The role and control of matrix metalloproteinases (MMPs) in equine cutaneous wounds: a focus on both endogenous and exogenous proteases

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By

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Abstract

Cutaneous wound healing is orchestrated by a number of physiological pathways which ultimately lead to re-formation of skin integrity and the production of functional scar tissue. Matrix metalloproteinases (MMPs) act to control the remodelling of the extracellular matrix (ECM). Regulation of MMPs is imperative for wound healing as excessive levels of MMPs can lead to disproportionate destruction of the wound ECM compared to ECM deposition. In addition to human MMPs, bacterial proteases have been found to be influential in tissue breakdown and as such have a role to play in the healing of infected wounds. Of particular interest in this thesis is *Pseudomonas aeruginosa* elastase, which has been reported in previous studies to induce degradation of host proteins including proteoglycans in chronic wounds and has also been shown to degrade host immune cell mediators. This evidence, along with recent data demonstrating the presence of *P. aeruginosa* biofilms in equine wounds, led to the hypothesis that *P. aeruginosa* proteases play a role in the non-healing of equine wounds and that the wound environment has an influence on the pathogenicity of *P. aeruginosa* in terms of protease expression.

In this thesis I identify that isolates of *P. aeruginosa* obtained from equine wounds display a highly variable protease expression and biofilm forming potential (BFP). Furthermore, environmental changes made to *P. aeruginosa* cultures including changes to the initial culture pH (i.e. changes to the initial pH of the media in a non-buffered system) and depletion of oxygen *in vitro* were shown to alter protease expression. Further experimentation has shown that the biofilm mode of growth causes changes in the proteolytic profiles of these isolates.

A further study was conducted to investigate the effects of exoproducts derived from *P. aeruginosa* in addition to purified *P. aeruginosa* elastase on the viability, growth and MMP/TIMP mRNA expression of equine dermal fibroblasts *in vitro*. Preliminary findings demonstrated that *P. aeruginosa* exoproducts, including purified elastase exert deleterious effects on both normal skin fibroblasts and granulation tissue fibroblasts in a concentration-dependent manner.

Immunohistochemistry analysis of wound tissue revealed the presence of bacterial clusters disseminated throughout the wound bed and this bacterial staining occurred in conjunction with moderate staining of MMPs -2, -3 and -13 throughout the tissue. Furthermore, whilst data is preliminary, QRT-PCR analysis showed that mRNA of each of the MMPs and TIMPs of interest were expressed in normal skin fibroblasts and granulation tissue fibroblasts to a higher level than GapDH, with cells from different equine donors responding differently in terms of MMP/TIMP mRNA expression when treated with *P. aeruginosa* conditioned media and *P. aeruginosa* elastase.

My results also identified the potential MMP modulating capacity of a novel foam dressing which incorporates polyphosphate. The dressing was effectively able to reduce the levels of MMP-2 and MMP-9 in both their active and latent forms and the dressing demonstrated its potential in the regulation of *P. aeruginosa* derived proteases.

In light of the results from these investigations, it is proposed that in future work the proteolytic phenotype and BFP of infecting bacteria should be considered when investigating the clinical nature of chronically infected wounds.

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List of Abbreviations

AE-IgG	Antielastase immunoglobulin G
ANOVA	Analysis of variance
ATCC	American type culture collection
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
BFP	Biofilm forming potential
BLAST	Basic local alignment search tool
$CaCl_2$	Calcium chloride
CBA	Columbian blood agar
CFU	Colony-forming unit
CLED	Cysteine lactose electrolyte deficient
DEAE	Diethylaminoethanol
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
ECR	Elastin Congo red
EDTA	Ethylenediaminetetraacetic acid
EGT	Exuberant granulation tissue
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polymeric substance
FCS	Foetal calf serum
GapDH	Glyceraldehyde 3-phosphate dehydrogenase
GT	Granulation tissue fibroblasts
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
H&E	Haematoxylin and eosin
IFN	Interferon

IL	Interleukin
I1	Isolate 1
I2	Isolate 2
LasB	*Pseudomonas aeruginosa* elastase B
LasA	*Pseudomonas aeruginosa* elastase A
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCA	MaConkey agar
MHB	Mueller Hinton broth
MMP	Matrix metalloproteinase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MT-MMP	Membrane-type matrix metalloproteinase
NaOH	Sodium hydroxide
NF	Normal skin fibroblasts
NOSF	Nano-oligosaccharide factor
OD	Optical density
OD_c	Cutt-off optical density
ORC	Oxidised regenerated cellulose
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	*Pseudomonas aeruginosa* elastase
PLEH	Phillip Leverhulme equine hospital
qRT-PCR	Quantitative real time polymerase chain reaction
QS	Quorum sensing
RAV	Relative activity value
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sIL-6R	Soluble interleukin-6 receptor
SMP	*Serratia marcescens* metalloproteinase
SNP	Single-nucleotide polymorphism
TCA	Trichloric acid
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TLC	Technology lipido-colloid
tPA	Tissue-type plasminogen activator
TNF	Tumour necrosis factor
TSA	Tryptone soy agar
uPA	Urokinase-type plasminogen activator

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1.0 Chapter 1

Introduction: Manuscript 1

The role of endogenous and exogenous enzymes in chronic wounds: a focus on the implications of aberrant levels of both host and bacterial proteases in wound healing

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1.1 Abstract

Cutaneous wound healing is orchestrated by a number of physiological pathways which ultimately lead to re-formation of skin integrity and the production of functional scar tissue. The remodelling of a wound is significantly affected by matrix metalloproteinases (MMPs) which act to control the degradation of the extracellular matrix (ECM). Regulation of MMPs is imperative for wound healing as excessive levels of MMPs can lead to disproportionate destruction of the wound ECM compared to ECM deposition. In addition to human MMPs bacterial proteases have been found to be influential in tissue breakdown and as such have a role to play in the healing of infected wounds. For example the zinc-metalloproteinase, elastase, produced by *Pseudomonas aeruginosa* has been shown to induce the degradation of fibroblast proteins including extracellular matrix component fibronectin and the proteoglycan decorin, and has also been shown to degrade host immune cell mediators, including complement components.

Microbial extracellular enzymes have also been shown to degrade proteins in human wound fluid and inhibit fibroblast cell growth. It is now being acknowledged that host and bacterial MMPs may act synergistically to cause tissue breakdown within the wound bed. Several studies have suggested that bacterial derived secreted proteases may act to up-regulate the levels of MMPs produced by the host cells. Together, these findings indicate that bacterial phenotype in terms of protease producing potential of bacteria should be taken into consideration during diagnostic and clinical intervention of infected wound management. Furthermore, both host MMPs and those derived from infecting bacteria need to be targeted in order to increase the healing capacity of the injured tissue.

The aim of this review is to investigate the evidence suggestive of a relationship between unregulated levels of both host and bacterial proteases and delayed wound healing.

1.2 Introduction

Cutaneous wound healing is the result of a complex process of physiological pathways, including many cell-cell and cell-matrix signalling pathways as well as communication between a number of cell lineages via soluble mediators, specifically between the epidermal and dermal layers (Werner & Grose, 2003; Martin, 1997; Singer & Clark, 1999). This is achieved through the careful balance between stimulating and inhibitory mediators during each stage of healing. When the wound does not heal in an orderly set of stages and in a short period of time they become chronic: chronic wounds are generally classified as those which fail to heal within three months of initial injury (Mustoe, 2005). It is those wounds which remain in one particular phase of healing, usually the inflammatory stage which is thought to synchronise the wound healing process (Harding, Morris & Patel, 2002), without a normal balance between deposition and degradation of extracellular matrix (ECM) components (most notably collagens) that become increasingly problematic (Diegelmann & Evans, 2004).

In normal physiology the connective tissue ECM is constantly being remodelled for growth and regeneration given its non-static nature (Bosman & Stamenkovic, 2003). In terms of the cellular interactions which occur in normal wound healing, dynamic signalling pathways trigger comparatively latent cell lineages at the wound margin to proliferate, invade the wound area and lay down new matrix proteins in order to replace the wound area with new tissue (granulation tissue and scar tissue) which ultimately leads to the healing of the wound (Martin, 1997). The process begins with the formation of a fibrin clot followed by a local inflammatory response with the infiltration of neutrophils and monocytes as a means to prevent infection from contaminating bacteria (Singer & Clark, 1999). Not only are these effector cells able to resist invading pathogens but they are also involved in tissue degradation and re-formation and in turn aid in the control of cell migration, proliferation and differentiation (Eming, Krieg & Davidson, 2007). Indeed, it has long been known that the inflammatory process following cutaneous injury is involved in the initiation of fibrosis, as well as its protective role (Simpson & Ross, 1972). Neutrophils are also involved in the debridement of necrotic tissue and phagocytosis of infectious agents, as they release a number of antimicrobial agents such as reactive oxygen

species (ROS), cationic peptides, and proteases (Eming, Krieg & Davidson, 2007) including neutrophil elastase (Yager & Nwomeh, 1999). Other important inflammatory cells involved in wound healing are mast cells which are ubiquitous effector cells involved in angiogenesis and the release of proinflammatory mediators (Nishikori, Shiota and Okunishi, 2014; Weller *et al*, 2006) and T lymphocytes which play a pivotal role in cell-mediated immunity. The inflammatory phase of healing begins shortly after injury and continues over the next 2-3 days in normally healing wounds (Falanga, 2005).The proliferation stage of healing follows the inflammatory stage, which involves the formation of granulation tissue (consisting of endothelial cells, macrophages and fibroblasts). Finally, the re-epithelisation of the wounded area along with angiogenesis and remodelling of the skin achieves tissue integrity and homeostasis (Martin, 1997; Singer & Clark, 1999). Keratinocytes detach from the basement membrane and migrate to cover exposed connective tissue with a gradual degradation of the wound clot and replacement by epithelial cells and granulation tissue. A selective release of signalling molecules produced by immune mediators acts to attract fibroblasts and keratinocytes to the wound bed, including interleukin-8 (Garner *et al*, 1994).

Given the primary function of skin to serve as a protective barrier against environmental pathogens and thus infection, the loss of integrity of this important organ may ultimately lead to massive disability or even mortality in extreme cases (Singer & Clark, 1999). It is reported that there are approximately 250,000 burn victims per year in the UK (Hettiaratchy & Dziewulski, 2004) and over 15% of general hospital in-patients develop a pressure ulcer (Courtney, Ruppman & Cooper, 2006). These wounds in particular can have serious implications for the patient, on both a physiological level causing pain, discomfort and immobility and also on the psychological and psychosocial level. Furthermore, given that chronic wounds mostly affect individuals later on in life (Mustoe, 2004), as the population ages the incidence of chronic wounds is set to rise (Supp & Boyce, 2005). In addition, not only can these wounds have significant implications on the health of the patient, they can have a huge financial impact on both the individual patient and the health care system as a whole. Cumulatively, this highlights the ever pressing need for improvements in clinical intervention strategies directed at efficient wound healing, with the primary goal of treatment being the rapid closure of the wound with a

resulting functional and aesthetically acceptable scar (Singer & Clark, 1999). Furthermore, continued progress in the comprehension of the diverse stages of wound repair and tissue regeneration will help to provide systems to manipulate and modulate other diseases with similar aetiology involving impaired tissue remodelling such as cancer.

The three main chronic wounds include pressure ulcers, diabetic ulcers and venous ulcers. With this in mind, it has been proposed that chronic wound pathogenesis is based primarily on three major pathological stages: the cellular and systemic changes of ageing, repeated ischemia-reperfusion injury and bacterial colonisation with an ensuing inflammatory host response (Mustoe, 2004).

The ECM is important for normal morphogenesis and structural support. It is composed of collagens, laminins, fibronectin, vitronectin, and proteoglycans among other structural proteins (Raghow, 1994). This structure supports cellular growth and proliferation and is important for normal skin regeneration and effective wound repair (Kleinman, Philp & Hoffman, 2003). The ECM is composed of two distinct regions: the basement membrane and the interstitial matrix (dermis) (Bosman & Stamenkovic, 2003). It is a complex mix of structural and functional proteins, glycoproteins and proteoglycans arranged in a tissue-specific ultrastructure (Badylak, 2002). During the healing of a wound, a provisional matrix is synthesised primarily of fibrin and fibronectin, acting as a scaffold for epithelial cell migration during repair (Clark *et al*, 1982; Greiling & Clark, 1997). An imbalance between ECM formation and destruction may have local and potentially systemic effects. The ECM is essentially a framework with signalling peptide sequences used to direct cells into the wound space and then activates these cells once bound to the matrix and is an ideal environment to support cellular proliferation and growth (Raghow, 1994). In chronic wounds the balance in ECM maintenance is disrupted partly due to an imbalance between proteinases and their inhibitors (Harding, Morris & Patel, 2002).

The normal end result of wound restoration is the formation of scar tissue which although provides a stable new barrier is subject to structural and aesthetically displeasing abnormalities. However, it is believed that in mammals, the 'normal' wound repair process has been evolutionarily optimised through a rapid inflammatory response for efficient healing to avoid complications such as infection (Bayat, McGrouther & Ferguson, 2003) and it is this level of skin restoration which is clinically important following the healing of a chronic wound.

Equine wounds have a characteristic elevated level of exuberant granulation tissue (EGT) which can lead to the formation of sarcoids (Cochrane, Freeman & Knottenbelt, 1996) and in some instances may resemble the human keloid scar, a benign fibroproliferative dermal lesion (Jacobs *et al*, 1984). In particular, it is the lower limb which encounters wounds which do not heal with efficiency in comparison to those wounds inflicted upon the head and trunk (Jacobs *et al*, 1984). It has been suggested that a limited local blood supply in the distal regions in both humans and horses due to limited tissue coverage over underlying bone and a poor vascular bed correlates to the poor wound healing observed in lower extremity wounds (Knottenbelt, 1997; Theoret & Wilmink, 2013). Exuberant and indolent equine wounds have distinct similarities with human leg ulcers, with these wound types more common in elderly patients in both humans and horses (Knottenbelt, 1997). Research has also revealed that wounds in ponies have been shown to heal more rapidly than those wounds observed in larger horses (Wilmink *et al*, 1999; Wilmink *et al*, 1999b; Stashak & Theoret, 2008; Theoret & Wilmink, 2013). The more rapid wound healing seen in wounds in ponies has been attributed in part to a greater contribution of wound contraction during wound healing, more effective myofibroblast organisation and a more efficient acute inflammatory response (Wilmink *et al*, 1999(a); Wilmink *et al*, 1999(b); Van Den Boom *et al*, 2002). In equine limb wounds, it has been shown that the wound retracts to nearly twice its original size within 2 weeks of the initial injury and the wound can take more than 3 months to completely heal (Wilmink *et al*, 1999). However, although epithelialisation begins a few hours after the initial injury, this is a slow process in equine limb wounds with epithelialisation occurring at a rate of 0.2mm per day in flank region equine wounds, compared with a rate as low as 0.09mm per day in lower limb wounds (Wilmink & Van Weeren, 2005; Stashak & Theoret, 2008).

Research into the pathology of these wounds may help to find clinically important targets against both functional and aesthetic problems associated with poor wound healing in horses and may lead to similar targets in human wound restoration. However, cell culture studies incur a number of limitations in generating data that can be related directly to the *in vivo* biological and genetic events occurring in the wound, given the variation in these environments (Brown & Bayat, 2009). Difficulties in studying genetic and molecular alterations in wound healing may also be hampered by the multifaceted and intricate pathological alterations involved in this complex process of events.

It is the aim of this review to analyse the current data within the literature which demonstrates either a positive or negative effect of MMPs or other relevant host proteases in the transformation from acute to chronic wound healing. Furthermore, considerations will be made into the possible detrimental effects of those proteases secreted from invading wound pathogens and the net effects on wound protease levels.

1.3 Proteases and wound remodelling

Cellular stimuli caused by injury causes the expression of several MMPs at the wound site (Toriseva *et al*, 2012). MMPs are believed to have a role in all phases of wound healing, contributing to the removal of cellular debris and detachment of keratinocytes from the basement membrane shortly after injury then remodelling of the provisional matrix (Gill & Parks, 2008; Torriseva & Kähäri, 2009; Mirastschijski, Schnabel & Tomasek, 2010). Proteolytic degradation of the ECM is vital for the efficient healing of cutaneous wounds (Werb, 1997) and the unregulated activity of such proteases is a major pathological mechanism underlying non-healing wounds (Palolhati *et al*, 1993; Barrick, Campbell & Owen, 1999). It is known that various activated cell types including activated keratinocytes at the wound edge, fibroblasts, and endothelial cells all share a common characteristic of protease expression, however it is the invading neutrophils and macrophages which are considered to be the major source of proteases at the wound site (Eming, Krieg & Davidson, 2007). While fibroblasts are a major source of matrix metalloproteinases (MMPs) in the healing wound, secreting proteases capable of degrading collagens, elastin and proteoglycans, neutrophils and macrophages recruited during the inflammatory phase release elastase and collagenase which degrade damaged extracellular matrix components (Tarnuzzer & Schultz, 1996). Furthermore, proteinases with broad specificity including elastase, cathepsin G and urokinase may have detrimental effects on growth factor function (Yager *et al*, 1997; Wlaschek *et al*, 1997). Neutrophil elastase has also been shown to have antimicrobial activity in the wound environment (Cole *et al*, 2001).

There are a number of proteolytic enzymes present at the wound site including the serine proteases, such as plasmin and various matrix metalloproteinases (MMPs). These proteases have an important role in the migration of keratinocytes to the wound bed. Immature keratinocytes produce MMPs and plasmin, allowing the keratinocytes to dissociate from the basement membrane thus facilitating migration (Simonetti *et al*, 2013). MMP-9 has been shown to be upregulated in wounded skin compared with unwounded skin in parallel with an increase in CD44 molecules which are involved in cell-cell and cell-matrix interactions (Simonetti *et al*, 2013).

MMP-1 is able to bind to α2β1 integrin within the wound bed, resulting in the migration of keratinocytes which ultimately contact interstitial collagens during epidermal wound healing (Sternlicht & Werb, 2001; Dumin *et al*, 2001). As studied by Kubo *et al* (2001), wound edge keratinocytes do not express the receptors required for fibrin/fibrinogen adhesion which is thought to be a mechanism for the removal of fibrin during tissue remodelling (Kubo *et al*, 2001). MMPs may play a role in this fibrinolytic pathway, allowing the epidermal cells to migrate across the wound bed. Plasminogen also plays a role in keratinocyte migration with one study utilising mice with an impaired plasminogen gene to demonstrate this function (Rømer *et al*, 1996). The study concluded that there was a reduced migration rate of keratinocytes from the wound margin during re-epithelialisation (Rømer *et al*, 1996). It is believed that this may be due to the decreased ability of these cells to navigate their way proteolytically through the fibrin-rich ECM in the deep wound tissue (Lund *et al*, 1999). Lund and colleagues (Lund *et al*, 1999) have also demonstrated the importance of plasmin and MMPs in wound healing with the residual wound healing capacity observed in the plasminogen-deficient mice possibly being a result of the ability of MMPs to perform alone the functions which they would perform together with plasmin in wild-type mice. However, a direct functional overlap between plasmin and MMPs was not demonstrated.

There is a generally high level of proteolytic activity in chronic wound fluid. Treatment aimed at the re-establishment of the natural balance of proteases as well as cytokines and growth factors and their inhibitors within the wound site, such as the application of combinations of selective inhibitors of metalloproteinases and growth factors may be beneficial (Tarnuzzer & Schultz, 1996). Conversely, it may be that combinations of certain growth factors applied to the wound at precisely timed intervals may be more effective in the promotion of healing (Singer & Clark, 1999).

Protease expression at the periphery of the wound aids leading edge keratinocytes to dissolve the fibrin (in the wound clot) through the fibrinolytic enzyme plasmin (Martin, 1997). This enzyme is derived from plasminogen and is activated by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) (Lijnen & Collen, 1987). The role of plasminogen in wound repair has been

demonstrated in transgenic mice where the plasminogen gene is knocked out and the re-epithelialisation of the wound is almost abolished (Rømer *et al*, 1996). Furthermore, key regulators of plasminogen activation are expressed by migrating keratinocytes (Rømer *et al*, 1996; Morioka *et al*, 1987; Rømer *et al*, 1994). Various classes of MMPs are also up-regulated by wound-edge keratinocytes and are involved in cell migration. MMP-9 (gelatinase), for example, has been shown to cleave basal lamina collagen type IV and anchoring fibril collagen type VII, which then releases keratinocytes to the basal lamina (Martin, 1997; Salo *et al*, 1994). On the contrary, the many experimental studies which have identified MMPs 2 and 9 as type IV collagenases should be interpreted with caution (Rowe & Weiss, 2008). In terms of basement membrane remodelling, the role of MMP2 and MMP9 is questionable given that the triple helical structure of collagen type IV under *in vivo* temperature is resistant to proteolytic breakdown (Rowe & Weiss, 2008). However, evidence has continued to suggest an elevated presence of these MMPs within the basement membrane during tissue repair (Gschwandtner *et al*, 2008; Matsubara, Zieske & Fini, 1991). In comparison, MMP-1 (interstitial collagenase) is only up-regulated in basal keratinocytes which have already migrated beyond the free edge of the basal lamina, suggesting that cell-matrix interactions control the expression of this MMP (Martin, 1997; Saarialho-Kere *et al*, 1992). MMP-1 is involved in the migration of keratinocytes on collagen and so is important in the initiation of re-epithelialisation (Pilcher *et al*, 1997; Toriseva *et al*, 2012). MMP-10 is also up-regulated by keratinocytes at the margin of the wound and is specifically increased during impaired/prolonged healing (Martin, 1997; Saarialho-Kere *et al*, 1994).

1.4 Structure and Function of MMPs

MMPs are a Zinc-dependent group of endopeptidases (proteolytic peptidases that break peptide bonds of non-terminal amino acids- i.e. within the molecule) and are part of a larger family of proteases called the metzincin superfamily (Ravanti & Kähäri, 2000; Parks, Wilson & López-Boado, 2004). Proteolysis aids in degradation of specific ECM proteins and can process bioactive molecules (Werb, 1997). The cleavage of cell surface receptors, release of apoptotic ligands (e.g. FAS ligand), chemokine/cytokine in/activation are all pathways initiated by MMPs which can regulate the wound healing process (Roeb, 2008). Importantly, they are involved in cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defence (Roeb, 2008). These enzymes are dependent on metal ions and other co-factors (i.e. non-protein chemical compounds bound to a protein required for the protein's biological activity, usually other enzymes). This distinguishes MMPs from other endopeptidases. They have a common domain structure: Prop-peptide, catalytic domain, and a haemopexin-like c-terminal domain (linked to catalytic domain by flexible hinge region) (Murphy & Knäuper, 1997; Van Wart & Birkedal-Hansen, 1990). Figure 1 illustrates the structure of the gelatinases, MMPs -2 and -9, which have the addition of fibronectin type II modules which bind to gelatin, collagens and laminin (Visse and Nagase, 2003).

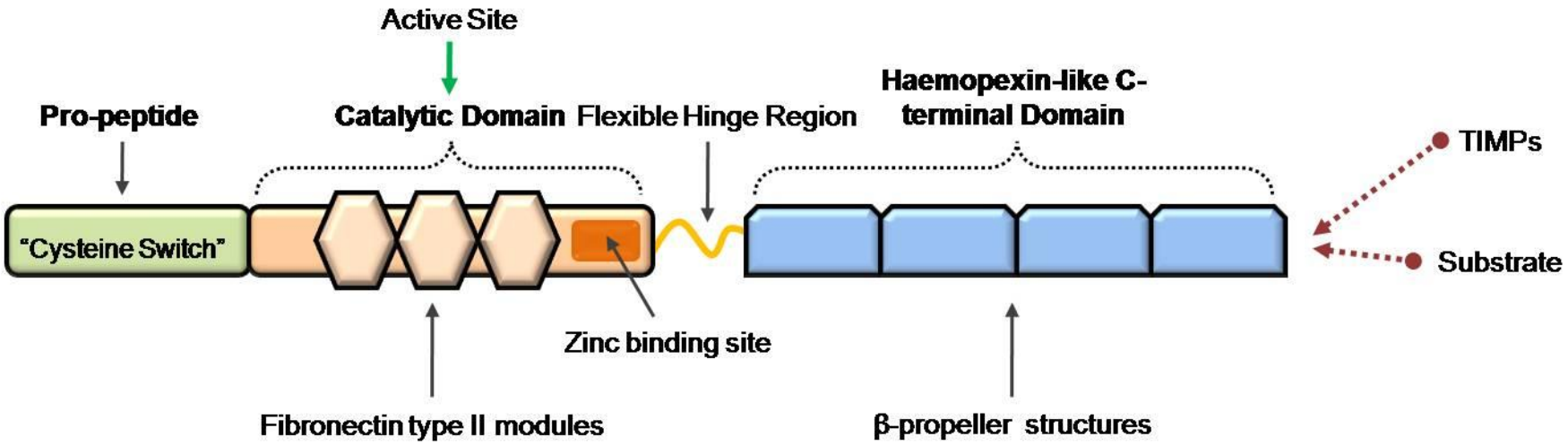

Figure 1: Structure of gelatinases (MMP-2/MMP-9). The three common domains are described as the pro-peptide, catalytic domain, and the hemopexin-like c-terminal domain (connected to the catalytic domain via a flexible hinge region). As MMPs are synthesized as inactive zymogens, the pro-peptide (part of the cysteine switch) must be cleaved to activate the protease. The cysteine switch contains a conserved cysteine residue which interacts with the zinc in the catalytic domain, preventing activation of the enzyme. The b-propeller structures in the c-terminal domain provide a substrate/TIMP recognition site.

MMPs are initially synthesised as inactive zymogens. For activation, the pro-peptide is removed. The pro-peptide domain is part of a "cysteine switch" which contains conserved cysteine residues and interacts with zinc in the active site, preventing binding/cleavage of the substrate (Van Wart & Birkedal-Hansen, 1990). This mechanism keeps the enzyme in an inactive state so as to control MMP expression during different healing demands of the skin. Additionally, some MMPs have a prohormone convertase cleavage site (Furin-like) in this pro-peptide domain which, when cleaved, activates the enzyme, particularly the membrane-type metalloproteinases (Stawowy *et al*, 2005). The catalytic domain is an active site running across catalytic domain with catalytically important Zn^{2+} ion, bound by 3 histidine residues (zinc-binding motif). The hinge region connects the catalytic domain to c-terminal domain and contains up to 75 amino acids, but with no determinable structure (Murphy & Knäuper, 1997). The haemopexin-like c-terminal domain is absent in MMPs 7, 23 and 26 (Parks, Wilson & López-Boado, 2004). It is composed of a four bladed β-propeller structure which provides a large flat surface ideal for protein-protein interactions. This part of the MMP structure determines substrate specificity and is also the site for interaction with TIMPs.

The classification of MMPs remains a subject of much debate, particularly in the identification of the MMP-2 and MMP-9 substrates (Rowe & Weiss, 2008). However, there are 4 classes of MMPs which have consistently been grouped according to substrate specificity: collagenases, which are thought to degrade triple-helical fibrillar collagens into shorter fragments and include MMP 1, 8, 13 and 18 (Nelson *et al*, 2000); gelatinases, which degrade ECM components including type IV collagen, gelatin and elastin (MMPs 2 and 9) (Steffensen, Hakkinen & Larjava, 2001; Bauvois, 2012); the stromelysins including MMP 3, 10 and 11 which have a broad substrate specificity being able to degrade several ECM components such as collagens, gelatin, laminin, elastin and proteoglycans (Steffensen, Hakkinen & Larjava, 2001; Chandler *et al*, 1997; Kerkelä *et al*, 2001; Rechardt et al, 2000) and the Membrane-type MMPs (MT-MMPs) comprising MMPs 14-17, 24 and 25 which also have a role in the degradation of ECM components (Marco, Fortin and Fulop, 2013).

1.5 Implications for MMPs in delayed wound healing

MMPs have a significantly profound effect on wound healing. In addition to the role of these enzymes in tissue remodelling, with a particular effect on the deposition and degradation of collagens and other ECM components, MMPs have a role in keratinocyte migration to the wound site and degradation of growth factors and their receptors (Stamenkovic, 2003). In normal response to injury, epithelial cells migrate across the wounded tissue to close the wound area and restore the barrier to infection (preventing the entry of environmental pathogens and leakage of fluids). Cell-matrix (intracellular and extracellular) interactions control migration. MMPs can facilitate and also suppress cell movement by affecting the state of these cell-matrix interactions and proliferation. At present, there are 26 MMPs known to exist.

Both MMPs and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs) exist in a state of dynamic equilibrium, with a slight excess of one or the other depending on the need for either ECM breakdown or synthesis. Pathological consequences stem from prolonged disruption to this balance in MMPs and their inhibitors (along with other mediators involved in wound healing such as growth factors and cytokines). Specific examples include laminitis in horses and cattle caused by the destruction of ECM in the lamellar basement membrane (Kyaw Tanner & Pollitt, 2004), and osteoarthritis in which the articular cartilage is progressively destroyed (Naito *et al*, 1999). However, a significant decrease in MMP activity can potentially lead to impeded wound healing by preventing fibrinolysis in fibrotic conditions (such as in the case of hypertrophic scarring and keloid formation) (Soo *et al*, 2000) and the resulting decrease in the removal of scar tissue in wounds.

It has been shown that the expression of various MMPs are upregulated during wound repair and regeneration with mRNAs encoding respective MMPs including gelatinase A (MMP-2), gelatinase B (MMP-9), collagenase-3 (MMP-13), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), MT-1 MMP (MMP-14) and macrophage metalloelastase (MMP-12) being upregulated (Lund *et al*, 1999). These have been detectable by *in situ* hybridization with probes specific for these MMPs. There is no detectable mRNA for any of these MMPs in normal skin compared to a

vast expression in wounded skin (Lund *et al*, 1999; Saarialho-Kere, 1998; Norgauer *et al*, 2002). Pro-inflammatory cytokines are considered as potent inducers of MMP expression in chronic wounds and it has been shown that they down regulate the expression of tissue inhibitor of metalloproteinases (TIMPs) which in turn creates an over-expression of MMPs (Eming, Krieg & Davidson, 2007). In particular, TNF-α and IL-1β are capable of increasing the levels of MMPs in wounds (Barrientos *et al*, 2008). Furthermore, important factors in healing such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are targeted by these proteases and are inactivated via proteolytic cleavage (Lauer *et al*, 2000; Roth *et al*, 2006).

It is also recognised that neoplastic progression is a realistic consequence of chronic wounding, with the risk of squamous cell carcinoma (SCC) significantly increased in chronic leg ulcers (Impola *et al*, 2005). Since MMPs are implicated in the pathogenesis of wound healing and are known to be involved in all stages of tumourigenesis it may be that epithelial MMP expression can influence hyperplasia in chronic wounds (Impola *et al*, 2005) and may be involved in tumour invasion and metastasis (Chin *et al*, 2005). The up-regulation of MMPs in most human cancers has been shown to be a reaction to the presence of tumour cells and is produced mainly by the stromal cells and inflammatory cells which infiltrate the tumour (Ramnath & Creaven, 2004). Indeed, Sternlicht *et al* (1999) and D'Armiento *et al* (1995) found MMP-1 and 3 alleles to be associated with an increased risk of cancer. MMP expression is also regulated by SNPs in the MMP gene promoter regions which create/abolish transcription factor binding sites (Overall & López-Otín, 2002).

The expression of MMPs in the various anatomical layers of wounded skin

Salo and colleagues (1994) demonstrated the localisation of various MMPs throughout wound repair. They found that the expression of MMP-2 was localised in connective tissue fibroblasts and endothelial cells during each of the phases of wound healing whereas MMP-9 mRNA was found to be located in the mucosal epithelium in the first few days of healing, yet the mucosal epithelium was mostly negative for MMP-2 expression. Comparatively, the non-wounded area of the skin expressed a high level of MMP-9 which was localised in the basal and suprabasal cell layers, with only the basal cell layer being MMP-9 positive in the migrating

epithelial sheet. In the later stages of healing, MMP-9 mRNA expression was significantly increased, specifically within the granulation tissue. Wound fluid also contained increased levels of MMP-9 (evident in the earlier stages) as compared to those levels of MMP-2. It was also discovered that a number of cytokines and growth factors are able to preferentially regulate the expression of MMP-9, in particular through keratinocyte production. Specifically, it was IL-1β, TGFβ-1 and tumour necrosis factor (TNF)-α which were able to enhance this production of MMP-9. In contrast, MMP-2 expression in cultured wound fibroblasts was not at all effected by the presence of any of these molecular mediators (Salo *et al*, 1994). Other important signalling pathways have a role in MMP expression within wounds, such as the mitogen-activated protein kinases (MAPKs) pathway and are possible therapeutic targets for wound healing disorders (Ravanti & Kähäri, 2000). Excessive collagenolytic activity in chronic wounds is also heightened due to reduced levels of tissue inhibitor metalloproteinase 1 (TIMP-1) (Armstrong & Jude, 2002).

The study by Ladwig *et al* (2002) was aimed at analysing pro and activated MMP-2 and MMP-9 in wound fluids and tissue biopsies from patients with chronic pressure ulcers. Patients were treated conventionally or with exogenous cytokine therapies over a period of 36 days. Quantitative assays were used to correlate the levels of these MMPs with the level of healing present in each wound. It was noted that the average ratio of MMP-9/TIMP-1 in fluids from chronic ulcers was significantly decreased as the ulcers healed. Additionally, in those wounds which ultimately healed well, fluids collected on day 0 of the wound had a significantly lower ratio of MMP-9/TIMP-1 compared with fluids from day 0 of wounds that healed poorly. Therefore, this study showed that the MMP-9/TIMP-1 ratio is a predictor of healing efficiency in pressure ulcers and shows that high levels of MMP activity along with comparatively low levels of TIMP activity, impairs wound healing in chronic pressure ulcers and is comparative to similar studies investigating other types of chronic abnormal cutaneous healing.

1.6 Biofilms- their role in chronic wound healing

Biofilms are communities of microbial aggregates which form as a survival mechanism due to diverse adverse environmental conditions. They form through a structured process of adherence and matrix forming mechanisms to create an intra and intercellular communication network for rapid adaptation to their changing environment. Biofilms are able to adhere to both biological and non-biological surfaces, mainly colonizing the solid-liquid interfaces (Costerton, Stewart & Greenberg, 1999; Donlan, 2002). As the bacterial bio-mass begins to proliferate on the surface a self-produced extracellular polymeric matrix is formed which offers protection from both mechanical and shear forces and, if formed on a living tissue, from host immune defences and antimicrobial agents. The ability of bacteria to form biofilms is an ancient and evolutionary emergent property which provides greater tensile strength and increased genetic potential. This formation of a functional community allows for metabolic co-operation between microbes and provides a wide source of nutritional recourses so that the bacteria can modify their own environment and proliferate.

As the biofilm-forming microcolony begins to grow, signals are passed between the cells after a certain density is reached. This is known as the quorum and the cells will then begin to release virulence factors as a defence system against the host's polymorphonuclear leukocytes (Widgerow, 2009). This cell-cell signalling mechanism is known as quorum sensing and is an important part of the biofilm's ability to produce both cell-associated and extra-cellular virulence factors for survival and to allow synchronization of activities of large groups of cells (Waters & Bassler, 2005). Quorum sensing mechanisms allow the population of microorganisms to change their phenotype to form a biofilm when a certain density is reached (Scali & Kunimoto, 2013). The quorum sensing controlled processes are only effective in the simultaneous signalling manner present within a large group of bacteria and are therefore exclusive to the biofilm communities (Waters & Bassler, 2005). This system is generally composed of a transcriptional activator protein, and small diffusible molecules which act as autoinducers that increase in concentration in response to an increase in cell density (Fuqua, Winans & Greenberg, 1994; Swift *et*

al, 1996; Scali & Kunimoto, 2013). These autoinducers are hormone-like molecules which form the basis of the chemical communication within the biofilm; this communication network involves the production, release, detection and response to these molecules (Waters & Bassler, 2005). This phenomenon allows the bio-mass to act as a single multicelluar organism (Fuqua, Winans & Greenberg, 1994) and the bacterial community is able to monitor and alter its behaviour on a population-wide scale in response to changes in the number of organisms, and/or the types of species present (Waters & Bassler, 2005). Cell-associated virulence factors include those for movement and attachment, such as the formation of flagellum for motility (Mahenthiralingham, Campbell & Speert, 1994), pilli and other adhesions (Simpson, Ramphal & Lory, 1992), and alginate (Chattoraj *et al*, 2010) particularly in the case of *Pseudomonas aeruginosa*, which aids the impairment of host innate immune defences imposed by phagocytes (Learn, Brestel & Seetharama, 1987; Krieg *et al*, 1988). The extracellular virulence factors include various enzymes and exotoxins. Important enzymes in terms of host tissue degradation and evasion/destruction of host physical barriers include elastase, alkaline protease and phopholipase C (Berka, Gray & Vasil, 1981; Leidal *et al* 2003; Lyczak, Cannon & Pier, 2000).

The mechanism of biofilm formation begins with the adherence and colonization of single cells of planktonic bacteria to a primed surface and, in terms of wound colonization, attachment to the exposed extracellular matrix of the injured tissue, developing into microcolonies (Widgerow, 2009). In acute wounds, these bacteria are normally rapidly inactivated and destroyed by neutrophils, antibodies and host wound bed preparations (Widgerow, 2009). However, in the chronic wound these bacteria often proliferate and produce an extracellular polymeric substance (EPS) which encases the population of bacteria forming the biofilm structure, providing the biofilm with additional protection from external factors including the host's immune system (Percival *et al*, 2011). The EPS can vary considerably in terms of pH, charge, ionic strength, physiochemical structure and the EPS can also vary depending on species (Percival *et al*, 2011). Figure 2 illustrates the processes involved in normal wound healing and the detrimental effects of biofilm formation on wound healing.

It is generally noted that biofilm formation is comprised of eight sequential steps: preparation of a conditioning film, transport, initial adhesion, co-adhesion, anchoring, coaggregation, growth and proliferation, and detachment. Dispersion of the individual bacteria from the biofilm matrix allows the activation of the host immune response. This action is a positive one in that the biofilm is able to gain nutrients from the host exudates produced by the resulting inflammatory response for the continual survival of the bacterial community (Widgerow, 2009). This is one example of how bacterial communities can take advantage of the host environment and in particular the innate host defences and benefit from the stimulation of the inflammatory response (Hornef *et al*, 2002). The resulting increase in wound exudate is detrimental to the regeneration of the wound as it continues to break down the extracellular matrix and blocks the effectiveness of intervention therapy including the use of growth factors and bioengineered skin (Falanga, 2002) and so a positive feedback mechanism is formed, leading the wound into a chronic mode of healing. Indeed, protease inhibitors have been shown to preserve the activity of growth factors within chronic wounds (Wlaschek *et al,* 1997). Deficiencies in growth factors in wounds are likely to be involved in the transformation from acute to chronic wounds. A recent study by Roy *et al* (2014) has also demonstrated the long-term impact of biofilm infection in chronic wounds. The study showed that biofilm infection can compromise skin barrier function in the closed wound, which could potentially lead to further complications post-closure including an increased risk of further infection (Roy *et al*, 2014).

The formation of a biofilm also incorporates channels to facilitate the efficient nutrient uptake and sharing by infusing fluid into the biofilm, optimizing nutrient delivery and metabolic waste-product removal (Hall-Stoodley, Costerton & Stoodley, 2004; Scali & Kunimoto, 2013). Biofilms can also be enhanced through their structure according to varying environments. Biofilms can be either flat or mushroom-shaped depending on the nutrient source (Hall-Stoodley, Costerton & Stoodley, 2004; Klausen *et al*, 2003).

The effects of biofilm culture on exoprotease production have previously been demonstrated in a number of pathogenic bacteria. For example, a quorum-sensing dependent regulation of exoprotease production in *Aeromonas hydophila,* affecting both the serine and metalloproteases has been established (Swift *et al*, 1999). Similarly, induction of the *LasB* gene in *Pseudomonas aeruginosa* encoding for elastase, along with other virulence genes, requires LasR and PAI-1 which are components of the quorum sensing activation pathway (Pearson, Pesci & Iglewski, 1997).

One of the major colonising bacteria found within the wound bed is *Pseudomonas aeruginosa* and it has consistently been shown to be a factor in the prognosis of cystic fibrosis (CF). This pathogen is known to secrete a range of harmful proteases which act as virulence factors in the lungs of CF patients (Cosgrove *et al*, 2009). Phenotypes which enable *P. aeruginosa* to be successful in the chronic infection of CF lungs include mucoidy and reduced twitching motility (Mayer-Hamblett *et al*, 2014). Since the bacteria residing in biofilms have limited supply of oxygen, anaerobic respiration is a necessary mode of growth which can occur via an inorganic terminal electron acceptor resulting in rapid growth or via substrate level phosphorylation using arginine, causing a slow mode of growth (Hassett *et al*, 2002). It has been demonstrated that the proteases secreted by *P. aeruginosa* differ under aerobic and anaerobic culture. Cosgrove and colleagues (2009) have shown that under aerobic growth, this organism primarily secretes elastase whereas under anaerobic culture the major secreted protease is alkaline protease. Furthermore, there seems to be a differential role of these proteases in the host's anti-protease screen (Cosgrove *et al*, 2009).

1.7 Bacterial Proteases and their Role in Wound Healing

The proteases of the serine, cysteine and metallo-type are produced by a wide range of pathogenic bacteria and are particularly important in the pathogenesis of wound healing. The functions of these proteases include colonisation and evasion of host defences, acquisition of nutrients for growth and proliferation, dissemination of the biofilm forming bacteria to different areas of the wound, and tissue degradation (Supuran, Scozzafava & Mastrolorenzo, 2001). However, bacterial proteases may have a clinically beneficial role via the debridement of non-viable tissue in the wound area or alternatively could be involved in the migration and stimulation of host cells (Supuran, Scozzafava & Clare, 2002). A number of specific and ubiquitous proteases have been characterised in both the Gram-positive and Gram-negative colonising bacteria of wounds (Supuran, Scozzafava & Mastrolorenzo, 2001). Since the majority of antibiotics used in the clinical setting are aimed at the mechanical inhibition of bacterial cell wall biosynthesis, resistance to these agents, especially when targeting biofilms, is a huge problem. Targeting the bacterial proteases as an alternative mode of action of antibiotics (i.e. bacterial protease inhibitors) may help to solve this problem in the future (Supuran, Scozzafava & Mastrolorenzo, 2001). However, at present few potent inhibitors of these proteases have been developed. Examples of protease inhibitors which may have potential in terms of infection eradication include inhibitors of signal peptidase, clostripain, *Clostridium histolyticum* collagenase, botulinum neurotoxin and tetanus neurotoxin inhibitors (Supuran, Scozzafava & Clare, 2002). However, no inhibitors of the critically important and ubiquitous AAA proteases (membrane-bound ATP-dependent proteases), degP or sortase have been reported (Supuran, Scozzafava & Mastrolorenzo, 2001; Supuran, Scozzafava & Clare, 2002).

Microorganisms produce a large array of proteases which act in an intracellular and/or extracellular way. Intracellular proteases are important in cellular and metabolic processes, for example sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. The extracellular proteases are vital for the hydrolysis of proteins in cell-free environments and enable the bacteria to absorb and utilise hydrolytic products

(Kalisz, 1988; Gupta, Beg & Lorenz, 2002). The proteases are also able to degrade growth factors and their receptors and are particularly involved in perturbation of host cytokine networks (Wilson, Seymour & Henderson, 1998; Schultz & Wysocki, 2009). For example, Theander *et al* (1988) showed that both the alkaline protease and the elastase of *Pseudomonas aeruginosa* were able to inhibit interleukin-2 (IL-2)-induced proliferation of murine lymphocytes. Furthermore, the alkaline protease of *Pseudomonas aeruginosa* has also been shown to degrade and inactivate IFN-γ (Horvat & Parmely, 1988) which is the principal T-cell product which activates macrophages. This mechanism therefore is an important example of how these bacterial proteases aid the evasion of host defences. In addition, some bacteria are able to produce potent modulins such as lipopolysaccharide (LPS) or exotoxins which can induce the release of proinflammatory cytokines from the host, creating a net proinflammatory affect (Wilson, Seymour & Henderson, 1998), keeping the wound in the chronic phase. Proteolytic cleavage of cell surface receptors for numerous ligands can also be a target of these bacterial proteases, including cytokine receptors. Cytokines that may be targeted include those that modulate leukocytes to produce proinflammatory responses and those that have the capacity to down-regulate inflammatory cells including macrophages and leukocytes (Wilson, Seymour & Henderson, 1998). For example, proteases from a number of organisms including *S. aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Serratia marcescens* have been shown to release the IL-6 receptor (IL-6R) from human monocytes (Vollmer *et al*, 1996). Perhaps one of the most well known examples of bacterial proteases in pathogenicity is the elastase produced by *Pseudomonas aeruginosa*, which degrades immunoglobulin G and components of the host's complement system. This provides the bacteria with a survival advantage in the host environment (Vollmer *et al*, 1996; Hase & Finkelstein, 1993; Schultz & Miller, 1974). In addition to substrate inactivation, bacterial proteases can have a biological effect through the release of membrane-bound proteins. These proteins are released and can have a negative effect in a variety of ways. For example, soluble receptors may act as competitive inhibitors of other ligands or could act as agonists. One example of this action is the release of the soluble interleukin-6 receptor (sIL-6R) which can bind to neighbouring cells through specific interactions which ultimately leaves these cells sensitive to the action of IL-6 (Rose-Jonh & Heinrich, 1994). Specifically, endogenous membrane-bound metalloproteinases have been shown to

shed various host signalling factors including tumour necrosis factor alpha and its receptors, IL-6R, and the FAS ligand (Kayagaki *et al*, 1995; Crowe *et al*, 1995; Mullberg *et al*, 1995). This shedding mechanism occurs through the proteolytic cleavage of membrane anchors at specific sites close to the membrane surface (Vollmer *et al*, 1996). These trans-signalling pathways can lead to the systemic inflammatory reactions (Bazil, 1995), particularly seen in chronic wounds. In turn, the effects of a heightened immune response can lead to excessive MMP production. Indeed, a number of studies have implicated bacterial-derived proteases in the activation or increased production of host MMPs. For example, Kernacki and colleagues (1997) concluded that mice immunized against *Pseudomonas aeruginosa* alkaline protease displayed lower proteolytic activity corresponding to host and bacterial proteases in an *In vivo* study of corneal infections. It was shown that resistant mice produced a lower overall level of proteolytic activity, corresponding to a decrease in host cell MMP-2 and MMP-9 production at various time points following infection. Using zymography it was demonstrated that those mice which were classified as susceptible to infection produced larger proteolytic bands for the inactive forms of both MMP-9 and MMP-2 along with the active form of MMP-9 in comparison to resistant mice. Likewise, a study by Matsumoto and colleagues (1992) found that purified elastase from *Pseudomonas aeruginosa* was able to cleave the inactive form of MMP-2 derived from human corneal fibroblasts into a biologically active form shown by the differences in gelatinolytic band weight using zymography following a direct in-tube incubation assay. A number of pathogenic bacteria, many of which are common wound pathogens, are producers of metalloproteinases including *Staphylococcus aureus*, *P. aeruginosa*, *Serratia marcescens, Listeria monocytogenes,* and *Bacillus subtilis* (Vollmer *et al*, 1996). *S. Marcescens* metalloproteinase (SMP), which is similar in structure to the elastase of *Pseudomonas aeruginosa*, was utilised (Vollmer *et al*, 1996) to demonstrate the ability of this enzyme to shed IL-6R from primary human monocytes using an enzyme-linked immunosorbent assay (ELISA) method, concluding that microbial proteases are capable of mimicking endogenous membrane-bound proteinases in their ability to shed mediator molecules from the host cell surface.

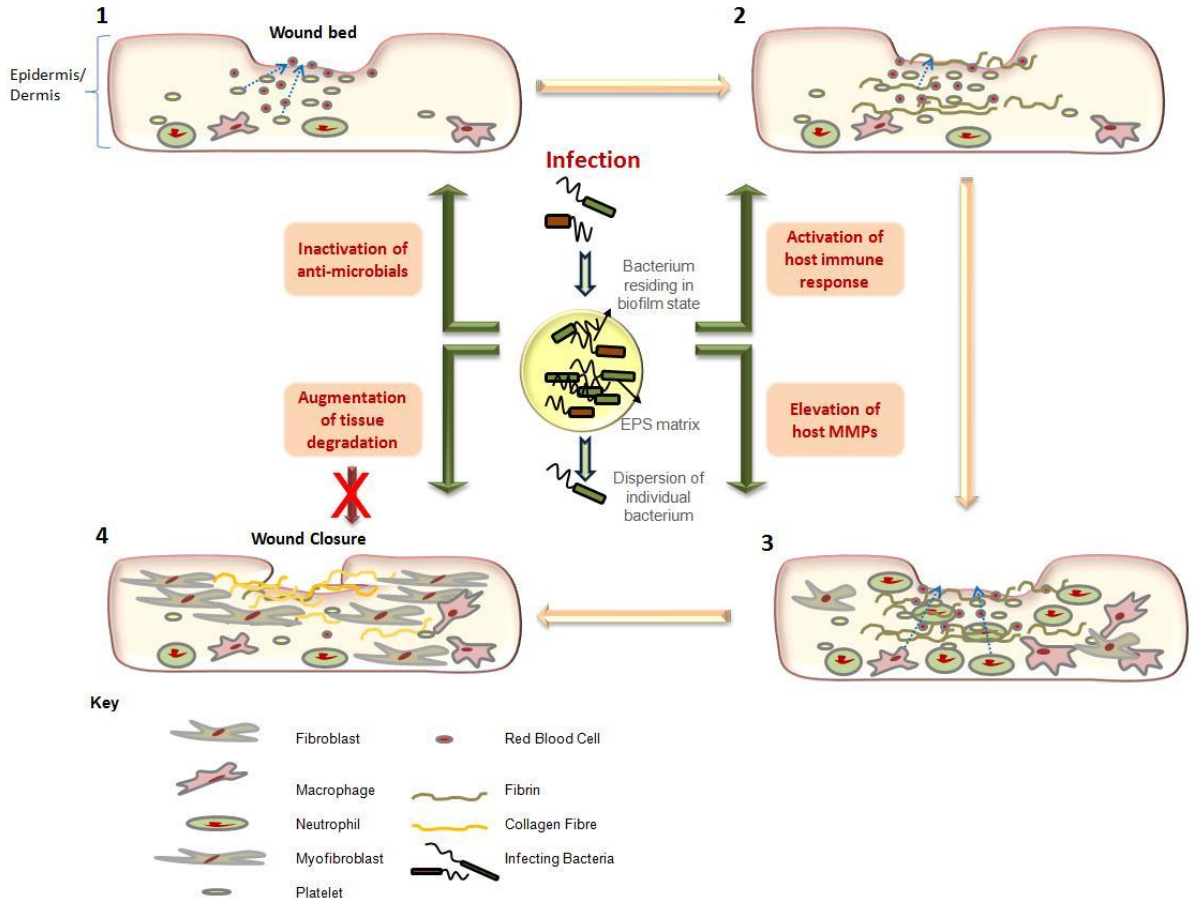

Figure 2: Schematic of the processes involved in normal wound healing and the detrimental effects of infection in terms of virulence factors associated with biofilm formation, including the induction of proteases. (1) In the initial stage of wound repair, platelets are recruited to the injury site, followed by an increase in fibrin fibers which create a clot serving as a barrier to invading pathogens (2). (3) Inflammatory cells are recruited to the wound bed to create a local inflammatory response. (4) Wound closure commences as the fibroblasts/myofibroblasts secrete collagen bundles into the wound site and the inflammatory response declines. During bacterial invasion of the wound, various cell-associated virulence factors direct the increase in tissue degeneration through the continued destruction of the extracellular matrix. This is achieved through the secretion of various bacterial proteases and further activation of the host's immune response. This process aids bacterial survival by providing nutrients in the form of matrix breakdown products/exudate, and also allows for the evasion of antimicrobial interventions.

1.8 Synthesis and Secretion of Bacterial Proteases

As mentioned previously, one of the major bacterial proteolytic enzymes studied in terms of host MMP activity is elastase B, a 33-kDa zinc-metalloprotease (Thibodeaux *et al*, 2007). The extracellular proteases of *Pseudomonas aeruginosa* are of particular interest given the opportunistic nature of this wide spread pathogen among individuals with a predilection to nosocomial infection, including sufferers of bacteremia in burn wounds, urinary tract infections and keratitis (Lyczak, Cannon & pier, 2000; Thibodeaux *et al*, 2007; Stover *et al*, 2000; Twining *et al*, 1993). Elastase is one of the many proteases produced by *Pseudomonas aeruginosa* which is secreted via the type II or general secretory pathway and is produced as a preproenzyme which is systematically processed into proelastase via autoproteolytic processing (Braun *et al*, 1998).

1.9 Management of MMPs in Wound Healing

At present, it seems to remain unclear as to why certain host MMP levels, particularly the gelatinases, are elevated. However, it has been generally accepted that by regulating MMPs in wounds this will help enhance the wound healing process. Given the large array of studies which have presented clear data to suggest an enhanced MMP activity in chronic wound tissue as compared to normally healing tissue and normal skin, the utilisation of protease-modulating treatments in chronic wounds has been shown to have beneficial effects on healing efficacy (Veves, Sheehan & Pham, 2002; Vin, Teot & Meaume, 2002; Kakagia *et al*, 2007; Chin *et al*, 2003; Timmons, 2010; Fitzgerald, 2009). Furthermore, studies have analysed ways of neutralising high levels of proteases in chronic wound fluids utilising topical doxycycline, a member of the tetracycline family of antibiotics which inhibits metalloproteinases (Chin *et al*, 2003). Veves *et al* (2002) have demonstrated the effectiveness of Promogran, a wound dressing composed of collagen and oxidised regenerated cellulose, in the inactivation of host proteases without affecting the activity of the growth factors involved in wound regeneration. Treatment of diabetic foot ulcers with this dressing resulted in an increased rate of healing when compared with moistened gauze. Similarly, UrgoCell Start is a dressing which is aimed at reducing MMP levels within the wound bed through the actions of the healing

accelerator nano-oligosaccharide factor (Timmons, 2010). Another novel wound dressing aimed at reducing MMP levels within the wound bed is Biostep, which contains a semi-denatured porcine collagen that acts to attract and bind excess MMPs in the wound environment, with EDTA permanently deactivating the MMPs by binding to the zinc ions of these proteases (Fitzgerald, 2009). However, the ability of these newly developed dressings to reduce or inhibit the activity and production of bacterial-derived proteases within the chronic wound site remains unclear.

1.10 Conclusions

It has been consistently and clearly demonstrated that there is a fundamental role for host cell MMPs in the healing of chronic wounds. However, there seems to be limited evidence to suggest that those extracellular proteases produced by the bacteria colonising chronic wounds have a significant influence on the levels of host MMPs within infected tissues. Generally there is a good understanding of the role human MMPs and other proteases have on wound healing. However, there appears to be a lack of research into the MMP inhibitors that could be used to influence the timely and systematic healing of chronic wounds. Excessive levels of MMPs are found in chronic wounds and constitute a concern warranting good management strategies along with differential clinical intervention depending on bacterial phenotype, and it is beginning to be noted that host and bacterial proteases could act synergistically to direct tissue degradation. Those studies that have aimed to prove the hypothesis that the downregulation of specific MMP related genes may enhance healing of chronic wounds have shown the positive actions that novel wound dressings and inhibitory treatments can have on chronic wound healing, demonstrating a role for these treatments in the restoration of affected tissues, the ultimate goal being the production of functionally viable and aesthetically pleasing scar tissue. However, there remains a pressing need for further research efforts in the development of treatments aimed at targeting both host and bacterial proteases within the wound environment. Research also needs to be focussed on the inter-communication between various bacterial, viral and fungal proteases among infected wounds and how these enzymes synergistically interact with host proteases and other mediators of the healing pathways involved in both acute and chronic wounds.

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1.11 Hypothesis

MMPs play a pivotal role in a range of processes that orchestrate cutaneous wound healing. The regulation of these proteases is an important part of the healing process since excessive levels of MMPs can shift the equilibrium of ECM degradation and deposition in favour of tissue breakdown and impaired wound healing. In chronic wounds an imbalance in the levels of proteinases and their inhibitors disrupt this balance of ECM deposition and degradation (Harding, Morris & Patel, 2002). A number of studies have recognised the elevated MMP activity within the wound fluid of chronic wounds and that this elevation in protease activity parallels a delay in wound closure (Trengove *et al*, 1999; Yager *et al*, 1996; Rayment, Upton and Shooter, 2008; Rowe & Weiss, 2008). However, there is little evidence on the impact of bacterial proteases on wound healing and host MMP expression despite the implications for wound infection on healing and evidence of biofilms in the wound milieu (Westgate *et al*, 2011). Furthermore, little is known about the ways in which the wound environment may alter this proteolytic activity. A particularly interesting pathogen in terms of wound healing is *Pseudomonas aeruginosa*, which is known for its prevalence in chronic wounds and demonstrates various virulence characteristics within the wound milieu (Morihara, 1964; Snell *et al*, 1978; Altoparlak *et al*, 2004; Engel & Balachandran, 2009). Further investigation into the response of *P. aeruginosa* isolates to changes in the culture environment in terms of protease production, and the effects of these proteases on wound closure will enhance our fundamental understanding of the role of bacterial proteases during the various phases of wound healing.

In this project I will address the hypothesis that clinical isolates of *P. aeruginosa* derived from equine wounds display high levels of proteolytic activity *in vitro* and that changes in the culture environment of these isolates (in particular, changes to the culture pH, oxygen availability and the biofilm versus the planktonic mode of growth) representative of the wound milieu will have an impact on protease production *in vitro*. I also aim to identify any effects of protease-rich *P. aeruginosa* conditioned media and *P. aeruginosa* elastase on host fibroblast viability, MMP/TIMP mRNA expression and wound closure using *in vitro* models.

1.12 Aims

The aims of this project are threefold:

(1) To investigate the production of proteases *in vitro* of *P. aeruginosa* isolates obtained from equine wounds throughout a 24 hour culture period and to assess the effects of the changing culture environment reflective of the wound milieu (i.e. changes in the culture pH and oxygen availability) on protease production using biochemical protease assays and gelatin zymography.

(2) To investigate the effect of the planktonic versus the biofilm mode of growth of *P. aeruginosa* equine wound isolates on protease production using *in vitro* culture models.

(3) To investigate the effects of protease-rich conditioned *P. aeruginosa* media and *P. aeruginosa* elastase on equine normal skin fibroblasts and equine granulation tissue fibroblasts in terms of cell viability, MMP/TIMP mRNA expression and wound closure, using *in vitro* models.

2.0 Chapter 2

Manuscript 2

Differential protease expression by isolates of *Pseudomonas aeruginosa* derived from equine wounds, under varying pH conditions and oxygen levels

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2.1 Abstract

P. aeruginosa is a common wound pathogen and is capable of inflicting a multitude of deleterious effects upon its host. This micro-organism has been shown to secrete a number of proteases in the wound milieu, which act to further delay the healing process. It was hypothesised that clinical isolates of *P.aeruginosa* obtained from equine cutaneous wounds display high levels of proteolytic activity *in vitro* and that changes to the *in vitro* culture conditions, in terms of pH and oxygen availability, would have an impact on protease production.

In the current study it has been shown that isolates of *P. aeruginosa* obtained from equine wounds cultured in the planktonic mode of growth display a highly variable protease expression. Furthermore, changes made to the initial culture pH (i.e. the starting pH of the broth in an un-buffered system) and depletion of oxygen in anaerobic (0% oxygen) cultures of *P. aeruginosa in vitro* caused changes in bacterial protease expression; however, different isolates responded differently to these changes. It is suggested that in future studies, the proteolytic phenotype of bacteria should be considered in terms of the clinical progression and outcome of chronically infected wounds.

2.2 Introduction

It is well documented that wound fluid derived from chronic wounds has an inherent ability to degrade important factors of healing both *in vitro* and *in vivo*. However, the mechanisms by which bacterial secretions, including protease production, influence tissue destruction and halted healing and the ways in which such mechanisms can be manipulated to aid healing requires further investigation.

A particularly interesting pathogen in terms of wound healing is *Pseudomonas aeruginosa*, which has long been known for its prevalence in chronic wounds and demonstrates various virulence characteristics within the wound milieu. Recent evidence has identified *P.aeruginosa* as one of the most commonly isolated bacteria from equine wound swabs, with 5.6% of bacterial isolates cultured from wound swabs tested in a study by Westgate *et al* (2011) identified as *P.aeruginosa* (Freeman *et al*, 2009; Westgate *et al*, 2011b). This micro-organism is a Gram negative pathogen which is difficult to eradicate despite the use of antimicrobial interventions once the infection has become established, as indicated in a number of pathologies. Indeed, *P. aeruginosa* has been identified as the most common cause of burn wound infections (Altoparlak *et al*, 2004) and multi-resistant strains have been identified in the hospital setting (Chitkara & Feierabend, 1981). As described by Engel & Balachandran (2009), *P. aeruginosa* uses a sophisticated secretion system to directly administer virulence factors to host cells. This system provides the pathogen with a tool to cause mucosal barrier injury and inhibit various aspects of the innate immune response and in turn intensifies the delay in wound repair (Engel & Balachandran, 2009).

In 1964, Morihara characterised the proteases produced by *P. aeruginosa*, specifically elastase and alkaline protease and following these observations many studies have focussed upon the pathogenic nature of *P. aeruginosa* isolates from various infection sites with particular interest placed on the protease profiles of such isolates. A study by Snell and colleagues (1978) demonstrated the role of exotoxin and proteases in the virulence of *P. aeruginosa* strains *in situ* in a mouse burn model.

Many confounding factors have been demonstrated to have a role in the augmentation or sequestration of enzyme activities within the wound environment, not least pH. The pH of the wound bed is indeed an important contributing factor in the healing process and different pH ranges are required for the various phases of healing (Schneider *et al*, 2007). Normal skin has an acidic pH in the range of pH4-6, whilst in wounds this acidic environment is disturbed and the underlying tissue with the body's internal pH of 7.4 is exposed (Schneider *et al*, 2007). Kaufman & Berger (1988) highlighted the relationship between wound healing and topical pH and concluded that wound healing could be controlled, in part, by changing pH levels at the wound site. Regarding protease production, the presence of these enzymes can both affect or be affected by changes in pH levels of the wound bed (Greener *et al*, 2005; Harjai *et al*, 2005). Quorum sensing systems, which act in part to direct virulence factor production, can also be affected by changes in pH (Horswill *et al*, 2007). A decrease in wound pH mirrors the healing process and thus a reduction in the nutrients needed for bacterial growth. In contrast, the persistence of slough within the wound bed is associated with an elevated pH and minimal reduction in the size of the wound (Gethin, Cowman & Conroy, 2008). Proteases produced by pathogenic bacteria within the wound act to liberate nutrients from the surrounding environment, aid attachment to the host surfaces and inactivate certain host defence components (Cicmanec & Holder, 1979; Lyczak, Cannon & Pier, 2000), and so this situation may cause an increase in measurable proteolytic activity. Yet, during nutrient limitation the mucoid phenotype may be promoted to protect the bacterial cells and reduce growth rate (Terry, Piña & Mattingly, 1991; Lyczak, Cannon & Pier, 2000).

Oxygen tension has also been shown to be an important factor in the healing of wounds, and in particular causes changes in bacterial colonisation and pathogenicity. In an *in vivo* animal model, Hunt and colleagues (1975) demonstrated that rabbit wounds infected with *P. aeruginosa* under hypoxic conditions (12% oxygen) displayed increased colonisation longevity than those wounds under hyperoxic (45% oxygen) conditions as confirmed by significantly higher bacterial counts following a 21 day infection period. Although the authors could not confirm a definite mechanism, they suggested that this effect may be due to depletion in the host's immune function under hypoxic conditions. The authors also noted that under

oxygen stress the toxicity of *P. aeruginosa* supernatants was greater towards a hybridoma cell line. In 2002, Sabra, Kim & Zeng carried out a study into the physiologic responses of *P. aeruginosa* to oxygen imitation using a computer-controlled bioreactor system. The authors found an increased growth rate of the bacteria under microaerobic conditions as compared to aerobic cultures. Furthermore, the total concentration of extracellular proteins was higher under microaerobic culture and also paralleled the growth curve. However, when the amount of secreted proteins was considered in terms of biomass, the actual protein yield was lower in the microaerobic cultures. Conversely, biochemical analysis revealed that only those cultures maintained under microaerobic conditions produced elastase with no significant elastase production under high dissolved oxygen concentrations. However, severe oxygen limitation led to a reduction in the production of virulence factors, including elastase.

A limited number of studies have highlighted the differences in *P. aeruginosa* virulence characteristics among different wound infections. Yet, as mentioned by Feltman *et al* (2001), many of the virulence factors produced by *P. aeruginosa* are variable traits, being produced by some clinical isolates but not others. Rumbaugh, Griswold & Hamood (1999) sought to investigate the production of virulence factors by *P. aeruginosa* isolates obtained from a number of clinical environments. They found no significant variation in elastase produced from 3 clinical sites. It was also noted that despite clinical isolation site, some of the *P. aeruginosa* isolates produced either significantly higher or lower levels of elastase, although no specific data was presented. However, no reference strain (non-clinical isolate) was used as a means of comparison. Nonetheless, it was concluded that both infection site and length of infection can influence the virulence of *P. aeruginosa* and that, although the mechanisms are not certain, the *in vivo* milieu (including antibiotic treatment) seems to have an influence on virulence factor production with such changes in pathogenicity being less likely associated with restriction site polymorphism or DNA rearrangement.

In 2001, Schmidtchen, Wolff & Hansson highlighted the variability in expression of elastase and alkaline protease by *P. aeruginosa* isolates derived from chronic leg ulcers, despite all isolates containing the genes for both proteases. The authors found that there was a phenotypic variation in protease production among the isolates, however protease levels over time was not considered and no reference (non-wound) strain was used.

The over abundance of granulation tissue in equine wounds occurs most frequently in wounds of the lower limbs and infection has been suggested as one of the influential factors, amongst others, in its generation. Furthermore, exuberant granulation tissue itself predisposes the wound to infection and further trauma (Engelen *et al*, 2004; Bertone, 1989). Lower limb equine wounds are particularly susceptible to complications caused by infection due to the limited soft tissue, muscle, blood supply and reduced tissue oxygenation to aid the healing process (Westgate *et al*, 2010). Wounds in these areas are also prone to bacterial contamination due to the ease of contaminant transfer from the surrounding environment. The prolonged inflammatory phase of healing seen in equine wounds which, in part, leads to the generation of exuberant granulation tissue (EGT) (Berry, Douglass & Sullins, 2003) may be augmented by the persistence of infection in turn contributing to longer healing times. Until recently, the microbial diversity and richness of equine wounds was relatively poorly identified. It is now clear, however, that a broad spectrum of bacteria can colonise and infect equine wounds and biofilms have also been identified in these wounds (Westgate *et al*, 2010; Cochrane *et al*, 2009; Westgate *et al*, 2011), with *P. aeruginosa* being one of the most commonly isolated bacteria from equine wounds and *Pseudomonas* spp being shown to be more prevalent among chronic wounds as compared with acute wounds (Westgate *et al*, 20011b). Resistant strains of *P. aeruginosa* to antimicrobials have been identified in the clinical setting (Pirnay *et al*, 2003). There has been little study into the roles of bacterial pathogenicity, with particular focus placed on bacterial proteases, on the delayed healing of equine wounds and how the local wound environment may augment or suppress these proteases in these wounds.

It is the aim of the current research study to investigate the production of proteases among clinical isolates of *P. aeruginosa* derived from equine wounds and to determine the effects, if any, of changes in initial culture pH and oxygen availability on enzyme productivity *in vitro* during the bacterial growth phases, with a particular focus upon *P. aeruginosa* elastase. Comparisons will also be made in terms of protease production and the biofilm forming phenotype (BFP) of these isolates.

2.3 Methods

2.3.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated.

2.3.2 Ethical Approval

Approval for this study was granted by the Ethics Committee, University of Liverpool and informed consent was obtained from the owners of the animals participating in this study.

Ethics Approval Number: RETH000355

2.3.3 Identification of *P. aeruginosa* Clinical Isolates

Standard microbiological culture techniques were used to confirm the identities of 7 previously collected equine wound isolates, which were obtained from equine skin wounds of horses within 1-3 days of attendance at the Philip Leverhulme Equine Hospital (PLEH) in Neston, South Wirral following the acquisition of informed consent from the horse owner (Westgate, 2011c) (table 1 gives equine case details from which *P. aeruginosa* isolates were obtained). Wounds were swabbed using a standardised method; briefly, a cotton-tipped swab was rubbed across a $1cm^2$ area of the wound for 5 seconds as described by Levine *et al* (1976) then stored in Stuart's transport medium (Oxoid Limited, Hampshire, UK) for the culture of aerobic bacteria which was performed within 24 hours of swab collection. Swab samples were stored at 4°C. Under sterile conditions, the swab was inoculated onto Tryptone soy agar (TSA) and also onto selective agar, namely MaConkey agar (MCA), Columbian blood agar (CBA) and Cysteine lactose electrolyte deficient (CLED) agar (all from LabM, Bury, UK) as described by Westgate *et al* (2011c). Agar plates were then incubated at 37°C (±2°C) for 24 hours. Colonies which visually resembled *P. aeruginosa* (i.e. round, convex, pearlescent appearance with a grape-like odour,

dependent on culture agar) were sub-cultured onto TSA plates and incubated for 24 hours at 37°C (±2°C). Pure bacterial colonies were identified using a Gram stain and isolates confirmed as Gram negative rods were further characterised using the oxidase test (using oxidase detection strips from Oxoid Limited, Hampshire, UK) and API 20 NE® identification strips, which utilise biochemical reactions with computer software-interpretation (Biomérieux UK Limited, Hampshire, UK). Confirmed *P. aeruginosa* isolates were stored on cryopreservation beads and frozen at -80°C until required for further investigation.

A *P. aeruginosa* reference strain (non-wound isolate) was used as a control; American Type Culture Collection (ATCC) 27853 (isolated from blood culture) (ATCC, Manassas, USA). This strain was chosen as it produces elastase and alkaline protease (O'Callaghan *et al,* 1996). An additional negative control wound isolate was used in the form of *Enterococcus faecium*, a Gram positive bacterium and common nosocomial pathogen. This isolate was identified using the API 20 Strep kit (Biomérieux UK Limited, Hampshire, UK) by the team at the Leahurst Microbiology Laboratory, University of Liverpool.

Table 1: Equine case details for each *P. aeruginosa* isolate. Cases included 5 chronic wounds, all of which were trauma wounds, and 2 acute surgical wounds.

***P. aeruginosa* Isolate**	**Case Number**	**Case Information**	**Location of Wound**	**Wound Type**	**Acute/Chronic**
Wound Isolate 1	37050	10 year old TB gelding	Unknown	Surgical	Acute
Wound Isolate 2	37682	2 year old Dx Arab gelding	Fetlock	Trauma	Chronic
Wound Isolate 3	Unknown	13 year old COBx mare	Unknown	Surgical	Acute
Wound Isolate 4	22222	7 year old gelding	Unknown	Trauma	Chronic
Wound Isolate 5	208642	13 year old COBx mare	Medial tarsus/ proximomedial metatarsus	Trauma	Acute
Wound Isolate 6	37592	3 year old TB gelding	TX of Limb	Trauma	Chronic
Wound Isolate 7	37509	21 year old TB gelding	Medial aspect of limb	Trauma	Chronic

2.3.4 *P. aeruginosa* Growth Curves

Sterile Mueller Hinton broth (MHB) (LabM, Bury, UK) was inoculated with a single loop of each corresponding *P. aeruginosa* isolate or *E. faecium* isolate (from TSA plate cultures) in 30ml portions cultured with shaking at 150rpm in a shaking incubator set at 37°C for 24 hours to stationary phase in sterile 75cm^3 culture flasks. The optical density (OD) of each culture was standardised to 0.85_{600nm} in a cuvette spectrophotometer (approximate McFarland standard 6). From each of the standardised cultures a 500µl aliquot was used to inoculate 75ml sterile MHB in 75cm^3 culture flasks. OD measurements were made in triplicate for each culture in a multi-well plate spectrophotometer at 600nm at various time points through the culture period: T0, T4, T8, T12 and T24 (corresponding to 0, 4, 8, 12 and 24 hours). The OD_{600nm} method described above was employed for the generation of growth curves for the *P. aeruginosa* isolates (and *E. faecium* isolate) grown at varied initial pH and under anaerobic conditions (0% oxygen). 'Initial' or 'standard' pH is defined as the pH at which the culture broth was set to prior to bacterial inoculation; a non-buffered system was used for the culture of *P. aeruginosa* isolates, however fluctuations in the pH of the cultures were minimal throughout the culture period (see Appendix 1).

2.3.5 Preparation of *P. aeruginosa* Conditioned Media

Sterile MHB (LabM, Bury, UK) was used as a non-buffered nutrient broth for all cultures and was made up in batches of 1 litre with distilled water. 75ml portions of MHB were inoculated with 500µl of stationary phase cultures corresponding to each individual *P. aeruginosa* isolate (and *E. faecium* isolate), as described above. Cultures were incubated with shaking at 150rpm in a shaking incubator set at 37°C (ambient O_2 conditions, ~21%) for a total culture period of 24 hours in 75cm^3 culture flasks. Culture samples were harvested at T0, T4, T8, T12 and T24 and centrifuged at 12000rpm for 10 minutes to pellet the cells. Conditioned culture supernatant was filtered through 0.45µm sterile filters to remove cell debris and stored at -80°C until required for further investigation.

For cultures with altered initial pH, MHB was prepared as done previously and pH was set using a pH meter and addition of concentrated sodium hydroxide (NaOH) or hydrochloric acid (HCl). Media was then autoclaved at 121°C and left to cool. Under sterile conditions, a sample of the sterile media was taken and the pH checked. All MHB stocks remained at the correct pH following autoclaving. The initial pH values in this study were chosen on the basis of previous research relating to the pH range for growth of *P. aeruginosa*. The pH values chosen were pH5.5, 6.0, 7.3 (standard/normal pH of MHB) and 8.0. A pH meter was used to monitor the pH of the cultures throughout each phase of growth; no significant pH fluctuations were noted throughout the entire culture period (see Appendix 1). Anaerobic cultures were prepared in an anaerobic chamber (du Scientific, Modular Atmosphere Controlled System); cultures were prepared in the same way as the aerobic cultures, with samples taken in a sterile manner at T0, T4, T8, T12, T24 and T48. Cultures were prepared once for each isolate and triplicate samples were taken as experimental replicates at each time point.

2.3.6 Polymerase Chain Reaction (PCR) Analysis of *P. aeruginosa* Elastase Gene

Genomic DNA Extraction from Bacterial Isolates

The extraction of genomic DNA from the *P. aeruginosa* and *E. faecium* isolates was performed using the boiling method, adapted from the methods described by Clarke, Millar & Moore (2003) and Sweeney, Whitlock & McAdams (2006); a loop of bacteria from an overnight culture from TSA was used to inoculate 1ml of molecular grade water in 1.5ml Eppendorf centrifuge tubes. Samples were boiled for 10 minutes in a heat block, allowed to cool and centrifuged at 12,000xg for 5 minutes. The supernatant was collected and stored at 4°C. Extracted DNA was used in PCR reactions within 48 hours of processing. DNA concentration and purity was determined using a Nanodrop (Thermo Scientific, MA, USA). The boiling extraction method yielded a range between 50 and 210ng/µl DNA for each of the isolates, and was found to be more efficient than a commercially available genomic DNA extraction kit.

PCR Conditions

Primers were designed using Primer Express 3.0 software and Primer BLAST (NCBI) was used to ensure gene specificity; there were no gene regions of similarity within the *P. aeruginosa* genome for the primers designed for the *P. aeruginosa* elastase (LasB) gene. The forward and reverse primers were 17bp and 24bp, respectively; Forward 5'- GCTGAACGACGCGCATT, Reverse 5'- AGTGCACCTTCATGTACAGCTTGT (Eurofins MWG Operon, London, UK). The primers yielded an amplicon of length 101bp. PCR was carried out in 50µl reactions in buffer containing 0.5µl Taq DNA polymerase (from Thermus aquaticus), 0.5µl dNTPs (each base), 1.65µl of each primer (300nM) and made up to the final volume with molecular grade water. PCR samples were heated at 95°C for 10 minutes before amplification. Parameters used were; 30 seconds at 94°C (denaturation), 45 seconds at 57°C (annealing), 90 seconds at 72°C (extension), 72°C for 10 minutes (final elongation). Samples were then held at 4°C. Amplified DNA was analysed by 1%

agarose gel electrophoresis and gels were visualised by staining with ethidium bromide using a 1kb molecular weight DNA ladder (Life Technologies, Paisley, UK).

2.3.7 Crystal Violet Assay for the Assessment of Biofilm Forming Phenotype (BFP)

The BFP of all bacterial isolates was determined using a microtitre plate assay based on the method described by Wakimoto *et al* (2004) and Westgate *et al* (2011b). Pure overnight cultures from TSA of each isolate were inoculated into 10ml of sterile MHB at 37°C in a shaking incubator set at 50rpm. Following incubation, a 1ml aliquot of each overnight culture was transferred to 10ml of fresh sterile MHB and the suspensions were adjusted with sterile MHB to 0.5 McFarland turbidity standard, giving a final suspension of 10^8 CFU/ml (Westgate *et al*, 2011b). ODs were also standardised at 600nm using a multi-well plate spectrophotometer. From each standardised inoculum, 250µl was transferred into triplicate wells of a Cellstar® 96-well culture plate. Blank wells contained sterile MHB only (made in triplicate). Plates were then incubated at 37°C for 24 hours. Following incubation, each well was carefully washed 3 times with 300µl of sterile physiological saline and the plates were shaken vigorously in a shaking incubator set at 150rpm for 5 minutes to remove all non-adherent bacteria. Attached bacteria were then fixed with 250µl of 96% ethanol per well for 15 minutes, after which time the plates were emptied and left to dry. For the visualisation of the biofilms, each well was stained with 200µl 2% crystal violet solution for 5 minutes. Excess stain was removed from the wells under running tap water and the biofilms were visible as purple rings formed on the sides of the wells. For quantitative assessment of biofilm formation, 200µl of 33% glacial acetic acid was added to each well and the plates were placed on a plate shaker for 5 minutes. The OD of the stain was measured using a microtitre plate reader at 570nm. The average OD_{570nm} of the MHB control wells was subtracted from the ODs of the test wells.

2.3.8 Milk-Casein Agar Plate Inoculation

Milk-casein agar was prepared with 25g skim milk powder (BD, Oxford, UK) 2.5g casein (BDH, VWR, Lutterworth, UK), 1.25g yeast extract (Oxoid, Basingstoke, UK), 0.5g D-glucose (BDH, Oxford, UK) and 6.25g No. 1 agar (LabM Bury, UK). Agar was sterilised and poured in 20ml portions into 9cm round petri dishes. A sterile 8mm punch biopsy was used to bore single wells in each of the milk plates. Supernatant samples recovered from the cultures were used to inoculate the milk-casein agar plates; 100µl aliquots of each sample were applied to the 8mm wells made in the milk plates in triplicate. Duplicate plates were prepared and incubated for 24 and 48 hours. Following incubation, the caseinolytic area was measured; two measurements were made (cm) at right angles to each other and an average measurement calculated, minus the 8mm well.

2.3.9 Azocasein Assay for General Protease Activity

To measure the general protease activity of each culture, a chromogenic assay utilising azocasein, a non-specific protease substrate conjugated to an azo-dye, was performed. Briefly, a working solution was made by adding 5mg/ml of azocasein to distilled water in a sterile glass bottle and mixed thoroughly on a stirring plate with a magnetic flea. A 50µl aliquot of each test culture was added to 50µl azocasein solution and 500µl sterile 1x phosphate buffered saline (PBS) in 1.5ml Eppendorf centrifuge tubes. Sterile MHB was used for the controls. All samples were made in triplicate. Samples were briefly vortexed and incubated overnight at 37°C. After the incubation period, 600µl of 5% Trichloric acid (TCA) was added to each sample to precipitate non-hydrolysed azocasein and left on ice for 30 minutes. The samples were then centrifuged at 11,000xg for 10 minutes at 4°C to pellet the undigested substrate. From each sample, 200µl of supernatant was added to a 96-well plate and the absorbance measured in a microtitre plate reader at 405nm.

2.3.10 Elastin-Congo Red Assay for Elastase Activity

To assess the level of elastase produced by each bacterial isolate, the elastin-congo red (ECR) method was employed, adapted from the studies by Petermann, Doetkott & Rust (2001) and Rust, Messing & Iglewski (1994). An ECR solution was made by adding 10mg of ECR powder to 1ml of assay buffer (10mM sodium phosphate buffer, pH7.0); the pH optimum of *P. aeruginosa* elastase is thought to be around physiologic pH, although the exact optimum pH seems to depend on the source of the *P. aeruginosa* (Mull & Callahan, 1965). A stock solution was made up in a sterile glass bottle for all of the samples and was mixed thoroughly on a stirring plate with a magnetic flea. In 1.5ml Eppendorf tubes, 1ml of the ECR solution was added along with 5µl of the bacterial test culture. Sterile MHB was used for the controls. The samples were made in triplicate and vortexed briefly. The samples were incubated for 24 hours at 37°C after which the samples were centrifuged at 1200rpm for 10 minutes at 4°C to pellet any undigested ECR. From each sample, 200µl of supernatant was added to a 96-well plate and the absorbance measured in a microtitre plate reader at 450nm.

2.3.11 Gelatin Zymography

Treatment of Samples

Samples were diluted with non-reducing Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK) at a ratio of 1:5 of the total sample volume and distilled water to an overall 1:20 dilution of the sample. Samples were then incubated in a 37°C water bath for 1 hour to activate the enzyme prior to application to the gel.

Gel Electrophoresis and Gel Imaging

A Miniprotean II gel system (Bio-Rad, Hemel Hempstead, UK) was employed for zymography gels. A 7.5% acrylamide/bis-acrylamide (Severn Biotech Ltd., Kidderminster, UK) resolving gel copolymerised with a 1% gelatin solution (EIA grade reagent gelatin) (Bio-Rad, Hemel Hempstead, UK) was prepared to create a 0.25% gelatin zymogram in a pH8.8 resolving buffer (1.5M Tris/HCl). A volume of 3.2ml of this was cast between ethanol-washed glass plates and overlain with isobutanol to level the gel. The gel was left to set. Once set, the resolving gel was layered with a stacking gel made up with a pH6.8 buffer (0.5M Tris/HCl) and was left to set with a 10-lane comb inserted in the top of the gel. The gels were placed in a tank filled with running buffer and 20µl aliquots of each sample were loaded into the gel and subjected to electrophoresis at 200 volts for 1 hour or until the lane marker had run off the end of the gel. Mark-12 high molecular weight lane markers (Invitrogen, LC5677, unstained standards) or purified MMP standards were run on each gel. Following electrophoresis, gels were carefully removed from the glass plates and the Mark-12 lane markers were removed and placed in 0.02% coomassie brilliant blue stain (BDH, Oxford, UK) for 15-20 minutes and then de-stained in diluted methanol/acetic acid until dark bands appeared on a light background. The gels were placed in 2.5% Triton X-100 and agitated on a plate shaker for 1 hour to remove all traces of sodium dodecyl sulphate (SDS). The gels were washed thoroughly in distilled water and placed in MMP incubation buffer/gelatin refolding buffer overnight. After incubation, the gels were stained for 1 hour in 0.5% coomassie stain and then destained in a solution of diluted methanol/acetic acid until

clear bands of digested gelatin were visible against a darkly stained blue background. Gels were visualised and imaged using Genesnap software. The gels were dried in cellophane sheets using a Hoeffer gel drying dock.

2.3.12 Passive elution of proteins from polyacrylamide gels

(Method adapted from ThermoScientific; Tech Tip #51)

To elute proteins from proteolytic bands in gelatin zymograms, gel bands were cut from the gel using a sterile scalpel. Excised bands (gel plugs) were placed in clean microcentrifuge tubes. To each band, 0.5ml of elution buffer (50mM Tris-HCl, 150mM NaCl, and 0.1mM EDTA; pH7.5) was added so that the gel pieces were completely immersed. The gel pieces were crushed using a clean pestle and the tubes were incubated for 2 hours at 30°C in a rotary incubator. Following incubation, tubes were centrifuged at 10,000 xg for 10 minutes and the supernatant was removed and placed into a new clean microcentrifuge tube. Supernatant samples were then run on an SDS-PAGE gel. Control gel plugs just below and to one side of the proteolytic band on the zymogram were excised and processed in separate microcentrifuge tubes.

2.3.13 Casein Zymography

The methodology for casein zymography was essentially the same as gelatin zymography, however a 2% solution of casein in dH_2O was used as the substrate for copolymerisation with the acrylamide gel to give an overall 0.2% casein/7.5% polyacrylamide gel. Casein refolding buffer (Tris/HCl, $CaCl_2$ [pH7.6]) was used in place of gelatin refolding buffer.

2.3.14 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A 15% acrylamide mini gel was prepared using 3.2mls resolving gel in a 1.5M Tris/HCl buffer, pH 8.8. Once set this gel was overlain with a stacking gel made up in a 0.5M Tris/HCl buffer, pH 6.8 in a Miniprotean gel system (Bio-Rad Hemel Hempstead, UK). Samples were prepared by adding 13µl original sample, 2µl DTT and 5µl non-reducing sample buffer with marker dye (Thermo Scientific, MA, USA). Samples were denatured by heating for 10 minutes in a heat block pre-set to 80°C. The samples were vortexed briefly. A 20µl aliquot of each sample was added to each corresponding lane of the gel. The gel tank was filled with a 1:20 dilution of NUPAGE MES SDS running buffer (Invitrogen, Paisley, UK) in distilled water. The gel was run at 15 volts for approximately 2 hours or until the lane marker ran to the bottom of the gel. The gels were carefully removed from the glass plates and placed in 0.02% Coomassie brilliant blue stain for 2 hours with agitation. Gels were then destained in a solution of diluted methanol/acetic acid until dark bands appeared against a stain-free background.

2.3.15 Silver Staining of polyacrylamide gels

For silver-stained SDS-PAGE gels, a Pierce® Silver Stain Kit (Thermo Scientific, MA, USA) was used according to the manufacturer's instructions. In brief, gels were washed in ultrapure water followed by a fixing step with a 30% ethanol: 10% acetic acid solution. Next, gels were washed in 10% ethanol, followed by washing in ultrapure water. Gels were then sensitized in a sensitizer working solution, followed by a wash step. Gels were stained with a stain working solution (0.5ml enhancer to 25ml stain) for 30 minutes. Gels were briefly rinsed in ultrapure water, then placed in a developer working solution (0.5ml enhancer to 25ml developer) for 2-3 minutes until bands appeared on the gel. The reaction was stopped with 5% acetic acid for 10 minutes.

2.3.16 Mass Spectrometry

Following SDS-PAGE of culture supernatant samples, gels were stained with silver stain and any bands of particular interest were excised using a sterile scalpel and processed for mass spectrometry (MS) analysis.

In-gel protein digest

Sample preparation

A small ($1mm^3$) plug of the stained protein band (containing typically 10ng-100ng of protein) was excised from the gel using a sterile scalpel. Since recoveries from in-gel digests are not 100% pure protein, two or three protein bands of the same molecular weight were pooled where possible.

Destaining

The gel plugs were washed with 50µl (per plug) Farmers reagent (15mM potassium ferricyanide/50mM sodium thiosulphate) for 15 minutes at 37°C until the bands were destained.

Reduction

To each Eppendorf tube, 25µl of ~10mM dithiothreitol (DTT) diluted in 25mM ammonium bicarbonate was added. Samples were vortexed and incubated for 60 minutes at 60°C. Samples were cooled to room temperature and briefly spun to return the liquid to the bottom of the tube. The liquid was aspirated and discarded.

Alkylation

To each Eppendorf tube, 25µl of ~60mM of iodoacetamide diluted in 25mM ammonium bicarbonate was added to prevent disulphide bonds from re-forming. Samples were then incubated in the dark at room temperature for 45 minutes.

Washing

Gel plugs were washed alternately with 25mM ammonium bicarbonate and 25mM ammonium bicarbonate: acetonitrile (ACN) (2:1). At each wash cycle gel plugs were incubated for 15 minutes at 37°C until destained. This step was finished with a wash in 25mM ammonium bicarbonate: ACN (2:1) to dehydrate the plug.

Digestion

To each gel plug, 10µl of molecular grade trypsin (12.5ng/µl diluted in 25mM ammonium bicarbonate) was added. Samples were incubated overnight (12-16 hours) at 37°C.

Peptide Recovery

Eppendorf tubes were briefly spun to collect the digest at the bottom of the tube. Formic acid (10% v/v) was added to attain a final concentration of 1% (this gave a final volume of 10µl per Eppendorf/plug). The solution containing the peptides was then aspirated and retained for MS analysis (peptides were pooled where appropriate). Samples were cleared of particulates with a further centrifugation step.

Protein digests were sent on dry ice to Dundee Cell Products Ltd (Dundee, Scotland) for matrix-assisted laser desorption/ionisation (MALDI) time of flight (TOF) MS analysis.

2.3.17 Statistical Analyses

Statistical analyses were performed on the milk-casein agar plate data and the data for the biochemical assays (azocasein and elastin-congo red) for the 24 hour culture time points for general protease or elastase activity under aerobic and anaerobic conditions; statistical differences in general protease and elastase activity between *P. aeruginosa* isolates were identified using a one-way analysis of variance (ANOVA) or a non-parametric test for data which was not normally distributed (Kruskal-Wallis test). For analysis of the effect of initial culture pH a general linear model was used. For effect of BFP on elastase activity, a one-way ANOVA was used. In all instances, Post Hoc analyses were applied with the Tukey test. All statistical analyses were performed using Minitab®, Version 16.

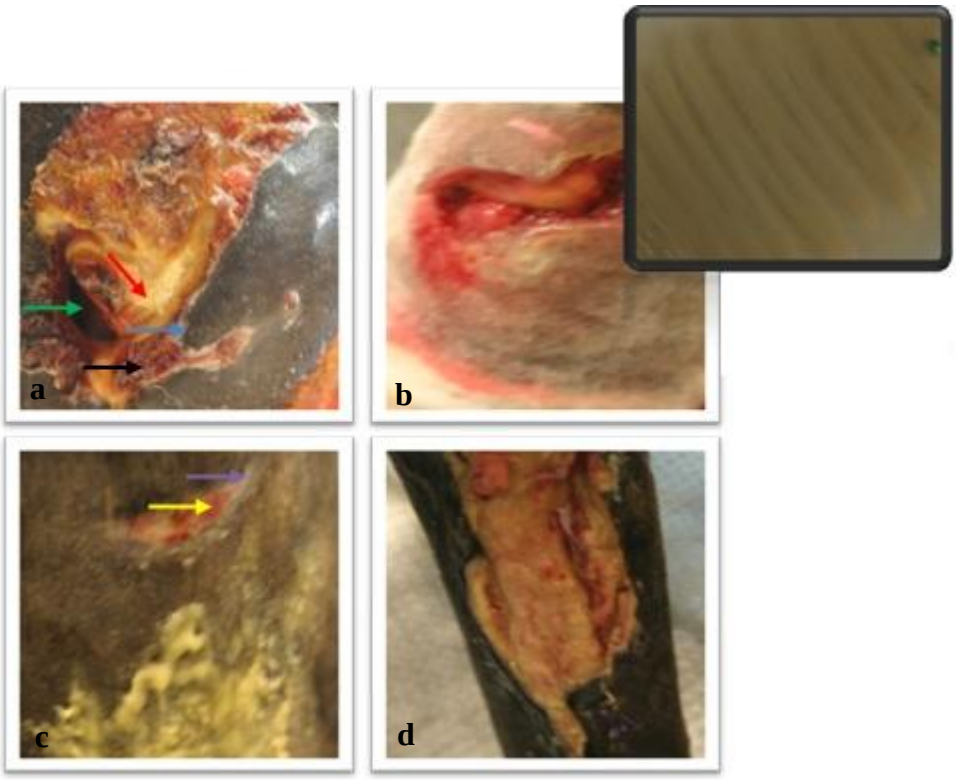


Figure 1: Equine wounds; a: a large, purulent stifle wound with a high degree of tissue loss and a cavity (green arrow). The wound exhibited minimal granulation tissue and was producing yellow exudate (red arrow). There was some evidence of epithelial proliferation/epithelial edge advancement (blue arrow). The black arrow indicates a healthier wound appearance with a vascularised granulation tissue bed; b: pastern wound with some tissue loss and minimal exudate. The wound bed appeared vascularised; c: a healing stifle wound with very minimal tissue loss and some healthy granulation tissue (yellow arrow). The wound margins appeared healthy and there was some evidence of epithelial proliferation/epithelial edge advancement (purple arrow); d: a cannon bone wound presenting with a pale wound bed and no signs of granulation tissue formation; Inset: *P. aeruginosa* isolated from a chronic equine wound.

2.4 Results

All error margins are displayed as the mean ± standard error of the mean (SEM).

Figure 1 gives examples of equine wounds.

2.4.1 Crystal Violet Assay for the Assessment of Biofilm forming phenotype (BFP)

The results of the crystal violet assay for BFP of the wound and non-wound isolates revealed a significant variation between isolates, $F(8, 71) = 162.06$ ($P = <0.001$) [Kruskal-Wallis Test: $P=<0.001$] (see figure 2). An OD reading at 570nm above 0.136 was indicative of a biofilm-forming isolate. A scale of biofilm forming phenotype was adapted from that described by Stepanovic *et al* (2000); the cut-off OD (ODc) for the assay was set at 3 standard deviations above the mean OD of the negative control (MHB only). Each strain was categorised according to the values given in table 2.

Table 2: Classifications of biofilm forming phenotype (BFP) according to OD_{570nm} Value.

Classification of Adherence	**OD_{570nm}**	**Interpretation (Category)**	**Category Code**
$OD \leq OD_c$	*0-0.136*	*Non-Adherent*	*0*
$OD_c < OD \leq 2xOD_c$	*0.136-0.272*	*Weakly Adherent*	+
$2xOD_c < OD \leq 4xOD_c$	*0.272-0.544*	*Moderately Adherent*	++
$4xOD_c < OD \leq 6xOD_c$	*0.544-0.816*	*Strongly Adherent*	+++
$OD \geq 6xOD_c$	*>0.816*	*Very Strongly Adherent*	++++

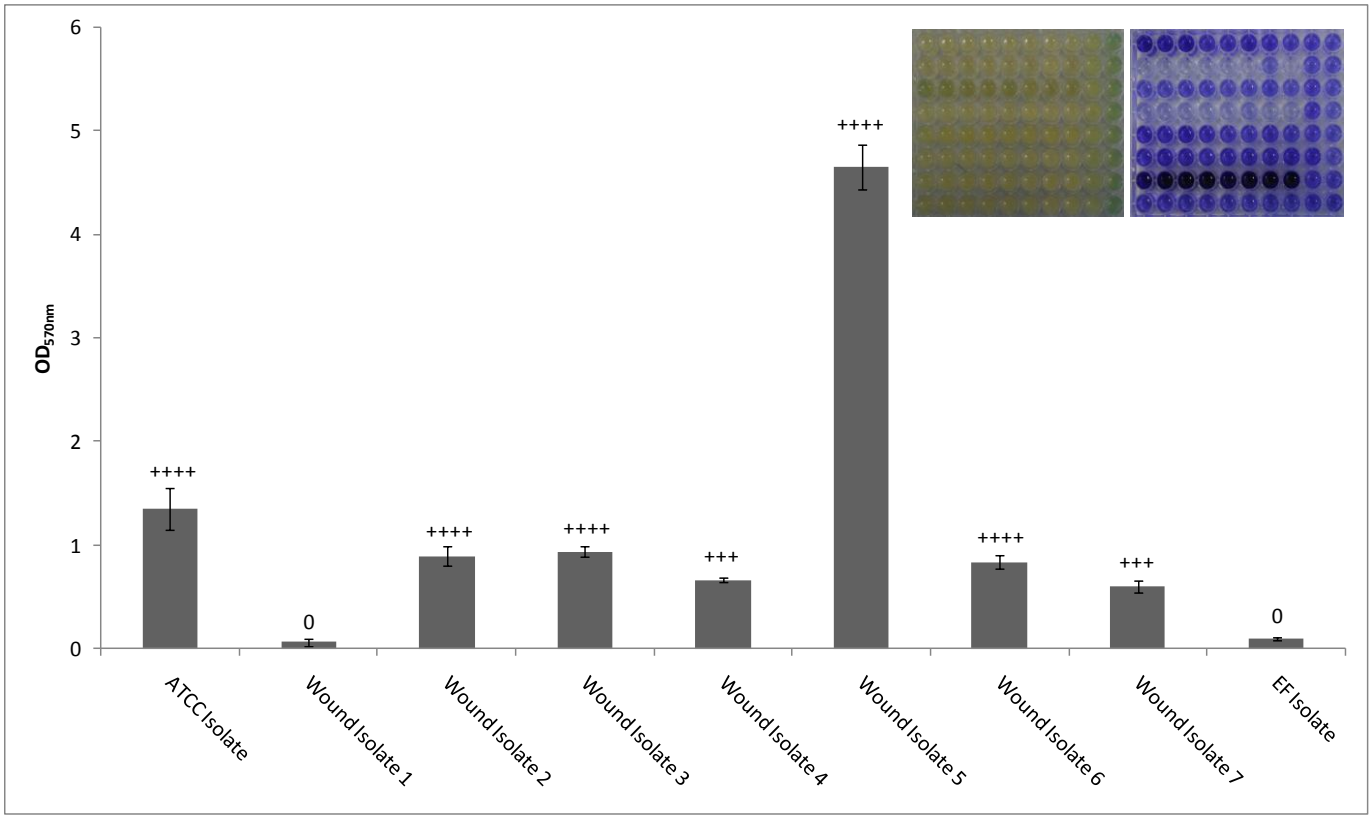


Figure 2: Biofilm forming phenotype (BFP) of the ATCC *P. aeruginosa* control isolate (ATCC Isolate), all 7 *P. aeruginosa* wound isolates (Wound Isolate 1- 7) and the *E. faecium* isolate (EF Isolate). Optical densities (ODs) were measured at 570nm. Inset: Left; 24 hour biofilm cultures in wells of a 96-well microtitre plate, Right; Biofilms stained with 2% crystal violet. Due to the complex relationships, statistical significance grouping information is detailed in Appendix 2. Data is presented as mean ± SEM.

2.4.2 PCR Analysis of *P. aeruginosa* Elastase Gene

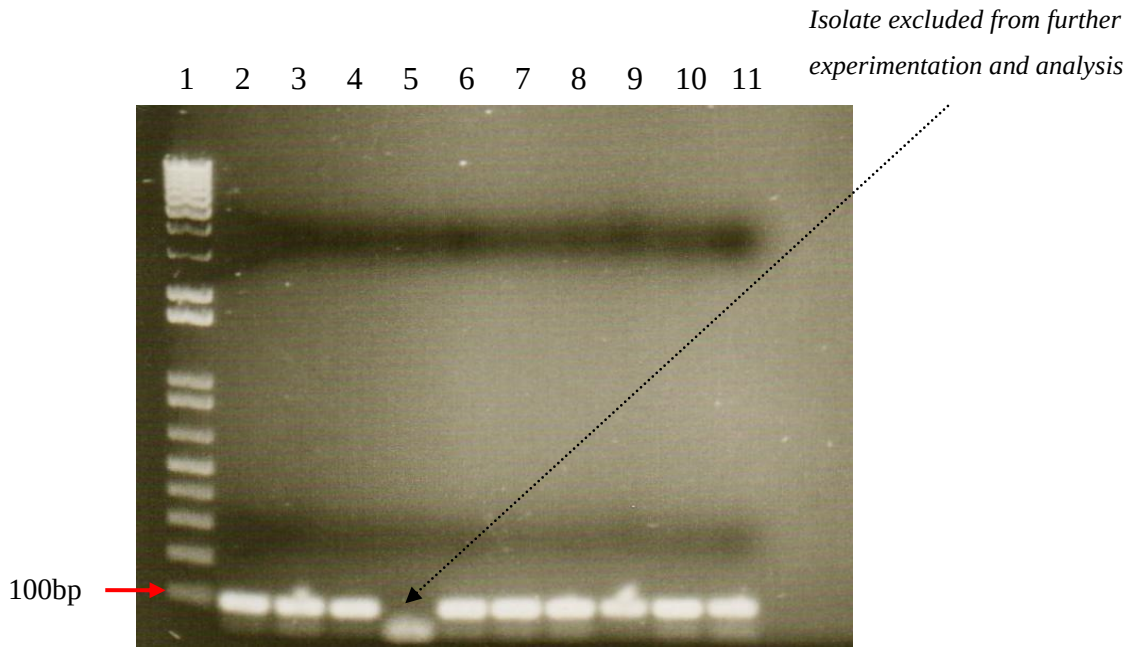


Figure 3: PCR agarose gel showing presence of the *P. aeruginosa* elastase (LasB) gene for the 8 *P. aeruginosa* isolates. Lane 1: 1kb Molecular weight marker; Lane 2: ATCC isolate; Lane 3: Wound isolate 1; Lane 5: *E. faecium* isolate (EF Isolate); Lane 6: Wound isolate 2; Lane 7: Wound isolate 3; Lane 8: Wound isolate 4; Lane 9: Wound isolate 5; Lane 10: Wound isolate 6; Lane 11: Wound isolate 7. The isolate in Lane 4 was excluded from further experimentation as it was found to be an impure sample.

The results of the PCR analysis clearly indicate that each of the *P. aeruginosa* isolates (including the ATCC reference isolate and all wound isolates) are positive for the 101bp amplicon indicative of the *P. aeruginosa* elastase gene (LasB). As expected, the EF isolate was negative for this gene.

2.4.3 Milk-Casein Agar Plate Analysis

Each of the *P. aeruginosa* isolates (both the wound isolates and the ATCC reference isolate) showed some degree of proteolytic activity on milk-casein agar plates when streaked on the plates and incubated for 24 hours. The effects of changes in the initial pH of broth cultures on the protease activity of the *P. aeruginosa* isolates following sub-culturing on milk-casein agar plates was investigated; broth cultures were grown at each initial pH over a 12 hour period and samples of each culture were taken at four-hourly intervals. Live bacterial culture samples were inoculated onto milk-casein agar plates as previously described. Resulting proteolytic activity was measured by the clearance zone, indicating hydrolysis of casein. The caseinolytic activity on milk-casein agar plates is considered as general protease activity; caseinolytic activity of *P. aeruginosa* is due mainly to the actions of LasB (elastase B), alkaline protease and protease IV (Caballero *et al*, 2001; Sonawane, Jyot & Ramphal, 2006). There was a general trend of increasing protease activity over the 48 hour milk-casein agar plate culture period, demonstrating that even after long culture periods the isolates maintained their caseinolytic activity (see figures 5-8).

Statistical analysis was performed for caseinolytic activity after initial 8 hour and 12 hour broth cultures and for 24 hour and 48 hour milk-casein agar plate cultures for each of the isolates, given that at earlier culture time points little caseinolytic activity was noted. Given the complex relationships, statistical significance is detailed in tables 3-6. There were significant variations in caseinolytic activity between wound isolates which was generally maintained over the culture periods and also when the initial pH of the culture broth was adjusted (see tables 3 and 4). Although not statistically significant, the ATCC reference isolate generally produced less caseinolytic activity than the wound isolates, the trend of which was maintained in all four initial broth pH cultures over time. Statistical analysis revealed that whilst the initial pH of the broth cultures prior to sub-culturing the isolates on milk-casein agar had an effect on caseinolytic activity of the isolates, further analysis identified that the pH affected different isolates in different ways (see tables 5 and 6). Since data was missing for the initial pH8.0 cultures, results for the pH8.0 cultures were excluded from the first set of statistical analyses concerning effects of pH on

caseinolytic activity (see table 5). Results from these analyses showed that whilst at 24 hours post milk-casein agar plate inoculation (initial 8 hour broth culture) there was no significant effect of pH on caseinolytic activity, $F(2, 65) = 2.88$, $P = 0.067$, at 48 hours post plate inoculation there was a significant effect of pH, $F(2, 63) = 11.58$, $P = <0.001$ which was maintained for the initial 12 hour broth cultures (see table 5). However, as previously mentioned, further analysis revealed very strong evidence to suggest that there was an interaction between isolate and pH when analysed together, i.e. pH had a differential effect on isolates (see table 5). Similarly, when the initial pH8.0 broth cultures and corresponding milk-casein agar plate cultures were included in to the statistical analyses, pH did not have a significant effect on caseinolytic activity at 24 hours post plate inoculation (initial 8 hour culture), $F(3, 62) = 0.91$, $P = 0.447$. However, at 48 hours post plate inoculation a significant effect was noted, $F(3, 61) = 4.84$, $P = 0.006$; again, this effect was not the same for all isolates, with strong evidence suggestive of a differential effect of pH on caseinolytic activity of different isolates (see table 6). Interval plots of the caseinolytic activity of the *P. aeruginosa* isolates at each initial broth culture pH are shown in figures 9 and 10. Due to the complex relationships, detailed statistical significance grouping information for each initial broth culture pH is presented in Appendix 3.

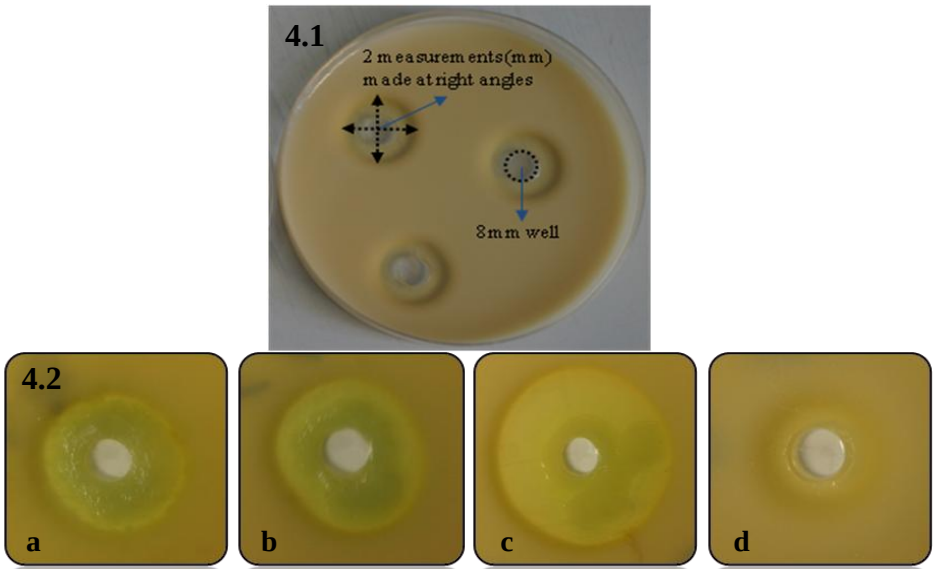


Figure 4: 4.1: Method of calculation of caseinolytic zone using the milk-casein agar plate method; **4.2:** Example milk-casein agar plate images. All isolates were grown in broth culture at standard initial pH (pH7.3) for 12 hours, followed by a 48 hour milk-casein agar plate culture; a: ATCC Isolate; b: Wound isolate 1; c: Wound isolate 3; d: *E. faecium* (EF) isolate.

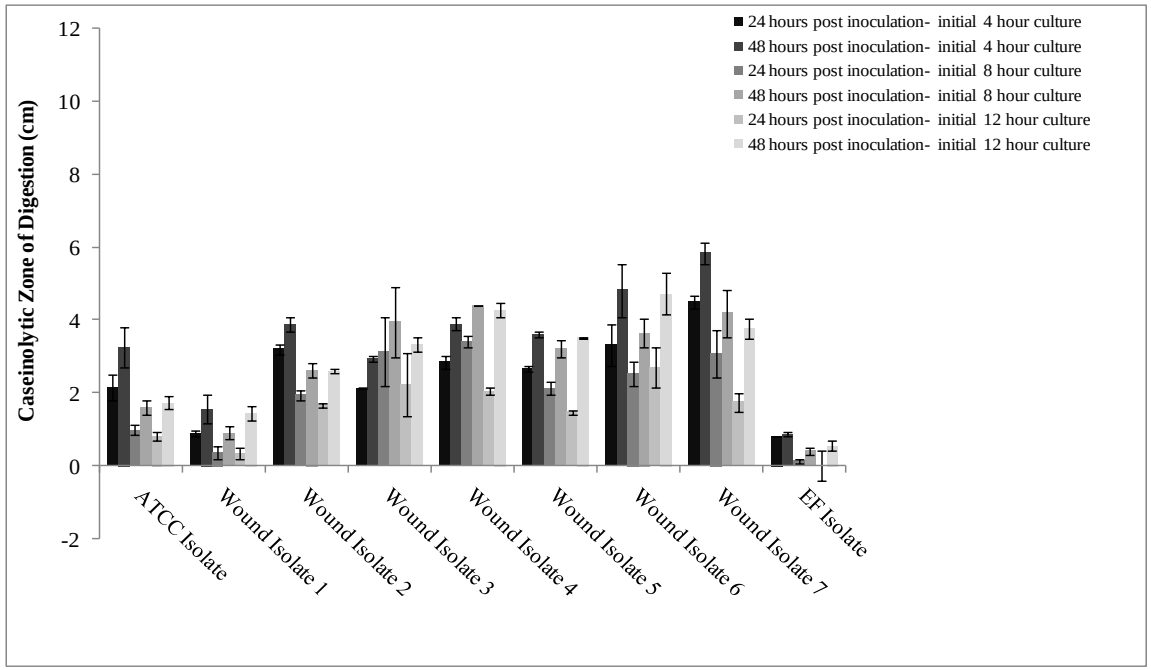


Figure 5: Milk-Casein agar plate assay for caseinolytic digest analysis of each isolate over 24 and 48 hour periods. Isolates were previously cultured in standard Mueller Hinton broth (MHB) (initial pH 7.3) over a 12 hour period. Data is presented as mean ± SEM. Statistical significance is detailed in tables 2-5.

*EF = *E. faecium*

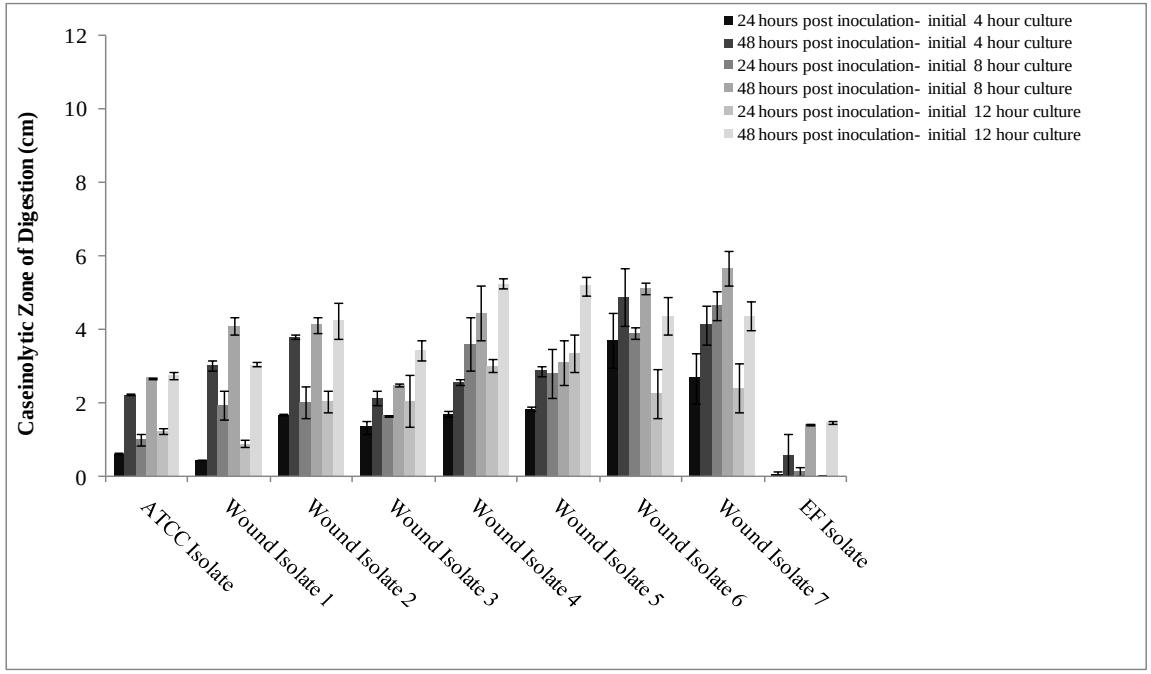


Figure 6 Milk-Casein agar plate assay for caseinolytic digest analysis of each isolate over 24 and 48 hour periods. Isolates were previously cultured in Mueller Hinton broth (MHB) adjusted to an initial pH of 5.5 over a 12 hour period. Data is presented as mean ± SEM. Statistical significance is detailed in tables 2-5.

*EF = *E. faecium*

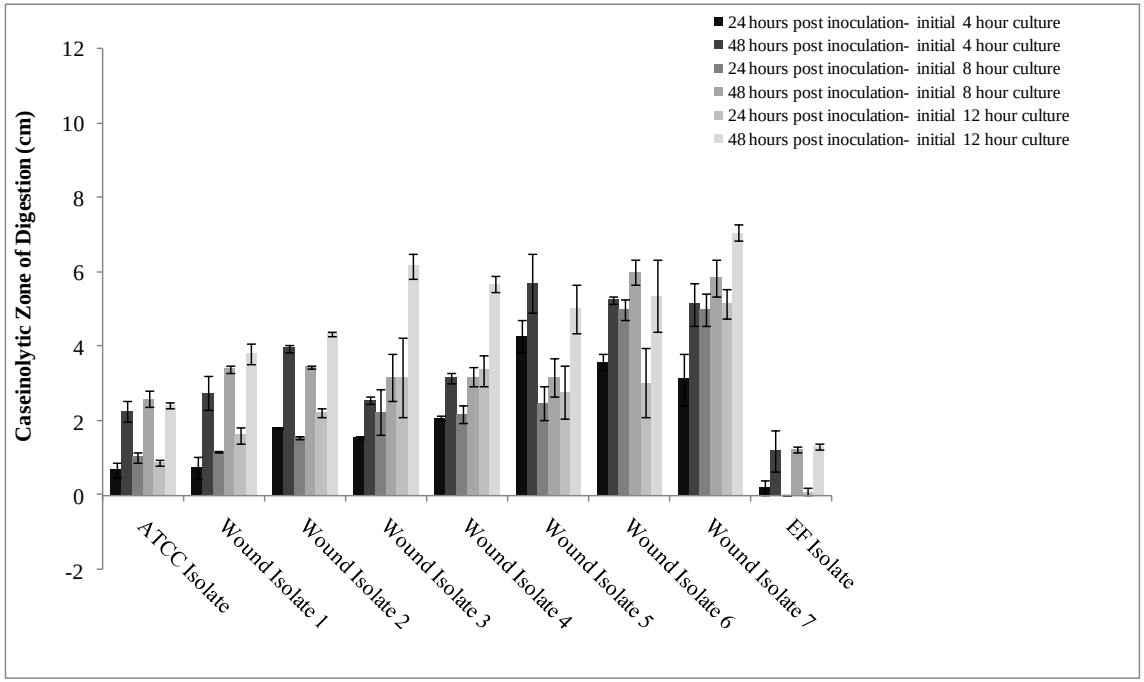


Figure 7: Milk-Casein agar plate assay for caseinolytic digest analysis of each isolate over 24 and 48 hour periods. Isolates were previously cultured in Mueller Hinton broth (MHB) adjusted to an initial pH of 6.0 over a 12 hour period. Data is presented as mean ± SEM. Statistical significance is detailed in tables 2-5.
*EF = *E. faecium*

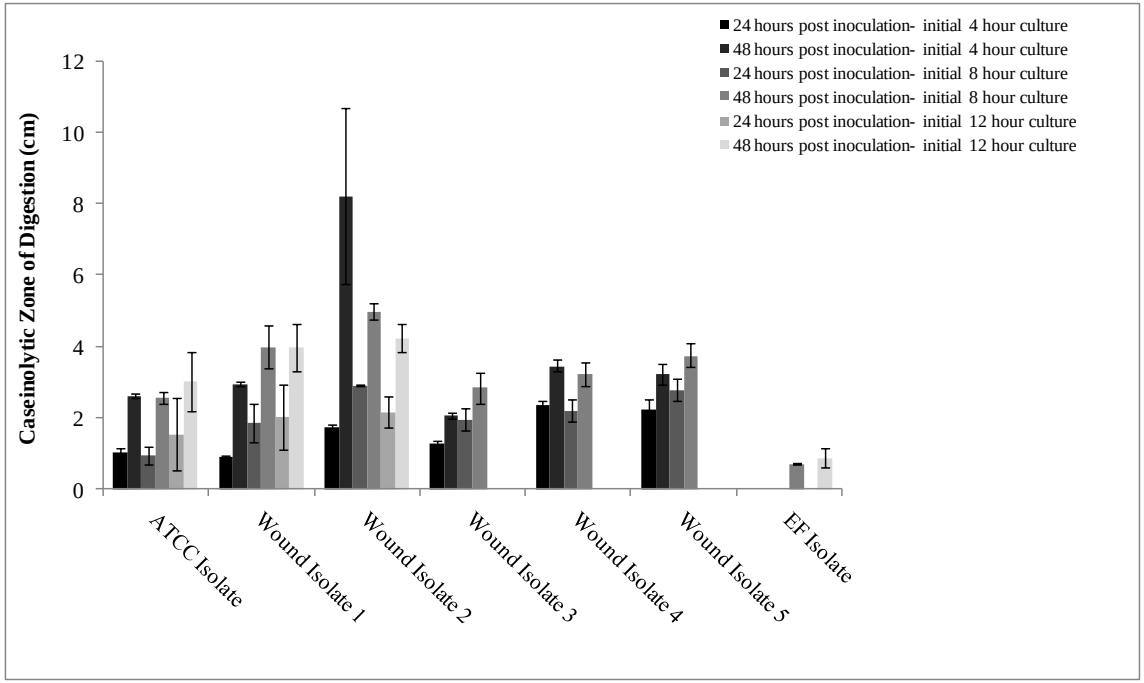


Figure 8: Milk-Casein agar plate assay for caseinolytic digest analysis of each isolate over 24 and 48 hour periods. Isolates were previously cultured in Mueller Hinton broth (MHB) adjusted to an initial pH of 8.0 over a 12 hour period. Data is presented as mean ± SEM. Statistical significance is detailed in tables 2-5.

Note: Missing data for isolates 6 and 7; missing data for T12 and T24 for isolates 3, 4 and 5.
*EF = *E. faecium*

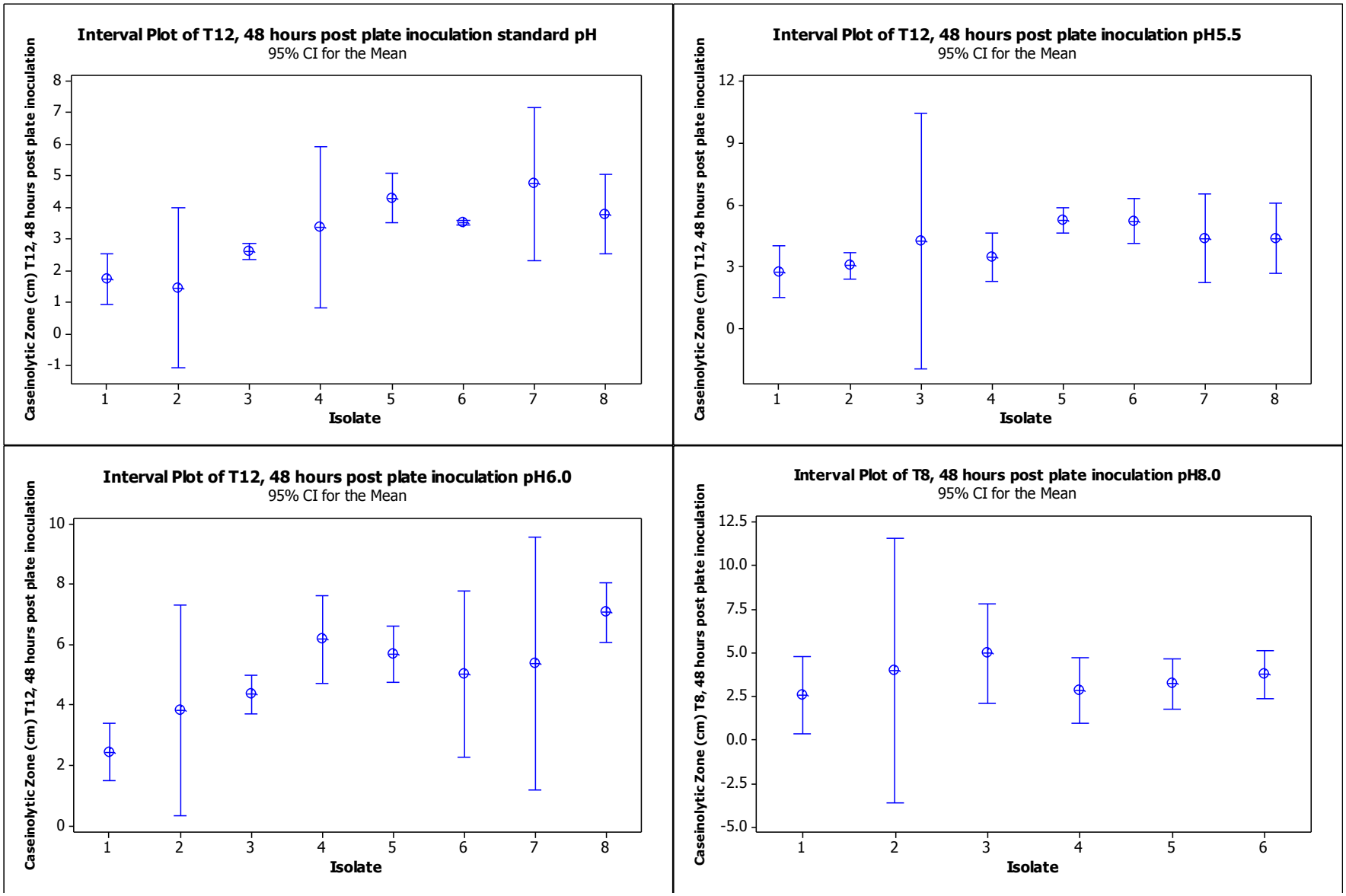


Figure 9: Interval plots for caseinolytic activity of the *P. aeruginosa* isolates at each initial broth culture pH following an initial 12 hour broth culture prior to a 48 hour milk-casein agar plate culture. For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-8 were numbered isolates 2-9; the *E. faecium* (EF) isolate was excluded from statistical analysis. For the initial pH8.0 broth cultures, due to missing data for the 12 hour broth cultures the 8 hour cultures were used for interval plots.

Table 3: *P. aeruginosa* isolates (ATCC isolate + 7 wound isolates): Differences in general protease activity between isolates, at each initial pH.

Initial Broth Culture Time (Hours)	Milk-Casein Agar Plate Culture Time (Hours)	Initial pH in Broth Culture	One-Way ANOVA Results	Non-Parametric (Kruskal-Wallis test) Results
8	24	Standard Broth (7.3)	F(7, 23) = 6.11, P = 0.001*	
8	48	Standard Broth (7.3)	F(7, 23) = 7.65, P = <0.001*	
12	24	Standard Broth (7.3)	F(7, 22) = 3.22, P = 0.027*	P = 0.025*
12	48	Standard Broth (7.3)	F(7, 21) = 16.94, P = <0.001*	P = 0.009*
8	24	5.5	F(7, 20) = 7.02, P = 0.001*	P = 0.037*
8	48	5.5	F(7, 20) = 6.60, P = 0.002*	
12	24	5.5	F(7, 20) = 2.06, P = 0.123	
12	48	5.5	F(7, 20) = 7.16, P = 0.001*	
8	24	6.0	F(7, 20) = 15.73, P = <0.001*	P = 0.014*
8	48	6.0	F(7, 18) = 9.62, P = 0.001*	P = 0.105
12	24	6.0	F(7, 20) = 3.07, P = 0.038	
12	48	6.0	F(7, 20) = 6.73, P = 0.002*	
8	24	8.0	F(5, 14) = 4.09, P = 0.033*	P = 0.096
8	48	8.0	F(5, 14) = 4.41, P 0.026*	P = 0.052
12	24	8.0	F(2, 5) = 0.16, P = 0.861	P = 0.682
12	48	8.0	F(2, 5) = 0.96, P = 0.477	P = 0.530

** Significant at the 0.05 level*

Table 4: *P. aeruginosa* wound isolates (7 wound isolates): Differences in general protease activity between isolates, at each initial pH.

Initial Broth Culture Time (Hours)	Milk-Casein Agar Plate Culture Time (Hours)	Initial pH in Broth Culture	One-Way ANOVA Results	Non-Parametric (Kruskal-Wallis test) Results
8	24	Standard Broth (7.3)	F(6, 20) = 5.00, P = 0.006*	
8	48	Standard Broth (7.3)	F(6, 20) = 6.23, P = 0.002*	
12	24	Standard Broth (7.3)	F(6, 19) = 2.49, P = 0.079	P = 0.082
12	48	Standard Broth (7.3)	F(6, 18) = 12.51, P = <0.001*	
8	24	5.5	F(6, 18) = 5.63, P = 0.005*	P = 0.066
8	48	5.5	F(6, 18) = 5.75, P = 0.005*	
12	24	5.5	F(6, 18) = 1.75, P = 0.192	
12	48	5.5	F(6, 18) = 5.39, P = 0.006*	
8	24	6.0	F(6, 18) = 14.52, P = <0.001*	P = 0.019*
8	48	6.0	F(6, 16) = 9.04, P = 0.001*	P = 0.114
12	24	6.0	F(6, 18) = 2.20, P = 0.115	
12	48	6.0	F(6, 18) = 3.72, P = 0.025*	
8	24	8.0	F(4, 12) = 1.92, P = 0.201	P = 0.195
8	48	8.0	F(4, 12) = 3.78, P = 0.052	P = 0.067
12	24	8.0	F(2, 5) = 0.16, P = 0.861	
12	48	8.0	F(2, 5) = 0.96, P = 0.477	

** Significant at the 0.05 level*

Table 5: General linear model: *P. aeruginosa* isolates (ATCC isolate + 7 wound isolates). Effects of initial broth culture pH on caseinolytic activity. Analysis includes standard broth pH (7.3), pH5.5 and pH6.0.

Initial Broth Culture Time (Hours)	Milk-Casein Agar Plate Culture Time (Hours)	Statistical Analysis
8	24	pH: F(2, 65) = 2.88, P = 0.067 Isolate: F(7, 65) = 20.31, P = <0.001 pH*Isolate: F(14, 65) = 3.11, P = 0.002
8	48	pH: F(2, 63) = 11.58, P = <0.001 Isolate: F(7, 63) = 14.83, P = <0.001 pH*Isolate: F(14, 63) = 3.68, P = 0.001
12	24	pH: F(2, 64) = 8.35, P = 0.001 Isolate: F(7, 64) = 5.71, P = <0.001 pH*Isolate: F(14, 64) = 1.50, P = 0.155
12	48	pH: F(2, 63) = 41.25, P = <0.001 Isolate: F(7, 63) = 19.56, P = <0.001 pH*Isolate: F(14, 63) = 3.29, P = 0.002

Table 6: General linear model: *P. aeruginosa* isolates (ATCC isolate + 5 wound isolates). Effects of initial broth culture pH on caseinolytic activity. Analysis includes standard broth pH (7.3), pH5.5, pH6.0 and pH8.0.

Initial Broth Culture Time (Hours)	Milk-Casein Agar Plate Culture Time (Hours)	Statistical Analysis
8	24	pH: $F(3, 62) = 0.91$, $P = 0.447$ Isolate: $F(5, 62) = 10.50$, $P = <0.001$ pH*Isolate: $F(15, 62) = 1.91$, $P = 0.041$
8	48	pH: $F(3, 61) = 4.84$, $P = 0.006$ Isolate: $F(5, 61) = 5.77$, $P = <0.001$ pH*Isolate: $F(16, 61) = 4.01$, $P = <0.001$

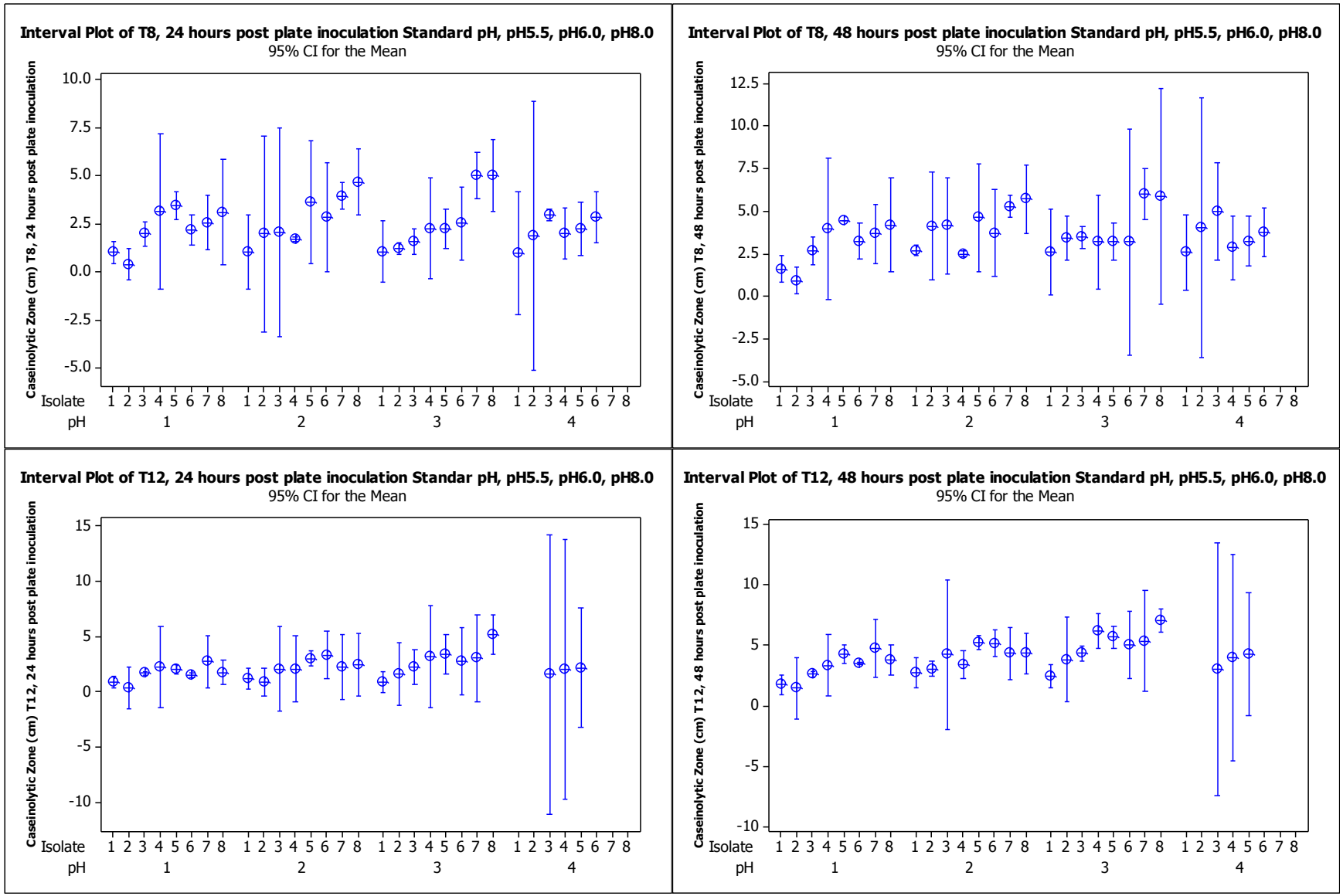


Figure 10: Interval plots for caseinolytic activity of the *P. aeruginosa* isolates at each initial broth culture pH following an initial 8 or 12 hour broth culture prior to a 24 or 48 hour milk-casein agar plate culture. For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-7 were numbered isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analysis. Standard pH=pH1; pH5.5=pH2; pH6.0=pH3; pH8.0=pH4.

2.4.4 Azocasein Assay for General Protease Activity

The azocasein substrate assay for non-specific protease activity was used to determine the variability or similarity in general protease activity among *P. aeruginosa* wound isolates in comparison to an ATCC reference *P. aeruginosa* isolate. The degree of proteolytic activity throughout a 24 hour liquid broth culture period was examined. Further, the effect if any, of alterations in the initial pH of the medium was assessed over the culture period. The azocasein method detects a range of enzymes, including metalloproteinases (for instance the elastolytic metalloproteinase, elastase B encoded by the LasB gene of *P. aeruginosa*) (Kuang *et al*, 2011) and serine proteases (Trengove *et al*, 1999); *P. aeruginosa* elastase and alkaline proteinase degrade azocasein (Schmidtchen, Wolff & Hansson, 2001; Döring *et al*, 1985). Protease activity was determined for each isolate at each time point; however, given the low protease levels at the start of each culture only results of protease activity at the end of the 24 hour culture were used for statistical analysis. The EF isolate was excluded from statistical analysis.

Given the complex relationships, statistical significance and grouping information is detailed in tables 7 & 8 and Appendix 4. As illustrated in figures 11-15, the protease activities varied significantly between isolates. In concordance with the milk-casein agar assays, wound isolate 1 was considerably less proteolytically active than the other wound isolates and the ATCC reference isolate. Wound isolate 2 demonstrated a considerably higher proteolytic activity than all other isolates, which was maintained when the initial pH was changed. However, when the initial culture pH was altered it was noted that those isolates, including the ATCC reference isolate, that produced little protease activity in the early stages of culture at a standard broth pH of 7.3, produced more protease activity at these early stages of culture. This was most prominent at more acidic initial culture pH (i.e. pH5.5 and pH6.0). However, a clear trend in increased protease activity at lower pH culture conditions could not be confirmed. Generally, the levels of protease activity increased over time, with levels commonly peaking after 12 hours. This observation applied to each of the different initial pH cultures. Statistical analysis revealed a significant difference between all isolates at each of the initial culture pHs. Interval plots (figures 16 and 17) illustrate the protease profiles of each isolate at each initial pH following a 24 hour culture period. Further analysis revealed that there was no statistical significance between

protease activities of the isolates (ATCC isolate plus 7 wound isolates) when cultured at either standard pH (pH7.3), pH5.5 or at pH6.0, $F(2, 70) = 2.30$, $P = 0.124$. However, when pH8.0 was considered there was a significant difference in protease activities of the isolates (ATCC isolate plus 5 wound isolates [no wound isolates 6 or 7]) when compared to the other initial pH cultures, $F(3, 69) = 3.55$, $P = 0.016$. Yet, as was the case for the milk-casein agar plates, there was strong evidence suggestive of an interaction between pH and isolate; alteration of the initial culture pH affected isolates in different ways, $F(15, 69) = 5.25$, $P = <0.001$.

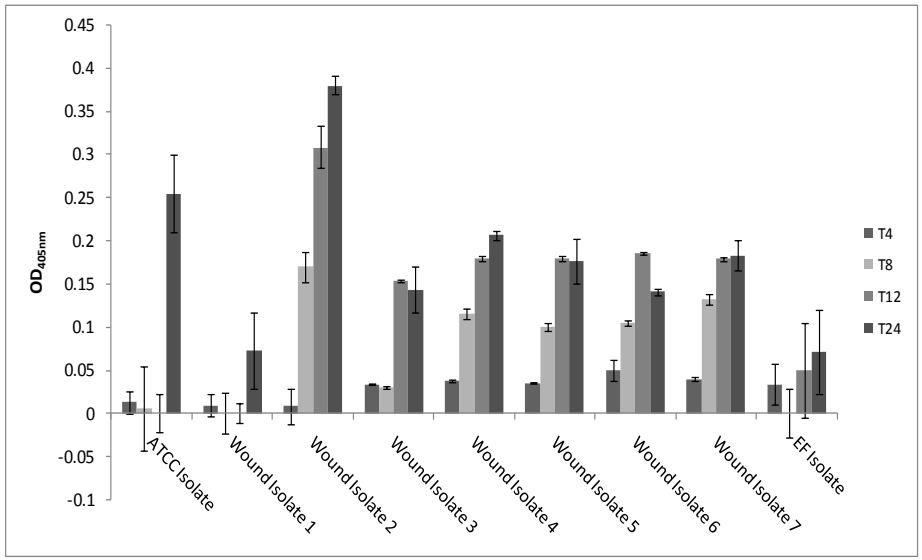


Figure 11: Azocasein assay for caseinolytic digest analysis of each isolate over a 24 hour period. Isolates were cultured in standard Mueller Hinton broth (MHB) broth (initial pH7.3). Data is presented as mean ± SEM. Statistical significance is detailed in tables 6 & 7.

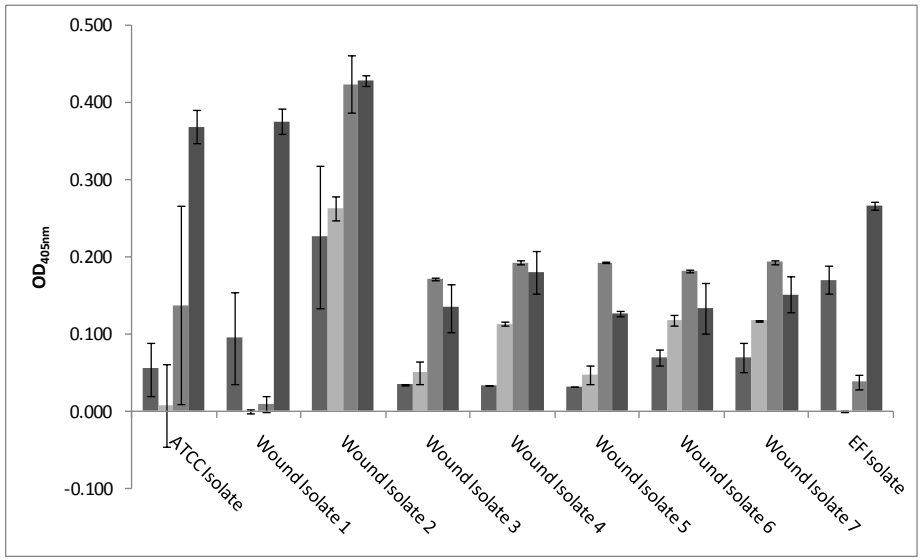


Figure 12: Azocasein assay for caseinolytic digest analysis of each isolate over a 24 hour period. Isolates were cultured in Mueller Hinton broth (MHB) adjusted to an initial pH of 5.5. Data is presented as mean ± SEM. Statistical significance is detailed in tables 6 & 7.

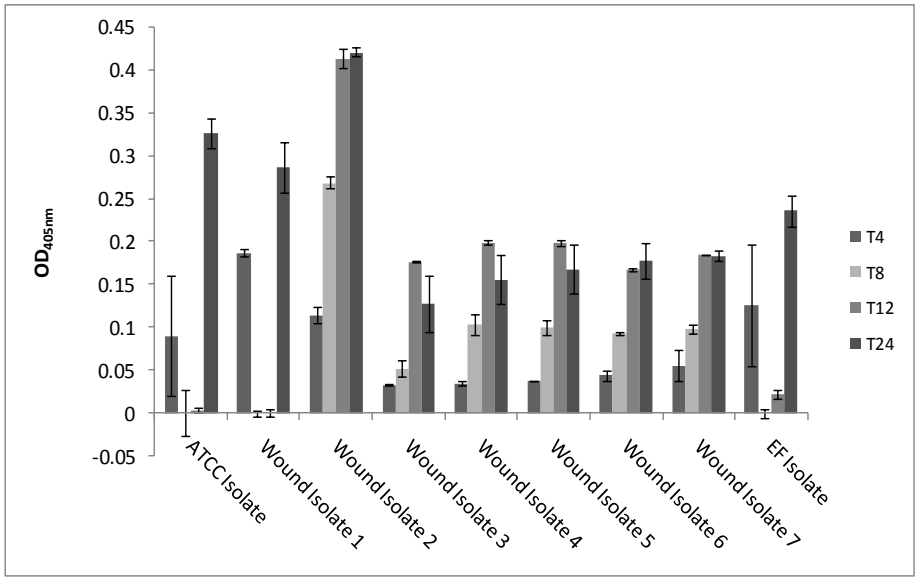


Figure 13: Azocasein assay for caseinolytic digest analysis of each isolate over a 24 hour period. Isolates were cultured in Mueller Hinton broth MHB adjusted to an initial pH of 6.0. Data is presented as mean ± SEM. Statistical significance is detailed in tables 6 & 7. *EF= *E.*

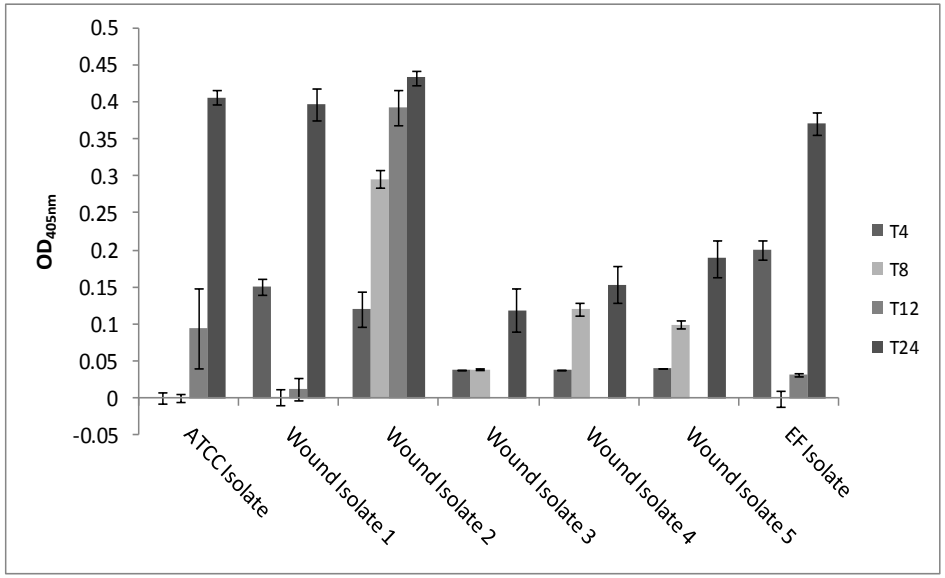


Figure 14: Azocasein assay for caseinolytic digest analysis of each isolate over a 24 hour period. Isolates were cultured in Mueller Hinton broth MHB adjusted to an initial pH of 8.0. Data is presented as mean ± SEM. Statistical significance is detailed in tables 6 & 7. *EF= *E.*

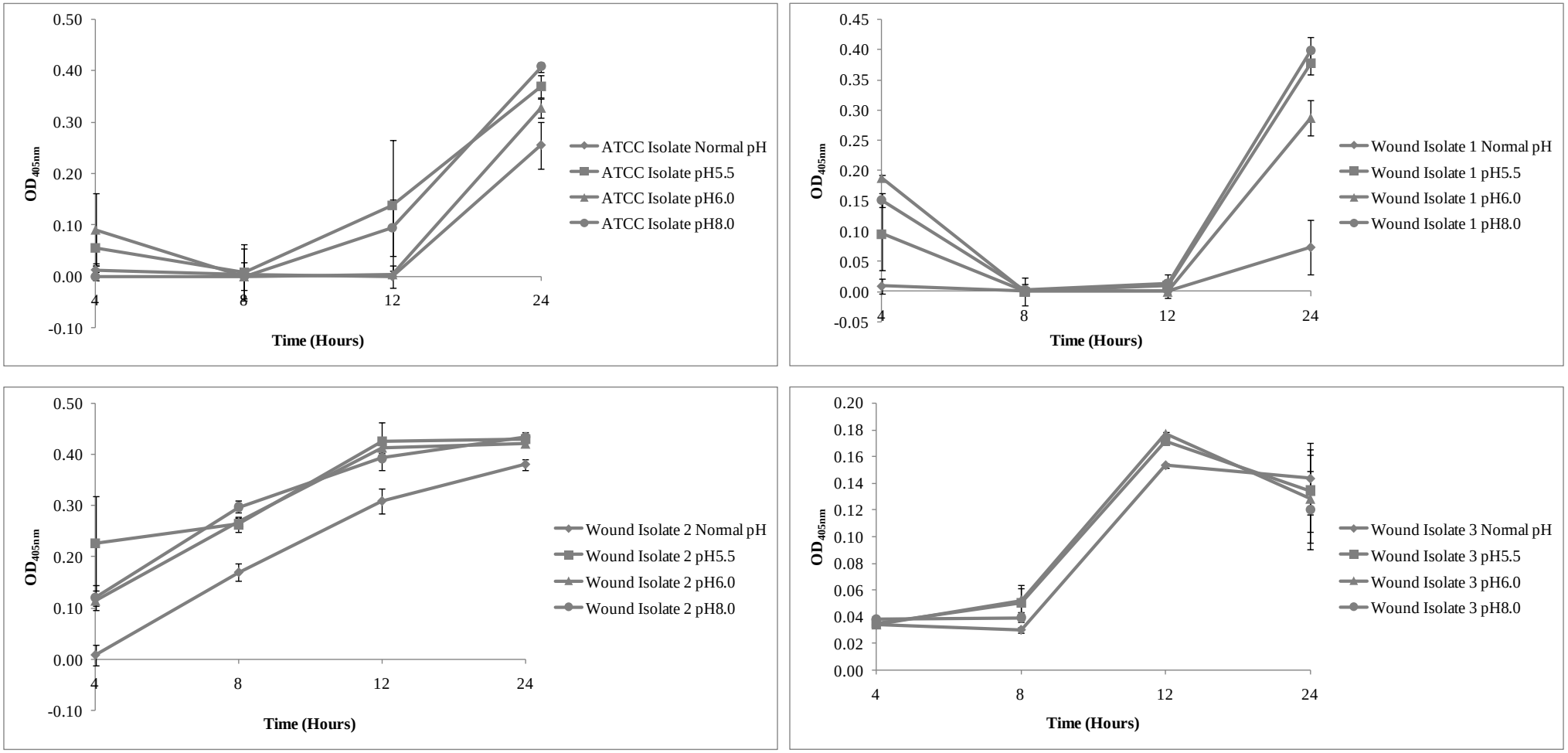


Figure 15: Azocasein assay data for 4 example isolates, showing variations in caseinolytic activity when cultured at different initial pHs. Data is presented as mean ± SEM. Statistical significance is detailed in tables 6 & 7.

Table 7: *P. aeruginosa* isolates (ATCC isolate + 7 wound isolates): Differences in general protease activity between isolates, at each initial pH.

Culture Time (Hours)	Initial Culture pH	Statistical Analysis	Post-Hoc Observations (using the Tukey method)	Kruskal-Wallis test
24	Standard Broth (7.3)	$F(7, 22) = 12.99$, $P = <0.001$*	Isolate 2 significantly different from each other isolate; ATCC isolate significantly different from wound isolates 1 & 6.	$P = 0.027$*
24	5.5	$F(7, 23) = 32.18$, $P = <0.001$*	ATCC isolate & wound isolates 1 & 2 are not significantly different from each other but each are significantly different from all other isolates.	$P = 0.010$*
24	6.0	$F(7, 23) = 19.22$, $P = <0.001$*	ATCC isolate & wound isolates 2 are not significantly different but each are significantly different from all other isolates; ATCC isolate & wound isolate 1 are not significantly different from each other; wound isolates 1, 6 & 7 are not significantly different from each other; wound isolates 3-7 are not significantly different from each other.	$P = 0.011$*
24	8.0 *(**note:** no wound isolates 6 or 7)*	$F(5, 16) = 38.33$, $P = <0.001$*	ATCC isolate & wound isolates 1 & 2 are not significantly different from each other but are significantly different from all other isolates; wound isolates 3-5 are not significantly different from each other but are significantly different from all other isolates.	$P = 0.022$*

** Significant at the 0.05 level*

Table 8: *P. aeruginosa* isolates (7 wound isolates): Differences in general protease activity between isolates, at each initial pH.

Culture Time (Hours)	Initial Culture pH	Statistical Analysis	Kruskal-Wallis test
24	Standard Broth (7.3)	F(6, 19) = 25.70, P = <0.001*	P = 0.030*
24	5.5	F(6, 20) = 30.69, P = <0.001*	P = 0.025*
24	6.0	F(6, 20) = 18.13, P = <0.001*	P = 0.030*
24	8.0	F(4, 13) = 31.46, P = <0.001*	P = 0.037*

** Significant at the 0.05 level*

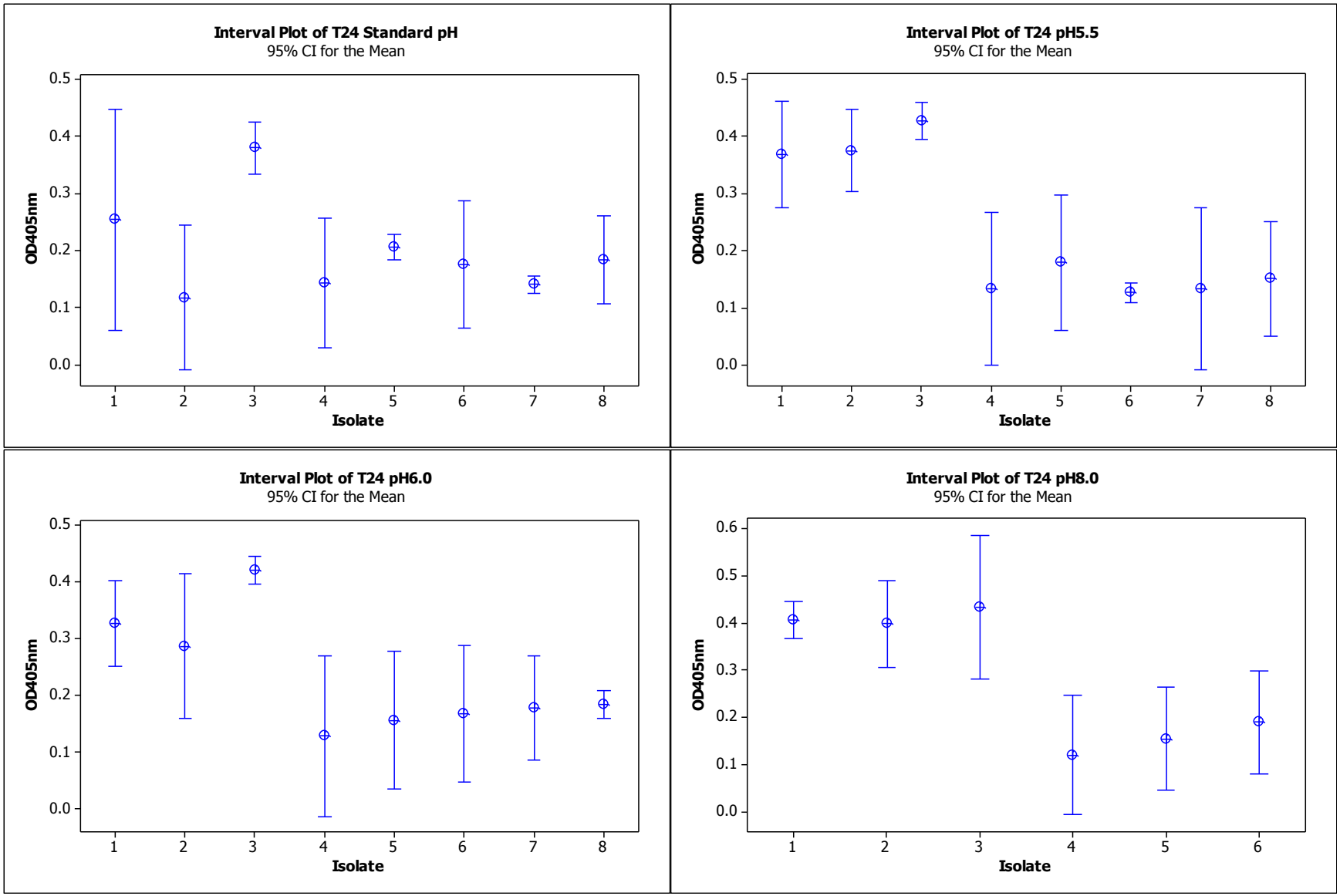


Figure 16: Interval plots for proteolytic activity of the *P. aeruginosa* isolates at each initial culture pH following a 24hour broth culture. For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-7 were numbered isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analysis.

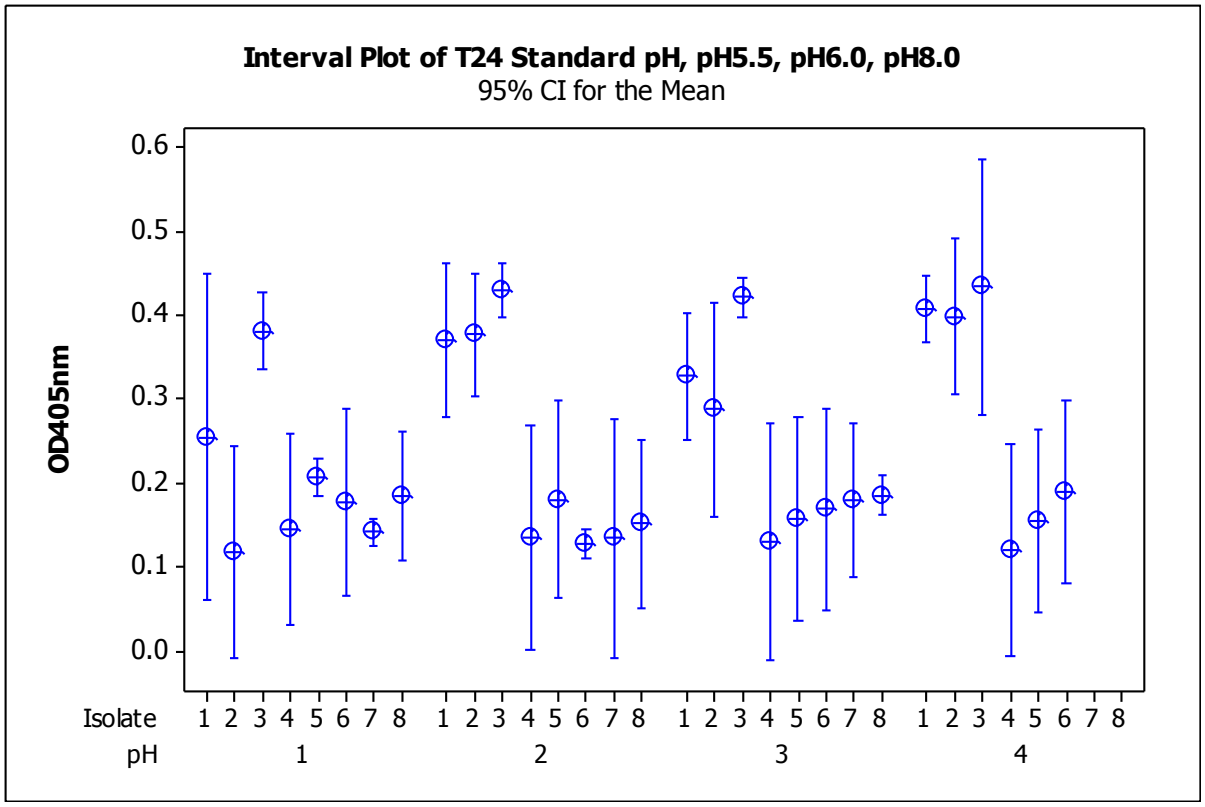


Figure 17: Interval plots for proteolytic activity of the *P. aeruginosa* isolates at each initial broth culture pH following a 24 hour broth culture. For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-7 were numbered isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analysis. Standard pH=pH1; pH5.5=pH2; pH6.0=pH3; pH8.0=pH4.

2.4.5 Elastin-Congo Red Assay for Elastase Activity

The elastolytic activities of each of the *P. aeruginosa* wound isolates and of the ATCC reference isolate were determined using the elastin-congo red (ECR) assay as described above; LasB-deficient and active strains can be differentiated using this assay (Schaber *et al*, 2004). Whilst both *P. aeruginosa* elastase A and elastase B degrade elastin, elastase B (an important virulence factor in wounds and burns) has been shown to degrade elastin to a greater extent than elastase A (Caballero *et al*, 2001). However, the ECR assay cannot definitively distinguish between the products of the LasB and LasA genes (Jones *et al*, 1993; Peters *et al*, 1992). *P. aeruginosa* alkaline protease and protease IV do not degrade elastin (Caballero *et al*, 2001). The assay demonstrated that the EF isolate, previously confirmed to be negative for the LasB gene (see Figure 3), exhibited very minimal elastolytic activity. Elastolytic activity was determined for each isolate at each time point; however, as with the azocasein assays elastolytic activities were low at the start of each culture, thus only results of elastolytic activity at the end of the 24 hour culture were used for statistical analysis.

Results demonstrated that there is a significant difference between isolates (see figures 18-22), including a large variation between wound isolates which was maintained at each initial culture pH after a 24 hour culture period (see tables 9 and 10). However, there was no clear trend in terms of an increase or decrease in protease activity of the isolates when the pH of the culture broth was changed. In concordance with both the milk-casein agar plate assays and the azocasein assays, wound isolate 1 demonstrated a low elastolytic activity at each initial culture pH when compared to the majority of the other isolates (ATCC reference isolate and wound isolates). Furthermore, wound isolate 1 did not produce measurable elastolytic activity until the 24 hour culture time point, in contrast to the other wound isolates. The ATCC reference strain also did not demonstrate elastolytic activity until the 24 hour time point at each initial culture pH. In contrast to the azocasein assays, wound isolates 2 and 3 did not produce significantly more elastase than the other wound isolates at 24 hours, however these isolates exhibited a steady increase in elastolytic activity throughout the cultures at each initial pH. Wound isolate 3 produced little elastolytic activity throughout each of the cultures, at each initial pH.

Generally, the levels of elastolytic activity increased over time, with levels highest at the end of the 24 hour culture. This observation applied to each of the different initial pH cultures. Interval plots (figures 23 and 24) illustrate the protease profiles of each isolate at each initial pH following a 24 hour culture period. Figure 25 illustrates the ECR assay plate format.

Further analysis revealed that there was a statistical significance between elastase activities of the isolates (ATCC isolate plus 7 wound isolates) when cultured at either standard pH (pH7.3), pH5.5 or at pH6.0, $F(2, 70) = 4.47$, $P = 0.015$. When pH8.0 was considered (ATCC isolate plus 5 wound isolates [no wound isolates 6 or 7]), however, there was a greater significant difference in elastase activities of the isolates (this does not, however, necessarily indicate a greater biological difference), $F(3, 70) = 7.39$, $P = <0.001$ with the greatest difference between elastolytic activities at the standard initial pH (pH7.3) and pH8.0, with more isolates producing low levels of elastase at an initial culture pH of 8.0. Yet, as was the case for the milk-casein agar plates and the azocasein assays, there was strong evidence suggestive of an interaction between pH and isolate and so these observations could not be validated; alteration of the initial culture pH affected isolates in different ways: $F(14, 70) = 32.39$, $P = <0.001$(comparison between standard pH (7.3), pH5.5 and pH 6.0); $F(15, 70) = 43.72$, $P = <0.001$ (comparison between standard pH (7.3), pH5.5, pH 6.0 and pH8.0). Given the complex relationships, Appendix 5 details the grouping information which shows how each individual isolate responded at each initial culture pH.

2.4.6 Association of BFP with Elastolytic Activity

Given the evidence within the literature suggestive of a relationship between a lack in the quorum sensing system, which controls the production of several virulence factors, and a reduction in LasB and LasA activity (Schaber *et al*, 2004) further statistical analysis was undertaken to define whether there is a relationship between the elastolytic activities of the *P. aeruginosa* wound isolates and BFP. At standard initial pH (7.3) there was a statistically significant relationship between BFP and elastolytic activity, $F(2, 19) = 16.73$, $P = <0.001$, with a significant difference between BFP scores of 0, 3 and 4 (the mean elastolytic activity for a BFP score of 3 was, however, higher than the mean for a BFP score of 4). Likewise, at an initial culture pH of 5.5 there was a statistically significant difference between the elastolytic activities of isolates identified by their BFP, $F(2, 20) = 6.98$, $P = 0.006$ [Kruskal-Wallis test: $P = 0.006$], with a significant difference between BFP scores of 0 and 3. However, at initial culture pHs of 6.0 and 8.0 there was no significant difference: $F(2, 20) = 2.85$, $P = 0.084$ (pH6.0), $F(2, 14) = 3.52$, $P = 0.063$. Results, however, were based on limited case numbers; there was only 1 non-adherent isolate and 1 moderately adherent isolate with the remaining isolates either highly or very highly adherent. Despite the lack of conclusive statistically significant data supportive of a link between BFP and protease activity given the limitations in terms of numbers of isolates for each BFP score, it was noted that wound isolate 1 (non-biofilm forming isolate) produced extremely low levels of elastolytic activity during the early stages of culture when cultured under each initial pH. Further, after the full 24 hour culture this wound isolate produced less elastolytic activity than all but one of the isolates at each initial pH. Conversely, there seemed to be no correlation between the intensity of biofilm formation and elastolytic activity, with wound isolate 6 (a very high BFP isolate) not demonstrating significantly more elastolytic activity than lower BFP isolates. Further investigation is needed using a larger number of isolates, with varying BFP.

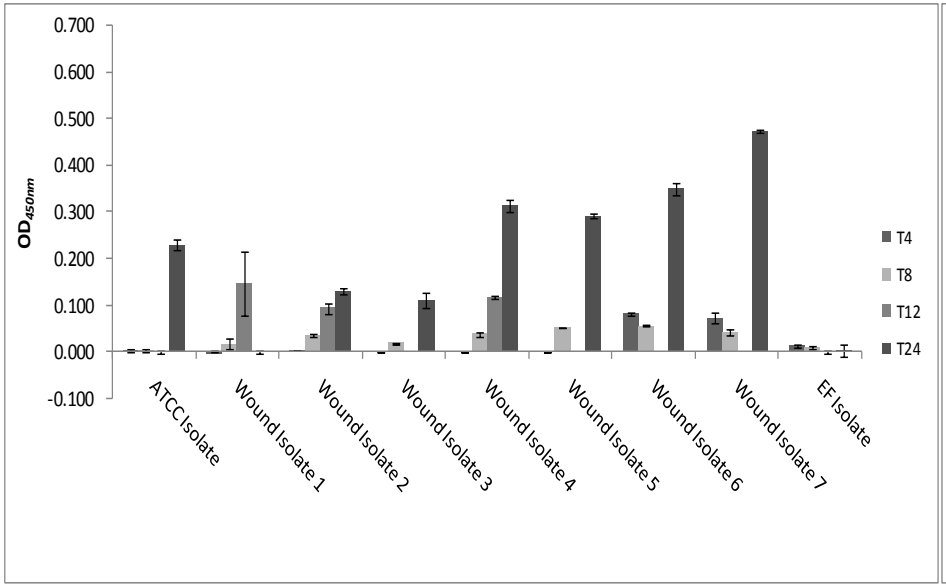


Figure 18: Elastin-Congo red (ECR) assay for elastinolytic activity analysis of each isolate over a 24 hour period. Isolates were cultured in standard Mueller Hinton broth (MHB) broth (initial pH7.3). Data is presented as mean ± SEM. Statistical significance is detailed in tables 8 & 9. *EF= *E. faecium*

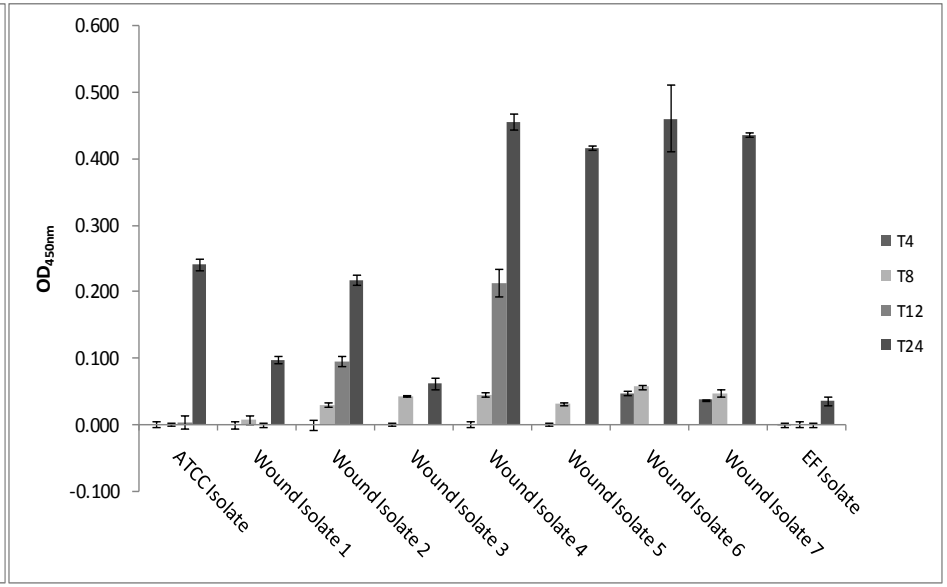


Figure 19: Elastin-Congo red (ECR) assay for elastinolytic activity analysis of each isolate over a 24 hour period. Isolates were cultured in Mueller Hinton broth (MHB) adjusted to an initial pH of 5.5. Data is presented as mean ± SEM. Statistical significance is detailed in tables 8 & 9. *EF= *E. faecium*

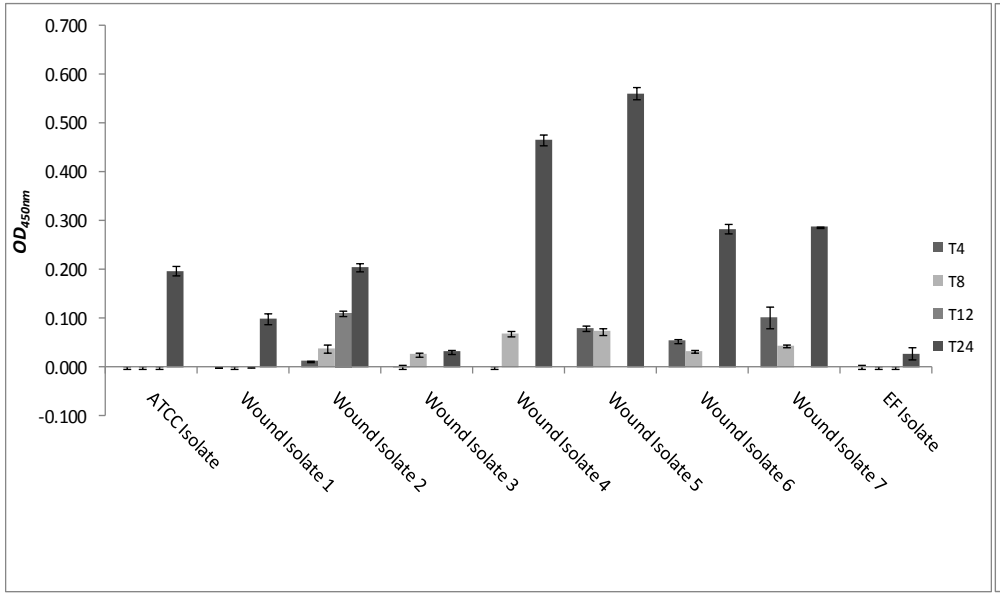


Figure 20: Elastin-Congo red (ECR) assay for elastinolytic activity analysis of each isolate over a 24 hour period. Isolates were cultured in Mueller Hinton broth (MHB) adjusted to an initial pH of 6.0. Data is presented as mean ± SEM. Statistical significance is detailed in tables 8 & 9. *EF= *E. faecium*

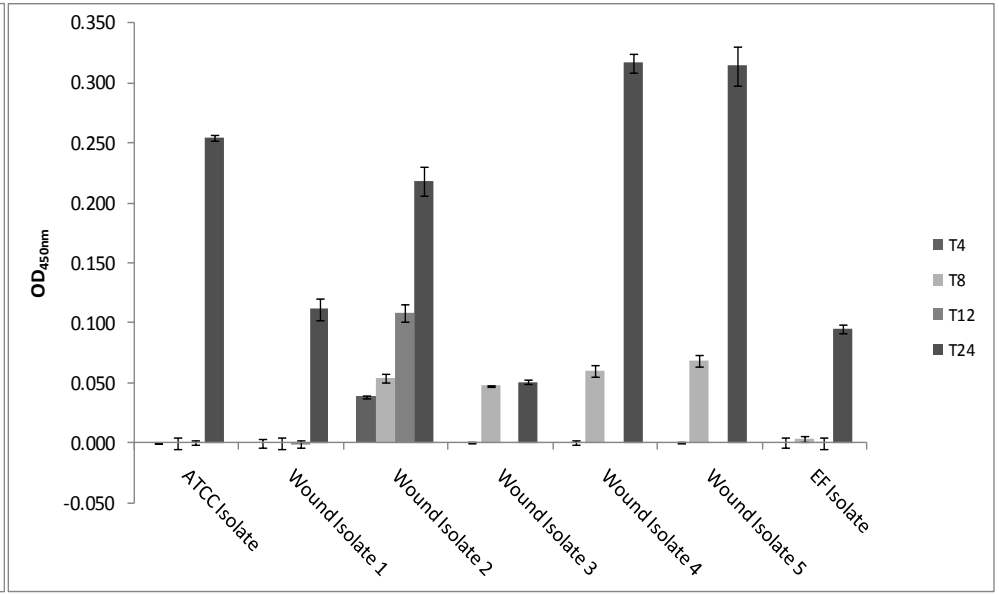


Figure 21: Elastin-Congo red (ECR) assay for elastinolytic activity analysis of each isolate over a 24 hour period. Isolates were cultured in Mueller Hinton broth (MHB) adjusted to an initial pH of 8.0. Data is presented as mean ± SEM. Statistical significance is detailed in tables 8 & 9. *EF= *E. faecium*

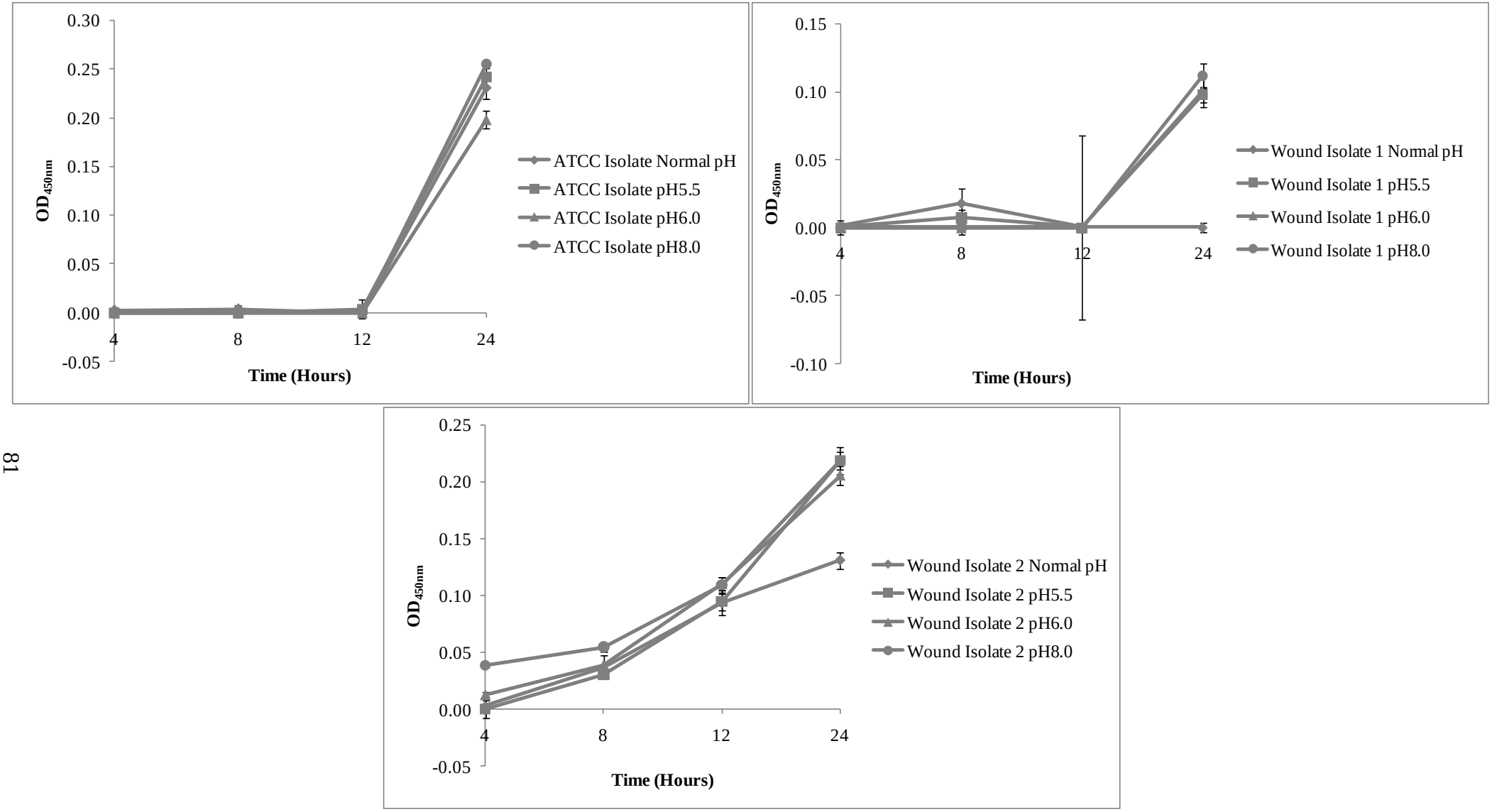


Figure 22: Elastin-Congo red (ECR) assay data for 3 example isolates, showing variations in elastinolytic activity when cultured at different initial pHs. Data is presented as mean ± SEM. Statistical significance is detailed in tables 8 & 9.

Table 9: *P. aeruginosa* isolates (ATCC isolate + 7 wound isolates): Differences in elastolytic activity between isolates, at each initial pH.

Culture Time (Hours)	Initial Culture pH	Statistical Analysis	Post-Hoc Observations (using the Tukey method)	Kruskal-Wallis test
24	Standard Broth (7.3)	F(7, 22) = 202.57, P = <0.001*	ATCC isolate is significantly different from all other isolates; wound isolate 1 is significantly different from all other isolates; wound isolate 7 is significantly different from all other isolates; wound isolates 2 &3 are not significantly different from each other but are significantly different from all other isolates; wound isolates 4 & 5 are not significantly different from each other; wound isolates 4 & 6 are not significantly different from each other.	
24	5.5	F(7, 23) = 74.11, P = <0.001*	ATCC isolate and wound isolate 2 are not significantly different from each other but are significantly different from all other isolates; Wound isolates 1 & 3 are not significantly different from each other but are significantly different from all other isolates; wound isolates 4, 5, 6 & 7 are not significantly different from each other but are significantly different from all other isolates.	P = 0.003*
24	6.0	F(7, 23) = 355.74, P = <0.001*	Wound isolate 5 significantly different from all other isolates; wound isolate 4 is significantly different from all other isolates; wound isolates 6 & 7 are not significantly different from each other but are significantly different from all other isolates; ATCC isolate and wound isolate 2 are not significantly different from each other but are significantly different from all other isolates; Wound isolate 1 is significantly different from all other isolates; wound isolate 3 is significantly different from all other isolates.	
24	8.0	F(5, 17) = 126.96, P = <0.001*	Wound isolates 4 & 5 are not significantly different from each other but are significantly different from all other isolates; ATCC isolate and wound isolate 2 are not significantly different from each other but are significantly different from all other isolates; Wound isolate 1 is significantly different from all other isolates; wound isolate 3 is significantly different from all other isolates.	P = 0.007*

** Significant at the 0.05 level*

Table 10: *P. aeruginosa* isolates (7 wound isolates): Differences in elastolytic activity between isolates, at each initial pH.

Culture Time (Hours)	Initial Culture pH	Statistical Analysis	Kruskal-Wallis test
24	Standard Broth (7.3)	$F(6, 19) = 237.62$, $P = <0.001$	
24	5.5	$F(6, 20) = 76.35$, $P = <0.001$	$P = 0.006$*
24	6.0	$F(6, 20) = 398.05$, $P = <0.001$	
24	8.0	$F(4, 14) = 128.32$, $P = <0.001$	$P = 0.012$*

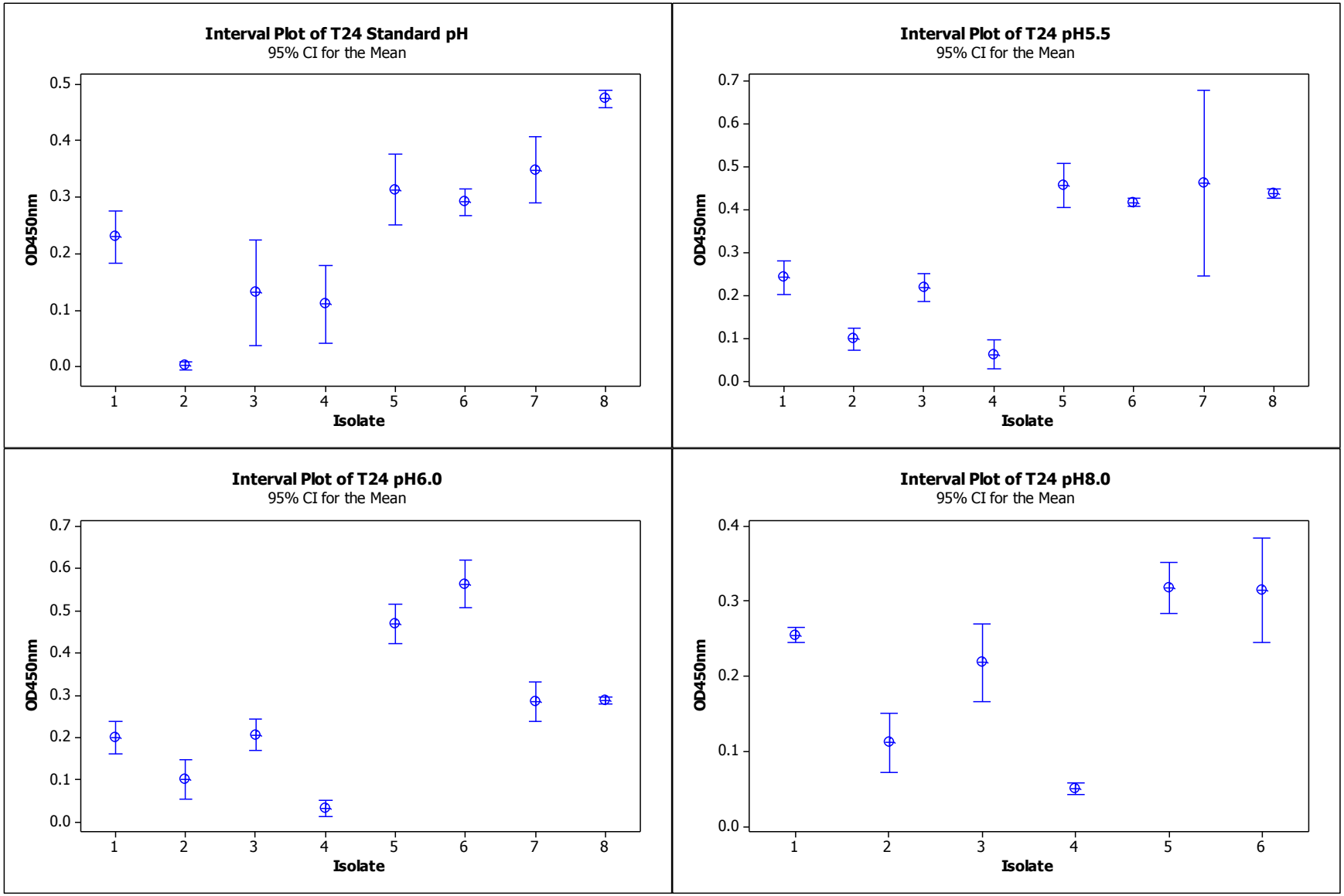


Figure 23: Interval plots for elastolytic activity of the *P. aeruginosa* isolates at each initial culture pH following a 24hour broth culture. For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-7 were numbered isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analysis.

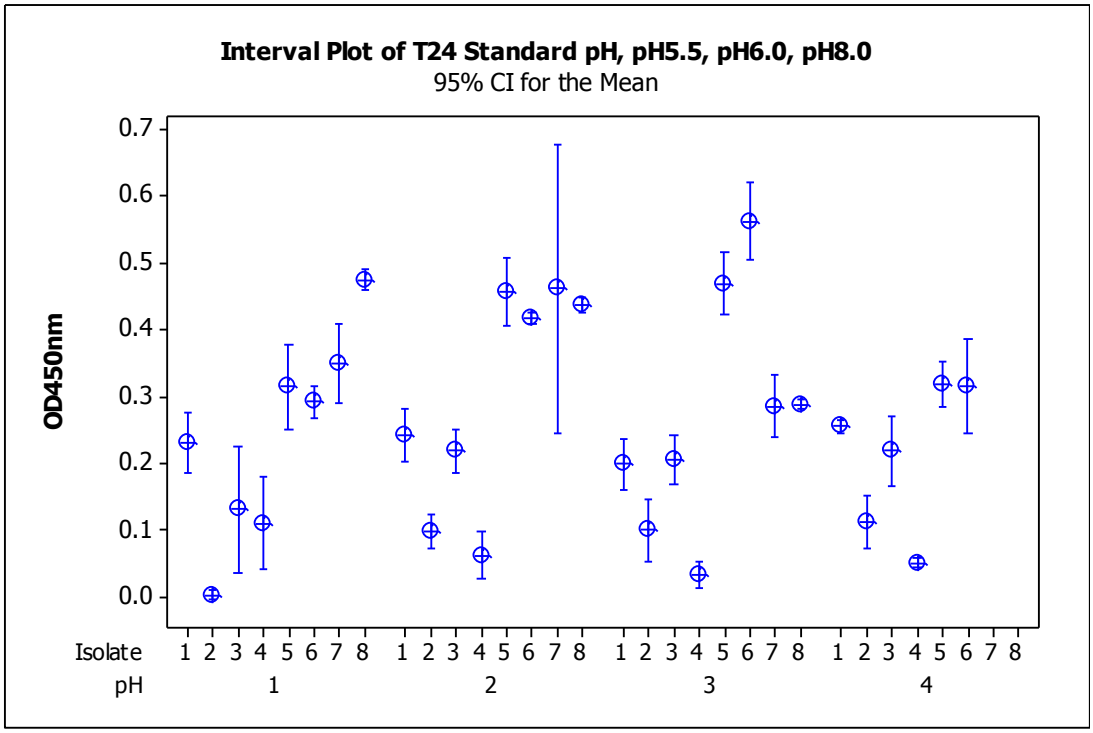


Figure 24: Interval plots for elastolytic activity of the *P. aeruginosa* isolates at each initial broth culture pH following a 24 hour broth culture. For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-7were numbered isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analysis. Standard pH=pH1; pH5.5=pH2; pH6.0=pH3; pH8.0=pH4.

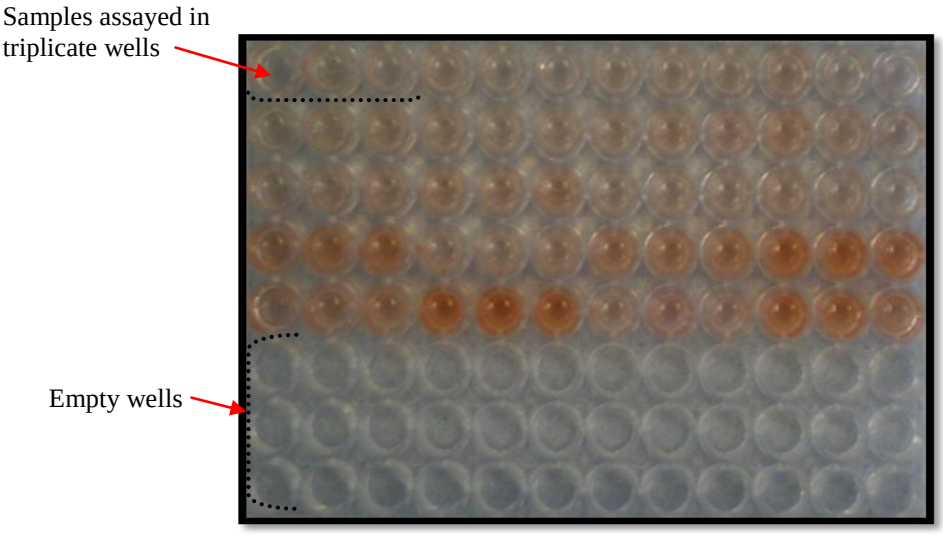


Figure 25: Example elastin-congo red (ECR) assay microtitre plate showing variations in elastase activity of different *P. aeruginosa* isolates. Samples were assayed in triplicate.

2.4.7 Intracellular Protease Activity

To determine any intracellular proteolytic activity, cell pellets from *P. aeruginosa* aerobic cultures (initial culture pH of 7.4) were lysed with lysis buffer and cells were sonicated using an ultrasonic homogeniser (Bandelin Electronic, Berlin, Germany) to disrupt cell walls/cell membranes and release cellular contents. Results revealed very minimal intracellular proteolytic activity for each of the *P. aeruginosa* isolates (see figures 26 and 27). However, a number of inherent limitations in this method may have led to an underestimation of this proteolytic activity. Firstly, the major secreted proteases of *P. aeruginosa* are activated upon autoproteolytic processing once secreted into the surrounding environment (Folders *et al*, 2000). Secondly, the subtle variations in the colour of the cell pellets may have influenced results of the colourimetric protease assays.

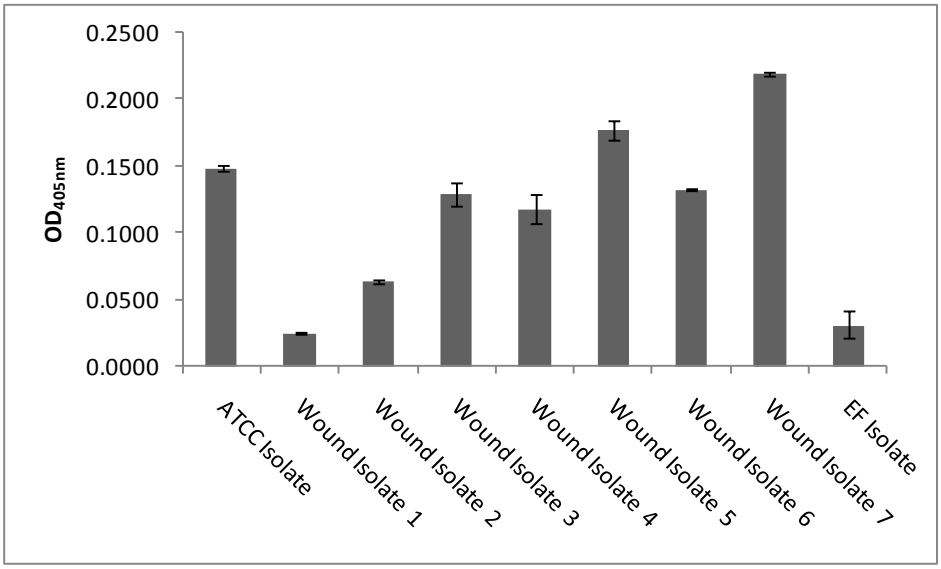


Figure 26: Azocasein assay for intracellular proteolytic activity for each *P. aeruginosa* isolate. Data is presented as mean ± SEM. *EF= *E. faecium*

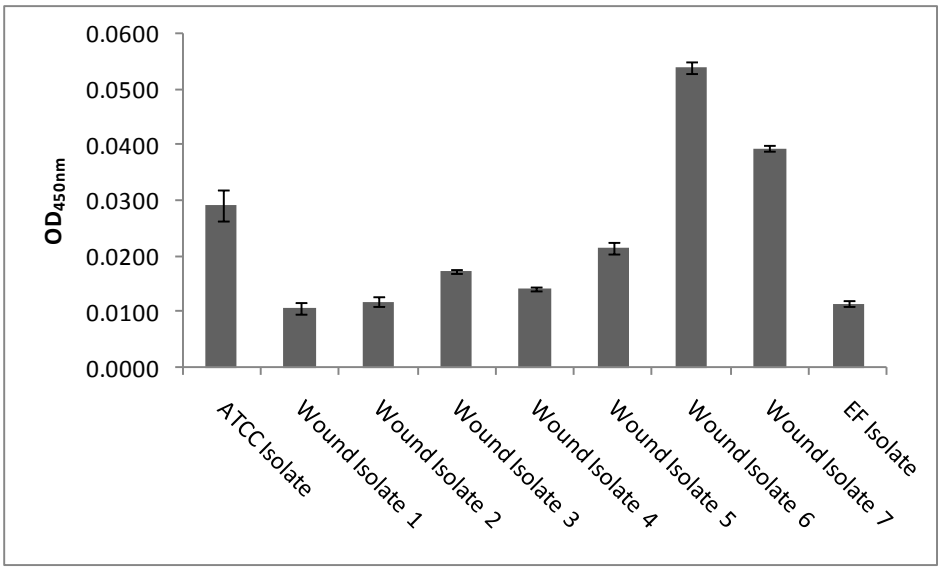


Figure 27: Elastin-Congo red (ECR) assay for intracellular elastolytic activity for each *P. aeruginosa* isolate. Data is presented as mean ± SEM. *EF= *E. faecium*

2.4.8 Anaerobic Cultures

Anaerobic cultures of each of the isolates were prepared in an anaerobic chamber (0% oxygen) to determine the effect of oxygen deprivation on growth and protease activity. All isolates were cultured using standard MHB (pH7.3). Protease activity was measured using the assays described above for the aerobic cultures over a 48 hour culture period. Statistical analysis was conducted for proteolytic activities at the 24 and 48 hour time points (the EF isolate was excluded from statistical analyses).

Results of the milk-casein agar plate assays (see figure 28) have shown that there is a significant difference between isolates (ATCC isolate plus wound isolates) at the 24 hour broth culture time point (followed by a 24 hour milk plate culture), $F(7, 47) = 16.03$, $P = <0.001$ which was maintained at the 48 hour time point (followed by a 24 hour milk plate culture), $F(5, 32) = 42.12$, $P = <0.001$. There was a general trend for all isolates, apart from wound isolate 3, showing that general protease activity peaked following an initial broth culture period of 4 hours followed by a 24 hour plate culture, with proteolytic activity declining over time following this time point; in comparison to the aerobic cultures, isolates were not able to maintain protease activity for extended periods. The difference between general protease activity, as demonstrated via the azocasein assay (see figure 26), of each isolate was significant at the 24 hour culture time point, $F(7, 31) = 9.98$, $P = <0.001$ which was maintained following a further 24 hours in culture, $F(7, 31) = 11.17$, $P = <0.001$. Azocasein assays demonstrated a lack of general protease activity of the ATCC reference isolate in comparison to the wound isolates (figure 29); however, elastolytic activity, as shown by the elastin-congo red assays (see figure 30), of the ATCC isolate was positive with enzyme levels increasing steadily over time. The difference between elastolytic activity, as demonstrated via the ECR assay, of each isolate was significant at the 24 hour culture time point, $F(7, 31) = 7.83$, $P = <0.001$. This difference between isolates, however, was not maintained following a further 24 hours in culture, $F(7, 31) = 0.88$, $P = 0.539$. As with the aerobic cultures, wound isolate 1 remained the least active isolate in terms of both general protease activity and elastolytic activity. In concordance with the aerobic cultures, wound isolate 3 was also a poor producer of proteolytic activity.

Differences between the growth of isolates remained similar to the aerobic cultures. Whilst statistical analysis of the bacterial densities of cultures at the 24 and 48 hour culture time points revealed a significant difference between isolates, the differences noted between proteolytic and elastolytic activities of isolates was not likely due to isolates growing at a faster rate (i.e. there was a true difference in proteolytic and elastolytic activity between isolates); those isolates which grew more slowly did not necessarily produce less proteolytic or elastolytic activity: for instance, wound isolate 2 was the slowest growing isolate under anaerobic conditions yet demonstrated the greatest elastolytic activity (figure 31). In contrast, wound isolate 3 was shown to have one of the fastest growth rates yet was a relatively poor producer of elastolytic activity. Furthermore, whilst the *P. aeruginosa* isolates continued to grow steadily over the culture period (apart from wound isolate 2), reaching stationary phase at 24 hours, general proteolytic or elastolytic activity did not always parallel this cell growth.

Whilst overall proteolytic and elastolytic activities of all isolates were lower than those of the aerobic cultures, all isolates had slower growth rates. Growth curve analysis has also shown that under anaerobic conditions the isolates have a longer lag phase with the logarithmic phase beginning at 8 hours. However, when the elastolytic activity of isolates cultured for 24 hours was considered in terms of the amount of bacteria, the isolates were actually generating more elastolytic activity than in aerobic culture.

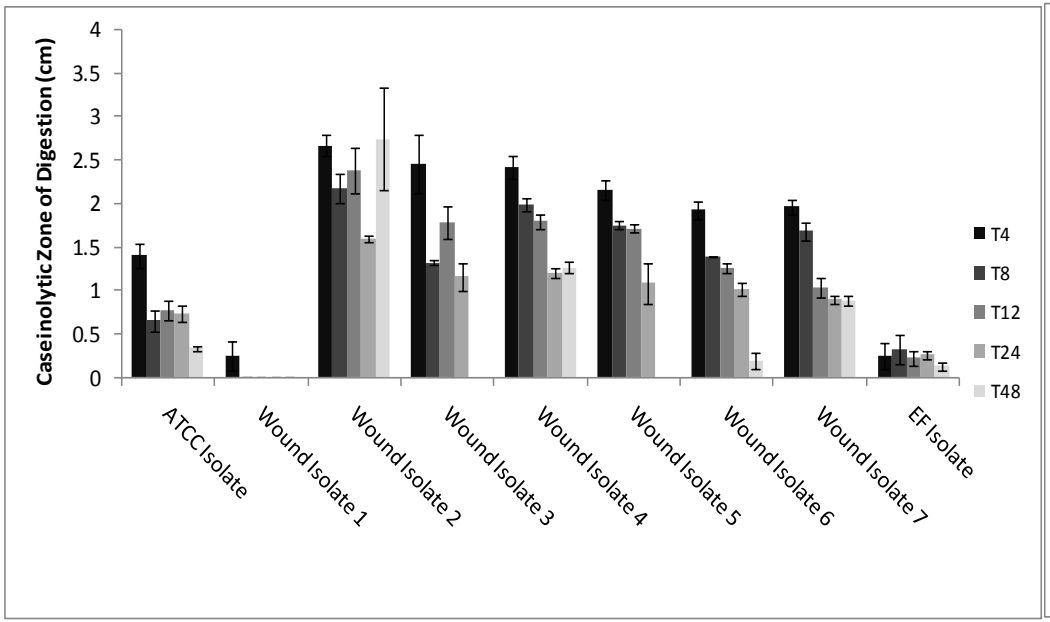


Figure 28: Milk-Casein agar plate assay for caseinolytic digest analysis of each isolate over a 48 hour period. Isolates were cultured in Mueller Hinton broth (MHB) under anaerobic conditions. Data is presented as mean ± SEM. *EF= *E. faecium*

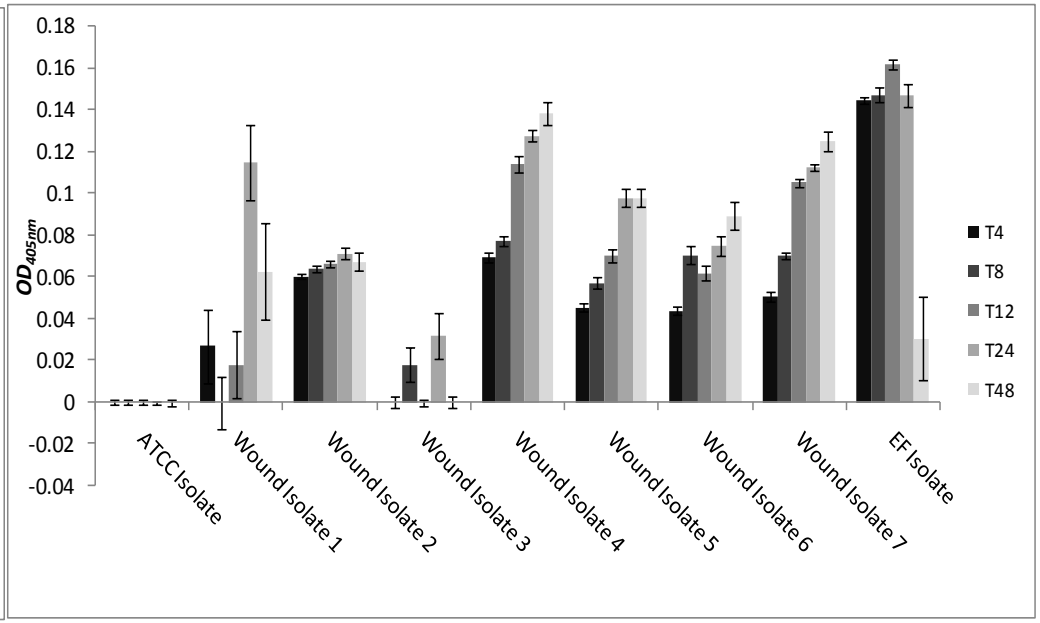


Figure 29: Azocasein assay for caseinolytic digest analysis of each isolate over a 48 hour period. Isolates were cultured in Mueller Hinton broth (MHB) under anaerobic conditions. Data is presented as mean ± SEM. *EF= *E. faecium*

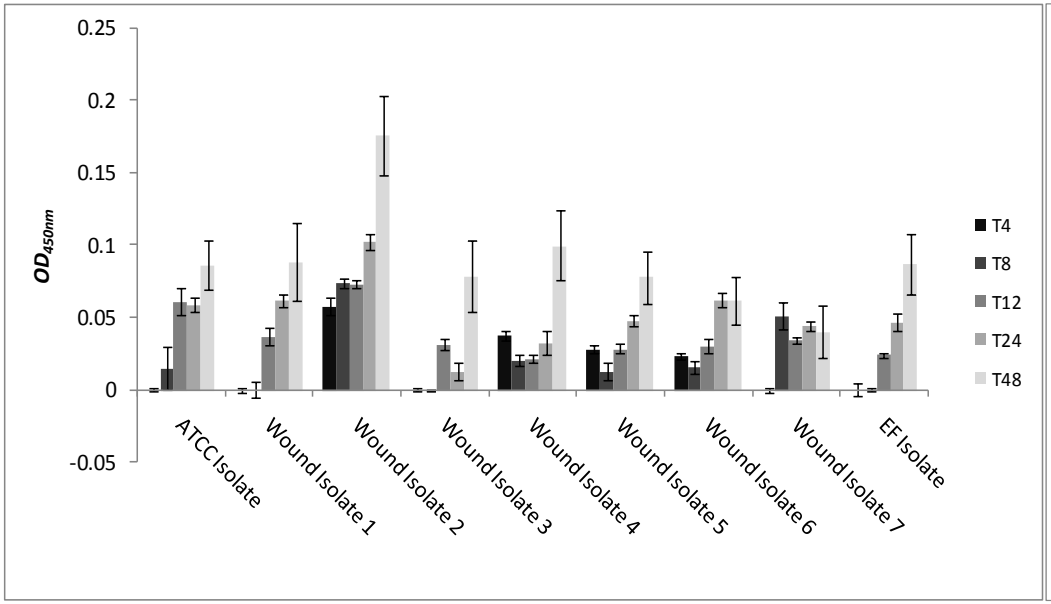


Figure 30: Elastin-Congo red assay for elastinolytic activity analysis of each isolate over a 48 hour period. Isolates were cultured in Mueller-Hinton broth (MHB) under anaerobic conditions. Data is presented as mean ± SEM.

*EF= *E. faecium*

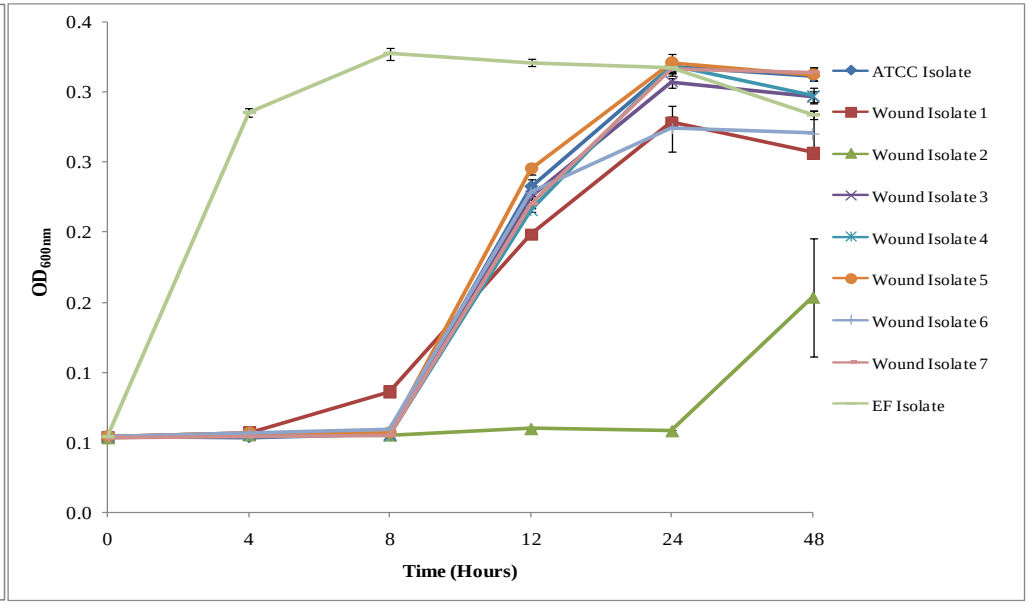


Figure 31: Growth curves of each isolate over a 48 hour period. Isolates were cultured in Mueller Hinton broth (MHB) under anaerobic conditions. Data is presented as mean ± SEM.

*EF= *E. faecium*

2.4.9 *P. aeruginosa* Growth Curves

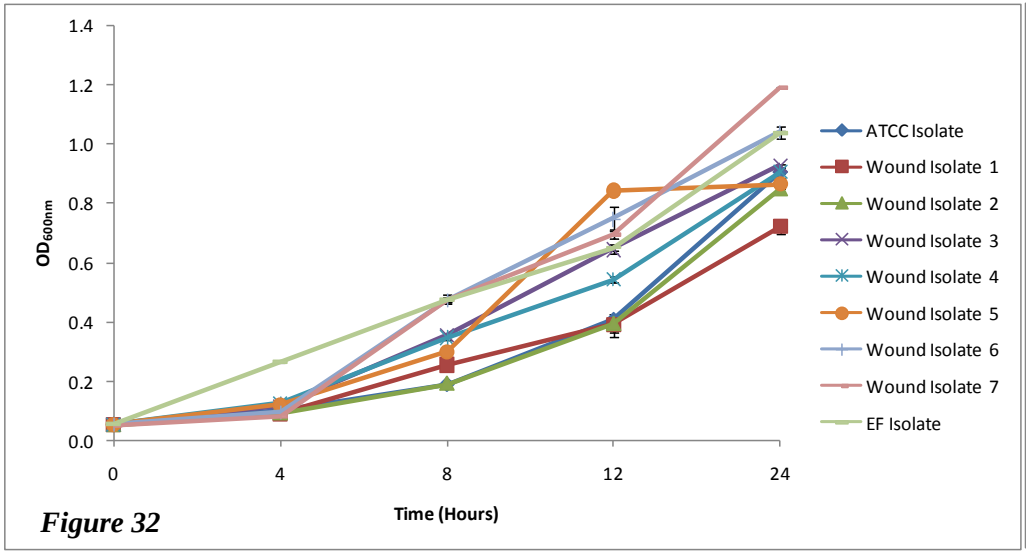


Figure 32

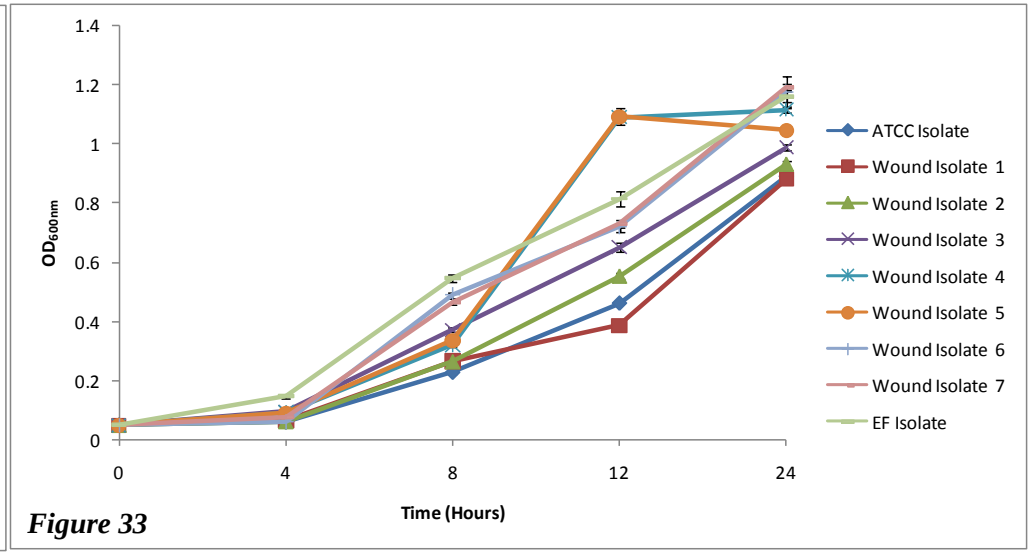


Figure 33

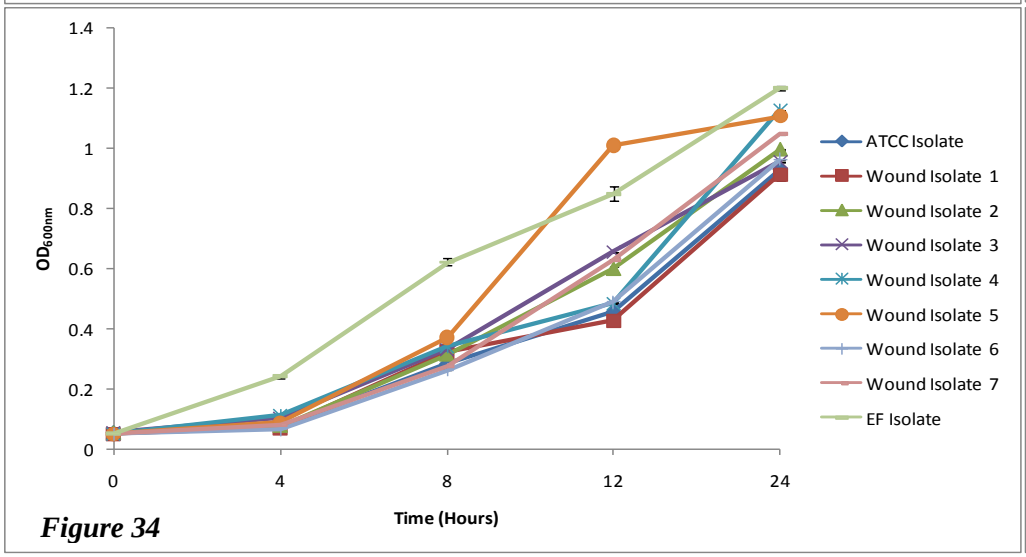


Figure 34

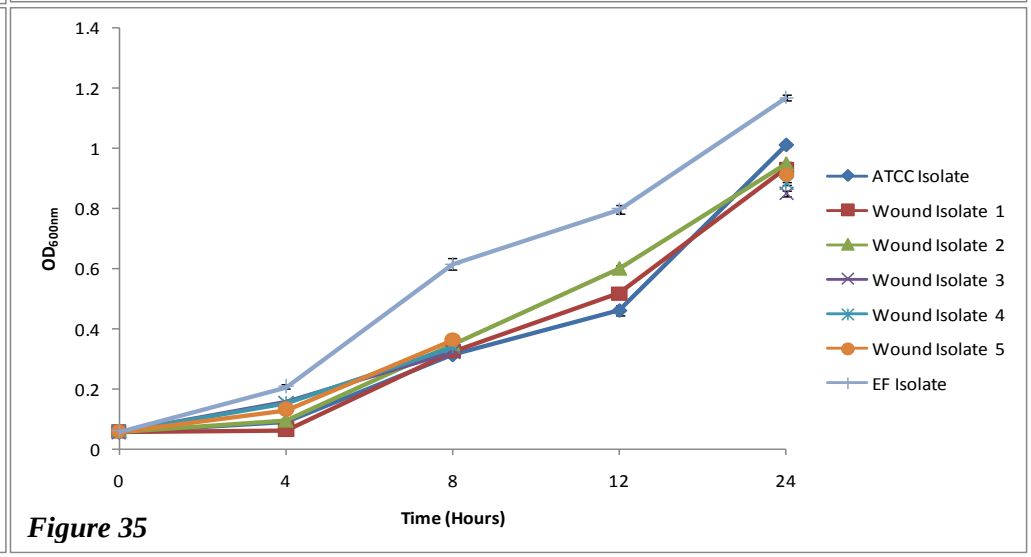


Figure 35

Figures 32-35: Bacterial growth curves; the 7 *P. aeruginosa* wound isolates, the ATCC *P. aeruginosa* isolate and the *Enterococcus faecium* (EF) wound isolate were grown over a 24 hour period in Mueller Hinton broth (MHB) at the 4 different initial pHs (standard broth at pH7.3, pH5.5, pH6.0 and pH8.0). At four-hourly intervals up to 24hours, samples of the culture broth were taken and measurements were made in triplicate of the OD_{600nm}. Figure 20: initial pH7.3; Figure 21: initial pH5.5; Figure 22: initial pH6.0; Figure 23: initial pH8.0. Data is presented as mean ± SEM. Statistical significance is detailed in Table 10.

Growth curves demonstrated that most of the *P. aeruginosa* isolates had similar *in vitro* growth patterns despite the differences in protease activity (see figures 32-35). Most isolates had a lag phase of 4 hours, after which time isolates entered the logarithmic phase of growth at each initial culture pH. Isolates had very similar growth curves at each initial culture pH. Whilst statistical analysis revealed a significant difference between the ODs of isolates at the 24 hour time point (see table 11), it is unlikely that the differences observed in protease/elastase activity between isolates was due to differences in bacterial density at this time point; those isolates which were strong producers of protease/elastase activity did not always have the highest OD at T24. Furthermore, differences between actual OD values at T24 were very small.

Table 11: *P. aeruginosa* wound isolates: Differences in the optical density (OD_{600nm}) of cultures following a 24 hour culture at each initial pH (difference between isolates).

Culture Time (Hours)	**Initial Culture pH**	**Statistical Analysis**	**Kruskal-Wallis test**
24	Standard Broth (7.3)	$F(7, 23) = 141.08$, $P = <0.001$*	$P = 0.003$*
24	5.5	$F(7, 23) = 31.06$, $P = <0.001$*	
24	6.0	$F(7, 23) = 13.71$, $P = <0.001$*	
24	8.0	$F(5, 17) = 52.20$, $P = <0.001$*	

2.4.10 Gelatin Zymography

Gelatin zymography confirmed the results of the biochemical enzyme assays in terms of increased protease activity over the 24 culture period; protease bands were most intense at the 24 hour time point. Bands were not visible at the 4 hour culture period and band intensity increased from 8 hours to 24 hours. All proteolytic bands present on the gelatin zymograms were at around the 50kDa mark, indicative of alkaline proteinase (Marquart *et al*, 2005). Whilst the elastin congo-red assay detected elastolytic activity of the culture effluents the level of activity may not have been high enough for detection on the zymogram gels. Whilst statistical analysis did not confirm a significant variation in proteolytic activity of isolates when cultured at varying initial pH, the proteolytic intensity seen in the gelatin zymograms appeared slightly greater for wound isolate 2 when cultured in broth with an initial pH of 5.5 which mirrors those results seen in the azocasein assay. Proteolytic bands were not as intense or clear for all other isolates, with no bands visible at the 4 hour time point. Proteolytic bands for anaerobic culture samples were also around the 50kDa mark, however bands were not as intense as those bands from aerobic culture samples.

In an attempt to identify the molecular weight of the bands seen in the gelatin zymograms, a method was used to purify and elute the protein from the bands in the zymogram gel matrix. The proteolytic band obtained following gelatin zymography of 24 hour culture supernatant from a wound isolate was excised from the gel. Supernatants from eluted proteins were analysed using SDS-PAGE. Unfortunately, no protein bands could be seen on the gel (see Appendix 6). It may be that, due to dilution of the enzyme during the elution process, the sample requires concentrating prior to running on a gel. An alternative elution method, such as electro-elution may give a higher protein yield. Furthermore, residual stain on the gel pieces excised from the zymogram may have had a negative effect on protein elution. A longer incubation period in elution buffer (for example, overnight) may also have produced better results. Excised gel plugs from the same sample could also be pooled to generate a higher protein yield.

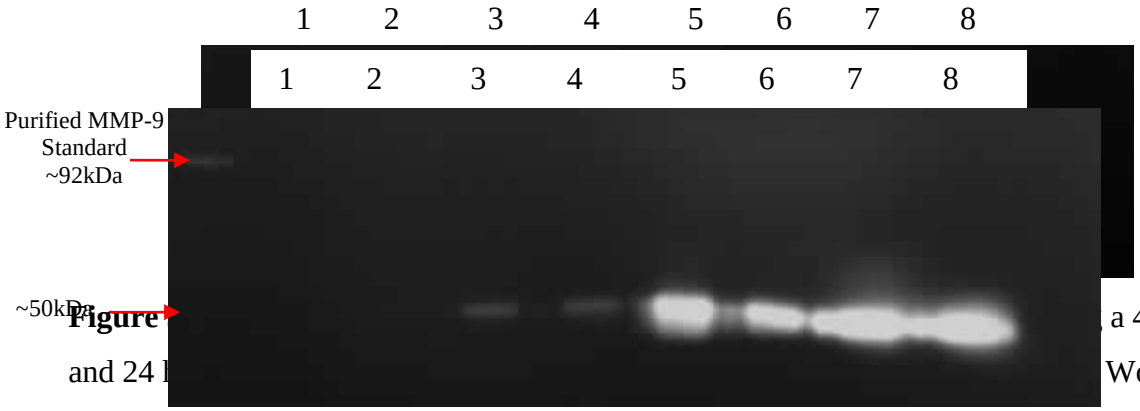


Figure a 4, 8, 12 and 24 h Wound isolate 2, T4; Lane 4: Wound isolate 2, T8; Lane 6: Wound isolate 2, T12. Samples were

Figure 36: Gelatin zymography of wound isolate 2 conditioned media following a 4, 8, 12 and 24 hour aerobic culture. All samples are from initial pH5.5 cultures. Lane 2: Wound isolate 2, T4; Lane 4: Wound isolate 2, T8; Lane 6: Wound isolate 2, T12; Lane 8: Wound isolate 2, T24. Lanes 1, 3, 5 & 7 are samples from the isolate found to be an impure culture, and were excluded from any analysis (see figure 3). Proteolytic bands were most intense at the 24 hour time point (Lane 8). Bands were not visible at the 4 hour time point (Lane 4). All proteolytic bands were at around the 50kDa mark.

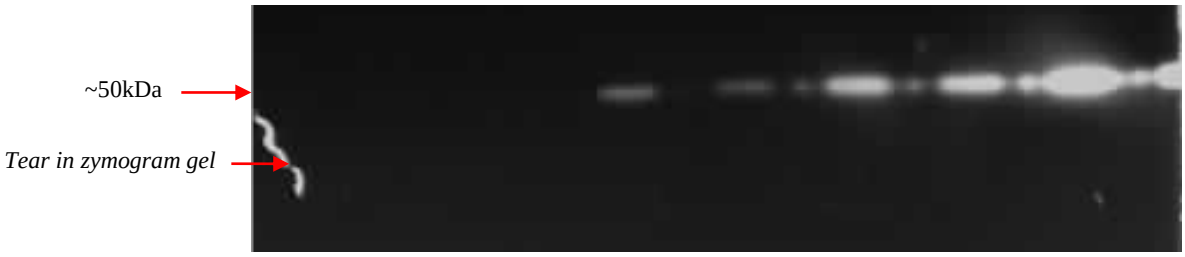


Figure 38: Gelatin zymography of wound isolate 2 conditioned media following a 4, 8, 12 and 24 hour aerobic culture. All samples are from initial pH8.0 cultures. Lane 2: Wound isolate 2, T4; Lane 4: Wound isolate 2, T8; Lane 6: Wound isolate 2, T12; Lane 8: Wound isolate 2, T24. Whilst the MMP-9 standard was used as in figure 24, the band could not be visualised on this gel. Lanes 1, 3, 5 & 7 are samples from the isolate found to be an impure culture, and were excluded from any analysis (see figure 3). Proteolytic bands were most intense at the 24 hour time point (Lane 8). Bands were not visible at the 4 hour time point (Lane 2). All proteolytic bands were at around the 50kDa mark.

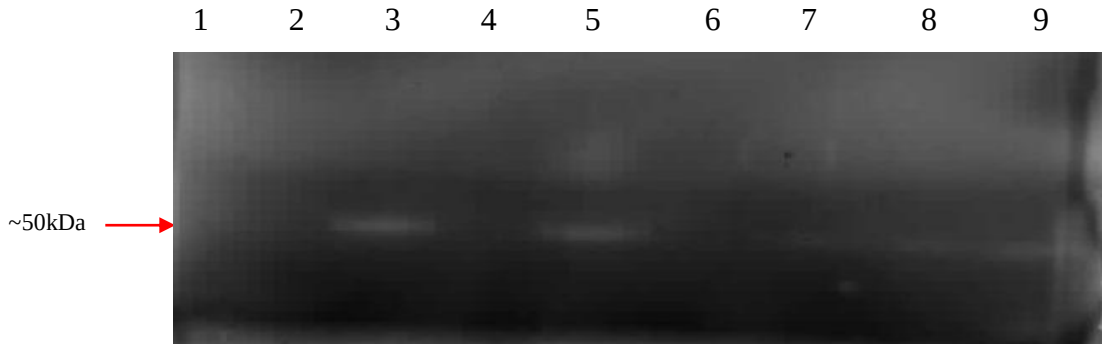


Figure 39: Gelatin zymography of *P. aeruginosa* conditioned media following 24 hour anaerobic culture. Lane 1: ATCC isolate; Lane 2: Wound isolate 1; Lane 4: EF isolate; Lane 5: Wound isolate 2; Lane 6: Wound isolate 3; Lane 7: Wound isolate 4; Lane 8: Wound isolate 5; Lane 9: Wound isolate 6. Lane 3 is a sample from the isolate found to be an impure culture, and was excluded from any analysis (see figure 3). All proteolytic bands were at around the 50kDa mark.

2.4.11 Casein Zymography

Casein zymography was conducted to confirm the results observed in the gelatin zymography gels. The principal for casein zymography is the same as gelatin zymography; the proteases present with the loaded sample degrade a substrate bound to the gel matrix. For casein zymography, a 2% casein solution is used as the substrate, as outlined in the Methods section. As observed for the azocasein assays, the proteolytic activity in the culture effluent varied between isolates. In general, the caseinolytic bands observed in the gels mirrored those seen in the gelatin zymograms. That is, proteolytic bands were visible at around the 50kDa mark, indicative of alkaline protease (see figure 40). However, proteolytic bands were much less prominent on the casein zymograms when compared to the gelatin zymograms. For casein zymography, fresh planktonic cultures were standardised to an OD_{600nm} of 1.0 prior to sample loading on to the gel, given that at lower ODs the proteolytic bands of the samples were not intense enough to visualise. As such, it was not possible to compare proteolytic bands over time.

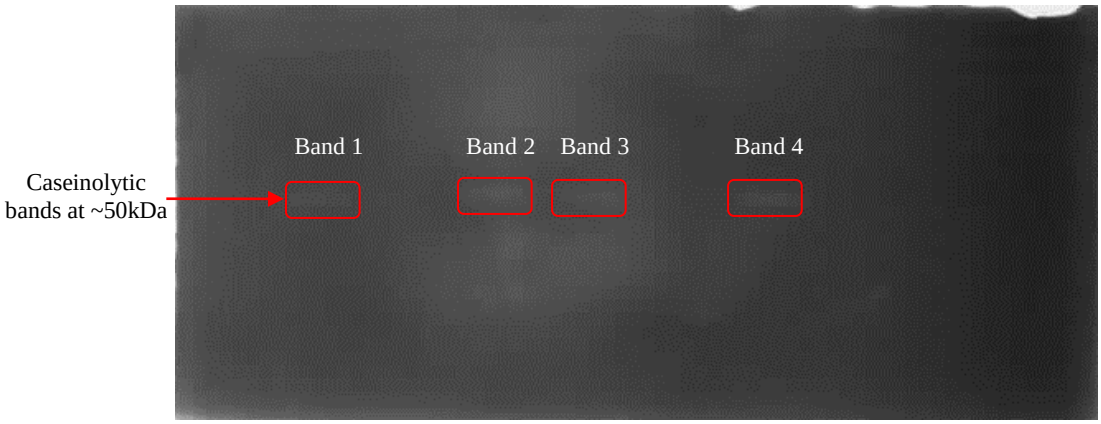


Figure 40: Casein zymography of culture effluent from 24 hour aerobic cultures (all samples were standardised to OD_{600nm} 1.0 prior to sample preparation). All samples are from initial pH7.4 cultures. Band 1: ATCC isolate; Band 2: Wound isolate 2; Band 3: Wound isolate 3; Band 4: Wound isolate 4. Whilst an MMP-2 standard was used, the band could not be visualised on the captured image of this gel. All proteolytic bands were at around the 50kDa mark.

2.4.12 SDS-PAGE (Coomassie staining)

SDS-PAGE analysis confirmed protein levels generally increased over the culture period, with protein bands most prominent at the 24 hour time point. In concordance with the gelatin zymography, protein bands were strongest for both wound isolate 2 when cultured in initial pH5.5 broth, with the strongest bands at around the 33kDa mark at each time point which corresponds with *P. aeruginosa* elastase. Strong bands were also noted at around the 51kDa mark which is suggestive of proelastase, the elastase precursor (alternatively, this band could be the 53kDa preproenzyme of elastase); although further investigation using protein analysis would be required to confirm these postulations. Another strong band was seen at the 10kDa mark. Although it is not possible to determine the protein without further analysis, this low molecular weight protein could be the 10kDa chaperonin of *P. aeruginosa*. The protein band observed at the 25kDa mark could be a cytotoxin, previously known as leukocidin, which contributes to eukaryotic cell invasion. Again, further investigation, is required for protein identification.

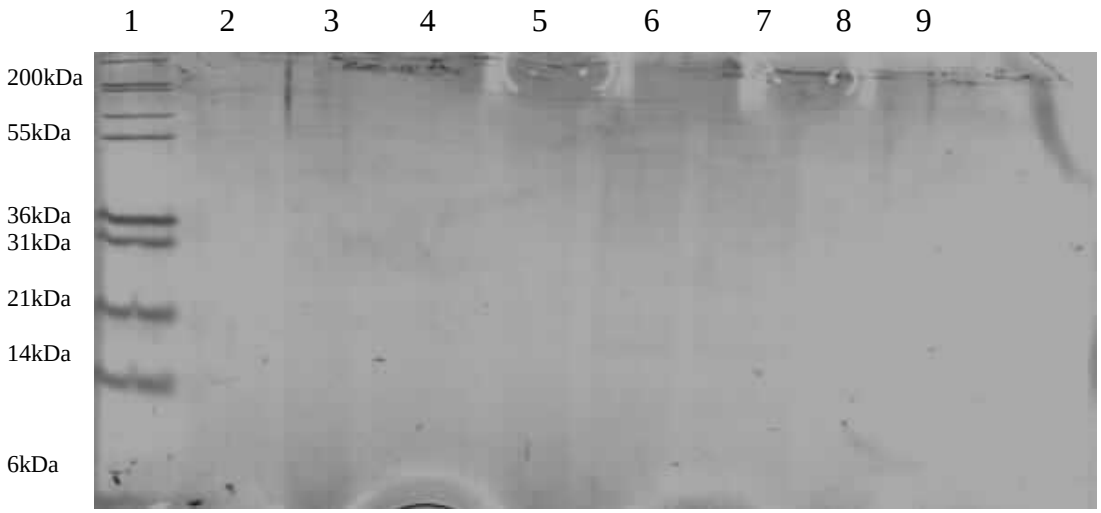


Figure 41: SDS-PAGE of wound isolate 2 following a 4, 8, 12 and 24 hour aerobic culture. All samples are from initial standard pH (pH7.4) cultures. Lane 2: Wound isolate 2, T4; Lane 4: Wound isolate 2, T8; Lane 6: Wound isolate 2, T12; Lane 8: Wound isolate 2, T24. Lanes 3, 5, 7 & 9 are samples from the isolate found to be an impure culture, and were excluded from any analysis (see figure 3).Whilst the protein bands in this gel were not clear, band intensity generally increased over the culture period.

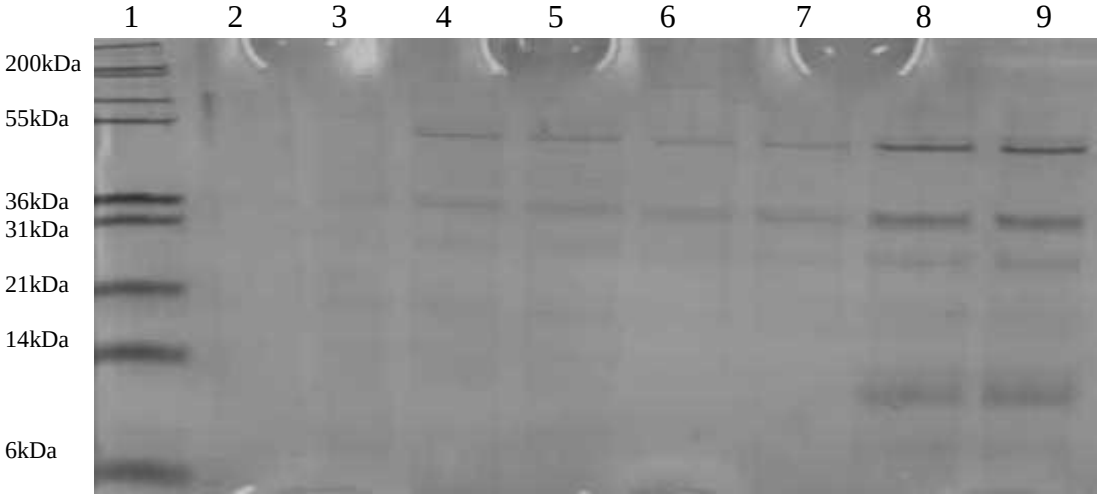


Figure 42: SDS-PAGE of wound isolate 2 following a 4, 8, 12 and 24 hour aerobic culture. All samples are from initial pH5.5 cultures. Lane 2: Wound isolate 2, T4; Lane 4: Wound isolate 2, T8; Lane 6: Wound isolate 2, T12; Lane 8: Wound isolate 2, T24. Lanes 3, 5, 7 & 9 are samples from the isolate found to be an impure culture, and were excluded from any analysis (see figure 3). Protein band intensity increased over the culture period. The most intense protein bands were noted at around the 33kDa mark at each time point.

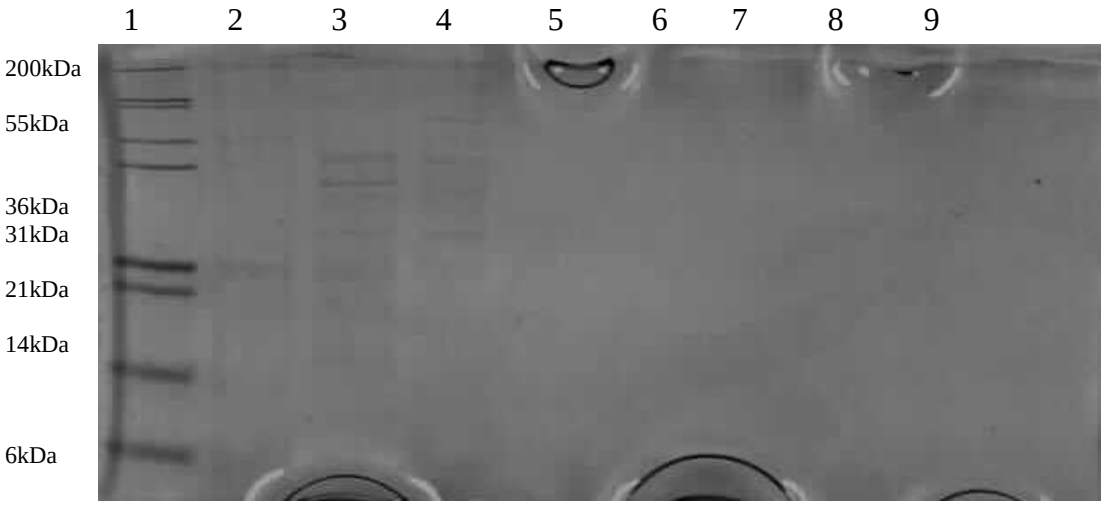


Figure 43: SDS-PAGE of wound isolate 2 following a 4, 8, 12 and 24 hour aerobic culture. All samples are from initial pH6.0 cultures. Lane 2: Wound isolate 2, T4; Lane 4: Wound isolate 2, T8; Lane 6: Wound isolate 2, T12; Lane 8: Wound isolate 2, T24. Lanes 3, 5, 7 & 9 are samples from the isolate found to be an impure culture, and were excluded from any analysis (see figure 3). Whilst the protein bands in this gel were not clear, band intensity generally increased over the culture period.

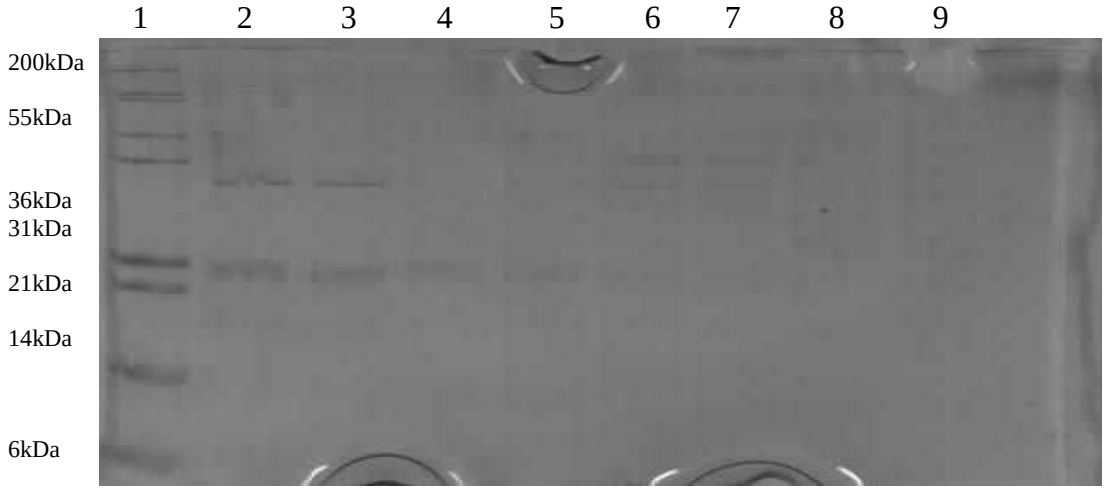


Figure 44: SDS-PAGE of wound isolate 2 following a 4, 8, 12 and 24 hour aerobic culture. All samples are from initial pH8.0 cultures. Lane 2: Wound isolate 2, T4; Lane 4: Wound isolate 2, T8; Lane 6: Wound isolate 2, T12; Lane 8: Wound isolate 2, T24. Lanes 3, 5, 7& 9 are samples from the isolate found to be an impure culture, and were excluded from any analysis (see figure 3). Whilst the protein bands in this gel were not clear, band intensity generally increased over the culture period.

2.4.13 SDS-PAGE (Silver-staining)

For further clarity of protein bands on SDS-PAGE gels, fresh 24 hour aerobic planktonic cultures for the ATCC *P. aeruginosa* strain and each of the 7 wound *P. aeruginosa* isolates were prepared using standard pH (pH7.3) MHB. Samples were run as previously described on SDS-PAGE gels and stained with silver stain. Distinct bands were seen between the 20kDa mark and the 30kDa mark and around the 45kDa mark. The intensities of these bands varied between isolates. Of particular interest were the protein bands at around the 20-30kDa mark (see figure 45). These bands were most intense for culture supernatant samples obtained from wound isolate 2 and wound isolate 4. These isolates were also two of the most proteolytic isolates, as noted from the azocasein and ECR assays. It was speculated that the bands evident at the 20-30kDa mark could be suggestive of mature elastase, which is secreted as a 33kDa protein into the culture media, or the 21kDa mature form of LasA, a staphylolytic protease. As such, protein bands from lanes 4 and 7 (wound isolates 2 and 4, respectively) were excised for MS analysis.

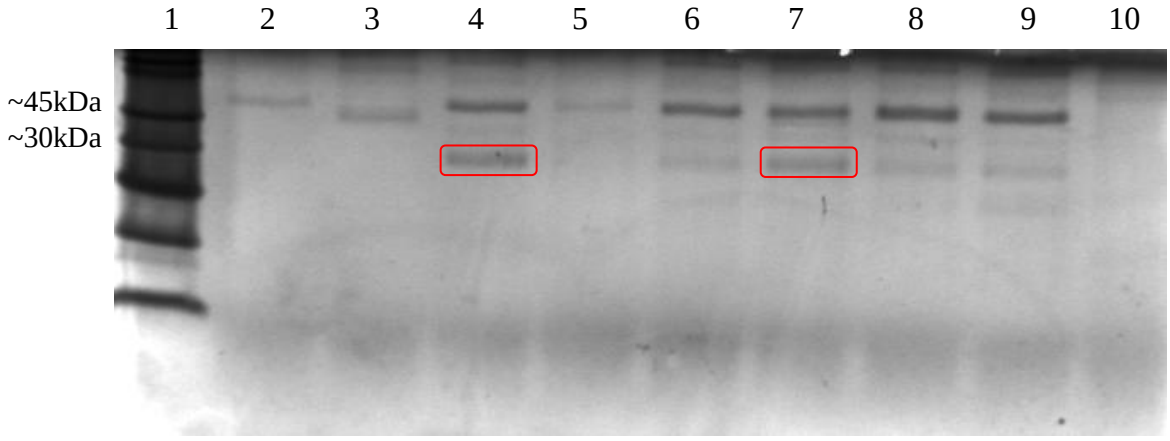


Figure 45: SDS-PAGE of ATCC isolate and the 7 wound isolates following a 24 hour aerobic culture. All samples are from initial pH7.4 cultures. Lane 1: molecular weight standards 2; Lane 2: ATCC isolate 2; Lane 3: Wound isolate 1; Lane 4: Wound isolate 2; Lane 5: Wound isolate 3; Lane 6: Wound isolate 5; Lane 7: Wound isolate 4; Lane 8: Wound isolate 6; Lane 9: Wound isolate 7; Lane 10: *E. faecium* (EF) isolate. Distinct bands were seen between the 20kDa mark and the 30kDa mark and around the 45kDa mark. The intensities of these bands varied between isolates. Protein bands from lanes 4 and 7 (wound isolates 2 and 4, respectively) were excised for mass spectrometry (MS) analysis.

2.4.14 Mass Spectrometry

Following the protein digest process for MS analysis, recovered peptide samples were run on an SDS-PAGE gel to verify that the gel plugs had been successfully digested (see Appendix 7). The MS sample was run against Uniprot Swall Bacteria and filtered using Mascot Significance Threshold of 0.05 as standard. Given that it was a general bacteria search carried out, any 'hits' from other species were homologues (i.e. protein #2). Table 12 shows the results from MS analysis. Results identified 2 proteins: protein #1 was an uncharacterised protein with a molecular weight of 22.1kDa. Given that protein #1 had the highest score and the required 2 peptides needed to confidently say that the protein is present in the sample, further investigation was made into the identity of this protein using the BLAST function in UniprotKB. Results revealed a 100% match with *Pseudomonas aeruginosa* Chitin-binding protein CbpD.

Table 12: Mass spectrometry results for the 2 proteins identified from the in-gel protein digest sample.

Protein#	Accession	Description	Score	Coverage	#Proteins	#Unique peptides	#Peptides	#PSMs	Molecular function	Biological process	Pfam IDs	#AAs	MW (kDa)	Calc. pI
1	G5FXA8	Putative uncharacterized protein OS=Pseudomonas sp. 2_1_26 GN=HMPREF1030_04111 PE=4 SV=1 - [G5FXA8_9PSED]	142.58	10.68	13	2	**2**	3			Pf03067	206	22.1	7.78
2	K8B37K	Rhamnulokinase OS=Cronobacter dublinensis 582 GN=BN133_3520 PE=4 SV=1 - [K8B3K7_9ENTR]	63.94	6.98	6	1	**1**	2	Catalytic activity	Metabolic process	Pf02782	129	14.1	6.89

2.5 Discussion

P. aeruginosa has been widely implicated in a number of pathologies, not least in chronic wound infections, and is one of the most recognised nosocomial pathogens (Altoparlak *et al*, 2004; Bielecki *et al*, 2008). *P. aeruginosa* exerts a number of secreted virulence factors upon its host, which have been shown to have several deleterious effects on host cells and tissues including the inhibition of various aspects of the host's immunity and induction of apoptosis (Engel & Balachandran, 2009; Bi *et al*, 2001). Indeed, proteases secreted by this organism act to degrade host proteins, in turn causing huge damage to the host (Travis & Maeda, 1995), including degradation of cytokines, growth factors, complement components, immunoglobulins and ECM components (Schmidtchen, Wolff & Hansson, 2001). It is, therefore, of importance to recognise the many factors involved in the pathogenesis of this organism and to address the potential effects they can have on various aspects of wound healing in the clinical setting.

The current study has highlighted an obvious variation in protease expression of *P. aeruginosa* isolates from equine wounds, which corroborates previous findings; Schmidtchen, Wolff & Hansson (2001) found that *P. aeruginosa* isolates from human leg ulcers displayed a highly variable expression of elastase and alkaline proteinase, whilst Jagger, Bahner & Warren (1983) demonstrated the variation in protease phenotypes of *P. aeruginosa* isolates obtained from cystic CF patients. Similarly, an early study by Woods and colleagues (1986) showed that the exoproduct levels of *P. aeruginosa* isolates, including levels of total protease, elastase, phospholipase C, exotoxin A and exoenzyme S, vary depending on the anatomical site of isolation. Likewise, Rumbaugh, Griswold & Hamood (1999) found that both the infection site and the duration of infection affects the pathogenic nature of *P. aeruginosa* by altering the production of extracellular virulence factors. Given the limited number of *P. aeruginosa* wound isolates for both chronic and acute wounds it was not possible in this study to compare protease levels of culture supernatant obtained from chronic versus acute wounds. In future studies it would be interesting to ascertain any differences in the proteolytic activity of isolates in the healing versus the non-healing wound and the contaminated versus the infected wound. Whilst the identification of the mechanism(s) behind such variation in the

protease profiles of the *P. aeruginosa* isolates was beyond the scope of this study, it has been suggested that deviation in protease expression could be related to epigenetic mechanisms such as transcriptional control or as a result of rearrangements within the structural genes or corresponding upstream regions (Schmidtchen, Wolff & Hansson, 2001).

In future studies it would be interesting to evaluate the protease profiles of *P. aeruginosa* isolates obtained from repeated cultures of the same wounds; as demonstrated by Schmidtchen and team in their 2001 study, isolates obtained from repeated cultures from skin ulcers maintained their protease expression patterns, suggesting that protease expression may be stable over extended periods *in vivo*. Comparatively, Jagger, Bahner & Warren (1983) found that a lower percentage of *P. aeruginosa* isolates from CF patients who were chronically infected with the organism were positive for alkaline protease expression and general protease activity compared with those isolates from colonised patients, suggesting that protease production from *P. aeruginosa* could be implicated in the initial colonisation of CF patients. The present study revealed that the *P. aeruginosa* isolates displayed caseinolytic activity (as demonstrated using the milk-casein agar substrate plate method) over an extended culture period, with most isolates showing a positive correlation of general protease/elastase activity throughout the growth of the bacteria, with all wound isolates exhibiting some level of protease activity. The caseinolytic and elastolytic activities measured in the culture supernatants of each of the isolates were generally maximal by the end of the exponential phase of growth, which was maintained when the isolates were grown under altered initial pH and also when grown anaerobically. It should also be noted that those isolates producing low levels of protease activity may be more proteolytically active under alternative growth conditions (Jagger, Bahner & Warren, 1983); for instance if different growth media was used, given that protease production is influenced by the complexity of the medium including cation concentration (Jagger, Bahner & Warren &, 1983). Indeed, *P. aeruginosa* is known to depend on a complex network of regulatory genes which act to control the production of various virulence factors in response to environmental stimuli (Rumbaugh, Griswold & Hamood, 1999). It is also worth noting that it was not possible to distinguish between true proteolytic activity of live cells and protease released from dead cells. One further limitation of this study is

that, whilst it is unlikely given the size of *P. aeruginosa* cells (about 1-5µm long and 0.5-1.0µm wide), the use of 0.45µm sterile filters may have allowed cells to escape into the media which could have altered the protease activity if any live cells were still present in the conditioned media. To confirm the absence of viable bacterial cells in the conditioned media, aliquots from each sample could be plated onto agar and cultured. Sterile filters with a pore size of 0.2µm would ensure complete removal of bacterial cells, however the time needed to double filter the conditioned media would need to be factored into the experimental plan. Furthermore, additional filtration steps may introduce more opportunities for contamination of samples from external sources. The variable SEMs for the milk-casein agar analysis and the biochemical protease assays highlight the need for further biological and experimental replicates in future studies.

In this study, the ATCC *P. aeruginosa* reference strain (non-wound isolate) generally showed lower protease expression throughout the culture period, despite changes in initial culture pH and oxygen depletion. This observation alone highlights the importance of carefully selecting the most appropriate isolate when studying the protease or general virulence characteristics of the bacteria in studies aiming to assess the effects of protease modulating therapies.

Additionally, this study has demonstrated that the biofilm forming phenotype (BFP) varies significantly between the clinical *P. aeruginosa* isolates derived from equine wounds. Whilst no correlation was observed in the current study in terms of the mucoid phenotype (BFP) of the *P. aeruginosa* isolates and their proteolytic profiles, it has been found that non-mucoid *P. aeruginosa* isolates obtained from CF patients are more proteolytic than mucoid isolates (Jagger, Bahner & Warren &, 1983).

Although the case number in this study was not large enough for observations to be made in terms of isolate protease profiles and *in vivo* wound characteristics, in future studies it would be of interest to determine whether there is a correlation between wound severity and progression with *P. aeruginosa* protease expression; the heterogeneity in protease activity of *P. aeruginosa* isolates from equine wounds could be explained by variations in clinical circumstances and patient status (Woods *et al*, 1986). For instance, Rumbaugh and colleagues (1999) noted that different

regulatory *P. aeruginosa* regulatory genes may respond to different signals within different anatomical sites of infection. However, it would be important to keep in mind any confounding variables such as antibiotic administration, other wound therapies utilised and the duration of infection (including multi-species infections) when making such interpretations (Schmidtchen, Wolff & Hansson, 2001). In some circumstances, serotyping the *P. aeruginosa* isolates may lead to correlations observed between serotype and protease expression, although this was not found to be the case in the early study by Woods *et al* (1986) who found no correlation between serotype and protease expression among *P. aeruginosa* isolates from a variety of clinical sources and site of isolation or exoproduct levels.

Whilst under anaerobic conditions each of the *P. aeruginosa* isolates (wound isolates and ATCC reference strain) expressed considerably less proteolytic activity relative to the aerobic cultures, growth curve analysis revealed that the bacteria were growing more slowly with an elongated lag phase as compared to the corresponding aerobic cultures for each isolate. Yet, further analysis confirmed that when considering the level of proteolytic activity for a given bacterial density the anaerobic cultures were generating a higher degree of protease activity. Sabra, Kim & Zeng (2002) found that the release of certain virulence factors, including elastase was significantly enhanced by *P. aeruginosa* PAO1 under microaerobic conditions. In terms of the wound environment, *P. aeruginosa* tends to live within the biofilm state, commonly subjected to microaerobic conditions thus prompting slow growth. Further investigation into the response of wound isolates to variations in oxygen tension in terms of protease production and how the biofilm mode of growth affects protease profiles is a topic of interest for future investigation.

Investigation into the effect of altering initial culture pH on bacterial growth and *P. aeruginosa* protease expression revealed that the isolates responded differently to pH change. Whilst it was not possible to conclusively determine the culture pH which generated the most protease activity, the results showed that all of the clinical isolates were more proteolytically active when compared with the ATCC type strain. These results have demonstrated the ability of *P. aeruginosa* equine wound isolates to resist changes in culture pH in terms of virulence in the form of protease expression. Indeed, this links in with the notable highly environmentally adaptable

nature of *P. aeruginosa* (Williams & Cámara, 2009); during infection, *P. aeruginosa* must survive environmental changes, including changes to the pH (Suh *et al*, 1999). However, the ways in which the increase in protease expression at a given pH reflects the wound milieu and changes in wound pH throughout the healing process remain to be elucidated. The ability of *P. aeruginosa* wound isolates to increase their protease expression may serve as a survival mechanism or stress response during the lowered pH of the skin observed during healing, however the mechanisms behind the observed pH and oxygen sensitivity require further characterisation. The observation made in this study may have implications in the use of topical therapeutic agents targeted at lowering the skin surface pH; it is apparent that *P. aeruginosa* is not only able to survive and grow but also readily produce proteases at a low pH. In addition, occlusive dressings commonly used in wound care act in part to lower the wound pH; whilst this will potentially lower the capacity for biofilm formation, elastase production may increase or be maintained, potentially allowing for the spread of bacteria to other parts of the wound or normal skin. *In situ* monitoring of the wound pH during the different healing phases and the extent of bacterial proteases produced may provide a better insight into the effects of pH on various aspects of healing.

The mass spectrometry analysis of the *P.aeruginosa* culture supernatants also produced some interesting results. One of the major proteins secreted by *P. aeruginosa* is a 43kDa protein, secreted via the type II secretion pathway (Braun *et al*, 1998; Folders *et al*, 2000; Folders *et al*, 2001). This protein is cleaved into smaller fragments, including a 30kDa and a 23kDa fragment, by elastase. The 23kDa fragment was originally classified as LasD, a staphylolytic protease. However, Folders *et al* (2000) found that the N-terminal half of the 43kDa protein is homologous to chitin-binding proteins of other bacterial species. As such, the authors renamed this protein as CbpD (chitin-binding protein D). CbpD is produced by many clinical isolates of *P. aeruginosa* (Folders *et al*, 2000) as is thought to be involved in pathogenicity (Frederiksen *et al*, 2013). Given that elastase is involved in the processing of the full length 43kDa protein into smaller fragments, it is speculated that the presence of the smaller fragments of CbpD could also act as a biomarker for the presence of *P. aeruginosa* elastase in the culture supernatant, given that the full length 43kDa protein is found in large amounts in the cell free supernatant of a LasB mutant strain (Folders *et al*, 2000). Furthermore, given that *P. aeruginosa* elastase is also secreted into the extracellular environment via the type II secretory pathway, the low levels elastase activity in the culture supernatant, noted in the ECR assays, is not likely a result of failings in its secretory pathway.

Collectively, the data obtained from this study would advocate a more thorough approach to wound diagnostics in terms of identifying the proteolytic and biofilm phenotype of bacteria isolated from persistent wounds to improve individual patient treatment plans. Yet, it should be noted that the true clinical *in vivo* setting is not completely represented in studies using *in vitro* cultures. For example, it has been documented that serum can affect protease activities *in vivo*. Future studies, therefore, should consider additional *in situ* observations. It would also be beneficial to assess the proteolytic profiles of *P. aeruginosa* isolates from both chronic and acute wounds along with more complex studies into the synergistic effects of introducing multiple bacterial species on protease expression.

2.6 Conclusion

The present study has demonstrated the highly variable expression of proteases, including elastase, between *P. aeruginosa* isolates derived from equine wounds, highlighting the apparent deviation in the virulence of this micro-organism amongst different host tissues. When the effects of an altered initial culture pH were examined *in vitro*, results showed that different isolates respond differently to different pHs. Furthermore, whilst a depletion of oxygen in cultures caused all isolates to grow at a slower rate and produce an overall lower level of protease activity, when proteolytic activity was considered in terms of biomass isolates grown anaerobically were more proteolytic. Overall, each isolate demonstrated a general linear increase in protease activity which paralleled the growth curve. The data generated from this study has emphasised the potential benefit of identifying the proteolytic phenotype of bacterial isolates obtained from chronic wounds in terms of improving individual treatment plans in the clinical setting. It is proposed that the bacterial phenotype should also be taken into account in future studies on the clinical nature of chronic infected wounds.

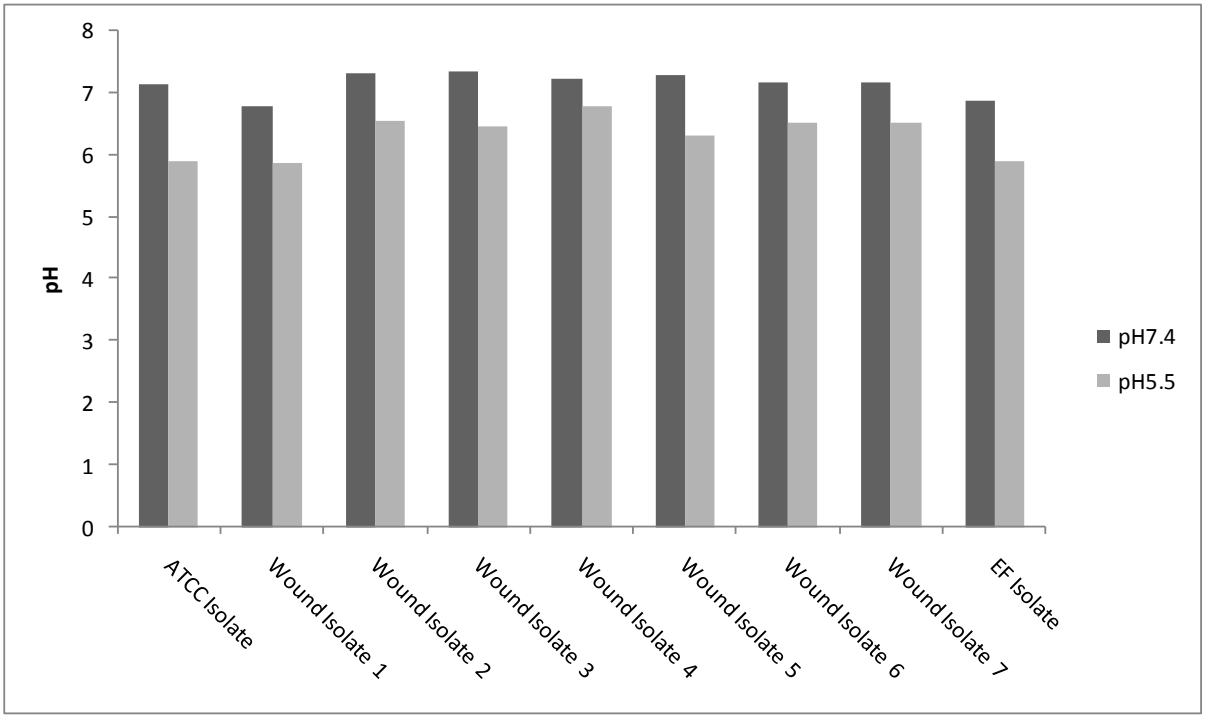
Acknowledgements

The authors would like to thank Advanced Medical Solutions Group PLC for their financial support for this project.

2.7 Appendices to Chapter 2

Appendix 1

pH Checks following a 24 hour aerobic culture for each *P. aeruginosa isolate (initial pH7.4 and initial pH5.5)*



Note: *For all analyses in Appendices 2-5, isolate 1 represents the ATCC isolate and isolates 2-8 represent wound isolates 1-7.*

Appendix 2

One-Way ANOVA Grouping information for BFP (ATCC isolate plus 7 wound isolates)

Isolate	N	Mean	Grouping
6	8	4.7739	A
1	8	1.4668	B
4	8	1.0565	B C
3	8	1.0121	B C
7	8	0.9570	B C
5	8	0.7827	C
8	8	0.7225	C
2	8	0.1816	D

Appendix 3

One-Way ANOVA Grouping information for milk-casein agar plate analysis (ATCC isolate plus 7 wound isolates). Means that do not share a letter are significantly different.

3.1 pH 7.4

3.1.1 T8, 24hrs

Isolate	N	Mean	Grouping
5	3	3.4167	A
4	3	3.1333	A
8	3	3.0833	A B
7	3	2.5333	A B
6	3	2.1333	A B C
3	3	1.9500	A B C
1	3	0.9833	B C
2	3	0.3667	C

3.1.2 T8, 48hrs

Isolate	N	Mean	Grouping
5	3	4.4000	A
8	3	4.1833	A
4	3	3.9500	A
7	3	3.6500	A B
6	3	3.2167	A B
3	3	2.6333	A B C
1	3	1.6000	B C
2	3	0.9000	C

3.1.3 T12, 24hrs

Isolate	N	Mean	Grouping
7	3	2.7167	A
4	3	2.2333	A B
5	3	2.0500	A B
8	3	1.7500	A B
3	3	1.6667	A B
6	3	1.4667	A B
1	3	0.8333	A B
2	2	0.3500	B

3.1.4 T12, 48hrs

Isolate	N	Mean	Grouping
7	3	4.7333	A
5	3	4.2833	A
8	3	3.7667	A B
6	3	3.5167	A B
4	2	3.3500	A B
3	3	2.6000	B C
1	3	1.7333	C
2	2	1.4500	C

3.2 pH5.5

3.2.1 T8, 24hrs

Isolate	N	Mean	Grouping
8	3	4.6500	A
7	3	3.9167	A B
5	3	3.6000	A B C
6	3	2.8000	A B C D
3	2	2.0250	B C D
2	2	1.9500	B C D
4	3	1.6500	C D
1	2	1.0000	D

3.2.2 T8, 48hrs

Isolate	N	Mean	Grouping
8	3	5.7167	A
7	3	5.2667	A
5	3	4.6000	A B
3	2	4.1250	A B
2	2	4.1000	A B
6	3	3.6833	A B
1	2	2.6750	B
4	3	2.4833	B

3.2.3 T12, 24hrs

Isolate	N	Mean	Grouping
6	3	3.3333	A
5	3	3.0167	A
8	3	2.4167	A
7	3	2.2500	A
4	3	2.0667	A
3	2	2.0500	A
1	2	1.2250	A
2	2	0.9000	A

3.2.4 T12, 48hrs

Isolate	N	Mean	Grouping
5	3	5.2333	A
6	3	5.1833	A
8	3	4.3667	A B
7	3	4.3667	A B
3	2	4.2350	A B
4	3	3.4333	B
2	2	3.0500	B
1	2	2.7500	B

3.3 pH6.0

3.3.1 T8, 24hrs

Isolate	N	Mean	Grouping
8	3	4.9833	A
7	3	4.9833	A
6	3	2.4833	B
4	3	2.2333	B
5	3	2.2000	B
3	2	1.5500	B
2	2	1.1750	B
1	2	1.0250	B

3.3.2 T8, 48hrs

Isolate	N	Mean	Grouping
7	3	6.0000	A
8	2	5.8500	A B
3	2	3.4500	B C
2	2	3.4000	C
5	3	3.1833	C
4	3	3.1833	C
6	2	3.1750	C
1	2	2.6000	C

3.3.3 T12, 24hrs

Isolate	N	Mean	Grouping
8	3	5.150	A
5	3	3.367	A B
4	3	3.183	A B
7	3	3.033	A B
6	3	2.767	A B
3	2	2.225	A B
2	2	1.625	A B
1	2	0.875	B

3.3.4 T12, 48hrs

Isolate	N	Mean	Grouping
8	3	7.0667	A
4	3	6.1667	A B
5	3	5.6833	A B
7	3	5.3667	A B
6	3	5.0167	A B C
3	2	4.3500	A B C
2	2	3.8250	B C
1	2	2.4250	C

3.4 pH8.0

3.4.1 T8, 24hrs

Isolate	N	Mean	Grouping
3	2	2.9250	A
6	3	2.8000	A
5	3	2.2167	A B
4	3	1.9667	A B
2	2	1.8500	A B
1	2	0.9500	B

3.4.2 T8, 48hrs

Isolate	N	Mean	Grouping
3	2	4.9750	A
2	2	4.0000	A B
6	3	3.7500	A B
5	3	3.2333	A B
4	3	2.8500	B
1	2	2.5750	B

3.4.3 T12, 24hrs

Isolate	N	Mean	Grouping
5	2	2.175	A
4	2	2.025	A
3	2	1.550	A

2.4.4 T12, 48hrs

Isolate	N	Mean	Grouping
5	2	4.2500	A
4	2	3.9750	A
3	2	3.0250	A

Appendix 4

One-Way ANOVA Grouping information for azocasein assays (ATCC isolate plus 7 wound isolates). Means that do not share a letter are significantly different.

4.1 pH7.4 T24

Isolate	N	Mean	Grouping
3	3	0.38033	A
1	3	0.25433	B
5	3	0.20660	B C
8	3	0.18330	B C
6	3	0.17617	B C
4	3	0.14313	B C
7	3	0.14080	C
2	2	0.11700	C

4.2 pH5.5 T24

Isolate	N	Mean	Grouping
3	3	0.42867	A
2	3	0.37600	A
1	3	0.36933	A
5	3	0.17960	B
8	3	0.15117	B
4	3	0.13430	B
7	3	0.13337	B
6	3	0.12683	B

4.3 pH6.0 T24

Isolate	N	Mean	Grouping
3	3	0.42133	A
1	3	0.32667	A B
2	3	0.28667	B C
8	3	0.18413	C D
7	3	0.17817	C D
6	3	0.16793	D
5	3	0.15633	D
4	3	0.12820	D

4.4 pH8.0 T24

Isolate	N	Mean	Grouping
3	2	0.43400	A
1	3	0.40700	A
2	3	0.39800	A
6	3	0.18937	B
5	3	0.15400	B
4	3	0.11973	B

Appendix 5

One-Way ANOVA Grouping information for ECR assays (ATCC isolate plus 7 wound isolates). Means that do not share a letter are significantly different.

5.1 pH7.4 T24 (ATCC isolate plus 7 wound isolates)

Isolate	N	Mean	Grouping
8	3	0.47446	A
7	3	0.34866	B
5	3	0.31375	B C
6	3	0.29208	C
1	3	0.23018	D
3	2	0.13085	E
4	3	0.11012	E
2	3	0.00165	F

5.2 pH5.5 T24

Isolate	N	Mean	Grouping
7	3	0.46079	A
5	3	0.45668	A
8	3	0.43656	A
6	3	0.41675	A
1	3	0.24125	B
3	3	0.21795	B
2	3	0.09748	C
4	3	0.06145	C

5.3 pH6.0 T24

Isolate	N	Mean	Grouping
6	3	0.56206	A
5	3	0.46758	B
8	3	0.28776	C
7	3	0.28462	C
3	3	0.20528	D
1	3	0.19822	D
2	3	0.09988	E
4	3	0.03268	F

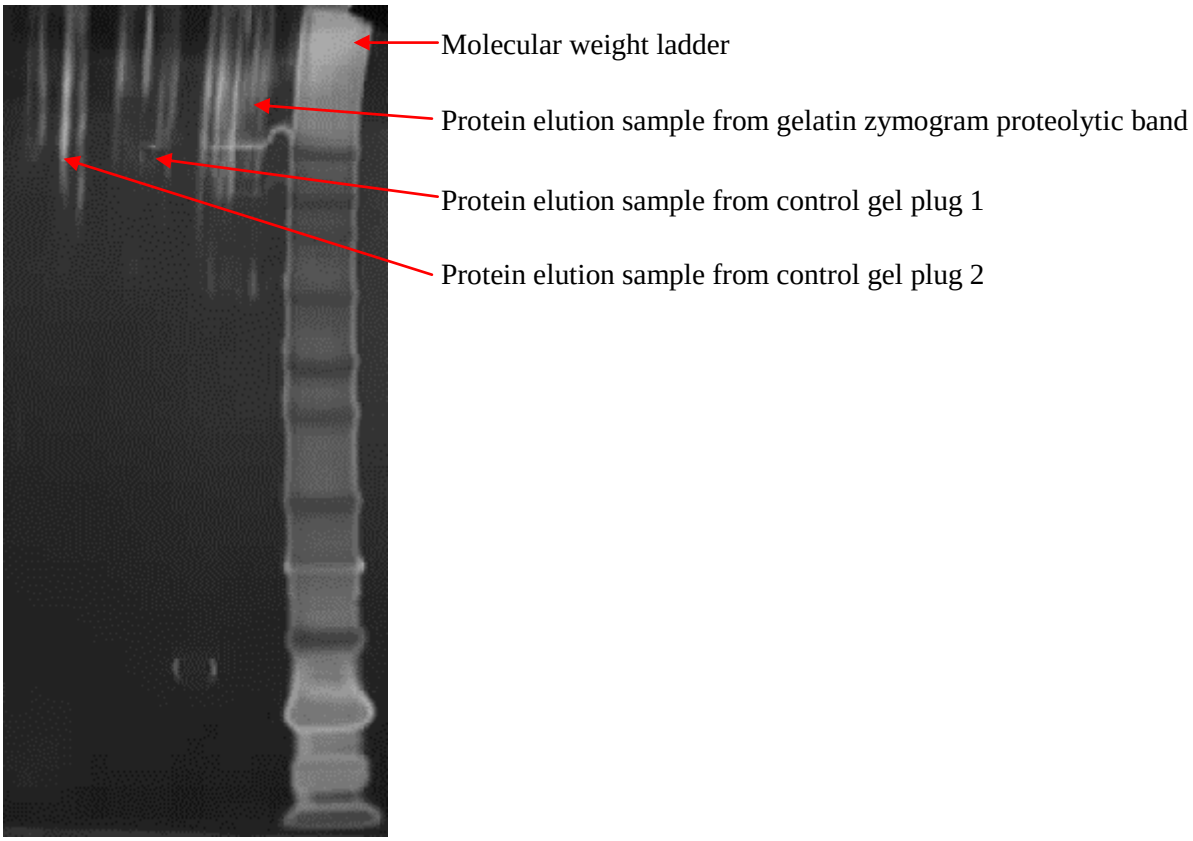
5.4 pH8.0 T24 (plus no isolate 7 or 8)

Isolate	N	Mean	Grouping
5	3	0.31712	A
6	3	0.31452	A
1	3	0.25462	B
3	3	0.21822	B
2	3	0.11188	C
4	3	0.05068	D

Appendix 6

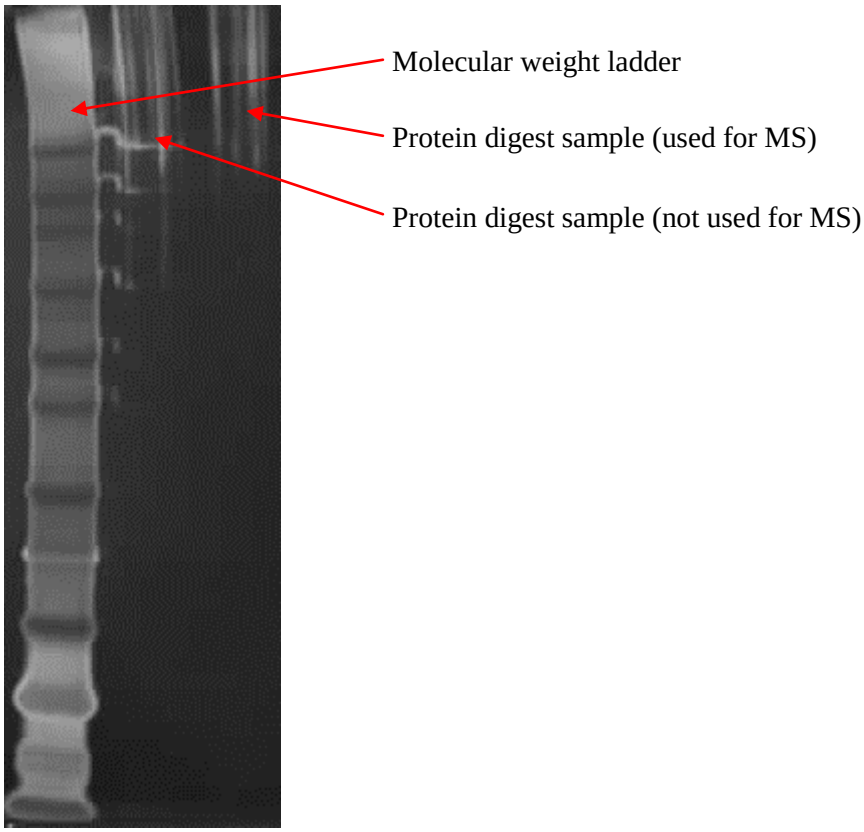
SDS-PAGE of eluted proteins from excised gelatin zymogram

The smears seen at the top of the gel are likely to be high molecular weight proteins from the gel matrix of the gelatin zymogram.



Appendix 7

SDS-PAGE verification of protein digest prior to MS analysis



3.0 Chapter 3

Manuscript 3

The role of biofilms in the virulence of *Pseudomonas aeruginosa*: a focus on protease production under varied pH conditions in biofilm culture

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Keywords: Wound healing, Matrix metalloproteinases (MMPs), *Pseudomonas aeruginosa* elastase, Biofilm, pH

3.1 Abstract

Pseudomonas aeruginosa is a widespread opportunistic pathogen commonly implicated in wound infections and has an inherent capacity for the development of biofilms. The combination of evasion from host defences and administered antimicrobials along with the production of multiple virulence factors, including several proteases, makes this pathogen a challenge in the management of chronic, infected wounds. In the previous chapter, the variable production of secreted proteases of clinical *P. aeruginosa* isolates obtained from equine wounds in the planktonic mode of growth was demonstrated. Furthermore, the effects of the changing environment (changes in initial culture pH and oxygen depletion) on the production of proteases by these isolates was demonstrated. Given the evidence in the literature of the presence of biofilms in chronic wounds, it was hypothesised that the biofilm versus the planktonic mode of growth has an impact on protease production *in vitro*.

In this chapter it is shown that isolates of *P. aeruginosa* obtained from equine wounds display a highly variable protease expression during the biofilm mode of growth. However, a change in the initial culture pH of developing biofilms *in vitro* did not cause significant fluctuations in the proteolytic profiles of the isolates. It is proposed that in future work the proteolytic phenotype should be considered in combination with the biofilm-forming potential of bacterial isolates when investigating the clinical nature of chronically infected wounds.

3.2 Introduction

Pseudomonas aeruginosa is an important opportunistic human and animal pathogen and is a particular concern in nosocomial infections, including wound infections (Hentzer *et al*, 2003; Bielecki *et al*, 2008). The inherent ability of *P. aeruginosa* to survive in a range of hostile environments with minimal nutritional requirements along with its renowned resistance to various antibiotics allows the microorganism to colonise and infect injured tissues (Rumbaugh *et al*, 1999; Estahbanati, Kashani & Ghanaatpisheh, 2002; Harjai *et al*, 2005). The virulence of *P. aeruginosa* results from a synergy of both cell-associated and extracellular factors, with the proteases LasB elastase, LasA elastase and alkaline protease all implicated in the pathogenic characteristics of this bacterium (Petermann, Doetkott & Rust, 2001; Van Delden & Iglewski, 1998). Indeed, these proteases have been a major focus in current research in terms of their action against host tissues; *P. aeruginosa* proteases are considered as important virulence factors given their capacity to damage host tissues and interfere with host antibacterial defence systems (Ołdak E & Trafny, 2005). These proteases have wide substrate specificity, with both LasA and LasB mediating the degradation of elastin and alkaline proteases and elastases degrading collagen (Petermann, Doetkott & Rust, 2001). *P. aeruginosa* proteases can also work synergistically to break down components of connective tissues and the immune system (Petermann, Doetkott & Rust, 2001). Research has shown that these proteases act to delay infection resolution, with protease-deficient strains showing almost avirulent characteristics (Tang *et al*, 1996). However, Preston and colleagues (1997) noted that neither LasR (the transcriptional activator of LasB and LasA) nor elastase production is required for the establishment or maintenance of corneal infection in a murine keratitis model (Preston *et al*, 1997). Nevertheless, it has been noted that there exists a wide variation in the proteolytic activity of *P. aeruginosa* between different infection sites and types of disease (Woods *et al*, 1986; Schmidtchen, Wolff & Hansson, 2001) and the wound healing responses to these variations in bacterial proteolytic activity in the *in vivo* environment are poorly understood.

The *P. aeruginosa* quorum sensing (QS) systems, las and rhl, direct the production of a number of virulence factors, including proteases and so contribute to the microorganism's pathogenicity (Rumbaugh *et al*, 1999). QS systems allow the microorganism to control the production and secretion of virulence factors in a co-ordinated, cell-density regulated manner (Van Delden & Iglewski, 1998). QS also has an important role in the development of biofilms, a growth behaviour which allows the bacteria to form surface-associated, multicellular structured and resistant complexes which aid persistence of infection (Hentzer *et al*, 2003; Davies *et al*, 1998). Once a critical bacterial density is established, extracellular signalling occurs which allows the bacterial population to change its phenotype (Thomson, 2011). Biofilms are a particularly problematic feature of chronic wounds, given their resilience to antibiotic treatment and host defence mechanisms which is, in part, a result of the alginate-containing extracellular polymeric matrix which accumulates at the attachment surface (Percival & Bowler, 2004).

Biofilms have been noted for their frequent occurrence within the wound milieu, particularly in chronic wounds, with strategies used to disrupt biofilms becoming an increasingly important part of wound management regimens (Thomson, 2011). Indeed, direct evidence of the presence of biofilms in chronic wounds has been provided in recent years; James *et al* (2008) examined both acute and chronic wounds for the presence of biofilms and found that 60% of chronic wound specimens contained evidence of biofilm compared to just 6% of acute wound specimens. Similarly, the presence of biofilms within equine chronic wounds has been identified (Westgate *et al*, 20011b), with evidence of bacterial clustering found in 8 out of 14 debrided wound tissue samples in a recent study (Westgate *et al*, 20011c). Furthermore, *P. aeruginosa* has been identified as one of the most commonly isolated bacteria from equine wounds with *Pseudomonas* spp identified as more prevalent among chronic wounds as compared with acute wounds (Westgate *et al*, 20011b; Westgate *et al*, 20011c).

In terms of the wound environment, the pH of the wound can affect a number of factors involved in healing including angiogenesis, oxygen release, bacterial toxicity and protease activity (Gethin, 2007). Whilst a more acidic environment is a feature of the inflammatory phase of healing, the pH of normally healing acute wounds gradually increases during the formation of granulation tissue prior to falling again during re-epithelialisation (Schneider *et al*, 2007). In contrast, the pH of chronic wounds maintains a prolonged alkaline pH (Schneider *et al*, 2007). *In vivo* environmental factors and host signals can also affect the formation of biofilms and the conversion of the non-mucoid to the mucoid phenotype. Indeed, a number of factors including nutrient limitation and osmotic stress have been found to induce the mucoid phenotype in previously non-mucoid bacterial strains (Terry, Piña & Mattingly, 1991). Mucoidy conversion may occur in response to environmental factors encountered during chronic infection or in *in vitro* adverse growth conditions (Terry, Piña & Mattingly, 1991). Furthermore, QS systems, which act in part to direct virulence factor production, can also be affected by changes in pH (Horswill *et al*, 2007).

Additionally, the production of proteases can both affect or be affected by changes in pH of the wound bed (Harjai *et al*, 2005; Greener *et al*, 2005). Other bacterial virulence factors can also be affected by the environmental pH, including the attachment rate to host tissues.

In relation to the cutaneous wound environment, the effects of the biofilm mode of growth and pH change during the healing of wounds on bacterial virulence have not been thoroughly investigated. In the previous chapter we demonstrated a highly variable protease expression among clinical *P. aeruginosa* isolates. However, it is important to consider how the *in vivo* mode of growth, i.e. biofilms, may affect virulence in terms of the production of proteases. The aim of the present study therefore is to investigate the effects of the biofilm mode of growth on the production of proteases using *in vitro* models. Additionally, the initial culture pH of these biofilms will be lowered to provide some insight into the pathogenic response of biofilm cells under the acidic conditions associated with the healing wound environment and to compare these responses between wound isolates and a non-clinical reference isolate.

3.3 Methods

3.3.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated.

3.3.2 Ethical Approval

Approval for this study was granted by the Ethics Committee, University of Liverpool and informed consent was obtained from the owners of the animals participating in this study.

3.3.3 Identification of *P. aeruginosa* Isolates

Standard microbiological culture techniques were used to confirm the identities of 7 previously collected equine trauma wound isolates (as described in Manuscript 2). Pure bacterial colonies were identified using a Gram stain and isolates confirmed as Gram negative rods were further characterised using the oxidase test (using oxidase detection strips from Oxoid Limited, Hampshire, UK) and API 20 E® identification strips, which utilise biochemical reactions with computer software-interpretation (Biomérieux UK Limited, Hampshire, UK). Confirmed *P. aeruginosa* isolates were stored on beads and frozen at -80°C until required for further investigation.

A *P. aeruginosa* reference strain (non-wound isolate) was used as a control; American Type Culture Collection (ATCC) 27853 (isolated from blood culture) (American Type Culture Collection [ATCC], Manassas, USA). This strain was chosen as it produces both elastase and alkaline protease (O'Callaghan *et al*, 1996). An additional negative control wound isolate was used in the form of *Enterococcus faecium* (EF isolate), a Gram positive bacterium and common nosocomial pathogen. This isolate was identified using the API 20 Strep kit (Biomérieux UK Limited, Hampshire, UK).

Polymerase chain reaction (PCR) analysis of the *Pseudomonas aeruginosa* elastase gene was performed for each isolate; all of the chronic wound isolates and the ATCC reference strain were positive for the elastase gene (as reported in Manuscript 2).

The biofilm forming phenotype (BFP) of each of the isolates was determined previously (as described in Manuscript 2) using the microtitre plate crystal violet staining method. *P. aeruginosa* isolates were also plated onto tryptone soy agar (TSA) to examine the mucoid/non-mucoid phenotype of each isolate (see figure 2).

3.3.4 Biofilm Cultures Using the Glass Microscope Slide Method

The protease activity of developing monospecies biofilms in liquid culture was assessed via the glass microscope slide method (adapted from the method described by Harrison-Balestra *et al* in 2003). Each of the chronic equine wound *P. aeruginosa* isolates, the ATCC reference *P. aeruginosa* strain and the EF isolate were cultured in sterile Mueller Hinton broth (MHB) to stationary phase with shaking at 150rpm in a shaking incubator set at 37°C; isolates were initially cultured on Tryptone Soy agar (TSA) and 50ml portions of MHB was inoculate with a single loop of a fresh overnight culture for each corresponding isolate. Stationary phase cultures were standardised to an optical density (OD) of 0.85_{600nm}, an approximate McFarland standard of 6. From each of the standardised cultures a 200µl aliquot was used to inoculate 30ml sterile MHB in sterile culture pots to create a standardised initial OD (as described in Manuscript 2). To each culture a glass microscope slide (frosted microscope slide [25x75x1mm], Thermo Scientific, MA, USA) was placed vertically. For each isolate, 2 biofilm cultures were prepared. Protease assays were all performed in triplicate for each culture. Biofilms were grown over a 24 hour period in a shaking incubator set at 150rpm at a constant temperature of 37°C. At the end of the culture period the microscope slides were removed using sterile disposable forceps and placed consecutively into 10ml aliquots of sterile phosphate buffered saline (PBS) and rinsed to remove any non-adherent bacteria. Test cultures were used to confirm the growth of biofilms on the microscope slides which were stained with 0.2% crystal violet for 2 minutes and visualised using light microscopy. Adherent bacterial cells were removed from the microscope slides via sonication;

microscope slides were placed into 10ml sterile MHB, ensuring full submersion of the microscope slide, and sonicated in a water bath sonicator (Ultrawave, [Q Series], Cardiff, Wales) at 100% power (highest sonication setting) for 20 minutes and cells were re-suspended with brief vortexing. The degree of sonication used is a standard protocol used in the laboratory and was previously standardised to ensure no effect on cell viability. Sonicated biofilm cells were then used for milk-casein agar analysis and colony-forming unit (CFU) counts. The spent media from each of the original cultures was sterile filtered and used for protease assays. Final optical densities (ODs) of the spent media of each of the original cultures were taken at 600nm.

For the analysis of protease production during biofilm development in an acidic environment, the MHB growth media was altered to an initial pH of 5.5 using a pH meter and concentrated hydrochloric acid (HCl). Media was then autoclaved at 121°C and left to cool. Under sterile conditions, a sample of the sterile media was taken and the pH checked. All MHB stocks remained at the correct pH following autoclaving. Litmus paper and a pH meter were used to monitor the pH of the cultures throughout each phase of growth; no significant pH fluctuations were noted throughout the entire culture period.

3.3.5 Current Downflow Contactor (CDC) Biofilm Reactor Cultures

For further *in vitro* assessment of protease productivity of *P. aeruginosa* isolates cultured under the biofilm mode of growth (mature biofilms), the current downflow contactor (CDC) reactor system was employed using a method adapted from Williams and Bloebaum (2010). The CDC reactor vessel (BioSurface Technologies, Bozeman, MT, USA) consists of a 1 litre glass vessel, into which the inoculated broth is poured, and a polyethylene lid which supports 8 polypropylene coupon holders is fixed into place. The unit has a side arm discharge port which limited the culture volume to 350mls. Each coupon holder holds three coupons (coupons had a diameter of 12.7mm and a depth of 3mm), providing a total of 24 sampling opportunities. In this study, however, alternate coupon holders were loaded with three coupons thus providing a total of 12 sampling opportunities. A magnetic stir bar was placed in the centre of the vessel, providing constant stirring of the inoculated broth during the culture period (48 hours) once placed on a magnetic stirrer (Hanna Instruments [HI 190M], Bedfordshire, UK) which was set at 50% power (i.e. the speed setting was set at the half way point on the magnetic stirrer) (see figure 1).

The vessel was filled with 300ml standard MHB. The entire CDC reactor, with the coupons in place, was autoclaved for 15 minutes at 121°C prior to the addition of the inoculate broth sample. The ATCC *P. aeruginosa* isolate, wound isolate 4 and wound isolate 6 were used for CDC bioreactor experiments. Wound isolates 4 and 6 were chosen since they both had a high BFP score; wound isolate 4 was strongly adherent and wound isolate 6 was very strongly adherent. As was done for the glass microscope slide biofilm cultures, pure *P. aeruginosa* isolates were cultured in sterile Mueller Hinton broth (MHB) to stationary phase with shaking at 150rpm in a shaking incubator set at 37°C; isolates were initially cultured on TSA and 50ml portions of MHB was inoculate with a single loop of a fresh overnight culture for each corresponding isolate. Stationary phase cultures were standardised to an OD of 0.85_{600nm}, an approximate McFarland standard of 6. From each of the standardised cultures a 2ml aliquot was used to inoculate 300ml sterile MHB in the CDC reactor to create the same standardised initial OD as for the glass slide cultures for each of

the *P. aeruginosa* isolates. The CDC reactor was placed on top of the magnetic stirrer in an incubator set a 37°C for 48 hours.

Following the 48 hour incubation, sterile techniques were used to recover the coupons from the coupon holders using sterile forceps. The position of each of the coupons and coupon holders was noted. For each isolate, 2 top coupons, 2 middle coupons and 2 bottom coupons (position from the top to the bottom of the coupon holder; 2 coupon holders at opposite positions in the reactor were used) were taken for CFU counts and 2 top coupons, 2 middle coupons and 2 bottom coupons were taken for milk-casein agar plate analysis of general proteolytic activity of live recovered biofilm cells.

Individual coupons were placed consecutively into 10ml aliquots of sterile PBS and rinsed to remove any non-adherent bacteria. Adherent bacterial cells were removed from the coupons via sonication; coupons were placed into 10ml sterile PBS, ensuring full submersion of the coupon, and sonicated in a water bath sonicator at 100% power for 20 minutes. Cells were re-suspended with brief vortexing. Sonicated biofilm cells were then used for milk-casein agar analysis and CFU counts.

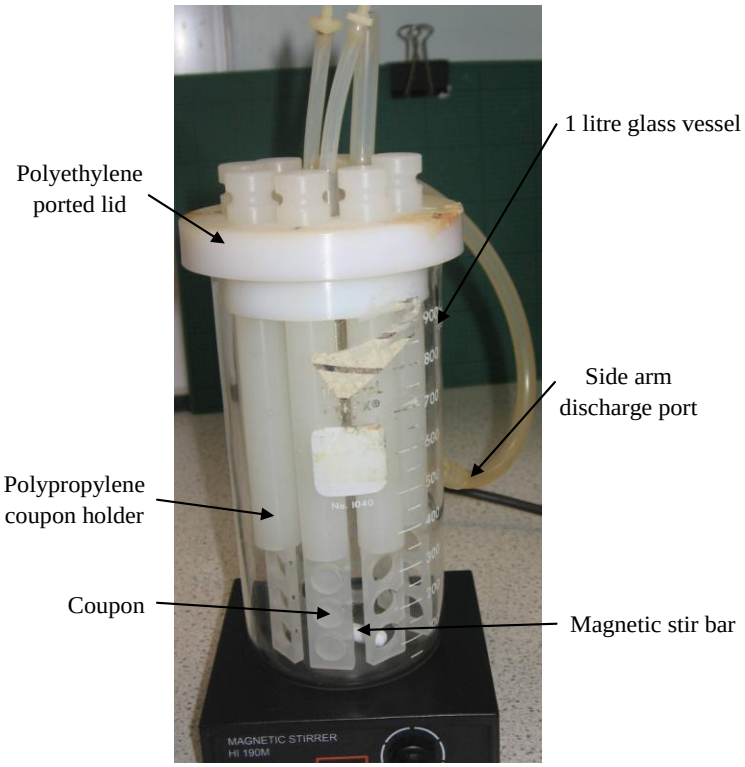

Figure 1: Photograph of the current downflow contactor (CDC) reactor system (BioSurface Technologies, Bozeman, MT, USA). Biofilms were grown on coupons over a 48 hour period, whilst subjected to shear forces produced by the rotating magnetic stir bar swirling the media around the glass vessel.

3.3.6 Milk-Casein Agar Analysis

Milk-casein agar was prepared with 25g skim milk powder (BD, Oxford, UK) 2.5g casein (BDH, VWR, Lutterworth, UK), 1.25g yeast extract (Oxoid, Basingstoke, UK), 0.5g D-glucose (BDH, Oxford, UK) and 6.25g No. 1 agar (LabM Bury, UK) (as described in Manuscript 2). A sterile 8mm punch biopsy was used to bore single wells in each of the milk plates. 100µl aliquots of the re-suspended biofilm cells were applied to the 8mm wells made in the milk plates in triplicate to determine the residual general protease activity of the biofilm cells of each *P. aeruginosa* isolate. Plates were incubated over a 48 hour period and measurements were made of the caseinolytic zone after 24 and 48 hours; two measurements were made in mm at right angles to each other and an average measurement made, minus the 8mm well.

3.3.7 Azocasein Assay for General Protease Activity

To measure the general protease activity of the spent media of each biofilm culture (at each initial pH), a chromogenic assay utilising azocasein, a non-specific protease substrate conjugated to an azo-dye, was performed (as described in Manuscript 2). Sterile MHB was used for the controls. All samples were made in triplicate. Media samples were briefly vortexed after addition of the azocasein solution and incubated overnight at 37°C. After the incubation period, 600µl of 5% Trichloric acid (TCA) was added to each sample to precipitate non-hydrolised azocasein and left on ice for 30 minutes. The samples were then centrifuged at 11,000xg for 10 minutes at 4°C to pellet the undigested substrate. From each sample, 200µl of supernatant was added to a 96-well plate and the absorbance measured in a microtitre plate reader at 405nm.

3.3.8 Elastin-Congo Red Assay for Elastase Activity

To assess the level of elastase produced by each biofilm culture (at each initial pH), the elastin-congo red (ECR) method was employed, adapted from the studies by Petermann, Doetkott & Rust (2001) and Rust, Messing & Iglewski (1994) and as described in Manuscript 2. Sterile MHB was used for the controls. Media samples were made in triplicate and vortexed briefly following addition of the ECR solution. The samples were incubated for 24 hours at 37°C after which the samples were centrifuged at 1200rpm for 10 minutes at 4°C to pellet any undigested ECR. From each sample, 200µl of supernatant was added to a 96-well plate and the absorbance measured in a microtitre plate reader at 495nm.

3.3.9 Gelatin Zymography

Gelatin zymography was used to confirm the presence/absence of enzyme activity in the spent media of the biofilm cultures at each initial pH. Briefly, sterile filtered spent media samples obtained from each of the developing biofilm cultures were diluted with non-reducing Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK) at a ratio of 1:5 and distilled water to an overall 1:5 dilution of the sample. Samples were then incubated in a 37°C water bath for 1 hour to activate the enzyme prior to application to the gel. A Miniprotean II gel system (Bio-RadHemel Hempstead, UK) was employed for zymography gels and gels were cast, run and stained according to the protocol outlined in Manuscript 2. 20µl aliquots of each sample were loaded into the gel. MMP-2 standards were run on each gel. Gels were visualised and imaged using Genesnap software.

3.3.10 SDS-PAGE Analysis of Conditioned *P. aeruginosa* Media

SDS-PAGE analysis was used to identify protein bands corresponding to *P. aeruginosa* proteases present in the spent media of the developing biofilm cultures. A 4-12% Bis-Tris gel was used (Novex, Life Technologies, Paisley, UK). Samples were prepared by adding 13µl original sample, 2µl DTT and 5µl non-reducing sample buffer with marker dye (Thermo Scientific, MA, USA). Samples were denatured by heating for 10 minutes in a heat block pre-set to 80°C. The samples were vortexed briefly. A 20µl aliquot of each sample was added to each corresponding lane of the gel. The gel tank was filled with a 1:20 dilution of NUPAGE MES SDS running buffer (Invitrogen, Paisley, UK) in distilled water. The gel was run at 15 volts for approximately 2 hours or until the lane marker ran to the bottom of the gel. The gels were carefully removed and stained using a Pierce® Silver Stain Kit (Thermo Scientific, MA, USA), as described in Manuscript 2.

3.3.11 Histology

Tissue sections taken from debrided equine wound granulation tissue were stained with both haematoxylin and eosin (H&E) and Gram stain in order to visualise any bacterial clusters within the tissues (see figure 30). Staining was performed by the team at the University of Liverpool Veterinary Pathology department.

3.3.12 Statistical Analyses

Statistical analyses were performed on the data for the biochemical assays (azocasein and elastin-congo red) for the 24 hour broth culture time points for general protease or elastase activity and the milk-casein agar plate data for the 24 hour initial broth culture (24 and 48 hour plate cultures); statistical differences in general protease and elastase activity between *P. aeruginosa* isolates were identified using a one-way analysis of variance (ANOVA) or a non-parametric test for data which was not normally distributed (Kruskal-Wallis test). The same analyses were performed for the data relating to bacterial growth (OD_{600nm}). For analysis of the effect of initial culture pH on general protease and elastase activity a general linear model was used. Statistical significance was defined as a P value of ≤ 0.005. In all instances, Post Hoc analyses were applied with the Tukey test. All statistical analyses were performed using Minitab®, Version 16.

3.4 Results

Each of the *P. aeruginosa* isolates were cultured on TSA plates and the mucoid appearance was observed. As shown in figure 2, all but one isolate (wound isolate 1, which showed slight mucoid appearance) demonstrated a distinct mucoid phenotype characterised by a smooth and slimy appearance.

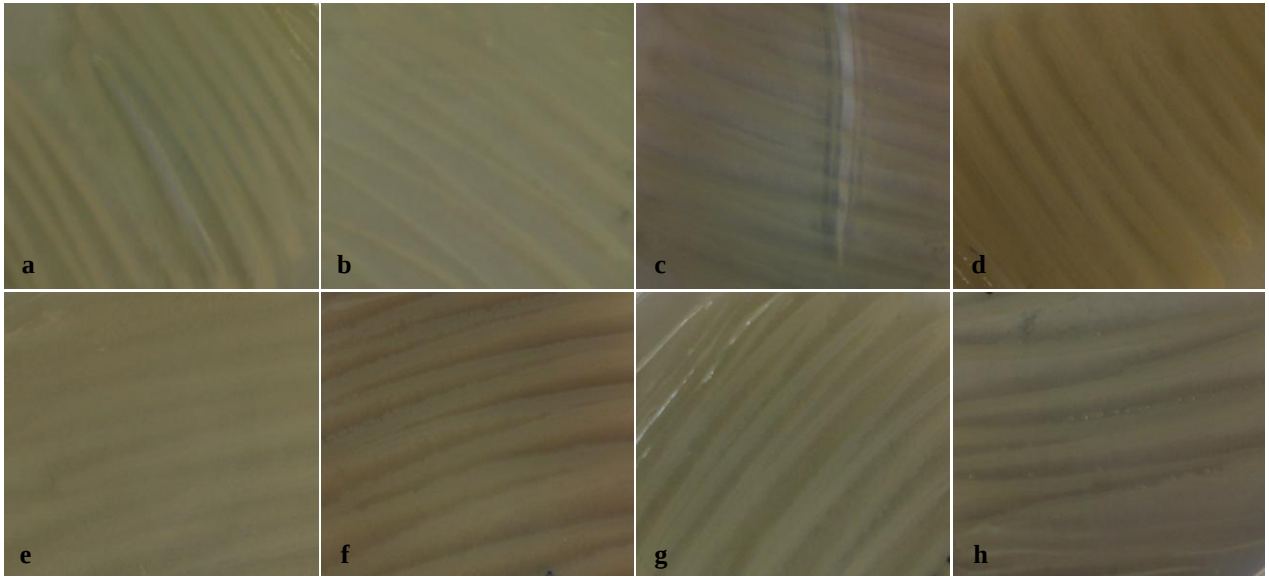

Figure 2: Tryptone soy agar (TSA) cultures of the ATCC reference *P. aeruginosa* isolate and the 7 wound *P. aeruginosa* isolates showing evidence of the mucoid phenotype; all isolates exhibited some degree of the mucoid phenotype, having a smooth and slimy appearance. a: ATCC isolate; b: Wound isolate 1; c: Wound isolate 2; d: Wound isolate 3; e: Wound isolate 4; f: Wound isolate 5; g: Wound isolate 6; h: Wound isolate 7. All but wound isolate 1 demonstrated a distinct mucoid phenotype; wound isolate 1 demonstrated a limited mucoid appearance. Wound isolate 1 appeared less slimy, with a more dry appearance.

3.4.1 Biofilm forming phenotype (BFP)

As demonstrated in Manuscript 2, the results of the crystal violet assay for biofilm forming phenotype of the wound and non-wound isolates revealed a significant variation between isolates, F (8, 71) = 162.06 (P = <0.001).

Table 1: Classifications of BFP according to the crystal violet assay.
* *Enterococcus faecium*= EF isolate

Isolate	**OD_{570nm}**	**BFP Interpretation**
ATCC Isolate	>0.816	Very Strongly Adherent
Wound Isolate 1	0-0.136	Non-Adherent
Wound Isolate 2	>0.816	Very Strongly Adherent
Wound Isolate 3	>0.816	Very Strongly Adherent
Wound Isolate 4	0.544-0.816	Strongly Adherent
Wound Isolate 5	>0.816	Very Strongly Adherent
Wound Isolate 6	>0.816	Very Strongly Adherent
Wound Isolate 7	0.544-0.816	Strongly Adherent
EF Isolate	0-0.136	Non-Adherent

3.4.2 Azocasein Assays for General Protease Activity

The azocasein substrate assay for non-specific protease activity was used to determine the variability in general protease activity among *P. aeruginosa* wound isolates and an ATCC reference *P. aeruginosa* isolate. In addition, the effect of a change in the initial pH of the growth media from pH7.3 to pH5.5 was assessed following a 24 hour broth culture. The azocasein assay used detects a range of enzymes, including *P. aeruginosa* elastase and alkaline proteinase. The EF isolate was excluded from statistical analysis.

Results have demonstrated that there is a significant variation in the general proteolytic profiles between the isolates when cultured as biofilms at an initial pH of 5.5. Given the complex relationships, statistical significance is detailed in tables 2 and 3. The general caseinolytic activity did vary between isolates when grown at an initial pH of 7.3, however this difference did not reach statistical significance (see tables 2 and 3). However, this could have been due to missing data and thus small sample sizes. The general trends remained similar between isolates when cultured at both initial pHs (see figures 3-7). The ATCC reference isolate and wound isolate 1 were the least proteolytically active isolates when cultured in MHB with an initial pH of either 7.3 or 5.5. Appendix 1 details the grouping information which shows the general caseinolytic activity of each individual isolate at each initial pH.

Further analysis revealed that the general proteolytic activity of isolates at the 24 hour time point did not significantly vary when the culture media was changed from an initial pH of 7.3 to an initial pH of 5.5, $F(1, 25) = 0.15$, $P = 0.703$.

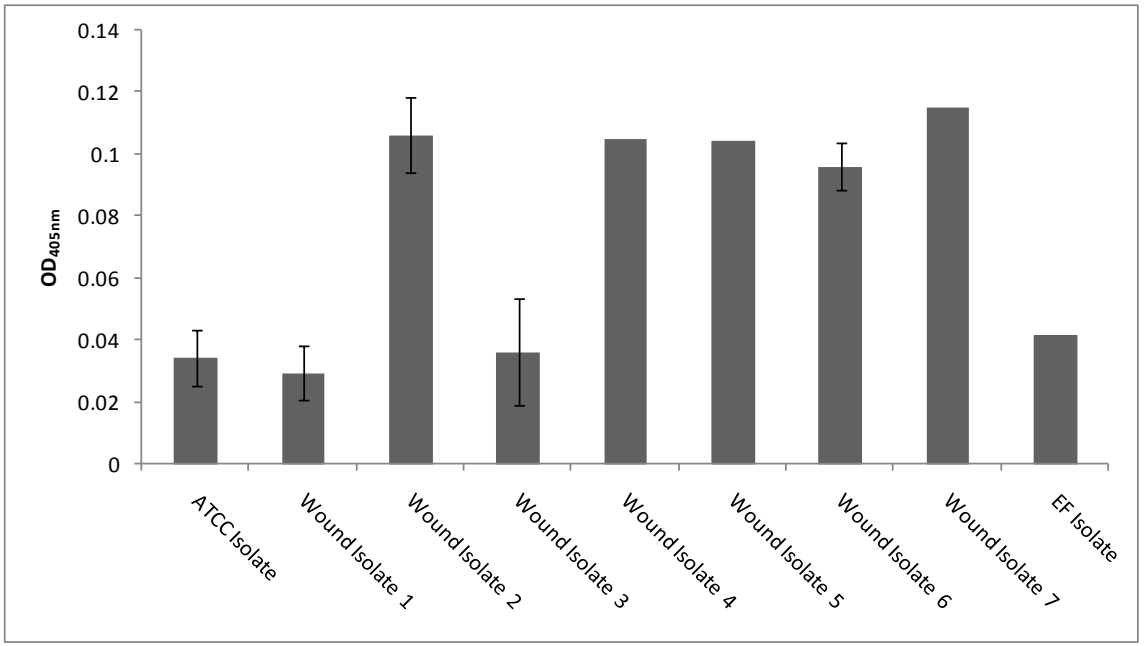

Figure 3: Azocasein assay for caseinolytic digest analysis of each isolate following a 24 hour culture period. Isolates were cultured in standard MHB (initial pH7.3). Data is presented as mean ± SEM. Statistical significance is detailed in Tables 2 and 3.
**Enterococcus faecium*= EF isolate

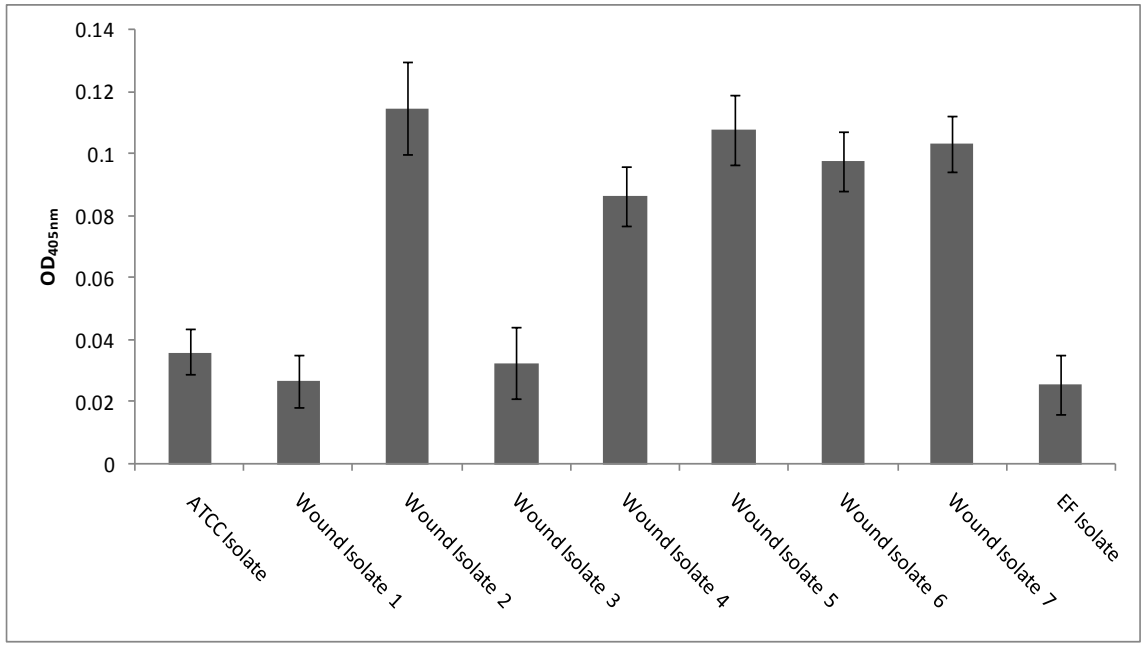

Figure 4: Azocasein assay for caseinolytic digest analysis of each isolate following a 24 hour culture period. Isolates were cultured in MHB adjusted to an initial pH of 5.5. Note: missing data for wound isolate 4, 5 and 7(n=1 for these isolates). Data is presented as mean ± SEM. Statistical significance is detailed in Tables 2 and 3.
**Enterococcus faecium*= EF isolate

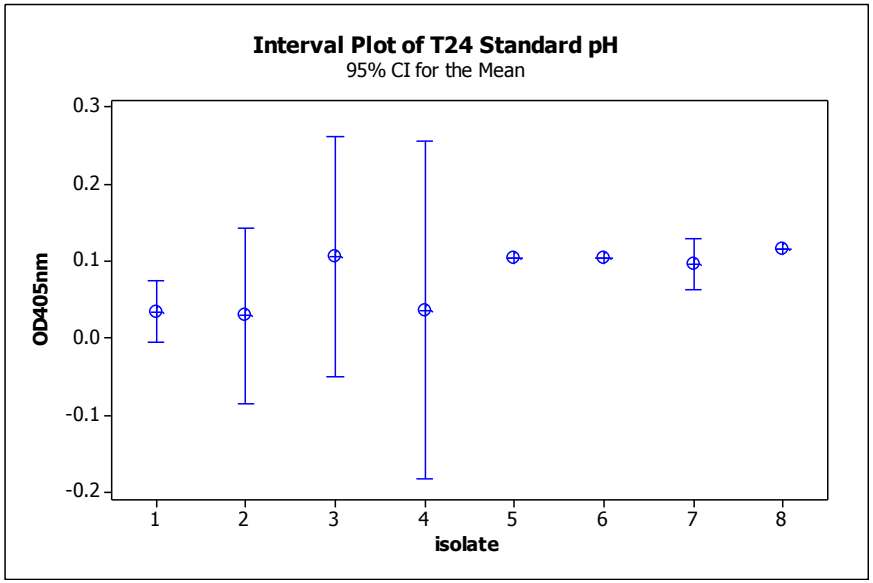

Figure 5: Interval plot for general proteolytic activity of each isolate following a 24 hour broth culture (standard initial pH7.3). For all statistical analyses and interval plots the ATCC isolate was named isolate 1; wound isolates 1-7 were named isolates 2-8; the *Enterococcus faecium* (EF) isolate was excluded from statistical analyses and interval plots.

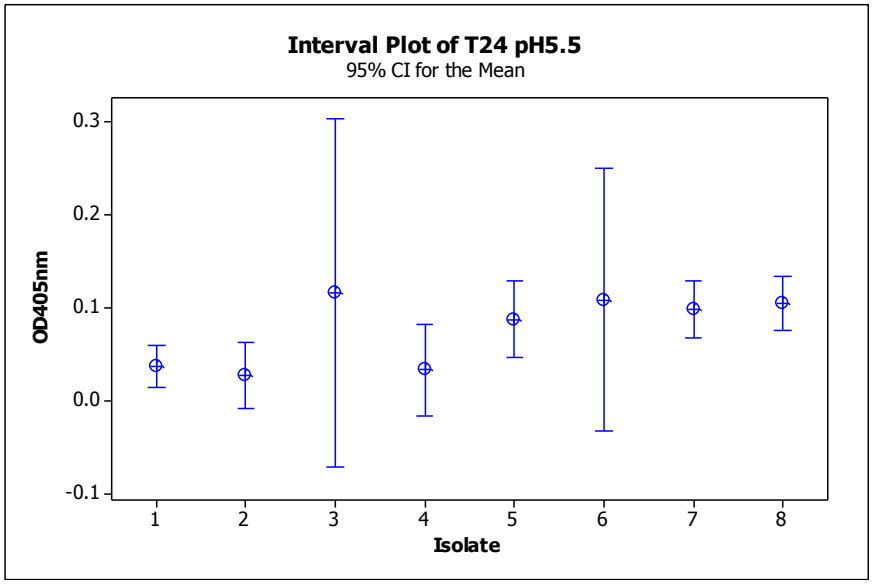

Figure 6: Interval plot for general proteolytic activity of each isolate following a 24 hour broth culture (initial pH5.5). For all statistical analyses and interval plots the ATCC isolate was named isolate 1; wound isolates 1-7 were named isolates 2-8; the *Enterococcus faecium* (EF) isolate was excluded from statistical analyses and interval plots.

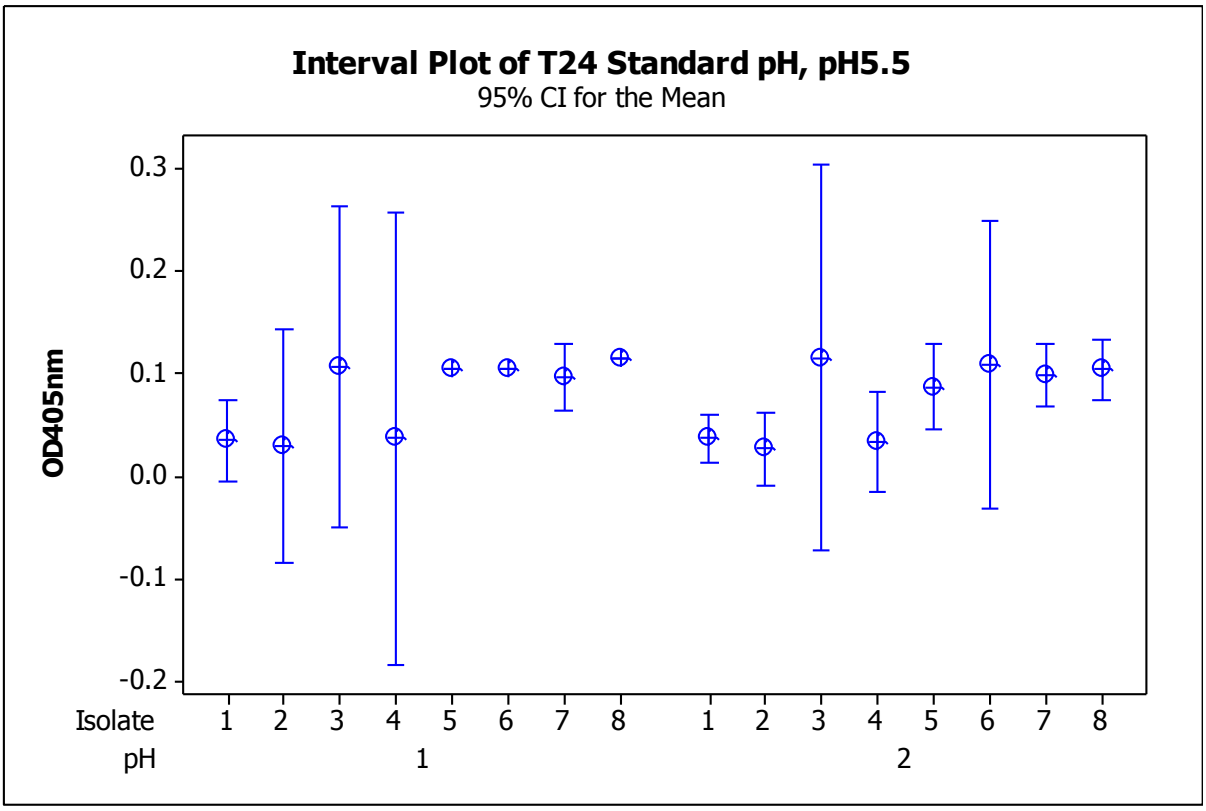

Figure 7: Interval plot for general proteolytic activity of the *P. aeruginosa* isolates at each initial broth culture pH following a 24 hour culture. For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-7 were numbered isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analysis. pH1=Standard pH (initial pH7.3); pH2=initial pH5.5.

Table 2: *P. aeruginosa* isolates (ATCC isolate + 7 wound isolates): Differences in general protease activity between isolates, at each initial pH.

Culture Time (Hours)	**Initial Culture pH**	**Statistical Analysis**	**Post-Hoc Observations (using the Tukey method)**	**Kruskal-Wallis test**
24	Standard Broth (7.3)	$F(7, 14) = 9.66$, $P = 0.004$*	Wound isolates 2 & 7 are not significantly different from each other but are significantly different from the ATCC isolate and wound isolate 1. All other isolates are not significantly different from each other.	$P = 0.134$
24	5.5	$F(7, 24) = 13.73$, $P = <0.001$*	ATCC isolate, wound isolate 1 and wound isolate 3 are not significantly different from each other but are from the rest of the isolates; the remaining wound isolates are not significantly different from each other.	

** Significant at the 0.05 level*

Table 3: *P. aeruginosa* isolates (7 wound isolates): Differences in general protease activity between isolates, at each initial pH.

Culture Time (Hours)	**Initial Culture pH**	**Statistical Analysis**	**Kruskal-Wallis test**
24	Standard Broth (7.3)	F(6, 11) = 8.01, P = 0.019*	P = 0.220
24	5.5	F(6, 20) = 11.69, P = <0.001*	

** Significant at the 0.05 level*

3.4.3 Elastin-Congo Red Assay for Elastase Activity

The elastolytic activities of each of the *P. aeruginosa* wound isolates were determined using the elastin-congo red assay as described above to identify LasB-deficient and active strains. As described in Manuscript 2, both *P. aeruginosa* elastase A and elastase B degrade elastin although elastase B (an important virulence factor in wounds and burns) has been shown to degrade elastin to a greater extent (Caballero *et al*, 2001). The EF isolate was shown to have some elastolytic activity; this is likely a result of the activity of the extracellular zinc metalloproteinase (GelE) which shares homologies with GelE of *Bacillus* species and *P. aeruginosa* elastase and has been shown to be an active virulence factor of human and animal EF isolates (although to a lesser degree than *Enterococcus faecalis*) (Gulhan *et al*, 2006; Su *et al*, 1991). The EF isolate however, was excluded from statistical analysis.

Results have shown that there is a small variation between isolates in terms of elastase activity (see figures 8-12), which was maintained at each initial culture pH after a 24 hour culture period. This difference did not reach statistical significance. However, the overall elastolytic activity observed in this assay was minimal for all isolates. Given the complex relationships, statistical significance is detailed in tables 4 and 5. In concordance with the azocasein assays, the ATCC reference isolate displayed less elastolytic activity when cultured in standard pH MHB (pH7.3) compared with the wound isolates.

Further analysis revealed that there was not a significant effect of pH on the elastolytic activity of the isolates and the 24 hour time point, $F(1, 39) = 2.24$, $P = 0.144$. Appendix 2 details the grouping information which shows the elastolytic activity of each individual isolate at each initial pH.

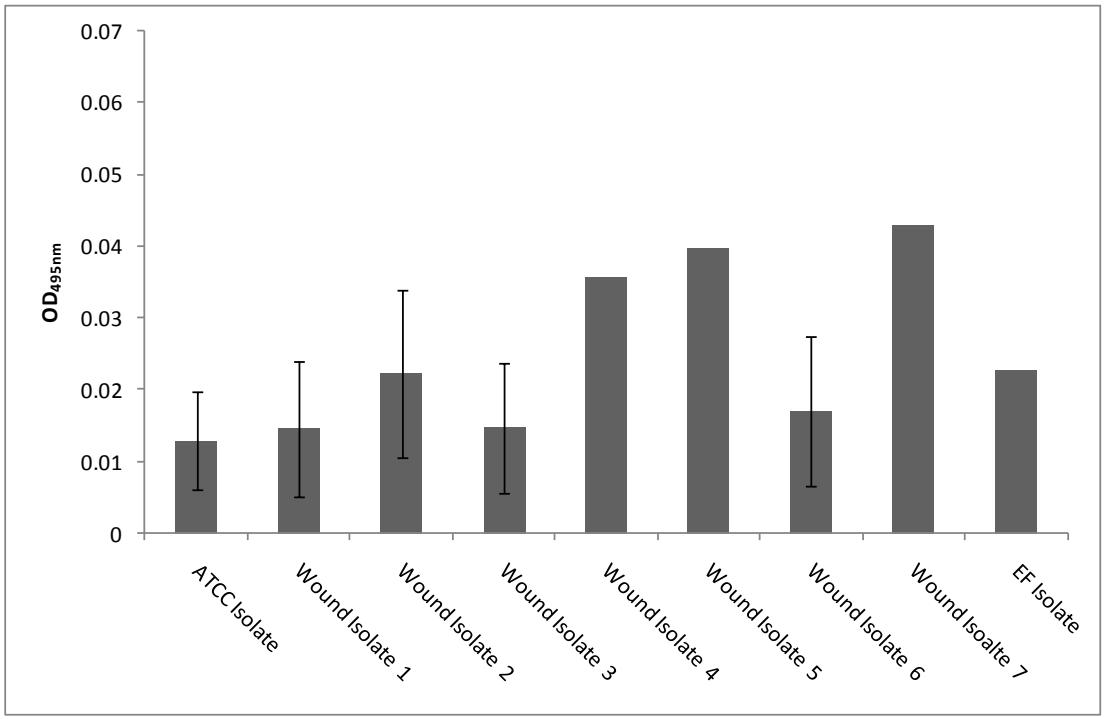

Figure 8: Elastin-Congor red (ECR) assay for elastolytic activity of each isolate following a 24 hour culture period. Isolates were cultured in standard MHB (initial pH7.3). Data is presented as mean ± SEM. Statistical significance is detailed in tables 4 and 5.

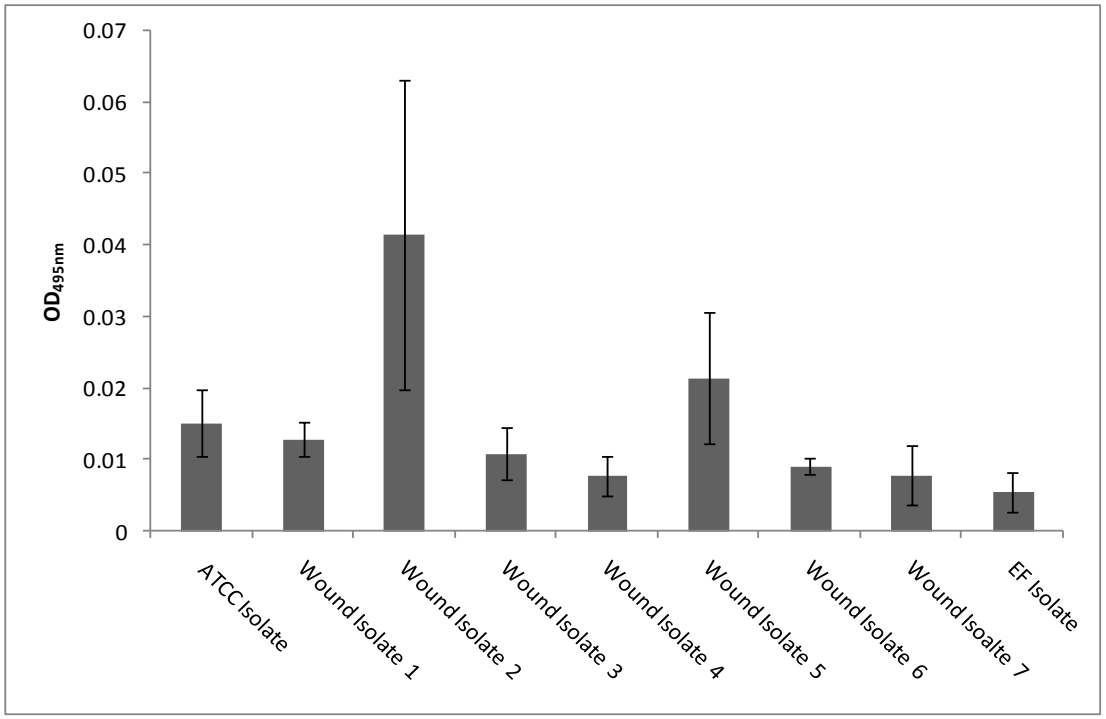

Figure 9: Elastin-Congo red (ECR) assay for elastolytic activity of each isolate following a 24 hour culture period. Isolates were cultured in MHB adjusted to an initial pH of 5.5. Data is presented as mean ± SEM. Statistical significance is detailed in tables 4 and 5.

**Enterococcus faecium*= EF isolate

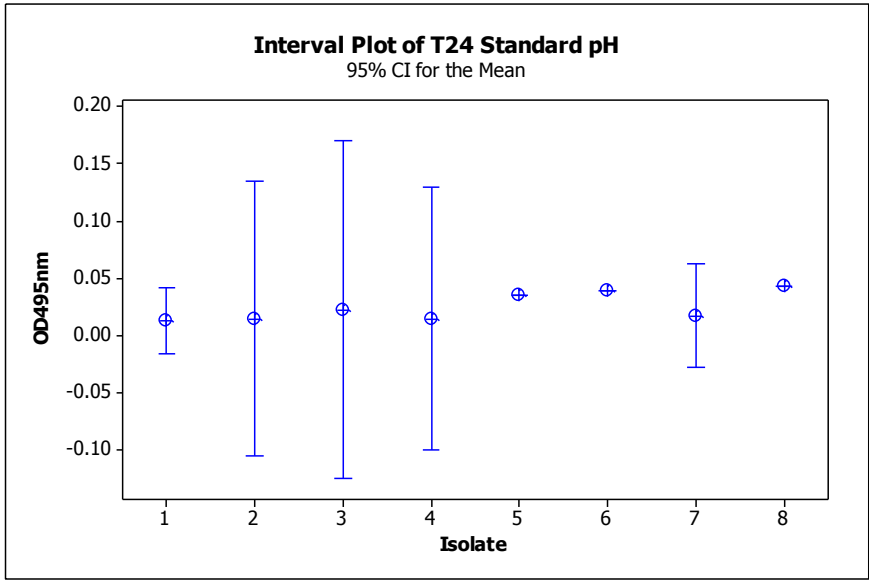

Figure 10: Interval plot for elastolytic activity of each isolate following a 24 hour broth culture (standard initial pH7.3). For all statistical analyses and interval plots the ATCC isolate was named isolate 1; wound isolates 1-7 were named isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analyses and interval plots.

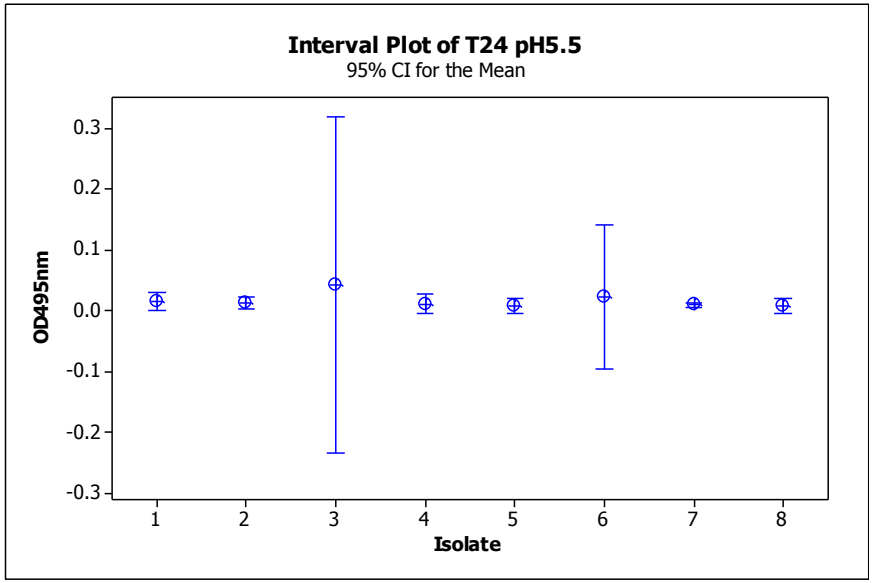

Figure 11: Interval plot for elastolytic activity of each isolate following a 24 hour broth culture (standard initial pH5.5). For all statistical analyses and interval plots the ATCC isolate was named isolate 1; wound isolates 1-7 were named isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analyses and interval plots.

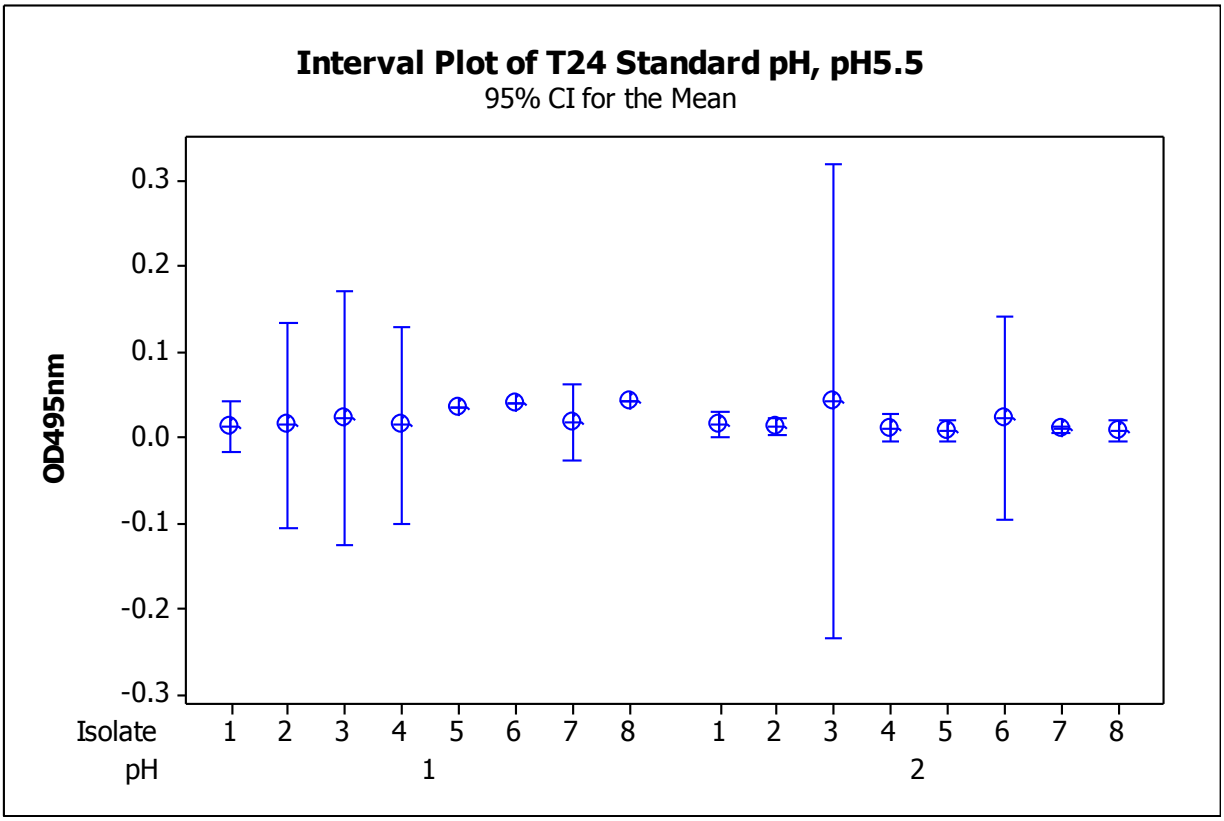

Figure 12: Interval plot for elastolytic activity of the *P. aeruginosa* isolates at each initial broth culture pH following a 24 hour culture. For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-7 were numbered isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analysis.

Table 4: *P. aeruginosa* isolates (ATCC isolate + 7 wound isolates): Differences in elastase activity between isolates, at each initial pH.

Culture Time (Hours)	**Initial Culture pH**	**Statistical Analysis**	**Post-Hoc Observations (using the Tukey method)**	**Kruskal-Wallis test**
24	Standard Broth (7.3)	F(7, 14) = 0.93, P = 0.536	None of the isolates are significantly different from each other.	
24	5.5	F(7, 24) = 2.78, P = 0.040*	Wound isolate 2 is significantly different from wound isolates 4, 6 & 7.	P = 0.214

** Significant at the 0.05 level*

Table 5: *P. aeruginosa* isolates (7 wound isolates): Differences in elastase activity between isolates, at each initial pH.

Culture Time (Hours)	**Initial Culture pH**	**Statistical Analysis**	**Kruskal-Wallis test**
24	Standard Broth (7.3)	F(6, 11) = 0.79, P = 0.612	
24	5.5	F(6, 20) = 3.14, P = 0.037*	P = 0.184

** Significant at the 0.05 level*

3.4.4 Milk-Casein Agar Plate Analysis

The milk-casein agar plate method was employed to compare the caseinolytic profiles of each of the *P. aeruginosa* isolates (both the ATCC reference strain and the wound isolates). The general protease activity of the isolates following sub-culturing of the sonicated cells onto milk-casein agar plates was investigated. As described above, sonicated biofilm cells were re-suspended in fresh MHB (standard pH) and inoculated onto the milk-casein agar plates and incubated for 24 hours. Proteolytic activity was measured by the clearance zone, indicating hydrolysis of casein, indicative of overall extracellular protease activity (as highlighted in Manuscript 2). Caseinolytic activity is caused mainly by elastase B (LasB), alkaline protease and protease IV (Caballero *et al*, 2001; Sonawane, Jyot & Ramphal, 2006). Whilst the clearance zones were minimal, each isolate displayed some caseinolytic activity at each initial pH (see figure 13).

The results of the milk-casein agar plate analysis have shown that following sub-culturing of the biofilm cells the isolates retained their caseinolytic activity, with similar trends seen between isolates as the azocasein and ECR assays; differences between isolates in terms of caseinolytic activity were noted, however these differences did not reach statistical significance (see tables 6 & 7). The ATCC isolate and wound isolate 1 were the least proteolytically active compared to each of the other isolates (except the EF isolate) (see figures 14-16). There was a general trend of increasing caseinolytic activity from 24 to 48 hours demonstrating that each of the isolates maintained their caseinolytic activity after an extended culture period.

Further statistical analysis using a general linear model revealed that pH did not have a significant effect on the caseinolytic activity of isolates at T24, $F(1, 39) = 0.44$, $P = 0.513$ or at T48, $(F1, 39) = 1.55$, $P = 0.223$.

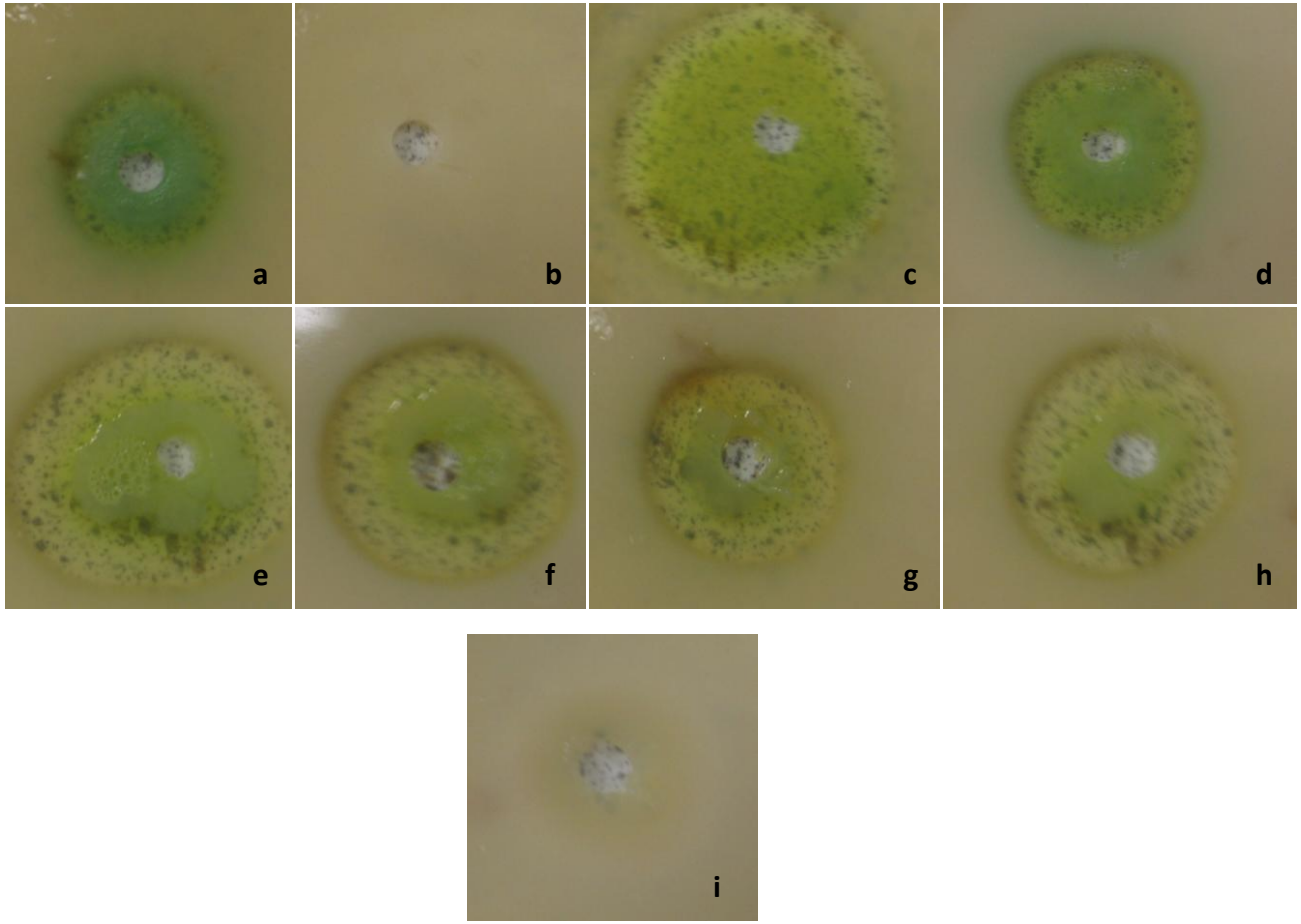

Figure 13: Milk-casein agar plates showing caseinolytic digestion of the sonicated/re-suspended 24 hour biofilm cells (initial pH5.5); a: ATCC isolate; b: Wound isolate 1; c: Wound isolate 2; d: Wound isolate 3; e: Wound isolate 4; f: Wound isolate 5; g: Wound isolate 6; h: Wound isolate 7; i: *E. faecium* (EF) isolate. Each milk-casein agar plate was cultured for 48hours.

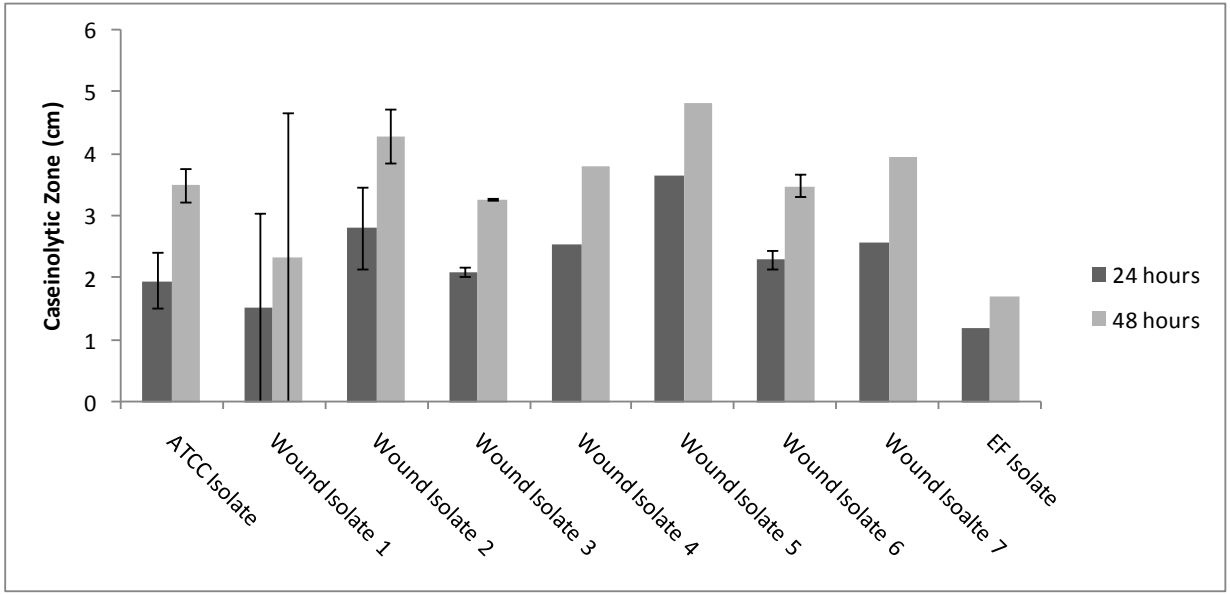

Figure 14: Milk-Casein agar plate assays for caseinolytic activity of each isolate following an initial 24 hour broth culture prior to a further 24 or 48 hour milk plate culture. Standard MHB (initial pH7.3) was used for broth cultures. Data is presented as mean ± SEM. Statistical significance is detailed in tables 6 and 7.

**E. faecium*= EF isolate

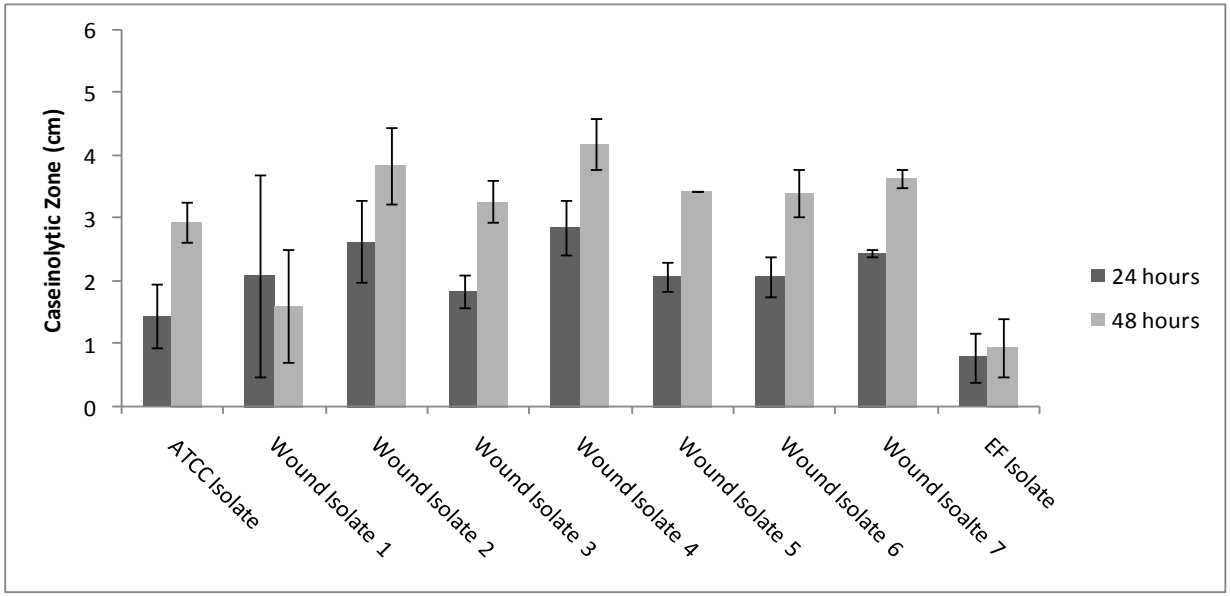

Figure 15: Milk-Casein agar plate assays for caseinolytic activity of each isolate following an initial 24 hour broth culture prior to a further 24 or 48 hour milk plate culture.) Initial pH5.5 MHB was used for broth cultures. Data is presented as mean ± SEM. Statistical significance is detailed in tables 6 and 7.

**E. faecium*= EF isolate

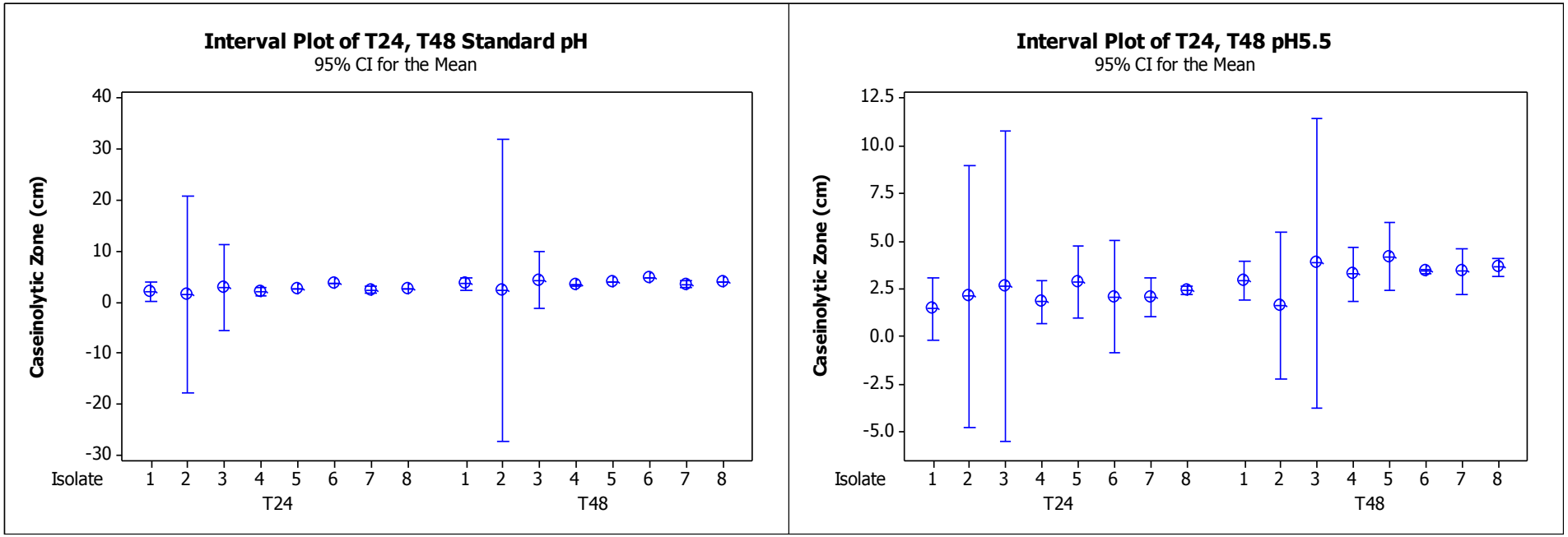

Figure 16: Interval plots for caseinolytic activity of the *P. aeruginosa* isolates following an initial 24 hour broth culture prior to a 24 or 48 hour milk plate culture (standard pH and pH5.5). For all statistical analyses the ATCC reference isolate was named isolate 1; wound isolates 1-7 were numbered isolates 2-8; the *E. faecium* (EF) isolate was excluded from statistical analysis.

Table 6: *P. aeruginosa* isolates (ATCC isolate + 7 wound isolates): Differences in caseinolytic activity between isolates.

pH	**Biofilm Broth Culture Time (Hours)**	**Milk-Casein Agar Plate Culture Time (Hours)**	**Statistical Analysis**	**Post-Hoc Observations (using the Tukey method)**	**Kruskal-Wallis test**
Standard pH (pH7.3)	24	24	F(7, 14) = 0.60, P = 0.743	None of the isolates are significantly different from each other.	
Standard pH (pH7.3)	24	48	F(7, 14) = 0.51, P = 0.803	None of the isolates are significantly different from each other.	P = 0.496
pH5.5	24	24	F(7, 24) = 0.51, P = 0.814	None of the isolates are significantly different from each other.	P = 0.329
pH5.5	24	48	F(7, 24) = 2.87, P = 0.036*	Wound isolates 1 & 4 are significantly different from each other.	P = 0.095

** Significant at the 0.05 level*

Table 7: *P. aeruginosa* isolates (7 wound isolates): Differences in caseinolytic activity between isolates.

pH	**Biofilm Broth Culture Time (Hours)**	**Milk-Casein Agar Plate Culture Time (Hours)**	**Statistical Analysis**	**Kruskal-Wallis test**
Standard pH (pH7.3)	24	24	F(6, 11) = 0.55, P = 0.758	P = 0.684
Standard pH (pH7.3)	24	48	F(6, 11) = 0.44, P = 0.826	P = 0.356
pH5.5	24	24	F(6, 20) = 0.28, P = 0.935	
pH5.5	24	48	F(6, 20) = 3.0, P = 0.042*	P = 0.113

** Significant at the 0.05 level*

3.4.5 Growth of Planktonic Bacteria (effluent)

Results have revealed that when the *P. aeruginosa* isolates were cultured at an initial pH of 7.3 there was no significant difference between the growths of the planktonic bacteria of each isolate within the effluent of the biofilm cultures (see figures 16-18). This also applied when the initial pH of the cultures was reduced to 5.5 (see table 8). Further analysis has shown that there is no statistically significant effect of pH on the growth of the planktonic bacteria within the effluent of the biofilm cultures, $F(1, 32) = 1.96$, $p = 0.173$. Furthermore, those isolates demonstrating a slightly higher OD_{600nm} within the effluent (whilst not statistically significant) did not always produce the greatest amount of proteolytic activity and so any variation in proteolytic activity was not likely a consequence of variations in growth of the planktonic bacteria (i.e. the growth of non-biofilm forming bacterial cells). Additionally, any variations observed between isolates in terms of proteolytic activity were not likely a result of increased or decreased growth of the planktonic bacteria.

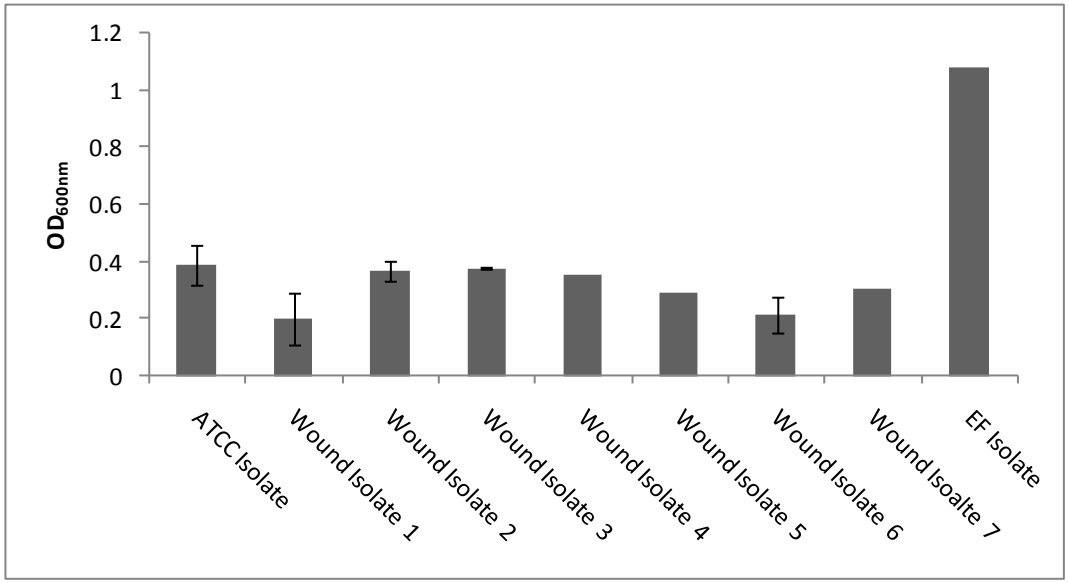

Figure 16: Optical density (OD_{600nm}) of planktonic bacteria in the effluent; samples from each biofilm culture were taken from the effluent to determine the growth of the non-adherent bacteria at an initial culture pH of 7.3. Data is presented as mean ± SEM. Statistical significance is detailed in table 8.
**E. faecium*= EF isolate

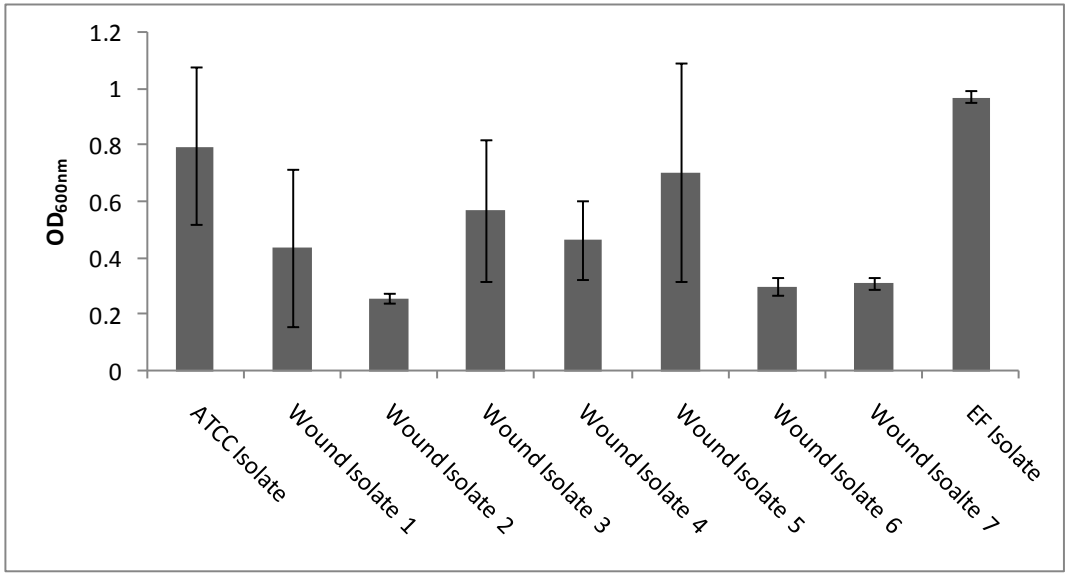

Figure 17: Optical density (OD_{600nm}) of planktonic bacteria in the effluent; samples from each biofilm culture were taken from the effluent to determine the growth of the non-adherent bacteria at an initial culture pH of 5.5. Data is presented as mean ± SEM. Statistical significance is detailed in table 8.
**E. faecium*= EF isolate

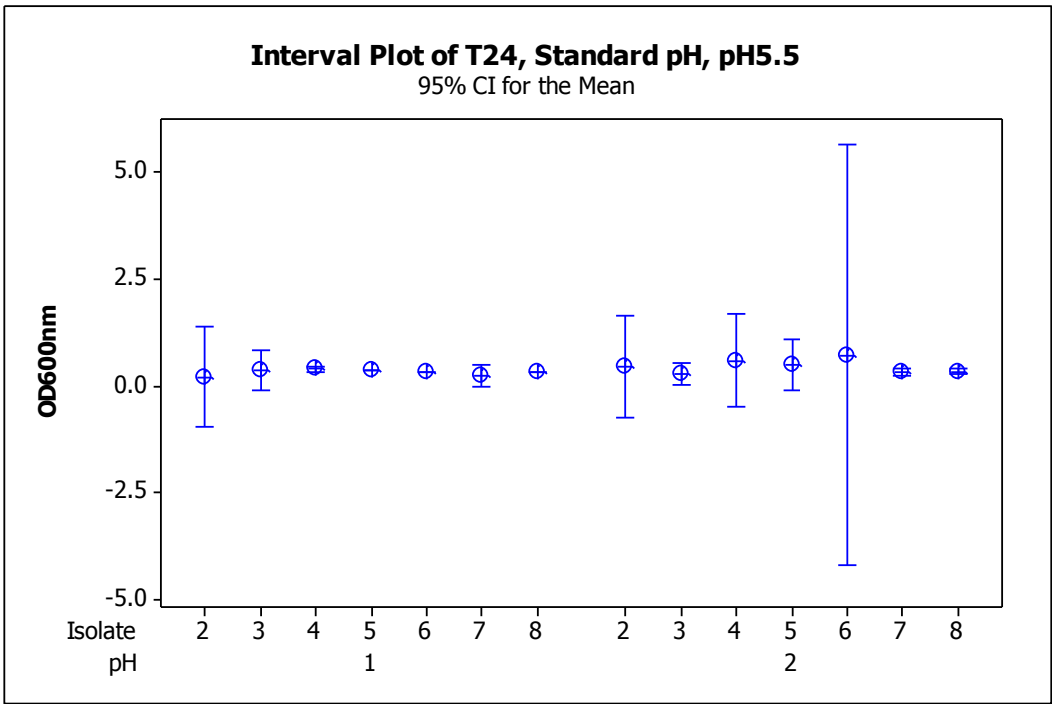

Figure 18: Interval plot of the growth trends of planktonic bacteria in the effluent for each isolate cultured at each initial pH. The *E. faecium* (EF) isolate was excluded from statistical analysis.

Table 8: *P. aeruginosa* isolates (7 wound isolates): Differences in growth of planktonic bacteria in the culture effluent at each initial culture pH.

Culture Time (Hours)	**Initial Culture pH**	**Statistical Analysis**	**Post-Hoc Observations (using the Tukey method)**	**Kruskal-Wallis test**
24	Standard Broth (7.3)	$F(6, 11) = 1.29$, $P = 0.399$	No significant difference between any of the isolates	$P = 0.233$
24	5.5	$F(6, 20) = 0.72$, $P = 0.641$	No significant difference between any of the isolates	$P = 0.533$

** Significant at the 0.05 level*

3.4.6 Growth of Biofilm Cells

The CFU counts were in concordance with the crystal violet BFP assays, apart from wound isolate 1. This isolate was found to be non-adherent in the BFP assay but did produce biofilms in the glass microscope slide biofilm cultures**.** One explanation for this variation could be the growth surface used; the BFP assay was conducted using polystyrene cell culture plates.

In concordance with the data for the OD_{600nm} of the planktonic cells in the biofilm culture effluent, there were no significant differences between the CFU counts of the adherent biofilm cells of the 7 wound isolates when cultured in MHB with an initial pH value of 7.3 (see figures 19-21 and table 9). The difference, however, between isolates was significant for the initial pH5.5 cultures (see table 9). Those isolates demonstrating a slightly higher biofilm CFU count (whilst not always statistically significant) did not always produce the greatest amount of proteolytic activity and so any variation in proteolytic activity was not likely a consequence of variations in bacterial proliferation within the biofilm. Additionally, any variations observed between isolates in terms of proteolytic activity were unlikely a result of increased or decreased biofilm growth. Further analysis has identified no significant variation in the CFU counts of the sonicated biofilm cells as a result of initial culture pH, $F(1, 38) = 2.44$, $P = 0.128$.

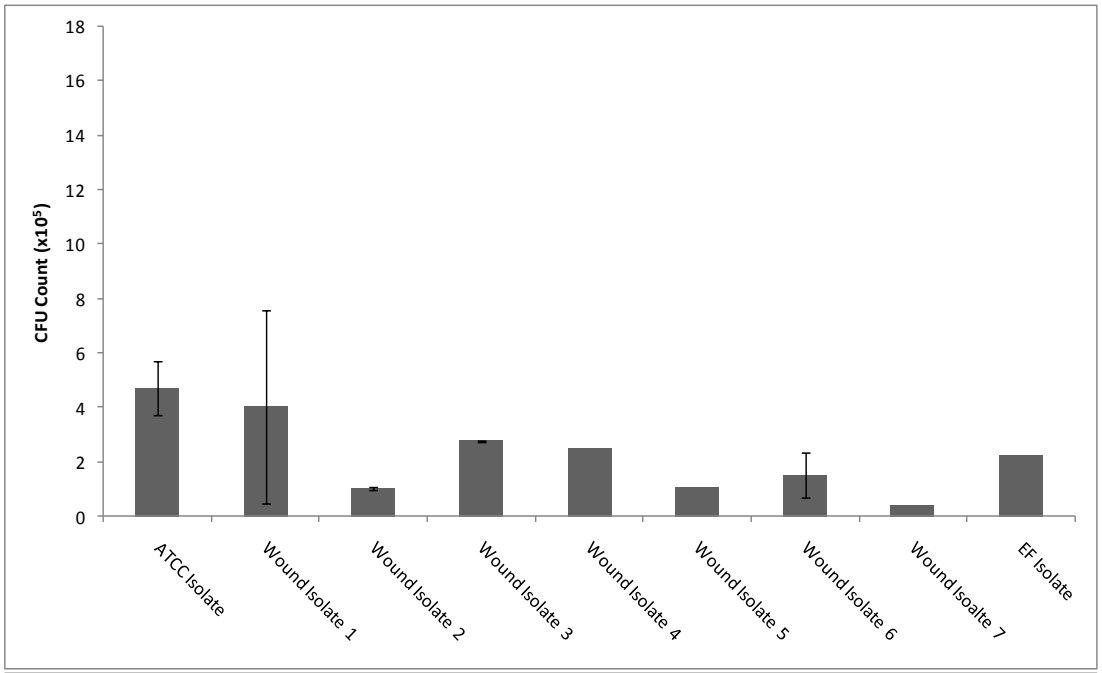

Figure 19: CFU counts of sonicated biofilm cells; samples from each biofilm culture were taken and CFU counted to determine the growth of the adherent bacteria at an initial culture pH of 7.3. Data is presented as mean ± SEM. Statistical significance is detailed in table 9.

**E. faecium*= EF isolate

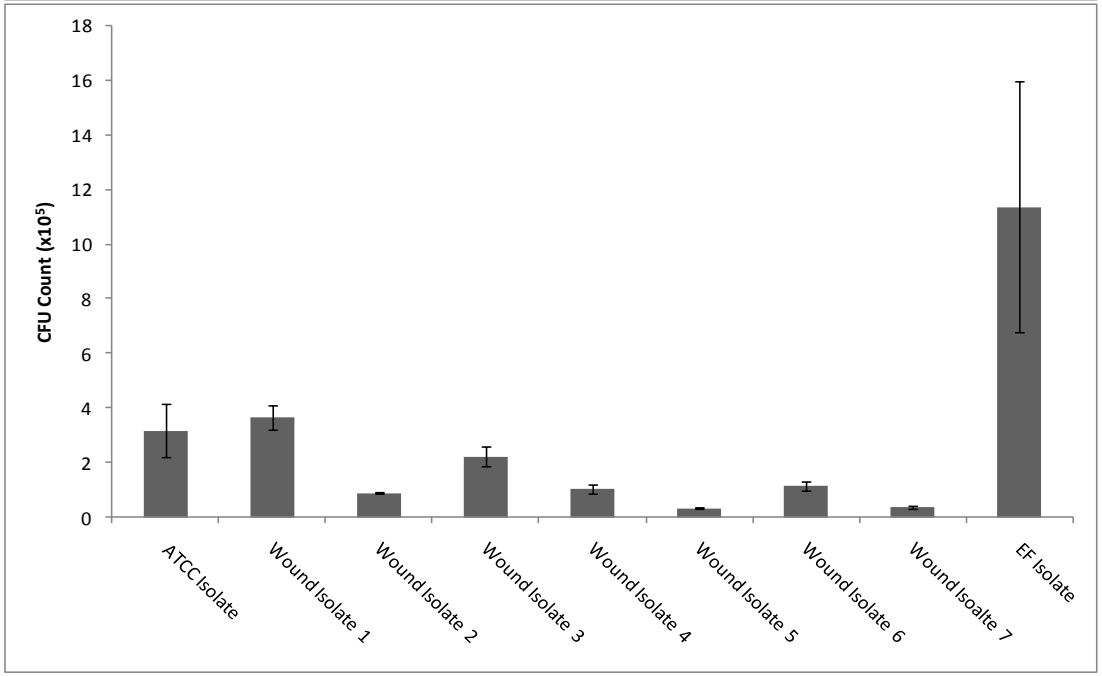

Figure 20: CFU counts of sonicated biofilm cells; samples from each biofilm culture were taken and CFU counted to determine the growth of the adherent bacteria at an initial culture pH of 5.5. Data is presented as mean ± SEM. Statistical significance is detailed in table 9.

**E. faecium*= EF isolate

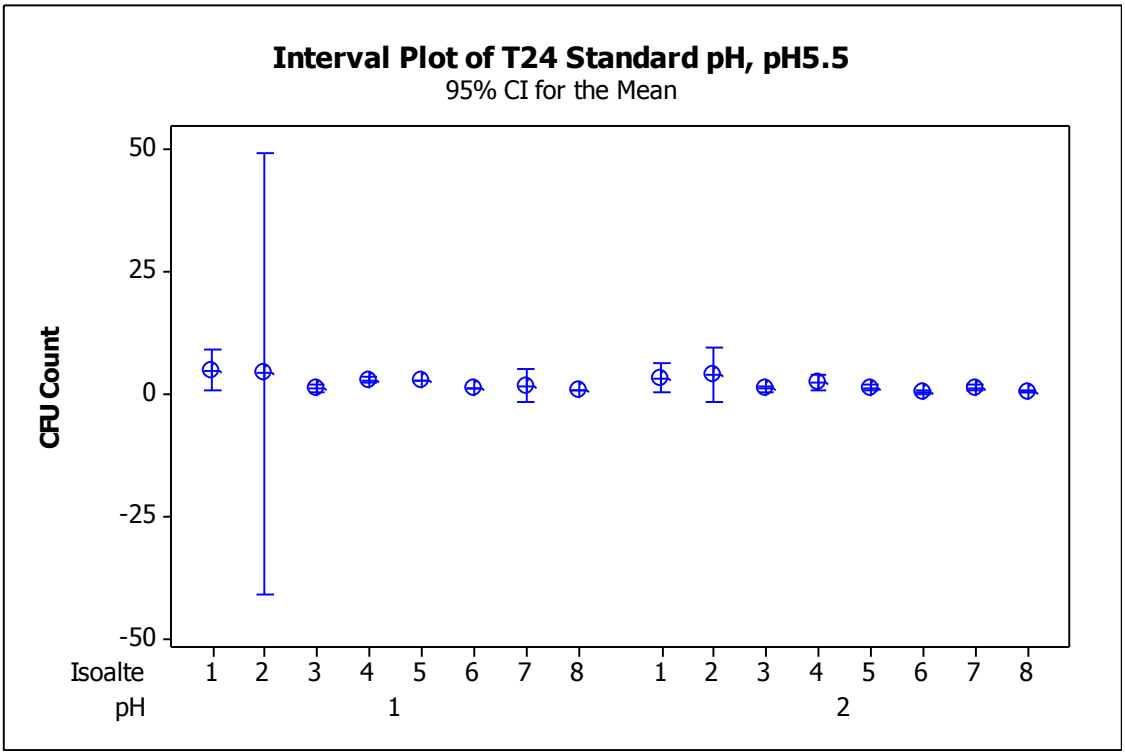

Figure 21: Interval plot of the growth trends of adherent bacteria in the biofilm culture for each isolate cultured at each initial pH. CFU counts are $x10^5$. pH1=Standard pH (pH7.3); pH2= pH5.5. *E. faecium* (EF) isolate was excluded from statistical analysis.

Table 9: *P. aeruginosa* isolates (7 wound isolates): Differences in growth of adherent bacteria in the culture effluent at each initial culture pH.

Culture Time (Hours)	**Initial Culture pH**	**Statistical Analysis**	**Post-Hoc Observations (using the Tukey method)**	**Kruskal-Wallis test**
24	Standard Broth (7.3)	F(7, 14) = 0.95, P = 0.528	No significant difference between isolates.	P = 0.446
24	5.5	F(7, 23) = 5.48, P = 0.002*	ATCC isolate and wound isolate 1 are significantly different from wound isolates 5 & 7.	P = 0.005*

** Significant at the 0.05 level*

3.4.7 CDC Biofilm Reactor Cultures

The CDC biofilm reactor system was used to compare the protease productivity of recovered biofilm-forming bacteria from mature 48 hour biofilms of two *P. aeruginosa* equine wound isolates and an ATCC *P. aeruginosa* isolate.

CFU counts of the sonicated biofilm cells revealed that the ATCC isolate had the highest CFU count compared with the wound isolates, which was consistent with the BFP assay conducted in Manuscript 2 (there was only a significant difference between the CFUs of the ATCC isolate and wound isolate 6: $F(2, 8) = 27.26$, $P = 0.001$). However, CFU counts from sonicated biofilm cells of wound isolate 6 were lower compared with wound isolate 4, $F(2, 8) = 27.26$, $P = 0.001$, which did not correlate with the BFP assay. Yet, results were inconclusive given the large error margins for CFU counts of the sonicated coupon biofilms (as a result of their only being one culture replicate for each isolate). There were no clear trends in the CFU counts from coupons taken from the top, middle or bottom of the coupon holder (see figure 21 and table 10). CFU counts are given in figures 22 and 23.

Table 10: Comparison of CFU counts in terms of coupon location from CDC reactor experiments.

Isolate	**Statistical Analysis**	**Post-Hoc Observations (using the Tukey method)**	**Kruskal-Wallis test**
ATCC isolate	$F(2, 17) = 2.75$, $P = 0.096$	No significant difference between coupons.	
Wound isolate 4	$F(2, 17) = 5.25$, $P = 0.019$*	Significant difference between top and bottom coupons.	$P = 0.019$*
Wound isolate 6	$F(2, 17) = 1.82$, $P = 0.197$	No significant difference between coupons.	$P = 0.300$

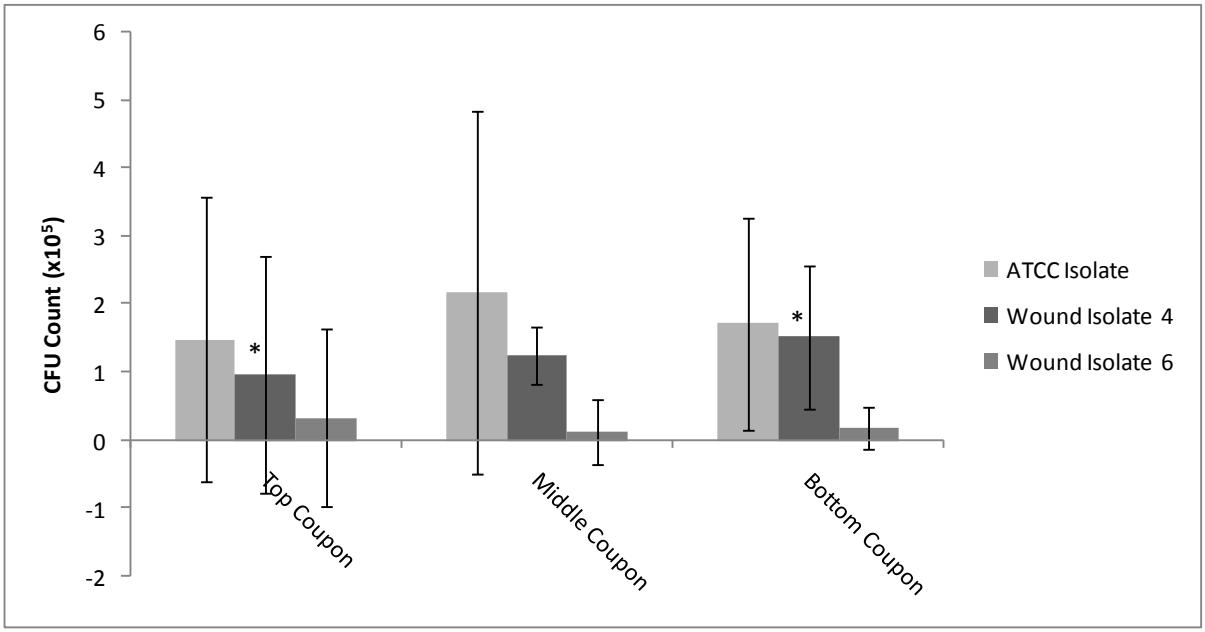

Figure 22: Colony forming unit (CFU) counts for sonicated biofilm cells from coupons taken from the top, middle and bottom of the coupon holders in the current downflow contactor (CDC) bioreactor for each *P. aeruginosa* isolate. Data is presented as mean ± SEM. Statistical significance is detailed in table 10.

** Significantly different from one another at the 0.05 level*

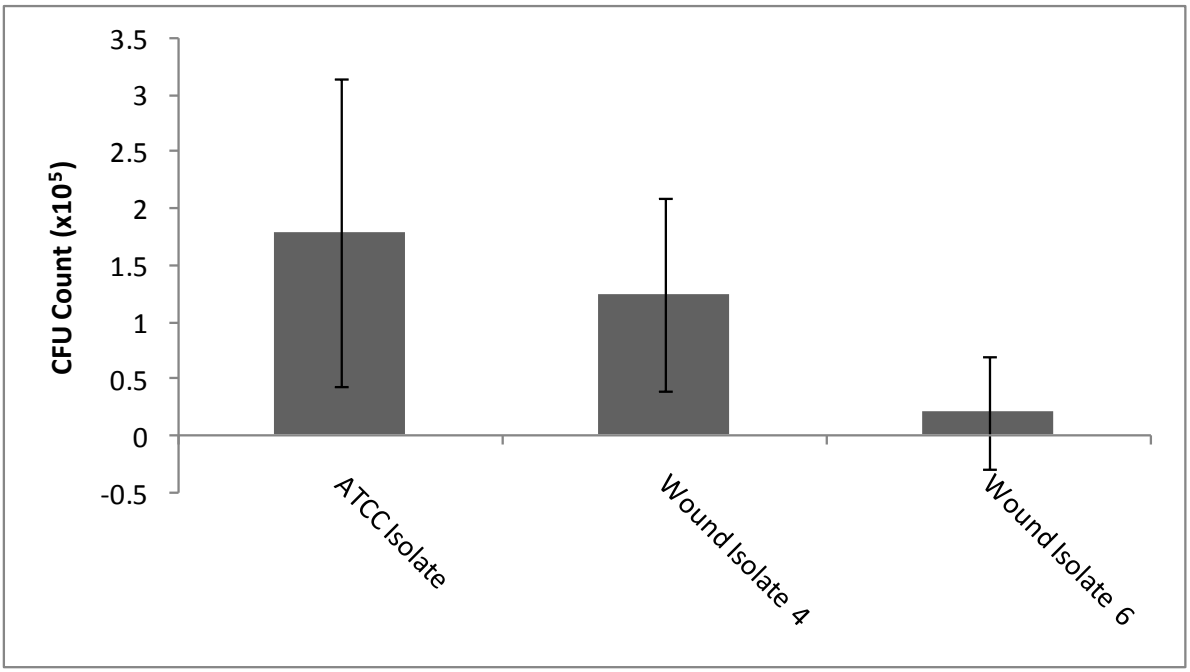

Figure 23: Combined colony forming unit (CFU) counts for sonicated biofilm cells from coupons taken from the top, middle and bottom of the coupon holders in the current downflow contactor (CDC) bioreactor for each *P. aeruginosa* isolate. Data is presented as mean ± SEM. Statistical significance is detailed in table 10.

Results from the milk-casein agar plate analysis (24 hour milk-casein agar plate incubation) revealed that there was a significant difference between the general proteolytic activity of re-suspended biofilm-forming cells following sonication from the mature 48 hour coupon biofilms: $F(2, 8) = 22.49$, $P = 0.002$, with the significance being between the ATCC isolate and the wound isolates (no significant difference between wound isolates 4 and 6) (see table 11 and figures 24 and 25). Indeed, wound isolates 4 and 6 were the most proteolytically active of the 3 isolates when compared in terms of biomass. This correlates well with the planktonic data from Manuscript 2 and generally with the glass slide biofilm data in that the ATCC isolate was less proteolytic than most of the wound isolates. The location of the coupons (i.e. top, middle or bottom) in the coupon holder did not have a significant effect on the proteolytic activity of the re-suspended biofilm-forming bacterial cells (see table 11).

Table 11: Comparisons between general protease activity (milk-casein agar plate analysis) of sonicated biofilm cells in terms of coupon location.

Isolate	**Statistical Analysis**	**Post-Hoc Observations (using the Tukey method)**	**Kruskal-Wallis test**
ATCC isolate	$F(2, 17) = 1.44$, $P = 0.269$	No significant difference between coupons.	$P = 0.420$
Wound isolate 4	$F(2, 17) = 0.58$, $P = 0.571$	No significant difference between coupons.	$P = 0.493$
Wound isolate 6	$F(2, 16) = 0.69$, $P = 0.516$	No significant difference between coupons.	

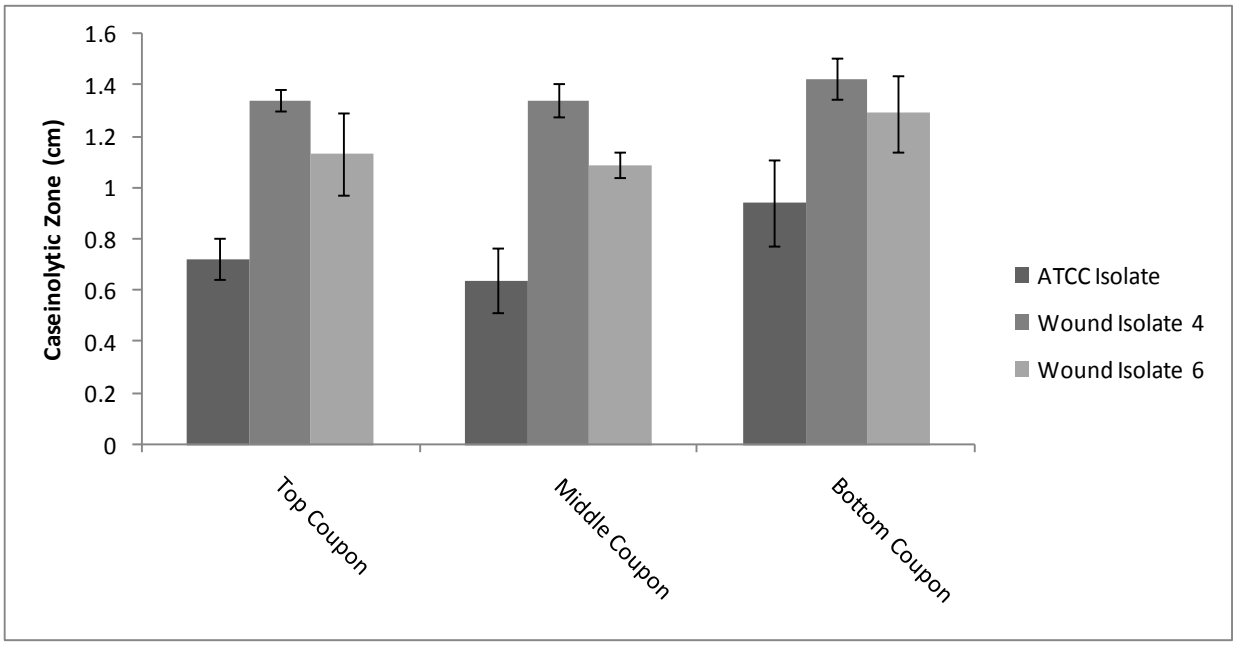

Figure 24: General protease activity of the 3 *P. aeruginosa* isolates following 24 hour incubation on milk-casein agar plates. Sonicated biofilm-forming cells from the 48 hour coupon biofilms (top, middle and bottom coupons) were re-suspended and plated on to milk-casein agar plates. Data is presented as mean ± SEM. Statistical significance is detailed in table 11.

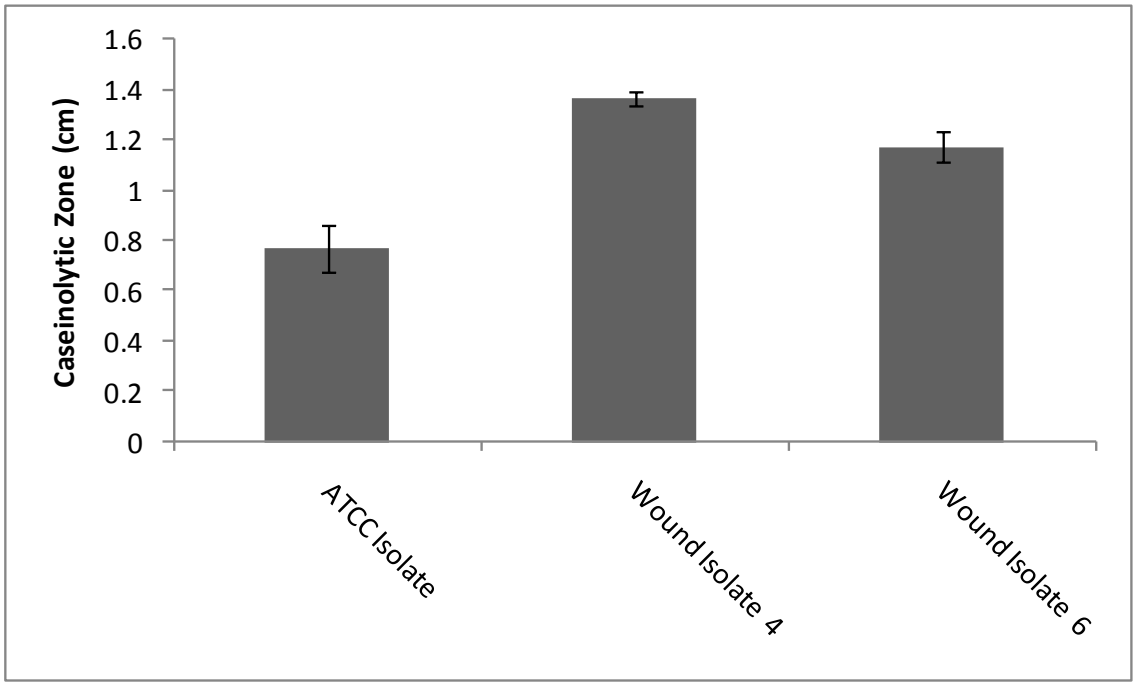

Figure 25: Combined general protease activity of the 3 *P. aeruginosa* isolates following 24 hour incubation on milk-casein agar plates. Sonicated biofilm-forming cells from the 48 hour coupon biofilms were re-suspended and plated on to milk-casein agar plates. Data is presented as mean ± SEM. Statistical significance is detailed in table 11.

3.4.8 Gelatin Zymography and SDS-PAGE

Gelatin zymography was conducted to confirm the results obtained from the biochemical assays and the milk-casein agar plates. As shown in figure 26, at an initial pH of 7.3 in glass microscope slide biofilm cultures, wound isolates 2, 4, 5, 6 and 7 produced proteolytic bands on the zymograms. The ATTC isolate, wound isolate 1 and wound isolate 3 did not produce any bands on the zymograms indicating a lack of proteolytic activity within the biofilm culture effluent. These results correlate well with the data obtained from both the azocasein and the elastin congo-red assays. All proteolytic bands present on the gelatin zymograms were at around the 50kDa mark, indicative of alkaline proteinase; whilst the ECR assay detected elastolytic activity of the biofilm culture effluents the level of activity may not have been high enough for detection on the zymogram gels. Trends in the proteolytic bands were the same for initial pH5.5 cultures (see figure 27). However, wound isolate 6 produced an additional proteolytic band, with a high molecular weight. Whilst further analysis would be needed to identify this enzyme, it may be representative of elastase A or elastase B (Caballero *et al*, 2001; Andrejko *et al*, 2012). Alternatively it could indicate a contaminant enzyme given the smear also seen for this isolate on gel 1 (figure 26). Results from the SDS-PAGE (figures 28 and 29) showed large variations in both the molecular weights of proteins present in the culture effluent and the intensity of the protein bands. There were also large variations in the protein bands present on the gels between the pH7.3 and pH5.5 biofilm culture samples. Further investigation would be needed to identify these variations in the proteins within the culture effluent.

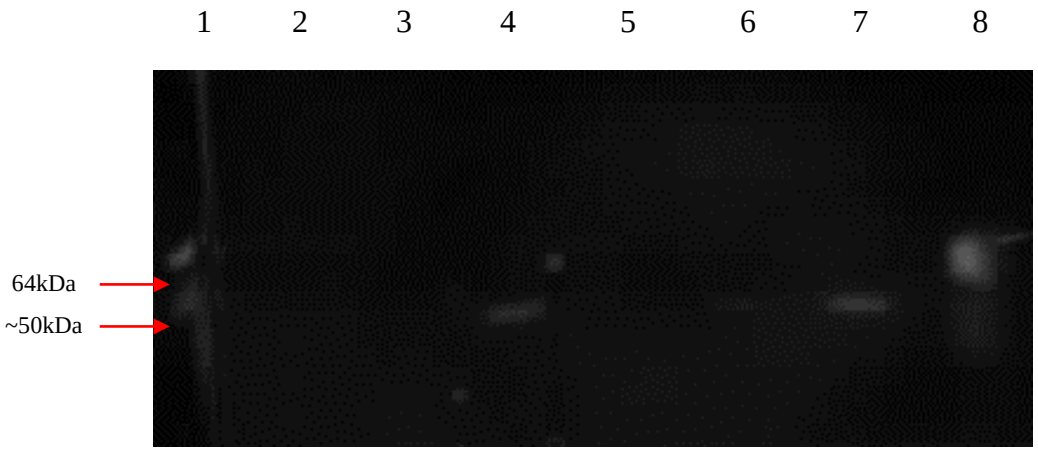

Figure 26: Gelatin zymography of glass microscope slide biofilm culture effluents following a 24 hour culture period at an initial pH of 7.3: Lane 1: MMP-2 marker (the lower molecular weight band indicates active MMP-2; the higher molecular weight band is indicative of latent MMP-2 [approximately 72kDa]); Lane 2: ATCC isolate; Lane 3: Wound isolate 1; Lane 4: Wound isolate 2; Lane 5: Wound isolate 3; Lane 6: Wound isolate 5; Lane 7: Wound isolate 4; Lane 8: Wound isolate 6. All effluent samples were loaded onto the gel at a final dilution of 1:20 (15µl per well). Wound isolates 2, 4, 5, 6 and 7 produced proteolytic bands on the zymograms. All proteolytic bands present on the gelatin zymogram were at around the 50kDa mark. The ATTC isolate, wound isolate 1 and wound isolate 3 did not produce any bands.

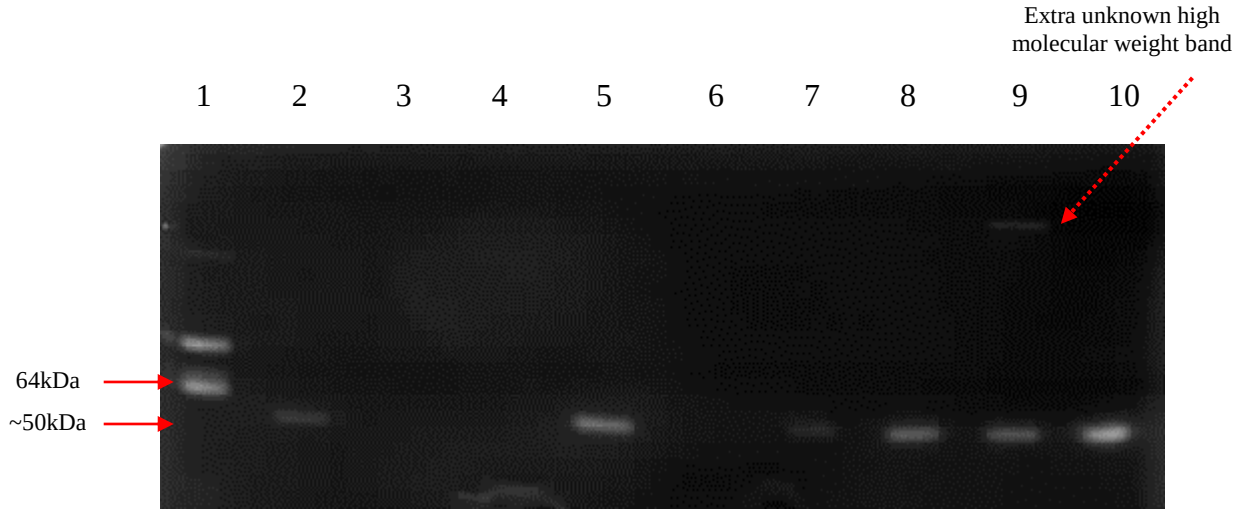

Figure 27: Gelatin zymography of glass microscope slide biofilm culture effluents following a 24 hour culture period at an initial pH of 5.5 (Lane 2: Wound isolate 7 from initial pH7.3 cultures): Lane 1: MMP-2 marker (the lower molecular weight band indicates active MMP-2; the higher molecular weight band is indicative of latent MMP-2 [approximately 72kDa]); Lane 2: Wound isolate 7 from initial pH7.3 cultures; Lane 3: ATCC isolate; Lane 4: Wound isolate 1; Lane 5: Wound isolate 2; Lane 6: Wound isolate 3; Lane 7: Wound isolate 5; Lane 8: Wound isolate 4; Lane 9: Wound isolate 6 (dashed red arrow indicates an unknown high molecular weight proteolytic band not identified in the other samples); Lane 10: Wound isolate 7. All effluent samples were loaded onto the gel at a final dilution of 1:20 (15µl per well). Wound isolates 2, 4, 5, 6 and 7 produced proteolytic bands on the zymograms. All proteolytic bands present on the gelatin zymogram were at around the 50kDa mark. The ATTC isolate, wound isolate 1 and wound isolate 3 did not produce any bands. Wound isolate 6 produced an additional proteolytic band, with a high molecular weight.

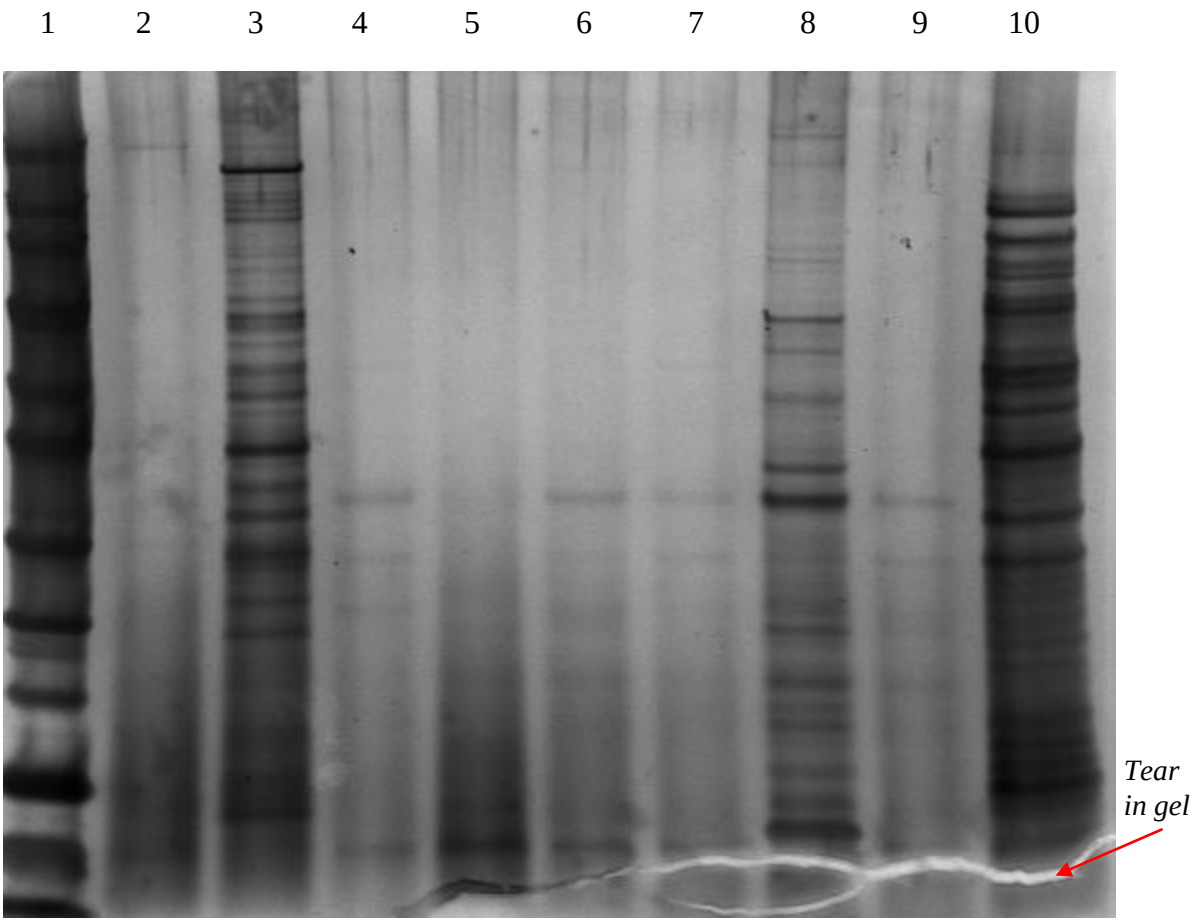

Figure 28: SDS-PAGE of glass microscope slide biofilm culture effluents following a 24 hour culture period at an initial pH of 7.3. Each well was loaded with 20µlculture supernatant (1:5 dilution). Lane 1: Molecular weight standards; Lane 2: ATCC isolate; Lane3: Wound isolate 1; Lane 4: Wound isolate 2; Lane 5: Wound isolate 3; Lane 6: Wound isolate 5; Lane 7: Wound isolate 4; Lane 8: Wound isolate 6; Lane 9: Wound isolate 7. There were large variations in both the molecular weights of proteins present in the culture effluent and the intensity of the protein bands.

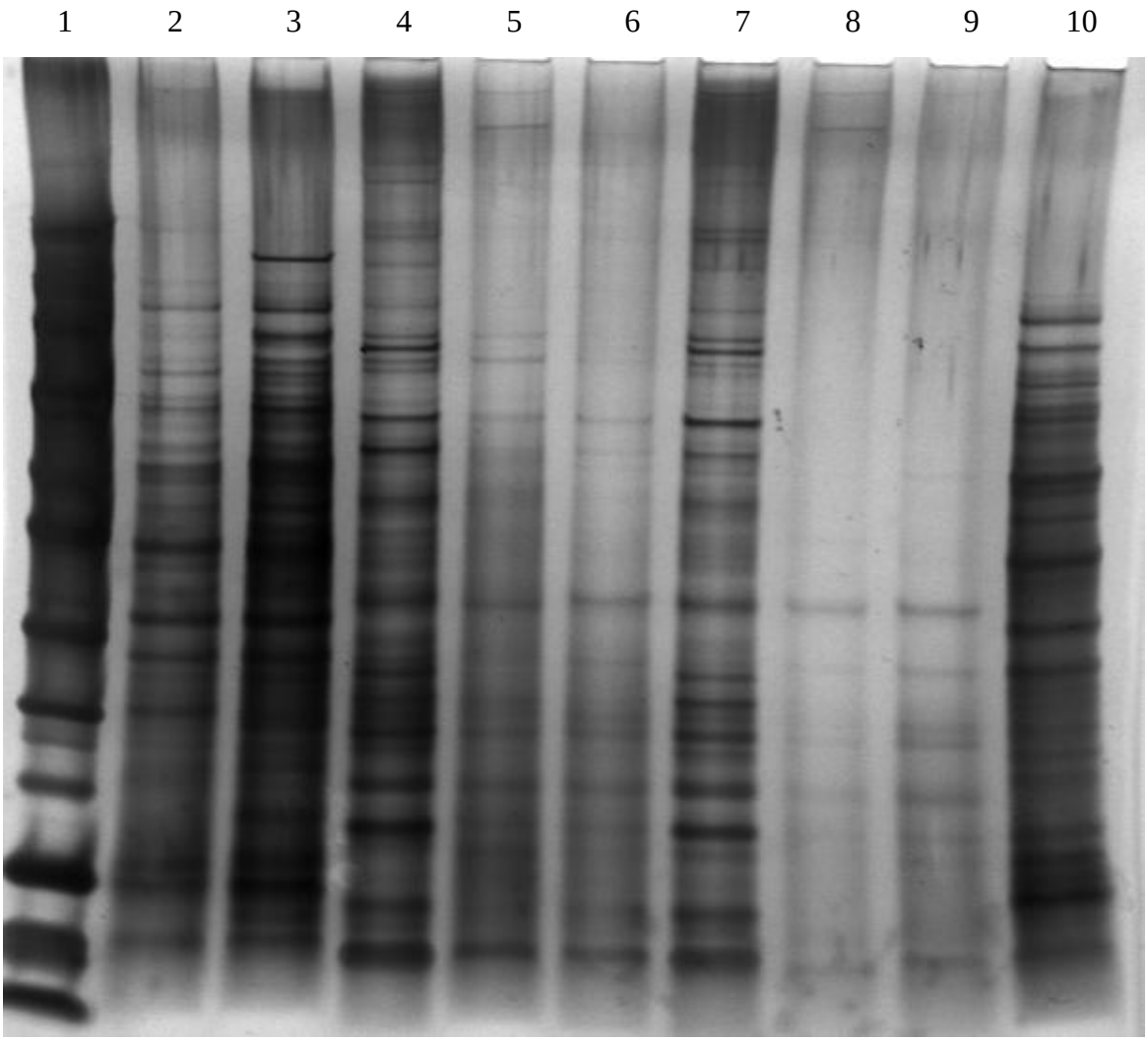

Figure 29: SDS-PAGE of glass microscope slide biofilm culture effluents following a 24 hour culture period at an initial pH of 5.5. Each well was loaded with 20µlculture supernatant (1:5 dilution). Lane 1: Moleular weight standards; Lane 2: ATCC isolate; Lane3: Wound isolate 1; Lane 4: Wound isolate 2; Lane 5: Wound isolate 3; Lane 6: Wound isolate 5; Lane 7: Wound isolate 4; Lane 8: Wound isolate 6; Lane 9: Wound isolate 7. There were large variations in both the molecular weights of proteins present in the culture effluent and the intensity of the protein bands.

3.4.9 Histology

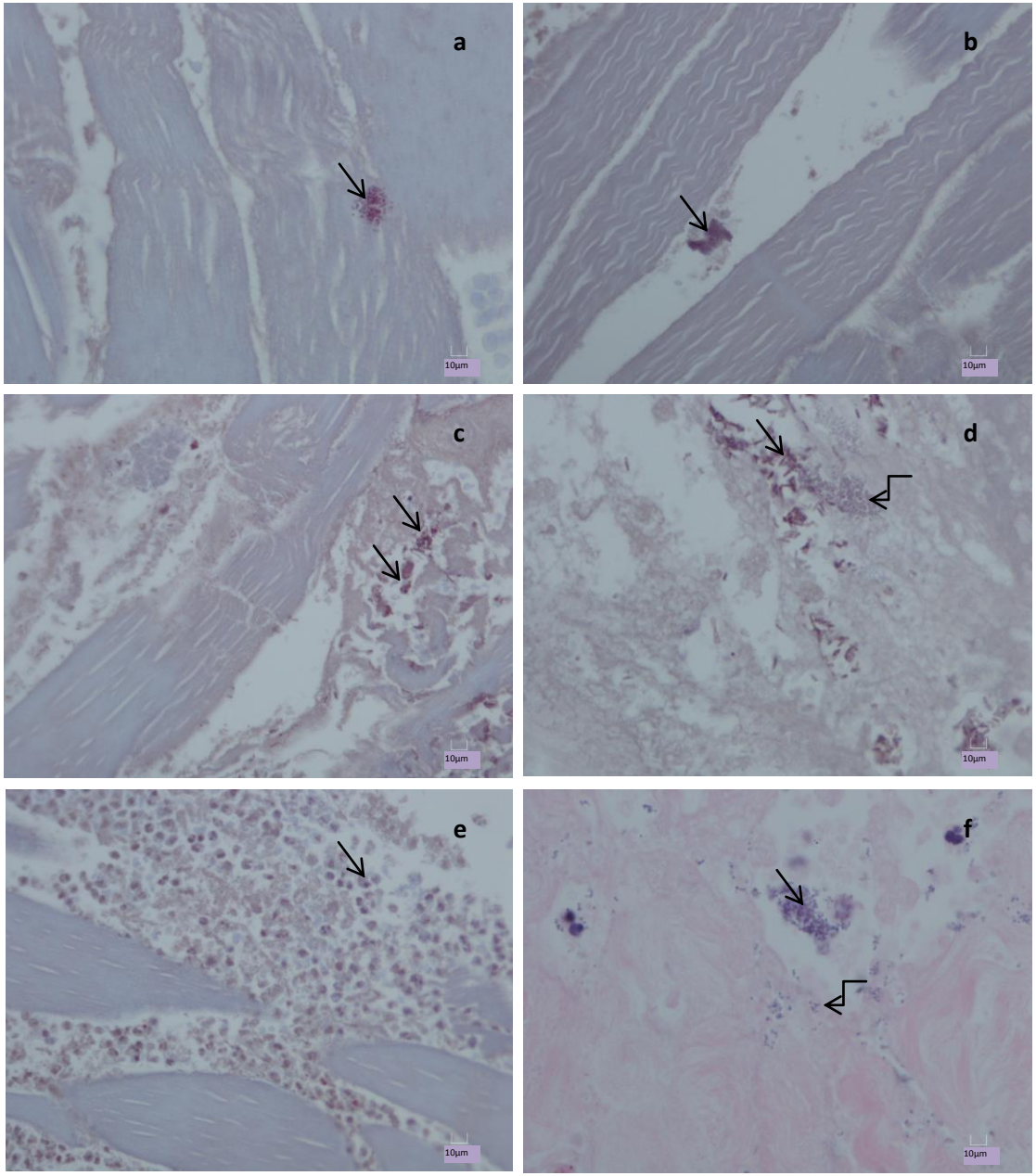

Figure 30: Histological evidence of bacterial clusters within equine wound tissue. a: Gram stain showing a round cluster of Gram positive cocci (see arrow) towards the edge of the tissue section; b: Gram stain showing a large cluster of predominantly Gram positive cocci at the edges of the tissue section. Smaller clusters of bacteria surround the large cluster (see arrows); c: Gram stain highlighting small clusters of both Gram positive and Gram negative bacteria (cocci and bacilli) (see arrows); d: Gram stain showing evidence of a mixed bacterial species cluster, including Gram positive and Gram negative cocci (see elbow arrow) and bacilli (see straight arrow); e: Inflammatory cell infiltration at the edge of the tissue section (see arrow); f: haematoxylin and eosin (H&E) stain showing a large cluster of Gram positive cocci (see straight arrow) surrounded by small bacterial clusters (see elbow arrow). Scale bars = 10µm

3.5 Discussion

Whilst it should be kept in mind that the *in vitro* experimental environment does not reflect the complex *in-vivo* situation, the results of this study have clearly highlighted the significant variation among *P. aeruginosa* isolates obtained from chronic equine wounds in terms of both growth rate and protease production within developing biofilms and mature biofilms. The study has also indicated that the *P. aeruginosa* biofilms did not respond significantly to changes in pH in terms of proteolytic activity *in vitro*.

The biofilm or sessile mode of growth and the mucoid phenotype of *P. aeruginosa* isolates has been implicated in the proteolytic activity of the microorganism in the clinical setting. Some research has suggested that the biofilm mode of growth supports a heightened level of proteolytic activity as compared to planktonic counterparts (Evans, Brown & Gilbert, 1994), with an increase in alginate paralleling an increase in protease production (Harjai *et al*, 2005). Yet, other studies have suggested protease production is inversely correlated with alginate production with alginate-producing strains producing lower yields of extracellular proteases than their respective alginate-negative strains. This may simply be a result of competition for energy or export systems given the large quantities of alginate secreted by mucoid strains (Ohman & Chakrabarty, 1982; Mathee *et al*, 1999). Whilst proteases are secreted by biofilm cells, the alginate matrix may 'trap' the enzymes thus preventing their release into the surrounding environment (Hoffman & Decho, 1999). However, it has also been noted that the reduction in protease activity of CF alginate-producing *P. aeruginosa* isolates was not due to a failure of the cells to release synthesised proteases (Ohman & Chakrabarty, 1982). Yet, the mucoid phenotype alone without a definitive biofilm mode of growth may not be enough to cause the entrapment of proteases as previously mentioned. It has also been noted that in CF *P. aeruginosa* proteases play a role in the initial stages of infection whereas the role of these enzymes later in the infection when biofilms are formed is questionable given the host's ability to produce neutralising antibodies against these proteases (Döring *et al*, 1984; Döring *et al*, 1983). Despite the widely accepted importance of biofilm formation in chronic wounds and also the destructive nature of certain bacterial proteases within wounds, the majority of current research into the

role that biofilms and extracellular bacterial proteases play in disease seems to focus upon CF and limited focus has been placed upon these factors in wounds.

In the present study, it has been noted that whilst the biofilm cultures did produce proteases as demonstrated by biochemical assays and milk-casein agar plate cultures, as measured in the culture effluent, the cultures were considerably less proteolytically active than corresponding planktonic cultures (as described in Manuscript 2). Given that protease activity was measured within the effluent this may be a result of protease entrapment within the biofilm matrix thus leading to an underestimation of protease levels. General proteolytic activity of the re-suspended biofilm cells was minimal on milk-casein agar, however proteolytic activity increased steadily following a 24 and 48 hour milk-casein agar plate culture, indicating that the bacteria are able to retain their proteolytic activity for extended periods following sub-culture.

It should be kept in mind, however, that the *in vitro* cultures used in this study may have limited the availability of nutrients when compared to the *in vivo* wound milieu and so protease activity as well as biofilm development may be somewhat over or under-estimated. The *in vitro* scenario does not take into consideration the changes in nutrient availability and the presence of host cells and their extracellular mediators including antibacterial secretions and proteases. Furthermore, it must be stressed that for the glass microscope slide biofilm cultures the proteases produced and measured during the biofilm cultures would have incorporated those proteases produced by planktonic daughter cells within the effluent.

The change in initial pH of the culture broth of biofilm cultures did not have a significant impact on the production of proteases from each of the *P. aeruginosa* isolates during early biofilm development (i.e. in the 24 hour glass microscope slide cultures). In future studies it would be interesting to compare results with cultures adapted using a wider range of initial culture pHs and nutrient availability. It may also prove useful to fully characterise the changes in alginate production together with changes in protease production and expression of other extracellular virulence factors in both early biofilm development and in mature biofilms in order to ascertain the relationship between biofilm development and proteolytic activity

within the wound milieu and compare findings with planktonic cultures. Mixed-species cultures may also cause changes in the proteolytic activity of biofilm cells and so a comparison of the results with cultures of a number of wound-related micro-organisms may produce some interesting data. Whilst in the present study it was not logistically possible to use continuous-flow biofilm cultures, in future studies it may be beneficial to use such systems alongside static biofilm assays such as the colony biofilm assay using nutrient agar for proteolytic analysis under varied environmental conditions.

3.6 Conclusion

This study has highlighted a clear variation among *P. aeruginosa* isolates derived from equine wounds in terms of both general proteolytic activity and elastase activity during early and late biofilm development. Results have also revealed that a change in the initial pH of the culture broth of developing biofilms from pH7.3 to pH5.5 does not have a significant effect on protease production. This could reflect the protective role of the biofilm matrix against environmental changes, given that the same isolates cultured in the planktonic mode of growth in Manuscript 2 were affected by pH fluctuations in terms of protease activity within the culture media. When comparing the results to those from Manuscript 2, the biofilm cultures were less proteolytically active than the planktonic cultures. In terms of translating these findings to the clinical setting, it may be worth considering that treatments aimed at disrupting biofilms within chronic wounds could lead to a possible initial increase in proteolytic activity from surviving planktonic cells.

Acknowledgements

The authors would like to thank Advanced Medical Solutions Group PLC for their financial support for this project.

3.7 Appendices to Chapter 3

Note: For all analyses, the first 2 digits in the IsolXpH (Isolate X pH) represent the initial culture pH; i.e. 73 represents pH 7.3 and 55 represents pH 5.5. The remaining 2 digits represent the isolate number.

Appendix 1

One-Way ANOVA for T24 (Azocasein assay)

Grouping Information Using Tukey Method

IsolXpH	N	Mean	Grouping
5506	6	0.20528	A
5508	6	0.20378	A
5504	6	0.20050	A B
7303	3	0.19927	A B
5507	6	0.19310	A B C
7304	3	0.18847	A B C D
5505	6	0.18708	A B C D
5503	6	0.18605	A B C D
7306	3	0.16577	B C D E
7307	3	0.16357	B C D E
7308	3	0.15590	C D E
5502	6	0.15590	D E
5501	6	0.12958	E F
7305	3	0.10423	F
7302	3	0.04050	G
7301	3	0.01997	G

Means that do not share a letter are significantly different.

Appendix 2

One-Way ANOVA for T24 (ECR assay)

Grouping Information Using Tukey Method

IsolXpH	N	Mean	Grouping
5504	6	0.24440	A
5507	6	0.23753	A
5503	6	0.21278	A B
5502	6	0.20328	A B
5506	6	0.19082	A B C
5508	6	0.17988	A B C
7307	3	0.17780	A B C D
5505	6	0.15468	B C D
7303	3	0.13690	B C D E
7306	3	0.13003	B C D E F
5501	6	0.12220	C D E F
7304	3	0.08120	D E F
7308	2	0.04205	E F
7302	3	0.04007	E F
7305	3	0.02140	F
7301	3	0.01987	F

Means that do not share a letter are significantly different.

Appendix 3

One-Way ANOVA for T24 (Growth of planktonic bacteria)

Grouping Information Using Tukey Method

IsolXpH	N	Mean	Grouping
7305	3	0.51427	A
7302	3	0.49093	A
7306	3	0.49053	A
7304	3	0.47773	A B
7303	3	0.47767	A B
7307	3	0.47330	A B
7308	3	0.46710	A B
5505	6	0.41298	B
5502	6	0.31432	C
5508	6	0.24560	D
5503	6	0.23372	D E
5504	6	0.23252	D E
5507	6	0.22847	D E
5506	6	0.19160	E

Means that do not share a letter are significantly different.

4.0 Chapter 4

Manuscript 4

The effect of *Pseudomonas aeruginosa* elastase on equine fibroblast viability *in vitro* and subsequent fibroblast MMP/TIMP expression

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Running Title: **Effect of *Pseudomonas aeruginosa* proteases on MMP/TIMP expression**

Keywords: Wound healing, Matrix metalloproteinases (MMPs), Tissue Inhibitors of Matrixmetalloproteinases (TIMPs), *Pseudomonas aeruginosa* elastase, Biofilm

4.1 Abstract

Previous findings in Chapters 2 and 3 demonstrated differential protease production *in vitro* of *Pseudomonas aeruginosa* clinical isolates derived from equine wounds. The aim of this chapter was to investigate the hypothesis that protease-rich *P. aeruginosa conditioned* media and purified *P. aeruginosa* elastase have an effect on the viability, growth and MMP/TIMP mRNA expression of equine dermal fibroblasts *in vitro*.

Preliminary findings have demonstrated that *P. aeruginosa* exoproducts, including purified elastase exert deleterious effects on both normal skin fibroblasts and granulation tissue fibroblasts in a concentration-dependent manner in terms of viability and growth. Immunohistochemistry analysis revealed the presence of bacterial clusters disseminated throughout the wound bed and this bacterial staining occurred in conjunction with moderate staining of MMPs -2, -3 and -13 throughout the tissue. qRT-PCR analysis showed that mRNA of each of the MMPs and TIMPs of interest were expressed by normal skin fibroblasts and granulation tissue fibroblasts at a higher level than GADH, with cells from different equine donors responding differently in terms of MMP/TIMP mRNA expression when treated with *P. aeruginosa* conditioned media and *P. aeruginosa* elastase. However, due to limited numbers of biological replicates it was not possible to confirm whether these changes in MMP/TIMP mRNA expression were statistically significant.

4.2 Introduction

Pseudomonas aeruginosa is an opportunistic bacterial pathogen and cause of community and nosocomial-acquired infections, including wound infections (Cowell *et al*, 2003; Hoge *et al*, 2010). Many virulence characteristics are attributed to this pathogen, not least the proteases elastase B and alkaline protease. These proteases play a role in the pathogenic interaction between bacterium and host, and protease-deficient strains of *P. aeruginosa* have been shown to be generally less virulent than protease-producing strains (Holder & Haidaris, 1979; Pavlovskis & Wretlind, 1979).

A range of bacterial proteases have been shown to affect host cells in a number of ways. For instance, it was reported by Uronowey and colleagues (2006) that proteases produced by the oral pathogen *Porphyromonas gingivalis* affected bacteria-host interactions and showed evidence of the promotion of apoptosis via intracellular proteolytic activation of caspase-3. Similarly, a purified cysteine proteinase from *P. gingivalis* has been shown to cause disruption of the basement membrane of human epithelial cell lines cultured in monolayer, but with no cytotoxic effects (Shah, Gharbia & O'Toole, 1992).

P. aeruginosa elastase B, a 33kDa protease also named LasB or pseudolysin, is one of the major proteins secreted by *P. aeruginosa* into the surrounding environment (Andrejko & Mizerska-Dudka, 2012). A number of investigations have highlighted the cytotoxic actions of *P. aeruginosa* towards host cells. Sawa *et al* (1998) demonstrated that an invasive strain of *P. aeruginosa* was cytotoxic to epithelial cells *in vitro* in a bacterial density-dependent manner which was correlated to increased elastase production. Supernatant from *P. aeruginosa* cultures has also been shown to slow the migration velocity of airway epithelial cells whilst delaying or inhibiting the re-establishment of epithelial cell integrity following wound closure (De Bentzmann *et al*, 2000).What is more, culture supernatant of *P. aeruginosa* caused over-activation of host MMP-2 and MMP-9. The over-activation of MMP-2, was also associated with a decrease in the inhibitor TIMP-2; with this effect being limited to elastase-producing *P. aeruginosa* strains (De Bentzmann *et al*, 2000).

Furthermore, LasB along with LasA has been implicated in the modulation of invasion of epithelial cells (Cowell *et al*, 2003).

P. aeruginosa has consistently been implicated in delayed wound healing and in particular LasB has been shown to have an important role in the pathogenicity of this bacterium within the wound milieu (Schmidtchen *et al*, 2003; Schmidtchen, Wolff & Hansson, 2001). In addition to the effects of elastase-producing strains of *P. aeruginosa* on the degradation of important matrix components and growth factors, LasB from ulcer-derived *P. aeruginosa* has also been reported to not only degrade proteins in wound fluid and human skin proteins during infection *ex vivo* as well as antiproteinases and other important wound healing molecules, but also inhibits human skin fibroblast cell growth (Schmidtchen *et al*, 2003). Whilst other mechanisms of *P. aeruginosa* pathogenicity including the secretion of exotoxins have been shown to exert an apoptotic effect on host cells (Morimoto & Bonavida, 1992; Valente *et al*, 2000; Keppler-Hafkemeyer, Brinkmann & Pastan, 1998; Andersson, Juell & Fodstad, 2004; Schmidtchen *et al*, 2003) Schmidtchen *et al* (2003) concluded that the *P. aeruginosa* strain studied in their investigation exerted most of its effect on fibroblast cell growth via an apoptosis-independent, proteinase-mediated mechanism, however the exact mechanisms involved were not studied.

P. aeruginosa elastase has been shown to inactivate human plasma alpha 1-proteinase inhibitor, an important inhibitor of tissue destruction by endogenous serine proteinases (Morihara, Tsuzuki & Oda, 1979). *P. aeruginosa* elastase has also been implicated in the direct and indirect degradation of type 1 collagen; Nagano *et al* (2001) demonstrated the deleterious effect of *P. aeruginosa* conditioned medium on collagen degradation in a 3-dimensional collagen gel *in vitro* model used to represent the collagen matrix in corneal tissue. It was *P. aeruginosa* elastase which caused degradation of collagen directly and also stimulated keratocyte-mediated collagen degradation. Comparatively, culture supernatant of elastase-deficient *P. aeruginosa* mutants did not affect collagen degradation in either the absence or presence of keratocytes. Additionally, *P. aeruginosa* elastase caused activation of the inactive precursors of keratocyte MMPs-1, -2, -3 and -9 (Nagano *et al*, 2001). MMPs, along with the serine, cysteine and aspartic proteases are important in normal physiologic and pathologic tissue remodelling processes, including during wound

healing (Dong *et al*, 2000) and yet augmented expression of these proteases within the wound environment has implications in delayed wound healing. In an early study, Twining and colleagues (1993) used an *in vitro* cornea culture model to assess the effect of *P. aeruginosa* conditioned media, elastase, alkaline protease and exotoxin A on corneal proteins and proteinases. They found that *P. aeruginosa* elastase activated host MMP-2 and MMP-9. At a higher concentration, however, the elastase degraded these host proteinases. Dong *et al* (2000) also reported on the upregulation of MMP-9 alongside alkaline protease in mouse corneas infected with *P. aeruginosa,* with this upregulation corresponding to the inflammatory response on a temporal basis. The induction of membrane-type MMPs (MT-MMPs) in mouse corneas infected with *P.aeruginsoa* (characterised by inflammation) during wound development at the mRNA and protein levels has also been demonstrated by Dong and colleagues (2000). Importantly, the MT-MMPs also play a role in the activation of latent MMPs and so the induction of MT-MMPs in the mouse cornea as a result of *P. aeruginosa* infection may also have implications in the damage of tissue caused by other activated proteases (Dong *et al*, 2000). In a later study, Dong and colleagues (2001) further reported on the correlation between over-expression of MT-MMPs in *P. aeruginosa* infected mouse corneas and the inflammatory response.

Interestingly, an early study demonstrated that the use of protease inhibitors may aid healing in *P. aeruginosa* infected wounds by inhibiting the proteolytic (but not elastolytic) activities of *P. aeruginosa* alkaline protease and elastase in burned skin (Holder, 1983). Similarly, the survival of mice with burns infected with protease-producing *P. aeruginosa* was enhanced via the administration of antiprotease serum, whereas this addition had no effect on burns infected with protease-deficient strains of *P. aeruginosa* (Pavlovskis & Wretlind, 1979). Conversely, antielastase immunoglobulin G (AE-IgG) showed no significant protective role against *P. aeruginosa* infection in a murine burn wound sepsis model (Cryz, Furer & Germainier, 1983).

MMPs -2, -3, -9 and -13 were chosen as MMPs of interest in this study given their importance in a range of processes involved in wound healing. The gelatinases MMPs -2 and -9 are particularly important in the remodelling and re-epithelialisation of wounds (Rayment, Upton and Shooter, 2008; Rowe & Weiss, 2008) and have consistently been shown to be upregulated in chronic wound fluids (Trengove *et al*, 1999; Yager *et al*, 1996; Rayment, Upton & Shooter, 2008; Rowe & Weiss, 2008). MMP-2 and MMP-9 have been shown to correspond with an increase in the degradation of epidermal growth factor in wound fluid samples (Trengove *et al*, 1999). An increase in MMP-9 in chronic wound fluid has also been linked with poor ulcer healing (Rayment, Upton & Shooter, 2008). MMP-3 is also induced by multiple cell types in the skin during normal and pathological tissue remodelling in the dermis and epidermis (McCrawley *et al*, 2008) and it has been shown to regulate the function of biologically active molecules (Vu & Werb, 2000). MMP-3 has also been shown to be important during wound contraction (Bullard *et al*, 1999; Gill and Parks, 2008) and MMPs -3 and -13 are involved in the remodelling of collagen (Parks *et al*, 2004). Epithelial and stromal cells in wounded tissue express multiple MMPs, including MMPs-2, -9 and -13 (Gill & Parks, 2008). Furthermore, the MMP/TIMP ratio is an important factor in wound healing, with a general decrease in the ratio being associated with healing (Ladwig *et al*, 2002).

Given the current evidence relating to the effects of bacterial proteases on host cell viability and MMP expression together with the identified gaps in the literature pertaining to the effects of bacterial proteases in cutaneous equine wounds, three main objectives were identified. The first aim was to assess the effects of *P. aeruginosa* protease-rich conditioned media from 3 isolates shown to be positive for the LasB gene in addition to purified *P. aeruginosa* elastase (dose-response) on the viability and growth of equine dermal fibroblasts. Secondly, the influence of *P. aeruginosa* elastase on wound closure *in vitro* using a scratch wound model was investigated. Finally, the effect of conditioned media and *P. aeruginosa* elastase on the levels of MMP-2, -3, -9, -13 and TIMP -1 and -3 mRNA expressed by equine dermal fibroblasts was explored.

4.3 Methods

4.3.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated.

4.3.2 Ethical Approval

Approval for this study was granted by the Ethics Committee, University of Liverpool and informed consent was obtained from the owners of the animals participating in this study.

4.3.3 Identification of *P. aeruginosa* Isolates

Standard microbiological culture techniques were used to confirm the identities of 2 previously collected equine trauma wound isolates (as described in Manuscript 2). Pure bacterial colonies were identified using a Gram stain and isolates confirmed as Gram negative rods were further characterised using the oxidase test (using oxidase detection strips from Oxoid Limited, Hampshire, UK) and API 20 E® identification strips, which utilise biochemical reactions with computer software-interpretation (Biomérieux UK Limited, Hampshire, UK). Confirmed *P. aeruginosa* isolates were stored on beads and frozen at -80°C until required for further investigation.

A *P. aeruginosa* reference strain (non-wound isolate) was used as a control; American Type Culture Collection (ATCC) 27853 (isolated from blood culture) (ATCC, Manassas, USA). This strain was chosen as it produces all of the major virulence factors of this organism, including elastase and alkaline protease (O'Callaghan *et al*, 1996).

Polymerase chain reaction (PCR) analysis of the *P. aeruginosa* elastase gene was performed for each isolate; the chronic wound isolates and the ATCC reference strain were positive for the elastase gene (as reported in Manuscript 2).

4.3.4 Preparation of *P. aeruginosa* conditioned media

Sterile Mueller Hinton broth (MHB) (LabM, Bury, UK) was used in 75ml aliquots for all cultures inoculated with 500µl of stationary phase cultures corresponding to each individual *P. aeruginosa* isolate, as described above. Cultures were incubated with shaking at 150rpm in a shaking incubator set at 37°C for 24 hours in 75cm^3 culture flasks. Culture samples were harvested and centrifuged at 12000rpm for 10 minutes to pellet the cells. Conditioned culture supernatant was filtered through 0.45µm sterile filters to remove cell debris. A bicinchoninic acid (BCA) protein assay (Pierce, Thermo Scientific, MA, USA) was used to standardise the amount of protein present in each sample of conditioned media; three total protein concentrations were used in the cell culture experiments to determine the dose response of the fibroblasts to the conditioned media in terms of cell viability and MMP/TIMP expression following treatment (50µl, 100µl and 300µl). The total protein concentrations used in the study were chosen on the basis of previous optimisation experimentation.

4.3.5 Purification of *P. aeruginosa* Elastase using DEAE-Sepharose Ion Exchange Chromatography

Supernatant from an overnight culture of *P. aeruginosa* (in MHB) was recovered by centrifugation at 15,000g for approximately 10 minutes. Supernatant was then filtered through 0.45µm filters. Supernatant was then concentrated using centrifugal ultrafiltration in Amicon filter devices (30kd cut-off) (Merck Millipore, Watford). The concentrates were then washed twice with 20mM sodium phosphate buffer, pH8.0, in Amicon filter devices (30kd cut-off).

A diethylaminoethanol (DEAE)-sepharose column was set up with a 20ml syringe and glass wool and was mounted on a stand out of direct sunlight and draughts (to help prevent temperature fluctuations). A 5ml aliquot of DEAE solution (pre-mixed with a starting buffer of 20mM sodium phosphate, pH8.0 as per the manufacturer's instructions) was poured into the column and allowed to flow gently down the side

of the column, avoiding bubble formation. Once the gel had set, the column was washed successively with a total of 350ml of 0.1M NaOH buffer and then a total of 350ml of 1M NaCl buffer and the flow-through discarded accordingly. The column was then equilibrated with 20mM sodium phosphate buffer, pH8.0. To check that the pH of the buffer going in to the column was the same as the pH of the buffer coming out of the column, pH indicator paper was utilised. The *P. aeruginosa* supernatant concentrates were passed through the column and eluted with equilibration buffer and several fractions collected. The fractions were then tested for the presence of elastase using SDS-PAGE. Where necessary, the fractions were concentrated, again using Amicon filter devices (30kd cut-off) and tested again using SDS-PAGE.

4.3.6 BCA Protein Assay

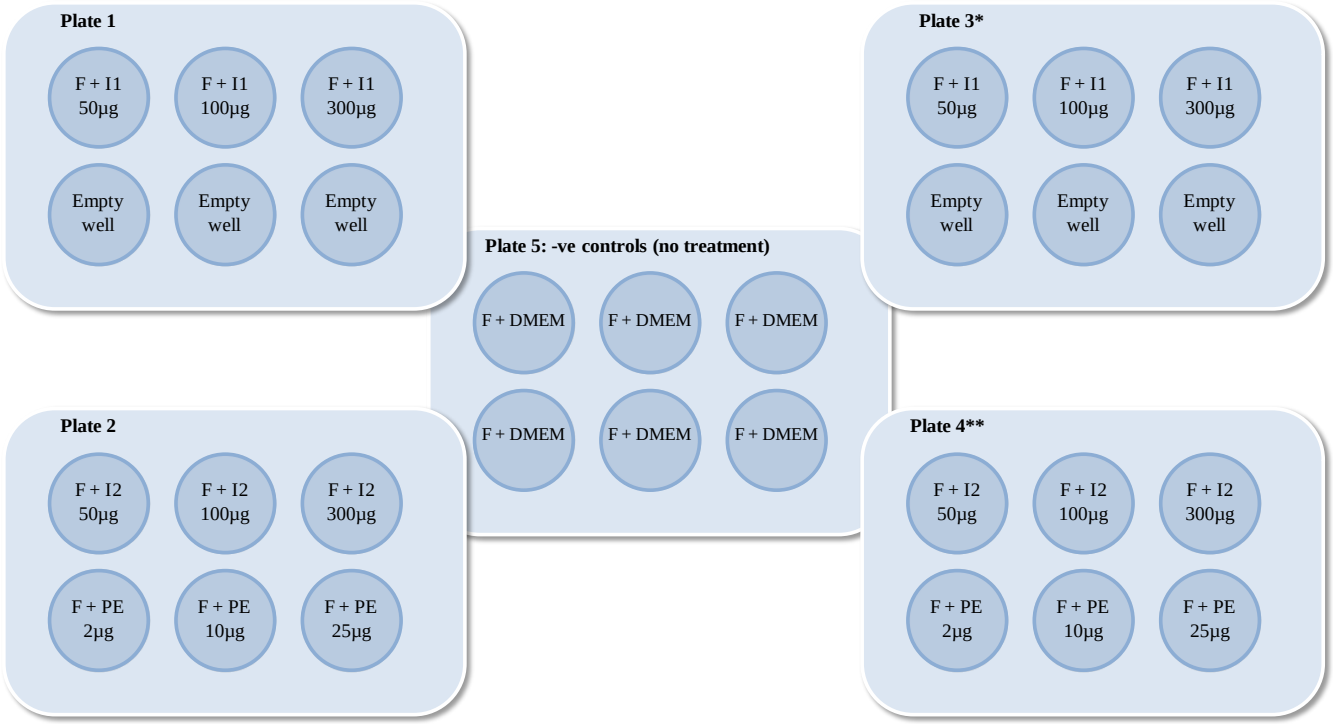
To determine and optimise the concentration of total protein in the eluted *P. aeruginosa* elastase fractions a BCA protein assay kit was utilised (Thermo Scientific, MA, USA). This kit is based on colorimetric detection using a plate reader at approximately 562nm. The concentration of total protein from pooled fractions (previously identified as purified *P. aeruginosa* elastase) was determined using the kit as per the manufacturer's instructions. Albumin standards, as supplied in the BCA protein assay kit, were used to generate a standard curve from which the concentration of total protein in the samples was determined.

4.3.7 Fibroblast Cell Culture for MMP/TIMP mRNA Expression Studies

Equine wound granulation tissue and normal equine skin were used to source fibroblasts for the cell culture experiments (2 donors for granulation tissue and 2 donors for normal skin). Equine dermal tissue was obtained either at post mortem of horses which had been euthanized for medical reasons or from tissue debrided during wound management procedures, with informed consent being given by the owner. All ethical considerations were made. Tissue sections were washed twice in 1x Hank's balanced salt solution (HBSS) (Gibco, Invitrogen, Paisley, UK) and minced into 3-5mm^2 pieces and placed into 25cm^2 culture flasks. Fibroblasts were cultured in DMEM (Gibco, Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum (FCS), 20mM Hepes buffer, 1% penicillin-streptomycin (Invitrogen, Paisley, UK) and 0.5µg/ml amphotericin B (fungizone). The cultures were incubated in 5% CO_2 in air at 37°C. Following a culture period of 5-10 days cells were examined microscopically (Nikon Eclipse, Melville, USA) for outgrowth and confluency. When confluent, cells were passaged in a ratio of 1:4 with 0.05% trypsin/EDTA and cells of passage 3-8 were used experimentally.

4.3.8 Experimental Design and Cell Treatment

Figure 1 illustrates the experimental design. Fibroblasts were seeded at a density of $0.3x10^6$ cells per well of a 6-well plate in a total of 4ml of DMEM (DMEM supplemented as described above) per well. Cells were incubated in 5% CO_2 in air at 37°C for 2 days or until cells were almost confluent. The media in each well was changed to serum-free DMEM and plates were incubated for a further 24 hours. Following incubation the media was removed and cells were washed with Hank's buffered salt solution (HBSS). Treatments were added to the corresponding wells as indicated in figure1 and plates were incubated as described above for 24 hours. Images of the cells in each cell culture plate well were taken microscopically prior to and following treatment using a Nikon Eclipse microscope (Melville, USA). Following the 24 hour incubation period of the cells with the relevant treatments, the conditioned media was removed and stored at -80°C (for gelatin zymography analysis). The cell layer in each well was washed twice with HBSS. To each well of the plate 1ml Tri reagent was added and using a disposable pipette tip the cell layer was removed from the bottom of each well (separate pipette tips were used for each well). Cells were stored in 1.5ml Eppendorf centrifuge tubes at -80°C until further investigation.



Key
F + I1: Fibroblasts + ATCC *P. aeruginosa* conditioned media
F + I2: Fibroblasts + chronic wound *P. aeruginosa* (high biofilm forming potential [BFP]) conditioned media
F + PE: Fibroblasts + *P. aeruginosa* elastase
-ve controls: Fibroblasts + DMEM, no treatment
*Plate 3 is a repeat of plate 1
**Plate 4 is a repeat of plate 2

Figure 1: Experimental design and cell treatment. Fibroblasts were cultured in 6-well culture plates and treatments applied to the cells for 24 hours. Conditioned media from cultures of an ATCC *P. aeruginosa* type strain (I1) and a chronic wound *P. aeruginosa* isolate (high biofilm forming potential [BFP]) (I2) were used to treat cells; 3 concentrations of total protein were used (50, 100 and 300µg) [equating to 12.5µg/ml, 25µg/ml and 75µg/ml]. Purified *P. aeruginosa* elastase was also used to treat cells (2, 10 and 25µg) [equating to 0.5µg/ml, 2.5µg/ml and 6.25µg/ml]. The concentrations of elastase used in the study were chosen on the basis of previous optimisation experimentation and studies by Twining *et al* (1993) and Miyajima *et al* (2001). Duplicate wells were used for each treatment type. A separate plate was set up to include negative controls (cells only, no treatments). A total of 5 culture plates were set up for each cell type; fibroblasts from normal equine skin from 2 different donors were used in addition to fibroblasts from equine granulation tissue from wounds of 2 different donors. The cells from each well following treatment were used for QRT-PCR analysis. For each cell type, an additional 5 culture plates were prepared in the same way and were used for crystal violet assays for cell viability following treatment.

4.3.9 Crystal Violet Assay for Cell Viability

The crystal violet assay is a colorimetric method of determining cell viability, proliferation or cytotoxicity caused by the application of a test treatment (Vega-Avila & Pugsley, 2011). The assay is used to obtain quantitative information about the relative cell density of monolayer cultures since the dye stains DNA; the amount of dye taken up by the monolayer and thus the colour intensity are proportional to cell number (Vega-Avila & Pugsley, 2011). This method was used to determine the effect of increased concentrations of *P. aeruginosa* elastase (2µg, 10µg and 25µg) and varying concentrations of total protein (50µg, 100µg and 300µg) from *P. aeruginosa* conditioned media from 2 different *P. aeruginosa* isolates on the viability of equine normal skin (NF) fibroblasts and equine wound granulation tissue (GT) fibroblasts following a 24 hour treatment period. Two biological replicates were used for normal equine skin fibroblasts (NF) and two biological replicates were used for equine wound granulation tissue fibroblasts (GT). For each cell type and treatment, 2 technical replicates were used.

A solution of 0.4% crystal violet was prepared in methanol; this was stirred using a magnetic flea until all of the crystal violet powder had dissolved. Following cell culture and the treatment period, the culture medium was carefully removed, without disturbing the cell layer on the bottom of the plate wells. Each of the plate wells were then gently washed with 2ml of warmed 1x HBSS. The crystal violet solution (500µl per well) was added to the plate wells and incubated for 10 minutes at room temperature. Following incubation, the plates were washed carefully in tap water by immersion in to a large, clean beaker changing the water between washes. The plates were allowed to drain upside down on paper towel and left to dry. Once the plates were dry, 1% SDS was added to each well (1ml per well) to solubilise the crystal violet stain and plates were agitated on a plate shaker for approximately 5 minutes until the colour was uniform in each well and no areas of dense colour on the sides/bottom of the wells were visible. From each of the plate wells, a 200µl aliquot of the solubilised crystal violet stain was added to a 96-well plate in triplicate. The absorbance of the resulting solution in each well was measured in a plate reader at 570nm.

4.3.10 qRT-PCR

RNA Extraction from Cells

To each of the cell samples (previously stored in Tri reagent) 200µl of chloroform was added and vortexed. Samples were left at room temperature for 10 minutes. Samples were then centrifuged at 4°C for 15 minutes at 13,000rpm. Following centrifugation, the clear supernatant was transferred into clean 1.5ml Eppendorf centrifuge tubes, ensuring that the DNA layer was not disturbed and the remaining solution was discarded as appropriate. Equal volumes of 70% ethanol (made up with molecular grade ethanol and DEPC water) were added to each sample and samples were mixed by pipetting. A Qiagen RNeasy mini kit (Qiagen, Manchester, UK) was used to continue the RNA extraction process according to the manufacturer's instructions. A DNase treatment stage was included in the process. This process generated 30-50µl RNA per sample.

cDNA Synthesis- Reverse Transcription

To 16µl of each of the RNA samples 1µl of random primers (Promega, WI, USA) were added and spun down for 5-10 seconds. Samples were placed into a PCR machine (2720 Thermal Cycler, Applied Biosystems, Life Technologies, CA, USA) and run for 5 minutes on a 70°C manual programme. Samples were then placed on ice. Master mix was prepared with dNTPs (Promega, WI, USA), 5x buffer (Promega, WI, USA) and reverse transcriptase (Promega, WI, USA) as per the manufacturer's instructions. The RNA samples (8.5µl of each sample) were added to individual Eppendorf tubes with 4µl mastermix. Samples were run in the PCR machine as per the reverse transcription protocol; 1 hour at 37°C, 10 minutes at 95°C and samples were then held at 4°C. DEPC water was added to the cDNA samples to dilute each sample 1:3.

Real-Time PCR

To each well of the qRT-PCR plate 17µl of master mix (made up with 5µl DEPC water, 2µl (3µM in DEPC water) forward and reverse primer mix and 10µl SYBR green (Thermo Scientific, MA, USA)) was added. To each well 3µl of the corresponding cDNA sample was added to create a total of 20µl per well of the 96-well plate. The plate was covered with a clear film and centrifuged for approximately 15 seconds. The plate was run in a QRT-PCR machine (7300 Real Time PCR System, Applied Biosystems, Life Technologies, CA, USA) using a relative quantification programme using 7300 system software. Samples were assayed for MMP-2, MMP-9, MMP-3, MMP-13, TIMP-1 and TIMP-3 mRNA expression (GapDH was used as the house-keeping gene). For analysis, the comparative CT method was used which compares the Ct value of one target gene to an internal control/reference gene (in this case GapDH) using the formula 2^^CT. Primer sequences are listed in Appendix 1.

4.3.11 Immunohistochemistry

Equine wound tissue samples were retrieved during standard debridement procedures. Tissue sections were collected into paraformaldehyde for immunohistochemistry staining using standard procedures and were processed by the University of Liverpool pathology department for staining; tissue sections were paraffin embedded and stained for MMP-2, MMP-9 and MMP-13 (method described in Appendix 2). Tissue sections were also stained with Gram stain for the identification of bacteria within the tissue, and Haematoxylin and Eosin (H&E).

4.3.12 Scratch Wound Assay

The most common *in vitro* model used to study cell migration and wound closure is the scratch wound method, given its simplicity, ease of use and cost efficiency (Liang, Park & Guan, 2007). Following the creation of a wound along the confluent monolayer, cells at the wound edge polarise and migrate into the wound area (Cory, 2011). Images can be taken of the wounded area before and after treatment and the change in surface area of the wounded site can be calculated (see figure 2a).

Granulation tissue fibroblasts from equine wounds (n=2) and normal equine skin fibroblasts (n=2) were sourced and put into primary culture as described previously. Fibroblasts were seeded at a density of $1x10^5$ cells per well in 12-well plates in a total of 1ml of DMEM (DMEM supplemented as described above) per well. Cells were incubated in 5% CO_2 in air at 37°C for 2 days or until cells were almost confluent. Cells were then serum-starved for 24 hours. Following incubation the media was removed and cells were washed with HBSS. Wounds were created manually using a 200µl sterile filter pipette tip. The pipette tip was scratched down the centre of the well and dislodged cells were removed via washing with HBSS. Wound size was found to be generally consistent, with a width of approximately 1mm. In order to inhibit fibroblast proliferation so that wound closure could be measured on the basis of fibroblast migration, fresh DMEM was supplemented with 2mM hydroxyurea. Treatments were added to the corresponding wells (see figure 2b) and plates were incubated as described above for a total of 72 hours. To ensure consistency of imaging, black markers were drawn in the centre of each well, indicating the section of the scratch wound to be imaged at each time point. Images of the scratch wounds in each cell culture plate well were taken microscopically prior to and following treatment at each time point (0, 36, 60 and 72 hours) using a Nikon Eclipse microscope (Melville, USA). The total wound surface area was measured using GNU Image Manipulation Program (GIMP), Version 2.8 and percentage wound closure over time was calculated in comparison to the surface area of the wound at time point zero.

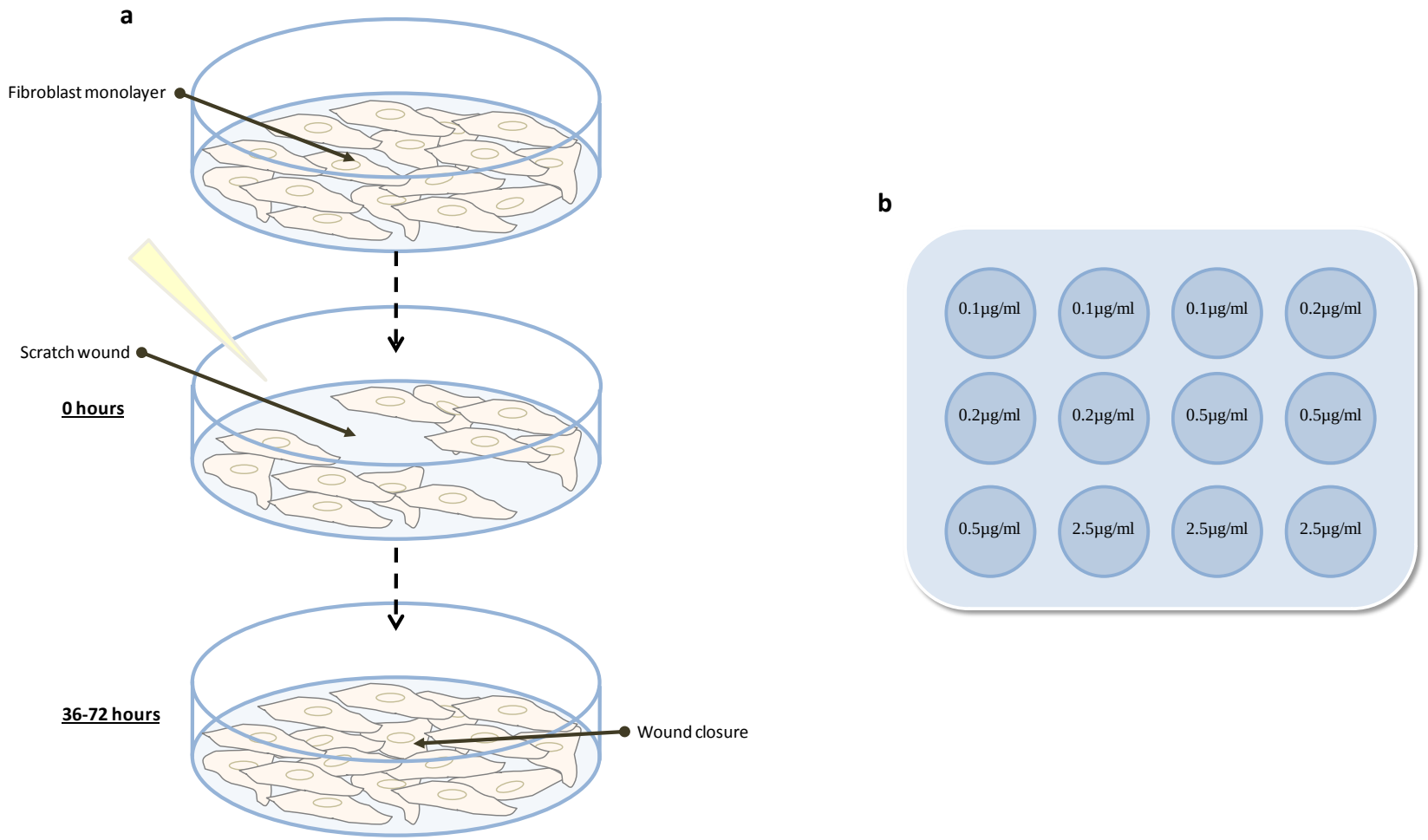


Figure 2: (a) Scratch wound assay method. A sterile filter pipette tip was used to create a scratch wound in each well of the cell culture plate. Dislodged cells were then rinsed away with HBSS and varying concentrations of *P. aeruginosa* elastase were added to the corresponding wells; (b) Fibroblasts were cultured in 12-well culture plates and treatments applied to the cells for a total of 72 hours following the creation of a scratch wound in each well. Triplicate wells were used for each treatment dose (0.1µg/ml, 0.2µg/ml, 0.5µg/ml and 2.5µg/ml). A separate plate was set up for each cell type (i.e. 2 normal fibroblast (NF) and 2 granulation tissue (GT) fibroblast cultures). Images of the wounded area were taken immediately after wounding (0 hours) and at 36, 60 and 72 hours post wounding for each well. Separate culture plates were prepared for controls (no treatment). Some wells in the control plates were used for crystal violet staining to monitor cell proliferation (the hydroxyl urea treatment of cells should have prevented proliferation).

4.4 Results

4.4.1 Crystal Violet Assay for Cell Viability

Results suggest that variations could exist between different *P. aeruginosa* isolates in terms of the effect of conditioned media on cell viability, particularly at higher concentrations of total protein (see tables 1 and 2, figures 3-6); it was noted that in general the conditioned media obtained from the ATCC *P. aeruginosa* type strain (I1) inflicted little effect on cellular viability to both the NF and GT cells as compared to the untreated control cells. Comparatively, the conditioned media from the chronic equine wound *P. aeruginosa* isolate (I2) caused a reduction in cellular viability of both cell types, which was dependent on concentration of total protein; as the concentration of total protein increased from 50µg to 300µg cell viability was reduced in a dose-dependent manner (see Figures 3-10). There was a statistically significant difference between I1 and I2 at concentrations 100µg ($F(1, 23) = 7.77$, $P = 0.050$) and 300µg ($F(1, 23) = 13.46$, $P = 0.001$) in terms of the mean NF fibroblast viability, with the I2 conditioned media resulting in a significantly reduced cell viability (see table 2).

P. aeruginosa elastase also had an effect on cell viability of both NF and GT cells (see Figures 3-10); whilst this effect was not as prominent as the effect observed on cell viability of those cells treated with total protein from *P. aeruginosa* conditioned media, as the concentration of *P. aeruginosa* elastase increased from 2µg to 10µg, cell viability decreased. However, this was not statistically significant (see table 3). Yet, there was a significant difference between GT1 cells treated with 2µg *P. aeruginosa* elastase and GT1 cells treated with 25µg *P. aeruginosa* elastase, with cell viability decreasing in a dose-dependent manner, $F(2, 3) = 16.81$, $P = 0.023$ (see table 3, figure 5). Figures 7 and 8 show interval plots for the percentage cell viability for both NF and GT cells following treatment with either *P. aeruginosa* conditioned media or *P. aeruginosa* elastase following the 24 hour treatment period as compared to untreated control cells.

Table 1: Statistical analysis of the effect of *P. aeruginosa* conditioned media on normal equine skin fibroblasts (NF) and equine granulation tissue fibroblasts (GT); analyses were conducted to determine whether differences exist between the 2 treatment types (conditioned media from 2 different *P. aeruginosa* isolates) in terms of the effect on cell viability at each concentration of total protein.

Cell Type	Concentration of Treatment	Statistical Analysis	
		One-Way ANOVA	Kruskal-Wallis
NF1	50µg	F(1, 11) = 0.03, P = 0.861	P = 0.873
NF1	100µg	F(1, 11) = 9.86, P = 0.011*	P = 0.010*
NF1	300µg	F(1, 11) = 7.16, P = 0.023*	P = 0.025*
NF2	50µg	F(1, 11) = 5.19, P = 0.046*	P = 0.150
NF2	100µg	F(1, 11) = 2.81, P = 0.124	P = 1.000
NF2	300µg	F(1, 11) = 8.06, P = 0.018*	P = 0.055
GT1	50µg	F(1, 11) = 15.89, P = 0.003*	
GT1	100µg	F(1, 11) = 2.39, P = 0.153	
GT1	300µg	F(1, 11) = 0.63, P = 0.446	P = 0.200
GT2	50µg	F(1, 11) = 2.55, P = 0.141	P = 0.423
GT2	100µg	F(1, 11) = 9.46, P = 0.012*	
GT2	300µg	F(1, 11) = 0.01, P = 0.914	P = 0.337

** Significant at the 0.05 level*

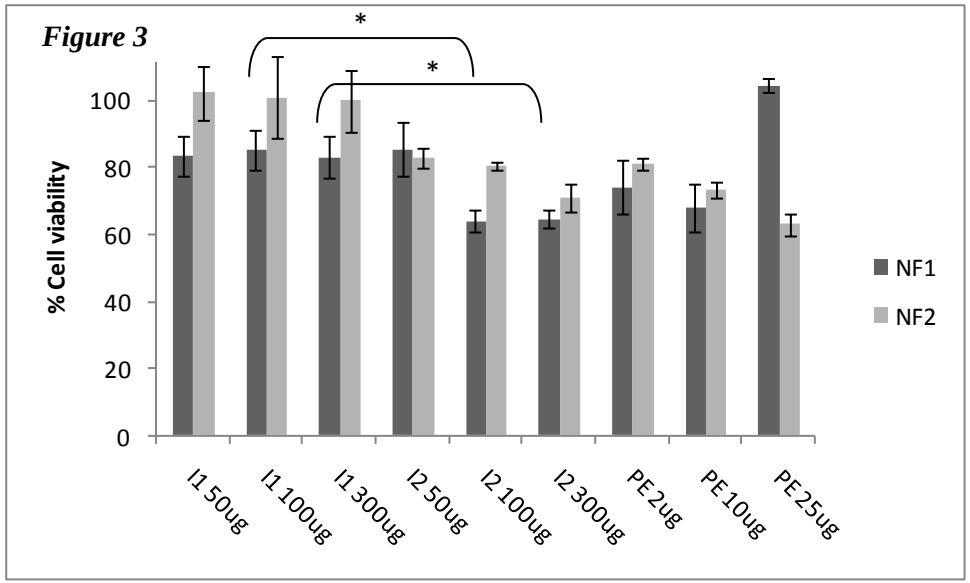
Table 2: As table 1; results for both normal skin fibroblast (NF) cell donors and both granulation tissue (GT) cell donors were averaged prior to statistical analysis.

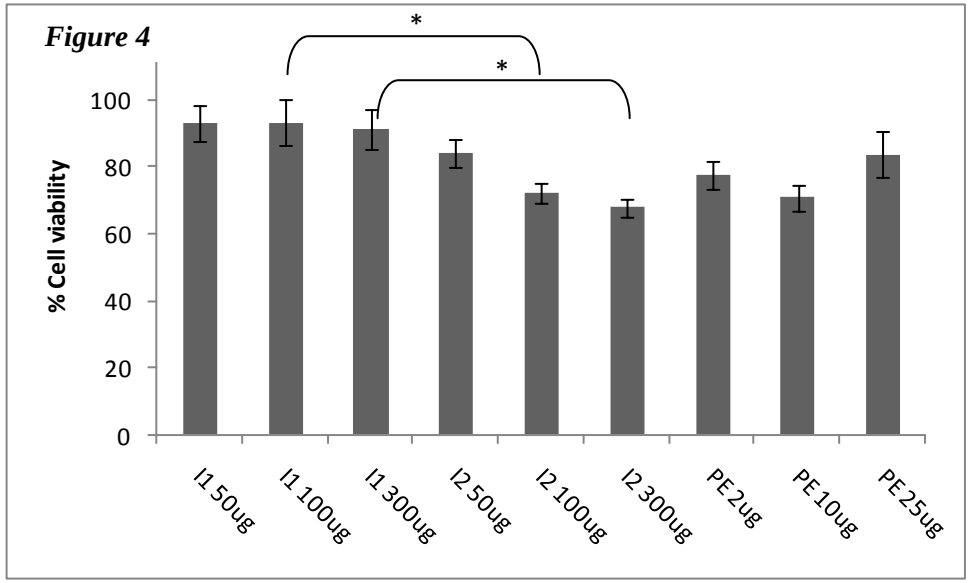
Cell Type	Concentration of Treatment	Statistical Analysis	
		One-Way ANOVA	Kruskal-Wallis
NF	50µg	F(1, 23) = 1.64, P = 0.213	
NF	100µg	F(1, 23) = 7.77, P = 0.011*	P = 0.050*
NF	300µg	F(1, 23) = 13.46, P = 0.001*	P = 0.003*
GT	50µg	F(1, 23) = 0.00, P = 0.999	P = 0.453
GT	100µg	F(1, 23) = 1.47, P = 0.239	P = 0.204
GT	300µg	F(1, 23) = 0.04, P = 0.837	P = 0.184

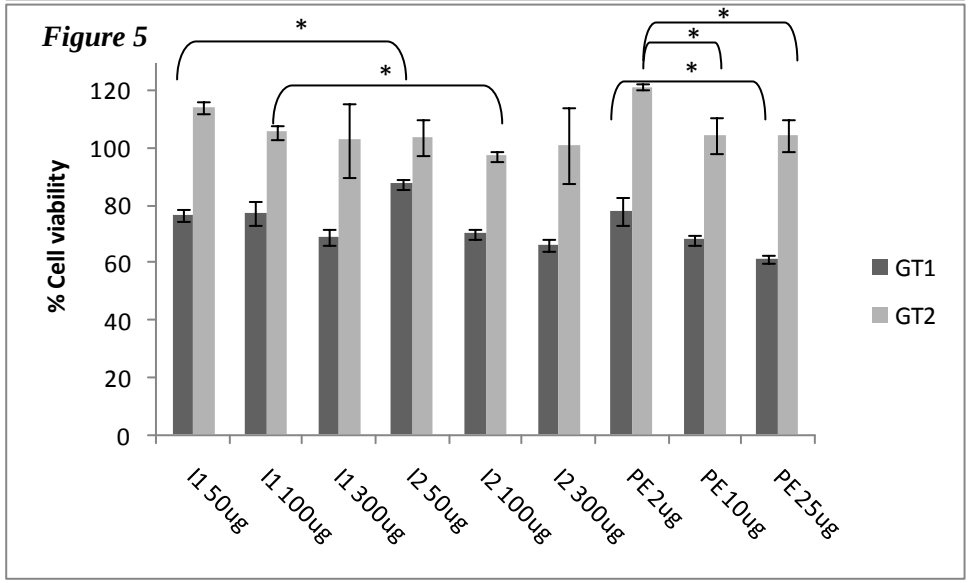
Table 3: Statistical analysis of the effect of *P. aeruginosa* elastase on equine normal skin fibroblasts (NF) and granulation tissue fibroblasts (GT); analyses were conducted to determine whether differences exist between the 3 different concentrations of *P. aeruginosa* elastase (2µg, 10µg and 25µg) in terms of the effect on cell viability.

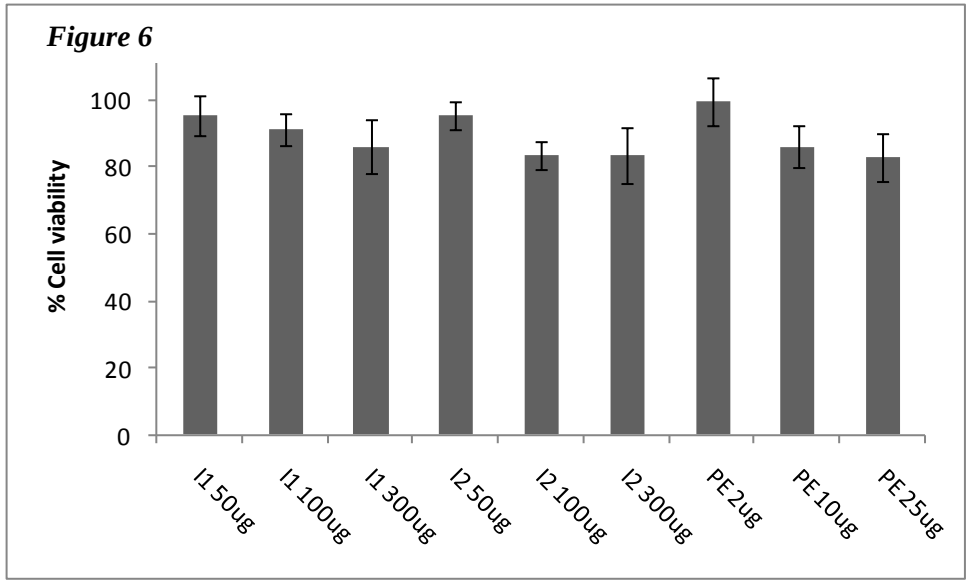
Cell Type	Statistical Analysis		
	One-Way ANOVA	**Post-Hoc Observations (using the Tukey method)**	**Kruskal-Wallis**
NF1	F(2, 3) = 1.86, P = 0.298		P = 0.156
NF2	F(2, 3) = 2.58, P = 0.223		P = 0.276
NF1/NF2 averaged	F(2, 3) = 0.28, P = 0.774		P = 0.565
GT1	F(2, 17) = 7.12, P = 0.007*	There is no statistically significant difference between 2µg and 10µg and there is no statistically significant difference between 10µg and 25µg. There is, however, a statistically significant difference between 2µg and 25µg.	P = 0.009*
GT2	F(2, 17) = 4.02, P = 0.040*	There is a statistically significant difference between 2µg and the other two concentrations of PE	P = 0.011*
GT1/GT2 averaged	F(2, 35) = 1.70, P = 0.199		P = 0.117

**Significant at the 0.05 level*









Figures 3-6: % cell viability of normal skin (NF) fibroblasts and granulation tissue (GT) fibroblasts following 24 hours treatment with varying concentrations of total protein from *P. aeruginosa* conditioned media (2 different isolates) and *P. aeruginosa* elastase (PE). Figure 3: % cell viability of NF1 and NF2 cells following treatment; Figure 4: % cell viability of NF1/NF2 cells (averaged data); Figure 5: % cell viability of GT1 and GT2 cells following treatment; Figure 6: % cell viability of GT1/GT2 cells (averaged data). Data is presented as mean ± SEM. * Significant at the 0.05 level. Statistical significance is detailed in Tables 1-3.

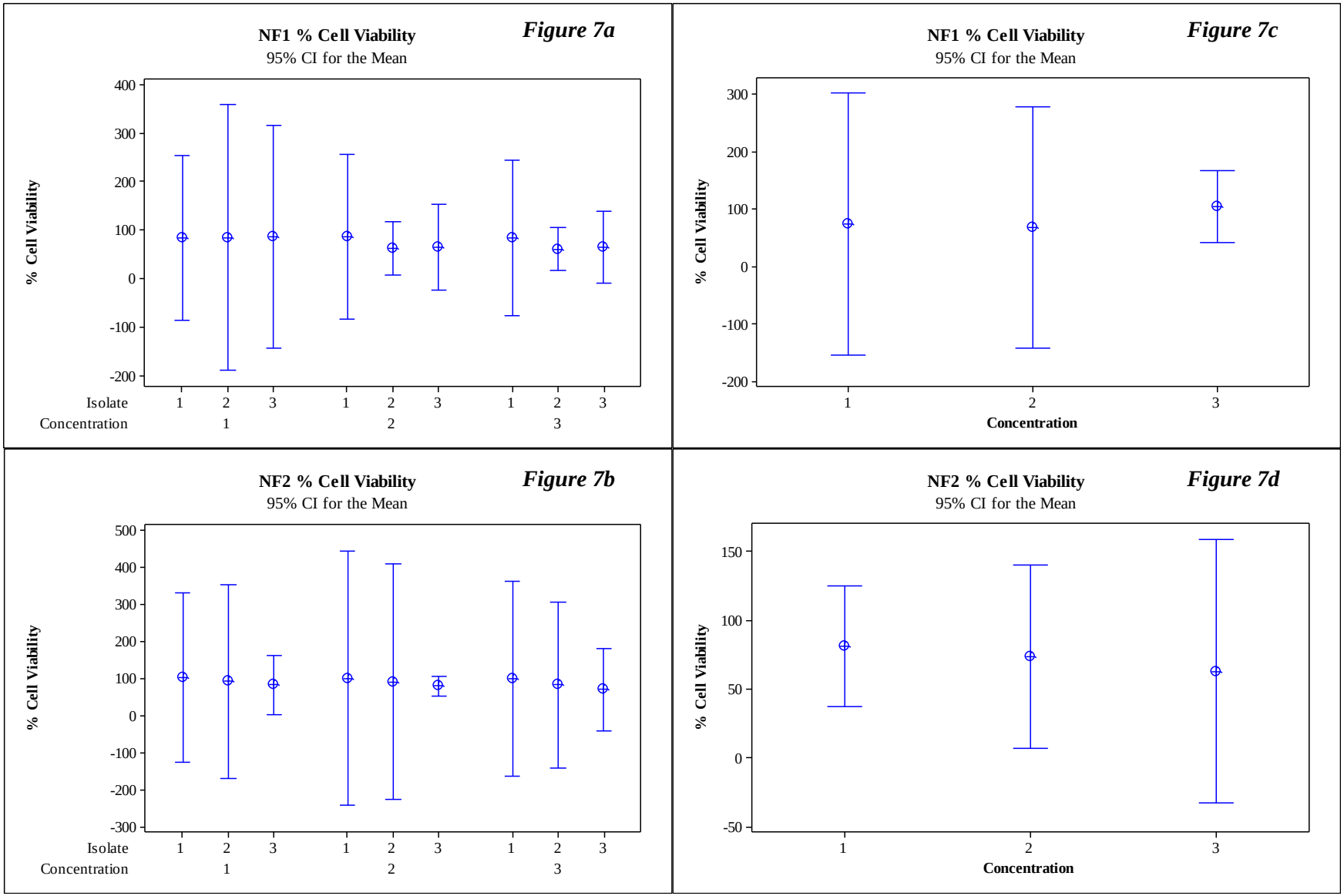


Figure 7: Interval plots for % cell viability of normal skin (NF) fibroblasts following 24 hours treatment with varying concentrations of total protein from *P. aeruginosa* conditioned media (3 different isolates). Figures 7a/7b: % cell viability of NF1 cells (Figure 7a) and NF2 cells (Figure 7b) following treatment with varying concentrations of total protein from *P. aeruginosa* conditioned media (3 different isolates); Concentration 1: 50µg; Concentration 2: 100µg; Concentration 3: 300µg. Figures 7c/7d: % cell viability of NF1 cells (Figure 7c) and NF2 cells (Figure 7d) following treatment with varying concentrations of *P. aeruginosa* elastase; Concentration 1: 2µg; Concentration 2: 10µg; Concentration 3: 25µg.

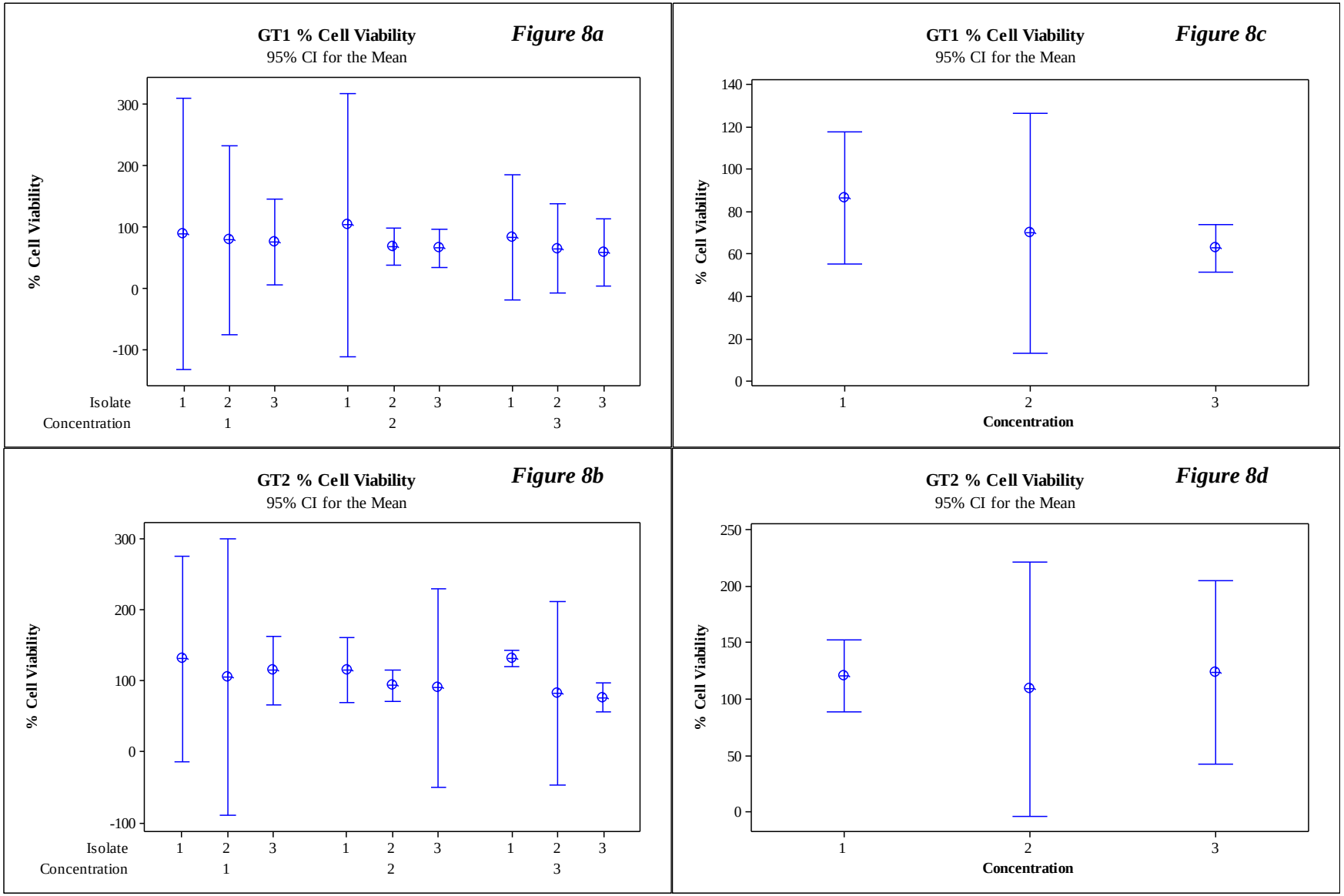


Figure 8: Interval plots for % cell viability of granulation tissue (GT) fibroblasts following 24 hours treatment with varying concentrations of total protein from P. aeruginosa conditioned media (3 different isolates). Figures 7a/7b: % cell viability of GT1 cells (Figure 8a) and GT2 cells (Figure 8b) following treatment with varying concentrations of total protein from P. aeruginosa conditioned media (3 different isolates); Concentration 1: 50µg; Concentration 2: 100µg; Concentration 3: 300µg. Figures 8c/8d: % cell viability of GT1 cells (Figure 8c) and GT2 cells (Figure 8d) following treatment with varying concentrations of *P. aeruginosa* elastase; Concentration 1: 2µg; Concentration 2: 10µg; Concentration 3: 25µg

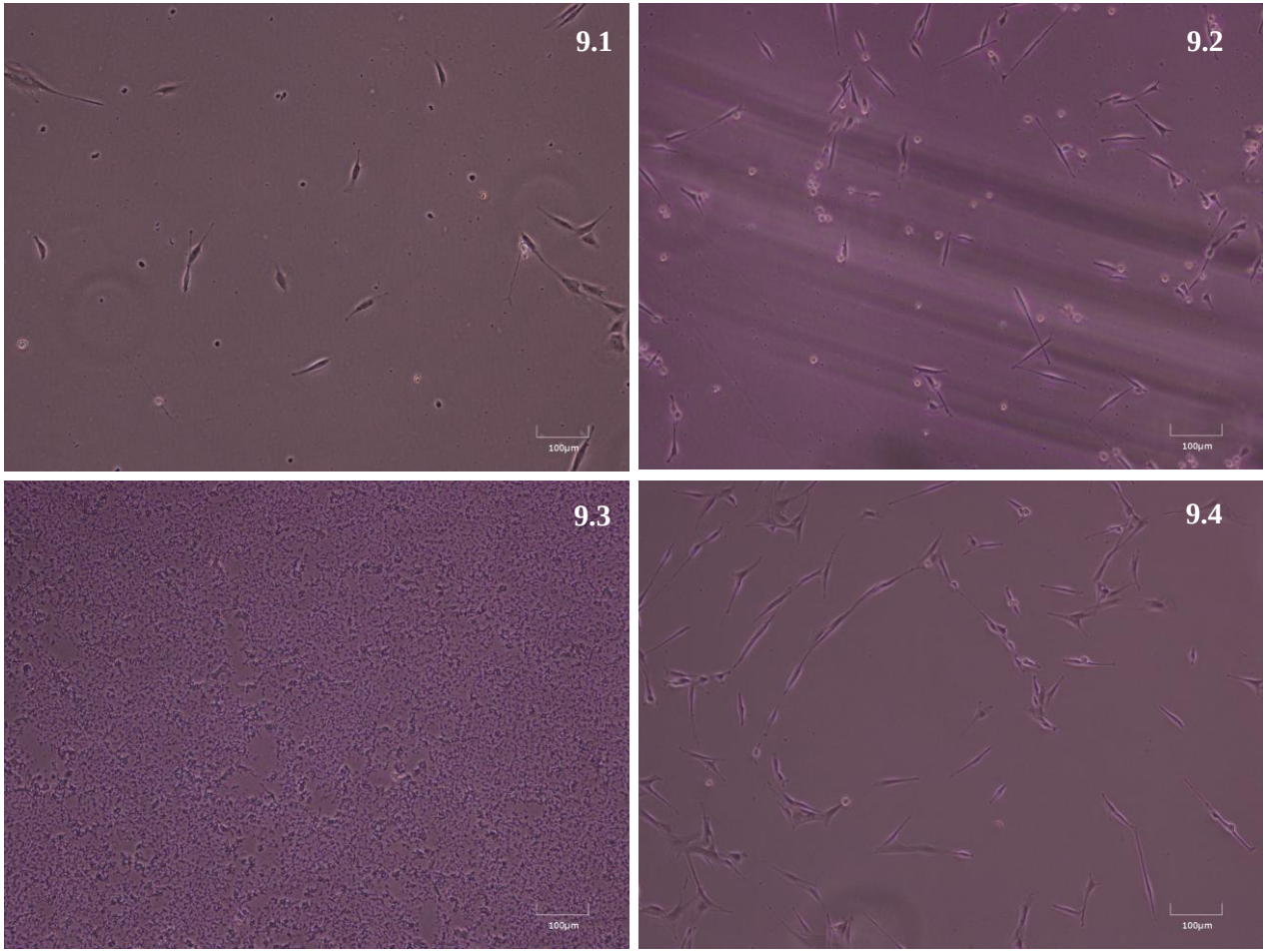


Figure 9: NF cell images following 24 hours of treatment with *P. aeruginosa* conditioned media (300µg) and 25µg *P. aeruginosa* elastase: 8.1: normal skin fibroblast (NF) cells treated for 24 hours with I1 conditioned media; 8.2 NF cells treated for 24 hours with I2 conditioned media; 8.3: NF cells treated for 24 hours with PE; 8.4: Un-treated control NF cells. Scale bars = 100µm

The images taken of NF cells following treatment illustrate variations in cell morphology between treatment types. At a concentration of 300µg, the conditioned media obtained from I1 had little effect on NF cell viability; as compared to the un-treated control NF cells (9.4), those NF cells treated at this concentration (9.1) showed a slight reduction in cell viability; cells appeared slightly less elongated with less contact between cells. Cells were also less densely populated. When NF cells were treated with 300µg I2 conditioned media (9.2) cells appeared much less elongated with little contact between cells, with fewer adherences to the bottom of the culture plate well. Cells were also less densely populated that the control cells. Cells treated with 25µg *P. aeruginosa* elastase (9.3) appeared non-viable; cells were less adherent to the surface of the culture plate well and whilst densely populated they were irregularly shaped, small and much more rounded than the control cells. Cells appeared ruptured, with blebbing of the cell membrane and a ridged appearance.

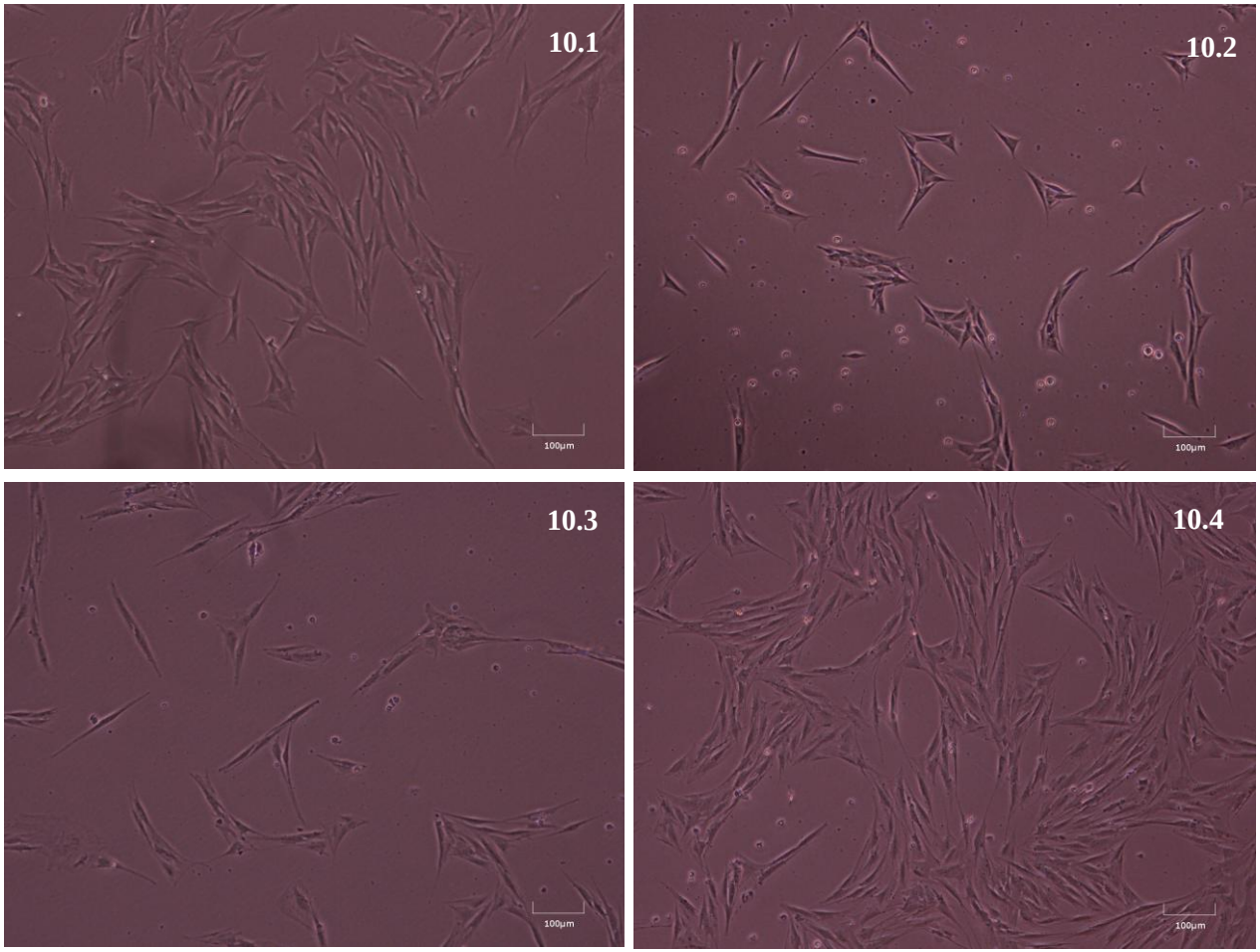


Figure 10: Granulation tissue (GT) cell images following 24 hours of treatment with *P. aeruginosa* conditioned media (300µg) and 25µg *P. aeruginosa* elastase: 10.1: GT cells treated for 24 hours with I1 conditioned media; 10.2 GT cells treated for 24 hours with I2 conditioned media; 10.3: GT cells treated for 24 hours with PE; 10.4: Un-treated control GT cells. Scale bars = 100µm

The images taken of GT cells following treatment illustrate variations in cell morphology between treatment types. As with the NF cells, at a concentration of 300µg, the I1 conditioned media had little effect on GT cell viability; as compared to the un-treated control GT cells (10.4), GT cells treated at this concentration (10.1) showed a slight reduction in cell density but viability did not seem to be affected. Cells demonstrated typical fibroblastic shape. GT cells treated with 300µg I2 conditioned media (10.2) appeared less elongated with little contact between cells and cells were much less densely populated than the control cells, with less contact between cells than un-treated control cells. A high proportion of cells were not adhered to the culture plate well; these cells appeared small and round. Cells treated with 25µg *P. aeruginosa* elastase (10.3) had similar morphology to those cells treated with I1 conditioned media but were less densely populated with little contact between viable cells. Overall, with each treatment, the GT cells did not seem to be affected to the same extent as the corresponding NF cells (see figures 9.1-9.4). For both cells types, the cells treated with I1 conditioned media were only slightly affected in terms of cell density and viability, whereas those cells treated with I2 conditioned media and PE seemed to be less densely populated with altered cell morphology as compared to un-treated cells.

4.4.2 qRT-PCR

Quantitative real time PCR (qRT-PCR) was conducted in this study to ascertain whether the treatment of equine dermal fibroblasts with protease-rich *P. aeruginosa* conditioned media and *P. aeruginosa* elastase at varying concentrations affected the expression of MMPs and their inhibitors, TIMPs *in vitro*. Given the limited number of cells available for experimentation along with some missing data from qRT-PCR results it was not possible to perform robust statistical analysis from the data obtained. However, some observational trends have been observed within the data in terms of MMP and TIMP expression of NF and GT cells following treatment. Figures 11 and 12 show the mRNA expression of each MMP and TIMP analysed for the NF cells. Figures 13 and 14 show the mRNA expression of each MMP and TIMP analysed for the GT cells. The mRNA expression of each MMP and TIMP analysed was up-regulated relative to the housekeeping gene, GapDH, for NF1, NF2, GT1 and GT2, regardless of treatment indicating a general expression of MMPs and TIMPs during fibroblast culture.

qRT-PCR revealed that relative mRNA expression relative to GapDH of un-treated, control NF cells of MMP-2, TIMP-1 and TIMP-3 were relatively high in comparison to the other MMPs measured. MMP-9 mRNA expression was also high but somewhat lower than the aforementioned mRNA levels. In contrast, MMP-13 and MMP-3 mRNA expression was considerably lower. However, whilst these trends were comparable between both NF cell types used, differences exist in terms of the overall levels of mRNA expression between cell types. It is, therefore, worth highlighting that results obtained from such data must be considered in terms of the cell type and variations between cell cultures from the same source/donor. In contrast, relative mRNA expression of un-treated, control GT cells of MMP-2, TIMP-1 and TIMP-3 were not as obviously different from those mRNA levels of other target MMPs; generally, MMP-9 and MMP-13 mRNA expression was lower than most other target MMPs and TIMPS, whilst MMP-3 mRNA expression was relatively high.

With the treatment of *P. aeruginosa* elastase, NF cells responded by a dose-dependent increase in MMP-13 mRNA expression, and a slight increase in MMP-3

mRNA and MMP-9 mRNA expression. TIMP-1 and TIMP-3 mRNA expression along with MMP-2 mRNA expression generally remained high at each concentration of *P. aeruginosa* elastase but did not differ considerably from the un-treated control cells. The relative mRNA expression of MMPs and TIMPs varied considerably between GT cell types. GT1 cells treated with *P. aeruginosa* elastase demonstrated a reduction in TIMP-3 mRNA expression; however TIMP-3 mRNA expression of GT2 cells remained relatively unchanged as compared to the untreated control cells. TIMP-1 and MMP-3 mRNA expression of GT1 cells was increased compared to untreated control cells. For the GT2 cells, MMP-9 and MMP-13 mRNA expression was up-regulated as compared to untreated control cells. MMP-9 mRNA expression was only slightly increased for the GT1 cells at a *P. aeruginosa* elastase concentration of 25µg. For GT cells, there was a general decline in TIMP-3 mRNA expression in most cases as the concentration of *P. aeruginosa* conditioned media increased. This was not observed for the NF cells.

Whilst the current study has identified variations in the effects of *P. aeruginosa* conditioned media and *P. aeruginosa* elastase on MMP and TIMP mRNA expression among different fibroblast cell types, further investigation with a larger number of NF and GT cells from different donors is needed along with more technical repeats in order to generate definitive conclusions as to which MMPs and TIMPs are expressed to the greatest extent when cells are treated with protease-rich *P. aeruginosa* conditioned media and *P. aeruginosa* elastase.

Figure 11

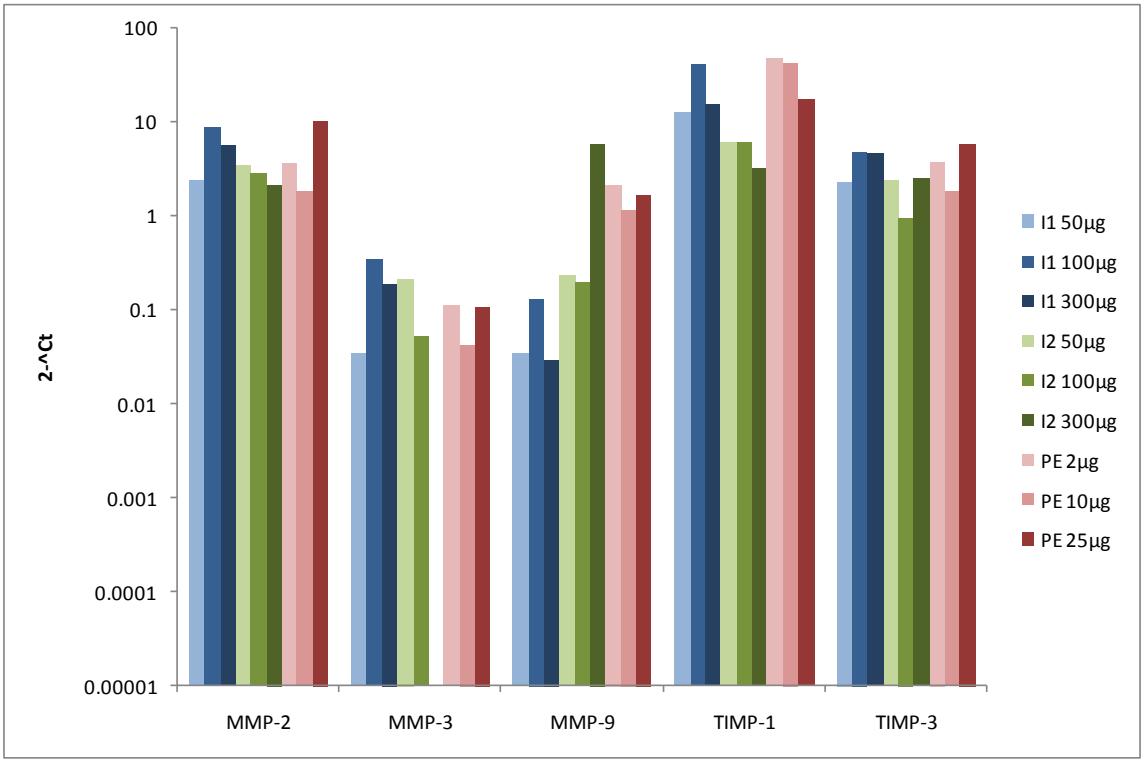
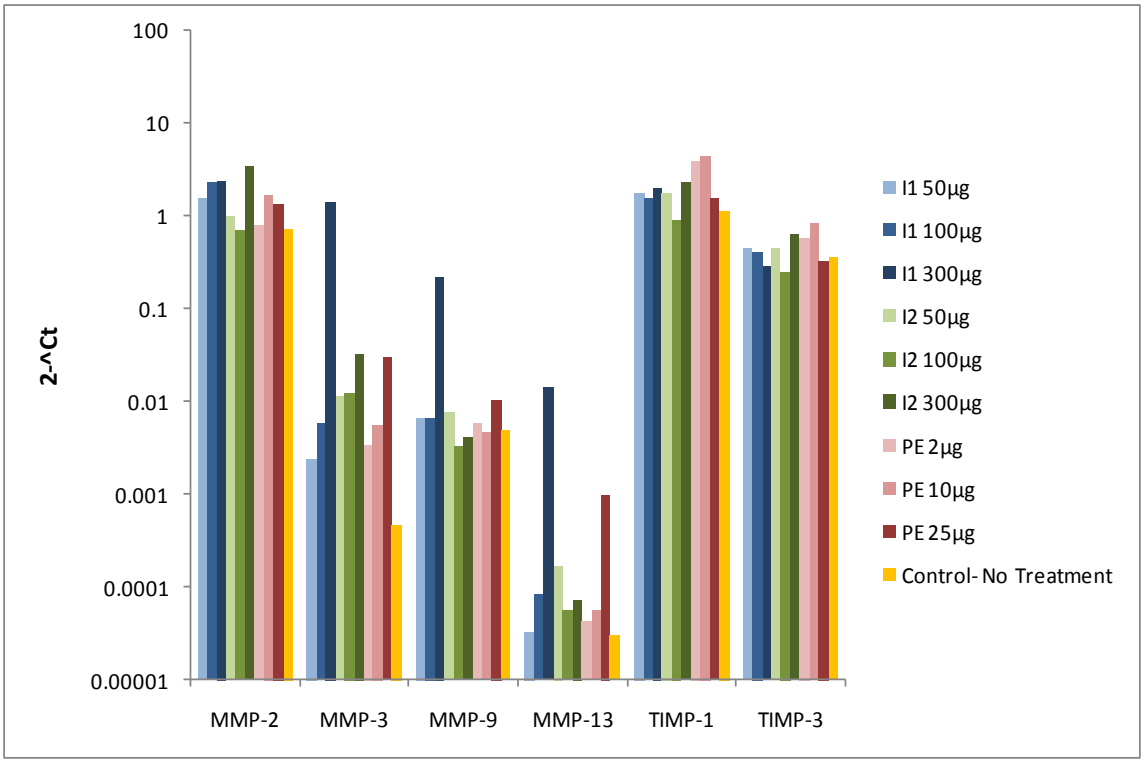


Figure 12



Figures 11-12: QRT-PCR results for MMP/TIMP mRNA expression of normal fibroblasts following treatment with increasing concentration of total protein of *P. aeruginosa* conditioned media or increasing concentrations of purified *P. aeruginosa* elastase (PE). Figure 11: Normal skin fibroblast (NF1) fibroblasts (for NF1 fibroblasts no data available for MMP-13 mRNA expression or for control [no treatment] cells); Figure 12:NF2 fibroblasts.

Figure 13

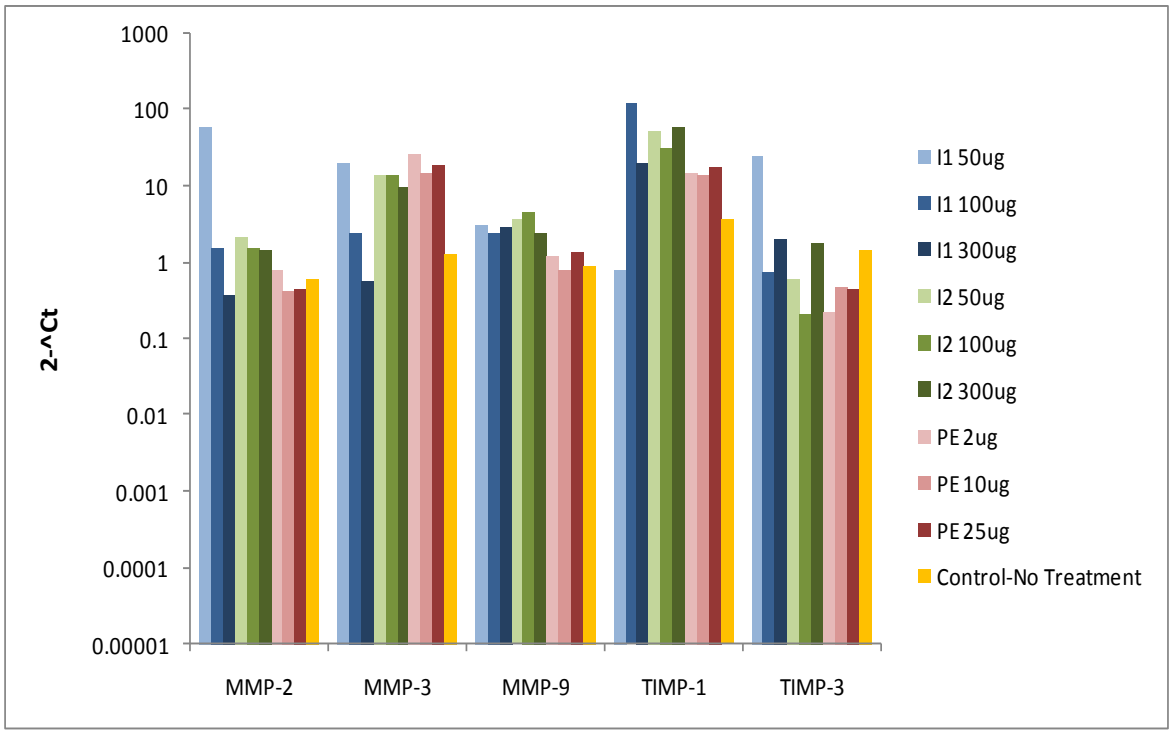
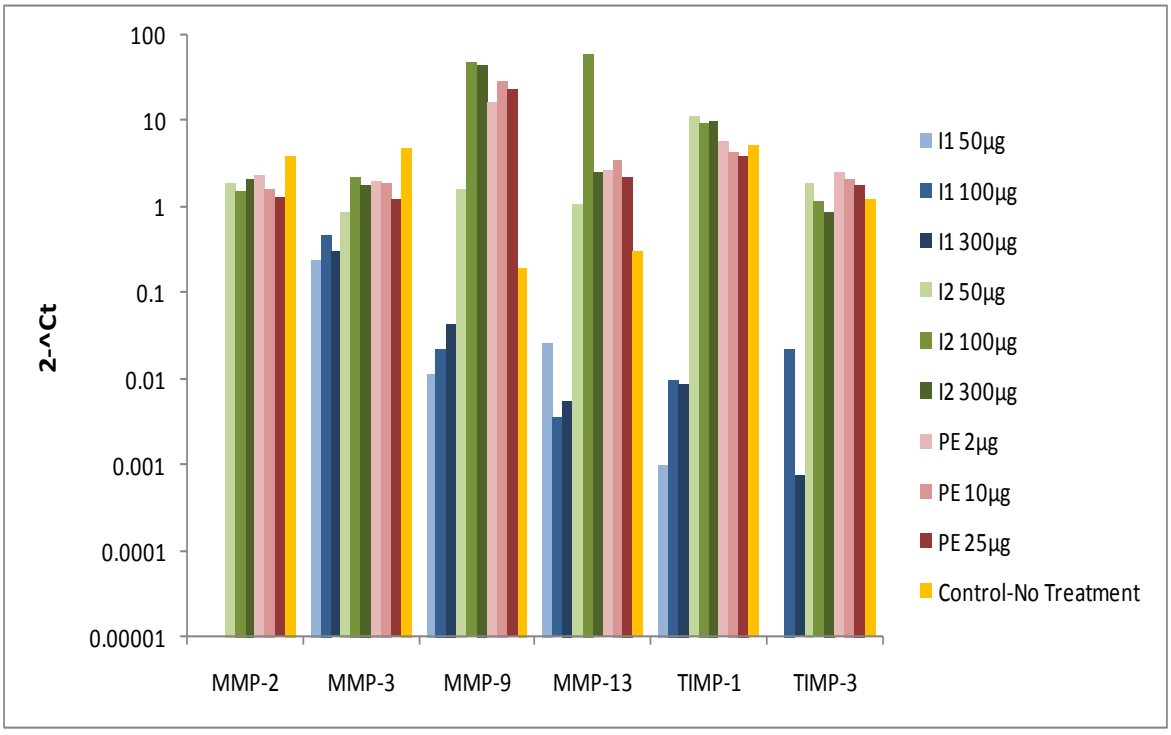


Figure 14



Figures 13-14: QRT-PCR results for MMP/TIMP mRNA expression of granulation tissue fibroblasts following treatment with increasing concentration of total protein of *P. aeruginosa* conditioned media or increasing concentrations of purified *P. aeruginosa* elastase (PE). Figure 13: Grnulation tissue fibroblast (GT1) fibroblasts (for GT1 fibroblasts no data available for MMP-13 mRNA expression); Figure 14:GT2 fibroblasts (for GT2 fibroblasts missing data for MMP-2 [I1 50µg, I1 100µg, I1 300µg] and missing data for TIMP-3 [I1 50µg]).

4.4.3 Immunohistochemistry

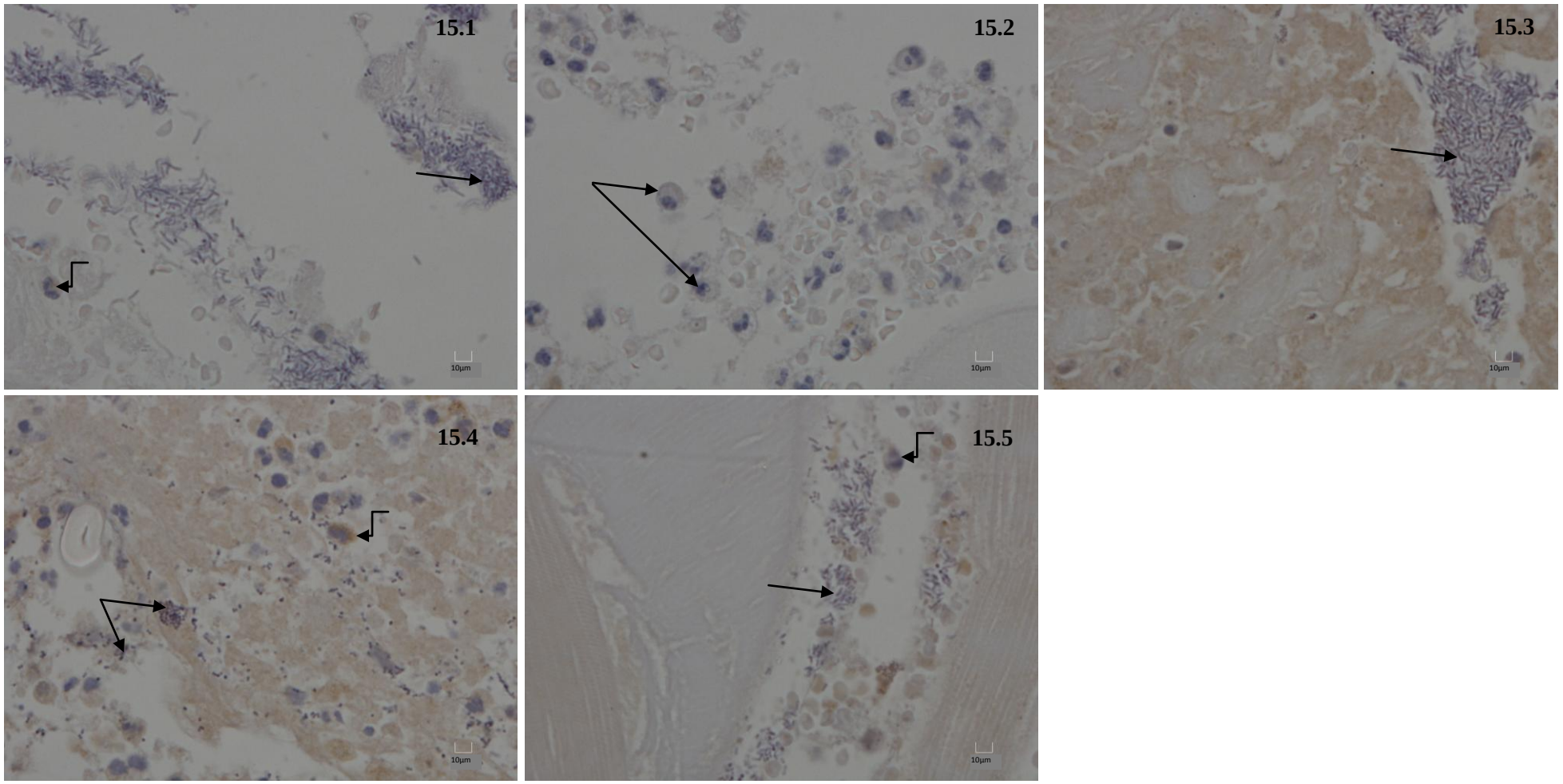


Figure 15: Immunohistochemistry images of equine wound granulation tissue taken from the middle of the wound area; tissue sections were stained for MMP-2, MMP-9 and MMP-13. MMPs are stained light brown. 15.1: MMP-2 staining with bacterial clusters (see arrow) at the outer edge of the tissue section and darkly stained inflammatory cell (see elbow arrow); 15.2: MMP-2 staining around inflammatory cells (see arrows) at the edge of the tissue section; 15.3: MMP-9 staining with a large bacterial cluster (see arrow); 15.4: MMP-9 staining showing small clusters of bacteria (see arrows) and dark staining around inflammatory cells (see elbow arrow); 15.5: MMP-13 staining with clusters of bacteria (see arrow) and dark staining around inflammatory cells (see elbow arrow). Scale bars = 10µm

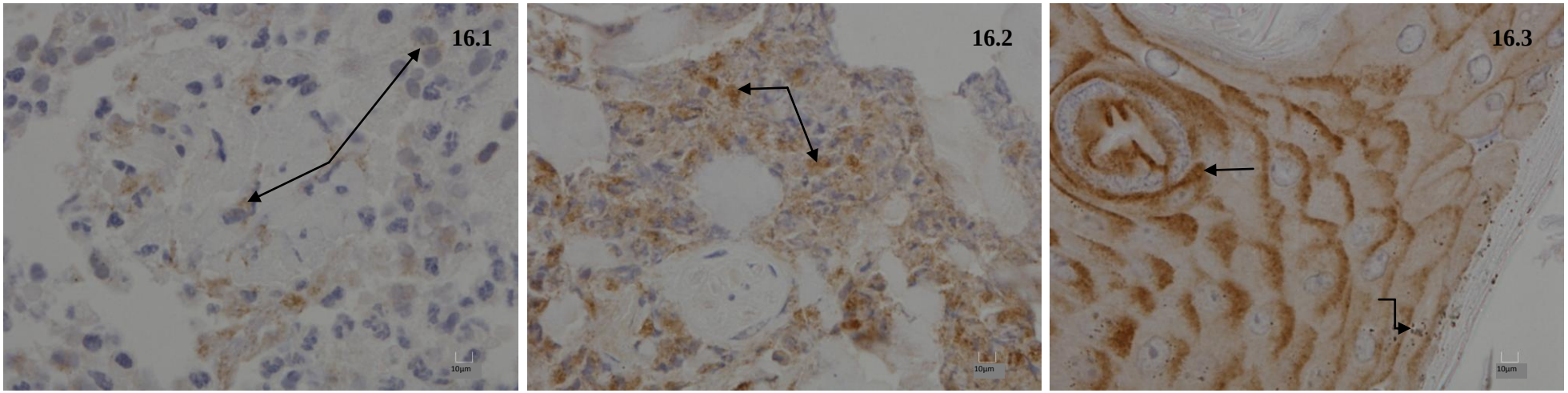


Figure 16: Immunohistochemistry images of equine wound granulation tissue taken from the outer edge of the wound area; tissue sections were stained for MMP-2, MMP-9 and MMP-13. MMPs are stained light brown. 16.1: MMP-2 staining towards the centre of the tissue section with dark staining around inflammatory cells (see arrows); 16.2: MMP-9 staining; dense areas of staining towards the middle of the tissue section (see arrows); 16.3: MMP-13 staining; dense staining at the edge of the tissue section (see arrow) with small bacterial clusters (see elbow arrow). Scale bars = 10µm

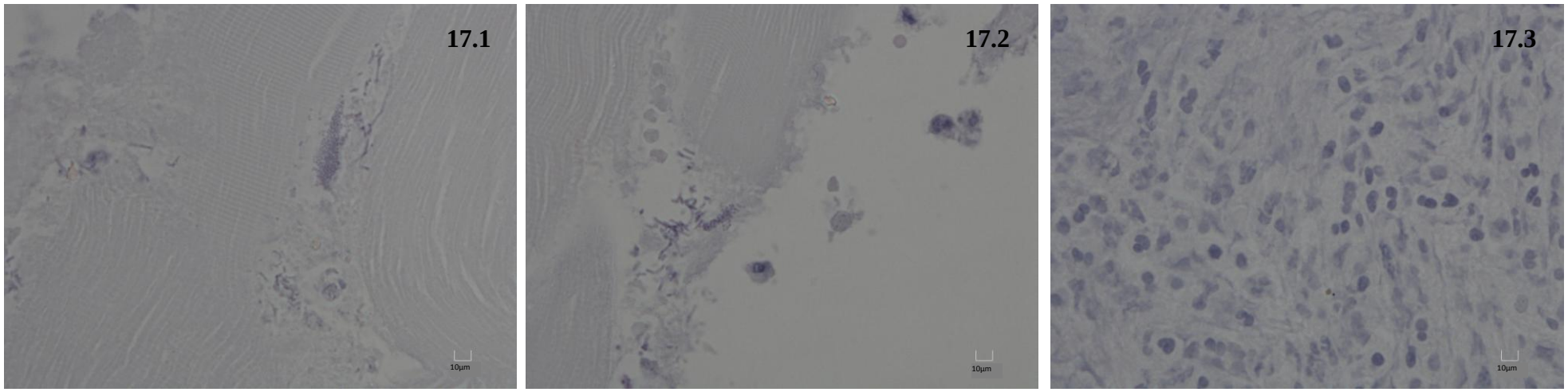


Figure 17: Immunohistochemistry images showing no secondary antibody staining controls for MMP-2 (17.1), MMP-9 (17.2) and MMP-13 (17.3). Scale bars = 10µm

Images taken from immunohistochemistry slides (figures 15-17) reveal bacterial clusters both within tissue sections taken from the centre of the wound area and from the edges of the wound, indicating bacterial dissemination throughout the wound bed with both cocci and rod-shaped bacteria evident. These tissue sections also stained positively for MMPs -2, -3 and -13, with dark staining around immune cells which seems to link evidence of infection, inflammation and protease expression throughout the wound tissue. More prominent staining for MMPs was noted on those tissue sections taken from the outer edges of the wound area as compared with the centre of the wound.

4.4.4 Scratch Wound Assay

The scratch wound assay was used to ascertain whether increasing concentrations of *P. aeruginosa elastase* has an effect on the wound healing capacity of both normal skin equine fibroblasts (NF) and equine granulation tissue fibroblasts (GT). Hydroxyurea was used as an inhibitor of proliferation so that wound closure was based on migration alone. Hydroxyurea (HU) is a metabolic inhibitor or ribonucleotide reductase (Stone *et al*, 2012) and inhibits DNA synthesis without affecting RNA or protein synthesis in a dose-dependent manner (Zungu *et al*, 2009). In the study by Jacobsen, Andersen and Krogfelt (2012), a concentration of 50µM HU inhibited fibroblast proliferation in an *in vitro* scratch wound assay. In the present study, a preliminary scratch wound assay was conducted and results revealed that this concentration of HU did not inhibit equine NF or GT fibroblast proliferation. Studies by Cai *et al* (2000), Sarkar *et al* (1996), Brown, Pan and Hassid (1999) and Zungu *et al* (2009) used concentrations in the range of 2mM-5mM HU. Yet, higher concentrations of HU appeared to completely inhibit wound closure and cell viability was lost in the present study and so a concentration of 2mM HU was used in further experimentation. Cai *et al* (2000) showed that at this concentration of HU proliferation was completely inhibited whilst having a minimal effect on migration of human umbilical vein endothelial cells (HUVECs). Brown, Pan and Hassid (1999) also showed that HU did not have a significant effect on cell migration of aortic smooth muscle cells.

Crystal violet staining of control scratch wound wells (containing only fibroblasts plus HU-supplemented DMEM) revealed that during the course of the study fibroblasts were still proliferating given an increase in crystal violet staining. As a result, it was not possible to differentiate between the effects of treatment on proliferation and migration. Alternative methods for the study of cell migration and wound closure are considered in the discussion section. Furthermore, control cells died after 36 hours of incubation for unknown reasons and so percentage wound closure at 36, 60 and 72 hours post wounding (0 hours) following treatment with each concentration with *P. aeruginosa* elastase could not be compared with no treatment controls, but comparisons were made between the four different concentrations of *P. aeruginosa* elastase to determine if there was a dose-dependent response to treatment.

Results revealed that both the GT1 and GT2 scratch wounds were between 25-70% healed by 36 hours and by 72 hours all wounds were completely or very nearly healed (see figures 18 and 19). Similar trends were noted for the NF scratch wounds (see figures 20 and 21), however the NF1 scratch wounds had reached a higher level of closure at 36 hours compared to NF2, GT1 and GT2 scratch wounds. Whilst not statistically significant (see table 4), it was noted that there was a wider variation in percentage wound closure at each time point between *P. aeruginosa* elastase treatment concentrations for the GT scratch wounds compared with the NF scratch wounds (see figures 18-21). Although it was not possible to determine if there was a significant effect of PE in general on wound closure compared with no treatment, results have shown that there was not a dose-dependent response to *P. aeruginosa* elastase treatment in terms of wound closure, i.e. an increase in *P. aeruginosa* elastase concentration did not adversely affect wound closure in this *in vitro* model. It should be kept in mind, however, that this model does not reflect the 'true' *in vivo* environment, where several matrix proteins, cell types and cellular mediators are involved in wound closure. Suggestions for future work in terms of mimicking the clinical milieu are considered in the discussion section.

Table 4: Statistical analysis of scratch wound assay results. Comparisons were made between the percentage wound closure following varying concentrations of *P. aeruginosa* elastase for normal equine skin fibroblasts (NF) and equine wound granulation tissue fibroblasts (GT).

Cell Type	Time Point (hours)	One-Way ANOVA	Kruskal-Wallis test
NF	36	F(3, 21) = 0.82, P = 0.500	P=0.944
	60	F(3, 21)= 1.52, P= 0.243	P=0.087
	72	F(3, 21)= 1.39, P= 0.277	P=0.261
GT	36	F(3, 23)= 0.27, P= 0.845	
	60	F (3, 23)= 0.45, P= 0.718	P=0.652
	72	F(3, 23)= 1.01, P= 0.408	P=0.564

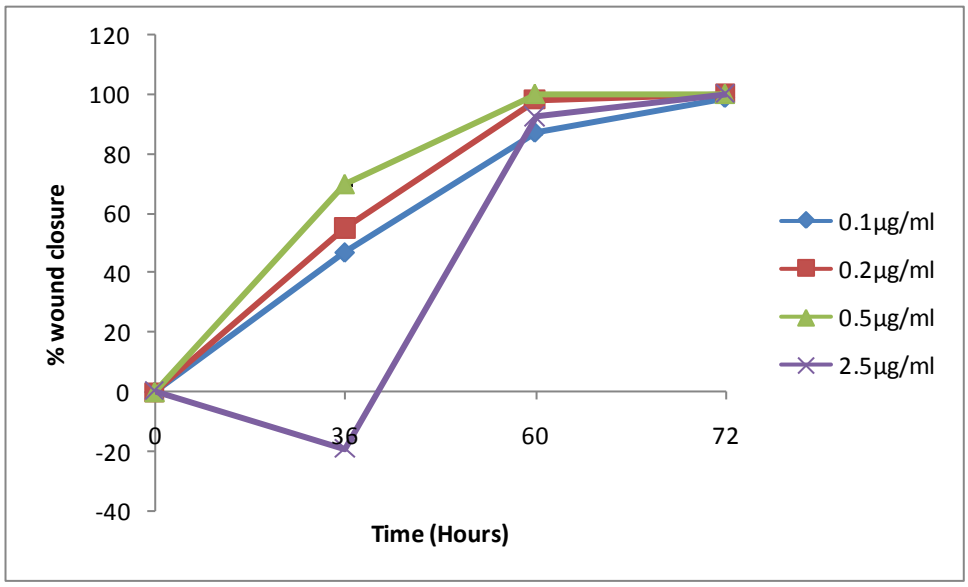


Figure 18: Effect of *P. aeruginosa* elastase (0.1µg/ml, 0.2µg/ml, 0.5µg/ml and 2.5µg/ml) on scratch wound healing of equine granulation tissue fobroblasts (GT1). The surface of the wound area was measured at 0, 36, 60 and 72 hours post-wounding and the percentage wound closure over time was calculated. Data is presented as mean ± SEM. Statistical significance is detailed in table 4).

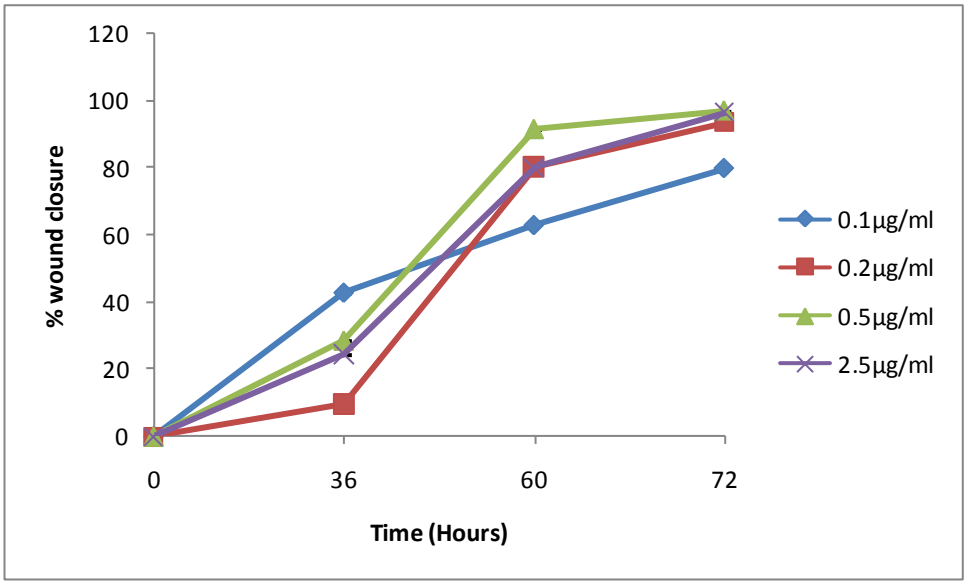


Figure 19: Effect of *P. aeruginosa* elastase (0.1µg/ml, 0.2µg/ml, 0.5µg/ml and 2.5µg/ml) on scratch wound healing of equine granulation tissue fobroblasts (GT2). The surface of the wound area was measured at 0, 36, 60 and 72 hours post-wounding and the percentage wound closure over time was calculated. Data is presented as mean ± SEM. Statistical significance is detailed in table 4).

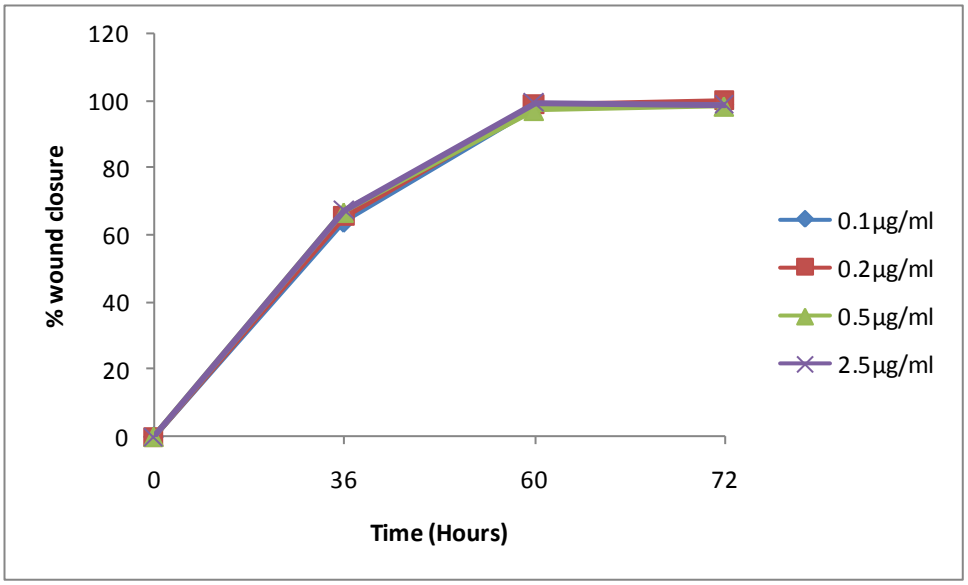


Figure 20: Effect of *P. aeruginosa* elastase (0.1µg/ml, 0.2µg/ml, 0.5µg/ml and 2.5µg/ml) on scratch wound healing of normal equine skin fobroblasts (NF1). The surface of the wound area was measured at 0, 36, 60 and 72 hours post-wounding and the percentage wound closure over time was calculated. Data is presented as mean ± SEM. Statistical significance is detailed in table 4).

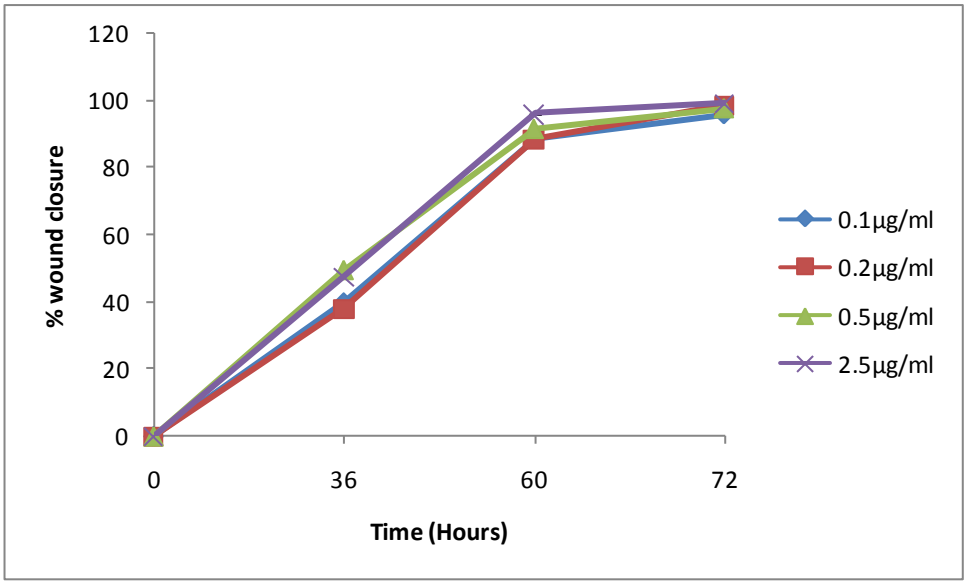


Figure 21: Effect of *P. aeruginosa* elastase (0.1µg/ml, 0.2µg/ml, 0.5µg/ml and 2.5µg/ml) on scratch wound healing of normal equine skin fobroblasts (NF2). The surface of the wound area was measured at 0, 36, 60 and 72 hours post-wounding and the percentage wound closure over time was calculated. Data is presented as mean ± SEM. Statistical significance is detailed in table 4).

4.5 Discussion

Despite the limited number of fibroblast cell donors available for use in the current study, clear detrimental effects of *P. aeruginosa* conditioned media and purified *P. aeruginosa* elastase on fibroblast viability and growth were highlighted. The crystal violet cell viability method has demonstrated that with increasing concentrations of total protein in *P. aeruginosa* conditioned media and increasing concentration of purified elastase generally the percentage decrease in cell viability became more prominent. This trend is consistent with earlier work conducted by Schmidtchen *et al* (2003) which demonstrated that LasB from ulcer-derived *P. aeruginosa* inhibited fibroblast growth. Microscopy images taken during the course of the experiment also confirmed that fibroblast growth and viability was hampered when cells were treated with *P. aeruginosa* elastase. In future experiments, further assays could be used in addition to the crystal violet staining method to assess cell changes following treatment, including assays to measure apoptosis and necrosis.

Equine fibroblasts were used in this study given the prevalence of non-healing wounds with the generation of exuberant granulation tissue (EGT) in horses (Engelen *et al*, 2004; Lepault *et al*, 2005; Stashak, 2008). Furthermore, equine wounds are susceptible to bacterial contamination; recent investigation has identified the presence of microbial diversity and biofilms within the equine wound milieu (Westgate *et al*, 2010; Cochrane *et al*, 2009; Westgate *et al*, 2011) and thus the presence of bacterial proteases is important in these wounds. The immunohistochemistry images presented in this study also identified the presence of bacterial clusters within equine granulation tissue. Evidence of bacterial contamination was not only present at the edges of the wound area but also within tissue sections taken from the centre of the wound, demonstrating that bacteria are disseminated throughout the wound area. Furthermore, this bacterial staining appeared alongside MMP-2, -3 and -13 staining; MMP staining was also more prominent on tissue sections derived from the edge of the wound area and particularly around immune cells highlighting the correlation between infection, inflammation and protease expression.

It would be interesting in further investigations to analyse the expression of MMPs via the staining patterns in tissue sections used in an *in vitro* organ culture model used to assess the effects of conditioned *P. aeruginosa* medium and purified elastase on regulation of host MMPs. This would help to establish the localisation of MMP staining within tissue sections following treatment. Furthermore, the effects of live *P. aeruginosa* inoculation of cultured fibroblasts on MMP expression from host fibroblasts could be assessed to provide a better insight into the *in vivo* environment. Alternatively, 3D matrix models can be used, such as the collagen gel method adopted by Nagano *et al* (2001). The use of *in vitro* wound healing models which incorporate matrix proteins may influence the effect of *P. aeruginosa* proteases on mRNA MMP/TIMP expression and wound healing. An organotypic co-culture wound model, such as that used by Schneider *et al* (2008), would also more closely reflect the *in vivo* environment. A chemical method of *in vitro* fibroblast wounding could be employed as an alternative to the scratch wound model (De Bentzmann *et al*, 2000). Alternative techniques can also be utilised to fully examine the effects of bacterial proteases on proliferation and migration, such as the Boyden Chamber method, whereby migration is analysed as the cells migrate through a simulated matrix (Decaestecker *et al*, 2007). The ways in which the migrating edge cells migrate to heal a wounded area *in vitro* can also be more closely monitored; random migration versus cohesive sheet migration can be measured, for example (Moreo-Bueno, 2009).

In terms of MMP and TIMP mRNA expression following treatment of equine dermal fibroblasts with *P. aeruginosa* conditioned medium and purified *P. aeruginosa* elastase, mRNA of each of the MMPs and TIMPs of interest were expressed by normal skin fibroblasts and granulation tissue fibroblasts at a higher level relative to GapDH. However, due to a limited number of fibroblast cell donors it was not possible to come to definitive conclusions about the effects of elastase on the regulation of host MMP and TIMP mRNA expression. Yet, it was highlighted that fibroblasts derived from different sources/donors behave and respond to treatments with *P. aeruginosa* conditioned media and purified elastase in different

ways and therefore it is important to appreciate this variation in future studies. Samples from the fibroblast culture media were collected for analysis of protease content using zymography. However, no detectable host-derived protease bands were noted on gelatin zymograms; the proteolytic activity of culture supernatants may need to be concentrated in future experiments in order to detect lower protease levels from short culture periods.

The use of specific protease inhibitors and specific inhibitors of toxins and other important *P. aeruginosa* virulence factors could be utilised in further investigations to establish the most important exoproducts within the *P. aeruginosa* conditioned media in terms of host cell growth and viability and MMP/TIMP mRNA expression. Since the regulation of MMPs is controlled at different levels including gene transcription, translation and pro-enzyme activation (Dong *et al*, 2000) it may be that *P. aeruginosa* elastase acts by activating pro-elastase rather than altering mRNA expression. As such, there are limitations in the use of immunohistochemistry and PCR techniques for the evaluation of MMP activity given the complex relationships between the presence of the protein, enzyme activation and the presence of MMP inhibitors. To further clarify the role of elastase on cell viability and MMP/TIMP mRNA production elastase-deficient strains of *P.aeruginsoa* could be utilised (Nagano *et al*, 2001).

4.6 Conclusion

Whilst the data presented in this study should be interpreted as preliminary findings, this study has highlighted clear evidence of the deleterious effects of *P. aeruginosa* exoproducts, including purified elastase on the viability and growth of equine dermal fibroblasts. *P. aeruginosa* conditioned media and *P. aeruginosa* elastase caused a reduction in the growth of both normal skin fibroblasts and granulation tissue fibroblasts in a dose-dependent manner. Immunohistochemistry analysis revealed the presence of bacterial clusters disseminated throughout the wound bed. Furthermore, bacterial staining occurred in conjunction with moderate staining of MMPs -2, -3 and -13 throughout the tissue. Whilst the study was limited in terms of cell donors available for qRT-PCR analysis, mRNA of each of the MMPs and TIMPs of interest were expressed by normal skin fibroblasts and granulation tissue fibroblasts at a higher level relative to GapDH, with cells from different sources/equine donors responding differently in terms of MMP/TIMP mRNA expression when treated with increasing concentrations of *P. aeruginosa* conditioned media or *P. aeruginosa* elastase.

4.7 Appendices to Chapter 4

Appendix 1

Primer sequences

Gene	Primer Sequence
MMP-2	F: GCCACTTGAGTTCGCCC R: ATCGCTGCGCCCTGTGTCTGTG
MMP-3	F: TCTTGCCGGTCAGCTTCATATAT R: CCTATGGAAGGTGACTCCATGTG
MMP-9	F: TTGGACATGCACGACGTCTT R: AGGGGTCCTGGGAGAAGTAA
MMP-13	F: GAGCATCCTTCCAAAGACCTT R: CATAACCATTAAGAGCCCAAAATT
TIMP-1	F: GCC AGG GCT TCA CCA AGA R: GGA TGG ATG AAC AGG GAA ACA
TIMP-3	F: CTGCAACTTCGTGGAGAGGT R: ACTCGTTCTTGGAGGTCACG
GAPDH	F: GCATCGTGGAGGGACTCA R: GCCACATCTTCCCAGAGG

Appendix 2

MMP-2 Immunohistochemistry Staining Method

1.	Xylene 2x 5mins 100% ethanol 2x 2mins 96% ethanol 1x 2mins
2.	Endogenous Block 360ml methanol + 6ml H_20_2 30mins Wash dH_20
3.	Citrate 6 Pre-treatment (30mins at 96°C, 20mins cool, then dH_20)
4.	Rack up in sequenza clips using TBS
5.	1x 5min TBS wash
6.	Block 50% swine serum in TBS, 10mins
7.	~100µl MMP-2 Primary Antibody Mouse MAb MMP-2 Ab-4 (A-Gel VC2), MS-806-P1, NeoMarkers 1:50 (dilute in TBS) 4°C overnight
8.	1x 5min TBS wash
9.	Apply ~100µl Secondary Antibody Rat anti Mouse (Jackson Immuno Research, 415-005-166) 1:100 in TBS 30mins, room temp
10.	1x 5min TBS wash
11.	Apply ~100µl PAP Mouse Mouse Peroxidase α Peroxidase, Jackson Immuno Research, 223-005-024 1:500 in TBS 30mins, room temp
12.	1x5min TBS wash
13.	Remove from sequenza clips to dH_20
14.	DAB 10mins 0.2g Diaminobenzidine in 400mls Imidazole Buffer: (280ml 0.1M Imidazole/120ml 0.42MHCl pH 7.2), filtered, activate with

	140µl 30% Hydrogen Peroxide H_20_2 (Fisher Scientific BP2633-500)
15.	3x 5min washes dH_20
16.	Counterstain with Papanicolaou's 1b Hematoxylin Solution (Merck 1.09254.0500) 20mls in 400mls dH_20 stain 1min "blue" running tap water 5min
17.	Dehydrate, Clear & Mount: 96% ethanol 1min 100% ethanol 2min, 100% ethanol 3min Xylene 2min, Xylene 3min, Xylene 3min Coverslip with DPX

MMP-9 Immunohistochemistry Staining Method

Steps 1-6 as protocol for MMP-2

7.	~100µl MMP-9 Primary Antibody Mouse MAb MMP-2 Ab-5 (IIA5), MS-817-P1, NeoMarkers 1:10 (dilute in TBS) 4°C overnight
8.	1x 5min TBS wash
9.	Apply ~100µl Secondary Antibody Rat anti Mouse (Jackson Immuno Research, 415-005-166) 1:100 in TBS 30mins, room temp
10.	1x 5min TBS wash
11.	Apply ~100µl PAP Mouse Mouse Peroxidase α Peroxidase, Jackson Immuno Research, 223-005-024 1:500 in TBS 30mins, room temp
12.	1x 5min TBS wash
13.	Remove from sequenza clips to dH_20
14.	DAB 10mins 0.2g Diaminobenzidine in 400mls Imidazole Buffer: (280ml 0.1M Imidazole/120ml 0.42MHCl pH 7.2), filtered, activate with 140µl 30% Hydrogen Peroxide H_20_2 (Fisher Scientific BP2633-500)
15.	3x 5min washes dH_20
16.	Counterstain with Papanicolaou's 1b Hematoxylin Solution (Merck 1.09254.0500) 20mls in 400mls dH_20 stain 1min "blue" running tap water 5min
17.	Dehydrate, Clear & Mount: 96% ethanol 1min 100% ethanol 2min, 100% ethanol 3min Xylene 2min, Xylene 3min, Xylene 3min Coverslip with DPX

MMP-13 Immunohistochemistry Staining Method

Steps 1-5 as protocol for MMP-2

6.	Block 20% swine serum in TBS, 10mins
7.	~100µl MMP-13 Primary Antibody Rabbit PAb MMP-13, Abcam ab39012 1:100 (dilute in 20% swine serum in TBS) 4°C overnight
8.	1x5min TBS wash
9.	Apply ~100µl Secondary Antibody Polyclonal Swine α Rabbit, Dako Z0196 1:100 (dilute in 20% swine serum in TBS) 30mins, room temp
10.	1x5min TBS wash
11.	Apply ~100µl PAP Rabbit PAP Rabbit, Covance SMI-4010L 1:250 in TBS 30mins, room temp
12.	1x5min TBS wash
13.	Remove from sequenza clips to dH_20
14.	DAB 10mins 0.2g Diaminobenzidine in 400mls Imidazole Buffer: (280ml 0.1M Imidazole/120ml 0.42MHCl pH 7.2), filtered, activate with 140µl 30% Hydrogen Peroxide H_20_2 (Fisher Scientific BP2633-500)
15.	3x5min washes dH_20
16.	Counterstain with Papanicolaou's 1b Hematoxylin Solution (Merck 1.09254.0500) 20mls in 400mls dH_20 stain 1min "blue" running tap water 5min
17.	Dehydrate, Clear & Mount: 96% ethanol 1min 100% ethanol 2min, 100% ethanol 3min Xylene 2min, Xylene 3min, Xylene 3min Coverslip with DPX

5.0 Chapter 5

Manuscript 5

The role of polyphosphate dressings in the sequestration of Matrix Metalloproteinases (MMPs) in chronic cutaneous wounds

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Keywords: Wound healing, Matrix Metalloproteinases (MMPs), Polyphosphate, Wound dressings, Chronic wounds

5.1 Abstract

The present study outlines the potential of a novel therapeutic dressing for the management of chronic wounds. The dressing incorporates polyphosphate, a non-toxic compound with a number of beneficial characteristics in terms of wound healing, in a foam matrix. The aim of this study was to explore the hypothesis that polyphosphate incorporated into a foam dressing is able to sequester the activity of MMPs and proteases derived from *Pseudomonas aeruginosa in vitro*. Methods used included gelatin zymography and milk-casein agar plate analysis. Results have shown that this dressing is effectively able to reduce the levels of MMP-2 and MMP-9 in both their active and latent forms using an *in vitro* model. The dressing also demonstrated its potential in the regulation of *Pseudomonas aeruginosa* derived proteases. Dressings that are able to alter proteolytic activity within the wound milieu, including the sequestration of bacterial proteases, could help to promote the healing of chronic wounds that remain in a state of prolonged inflammation characterised by an increase in protease levels and tissue degeneration.

5.2 Introduction

Cutaneous wound healing is a complex process which involves a careful balance between tissue synthesis, deposition and breakdown. A number of cell types and molecular mediators are involved in the process, which work synergistically to achieve rapid restoration of tissue architecture and function (Ribeiro *et al*, 2009). If this balance is disrupted, however, healing is hampered leading the wound into a state of chronicity (Cullen *et al*, 2002; Wright *et al*, 2002). Such instances include chronic ulcers in which excessive tissue degradation prevails and keloid scars which are instigated by excessive extracellular matrix production (Ravanti & Kahari, 2000). Proteases including the matrix metalloproteinases (MMPs) and their inhibitors, particularly the natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs), play a vital role in this equilibrium and are important factors of the healing process (Cullen *et al*, 2002). A number of cell types including keratinocytes, fibroblasts and endothelial cells contribute to the proteolytic environment within the wound bed. Whilst fibroblasts produce MMPs, invading neutrophils and macrophages produce elastase and collagenase (Trengove *et al*, 1999; Tarnuzzer & Schultz, 1996; McCarty *et al*, 2012).

All chronic wounds are characterised by an elevated inflammatory response, resulting in increases in protease activity and abundance and decreased growth factor activity (Cullen *et al*, 2002). MMPs are able to break down peptide links of growth factors thus indirectly inhibiting matrix remodelling (Timmons, 2010). This dual biochemical imbalance in wound tissues is, in part, responsible for the failing of healing in such wounds, given the net loss of tissue (Cullen *et al*, 2002). MMPs have also been implicated in the pathogenesis of cancer metastasis, as a result of increased tissue breakdown and remodelling during invasive tumour growth and angiogenesis (Komorowski *et al*, 2002). Indeed, raised blood plasma levels of MMP-3 and MMP-9 have been found in patients with medullary carcinoma (Komorowski *et al*, 2002).

MMPs are of particular interest in terms of cutaneous wound healing since a number of MMPs have been shown to be upregulated within the tissues of chronic wounds which fail to heal adequately. Indeed, research has revealed that the expression of various MMPs are upregulated during wound repair and regeneration, with mRNAs encoding specific MMPs being upregulated in wounded skin as compared to normal uninjured skin. For example, a study by Lund *et al* (1999) demonstrated via a murine wound model that MMP-2, -9, -3, -13, -11, 12 and -14 were all upregulated in terms of mRNA expression in skin wounds when compared to normal control skin (Lund *et al*, 1999).

Given the extensive evidence which suggests excessive MMP levels as a central pathological feature of chronic wounds, much research interest has been aimed at redressing this proteolytic imbalance and is an important goal of medical material science (Edwards & Howley, 2007). While many wound dressings are aimed at promoting healing through the absorption of exudates and reduction in bioburden through antimicrobial actions (Timmons, 2010), a number of dressings are now available which are aimed at sequestering MMP levels in the wound bed. Such dressings are aimed at modulating the biological molecules including growth factors and MMPs involved in wound healing (Veves, Sheehan & Pham, 2002).

A limited number of studies have demonstrated the ability of polyphosphate dressings to sequester various proteinases within the wound bed. Unbound polyphosphate has been shown to promote healing of chronic wounds though the inhibition of proteases, in turn maintaining a robust extracellular matrix (Richardson *et al*, 2009). A number of polyphosphates have been used including trimetaphosphate and hexametaphosphate. Such polyphosphates may be used externally in wound dressings or may be administered enterally and have been shown to be effective against pepsin, collagenase, elastase and hyaluronidase and are active at a broad pH range of 2-6. A number of enzyme dose response assays revealed that polyphosphates are active against wound associated proteases in a concentration-dependant manner (Richardson *et al*, 2009). Such dressings may prove to be more cost effective for the patient and health care services alike, when compared to other high technology approaches such as skin substitutes used to balance tissue degeneration (Hart *et al*, 2002).

The aim of the present study was to examine the potential MMP modulating capacity of a foam dressing containing polyphosphate compared with a commercially available sterile, freeze-dried dressing with a collagen/oxidised regenerated cellulose (ORC) composition, a soft adherent foam dressing with Technology Lipido-Colloid (TLC) with the healing accelerator nano-oligosaccharide factor (NOSF) and a standard polyurethane foam dressing. Given the evidence which suggests an elevated level of MMPs -2 and -9 within the chronic wound milieu (Wysocki, Staiano-Coico & Grinnell, 1993), this study has focussed specifically on the ability of the selected dressings to sequester these MMPs.

5.3 Methods

5.3.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated.

5.3.2 Ethical Approval

Approval for this study was granted by the Ethics Committee, University of Liverpool and informed consent was obtained from the owners of the animals participating in this study.

5.3.3 Dressings

Dressings used in this study included a standard polyurethane foam dressing (Smith and Nephew), a Collagen/ORC dressing (Johnson and Johnson), a TLC-NOSF dressing (Urgo Medical) and a foam dressing containing polyphosphate (Advanced Medical Solutions Plc).

5.3.4 MMP-2 Isolation

Equine dermal tissue was obtained either at post mortem of horses which had been euthanised for medical reasons or from tissue debrided during wound management procedures, in which case consent was given by the owner. All ethical considerations were made. Tissue sections were washed twice in 1x Hank's balanced salt solution (HBSS) (Gibco, Invitrogen, Paisley, UK) and minced into 3-5mm^2 pieces and placed into 25cm^2 culture flasks. Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum (FCS), 20mM Hepes buffer, 1% penicillin-streptomycin (Invitrogen, Paisley, UK) and 0.5µg/ml amphotericin B (Fungizone). The cultures were humidified in 5% CO_2 in air at 37°C. Following a culture period of 5-10 days cells were examined microscopically (Nikon Eclipse, Melville, USA) for outgrowth and

confluency. When confluent, cells were passaged in a ratio of 1:4 with 0.05% trypsin/EDTA and cells of passage 3-8 were used experimentally. Once cells regained confluency the media was removed and the cell layer washed with HBSS to remove all traces of FCS. Cells were then cultured in serum-free DMEM with 20mM Hepes buffer and 1% penicillin-streptomycin (Invitrogen, Paisley, UK) to confluency (approximately 5 days). The media was removed and cells were centrifuged at a speed of 1400rpm for 5 minutes. The supernatant media was removed and stored at -80°C ready for enzyme purification and the pellet discarded appropriately.

5.3.5 MMP-9 Isolation

Equine peripheral blood was collected by venepuncture, anticoagulated with heparin and refrigerated for 30 minutes. Monocytes were isolated using Histopaque solution according to the manufacturer's instructions. In brief, the anticoagulated blood was layered on top of the Histopaque at a 1:1 ratio in 50ml centrifuge tubes. Blood samples were centrifuged at 400xg for 30 minutes to separate the monocyte layer from the blood plasma and red blood cells. Differential migration resulted in the clearly identifiable monocyte cell layer which was removed and successively washed in 1x HBSS. The monocytes were pelleted and stored in 0.5ml 1x HBSS. Cells were lysed with 1ml 0.1% triton X-100 per pellet and freeze-thawed 4 times to release the enzyme. The lysed cells were stored at -80°C ready for enzyme purification.

5.3.6 MMP-2 and MMP-9 Purification using Gelatin Sepharose Chromatography

A gelatin sepharose (GE Healthcare, Little Chalfont, UK) chromatography column was prepared using a 20ml disposable syringe (without needle) with a small amount of glass wool applied to the bottom and overlaid with 5mls gelatin sepharose solution. A separate column was prepared for MMP-2 and for MMP-9. The column was washed twice with equilibration buffer; 0.05M Tris base, 0.5M NaCl2 (Fisher Bioreagents, Fisher Scientific, Loughborough, UK), 0.005M $CaCl_2$, 0.05% Brij, 0.02% sodium azide, pH7.6. The fibroblast spent media or lysed cell suspensions were passed through the appropriate column and discarded. The column was washed with 5x 5ml equilibration buffer. The gelatin sepharose-bound enzyme was eluted with 10ml 10% dimethyl sulphoxide in 80% elution buffer (0.05M Tris hydrochloride, 1M NaCl, 0.005M $CaCl_2$, 0.05% Brij 35%, 0.02% sodium azide) and collected in 15ml centrifuge tubes. The eluent was then dialysed overnight in MMP dialysis buffer (0.05M Tris hydrochloride, 0.005M $CaCl_2$, 0.05% Brij 35, 0.02% sodium azide) at 4°C. Dialysed aliquots were then stored at -80°C until required for dressing incubation experiments. The columns were rehydrated with equilibration buffer, sealed and stored at 4°C for further use.

5.3.7 Dressing Interaction Studies Using Purified MMPs

In order to assess the ability of each dressing to sequester MMP-2 and MMP-9 activity an *In vitro* model was used, as adapted from the study by Walker *et al* (2005). Dressing samples weighing 0.01g were prepared and incubated with 250µl of either MMP-2 or MMP-9 in triplicate for each of the four dressings in 24-well culture plates. Separate samples were incubated for 0 (1 minute), 3, 6, 24 and 48 hours. At each corresponding time point 500µl 1x sterile phosphate buffered saline (PBS) was added to each well of the plate, to stop the action of the dressing and to aid in the elution of the enzyme solution from the dressing samples, and the enzyme samples were recovered and the supernatant stored at -80°C until further analysis of enzyme activity using gelatin zymography.

5.3.8 Dressing Interaction Studies Using *Pseudomonas aeruginosa* Conditioned Media

Bacterial Isolation and Identification

P. aeruginosa isolates were obtained from chronic equine wounds using an optimised swab method and stored in Stuart's transport medium (Oxoid, Basingstoke, UK). Individual colonies were isolated on tryptone soy agar (TSA) (LabM, Bury, UK) plates and colonies were confirmed as *P. aeruginosa* on 5% blood agar plates and MacConkey agar (LabM, Bury, UK), followed by Gram staining and analysis under oil immersion. Oxidase tests and API identification strips (API 20 E, Biomerieux, Basingstoke, UK) were used for final identification. An ATCC type strain of *P. aeruginosa* (ATCC 27853) was used as a control.

P. aeruginosa Conditioned Media Preparation

Sterile Mueller Hinton broth (MHB) was inoculated in 30ml portions with each of the corresponding *P. aeruginosa* isolates and cultured with shaking at 150rpm in a shaking incubator set at 37°C for 24 hours in 75cm^3 culture flasks. Cultures were harvested and centrifuged at 12000rpm for 10 minutes to pellet the cells. Conditioned culture supernatant was filtered through 0.45µm sterile filters.

Dressing Interaction Studies

In order to assess the ability of each dressing to sequester *P. aeruginosa* derived protease activity *in vitro*, 0.01g dressing samples were prepared and incubated with 250µl of each corresponding *P. aeruginosa* conditioned media in triplicate for each of the four dressings in 24-well culture plates. Separate samples were incubated for 0 (1 minute), 3, 6, 24 and 48 hours. At each corresponding time point 500µl sterile PBS was added to each well of the plate and the enzyme samples were recovered and the supernatant stored at -80°C until further analysis of enzyme activity. Dressing interaction studies were also performed with a combination of MMP-2 and *P. aeruginosa* conditioned media: 125µl of MMP-2 was added to the plate wells with

50µl conditioned *P. aeruginosa* media. Samples were treated as previously described following incubation, and supernatants harvested ready for analysis via the milk-casein agar plate method.

5.3.9 Milk-Casein Agar Plate Inoculation

Whilst the milk-casein agar plate method for detecting proteolytic activity is only semi-quantitative, it provides an initial visual representation of the levels of general proteolytic activity of different bacterial isolates over time prior to further investigation. This method is commonly used for the screening of general proteases produced by both Gram negative and Gram positive bacteria, as reviewed by Kasana, Salwan & Yadav, (2011). Whilst some studies have measured the proteolytic activity of various microorganisms from the proteolytic halo zone formed around colonies from microorganisms isolated from various settings (Sokol, Ohman & Iglewski, 1979; Howe & Iglewski, 1984; Nicas & Iglewski, 1984; Wery *et al*, 2003; Ponmurugan, 2007), in this study the method was standardised using wells cut into the agar plates into which an aliquot of conditioned bacterial media from a culture sample normalised to a set optical density (OD) was placed. This method has been used previously to detect the presence of bacterial proteolytic activity in the clinical setting (Palolahti *et al*, 1993; Wladyka *et al*, 2008).

Milk-casein agar was prepared with 25g skim milk powder (BD, Oxford, UK) 2.5g casein (BDH, VWR, Lutterworth, UK), 1.25g yeast extract (Oxoid, Basingstoke, UK), 0.5g D-glucose (BDH, Oxford, UK) and 6.25g No. 1 agar (LabM, Bury, UK). Agar was sterilised by autoclaving and poured to equal levels into 9cm round petri dishes. A sterile 8mm punch biopsy was used to bore single wells in each of the milk plates. Samples recovered from the dressing incubations with *P. aeruginosa* conditioned cell-free media were used to inoculate milk-casein agar plates. A 100µl aliquot of each sample was applied to the 8mm wells made in milk plates in triplicate. Plates were incubated for 24 and 48 hours. Following incubation, the caseinolytic area was measured; two measurements were made in mm at right angles to each other and an average measurement made, minus the 8mm well (see Figure 1). For calculations of percentage decrease in caseinolytic activity, the average measurement of the clear caseinolytic zone (mm) (minus the 8mm well) for each

sample was given as a percentage of the average measurement of the clear caseinolytic zone (mm) (minus the 8mm well) for the wells containing conditioned media which was previously incubated without any of the test dressings (control wells). Percentage decrease in the caseinolytic zone, as compared with the control wells, was then calculated.

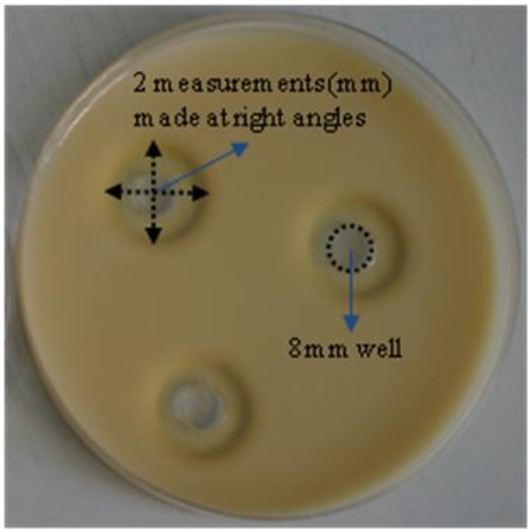

Figure 1: Method of calculation of caseinolytic zone using the milk-casein agar plate method.

5.3.10 Dressing Interaction Studies with *P. aeruginosa* Live cultures

P. aeruginosa cultures, both wound-isolates and ATCC type strain, were prepared as previously described in Chapter 2. Live cultures were standardised to an optical density of 0.85 at 600nm using a spectrophotometer. 100µl aliquots of each individual culture were applied, in triplicate, to 8mm wells in milk-casein agar plates, prepared as previously described. 0.01g portions of each dressing were overlaid over the cultures in the corresponding wells. Plates were incubated at 37°C for 24 and 48 hours. Caseinolytic zones were measured as described above and averages taken.

5.3.11 Treatment of Samples for Gelatin Zymography

Samples were diluted with non-reducing Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK) at a ratio of 1:5 and distilled water to an overall 1:5 dilution of the sample. Samples were then incubated in a 37°C water bath for 1 hour to activate the enzyme prior to application to the gel. A Miniprotean II gel system (Bio-Rad, Hemel Hempstead, UK) was employed for zymography gels. A 7.5% acrylamide/bis-acrylamide (Severn Biotech Ltd., Kidderminster, UK) resolving gel copolymerised with a 1% gelatin solution (EIA grade reagent gelatin) (Bio-Rad, Hemel Hempstead, UK) was prepared to create a 0.25% gelatin zymogram in a pH8.8 resolving buffer (1.5M Tris/HCl). A volume of 3.2ml of this was cast in between ethanol washed glass gel plates and overlain with isobutanol to level the gel. The gel was left to set. Once set, the resolving gel was layered with a stacking gel made up with a pH6.8 buffer (0.5M Tris/HCl) and was left to set with a 10 lane comb inserted in the top of the gel. The gels were placed in a tank filled with running buffer and 20µl portions of each sample were loaded into the gel and subjected to electrophoresis at 200 volts for 1 hour or until the lane marker had run off the end of the gel. Mark-12 high molecular weight lane markers (Invitrogen, LC5677, unstained standards) were run on each gel. Following electrophoresis, gels were carefully removed from the glass plates and the lane markers were removed and placed in 0.02% coomassie brilliant blue stain (BDH, Oxford, UK) for 15-20 minutes. The gels were placed in 2.5% Triton X-100 and agitated on a plate shaker at rpm for 1 hour to remove all traces of SDS. The gels were washed thoroughly in distilled water and placed in MMP incubation buffer/gelatine refolding buffer overnight. After incubation, the gels were stained for 1 hour in 0.5% coomassie stain and then destained in a solution of diluted methanol/acetic acid until clear bands of digested gelatin were visible against a darkly stained blue background. Gels were visualised and imaged using Genesnap software. The gels were dried in cellophane sheets using a Hoeffer gel drying dock.

5.3.12 Gel Quantification

Gel images were captured and analysed using the GeneGenius Gel Documentation System (Syngene, Cambridge, UK). The relative activity value (RAV) was calculated using the following equation:

$$RAV = \frac{\text{Peak area of sample}}{\text{Peak area of positive control}}$$

5.3.13 SDS-PAGE Analysis of Conditioned *P. aeruginosa* Media

A 15% acrylamide mini gel was prepared using 3.2mls resolving gel in a 1.5M Tris/HCl buffer, pH 8.8. Once set this gel was overlaid with a stacking gel made up in a 0.5M Tris/HCl buffer, pH 6.8 in a Miniprotean gel system (Bio-Rad Hemel Hempstead, UK). Samples were prepared by adding 13µl original sample, 2µl DTT and 5µl non-reducing sample buffer with marker dye (Thermo Scientific,). Samples were denatured by heating for 10 minutes in a heat block pre-set to 80°C. The samples were vortexed briefly. A 20µl aliquot of each sample was added to each corresponding lane of the gel. The gel tank was filled with a 1:20 dilution of NUPAGE MES SDS running buffer (Invitrogen, Paisley, UK) in distilled water. The gel was run at 15 volts for approximately 2 hours or until the lane marker ran to the bottom of the gel. The gels were carefully removed from the glass plates and placed in 0.02% Coomassie brilliant blue stain for 2 hours with agitation. Gels were then destained in a solution of diluted methanol/acetic acid until dark bands appeared against a stain-free background.

5.4 Results

5.4.1 Dressing Incubations with MMP-2 and MMP-9

Following incubation at 37^{o}C with all of the dressings tested, the residual proteinase activity was assayed at 0 (1 minute), 1, 3, 6, 24 and 48 hours to evaluate the capacity for each dressing to inactivate both purified MMP-2 and MMP-9. All of the dressings tested were able to reduce the levels of active and latent enzymes when compared to the control (no dressing) from 1 minute onwards. The standard foam dressing was less effective than the other dressings, yet was still able to actively reduce MMP levels. This could be attributed to the dressing's active fluid management system. Samples incubated with the Collagen/ORC dressing demonstrated complete inhibition of both MMP-2 and MMP-9 in their latent (figures 2 and 4) and active forms (figures 3 and 5) from 1 hour onwards. The foam dressing containing polyphosphate consistently outperformed both the standard foam dressing and the TLC-NOSF dressing in terms of the sequestration of the active and latent forms of MMP-2 and MMP-9. Samples treated with the foam dressing containing polyphosphate demonstrated a time-dependant reduction in MMP activity, decreasing MMP-2 and MMP-9 by more than 50% by 48 hours in both the active and latent forms.

Incubations with MMP-2 and 9 showed that the foam dressing containing polyphosphate sequestered significantly more latent enzyme than the TLC-NOSF dressing at 3 and 48 hours ($P<0.005$). The performance of the foam dressing containing polyphosphate was significantly greater when compared to the standard foam dressing following 1, 3, 6, 24 and 48 hours incubation with the enzymes ($P<0.001$) (see figures 2-7).

The foam dressing containing polyphosphate significantly reduced the active enzymes (both MMP-2 and MMP-9) when compared to the TLC-NOSF dressing at 1, 3, 6 and 24 hours ($P<0.005$). Likewise there was a significant reduction ($P<0.001$) in active MMP-2 and 9 when tested against the standard foam dressing at 1, 3, 6, 24 and 48 hours.

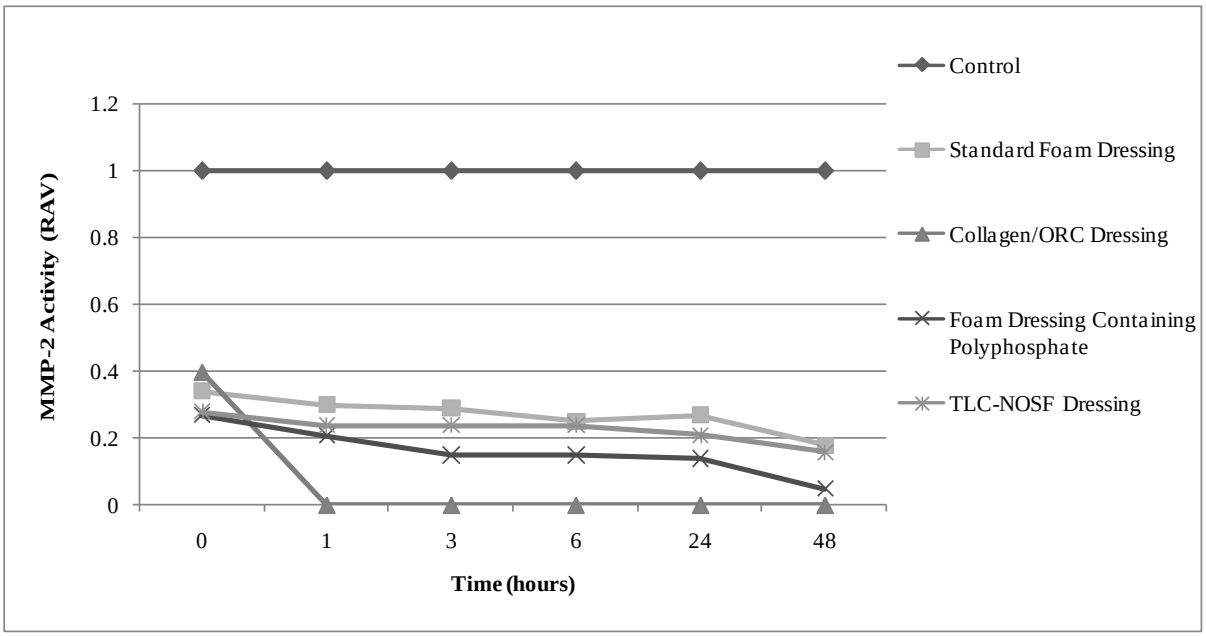

Figure 2: Action of each of the test dressings against the activity of MMP-2 in the latent form. Dressings were incubated with MMP-2 (n=3) for 0, 1, 3, 6, 24 and 48 hours.

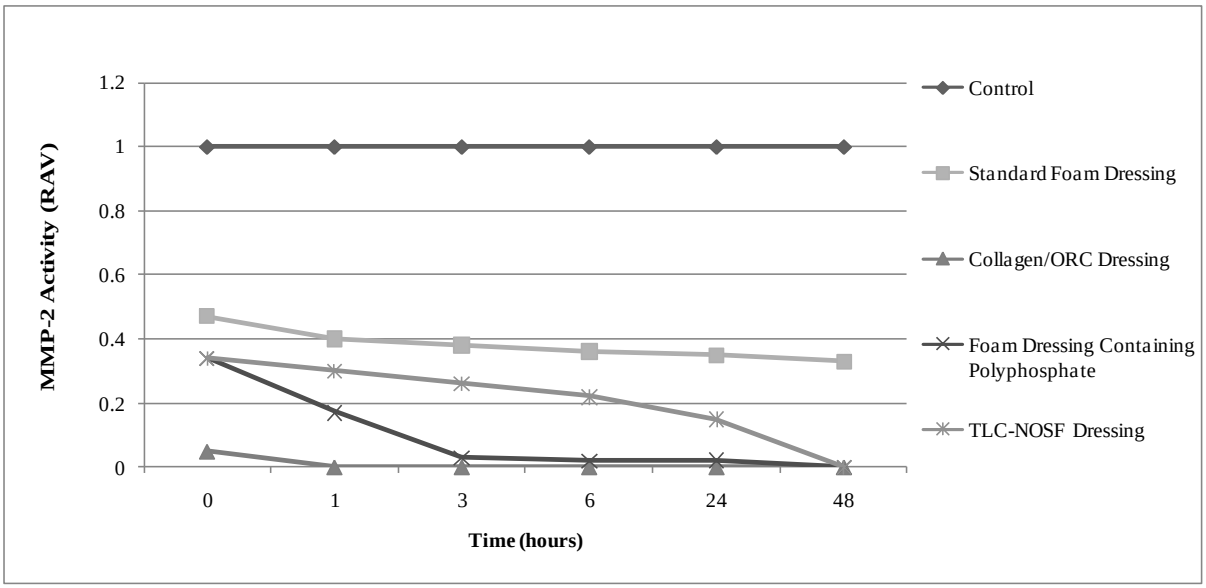

Figure 3: Action of each of the test dressings against the activity of MMP-2 in the active form. Dressings were incubated with MMP-2 (n=3) for 0, 1, 3, 6, 24 and 48 hours.

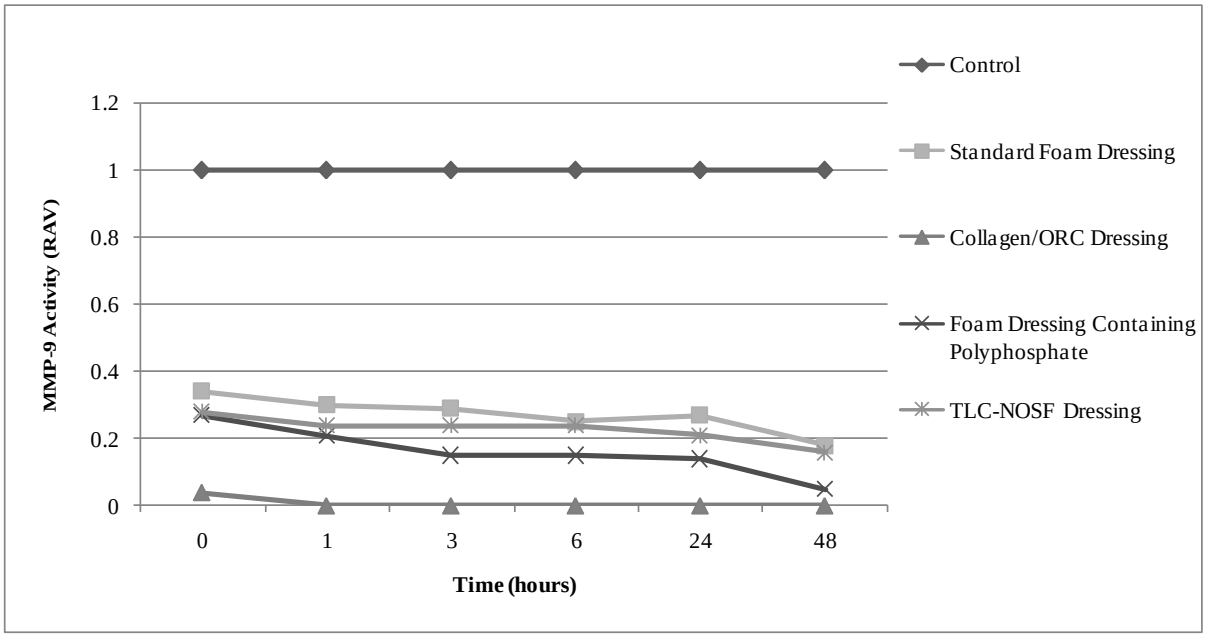

Figure 4: Action of each dressing against the activity of MMP-9 in the latent form. Dressings were incubated with MMP-9 (n=3) for 0, 1, 3, 6, 24 and 48 hours.

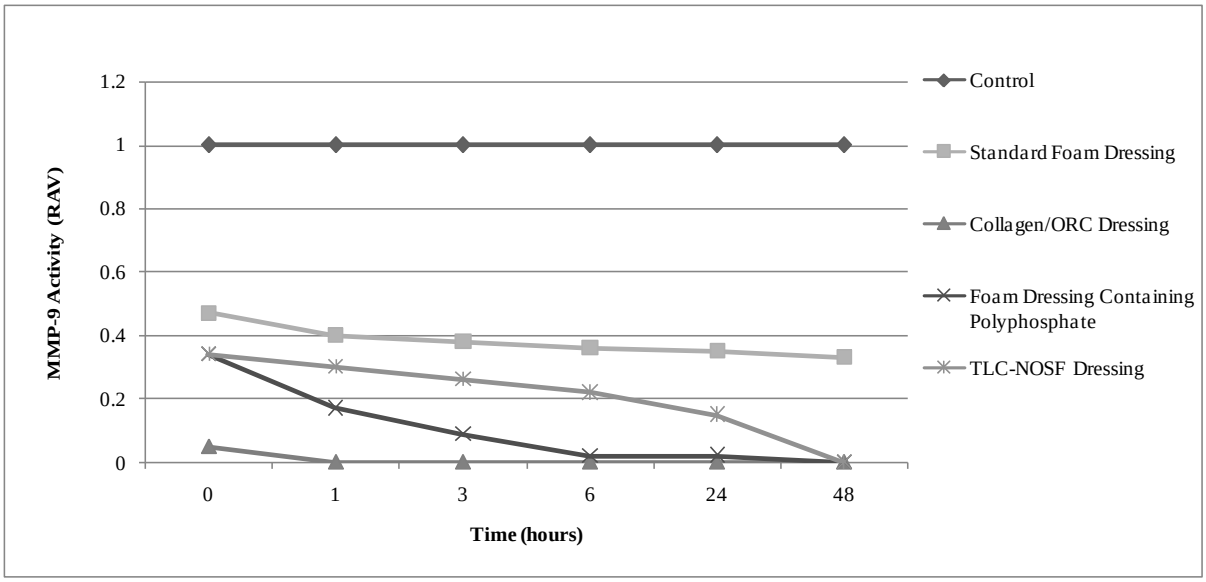

Figure 5: Action of each dressing against the activity of MMP-9 in the active form. Dressings were incubated with MMP-9 (n=3) for 0, 1, 3, 6, 24 and 48 hours.

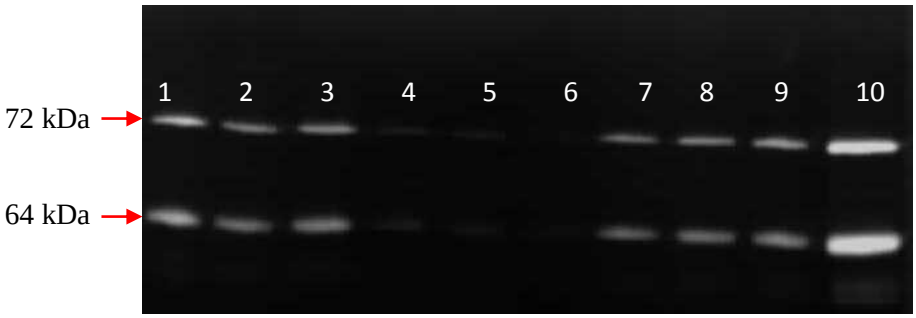

Figure 6: Example zymogram of samples from dressing incubations with MMP-2. The 72 kDa bands are indicative of latent MMP-2 whereas the 64 kDa bands are indicative of active MMP-2. Lanes 1-3: T0, standard foam dressing; Lanes 4-6: T0, Collagen/ORC dressing; Lanes 7-9: T0, Polyphosphate; Lane 10: T0, Control MMP-2 (no dressing treatment). There was an initial reduction in the level of MMP-2 activity (latent and active enzyme) following incubation with each dressing. The Collagen/ORC dressing resulted in the greatest reduction in MMP-2 activity at 0 hours (1 minute).

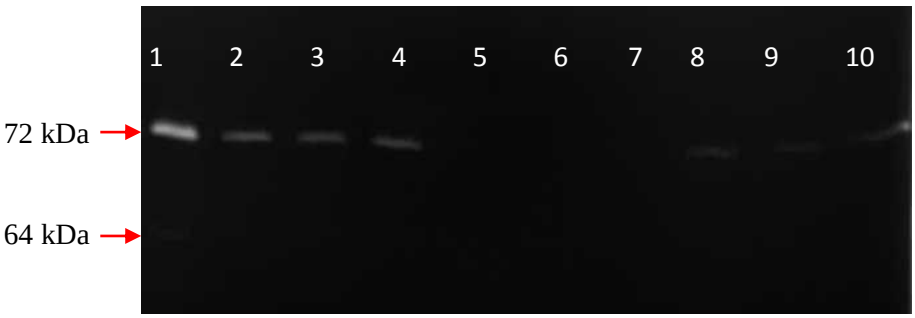

Figure 7: Example zymogram of samples from dressing incubations with MMP-2. The 72 kDa bands are indicative of latent MMP-2 whereas the 64 kDa bands are indicative of active MMP-2. Lane 1: T3, Control MMP-2 (no dressing treatment), Lanes 2-4: T3, standard foam dressing; Lanes 5-7: T3, Collagen/ORC dressing; Lanes 8-10: T3, Polyphosphate. There was a reduction in the level of MMP-2 activity (latent and active enzyme, but particularly the active enzyne) following 3 hours incubation with each dressing. The Collagen/ORC dressing resulted in the greatest reduction in MMP-2 activity at 3 hours, followed by the foam dressing containing polyphosphate.

5.4.2 Dressing Interaction Studies Using *Pseudomonas aeruginosa* Conditioned Media

Each of the test dressings were incubated as described for the MMP incubations, with 250µl filtered 24 hour conditioned culture media from 1 ATCC type strain and 2 chronic wound-isolated strains of *P. aeruginosa*. Milk-casein agar plate evaluation demonstrated a reduction in caseinolytic activity following dressing incubation with each of the dressings when compared with the bacterial conditioned media alone. The percentage decrease in caseinolytic zone area showed that the foam dressing containing polyphosphate sequestered *P. aeruginosa* derived proteases to a greater extent when compared to the standard foam dressing and the TLC-NOSF dressing after 24 hours (figure 8) when incubated with the ATCC type strain derived conditioned media. In contrast, the proteolytic activity of the ATCC *P. aeruginosa* conditioned media incubated with the TLC-NOSF dressing and the standard foam dressing returned after 24 hours. The Collagen/ORC dressing had the greatest effect against the proteases of this strain, reducing the proteolytic activity from 1 hour onwards when compared in terms of percentage decrease with the other dressing types. The foam dressing containing polyphosphate showed an optimum activity at each time point against the wound-isolated strain with high biofilm forming capacity, when compared to each of the other 3 dressings.

The activity of the Collagen/ORC dressing peaked following 3 hours incubation with the conditioned media of each strain but this dressing demonstrated the greatest activity against the ATCC *P. aeruginosa* reference strain derived conditioned media when compared to the chronic-wound isolated *P. aeruginosa* cultures. Results showed that following 24 hours incubation, the foam dressing containing polyphosphate was more active at reducing caseinolytic zones from the high biofilm-forming *P. aeruginosa* isolate derived proteases than the Collagen/ORC dressing. The foam dressing containing polyphosphate was also significantly better at reducing proteolytic activity than the TLC-NOSF dressing after 3 hours of incubation with the conditioned media from the high biofilm-forming isolate ($P<0.05$). SDS-PAGE analysis was conducted to identify the major proteases produced by *P. aeruginosa* isolates *in vitro* (see figure 9).

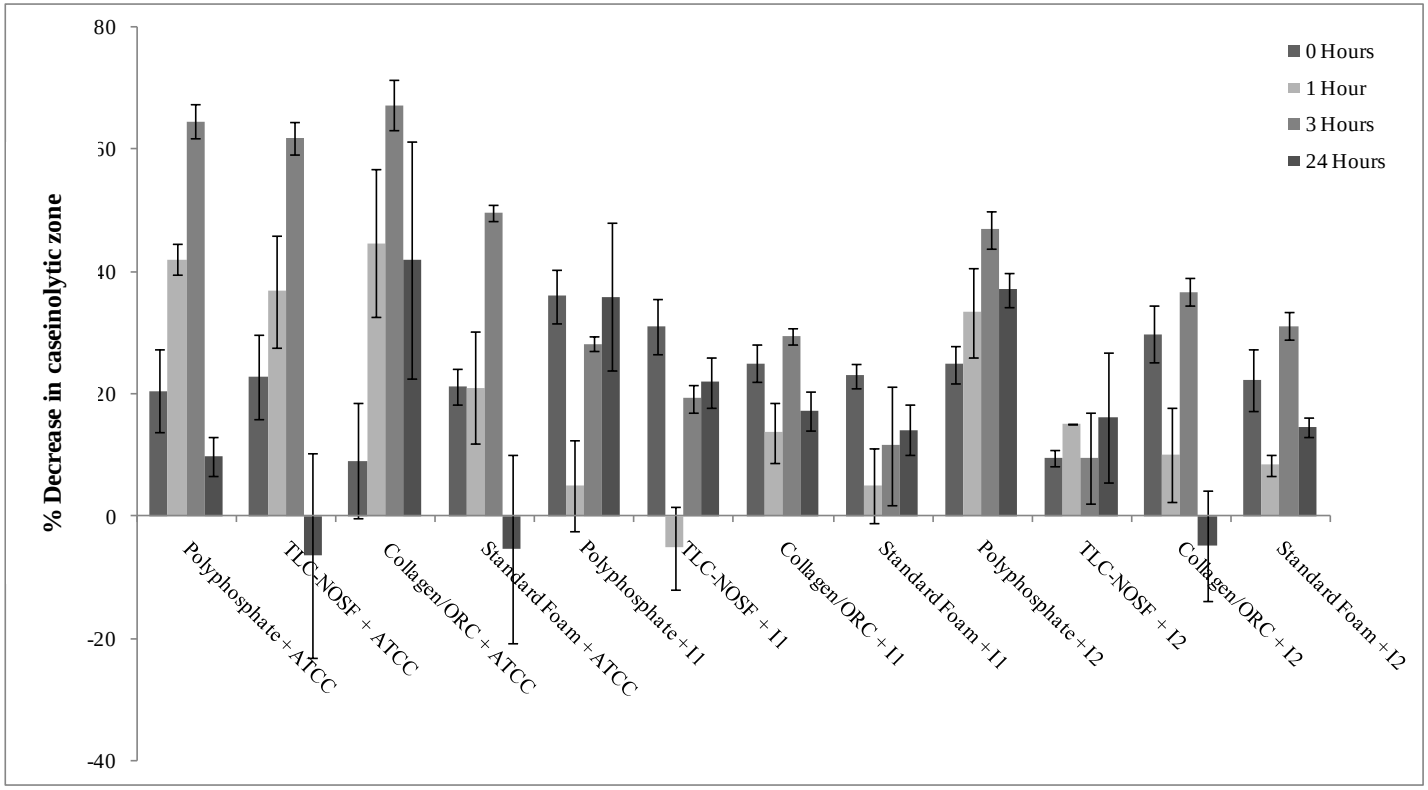

Figure 8: Percentage decrease in caseinolytic zone of milk-casein agar when incubated with each dressing and protease-rich conditioned media of an ATCC type strain and 2 chronic wound-isolated *P. aeruginosa*. Milk-casein agar plates were incubated (n=3) for 0, 1,3, 6 and 24 hours. I1: *P. aeruginosa* Wound strain 1 (low biofilm potential); I2: *P. aeruginosa* Wound strain 2 (high biofilm potential). 'Polyphosphate' refers to the foam dressing containing polyphosphate. Data is presented as mean ± SEM.

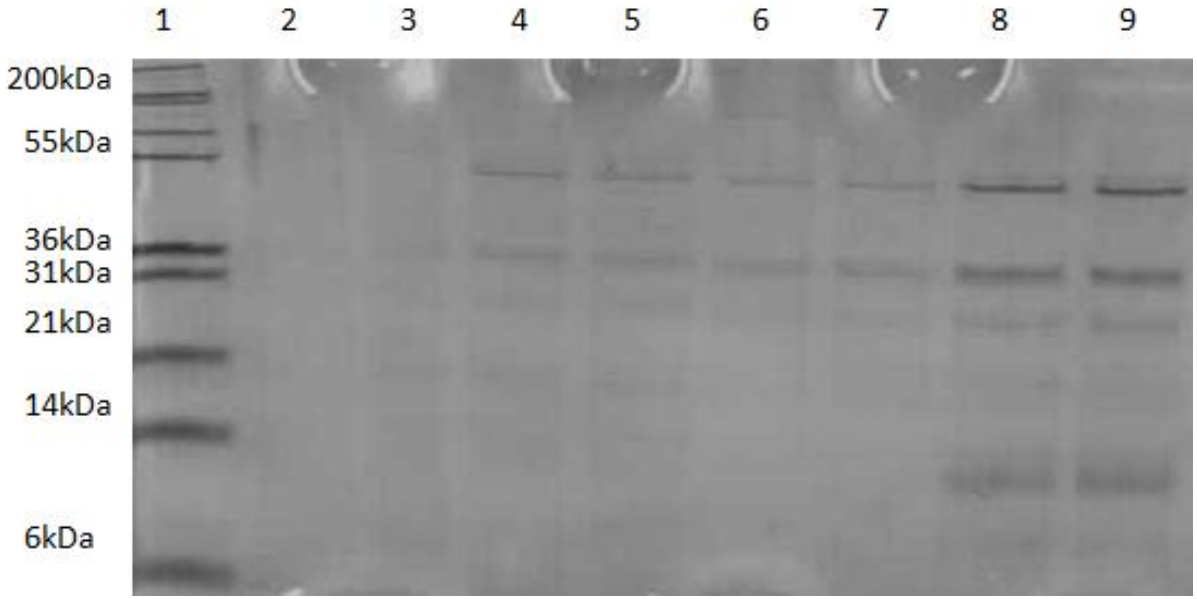

Figure 9: Example SDS-PAGE showing the major protein bands produced by *P. aeruginosa* chronic equine wound isolates at 4 hours (Lanes 2 & 3), 8 hours (Lanes 4 & 5), 12 hours (Lanes 6 & 7) and 24 hours (Lanes 8 & 9) after MHB inoculation. Lane 1: Protein markers; Lanes 2, 4, 6 & 8: Low biofilm forming isolate; Lanes 3, 5, 7, & 9: High biofilm forming isolate. The second bands from the top of the gel may represent the 33kDa protein, mature elastase whereas the top bands seen on the gel may represent the 51kDa proelastase or the 53kDa preproenzyme of elastase (McIver, Kessler & Ohman, 1991). However, for confirmation further protein analysis would be required.

5.4.3 Dressing Incubation with Live *P. aeruginosa* Cultures

Each of the dressings were tested for their potential ability in sequestering the proteases secreted into the media of a number of live *P. aeruginosa* cultures. Results showed that after 24 hours incubation the foam dressing containing polyphosphate reduced the caseinolytic zones of milk-casein agar plates inoculated with each of the clinical *P. aeruginosa* isolates when compared with the test plates with no dressing and compared to each of the other dressings (see figure 10).

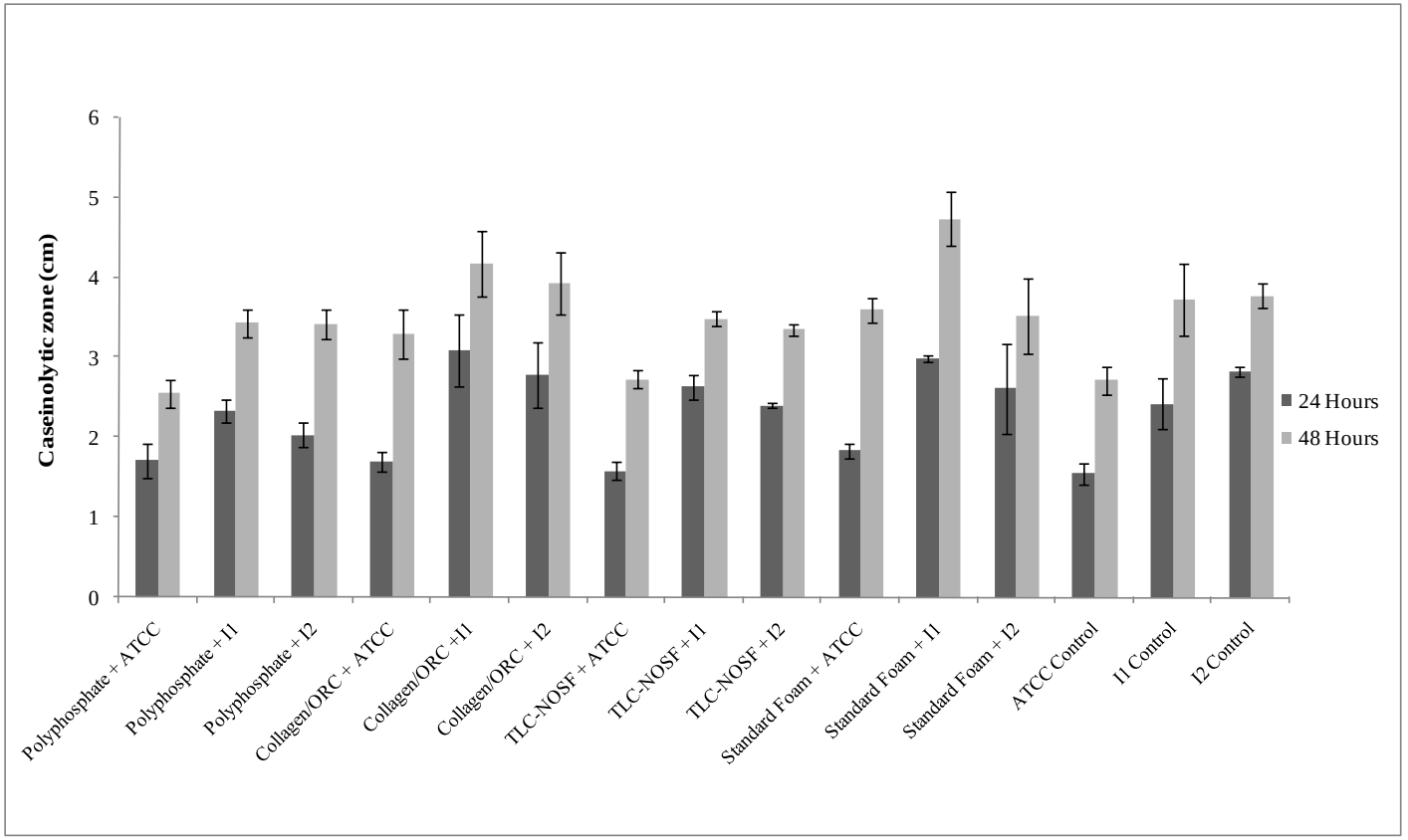

Figure 10: Caseinolytic zone measurements (average of 2 measurements) (n=3) of milk-casein agar plates inoculated with *P. aeruginosa* live cultures, overlaid with each of the 4 dressings. Plates were incubated for 24 hours and 48 hours and measurements made at each time point. ATCC: *P. aeruginosa* reference strain; I1: *P. aeruginosa* Wound strain 1 (low biofilm potential); I2: *P. aeruginosa* Wound strain 2 (high biofilm potential). 'Polyphosphate' refers to the foam dressing containing polyphosphate. Data is presented as mean ± SEM.

5.4.4 Dressing incubations with a combination of purified MMP-2 and *P. aeruginosa* conditioned media

Each of the dressings were tested for their potential ability in sequestering the proteases of *P. aeruginosa* conditioned media in combination incubations with purified MMP-2 (see figures 11 and 12). Results showed that the foam dressing containing polyphosphate reduced the activity of both MMP-2 and the *P. aeruginosa* derived protease steadily over a 6 hour period. However, following further incubation, the activity of the bacterial derived protease increased. This also occurred in the other dressing incubations, apart from the Collagen/ORC dressing which maintained its activity over the 24 hour period. The foam dressing containing polyphosphate performed better than the standard foam dressing throughout the incubation period.

Results from the zymography analysis revealed that the dressings had an immediate action in terms of sequestration of the active MMP-2 enzyme when in combination with *P. aeruginosa* derived protease. Similarly, the levels of *P. aeruginosa* protease activity decreased rapidly throughout the incubation period for all dressings tested. However, when incubated with the standard foam dressing the activity of *P. aeruginosa*-derived protease increased after 3 hours before reaching an activity that was over 80% of the control after 24 hours incubation. This also occurred when samples were incubated with both the foam dressing containing polyphosphate and the TLC-NOSF dressing but only after a 6 hour incubation period. The reason for this increase is not known, but may suggest that the dressings are only able to alter the activity of these enzymes for a limited period. The Collagen/ORC dressing was effectively able to reduce the activities of both MMP-2 and *P. aeruginosa* derived protease following 0 (1 minute), 3, 6 and 24 hours incubation when tested in this *In vitro* model.

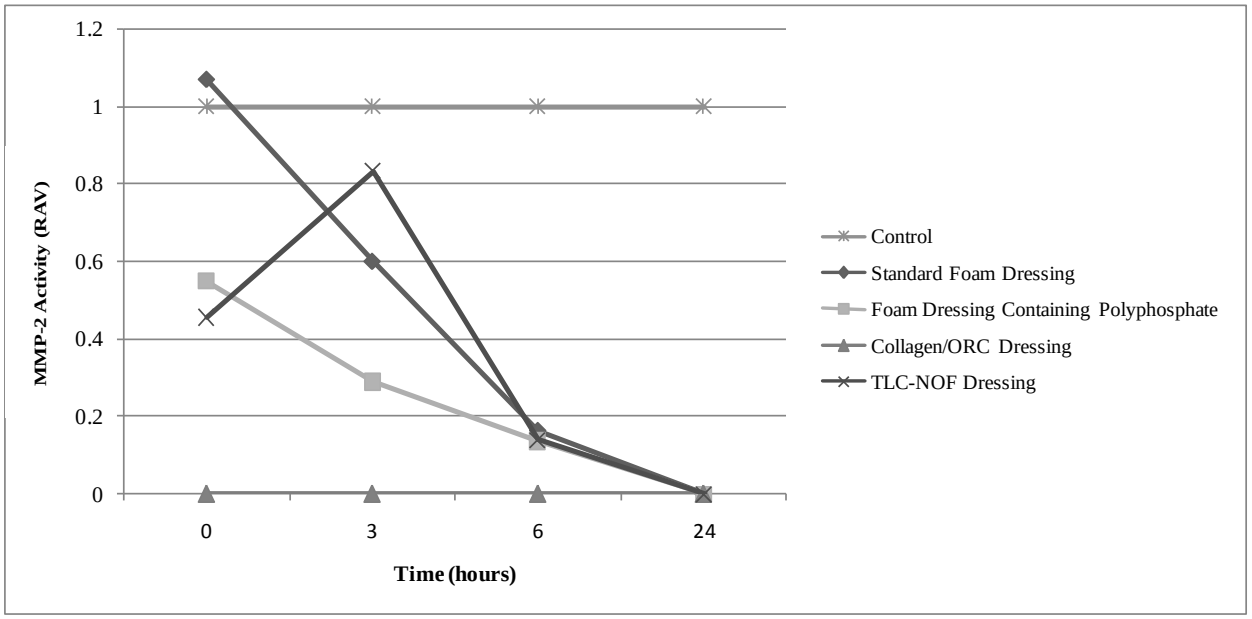

Figure 11: Action of each dressing against the activity of MMP2. Dressings were incubated with MMP2 (n=3) for 0, 3, 6 and 24 hours.

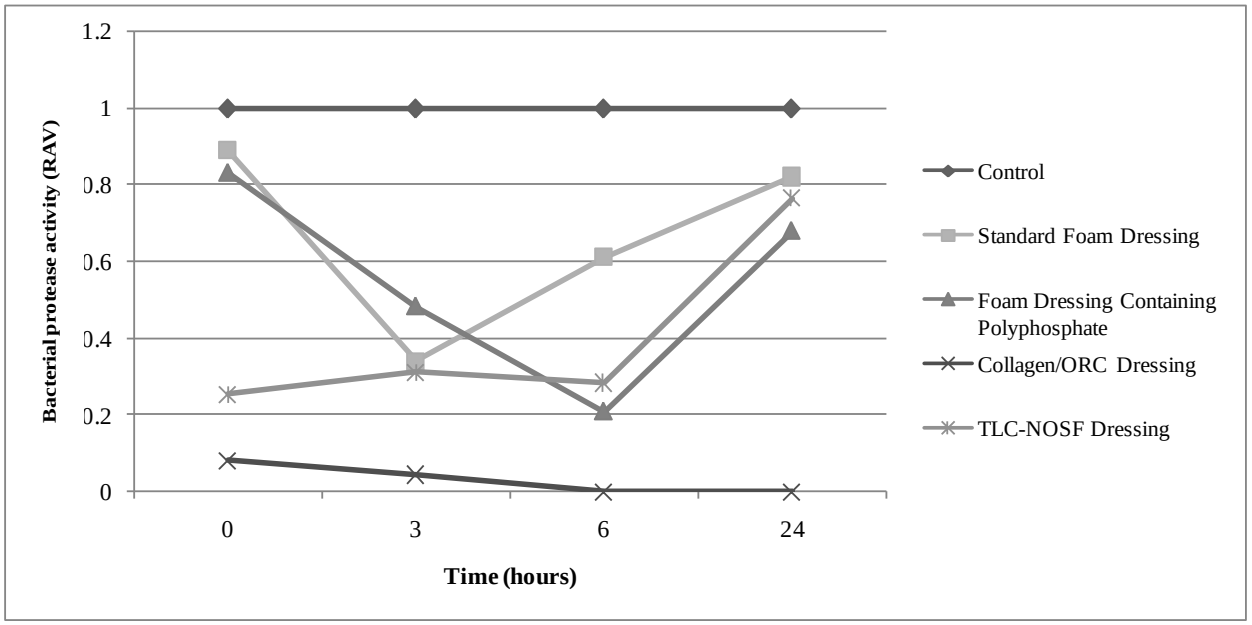

Figure 12: Action of each dressing against the activity of *P. aeruginosa*-derived protease. Dressings were incubated with bacterial protease (n=3) for 0, 3, 6 and 24 hours.

5.5 Discussion

It has been consistently reported that elevated levels of proteases, particularly the metalloproteases MMP-2 and MMP-9 are present in chronic wound fluids in a range of mammalian models (Cullen *et al*, 2002; Lund *et al*, 1999; Cochrane, 1997). It is likely that the sequestration of these harmful levels of MMPs within the wound bed could enhance healing and encourage matrix reformation. While it may not be possible, and would not be necessary, to completely remove MMPs from the wound environment or to completely inhibit their action, their activity may be contained sufficiently.

A number of dressings have been developed with the aim to retain MMPs within the wound bed or to sequester protease activity, including silver dressings (Walker, Bowler & Cochrane, 2007), collagen-containing dressings which act as an alternative substrate for MMPs (Cullen *et al*, 2002) and dressings which act to absorb and retain proteases within the matrix of the dressing whilst immobilising bacteria (Walker *et al*, 2003). While collagen is useful in wound healing as a mechanical scaffold to promote fibroblast migration (Hart *et al*, 2002) and can enhance the metabolic activity of newly formed granulation tissue, regenerated cellulose is able to effectively reduce levels of proteases. Another type of dressing incorporates a compound aimed at improving wound closure primarily through MMP inhibition, namely nano-oligosaccharide factor or NOSF. Polyphosphates have been used for their ability to chelate alginate, a product of biofilm forming bacteria. Molecularly dehydrated polyphosphates have been added to wound dressings to reduce the alginate present in infected wounds and they have proved more successful than organic surfactants in terms of their toxicity to the skin. Indeed, polyphosphates have been shown to be more effective at chelating and solubilising alginate than other commercially available chelating agents including EDTA, malonic acid and acetic acid. Furthermore, these other chelating agents require stabilisation of pH to ensure cytoprotection, which is not always possible during wound management.

Ribeiro *et al* (2009) described a chitosan hydrogel dressing which incorporated polyphosphate as an anticoagulant along with silver as an antibacterial agent. While many research studies have focussed on the anticoagulant properties of polyphosphates in wound dressings, the potential of this substance in protease sequestration is emerging. Since polyphosphates are negatively charged, they associate with and bind cations, including zinc ions and so prove useful in the sequestration of metalloproteinases. Furthermore, polyphosphate has also been shown to have antibacterial properties, although this has been insufficiently studied experimentally.

In the present study, it has been demonstrated that a foam dressing containing polyphosphate was able to sequester both MMP-2 and MMP-9 in their active and latent states as visualised using gelatin zymography. The results from this study also showed that the Collagen/ORC dressing effectively inhibited the activity of both MMP-9 and MMP-2, corresponding well to published data on the efficacy of this dressing (Cullen *et al*, 2002b). The Collagen/ORC dressing functions to actively bind and inactivate proteases involved in wound healing including MMPs, plasmin and neutrophil elastase (Cullen *et al*, 2002b). As in other studies, this dressing sustained its activity throughout the 48 hour test period. This coincides with the dressing change time period recommended for this type of dressing (Cullen *et al*, 2002b). The collagen component of this dressing acts as an alternative substrate, binding the proteases and thus inactivating them. The Collagen/ORC active agent is negatively charged, and is able to physically bind positively charged molecules including metal ions important for MMP structure and stability.

The current study represents significant data relating to the efficacy of a foam dressing containing polyphosphate in the sequestration of MMPs -2 and -9: the dressing was effectively able to reduce MMP-2 and MMP-9 activity throughout the 48 hour test period, with optimal activity observed after 6 hours of incubation with these enzymes. Furthermore, the foam dressing containing polyphosphate out-performed the standard foam dressing and the TLC-NOSF dressings in terms of reducing the levels of active MMP-2 and -9 over the entire incubation period and until 24 hours incubation, respectively. Future studies should aim to assess the capacity of the foam dressing containing polyphosphate to sequester other important proteases involved in wound healing, including neutrophil elastase and also any effect of this dressing on the levels of relevant protease inhibitors.

The foam dressing containing polyphosphate also demonstrated potential in the inactivation of *P. aeruginosa* derived proteases, both alone and in combination with MMP-2. Moreover, the foam dressing containing polyphosphate was more effective in the inhibition of proteases derived from a *P. aeruginosa* chronic wound-derived isolate with high biofilm-forming capacity when compared to a type strain and a low biofilm forming isolate. This may be important in relation to chronic wounds in which high levels of bacterial bioburden exist, particularly in the biofilm state. There is very little evidence in the literature to suggest a role of therapeutic wound dressings in inhibition of bacterial derived proteases despite evidence suggesting that these proteases play a vital pathological role in wound chronicity.

Whilst the methods employed in this study for the analysis of protease activity are semi-quantitative, the study demonstrates the potential of a novel foam dressing containing polyphosphate to inhibit both host MMPs -2 and -9 and also those exogenous proteases found within the wound bed. However, after longer incubation times the activity of the *P.aeruginosa* derived proteases increased for all dressing incubations apart from the Collagen/ORC dressing. This may be due to the limited effectiveness of dressings over extended time periods or may be a result of proteases being released back into the surrounding supernatant from the dressing samples. The dressings had an immediate action in terms of sequestration of the active MMP-2 enzyme when in combination with *P. aeruginosa* derived protease. Whether this is a direct synergistic effect of the presence of bacterial protease remains to be elucidated.

In future experimentation, fully quantitative methods should be used to confirm the levels of specific bacterial derived proteases; substrate assays can be utilised to assess specific protease activity. For instance, the elastin Congo red (ECR) assay can be used for the detection of *P. aeruginosa* elastase. Fluorescent substrate assays can also be used for quantitative analysis of proteolytic activity. Furthermore, other bacterial proteases should be considered in future studies. For example, *Staphylococcus aureus* produces a number of proteases (including the serine protease, V8 protease) which are considered important virulence factors within the wound milieu. Different combinations of host and bacterial proteases could be used to assess the ability of the foam dressing containing polyphosphate to sequester those proteases found within the chronic wound. Although the study of the mechanism(s) of action of the polyphosphate foam dressing was beyond the scope of the current study, the effect seen could relate to the inhibition of biofilm formation through the binding of zinc which is also the key pathway in the inhibition of metalloproteinases. Furthermore, chelation of Zn^{2+} specifically prevents biofilm formation by *S. epidermidis* and methicillin-resistant *S.aureus* (MRSA) (Conrady *et al*, 2008). However, the exact mechanism which allows for both MMP and biofilm derived proteases in combination remains to be elucidated. Yet, the therapeutic inhibition of zinc molecules within the wound bed may prove useful in inhibiting both biofilm formation (Conrady *et al*, 2008) and in the inactivation of proteases, both bacterial and host-derived.

Further investigation into the potential of the foam dressing containing polyphosphate to sequester proteases should also consider dressing absorption; proteases which are free within the dressing once absorbed may still be active and thus would be free to migrate back into the wound bed. It is important to investigate the mechanism of action of the dressing in terms of reduction of protease activity and to address the questions of whether proteases are bound within the dressing and whether they are permanently inactivated. In figures 2-5 and figures 11-12, there is evidence of protease activity returning over time following incubation of the proteases with the test dressing samples. This may be indicative of an initial decrease in protease activity due to dressing absorption; in future experimentation it may be useful to pre-wet the dressing samples so as to exclude absorption as an influential factor.

In terms of transposing the data presented to the human chronic wound, the equine model is an ideal model in which to study the naturally occurring human chronic wound (Cochrane, 1997; Cochrane, Pain & Knottenbelt, 2003). The pathophysiology of the equine chronic wound is very similar to that of the human chronic wound. For example, the equine indolent wound is similar in appearance to human venous leg ulcers; they produce little granulation tissue and are often inflamed and infected (Cochrane, 1997). The equine wounds used to isolate bacteria used in the present study were naturally occurring chronic wounds. Furthermore, gelatinase activity in equine tissue homogenates show that the major bands of activity in equine granulation tissue correspond to human MMP-2 and MMP-9 and proteolytic bands have been shown to be less prominent in normal tissue homogenates compared with granulation tissue homogenates (Cochrane, 1997). Whilst no *In vitro* model can mimic the true clinical environment in terms of the levels of proteolytic activity, future experimentation could utilise wound fluids collected from acute and chronic wounds to test the ability of wound dressings to sequester various proteases.

5.6 Conclusion

To conclude, this study has presented evidence to suggest a role for polyphosphate when incorporated into a foam wound dressing for the management of chronic wounds with elevated protease activity. A foam dressing incorporating polyphosphate has been shown to reduce the activity of both host MMPs -2 and -9 and *P. aeruginosa* derived proteinases in an *in vitro* model.

Acknowledgements

This work was funded by an educational grant from Advanced Medical Solutions Ltd.

6.0 Chapter 6

6.1 General Discussion and Future Direction

The main aims of this study were to investigate whether *P. aeruginosa* isolates from equine wounds display elevated levels of proteolytic activity *in vitro* and what effects changing the growth environment of these isolates, with relevance to the chronic wound environment, have in terms of protease production of these isolates. This study highlighted a clear variation in the protease expression of isolates derived from chronic equine wounds which corresponded well with previous work by Schmidtchen, Wolff & Hansson (2001). Furthermore, the reference, non-wound ATCC *P. aeruginosa* strain used in all experiments generally demonstrated lower overall protease secretion and lower elastase secretion in all culture conditions. These results collectively highlight the need for caution when interpreting results from reference strains and from bacterial isolates obtained from varied sources.

Following the work of Morihara (1964), which characterised *P. aeruginosa* proteases, many studies have focussed upon the pathogenic nature of *P. aeruginosa* isolates from various infection sites in terms of protease secretions. It has long been known that the pH of the wound bed is an important factor in the healing process, with different pH ranges required for different phases of healing. Oxygen tension also has an impact on healing and can cause alterations in patterns of bacterial pathogenicity and colonisation (Hunt *et al*, 1975; Sabra, Kim & Zeng, 2002; Sabra, Lünsdorf & Zeng, 2003). In order to investigate the effects of the culture environment of *P. aeruginosa* wound isolates on protease secretion into the surrounding environment, studies were conducted using *in vitro* culture models as described in Chapter 2. The study highlighted both the variation in protease activity between *P. aeruginosa* isolates and the variation in the response of isolates to changes in initial culture pH in terms of protease activity in the spent culture medium.

The study also revealed that protease production from the clinical isolates and the ATCC reference strain generally increased over the culture period, with caseinolytic and elastolytic activities generally maximal by the exponential phase of growth. This pattern of proteolytic activity was also maintained when the pH of the culture medium and oxygen availability was adjusted. It would be interesting to develop this study further by using repeated cultures from the same wounds over a period of time to assess the proteolytic profiles of *P. aeruginosa* isolates over the course of the wound healing process.

Given that *P. aeruginosa* virulence factor production, including protease production, is influenced by the nutritional complexity of the growth environment, particularly the presence or absence of metal ions (Twining, Davis & Hyndiuk, 1986; Olson & Ohman, 1992; Wagner *et al*, 2003), in future experiments alteration to the growth medium could be made in order to increase assay sensitivity since higher amounts of both alkaline protease and elastase are produced when such components are added to the growth medium (Twining, Davis & Hyndiuk, 1986). Indeed, a recent study has highlighted the significance of nitrogen and carbon sources as well as the pH, incubation temperature and incubation time in the production of bacterial proteases *in vitro* (Olajuyigbe, 2013). Furthermore, concentrated conditioned growth media could be used in assays used to detect proteolytic activity (Kadurugamuwa & Beveridge, 1995).

Production of virulence factors can be influenced by oxidative stress, as shown by recent evidence that virulence factor production, including rhamnolipid, elastase and alkaline protease, is affected by the aeration surface when cultured in shaken flasks (Pacheco *et al*, 2012). This work, along with work by Kronemberger *et al* (2008) who demonstrated an elevated production of proteases in cultures with a higher level of dissolved oxygen, ties in with the results from the present study whereby anaerobic *P. aeruginosa* cultures generally produced a lower total yield of proteases. However, when levels of protease activity were considered in terms of bacterial density, anaerobic cultures produced a higher level of proteolytic activity. Given the reports of increased protease activity under microaerobic conditions (Sabra, Kim & Zeng, 2002) the culture of *P. aeruginosa* isolates under varied oxygen tensions incorporating microaerobic conditions may reveal further differences in proteolytic activity in these changing environments.

In terms of the clinical *in vivo* environment, the use of multi-species bacterial cultures to reflect the microbial diversity of the chronic wound milieu could be utilised to further consider the synergistic effects of introducing multiple bacterial species on overall protease activity. The effects of commonly isolated wound bacteria, yeasts and fungi grown in mono-culture and in combination cultures can be used to establish which proteases are produced throughout the various growth phases. A recent study by Vandecandelaere *et al* (2014) generated some interesting data relating to the effect of protease production by *Staphylococcus epidermidis* on *Staphylococcus aureus* biofilms. They found that treating *S.aureus* biofilms with the protease-rich supernatant of *S.epidermidis* caused a decrease in the biomass of the biofilm, which was found to be mainly due to the actions of serine proteases causing a thinner biofilm with almost no ECM. The relationship between different bacterial species within a clinical biofilm and the implications on protease levels within a multi-species biofilm would be a basis for future research to more closely reflect the clinical situation.

Further investigation into possible differences in protease expression by *P. aeruginosa* isolates obtained from acute and chronic wounds could be conducted, relating such data to the clinical outcome of the wound. Dressings obtained from acute and chronic wounds can also be utilised to detect the presence and abundance of both host and bacterial proteases as the wounds progress. To more closely mimic a chronically infected wound and to investigate the effects that bacterial proteases have on tissue damage, a model such as the one used in the recent study by Harrison *et al* (2014) would be a useful tool for the assessment of the ways in which the wound environment, including during polymicrobial infection, influences protease levels.

It has been demonstrated that *P. aeruginosa* secretes some of its exoproducts within membrane vesicles during normal growth (Kadurugamuwa & Beveridge, 1995). These vesicles 'bleb-off' into the culture medium and it is proposed that they act as a mechanism by which *P. aeruginosa* secretes virulence factors, including proteases, into the surrounding environment (Kadurugamuwa & Beveridge, 1995). Indeed, more recent data has identified LasA protease as one of many extracellular proteins identified from *P.aeruginosa* outer membrane vesicles (Choi *et al*, 2011). *Pseudomonas* metalloproteases, elastase and alkaline protease have also been identified as outer membrane vesicle proteins in cultures challenged with the antibiotic ciprofloxacin (Maredia *et al*, 2012). Furthermore, these vesicles have been found to increase following exposure to gentamicin, a surface-active aminoglycoside antibiotic (Kadurugamuwa & Beveridge, 1995). In future studies, the presence of membrane vesicles in the supernatant of *P. aeruginosa* cultures should be considered; in culture supernatants a proportion of the proteolytic activity may be missed due to retained proteases within the membrane vesicles and thus the use of high-speed centrifugation to distinguish between membrane vesicle protease content and soluble proteases would allow for a more thorough evaluation of the proteolytic activity that is available to act upon the host tissues (Kadurugamuwa & Beveridge, 1995). In addition, in terms of the clinical situation, where antibiotics are administered the increase in membrane vesicles and their content should be appreciated. Enzymatically active elastase was not found in these membrane vesicles in the aforementioned study by Kadurugamuwa & Beveridge (1995); elastase is produced in an inactive form and the proenzyme only becomes active as it crosses

the outer membrane (Kadurugamuwa & Beveridge, 1995). Since the proenzyme entrapped within the membrane vesicle would not cross the outer membrane it would remain inactive (Kadurugamuwa & Beveridge, 1995). Thus, assays such as those used in the present study would not detect these enzymes unless they were activated. Given that bacteria produce outer membrane vesicles in response to stress factors (Maredia *et al*, 2012; Kuehn & Kesty, 2005), it would seem plausible to further investigate the effects of the wound environment on vesicle production. In addition, the abundance of proteases within the supernatant of biofilm cultures could have been underestimated due to entrapment of these enzymes within the alginate matrix. Therefore, further studies should explore the proteolytic content of the biofilm matrix as well as within the culture supernatant.

Given recent evidence that suggests a role of the type II secretion system, which is involved in the production of proteases, in the type III secretion pathway (Golovkine *et al*, 2014) it would also be interesting to investigate the impact of an active type II secretion system on other virulence factor production and the synergistic effect of these virulence factors on wound healing.

The present study demonstrated the variability in the BFP of *P. aeruginosa* isolates from chronic equine wounds; whilst most isolates were able to form biofilms to some extent, some isolates were unable to produce robust biofilms, as demonstrated via the crystal violet assay. Whilst it was not possible to make definitive conclusions with regards to a possible relationship between BFP and protease production in the present study, in future work the use of more *P. aeruginosa* isolates with varying BFPs should be used to investigate the effects of biofilm formation on the production of *P. aeruginosa* proteases. Previous studies have reported that the sessile or biofilm mode of growth and the mucoid phenotype affects bacterial proteolytic activity (Evans, Brown & Gilbert, 1994; Ohman & Chakrabarty, 1982; Mathee *et al*, 1999; Mohr *et al*, 1990). For instance, Mohr *et al* (1990) explored possible coregulation at the transcriptional level of LasB (encoding elastase) and algD (encoding alginate) and found that LasB and algD genes showed inverse levels of promoter activity. Henderson *et al* (2014) also found that *P.aeruginosa* growing in a thick mucus with biofilm physiology secreted proteases caused cleavage of mucin macromolecules and that proteases can cleave antigenically exposed regions of mucins in CF

secretions. Comparatively, Yu *et al* (2014) found that LasB elastase has a role to play in *P.aeruginosa* biofilm formation; a LasB deletion mutant displayed decreased bacterial attachment, microcolony formation and ECM linkage which was also associated with a decreased biosynthesis of rhamnolipids. In chapter 3 an *in vitro* biofilm culture model was described; the glass microscope slide biofilm model was used to assess the effect of the biofilm mode of *P. aeruginosa* growth on the production of proteases. The present study highlighted a clear difference between *P. aeruginosa* isolates from equine wounds in terms of both growth rate and protease production within developing biofilms *in vitro* but there was generally a low level of protease activity in these cultures. However, when compared in terms of biomass the biofilm cultures were actually producing higher protease levels relative to biomass than the planktonic cultures. Given the these findings, along with the current literature, it may be reasonable to suggest that proteases secreted during the early stages of biofilm formation aid in their development. Further characterisation of the *P.aeruginosa* isolates used in this study in terms of alginate production along with the development of biofilm models of varying maturity would further support the evidence suggestive of a relationship between protease activity and the biofilm mode of growth and the clinical implications this may have in terms of wound healing.

Given the evidence that heterogeneity exists among clinical *P. aeruginosa* isolates in terms of biofilm formation (Lee *et al*, 2005), it is likely that differences also exist in terms of the proteolytic activity of biofilms formed from different *P. aeruginosa* isolates obtained various chronic and acute wounds throughout their development. The present study, to some extent, corroborated this hypothesis in terms of general proteolytic activity of early developing biofilms *in vitro*, however further investigation is needed to fully characterise the proteolytic profiles of *P. aeruginosa* wound isolates during growth within mature biofilms. Microarray analysis would also allow investigation into the virulence genes which are up or down-regulated when bacteria are grown within the biofilm state (Jesaitis *et al*, 2003). Genomic characterisation of the isolates used in this study would give a much clearer understanding of the reasons for the elevated protease production of some isolates compared with others.

The aim of Chapter 4 was to investigate the effects of cell-free conditioned media from *P. aeruginosa* chronic wound isolates and purified *P. aeruginosa* elastase on the viability and growth of equine dermal fibroblasts. Given the limited evidence when this study was initiated suggestive of an effect of *P. aeruginosa* proteases in cutaneous wound healing with respect to the regulation of host proteases within the wound milieu, this study also aimed to further investigate the potential actions of *P. aeruginosa* exoproducts, including purified *P. aeruginosa* elastase, on host fibroblasts MMP and TIMP expression. Whilst there was a limitation of cell donors, clear trends in the inhibition of fibroblast growth and diminished fibroblast viability were observed with increasing concentrations of total protein concentration of *P. aeruginosa* conditioned media and purified *P. aeruginosa* elastase. These results corroborated previous findings by Schmidtchen *et al* (2003) who demonstrated that LasB from ulcer-derived *P. aeruginosa* inhibited fibroblast growth. Whilst it was not possible to analyse the results of the qRT-PCR data statistically due to a limitation in cell donors, each of the MMPs and TIMPs of interest were up-regulated relative to GapDH. It was also noted that fibroblasts derived from different donors and those from either granulation tissue or normal skin responded to treatment with *P. aeruginosa* conditioned media and purified *P. aeruginosa* elastase in different ways.

In order to fully characterise the effects of *P. aeruginosa* exoproducts on cell viability, assays such as the MTT assay (a colorimetric assay using a tetrazoilum dye) for cell activity and the caspase-3 assay for apoptosis can be utilised. The propidium iodide assay could also be used to assess any effects of the *P. aeruginosa* exoproducts on necrosis of dermal fibroblasts. Additionally, immunocytochemistry techniques can be employed to further investigate the effects of *P. aeruginosa* conditioned media and elastase on cultured fibroblasts including potential effects on cell surface components and ECM (Azghani, Kondepudi & Johnson, 1992).

Schmidtchen and colleagues (2003) demonstrated that *P. aeruginosa* growth and elastase production was similar when isolates were grown in bacterial growth medium, wound fluid and in the presence of human skin. Significant degradation of wound fluid components was noted in wound fluids incubated with elastase-producing *P. aeruginosa* and similar findings were observed when the *P. aeruginosa* isolates were cultured with human skin biopsies. In future work, it would be interesting to use *ex vivo* culture models with metabolic labelling of equine fibroblasts in the presence of live *P. aeruginosa* cultures, *P. aeruginosa* conditioned media and purified elastase to establish the effects of *P. aeruginosa* proteases on components of wound fluids derived from both acute and chronic equine wounds and how changes in the growth environment of *P. aeruginosa* isolates affects such degradation of wound fluid components. Fluorescence microscopy can also be employed as an advanced method to investigate further the effects of *P. aeruginosa* proteases on fibroblast morphology.

The immunohistochemistry images presented in Chapter 4 confirm the presence of bacterial clusters within equine wound tissue both at the outer edges of the wound area and within tissue sections taken from the middle of the wound, indicating that bacteria are disseminated throughout the wound bed. Furthermore, bacterial staining occurred in union with moderate staining of MMP-2, -3 and -13, with dark staining around immune cells which seems to link evidence of infection, inflammation and protease expression throughout the tissue. It may prove useful to further investigate the localisation of MMP/TIMP expression throughout larger granulation tissue and normal skin sections upon culture with *P. aeruginosa* elastase and other exoproducts using an *in vitro* organ culture model. Live *P. aeruginosa* cultures, incorporating specific elastase-producing and elastase-deficient strains, used in equine dermal fibroblast infection models would help to further establish the effects of *P. aeruginosa* secretions on fibroblast viability, growth and MMP/TIMP mRNA expression in a culture model which more closely reflects the *in vivo* environment. Given that *P. aeruginosa* proteases are regulated at different levels, including gene transcription, translation and pro-enzyme activation (Dong *et al*, 2000) zymographic analysis of concentrated fibroblast culture supernatants would help to establish at which level bacterial proteases enhance host fibroblast proteolytic activity.

In Chapter 5 the potential MMP modulating capacity of a novel foam wound dressing containing polyphosphate was investigated using an *in vitro* model. It has been previously shown that unbound polyphosphate can help to maintain a robust ECM and promote wound healing via the inhibition of proteases in a concentration dependent manner (Richardson *et al*, 2009). The present study examined the ability of a foam wound dressing containing polyphosphate to sequester MMPs -2 and -9 *in vitro* in comparison with 3 commercially available wound dressings. Using gelatin zymography, it was observed that the foam wound dressing containing polyphosphate was able to effectively sequester both active and latent forms of MMP-2 and MMP-9 throughout a 48 hour test period, with optimal activity of the dressing observed after 6 hours of incubation with the enzymes. Furthermore, this dressing proved effective in the minimisation of *P. aeruginosa* derived proteases; the polyphosphate-containing foam dressing was able to reduce protease levels of *P. aeruginosa* conditioned media obtained from chronic wound *P. aeruginosa* isolates with both high a low BFP. This study, therefore, demonstrates the potential of a polyphosphate foam dressing to inhibit both host proteases and exogenous proteases found within the wound bed.

Certainly, the data presented in this *in vitro* study should be interpreted as preliminary findings and future work should aim to corroborate these findings and to further characterise the mechanisms by which bacterial derived proteases affect the wound healing process *in vivo*. Nonetheless, the data presented in this study has highlighted a clear variation in the proteolytic profiles of *P. aeruginosa* isolates obtained from various equine chronic wounds. Furthermore, alterations in the growth environment of these isolates has demonstrated that both initial pH and oxygen availability, which are known to be important factors which affect the wound healing process, along with the growth mode of the bacteria (i.e. the planktonic versus the biofilm mode of growth) affects protease production by different *P. aeruginosa* isolates in different ways. It is important, therefore, in future studies to appreciate that there exists some deviation in the virulence of *P. aeruginosa* isolates derived from different host environments and in particular that the proteolytic activity of type strains varies from clinical isolates. The study has also demonstrated the potential effects of *P. aeruginosa* exoproducts on host fibroblast viability, growth and MMP/TIMP expression. The ways in which proteolytic activities can be manipulated within the wound milieu have been explored, and a novel foam dressing which incorporates polyphosphate has proven to be effective in its ability to sequester not only equine MMPs but also *P. aeruginosa* derived proteases.

Conclusions

Whilst there were a number of limitations with this study, inherent in all *in vitro* investigations, plus a limited number of bacterial isolates and tissue donors the study has clearly highlighted a variation in the proteolytic profiles of *P.aeruginosa* equine wound isolates and the detrimental effect that these proteases can have on host fibroblasts viability. Changes in the culture environment of these isolates, which represent the changes observed during wound healing, have been shown to have an effect on the proteolytic productivity of these isolates. Different isolates responded to these environmental changes in different ways in terms of proteolytic activity. The study has also demonstrated the ability of a novel foam dressing incorporating polyphosphate to sequester both host and bacterial proteases in an *in vitro* model.

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