiency of common ions and trace elements may also act as triggers for dispersal[127].

2.5 The chemical composition of bacterial exopolymeric substance (EPS)

Although bacterial EPS has been studied extensively, knowledge of the chemical composition remains ill defined. Bacterial EPS composition varies from species to species, and within each species in accordance with the metabolic requirements and the genetic expression of the microorganism. In general, EPS accounts for the greater part of the biofilm and contributes 85-98% of the total volume, with bacterial cells accounting for the remaining 2-15%[29]. The EPS is well hydrated and up to 97% of its volume consists of water[129]. The EPS that surrounds bacteria was originally described as a homogeneous gel consisting of a random arrangement of hydrated polysaccharides fibres that served as an adhesive, to attach the bacteria to solid surfaces and to each other. It was initially thought that each species produced a limited range of polysaccharides[22]. However, subsequent studies on environmental and laboratory biofilms have that the preponderance of polysaccharides in EPS is not a universal finding[11,130]. Bacteria develop an EPS matrix by producing a vast and complex dynamic array of insoluble gelatinous extracellular polymers from different chemical groups; these include a wide variety of proteins, glycoproteins, glycolipids and extracellular DNA[70,131,132]. Microbial cells, cell debris and cell appendages (e.g. fimbriae, pili, and flagella), outer membrane proteins and inorganic molecules are also present as part of the biofilm matrix[25,29]. Polysaccharides and polyamides (proteins) are the main structural exopolymers in EPS and function as a scaffold onto which many of the other constituents are attached[11,29]. In addition, the lipids, glycoproteins, glycolipids and extracellular DNA also contribute towards the structural integrity of the biofilm[132,133]. Collectively the organic and inorganic constituents of the EPS facilitate bacterial adhesion and aggregation, provide a nutrient source and afford protection from the external environment, including antibiotics and biocides, and against predation[6,8,28,134]. It should also be noted that non-structural molecules and biopolymers, such as those involved in quorum-sensing, adhesion, maturation and dispersal, will also form part of the EPS[75,86,125,128]. Some components of the biofilm matrix are not of bacterial origin and are derived directly from the surrounding environment. For example, biofilms resident on a living surface may also contain adsorbed host factors such as fibrin, cells and cellular debris,

albumin and glycoproteins[6]. During infection the EPS of pathogenic bacteria also contains host-derived inflammatory response proteins in addition to bacterial matrix proteins[40].

a) *S. aureus* EPS

Recent advances in molecular biology have enabled more detailed analysis of the chemistry of *S. aureus* biofilm, however, considerable knowledge in the area is still lacking. The prevailing view regarding the composition of *S. aureus* EPS is that the exopolysaccharide PIA/PNAG (Figure 7) forms the greater part of the solid component of the matrix. PIA/PNAG is a linear homopolymer of partially de-acetylated *β*-1-6-linked *N*-acetylglucosamine; with 80–85% consisting of *N*-acetylglucosamine, and 15–20% of non-*N*-acetylated D-glucosaminyl residues[24,135].

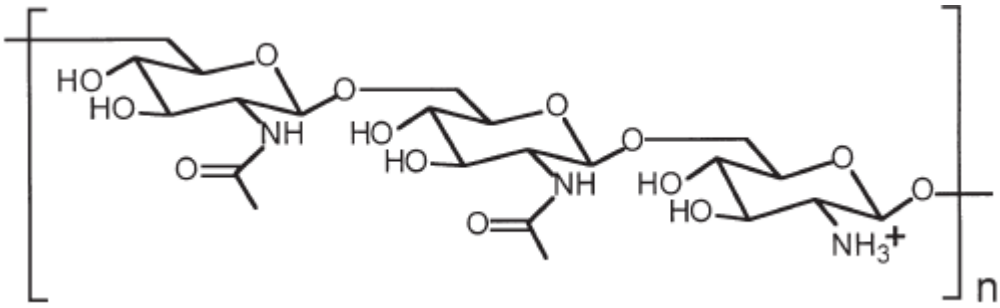

Figure 7: Structure of polysaccharide intercellular adhesin[135].

Initially *β*-1-6-linked *N*-succinylglucosamine (PNSG) was regarded as the main EPS polysaccharide produced both *in vitro* and *in vivo*[83,136]. However, later studies refuted this and believed that the presence of PNSG was merely an artefact[135,137].

Other important components of the EPS include Bap, eDNA, lipopolysaccharides and constituents of the cell wall. Bap is essentially a surface associated protein but may also be found in the EPS. It is a large protein molecule with a high molecular weight of 254 kilodaltons[81,83]. Along with polysaccharides and proteins in the *S. aureus* biofilm matrix, it has now emerged that eDNA is an important structural component of the matrix[86,87]. Lipopolysaccharides and related precursors (e.g. *β*-hydroxy fatty acids and 2-keto-3-deoxyoctulosonic acid), that are more commonly associated with Gram-negative bacteria, have also been identified in the EPS of *S. aureus*[138,139]. Techoic acids derived from the bacterial cell wall and host-derived proteins are presenting the EPS[24].

b) *P. aeruginosa* EPS

The full spectrum of extracellular and membrane bound molecules responsible for the structural integrity of *P. aeruginosa* biofilms has not been established to date. *P. aeruginosa* bacteria have the ability to produce the polysaccharides alginate, Psl and Pel[64]. A lack of Psl has been shown to enhance the production of Pel, whereas the absence of Pel enhances the production of alginate. Strains that are deficient in Psl and Pel, and produce only alginate, are not able to form biofilms[66,97]. Alginate is a polysaccharide composed of variable sequences of 1, 4-linked *β*-D-mannuronic acid (M) and α-L-guluronic acid (G) in linear unbranched chains of about 100 to 3000 monomers with *O*-acetyl side groups found on C2 and C3 of the mannuronic acid residues (Figure 8)[64,140]. It is currently the most extensively studied of the three polysaccharides, however, large amounts of alginate are secreted almost exclusively by subset of mucoid strains of *P. aeruginosa* isolated from the lungs of patients with cystic fibrosis[65]. In contrast, non-mucoid phenotypes of *P. aeruginosa* like PAO1 and PA14, that are commonly used to study biofilms, do not express alginate biosynthetic genes and do not require alginate during the formation of biofilms[64,141].

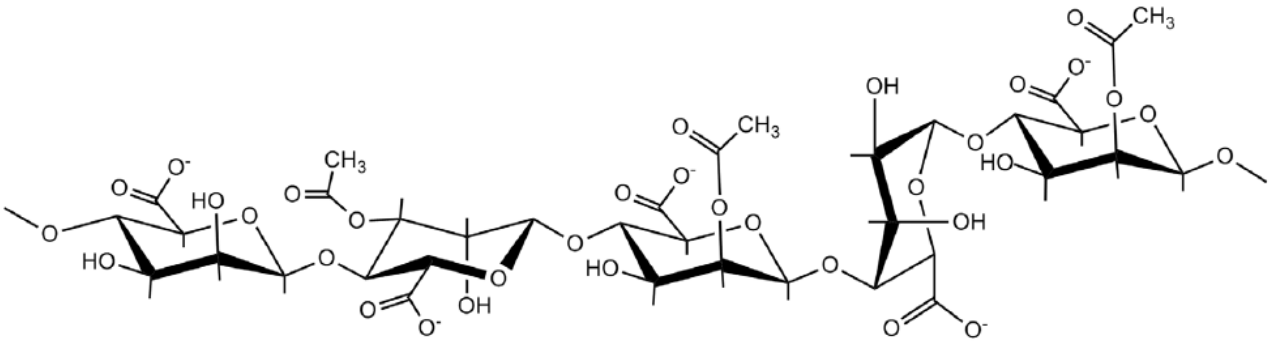

Figure 8: Linear alginate segment with M-M-M-G-M monomer sequence[64].

Recent studies on the structure of Psl polysaccharide have presented differences in the range of monomers that are present. One set of studies has described Psl as a polymer of repeating pentamer blocks composed of mannose (most abundant residue), rhamnose, and glucose (Figure 9)[62,64,142]. Other studies, however, report the additional finding of galactose and xylose and show Psl to be rich in both mannose and galactose residues with a preponderance of galactose[61,143]. The structure of Pel remains unknown; nevertheless, it is thought to be a glucose-rich polysaccharide[139,142].

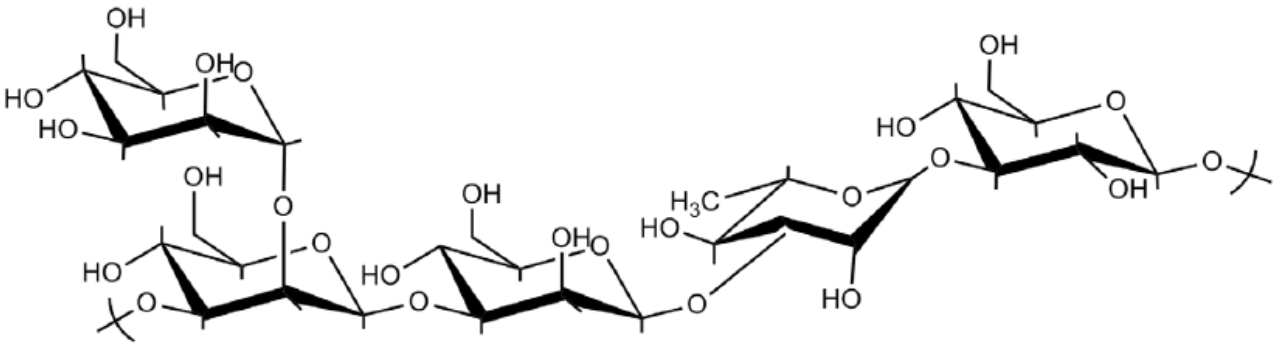

Figure 9: Branched Psl pentamer unit consisting of M-M-M-R-G residues[64].

In addition to the aforementioned polysaccharides, *P. aeruginosa* synthesizes lipopolysaccharides (LPS) and glycerophosphorylated polysaccharides (Figure 10)[139,144]. LPS has a structure that consists of a lipid moiety to which a core oligosaccharide with an O-polysaccharide chain (O-antigen) is attached[145]. The O-antigen is a repetitive polysaccharide polymer with a composition that varies from strain to strain. The chemical structure of the O-antigen in two strains of *P. aeruginosa* was characterized as a linear trisaccharide with repeating units of 2-acetamido-2-deoxy-α-L-galacturonic acid, 2-diacetamido-2,6-dideoxy-α-D-glucose and α-L-rhamnose[139,145]. Lipopolysaccharide, O-antigen and a cyclic glycerophosphorylated *β*-(1,3)-D-glucose polysaccharide were found to be the three major EPS biopolymers in *P. aeruginosa* PA14 biofilm[139]. However, their contribution towards biofilm formation and structure is still uncertain.

Quorum-sensing molecules, eDNA, rhamnolipids and cis-2-decenoic also form part of the EPS.

2.6 The functions of the exopolymeric substance

Research has revealed that biofilm EPS is a dynamic, three-dimensional, highly organised assembly of biopolymers[70,131,132]. The EPS has several functions that give microorganisms the ability to exhibit a biofilm phenotype and derive benefit from close proximity in a communal lifestyle (Table 1). However, the precise mechanisms by which the EPS functions, and the specific contributions made by each of the components, are poorly understood[25].

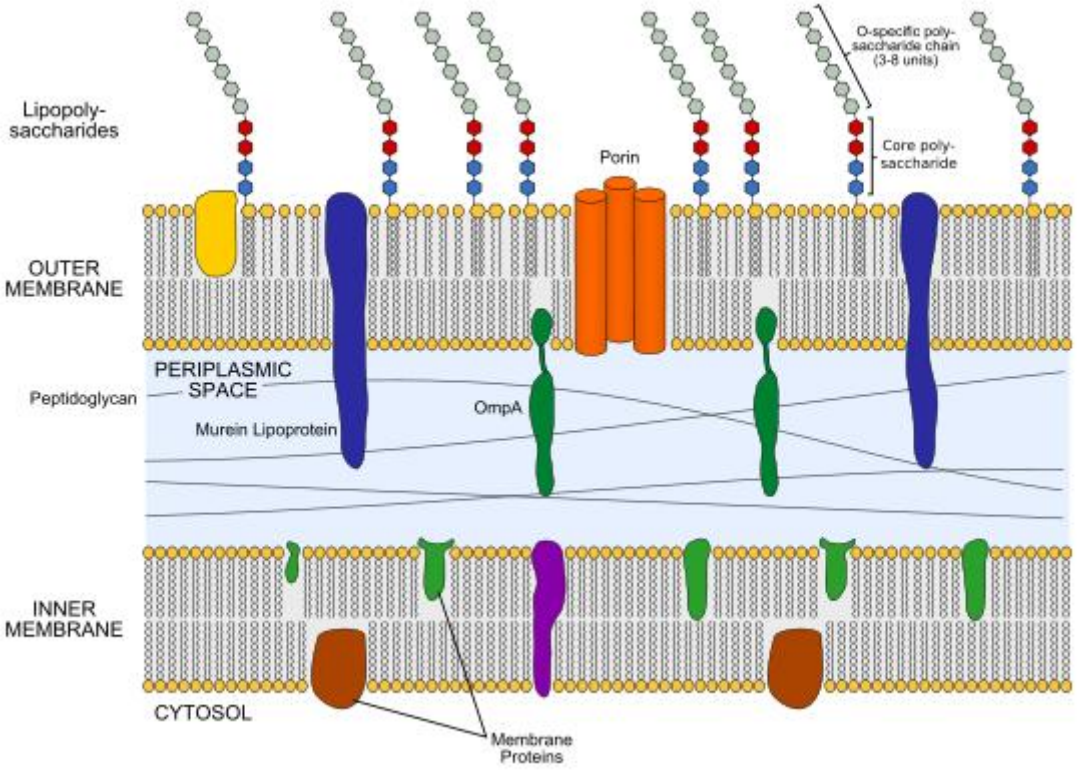

Figure 10: Schematic diagram of the cell wall structure of Gram-negative bacteria [144]

2.7 Bacterial resistance to antimicrobial agents

The term "antimicrobial agent" encompasses both antibiotics and biocides. Antibiotics are defined as organic substances (natural or synthetic) that are selectively toxic to microorganisms, generally at low concentrations, and which are suitable for administration to animals and humans. Biocides are a diverse collection of chemical substances which are classified as antiseptics, disinfectants or preservatives. Antiseptics are used to destroy or inhibit the growth of microorganisms in or on living tissue; disinfectants are applied on inanimate objects; and preservatives are usually added to food, pharmaceuticals and cosmetics, to protect against decay or decomposition[146].

Antimicrobial agents are essential in the prevention and treatment of bacterial infections. They exert their lethal action in several possible ways including inhibition of cell wall synthesis (e.g. β-lactams, glycopeptides), inhibition of protein synthesis (e.g. aminoglycosides, tetracyclines, chloramphenicol, macrolides and clindamycin), inhibition of nucleic acid synthesis (e.g. fluoroquinolones, metronidazole), disruption of the cell wall (e.g. polymyxins, daptomycin, telavancin, biocides) and antimetabolite activity (e.g. sulfonamides, dapsone, trimethoprim)[147].

Table 1: Functions of the extracellular polymeric substance in bacterial biofilm. [adapted with permission from "The biofilm matrix" (Flemming and Wingender 2010)[25]]

Function	Relevance for biofilms	EPS components involved
Adhesion	Promotes the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, eDNA and amphiphilic molecules[86,139]
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial cells	Polysaccharides, proteins and eDNA[85,97]
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell-cell communication	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and eDNA[64,66]
Retention of water	Maintains a highly hydrated microenvironment around biofilm microorganisms, leading to their tolerance of desiccation in water-deficient environments	Hydrophilic polysaccharides and, possibly proteins[64,148]
Protective barrier	Confers tolerance to various antimicrobial agents (e.g. disinfectants and antibiotics), as well as protecting bacteria from the harmful effects of UV light, pH fluctuation, oxidative stress, osmotic flux and protecting against some grazing protozoa	Polysaccharides and proteins[65,149,150]
Virulence	Confers resistance to nonspecific and specific host defences during infection	Polysaccharides and proteins[137,149]
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins[148,151]
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate[151]

Function	Relevance for biofilms	EPS components involved
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins[111,118]
Nutrient source	Provides an endogenous source of carbon-, nitrogen- and phosphorous-containing compounds for utilization by the biofilm bacteria	Potentially all EPS components[25]
Circulatory system	Transport and distribution of solutes (including nutrients and waste) by osmosis and diffusion within cell clusters and by bulk flow through water channels	Polysaccharides, proteins and water[152]
Exchange of genetic information	Facilitates horizontal gene transfer between biofilm cells	DNA[153]
Quorum-sensing	Enables close proximity of single and mixed species of bacteria which facilitates cell-cell communication through signalling systems	AHLs and oligopeptides[75,76]
Cytoplasmic transfer of intracellular molecules	Enables stable close proximity of bacteria which allows the development of nanotubules (cytoplasmic bridging) through which intercellular exchange occurs	Cell membrane (proteins, lipids and polysaccharides)[80]
Electron donor or acceptor	Permits redox activity in the biofilm matrix and, in photosynthetic communities, EPS molecules can fluoresce and provide photons to microorganisms located deep within the matrix.	Proteins (for example, those forming pili and nanowires) and, possibly humic substances[25,151]
Export of cell components	Releases extracellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids[154]
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides[25]
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides	Polysaccharides and enzymes[151]

Upon exposure to antimicrobials, bacteria respond by developing and expressing systems that enable them to become either resistant or tolerant to the agents. The term "*resistance*" is defined as the ability of a microorganism to continue to grow in the presence of an antimicrobial agent; whereas "*tolerance*" is defined as the ability of a microorganism to survive but not grow in the presence of an antimicrobial agent[155]. Antimicrobial resistance is a worldwide problem that continues to evolve at an alarming rate. Resistance is particularly towards antibiotics and, to a lesser extent, to biocides[1,156]. This resistance is compounded by an increase in their usage, the evolution of new resistance mechanisms, the emergence of multidrug resistant strains, rapid geographic spread of bacteria by way of global travel and the slow pace in the development of new therapeutic alternatives[1–3,157]. A number of scientific publications on antibiotic resistance in planktonic bacteria provide a wealth of information about the well known and the newly discovered resistance patterns, and give in-depth discussions on the mechanisms that are involved[158,159]. In general, the resistance mechanisms act either on the antibiotic or on its target site and are primarily directed towards preventing the antibiotic from effectively binding to its site of action[32].

Biocides are widely used in the clinical, industrial and domestic environment as a means with which to achieve effective hygiene[146]. In contrast to antibiotics, there is a relative lack of information with regards to the mechanisms by which biocides act, and no clear explanation of the mechanisms that mediate bacterial resistance and tolerance to these agents[158,160]. Studies have shown that biocides act at several non-specific sites, either within the cytoplasm and/or on the cell membrane, and in this regard thus differ from antibiotics which mainly act at specific targets[146,160]. Although bacterial resistance to biocides is a well known phenomenon, it is a less common occurrence than resistance to antibiotics. The mechanisms that mediate the resistance are not clearly understood but are likely to involve the multiple targets within the cell[156,157,161]. Studies have shown that exposure to low concentrations of a biocide can result in a reduced susceptibility to the agent. Resistance to biocides is largely attributed to the actions of multi-drug efflux pumps that actively remove a wide assortment of chemical compounds from the interior of the cells[162]. There is growing concern that the widespread use of biocides could contribute towards an increase in the prevalence of biocide resistance in bacteria and, through shared efflux-mediated resistance mechanisms, could be linked to antibiotic resistance[160,163]. However, evidence collated from studies is not conclusive and neither confirms nor refutes the possibility of biocide-induced resistance to antibiotics. Where resistance mechanisms may be shared, this may not be of any practical concern since biocides are most often used at very high bactericidal concentrations[146,163].

a) *S. aureus* antimicrobial resistance mechanisms

S. aureus exhibits a remarkable ability to adapt rapidly to antimicrobial challenge by the acquisition and/or development of resistance mechanisms. Although, resistance to antibiotics and biocides may be inherent, its is usually acquired either through endogenous chromosomal mutation with positive selection or via the acquisition of exogenous resistance genes (i.e. plasmids, transposons and integrons) by horizontal transfer[164]. Some of the known mechanisms of antimicrobial resistance exhibited by *S. aureus* include:

i) Antibiotic and biocide deactivation

- Enzymatic destruction e.g. hydrolysis of both penicillin and ampicillin by bacterial *β*-lactamase[164].
- Modification of the antibiotic e.g. aminoglycoside-modification enzymes express nucleotidyltranferase, phosphotransferase, and acetyltransferase activity which mediate specific substitutions of the hydroxyl (OH) and amine (NH_2) moieties on the aminoglycosides thereby inactivating the drugs[165].

ii) Reduced intracellular accumulation of antibiotics and biocides

- Restricted permeability e.g. entry of vancomycin and chlorohexidine into the cytoplasm is hindered by entrapment of the drug within a bacterial cell membrane that is proactively thickened by excess amounts of peptidoglycan[146,166].
- Increased efflux

  Multidrug efflux pumps, regulated by plasmids and mutant regulatory genes, act on a range of antibiotics (e.g. tetracycline, erythromycin and ciprofloxacin) and biocides (e.g. benzalkonium chloride, chlorohexidine and triclosan)[162].

  Drug-specific pumps regulated via plasmids, transposons and integrons[167].

iii) Alteration of target site resulting in reduced affinity for the antibiotic

- Genetic mutation: Ciprofloxacin stops the synthesis of DNA and protein and kills bacteria by interfering with enzymes (DNA gyrase and topoisomerase IV) that are essential for DNA replication. Resistance occurs due to spontaneous mutations that encode amino acid changes in these target enzymes which render them non-susceptible to ciprofloxacin[164].
- Substitution (expression of an alternative target): Excess unlinked peptidoglycan chains on the cell membrane of methicillin-resistant *S. aureus* contains vancomycin

binding sites to which the drug attaches and is then prevented from entering into the cell[166].

- Enzyme-mediated change: Penicillin-binding proteins (PBPs) are enzymes that are required for cell wall biosynthesis. Normal PBPs are susceptible to modification by *β*-lactamases resulting in death by cell lysis. Methicillin-resistant *S. aureus* produces PBP2a that is refractory to *β*-lactam antibiotics (penicillins, cephalosporins, carbapenems, and monobactams)[168].

iv) Phenotypic variation

- *S. aureus* undergoes gene transformation with the formation of antimicrobial-resistant phenotypes[169].

b) *P. aeruginosa* antimicrobial resistance mechanisms

P. aeruginosa possesses innate chromosomally-encoded resistance to a wide range of antibiotics from many different classes, but remains sensitive to a limited number of drugs which include: a selection of penicillins, cephalosporins, carbapenems, monobactams, aminoglycosides, fluoroquinolones, and polymyxins[170]. Unfortunately, *P. aeruginosa* has the additional ability to rapidly develop resistance to all reliable antibiotics either by undergoing genetic mutation or through horizontal transfer of antibiotic resistance genes[170–172]. *P. aeruginosa* utilizes all the resistance mechanisms expressed by *S. aureus* and also undergoes transformation with the formation of antimicrobial resistant phenotypes[173,174]. The impressive complement of innate and acquired resistance mechanisms provide the means for high-level multidrug resistance to emerge, even during the course of therapy at recommended doses[171,172] The innate resistance is mainly due to the action of several proteinaceous efflux pumps which actively export multiple classes of antibiotics (except polymyxins), as well as various dyes and detergents, from the cytoplasm and periplasmic space to the exterior[171,175]. These efflux pumps are located in the cytoplasmic portion of the bacterial cell membrane and are linked via lipoproteins to auxiliary proteins that form an exit portal in the outer membrane (Figure 11)[170,171].

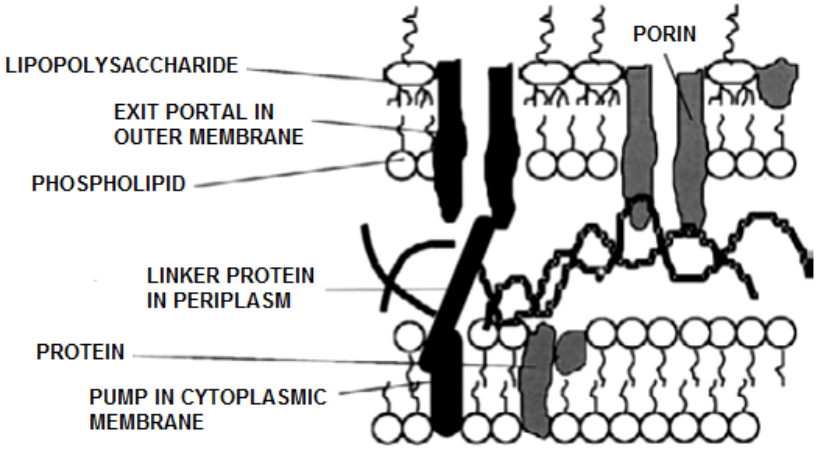

Figure 11: Typical structure of a three-component efflux pump[171].

2.8 Antimicrobial resistance and tolerance in bacterial biofilms

Over and above the formidable antimicrobial drug resistant capabilities of individual bacterial cells, the biofilm phenotype and EPS envelope confer additional unique resistance and tolerance properties upon the bacteria[176,177]. From the earliest stages of biofilm formation, bacteria encased within biofilms have been found to be 10 to 1000 times more resistant to antimicrobial agents than their own free-floating planktonic phenotype[8,169,178]. This biofilm-mediated bacterial resistance to a whole range of antimicrobial agents, disinfectants and host immune factors depends upon protective mechanisms that are only made possible through their strategy of close communal co-existence[5,178]. The precise mechanisms involved in biofilm-mediated resistance to antimicrobials and biocides are not fully understood, however, variable interplay between several mechanisms may be responsible[156,176]. Although there is a wealth of scientific reports on biofilm-mediated resistance, a large proportion of the work has used *P. aeruginosa* as the model microorganism. This section of the review will be limited largely to papers which report on aspects of antimicrobial resistance mechanisms exhibited by *S. aureus* and *P. aeruginosa* biofilms. Some of the possible mechanisms are described below.

a) Presence of dormant persister cells

Persisters are a small sub-population of cells in a bacterial culture that either divide slowly or temporarily exist as dormant non-dividing cells[177,179]. They are not exclusive to biofilms, but are found in a higher proportion in biofilms than amongst planktonic cells[179]. Persisters are

specialized non-mutant cells that neither grow nor die in the presence of bactericidal antibiotics but pre-exist as part of a bacterial population (Figure 12)[155].

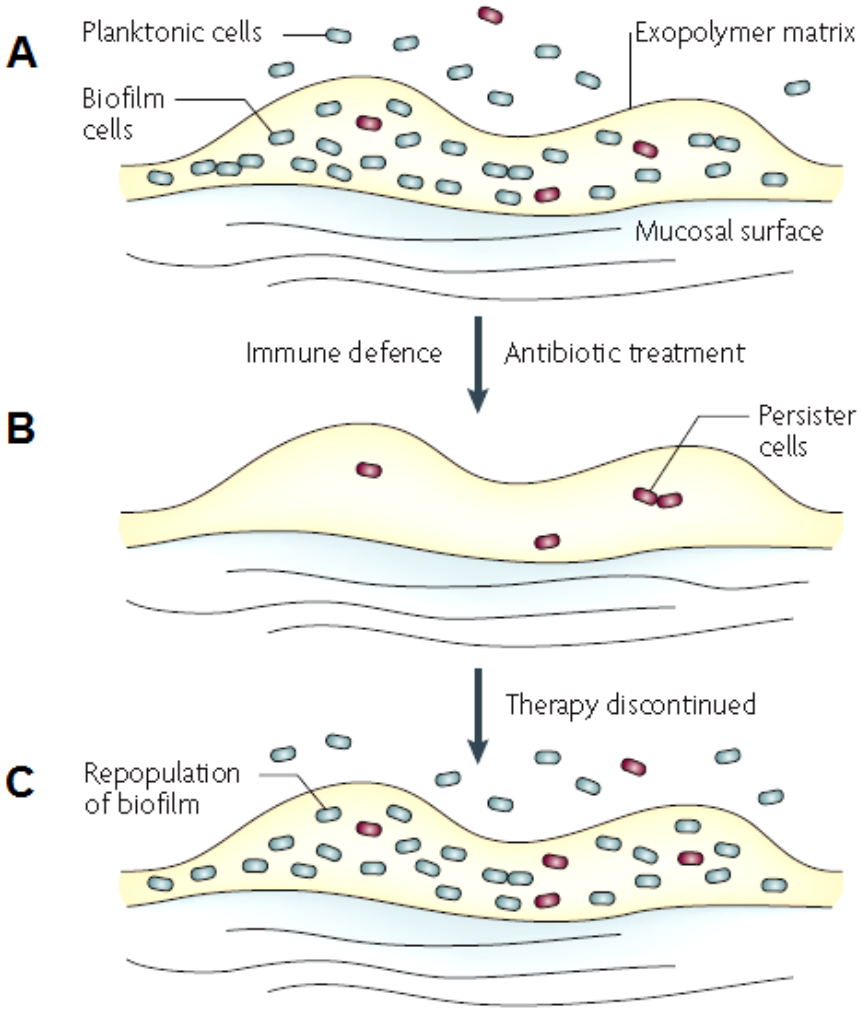

Figure 12: Model of biofilm resistance to bactericidal antibiotics based on persister survival[32].

New persisters are formed during all stages of the growth of a colony, although the precise regulatory mechanisms that underlie persister cell formation are unknown. One hypothesis regarding the mechanism of persister formation is that the transformation of regular active cells into the persister phenotype, as well as phenotypic switching between the two forms, occurs spontaneously and without gene regulation[32,177]. However, recent studies on *P. aeruginosa* have identified a number of regulator genes and quorum-sensing molecules that may have a prominent role in persister formation[155,173,180]. So far, evidence suggests that the rate of persister formation is independent of antibiotic exposure[177,180]. The relative number of persisters and their rate of formation varies for each growth stage. Whereas only a few

persisters are present during the early exponential growth phase, their numbers rapidly increase through the mid-exponential phase to reach a maximum during the stationary phase[32,177]. Their presence in the stationary phase ensures the survival of this non-growing population. Stationary cultures and biofilms of *S. aureus* have a relatively higher number of persisters than *P. aeruginosa* biofilms.

Most antibiotics with bactericidal activity require that the bacteria are actively dividing and almost all regular biofilm cells are thus susceptible[147,181]. However, persisters are non-proliferating and have virtually no ongoing cell-wall synthesis or nuclease activity. Therefore, if antibiotics bind to target sites that remain inactive, they will not be able to corrupt the function of the targets, and the cells will not die[32]. A study by Spoering and Lewis[182] compared the resistance of planktonic and biofilm cells of *P. aeruginosa* against four antimicrobial agents (ofloxacin, tobramycin, carbenicillin and peracetic acid). Logarithmic-phase, stationary-phase and biofilm cultures were challenged with the antimicrobials for 6 h with concentrations that ranged from minimum inhibitory concentration (MIC) value, 0.5-30 µg ml^{-1}, up to 600-1000 µg ml^{-1}. The majority of planktonic and biofilm cells were eradicated by the antimicrobial agents, leaving behind a small number of invulnerable persisters. The exception was carbenicillin, a β-lactamase, that was ineffective against the slow-growing cells in the stationary-phase and biofilms as a direct consequence of the nature of its mechanism of action[147]. The authors concluded that tolerance to antibiotics in stationary-phase and biofilm cultures was largely dependent on the presence of persister cells. Furthermore, they suggested that biofilm regular (non-persister) cells do not have greater resistance than their planktonic counterparts[182]. Recently the association between antibiotic resistance and the presence of persisters in *S. aureus* cultures *in vitro* was determined[169]. Planktonic and biofilm cultures were exposed to five antibiotics (oxacillin, cefoxatime, amikacin, ciprofloxacin and vancomycin) at concentrations of 0-100 µg ml^{-1} for 48 h at 37°C. All planktonic cells and > 99% of biofilm cells were killed. The small sub-population (0.24–0.4%) of biofilm persisters that survived, reverted back to the original phenotype upon antibiotic withdrawal and were found to be susceptible upon re-exposure. These studies provide evidence that supports the view that the vast majority of cells in a biofilm are not more resistant to bactericidal antibiotics than planktonic cells, but that a few persisters survive and are paradoxically preserved in the presence of the antibiotics[169,179,182].

There is growing acceptance that biofilm survival, and the associated high levels of tolerance to antimicrobials, is largely due to the presence of persisters and not due to the expression of biofilm-specific resistance mechanisms[182]. The concept of the relative nature of persisters

offers one possible explanation for the occurrence of chronic and persistent biofilm infections[6,23]. When appropriate antibiotics are used to treat an infection, the majority of cells (i.e. regular) are killed and the immune system eliminates both regular cells and persisters from the general circulation. The only remaining live cells would be persisters that are resident in biofilms in the infected tissues. Upon cessation of treatment the antibiotic level drops to sub-lethal concentrations and persisters revert to the active phenotype, re-populate the biofilm, and the infection relapses (Figure 12)[155]. Furthermore, persisters could promote the generation of antibiotic-resistant mutants by maintaining a reservoir of viable bacteria that can, upon re-activation, acquire resistance by random mutation or horizontal gene transfer[183].

b) Induction of unique resistant phenotypes; small colony variants (SCV)

Subtle molecular and genetic changes occur in response to environmental challenges (e.g. temperature fluctuations, nutrient deficiencies and antimicrobials), which can cause major alterations in behaviour resulting in the generation of a resistant phenotype known as small colony variants (SCVs)[184]. SCVs have up to 10 times smaller colony sizes than their parent strains and, unlike persisters (that are considered to be normal variants), they are regarded as genetic mutants which are defective in cellular mechanisms that have essential metabolic roles[185]. *S. aureus* SCVs are not able to synthesize thiamine, menadione and/or hemin, which are required for the biosynthesis of components of the electron transport chain and therefore have reduced levels of adenosine triphosphate (ATP). ATP is a nucleoside triphosphate that stores and transports chemical energy within cells, and is used as an energy source by numerous cellular processes. Reduced levels of ATP and reduced cell metabolism manifests as slow growth, decreased respiration, decreased pigmentation, decreased haemolytic activity, decreased coagulase activity, increased resistance to aminoglycosides, and an unstable colony phenotype[185].

Exposure of *S. aureus* and *P. aeruginosa* to antibiotics results in the development and/or selection of SCV. Their SCVs exhibit a high biofilm forming capacity and are frequent findings in biofilm-associated infections in which their innate antibiotic resistance may also enhance the resistance of the biofilms that are formed[169,174]. The biofilms formed by SCVs are almost completely resistant to antibiotics. The type of antimicrobial used has been found to have an influence on the formation of SCVs. Observations from in vitro studies have revealed that exposure of *S. aureus* to amikacin, ciprofloxacin and vancomycin results in the formation of SCVs in addition to persisters. Ciprofloxacin treatment was associated with the immediate

formation of biofilm SCVs whereas amikacin and vancomycin initially formed planktonic SCVs which subsequently formed biofilms[169]. No SCVs were formed on treatment with oxacillin and cefotaxime. *S. aureus* and *P. aeruginosa* are common pulmonary pathogens in patients with cystic fibrosis (CF) disease and methicillin-resistant strains account for up to 60% of *S. aureus* isolates[56,186,187]. Whereas *S. aureus* may not be the primary cause of lung damage, its eradication is advised because it predisposes to infection by *P. aeruginosa* which is associated with significant lung damage, increased morbidity and mortality[188]. Persistence of *S. aureus* in CF and other chronic infections has been associated with the isolation of SCVs Despite rigorous infection control and early aggressive antibiotic treatment, eradication of infection by *S. aureus* is not always successful[187]. In addition to the prospect of failed treatment, there is evidence which suggests that continuous or frequent administration of antistaphylococcal antibiotics is associated with a higher rate of acquisition of *P. aeruginosa*[189]. The unique environment of the CF lung, selects a particularly aggressive and resistant SCV of *P. aeruginosa*. SCVs isolated from CF patients differ from the parent "wild" strain in that the colonies are smaller and the cells express abundant pili, have increased twitching motility and have a propensity to form dense stable biofilms[174].

c) Physiological heterogeneity and adaptive gene expression

Within the biofilm each bacterium occupies its own unique physical and chemical microenvironment composed of adjacent cells, water channels and complex EPS. Each of these distinct environmental niches are located at different depths in relation to the periphery of the biofilm[25,152,190]. Each bacterium is exposed to a different degree to bulk fluid flow, metabolic activity by adjacent cells, pH, oxygen tension, nutrient gradients and waste accumulation. This environmental heterogeneity, which may vary drastically from one point to another within the biofilm, encourages the formation of a heterogeneous population of cells, each with different growth characteristics and different levels of antimicrobial resistance[178,190]. The oxygen tension is the most significant of the environmental parameters that affects antimicrobial resistance. Other factors such as nutrient deprivation, pH variation and high cell density may induce transformation to a resistant phenotype or act by slowing the metabolic rate of the cells[178].

The role of oxygen limitation in the protection *P. aeruginosa* biofilms from being killed by antibiotics has been investigated *in vitro*. The result of microelectrode analysis of *P. aeruginosa* biofilm has demonstrated that oxygen penetrates about 50 to 90 µm into the

biofilm from the air interface, beyond which the local environment remains relatively anoxic. The oxygenated region of the biofilm is associated with active bacterial metabolic activity whereas the bacteria in the oxygen-poor regions in the interior of the biofilm have low metabolic activity[191,192]. Transmission electron microscopic observations after exposure to antibiotic treatments, with either 10.0 µg/ml of tobramycin or 1.0 µg/ml of ciprofloxacin, showed the presence of cell damage near the air interface. Although both antibiotics permeated throughout the biofilm, the cells further away from the air interface were less affected by the antibiotics[192]. Borriello *et al.*[191] observed that 48-h-old *P. aeruginosa* biofilms were physiologically heterogeneous and that the majority of cells which occupied the oxygen-limited zones were either slow-growing or non-growing. They found that the biofilms remained viable after 12 h exposure to several antibiotics namely; tobramycin, ciprofloxacin, carbenicillin, ceftazidime, chloramphenicol, and tetracycline. In contrast, planktonic *P. aeruginosa* and metabolically active cells in young (4-h-old colony) biofilms were found to be susceptible to antibiotics when grown aerobically. However, when challenged under anaerobic conditions, the efficacy of the antibiotics was significantly reduced[191,193]. All of these studies provid evidence that local oxygen limitation is one of the main factors behind the protection afforded to bacteria in mature biofilm colonies.

Bacteria sense changes in their environment and rapidly adapt by modulating the transcription of genes whose expression is deemed appropriate for the purpose. In this regard new evidence has emerged that links gene expression to the ability for *P. aeruginosa* to survive in an oxygen-depleted environment. Alvarez-Ortega and Harwood, and later Folsom *et al.*, studied the complete set RNA molecules (transcriptome) in *P. aeruginosa* and identified a set of genes that were strongly induced by low oxygen, and whose rate of transcription increased consistently with decreasing oxygen concentration[194,195]. Results from other investigations have shown that over 100 genes that are induced when *P. aeruginosa* is subjected to anaerobic conditions[196].

d) Quorum sensing and horizontal gene transfer

Most natural biofilms are formed by several species of microorganisms that live in close proximity to each other. These microorganisms interact, through extracellular secreted molecules and species-specific physical interactions, to either support or inhibit growth of one another[25,73]. Bacteria are known to have the ability to absorb DNA (plasmids, transposons and integrons) from their immediate environment and to integrate it into their genome. The

available DNA is derived from the diverse community of cohabiting species and also from host (eukaryotic) cells. The close proximity afforded by the biofilm increases the opportunity to acquire DNA and, through this process of horizontal gene transfer, the entire metabolic capacity to resist antimicrobial agents can be readily transferred from one bacterial cell to another[80,153,197].

According to *in vitro* evidence, quorum-sensing systems could contribute towards the increased antibiotic resistance seen in biofilm bacteria[76,180]. In contrast to antibiotics, the role of quorum sensing in biocide resistance is not yet established[178]. Yarwood *et al.*[76] investigated the involvement of the quorum-sensing accessory gene regulator (*agr*) on *S. aureus* biofilm resistance to antibiotics. In their study, biofilms of *agr*-defective mutants and non-mutant strains were challenged with oxacillin and rifampicin. Non-mutant strains showed appreciable resistance to both antibiotics; *agr*-defective mutants were much more sensitive to rifampin but not to oxacillin. The researchers concluded that *agr* expression is involved in *S. aureus* biofilm resistance but only towards selected antibiotics[76]. It has been shown that genes that encode the synthesis of N-(3-oxododecanoyl)-l-homoserine lactone by *P. aeruginosa* are up-regulated during hypoxia, and that pyocyanin (a secreted toxin) is mainly produced during the stationary phase of growth[194]. Möker *et al.*[180] observed that both of these substances trigger a increase in the number of persister cells in rapidly growing cultures of *P. aeruginosa*. These findings suggest that quorum sensing molecules may mediate the antibiotic resistance effects of hypoxia by promoting the formation of persisters[180,191,194].

e) Multispecies biofilms

In vitro studies have demonstrated that multispecies bacterial biofilms possess greater antimicrobial resistance and physical stability than single species biofilm[198–200]. The precise mechanisms regulating this phenomenon still remain unclear. Based on recent reports, it is possible that cell-to-cell communication and shared genetic information may play important roles in influencing the expressed phenotype[75,153].

Simŏes *et al.*[199] studied the effect of 0.9 mM concentrations of two biocides, glutaraldehyde and cetrimonium bromide, on the adhesion and cell viability of single and dual species biofilms of *Bacillus cereus* and *Pseudomonas fluorescens*. After treatment, dual species biofilms were found to be more adherent to the substratum and contained a significantly greater proportion of viable cells than those of single species biofilms. Similar findings were

reported by Millezi *et al.*[200] who investigated the effects of essential oils of citronella and lemon on the growth of *S. aureus* and *Escherichia coli* biofilms. They noted that the enhanced resistance exhibited by the dual species biofilm occurred in spite of the fact that *E. coli* antagonized the growth of *S. aureus* within the biofilm. In a study by Burmølle *et al.*[198], several bacterial strains were isolated from a marine environment and different combinations multispecies biofilms were grown. *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis*, and *Acinetobacter lwoffii*, were found to interact synergistically within a four-species biofilm and that a marked increase in cell activity and biofilm biomass formation, of over 167%, was observed. When compared to the biofilms formed by single species, the four-species biofilm was less susceptible to the microbicidal actions of hydrogen peroxide and tetracycline, and resisted invasion by a non-resident bacterium, *Pseudoalteromonas tunicata*, to a greater extent. Substitution of each strain by its cell-free culture supernatant yielded similar experimental results which suggested that the observed synergy was dependent on extracellular secreted factors in addition to other possible interactions between cells[198].

f) Poor penetration of antimicrobials into the biofilm

Biofilm bacteria are embedded in a self-produced EPS matrix composed of a hydrated gel of various types of molecules[25,29]. Studies have shown that the matrix may act as a physical barrier and restrict diffusion of antimicrobial agents[201–203]. As a consequence, antimicrobials are prevented from attaining effective concentrations in the deeper reaches of the biofilm. Evidence in favour of this concept was provided by Anderl *et al.*[202] who studied the diffusion of ampicillin and ciprofloxacin through *Klebsiella pneumoniae* biofilm *in vitro*. They observed that ampicillin did not penetrate through biofilm, however, penetration by ciprofloxacin was rapid. Furthermore they noted that biofilms were more resistant to both drugs as compared to planktonic bacteria and concluded that poor penetration of the antibiotic may have contributed towards biofilm resistance to ampicillin but not to ciprofloxacin[202]. However, given the diverse and dynamic nature of EPS, between and within species, it is hardly surprising that there are also credible studies that find no limitation to the diffusion of antibiotics into bacterial biofilms[29,192].

There are very few reports in the literature that address the role of *S. aureus* biofilm matrix in retarding the diffusion of antimicrobials. Singh *et al.*[204] reported on the penetration through *S. aureus* and *Staphylococcus epidermidis* biofilms by five of antimicrobials namely; amikacin

(aminoglycoside), ciprofloxacin (fluroquinolone), vancomycin (glycopeptides), oxacillin and cefoxatime (*β*-lactams). They observed that the penetration by vancomycin, oxacillin and cefoxatime was reduced whereas this did not occur with either amikacin or cefoxatime. There appears to be no study that specifically investigates the diffusion of amoxicillin-clavulanate, a *β*-lactam antibiotic, through staphylococcal biofilm.

Laboratory studies have investigated the diffusion of *β*-lactams, aminoglycosides and fluoroquinolones through *P. aeruginosa* biofilms[13,203,205]. The experimental model used in some of these studies did not take into consideration the numerous variables that are involved in natural biofilms and have led researchers to obtain inconsistent results and draw conclusions of limited clinical value. In general the diffusion of antibiotics is rapid and unhindered, although there are reports to the contrary. As an example, there are opposing views on whether or not ciprofloxacin, piperacillin (a *β*-lactam) and tobramycin (an aminoglycoside) readily penetrate *P. aeruginosa* biofilm[192,201,203,205]. Hatch and Schiller[205] demonstrated that the diffusion of aminoglycosides (gentamicin and tobramycin) was prevented by a 2% w/v suspension of *P. aeruginosa* alginate, but not that of carbenicillin. This study also found that enzymatic degradation of alginate by alginate lyase, enhanced the diffusion of both aminoglycosides. Supportive evidence for a protective role of alginate is deduced from a study in which degradation of alginate in *P. aeruginosa* biofilms was found to enhance the bactericidal action of gentamicin[13]. On the other hand, Walters *et al.*[192] observed that tobramycin and ciprofloxacin easily penetrated *P. aeruginosa* biofilms but failed to effectively kill the bacteria. They suggested that protective mechanisms, other than reduced antibiotic penetration, were primarily responsible for the survival of the bacteria.

2.9 Biofilm infections in clinical practice

Bacterial biofilms are ubiquitous in nature and form wherever there is a surface that is exposed to water and provides sufficient nutrients to meet their needs. This includes the occurrence of intracellular and extracellular biofilms of commensal and pathogenic bacteria in man[7]. All epithelial surfaces of the body that are in contact with the external environment (i.e. skin, mucous membranes and gastro-intestinal tract) are normally inhabited by bacteria. Colonization takes place at and/or soon after birth[206,207]. Endothelial tissues are protected from bacterial invasion by tissue-associated antibodies and cell mediated immune response[208,209]. Commensal and pathogenic bacteria may cause infection when the body defences are compromised by a physical breach of natural barriers (e.g. injury, invasive

procedures and surgery), immunosuppression (e.g. Human immunodeficiency virus [HIV], diabetes, chemotherapy) or exposure to virulent bacterial strains. *S. aureus* and *P. aeruginosa* form biofilms, neutralize antibodies, inhibit complement activation, inhibit neutrophil chemotaxis, lyse neutrophils, and prevent phagocytosis[134,210,211]. It has also been observed that neutrophils, which constitute an important part of the natural defence against bacteria, may paradoxically promote bacterial biofilm formation[212].

Biofilms are associated with acute, recurrent and chronic infections in man. Typically, biofilm-related infections tend to be chronic and associated with mild symptoms. However, when seeding occurs, the sudden release of planktonic bacteria may cause acute symptoms[6]. Medical practitioners are gradually adjusting to the reality that biofilms are associated with virtually all bacterial infections and the recent upsurge of clinical data in published medical literature is a reflection of this growing awareness[213–220]. A partial list of some biofilm-associated infections is presented in Table 2.

Insertion of synthetic implants and indwelling devices (e.g. intravascular cannulas, urinary catheters, cerebrospinal fluid shunts, internal bone implants, joint prostheses, prosthetic heart valve) is now an everyday part of modern medical practice. Virtually all these devices are prone to infection irrespective of the sophistication of the device, the preventive sterility measures taken during implantation or the site of placement of the device within the body (Table 3)[35]. Their susceptibility to infection is mainly because they lack effective antibacterial mechanisms, and on insertion they are exposed to nutrient-rich body fluids and become conditioned with adsorbed proteins which provide a surface for bacterial attachment[35,220]. Microorganisms most commonly isolated from indwelling medical devices include *E. faecalis, S. aureus, S. epidermidis, Streptococcus viridans, E. coli, K. pneumoniae, Proteus mirabilis, P. aeruginosa* and *Candida* species[221]. Acute and chronic inflammatory responses surround infected devices due to the foreign body reaction to the implant and the immune response to the bacteria. The prolonged inflammation that occurs eventually leads to neutrophil and macrophage-mediated collateral damage in adjacent healthy tissues[35].

2.10 Management of biofilms in clinical practice and clinical research

Biofilms are present in over 60% of bacterial infections[23]. The appreciation of the complex nature of biofilm bacterial behaviour has led to the development of an integrated multi-disciplinary management approach to biofilm-associated disease[34].

Table 2: Partial list of human infections involving biofilms[23,222]

Infection	Common microorganisms
Dental plaque, dental caries, periodontitis and gingivitis	Gram-positive cocci (mainly *Streptococcus* species) and Gram-negative anaerobic oral bacteria[223,224]
Otitis media and cholesteatoma	*H. influenzae, P. aeruginosa, Streptococcus pneumoniae, Moraxella catarrhalis*[225,226]
Sinusitis	*H. influenzae, S. pneumoniae, S. aureus, P. aeruginosa*, other bacterial species and fungi[227,228]
Chronic tonsillitis	*Staphylococcus* species, *Streptococcus* species and various other Gram-positive and Gram-negative bacteria[229,230]
Conjunctivitis, keratitis, endophthalmitis, eyelid abscess	*S. aureus, P. aeruginosa, Bacillus species, fungi, Staphylococcus haemolyticus, Candida tropicalis*[231,232]
Cystic fibrosis pneumonia	*P. aeruginosa, S. aureus, Burkholderia cepacia, H. influenzae, Stenotrophomonas maltophilia* and *Alcaligenes xylosoxidans*[186]
Endocarditis	Viridans group *streptococci*, *β*-haemolytic *streptococci* (group A, G), *streptococcal* species, *coagulase-negative staphylococci, S. aureus, Enterococcus faecalis*[219,233,234]
Necrotizing fasciitis	Group A *streptococci (Streptococcus pyogenes)*[23,235]
Musculoskeletal infections	*S. aureus*, group *A β-haemolytic streptococci,* group *B streptococcus, H. influenzae, S. pneumoniae, Kingella kingae, Salmonella* species, *P. aeruginosa, E. coli*[236]
Osteomyelitis	*S. aureus, Streptococcus agalactiae, E coli, Streptococcus pyogenes, H. influenzae, Enterococcus* species, *Streptococcus* species, *P. aeruginosa*, *Enterobacter* species, *Mycobacterium* species, anaerobic bacteria and *Candida* species[213]
Pancreatic and biliary tract infection	Enteric bacteria (e.g. *E. coli*) [237]
Infectious kidney stones	Gram-negatve rods (including *Proteus, Providencia, Klebsiella*, and *Pseudomonas* species)[238]
Bacterial prostatitis	*E. coli, Klebsiella, enterobacteria, Proteus, Serratia, P. aeruginosa, coagulase-negative staphylococci, E. faecalis, Bacteroides* species, *Gardnerella* species, *Corynebacterium* species[33,239]

Despite widespread awareness of the medical importance of biofilms, only a very small number of structured clinical studies have ever been conducted on biofilm eradication. Most pharmacological research has involved laboratory studies that were conducted using a number of *in vitro* systems and *in vivo* animal models from which clinical inferences have been made. To date, most of the research has not translated into clinical application[179,240].

The current treatment paradigm of bacterial infections in clinical practice is the identification of pathogenic bacteria, laboratory confirmation of their antibiotic susceptibility and the use of an appropriate agent to either kill the bacteria or retard their growth. Unfortunately, in biofilm-related infections, it is not uncommon for the offending pathogens to be unculturable and antimicrobial treatment is then rendered empirically[241]. In addition biofilm bacteria that are identified have a decreased susceptibility to antibiotics and possess a remarkable ability to evade the immune defences of the body[178,211,242]. As a consequence of this, antibiotic therapy is often found to be ineffective even when the choice of agent is justified on the basis of confirmed laboratory susceptibly results[178]. The typical clinical picture encountered during antibiotic treatment of biofilm-related disease is one in which the patient responds positively to initial therapy only for the same infection to recur soon after cessation of treatment[23].

Due to the frequent failure of conventional antibiotic therapy, and a limited choice of available clinical management options, there is an urgent need to develop new antibiotics, to re-evaluate the antibacterial efficacy of existing drugs, including drug combinations, and to develop new ways of enhancing the action of available antibiotics. Some biofilm treatment options were researched and were utilized in patients, or were noted to have a strong potential for future clinical applications. The most effective treatments are based on the use of antibiotics in conjunction with therapies that cause mechanical disruption (or removal) of the biofilms. For example, when the focus of infection is localized and amenable to surgery, surgical debridement with removal of the infected tissues, including infected implants, is recommended in addition to antibiotic therapy[34,35,220]. In some cases, physical removal of the focus of pathogenic biofilm is not always the most prudent option and in such cases antibiotic therapy remains the treatment of choice[243].

Both conventional and novel antibiotics are used either singly, or in various combinations, for the prevention and treatment of intransigent biofilm-related bacterial infections in clinical medicine and research.

Table 3: Biofilm-associated microorganisms commonly isolated from selected indwelling medical devices and foreign materials[23,222]

Medical Device	**Common microorganisms involved**
Contact lenses	*Coagulase-negative staphylococci*, *Propionibacterium* species, *Corynebacteria* species, *S. aureus*, *S. viridans*, *S. pneumoniae*, *Micrococcus* species, *Serratia marcescens*, *P. aeruginosa*, other Gram-positive and Gram-negative bacteria, fungi[244]
Surgical sutures	*S. aureus*, *coagulase-negative staphylococci*, *Enterococcus* species[245]
Tympanostomy tubes	*P. aeruginosa*, *S. aureus*[246]
Endotracheal tubes	*P. aeruginosa*, *H. parainfluenzae*, *S. aureus*, *Fusobacterium nucleatum*, *Neisseria perflava*, *Prevotella nigrescens*[247]
Ventilation-associated pneumonia	*P. aeruginosa*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, *Enterobacteriaceae* and other Gram-negative bacilli[248]
Artificial voice prosthesis	*Candida. albicans*, *Candida tropicalis*, *Streptococcus mitis*, *Streptococcus salivarius*, *Rothia dentrocariosa*, *Streptococcus sobrinus*, *S. epidermidis*, *Stomatococcus mucilaginous*[249]
Mechanical heart valves	*S. epidermidis*, *S. aureus*, *Streptococcus* species, gram-negative bacilli, *diphtheroids*, *enterococci*, *Candida* species[221]
Vascular grafts	S. *epidermidis*, methicillin-resistant *S. aureus* (MRSA) , *P. aeruginosa* and other Gram-negative organisms[250]
Arteriovenous shunts	*S. aureus*, *E. faecalis*, *P. mirabilis*, fungal cells[23]
Endovascular catheter infections	*S. epidermidis* , *S. aureus*, *coagulase negative staphylococci*, *P aeruginosa*, *C. albicans*, *K. pneumoniae*, *E. faecalis*[221,251]
Ventriculoperitoneal shunts and external ventricular drains	*S. aureus*, *S. epidermidis*[252]
Continuous ambulatory peritoneal dialysis (CAPD) peritonitis	*S. aureus*, *S. epidermidis*, *P. aeruginosa*, *E. coli*[253]
Urinary catheter infections	*S. epidermidis*, *E. coli*, *E. faecalis*, *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae* and other Gram-negative organisms[249,254]
Intrauterine devices (IUDs)	*S. aureus*, *S. epidermidis*, *P. aeruginosa*, *E. coli*, *Neisseria gonorrhoeae*, *C. albicans*, *Candida dubliniesis*, *Actinomyces israelii*, *Corynebacterium* species, *Micrococcus* species, *Lactobacillus plantarum*, group B *streptococci*, *Enterococcus* species and others[23,249,255]
Penile prostheses	*Staphylococci* and other Gram-positive cocci, Gram-negative bacilli, fungi[256]
Orthopaedic prosthesis	*S. aureus*, *coagulase-negative staphylococci*, *P. aeruginosa*, gram-negative bacilli, anaerobes, *E. coli*, *Viridans streptococci*, *β-haemolytic streptococci*, *P. mirabilis*, *Bacteroides* species[249,257]

Different strategies have been proposed and tried, with varying success; most involve variations in the choice of agent (or agents), the dose and frequency of administration, the route of administration and the duration of treatment. Current antibiofilm strategies can be grouped under six headings namely; prolonged or recurrent antibiotic treatment, systemic antibiotic prophylaxis, topical antibiotic prophylaxis and treatment, antibiotic combination therapy, new anti-biofilm antibiotics and, novel (experimental) therapies.

2.10.1 Prolonged or recurrent antibiotic treatment

A common therapeutic approach to the treatment of persistent biofilm infections is to administer a prolonged course of antibiotics (based where possible on culture and sensitivity results) until the clinical symptoms resolve and laboratory-based inflammatory markers (e.g. leukocyte count and C-reactive protein levels) normalize[222,243]. Depending on the site and severity of the disease and the patients innate response, the duration of treatment could range from 2 to 6 weeks up to 6 months[243,258]. During the early stages of treatment all bacteria that are susceptible to the antibiotic are killed, however, a subpopulation of dormant naturally-resistant cells (i.e. persisters and small cell variants) will survive[155,184]. In order for bacteria to propagate they are compelled to undergo phenotypic transformation into a metabolically active planktonic state[7]. This transformation is associated with an increase in susceptibility to antibiotics, and a sustained therapeutic level of antibiotic will effectively kill the active bacteria[222]. As an alternative to prolonged therapy, short courses of antibiotics are administered for up to 2 weeks, and repeated if the disease recurs[243]. The logical benefit of recurrent treatment is that, on cessation of antibiotic treatment, a population of biofilm persister cells that survive the exposure to antibiotics will undergo phenotypic transformation to a planktonic state. This transformation is associated with increased susceptibility to the antibiotic and subsequent re-exposure will effectively kill the planktonic bacteria[32,169]. With passage of time, prolonged and recurrent treatments should eventually deplete the population of persister cells and eradicate the biofilm infection. Unfortunately this treatment approach is not without undesirable side effects which include the alteration of the normal bacterial flora of the body and the development of antibiotic resistant commensal and pathogenic strains[1,259].

2.10.2 Systemic antibiotic prophylaxis

Prophylactic treatment consists of an empiric course of antibiotics, commenced in the absence of an established bacterial infection, in order to prevent the occurrence of infection where there is either evidence of, or the perceived potential for, pathogenic bacterial contamination of body tissues and/or implanted medical devices[260]. This underlying principle of prophylaxis was established during the 1960's and based on the assumption that an infection is established and maintained solely by planktonic bacteria[26]. Ideally, the use of prophylactic antibiotics at levels exceeding the MIC of the antibiotic for the likely contaminating bacteria would eradicate invading bacteria on entry and also prevent the emergence of antimicrobial resistance. Current knowledge, however, reveals that bacteria naturally occur as biofilms and that biofilm dispersal with subsequent contamination of new sites occurs by means of planktonic cells and cell clusters that contain a varying population of bacteria[102,103,261]. Furthermore, studies have also demonstrated that large cell clusters maintain the enhanced antimicrobial resistance profile of their parent biofilm, which could partly explain the occasional failure of antibiotic prophylaxis[102].

Biofilm formation may be prevented in non-surgical high-risk patients, who are predisposed to developing infectious foci (e.g. valvular heart disease, bronchiectasis and indwelling medical devices), through the use of long-term suppressive therapy with low doses of oral antibiotics. Although still commonly practiced, this approach is discouraged by some scientists and clinicians because of the lack of evidence of its efficacy and the risk of promoting the emergence of antibiotic resistant microorganisms[262]. In surgical and dental patients, biofilm formation may be prevented at local operation and injury sites, and at remote sites, by early aggressive perioperative antibiotic prophylaxis[260,263,264]. The recommended perioperative practice is to establish therapeutic concentrations of antibiotic before surgery, maintain the levels throughout the procedure, and discontinue the medication within 24 hours after surgery[264,265]. Although numerous guidelines are available, for the most part the choice of the most suitable antibiotic and duration of administration remains ill defined[260].

2.10.3 Topical antibiotic prophylaxis and treatment

Infections that are associated with implanted medical devices are due to the formation of biofilms on the surface of the device. Primary bacterial inoculation with subsequent colonization of the devices most probably occurs at the time of surgical placement[35]. Topical

application of antimicrobial agents is commonly used in an effort to prevent organisms from colonizing the implant and/or contaminating the tissues adjacent to the implant. Four methods constitute the prevailing clinical practice in the prevention of infections associated with surgical implants namely; antimicrobial irrigation of surgical field, placement of antibiotic-impregnated drug carrier implants, antimicrobial pre-placement washing of implanted medical device and antimicrobial coating of implant devices[266].

With all these methods, there is neither a standardized measure of dose delivery (or maintenance) nor convincing evidence of clinical efficacy. The high drug levels can be achieved by irrigation and washing, but these levels are not maintained for more than a few hours. A potential disadvantage of antibiotic-coated and impregnated implants is that the continuous slow release of sub-therapeutic doses of antibiotic from the implants may promote the development of antibiotic resistant microbial strains[266,267].

2.10.4 Antibiotic combination therapy

Biofilm bacteria are known to be highly resistant to antibiotics and antibiotic combinations[178,268]. Nevertheless, *in vitro* studies have found that, by comparison, antibiotic combination therapy is more effective than monotherapy in the treatment of biofilm infections[106,269,270]. The rationale for administering more than one antibiotic is that naturally-occurring mixed species biofilms are susceptible when treated by a collection of antibiotics which target each of the individual bacterial species. Furthermore, the actions of combinations of two or more antibiotics could also have additive and synergistic actions on individual bacterial species[106,270]. It has also been suggested that co-administration of several antibiotics may delay the emergence of bacteria resistance to each antibiotic; however, there is an overall paucity of supporting clinical evidence.

2.10.5 New anti-biofilm antibiotics

Daptomycin and telavancin are new lipopeptide antibiotics which act directly on the bacterial membrane by disrupting membrane function and causing leakage of essential potassium ions, ultimately leading to loss of membrane potential and cell death[147,271,272]. Because the bactericidal activities of these drugs are not limited to the interruption of cell-wall synthesis or nuclease activity in actively dividing cells, both agents effectively kill dormant biofilm-

embedded Gram-positive pathogens *in vitro* and *in vivo* including *Streptococci*, methicillin-resistant *S. aureus*, and vancomycin-resistant *Enterococci*[271–274]. Clinical evaluation of daptomycin and telavancin, for potential use across a wide spectrum of infections, is an ongoing field of research that is still at a relatively early stage. Daptomycin has been shown to be safe and effective in treating complicated skin and soft-tissue infections, and in the management of orthopaedic prosthetic infections[273,275]. Telavancin is currently approved for the treatment of complicated skin infections and is being evaluated for the treatment of other infections caused by *S. aureus* and other Gram-positive bacteria, with particular emphasis towards methicillin-resistant and vancomycin-resistant strains of *S. aureus*; MRSA and VRSA, respectively[274,276].

2.10.6 Novel (experimental) therapies

In medical research, there are diverse efforts towards the development of new biofilm modifying or disruptive agents. The discovery and elucidation of QS has fostered research into ways with which to manipulate bacterial communication for therapeutic benefit, and several new classes of chemical compounds have been identified (e.g. AHL analogues and AHL-degrading enzymes). The majority of these chemicals exert their anti-biofilm action through the disruption of QS thereby making biofilms less virulent and more susceptible to antimicrobial treatments[277,278]. Macrolide antibiotics (azithromycin and clarithromycin), *N*-acetylcysteine, honey, traditional herbal recipes and excess elemental iron have all demonstrated the ability to inhibit bacterial biofilms *in vitro*[278–281]. The effects of physical therapies have also been studied. *In vitro* studies have revealed that, in the presence of an electric current, ultrasonic agitation or photodynamic therapy, the efficacy of selected antibiotics against bacterial biofilms is increased[282–285]. The research into the discovery of new anti-biofilm treatments is still in its infancy and, so far, none of the novel chemical and physical agents are currently recommended for clinical application.

2.11 Anti-biofilm actions of EPS matrix degrading enzymes

Biofilm EPS is a complex structural and functional matrix in which bacterial cells thrive in a stable synergistic consortium with integrated functions that favour their survival and propagation[25]. Biofilm-embedded bacteria are highly resistant to antimicrobials and biocides and are able to evade host immune responses and remain viable in the body as a persistent

and/or chronic infection[23,150,156,242]. Although sessile biofilm bacteria are more resistant to antimicrobial agents than their planktonic phenotype, upon dispersal from the biofilm, they quickly revert back to the planktonic state and antibiotic susceptibility is rapidly and fully restored[8,195]. It is believed that dispersal may render pathogenic bacteria more susceptible to the cellular immune responses in an infected host[286]. Disruption of bacterial community behaviour by promoting biofilm dispersal presents a novel therapeutic and prophylactic approach to the management of biofilm infections. The predicted benefit is that induced dispersion would invariably interrupt the numerous biological functions that normally occur within an intact biofilm and also trigger change in the bacterial phenotype from biofilm to planktonic[7,25]. In keeping with this line of thinking, recent research has led to the identification of several classes of chemical compounds that promote dispersal through a variety of mechanisms, and which may have future clinical application: namely biofilm-matrix-degrading enzymes, quorum-sensing signals, surfactants, chelating agents and bacterial diguanylate cyclase inhibitors[7,109].

Biofilm-matrix degrading enzymes prevent the formation of new biofilm, disrupt the structural stability and promote detachment of established biofilms. A number of studies have documented the biofilm degrading abilities of bacterial-secreted and fungal-derived enzymes (Table 4). The primary mode of action is one in which the enzymes decrease biofilm biomass by removing EPS and cells from various substrata without killing the bacteria. In addition, enzymes may also degrade essential adhesion molecules and subsequently disrupt cell-to-substratum adhesion and intercellular cohesion[287]. However, some investigators have observed that enzymes can be bactericidal and that certain enzyme combinations may also interact with each other to enhance or inhibit the bactericidal actions in enzyme mixtures[10,288]. Conversely, there are a few studies in which enzymes have been shown to promote the formation, growth and adhesion of biofilm[289,290]. An additional important finding in several non-clinical studies, is the ability of some enzymes to enhance the efficacy of concurrently and sequentially administered antimicrobial agents[13,15,17,291,292].

Enzymes are generally found to be safe and have the advantage of being highly selective for their substrates, biodegradable and relatively non toxic to humans and animals. Potential benefits in the treatment and/or prevention of bacterial infections appears in scientific reports from *in vivo* studies conducted on laboratory mice in the 1930's[293]. In recent times, scientists have taken a particularly active interest in developing the use of enzymes as biofilm control agents in industrial applications and in their potential use as therapeutic anti-biofilm agents[107,294,295]. A unique advantage of EPS matrix-degrading enzymes is that their site of

action is the matrix and not the bacterial cell. Therefore, enzymes do not issue direct provocation to bacteria which may translate into a reduced likelihood for the emergence of resistance to the enzyme[7]. However, the majority of studies that have investigated the therapeutic properties of enzymes are *in vitro* laboratory studies with only a handful of *in vivo* animal-model studies appearing in medical literature[14,293,296]. One study investigated the use of a natural mixture of digestive proteases (Krillase®) in the control of dental plaque and reported a successful outcome when the preparation was used as the only antimicrobial treatment[14].

2.12 Rationale for the study of fungal-derived commercial enzymes

It has been known for a long time that certain species of fungi and bacteria have the ability to produce and secrete large quantities of many different extracellular enzymes that have the ability to degrade complex polysaccharides and proteins[297]. Microbial-derived enzymes have acquired important roles in a wide range of industrial applications including the processing of food and drink, pharmaceuticals, textiles and leather, water purification, waste management and paper manufacturing, with more uses still being developed[298]. Several species belonging to the fungal genus *Aspergillus*, have a high enzyme production capacity and are a leading source of commercial enzymes, many of which have a long safety record particularly in the food and drink industries, and are classified as GRAS ("Generally Recognized As Safe") by the United States Food and Drug Administration (FDA)[299].

A major therapeutic problem is that most biofilms in nature are multispecies, the chemical constituents found in biofilm EPS are complex and numerous and the precise composition of EPS is dynamic, varying widely and continuously between and within species[16,132]. Enzymes often bind and act on their targets with great affinity and specificity and it is unlikely that the action spectrum of any single enzyme will provide adequate coverage for the dynamic heterogeneous chemistry of bacterial EPS. Researchers have suggested that complete degradation is more likely to require a complex cocktail of enzymes or suitable enzyme-antibiotic combinations[10,13,17,296,300]. One such enzyme preparation is Pectinex (Pectinex Ultra SP-L, Novozymes, SA; Appendix A), a readily available product that is derived from *Aspergillus aculeatus*. The manufacturer's declared enzyme activity in Pectinex is polygalacturonase (Appendix A), and no other proprietary information on enzyme activity was made available. However, scientific studies have demonstrated that Pectinex possesses a wide range of carbohydrase activities that includes pectinase, arabanase, cellulase, hemicellulase, *β*-glucanase, xylanase, fructosyltransferase and α-amylase[10,299,301,302].

Table 4: Selected enzymes and their actions on biofilms of common bacteria[303]

Enzyme	Biofilms	Action
Alginate lyase	*P. aeruginosa* biofilm	Degrades alginate, an exopolysaccharide produced by the bacteria, and causes enhanced sloughing of biofilm cells[118]. Co-administration of antibiotics (gentamicin or ceftazidime) with alginate lyase increases the efficacy of the antibiotics[13].
Amylases (amyloglucosidase and bacterial amylase novo)	*P. fluorescens* biofilm grown on a glass susbstratum.	Amylases were not effective in degrading the EPS mainly because protein was the main component of EPS. [11].
Cellulase	*P. aeruginosa* on glass	Partial inhibition of biofilm formation[304].
Crude fungal enzyme extracts	*P. fluorescens* biofilm	Significant degradation of biofilm matrix by enzymes from *Aspergillus niger*, *Trichoderma viride* and *Penicillium* species[12].
Dispersin B (β-hexosaminidase)	*E. coli*, *S. epidermidis*, *Yersinia pestis*, *P. aeruginosa*, *P. fluorescens* and *S. enterica serovar Typhimurium* biofilm.	Dispersin B hydrolyses the intercellular adhesin, *β*-1,6-*N*-acetylglucosamine and inhibits biofilm formation by *E. coli*, *S. epidermidis*, *P. fluorescens* and *Y. pestis* but not *S. enterica* or *P. aeruginosa*. Mature biofilm of *E. coli*, *P. fluorescens* and *S. epidermidis* are dispersed, but not *Y. pestis*[287].
	S. epidermidis biofilms.	Efficient removal of biofilm by degrading EPS or interfering with intercellular adhesion[17,105].
	S. aureus and *S. epidermidis* biofilms.	Dispersin B improves the diffusion of cefamandole into biofilms and enhances the drugs bactericidal activity[300].
DNAase I	*P. aeruginosa* biofilm	DNase effectively removed young biofilms (< 60 h old); established biofilms (>84 h) were affected to a minor degree[133].
Bovine pancreatic DNase I	*E. coli*, *H. influenzae*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. pyogenes* and *Acinetobacter baumannii*	DNase degradation of eDNA altered biofilm formation and enhanced the effect of antibiotics, resulting in decreased biofilm biomass and cell count[292].

Enzyme	Biofilms	Action
Glucose oxidase and lactoperoxidase combination	*S. aureus, S. epidermidis, P. fluorescens, P. aeruginosa, Streptococcus mutans, Actinomyces viscosus* and *Fusobacterium nucleatum* biofilms.	Bactericidal *in vitro* but did not remove biofilm biomass[10].
Krillase® (protease activity)	*Streptococcus mitis, Streptococcus sanguis, Streptococcus gordonii* and *Actinomyces naeslundii* on hydroxyapatite. Dental plaque.	Decreased bacterial adhesion by over 70%; the decrease in adherent cells varied with the bacterial strain[14]. Removed plaque accumulated on dentures *in vivo* and *in vivo*[14].
Pectinex Ultra SPL®	*S. aureus, S. epidermidis, P. fluorescens, P. aeruginosa* on stainless steel and polypropylene substrata. *S. epidermidis (ATCC 35984),* and *clinical isolates of S. epidermidis, S. aureus* and *Staphylococcus lugdunensis*	Degraded EPS polysaccharides and removed bacterial biofilm but did not exhibit bactericidal activity[10]. Efficiently removed the biofilm of *S. aureus* but had a negligible effect on other staphylococcal strains[17].
Pronase® in combination with pectin methyl esterase, cellulase or pectin lyase	*P. fluorescens* biofilm on glass.	Effectively reduced cells and removed biofilm biomass[305].
Proteases (savinase, everlase and polarzyme)	*P. fluorescens* biofilm on glass.	Proteases (savinase and everlase) were highly effective in removing biofilms and degrading the EPS[11].
Serratiopeptidase	Sessile cultures of *P. aeruginosa* and *S. epidermidis* on glass. *S. epidermidis* biofilms on stainless steel and polymethylmethacrylate implants in Sprague-Dawley rats.	Enhanced activity of co-administered ofloxacin[291] Effective eradication of infection and enhanced efficacy of antibiotics[296].
Varidase (Streptokinase)	*S. aureus* biofilm.	Removed biomass and reduced bacterial cell count and enhanced the effect of ofloxacin[15].

Pectinex has also been shown to exhibit protease activity[17]. The wide range of enzyme activities in Pectinex makes it a suitable candidate to investigate for a role in the management of medical biofilm-related infections across the diverse range of bacterial species associated with medical infections. Pectinex is GRAS-designated and used extensively in the food and beverage industry to catalyse enzymatic maceration of plant cells in the preparation of mash (puree) foods and in the extraction of juices and oils from fruits, vegetables and grain[299]. The manufacturer's product information brochure (Appendix B) states that Pectinex is non-irritating to the skin and eyes and warns that inhalation of aerosolized enzyme may induce sensitization and cause allergic reactions in susceptible individuals. The brochure also states that ingestion of Pectinex is not known to cause significant adverse health effects and cites an oral lethal dose of more than 2 g/kg body weight in 50% of rats ($LD_{50)}$ which it regards as being "non-toxic". Pectinex has already demonstrated the ability to remove biofilms of two medically significant bacteria, *S. aureus* and *P. aeruginosa*, without manifesting any bactericidal or bacteriostatic effects. The antibiofilm action was probably due to the enzymatic degradation of exopolysaccharides, proteins and other undetermined substances within the EPS matrix[10,17].

As part of the ongoing investigations for new antibacterial treatment strategies, Pectinex presents itself as an agent that warrants further attention. To date there are no studies that examine the effect of the co-administration of antibiotics with Pectinex on either bacteria or bacterial biofilms. Furthermore, there are no published *in vitro* cytotoxicity studies on the effects of prolonged exposure, of either animal or human tissues, to pharmacological doses of Pectinex. The absence of such studies may stem from the fact Pectinex has a long-term safety record in various industrial applications but not been investigated as a potential therapeutic agent[299].

2.13 Aim

The aim of this study is to investigate a novel antibacterial treatment regimen, for the treatment of *S. aureus* and *P. aeruginosa* biofilm infections, in which conventional microorganism-targeted antibacterial therapy is supplemented by degradation of biofilm exopolymeric substance using Pectinex Ultra-SP-L enzyme.

2.14 Objectives

(i) To evaluate the effect of therapeutic and sub-therapeutic concentrations of amoxicillin-clavulanate and ciprofloxacin on *S. aureus* and *P. aeruginosa* with respect to the growth and viability of bacteria , and the establishment, growth, removal and disinfection of biofilms.

(ii) To investigate the effects of a range of concentrations of Pectinex on *S. aureus* and *P. aeruginosa* with respect to the growth and viability of bacteria, and the establishment, growth, removal and disinfection of biofilms.

(iii) To examine the influence of the concurrent administration of Pectinex with amoxicillin-clavulanate on *S. aureus*, and Pectinex with ciprofloxacin on *P. aeruginosa*, with respect to the growth and viability of bacteria, and the establishment, growth, removal and disinfection of biofilms.

(iv) To examine the effects of amoxicillin-clavulanate, ciprofloxacin, Pectinex and combinations of Pectinex with antibiotics on the electron microscopic structure of newly forming and preformed biofilms of *S. aureus* and *P. aeruginosa*.

(v) To investigate the cytotoxicity profile of Pectinex on human epithelial cells, lymphocytes and neutrophils *in vitro*.

CHAPTER 3

MATERIALS AND METHODS

3.1 Research and ethics approval

Approval to conduct this study on the commercial cell lines was obtained from the University of Pretoria Research Ethics Committee, Faculty of Health Sciences [Appendix C]. Ethical approval was obtained to collect blood from health donors and to isolate lymphocytes and neutrophils [Appendix D].

3.2 *In vitro* antimicrobial susceptibility tests on biofilm-forming pathogenic bacteria

3.2.1 Selection of microorganisms

S. aureus and *P. aeruginosa* were chosen for this study on the basis of their proven ability to form biofilm under test conditions and their importance as clinical pathogens; both clinical and laboratory strains were used[23,222]. American Type Culture Collection (ATCC) laboratory strains of *S. aureus* (ATCC 12600) and *P. aeruginosa* (ATCC 9027) were purchased from Davies Diagnostics (Randburg, SA) and clinical strains were sourced from the National Health Laboratory Service at the Steve Biko Academic Hospital (Department of Microbiology of the University of Pretoria).

Pure cultures of all selected strains of *S. aureus* and *P. aeruginosa* were grown and maintained on Mueller-Hinton Agar (MHA) plates. Stock cultures of the bacteria were stored at 4ºC and used sparingly to prepare fresh 24 h cultures. At each sub-culture, evidence of culture purity was assessed by the uniformity of colony form (shape and colour) on the plate, similarity with the gross appearance of the colonies seen on the original culture and by light microscopy. Mueller-Hinton Broth (MHB) was used as the nutrient medium during antimicrobial testing and as the diluent in the preparation of bacterial suspensions and antimicrobial solutions. The incubation condition for bacterial growth during all stages of the study was in an incubator with a temperature of 37ºC with 100% relative humidity.

3.2.2 Selection, storage and dose of antimicrobial test agents

The commercial enzyme Pectinex was chosen on the basis of prior evidence of antibiofilm activity from *in vitro* studies[10,17]. Pectinex (Pectinex Ultra SPL®) was a gift from Novozymes (Pty) Ltd, (Johannesburg, SA). The enzyme was supplied as a clear sterile, water-soluble liquid and was divided into multiple stock solutions and kept in a refrigerator at 4ºC as per the manufacturers instructions. The declared active ingredient in Pectinex is *polygalacturonase*; an enzyme produced by the fungus *Aspergillus aculeatus*. The stated enzyme activity was 9500 polygalacturonase units per millilitre (PGU/ml) of solution (Appendix A); where 1 PGU is defined as the amount of enzyme that liberates 1 µmol of galacturonic acid substrate per minute[306]. A dose range of 7.42 – 950 PGU/ml was chosen in order to span from below to above the concentrations that were previously shown to be effective against both *S. aureus* and *P. aeruginosa*[10].

The antimicrobial agents amoxicillin-clavulanate and ciprofloxacin were selected for use because of their known effectiveness against *S. aureus* and *P. aeruginosa*, respectively[307,308]. Amoxicillin-clavulanate (Co-Amoxiclav, Sandoz, SA) was purchased as a sterile powder and ciprofloxacin (Sabax Ciprofloxacin, Adcock Ingram, SA) as a sterile solution (2.0 mg/ml). Frozen stock solutions containing 1000 µg/ml were prepared for both drugs, by appropriate dilution with sterile distilled water and stored at -70ºC temperature; frozen stocks were periodically thawed for use as working stocks which were stored at 4ºC and used over a period of 4 – 8 weeks[309]. The bactericidal dose ranges, 0.03 – 32.0 µg/ml for amoxicillin-clavulanate and 0.03 – 8 µg/ml for ciprofloxacin, were chosen to incorporate a range that was intended to span from below to above the known MIC for each agent; 1.0 – 16.0 µg/ml and 0.25 – 1.0 µg/ml for amoxicillin-clavulanate and ciprofloxacin, respectively[307,308].

3.2.3 Spectrophotometric (turbidimetric) technique for preparation of standard bacterial inocula

All antimicrobial susceptibility experiments were conducted using a standard bacterial inoculum of 5 x 10^5 colony-forming units per millilitre (CFU/ml)[310]. To prepare the bacterial inocula, fresh colonies were cultured on MHA by overnight incubation for 12 to 18 h at 37ºC in air with 100% relative humidity. Four to five discrete colonies were harvested with a sterile loop and suspended in 5 ml of MHB and then incubated for a further 3 to 5 h. During

incubation, bacteria multiplied rapidly (logarithmic growth phase) and transformed the MHB from an initially clear solution into a turbid suspension as the bacterial count increased. The bacterial suspension was vortexed (Model 750, Heidolph Instruments, Germany) after which the optical density at 520 nm wavelength (OD520nm) of the suspension was measured using a colorimeter (Sherwood colorimeter 254, Sherwood Scientific Ltd, UK). The desired end-points were an OD520nm of 0.07 and 0.1 for *S. aureus* and *P. aeruginosa*, respectively; the OD equivalents of the 0.5 McFarland turbidity standard. It was accepted that a bacterial suspension with the OD520nm equivalent of a 0.5 McFarland turbidity standard had a cell density of approximately 1 x 10^8 CFU/ml. The suspension was diluted 100-fold with MHB to achieve a cell density of 1.0 x 10^6 CFU/ml. This diluted suspension was used in 100 µl aliquots such that a further 2-fold dilution, which occurred by the addition of 100 µl of test agents and/or MHB, produced a final inoculum cell density of 5 x 10^5 CFU/ml.

3.2.4 Viable colony count (VCC) and calculation of bacterial cell density

A VCC is done by plating out a small amount of serially diluted liquid culture onto nutrient agar, and after appropriate incubation, the colonies are counted. The underlying assumption is that each colony has risen from one single bacterium; a colony forming unit (CFU)[311]. A VCC was performed on each freshly prepared batch of bacterial suspension in order to obtain a quantitative estimate of the concentration of viable bacteria in the prepared 0.5 McFarland suspensions. In addition, the value obtained was also used to calculate the end-point for determining the minimum bactericidal concentration (MBC) of the selected test agents. In order to determine the VCC, each of the 0.5 McFarland bacterial suspensions was diluted 20000-fold (Table 5) and a volume of 10 µl of the diluted suspension was spread over the surface of drug free MHA plates. The agar plates were prepared in duplicate and incubated for 24 h at 37ºC in air with 100% relative humidity after which a manual colony count was performed with the plates illuminated by transmitted light. Values of between 5 and 200 colonies were deemed acceptable whilst values outside the range negated the validity of the experiment results obtained from the specific inoculum[311].

Table 5: Cumulative dilution and MHA plate inoculation of bacterial suspension

Procedure (steps 1 to 5)	Dilution		Expected cell density (CFU/ml)
	Per step	Cumulative	
1. Bacterial suspension (0.5 McFarland)	0	0	1×10^8
2. 100 µl of undiluted bacterial suspension added to 9.9 ml MHB	1:100	1:100	1×10^6
3. 50 µl of diluted (1:100) suspension added to 50 µl MHB (Final working concentration)	1:2	1:200	5×10^5
4. 100 µl of diluted (1:200) suspension added to 9.9 ml MHB	1:100	1:20000	5×10^3
5. 10 µl spread evenly onto MHA plate			

The average of duplicate VCC values were used to estimate the actual concentration (CFU/ml) of viable bacteria in each of the original 0.5 McFarland solutions and the results were compared with the expected estimate (Table 5). The cell concentrations were calculated by using the formula:[310,312]

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} = \frac{\text{Number of CFU}}{\text{mL}}$$

3.2.5 Microtitre plate setup for antimicrobial susceptibility tests

All susceptibility testing on planktonic bacteria and biofilm, was performed in clear, flat-bottomed 96-well microtitre plates with lids (Nunc Microwell Plate; Product No. 269787). The wells on the microtitre plate are arranged in a rectangular grid of 8 rows, labelled A though to H, and 12 columns, labelled 1 through to12. Bacteria were cultured in MHB until biofilms adhered to the walls of the microtitre plate wells.

3.2.6 Serial dilution of drugs and regimen for inoculation of microtitre challenge plates

Solutions of the test agents were prepared to a concentration of 10 times the highest concentration to be used; antibiotics solutions had concentrations of 320 and 80 µg/ml for amoxicillin-clavulanate and ciprofloxacin, respectively, and pectinex was not diluted (i.e. 9500 PGU/ml). Using each of these solutions as the start of a unique series, 8 two-fold dilutions of each were prepared by macrodilution in 5 ml test tubes to provide a volume of 2 ml per dilution. The required 10-fold dilution was achieved in a volume of 200 µl in the individual wells of the microtitre plates, where 20 µl aliquots of the dilutions were added to 180 µl of solution (a mixture of MHB, bacterial suspension with or without co-administered test agent).

All wells on a microtitre plate were used; columns 1 to 4 (rows A to H) were used for control experiments and columns 5 to 12 (rows A to H) were used for the antimicrobial challenge tests (Table 6, Figures 13, 14 and 15).

Table 6: Layout of microtitre antimicrobial challenge plates

Column Number(s)	Contents
1	Nutrient sterility control with 200 µl MHB per well; without bacteria or test agents
2	Sterility control with 200 µl of sterile distilled water
3	Negative control containing 100 µl of bacterial suspension (1 x 10^6 CFU/ml) plus 100 µl MHB without antimicrobial agents
4	Additional negative control when antibiotics were assessed without the addition of Pectinex (Figure 13) Positive control containing 100 µl of bacterial suspension (1 x 10^6 CFU/ml) plus 80 µl MHB and 20 µl of either amoxicillin-clavulanate 32.0 µg/ml or ciprofloxacin 8.0 µg/ml (Figures 14 and 15)
5 to 12	Antimicrobial challenge wells containing 100 µl of bacterial suspension and 20 µl aliquots of 8 concentrations of: antibiotics in decreasing concentrations down the plate from row A to H (Figure 13) plus 80 µl MHB Pectinex in decreasing concentrations across the plate from column 5 to 12 in rows A through to H (Figure 14) plus 80 µl MHB an antibiotic and Pectinex in a checkerboard arrangement (Figure 15) plus 60 µl MHB

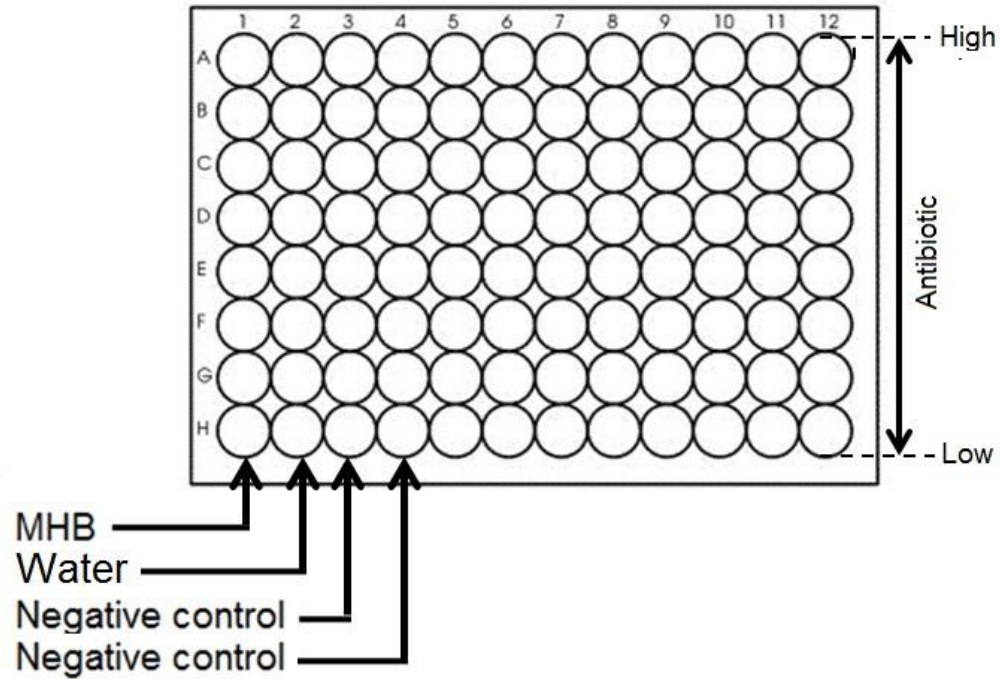

Figure 13: Microtitre plate layout using antibiotics

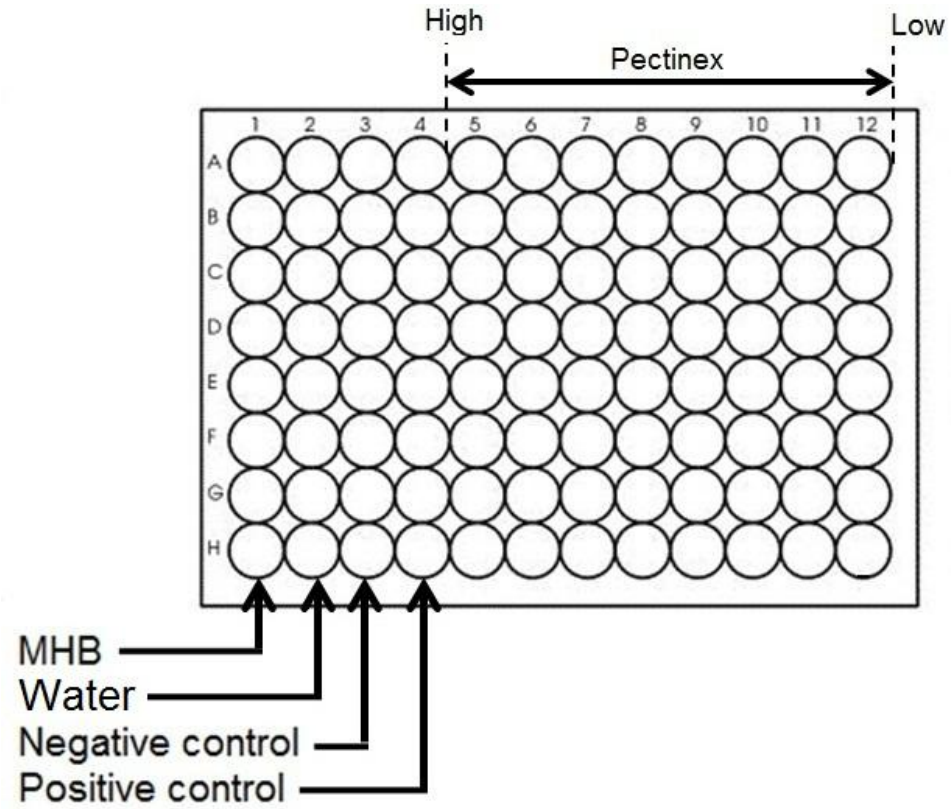

Figure 14: Microtitre plate layout using Pectinex

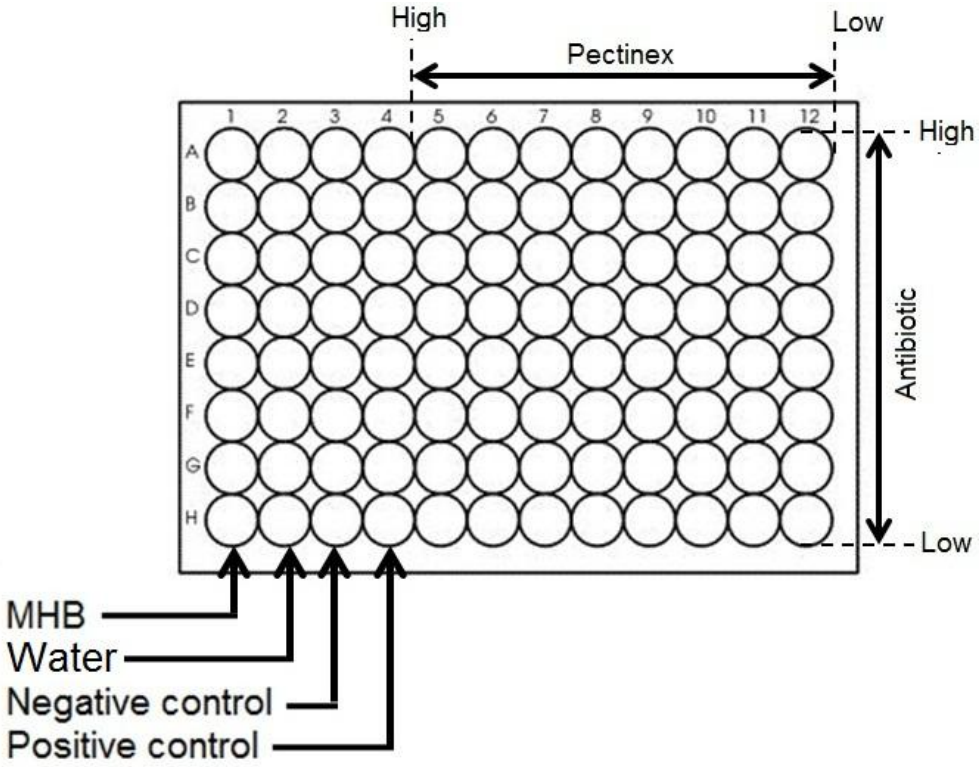

Figure 15: Microtitre plate layout using antibiotics and Pectinex (checkerboard assay)

3.2.7 Antimicrobial microtitre susceptibility tests

Methods were initially evaluated in pilot experiments to verify that satisfactory results could be obtained. Amoxicillin-clavulanate and ciprofloxacin were tested against *S. aureus* and *P. aeruginosa*, respectively, and Pectinex against both microorganisms. Susceptibility tests using single agents were performed in octuplet, and each experiment was repeated at least three times on different days. Thereafter the effect of antibiotic-Pectinex combinations was assessed against the designated microorganisms for the selected antibiotic. A checkerboard synergy assay, in an 8-column x 8-row configuration (Figure 15), was used to evaluate the effects of 8 doubling concentrations of Pectinex, from 7.42 to 950 PGU/ml, on the antibacterial and antibiofilm efficacy across a range of 8 concentrations of amoxicillin-clavulanate and ciprofloxacin. These experiments were performed in triplicate and repeated at least three times. The results obtained from this assay were compared with those obtained by exposure to the individual agents alone and used to determine the interaction index of each combination. No antibiotic-antibiotic combinations were evaluated. Growth in untreated cultures represented 0% inhibition. In treated cultures, a value > 0% indicated positive inhibition (i.e. reduced bacterial and/or biofilm growth) and a value < 0% indicated negative inhibition (i.e. increased growth).

a) *p*-Iodo-nitrotetrazolium violet (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; *p*-INT) MIC assay

A modified *p*-INT microplate MIC assay of Eloff[313] was used to assess the antimicrobial activity of the selected antimicrobial agents on the growth and viability of the planktonic forms of bacteria. The results of this assay were used as a guide to select the most appropriate dose ranges for all subsequent antimicrobial tests. In this assay, after exposure to serial dilutions of an antimicrobial agent in a 96-well microtitre plate, the presence of viable planktonic micro-organisms was determined by their ability to reduce *p*-INT from a colourless solution to a visible red formazan dye.

Microtitre challenge plates were set up as previously described (Table 6). The concentration ranges for the test agents were:

- Amoxicillin-clavulanate; 0.25 – 32.0 µg/ml
- Ciprofloxacin; 0.06 – 8.0 µg/ml
- Pectinex; 7.42 – 950 PGU/ml

Following incubation at 37ºC for 24 h, 20 µl of 0.20 mg/ml *p*-INT aqueous solution (Sigma-Aldrich, St Louis, Missouri, USA) was added to all the wells and the plates incubated for a further 6 h at room temperature. After incubation, each well was examined for colour and the MIC was determined. The MIC was taken as the concentration in the well with the lowest concentration that inhibited the reduction of *p*-INT. This was indicated by the first clear well to appear in the ascending order of the serial concentrations of the antimicrobial agent. The results of the MIC assay were used as a guide to determine the appropriate concentration range that was subsequently used throughout the remainder of the study.

b) Minimum bactericidal concentration (MBC) assay

The MBC is defined as the lowest concentration of an antimicrobial agent which kills at least 99.9% of the starting (pre-incubation) planktonic bacterial population in an inoculum (the equivalent of a 3 $\log_{10}$ reduction)[314]. The MBC assay followed on from the MIC assay and depended on the whether or not the antimicrobial agent inhibited planktonic bacterial growth. The MBC assessment was not performed on cultures exposed exclusively to Pectinex because

it did not display any ability to inhibit bacterial growth. Where the antimicrobial agent demonstrated an inhibitory effect on bacterial growth then, after determining the MIC, a subculture was grown from the contents of two of the first clear wells and the first red-coloured well in the same column. An inoculum of 100 µl was obtained from the selected wells and diluted 100-fold after which 10 µl was spread over an agar plate. This procedure was performed in duplicate. The inoculated agar plates were then incubated at 37ºC for 24 h. After incubation, the subculture plates were examined for growth and, if any colonies were formed, a colony count was performed and used to determine the antimicrobial concentration at which 99.9% killing of bacteria in the inoculum was achieved. The formula, $\mathbf{n + 2\sqrt{n}}$, is used to determine a quantitative endpoint that includes the 95% confidence limits for 99.9% killing. In this calculation, n is equal to 0.1% of the VCC and $n + 2\sqrt{n}$ is the corrected MBC Cut-off Colony Number (CCN). The first drug concentration, in the order of ascending drug concentration, that grows less than or equal to the number of colonies determined by this calculation (i.e. CCN) was considered the MBC.

c) Dose regimen for antimicrobial test agents

After evaluating the MIC and MBC for amoxicillin-clavulanate and ciprofloxacin against *S. aureus* and *P. aeruginosa*, respectively, and pectinex against both bacteria, the serial dilution sequences were adjusted to 11 for amoxicillin-clavulanate and 9 for ciprofloxacin; pectinex remained unchanged at 8. A dose regimen was established for the antibiotics against all four strains of microorganisms (Table 7).

Table 7: Dose ranges for amoxicillin-clavulanate, ciprofloxacin and Pectinex

Microorganism	Concentration range of test agents
S. aureus ATCC	Amoxicillin-clavulanate; 0.03 – 4.0 µg/ml
S. aureus clinical strain	Amoxicillin-clavulanate; 0.125 – 16.0 µg/ml
P. aeruginosa ATCC	Ciprofloxacin; 0.03 – 4.0 µg/ml
P. aeruginosa clinical strain	Ciprofloxacin; 0.03 – 4.0 µg/ml
All strains	Pectinex; 7.42 – 950 PGU/ml

d) Modified crystal violet (CV) assay

A modified CV assay of Pitts *et al.*[315] was used to measure the total biofilm mass which included both the bacteria (dead or alive) and the exopolysaccharide matrix. The assay was used to evaluate the effects of the test agents on the formation, growth and removal of biofilm. Microtitre challenge plates were prepared (Table 6) using the newly established dose regimen (Table 7). Biofilms were cultured and challenged using four different protocols to evaluate the effect of the test agents on:

i. biofilm formation after a 6 h exposure to the test agents
ii. biofilm formation after a 24 h exposure
iii. preformed 6 hour-old biofilms after an 18 h exposure
iv. preformed 24 hour-old biofilms after a 24 h exposure

To assess the ability of the test agents to inhibit formation of biofilm, microtitre plates prepared with both bacterial suspension and test agents were incubated for 6 and 24 h at 37°C. In order to test the ability of the test agents to prevent biofilm development or to remove mature biofilms, biofilms were pre-formed in 96-well microtitre plates by aliquoting 100 µl of standardized (1.0×10^6 CFU/ml) *S. aureus* or *P. aeruginosa* suspension and 80 (or 60) µl of MHB into the wells without the addition of test agents and incubating the suspension for either 6 or 24 h at 37°C. Thereafter, the test agents were added and the plates incubated for a further 18 h following a 6 h growth period, or 24 h following the 24 h growth period.

At the end of the incubation period, medium and planktonic bacteria were removed from each microtitre plate by briskly shaking the plates out over a waste tray. Then, to remove loosely attached bacteria, the plates were rinsed three times by submerging the plates in a tray filled with microfiltered tap water and vigorously shaking out the contents over the waste tray. The plates were air dried for 45 to 60 minutes. Thereafter, the microplate wells were filled with 200 µl of 0.1% (w/v) crystal violet (Sigma-Aldrich, St Louis, Missouri, USA) and plates were allowed to stand for 30 min at room temperature with the lid in place to allow staining of any adherent material on the walls. Plates were then washed three times to remove all unabsorbed stain by successive submersion through the series of water trays and shaking out as much liquid as possible after each wash[316]. The washing steps were performed to remove any crystal violet that was not specifically staining the adherent biofilm. Following the final washing step, the plates were inverted and vigorously tapped on paper towels to remove any excess

liquid, after which the plates were air-dried overnight at room temperature. Stain that was adsorbed onto the biofilm was solubilized by adding 200 µl of 95% ethanol to each well and allowing the plates to stand for 15-20 min at room temperature. The OD560nm of solubilised crystal violet in all the wells was measured with a microplate reader (GLR 1000, Genelabs Diagnostics, Singapore). All raw absorbance values were corrected by subtracting the mean of absorbance readings of the water sterility control (regarded as a blank control with no biofilm and no treatment). The readings obtained from the antimicrobial challenge plates were presented as the percentage reduction in optical density when compared on a scale represented by the mean absorbencies of the negative control at 100% and the water sterility controls at 0%. A percentage reduction of equal to or greater than 40% was regarded as significant. The percentage reduction in absorbance was derived by using the equation[315]:

$$\text{Percentage Reduction} = \left[\frac{(C - B) - (T - B)}{(C - B)}\right] 100\%$$

Where;

C = mean absorbance of the negative control (bacteria in MHB with no antimicrobial agent present)

B = mean absorbance of the water sterility controls (no bacteria and no treatment)

T = mean absorbance of the treatment wells at a specified agent concentration (bacteria in MHB plus antimicrobial agent)

e) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay of antimicrobial efficacy against biofilm bacteria

The ability of all three test agents (i.e. amoxicillin-clavulanate, ciprofloxacin and Pectinex) to disinfect bacterial biofilms was evaluated using a quantitative spectrophotometric technique based on intracellular metabolism of MTT[317]. Microtitre challenge plates were prepared as described earlier (Table 6) and inoculated using the newly established dose regimen (Table 7). Bacteria were cultured and challenged using four different protocols to evaluate the ability of the test agents to kill bacteria embedded within biofilms at various stages of development [Section 3.2.7 (d)]. Following the scheduled periods of incubation 20 µl of MTT was added to all the wells on the microtitre plates. Based on the premise that biofilm bacteria are either slow-growing or non-growing, the bacterial cultures were incubated for a further 2 h at 37°C to provide reasonable time for MTT metabolism to occur within the biofilm bacteria. After

incubation the plates were emptied, then washed three times in microfiltered tap water to remove medium and loosely adherent bacteria [Section 3.2.7 (d)]. Thereafter the plates were left to dry overnight in a dark cupboard at room temperature and ambient humidity.

Intracellular MTT formazan precipitate was solubilised by adding 200 µl of DMSO to each well, replacing the lids and allowing the plates to stand in the dark for 60 min at room temperature. The OD560nm of solubilised MTT formazan in all the wells was measured with a microplate reader. The percentage reduction in viable bacteria was determined as the difference in absorbance between the treatment wells and the negative control and expressed as a percentage of the absorbance of the negative control.

3.3 *In vitro* cytotoxicity testing of Pectinex on human cell cultures and isolated cells

An assessment of Pectinex cytotoxicity was conducted on three selected human cell types: human cervical adenocarcinoma cells (HeLa cells, ATCC CCL-2), lymphocytes and neutrophils. Where modified experimental procedures were used, evaluation experiments were conducted to verify that satisfactory results could be obtained before conducting experiments for data collection. HeLa cell lines were selected because of their ability to adapt to growth in microtitre tissue culture plates and to replicate rapidly and indefinitely when suitable nutritional and incubator conditions are provided[318]. Human peripheral blood lymphocytes and neutrophils from healthy volunteers (Appendix D) were used to represent normal tissue[319]. Cells were incubated in cell culture medium at a temperature of 37ºC, 5% CO_2 in 95% air and, 100% relative humidity[320]. HeLa cells were incubated for 7 days, lymphocytes for 3 days and neutrophils for 4 h as well as 24 h[318,321,322]. During incubation the cells were challenged with 8 two-fold serial dilutions of Pectinex (7.42 - 950 PGU/ ml). Mitomycin C (10 µg/ml) and nutrient medium were used as positive and negative controls, respectively. Nutrient medium without cells was used as the experimental blank for optical density measurements.

After incubation, cell viability was determined using two modified spectrophotometric MTT assays based on two different protocols, each with its own merits and limitations[323,324]. The underlying mechanism in both methods is that viable, actively metabolising cells reduce MTT from a pale yellow solution to an insoluble dark purple formazan compound that is amenable to optical density measurement[317]. The essential difference between the two was that one

method, derived from a study by Denizot and Lang[324], included cell washing steps that were used to remove excess culture medium. A disadvantage of this method is that cell washing has been shown to be associated with inevitable loss of cells, which results in a significant reduction in optical density[323]. The other method, previously described by Hansen *et al.*[323] did not include washing steps but has been shown to be associated with increased background optical density due to the presence of the medium. The assays were conducted with and without cell washing steps in round-bottomed (300 µl per well; Nunc Microwell Plate; Product No. 163320) and flat bottomed (400 µl per well; Nunc Microwell Plate; Product No. 269787) microtitre plates, respectively.

3.3.1 HeLa cell growth and Pectinex microtitre challenge

HeLa cells were grown and maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin (EMEM+). The cultures were grown in 250 ml (75 cm^3) sealed polystyrene tissue culture flasks (Griener Bio-One, SPL Life Sciences Inc) at 37ºC in 5% CO_2 and 95% air. Culture medium was changed every 3-4 days to maintain optimal cell growth. When adherent cells reached 70-80% confluency, the culture medium was removed and about 3-5 ml of 0.25% trypsin/versene solution was added to the flask in order to induce cell detachment. The flask was incubated at 37ºC for 10-15 min until the cells detached from the flask walls. The resulting cell suspension was transferred into a 15 ml centrifuge tube which was then filled with EMEM+ and centrifuged at 200 g for 10 min. The supernatant was discarded and the cell pellet re-suspended in 1 ml of medium and the suspension thereafter homogenized by gentle pipetting.

The cell concentration of viable cells in the suspension was determined by the trypan blue exclusion method. The latter technique is based on the principle that living cells possess intact cell membranes that excludes trypan blue dye, whereas dead cells do not[325]. A volume of 50 µl of the cell suspension was added to 450 µl of cell counting fluid composed of trypan blue plus either 2% acetic acid or 1% hydrochloric acid. Manual cell counting of viable cells was done using a haemocytometer under light microscopy at x400 magnification. A cell suspension with a final concentration of 2.5 x 10^4 cells/ml was prepared by appropriate dilution in EMEM+.

a) Standardization of techniques for challenge of HeLa cells with Pectinex

No prior information was available on the pH of Pectinex, therefore, its value was measured using a pH meter (Crison pH meter, Crison Instruments, SA), and was found be 4.8. It was considered plausible that, at relatively high concentrations, Pectinex could substantially lower the pH of the culture medium and adversely affect cell growth and viability. Therefore, the effect of Pectinex on the viability of HeLa cells was evaluated using the MTT assays and, with the Pectinex solution at its original pH of 4.8 and when adjusted to 7.0 prior to inoculation of the challenge plates.

One hundred microlitre aliquots of cell suspension were seeded into all but three of the wells in both flat and round-bottomed 96-well microtitre plates. Three wells (row H, columns 1, 2 and 3) served as experiment blanks without any HeLa cells; column 1 in row H contained 200 µl of EMEM+ and columns 2 and 3 in row H each contained 180 µl EMEM+ plus 20 µl of Pectinex 9500 PGU/ml (Figure 16). The plates were incubated for 60 min at 37°C. After incubation the experiment layout was completed and the final volume achieved in all the wells was 200 µl:

- 100 µl of EMEM+ was added to the negative controls wells (columns 1 to 3)
- 80 µl EMEM+ and 20 µl mitomycin C (200 µg/ml) to the positive controls wells (column 4)
- 80 µl EMEM+ and 20 µl Pectinex to the challenge wells (columns 5 to 12)

Challenge wells were prepared in 2 groups, acidic (columns 5 to 8) and neutral (columns 9 to 12), with each group consisting of 4 replicates of 8 doubling dilutions (rows A to H) of Pectinex (7.42-950 PGU/ml) using EMEM+ as the diluent (Figure 16). At the start of each serial dilution, the pH of Pectinex in the acidic group was left unchanged at 4.8 whilst that of the neutral group was adjusted to 7.0 by titration with fine drops of 0.01M sodium hydroxide (NaOH) and continuous pH measurement. The microtitre plates were incubated for 7 days at 37°C, 5% CO2, 95% air and 100% relative humidity without changing the medium. All the experiments were repeated three times.

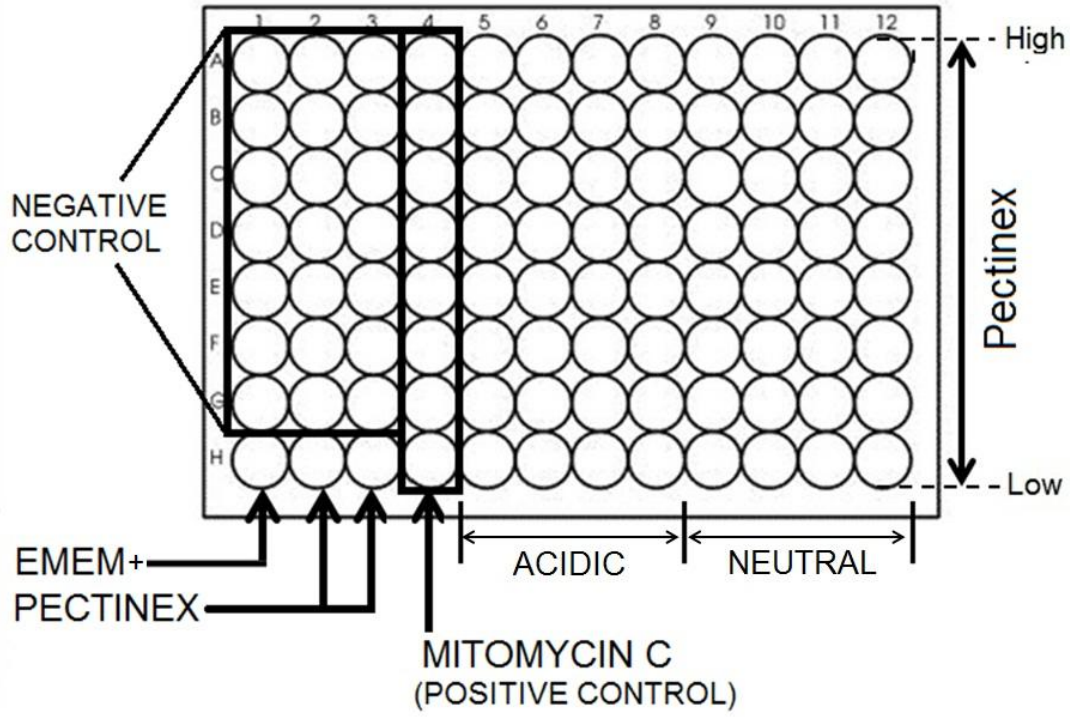

Figure 16: Microtitre plate layout for challenge of HeLa cells with Pectinex

b) MTT cytotoxicity assay

Following incubation, 20 µl of MTT (1 mg/ml) was added to the wells and the plates were incubated for a further 4 h under the same conditions. Mitochondrial dehydrogenase in viable, actively metabolising cells transformed MTT from a pale yellow solution to an insoluble dark purple formazan compound. Two methods were used to solubilize MTT formazan prior to optical density measurement:

i) Method 1: Round-bottomed tissue culture plates were used because they promoted pellet formation at the bottom of the wells during centrifugation which enabled removal of culture medium and facilitated cell washing with PBS. The plates were centrifuged at 850 g for 10 min after which the supernatant was removed by careful pipetting without disturbing the cell pellet. The pellets were washed by adding 150 µl of PBS to the wells, repeat centrifugation and then discarding the supernatant. Plates were air-dried overnight without exposure to light. After drying the plates, 100 µl of DMSO was added to each well, and the plates were agitated on a shaker (VRN-200 Orbital Shaker, Gemmy Industrial Corp, USA) for 1 h[324,326].

ii) Method 2: A 100 ml stock of cell lysis buffer solution was prepared by dissolving 30% w/v sodium dodecyl sulphate (SDS) in 50% v/v *N*,*N*-dimethyl formamide (DMF) in de-mineralized water at 35-37°C, using a combined hot-plate and magnetic-stirrer (Isotemp, Fischer Scientific). The pH of the solution was adjusted to about 4.5 by titration with 1% acetic acid and 1 M hydrochloric acid (HCl). A 100 µl aliquot of lysing buffer was added to all wells in the flat-bottomed microtitre plates and the plates were incubated overnight at 37°C in 5% CO_2. The lysis buffer dissolved the lipids and proteins of the cell membrane, and within the nutrient medium, to create a uniform suspension; cell washing procedures to remove protein and cell debris were not required. MTT formazan was released from the cells and solubilized by DMF[323].

After dissolving the MTT formazan, light optical density of each well was measured with an ELx800 Universal Microplate Reader (BioTek Instruments Inc, Bedfordshire, UK) at a test wavelength 570 nm and reference wavelength of 630 nm. The results were expressed as the percentage viability in comparison to the negative (untreated) control by using the formula:

$$\% \text{ cell viability} = \frac{\text{mean absorbance of sample x 100}}{\text{mean absorbance of control}}$$

c) Standardized protocol for cell viability assay

Analysis of results revealed that adjusting the pH of Pectinex from 4.8 to 7.0 had no significant influence on the outcome of the experiments. All subsequent cytotoxicity tests were conducted without pH manipulation. A further set of experiments was performed using HeLa cells, in which each concentration of Pectinex was assessed in triplicate and the experiments were repeated three times.

3.3.2 Isolation of lymphocytes and neutrophils and Pectinex microtitre challenge

Lymphocytes and neutrophils were isolated from freshly drawn blood by continuous density-gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, St Louis, Missouri, USA) using a modified method adapted from a study by Pretlow and Luberoff[319]. The method is

known to achieve a high degree of cell purity; 99.9 ± 0.1% in the case of lymphocytes and 90.1 ± 38.0% for granulocytes. In this procedure, a 90 ml sample of venous blood was obtained via phlebotomy into 10 ml ethylenediaminetetraacetic acid (EDTA) vacutainer tubes from each of four drug naive healthy human volunteers. After collection, the tubes were inverted 5-10 times then gently agitated on a tube rocker until processed within 1 h from collection. Each sample was poured directly from the vacutainer tubes onto 15 ml of Histopaque in a 50 ml conical centrifuge tube. The tube was centrifuged at 650 g for 25 min during which time the contents separated into five main layers which consisted, from top to bottom, of plasma, lymphocyte-rich peripheral blood mononuclear cells (PBMCs), Histopaque solution, neutrophil-rich granulocytes and erythrocytes (Figure 17) .

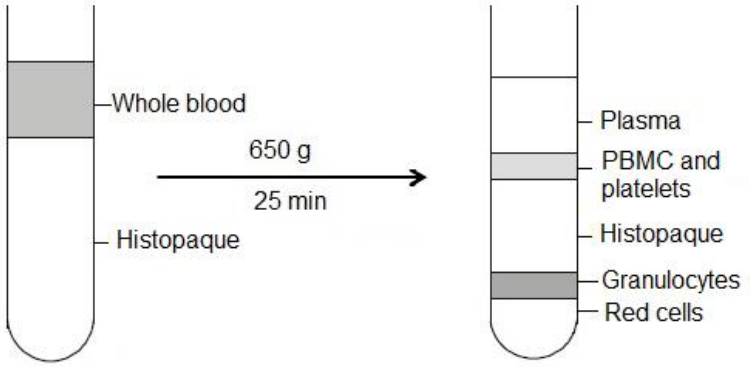

Figure 17: Schematic illustration of blood component separation by Histopaque continuous density-gradient centrifugation

a) Isolation of lymphocyte-rich PBMC's

The plasma layer was removed by pipetting and discarded. The entire PBMC layer was extracted and transferred into a sterile 50 ml conical centrifuge tube. To wash the cells in order to remove plasma and contaminating platelets, the tube was filled with 10% Roswell Park Memorial Institute Medium (RPMI-1640; Sigma-Aldrich, St Louis, Missouri, USA) and centrifuged at 200 g for 15 min. After centrifugation a cell pellet formed at the bottom of the tube and the supernatant was removed and discarded. The wash cycle was repeated with a shorter centrifuge time of 10 min. Thereafter, contaminating erythrocytes were lysed by filling the tube with cold (2-8ºC) ammonium chloride lysing solution, incubation on ice for 10 min, followed by centrifugation at 200 g for 10 min. The supernatant was discarded and the

cell pellet was re-suspended in 1 ml of RPMI supplemented with 10% v/v autologous serum. A viable cell concentration was determined by manual counting using a haemocytometer and light microscope as earlier described [Section 3.3.1]. Finally, a working concentration of about 2 x 10^6 cells/ml was made by appropriate dilution with RPMI.

b) Isolation of neutrophil-rich granulocytes

After removing the plasma, PBMC and Histopaque layers, the granulocyte layer was pipetted out and transferred into a 50 ml tube. The cells were cleared of platelets and erythrocytes by washing and erythrolysis as previously described [Section 3.3.2 (a)]. Then the cell pellet that was formed after centrifugation was re-suspended in 1 ml RPMI culture medium supplemented with 10% autologous serum and 1% penicillin-streptomycin. A working concentration of 2 x 10^6 cells per ml was prepared by cell counting and dilution as described.

c) Lymphocyte and neutrophil cytotoxicity challenge plates

Two Pectinex challenge plates with experimental controls were prepared to facilitate the two MTT assay protocols (Figure 18). An aliquot of 100 µl of cell suspension was dispensed into each of the wells of the 96-well plates except for the wells in column 1 (row A to H). The plates were incubated for 30 min at 37°C to allow the cells to adapt to culture conditions. Thereafter, Pectinex (950 – 7.42 PGU/ml) cytotoxicity was investigated in lymphocytes and neutrophils in both a resting and activated state. Cell in quadruplet wells at each concentration and a negative control were stimulated by adding 20 µl of 0.2% w/v *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) into each well. FMLP is a synthetic polypeptide that mimics the effects of bacterial cell wall and host mitochondrial proteins, thereby simulating bacterial infection and tissue damage *in vitro*, and activating lymphocytes and neutrophils[327,328]. This enabled the susceptibility of these cells to be studied during conditions that represented an infection.

The challenge plates were completed by adding to the wells:

- Column 1: 200µl of RPMI into the all the wells of to constitute the experimental blank
- Column 2: 100 µl of RPMI

- Column 3: 80 µl of RPMI plus 20 µl fMLP
- Column 4: 80 µl of RPMI plus 20 µl mitomycin C (200 µg/ml)
- Columns 5 to 8: 80 µl RPMI to all the wells and 20 µl Pectinex in decreasing concentrations down the columns
- Columns 9 to 12: 60 µl RPMI and 20 µl fMLP to all the wells and 20 µl Pectinex in decreasing concentrations down the columns

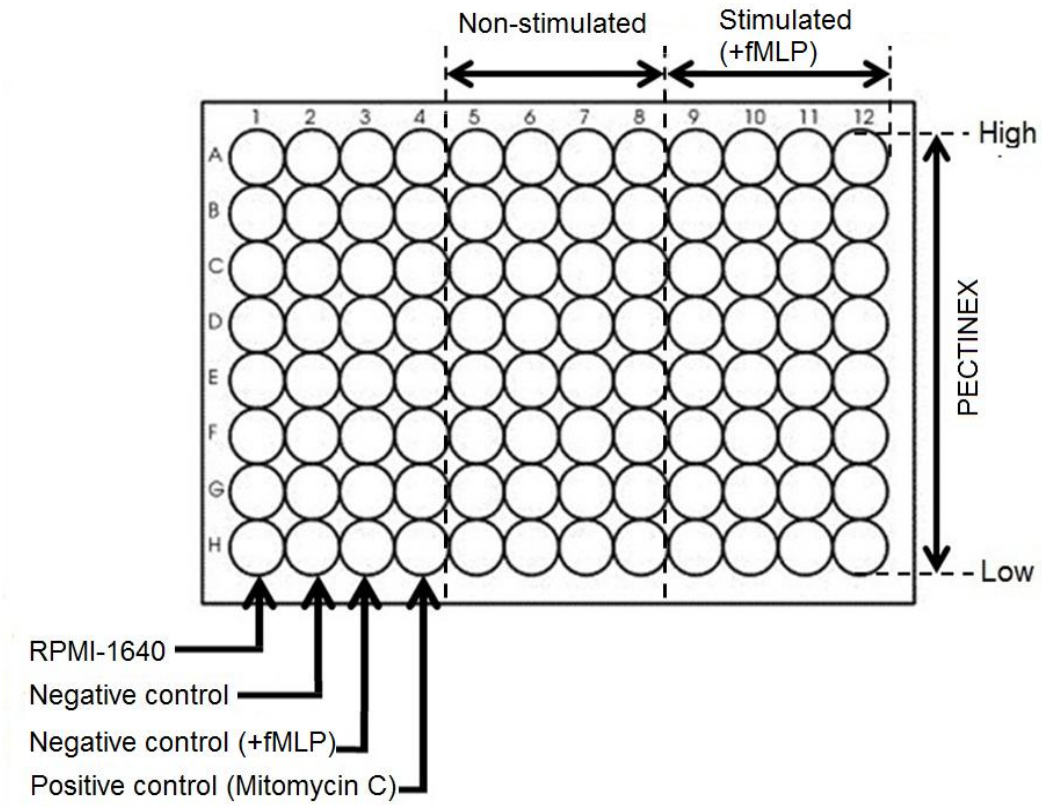

Figure 18: Microtitre plate layout for Pectinex cytotoxicity assay against lymphocytes and neutrophils

3.4 Examination of human cell cultures by polarization-optical transmitted light differential interference contrast microscopy (PlasDIC) after exposure to Pectinex

Suspensions of viable HeLa cells (2.5 x 10^4 cells/ml), lymphocytes and neutrophils (2.0 x 10^6 cells/ml) were prepared. Aliquots of 250 µl were seeded into separate 24-well polystyrene cell culture plates (Costar®, Product No: 3524, Corning Incorporated, USA). The cells were exposed to eight 2-fold serial dilutions of Pectinex (7.42-950 PGU/ml) for 24 h at 37°C. Mitomycin C (10 µg/ml) and staurosporine (1 µg/ml) served as primary and secondary

positive controls, respectively, and untreated cells were used as negative controls. The final layout of the plates consisted of 250 µl of cell suspension to all the test wells and completed by adding:

- 50 µl mitomycin C and 200 µl culture medium to primary positive controls (2 wells)
- 50 µl staurosporine and 200 µl culture medium to secondary positive controls (2 wells)
- Negative controls
    - HeLa cells: 250 µl culture medium to untreated cells (4 wells)
    - Lymphocytes and neutrophils: 50 µl fMLP and 200 µl culture medium to fMLP-stimulated cells (2 wells) and non-stimulated cells (2 wells)
- Challenge wells
    - HeLa cells: 50 µl Pectinex plus 200 µl culture medium (8 wells)
    - Lymphocytes and neutrophils: 50 µl Pectinex, 50 µl fMLP and 200 µl culture medium to fMLP-stimulated cells (8 wells) and non-stimulated treatment wells (8 wells)

Following incubation, morphological characteristics of the cells were observed by PlasDIC at x400 magnification using a Zeiss Axiovert-40 microscope (Göttingen, Germany) and images were captured by AxioVision camera software[329]. Evidence of cell death was determined by the presence of morphological changes that included cell shrinkage, plasma membrane blebbing, apoptotic bodies, cytoplasmic vacuolization and disruption of the cell membrane[330].

3.5 Scanning electron microscopy (SEM) of bacterial biofilms after exposure to antibiotics and Pectinex

SEM examination of biofilm ultra-structure after antimicrobial challenge was performed at the Central Laboratory for Microscopy and Microanalysis (UP). To observe the effects of selected antibiotic and enzyme doses on biofilm formation, biofilms were cultured for 24 h on glass microscope coverslips in the wells of 6-well microtitre plates (Product No. 3335, Corning, Amsterdam, Netherlands). During incubation, each microorganism was challenged with the designated antibiotic at its established MIC (Table 8), Pectinex 118.75 PGU/ml (serial dilution below the IC_{50} of Pectinex for HeLa cells) and the combination of antibiotics with Pectinex. Positive (amoxicillin-clavulanate 32 µg/ml, ciprofloxacin 8.0 µg/ml and pectinex 950 PGU/ml) and negative (untreated) controls were used. To assess the effects of

the test agents on established biofilms, 24 h-old biofilms were cultured on microscope coverslips in 6-well microtitre plates then exposed to the same regimen of test agents over the following 24 h.

The final layout of the plates contained a total volume of 5000 µl which was achieved by adding 2500 µl of bacterial suspension to all the wells and then completed as follows:

- Positive control: 500 µl of antibiotic (either amoxicillin-clavulanate 32.0 µg/ml or ciprofloxacin 8 µg/ml) plus2000 µl culture medium
- Negative control: 2500 µl culture medium to untreated cells
- Challenge wells:
  - 500 µl Pectinex (950 PGU/ml) plus 2000 µl culture medium
  - 500 µl Pectinex (118.75 PGU/ml) plus 2000 µl culture medium
  - 500 µl antibiotic (at MIC for specific bacteria) plus 2000 µl culture medium
  - 500 µl antibiotic (MIC) and 500 µl Pectinex (118.75 PGU/ml) plus 1500 µl medium

Table 8: MIC values of antibiotics against microorganisms

Microorganism	MIC
S. aureus ATCC	Amoxicillin-clavulanate; 0.5 µg/ml
S. aureus clinical strain	Amoxicillin-clavulanate; 2.0 µg/ml
P. aeruginosa ATCC	Ciprofloxacin; 0.125 µg/ml
P. aeruginosa clinical strain	Ciprofloxacin; 1.0 µg/ml

After incubation, biofilm specimens were prepared for electron microscopy using the laboratory standard operating procedure. The medium was pipetted out from all the well of the microtitre plates and discarded. The wells and coverslips were rinsed three times; at each rinse 5 ml of PBS was added to the wells and then discarded after 10 min. After rinsing, the biofilm specimens that remained adherent to the coverslips were prepared for electron microscopy using the described method of Kim *et al.*[331] with modifications. Biofilm on both the control and treatment coverslips were fixed in 2.5% glutaraldehyde in 0.075M PBS for 1 h

at room temperature. Secondary fixation was done with 0.5% aqueous osmium tetroxide for 1-2 h at room temperature, followed by a repeat sequence of rinsing in PBS. Fixed biofilm specimens were dehydrated by transfer through a series of increasing concentrations of ethanol (30, 50, 70, 90, 100, 100 and 100%) for 10 min at each concentration. Final preparation for microscopy was by critical point drying with liquid CO_2 [Critical Point Dryer: Bio-Rad E3000, Watford, England] and sputter coating with gold [Emitech K550X Sputter Coater, Ashford, England]. The specimens were examined by scanning electron microscopy [SEM; JEOL JSM-840, Tokyo, Japan] and images were captured. Biofilm formation was determined by the presence of adherent cells which, if present, were characterized as weak, moderately adherent or fully established as described by Smith *et al.*[332]. The experiments were performed in duplicate, and features of biofilm morphology were noted and compared with untreated controls.

3.6 Statistical analyses and presentation of data

All *in vitro* microbiology tests were carried out at least in triplicate and on separate occasions and repeated as necessary to obtain complete datasets. The MIC and MBC of three test agents (amoxicillin-clavulanate, ciprofloxacin and Pectinex) against planktonic bacterial cultures were determined visually and from colony growth on agar plates. The quantity of biofilm and the viability of biofilm bacteria were determined by spectrophotometric assays and the results were expressed as the mean percentage of the negative controls ± standard error of the mean (S.E.M). Pectinex cytotoxicity results on cell viability in human cells were expressed as the mean percentage cell survival ± S.E.M. as compared to the negative controls. Morphological changes in bacteria and human cells were documented as images and the findings were described. Numeric data were captured and manipulated using software Microsoft Excel 2007, IC_{50} values were calculated using ED50plus version 1.0 software, and data were analyzed for statistical significance using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, California, USA). Significance testing was performed in conjunction with the Department of Statistics (UP). Analysis on variables was carried out using Kruskal-Wallis one-way analysis of variance with Dunn's multiple-comparison procedure, and regarded as statistically significant at a level of $p \leq 0.05$.

CHAPTER 4

RESULTS

4.1 Growth and viability of planktonic bacteria

The MIC and MBC for the antibiotics and Pectinex against *S. aureus* and *P. aeruginosa* are presented in Table 9. The MBC was of the same order of magnitude as the MIC in *S. aureus*. For the clinical and standard strains of *P. aeruginosa*, the MBC was found to be higher than the MIC. Following incubation with Pectinex (7.42 – 950 PGU/ml), no bactericidal effects was observed in any of the bacterial cultures; therefore no MIC or MBC values were obtained. The checkerboard MIC and MBC, which represents the concentration of the antibiotic in an antibiotic-Pectinex mixture, had values similar to the MIC and MBC of the antibiotic alone in standard cultures of *S. aureus* and *P. aeruginosa*. In the clinical cultures, an increase was observed in both the MIC and MBC.

Table 9: MIC and MBC of amoxicillin-clavulanate and ciprofloxacin against *S. aureus* and *P. aeruginosa*, respectively.

	Amoxicillin-clavulanate (µg/ml)		Ciprofloxacin (µg/ml)	
	S. aureus ATCC	*S. aureus* clinical strain	*P. aeruginosa* ATCC	*P. aeruginosa* clinical strain
MIC	0.42 ± 0.08	2.0 ± 0.0	0.13 ± 0.0	1.0 ± 0.0
MBC	0.50 ± 0.0	2.0 ± 0.0	0.33 ± 0.08	1.67 ± 0.33
Checkerboard MIC	0.42 ± 0.04	2.56 ± 0.38	0.12 ± 0.01	1.89 ± 0.11
Checkerboard MBC	0.56 ± 0.09	2.56 ± 0.38	0.32 ± 0.05	3.22 ± 0.40

Results expressed as mean ± S.E.M. of at least three experiments carried out in triplicate.

4.2 Effect of Pectinex, amoxicillin-clavulanate and ciprofloxacin on biofilm biomass and viability

Clinical and reference strains of *S. aureus* and *P. aeruginosa* were exposed to Pectinex and antibiotics (amoxicillin-clavulanate and ciprofloxacin) as single agents and in combination. Biofilm biomass was quantified by CV staining and viability of biofilm bacteria was quantified by MTT spectrophotometric assay. Quantitative values of biomass and viability were expressed as the mean percentage reduction ± S.E.M. of the value in untreated cultures. Detailed results of the mean percentage reduction ± S.E.M. for the biomass and viability assays are found in Appendix E (E1 – E16) and Appendix F (F1 – E16), respectively. In the statistical analysis of each assay, Pectinex, antibiotics and Pectinex – antibiotic combinations were compared with untreated controls, and then Pectinex and antibiotics were compared with Pectinex – antibiotic combinations. The statistical significance of these comparisons is presented for each bacterium. The figures presented in the text show the results for Pectinex, the antibiotic and a combination of the antibiotic with the highest (950 PGU/ml) and lowest (7.42 PGU/ml) concentration of Pectinex; exceptions were made in order to highlight specific data. The results for the rest of the combinations are provided in Appendix E and F.

4.2.1 Biofilm biomass and viability after 6 h incubation of bacterial cultures with Pectinex and antibiotics

a) *S. aureus* ATCC

Pectinex concentrations from 7.42 to 237.5 PGU/ml were associated with significant ($p \leq 0.05$) negative inhibition (i.e. stimulation) of biofilm formation as compared to the untreated control (Figure 19A). Non-significant ($p > 0.05$) inhibition of -117.3 ± 30.2% and 29.5 ± 11.3% was observed with Pectinex 475 and 950 PGU/ml, respectively. Significant ($p \leq 0.05$) inhibition occurred with amoxicillin-clavulanate 0.5 – 4.0 µg/ml (Figure 19B). Non-significant ($p > 0.05$) inhibition was found at 0.125 and 0.25 µg/ml. Negative inhibition was observed at 0.03 and 0.06 µg/ml; however, it was not statistically significant ($p > 0.05$). The combination of Pectinex 475 PGU/ml and amoxicillin-clavulanate 4.0 µg/ml significantly ($p \leq 0.05$) inhibited (103.6 ± 5.8%) biofilm formation as compared to untreated controls. With all other combinations, biofilm biomass was not significantly ($p > 0.05$) different from the untreated control.

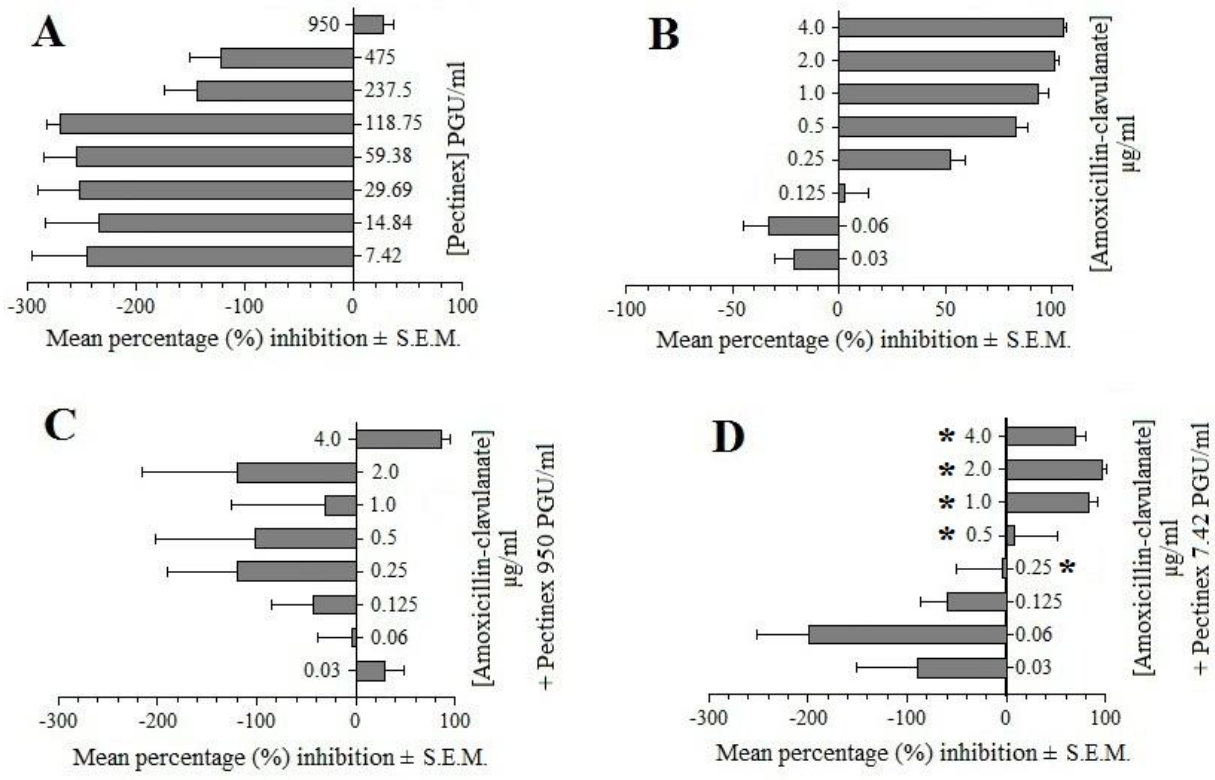

Figure 19: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *S. aureus* ATCC strain after 6 h incubation with [A] Pectinex, [B] amoxicillin-clavulanate, [C] Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E1].

Treatments with Pectinex 950 PGU/ml and amoxicillin-clavulanate (0.03 – 4.0 µg/ml) were not significantly ($p > 0.05$) different from that of singly administered Pectinex (Figures 19A and 19C). At lower Pectinex concentrations, the following combined treatments were found to have significantly ($p \leq 0.05$) greater inhibition than Pectinex alone:

- Pectinex 29.69 – 475 PGU/ml with amoxicillin-clavulanate 2.0 and 4.0 µg/ml
- Pectinex 14.84 PGU/ml with amoxicillin-clavulanate 1.0 – 4.0 µg/ml
- Pectinex 7.42 PGU/ml with amoxicillin-clavulanate 0.25 – 4.0 µg/ml (Figures 19A and 19D)

No significant ($p > 0.05$) differences in biomass were found following treatment with amoxicillin-clavulanate alone and when it was used in combination with Pectinex.

A significant ($p \leq 0.05$) increase in biofilm bacterial viability was noted at low concentrations of Pectinex (7.42 – 59.38 PGU/ml) and a significant ($p \leq 0.05$) decrease was found at 950

PGU/ml (Figure 20A). There was no significant ($p > 0.05$) difference between biofilm viability in the untreated control and in cultures exposed to Pectinex at 118.75 – 475 PG/ml. Amoxicillin-clavulanate 0.06 – 4.0 µg/ml reduced biofilm viability, and 0.03 µg/ml was associated with an increase (Figure 20B). However, these findings were not significantly ($p > 0.05$) different from those in untreated controls.

Pectinex 950 PGU/ml in combination with all concentrations of amoxicillin-clavulanate (0.03 – 4.0 µg/ml) significantly ($p \leq 0.05$) reduced biofilm viability (Figure 20C). A significant ($p \leq 0.05$) reduction in biofilm viability also occurred when Pectinex 475 PGU/ml was used in combination with amoxicillin-clavulanate at 0.03, 0.25 – 1.0 and 4.0 µg/ml. Pectinex 475 PGU/ml plus amoxicillin-clavulanate 0.06 and 2.0 µg/ml produced non-significant ($p > 0.05$) inhibitions of 35.2 ± 12.1% and 40.3 ± 19.2%, respectively. Most combinations of Pectinex 7.42 – 237.5 PGU/ml with amoxicillin-clavulanate (0.03 – 4.0 µg/ml) were associated with non-significant ($p > 0.05$) increases in viability (stimulation) of biofilm bacteria. The exceptions in which non-significant ($p > 0.05$) inhibition was found were Pectinex 237.5 PGU/ml with amoxicillin-clavulanate 0.125, 0.5 and 1.0 µg/ml.

No significant ($p > 0.05$) differences in biofilm viability were found between cultures incubated with Pectinex (7.42 – 950 PGU/ml) alone and when Pectinex was combined with amoxicillin-clavulanate (0.03 – 4.0 µg/ml) (Figures 20A, 20C and 20D). When the effects of combinations were compared with those of singly administered amoxicillin-clavulanate, biofilm viability was significantly ($p \leq 0.05$) less when amoxicillin-clavulanate 0.03 and 1.0 µg/ml was combined with Pectinex 950 PGU/ml than when either concentration was used alone (Figures 20B and 20C). The combination of amoxicillin-clavulanate 0.06 µg/ml with Pectinex 7.42 PGU/ml was found to promote biofilm viability to a significantly ($p \leq 0.05$) greater extent than the antibiotic alone (Figures 20B and 20D). There were no significant ($p > 0.05$) differences between biofilm viabilities found in cultures exposed to amoxicillin-clavulanate (0.03 – 4.0 µg/ml) alone and when it was combined with Pectinex 14.84 – 475 PGU/ml.

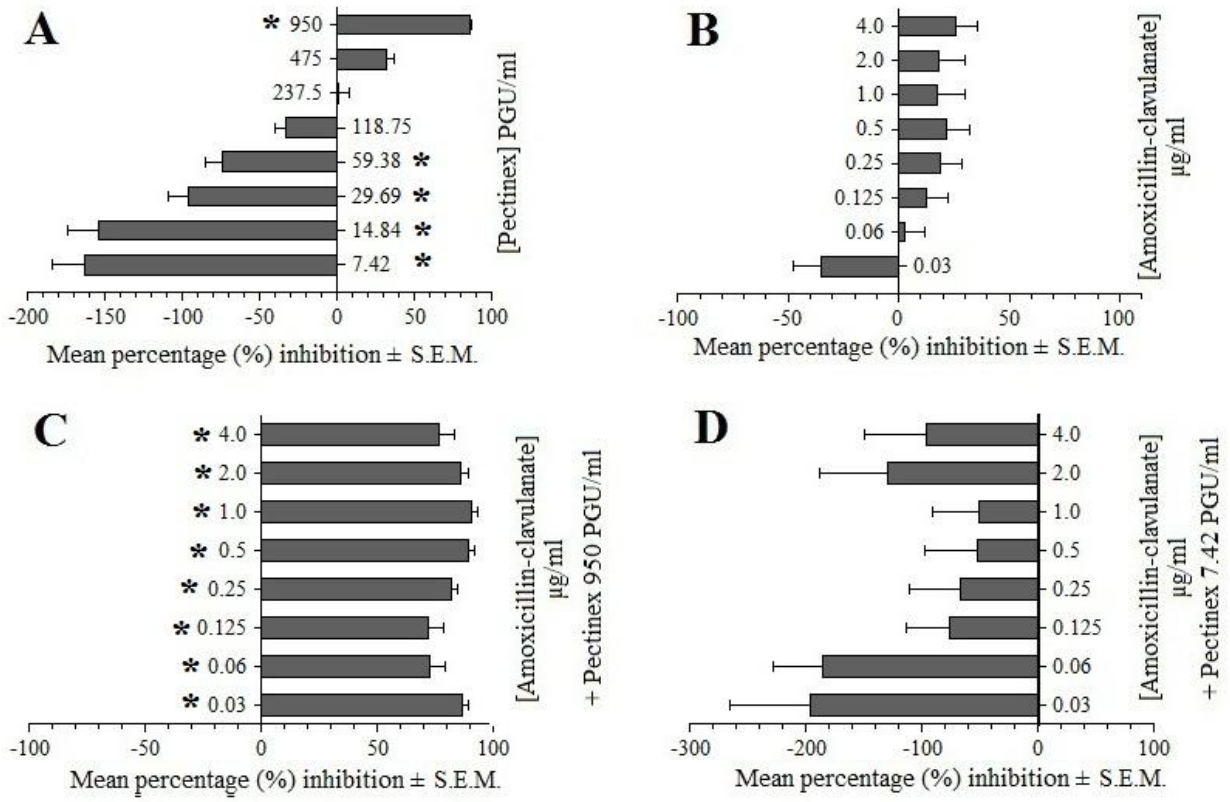

Figure 20: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *S. aureus* ATCC cultures after 6 h incubation with [A] Pectinex, [B] amoxicillin-clavulanate, [C] combination of Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] combination of Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F1].

b) *S. aureus* clinical strain

Pectinex concentrations from 7.42 to 118.75 PGU/ml were associated with statistically significant ($p \leq 0.05$) increases in biomass (Figure 21A). At 475 and 950 PGU/ml, Pectinex inhibited biofilm development by 20.1% ± 10.1% and 68.4 ± 2.9% respectively; however, this was not statistically significant ($p > 0.05$). With Pectinex 237.5 PGU/ml, there was negative inhibition (-109.5 ± 26.0%) that was not significant ($p > 0.05$). In cultures incubated with amoxicillin-clavulanate, a significant ($p \leq 0.05$) reduction in biofilm formation occurred at 2.0 – 16.0 µg/ml (Figure 21B). At lower concentrations (0.125 – 1.0 µg/ml) the inhibitory effects decreased in a dose-dependent manner and were not statistically significant ($p > 0.05$). When the two agents were co-administered (Figures 21C and 21D), inhibition was observed with those combinations that had high concentrations of Pectinex (475 and 950 PGU/ml), whilst enhanced growth was observed at lower concentrations (7.42 – 237.5 PGU/ml). However,

none of the combined treatments were significantly ($p > 0.05$) different from untreated controls.

The combinations of amoxicillin-clavulanate 16.0 µg/ml with either of Pectinex 118.75 PGU/ml or 237.5 PGU/ml inhibited biofilm formation by 42.5 ± 53.8% and 74.8 ± 13.5%, respectively. The effects of these combinations were significantly ($p \leq 0.05$) greater than with Pectinex alone. No statistically significant ($p > 0.05$) differences were found with any of the other combinations. When the effects of combinations were compared with those of amoxicillin-clavulanate administered alone, significantly greater inhibition was observed with combinations of amoxicillin-clavulanate 4.0 µg/ml plus Pectinex 7.42 – 118.75 PGU/ml, amoxicillin-clavulanate 2.0 µg/ml plus Pectinex 118.75, and amoxicillin-clavulanate 0.5 µg/ml plus Pectinex 59.38 PGU/ml.

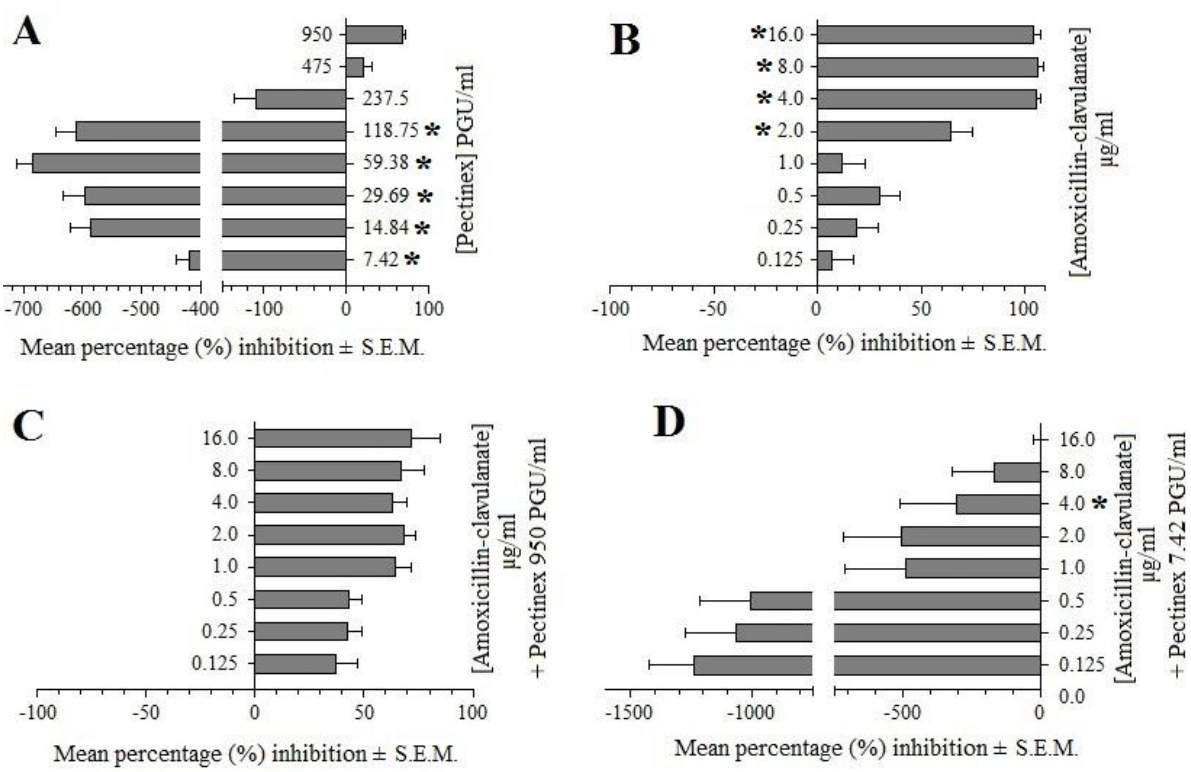

Figure 21: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *S. aureus* clinical strain after 6 h incubation with [A] Pectinex, [B] amoxicillin-clavulanate, [C] Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate(* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E2].

Pectinex 475 and 950 PGU/ml caused a significant ($p \leq 0.05$) reduction of biofilm bacterial viability of 68.1 ± 3.8% and 79.1 ± 3.4%, respectively (Figure 22A). Enhanced biofilm viability was observed after incubation with Pectinex 59.38 and 118.75 PGU/ml and inhibition occurred with 237.5 PGU/ml; however, the effects were not significantly ($p > 0.05$) different from the untreated control. The lower concentrations of Pectinex (7.42 – 29.69 PGU/ml) significantly ($p \leq 0.05$) enhanced biofilm viability. The enhancement and reduction of biofilm viability that was observed with amoxicillin-clavulanate (0.125 – 16.0 µg/ml) was not significantly ($p > 0.05$) different from the untreated control (Figure 22B).

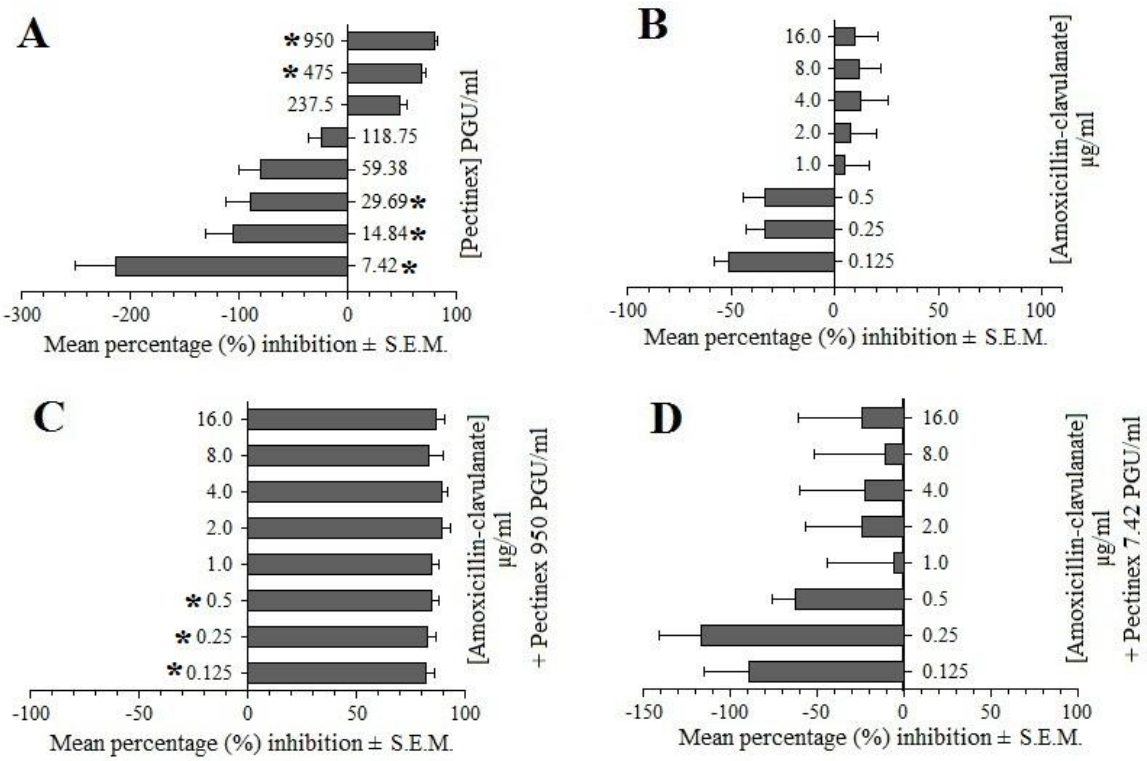

Figure 22: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *S. aureus* clinical strain cultures after 6 h incubation with [A] Pectinex, [B] amoxicillin-clavulanate, [C] combination of Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] combination of Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F2].

No statistically significant ($p > 0.05$) differences in the effects on biofilm viability were observed between the combined treatments and singly administered Pectinex (Figures 22A, 22C and 22D). The combinations of amoxicillin-clavulanate 0.125, 0.25 and 0.5 µg/ml paired with each of Pectinex 237.5, 475 and 950 PGU/ml (Figures 22B and 22C) reduced biofilm viability to a significantly ($p \leq 0.05$) greater extent than singly administered amoxicillin-clavulanate.

c) *P. aeruginosa* ATCC

Biofilm inhibition by Pectinex 237.5 – 950 PGU/ml was significantly ($p \leq 0.05$) greater when compared with the negative control (Figure 23A). Inhibition of 1.2 ± 2.9% and 11.4 ± 2.6% occurred at 59.38 and 118.75 PGU/ml, respectively, but was not significant ($p > 0.05$). Concentrations of 7.42 – 29.69 PGU/ml were associated with increased biofilm growth; however, this was not significantly ($p > 0.05$) different from the untreated control. Ciprofloxacin significantly ($p \leq 0.05$) prevented biofilm formation at the concentrations 0.25 – 4.0 µg/ml (Figure 23B).

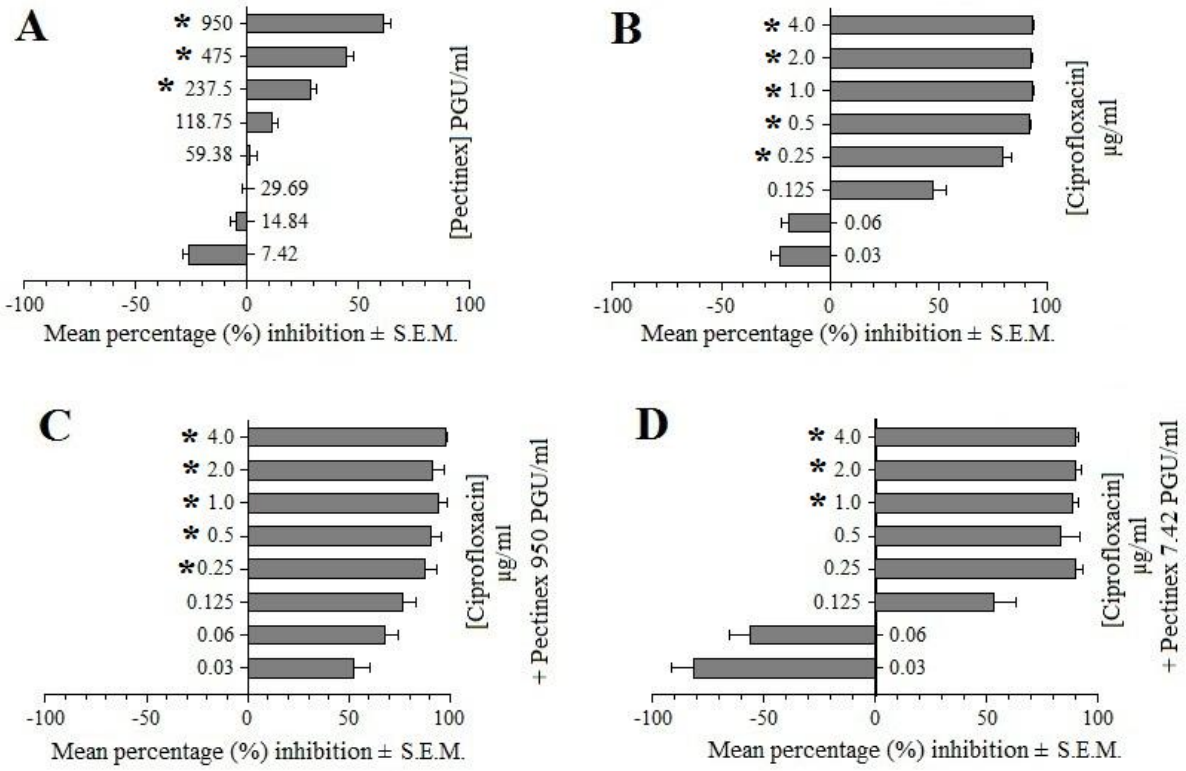

Figure 23: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *P. aeruginosa* ATCC strain after 6 h incubation with [A] Pectinex, [B] ciprofloxacin, [C] Pectinex 950 PGU/ml plus ciprofloxacin, and [D] Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E3].

There was also a 47.6 ± 5.8% mean reduction in biofilm formation at 0.125 µg/ml, and enhanced growth of -22.8 ± 4.4% and -19.0 ± 3.3% that occurred at 0.03 and 0.06 µg/ml, respectively; however none of these findings were statistically significant ($p > 0.05$). Significant ($p \leq 0.05$) inhibition of biofilm biomass occurred with combinations of:

- Pectinex 59.38 – 950 PGU/ml and ciprofloxacin 0.25 – 4 µg/ml
- Pectinex 29.69 PGU/ml plus ciprofloxacin 0.5 – 4.0 µg/ml
- Pectinex 14.84 PGU/ml plus ciprofloxacin 1.0 – 4.0 µg/ml

None of the combinations with Pectinex 7.42 PGU/ml were significantly ($p > 0.05$) different from the untreated controls.

The combinations that inhibited biofilm formation to a significantly ($p \leq 0.05$) greater extent than Pectinex alone were Pectinex (7.42 – 950 PGU/ml) with ciprofloxacin 1.0 – 4.0 µg/ml, Pectinex 7.42 – 118.75 PGU/ml with ciprofloxacin 0.5 µg/ml, and Pectinex 59.38 PGU/ml with ciprofloxacin 0.25 µg/ml (Figures 23A, 23C and 23D). No significant ($p > 0.05$) difference in biofilm biomass was observed after treatment with combined regimens and treatment with ciprofloxacin alone.

P. aeruginosa ATCC biofilm viability was reduced by Pectinex at concentrations 59.38 – 950 PGU/ml, and enhanced by low concentrations (7.42 – 29.69 PGU/ml), in a dose dependent manner (Figure 24A). Significant ($p \leq 0.05$) reduction of viability occurred with Pectinex 237.5, 475 and 950 PGU/ml with percentage inhibition values of 38.5 ± 1.9%, 53.0 ± 1.6% and 73.8 ± 1.3%, respectively. The effect at all other concentrations of Pectinex (7.42 – 118.75 PGU/ml) was not significantly ($p > 0.05$) different from the untreated control. Ciprofloxacin significantly ($p \leq 0.05$) reduced biofilm viability at the concentrations 0.25 – 4.0 µg/ml (Figure 24B). At 0.125 µg/ml, ciprofloxacin caused a 76.6 ± 1.2% reduction in viability; however, this was not statistically significant ($p > 0.05$). Non-significant ($p > 0.05$) enhancement of biofilm viability was observed in cultures incubated with ciprofloxacin 0.03 and 0.06 µg/ml.

When cultures were incubated with both agents, statistically significant ($p \leq 0.05$) reduction in viability was found with combinations of:

- Pectinex 29.69 – 950 PGU/ml plus ciprofloxacin 0.25 – 4.0 µg/ml; with the exceptions of Pectinex 29.69 and 59.38 PGU/ml in combination with ciprofloxacin 2.0 µg/ml (Figure 24C)
- Pectinex 14.84 PGU/ml plus ciprofloxacin 0.25 and 1.0 µg/ml
- Pectinex 7.42 PGU/ml plus ciprofloxacin 1.0 and 4.0 µg/ml (Figure 24D).

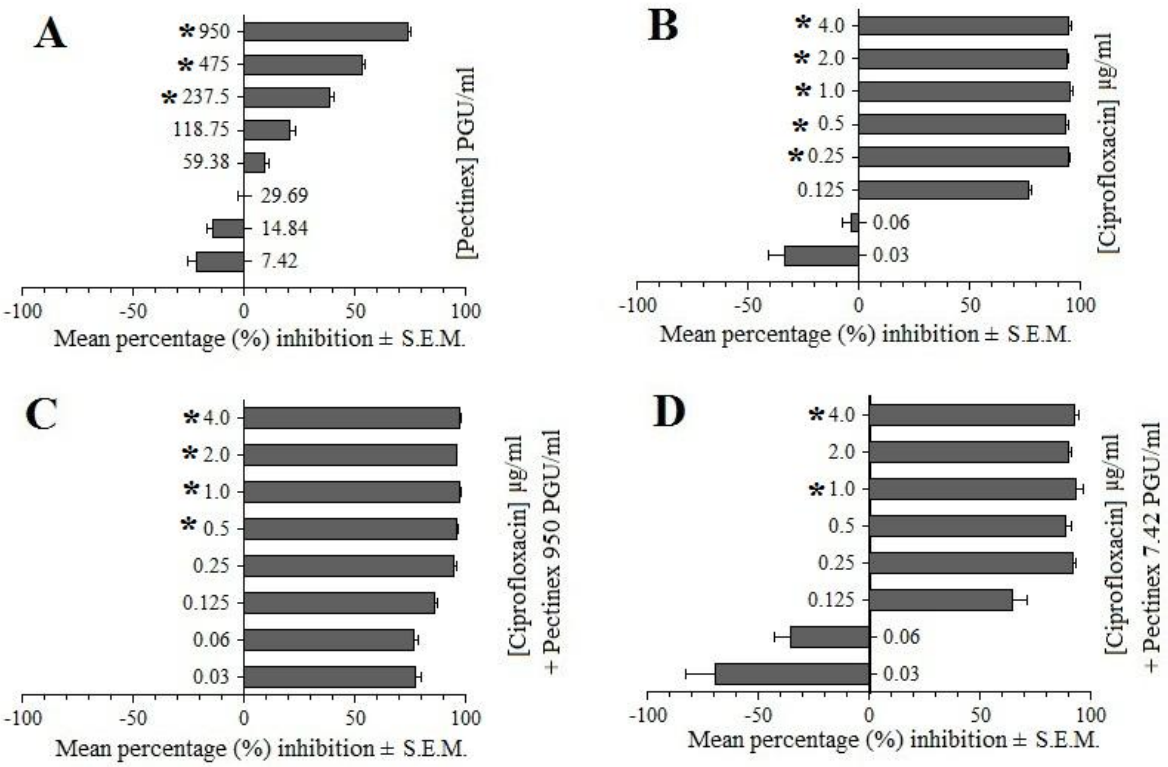

Figure 24: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *P. aeruginosa* ATCC cultures after 6 h incubation with [A] Pectinex, [B] ciprofloxacin, [C] combination of Pectinex 950 PGU/ml plus ciprofloxacin, and [D] combination of Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F3].

The reduction in biofilm viability was significantly ($p \leq 0.05$) greater after treatment with Pectinex (7.42 – 950 PGU/ml) in combination with ciprofloxacin at 0.25 – 4.0 µg/ml than when Pectinex was used alone. There was no significant ($p > 0.05$) difference between the effects of singly administered Pectinex and the combinations with the lowest concentrations of ciprofloxacin (0.03 – 0.125 µg/ml). At all concentrations of ciprofloxacin (0.03 – 4.0 µg/ml), there was no significant ($p > 0.05$) difference between the effects observed when the drug was combined with Pectinex (7.42 – 950 PGU/ml) and when it was used as a single agent.

d) *P. aeruginosa* clinical strain

Pectinex was found to have a dose-dependent effect on biofilm formation (Figure 25A). Significant ($p \leq 0.05$) inhibition (40.4 ± 6.2%) and significant ($p \leq 0.05$) stimulation (-31.7 ± 5.4%) was exhibited by 950 and 7.42 PGU/ml, respectively.

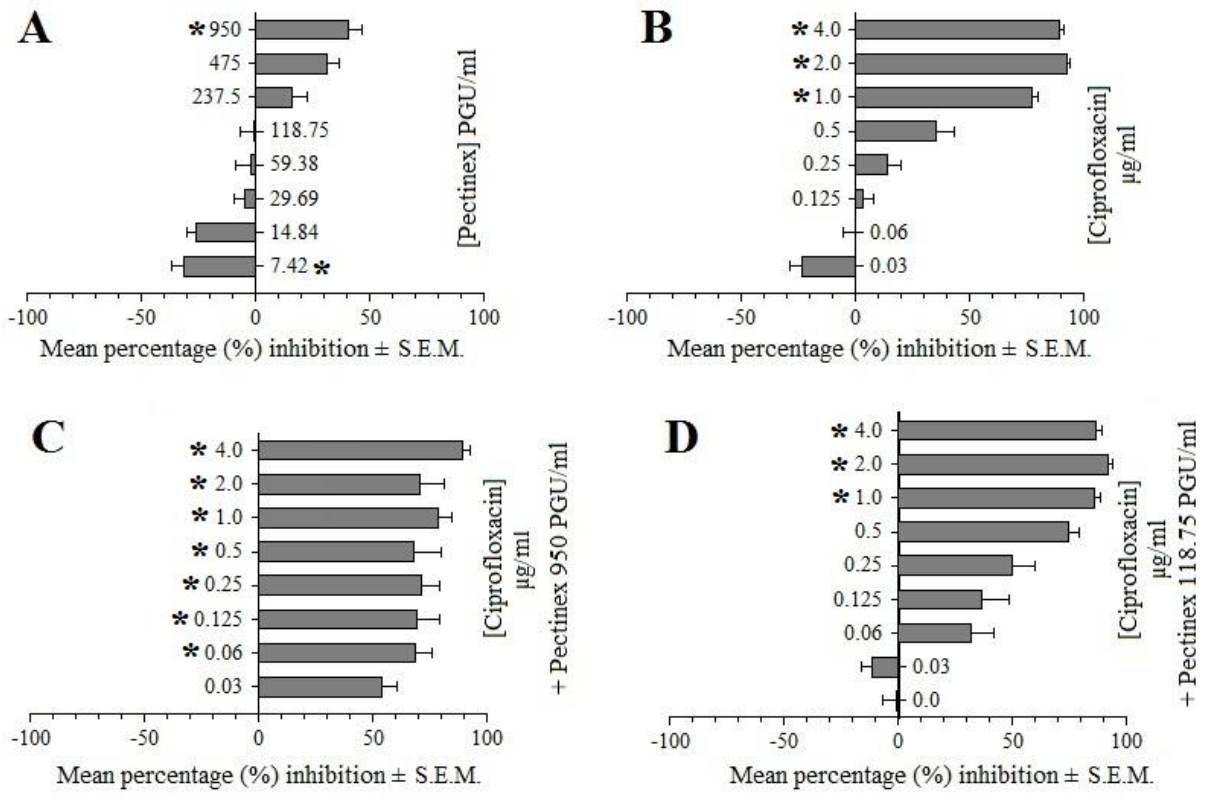

Figure 25: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *P. aeruginosa* clinical strain after 6 h incubation with [A] Pectinex, [B] ciprofloxacin, [C] Pectinex 950 PGU/ml plus ciprofloxacin, and [D] Pectinex 118.75 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E4].

The effects at the other concentrations ranged from -26.2 ± 3.9% stimulation at 14.84 PGU/ml to 31.0 ± 5.4% inhibition at 475 PGU/ml; however, none were found to be significantly ($p > 0.05$) different from the untreated controls. Significant ($p \leq 0.05$) biofilm inhibition was found at ciprofloxacin concentrations of 1.0 – 4.0 µg/ml (Figure 25B). When compared with the untreated control, significant ($p \leq 0.05$) inhibition of biofilm biomass occurred with combinations of:

- Pectinex 7.42 – 950 PGU/ml and ciprofloxacin 1.0 – 4.0 µg/ml
- Pectinex 950 PGU/ml and ciprofloxacin 0.06 – 0.5 µg/ml (Figure 25C)
- Pectinex 475 PGU/ml and ciprofloxacin 0.5 and 0.5 µg/ml
- Pectinex 118.75 and ciprofloxacin 0.5 µg/ml (Figure 25D).

Several combinations of Pectinex and ciprofloxacin exhibited biofilm inhibition that was significantly ($p \leq 0.05$) than when the same concentrations of Pectinex were used alone. These concentrations were:

- Pectinex 7.42 – 237.5 PGU/ml with ciprofloxacin 1.0 – 4.0 µg/ml
- Pectinex 14.84 – 118.75 (Figure 25D) with ciprofloxacin 0.5 µg/ml.

With Pectinex 475 and 950 PGU/ml, no significant ($p > 0.05$) difference in biofilm inhibition was observed with combinations and when Pectinex was administered alone. When the effects of combined treatments were compared with the effects of singly administered ciprofloxacin, the magnitude of biofilm inhibition was found to be greater ($p \leq 0.05$) when Pectinex 950 PGU/ml was combined with ciprofloxacin 0.03 and 0.06 µg/ml (Figures 25B and 25C).

Statistically significant ($p \leq 0.05$) reduction in biofilm bacterial viability was observed after incubation with Pectinex 118.73, 237.5, 475 and 950 PGU/ml (Figure 26A). Reduction in viability occurred with Pectinex 59.38 PGU/ml, and increased viability was found at 7.42 – 29.69 PGU/ml; however, these findings were not statistically significant ($p > 0.05$). Ciprofloxacin 1.0 – 4.0 µg/ml significantly ($p \leq 0.05$) reduced biofilm viability. Non-significant ($p > 0.05$) reduction in viability occurred with ciprofloxacin 0.25 and 0.5 µg/ml, and non-significant increases in viability were observed 0.03 and 0.06 µg/ml.

The effects of bacterial cultures that were incubated with combinations of the two agents were compared with the effect observed in untreated controls. Significant ($p \leq 0.05$) reduction in biofilm viability occurred with all combinations of:

- Pectinex 950 PGU/ml plus ciprofloxacin 0.25 µg/ml
- Pectinex 475 PGU/ml plus ciprofloxacin 1.0 – 4.0 µg/ml
- Pectinex 29.69 – 237.5 PGU/ml (Figure 26C) and Pectinex 950 plus ciprofloxacin 0.50 µg/ml
- Pectinex 7.42 – 950 plus ciprofloxacin 1.0 – 4.0 µg/ml (Figures 26C and 26D)

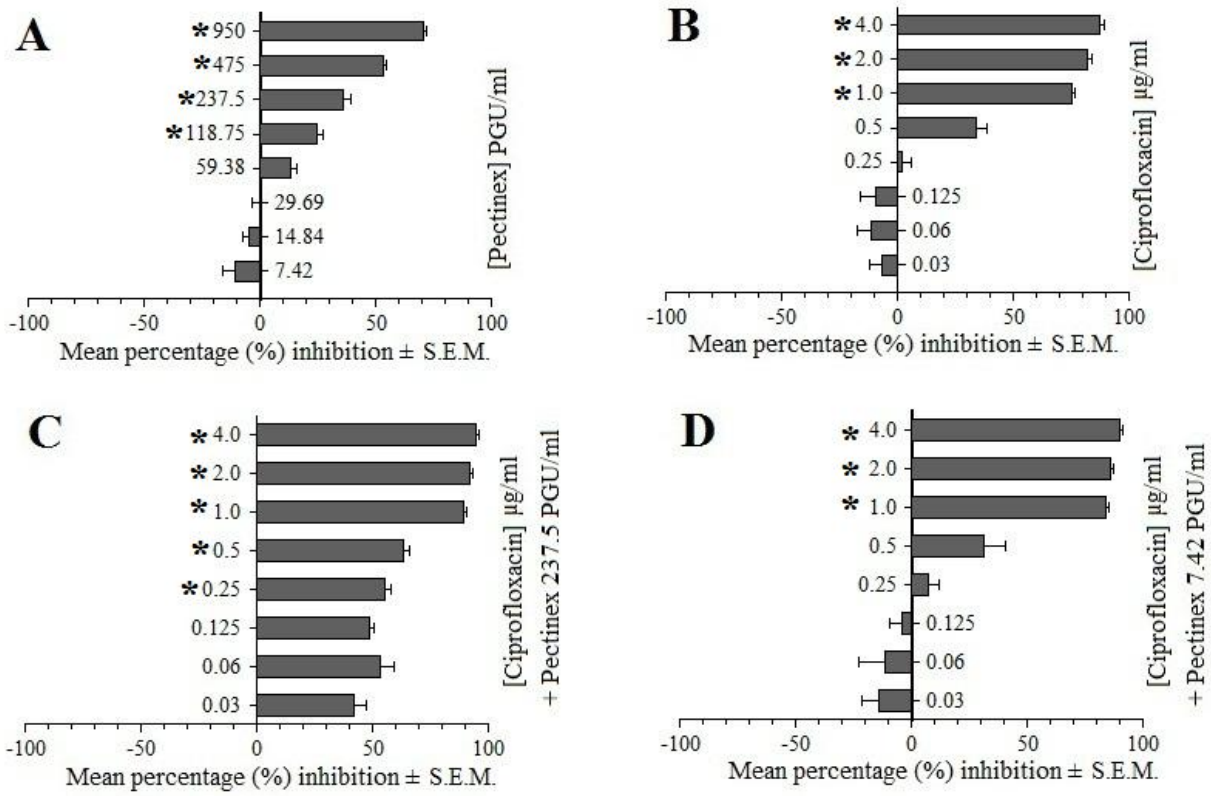

Figure 26: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *P. aeruginosa* clinical strain cultures after 6 h incubation with [A] Pectinex, [B] ciprofloxacin, [C] combination of Pectinex 237.5 PGU/ml plus ciprofloxacin, and [D] combination of Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F4].

Non-significant ($p > 0.05$) reduction in viability occurred with all other combinations, except for Pectinex 7.42 plus ciprofloxacin 0.03 – 0.125 µg/ml where a slight increase in viability was observed. Compared with the effect of singly administered Pectinex, significantly ($p \leq 0.05$) greater reduction in biofilm viability was found at concentrations of Pectinex 7.42 – 237.5 PGU/ml when combined with ciprofloxacin 1.0 – 4.0 µg/ml (Figure 26C and 26D). Combined treatments with ciprofloxacin (0.03 – 4.0 µg/ml) and either Pectinex 475 or 950 PGU/ml were not significantly ($p > 0.05$) different from singly administered Pectinex. The effects of ciprofloxacin 0.06 µg/ml plus Pectinex 118.75 – 950 PGU/ml, ciprofloxacin 0.125 µg/ml plus Pectinex 457 and 950 PGU/ml, and the effects of ciprofloxacin 0.25 µg/ml plus Pectinex 950 PGU/ml, were significantly ($p \leq 0.05$) greater than those observed when ciprofloxacin was administered alone. With all other combinations there were no significant ($p > 0.05$) differences between the effects of combinations and the effects of singly administered ciprofloxacin.

4.2.2 Biofilm biomass and viability after 24 h incubation of bacterial cultures with Pectinex and antibiotics

a) *S. aureus* ATCC

Biofilm biomass was greater in *S. aureus* ATCC cultures exposed to all concentrations of Pectinex than in the untreated controls (Figure 27A); however, the differences were not statistically significant ($p > 0.05$).

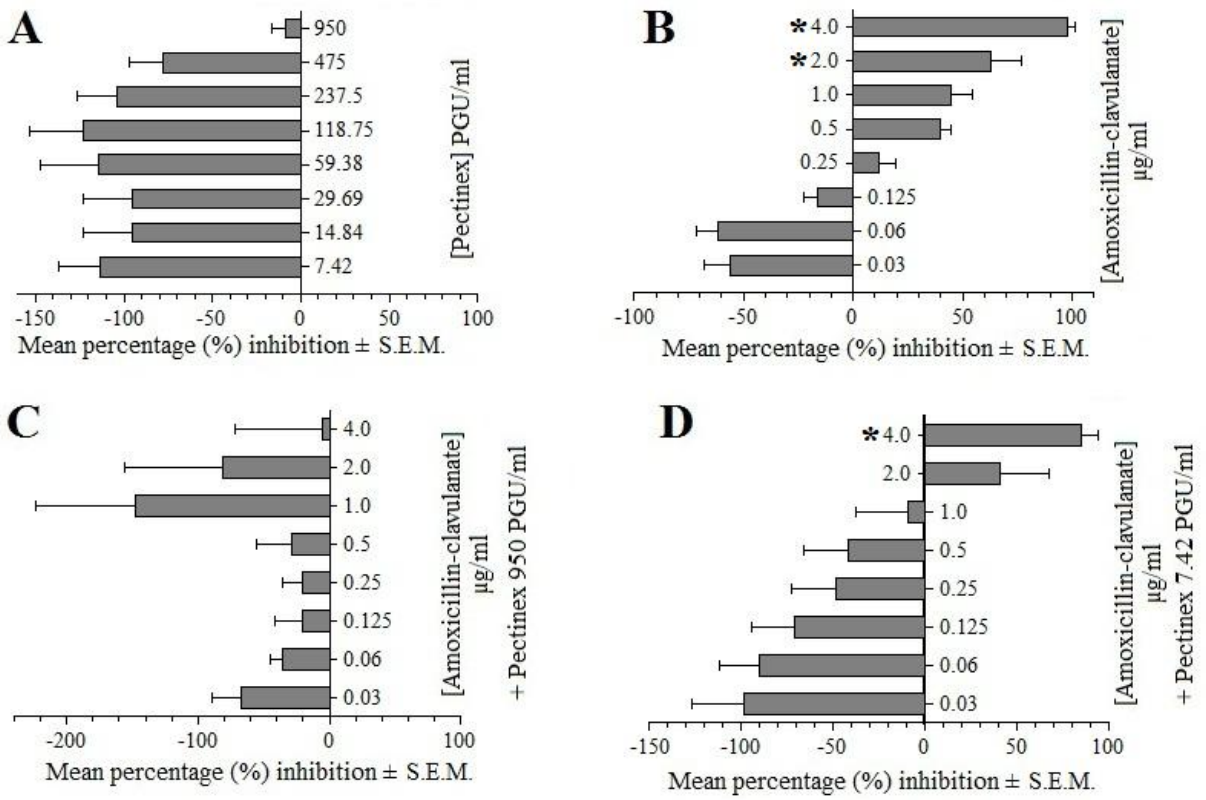

Figure 27: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *S. aureus* ATCC strain after 24 h incubation with [A] Pectinex, [B] amoxicillin-clavulanate, [C] Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E5].

A decrease in biofilm biomass was observed with increasing amoxicillin-clavulanate concentrations with a significant ($p \leq 0.05$) decrease of 63.0 ± 13.7% and 98.0 ± 3.6% in biofilm occurring at 2.0 and 4.0 µg/ml, respectively (Figure 27B). A non-significant ($p > 0.05$) increase in biofilm was seen at the lowest three antibiotic concentrations (0.03 – 0.125 µg/ml). The effect of combined treatment with high concentrations of Pectinex (237.5 – 950 PGU/ml) and amoxicillin-clavulanate (0.03 – 4.0 µg/ml) was not significantly ($p > 0.05$)

different from the untreated controls. However, significant ($p \leq 0.05$) biofilm inhibition was observed when lower concentrations of Pectinex (7.42 – 118.75 PGU/ml) were administered along with amoxicillin-clavulanate 4.0 µg/ml (Figure 27D).

The combinations of Pectinex7.42 – 237.5 PGU/ml with amoxicillin-clavulanate 4.0 µg/ml and Pectinex 7.42 PGU/ml with amoxicillin-clavulanate 2.0 µg/ml (Figure 27D), exhibited a decrease in biofilm biomass that was greater extent than with Pectinex alone ($p \leq 0.05$). The effect of treatment with Pectinex 475 or 950 PGU/ml plus amoxicillin-clavulanate (0.03 - 4.0) was not significantly ($p > 0.05$) different from that of Pectinex alone. When combinations were compared with reference to the effects of singly administered amoxicillin-clavulanate, all concentrations of amoxicillin-clavulanate (0.03 – 4.0 µg/ml) were less effective at reducing biofilm biomass when in combination with Pectinex (7.42 – 950 PGU/ml). The difference was statistically significant ($p \leq 0.05$) in the combinations that had Pectinex 237.5 PGU/ml with amoxicillin-clavulanate 0.5 µg/ml and Pectinex 475 PGU/ml with amoxicillin-clavulanate 1.0 µg/ml.

Pectinex 7.42 – 237.5 PGU/ml significantly ($p \leq 0.05$) enhanced biofilm viability (Figure 28A). The largest increase in viability (-155.4 ± 57.0%) occurred with Pectinex 118.75 PGU/ml, and the magnitude progressively decreased at concentrations both above and below this concentration. Enhanced biofilm viability (-33.0 ± 14.0%) and reduced viability (44.7 ± 6.0%) occurred with Pectinex 475 and 950 PGU/ml, respectively; however, these findings were not statistically significant ($p > 0.05$). When bacterial cultures were incubated with amoxicillin-clavulanate, increased biofilm viability was observed at all concentrations with significant ($p \leq 0.05$) increases occurring at 0.03, 2.0 and 4.0 µg/ml (Figure 28B).

When bacterial cultures were incubated with Pectinex and amoxicillin-clavulanate in combination, significant ($p \leq 0.05$) increases in biofilm viability were observed with:

- Pectinex 237.5 PGU/ml plus amoxicillin-clavulanate 0.5 and 1.0 µg/ml
- Pectinex 118.75 PGU/ml plus amoxicillin-clavulanate 0.03 – 0.5 µg/ml, and also with 2.0 µg/ml (Figure 28C)
- Pectinex 59.38 PGU/ml plus amoxicillin-clavulanate 0.03 – 0.25 µg/ml
- Pectinex 29.69 PGU/ml plus amoxicillin-clavulanate 0.03, 0.06 and 0.5 µg/ml
- Pectinex 14.84 PGU/ml plus amoxicillin-clavulanate 0.03 – 0.5 µg/ml
- Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate 0.06 – 0.125 µg/ml

There were no significant ($p > 0.05$) differences in biofilm viability between the untreated controls and cultures that were incubated with combinations of Pectinex at the concentrations 475 and 950 PGU/ml with amoxicillin-clavulanate (0.03 – 4.0 µg/ml).

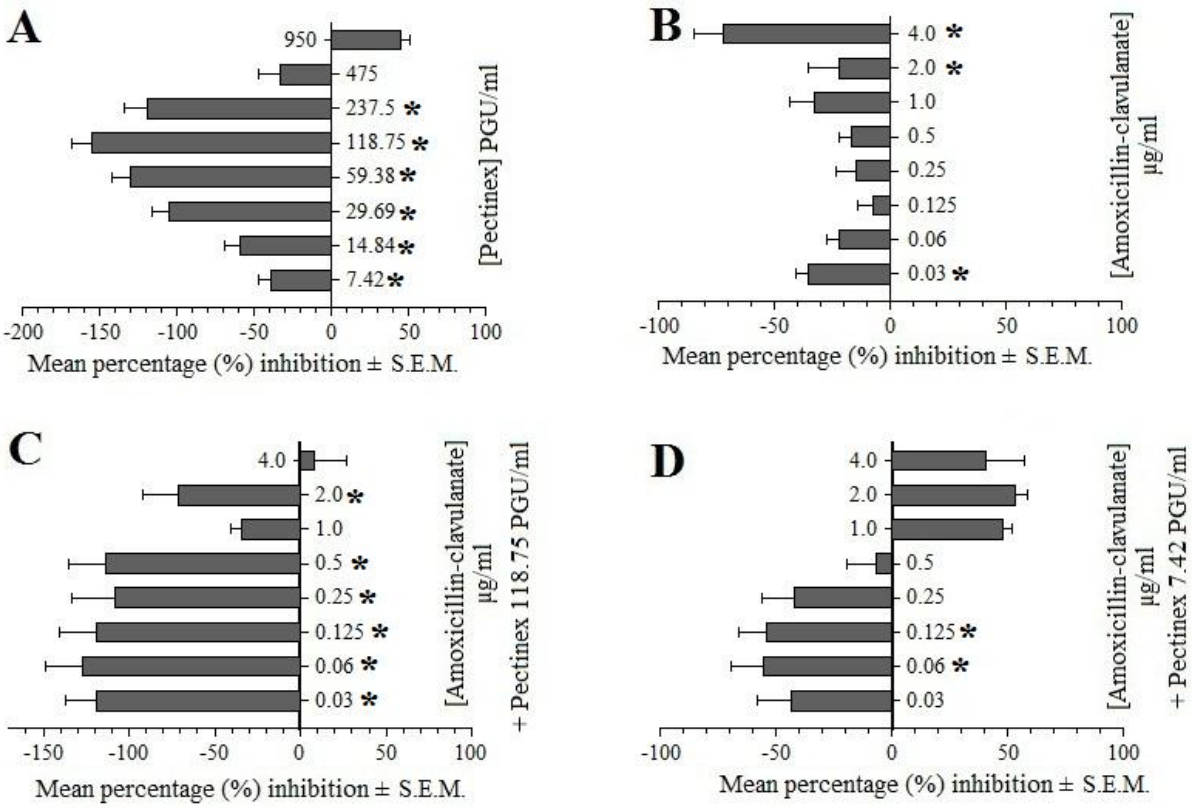

Figure 28: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *S. aureus* ATCC cultures after 24 h incubation with [A] Pectinex, [B] amoxicillin-clavulanate, [C] combination of Pectinex 118.75 PGU/ml plus amoxicillin-clavulanate, and [D] combination of Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F5].

There were no statistically significant ($p > 0.05$) differences in biofilm viability between singly administered Pectinex and the combinations where Pectinex 475 or 950 PGU/ml was combined with any of the concentrations of amoxicillin-clavulanate. A reduction in biofilm viability was observed with the combination amoxicillin-clavulanate 4.0 µg/ml and Pectinex 14.84 – 237.5 PGU/ml (Figure 28C), and with Pectinex 7.42 with amoxicillin-clavulanate either 1.0 or 2.0 µg/ml (Figure 28D). Whilst enhanced viability occurred with Pectinex 29.69 and 59.38 PGU/ml plus amoxicillin-clavulanate 2.0 and 1.0 µg/ml, respectively. The effects these combinations were significantly ($p \leq 0.05$) different from the viability that was observed with singly administered Pectinex (7.42 – 237.5 PGU/ml). Compared to singly administered

amoxicillin-clavulanate, the combinations of antibiotic plus Pectinex that significantly ($p \leq 0.05$) reduced biofilm viability were Pectinex 950 PGU/ml plus amoxicillin-clavulanate 0.03, 0.06 and 0.5 µg/ml, Pectinex 7.42 – 950 PGU/ml plus amoxicillin-clavulanate 4.0 µg/ml, and Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate 1.0 and 2.0 µg/ml. Significant increases in biofilm viability occurred with Pectinex 118.75 PGU/ml with amoxicillin-clavulanate 0.125 and 0.5 µg/ml, and either of Pectinex 14.84 and 59.38 PGU/ml with amoxicillin-clavulanate 0.125 and 0.25 µg/ml.

b) *S. aureus* clinical strain

Pectinex caused a significant ($p \leq 0.05$) increase in biofilm formation at 7.42 PGU/ml (Figure 29A). At all other concentrations there was no significant ($p > 0.05$) difference in biofilm biomass between the exposed cultures and the untreated controls. A statistically significant ($p \leq 0.05$) reduction in biofilm of 86.7 ± 5.0% was observed after exposure to 16.0 µg/ml amoxicillin-clavulanate (Figure 29B); all other concentrations had mixed results with inhibition and enhancement that were, however, not significant ($p > 0.05$). When the two agents were used in combination and compared with the untreated control, significant ($p \leq 0.05$) decreases in biofilm biomass were observed with several combinations; namely:

- Pectinex 29.38 – 950 PGU/ml with amoxicillin clavulanate 16.0 µg/ml
- Pectinex 950 PGU/ml with amoxicillin clavulanate 4.0 µg/ml
- Pectinex 237.5 PGU/ml with amoxicillin clavulanate 0.5 and 1.0 µg/ml
- Pectinex 118.75 PGU/ml with amoxicillin clavulanate 0.25 – 8.0 µg/ml
- Pectinex 59.38 PGU/ml with amoxicillin clavulanate 4.0 µg/ml

Pectinex 237.5 PGU/ml plus amoxicillin-clavulanate 16.0 µg/ml produced a significantly ($p \leq 0.05$) greater reduction in biofilm biomass than Pectinex alone (Figure 29C). When combinations were compared with singly administered amoxicillin-clavulanate, significantly ($p \leq 0.05$) more inhibition was found when Pectinex 59.38 and 950 PGU/ml were combined with amoxicillin-clavulanate 4.0 µg/ml, with Pectinex 118.75 and amoxicillin-clavulanate 0.25 – 8.0 µg/ml, also when Pectinex 237.5 PGU/ml was added to amoxicillin-clavulanate 0.5 µg/ml. On the other hand, significantly ($p \leq 0.05$) more biofilm growth occurred with the combination of 7.42 PGU/ml and 0.125 µg/ml of Pectinex and amoxicillin-clavulanate, respectively (Figure 29D).

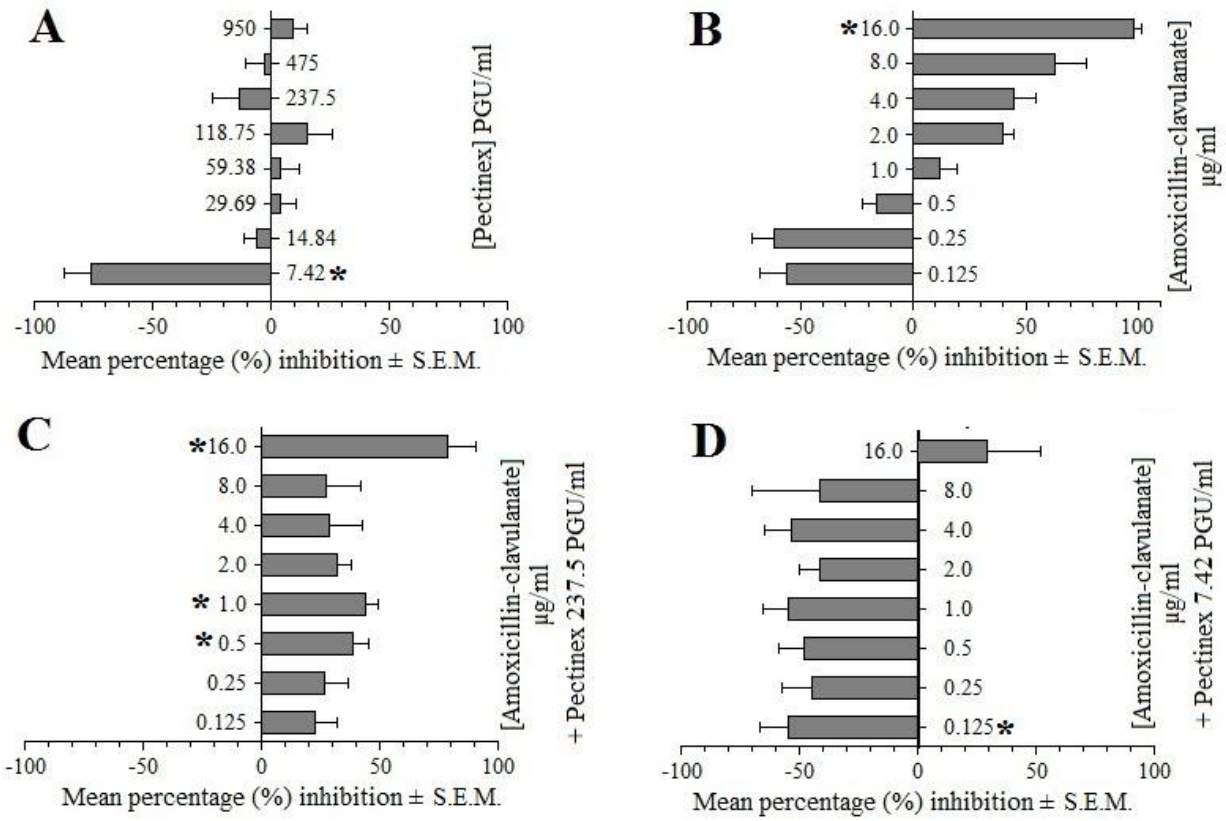

Figure 29: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *S. aureus* clinical strain after 24 h incubation with [A] Pectinex, [B] amoxicillin-clavulanate, [C] Pectinex 237.5 PGU/ml plus amoxicillin-clavulanate, and [D] Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E6].

Statistically significant ($p \leq 0.05$) reduction in biofilm viability occurred with Pectinex 14.84, 59.38 – 237.5, and 950 PGU/ml. Non-significant ($p > 0.05$) reduction followed incubation with Pectinex 7.42, 29.69 and 475 PGU/ml. (Figure 30A). With the exception of amoxicillin 0.25 µg/ml, there was no statistically significant ($p > 0.05$) difference between the biofilm viability observed in cultures treated with amoxicillin-clavulanate and the viability found in untreated controls (Figure 30B). Biofilm viability was reduced by all combinations of Pectinex (7.42 – 950 PGU/ml) and amoxicillin clavulanate (0.125 – 16.0 µg/ml) except for Pectinex 59.38 PGU/ml with amoxicillin-clavulanate 0.5 µg/ml. Those that were significantly ($p \leq 0.05$) less than the untreated controls were:

- Pectinex 950 PGU/ml plus amoxicillin-clavulanate 1.0 – 4.0 µg/ml, and 16.0 µg/ml
- Pectinex 475 PGU/ml plus amoxicillin-clavulanate 0.125, 2.0, 8.0 µg/ml, and 16.0 µg/ml

- Pectinex 237.5 PGU/ml plus amoxicillin-clavulanate 0.5 and 1.0 µg/ml
- either of Pectinex 59.38 and 118.75 PGU/ml plus amoxicillin-clavulanate 2.0 µg/ml
- Pectinex 29.69 PGU/ml plus amoxicillin-clavulanate 16.0 µg/ml
- Pectinex 14.84 PGU/ml plus amoxicillin-clavulanate 0.25, 2.0, 8.0 and 16.0 µg/ml
- Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate 0.5 – 16.0 µg/ml

There was no significant ($p > 0.05$) difference between the effect on biofilm viability caused when either Pectinex or amoxicillin-clavulanate were administered as the sole agents and the effects caused by the combination of both agents.

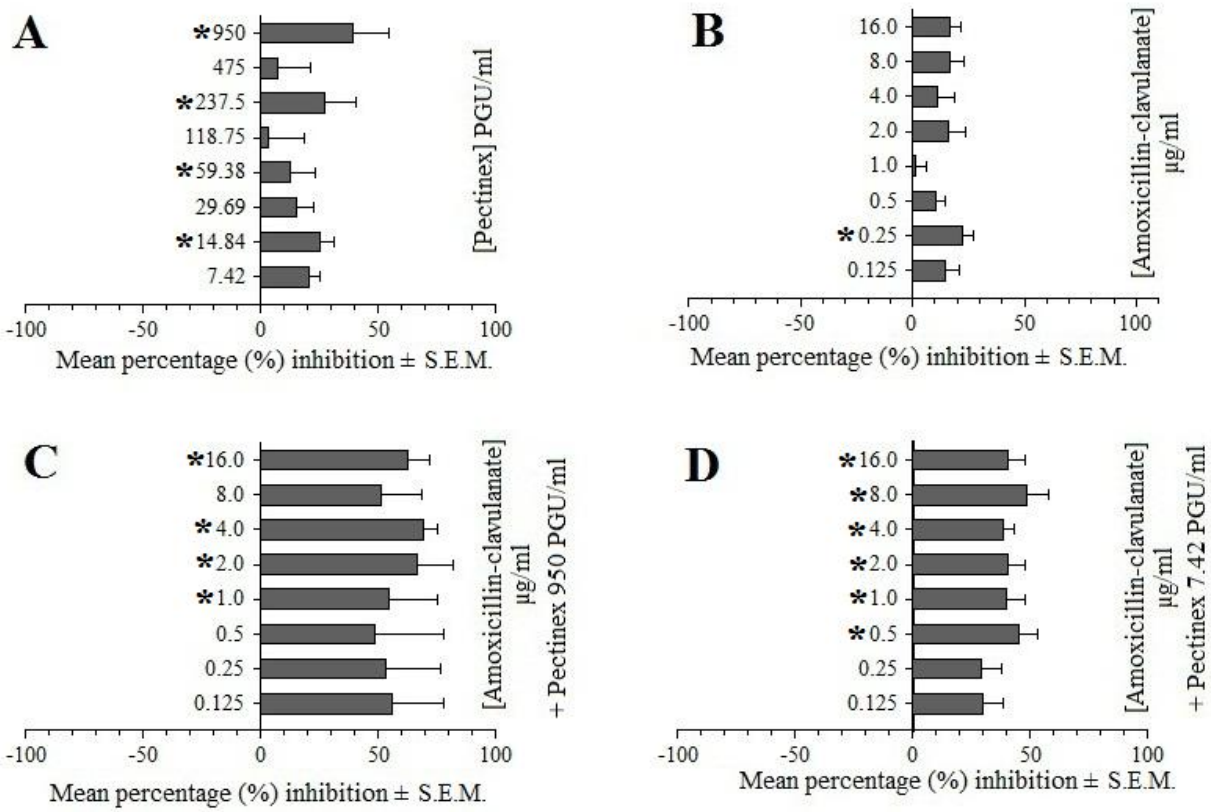

Figure 30: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *S. aureus* clinical strain cultures after 24 h incubation with [A] Pectinex, [B] amoxicillin-clavulanate, [C] combination of Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] combination of Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F6].

c) *P. aeruginosa* ATCC

Significant ($p \leq 0.05$) enhancement of biofilm occurred in *P. aeruginosa* ATCC cultures at all concentrations of Pectinex (Figure 31A), and significant ($p \leq 0.05$) inhibition was observed

with ciprofloxacin at concentrations of 0.25 – 4.0 µg/ml (Figure 31B). When both agents were used simultaneously and compared with the untreated controls and also with singly administered Pectinex, statistically significant ($p \leq 0.05$) inhibition of biofilm could be found with Pectinex 7.42 – 950 PGU/ml in combination with ciprofloxacin 0.5 – 4.0 µg/ml, and with Pectinex 7.42 – 29.69 PGU/ml and ciprofloxacin 0.25µg/ml (Figure 31C and 31D). When the effects of combined regimens were compared with those of singly administered ciprofloxacin, none of the combinations were found to be significantly ($p > 0.05$) different from ciprofloxacin alone.

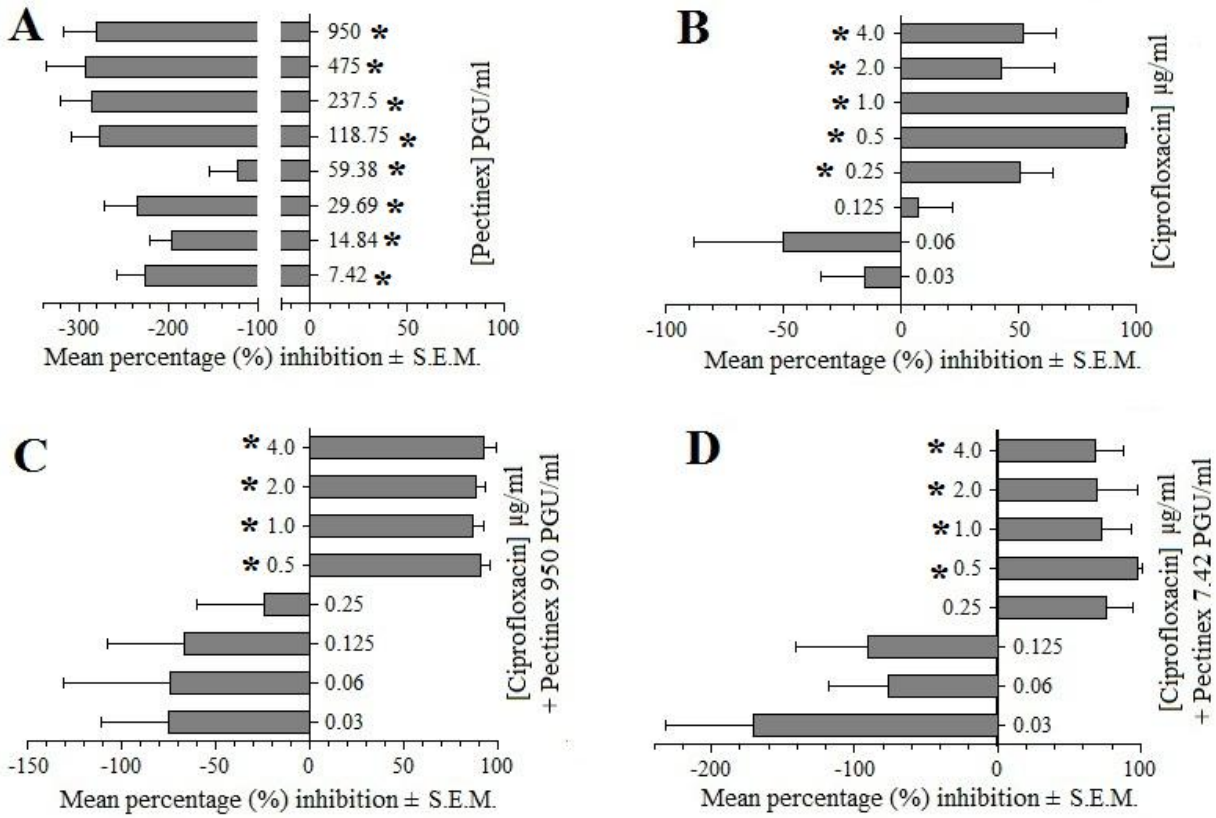

Figure 31: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *P. aeruginosa* ATCC strain after 24 h incubation with [A] Pectinex, [B] ciprofloxacin, [C] Pectinex 950 PGU/ml plus ciprofloxacin, and [D] Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E7].

All concentrations of Pectinex promoted an increase in the viability of biofilm bacteria in cultures of *P. aeruginosa* ATCC strain. A statistically significant ($p \leq 0.05$) increase occurred with Pectinex 29.69 PGU/ml (Figure 32A). On the other hand, ciprofloxacin 0.25 – 4.0 µg/ml caused a significant ($p \leq 0.05$) reduction biofilm viability (Figure 32B). When the two agents

were combined, significant ($p \leq 0.05$) differences in biofilm viability were found between the untreated controls and cultures treated with:

- Pectinex 7.42 – 950 PGU/ml plus ciprofloxacin 4.0 µg/ml
- Pectinex 29.69, 118.75 and 237.5 PGU/ml with ciprofloxacin 2.0 µg/ml
- Pectinex 29.69 – 950 PGU/ml and ciprofloxacin 1.0 µg/ml
- Pectinex 237.5 – 950 PGU/ml and ciprofloxacin 0.5 µg/ml

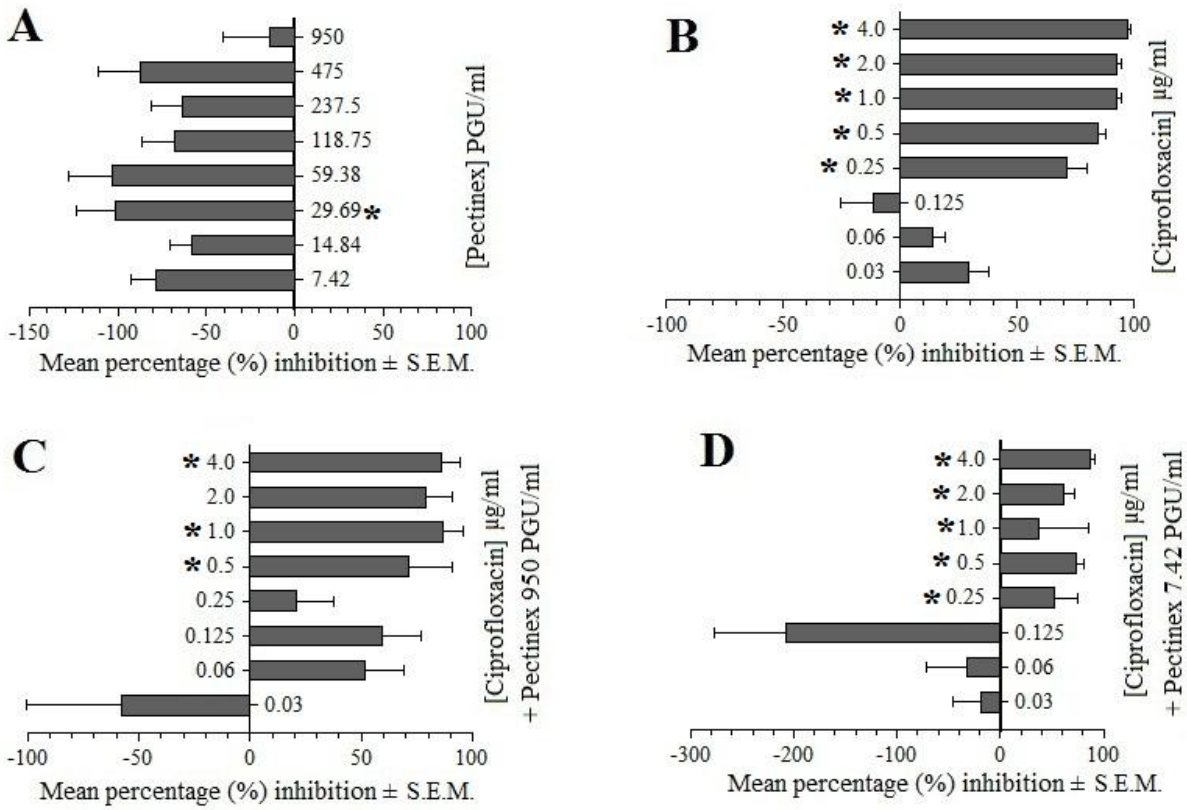

Figure 32: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *P. aeruginosa* ATCC cultures after 24 h incubation with [A] Pectinex, [B] ciprofloxacin, [C] combination of Pectinex 950 PGU/ml plus ciprofloxacin, and [D] combination of Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F7].

When the effects of combined treatments were compared with the effects of singly administered Pectinex, significantly ($p \leq 0.05$) greater reductions in biofilm viability were observed with:

- Pectinex 7.42 – 475 PGU/ml plus ciprofloxacin 0.5 – 4.0 µg/ml
- Pectinex 950 PGU/ml plus either of ciprofloxacin 1.0 or 4.0 µg/ml
- Pectinex 7.42, 14.84 and 59.38 – 237.5 PGU/ml plus ciprofloxacin 0.25 µg/ml

There were no statistically significant ($p > 0.05$) differences in viability in cultures exposed to any combination of Pectinex plus ciprofloxacin, and in cultures treated with ciprofloxacin alone.

d) *P. aeruginosa* clinical strain

In cultures of *P. aeruginosa* clinical strains, Pectinex (7.42 – 950 PGU/ml) promoted significantly ($p \leq 0.05$) more biofilm formation than that found in untreated controls (Figure 33A); the exception was Pectinex 237.5 PGU/ml. Ciprofloxacin significantly ($p \leq 0.05$) inhibited biofilm formation at 0.5, 2.0 and 4.0 µg/ml, but was associated with significantly enhanced ($p \leq 0.05$) biofilm formation at 1.0 µg/ml (Figure 33B). The inhibition observed at 0.25 µg/ml was not statistically significant ($p > 0.05$). Pectinex at 14.84 PGU/ml plus ciprofloxacin 4.0 µg/ml significantly ($p \leq 0.05$) inhibited biofilm growth; however, at all other combinations no statistically significant ($p > 0.05$) differences were found.

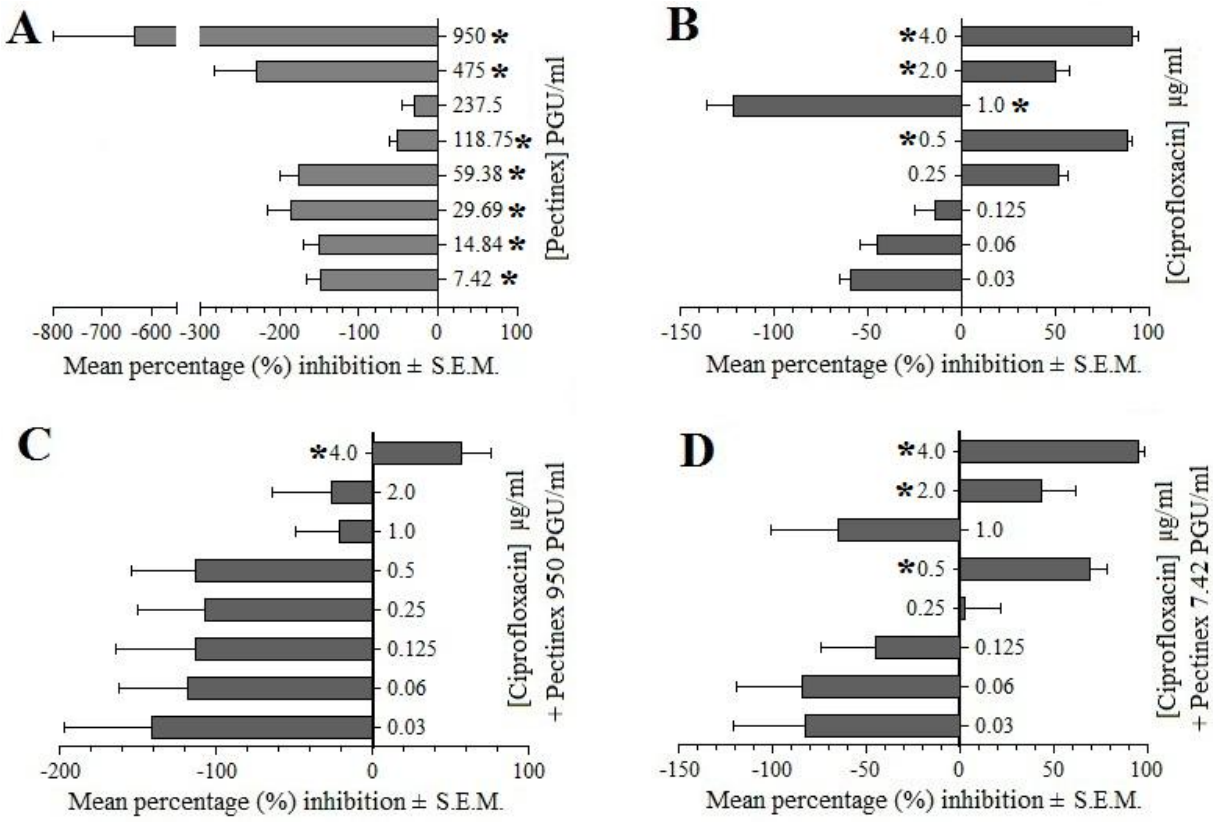

Figure 33: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *P. aeruginosa* clinical strain after 24 h incubation with [A] Pectinex, [B] ciprofloxacin, [C] Pectinex 950 PGU/ml plus ciprofloxacin, and [D] Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E8].

Pectinex-ciprofloxacin combinations inhibited biofilm formation to a greater extent than Pectinex alone. Those that demonstrated statistically significantly ($p \leq 0.05$) differences were Pectinex:

- 950 PGU/ml with ciprofloxacin 4 µg/ml (Figure 33C)
- 475 PGU/ml with ciprofloxacin 0.5, 1.0 and 4.0 µg/ml
- 237 PGU/ml with ciprofloxacin 1.0 and 4.0 µg/ml
- 7.42 (Figure 33D), 14.84 and 118.75 PGU/ml with ciprofloxacin 0.5, 2.0 and 4.0 µg/ml
- 29.69 and 59.38 PGU/ml with ciprofloxacin 0.25, 0.5, 2 and 4 µg/ml

Ciprofloxacin 1.0 µg/ml with each of Pectinex 118.75, 237.5 and 475 PGU/ml, caused biofilm inhibition that was significantly ($p \leq 0.05$) different from singly administered ciprofloxacin (1.0 µg/ml). The effects observed at all other combinations of Pectinex with ciprofloxacin did not differ significantly ($p > 0.05$) from those of singly administered ciprofloxacin.

All concentrations of Pectinex enhanced biofilm bacterial viability, however, this effect was significantly ($p \leq 0.05$) different from the untreated controls only at the concentrations of 7.42 – 29.84, 118.75, 475 and 950 PGU/ml (Figure 34A). Ciprofloxacin significantly ($p \leq 0.05$) reduced biofilm viability at 0.125, 0.25, 0.5, 2.0 and 4.0 µg/ml (Figure 34B). Non-significant ($p > 0.05$) enhancement of biofilm viability was observed with ciprofloxacin 1.0 µg/ml (-10.3 ± 19.0%) and to a lesser extent with 0.06 µg/ml (-1.7 ± 4.9%).

Compared to the untreated controls, significant ($p \leq 0.05$) reduction in biofilm viability occurred with the combinations of Pectinex 950 PGU/ml plus ciprofloxacin 0.25 – 4.0 µg/ml (Figure 34C), Pectinex 7.42 – 475 PGU/ml plus either of ciprofloxacin 0.5 or 4.0 µg/ml, and with Pectinex 14.84 – 237.5 PGU/ml plus ciprofloxacin 2.0 µg/ml. When the effects of combinations of Pectinex and ciprofloxacin were compared with the effects of singly administered Pectinex, the combinations that were found to significantly ($p \leq 0.05$) reduce biofilm viabilities were:

- Pectinex 7.42 – 950 PGU/ml plus ciprofloxacin 0.5, 2.0 and 4.0 µg/ml (Figures 34 C and 34D)
- Pectinex 950 PGU/ml plus ciprofloxacin 0.25 and 1.0 µg/ml (Figure 34C)
- Pectinex 475 PGU/ml plus ciprofloxacin 0.25 µg/ml

No statistically significant ($p > 0.05$) differences were found between bacterial cultures exposed to Pectinex (7.42 – 950 PGU/ml) plus ciprofloxacin (0.03 – 4.0 µg/ml) and in cultures treated with ciprofloxacin alone.

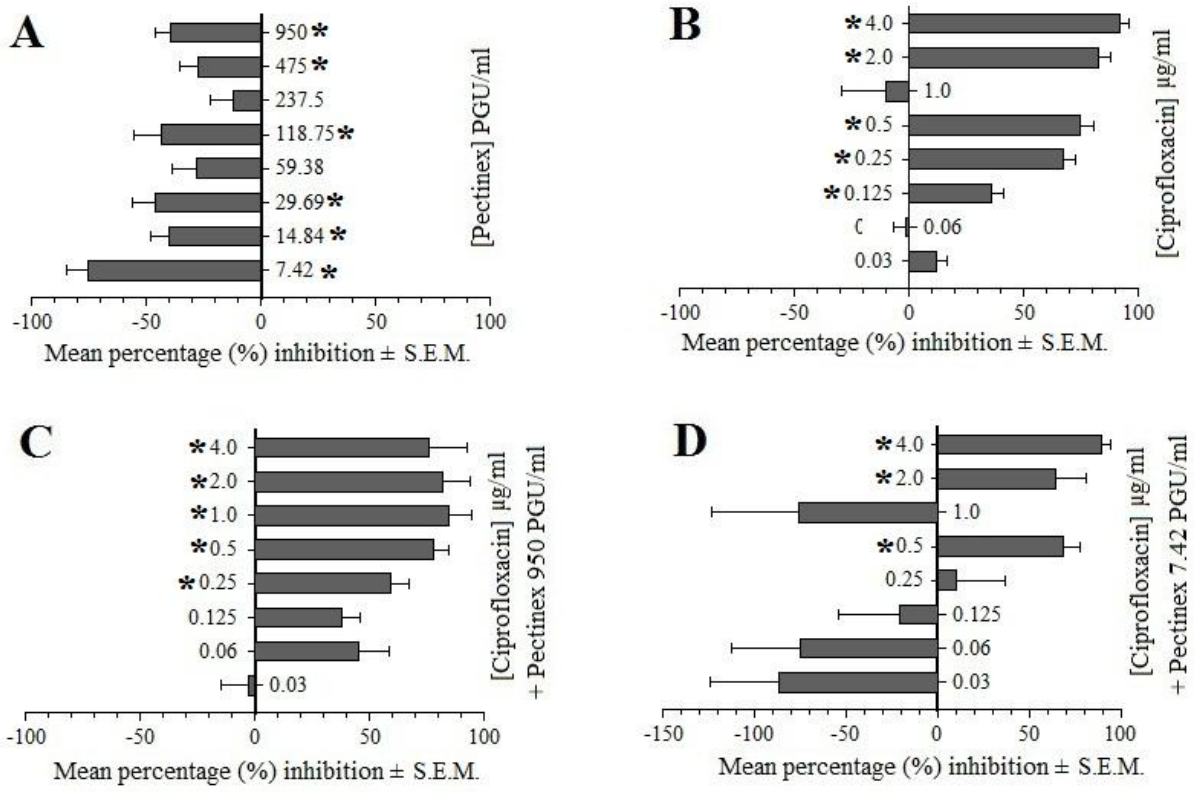

Figure 34: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *P. aeruginosa* clinical strain cultures after 24 h incubation with [A] Pectinex, [B] ciprofloxacin, [C] combination of Pectinex 950 PGU/ml plus ciprofloxacin, and [D] combination of Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F8].

4.2.3 Biofilm biomass and viability after 18 h incubation of 6 h-old bacterial cultures with Pectinex and antibiotics

a) *S. aureus* ATCC

Pectinex (7.42 – 950 PGU/ml) was associated with enhanced growth and development of biofilm (Figure 35A) that was significantly ($p \leq 0.05$) greater than in the untreated control. Amoxicillin-clavulanate decreased biofilm formation at 0.03 µg/ml (22.4 ± 13.5%) and at concentrations 0.25 – 4.0 µg/ml; however, statistical significance ($p \leq 0.05$) was only found at 0.25, 0.5 and 4.0 µg/ml (Figure 35B). Non-significant ($p > 0.05$) enhanced biofilm growth was observed at the concentrations of 0.03, 0.06 and 0.125 µg/ml.

Most combinations of Pectinex and amoxicillin-clavulanate promoted biofilm growth. Compared to untreated controls, statistically significant ($p \leq 0.05$) growth was found with the combination of Pectinex 14.84 PGU/ml with amoxicillin-clavulanate 0.06 µg/ml (-150.6 ± 27.5%). No significant ($P > 0.05$) differences were found with all other combinations. Combinations that differed significantly ($p \leq 0.05$) from singly administered Pectinex were:

- Pectinex 7.42 – 118.75 PGU/ml with amoxicillin-clavulanate 4.0 µg/ml
- Pectinex 59.38 and 118.75 PGU/ml with amoxicillin-clavulanate 2.0 µg/ml
- Pectinex 118.75 PGU/ml with amoxicillin clavulanate 1.0 µg/ml

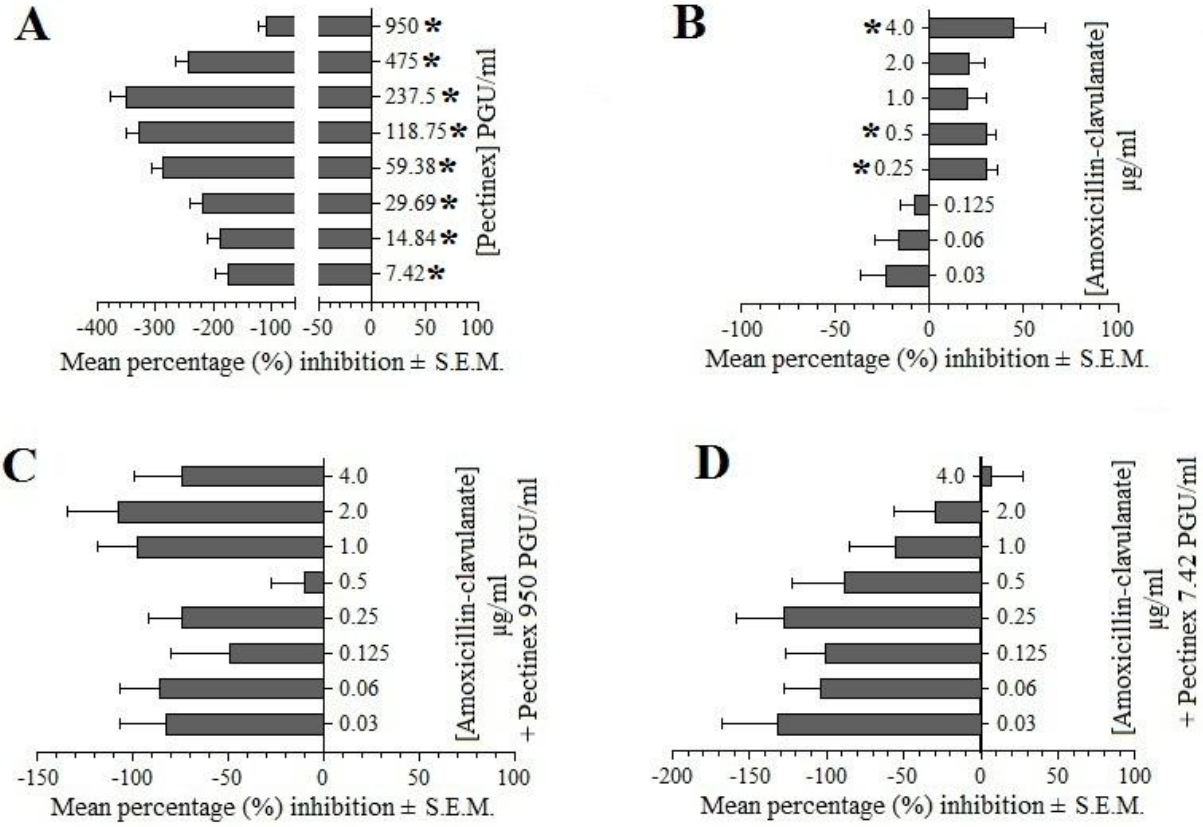

Figure 35: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *S. aureus* ATCC strain after 18 h incubation of 6 h-old cultures with [A] Pectinex, [B] amoxicillin-clavulanate, [C] Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E9].

None of the combinations of amoxicillin-clavulanate (0.03 – 4.0 µg/ml) with Pectinex 475 and 950 PGU/ml produced results that were significantly ($p > 0.05$) different from Pectinex alone (Figure 35C). The combinations with amoxicillin clavulanate 4.0 µg/ml inhibited biofilm growth. The combinations with amoxicillin clavulanate 1.0 and 2.0 µg/ml enhanced biofilm growth; however, this was to a lesser degree than with Pectinex alone. Enhanced

growth that was significantly ($p \leq 0.05$) more than with singly administered amoxicillin-clavulanate was noted with Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate 0.25 µg/ml (Figures 35B and 35D). Where combinations were found to cause inhibition of biofilm growth, the effects were not statistically different ($p > 0.05$) from that of the singly administered antibiotic.

Biofilm bacterial viability was significantly ($p \leq 0.05$) higher at all concentrations of Pectinex than in the untreated control (Figure 36A). Amoxicillin-clavulanate promoted biofilm viability, however, the observations were not found to be significantly ($p > 0.05$) different from the untreated control (Figure 36B). The combination of amoxicillin-clavulanate 0.03 µg/ml with each of Pectinex 59.38, 118.75 and 237.5 PGU/ml significantly ($p \leq 0.05$) enhanced (negative inhibition) biofilm viability. All other combinations were not found to be significantly ($p > 0.05$) different from the untreated control.

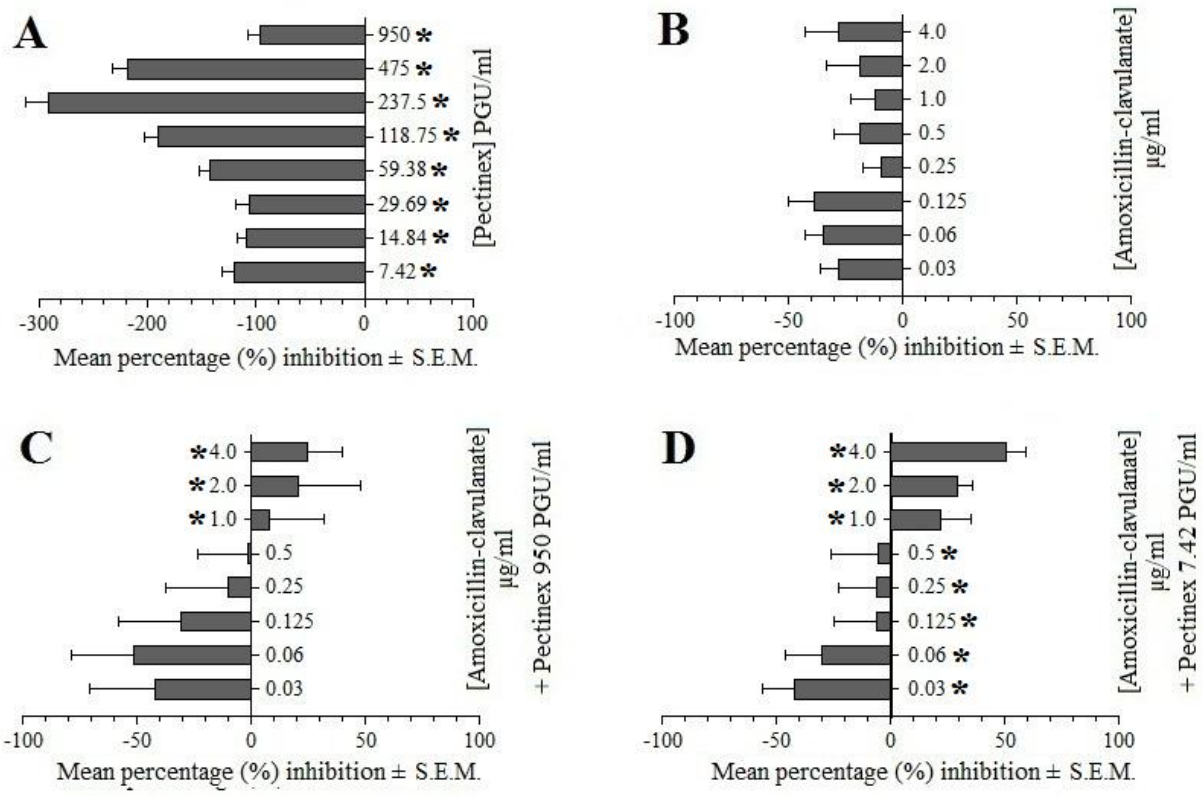

Figure 36: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *S. aureus* ATCC cultures after further 18 h incubation of 6 h-old cultures with [A] Pectinex, [B] amoxicillin-clavulanate, [C] combination of Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] combination of Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F9].

Combined treatments that were significant ($p \leq 0.05$) different from Pectinex alone were Pectinex 7.42 – 950 PGU/ml plus amoxicillin-clavulanate 1.0 – 4.0 µg/ml, each of Pectinex 7.42, 118.75 and 475 PGU/ml plus amoxicillin-clavulanate 0.125 – 0.5 µg/ml, and Pectinex 59.38 PGU/ml plus amoxicillin-clavulanate 0.25 and 0.5 µg/ml. With combinations of Pectinex (7.42 – 950 PGU/ml) and amoxicillin-clavulanate 0.03 – 0.5 µg/ml, biofilm viability was significantly ($p \leq 0.05$) different from amoxicillin-clavulanate alone (Figure 36C and 36D). Combinations Pectinex (7.42 – 950 PGU/ml) with amoxicillin-clavulanate 1.0 – 4.0 µg/ml were not significantly ($p > 0.05$) different from amoxicillin-clavulanate alone.

b) *S. aureus* clinical strain

Pectinex produced statistically significant ($p \leq 0.05$) inhibition of biofilm growth at the concentrations 7.42 – 237.5 PGU/ml. (Figure 37A). Non-significant ($p > 0.05$) inhibition was seen at 475 and 950 PGU/ml.

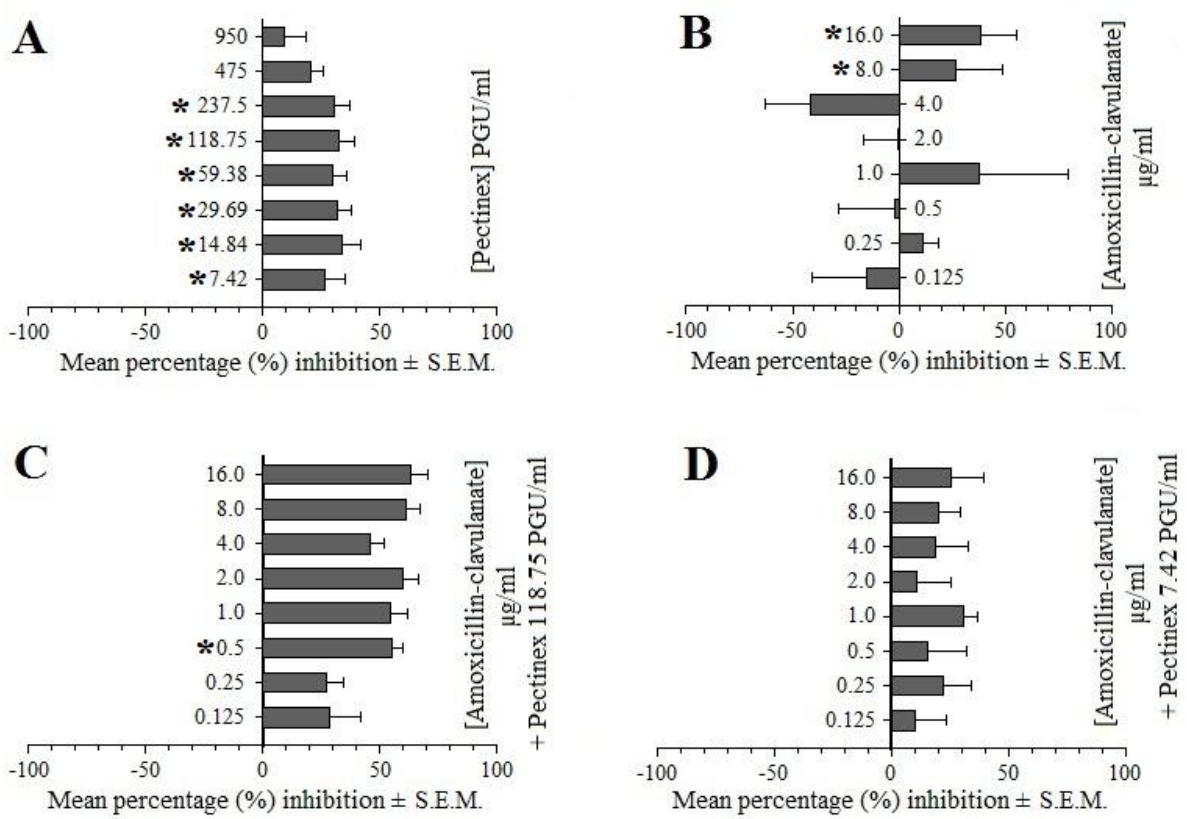

Figure 37: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *S. aureus* clinical strain after 18 h incubation of 6 h-old cultures with [A] Pectinex, [B] amoxicillin-clavulanate, [C] Pectinex 118.75 PGU/ml plus amoxicillin-clavulanate, and [D] Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E10].

Amoxicillin-clavulanate achieved significant ($p \leq 0.05$) inhibition of biofilm growth at 8.0 and 16.0 µg/ml (Figure 37B). The effect at other concentrations of amoxicillin-clavulanate (0.125 – 4.0 µg/ml) was not significantly ($p > 0.05$) different from the untreated control. Statistically significant ($p \leq 0.05$) inhibition was found with combinations of:

- Pectinex 29.69 – 237.5 PGU/ml and amoxicillin-clavulanate 0.1 – 16.0 µg/ml
- Pectinex 475 PGU/ml with amoxicillin-clavulanate 8.0 and 16.0 µ/ml
- Pectinex 950 PGU/ml and amoxicillin-clavulanate 0.5 µg/ml

When the effects of combinations were compared with the effect of singly administered Pectinex (Figures 37C and 37D), no statistically significant ($p > 0.05$) difference was found at all concentrations. Amoxicillin-clavulanate (4.0 µg/ml) promoted biofilm growth (-41.9 ± 20.9%) when administered alone but inhibited growth (55.7 ± 5.5%) when combined with Pectinex 237.5 PGU/ml (Figure 37B); the difference between the two regimens was significant ($p \leq 0.05$). At all other combinations, there was no significant ($p > 0.05$) difference between the effects of the combinations and those of singly administered amoxicillin-clavulanate.

Pectinex 29.69 and 59.38 PGU/ml significantly ($p \leq 0.05$) reduced biofilm viability (Figure 38A). The reduction and enhancement observed at other concentrations of Pectinex were not significantly ($p > 0.05$) different from the untreated controls. Amoxicillin-clavulanate reduced biofilm viability at concentrations of 0.5 – 16.0 µg/ml and increased viability at concentrations of 0.125 and 0.25 µg/ml; however, these findings were not significantly ($p > 0.05$) different from the untreated controls (Figure 38B).

Several combinations of Pectinex with amoxicillin-clavulanate significantly ($p \leq 0.05$) reduced biofilm viability, namely:

- Pectinex 950 PGU/ml plus amoxicillin-clavulanate at 1.0 – 4.0 µg/ml (Figure 38C)
- Pectinex 29.69 PGU/ml plus amoxicillin-clavulanate at 4.0 – 16.0 µg/ml
- Pectinex 14.84 PGU/ml plus amoxicillin-clavulanate at 2.0 – 16.0 µg/ml

Biofilm bacterial viability found at all the other combinations was not significantly ($p > 0.05$) different from the untreated controls. Pectinex 950 PGU/ml plus 2.0 µg/ml amoxicillin-clavulanate (38.4 ± 6.0%), and Pectinex 475 PGU/ml plus amoxicillin-clavulanate 16.0 µg/ml

(29.7 ± 13.5%), were found to reduce biofilm viability to a significantly ($p \leq 0.05$) greater extent than Pectinex 950 PGU/ml alone (-3.8 ± 5.7%). Viability after exposure to all other combinations was not significantly ($p > 0.05$) different from that of Pectinex alone (Figure 38D). When the effects of combinations were compared with the effects of singly administered amoxicillin-clavulanate, the only combination that was found to exhibit a statistically significant ($p \leq 0.05$) difference was Pectinex 950 PGU/ml plus amoxicillin-clavulanate 0.125 µg/ml which had a mean percentage inhibition of 20.9 ± 4.0%, as compared to amoxicillin-clavulanate (0.125 µg/ml) with a value of -23.4 ± 6.4% (Figures 38B and 38C).

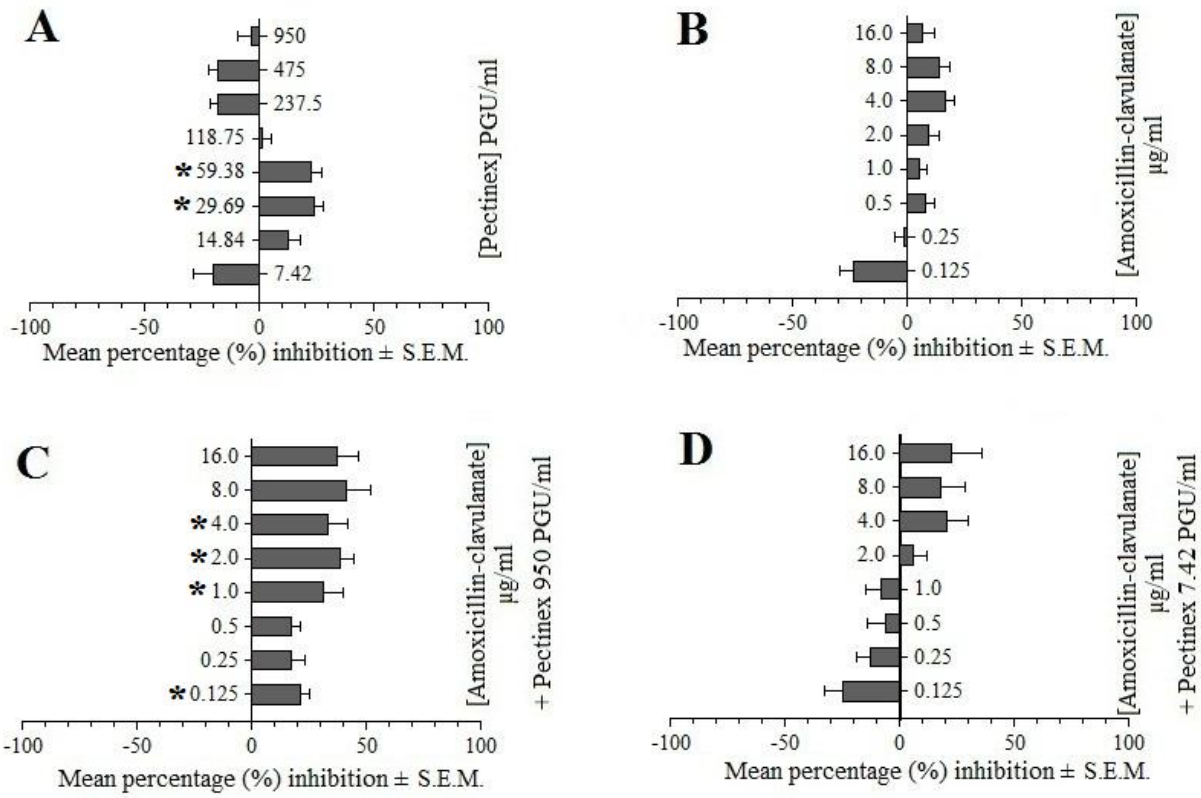

Figure 38: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *S. aureus* clinical strain cultures after further 18 h incubation of 6 h-old cultures with [A] Pectinex, [B] amoxicillin-clavulanate, [C] combination of Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] combination of Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F10].

c) *P. aeruginosa* ATCC

In *P. aeruginosa* ATCC cultures, significant ($p \leq 0.05$) biofilm growth enhancement was observed at all concentrations of Pectinex (Figure 39A). Ciprofloxacin caused significant ($p \leq 0.05$) inhibition at 0.06 µg/ml (Figure 39B). Prominent but non-significant ($p > 0.05$)

inhibition and enhancement were observed at 0.03 and 4.0 µg/ml, respectively (Figure 39B). The combination of Pectinex 950 PGU/ml with ciprofloxacin 0.03, 0.06, 0.5 and 1.0 µg/ml, significantly ($p \leq 0.05$) enhanced of biofilm growth. All other combinations were not found to be different ($p > 0.05$) from the untreated controls.

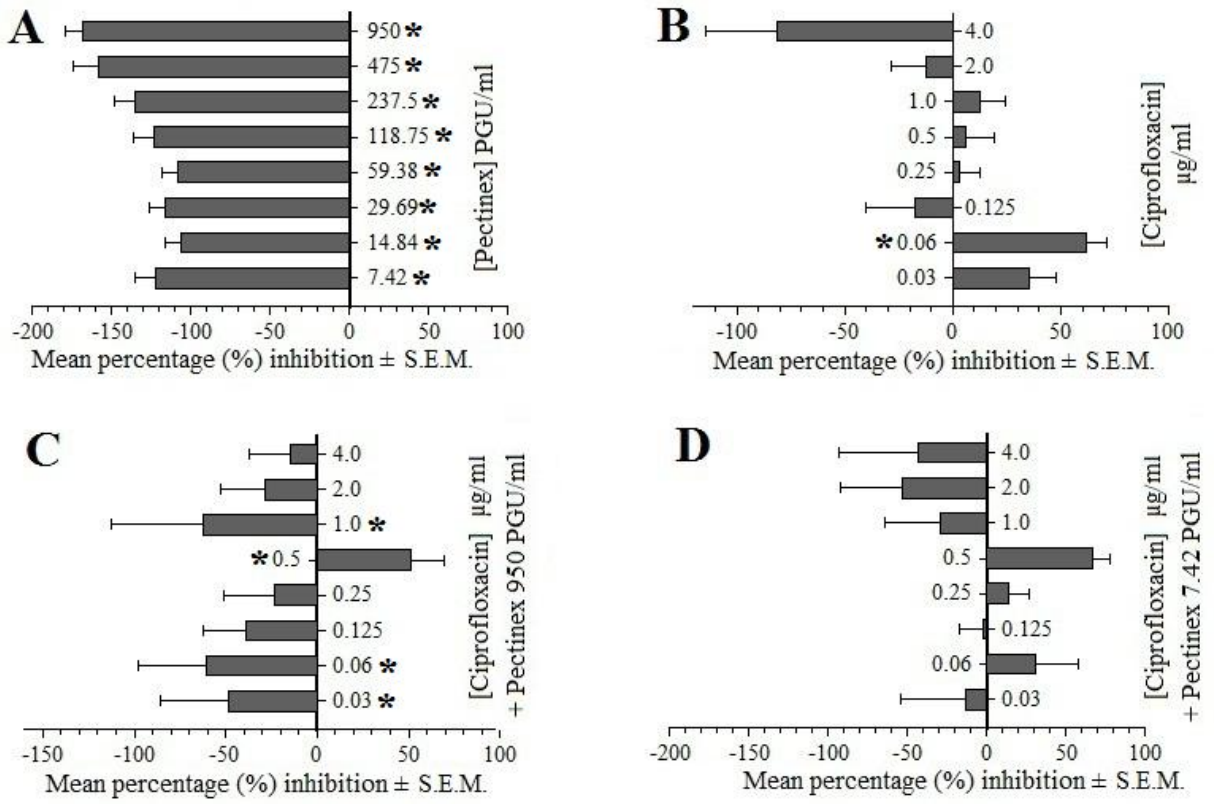

Figure 39: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *P. aeruginosa* ATCC strain after 18 h incubation of 6 h-old cultures with [A] Pectinex, [B] ciprofloxacin, [C] Pectinex 950 PGU/ml plus ciprofloxacin, and [D] Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E11].

The effect of several combinations of Pectinex and ciprofloxacin were found to be significantly ($p \leq 0.05$) different from the effects of Pectinex alone (Appendix E11). Ciprofloxacin (0.03 µg/ml) inhibited biofilm growth by 35.3 ± 12.3%, however, enhanced growth of -202.8 ± 51.2% occurred when this concentration of ciprofloxacin was combined with Pectinex 950 PGU/ml. The difference between the two regimens was significant ($p \leq 0.05$). Similarly, ciprofloxacin at 0.06 µg/ml inhibited biofilm growth by 61.6 ± 9.2% but when added to Pectinex 118.75 – 950 PGU/ml the combination significantly enhanced biofilm growth to between -60.3 ± 34.7% and -121.6 ± 69.8%. At all other combinations, there was no significant ($p > 0.05$) difference between the effects of the combinations and those of singly administered ciprofloxacin.

Biofilm bacterial viability was enhanced in *P. aeruginosa* ATCC cultures at all concentrations of Pectinex (Figure 40A). Viability after incubation with Pectinex 7.42 – 59.38 PGU/ml was significantly ($p \leq 0.05$) greater than in the untreated controls. There was no significant ($p > 0.05$) difference in the biofilm viability between the untreated control and in cultures incubated with Pectinex 118.75 – 950 PGU/ml. Treatment with ciprofloxacin (0.03 – 4.0 µg/ml) reduced biofilm viability; significant ($p \leq 0.05$) reductions were observed at 0.25 – 4.0 µg/ml (Figure 40B). When bacterial cultures were incubated with combinations of Pectinex and ciprofloxacin, significant ($p \leq 0.05$) reduction in biofilm bacterial viability was observed with a number of combinations of Pectinex 29.69 – 950 PGU/ml plus ciprofloxacin 0.06 – 4.0 µg/ml (Appendix F11). There was no significant ($p > 0.05$) difference observed between the untreated controls and combinations that contained Pectinex 7.42 and 14.84 PGU/ml.

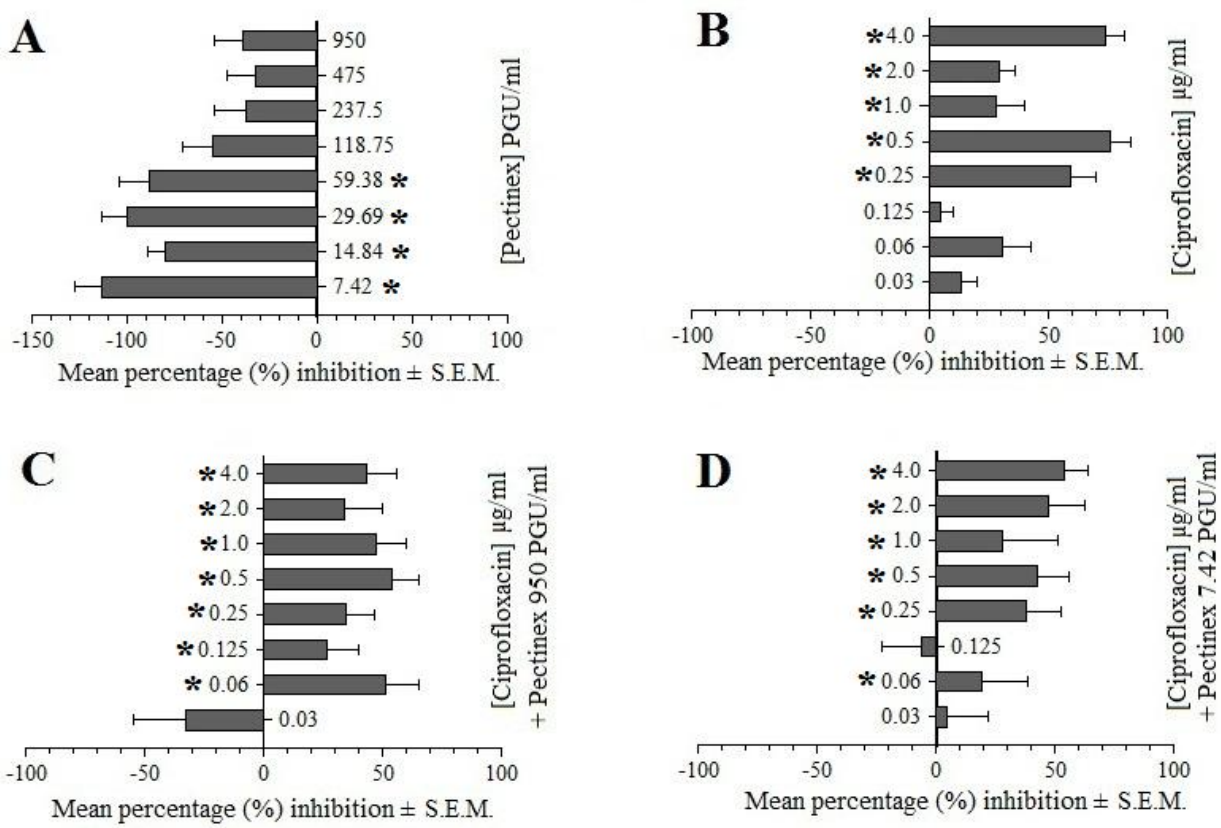

Figure 40: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *P. aeruginosa* ATCC cultures after further 18 h incubation of 6 h-old cultures with [A] Pectinex, [B] ciprofloxacin, [C] combination of Pectinex 950 PGU/ml plus ciprofloxacin, and [D] combination of Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F11].

When biofilm viability after exposure to Pectinex and ciprofloxacin combinations were compared with the effect at the same concentrations of Pectinex administered singly, significantly greater reduction in viability was observed with the combinations of:

- Pectinex 950 PGU/ml plus either of ciprofloxacin 0.06 or 0.5 µg/ml (Figure 40C)
- Pectinex 7.42 – 475 PGU/ml plus ciprofloxacin 0.06 and 0.25 – 4.0 µg/ml (Figure 40D)
- both Pectinex 14.84 and 29.69 PGU/ml plus ciprofloxacin 0.03 µg/ml

However, when the effect of combinations were compared with the effect of singly administered ciprofloxacin, there was no statistically significant ($p > 0.05$) difference between the biofilm viability found at all combinations and the viability observed after exposure the ciprofloxacin alone.

d) *P. aeruginosa* clinical strain

All concentrations of Pectinex enhanced the growth of biofilm in cultures of *P. aeruginosa* clinical strain. The effect at 7.42 PGU/ml and from 237.5 to 950 PGU/ml was found to be significantly ($p \leq 0.05$) different from the untreated controls (Figure 41A). Ciprofloxacin significantly ($p \leq 0.05$) inhibited biofilm growth in the concentration range of 0.06 – 1.0 µg/ml (Figure 41B). In addition, non-significant ($p > 0.05$) inhibition by ciprofloxacin occurred at 4.0 µg/ml, and non-significant ($p > 0.05$) growth enhancement occurred at 0.03 and 2.0 µg/ml. None of the combined treatments were significantly ($p > 0.05$) different from the untreated controls (Figures 41C and 41D).

When compared with effects of singly administered Pectinex, significant ($p \leq 0.05$) inhibition of 64.1 ± 17.8%, 70.9 ± 17.4%, 60.1 ± 20.7 and 66.9 ± 51.8% was observed with the combination ciprofloxacin 0.06 µg/ml and Pectinex 7.42 (Figure 41D), 14.84, 29.69 and 59.38 PGU/ml, respectively. Similarly, significant ($p \leq 0.05$) inhibition occurred when ciprofloxacin 0.125 µg/ml was added to Pectinex 14.84, 59.38, 118.75 PGU/ml and 475 PGU/ml. The addition of Pectinex 950 PGU/ml to ciprofloxacin was found to cause increased biofilm growth to a greater extent ($p \leq 0.05$) than singly administered ciprofloxacin at the concentrations of 0.25 – 4.0 µg/ml (Figure 41B and 41C).

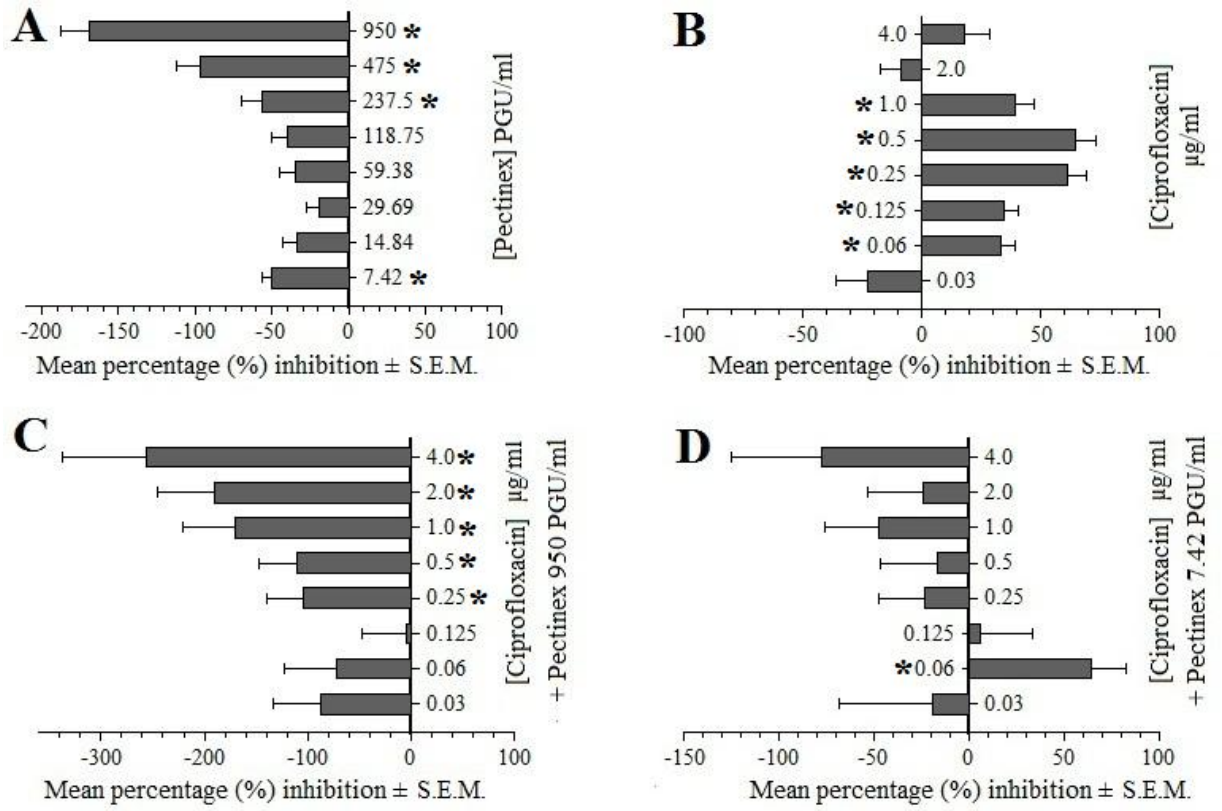

Figure 41: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *P. aeruginosa* clinical strain after 18 h incubation of 6 h-old cultures with [A] Pectinex, [B] ciprofloxacin, [C] Pectinex 950 PGU/ml plus ciprofloxacin, and [D] Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E12].

Pectinex (7.42 – 950 PGU/ml) increased biofilm bacterial viability (Figure 42A). With Pectinex 7.42 PGU, the observed viability was significantly ($p \leq 0.05$) greater than in the untreated controls. A significant ($p \leq 0.05$) reduction in biofilm viability (50.6 ± 9.2%) occurred in cultures that were incubated with ciprofloxacin 0.5 µg/ml (Figure 42B). The effects at all other concentrations of ciprofloxacin were not significantly ($p > 0.05$) different from the untreated control. Biofilm viability in bacterial cultures exposed to all combinations of Pectinex and ciprofloxacin were not found to be significantly ($p > 0.05$) different from the viability observed in untreated controls, with one exception, The exception was Pectinex 29.69 PGU/ml plus ciprofloxacin 0.5 µg/ml which was found to have a significantly ($p \leq 0.05$) greater reduction (52.6 ± 8.2%) in viability.

Several combinations were found to have a significantly ($p \leq 0.05$) lower biofilm bacterial viability than those exposed to Pectinex alone. These combinations were:

- Pectinex 7.42 – 950 PGU/ml plus ciprofloxacin 0.5 µg/ml (Figures 42C and 42D)
- Pectinex 7.42 (Figure 42D) and 59.38 – 237.5 PGU/ml plus ciprofloxacin 4.0 µg/ml
- Pectinex 7.42, 118.75 and 950 PGU/ml plus ciprofloxacin 0.25 µg/ml (Figures 42C and 42D)

When the effect of Pectinex and ciprofloxacin combinations were compared with the effect of singly administered ciprofloxacin, there was no statistically significant ($p > 0.05$) difference between the biofilm viability found at all combinations and the viability observed after exposure the same concentration of ciprofloxacin was administered alone.

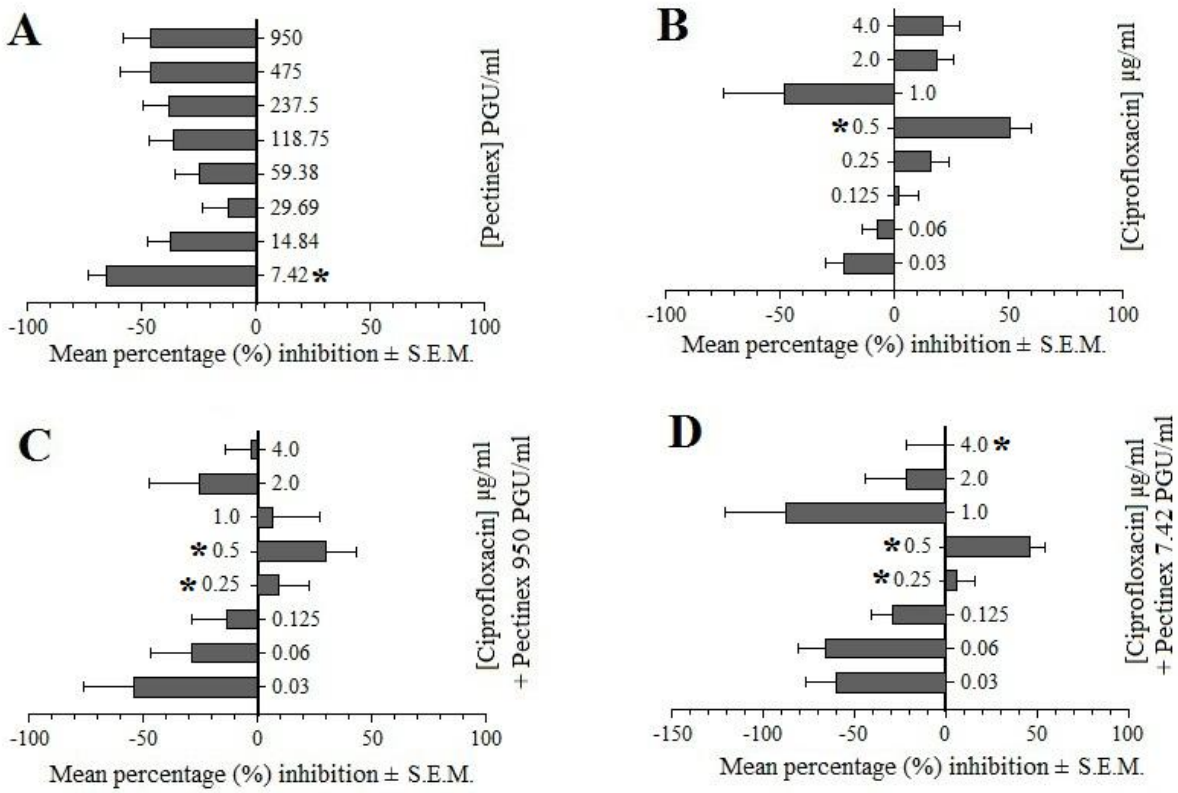

Figure 42: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *P. aeruginosa* clinical strain cultures after further 18 h incubation of 6 h-old cultures with [A] Pectinex, [B] ciprofloxacin, [C] combination of Pectinex 950 PGU/ml plus ciprofloxacin, and [D] combination of Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F12].

4.2.4 Biofilm biomass and viability after 24 h incubation of 24 h-old bacterial cultures with Pectinex and antibiotics

a) *S. aureus* ATCC

Pectinex promoted a significant ($p \leq 0.05$) increase in biofilm biomass at the concentration range 7.42 – 59.38 PGU/ml and also at 237.5 PGU/ml (Figure 43A). A non-significant ($p > 0.05$) increase in biomass occurred at 118.75 PGU/ml. Inhibition occurred at 475 and 950 PGU/ml; however, this was not significantly ($p > 0.05$) different from the untreated control. Biofilm biomass was increased (negative inhibition) at all concentrations of amoxicillin-clavulanate (Figure 43B). Growth in cultures exposed to amoxicillin-clavulanate concentrations 0.03 – 2.0 µg/ml were significantly ($p \leq 0.05$) different from the untreated control.

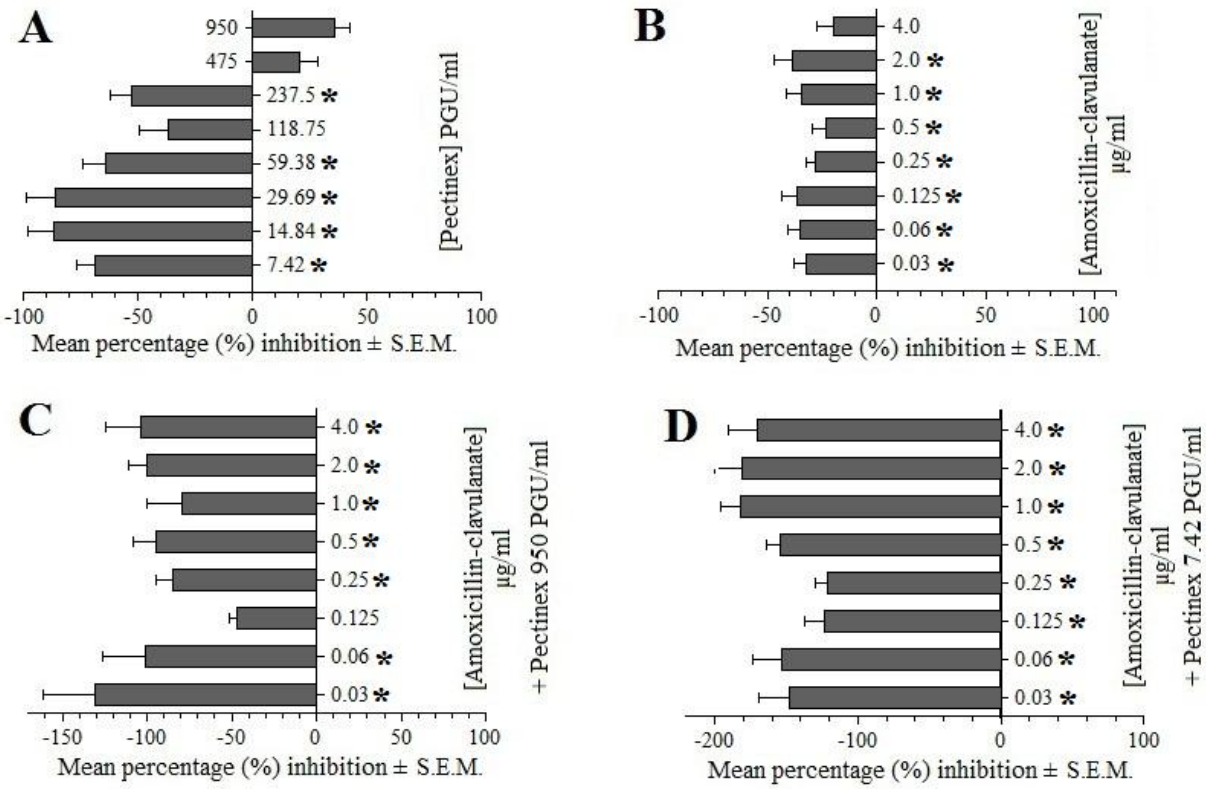

Figure 43: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *S. aureus* ATCC strain after 24 h incubation of 24 h-old cultures with [A] Pectinex, [B] amoxicillin-clavulanate, [C] Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E13].

All combinations of Pectinex and amoxicillin-clavulanate appeared to promote biofilm growth (Figures 43C and 43D). All combinations, except both Pectinex 475 and 950 PGU/ml with amoxicillin-clavulanate 0.125 µg/ml, were found to be significantly ($p \leq 0.05$) different from the untreated control (Figure 43C and Appendix E13).

Pectinex (7.42 – 950 PGU/ml) enhanced biofilm bacterial viability at all concentrations, and viability was significantly ($p \leq 0.05$) different from untreated controls at the concentrations 7.42 and 59.38 – 950 PGU/ml (Figure 44A).

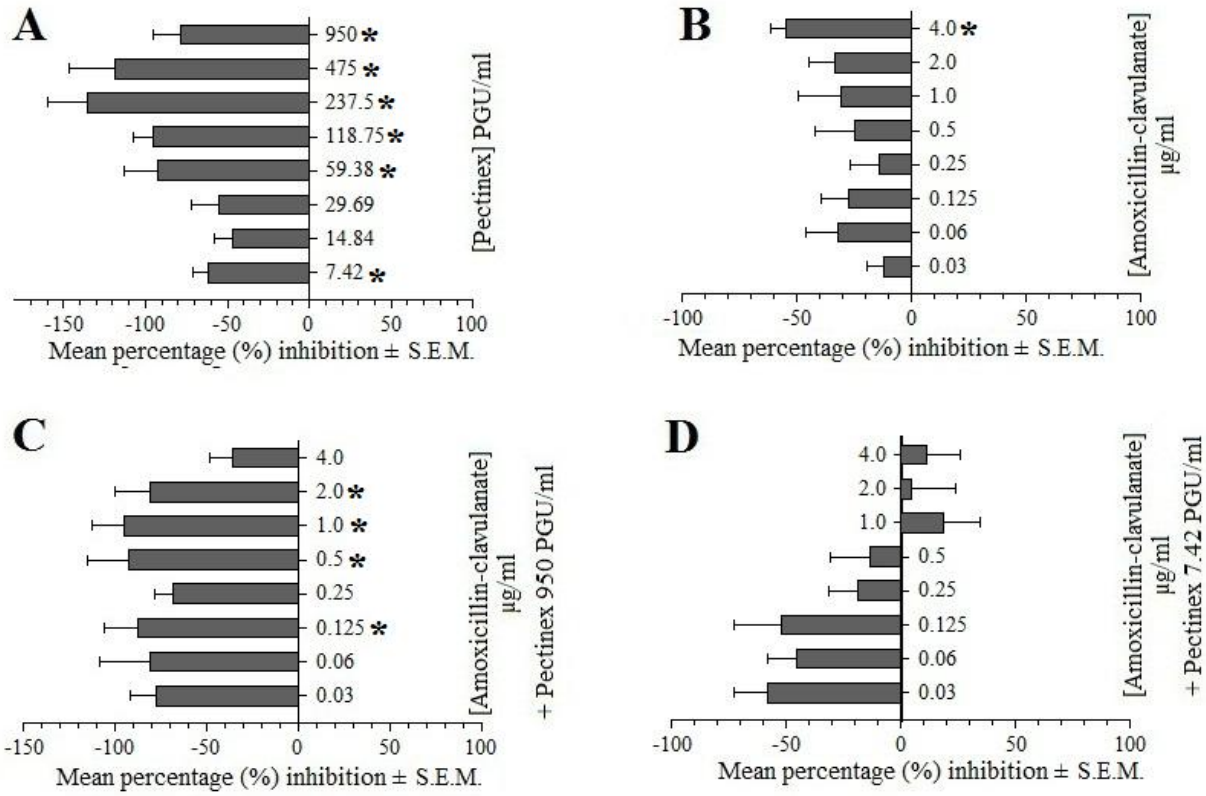

Figure 44: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *S. aureus* ATCC cultures after further 24 h incubation of 24 h-old cultures with [A] Pectinex, [B] amoxicillin-clavulanate, [C] combination of Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] combination of Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F13].

Amoxicillin-clavulanate (0.03 – 4.0 µg/ml) also enhanced biofilm viability at all concentrations; however only amoxicillin-clavulanate 4.0 µg/ml was significantly ($p \leq 0.05$) different from untreated controls (Figure 44B). A mixed response of increased and decreased viability was found following combined treatment. Pectinex (118.75 – 950 PGU/ml) with

amoxicillin-clavulanate (0.03 – 2.0 µg/ml) significantly ($p \leq 0.05$) increased the viability of biofilm bacteria at several combinations as compared to the untreated control (Appendix F13). All combinations with Pectinex 7.42 – 59.38 PGU/ml were not significantly ($p > 0.05$) different from the untreated control.

When the effects of combinations were compared with the effect of singly administered Pectinex, the combinations that showed a significantly ($p \leq 0.05$) lower biofilm viability than Pectinex alone were:

- Pectinex 237.5 PGU/ml plus amoxicillin-clavulanate 4.0 µg/ml
- Pectinex 118.75 PGU/ml plus amoxicillin-clavulanate 1.0 – 4.0 µg/ml
- Pectinex 14.84 PGU/ml plus amoxicillin-clavulanate 2.0 µg/ml

The observed biofilm viability was not significantly ($p > 0.05$) different from the effects of singly administered Pectinex in cultures that were incubated with all other combinations (Figure 44C and 44D). When the effects of combinations were compared with the effect of singly administered amoxicillin-clavulanate, the increase in biofilm viability was significantly ($p \leq 0.05$) higher with the combinations of Pectinex 237.5 PGU/ml plus amoxicillin-clavulanate 0.25 µg/ml. There was no statistically significant ($p > 0.05$) difference between the effects of singly administered amoxicillin-clavulanate and any of the remaining combinations.

b) *S. aureus* clinical strain

Neither Pectinex nor amoxicillin-clavulanate exerted any influence on biofilm biomass that was significantly ($p > 0.05$) different from the untreated controls (Figures 45A and 45B). None of the combinations Pectinex with amoxicillin-clavulanate were significantly ($p > 0.05$) different from the untreated controls (Figure 45C and 45D). When bacteria were cultured with combinations of Pectinex and amoxicillin-clavulanate, no significant ($p > 0.05$) difference was found between the effects that occurred with the combinations and those that occurred across all concentrations of either singly administered Pectinex or singly administered amoxicillin-clavulanate (Figures 45C and 45D).

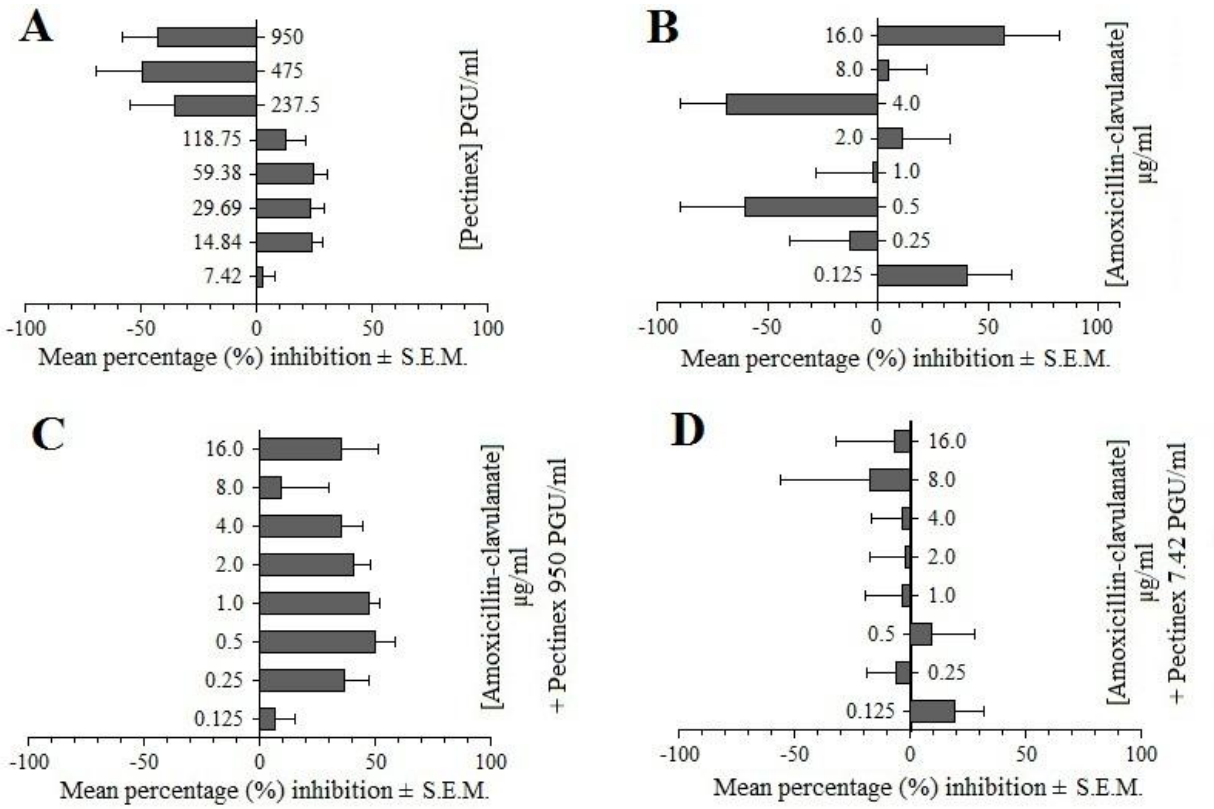

Figure 45: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *S. aureus* clinical strain after 24 h incubation of 24 h-old cultures with [A] Pectinex, [B] amoxicillin-clavulanate, [C] Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate [Detailed results are provided in Appendix E14].

Pectinex 29.69 – 950 PGU/ml increased biofilm bacterial viability of *S. aureus* clinical strain, whilst a reduction in viability was observed at the concentrations of 7.42 and 14.84 PGU/ml (Figure 46A). Significantly ($p \leq 0.05$) enhanced biofilm viabilities occurred at Pectinex concentrations of 118.75 and 237.5 PGU/ml. Biofilm viability found in all cultures incubated with either amoxicillin-clavulanate, or with combinations of amoxicillin-clavulanate and Pectinex (7.42 – 950 PGU/ml), was not significantly ($p > 0.05$) different from the untreated control (Figure 46B)

No statistically significant ($p > 0.05$) difference in the biofilm viability was found between cultures incubated with Pectinex and in those cultured with a combination of Pectinex and amoxicillin-clavulanate (Figure 46C). When combinations were compared with singly administered amoxicillin-clavulanate, there was no significant ($p > 0.05$) difference between the groups (Figure 46B and 46C).

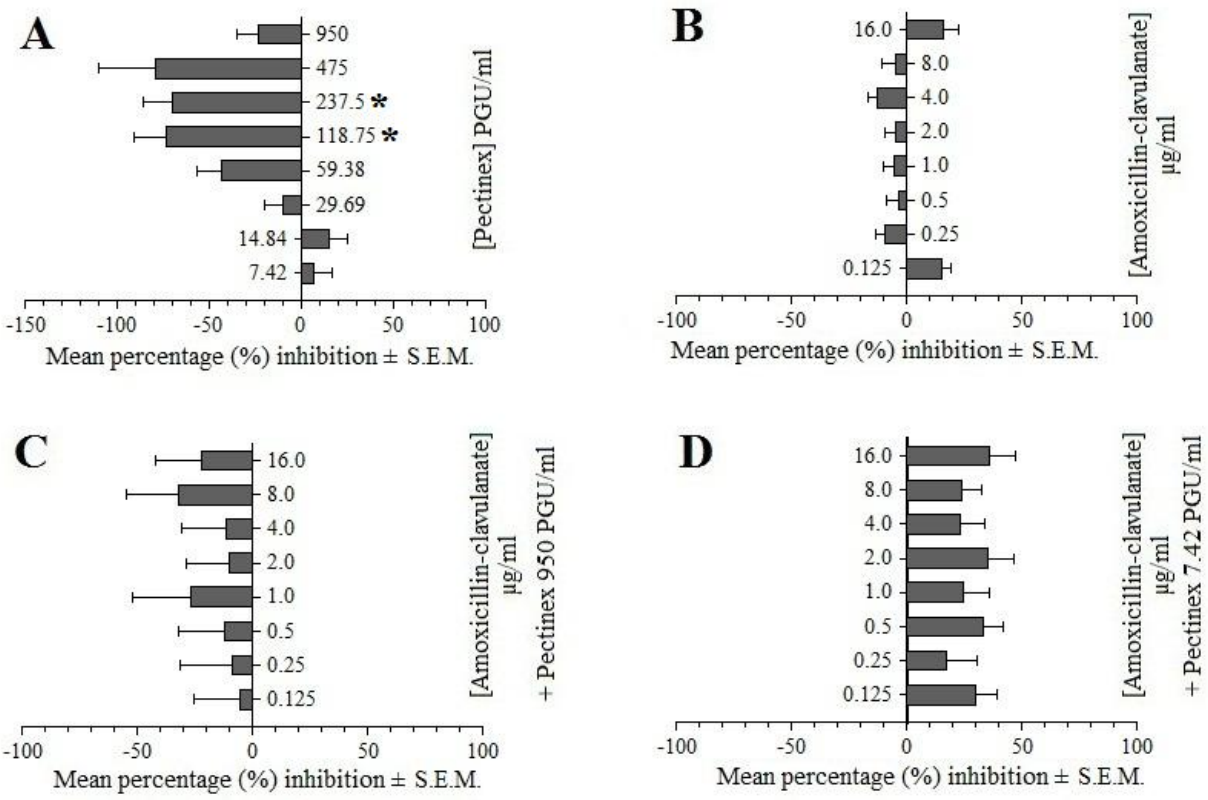

Figure 46: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *S. aureus* clinical strain cultures after further 24 h incubation of 24 h-old cultures with [A] Pectinex, [B] amoxicillin-clavulanate, [C] combination of Pectinex 950 PGU/ml plus amoxicillin-clavulanate, and [D] combination of Pectinex 7.42 PGU/ml plus amoxicillin-clavulanate (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F14].

c) *P. aeruginosa* ATCC

Biofilm biomass in cultures of *P. aeruginosa* ATCC was reduced by Pectinex 7.42 – 118.75 PGU/ml. Biofilm biomass was increased at concentrations ≥ 237.5 PGU/ml, however, the findings did not differ significantly ($p > 0.05$) from untreated controls (Figure 47A). In comparison to untreated controls, significantly ($p \leq 0.05$) reduced biofilm growth was observed at 14.48, 29.69 and 59.58 PGU/ml (Figure 47A). Non significant ($p > 0.05$) inhibition of 8.9 ± 4.1% was observed with Pectinex 7.42 PGU/ml. Enhanced growth occurred with Pectinex 237.5 – 950 PGU/ml, however, it was not statistically significant ($p > 0.05$). Ciprofloxacin significantly ($p \leq 0.05$) reduced biofilm biomass at the concentrations of 0.06, 0.25 and 0.5 µg/ml (Figure 47B). Ciprofloxacin 4.0 µg/ml increased biofilm formation; however, this was not statistically significant ($p > 0.05$). Combined treatments were not significantly ($p > 0.05$) different from the untreated control and from treatment with either Pectinex or ciprofloxacin administered alone.

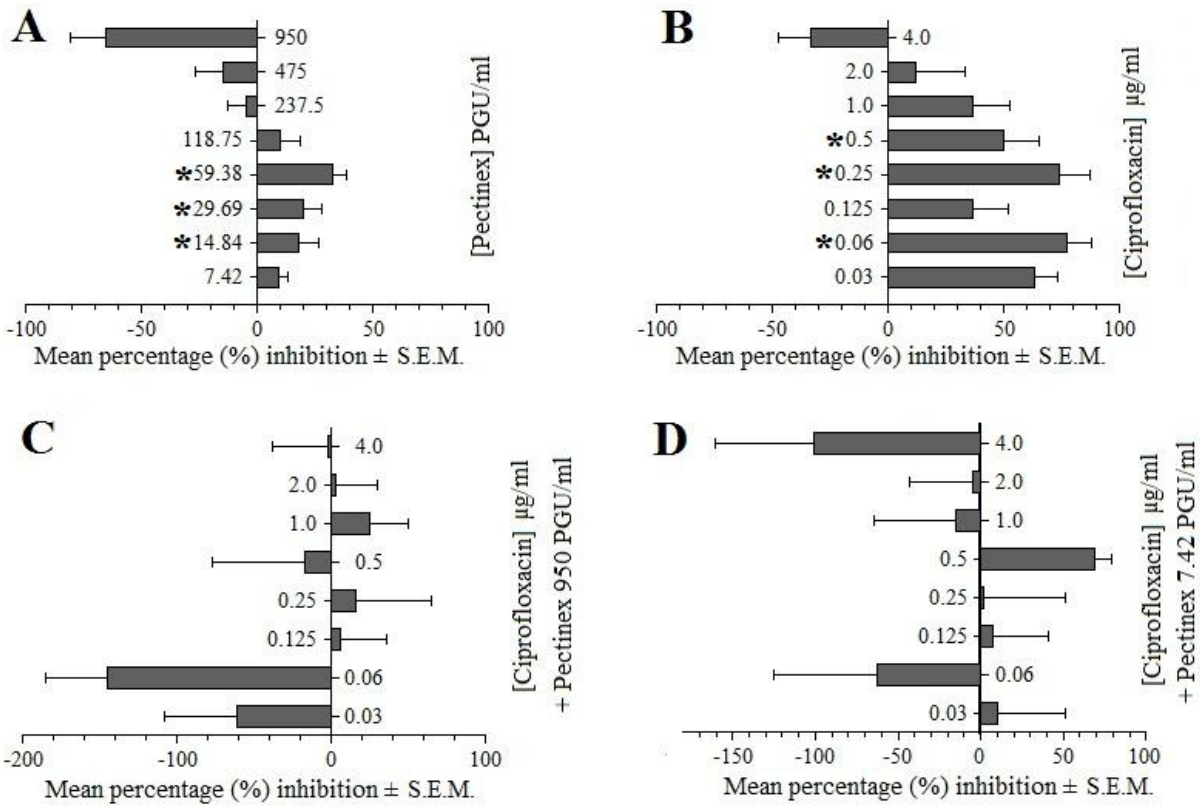

Figure 47: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *P. aeruginosa* ATCC strain after 24 h incubation of 24 h-old cultures with [A] Pectinex, [B] ciprofloxacin, [C] Pectinex 950 PGU/ml plus ciprofloxacin, and [D] Pectinex 7.42 PGU/ml plus ciprofloxacin(* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E15].

All concentrations of Pectinex were found to promote biofilm bacterial viability (Figure 48A). However, there were no statistically significant ($p > 0.05$) differences in biofilm viability between the Pectinex-treated cultures and the untreated control, with the exception of Pectinex 237.5 PGU/ml where the difference was found to be significant ($p \leq 0.05$).

Biofilm viability was significantly ($p \leq 0.05$) reduced in cultures that were incubated with ciprofloxacin at concentrations of 0.06 – 4.0 µg/ml (Figure 48B). Reduced biofilm viability was observed in cultures that were treated with ciprofloxacin 0.03 µg/ml, however, this was not found to be significant ($p > 0.05$).

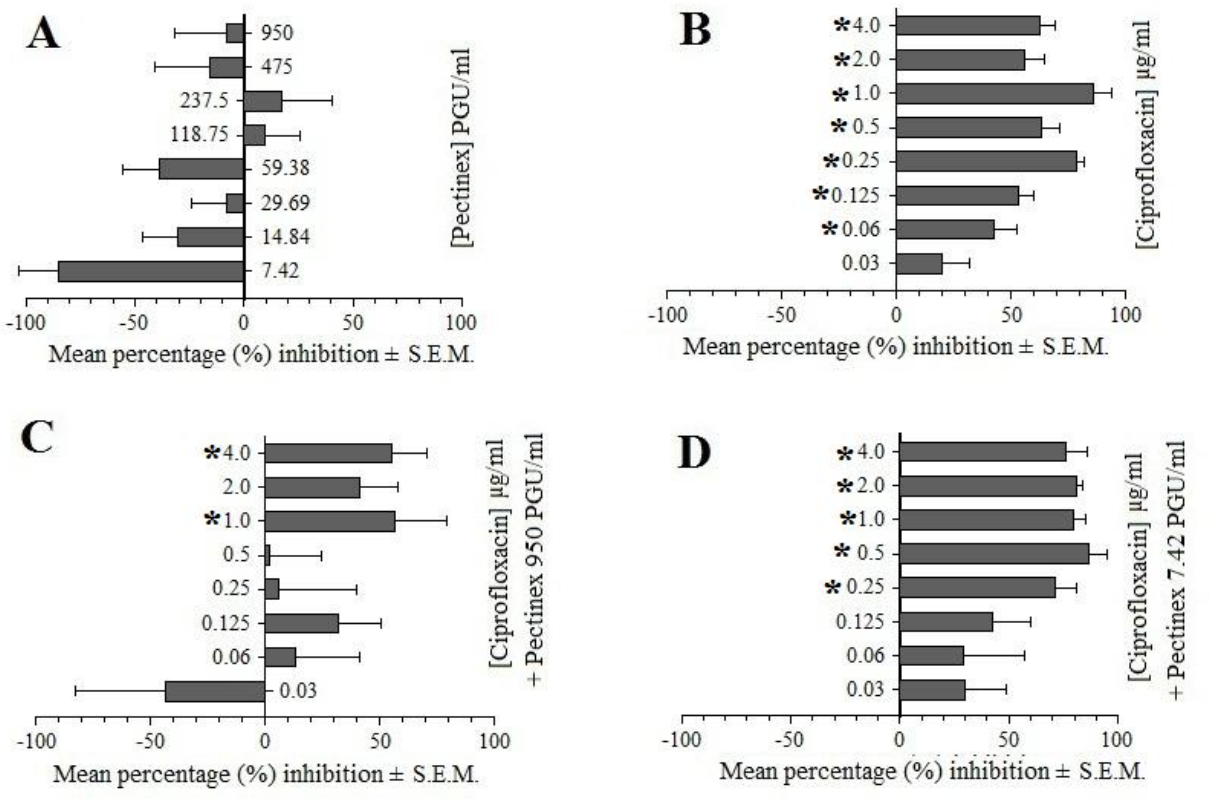

Figure 48: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *P. aeruginosa* ATCC cultures after further 24 h incubation of 24 h-old cultures with [A] Pectinex, [B] ciprofloxacin, [C] combination of Pectinex 950 PGU/ml plus ciprofloxacin, and [D] combination of Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F15].

When bacterial cultures were incubated with a combination of Pectinex and ciprofloxacin, statistically significant ($p \leq 0.05$) reductions in viability were observed with:

- Pectinex 950 PGU/ml plus either of ciprofloxacin 1.0 or 4.0 µg/ml
- Pectinex 475 PGU/ml plus either of ciprofloxacin 1.0 or 4.0 µg/ml
- Pectinex 59.38 PGU/ml plus ciprofloxacin 0.25 – 4.0 µg/ml
- Pectinex 29.69 PGU/ml plus ciprofloxacin 0.06 and 0.5 – 4.0 µg/ml
- Pectinex 14.84 PGU/ml plus ciprofloxacin 0.06 – 4.0 µg/ml
- Pectinex 7.42 PGU/ml plus ciprofloxacin 0.25 – 4.0 µg/ml

At all other combinations, there was no significant ($p > 0.05$) difference between the biofilm viability in the treated cultures as compared to the untreated controls.

Biofilm bacterial viability in cultures exposed to Pectinex 29.69 and 118.75 – 950 PGU/ml in combination with all concentrations of ciprofloxacin was not significantly ($p > 0.05$) different from the viability observed in cultures treated with Pectinex alone (Figure 48C). Compared to singly administered Pectinex, significantly ($p \leq 0.05$) more reduction in viability was observed with the combination of Pectinex 7.42 and 14.84 PGU/ml plus ciprofloxacin 0.25 – 4.0 µg/ml, and also with the combination of Pectinex 7.42 PGU/ml and ciprofloxacin 0.125 µg/ml (Figure 48D and Appendix F15). The biofilm viability found in bacterial cultures incubated with all combinations of Pectinex and ciprofloxacin was not significantly ($p > 0.05$) different from the viability observed after exposure to ciprofloxacin alone.

d) *P. aeruginosa* clinical strain

In cultures of *P. aeruginosa* clinical strain, none of the concentrations of Pectinex produced any significant ($p > 0.05$) difference in biofilm biomass when compared to the untreated controls (Figure 49A).

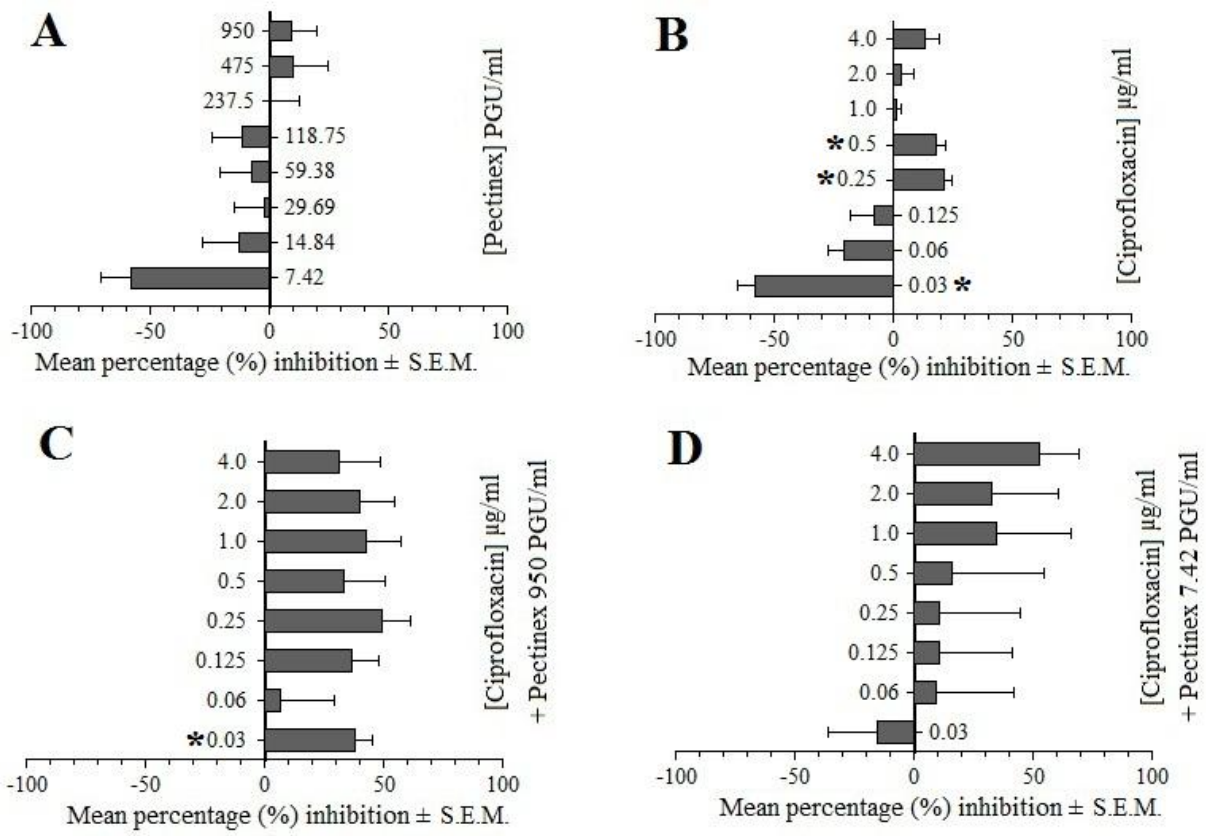

Figure 49: Mean percentage (%) inhibition ± S.E.M. of biofilm formation by *P. aeruginosa* clinical strain after 24 h incubation of 24 h-old cultures with [A] Pectinex, [B] ciprofloxacin, [C] Pectinex 950 PGU/ml plus ciprofloxacin, and [D] Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix E16].

Ciprofloxacin 0.03 µg/ml significantly ($p \leq 0.05$) increased biofilm biomass whilst a significant ($p \leq 0.05$) reduction was observed with 0.25 and 0.5 µg/ml (Figure 49B). When bacterial cultures were exposed to combinations of Pectinex and ciprofloxacin, no significant ($p > 0.05$) differences were found between the effect of the combinations and the untreated control.

At all concentrations of Pectinex, no statistically significant ($p > 0.05$) differences were found between the effects of singly administered concentrations and when Pectinex was combined with ciprofloxacin (Figures 49C and 49D). When the effect of combinations were compared with the effects of ciprofloxacin alone, reduction of biofilm biomass was significantly ($p > 0.05$) greater when ciprofloxacin 0.03 µg/ml was combined with Pectinex 14.84 – 950 PGU/ml, and also when ciprofloxacin 0.06 µg/ml was combined with Pectinex 29.69 and 475 PGU/ml.

Biofilm viability was enhanced following exposure to Pectinex at concentrations of 7.42 – 237.5 PGU/ml (Figure 50A). When compared to untreated controls, viability was significantly ($p \leq 0.05$) enhanced by Pectinex 7.42 and 59.38 PGU/ml. Reduced viabilities were found after exposure to Pectinex 475 and 950 PGU/ml; however, neither was found to be statistically significant ($p > 0.05$) (Figure 50A). Ciprofloxacin, at concentrations of 0.25 – 4.0 µg/ml, significantly ($p \leq 0.05$) reduced biofilm viability (Figure 50B).

When Pectinex and ciprofloxacin were administered in combination, Pectinex 7.42 (Figure 50D) and 29.69PGU/ml in combination with ciprofloxacin 1.0 µg/ml significantly ($p \leq 0.05$) reduced biofilm viability by 77.7 ± 11.8% and 73.3 ± 10.2%, respectively. Biofilm viability observed at all other combinations was not significantly ($p > 0.05$) different from the untreated controls. When the effects after incubation with both Pectinex and ciprofloxacin were compared that Pectinex administered alone (Figures 50C and 50D), biofilm bacterial viability was significantly ($p \leq 0.05$) reduced by the following combinations:

- Ciprofloxacin 0.5 µg/ml plus Pectinex 7.42, 14.84, 59.38 and 237.5 PGU/ml
- Ciprofloxacin 1.0 µg/ml plus Pectinex 7.42 – 237.5 PGU/ml
- Ciprofloxacin 2.0 µg/ml plus Pectinex 14.84 and 29.69 PGU/ml
- Ciprofloxacin 4.0 µg/ml plus Pectinex 7.42, 26.69 and 237.5 PGU/ml

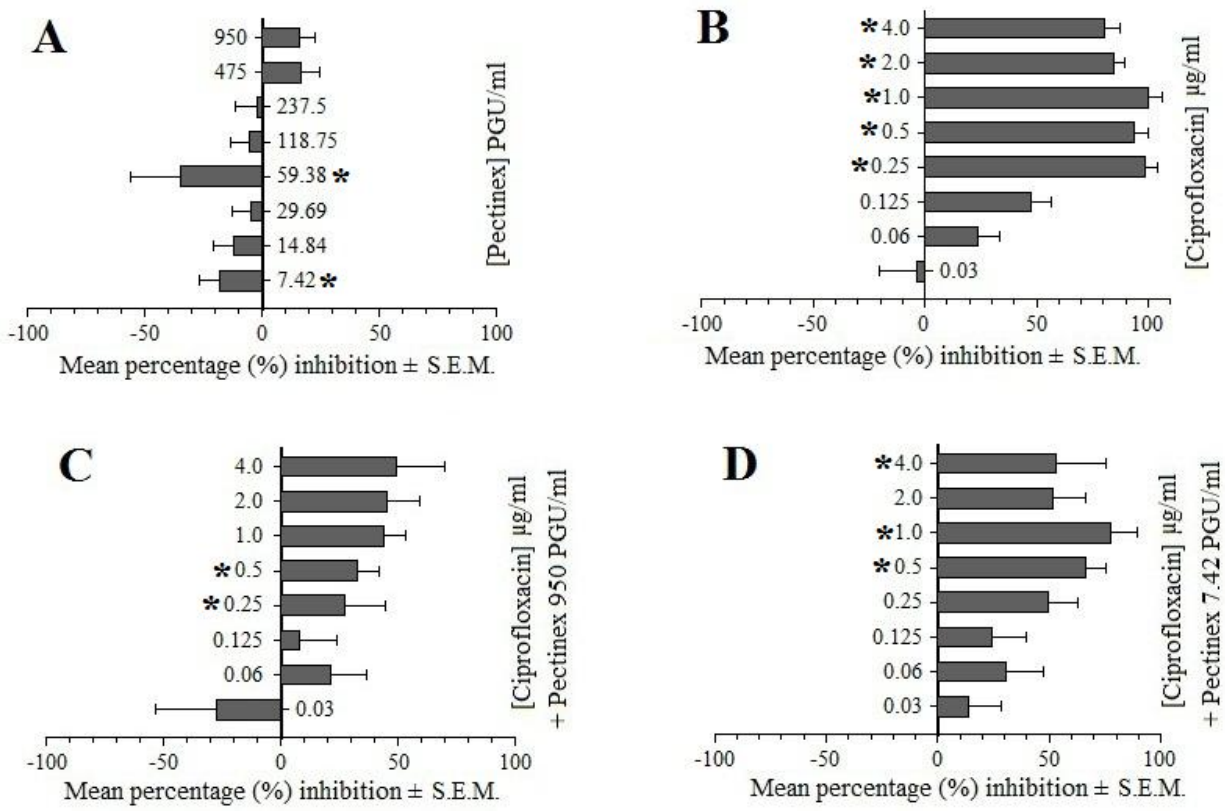

Figure 50: Mean percentage (%) inhibition ± S.E.M. of biofilm viability in *P. aeruginosa* clinical strain cultures after further 24 h incubation of 24 h-old cultures with [A] Pectinex, [B] ciprofloxacin, [C] combination of Pectinex 59.38 PGU/ml plus ciprofloxacin, and [D] combination of Pectinex 7.42 PGU/ml plus ciprofloxacin (* indicates where $p \leq 0.05$) [Detailed results are provided in Appendix F16].

When the effects of combined treatments were compared with the effects observed after singly administered ciprofloxacin, significantly ($p \leq 0.05$) greater reductions in biofilm viability were observed when Pectinex 475 PGU/ml was combined with ciprofloxacin 0.25 µg/ml, and Pectinex 950 PGU/ml was combined with either of ciprofloxacin 0.25 or 0.5 µg/ml. The effects observed at all other combinations were not significant ($p > 0.05$) different from the effects of attributable to ciprofloxacin alone.

4.3 *In vitro* cytotoxicity assay of Pectinex on HeLa cells, lymphocytes and neutrophils

The effect of Pectinex (7.42 – 950 PGU/ml) on the viability of HeLa cells, lymphocytes and neutrophils was determined using two parallel MTT assays; Method 1 with cell washing steps and Method 2 without washing steps [Section 3.3.1 (b)]. The results are expressed as the mean

percentage inhibition as compared to untreated negative controls. For comparison, the inhibition of cell viability in the untreated control was assigned a value of 0%. With each of the two MTT assay methods, six replicate experiments were performed for each of the three cell types. In addition, three more replicates were conducted on HeLa cells in order to determine the significance of the pH of Pectinex on the experimental outcomes. For each assay method, the percentage inhibition (between untreated control and treated groups) of all the individual data points in all replicates were combined and the mean percentage inhibition, for the designated cell type, was calculated for each concentration of Pectinex (7.42 - 950 PGU/ml) and mitomycin C 10 µg/ml (positive control). Detailed results of the mean percentage inhibition ± S.E.M. are shown in Appendix G. Statistical comparisons were made between the mean percentage concentrations after exposure to Pectinex and the untreated controls, and between mitomycin C and the untreated controls. Comparisons were also made between Pectinex and mitomycin C; however, these were only done with HeLa cells. Mean percentage inhibitions were calculated for Pectinex concentrations within the individual replicate experiments. Thereafter, the IC_{50} values of Pectinex (expressed as PGU/ml) for each replicate experiment were independently calculated, and the mean IC_{50} ± standard error of the mean (S.E.M.) of the replicates was taken as the final value (Table 10).

Table 10: IC_{50} ± S.E.M. of Pectinex on HeLa cells, lymphocytes and neutrophils

Cell cultures[incubation time]	IC_{50} S.E.M. (PGU/ml)	
	Method 1 with cell washing	Method 2 without cell washing
HeLa cells with Pectinex pH 4.8 [7d]	193.9 ± 22.2	237.6 ± 31.0
HeLa cells with Pectinex pH 7.0 [7d]	242.5 ± 35.8	330.4 ± 19.0
non-stimulated lymphocytes [3d]	629.6 ± 62.8	319.9 ± 69.1
fMLP-stimulated lymphocytes [3d]	383.4 ± 81.5	369.7 ± 91.0
non-stimulated neutrophils [4 h]	529.7 ± 40.7	267.6 ± 1.2
fMLP-stimulated neutrophils [4 h]	245.9 ± 9.4	-
non-stimulated neutrophils [24 h]	276.9 ± 17.8	230.4 ± 38.4
fMLP-stimulated neutrophils [24 h]	171.4 ± 22.7	146.9 ± 13.3

Replicate IC_{50} values that were found to have a value of ≤ 0 PGU/ml were not used to calculate mean IC_{50} values; the assumption being that, although mathematically achievable, such concentrations did not constitute logical entities. Furthermore, extrapolated IC_{50} values

that occurred outside the range 7.42 – 950 PGU/ml were not used such that the mean Pectinex IC_{50} ± S.E.M. was derived by interpolation of generated data rather than by extrapolation.

Cell viability was assessed in HeLa cell cultures after exposure to Pectinex (7.42 – 950 PGU/ml) and mitomycin C (10 µg/ml) for 7 days. The mean percentage reductions in HeLa cell viability, observed at different concentrations of Pectinex, were compared with the effects of untreated controls and mitomycin C. There was no significant ($p > 0.05$) difference between the mean percentage inhibition in the untreated controls and at Pectinex concentrations ≤ 59.38 PGU/ml. Significant ($p \leq 0.05$) inhibition was observed at all Pectinex concentrations ≥ 118.75 PGU/ml with the greatest effect occurring at 475 and 950 PGU/ml (Figure 51).

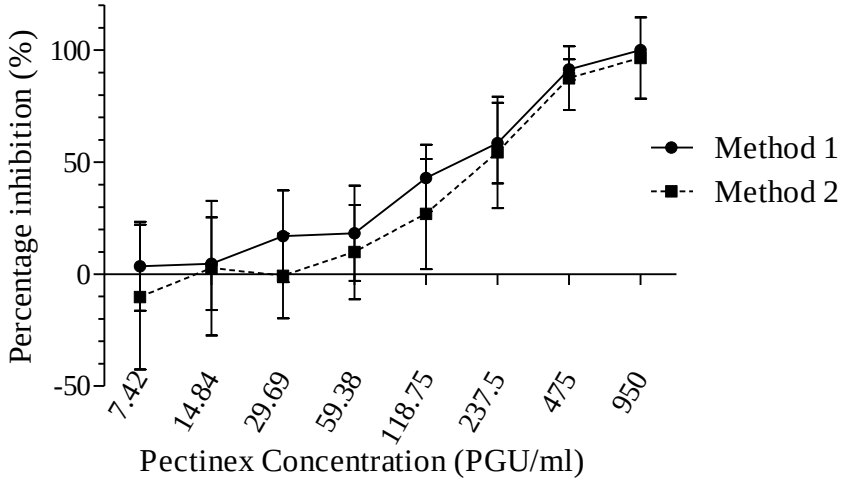

Figure 51: Mean percentage inhibition± S.E.M. of HeLa cells after 7 day incubation with Pectinex using Method 1 with cell washing, and Method 2 without cell washing [Detailed results are provided in Appendix G]

Mitomycin C was an effective positive control for HeLa cells and was found to inhibit viability by 99.0 ± 1.6% and 95.1 ± 9.8% when using Methods 1 and 2, respectively. With both MTT assay methods, there was no significant ($p > 0.05$) difference between the mean percentage inhibition of HeLa cells by mitomycin C and by Pectinex at the concentrations of 237.5, 475 and 950 PGU/ml (Figure 51). However, at Pectinex concentrations of 7.42 – 118.75 PGU/ml, the values observed were significantly ($p \leq 0.05$) lower than that of mitomycin C. The mean IC_{50} ± S.E.M. of Pectinex on HeLa cells was found to be 193.9 ±

22.2 and 237.6 ± 31.0 PGU/ml using Method 1 and Method 2, respectively (Table 10). There was no significant difference ($p > 0.05$) between the values obtained from either technique. When the contributions of the pH of Pectinex on HeLa cell viability were examined, no significant differences ($p > 0.05$) were found in the mean IC_{50} ± S.E.M. of Pectinex in HeLa cell cultures that were incubated with Pectinex solutions with initial pH values of either 4.8 (acidic) or 7.0 (neutral).

Lymphocytes were exposed to Pectinex for 3 days after which cell viability was determined by MTT assay. Significant ($p \leq 0.05$) reduction of cell viability in non-stimulated lymphocytes only occurred with Pectinex 950 PGU/ml (Figure 52). In contrast, with fMLP-stimulated cells, significant ($p \leq 0.05$) inhibition was also observed at lower concentrations; ≥ 59.38 PGU/ml using Method 1, and 7.42 PGU/ml (Figure 52) and 118.75 – 950 PGU/ml using Method 2 (Figure 53). A higher Pectinex IC_{50} was observed with non-stimulated lymphocytes as compared to fMLP-stimulated lymphocytes (Table 10). With MTT assay Method 1, the IC_{50} ± S.E.M. of Pectinex on non-stimulated and fMLP-stimulated lymphocytes was 629.6 ± 62.8 and 383.4 ± 81.5 PGU/ml, respectively.

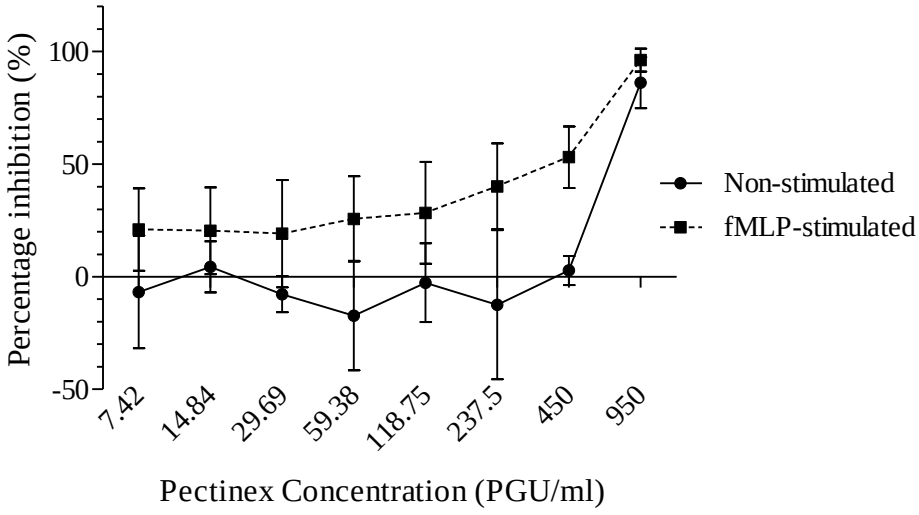

Figure 52: Mean percentage inhibition± S.E.M. of non-stimulated and fMLP stimulated lymphocytes after 3 day incubation with Pectinex using Method 1; with cell washing [Detailed results are provided in Appendix G]

Using Method 2, comparable IC_{50}values of 319.9 ± 69.1 and 369.7 ± 91.0 PGU/ml were observed for non-stimulated and fMLP-stimulated lymphocytes, respectively. However, these differences were not statistically significant ($p > 0.05$). When the IC_{50} concentrations derived from the two different assay methods were compared with each other, no statistically

significant ($p > 0.05$) differences were found between the equivalent values in each assay for both non-stimulated and fMLP-stimulated lymphocytes.

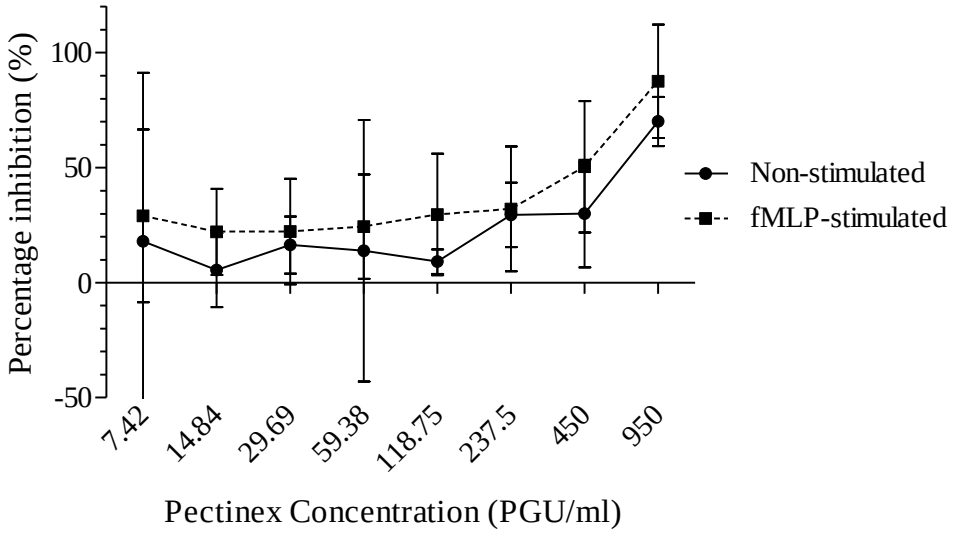

Figure 53: Mean percentage inhibition± S.E.M. of non-stimulated and fMLP stimulated lymphocytes after 3 day incubation with Pectinex using Method 2; without cell washing [Detailed results are provided in Appendix G]

Neutrophils were incubated with Pectinex for two different time periods; 4 h and 24 h. After a 4 h incubation period, cell viability in both non-stimulated and fMLP-stimulated neutrophils was significantly reduced by Pectinex 475 and 950 PGU/ml as determined by Method 1 (Figure 54). The results from Method 2 differed in that stimulated lymphocytes were significantly inhibited by lower Pectinex concentrations (29.69 – 237.5 PGU/ml) in addition to 950 PGU/ml (Figure 55). With both MTT assays, the mean IC_{50} values were found to be lower with non-stimulated neutrophils than with stimulated neutrophils (Table 10). Using Method 1, the mean IC_{50} ± S.E.M. values were 529.7 ± 40.7 and 245.9 ± 9.4 PGU/ml, and with Method 2, the value was 267.6 ± 1.2 PGU/ml for non-stimulated; however, an IC_{50} could not be achieved in fMLP-stimulated neutrophils (Table 10).

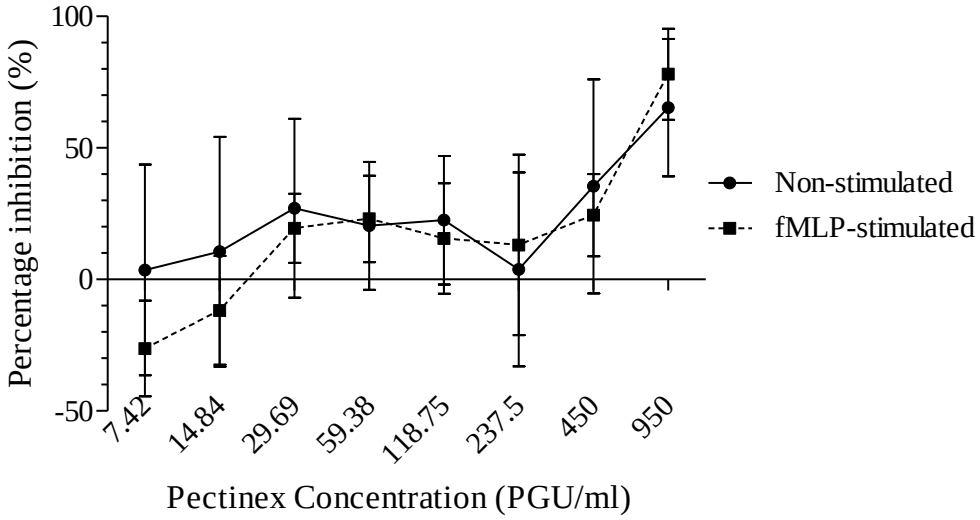

Figure 54: Mean percentage inhibition± S.E.M. of non-stimulated and fMLP stimulated neutrophils after 4 h incubation with Pectinex using Method 1; with cell washing [Detailed results are provided in Appendix G]

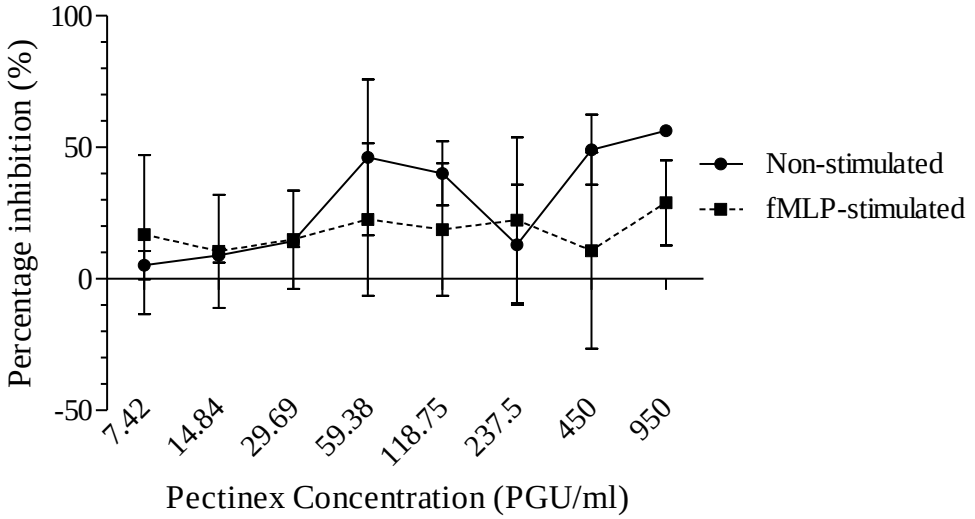

Figure 55: Mean percentage inhibition± S.E.M. of non-stimulated and fMLP stimulated neutrophils after 4 h incubation with Pectinex using Method 2; without cell washing [Detailed results are provided in Appendix G]

After a 24 h exposure, Method 1 assay demonstrated a significant ($p \leq 0.05$) reduction in non-stimulated neutrophil viability with Pectinex 950 PGU/ml, and in fMLP-stimulated with 237.5 – 950 PGU/ml (Figure 56). With Method 2, statistically significant reduction in cell viability was observed at Pectinex concentrations of 14.84 – 950 PGU/ml in both non-

stimulated and fMLP-stimulated neutrophils (Figure 57). The IC_{50} ± S.E.M. of Pectinex on neutrophils was found to be 276.9 ± 17.8 and 171.4 ± 22.7 PGU/ml using Method 1, and 230.4 ± 38.4 and 146.9 ± 13.3 PGU/ml using Method 2, for non-stimulated and fMLP-stimulated neutrophils, respectively (Table 10). There were no statistically significant differences between any of these observations.

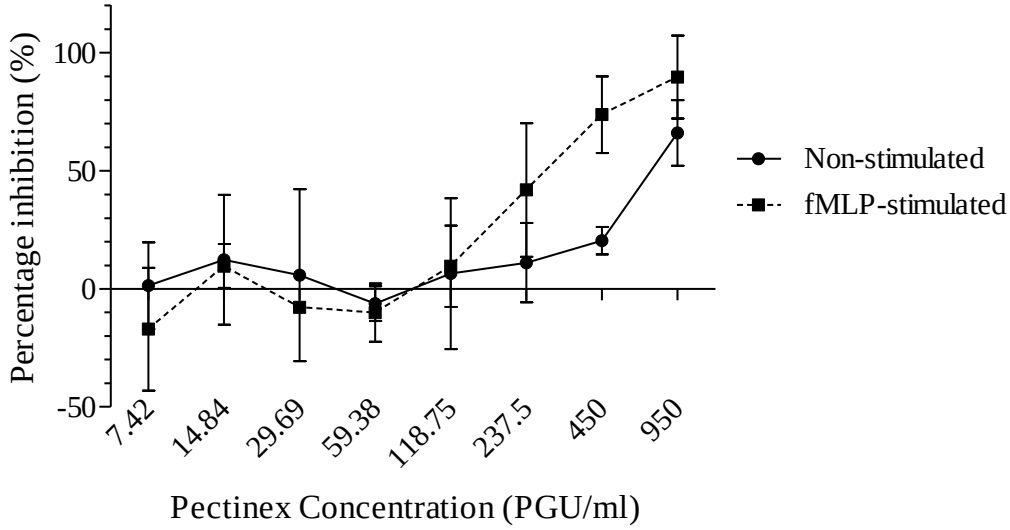

Figure 56: Mean percentage inhibition± S.E.M. of non-stimulated and fMLP stimulated neutrophils after 24 h incubation with Pectinex using Method 1; with cell washing [Detailed results are provided in Appendix G]

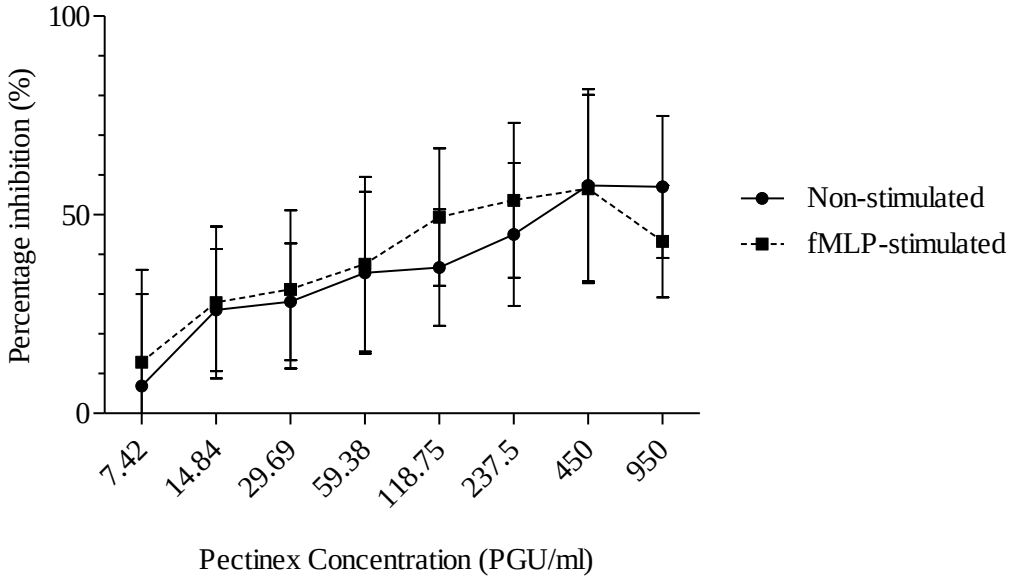

Figure 57: Mean percentage inhibition± S.E.M. of non-stimulated and fMLP stimulated neutrophils after 24 h incubation with Pectinex using Method 2; without cell washing [Detailed results are provided in Appendix G]

The mean IC_{50} ± S.E.M. concentrations of Pectinex were compared between all experimental subgroups of HeLa cells, lymphocytes and neutrophils (Table 10). No statistically significant differences ($p > 0.05$) were found in the values of the mean IC_{50} ± S.E.M. derived from any of the cell types after exposure to Pectinex.

4.4 PlasDIC microscopy of human cell cultures

Cultures of HeLa cells, lymphocytes and neutrophils were exposed to Pectinex (7.42 – 950 PGU/ml) for 24 h and compared with positive controls (i.e. mitomycin C and staurosporine) and untreated controls. PlasDIC microscopy at x400 magnification revealed that Pectinex treatment was associated with a dose-dependent increase in cell apoptosis and necrosis in all three cell lines (Figures 58 – 62). The morphological changes that were observed included shrunken rounded cells, cell membrane blebs (zeiosis), apoptotic bodies, cytoplasmic vacuoles and cell debris. In both lymphocyte and neutrophil cultures there was a decrease in the number of cells seen per microscopic field as the concentration of Pectinex increased. HeLa cells (Figure 58) were affected by concentrations upwards from 237.5 PGU/ml. No normal cells were found after exposure to Pectinex at 950 PGU/ml and, at this concentration only cell fragments and apoptotic bodies were visualized. Pectinex concentrations of 7.42 through to 118.75 PGU/ml did not have any visible effect on the morphology of HeLa cells, however, a rare instance of cytoplasmic vacuolization was observed at 59.38 PGU/ml.

In comparison to untreated controls, non-stimulated lymphocytes were adversely affected at all concentrations of Pectinex and features of cell death along with clumping (aggregation of cells and cell debris) were observed (Figure 59). A marked decrease in cell numbers and a corresponding increase in cell debris occurred at and above 29.69 PGU/ml. At 950 PGU/ml shrunken cells, apoptotic bodies and cell debris were seen, and no normal cells were found. Lymphocytes that were stimulated with fMLP exhibited apoptotic and necrotic changes in morphology and excessive clumping at all concentrations of Pectinex, with mitomycin C (10 µg/ml) and also in the untreated controls (Figure 60). Significantly less cell clumping by fMLP-stimulated lymphocytes was encountered after exposure to Pectinex at 950 PGU/ml and to staurosporine (1 µg/ml). Both of these treatments appeared to cause a similar magnitude of cell damage.

Cultures of non-stimulated neutrophils (Figure 61) were unaffected by Pectinex at concentrations below 118.57 PGU/ml. However, upwards from 237.5 PGU/ml, there was a dose-dependent increase in the amount of cell debris and the number of shrunken and apoptotic cells; there was a corresponding decrease in the number of neutrophils. At 950 PGU/ml, Pectinex appeared to cause more extensive cell necrosis than either mitomycin C or staurosporine, as evidenced by the comparatively greater extent of the loss of cell membrane integrity. Stimulated neutrophils (Figure 62) exhibited features of cell death across all concentrations of Pectinex; the effects were more prominent from 29.69 PGU/ml and, below this concentration the cell morphologies were comparable to the untreated controls.

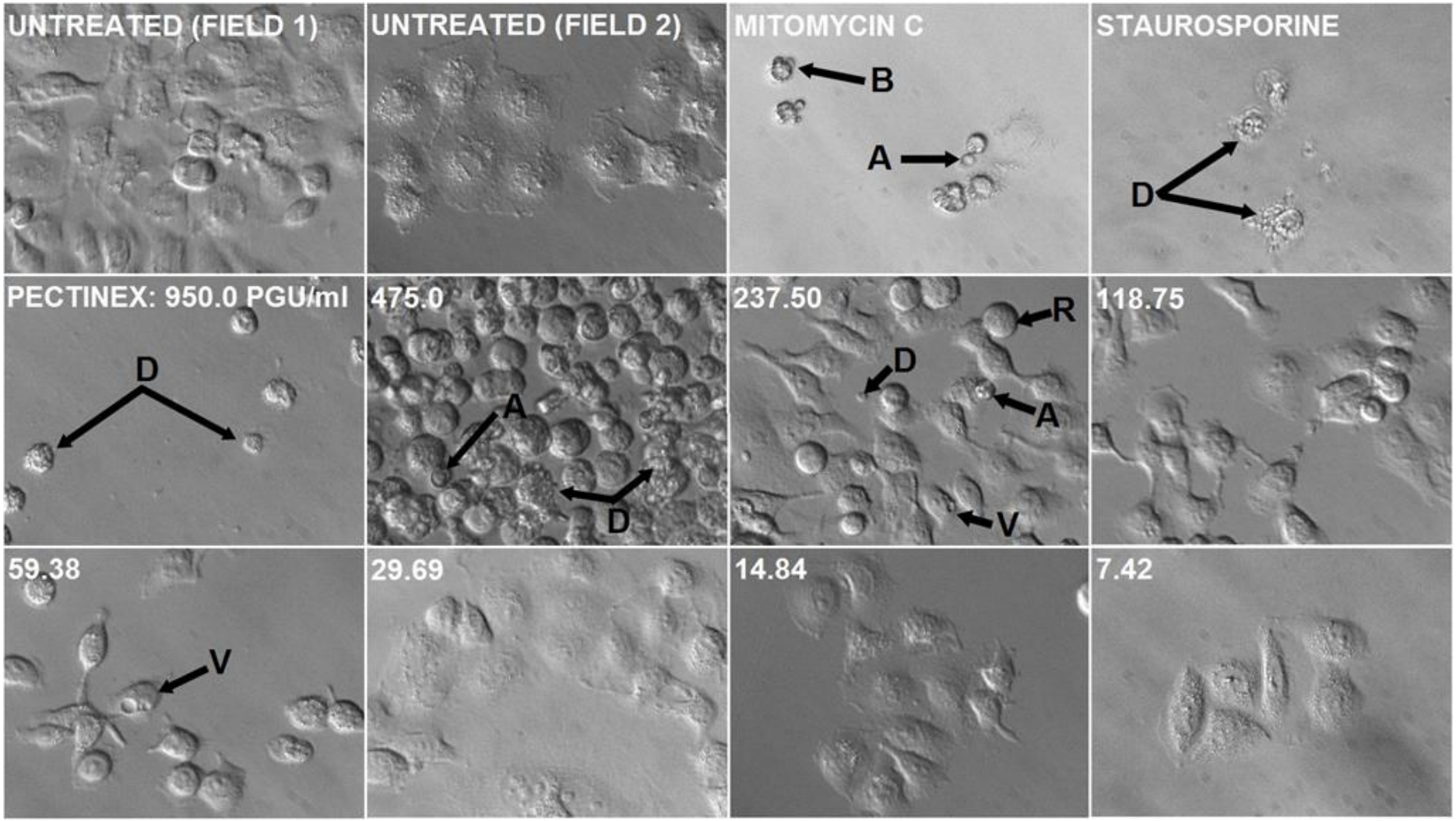

Figure 58: PlasDIC microscopy images (x400 magnification) of HeLa cells exposed to Pectinex (7.42-950 PGU/ml) and controls for 24 h. [A, apoptotic bodies; B, blebs; D, cell debris; R, shrunken rounded cells; V, cytoplasmic vacuoles]

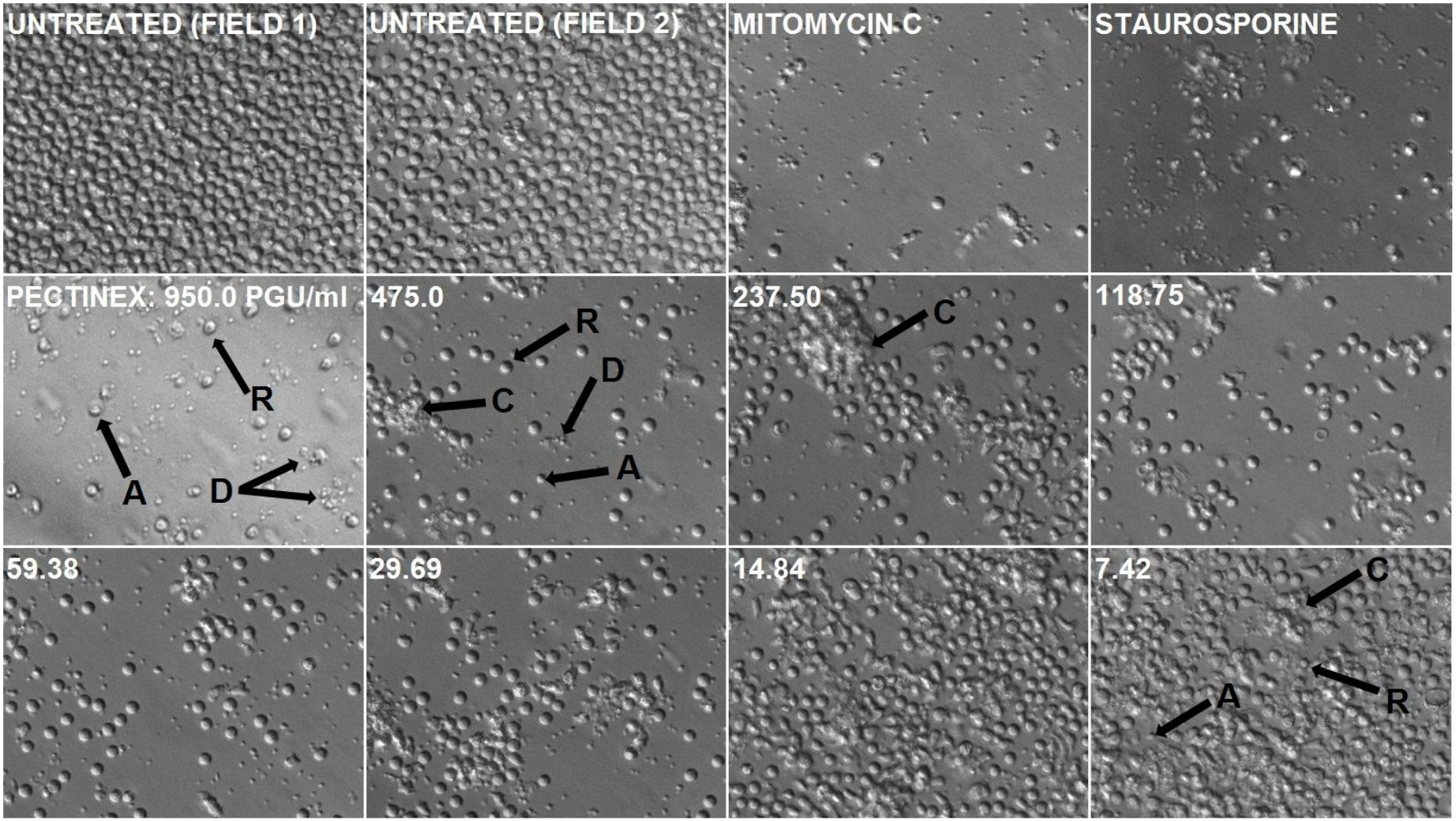

Figure 59: PlasDIC microscopy images (x400 magnification) of non-stimulated lymphocytes exposed to Pectinex (7.42-950 PGU/ml) and controls for 24 h. [A, apoptotic bodies; C, clumped cells; D, cell debris; R, shrunken rounded cells]

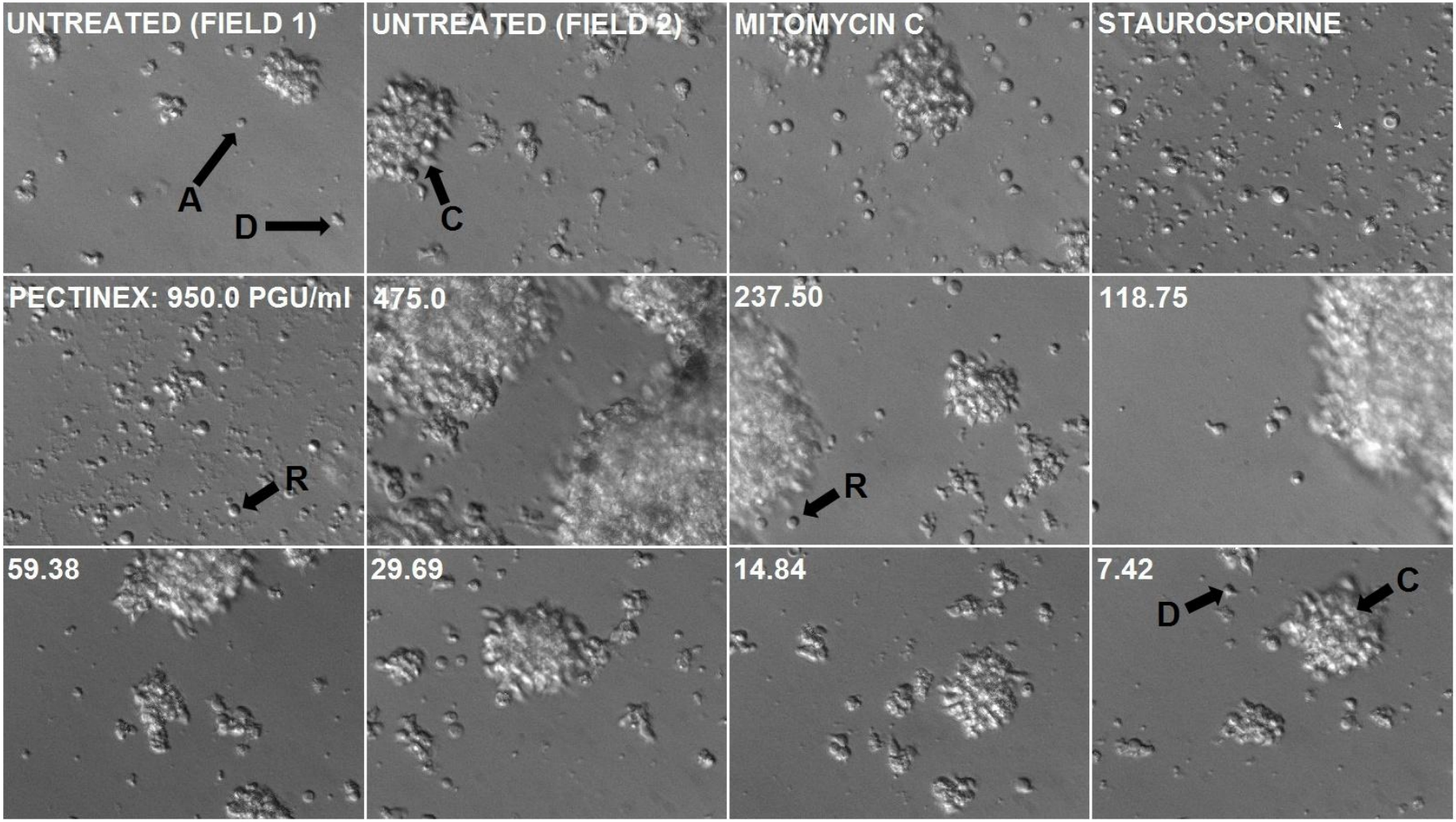

Figure 60: PlasDIC microscopy images (x400 magnification) of fMLP-stimulated lymphocytes exposed to Pectinex (7.42-950 PGU/ml) and controls for 24 h. [A, apoptotic bodies; C, clumped cells; D, cell debris; R, shrunken rounded cells]

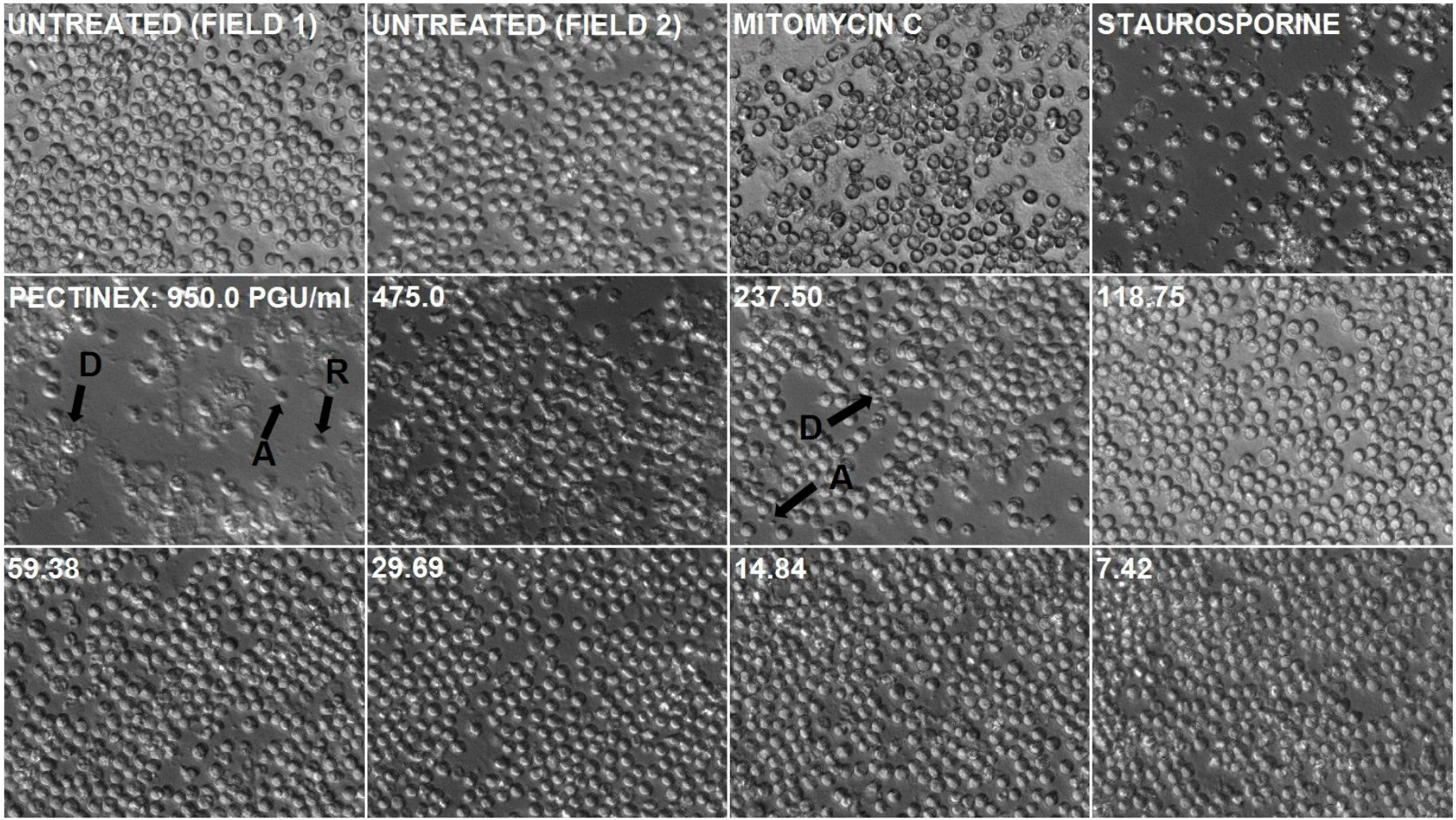

Figure 61: PlasDIC microscopy images (x400 magnification) of non-stimulated neutrophils exposed to Pectinex (7.42-950 PGU/ml) and controls for 24 h. [A, apoptotic bodies; D, cell debris; R, shrunken rounded cells]

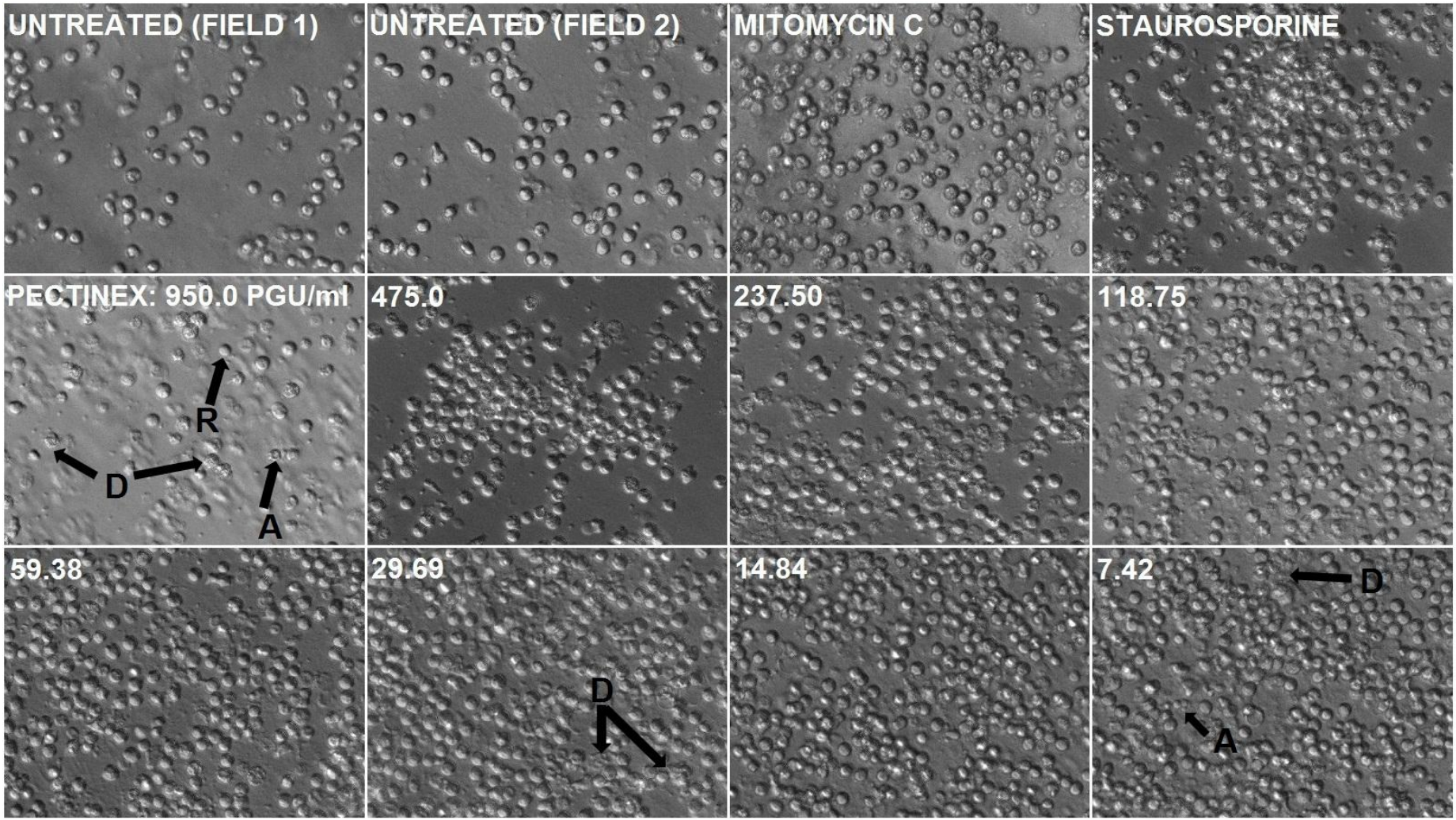

Figure 62: PlasDIC microscopy images (x400 magnification) of fMLP-stimulated neutrophils exposed to Pectinex (7.42-950 PGU/ml) and controls for 24 h. [A, apoptotic bodies; D, cell debris; R, shrunken rounded cells]

4.5 SEM of bacterial biofilm

S. aureus and *P. aeruginosa* biofilms were cultured on microscope coverslips and challenged with antibiotics (amoxicillin-clavulanate and ciprofloxacin), Pectinex or a combination of Pectinex with an antibiotic [Section 3.5] for 24 h. Two different starting points were used; fresh cultures were challenged from inoculation (i.e. from time 0 to the 24th h) and, mature cultures were challenged after 24 h of prior incubation (i.e. from the 24th to 48th h). After incubation, the coverslips were examined by SEM and biofilm formation was indicated by the presence of adherent bacteria and/or cell debris on the coverslips. Representative SEM images of biofilm were acquired at x5000 (Figures 56 – 59) and x2500 magnification (Figures 60 – 63) for *S. aureus* and *P. aeruginosa*, respectively. Biofilms were characterized as being weak, moderate or strong based on their subjective appearance, as described by Smith *et al.*[332].

a) Staphylococcal biofilms

The untreated controls of fresh (0 – 24 h) and mature (24 – 48 h) cultures of *S. aureus* ATCC and clinical strains formed moderate biofilms (Figures 63 – 66) that consisted of cells and cell clusters of variable size, however, the observed biofilms were unevenly distributed over the surface of the coverslips and large proportions remained devoid of cells. Numerous small particles and a few cell fragments were also visualized. Fresh cultures of *S. aureus* ATCC that were exposed to Pectinex at 118.75 and 950 PGU/ml (Figure 63) grew moderate biofilms that had greater cell numbers than were found in the untreated control. Biofilms were absent after treatment with amoxicillin-clavulanate at 0.5 and 32 µg/ml, and also when amoxicillin-clavulanate 0.5 µg/ml was combined with Pectinex 118.75 PGU/ml. Only cells without a clear cell wall ("ghost cells") were found after treatment with amoxicillin-clavulanate 32 µg/ml. Mature cultures of *S. aureus* ATCC (Figure 64) were found to have moderate biofilm attachment when treated with amoxicillin clavulanate, at 0.5 and 32 µg/ml. This was in sharp contrast to the effect of the antibiotic on the fresh bacterial cultures in which biofilm was notably absent. Treatment of mature biofilms with Pectinex 118.75 PGU/ml also produced moderate biofilms, however, weak biofilm attachment was found with Pectinex 950 PGU/ml. These weak staphylococcal biofilms were consisted of a widely dispersed monolayer of a few cells, cells with disrupted cell membranes and cell fragments. Weak biofilm were observed with the combined treatment of Pectinex 118.75 PGU/ml and amoxicillin-clavulanate 0.5 µg/ml, although neither agent had independently achieved this.

Fresh cultures of the clinical strain of *S. aureus* produced moderate biofilm growth when untreated and when challenged with Pectinex 950 PGU/ml (Figure 65). Weak biofilms were seen after treatment with all other regimen, and were absent from cultures treated with amoxicillin-clavulanate 2.0 µg/ml. The mature cultures (Figure 66) exhibited moderate growth in the untreated control and also after treatment with amoxicillin-clavulanate at both 2.0 and 32 µg/ml. However, in contrast to the moderate biofilm produced by fresh cultures, mature cultures produced a weak biofilm when treated with Pectinex 118.75 and 950 PGU/ml, and also with the amoxicillin-clavulanate 2.0 µg/ml plus Pectinex 118.75 PGU/ml combination.

b) Pseudomonal biofilms

Untreated controls of the ATCC and clinical strains of *P. aeruginosa* were found to have weak biofilm attachment in both fresh and mature cultures (Figures 60 – 63). Fresh cultures of the ATCC strain (Figure 67) that were treated with Pectinex 118.75 PGU/ml alone and in combination with ciprofloxacin 0.125 µg/ml developed thick biofilm. The former was associated with increased cell numbers and cell fragments and latter was associated with an increased cell count and the formation of thick 3-dimensional cell clusters. Virtually no biofilm was formed after treatment with either ciprofloxacin 8.0 µg/ml or Pectinex 950 PGU/ml. Treatment with ciprofloxacin 0.125 µg/ml produced weak biofilm of a similar appearance to that of the untreated control. In mature cultures of *P. aeruginosa* ATCC (Figure 68), scanty cell attachment was observed with ciprofloxacin 8.0 µg/ml, Pectinex 118.75 PGU/ml and Pectinex plus ciprofloxacin 0.125 µg/ml. No biofilm attachment was observed after treatment with all regimens.

Fresh clinical strains of *P. aeruginosa* (Figure 69) formed 3-dimensional cell clusters when treated with ciprofloxacin 1.0 µg/ml alone and when combined with Pectinex 118.75 PGU/ml. These treatments were also associated with more cell debris and other particulate matter than was seen in the untreated control. Weak biofilms were found after treatment with ciprofloxacin 8.0 µg/ml and Pectinex; 118.75 and 950 PGU/ml. A greater number of attached cells were observed following treatment with ciprofloxacin 8.0 µg/ml than were found in the untreated control. Mature cultures produced weak biofilms with sparsely distributed cells in the untreated control and at all treatment regimens (Figure 70).

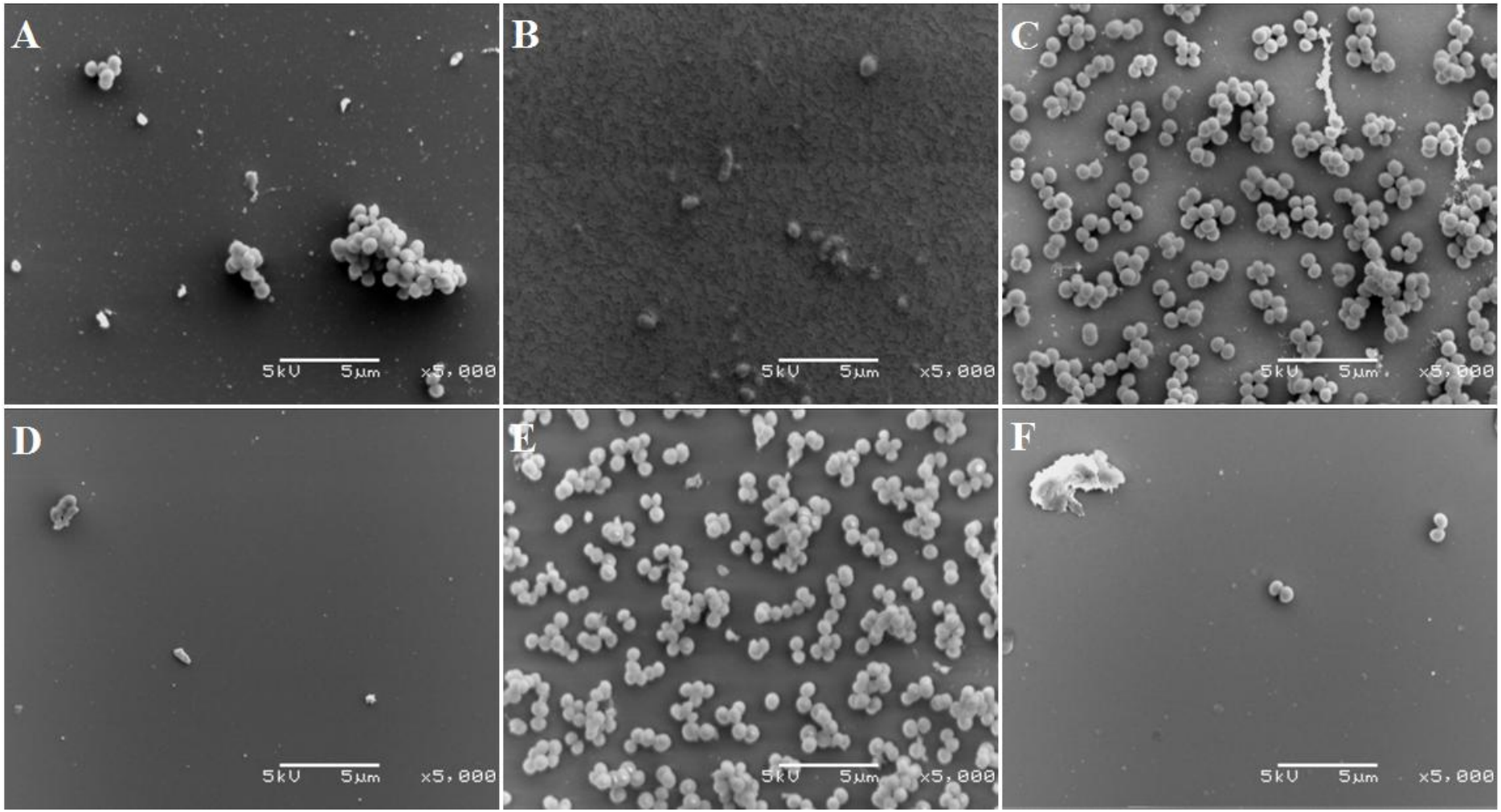

Figure 63: SEM images (x5000 magnification) of S. aureus ATCC biofilms following exposure of fresh cultures to test agents for 24 h. [A], untreated control; [B], amoxicillin-clavulanate 32 µg/ml; [C], Pectinex 950 PGU/ml; [D], amoxicillin-clavulanate 0.5 µg/ml (MIC); [E], Pectinex 118.75 PGU/ml; [F], amoxicillin-clavulanate 0.5 µg/ml plus Pectinex 118.75 PGU/ml.

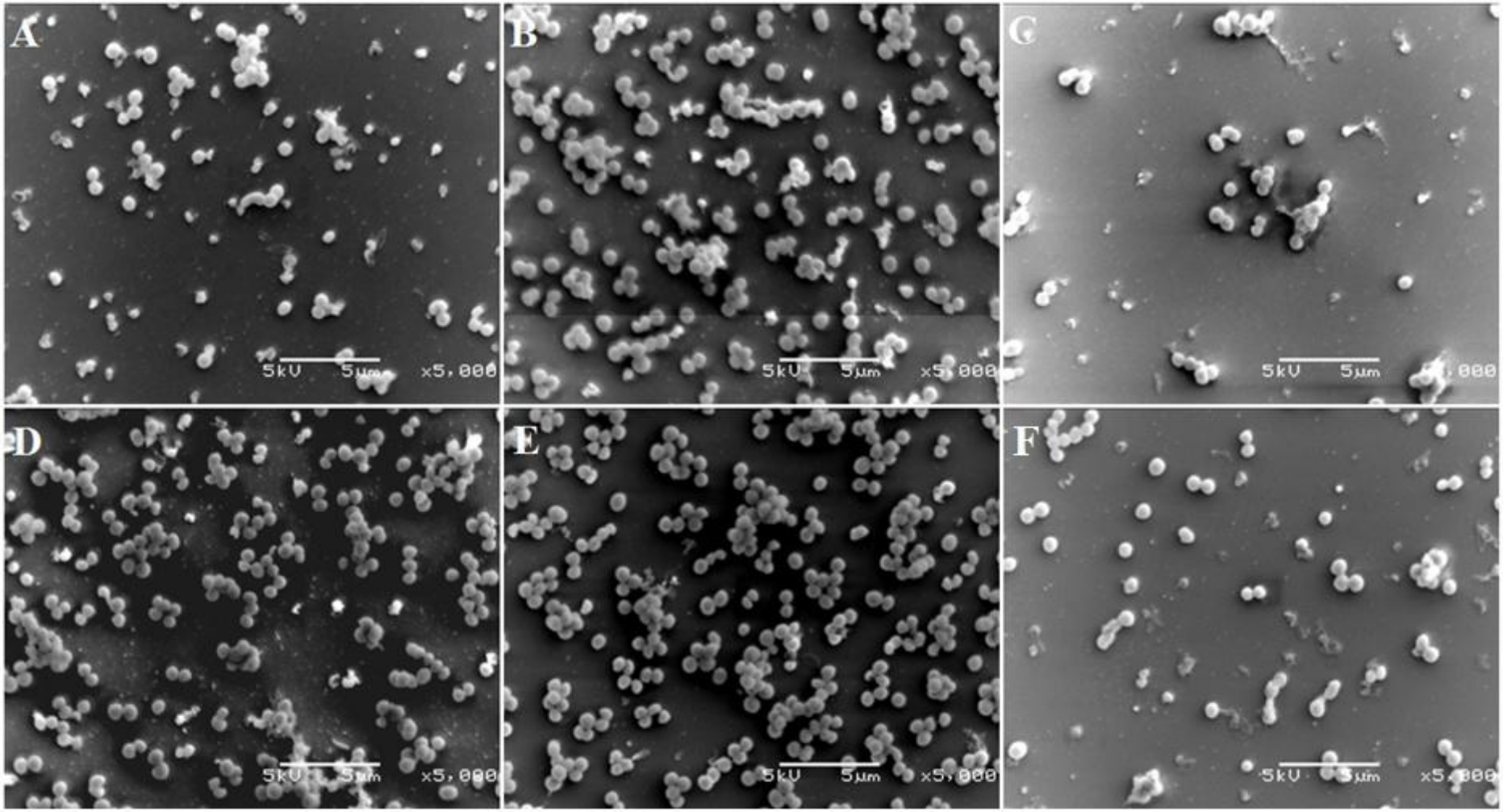

Figure 64: SEM images (x5000 magnification) of *S. aureus* ATCC biofilms following exposure of 24-h old biofilms to challenge agents for 24 h. [A], untreated control; [B], amoxicillin-clavulanate 32 µg/ml; [C], Pectinex 950 PGU/ml, [D], amoxicillin-clavulanate 0.5 µg/ml (MIC); [E], Pectinex 118.75 PGU/ml; [F], amoxicillin-clavulanate 0.5 µg/ml plus Pectinex 118.75 PGU/ml.

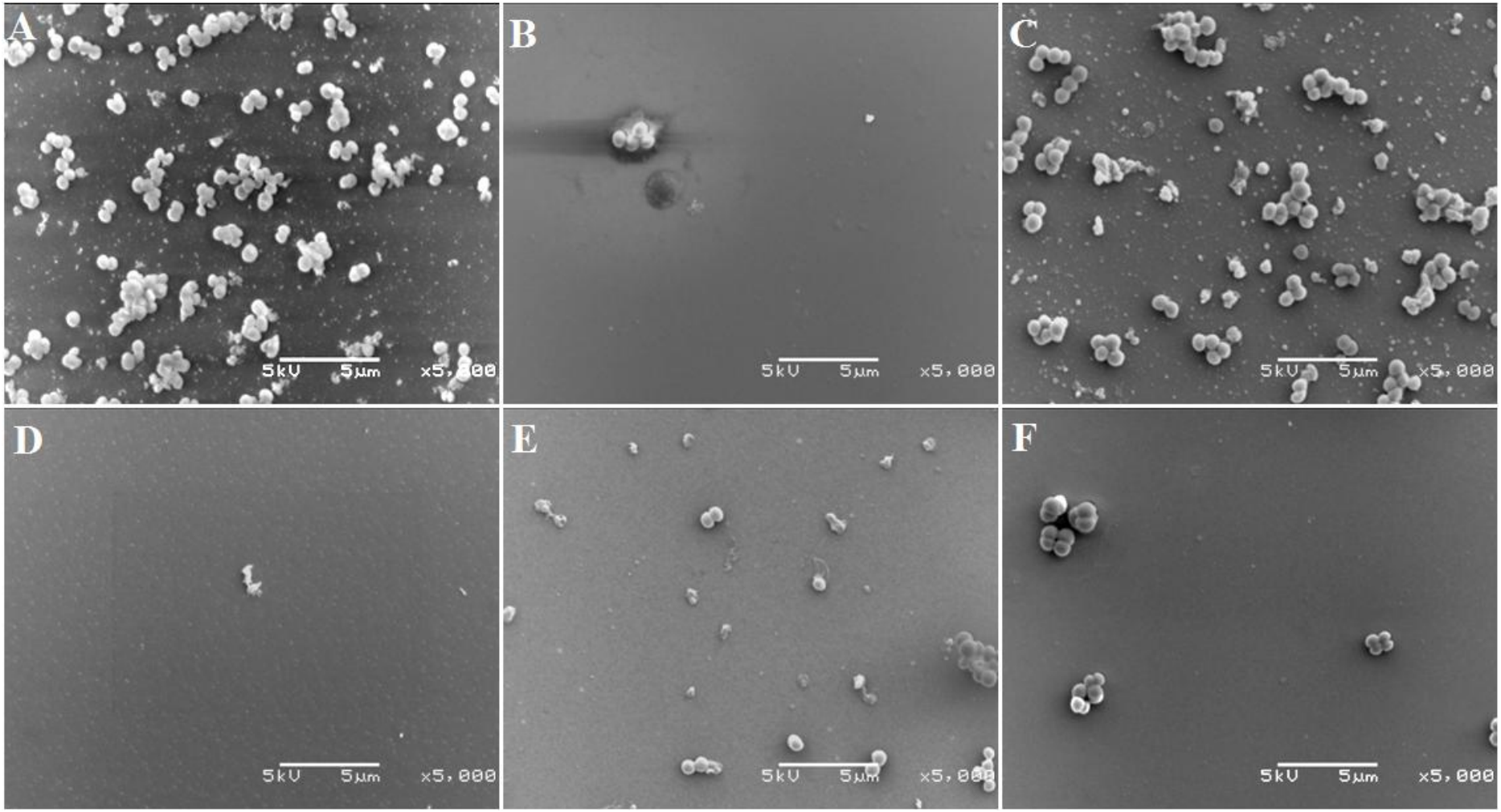

Figure 65: SEM images (x5000 magnification) of *S. aureus* clinical strain biofilms following exposure of fresh cultures to challenge agents for 24 h [A], untreated control; [B], amoxicillin-clavulanate 32 µg/ml; [C], Pectinex 950 PGU/ml; [D], amoxicillin-clavulanate 2.0 µg/ml (MIC); [E], Pectinex 118.75 PGU/ml; [F], amoxicillin-clavulanate 2.0 µg/ml plus Pectinex 118.75 PGU/ml.

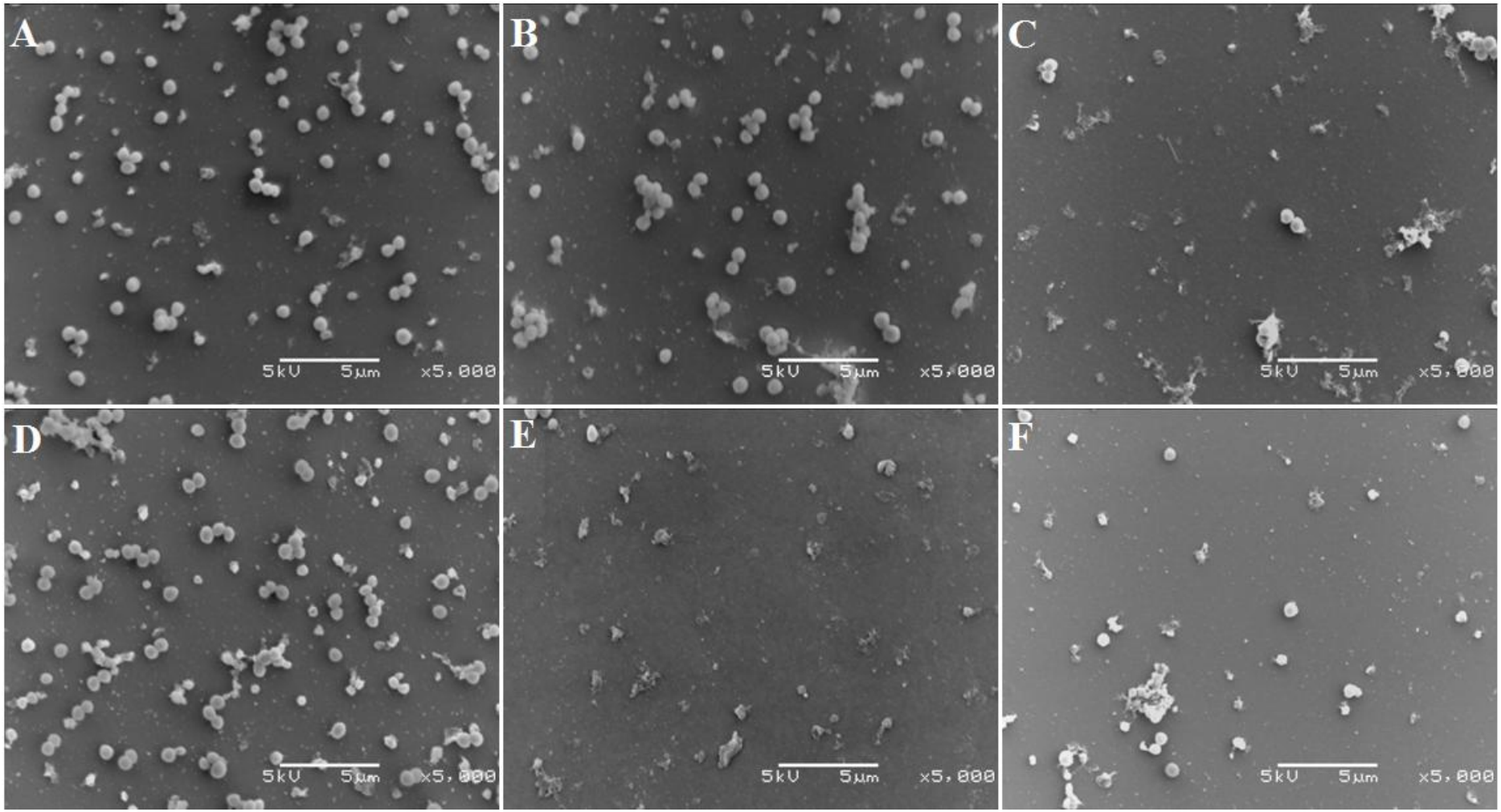

Figure 66: SEM images (x5000 magnification) of *S. aureus* clinical strain biofilms following exposure of 24-h old biofilms to challenge agents for 24 h. [A], untreated control; [B], amoxicillin-clavulanate 32 µg/ml; [C], Pectinex 950 PGU/ml, [D], amoxicillin-clavulanate 2.0 µg/ml (MIC); [E], Pectinex 118.75 PGU/ml; [F], amoxicillin-clavulanate 2.0 µg/ml plus Pectinex 118.75 PGU/ml.

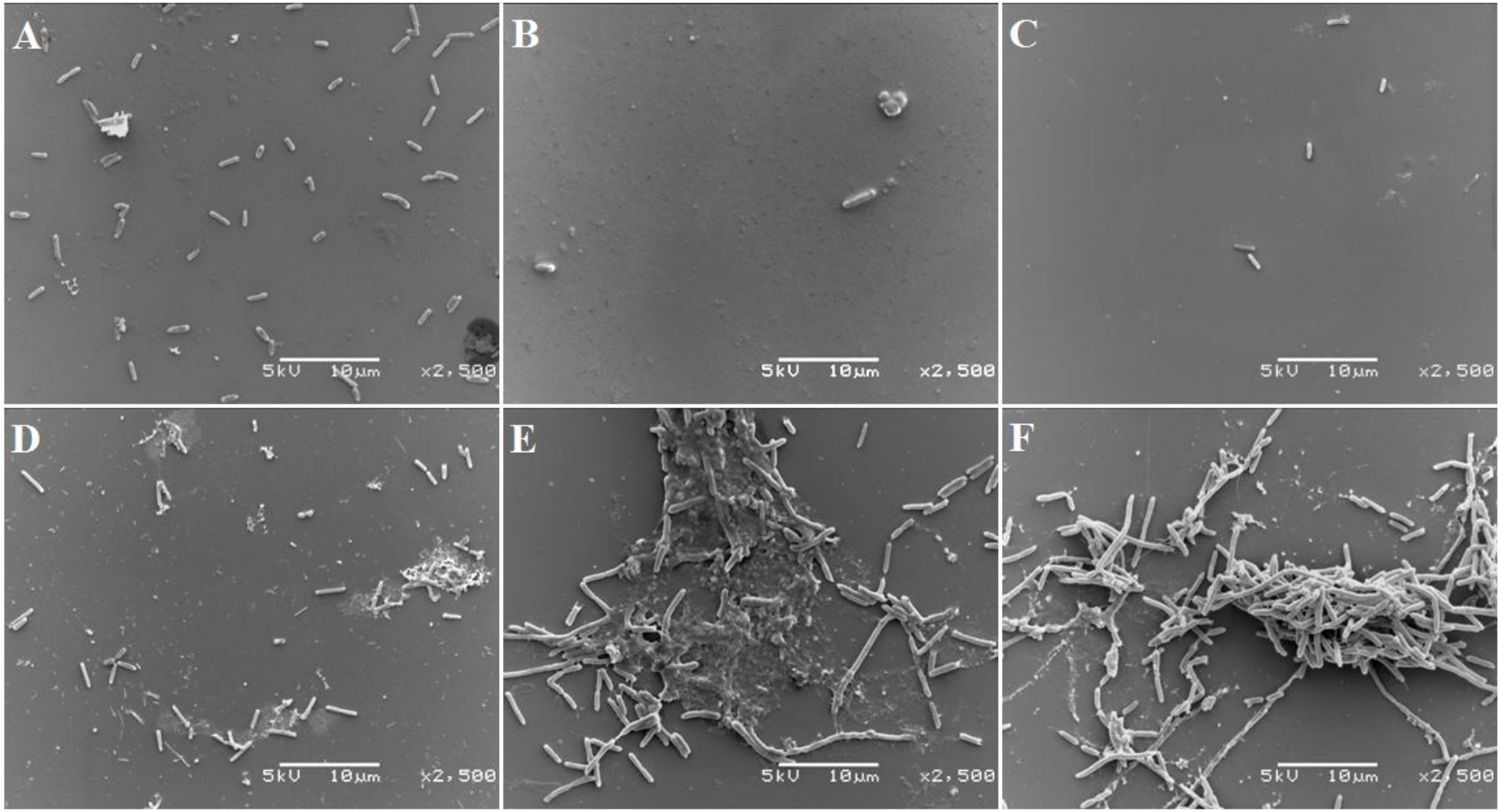

Figure 67: SEM images (x2500 magnification) of *P. aeruginosa* ATCC biofilms following exposure of fresh cultures to challenge agents for 24 h [A], untreated control; [B], ciprofloxacin 8 µg/ml; [C], Pectinex 950 PGU/ml; [D], ciprofloxacin 0.125 µg/ml (MIC); [E], Pectinex 118.75 PGU/ml; [F], ciprofloxacin 0.125 µg/ml plus Pectinex 118.75 PGU/ml.

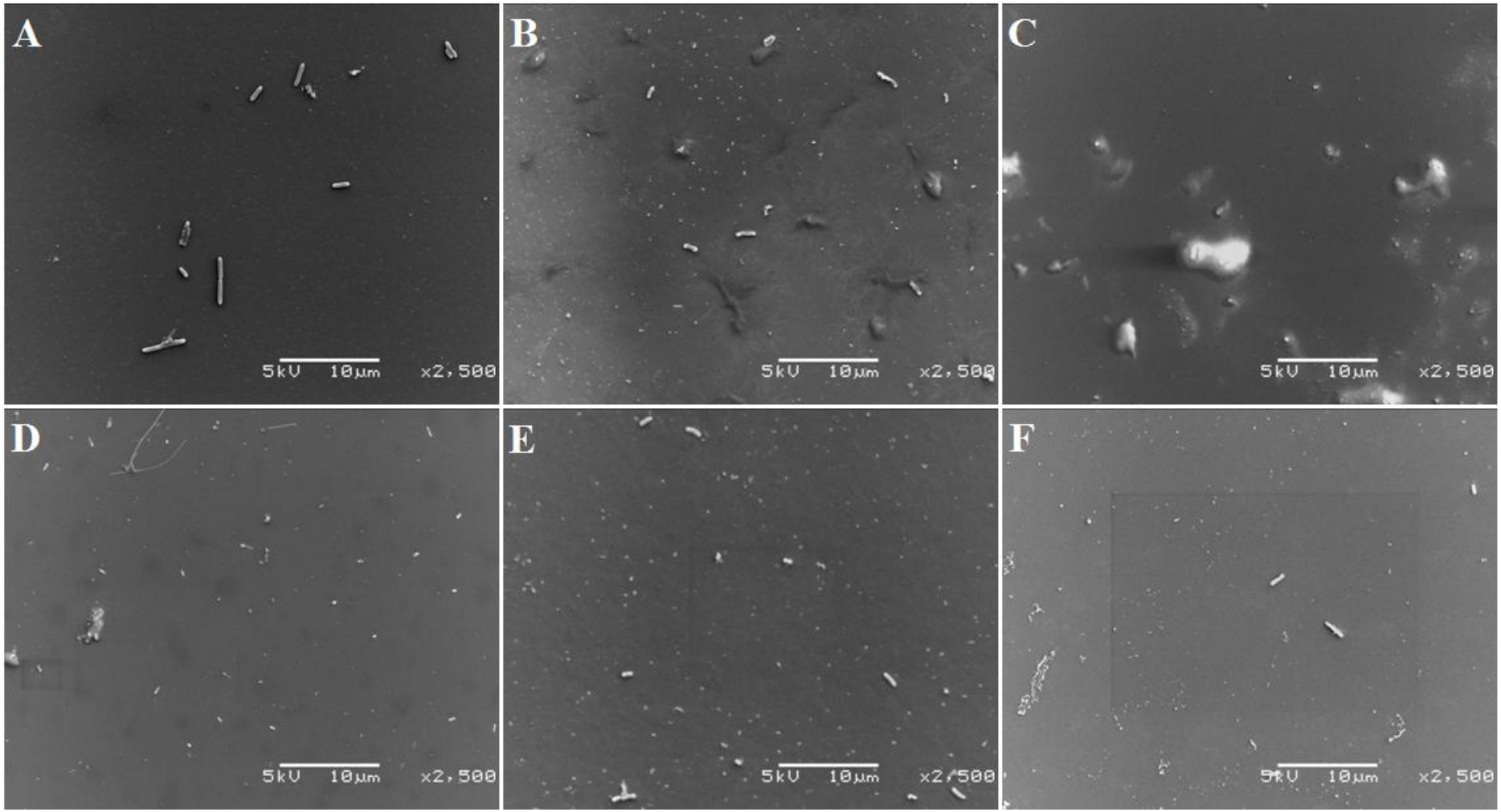

Figure 68: SEM images (x2500 magnification) of *P. aeruginosa* ATCC biofilms following exposure of 24-h old biofilms to challenge agents for 24 h. [A], untreated control; [B], ciprofloxacin 8 µg/ml; [C], Pectinex 950 PGU/ml, [D], ciprofloxacin 0.125 µg/ml (MIC); [E], Pectinex 118.75 PGU/ml; [F], ciprofloxacin 0.125 µg/ml plus Pectinex 118.75 PGU/ml.

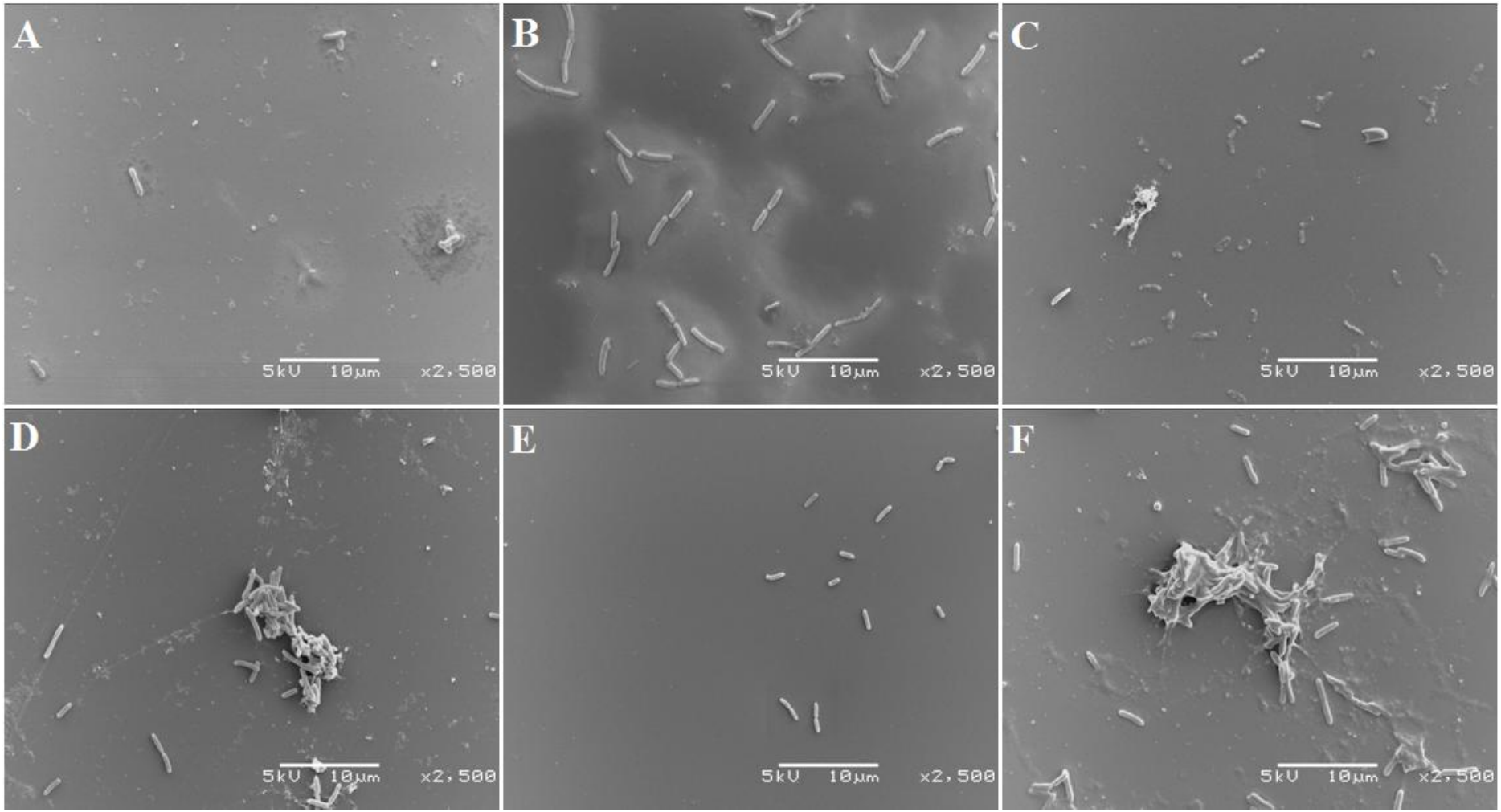

Figure 69: SEM images (x2500 magnification) of *P. aeruginosa* clinical strain biofilms following exposure of fresh cultures to challenge agents for 24 h [A], untreated control; [B], ciprofloxacin 8 µg/ml; [C], Pectinex 950 PGU/ml; [D], ciprofloxacin 1.0 µg/ml (MIC); [E], Pectinex 118.75 PGU/ml; [F], ciprofloxacin 1.0 µg/ml plus Pectinex 118.75 PGU/ml.

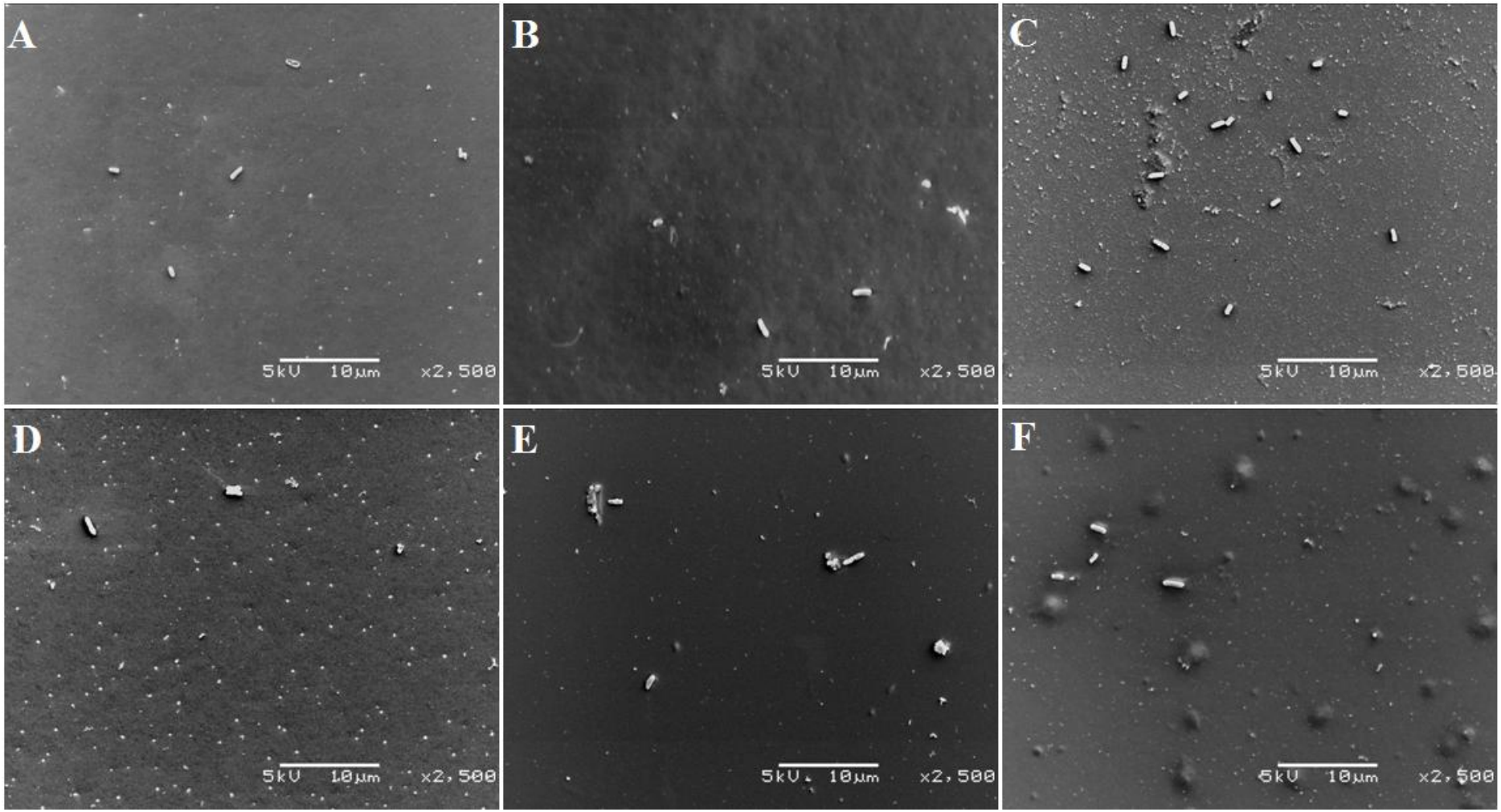

Figure 70: SEM images (x2500 magnification) of *P. aeruginosa* clinical strain biofilms following exposure of 24-h old biofilms to challenge agents for 24 h. [A], untreated control; [B], ciprofloxacin 8 µg/ml; [C], Pectinex 950 PGU/ml, [D], ciprofloxacin 1.0 µg/ml (MIC); [E], Pectinex 118.75 PGU/ml; [F], ciprofloxacin 1.0 µg/ml plus Pectinex 118.75 PGU/ml.

CHAPTER 5

DISCUSSION

Antibiotic drugs, that were once regarded as "*miracles*" of modern medicine, are well on the way to becoming obsolete[1,5]. The stark reality of this situation has prompted some decision-making authorities to regard the research and development of new antibacterial drugs as being an issue of high priority[4]. Although much of the current microbiological research is still centred on planktonic bacteria in pure cultures, scientists now recognize that bacteria naturally occur as multispecies biofilms[16,22]. In this regard, cognisance has been taken of the fact that biofilms are responsible for the majority of bacterial infections[23,222]. As a result, scientists are increasingly engaged in research into the development of antimicrobial drugs that also have the ability to interfere with biofilm production and/or to disrupt established biofilms[34,278,295,303]. A relatively new area of research involves the use of biofilm-degrading enzymes as antimicrobial and antibiofilm agents. In support of this concept, several *in vitro* studies have identified biofilm-degrading enzymes that have the ability to remove bacterial biofilms, potentiate the effects of co-administered antibiotics and exert antibacterial actions of their own[303].

Pectinex Ultra SP-L (Pectinex) was selected as the investigational drug for this study on strength of evidence from previous research by Johansen *et al.*[10] and Chaignon *et al.*[17]. In the study by Johansen *et al.*[10], using fluorescence microscopy and DAPI (4',6-diamidino-2-phenylindole) staining to determine the total bacterial cell counts, the researchers found that Pectinex effectively removed biofilms of *S. aureus*, *S. epidermidis*, *P. fluorescens* and *P. aeruginosa* from a stainless steel substratum. With further staining of residual adherent cells of *P. aeruginosa* biofilms, using CTC (5-cyano-2,3-ditolyl tetrazolium chloride) to determine viable cell counts, the authors demonstrated that the cells remained viable. The removal of complex heterogeneous biofilms as a possible use for Pectinex was suggested. Chaignon *et al.*[17], assessed the effect of Pectinex on clinical isolates of three staphylococcal species and found the enzyme to be highly effective against *S. aureus* biofilms but comparatively less effective against *S. epidermidis* and *S. epidermidis*. The variation in the response was attributed to differences in the chemical composition between the isolates. However, the authors declined to recommend the use of Pectinex for the removal of biofilms in clinical infections. Together these studies demonstrated that Pectinex was a biofilm matrix-degrading

enzyme, with protease and carbohydrase activity, that lacked bactericidal and bacteriostatic activity but had the ability to inhibit bacterial biofilm formation and effectively remove pre-established biofilms in selected bacterial species[10,17]. On the other hand, a study by Leroy *et al.*[290], demonstrated that Pectinex increased biofilm formation in marine bacteria. Another study by Augustin *et al.*[333] examined the disinfectant and biofilm removing properties of Pectinex (along with 14 other agents) and considered it to be ineffective in comparison to other enzymes. However, they did not provide substantive detail on its actions. Further review of the literature revealed that the bulk of the existing studies were focused on the use of Pectinex as a macerating agent in the food processing industry and there were no other studies that critically examined the role Pectinex as an antibacterial agent[298].

A search of the existing literature revealed that there were no peer-reviewed studies that had evaluated the cytotoxic potential of Pectinex. In addition, the nature and possible benefit of the interactions between Pectinex and conventional antimicrobial agents was not also examined. Furthermore, none of the previous studies examined the effectiveness of Pectinex in treating biofilms without prior removal of growth media. Therefore, this study set out with the aim of investigating the effectiveness and safety of Pectinex, when used as a single agent, and also when used in conjunction with amoxicillin-clavulanate and ciprofloxacin, respectively, as a regimen for the treatment of *S. aureus* and *P. aeruginosa* infections. The argument in favour of the choice of test agents, Pectinex, amoxicillin-clavulanate and ciprofloxacin, was based on the knowledge that each agent contributes a unique mechanism of antibacterial action and that, in selected combinations, their actions were likely to complement each other. Pectinex acts by degrading the matrix of biofilm and large cell clusters, thereby promoting cell dispersal and the bulk removal of bacteria[10,17]. Amoxicillin-clavulanate and ciprofloxacin are effective bactericidal drugs against actively multiplying *S. aureus* and *P. aeruginosa*, respectively[307,308]. Amoxicillin, a *β*-lactam antibiotic, kills *S. aureus* by inhibiting bacterial transpeptidase enzyme. This prevents the formation of peptidoglycan cross-links, that are essential to bacterial cell wall integrity, and leads to cell lysis. Clavulanic acid has virtually no antimicrobial activity of its own; however, it acts by inhibiting bacterial *β*-lactamase enzyme thereby preventing the deactivation of amoxicillin, and other *β*-lactam antibiotics, prolonging their action and enabling them to kill bacteria[164,334]. Ciprofloxacin, a fluoroquinolone antibiotic, acts by preventing cell division in *P. aeruginosa* by promoting the breakage of double-stranded bacterial DNA, and by inhibiting the enzymes, DNA gyrase and topoisomerase IV, which are required for the synthesis of DNA[335]. On the

basis of supporting evidence derived from prior studies, the dispersal of cells was thought to promote an increase in bacterial susceptibility to co-administered antibiotics[8,102,195].

In this study, during the *in vitro* challenge with the test agents, bacterial cultures were incubated under favourable conditions of nutrition, temperature and pH, so as to simulate an *in vivo* physical environment. Firstly, the MIC and MBC for each of Pectinex, amoxicillin-clavulanate and ciprofloxacin, were determined using *p*-INT (p-iodo-nitrotetrazolium violet) staining of bacterial cultures and regrowth of subcultures, respectively. Thereafter, the antibacterial efficacy of Pectinex-antibiotic combinations was assessed. This was followed by assessing the ability of the test agents to remove and disinfect biofilm, from the early stages of biofilm development through to late stages, using CV and MTT colorimetric assays, respectively [Sections 3.2.7 (d) and (e)]. The CV assay provided a quantitative estimate of the total biofilm biomass, whilst the MTT assay provided an estimate of the number of viable bacteria. The results of these two assays are discussed together. To evaluate the safety of Pectinex, its cytotoxicity on human cell lines (HeLa cells, lymphocytes and neutrophils) was evaluated *in vitro* using the MTT assay and PlasDIC (polarization-optical transmitted light differential interference contrast) microscopy. Finally, the antibiofilm effects of the highest non-toxic concentration of Pectinex, in combination with the MIC of amoxicillin-clavulanate and ciprofloxacin, were evaluated by SEM in *S. aureus* and *P. aeruginosa* biofilms, respectively.

In contrast to this approach, a number of *in vitro* studies, that have evaluated the use of enzymes as industrial disinfectants and cleaning agents, have employed methods in which nutrient media was removed and incubation conditions rendered unsuitable for bacterial growth prior to the administration of enzyme treatments[10–12,17,118]. These studies present a logical argument in favour of the removal of nutrients prior to treatment on the basis of evidence which strongly suggests that nutrients may inhibit the actions of some enzymes and compromise the industrial cleaning processes[333]. In this regard, the results obtained from the antibacterial/antibiofilm experimental model used in this study are more likely to bear a closer resemblance to the events that would occur during the treatment of a clinical infection. However, the findings of this study do not take into consideration the added antibacterial contribution that would be derived from the humoral and cell mediated immunity mechanisms *in vivo*.

5.1 Antibacterial effects of Pectinex, amoxicillin-clavulanate and ciprofloxacin

The results of this study showed that the MIC/MBC of amoxicillin-clavulanate against standard and clinical strains of *S. aureus* was 0.42/0.50 and 2.0/2.0 µg/ml, respectively, and against standard and clinical strains of *P. aeruginosa* the values for ciprofloxacin were found to be 0.13/0.33 and 1.0/1.67 µg/ml, respectively (Table 9). The MIC results were within acceptable quality control ranges for the respective antibiotics against *S. aureus* (amoxicillin-clavulanate, 1.0 – 16.0 µg/ml) and *P. aeruginosa* (ciprofloxacin, 0.25 – 1.0 µg/ml)[307,308]. Pectinex (7.42 – 950 PGU/ml) did not inhibit the growth of any of the tested bacterial strains. This finding is in agreement with the findings of Johansen *et al.*[10] who noted that Pectinex was not bactericidal at concentrations of between 0.18 and 1800 PGU/ml. When the antimicrobial susceptibilities towards singly administered antibiotics were compared with those of the Pectinex-antibiotic combinations, there was a 28.0% increase (from 2.0 to 2.56 µg/ml) in both the MIC and MBC of amoxicillin-clavulanate against *S. aureus* clinical strain, and in cultures of *P. aeruginosa* clinical strain, there was an 89.0% increase (from 1.0 to 1.89 µg/ml) in the MIC and a 92.8% increase (from 1.67 to 3.22 µg/ml) in the MBC of ciprofloxacin (Table 9). Pectinex (7.42 – 950 PGU/ml) had no bactericidal effects and no MIC could be obtained for Pectinex. Therefore, the nature of the interaction between Pectinex and either one of the antibiotics could not be determined using conventional *in vitro* methods for detecting synergy; checkerboard, time-kill, and E test[336]. In standard ATCC cultures of both *S. aureus* and *P. aeruginosa*, the addition of Pectinex did not change the susceptibilities to either amoxicillin-clavulanate (MIC/MBC = 0.42/0.56 µg/ml) or ciprofloxacin (MIC/MBC = 0.12/0.32 µg/ml), respectively.

A number of *in vitro* studies have demonstrated an increase in antibiotic susceptibility associated with various different antibiotic and enzyme combinations[13,15,300]. However, conflicting results have also been found in studies in which the same combination of enzyme, antibiotic and bacterial species was investigated. One such study, by Alkawash *et al.*[13], found that alginate lyase increased the susceptibility of *P. aeruginosa* to gentamicin and alginate lyase in *P. aeruginosa* cultures. However, another study, by Diaz *et al.*[337], showed that the susceptibility of *P. aeruginosa* to gentamicin decreased despite treatment with alginate lyase, and found no difference between the treated cultures and untreated controls. The anticipated outcome of this study was that Pectinex-antibiotic combinations would complement each other in a similar manner such that both amoxicillin-clavulanate and ciprofloxacin were able to kill bacteria more effectively. However, contrary to this expectation, combinations reduced

the susceptibility of all clinical strains (*S. aureus* and *P. aeruginosa*) and had no effect on standard cultures; a trend similar to that which was previously reported by Diaz *et al.*[337]. A possible explanation for this observation may lie in the fact that Pectinex is a multi-component enzyme of fungal (*Aspergillus aculeatus*) origin[10]. In this regard, the actions of Pectinex should be viewed against the background knowledge that fungi are known to produce a wide variety of biologically active compounds (e.g. extracellular enzymes) that can be beneficial, neutral or harmful to the growth of bacteria[338]. The findings of this study suggest that Pectinex promoted antibiotic resistance in clinical strains of both *S. aureus* and *P. aeruginosa*. From the evidence of previous studies, possible resistance mechanisms with which the bacteria may have responded include; the expression of resistance genes, the stimulation of metabolism and growth by fungal growth factors, and the utilization of components of Pectinex as a source of nutrients[338,339]. In addition, it is possible that the beneficial actions of Pectinex may have been compromised, or the resilience of the bacteria enhanced, in the presence of nutrient-rich growth media (Mueller Hinton broth)[333].

5.2 Effect of Pectinex, amoxicillin-clavulanate and ciprofloxacin on formation, growth, removal and disinfection of bacterial biofilms

a) Prevention of early formation (bacterial adhesion) and disinfection of biofilm

The results of this study indicated that the effects of the test agents on early (0 – 6 h) biofilm formation varied between the bacterial species and also between the different strains of the same species. Pectinex was found to be more effective at preventing biofilm formation in both the standard (Figure 23A) and clinical (Figure 25A) strains of *P. aeruginosa* than in either strain of *S. aureus* (Figures 19A and 21A). Amongst all the tested bacterial strains, *P. aeruginosa* ATCC was the most susceptible to Pectinex and *S. aureus* ATCC, was the least susceptible. Low concentrations of Pectinex were found to be associated with increased biofilm adhesion of both the ATCC and clinical strains of *S. aureus* to a greater extent than in untreated controls. At higher concentrations, Pectinex (475 and 950 PGU/ml) was not effective at preventing biofilm formation and the findings were comparable to untreated controls. In contrast, Pectinex effectively inhibited biofilm attachment in cultures of *P. aeruginosa* ATCC and clinical strains at concentrations of 237.5 – 950 PGU/ml and 950 PGU/ml, respectively. Pectinex concentrations of ≤ 118.75 PGU/ml were associated with bacterial adhesion that was comparable to the untreated controls in cultures of both

pseudomonal strains, with the exception of 7.42 PGU/ml which was associated with a significant increase in biofilm formation by the clinical strain. In general, Pectinex was found to have a graduated dose-dependent effect on biofilm formation in all bacterial strains. There was a consistent trend in which low concentrations of Pectinex promoted bacterial adhesion, and high concentrations either inhibited adhesion or promoted it to a lesser degree than at low concentrations. Following exposure to Pectinex, quantitative estimates of viable biofilm bacteria and the biofilm biomass appeared to follow a similar trend. This observation is in keeping with the knowledge that Pectinex is not bactericidal[10]. Therefore, the quantity of adherent biofilm was also representative of the quantity of viable bacteria.

This study found that Pectinex exhibited a dose-response that ranged from significant reduction in biofilm viability at high concentrations to enhanced viability at low concentrations. *P. aeruginosa* were found to be more susceptible to Pectinex than biofilm of either strain of *S. aureus*. Cell viability in ATCC and clinical biofilms of *P. aeruginosa* was significantly reduced by Pectinex (237.5 – 950 PGU/ml) and lower concentrations had no significant effect. The viability in *S. aureus* biofilms was significantly reduced only at the highest concentrations of Pectinex; 950 PGU/ml and 475 – 950 PGU/ml in ATCC and clinical strains, respectively. However, in contrast to *P. aeruginosa*, low concentrations of Pectinex caused significant enhancement of cell viability in *S. aureus* biofilms. The findings are also in keeping with existing knowledge which recognizes the varied responses, including enhanced growth, which bacteria may have towards fungal extracts (e.g. Pectinex)[338,339]. The observed variations in the response to Pectinex by the different bacteria species and strains may be explained by differences that may exist in the nature of their chemical constituents coupled with the substrate specificity of the enzyme[17].

This study showed that concentrations of antibiotics at and above the MIC effectively prevented biofilm formation in all bacterial strains. Amoxicillin-clavulanate 0.5 – 4.0 µg/ml and 4.0 – 16.0 µg/ml, respectively, inhibited biofilm formation in standard and clinical cultures of *S. aureus*. Ciprofloxacin, 0.25 – 4.0 µg/ml and 1.0 – 4.0 µg/ml, effectively inhibited biofilm formation in cultures of *P. aeruginosa* ATCC and clinical strains, respectively. At the time of inoculation the bulk of the bacterial population were considered to be in the planktonic state and were rapidly killed by the antibiotic. However, it is known that within bacterial suspension there are some cells that are naturally resistant and survive the lethal effects of the antibiotics[177]. In addition, pre-existing clusters of bacteria, that have acquired the characteristics of sessile biofilm bacteria, are known to be present in bacterial

suspensions[103]. Furthermore, during the time of incubation the bacteria were in the logarithmic growth phase, and possessed the ability to rapidly re-populate and increase the numbers of surviving resistant bacteria. Therefore it was not unexpected to find that even the highest antibiotic concentrations used in this study did not prevent cell attachment by 100%. Antibiotic concentrations below their respective MIC's had no statistically significant effect on biofilm formation. In cultures of *S. aureus* clinical strains and *P. aeruginosa* ATCC, the lowest antibiotic concentrations with significant inhibition were equivalent to the MIC x 2. When the effects of antibiotics on cell viability were determined, the results of this study showed that ciprofloxacin 0.25 – 4.0 µg/ml and 1.0 – 4.0 µg/ml, respectively, reduced cell viability in standard and clinical biofilms of *P. aeruginosa*. This finding corresponded to a significant reduction in biofilm biomass that occurred at the same concentrations of ciprofloxacin. In contrast to this, amoxicillin-clavulanate had no significant effect on cell viability in *S. aureus* biofilms. Both of these findings are supported by previous research that has shown biofilm bacteria to be more resistant to the effects of antibiotics[176].

Pectinex was found not to enhance the actions of either amoxicillin-clavulanate or ciprofloxacin. In *P. aeruginosa* cultures, the effect of combinations mirrored the effects of singly administered ciprofloxacin, except at low concentrations of Pectinex (7.42 and 14.84 PGU/ml) where the effect of increased biofilm growth at these concentrations negated the inhibitory actions of otherwise effective concentrations of ciprofloxacin in cultures of standard (0.25 – 4.0 µg/ml) and clinical (1.0 – 4.0 µg/ml) strains. After treatment with Pectinex-antibiotic combinations, there were differences in cell viability considering bacterial species and the antimicrobial agents. The reduction in cell viability in *S. aureus* biofilm was directly related to the concentration of Pectinex whereas cell viability in *P. aeruginosa* biofilm was directly related to the concentration of ciprofloxacin.

b) Prevention of formation (bacterial adhesion and maturation) and disinfection of biofilm

Bacterial cultures were incubated for 24 h from the outset with Pectinex, antibiotics and combinations. These experiments were used to evaluate the development of biofilm by bacteria that manage to survive the early (6 h) stage of growth and determine the efficacy of the antimicrobial agents over 24 h. According to published literature, bacteria multiply rapidly (logarithmic growth phase) during the first 12 h of incubation and corresponding biofilm growth occurs. Beyond 12 h, growth and development are centred around biofilm

maturation[340]. This study found that bacterial cultures were more resistant to the effects of the antimicrobial agents in 24 h cultures as compared to 6 h cultures. The patterns of biofilm growth exhibited by the different bacterial species were similar. Pectinex (7.42 – 950 PGU/ml) promoted biofilm growth and increased cell viability in all bacterial cultures. The inhibitory effects of Pectinex, which were observed in 6 h *P. aeruginosa* cultures, were not observed in 24 h cultures. As compared to the results from the 6 h incubation period, antibiotics achieved significantly reduced biofilm biomass at comparatively higher concentrations in *S. aureus*, but not *P. aeruginosa*, cultures. Amoxicillin-clavulanate 2.0 – 4.0 µg/ml and 16.0 µg/ml significantly reduced biofilm biomass in standard and clinical cultures of *S. aureus*, respectively. Amoxicillin-clavulanate failed to reduce cell viability in *S. aureus* biofilm. In standard cultures of *S. aureus*, cell viability was significantly increased at all concentrations of amoxicillin-clavulanate (0.03 – 4.0 µg/ml). In comparison, the viability in *S. aureus* clinical cultures was not significantly affected by amoxicillin-clavulanate (0.125 – 16.0 µg/ml).

These findings may be explained by the fact that naturally resistant bacteria survive the initial antibiotic attack and go on to multiply and repopulate the colonies (planktonic and biofilm) with a selection of antibiotic resistant bacteria[169]. Combinations of Pectinex and amoxicillin-clavulanate were not effective. Pectinex reduced the effectiveness of the antibiotic against *S. aureus* except in clinical cultures where effects of amoxicillin-clavulanate 16.0 µg/ml remained prominent. Ciprofloxacin was effective in reducing biofilm biomass at 0.25 – 4.0 µg/ml in standard cultures of *P. aeruginosa*, and at 0.5 and 4.0 µg/ml in the clinical cultures. Cell viability of both strains of *P. aeruginosa* biofilm was significantly reduced by ciprofloxacin (0.25 – 4.0 µg/ml). Since Pectinex was associated with increased viability, it is most likely that the observed reduction in cell viability, after treatment with Pectinex-ciprofloxacin combinations, was due to the bactericidal actions of ciprofloxacin. When the agents were combined, Pectinex negated the inhibitory effect of ciprofloxacin in *P. aeruginosa* cultures. Significant increases in biofilm growth were observed with high concentrations of Pectinex (950 PGU/ml) in combination with low concentrations of ciprofloxacin (0.03 and 0.06 µg/ml). Combination therapy was associated with an increase in the viability of *S. aureus*, particularly when low concentrations were used. In *P. aeruginosa* biofilm (standard and clinical), combination therapy was not effective which suggested the inhibitory effects of ciprofloxacin (0.25 – 4.0 µg/ml) were neutralized by the growth stimulating effects of Pectinex.

c) Inhibition of growth in preformed 6 h-old biofilms and disinfection of adherent biomass

Bacteria were allowed to grow for 6 h to establish biofilms before being treated with antimicrobial agents. At the start of the antimicrobial challenge, two prominent growth states were presumed to exist simultaneously. On the one hand, planktonic bacteria that were rapidly multiplying in suspension, and on the other hand, sessile (biofilm) bacteria that were attached to the walls of the microtitre plates. This study found that all concentrations of Pectinex were associated with an increase in biofilm biomass and cell viability in all bacterial cultures except for the *S. aureus* clinical strain. Surprisingly, incubation with Pectinex (7.42 – 237.5 PGU/ml) caused significant inhibition of growth in clinical cultures of *S. aureus* and an unexpected reduction in cell viability at 29.69 and 59.38 PGU/ml. Amoxicillin-clavulanate, at concentrations of 0.25 – 4.0 µg/ml and 8.0 – 16.0 µg/ml, significantly reduced biofilm biomass in cultures of standard and clinical strains of *S. aureus*, respectively. The lowest effective concentrations were less than those observed when the bacteria were cultured with amoxicillin-clavulanate for 24 h without prior incubation. However, cell viability was significantly increased in standard cultures of *S. aureus* and unaffected in clinical cultures. One possible explanation for this difference is that, with cultures that were continuously challenged for 24 h, biofilm and bacterial suspension were populated with resistant bacteria from the outset and were therefore more resistant. In contrast, prior 6 h incubation populated growing biofilm and planktonic suspension with unchallenged susceptible bacteria, thereby rendering the cultures vulnerable at a relatively advanced stage of growth and without sufficient time to recover. The response to ciprofloxacin in *P. aeruginosa* cultures presented unexpected observations. In cultures of *P. aeruginosa* ATCC there was mixed inhibition and enhancement that appeared to be randomly distributed; however, the only concentration that exhibited significant ($p \leq 0.05$) inhibition was 0.06 µg/ml (Figure 39B). In clinical cultures of *P. aeruginosa*, the reduction of biofilm growth was observed at all concentrations of ciprofloxacin with the greatest reduction occurring at the concentration range of 0.06 – 1.0 µg/ml. The effects at higher concentrations of ciprofloxacin (2.0 and 4.0 µg/ml) were not found to be statistically significant. This occurrence of a paradoxically lower antibacterial response at higher concentrations (> MIC) of ciprofloxacin has previously been described ("Eagle effect")[341]. Clinical cultures of *S. aureus* appeared to be susceptible to the Pectinex-amoxicillin combinations; however, this was largely due to a lack of enhanced biofilm growth by Pectinex and, therefore, there was no inhibition of the effects of amoxicillin-clavulanate (Appendix E10). With the other three bacterial strains, combinations of Pectinex with either

of the antibiotics did not inhibit biofilm growth; however, significantly enhanced growth occurred at combinations with low concentrations of both agents. Cell viability observed after treatment with combinations of Pectinex (7.42 – 950 PGU/ml) and ciprofloxacin (0.03 – 4.0 µg/ml) was not different from singly administered ciprofloxacin. This result indicated that the main antibacterial action in *P. aeruginosa* biofilm was due to ciprofloxacin.

d) Inhibition of growth and removal of established 24 h-old biofilms and disinfection of adherent biomass

Biofilms were grown for 24 h without exposure to antimicrobial agents and, according to a study by Sun *et al.*[340], the biofilms were mature and the bacteria were in a steady state characterized by relatively slow growth rates. Thereafter, bacterial cultures were challenged for a further 24 h with Pectinex and antibiotics (amoxicillin-clavulanate and ciprofloxacin) in order to determine their ability to remove adherent biofilm. This study found that mature biofilms in all the bacterial cultures were more resistant to the effects of the test agents than those that were treated at earlier stages of biofilm development. The results showed that Pectinex (7.42 – 950 PGU/ml) failed to reduce biomass and was associated with increased cell viability in biofilms of all the bacterial strains. Similarly, amoxicillin-clavulanate, ciprofloxacin and Pectinex-antibiotic combinations were ineffective against the biofilms in their respective challenge tests. Amoxicillin-clavulanate, on its own and in combination with Pectinex, promoted biofilm growth and cell viability in *S. aureus* ATCC biofilms but had no significant effect in biofilms of the clinical strain. In contrast, *P. aeruginosa* biofilms were susceptible to the effects of ciprofloxacin and Pectinex-ciprofloxacin combinations; however the effect of combination were mainly due to the actions of ciprofloxacin.

5.3 Cytotoxicity of Pectinex on HeLa cells, lymphocytes and neutrophils

Pectinex is a GRAS-designated substance; its classification as a safe product is based upon its current use in the processing of food and beverages. The fact that Pectinex has no clinical applications, coupled with the historical safety record of fungal enzymes, may explain why there has been no prior interest in evaluating the cytotoxicity of Pectinex, and many other similar substances, on human tissues. Scientific evidence, which supported the use of Pectinex as a pharmacological biofilm control agent, has prompted the need for preclinical evaluation

of its cytotoxicity[10,17]. This study determined the cytotoxicity of Pectinex (7.42 – 950 PGU/ml) on HeLa cells, lymphocytes and neutrophils by MTT staining of viable cells using two simultaneous modified methods by Denizot and Lang[324] and by Hansen *et al*.[323] (i.e. with and without cell washing stages). HeLa cells divide indefinitely and grow in number, whereas circulating lymphocytes and neutrophils are end-stage cells which do not divide and have a short lifespan. Therefore, unlike HeLa cell cultures, *in vitro* cell numbers in lymphocytes and neutrophils cultures are subject to ongoing spontaneous apoptosis and subsequent necrosis. Different incubation periods were chosen for each cell type (HeLa cells, 7 days; lymphocytes, 3 days; neutrophils, 4 and 24 h) to provide adequate exposure time but with limited distortion of results that could arise from spontaneous cell death in lymphocytes and neutrophils[318,321,322]. The quantity of MTT formazan produced by viable cells was both a measure of the cell numbers and of the level of metabolic activity within the cells.

The results obtained by the two assay methods were comparable across all experiments, and each method served to validate the results of the other. This study showed that high concentrations of Pectinex (237.5 – 950 PGU/ml) significantly reduced the viability of cells in all three cell cultures, and were comparable to mitomycin C 10 µg/ml (positive control). The mean IC_{50} of Pectinex was found to be above 118.75 PGU/ml in all three cell cultures under their respective experimental conditions of exposure. Slightly lower mean IC_{50}'s were found in fMLP-stimulated lymphocytes and 24 h neutrophils as compared to non-stimulated cells under the same conditions of incubation (Table 11). However, the opposite was true in neutrophils after 4h incubation. A few studies have shown that fMLP stimulation may delay apoptosis within neutrophils and maintain mitochondrial function[342]. This was not found to be the case in this experiment. The mean IC_{50} ± S.E.M. concentrations of Pectinex in all three cell types were not significantly ($p > 0.05$) different from each other. This finding suggests that the cell types are equally susceptible to Pectinex and their unique metabolic characteristics (e.g. cell division and immune response) probably do not play an important part in the mechanism of Pectinex cytotoxicity.

5.4 PlasDIC microscopy of human cell cultures

PlasDIC microscopic examination of HeLa cells, lymphocytes and neutrophils after 24 h incubation with Pectinex (7.42 – 950 PGU/ml) provided good correlation with the corresponding results obtained from the MTT assay. All cell lines exhibited evidence of cell

death, characterized by features of both apoptosis and necrosis, when exposed to high concentrations of Pectinex (237.5 – 950 PGU/ml). These features have previously been described and include; shrunken rounded cells, cell membrane blebs, apoptotic bodies, cytoplasmic vacuoles and cell debris[330]. Lymphocytes and stimulated neutrophils were visibly affected by all concentrations of Pectinex; non-stimulated neutrophils were more resilient and were affected by concentrations ≥ 237.5 PGU/ml. However, clumping by fMLP-stimulated lymphocytes obscured the greater part of the morphological changes at all concentrations, and in the untreated controls, with the exception of 950 PGU/ml where extensive cell death was evident. The cytotoxic efficacy of Pectinex at 950 PGU/ml appeared to be the same as that of both positive controls; mitomycin C (10 µg/ml) and staurosporine (1 µg/ml). Pectinex, at concentrations of ≤ 118.75 PGU/ml, was non-toxic and the visual fields in all cell cultures were dominated by normal cells at these concentrations. This study found that apoptosis, as evidenced by shrunken rounded cells, cell membrane blebs and apoptotic bodies, was the predominant form of cell death at all Pectinex concentrations. It is likely that Pectinex acted on one or more of the basic structural components of the cell membrane (i.e. proteins and lipids) that are common to all cells, and triggered apoptosis as a stress response to cell membrane damage. Necrosis, as evidenced by cytoplasmic vacuolization and cell debris, was greater at the highest concentrations (475 and 950 PGU/ml).

A possible mechanism for non-selective Pectinex cytotoxicity may be due to enzymatic activity on the surface of the cells. The outermost layer of the mammalian cell membrane is covered by a carbohydrate coat (glycocalix) formed by the oligosaccharides of glycolipids and transmembrane glycoproteins[343]. These oligosaccharides have functions that are essential to the survival of the cells[344]. Enzymatic degradation of membrane oligosaccharides by Pectinex may disrupt the function and/or integrity of the cell membrane and lead to cell death. However, the mechanisms associated with Pectinex toxicity are unknown.

5.5 SEM of bacterial biofilms after exposure to non-toxic concentrations of Pectinex and antibiotics at MIC

Bacterial biofilms were examined by SEM after exposure to Pectinex, antibiotics (amoxicillin-clavulanate and ciprofloxacin) and their combinations. Pectinex, at a concentration of 118.75 PGU/ml, was chosen as the highest non-toxic dose on the basis of the results of the cytotoxicity MTT assays and PlasDIC microscopy. Antibiotics were used at

their respective MIC values (Table 9). Both Pectinex (950 PGU/ml) and antibiotics (amoxicillin-clavulanate 32 µg/ml and ciprofloxacin 8 µg/ml) were used as positive controls. No correlation was found between the subjective SEM appearance of biofilms and the quantitative results of both the CV and MTT biofilm assays. The SEM images of the untreated controls showed that, amongst the selected bacterial strains, *S. aureus* formed more robust biofilms than did *P. aeruginosa*. Treatment with Pectinex was shown to be associated with an increase in the number of adherent, morphologically normal bacteria, and in the complexity of the cell arrangements, in both *S. aureus* and *P. aeruginosa* cultures. However, the precise responses were found to vary between the species (and strains). *S. aureus* biofilms from mature cultures (24 – 48 h) were more resistant to the effect of the antimicrobial agents than the fresh (0 – 24 h) biofilms whereas the converse appeared to be true for *P. aeruginosa* biofilms.

5.6 Analysis of Pectinex antibacterial therapy

This study re-evaluated the effect of Pectinex on planktonic and biofilms cultures of standard and clinical strains of *S. aureus* and *P. aeruginosa*. It was found that the effect of Pectinex on bacterial biofilms varied between the different bacterial strains and also with the maturity of the biofilms. In this regard the findings were consistent with the study by Chaignon *et al.*[17], which found that differences occurred in the response to Pectinex between different strains of *S. aureus*, *S. epidermidis* and *S. lugdunensis*. In general, lower concentrations of Pectinex (≤ 118.75 PGU/ml) were found to be associated with increased biofilm formation and cell viability whereas higher concentrations were associated with either inhibition or no effect. The findings of the current study were consistent with those of Leroy *et al.*[290]. These researchers found that the activity of low concentrations of Pectinex, and several other enzymes (including α-amylase, and cellulase), significantly increased bacterial adhesion in 3 h-old cultures of *Pseudoalteromonas* species, whereas higher concentrations reduced adhesion. The findings of the current study do not support the previous research by Johansen *et al.*[10] and Chaignon *et al.*[17], which found Pectinex to be effective against biofilms of *P. aeruginosa* and *S. aureus*. Johansen *et al.*[10] investigated the use of Pectinex for its potential as an industrial cleaning agent and demonstrated that Pectinex concentrations as low as 0.18 – 1.8 PGU/ml effectively removed biofilms of *S. aureus. S. epidermidis, P. aeruginosa* and *P. fluorescens*. The methodology of their study, and also that of Chaignon *et al.*[17], differed from the current study in that the biofilms were removed from their nutrient source during exposure

to the enzyme. It is likely, therefore, that the findings were representative of the enzyme activity and the "hostile" nutrient-deprived conditions during exposure. In support of this notion, Augustin *et al.*[333] observed that the presence of milk diminished the effectiveness of several proteolytic enzymes against *P. aeruginosa* biofilms. The *in vitro* effects of Pectinex have been evaluated in a very limited number of bacterial species and the effects on other microorganisms of medical importance (e.g. *H. influenzae*, *E. coli* and *K. pneumoniae*) still needs to be investigated.

There are no prior studies that have evaluated the effect of Pectinex on bacterial cultures or biofilms when used in combination with antibiotic drugs. This study did not demonstrate any beneficial interaction between Pectinex and either amoxicillin-clavulanate or ciprofloxacin. More often than not, Pectinex reduced the effectiveness of antibiotics by promoting growth in bacterial biofilms. Longer incubation periods and delayed onset of treatment were associated with increased resistance to the effects of Pectinex, antibiotic and Pectinex-antibiotic combinations. This was probably due, in part, to Pectinex-promoted bacterial growth associated with multiplication of resistant phenotypes. However, a number of other studies using different enzymes (alginate lyase, serratiopeptidase, DNase, dispersin B and streptokinase) have demonstrated additive and synergistic interactions between the various enzymes and conventional antibiotics[303]. Some researchers have gone further to propose the use of enzyme-antibiotic combinations as a possible strategy for the management of biofilm-related infections. However, the use of enzymes has not become part of routine clinical practice. No other studies have investigated the cytotoxicity of Pectinex on human tissues. This study demonstrated that Pectinex exhibits dose-dependent cytotoxic effects. Concentrations $\geq$ 237.5 PGU/ml were found to be toxic to HeLa cells, lymphocytes and neutrophils to a similar extent as that observed with known cytotoxic drugs (mitomycin C and staurosporine). Although the precise mechanism of cytotoxicity was not investigated in this study, the PlasDIC microscopy findings (apoptotic bodies and cell debris) and the comparable IC_{50} values found in the different cell types, suggest that the loss of cell membrane integrity through enzymatic activity may either trigger or modulate apoptosis.

CHAPTER 6

CONCLUSION

The prevalence of bacterial drug resistance has been on the increase and has now become a cause of global concern[1]. Unfortunately, as the problem of drug resistance continues to evolve, the pace of development and subsequent release of new antibiotics has been relatively slow[3]. Scientists have responded to this challenge by advancing their research into bacterial drug resistance mechanisms, re-evaluating the efficacy of existing drugs, and investigating a variety of new antibiotic compounds. Although the focus of much research is still directed towards the eradication or control of free-floating planktonic bacteria, it is now clearly understood that the vast majority of bacteria naturally exist as biofilms; dynamic, integrated, heterogeneous (spatial, genetic and physiologic) communities, encased in a complex exopolymeric matrix, that are either surface-bound or free-floating in cohesive cell-to-cell clusters[7,102]. Published research has shown that biofilms are highly resistant to conventional antibiotics, and are a cause of persistent infections and contaminations that are difficult to eradicate[23,222]. This thesis reviewed the scientific literature pertaining to the microbiology and molecular biology of bacterial biofilms, mechanisms of antimicrobial resistance in both planktonic and biofilm bacteria, and the current and emergent biofilm control strategies. Amongst the different antibiofilm agents that have been investigated, biofilm matrix-degrading enzymes appear to hold great promise for the future management of biofilms[10–12]. In a number of studies, upon which the assumptions of this thesis were formulated, enzymes have been shown to prevent the formation and facilitate the removal of biofilm, and to potentiate the action of co-administered antibiotics[303].

Previous studies have demonstrated that Pectinex (Pectinex Ultra SP-L), a multi-substrate commercial enzyme complex, has the ability to degrade the exopolymeric matrix of two major human pathogens; namely, *S. aureus* and *P. aeruginosa*[10,17]. This thesis argued that Pectinex, either alone or in combination with an antibiotic, could be useful as a therapeutic agent in the management of clinical infections. Therefore, the aim of this study was to investigate the use of Pectinex, administered either singly or as an adjunct to antibiotics, as a potential safe treatment option in the management of biofilm-related infections. The objectives of this study were designed to evaluate the antibiofilm effects of the, Pectinex, with respect to the formation, growth, removal and disinfection of the biofilms of standard and clinical strains of *S. aureus*

and *P. aeruginosa*. In addition, the antibiofilm effects of the conventional antibiotics, amoxicillin-clavulanate and ciprofloxacin, were determined in *S. aureus* and *P. aeruginosa* biofilms, respectively. Thereafter, the effects of co-administered Pectinex plus antibiotics were investigated, and the findings were interpreted mainly in relation to the effect of Pectinex on the actions of the antibiotics. Finally, to fill a void in current scientific literature, and in preparation for possible future experiments *in vivo*, this study investigated the *in vitro* cytotoxicity profile of Pectinex on human epithelial cancer cells (HeLa cells), lymphocytes and neutrophils.

This study has shown that Pectinex either enhanced or inhibited the extent of biofilm formation and cell viability depending on the concentration of Pectinex, the maturity of the biofilms (i.e. duration of incubation), and the bacterial species and strain. In all bacterial cultures, enhanced bacterial attachment and increased cell viability were generally found at Pectinex concentrations of $\leq$ 118.75 PGU/ml. Increased incubation times of $\geq$ 6 h were associated with increased biofilm formation and cell viability irrespective of the concentration of Pectinex in all cultures. This time-dependent enhancement of biofilm growth was found to progress to such an extent that all biofilms not only persisted, but appeared to thrive in the presence of Pectinex (7.42 – 950 PGU/ml), after 24 h incubation. In 6 h cultures, *P. aeruginosa* strains were susceptible to inhibition by Pectinex at concentrations between 237.5 – 950 PGU/ml with the ATCC strain being comparatively more susceptible. Growth in *S. aureus* ATCC cultures, and in the clinical strain to a lesser extent, was promoted by Pectinex and inhibition was not observed. The second major finding was that Pectinex attenuated the antibacterial and antibiofilm actions of amoxicillin-clavulanate and ciprofloxacin in cultures of *S. aureus* and *P. aeruginosa*, respectively. The individual bacterial strains varied in their responses to the enzyme-antibiotic combinations. The MIC and MBC of the respective antibiotics remained unchanged by the addition of Pectinex to standard cultures of both *S. aureus* and *P. aeruginosa*. However, in clinical cultures of both bacterial species significant increases in both of the bactericidal indices were demonstrated.

Taken together these results indicate that Pectinex was most effective against bacterial biofilms following early administration in cultures of selected species and strains. In a clinical scenario, this would translate into the appropriate use of Pectinex as a prophylactic agent at/close to the time of bacterial inoculation. However, contrary to the evidence from previous studies, this current study did not find Pectinex to be suitable for the management of mature biofilms of *S. aureus* and *P. aeruginosa* under the prevailing conditions of the experiment. There was a difference in the methodologies used in this study and those in which Pectinex

was reported to be a highly effective antibiofilm agent[10,17,290]. In this study, conditions to support bacterial growth (i.e. nutrient media and temperature) were maintained during the time of exposure to Pectinex whereas in the other studies these conditions were removed. The purpose of this approach was to approximate *in vivo* environmental conditions during the growth and challenge of bacterial cultures. The results from this study suggested that a logical explanation for the ineffectiveness of Pectinex was that, by maintaining suitable conditions for growth, the bacteria were better equipped to adapt and grow in the presence of the enzyme (and enzyme-antibiotic combinations) using mechanisms that are not yet clearly defined. Therefore, although this study did not confirm Pectinex as a suitable candidate for immediate evaluation as an *in vivo* therapeutic agent, it does not discount the potential role of Pectinex as an *in vitro* antibiofilm agent. Notwithstanding, the significance of the finding of this study has gone some way towards enhancing an understanding of the growth of bacterial cultures in the presence of matrix-degrading enzymes under different experimental conditions and, hopefully, may serve as a basis upon which to design future experiments and to re-evaluate some of the previous studies.

Another significant finding that emerged from this study was that Pectinex was found to be cytotoxic to human epithelial cancer cells (HeLa), lymphocytes and neutrophils, at concentrations ≥ 237.5 PGU/ml. Apoptosis was recognized as the main mode of cell death in all three cell types; however, the underlying cytotoxic mechanisms were not investigated. Nevertheless, the observed loss of cell membrane integrity, coupled with past evidence suggestive of protease activity, supports the notion that apoptosis may have been due to enzymatic degradation of the cell plasma membrane. The current classification of Pectinex as a GRAS-designated substance is conditional upon its recommended use as a macerating agent in the food industry and, therefore, has no relation to its properties when used as a pharmacological agent. At present, there are no studies that have investigated the cytotoxicity of Pectinex on human tissues. Therefore, this study contributes to the existing body of literature by providing new information on the cytotoxicity of Pectinex.

6.1 Limitations and recommendations

There are a number of important limitations that should be considered. This study was structured and conducted within the limits of the available resources; namely funding, equipment and personnel. Strict adherence to standard laboratory practices and techniques

(microbiological and cytological) determined access to the laboratory, and the time and purpose for which available equipment was used. Standard laboratory operating procedures were given preference for all experiments. Alternative procedures were developed as dictated by the requirements of the study. In this study, bacterial cell viability in the MIC and MBC assays was determined visually using a *p*-INT assay. However, the use of *p*-INT was not carried over spectrophotometric measurements due to limitations of available wavelength filters. MTT spectrophotometric measurements were recorded on two different microplate readers located in separate sections of the laboratory. As a result, spectrophotometric readings were made at 560 nm for bacteria and 570 nm and human cells.

In this study, the choice of Pectinex was based on the assumption that it had the capacity to act at multiple substrate sites within biofilms and catalyze the degradation of their exopolymeric matrices. However, the nature and quantity of the enzymatic degradation products were not determined. In addition, the study did not consider the possibility that some of the functional moieties on the enzyme molecule may interact with the bacteria and stimulate biofilm growth as opposed to breaking down the biofilm matrix. Although, the study objectives addressed the question of the effect of Pectinex on bacterial cultures, no consideration was given to the effect of the bacteria on Pectinex, given that the possibility exists that the enzyme itself may be utilized by the bacteria. As is common to most *in vitro* studies, the findings of this study cannot be readily extrapolated to the *in vivo* setting. The possible outcomes of a complex interaction between the bacteria, Pectinex and the host immune responses (humoral and cellular) are difficult to predict.

Although the experimental methodologies that were used in the quantitative biofilm assays were scientifically acceptable, wide variations in the results of repeat experiments were obtained as is the norm rather than the exception. One of the sources of variation included natural fluctuations in the growth of bacterial populations (e.g. rate of growth and chemical composition of exopolymeric substance). In the experimental methodology, an inevitable variation is introduced at the stages in which biofilms are isolated by discarding bacterial suspensions and washing off loosely attached bacteria. Given both the vast diversity of bacterial growth patterns and the methodology related variations, the sample size could be regarded as small. However, it still remains unlikely that a significant multiple of repeat experiments would alter the overall outcome of the findings of this study.

The results of SEM evaluation of bacterial biofilms were vulnerable to distortion by the standard procedures used during sample preparation. Water accounts for up to 97% of the biofilm EPS matrix and, as a consequence of this, the dehydration and fixation stages used in SEM invariably caused shrinking of the matrix and may have also resulted in its destruction[129]. Technologies, such as confocal laser scanning microscopy, that could have avoided this shortcoming were not readily available for use during this study.

As an integral part of the search for new antibiotic drugs, the research into the use of biofilm disrupting enzymes is still relatively new. This current study has raised a number of issues that warrant attention:

1. No MIC value was attained for Pectinex (7.42 – 950 PGU/ml); therefore statistical assessment of synergism and antagonism could not be determined. Therefore, further studies using a wider dose range of Pectinex (0 – 9500 PGU/ml) should be conducted and used to determine whether or not other combinations of Pectinex and antibiotics could exhibit antagonistic or synergistic effects.
2. Further studies are still needed in order to determine the actions of Pectinex on other common clinical pathogens (e.g. *H. influenzae*, *S. pneumonia* and *E. coli*).
3. Because enzymes are substrate specific and bacteria are numerous and diverse, the search for effective enzymes should entail testing each enzyme against a wide range of bacteria. In the same regard, potentially effective enzyme-antibiotic combinations should be studied.
4. Enzymes are influenced by environmental conditions such as temperature, pH and the presence of substrates. Therefore, in order to make therapeutic predictions from *in vitro* experiments, future research should strive to create conditions that closely approximate the *in vivo* setting.
5. The mechanism of Pectinex cytotoxicity still needs to be investigated.
6. *In vitro* cytotoxicity testing should be performed on all enzymes that are potentially useful.

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APPENDIX A

Product Data Sheet

Pectinex Ultra SP-L

Valid From 2007-10-04

Product Characteristics:

Declared Enzyme	Polygalacturonase
Declared Activity	9500 PGU/ml
Colour	Brown

Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity.

Physical form	Liquid
Approximate Density (g/ml)	1.16
Stabilisers	Glycerol Potassium chloride
Preservatives Comment	No preservatives added
Odour	Slight fermentation odour
Solubility	Active component is readily soluble in water at all concentrations that occur in normal usage. Standardisation components can cause turbidity in solution.
Production organism	Aspergillus aculeatus

Produced by submerged fermentation of a micro organism.
The enzyme protein is separated and purified from the production organism.

Product Specification:

	Lower Limit	Upper Limit	Unit
Polygalacturonase PGU	9500		/ML
Density	-	-	G/ML
Total Viable Count		50000	/G
Coliform Bacteria		30	/G
Enteropathogenic E.Coli	Not Detected		/25 g
Salmonella	Not Detected		/25 g

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Packaging: See the standard packaging list for more information.

Recommended Storage:

Best before	Best before date appears from label or CoA as requested.
Storage at customer's warehouse	0-10°C (32°F-50°F)
Storage Conditions	In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.

Safety and Handling Precautions

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. The product may create easily inhaled aerosols if splashed or vigorously stirred. Spilled product may dry out and create dust. Spilled material should be flushed away with water. Avoid splashing. Left over material may dry out and create dust. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes. A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information regarding how to handle the product safely.

Distributed in the USA by:

Gusmer Enterprises, Inc.

Fresno, CA - 559.485.2692
Napa, CA - 707.224.7903
Mountainside, NJ - 908.301.1811
Waupaca, WI - 715.258.5525

APPENDIX B

Enzyme Material Safety Data Sheet

1. CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Commercial Product Name:	Pectinex Ultra SP-L
Chemical characterization:	Enzyme preparation
Product Use:	Novozymes' enzyme preparations are biocatalysts used in a variety of industrial processes and in certain consumer products.
Responsible Company	NOVOZYMES NORTH AMERICA, INC. 77 Perry Chapel Church Rd., Box 576, Franklinton, NC 27525
Information Phone Number:	(919) 494-3000 8 am - 4:30 pm EST M-F
Chemtrec Phone Number:	(800) 424-9300 24 hours everyday

2. INFORMATION ON INGREDIENTS

Active component:	Enzyme protein
Common name:	Polygalacturonase
CAS Number:	9032-75-1
Hazardous Ingredients:	Enzyme protein (1 - 10%), Xn,R42.
Exposure Limit:	Not established

3. HAZARDS IDENTIFICATION

Emergency Overview

Description :	Aqueous enzyme preparation
Appearance:	Brown liquid.
Odor:	Slight fermentation odor

Liquid enzyme preparations are dustfree preparations. However, inappropriate handling may cause formation of dust or aerosols. For appropriate handling, see section 6 and 7. Inhalation of enzyme dust or aerosols resulting from handling may induce sensitization and may cause allergic reactions in sensitized individuals.

Potential Health Effects:

INHALATION: Inhalation of dust/aerosols may induce sensitization in susceptible individuals. Subsequent exposures may cause allergic reactions in sensitized individuals. Avoid inappropriate handling which may result in aerosol/dust generation.

EYE CONTACT: Non-irritating to the eyes. However, it is recommended that direct contact with the eyes be avoided.

SKIN CONTACT: Non-irritating to the skin. However, it is recommended that prolonged contact with the skin be avoided.

INGESTION: Ingestion of material is not known to cause significant adverse health effects.

4. FIRST AID MEASURES

Skin Contact: Wash skin with plenty of water.

Eye Contact: Rinse eyes with plenty of water.

Ingestion: Rinse mouth and throat thoroughly with water. Drink water.

Inhalation: Remove from exposure. If symptoms of irritation or sensitization occur (shortness of breath, wheezing or laboured coughing), call a doctor.

5. FIRE-FIGHTING MEASURES

Protection against Fire and Explosions:	No special requirements	HMIS Rating:	Health = 1
Suitable Fire Extinguishing Media:	Water, foam		Flammability = 1
Non Suitable Media:	None		Reactivity = 0
Special Exposure Hazards:	None		

6. ACCIDENTAL RELEASE MEASURES

Spilled preparation should be removed immediately to avoid formation of dust from dried preparation. Take up by mechanical means preferably by a vacuum cleaner equipped with a high efficiency filter. Flush remainder carefully with plenty of water. Avoid splashing and high pressure washing (avoid formation of aerosols). Ensure sufficient ventilation. Wash contaminated clothing.

7. HANDLING AND STORAGE

Avoid formation of aerosols and dust from drying out of spilled preparation. Avoid splashing and high pressure washing. Ensure good ventilation of the room, when handling this preparation.

STORAGE REQUIREMENTS: Store container in a cool place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

ENGINEERING CONTROLS:

Maintain good conditions of industrial hygiene. Some processes may require enclosures, local exhaust ventilation, or other engineering controls to control airborne levels. Additional handling and health/safety information is available upon request.

RECOMMENDED PERSONAL PROTECTIVE EQUIPMENT:

Respiratory Protection:	Not required under usual conditions of use. However, if exposure potential exists, refer to NIOSH Criteria Guides to determine appropriate unit.
Hand Protection:	Impermeable gloves recommended.
Eye Protection:	Protective glasses or eye shield
Protection:	Wear suitable protective clothing

EXPOSURE GUIDELINES:

PEL (OSHA) for enzyme protein:	Not established.
TLV (ACGIH) for enzyme protein:	Not established

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance: Brown liquid.

Odor, pH, Boiling point, Melting point, Flash point, Ignition temperature, Vapour pressure, Density and Solubility are not relevant to safety.
For further information see the Product Data Sheet for this preparation.

10. STABILITY AND REACTIVITY

This material is stable under normal conditions of use.

Conditions to Avoid:	None
Materials to Avoid:	None
Hazardous Decomposition Products:	None

11. TOXICOLOGICAL INFORMATION

Inhalation of aerosols or dust resulting from inappropriate handling may induce sensitization and may cause allergic reactions in sensitized individuals. Oral rat LD-50 > 2g/kg b.w. classifies the preparation as "non-toxic".

12. ECOLOGICAL INFORMATION

LC-50(fish) > 100 mg/l, EC-50(daphnia) > 100 mg/l and IC-50(algae) > 100 mg/l, which classifies the preparation as "non-dangerous" to the environment.

The preparation is biodegradable.

13. DISPOSAL CONSIDERATIONS

WASTE DISPOSAL
No special disposal method required, except that in accordance with current local authority regulations.

14. TRANSPORT INFORMATION

SHIPPING INFORMATION:

Not classified as hazardous materials under U.S. Department of Transportation regulations (49 CFR Parts 100-185). Therefore, transport regulations do not apply.

15. REGULATORY INFORMATION

This product complies with FAO/WHO JECFA and FCC recommended specifications for food grade enzymes.

The active ingredient and all components of the enzyme preparation are listed on the TSCA inventory.

Canadian WHMIS: Controlled Product Hazard Class D2 A (respiratory sensitizer).

This product has been classified in accordance with the hazard criteria of the CPR and the MSDS contains all the information required by the CPR.

16. OTHER INFORMATION

The information contained in this Enzyme Material Safety Data Sheet, as of the issue date, is believed to be true and correct. However, the accuracy or completeness of this information and any recommendations or suggestions are made without warranty or guarantee.
Since the conditions of use are beyond the control of our company, it is the responsibility of the user to determine the conditions of safe use of this preparation. The information in this sheet does not represent analytical specifications, for which please refer to our Product Data Sheet.

Novozymes North America, Inc. Information Phone Number (919) 494-3000 77 Perry Chapel Church Rd.
Chemtrec Phone Number (800) 424-9300 Box 576

Franklinton NC 27525

APPENDIX C

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

DATE: 31/08/2011

TO:
Prof V Steenkamp
Dept of Pharmacology

Best Prof V Steenkamp

RE.: Commercial Lines: The use of Commercial lines ~ Dr I P Olwoch

During the meeting held on 31/08/2011, the use of Commercial Lines were discussed.

The Faculty of Health Science Ethics Committee approved the use of the cell lines for the In vitro component only. The In vivo study a protocol must be submitted.

With regards

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

♦Tel:012-3541330 ♦Fax:012-3541367 / 0866515924 ♦E-Mail: manda@med.up.ac.za
♦Web: //www.healthethics-up.co.za ♦H W Snyman Bld (South) Level 2-34 ♦P.O.BOX 667, Pretoria, S.A., 0001

APPENDIX D

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 27/08/2009

Prof V Steenkamp
Department of Pharmacology
University of Pretoria

Best Prof Vanessa Steenkamp

RE: Application for Blood Collection utilizing lymphocytes, macrophage, neutrophils and plasma.

Herewith acknowledgement that the above Application for blood collection has been received and tabled on 26/08/2009, and found to be acceptable by the Faculty of Health Sciences Research Ethics Committee.

With regards

DR R SOMMERS; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

31 Bophelo Road ♦ H W Snyman Building (South) Level 2-34 ♦ P.O.BOX 667, Pretoria, South Africa, 0001 ♦ Tel:(012)3541330 ♦

APPENDIX E: BIOFILM BIOMASS CRYSTAL VIOLET ASSAY

Appendix E1: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) in newly forming (0 – 6 h) biofilms of *S. aureus* ATCC after a 6-h exposure to amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-244.3 ± 51.0	-233.9 ± 49.3	-251.4 ± 39.2	-255 ± 29.3	-270.1 ± 11.7	-143.1 ± 31.4	-117.3 ± 30.2	29.5 ± 11.3
0.03	-20.9 ± 9.2	-90.0 ± 60.8	-112.8 ± 72.1	-185.4 ± 77.8	-244.1 ± 89.8	-272.3 ± 103.5	-217.0 ± 114.9	-206.2 ± 105.8	17.3 ± 19.8
0.06	-33.0 ± 12.1	-198.6 ± 52.6	-262.8 ± 50.7	-409.5 ± 51.7	-448.6 ± 66.9	-487.6 ± 48.0	-358.3 ± 80.2	-271.4 ± 80.1	-3.9 ± 34.2
0.125	2.5 ± 11.3	-60.2 ± 25.9	-122.0 ± 26.7	-218 ± 54.1	-402.8 ± 42.6	-352.5 ± 46.9	-369.4 ± 82.4	-285.3 ± 87.5	-19.8 ± 40.0
0.25	52.4 ± 6.7	-3.3 ± 47.1	-40.2 ± 52.6	-94.0 ± 70.4	-179.2 ± 91.8	-245.5 ± 85.0	-265.6 ± 85.5	-274.7 ± 84.5	-70.6 ± 58.8
0.5	83.0 ± 6.1	42.1 ± 31.1	-18.3 ± 49.6	-93.2 ± 84.6	-123.5 ± 99.7	-150.1 ± 115.4	-113.3 ± 115.5	-105.7 ± 109.5	-12.7 ± 52.5
1	93.9 ± 5.0	83.6 ± 7.8	63.7 ± 12.7	49.9 ± 21.2	-5.6 ± 54.4	-64.4 ± 97.1	-80.5 ± 118.3	-96.8 ± 124.4	63.3 ± 19.0
2	101.2 ± 2.3	96.0 ± 3.6	96.9 ± 2.3	93.9 ± 3.4	91.8 ± 2.2	95.5 ± 2.5	91.6 ± 5.7	88.1 ± 10.2	3.6 ± 62.6
4	105.3 ± 1.7	69.5 ± 10.3	80.7 ± 13.0	95.7 ± 3.2	91.5 ± 5.1	100.1 ± 2.5	99.4 ± 3.7	103.6 ± 5.8	85.6 ± 10.0

[Appendix E1 provides a cross-reference of all data in both the text and Figure 19 of Section 4.2.1(a)]

Appendix E2: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) in newly forming biofilms of *S. aureus* clinical strain after a 6-h exposure to amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-418.2 ± 24.2	-585 ± 36.5	-595.8 ± 37.7	-685.3 ± 26.5	-609.8 ± 36.6	-109.5 ± 26.0	20.1 ± 10.1	68.4 ± 2.9
0.125	6.5 ± 11.1	-1237.8 ± 187.3	-1215.2 ± 174.4	-1091.9 ± 156.7	-1066.4 ± 131.2	-818.2 ± 107.8	-11.6 ± 25.3	-8.5 ± 12.0	37.4 ± 9.4
0.25	18.5 ± 10.6	-1066.9 ± 206.5	-1070.8 ± 159.0	-1185.4 ± 157.5	-1157.0 ± 112.0	-959.3 ± 67.3	-75.1 ± 45.1	24.9 ± 13.1	42.1 ± 6.9
0.5	30.2 ± 9.4	-1003.2 ± 210.6	-1088.1 ± 1350	-1079.7 ± 141.3	-1096.7 ± 83.7	-803.5 ± 186.8	-108.5 ± 49.0	30.5 ± 16.2	42.9 ± 6.4
1	11.5 ± 11.7	-493.2 ± 217.3	-834.2 ± 259.7	-813.4 ± 222.1	-772.4 ± 158.3	-678.2 ± 97.8	-86.1 ± 44.4	44.9 ± 14.3	64.1 ± 7.6
2	63.9 ± 11.1	-507.5 ± 211.3	-612.0 ± 216.3	-650.4 ± 224.7	-667.1 ± 199.9	-561.3 ± 160.6	-62.6 ± 44.9	49.4 ± 10.6	68.3 ± 5.7
4	105.9 ± 1.8	-308.4 ± 203.5	-318.2 ± 194.0	-408.4 ± 217.0	-440.7 ± 181.8	-278 ± 126.9	-21.4 ± 49.4	53.8 ± 20.4	63.3 ± 6.6
8	106.3 ± 2.5	-169.5 ± 155.8	-102.2 ± 112.0	-192.5 ± 155.9	-185.3 ± 164.4	-198.2 ± 149.7	47.2 ± 15.7	47.7 ± 20.5	67.1 ± 10.7
16	104 ± 3.9	9.4 ± 35.1	14.2 ± 50.2	-14.0 ± 70.4	-27.2 ± 78.2	42.5 ± 53.8	74.8 ± 13.5	65.8 ± 18.1	71.6 ± 13.5

[Appendix E2 provides a cross-reference of all data in both the text and Figure 21 of Section 4.2.1(b)]

Appendix E3: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) in newly forming (0 – 6 h) biofilms of *P. aeruginosa* ATCC after a 6-h exposure to ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-26.0 ± 3.2	-5.1 ± 2.4	-0.1 ± 2.4	1.2 ± 2.9	11.4 ± 2.6	28.6 ± 2.6	44.5 ± 3.3	61.4 ± 2.9
0.03	-22.8 ± 4.4	-81.6 ± 9.8	-48.7 ± 4.6	-33.0 ± 8.1	-19.7 ± 8.2	-13.6 ± 15.2	6.8 ± 14.1	25.9 ± 10.6	52.2 ± 8.3
0.06	-19.0 ± 3.3	-56.5 ± 8.7	-27.0 ± 7.1	-11.8 ± 7.4	3.4 ± 6.8	21.2 ± 12.4	42.7 ± 9.9	49.6 ± 9.3	67.8 ± 6.5
0.125	47.6 ± 5.8	53.3 ± 9.8	59.3 ± 12.1	37.8 ± 16.5	42.4 ± 14.3	54.1 ± 12.0	68.8 ± 9.2	64.0 ± 8.7	76.2 ± 6.6
0.25	79.8 ± 4.1	90.0 ± 3.2	77.2 ± 13.8	66.4 ± 15.4	69.8 ± 13.8	80.5 ± 8.7	82.7 ± 8.3	83.4 ± 7.3	87.6 ± 5.9
0.5	91.9 ± 0.7	83.0 ± 8.9	86.7 ± 5.2	85.1 ± 5.3	86.0 ± 4.2	89.4 ± 3.7	88.2 ± 5.5	85.1 ± 7.9	90.5 ± 4.9
1	93.5 ± 0.5	88.7 ± 2.4	90.4 ± 2.4	93.3 ± 1.1	92.4 ± 1.4	92.8 ± 1.9	97.5 ± 1.7	84.1 ± 10.1	94.3 ± 4.3
2	92.6 ± 0.8	90.1 ± 2.4	92.0 ± 1.4	90.5 ± 1.1	92.8 ± 1.3	92.3 ± 1.6	95.9 ± 1.7	93.7 ± 3.7	90.8 ± 6.5
4	93.0 ± 0.6	90.1 ± 1.3	91.2 ± 1.7	92.7 ± 1.2	92.4 ± 0.9	92.0 ± 2.0	96.1 ± 1.4	98.1 ± 1.8	97.5 ± 1.1

[Appendix E3 provides a cross-reference of all data in both the text and Figure 23 of Section 4.2.1(c)]

Appendix E4: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) in newly forming (0 – 6 h) biofilms of *P. aeruginosa* clinical strain after a 6-h exposure to ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-31.7 ± 5.4	-26.2 ± 3.9	-4.6 ± 5.1	-2.2 ± 6.5	-1.0 ± 5.8	16.0 ± 6.1	31.0 ± 5.4	40.4 ± 6.2
0.03	-23.8 ± 5.0	-54.6 ± 11.4	-37.1 ± 11.4	-28.5 ± 8.0	-26.0 ± 3.7	-11.7 ± 4.3	11.1 ± 7.9	29.2 ± 11.0	53.8 ± 6.9
0.06	-0.3 ± 5.6	-12.3 ± 8.6	0.6 ± 7.5	10.2 ± 7.8	21.8 ± 8.1	31.8 ± 10.3	41.4 ± 8.7	54.1 ± 8.7	68.3 ± 7.6
0.125	2.8 ± 4.8	1.5 ± 16.1	24.1 ± 14.1	33.9 ± 12.1	32.3 ± 13.9	36.7 ± 11.9	46.9 ± 12.3	63.9 ± 8.8	69.1 ± 10.2
0.25	13.8 ± 5.8	15.3 ± 22.0	40.7 ± 16.5	39.4 ± 11.8	45.4 ± 12.2	49.8 ± 9.9	55.2 ± 9.3	64.0 ± 9.2	71.4 ± 7.5
0.5	35.3 ± 8.2	39.0 ± 17.7	58.1 ± 10.9	59.8 ± 8.2	70.2 ± 4.9	74.2 ± 5.1	54.8 ± 14.3	64.1 ± 7.9	68.0 ± 11.9
1	76.9 ± 3.0	82.6 ± 3.0	84.4 ± 3.9	85.0 ± 3.7	83.7 ± 2.1	85.6 ± 3.0	71.2 ± 12.1	42.5 ± 11.9	78.3 ± 6.1
2	92.4 ± 1.5	89.6 ± 2.5	87.1 ± 4.5	91.1 ± 1.8	90.4 ± 1.5	91.7 ± 2.0	85.9 ± 5.2	66.7 ± 11.5	70.2 ± 10.6
4	89.2 ± 2.2	87.4 ± 3.0	88.7 ± 2.6	88.7 ± 2.6	86.8 ± 2.8	86.4 ± 3.0	84.2 ± 4.7	86.4 ± 5.1	89.2 ± 3.3

[Appendix E4 provides a cross-reference of all data in both the text and Figure 25 of Section 4.2.1(d)]

Appendix E5: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) in *S. aureus* ATCC biofilms after a 24-h incubation with amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-113.8 ± 23.5	-95.4 ± 27.3	-95.3 ± 27.5	-114.7 ± 32.6	-122.9 ± 30.2	-103.7 ± 22.4	-78.3 ± 19.1	-8.4 ± 8.3
0.03	-56.5 ± 11.5	-99.0 ± 28.2	-124.1 ± 34.2	-169.4 ± 40.8	-187.1 ± 43.4	-234.6 ± 49.9	-233.8 ± 33.7	-195 ± 42.8	-67.8 ± 21.5
0.06	-61.4 ± 10.3	-90.7 ± 21.4	-103.8 ± 22.6	-140.4 ± 32.4	-131.8 ± 34.2	-184.6 ± 42.7	-247.3 ± 34.6	-161.1 ± 33.3	-36.0 ± 9.4
0.125	-16.1 ± 6.4	-70.9 ± 23.6	-73.9 ± 23.7	-85.8 ± 29.4	-147.4 ± 42.1	-136.5 ± 37.8	-173.6 ± 37.5	-271.3 ± 73.6	-20.9 ± 20.1
0.25	12 ± 7.7	-49.0 ± 23.6	-39.9 ± 17.1	-63.5 ± 25.8	-97.3 ± 30.9	-123.6 ± 39.9	-151.6 ± 44.6	-322.5 ± 86.1	-20.9 ± 14.4
0.5	39.6 ± 5.2	-42.4 ± 23.4	-41.1 ± 25.3	-46.3 ± 27.5	-49.6 ± 24.1	-80.4 ± 27.5	-130.0 ± 38.9	-178.6 ± 95.9	-28.7 ± 27.0
1	44.9 ± 9.9	-9.3 ± 28.2	-28.1 ± 31.0	-22.2 ± 27.7	-6.4 ± 22.5	-24.4 ± 28.9	-53.4 ± 32.3	-259.4 ± 86.7	-148.2 ± 75.6
2	63.0 ± 13.7	40.5 ± 26.4	33.4 ± 26.9	23.9 ± 29.1	33.6 ± 24.1	24.4 ± 29.5	27.3 ± 31.1	-34.8 ± 47.4	-81.2 ± 74.5
4	98.0 ± 3.6	85.1 ± 8.7	83.3 ± 9.4	85.7 ± 8.4	78.4 ± 11.3	83.3 ± 9.1	70.6 ± 15.2	34.9 ± 31.0	-5.2 ± 67.0

[Appendix E5 provides a cross-reference of all data in both the text and Figure 27 of Section 4.2.2(a)]

Appendix E6: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) in *S. aureus* clinical strain biofilms after a 24-h incubation with amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-75.9 ± 11.8	-6.4 ± 5.0	3.5 ± 7.2	3.8 ± 7.9	15.4 ± 10.7	-13.5 ± 11.2	-2.8 ± 7.8	9.4 ± 6.0
0.125	7.3 ± 6.0	-54.8 ± 11.8	-31.3 ± 14.6	-1.9 ± 11.0	-4.0 ± 20.2	30.2 ± 6.3	22.5 ± 9.4	-31.6 ± 10.9	2.1 ± 15.8
0.25	-8.9 ± 5.2	-45.1 ± 12.4	-29.6 ± 17.6	10.1 ± 13.4	5.2 ± 13.4	47.7 ± 6.0	26.4 ± 10.3	-9.6 ± 17.7	1.7 ± 23.3
0.5	-10.4 ± 6.1	-48.5 ± 10.5	3.7 ± 9.6	14.4 ± 8.4	18.3 ± 11.3	43.4 ± 6.1	38.4 ± 6.4	-16.8 ± 15.7	19.5 ± 8.8
1	-2.6 ± 4.5	-55.0 ± 10.4	-5.3 ± 10.4	25.9 ± 9.4	35.9 ± 6.7	52.3 ± 6.9	43.5 ± 5.8	-24.3 ± 14.9	19.8 ± 9.5
2	8.0 ± 3.8	-41.8 ± 8.5	-1.8 ± 8.3	18.8 ± 9.2	30.8 ± 8.3	50.4 ± 7.6	31.7 ± 6.1	-30.5 ± 22.7	13.7 ± 12.5
4	-17.7 ± 8.0	-53.8 ± 11.2	4.9 ± 10.2	12.5 ± 10.6	39.5 ± 6.3	52.0 ± 6.7	28.5 ± 14.2	-19.4 ± 17.6	38.9 ± 3.9
8	-1.3 ± 11.4	-41.8 ± 28.4	-22.9 ± 13.7	4.0 ± 7.1	24.9 ± 8.3	55.5 ± 6.3	27.1 ± 14.9	18.2 ± 18.6	14.5 ± 12.2
16	86.7 ± 5.0	28.9 ± 22.7	55.6 ± 19.9	71.3 ± 15.3	81.0 ± 13.8	81.7 ± 13.0	78.4 ± 12.3	54.3 ± 32.1	77.5 ± 15.2

[Appendix E6 provides a cross-reference of all data in both the text and Figure 29 of Section 4.2.2(b)]

Appendix E7: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) in *P. aeruginosa* ATCC biofilms after a 24-h incubation with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-226.5 ± 30.6	-195.4 ± 25.9	-234.5 ± 37.8	-123.1 ± 31.7	-276.9 ± 31.5	-285.8 ± 35.5	-293.3 ± 43.8	-280.1 ± 37.2
0.03	-15.3 ± 18.8	-170.6 ± 61.6	-54.0 ± 40.0	-17.3 ± 31.3	-4.2 ± 24.4	-23.0 ± 35.5	-31.3 ± 42.9	-53.7 ± 43.2	-75.0 ± 36.0
0.06	-50.5 ± 37.8	-76.4 ± 41.2	22.6 ± 20.8	36.2 ± 26.6	32.6 ± 25.6	35.2 ± 24.7	37.9 ± 19.2	34.6 ± 17.6	-74.8 ± 56.5
0.125	7.0 ± 15.0	-90.6 ± 50.5	-101.9 ± 48.1	-22.7 ± 44.6	-49.1 ± 44.1	-1.8 ± 34.2	33.4 ± 14.3	37.2 ± 17.3	-31.2 ± 21.3
0.25	50.4 ± 14.4	75.3 ± 18.5	93.2 ± 4.1	81.4 ± 13.4	61.9 ± 17.1	54.4 ± 18.3	55.2 ± 16.6	25.7 ± 34.1	-24.3 ± 36.0
0.5	95.3 ± 0.8	97.3 ± 2.1	72.1 ± 24.5	80.9 ± 13.7	83.3 ± 15.2	97.4 ± 1.1	84.1 ± 10.1	98.3 ± 2.4	94.0 ± 5.1
1	95.5 ± 0.9	72.1 ± 20.9	84.7 ± 12.8	93.0 ± 4.1	83.9 ± 14.9	97.1 ± 1.7	77.7 ± 14.8	103.2 ± 2.9	89.3 ± 6.5
2	42.2 ± 22.7	69.7 ± 28.1	77.5 ± 19.0	73.7 ± 23.8	84.8 ± 12.2	74.5 ± 24.6	89.3 ± 12.1	101.2 ± 2.5	91.4 ± 5.1
4	51.6 ± 14.0	67.9 ± 19.8	84.1 ± 8.3	80.3 ± 14.7	77.9 ± 13.2	94.4 ± 3.8	86.3 ± 13.0	81.2 ± 19.6	95.6 ± 7.0

[Appendix E7 provides a cross-reference of all data in both the text and Figure 31 of Section 4.2.2(c)]

Appendix E8: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) in *P. aeruginosa* clinical strain biofilms after a 24-h incubation with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-155.0 ± 19.4	-173.3 ± 30.7	-186.1 ± 29.5	-175.8 ± 24.0	-51.1 ± 11.3	-29.9 ± 16.0	-228.2 ± 53.8	-634.6 ± 165.8
0.03	-59.8 ± 5.9	-82.8 ± 38.0	-103.1 ± 34.5	-104.9 ± 28.1	-98.1 ± 30.0	-92.8 ± 32.4	-40.8 ± 21.2	-90.7 ± 46.5	-178.9 ± 59.7
0.06	-45.5 ± 9.1	-84.7 ± 34.6	-67.4 ± 28.8	-68.2 ± 19.2	-98.2 ± 29.8	-56.6 ± 15.8	-38.2 ± 17.0	-81.4 ± 42.3	-118.4 ± 44.4
0.125	-14.8 ± 10.4	-45.1 ± 29.4	-26.6 ± 25.9	-36.9 ± 23.6	-0.5 ± 18.9	-15.8 ± 13.0	-21.7 ± 22.0	-73.7 ± 48.0	-113.3 ± 51.3
0.25	51.2 ± 5.2	2.5 ± 19.3	20.5 ± 22.2	24.5 ± 18.0	44.4 ± 13.0	40.0 ± 18.7	38.9 ± 16.9	-29.2 ± 27.8	-106.9 ± 43.8
0.5	88.0 ± 2.5	68.5 ± 9.5	80.9 ± 4.2	72.3 ± 12.3	81.4 ± 4.0	65.3 ± 17.8	82.9 ± 6.0	52.9 ± 10.8	-113.5 ± 40.6
1	-126.7 ± 14.8	-64.9 ± 36.4	-47.4 ± 44.6	-6.0 ± 24.5	1.4 ± 23.2	44.5 ± 14.5	74.3 ± 18.9	82.5 ± 9.5	-21.7 ± 28.0
2	69.4 ± 7.7	43.2 ± 18.2	32.1 ± 28.5	23.0 ± 18.1	74.2 ± 5.7	65.0 ± 21.3	32.9 ± 16.6	-2.4 ± 20.2	-26.3 ± 38.4
4	90.6 ± 3.4	94.4 ± 3.6	98.1 ± 3.2	72.1 ± 24.3	73.4 ± 25.4	63.9 ± 32.7	71.0 ± 23.9	87.0 ± 8.3	57.0 ± 18.6

[Appendix E8 provides a cross-reference of all data in both the text and Figure 33 of Section 4.2.2(d)]

Appendix E9: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) after an 18 h exposure of preformed 6 h-old *S. aureus* ATCC biofilms to amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml)	Pectinex (PGU/ml)								
	0.0	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-173.2 ± 23.2	-188.0 ± 20.8	-217.7 ± 23.9	-287.0 ± 18.3	-327.4 ± 22.2	-348.4 ± 28.5	-243.7 ± 21.6	-108.3 ± 14.7
0.03	-22.4 ± 13.5	-132.0 ± 35.4	-70.2 ± 37.2	-88.3 ± 20.1	-112.6 ± 35.8	-173.5 ± 27.2	-255.3 ± 35.4	-200.8 ± 45.9	-132 ± 35.4
0.06	-16.4 ± 13.0	-104.2 ± 23.3	-150.6 ± 27.5	-120.8 ± 36.7	-107.5 ± 27.2	-177.2 ± 46.0	-231.4 ± 41.2	-191.7 ± 37.4	-104.2 ± 23.3
0.125	-7.8 ± 7.9	-101.2 ± 25.1	-131.5 ± 20.1	-107.0 ± 33.1	-88.7 ± 48.1	-134.3 ± 43.3	-119.8 ± 38.8	-124.6 ± 25.8	-101.2 ± 25.1
0.25	30.0 ± 6.2	-127.5 ± 31.6	-65.6 ± 27.7	-76.0 ± 29.4	-59.5 ± 26.8	-90.3 ± 43.4	-100.9 ± 42.0	-66.4 ± 31.3	-127.5 ± 31.6
0.5	30.0 ± 5.6	-87.9 ± 34.1	-47.2 ± 32.2	-38.1 ± 30.1	-70.6 ± 28.7	-80.3 ± 29.4	-109.5 ± 47.8	-115.8 ± 36.3	-87.9 ± 34.1
1	20.2 ± 7.4	-54.9 ± 30.4	-48.6 ± 28.2	-45.6 ± 28.0	-42.6 ± 20.8	-14.6 ± 33.0	-81.0 ± 33.5	-116.7 ± 47.9	-54.9 ± 30.4
2	20.8 ± 5.1	-29.8 ± 26.3	-65.5 ± 32.9	-30.9 ± 24.9	-5.7 ± 20.2	-2.8 ± 28.0	-44.9 ± 20.4	-83.0 ± 29.8	-29.8 ± 26.3
4	39.2 ± 6.6	6.9 ± 20.8	14.6 ± 13.7	32.1 ± 17.7	8.7 ± 19.0	3.8 ± 18.1	-5.8 ± 17.7	-49.5 ± 33.1	6.9 ± 20.8

[Appendix E9 provides a cross-reference of all data in both the text and Figure 35 of Section 4.2.3(a)]

Appendix E10: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) after an 18 h incubation of preformed 6 h-old *S. aureus* clinical strain biofilms with amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	26.6 ± 8.6	33.7 ± 8.4	31.9 ± 6.1	29.7 ± 6.0	32.6 ± 6.7	30.1 ± 6.8	20.1 ± 5.9	9.1 ± 9.2
0.125	-15.3 ± 25.7	10.4 ± 13.5	-2.5 ± 18.8	-24.3 ± 27.1	18.5 ± 17.2	28.5 ± 13.4	-1.3 ± 13.4	-13.4 ± 16.6	-7.4 ± 15.3
0.25	11.5 ± 6.7	22.3 ± 12.3	45.5 ± 32.9	12 .0± 12.0	36.6 ± 13.9	27.0 ± 7.4	19.6 ± 9.7	11.3 ± 12.1	14.5 ± 12.3
0.5	-2.2 ± 26.0	15.6 ± 16.8	26.8 ± 28.8	19.6 ± 31.3	29.2 ± 14.3	55.2 ± 4.9	22.8 ± 11.6	3.7 ± 12.0	30.7 ± 8.6
1	37.8 ± 41.5	31.3 ± 5.9	54.4 ± 29.7	36.2 ± 10.6	57.6 ± 6.8	54.4 ± 7.2	45.9 ± 8.5	30.1 ± 17.1	33.5 ± 17.8
2	-0.7 ± 16.1	10.8 ± 15.0	31.5 ± 9.3	64.3 ± 25.4	45.0 ± 8.4	59.7 ± 6.6	45.6 ± 9.7	19.4 ± 18.1	33.9 ± 18.8
4	-41.9 ± 20.9	19.1 ± 14.2	27.8 ± 10.6	40.1 ± 1.00	42.5 ± 6.9	45.7 ± 6.2	55.7 ± 5.5	29.5 ± 11.8	43.5 ± 13.2
8	26.8 ± 21.7	20.5 ± 9.4	29.7 ± 9.6	45.9 ± 10.3	61.5 ± 4.2	61.2 ± 5.9	63.8 ± 7.1	56.1 ± 9.4	34.2 ± 14.4
16	38.0 ± 17.0	25.9 ± 13.9	50.8 ± 29.5	59.0 ± 9.1	62.7 ± 7.5	63.1 ± 7.1	62.4 ± 6.6	72.9 ± 14.2	40.1 ± 12.5

[Appendix E10 provides a cross-reference of all data in both the text and Figure 37 of Section 4.2.3(b)]

Appendix E11: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) after an 18 h incubation of preformed 6 h-old *P. aeruginosa* ATCC biofilms with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-122.3 ± 13.5	-106.1 ± 9.9	-115.9 ± 10.2	-108.8 ± 9.1	-122.9 ± 13.0	-134.9 ± 13	-158.2 ± 15.9	-168.1 ± 10.9
0.03	35.3 ± 12.3	-13.3 ± 41.5	**10.8 ± 24.4**	**-3.8 ± 17.6**	-59.4 ± 31.1	-48.5 ± 36.8	-78.2 ± 31.2	-106.5 ± 48.5	-202.8 ± 51.2
0.06	61.6 ± 9.2	**30.8 ± 26.7**	**33.6 ± 15.3**	**11.0 ± 24.1**	-22.1 ± 29.6	-60.3 ± 37.4	-69.0 ± 39.2	-91.6 ± 48.6	-121.6 ± 69.8
0.125	-17.6 ± 22.7	**-2.4 ± 14.6**	**31.1 ± 9.6**	**28.8 ± 12.2**	-32.6 ± 41.5	-38.9 ± 23.6	-41.1 ± 40.7	-38.3 ± 43.4	-82.9 ± 73.5
0.25	3.3 ± 9.1	**13.7 ± 13.4**	**26.5 ± 14.8**	**24.3 ± 11.4**	**-0.9 ± 17.9**	-23.0 ± 28.3	-54.6 ± 34.9	**-27.6 ± 44.0**	-98.5 ± 66.2
0.5	5.6 ± 13.5	**67.0 ± 10.4**	**64.7 ± 14.7**	**63.1 ± 12.9**	**52.9 ± 22.2**	**51.5 ± 18.1**	**48.1 ± 15.4**	-41.2 ± 45.6	-123.7 ± 64.1
1	12.3 ± 11.8	-29.3 ± 35.4	-54.5 ± 35.9	**-19.3 ± 52.9**	-24.6 ± 33.8	-62.0 ± 50.5	-33.0 ± 37.1	**13.7 ± 24.9**	-110.0 ± 50.4
2	-12.7 ± 15.7	-53.7 ± 38.3	**-9.6 ± 23.3**	**-8.1 ± 20.3**	**17.4 ± 15.0**	-28.7 ± 24.2	**9.4 ± 16.7**	-20.0 ± 26.2	-105.0 ± 45.8
4	-81.3 ± 32.8	-43.3 ± 49.9	**-16.6 ± 23.5**	-43.7 ± 36.2	**19.5 ± 26.6**	-14.6 ± 23	**-3.5 ± 24.1**	-16.2 ± 27.2	-85.8 ± 36.7

Shaded cells indicate values that are significantly ($p \leq 0.05$) different from the untreated control (0.0)

[Appendix E11 provides a cross-reference of all data in both the text and Figure 39 of Section 4.2.3(c)]

Appendix E12: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) after 18 h incubation of preformed 6 h-old *P. aeruginosa* clinical strain biofilms with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-49.9 ± 6.5	-33.4 ± 9.2	-19.4 ± 7.9	-34.5 ± 10.0	-39.5 ± 11.1	-56.4 ± 13.6	-96.9 ± 15.7	-169.3 ± 17.9
0.03	-22.7 ± 13.5	-19.6 ± 49.4	-5.8 ± 27.6	5.3 ± 18.8	-4.9 ± 20.4	-38.6 ± 29.6	-62.9 ± 37.1	-89.7 ± 33.2	-88.1 ± 45.7
0.06	32.9 ± 6.4	64.1 ± 17.8	70.9 ± 17.4	60.1 ± 20.7	66.9 ± 17.3	37.2 ± 20.3	5.8 ± 30.0	-26.7 ± 41.4	-72.0 ± 50.2
0.125	34.3 ± 6.4	5.3 ± 27.9	65.6 ± 14.4	51.1 ± 22.5	51.5 ± 18.8	58.0 ± 19.6	26.0 ± 30.7	46.0 ± 20.9	-5.5 ± 42.8
0.25	61.4 ± 7.9	-23.9 ± 23.7	22.1 ± 8.9	28.1 ± 8.7	-5.3 ± 16.0	20.0 ± 19.9	-1.4 ± 30.3	-10.5 ± 33.9	-104.2 ± 36
0.5	64.4 ± 8.6	-17.0 ± 30.2	19.3 ± 19.7	-3.9 ± 29.2	22.1 ± 16.4	31.3 ± 15.6	7.9 ± 22.6	8.9 ± 16.9	-110.7 ± 36.4
1	38.9 ± 8.2	-47.6 ± 28.6	1.7 ± 21.8	-4.5 ± 27.8	16.1 ± 16.7	14.4 ± 17.4	24.2 ± 18.8	-33.3 ± 31.8	-171.0 ± 50.6
2	-9.1 ± 8.5	-24.3 ± 29.4	-112.6 ± 44.7	-65.8 ± 39.4	-70.4 ± 55.2	-11.5 ± 13.4	-35.3 ± 36.1	-89.4 ± 40.3	-190.5 ± 55.3
4	24.2 ± 8.9	-77.5 ± 47.8	-100.2 ± 36.2	-102.4 ± 39.6	-76.5 ± 34.0	-139.9 ± 52.0	-90.7 ± 36.9	-52.3 ± 39.0	-256.3 ± 81.9

[Appendix E12 provides a cross-reference of all data in both the text and Figure 41 of Section 4.2.3(d)]

Appendix E13: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) after a 24 h exposure of preformed 24 h-old *S. aureus* ATCC biofilms to amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-69.1 ± 7.7	-86.6 ± 11.5	-86.0 ± 12.6	-64.0 ± 10.5	-36.8 ± 12.9	-52.7 ± 9.7	20.6 ± 7.9	36.0 ± 6.4
0.03	-32.1 ± 5.8	-147.6 ± 21.5	-114.2 ± 9.4	-108.2 ± 14.3	-149.2 ± 5.3	-184.5 ± 28.4	-283.7 ± 21.3	-188.3 ± 41.9	-130.9 ± 30.3
0.06	-35.0 ± 6.0	-153.5 ± 20.0	-115.9 ± 23.9	-127.6 ± 21.1	-137.3 ± 23.4	-249.7 ± 63.9	-273.1 ± 36.2	-100.1 ± 27.8	-100.8 ± 25.2
0.125	-36.2 ± 7.0	-122.9 ± 14.6	-109.7 ± 15.6	-113.5 ± 31.7	-136.8 ± 33.5	-143.2 ± 8.4	-207.6 ± 50.2	-118.8 ± 59.1	-46.9 ± 4.2
0.25	-28.1 ± 4.5	-121.6 ± 8.6	-136.6 ± 17.7	-131.2 ± 18.9	-121.9 ± 31.5	-150.9 ± 18.5	-257.0 ± 28.3	-138.3 ± 21	-84.9 ± 9.6
0.5	-23.4 ± 6.4	-154.3 ± 9.7	-124.3 ± 19.0	-126.7 ± 15.8	-144.5 ± 20.2	-154.9 ± 23.1	-267.2 ± 14.3	-119.5 ± 17.1	-95.1 ± 13.1
1	-34.2 ± 6.9	-181.8 ± 14.1	-145.2 ± 17.6	-103.7 ± 12.8	-119.2 ± 18.3	-204.3 ± 17.1	-245.3 ± 8.8	-160.8 ± 40.8	-79.8 ± 20.1
2	-39.0 ± 8.3	-180.4 ± 19.9	-180.6 ± 24.2	-129.0 ± 21.8	-141.0 ± 34.8	-160.1 ± 25.5	-231.2 ± 22.5	-184.7 ± 30.9	-100.0 ± 11.0
4	-19.9 ± 7.8	-170.0 ± 20.8	-121.1 ± 22.9	-93.0 ± 22.8	-89.9 ± 14.6	-154.5 ± 16.1	-206.9 ± 24.1	-269 ± 33.2	-103.9 ± 20.3

[Appendix E13 provides a cross-reference of all data in both the text and Figure 43 of Section 4.2.4(a)]

Appendix E14: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) after a 24 h exposure of preformed 24 h-old *S. aureus* clinical strain biofilms with amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	2.4 ± 5.6	23.8 ± 4.9	22.8 ± 6.2	24.6 ± 6.1	12.4 ± 8.8	-35.9 ± 18.9	-49.8 ± 19.6	-42.6 ± 15.7
0.125	40.6 ± 20.0	18.9 ± 12.8	26.0 ± 13.7	22.5 ± 15.0	7.4 ± 13.6	38.9 ± 13.5	31.3 ± 12.5	21.6 ± 18.5	6.8 ± 8.1
0.25	-12.8 ± 27.5	-6.2 ± 12.9	23.8 ± 11.5	0.0 ± 32.2	10.6 ± 14.7	15.3 ± 13.4	13.1 ± 13.8	27.2 ± 19.5	36.2 ± 11.2
0.5	-60.3 ± 29.5	9.0 ± 18.7	15.7 ± 18.2	46.7 ± 7.4	40.9 ± 9.1	43.7 ± 9.7	33.0 ± 5.6	44.2 ± 2.4	49.7 ± 9.0
1	-2.3 ± 25.6	-3.5 ± 16.2	6.4 ± 17.8	16.6 ± 11.4	30.6 ± 13.6	21.2 ± 13.6	16.2 ± 17.2	44.3 ± 9.4	47.2 ± 4.9
2	11.3 ± 21.7	-2.1 ± 15.3	0.5 ± 17.8	-11.3 ± 46.6	19.6 ± 9.8	18.1 ± 7.8	29.6 ± 8.3	42.5 ± 7.2	40.2 ± 7.3
4	-68.5 ± 21.5	-3.5 ± 13.4	5.3 ± 21.9	18.2 ± 8.2	8.0 ± 25.8	32.3 ± 7.4	37.5 ± 4.8	22.1 ± 12	34.9 ± 9.8
8	4.5 ± 17.5	-17.3 ± 38.6	13.2 ± 15.9	20.0 ± 18.5	2.0 ± 35.6	11.9 ± 18.8	13.8 ± 12	30.7 ± 12.5	9.3 ± 20.2
16	57.1 ± 25.5	-7.0 ± 25.5	24.8 ± 24.7	34.6 ± 12.1	24.7 ± 15.7	36.6 ± 18.2	47.3 ± 9.7	29.0 ± 18.4	35.4 ± 15.5

[Appendix E14 provides a cross-reference of all data in both the text and Figure 45 of Section 4.2.4(b)]

Appendix E15: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) after a 24 h exposure of preformed 24 h-old *P. aeruginosa* ATCC biofilms with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	8.9 ± 4.1	17.9 ± 8.7	20 ± 7.5	32.2 ± 5.9	9.9 ± 8.4	-4.9 ± 8.1	-14.6 ± 12.1	-65.2 ± 15.9
0.03	62.9 ± 10.3	10.4 ± 40.6	21.1 ± 15.1	30.6 ± 19.4	35.5 ± 32	42.6 ± 17.1	17.4 ± 24.9	-20.7 ± 31.3	-61.1 ± 47.5
0.06	76.9 ± 10.8	-62.8 ± 62.6	24.4 ± 22.8	45.1 ± 28.6	76.2 ± 15.1	76.6 ± 15.9	44.1 ± 23.0	-32.2 ± 82.4	-45.0 ± 4.8
0.125	36.1 ± 15.3	7.4 ± 33.1	-15.3 ± 61.0	41.1 ± 30.5	69.3 ± 16.4	57.6 ± 19.1	48.2 ± 27.3	76.4 ± 8.8	5.6 ± 30.5
0.25	73.9 ± 13.0	1.4 ± 50.1	10.8 ± 53.5	31.4 ± 38.4	43.7 ± 26.2	75.1 ± 15.5	90.9 ± 0.8	83.8 ± 5.6	15.8 ± 48.4
0.5	50.0 ± 14.8	68.7 ± 10.1	50.2 ± 31.2	62.2 ± 18.4	33.9 ± 56.0	77.8 ± 7.3	62.9 ± 15.7	75.9 ± 9.1	-17.0 ± 60.2
1	36.3 ± 16.1	-15 ± 49.2	68.4 ± 11.0	22.8 ± 41.6	78.2 ± 6.0	73.5 ± 9.3	61.6 ± 11.3	45.8 ± 19.7	25.0 ± 24.2
2	12.1 ± 21.1	-5.0 ± 38.2	28.1 ± 40.0	57.8 ± 17.1	74.9 ± 6.9	67.9 ± 16.3	78.4 ± 5.2	35.6 ± 16.5	2.3 ± 27.4
4	-33.5 ± 14.3	-101.0 ± 59.7	1.6 ± 36.7	47.1 ± 32.0	49.7 ± 22.4	58.3 ± 20.6	73.4 ± 7.0	44.9 ± 23.6	-2.3 ± 35.9

[Appendix E15 provides a cross-reference of all data in both the text and Figure 47 of Section 4.2.4(c)]

Appendix E16: Mean percentage (%) reduction ± S.E.M. in CV OD560nm (compared to the untreated control) after a 24 h exposure of preformed 24 h-old *P. aeruginosa* clinical strain biofilms with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-58.1 ± 12.9	-13.0 ± 15.4	-2.2 ± 12.9	-7.2 ± 13.9	-11.3 ± 13.1	0.3 ± 11.9	9.8 ± 14.7	9.4 ± 10.2
0.03	-58.4 ± 7.4	-15.5 ± 20.4	32.8 ± 14.6	45.8 ± 12.5	46.6 ± 14.4	44.4 ± 16.1	62.4 ± 10.5	55.0 ± 10.5	37.9 ± 7.4
0.06	-20.8 ± 6.6	8.9 ± 32.8	18.8 ± 28.2	44.2 ± 15.3	46.3 ± 16.6	44.0 ± 19.7	46.1 ± 16.9	55.5 ± 9.4	6.3 ± 23.2
0.125	-8.0 ± 10.1	10.5 ± 30.7	19.7 ± 300	31.3 ± 22.8	33.6 ± 23.5	45.9 ± 16.6	43.9 ± 19.2	53.2 ± 11.5	36.2 ± 11.2
0.25	21.1 ± 3.6	10.2 ± 34.4	14.9 ± 31.0	29.9 ± 26.7	40.4 ± 21.6	52.9 ± 16.7	53.8 ± 15.2	46.8 ± 10.6	49.4 ± 12.0
0.5	17.8 ± 3.6	16.1 ± 38.1	25.5 ± 33.3	38.1 ± 27.3	44.4 ± 21.2	39.1 ± 20.0	55.2 ± 16.7	56.2 ± 10.7	33.0 ± 17.2
1	1.0 ± 2.2	34.5 ± 31.2	38.5 ± 32.9	37.7 ± 28.2	37.1 ± 30.1	54.4 ± 20.2	50.4 ± 18.7	49.7 ± 14.4	42.5 ± 14.4
2	3.3 ± 5.5	32.5 ± 27.9	34.1 ± 24.9	28.1 ± 27.6	35.5 ± 25.6	37.8 ± 21.7	43.7 ± 17.2	46.2 ± 19.5	39.5 ± 15.2
4	13.3 ± 5.7	52.2 ± 17.0	34.4 ± 31.6	38.3 ± 30.8	41.5 ± 29.1	47.4 ± 22.1	60.0 ± 13.6	41.0 ± 17.9	31.4 ± 16.9

[Appendix E16 provides a cross-reference of all data in both the text and Figure 49 of Section 4.2.4(d)]

APPENDIX F: BIOFILM DISINFECTION MTT ASSAY

Appendix F1: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) in newly forming (0 – 6 h) biofilms of *S. aureus* ATCC after a 6-h exposure to amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-163.6 ± 20.8	-154.7 ± 19.4	-96.6 ± 12.6	-73.9 ± 11.4	-33.2 ± 7.1	0.8 ± 7.1	31.5 ± 4.7	85.8 ± 1.0
0.03	-34.9 ± 13.0	-196.9 ± 69.2	-50.7 ± 38.9	-79.0 ± 42.9	-103.8 ± 35.2	-49.5 ± 16.7	-34.1 ± 14.2	51.6 ± 8.2	86.4 ± 2.6
0.06	2.6 ± 9.3	-185.5 ± 42.9	-100.0 ± 30.4	-61.2 ± 17.8	-65.4 ± 23.1	-50.7 ± 16.5	-2.0 ± 15.0	35.2 ± 12.1	72.5 ± 6.5
0.125	12.5 ± 9.7	-76.0 ± 38.3	-79.4 ± 38.1	-43.4 ± 30.5	-37.3 ± 23.3	-18.0 ± 20.2	3.3 ± 16.0	57.4 ± 6.8	71.5 ± 7.3
0.25	18.7 ± 10.2	-67.0 ± 43.6	-69.3 ± 40.4	-37.1 ± 34.8	-24.7 ± 25.8	-13.9 ± 19.9	10.7 ± 17.8	60.3 ± 7.6	82.0 ± 2.1
0.5	21.5 ± 10.4	-52.4 ± 44.8	-33.6 ± 33.9	-22.0 ± 30.5	-25.8 ± 28.7	-5.6 ± 19.7	6.8 ± 17.8	52.9 ± 10	89.3 ± 2.5
1	17.3 ± 12.3	-50.9 ± 40.0	-13.9 ± 33.8	-20.0 ± 31.5	-22.0 ± 30.7	-3.4 ± 19.9	-1.5 ± 19.4	60.5 ± 9.7	90.7 ± 2.2
2	18.3 ± 11.7	-129.5 ± 58.5	-72.8 ± 39.5	-49.9 ± 38.1	-36.1 ± 33.7	-12.1 ± 20.0	-60.2 ± 36.4	40.3 ± 19.2	85.5 ± 3.6
4	25.5 ± 10.3	-96.5 ± 53.6	-97.5 ± 48.4	-97.5 ± 47.4	-52.1 ± 36.7	-55.5 ± 32.9	-72.9 ± 35.6	78.3 ± 10.3	76.7 ± 6.3

[Appendix F1 provides a cross-reference of all data in both the text and Figure 20 of Section 4.2.1(a)]

Appendix F2: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) in newly forming (0 – 6 h) biofilms of *S. aureus* clinical strain after a 6-h exposure to amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml)	Pectinex (PGU/ml)								
	0.0	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-213.8 ± 36.8	-105.4 ± 26.1	-90.1 ± 22.7	-80.0 ± 19.8	-24.0 ± 13.0	47.0 ± 7.5	68.1 ± 3.8	79.1 ± 3.4
0.125	-51.0 ± 7.4	-89.4 ± 26.2	-112.1 ± 19.8	-110.7 ± 19.3	-66.0 ± 20.8	-29.9 ± 8.4	48.1 ± 7.8	66.0 ± 5.2	81.8 ± 4.2
0.25	-33.9 ± 8.7	-117.3 ± 23.6	-79.8 ± 16.3	-98.1 ± 33.0	-34.8 ± 18.7	2.3 ± 6.9	64.5 ± 4.3	76.1 ± 3.2	82.6 ± 3.9
0.5	-33.7 ± 10.4	-62.8 ± 13.6	-143.4 ± 88.8	-27.0 ± 10.0	-14.5 ± 19.0	12.8 ± 7.1	63.0 ± 5.6	78.6 ± 4.5	84.5 ± 3.5
1	4.8 ± 11.9	-6.3 ± 37.9	18.5 ± 30.2	10.5 ± 22.9	8.4 ± 22.6	19.1 ± 15.3	68.1 ± 6.6	80.8 ± 4.4	84.4 ± 3.6
2	7.8 ± 12.5	-24.3 ± 32.2	-17.6 ± 30.2	10.3 ± 26.6	15.0 ± 23.2	20.7 ± 15.1	69.8 ± 6.3	78.4 ± 3.3	89.4 ± 3.5
4	12.7 ± 12.8	-23.0 ± 37.4	-12.1 ± 28.8	35.8 ± 21.4	-18.5 ± 26.5	17.2 ± 16.7	68.4 ± 8.1	80.2 ± 4.8	89.1 ± 2.8
8	11.6 ± 10.9	-11.4 ± 40.2	-16.5 ± 29.9	4.0 ± 24.9	-6.5 ± 24.1	10.9 ± 18.2	65.4 ± 8.3	81.2 ± 4.2	83.0 ± 6.8
16	9.4 ± 11.2	-24.1 ± 37	-39.7 ± 41.3	-16.9 ± 30.2	-25.0 ± 37.3	22.8 ± 15.6	69.0 ± 7.2	77.9 ± 4.7	86.1 ± 4.2

[Appendix F2 provides a cross-reference of all data in both the text and Figure 22 of Section 4.2.1(b)]

Appendix F3: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) in newly forming (0 – 6 h) biofilms of *P. aeruginosa* ATCC after a 6-h exposure to ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-21.4 ± 4.0	-14.2 ± 2.6	-0.5 ± 2.7	9.4 ± 2.0	20.6 ± 2.2	38.5 ± 1.9	53.0 ± 1.6	73.8 ± 1.3
0.03	-33.5 ± 7.6	-69.4 ± 13.3	-54.3 ± 12	-29.1 ± 13.7	-14.3 ± 11.1	10.6 ± 8.8	34.8 ± 4.4	48.5 ± 2.9	76.9 ± 2.9
0.06	-3.7 ± 4.0	-35.6 ± 7.5	-13.7 ± 9.1	-4.4 ± 10.8	-5.4 ± 11.3	27.5 ± 6.9	56.5 ± 4.6	60.6 ± 4.5	76.8 ± 1.6
0.125	76.6 ± 1.2	64.4 ± 6.6	79.0 ± 2.1	80.8 ± 1.0	75.0 ± 8.7	85.3 ± 5.0	87.1 ± 3.3	83.9 ± 2.0	85.9 ± 1.1
0.25	94.1 ± 0.9	91.6 ± 1.3	93.1 ± 1.6	93.2 ± 1.1	93.3 ± 1.3	101.2 ± 6.4	94.9 ± 0.6	101.2 ± 6.5	94.4 ± 1.2
0.5	93.4 ± 1.1	88.6 ± 2.7	91.6 ± 0.9	92.9 ± 0.8	92.7 ± 1.1	97.1 ± 5.7	92.9 ± 1.0	98.0 ± 4.4	95.5 ± 0.8
1	95.4 ± 0.9	93.3 ± 3.3	92.6 ± 1.3	93.8 ± 0.9	93.3 ± 1.0	97.8 ± 1.9	96.2 ± 1.2	100.2 ± 6.0	97.1 ± 0.9
2	93.6 ± 0.6	90 .0± 1.4	90.1 ± 1.5	90.4 ± 1.8	91.7 ± 1.3	97.1 ± 4.9	94.0 ± 1.3	97.6 ± 2.7	95.6 ± 0.5
4	94.7 ± 0.8	92.5 ± 1.8	91.1 ± 1.4	93.0 ± 1.6	93.0 ± 1.3	102.0 ± 6.3	95.1 ± 1.3	98.9 ± 3.0	96.9 ± 0.8

[Appendix F3 provides a cross-reference of all data in both the text and Figure 24 of Section 4.2.1(c)]

Appendix F4: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) in newly forming (0 – 6 h) biofilms of *P. aeruginosa* clinical strain after a 6-h exposure to ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-11.1 ± 4.9	-5.0 ± 2.9	-0.1 ± 3.2	13.2 ± 2.8	24.2 ± 2.8	35.9 ± 3.2	53.3 ± 1.4	70.4 ± 1.3
0.03	-7.0 ± 4.9	-14.2 ± 7.6	11.9 ± 6.4	18.8 ± 4.7	20.9 ± 4.1	25.9 ± 4.3	41.7 ± 5.5	47.4 ± 8.2	56.9 ± 8.4
0.06	-11.7 ± 5.7	-11.8 ± 10.8	7.1 ± 6.1	25.0 ± 4.0	25.0 ± 3.9	44.4 ± 6.3	53.3 ± 5.6	60.9 ± 5.6	70.9 ± 2.9
0.125	-9.7 ± 6.2	-4.1 ± 5.2	7.6 ± 4.2	19.4 ± 3.3	23.7 ± 4.2	38.6 ± 2.6	48.2 ± 2.1	58.6 ± 1.5	65.1 ± 5.2
0.25	1.7 ± 4.1	7.1 ± 4.9	23.2 ± 3.5	26.6 ± 2.4	34.6 ± 2.4	41.8 ± 3.3	55.1 ± 2.8	60.9 ± 3.5	74.7 ± 2.0
0.5	33.7 ± 4.9	31.2 ± 8.9	50.5 ± 5.2	56.0 ± 4.5	56.7 ± 3.8	61.3 ± 2.4	63.0 ± 2.8	60.6 ± 1.9	74.8 ± 2.3
1	74.9 ± 1.5	83.5 ± 1.8	82.8 ± 3.6	88.1 ± 1.9	89.5 ± 3.6	89.3 ± 1.6	89.3 ± 1.0	75.7 ± 3.0	73.7 ± 5.5
2	81.7 ± 2.0	85.9 ± 1.3	88.5 ± 0.8	88.9 ± 1.3	91.5 ± 0.6	92.5 ± 1.4	92.1 ± 0.9	89.1 ± 0.9	86.3 ± 1.9
4	86.8 ± 2.3	89.8 ± 1.5	90.4 ± 1.7	93.6 ± 0.8	85.7 ± 8.6	94.3 ± 1.7	94.7 ± 0.9	91.8 ± 1.1	92.6 ± 1.3

[Appendix F4 provides a cross-reference of all data in both the text and Figure 26 of Section 4.2.1(d)]

Appendix F5: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) in *S. aureus* ATCC biofilms after a 24-h incubation with amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-39.3 ± 8.6	-59.7 ± 9.2	-105.2 ± 11.0	-130.5 ± 12.2	-155.4 ± 12.4	-119.0 ± 15.2	-33.0 ± 14.0	44.7 ± 6.0
0.03	-35.8 ± 5.0	-43.2 ± 14.8	-84.5 ± 17.5	-91.5 ± 20.6	-97.6 ± 20.4	-118.6 ± 18.7	-91.9 ± 17.3	-30.3 ± 20.0	45.7 ± 7.1
0.06	-22.4 ± 4.9	-55.3 ± 14.1	-99.3 ± 25.2	-92.8 ± 19.3	-89.2 ± 10.3	-127.3 ± 21.4	-42.1 ± 37.2	-39.9 ± 26.4	51.1 ± 5.1
0.125	-7.6 ± 6.3	-54.0 ± 12.4	-95.2 ± 23.8	-73.8 ± 28.6	-123.5 ± 17.3	-119.3 ± 21.7	-68.0 ± 26.0	-34.0 ± 24.7	58.3 ± 3.0
0.25	-15.0 ± 8.7	-42.5 ± 13.6	-114.0 ± 26.6	-97.0 ± 22.9	-114.5 ± 23.7	-108.2 ± 24.8	-43.5 ± 50.7	-28.6 ± 22.8	48.1 ± 7.3
0.5	-16.7 ± 5.4	-6.6 ± 13.0	-86.3 ± 27.7	-89.4 ± 39.5	-67.9 ± 20.4	-114 ± 21.0	-80.3 ± 28.1	-71.3 ± 30.3	64.7 ± 2.7
1	-32.7 ± 10.8	47.6 ± 4.3	7.9 ± 24.9	-37.8 ± 32.0	-16.2 ± 17.9	-34.3 ± 6.8	-77.7 ± 21.6	-72.7 ± 32.3	31.8 ± 19.6
2	-22.4 ± 13.3	53.4 ± 4.8	-24.8 ± 25.7	-25.3 ± 27.6	-59.8 ± 26.2	-71.6 ± 20.6	-47.1 ± 12.7	-36.9 ± 14.9	35.7 ± 10.3
4	-72.4 ± 12.8	40.5 ± 16.7	15.8 ± 13.6	16.8 ± 17.1	15.7 ± 16.6	8.3 ± 18.5	22.9 ± 18.6	28.3 ± 15.2	27.7 ± 7.6

[Appendix F5 provides a cross-reference of all data in both the text and Figure 28 of Section 4.2.2(a)]

Appendix F6: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) in *S. aureus* clinical strain biofilms after a 24-h incubation with amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	20.5 ± 4.6	25.2 ± 5.8	15.3 ± 7.2	12.6 ± 10.6	3.3 ± 15.4	27.4 ± 13.2	7.0 ± 14.0	39.4 ± 15.4
0.125	14.5 ± 6.1	30.0 ± 8.2	26.1 ± 7.7	35.4 ± 11.2	21.0 ± 15.4	52.6 ± 9.3	42.4 ± 7.9	**41.3 ± 6.8**	55.9 ± 21.9
0.25	22.2 ± 4.7	29.3 ± 8.4	**39.5 ± 8.4**	30.1 ± 12.8	15.0 ± 20.2	16.1 ± 25.4	31.7 ± 7.8	34.9 ± 8.1	53.1 ± 23.4
0.5	10.4 ± 4.5	**45.0 ± 8.1**	34.8 ± 10.3	22.8 ± 10.5	-3.6 ± 22.8	22.6 ± 14.5	**48.3 ± 10.5**	24.9 ± 7.2	48.4 ± 29.5
1	1.2 ± 5.3	**39.6 ± 8.0**	27.2 ± 11.2	26.1 ± 11.0	19.4 ± 16.2	12.8 ± 24.1	**41.6 ± 4.9**	27.5 ± 7.1	**54.3 ± 20.9**
2	16.0 ± 7.4	**40.3 ± 7.4**	**38.1 ± 8.3**	11.5 ± 28.6	**40.1 ± 9.9**	**44.3 ± 10.9**	42.6 ± 11.1	**43.6 ± 10.8**	**66.4 ± 15.6**
4	11.2 ± 4.6	**38.2 ± 5.1**	30.0 ± 5.3	27.5 ± 7.6	8.0 ± 18.1	26.7 ± 15.8	32.0 ± 7.4	33.8 ± 5.8	**69.3 ± 6.0**
8	16.5 ± 6.7	**48.7 ± 9.0**	**50.2 ± 6.9**	19.8 ± 5.6	31.6 ± 12.2	9.1 ± 22.9	32.4 ± 9.0	**41.3 ± 6.4**	51.0 ± 17.6
16	16.3 ± 5.2	**40.5 ± 7.1**	**52.5 ± 8.3**	**36.9 ± 3.8**	21.9 ± 8.8	29.4 ± 17.7	**44.3 ± 8.7**	**55.7 ± 4.1**	**62.7 ± 9.2**

Shaded cells indicate values that are significantly ($p \leq 0.05$) different from the untreated control (0.0)

[Appendix F6 provides a cross-reference of all data in both the text and Figure 30 of Section 4.2.2(b)]

Appendix F7: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) in *P. aeruginosa* ATCC biofilms after a 24-h incubation with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-78.7 ± 14.0	-58.2 ± 12.7	-101.6 ± 21.9	-103.7 ± 24.0	-68.4 ± 18.3	-63.5 ± 17.7	-87.1 ± 24.0.0	-14.5 ± 25.9
0.03	29.1 ± 8.8	-18.1 ± 28.4	-27.1 ± 33.7	-56.0 ± 32.5	-34.1 ± 35.0	-22.0 ± 38.1	-2.5 ± 23.9	-14.4 ± 34.5	-57.8 ± 42.9
0.06	13.6 ± 5.4	-32.5 ± 39.1	-32.6 ± 30.8	-13.0 ± 27.2	31.3 ± 20.7	33.8 ± 20.5	44.1 ± 22.2	60.5 ± 15.3	51.6 ± 17.1
0.125	-11.5 ± 14.3	-208.2 ± 68.3	-51.9 ± 30.0	-41.5 ± 30.2	-66.7 ± 34.9	-53.5 ± 26.7	-108.4 ± 56.1	25.7 ± 17.5	59.2 ± 17.5
0.25	71.1 ± 8.8	52.3 ± 22.2	48.9 ± 19.4	-6.8 ± 49.7	55.7 ± 19.7	66.1 ± 14.4	61.2 ± 20.8	44.1 ± 25.7	21.0 ± 16.7
0.5	84.3 ± 3.4	73.9 ± 7.2	80.8 ± 4.3	73.7 ± 7.5	70.3 ± 8.2	80.5 ± 5.1	**90.1 ± 5.2**	**88.4 ± 4.3**	**70.7 ± 19.7**
1	92.7 ± 1.7	37.8 ± 47.5	46.6 ± 43.1	**56.4 ± 36.3**	**53.0 ± 41.7**	**64.4 ± 35.7**	**64.5 ± 22.0**	**69.4 ± 17.3**	**86.6 ± 9.2**
2	92.4 ± 1.8	61.1 ± 10.5	52.4 ± 21.7	**84.5 ± 5.9**	59.8 ± 23.5	89.3 ± 3.8	**84.8 ± 5.8**	54.4 ± 24.9	79.0 ± 11.9
4	97.2 ± 1.0	**86.6 ± 4.5**	**83.4 ± 9.2**	**79.0 ± 8.8**	**89.0 ± 5.4**	**71.7 ± 17.5**	**91.5 ± 4.6**	**76.0 ± 17.4**	**85.8 ± 8.7**

Shaded cells indicate values that are significantly ($p \leq 0.05$) different from the untreated control (0.0)

[Appendix F7 provides a cross-reference of all data in both the text and Figure 32 of Section 4.2.2(c)]

Appendix F8: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) in *P. aeruginosa* clinical strain biofilms after a 24-h incubation with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-77.1 ± 10.4	-45.0 ± 7.9	-53.3 ± 10.0	-32.9 ± 11.4	-47.8 ± 12.9	-11.0 ± 10.9	-24.4 ± 9.1	-36.4 ± 7.1
0.03	11.9 ± 4.7	-86.8 ± 37.4	-63.6 ± 40.8	-79.8 ± 39.7	-98.6 ± 34.0	-58.8 ± 27.6	-11.7 ± 19.8	-16.0 ± 15.1	-3.2 ± 11.5
0.06	-1.7 ± 4.9	-75.7 ± 37.0	-39.9 ± 25.8	-97.0 ± 38.9	-44.1 ± 38.0	-30.3 ± 29.9	-2.0 ± 28	27.1 ± 13.2	44.9 ± 13.7
0.125	35.5 ± 5.6	-20.8 ± 33.4	-30 ± 25.6	-31.8 ± 18.3	-39.2 ± 21.9	-65.2 ± 16.9	-6.4 ± 10.3	14.4 ± 11.3	37.9 ± 8.0
0.25	66.8 ± 5.4	10.0 ± 26.1	31.9 ± 19.0	29.6 ± 18.3	30.0 ± 18.7	31.8 ± 16.8	54.2 ± 10.4	54.5 ± 17.0	59.2 ± 7.9
0.5	74.4 ± 6.2	67.7 ± 9.6	78.1 ± 6.6	83.6 ± 4.4	75.6 ± 4.7	80.5 ± 5.8	73.6 ± 8.5	88.9 ± 3.7	78.1 ± 6.6
1	-10.3 ± 19.0	-75.7 ± 47.7	-30.5 ± 41.6	-22.6 ± 39.1	-44.0 ± 34	-18.7 ± 36.7	50.4 ± 21.5	43.6 ± 19.7	84.4 ± 9.8
2	82.5 ± 5.3	64.1 ± 16.6	73.7 ± 10.7	80.4 ± 9.1	74.7 ± 13.4	74.4 ± 9.3	76.2 ± 9.7	47.1 ± 22.2	81.9 ± 11.9
4	91.5 ± 4.5	88.7 ± 5.4	82.6 ± 7.7	80.5 ± 8.3	86.9 ± 7.1	89.8 ± 5.5	91.3 ± 6.7	84.9 ± 6.5	75.7 ± 16.8

[Appendix F8 provides a cross-reference of all data in both the text and Figure 34 of Section 4.2.2(d)]

Appendix F9: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) after an 18 h exposure of preformed 6 h-old *S. aureus* ATCC biofilms to amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-119.8 ± 11.1	-108.5 ± 9.2	-106.9 ± 12.0	-142.8 ± 9.6	-190.8 ± 11.9	-290.4 ± 22.0	-218.3 ± 14.3	-97.0 ± 10.6
0.03	-27.9 ± 8.5	-42.2 ± 14.0	-69.4 ± 18.2	-70.6 ± 13.3	-85.5 ± 13.5	-104.2 ± 14.4	-160.7 ± 38.0	-69.8 ± 12.7	-42.5 ± 28.4
0.06	-34.6 ± 8.0	-30.1 ± 16.2	-52.8 ± 23.2	-57.0 ± 21.0	-68.2 ± 22.2	-74.1 ± 24.3	-130.6 ± 44.9	-28.6 ± 27.2	-51.5 ± 27.0
0.125	-38.9 ± 11.0	-5.9 ± 19.3	-24.3 ± 21.6	-21.7 ± 22.5	-33.5 ± 25.1	-37.6 ± 34.9	-89.7 ± 52.3	7.1 ± 33.7	-30.9 ± 27.3
0.25	-9.9 ± 7.4	-6.4 ± 16.5	-30.1 ± 20.9	-22.6 ± 20.6	-20.6 ± 25.5	-37.2 ± 35.6	-80.2 ± 52.4	32.6 ± 27.2	-10.3 ± 27.4
0.5	-18.7 ± 11.6	-5.5 ± 20.8	-25.8 ± 21.4	-19.9 ± 23.9	-24.9 ± 24.0	-27.9 ± 33.5	-59.9 ± 46.0	4.9 ± 31.9	-1.3 ± 22.1
1	-12.1 ± 10.6	21.7 ± 13.2	4.0 ± 16.9	20.5 ± 12.7	4.9 ± 19.2	11.4 ± 25.3	28.6 ± 36.6	25.3 ± 25.2	8.0 ± 23.7
2	-19.1 ± 14.2	29.4 ± 6.3	8.8 ± 12.7	22.8 ± 13.5	28.8 ± 10.0	19.8 ± 13.6	1.3 ± 16.9	0.5 ± 27.0	21.1 ± 27.4
4	-28.4 ± 14.7	50.2 ± 8.9	23.1 ± 15.3	7.5 ± 13.9	21.9 ± 10.2	36.3 ± 8.8	34.5 ± 10.9	59.4 ± 12.8	24.2 ± 15.5

[Appendix F9 provides a cross-reference of all data in both the text and Figure 36 of Section 4.2.3(a)]

Appendix F10: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) after an 18 h incubation of preformed 6 h-old *S. aureus* clinical strain biofilms with amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-19.9 ± 9.0	12.6 ± 4.9	23.8 ± 4.3	22.6 ± 4.7	1.1 ± 4.2	-18.1 ± 3.7	-18.1 ± 4.5	-3.8 ± 5.7
0.125	-23.4 ± 6.4	-24.6 ± 8.1	16.0 ± 7.2	13.3 ± 6.3	4.3 ± 5.6	-9.5 ± 9.2	-15.0 ± 7.5	-2.4 ± 8.3	20.9 ± 4.0
0.25	-1.9 ± 3.6	-13.0 ± 6.2	1.0 ± 3.7	20.9 ± 5.3	8.9 ± 7.3	-12.9 ± 10.9	-28.8 ± 9.9	-11.5 ± 8.8	17.0 ± 6.2
0.5	7.8 ± 3.7	-6.1 ± 8.1	11.6 ± 5.4	19.5 ± 4.8	2.0 ± 10.5	-1.5 ± 9.8	-18.2 ± 13.7	-10.1 ± 9.9	17.2 ± 3.7
1	4.8 ± 3.9	-8.3 ± 6.3	17.9 ± 4.1	22.5 ± 5.1	11.2 ± 8.7	-6.5 ± 14.5	-27.3 ± 17.2	-4.4 ± 11.5	31.4 ± 8.1
2	9.1 ± 4.3	6.0 ± 6.1	23.9 ± 3.9	13.7 ± 12.5	8.9 ± 13.9	-6.0 ± 15.0	-5.9 ± 15.1	5.9 ± 16.6	38.4 ± 6.0
4	16.3 ± 4.5	20.6 ± 9.3	33.7 ± 8.7	38.3 ± 9.1	16.3 ± 12.6	27.3 ± 18.6	7.1 ± 18.3	21.9 ± 17.1	33.3 ± 8.3
8	13.7 ± 4.5	18.0 ± 10.6	31.6 ± 8.5	38.2 ± 8.9	6.4 ± 12.6	-2.9 ± 23.0	11.6 ± 17.0	21.4 ± 16.3	41.0 ± 10.5
16	6.6 ± 5.2	22.5 ± 13.4	34.0 ± 10.1	39.0 ± 10.4	15.2 ± 10.7	21.2 ± 16.8	17.5 ± 16.5	29.7 ± 13.5	36.9 ± 9.9

[Appendix F10 provides a cross-reference of all data in both the text and Figure 38 of Section 4.2.3(b)]

Appendix F11: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) after an 18 h incubation of preformed 6 h-old *P. aeruginosa* ATCC biofilms with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-113.3 ± 14.6	-80.0 ± 9.7	-100.2 ± 13.1	-88.5 ± 16.3	-55.2 ± 16.2	-37.8 ± 16.9	-32.5 ± 15.4	-39.3 ± 15.6
0.03	13.0 ± 6.9	4.7 ± 17.0	29.4 ± 15.7	39.2 ± 11.5	20.9 ± 14.3	34.8 ± 12.5	27.6 ± 17.9	33.2 ± 16.6	-32.7 ± 22.4
0.06	30.6 ± 15.1	19.3 ± 18.9	50.5 ± 12.4	**73.8 ± 7.4**	**68.6 ± 9.7**	**65.7 ± 10.3**	**71.9 ± 7.8**	**59 ± 13.5**	**50.9 ± 14.1**
0.125	4.4 ± 4.1	-6.4 ± 16.1	-4.1 ± 22.4	-5.1 ± 26.2	1.9 ± 25.2	9.3 ± 22.0	10.5 ± 19.2	26.5 ± 12.9	26.7 ± 13.0
0.25	**58.9 ± 12.1**	37.8 ± 14.6	41.8 ± 12.7	31.0 ± 17.3	48.5 ± 12.8	52.0 ± 8.8	**54.6 ± 13.9**	**54.6 ± 12.9**	34.4 ± 11.8
0.5	**75.6 ± 8.4**	42.6 ± 13.0	44.1 ± 13.8	43.2 ± 16.8	55.4 ± 13.0	**68.6 ± 8.9**	**72.1 ± 7.4**	**64.1 ± 9.7**	**53.9 ± 11.3**
1	**28.5 ± 13.0**	27.9 ± 23.2	32.7 ± 20.3	17.5 ± 21.6	46.0 ± 16.8	45.0 ± 17.2	**44.9 ± 18.4**	46.2 ± 14.0	47.0 ± 12.5
2	**29.3 ± 9.4**	47.2 ± 15.5	55.4 ± 9.3	41.7 ± 17.2	**62.2 ± 8.4**	**62.2 ± 9.7**	**66.6 ± 7.5**	**57.5 ± 11.1**	33.7 ± 16.1
4	**73.9 ± 7.9**	53.8 ± 9.6	59.0 ± 8.7	50.1 ± 17.9	**64.3 ± 8.6**	**68.2 ± 7.1**	**52.5 ± 16.7**	**59.2 ± 9.0**	43.3 ± 12.7

Shaded cells indicate values that are significantly ($p \leq 0.05$) different from the untreated control (0.0)

[Appendix F11 provides a cross-reference of all data in both the text and Figure 40 of Section 4.2.3(c)]

Appendix F12: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) after an 18 h incubation of preformed 6 h-old *P. aeruginosa* clinical strain biofilms with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-65.5 ± 8.3	-37.5 ± 9.9	-12.4 ± 11.1	-25.1 ± 10.7	-36.5 ± 10.4	-38.5 ± 10.8	-46.0 ± 13.8	-46.0 ± 12.4
0.03	-22.3 ± 8.0	-60.4 ± 16.8	-34.4 ± 14.4	-29.3 ± 13.7	-56.8 ± 15.6	-41.2 ± 13.0	-43.9 ± 16.7	-48.7 ± 20.2	-54.4 ± 21.6
0.06	-7.5 ± 6.8	-66.5 ± 14.8	-35.5 ± 17.8	-13.9 ± 15.2	-14.1 ± 12.5	-14.3 ± 10.0	-29.3 ± 21.1	-39.6 ± 22.4	-28.8 ± 18.4
0.125	1.6 ± 8.6	-29.2 ± 12.2	-14.6 ± 15.8	-11.3 ± 16.7	10.7 ± 7.5	-7.1 ± 10.3	-1.8 ± 13.9	-19.2 ± 16.4	-13.3 ± 15.8
0.25	16.0 ± 7.9	5.9 ± 9.6	15.8 ± 13.4	25.7 ± 18.7	17.9 ± 10.9	31.1 ± 12.7	19.4 ± 9.1	6.4 ± 15.7	9.3 ± 13.2
0.5	50.6 ± 9.2	45.4 ± 8.9	44.4 ± 8.4	52.6 ± 8.2	47.4 ± 9.2	46.1 ± 9.8	48.5 ± 10.8	29.9 ± 17.3	30.0 ± 13.1
1	-48.3 ± 26.7	-87.6 ± 33.2	-59.9 ± 35.6	-34.0 ± 25.9	-57.3 ± 27.5	-71.2 ± 39.2	-38.0 ± 28.1	-47.8 ± 33.2	6.8 ± 20.2
2	18.2 ± 7.5	-21.7 ± 22.5	5.2 ± 12.4	29.2 ± 20.3	8.9 ± 16.5	6.5 ± 15.8	8.6 ± 18.0	49.1 ± 73.6	-25.4 ± 22.4
4	21.2 ± 7.3	-0.9 ± 21.0	28.2 ± 10.2	36.1 ± 12.6	47.8 ± 7.9	37.5 ± 11.6	30.4 ± 16.5	20.3 ± 12.8	-3.1 ± 11.2

[Appendix F12 provides a cross-reference of all data in both the text and Figure 42 of Section 4.2.3(d)]

Appendix F13: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) after a 24 h exposure of preformed 24 h-old *S. aureus* ATCC biofilms to amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	**-62.1 ± 9.3**	-46.7 ± 11.6	-55.4 ± 16.7	**-92.2 ± 20.5**	**-95.7 ± 12**	**-135.1 ± 24.4**	**-118.7 ± 28.4**	**-78.7 ± 16.5**
0.03	-12.1 ± 7.5	-58.2 ± 14.7	5.3 ± 14.5	-52.8 ± 29.0	-46.1 ± 15.4	-79.2 ± 19.9	-93.1 ± 21.6	**-144.2 ± 23.5**	-77.9 ± 14.2
0.06	-32.3 ± 13.9	-45.2 ± 12.8	0.3 ± 18.4	-46.8 ± 30.5	-44.5 ± 8.0	**-107.0 ± 16.2**	**-110.6 ± 13.6**	**-122.2 ± 29.7**	-81.2 ± 27.5
0.125	-27.9 ± 11.3	-52.2 ± 20.6	5.7 ± 15.5	-74.2 ± 37.4	-34.1 ± 10.5	**-106.1 ± 10.9**	-88.3 ± 37.6	**-130.1 ± 21.6**	**-87.8 ± 18.3**
0.25	-14.4 ± 12.6	-19.0 ± 12.9	2.6 ± 17.2	-66.5 ± 39.7	-56.6 ± 16.2	**-105.7 ± 13.0**	**-147.5 ± 14.7**	**-125.6 ± 26.6**	-68.2 ± 10.4
0.5	-25 ± 17.3	-13.5 ± 17.1	10.2 ± 19.7	-26.9 ± 37.3	-21.4 ± 17.0	-50.2 ± 18.9	**-120.9 ± 22.4**	**-153.6 ± 48.1**	**-92.8 ± 22.4**
1	-30.9 ± 18.7	18.7 ± 15.7	30.0 ± 18.0	19.5 ± 28.5	11.1 ± 17.4	2.7 ± 10.6	-85.8 ± 23.1	**-130.4 ± 36.9**	**-95.5 ± 17.2**
2	-33.4 ± 11.8	4.3 ± 19.4	34.5 ± 12.7	22.5 ± 20.7	5.9 ± 23.0	7.1 ± 19.4	-45.3 ± 17.5	**-116.7 ± 29.9**	**-81.3 ± 18.5**
4	-54.6 ± 7.1	10.9 ± 14.7	-33.9 ± 36.9	-40.4 ± 40.6	-28.6 ± 33.0	-2.5 ± 25.1	-1.3 ± 16.1	-90.3 ± 23.3	-36.3 ± 12.3

Shaded cells indicate values that are significantly ($p \leq 0.05$) different from the untreated control (0.0)

[Appendix F13 provides a cross-reference of all data in both the text and Figure 44 of Section 4.2.4(a)]

Appendix F14: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) after a 24 h exposure of preformed 24 h-old *S. aureus* clinical strain biofilms with amoxicillin-clavulanate, Pectinex and a checkerboard matrix of combinations of the two agents.

Amoxicillin-clavulanate (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	6.5 ± 9.8	14.8 ± 9.9	-10.0 ± 10.0	-43.8 ± 13.6	-73.2 ± 17.7	-70.4 ± 15.7	-79.8 ± 30.7	-23.9 ± 11.7
0.125	14.9 ± 3.8	30.0 ± 8.8	9.3 ± 12.8	-6.1 ± 19.2	-8.2 ± 12.7	-34.0 ± 20.5	-37.7 ± 25.8	-37.2 ± 17.9	-5.5 ± 20.1
0.25	-9.8 ± 4.1	17.3 ± 13.2	26.5 ± 13.2	-25.9 ± 14.3	-41.0 ± 24.5	-39.4 ± 29.5	-29.4 ± 24.8	-49.4 ± 24.6	-9.0 ± 22.4
0.5	-3.8 ± 5.0	33.3 ± 8.6	0.3 ± 13.0	3.3 ± 16.8	-38.8 ± 18.1	-59.2 ± 24.2	-56.3 ± 25.4	-42.7 ± 17.5	-12.2 ± 20.2
1	-5.8 ± 4.5	24.5 ± 11.4	1.7 ± 13.9	-6.4 ± 18.7	-55.3 ± 22.9	-57.9 ± 23.8	-52.8 ± 30.3	-35.5 ± 18.1	-27.1 ± 25.1
2	-5.2 ± 4.0	35.0 ± 11.7	3.6 ± 13.5	-10.3 ± 19.5	-24.0 ± 19.3	-51.7 ± 25.7	-50.4 ± 23.7	-46.8 ± 19	-10.0 ± 18.6
4	-12.8 ± 3.9	23.3 ± 10.3	11.7 ± 13.3	7.3 ± 17.3	-50.6 ± 20.3	-58.3 ± 27	-50.0 ± 24.2	-64.0 ± 22.0	-11.4 ± 19.3
8	-5.1 ± 5.8	23.6 ± 8.9	11.6 ± 14.5	-14.5 ± 16.6	-15.9 ± 22.7	-12.0 ± 24.7	-57.4 ± 21.4	-54.7 ± 17.7	-32.2 ± 22.5
16	15.9 ± 6.5	35.5 ± 11.7	23.0 ± 15.2	9.9 ± 17.1	-1.8 ± 22.3	-35.6 ± 25.6	-46.3 ± 26.9	-41.7 ± 16.1	-22.3 ± 19.7

[Appendix F14 provides a cross-reference of all data in both the text and Figure 46 of Section 4.2.4(b)]

Appendix F15: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) after a 24 h exposure of preformed 24 h-old *P. aeruginosa* ATCC biofilms with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-84.9 ± 18.4	-30.1 ± 16.5	-8.1 ± 15.9	-38.6 ± 17.3	9.7 ± 15.8	17.0 ± 23.5	-16.0 ± 25.1	-7.7 ± 24.0
0.03	19.9 ± 11.6	29.6 ± 19.3	58.0 ± 16.8	53.7 ± 12.1	34.6 ± 18.8	7.1 ± 18.4	-13.8 ± 23.6	-33.0 ± 31.2	-43.6 ± 39.3
0.06	42.2 ± 10.2	29.1 ± 27.7	64.9 ± 14.6	70.9 ± 13.4	47.4 ± 17.2	33.0 ± 25.3	31.4 ± 26.4	30.4 ± 18.6	12.9 ± 28.5
0.125	53.2 ± 6.4	42.3 ± 17.5	59.0 ± 14.9	53.7 ± 11.8	55.9 ± 16.3	31.2 ± 20.4	18.0 ± 19.9	31.7 ± 19.9	32.1 ± 18.4
0.25	78.1 ± 3.5	71.1 ± 10.1	80.1 ± 6.7	54.7 ± 16.7	46.0 ± 26.8	28.9 ± 21.4	16.4 ± 22.3	19.3 ± 22.8	5.8 ± 34.0
0.5	63.2 ± 8.1	86.7 ± 8.0	89.4 ± 7.7	79.1 ± 16.3	78.7 ± 21.3	31.7 ± 29.6	23.8 ± 26.0	47.4 ± 15.5	1.7 ± 22.8
1	85.5 ± 8.3	79.8 ± 5.5	90.8 ± 6.1	62.6 ± 19.4	75.9 ± 23.0	33.0 ± 27.0	56.0 ± 28.8	47.0 ± 22.0	56.2 ± 23.0
2	55.6 ± 9.0	80.8 ± 2.9	90.1 ± 6.7	56.6 ± 24.1	69.8 ± 18.2	19.3 ± 28.3	43.8 ± 23.3	30.9 ± 23.6	41.1 ± 16.3
4	62.7 ± 6.1	76.1 ± 9.5	73.9 ± 13.4	66.9 ± 14.8	79.9 ± 14.2	35.3 ± 21.3	66.0 ± 13.5	51.9 ± 17.4	55.1 ± 15.4

[Appendix F15 provides a cross-reference of all data in both the text and Figure 48 of Section 4.2.4(c)]

Appendix F16: Mean percentage (%) reduction ± S.E.M. in MTT OD560nm (compared to the untreated control) after a 24 h exposure of preformed 24 h-old *P. aeruginosa* clinical strain biofilms with ciprofloxacin, Pectinex and a checkerboard matrix of combinations of the two agents.

Ciprofloxacin (µg/ml) \ Pectinex (PGU/ml)	**0.0**	**7.42**	**14.84**	**29.69**	**59.38**	**118.75**	**237.5**	**475**	**950**
0.0	0.0	-18.3 ± 8.5	-12.5 ± 8.2	-4.8 ± 8.4	-35.0 ± 21.5	-5.4 ± 8.4	-2.4 ± 9.3	16.6 ± 7.7	16.0 ± 6.4
0.03	-3.7 ± 16.9	13.7 ± 14.4	12.2 ± 15.3	22.4 ± 22.1	36.2 ± 14.7	31.8 ± 19.7	18.0 ± 21.8	14.5 ± 15.3	-27.8 ± 25.8
0.06	23.8 ± 9.9	30.2 ± 17.3	31.2 ± 15.3	38.3 ± 17.9	37.5 ± 14.5	29.8 ± 15.5	35.0 ± 16.2	30.0 ± 12.3	21.0 ± 15.5
0.125	47.4 ± 9.3	24.1 ± 15.7	7.7 ± 29.9	31.9 ± 16.0	24.7 ± 13.7	4.7 ± 19.7	34.7 ± 12.8	34.6 ± 9.6	7.6 ± 16.3
0.25	98.4 ± 5.5	49.5 ± 13.2	11.9 ± 54.0	47.5 ± 12.0	55.2 ± 8.1	49.6 ± 11.0	53.4 ± 6.5	30.8 ± 12.7	27.3 ± 16.9
0.5	93.9 ± 6.0	66.4 ± 8.7	68.6 ± 7.2	63.3 ± 6.2	62.1 ± 7.3	56.7 ± 7.0	64.3 ± 11.0	45.5 ± 12.5	32.6 ± 8.8
1	99.9 ± 6.0	77.7 ± 11.8	64.5 ± 13.9	73.3 ± 10.2	62.2 ± 12.8	65.1 ± 12.5	72.6 ± 8.6	52.4 ± 11.5	43.6 ± 9.7
2	84.6 ± 4.6	51.3 ± 15.1	64.0 ± 12.1	64.5 ± 13.5	36.0 ± 18.4	48.3 ± 16.4	53.7 ± 21.5	34.3 ± 24.6	45.2 ± 13.8
4	80.2 ± 6.9	53.0 ± 22.4	46.5 ± 21.9	67.1 ± 13.1	27.5 ± 28.3	5.5 ± 43.0	57.3 ± 19.8	39.7 ± 26.6	49.2 ± 20.6

[Appendix F16 provides a cross-reference of all data in both the text and Figure 50 of Section 4.2.4(d)]

APPENDIX G: CYTOTOXICITY ASSAY ON HELA CELLS, LYMPHOCYTES AND NEUTROPHILS

Mean percentage (%) inhibition ± S.E.M. of Pectinex (7.42 – 950 PGU/ml) and mitomycin C (10 µg/ml) derived as the mean of the percentage inhibitions of the individual data points of 6 replicate experiments.

Cell culture(s)	Mean % Inhibition± S.E.M. of Pectinex and Mitomycin C (10 µg/ml)								
	7.42	14.84	29.69	59.38	118.75	237.50	475.00	950.00	Mitomycin
[1] H pH 4.8	3.5 ± 19.8	4.6 ± 20.7	16.9 ± 20.5	18.2 ± 21.3	42.9 ± 14.9	58.5 ± 18.0	91.3 ± 4.6	99.9 ± 0.9	99.0 ± 1.6
[1] H pH 7.0	15.5 ± 14.4	5.5 ± 15.6	13.6 ± 25.2	15.6 ± 18.6	24.7 ± 15.2	44.8 ± 10.5	87.2 ± 8.5	99.8 ± 1.1	
[2] H pH 4.8	-10.2 ± 32.3	2.6 ± 30.0	-0.8 ± 18.9	9.8 ± 21.0	26.8 ± 24.6	54.3 ± 24.9	87.4 ± 14.2	96.4 ± 18.1	95.1 ± 9.9
[2] H pH 7.0	2.5 ± 8.5	-4.4 ± 12.1	-2.0 ± 10.5	-7.4 ± 7.9	8.4 ± 9.8	33.6 ± 14.7	72.1 ± 11.4	91.0 ± 4.0	
[1] L ns	-6.8 ± 25.0	4.3 ± 11.3	-7.8 ± 8.0	-17.3 ± 24.3	-2.7 ± 17.5	-12.5 ± 33.1	2.7 ± 6.5	86.1 ± 11.3	32.8 ± 30.4
[1] L fMLP	20.9 ± 18.3	20.3 ± 19.3	19.0 ± 23.8	25.6 ± 18.9	28.3 ± 22.6	40.1 ± 19.1	53.0 ± 13.7	96.1 ± 5.1	
[2] L ns	17.9 ± 73.2	5.4 ± 16.1	16.3 ± 12.4	13.8 ± 56.8	9.1 ± 5.3	29.4 ± 14.0	30.0 ± 23.4	70.0 ± 10.7	50.3 ± 48.2
[2] L fMLP	29.0 ± 37.5	22.0 ± 18.7	22.2 ± 23.0	24.3 ± 22.6	29.6 ± 26.4	32.0 ± 27.1	50.3 ± 28.5	87.5 ± 24.7	
[1] N 4 h ns	3.6 ± 40.1	10.5 ± 43.7	27.1 ± 34.0	20.4 ± 24.3	22.5 ± 24.4	3.8 ± 36.9	35.4 ± 40.7	65.3 ± 26.1	20.6 ± 33.6
[1] N 4 h fMLP	-26.3 ± 18.2	-11.8 ± 20.7	19.4 ± 13.1	23.0 ± 16.4	15.5 ± 21.0	13.1 ± 34.3	24.4 ± 15.6	78.0 ± 17.3	
[2] N 4 h ns	5.2 ± 5.4	8.9 ± 2.7	14.3 ± 2.3	46.2 ± 29.6	40.1 ± 12.2	12.9 ± 22.9	49.1 ± 13.3	56.3 ± 0.6	16.0 ± 27.5
[2] N 4 h fMLP	16.8 ± 30.3	10.4 ± 21.5	14.9 ± 18.7	22.5 ± 29.0	18.7 ± 25.2	22.3 ± 31.5	10.7 ± 37.3	28.9 ± 16.2	
[1] N 24 h ns	1.3 ± 18.4	12.3 ± 27.5	5.8 ± 36.4	-6.3 ± 7.3	6.4 ± 31.9	11.0 ± 16.8	20.3 ± 5.8	66.0 ± 13.9	-5.2 ± 16.1
[1] N 24 h fMLP	-17.1 ± 26.0	9.6 ± 9.3	-7.9 ± 1.8	-10.1 ± 12.3	9.5 ± 17.2	41.8 ± 28.2	73.7 ± 16.2	89.6 ± 17.6	
[2] N 24 h ns	6.9 ± 29.2	26.0 ± 15.4	28.1 ± 14.7	35.4 ± 20.4	36.7 ± 14.7	45.0 ± 18.0	57.4 ± 24.2	57.0 ± 17.9	24.5 ± 25.2
[2] N 24 h fMLP	12.9 ± 17.1	27.9 ± 19.1	31.2 ± 19.9	37.5 ± 22.0	49.4 ± 17.3	53.6 ± 19.5	56.5 ± 23.7	43.3 ± 14.1	

Shaded values = $p \leq 0.05$, compared with untreated control (0%); [1] = Method 1, with cell washing stages; [2] = Method 2, without cell washing; H = HeLa cells; L = Lymphocytes; N = Neutrophils; ns = non-stimulated; fMLP = fMLP-stimulated; 4h and 24 h = neutrophil incubation time(s). [Appendix G provides a cross-reference of all data in Figures 51 – 57 in Section 4.3]