An Investigation into the Significance of Tissue pH and Biofilms on Wound Healing.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by:

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Abstract

A new paradigm is coming to the forefront of chronic wound research and a potential treatment target; this is the wound pH. Non-healing wounds typically have a more alkaline pH, and from previous experimental studies, it appears that attempts should be made to reduce pH and shift the wound toward an acidic environment. This investigation aimed to determine the effects of pH alone and the combination of altering pH and bacterial biofilms on key wound healing processes *in vitro*. Normal healthy equine skin fibroblasts (NF) and equine chronic wound fibroblasts (CF) were cultured in pH 6, 7.5 and 9 conditions or planktonic conditioned media (PCM) and biofilm conditioned media (BCM) titrated to pH 6, 7.5 and 9. After 24 hours conditioned media was collected and analysed for the presence of collagen, fibronectin, glycosaminoglycans (GAGs) and tropoelastin. Cell migration was analysed over 48 hours: a scratch was created on a confluent layer of cells, and then cells were incubated in the different conditions and images taken at 0, 24 and 48hours. Scratch images were analysed using ImageJ and percentage wound closure determined.

Results revealed that pH had no effect on ECM abundance from CF, however, it was evident that there was a lower amount of these ECM molecules from CF compared to NF. BCM alone affected ECM abundance with no further effects seen with pH alterations. Migration of CF was significantly diminished in pH 9 conditions compared with pH 6 and pH 7.5. NF were not affected by alkaline pH, with similar migration rates observed in pH 7.5 and pH 9 conditions. The reduced rate of migration observed in alkaline conditions may be explained by the reduced abundance of ECM from CF. Cell migration was significantly affected by the combination of BCM and alkaline pH, with significant inhibition compared to other pH and PCM conditions. To my knowledge this is the first study which has investigated the effect of pH on wound related parameters and the combined effect of pH and bacterial biofilms on wound healing. This study demonstrates that pH does play a role in wound healing. However, it is important to note that many other factors are involved in chronic wounds and should be taken into account when developing future research. Findings here warrant further investigation into pH and wound healing *in vivo*. Simply making sure that the wound remains at neutral pH and does not increase to unfavourable alkaline levels may be sufficient to prevent wounds from progressing to a chronic non-healing state.

Declaration

I declare that this PhD thesis entitled 'An investigation into the significance of tissue pH and biofilms on wound healing' and the work presented in it are my own and has been generated by me as the result of my own original research.

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Ac yn olaf, ond yn wir yn llawn mor bwysig, hoffwn ddiolch i fy nheulu, Mam, Dad, Meinir, Nain a Taid Mynydd a Nain Gela am eu cymorth, cefnogaeth ac hanogaeth dros y blynyddoedd diwethaf yn ogystal ag ymddiried ynof i orffen ysgrifennu y traethawd hir yma.

List of Abbreviations

Abbreviation	Meaning
BCM	Biofilm Conditioned Media
COL1A1	Collagen Type1A1
COL1A2	Collagen Type1A2
CF	Chronic Fibroblast
CM	Conditioned Media
DCN	Decorin
ECM	Extracellular Matrix
EPS	Extracellular Polymeric Substance
FN	Fibronectin
GAG	Glycosaminoglycan
HA	Hyaluronan
HaCaT	Immortalised Epithelial Keratinocytes
HDF	Human Dermal Fibroblast
IL	Interleukin
LUM	Lumican
MMP	Matrix Metalloproteinases
NF	Normal Fibroblast
NHE	Sodium-Hydrogen Exchanger
PBS	Phosphate Buffered Saline
PCM	Planktonic Conditioned Media
PDGF	Platelet Derived Growth Factor
PG	Proteoglycan
pHi	Intracellular pH
SLRP	Small Leucine Rich Proteoglycan
TGF	Tumor Growth Factor
TIMP	Tissue Inhibitor of Matrix Metalloproteinases
TNF	Tumor Necrosis Factor
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
VCAN	Versican

Chapter 1:

Literature Review

1   Literature Review

1.1   Skin

Skin is often overlooked as being the largest and most extensive organ of the human body. It acts as a barrier to provide protection from physical, chemical, and microbial injury and its sensory components perceive heat, cold, pain, touch, and pressure. The skin is divided into an external layer of the epidermis, an inner layer the dermis and the subcutaneous hypodermis (Figure). The epidermis is composed of four different cell types: keratinocytes, melanocytes, Langerhans, and Merkel cells. Keratinocytes are the predominant cell of the epidermal layer and get their name from the protein they produce 'keratin'. Below the epidermis is the dermal layer (dermis) formed by cells and fibres and unlike the epidermis is richly innervated and vascularized. The most abundant cell of the dermis is the fibroblast; which is responsible for synthesising the molecules that make up its extracellular matrix (ECM). Finally, the hypodermis is a subcutaneous layer below the dermis that acts as an energy store.

1.2   Wound Healing

A wound is a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of underlying normal tissue (Enoch et al. 2006). A wound occurs as a result of disruption to the tissue and is either surgical or due to trauma. Wound healing is the process by which this damaged tissue is restored. Wounds typically heal in a timely and efficient manner, following distinct and overlapping phases of haemostasis, inflammation, proliferation and re-modelling (Beldon 2010).
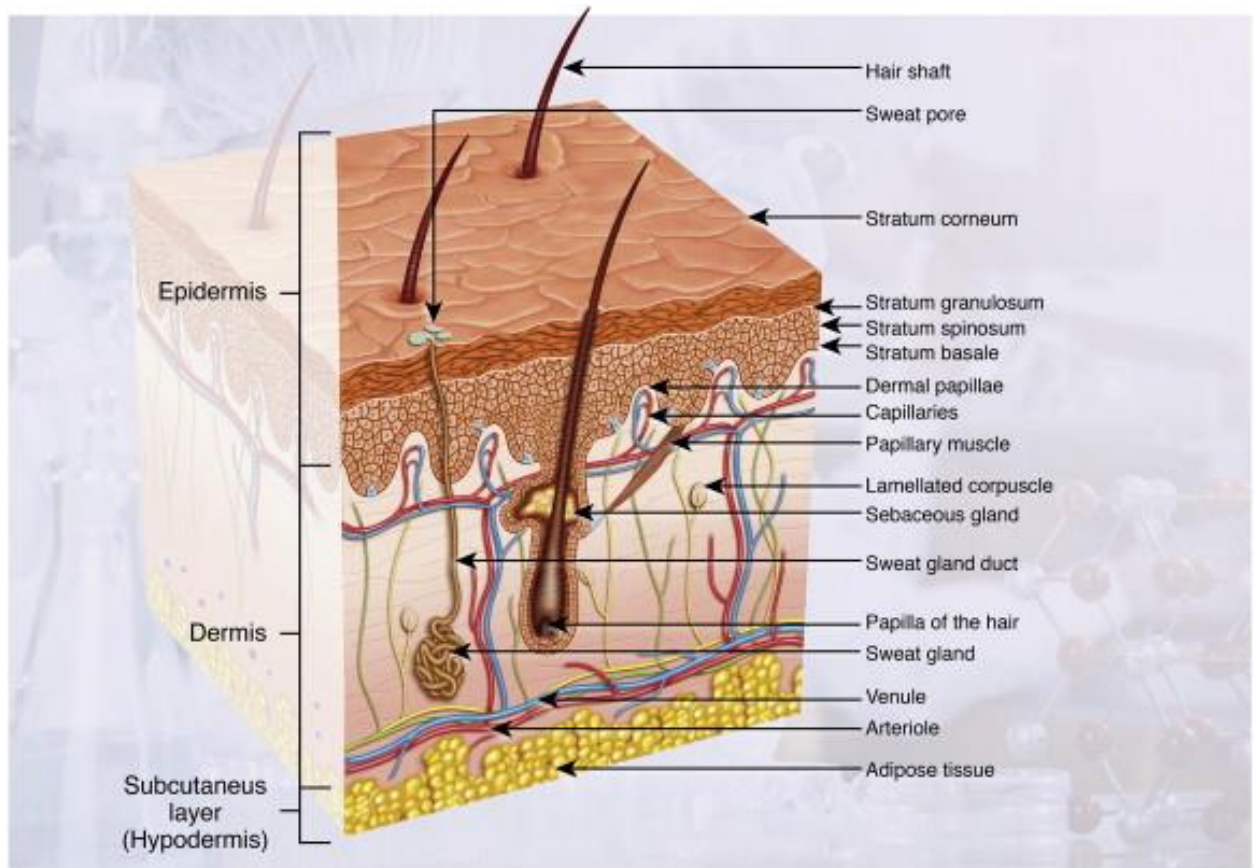
Figure 1.1 Schematic structure of the skin.

Image shows a diagram of human skin sub-classified into three main compartments: epidermis, dermis and hypodermis. Image taken from (Mathes et al. 2013).

1.2.1 Haemostasis

Within seconds of injury the initial phase of wound healing begins with vascular constriction and the activation of platelets. The platelets release several cytokines and growth factors and aggregate together triggering the coagulation cascade (Beldon 2010). This results in the activation and formation of a fibrin clot which serves to slow and prevent further bleeding. Chemokines released by platelet activation attract inflammatory cells to the wound area leading to the next phase in the healing process.

1.2.2 Inflammation

Inflammation begins within 24 hours of wounding; the aim of this stage is to debride and remove necrotic tissue in order to prevent infection. Regardless of the aetiology of the wound, the mechanical barrier which was the frontline against invading microorganisms is no longer intact. Neutrophils accumulate within the wound and act to cleanse the wound of bacteria and necrotic matter, this is shown by the appearance of pus and slough in the wound bed (Beldon 2010). The neutrophils then begin to disappear from the wound bed via apoptosis; they are replaced by monocytes which undergo a phenotypic change to become macrophages. Macrophages are the most important cells in the later stages of the inflammatory phase (Enoch et al. 2006). They function as a phagocytic cell and remove all non-viable cells, damaged ECM and any bacteria present within the wound site. Another biochemical event associated with debridement of the wound during the inflammation stage is the activation of matrix metalloproteinases (MMPs). In the absence of injury these proteolytic enzymes are quiescent mainly due to tissue inhibitors of matrix metalloproteinases (TIMPs). Following injury, TIMP activity significantly decreases and MMP activity is increased as they actively break down devitalized tissues (Enoch et al. 2006). Macrophages release platelet-derived growth factor (PDGF) and tumor growth factor-β (TGF-β) which help to promote proliferation of fibroblasts into the wound for production of ECM, proliferation of smooth muscle cells and endothelial cells to promote angiogenesis (Enoch et al. 2006).

1.2.3 Proliferation

Once haemostasis has been achieved, the inflammatory response is balanced and the wound is fully debrided, the proliferative stage of the healing cascade can begin to repair the wound. The proliferation phase is characterised by fibroblast migration, deposition of ECM and formation of granulation tissue that replaces the original temporary fibrin clot (Enoch et al. 2006). Fibroblasts proliferate and synthesise proteins including fibronectin, hyaluronan (HA), collagen and proteoglycans; these form the new ECM, which supports further growth of cells essential for wound repair. Formation of granulation tissue indicates optimal healing with a pink, soft appearance. The process of angiogenesis is dependent on the production of ECM and granulation tissue which acts as a scaffold to support the newly formed blood vessels. Wound contraction occurs through the interactions between fibroblasts and the surrounding extracellular matrix and is influenced by a number of cytokines (Enoch et al. 2006). Contraction in open wounds is desirable so that less connective tissue is required to fill the wound and the wound will heal quicker. Epithelialisation is the final visible stage of wound healing; epithelial cells migrate from the wound edges to resurface the wound area. Epithelialisation requires a moist environment, adequate nutrition, and bacteriological control, which is modulated by several growth factors (Enoch et al. 2006).

1.2.4 Remodeling

The remodeling phase of healing can continue for up to a year. Inflammatory cells disappear, and growth factors decrease. Fibroblast numbers decrease but they continue to deposit collagen. There is continuous synthesis and breakdown of collagen as the extracellular matrix is constantly remodeled, eventually equilibrating to a steady state. Ultimately the granulation tissue evolves into a scar, but the scar tissue is never more than 80% of the tensile strength of unwounded skin (Enoch et al. 2006). Occasionally an imbalance can occur during the remodeling phase disrupting the synthesis and degradation of the ECM resulting in abnormal hypertrophic scars or keloid formation (Beldon 2010).

1.3 Chronic Wounds

Some wounds fail to heal in a timely and orderly manner, resulting in non-healing wounds that require continued medical or veterinary management. A chronic wound occurs when it fails to progress through the normal phases of healing and remains in the inflammatory phase, causing an increase in levels of degrading proteases which destroys components of the ECM that are essential for the wound healing process (Broderick 2009). As a result re-epithelialisation cannot occur affecting the anatomic and functional integrity of the skin.

The most common types of chronic wounds are venous leg ulcers, diabetic foot ulcers and pressure ulcers (Mustoe et al. 2006) (Figure 1.2). Leg ulcers are commonly caused by venous hypertension which causes swelling, restricted blood flow and skin damage (Beldon 2010). A common complication of diabetes is foot ulcers, loss of sensation makes the foot susceptible to minor trauma. Patients are susceptible to infection which inhibits healing once injured and often leads to gangrene and amputation of the foot (Posnett & Franks 2008). Pressure ulcers (also referred to as bed sores) result from prolonged pressure or friction to an area of skin that becomes damaged and infected. Chronic wounds are a major burden to the patient causing pain, suffering, sepsis, infection, nausea, fatigue, depression, loss of function and psychological disturbances (Hurd 2013).

Chronic wounds are a major health problem worldwide with an estimated 200,000 patients suffering in the UK alone (Posnett & Franks, 2008). The majority of chronic wounds occur in older patients, the population is ageing rapidly and the percentage of people over the age of 65 is expected to increase from 15.9% to 26.5% by the year 2050 (Hurd 2013). Due to this ageing population and the growing incidence of diabetes and obesity worldwide the burden is fast increasing (Sen et al. 2009). Chronic wounds will soon become a significant problem not only for the welfare of patients but also the cost of treatment to governments and health care organisations. It is estimated that chronic wounds cost the NHS around £2.3 – 3.1 billion per year, which is 3% of their budget (Posnett & Franks 2008). With proper diagnosis and treatment this burden could be avoided.

Figure 1.2 Images of Chronic Wounds

The most common type of chronic wounds are: diabetic foot ulcer (A), venous leg ulcer (B) and pressure ulcers (C and D). In D the wound bed appears sloughy due to clearance of debris and presence of bacteria. Images obtained from Beldon 2010.

1.4 Equine Wounds

Equine chronic wounds have a similar pathophysiology to human chronic wounds (Westgate et al. 2011). This is of particular interest as laboratory rats do not commonly suffer from chronic wounds, thus there is currently no adequate animal model for human chronic wounds (Dorsett-Martin & Wysocki 2008). Wounds can be traumatic or surgical and often fail to heal despite extensive veterinary intervention. They are then described as chronic. Chronic wounds are highly prevalent in horses and represent a significant challenge to veterinary surgeons (Figure 1.3). The process of equine wound healing is fundamentally the same as all mammalian wounds, for example, wounds on the equine lower limb heal significantly slower than wounds in the thoracic region (Theoret & Wilmink 2013), which is due to poor blood supply and reduced tissue oxygenation in the limb. Wound closure is more rapid in ponies than in horses; this is because ponies have a better local defence mechanisms which means the inflammatory phase is shorter, thus a shorter time frame for the wound healing process (Westgate et al. 2011). Equine wounds are characterised by an elevated level of excessive granulation tissue during wound healing. Chronic wounds can be a risk factor for formation of the local invasive neoplasm, sarcoid, and in some instances may resemble the human keloid scar (Cochrane et al. 1996). Equine wounds, specifically lower limb wounds are a particular concern for the risk of infection. The risk of infection is greater in horses than in humans, due to the horses local environment which provides a reservoir of microorganisms that have the ability to colonise open wounds (Westgate et al. 2010). Furthermore, it has been shown that equine chronic wounds similarly to human chronic wounds are contaminated with bacterial biofilms that inhibit wound healing (Westgate et al. 2011).

Figure 1.3 Equine Chronic Wound

Images of chronic wounds on equine lower limbs. Wound shows multiple factors that could affect wound healing including infection and necrotic tissue (Westgate et al. 2011).

1.5 The Extracellular Matrix

The synthesis of ECM from dermal fibroblasts plays a vital role in supporting normal wound healing. Fibroblasts migrate to the wound area and are present in a healing wound from late inflammatory phase until re-epithelialisation is complete (Bainbridge 2013). Fibroblasts migrate into the wound site secreting ECM proteins including collagen, glycoproteins, proteoglycans and glycosaminoglycans (GAGs). The ECM supports and directs a specific organised response throughout the four phases of healing influenced by specific cellular interactions and growth factors (Schultz & Wysocki 2009). This allows for successful angiogenesis, granulation-tissue formation, re-epithelialisation and re-modelling of the skin. However, in a chronic non-healing wound, the fibroblasts become unresponsive and the ECM molecules become disorganised, leading to a disruption in the phases of wound healing (Broderick 2009; Jones et al. 2014).

The ECM is a highly complex non-cellular component of all tissues within the body. It is the largest component of the skin and is fundamental in the process of wound healing. Not only does it provide the cells with an essential scaffold for support, it is key in initiating and directing biochemical and biomechanical processes crucial for tissue differentiation and homeostasis. In the context of wound healing the ECM consists of four main groups of proteins: structural proteins, adhesive glycoproteins, GAGs and matricellular proteins (Schultz & Wysocki 2009) (Table 1.1).

Table 1.1 The major classes and Extracellular Matrix components involved in wound healing.

Table summarises the major protein classes and subsequent ECM molecules involved in wound healing (Schultz & Wysocki 2009; Iorio et al. 2015)

Major Proteins Classes	Extracellular Matrix proteins		
Structural Proteins	Collagen I, III, IV and V Elastin		
Glycoproteins	Fibronectin Vitronectin Laminin 3A32, 3B32, 411, 3B11, 511 and 521		
Glycosaminoglycans and Proteoglycans	**GAGs**	**Small PGs**	**Large PGs**
	Dermatan Sulfate	Decorin	Versican
	Chondroitin Sulfate	Biglycan	Perlecan
	Heparin Sulfate	Fibromodulin	Aggrecan
	Keratan sulphate	Lumican	
	Hyaluronic acid		
Matricellular Proteins	SPARC (also known as osteonectin) Thrombospondin-1 Tenascin-C Osteopontin		

1.5.1 Collagen

Collagen is the most abundant ECM protein; the skin consists of mainly collagen type I, along with small amounts of collagen III, IV and V (Lovell et al. 1987). Collagen provides the skin with tensile strength and stability (Schultz et al. 2005). During early phases of wound healing collagen III is secreted which is eventually replaced by collagen I in normal wound healing (Lovell et al. 1987). Collagen type IV is found along the basement membrane where it interacts with other ECM proteins and where the epidermal cells attach (Schultz et al. 2005). Collagen molecules must bind specifically with each other in order to form a collagen fibril. Partially degraded collagen molecules will not bind with newly synthesised collagen molecules during scar formation, resulting in a weak and disorganised ECM (Schultz et al. 2005). During the process of wound healing it is vital that degraded collagen molecules are removed by the controlled action of MMPs. In a chronic wound the synthesis of collagen becomes disorganised, due to excess MMPs, resulting in excess granulation tissue. When the wound eventually heals the resulting scar tissue never achieves the same tensile strength (Schultz & Wysocki 2009). Studies found that chronic fibroblasts are not able to synthesise collagen to the same extent as normal healthy fibroblasts (Herrick et al. 1996), other investigators however found no differences unless cells were stimulated with TGF-β (Hasan et al. 1997). In contrast to this, histological studies found chronic wound tissues are abundant in collagen (Herrick et al. 1992).

1.5.2 Elastin

Elastin is an important ECM protein as its function in the skin is to provide elasticity and give such tissues their recoil ability following stretching (Schultz et al. 2005). The presence of elastin has not been found in chronic wound granulation tissue to date and data on elastin in chronic wound healing is lacking (Schultz & Wysocki 2009). Details of elastin degradation is limited within chronic wounds, but in burn wounds it is known that elastase is responsible for the degradation of fibronectin (Grinnell & Zhu 1994). Elastin derived peptides have

shown beneficial effects on wound healing; proteases act on elastin to release these proteins resulting in increased fibroblast proliferation and an increase in collagen and tropoelastin synthesis (Antonicelli et al. 2009). Fibroblasts secrete monomers of tropoelastin into the extracellular space where they are then transformed into elastic fibres (Muiznieks & Keeley 2013). Tropoelastin has been shown to promote fibroblast attachment and proliferation through the binding of integrins in an *in vitro* model (Tracy et al. 2014).

1.5.3 Fibronectin

Fibronectin is an adhesive glycoprotein synthesised by local cells. It exists in either a modified soluble form primarily found in plasma or in an insoluble form found in the ECM. It is a multifunctional protein which can act as a biological glue allowing it to interact with other ECM components to direct cell attachment and organisation (Clark 1990). Fibronectin is essential for cellular migration and wound closure. Upon injury to the skin fibronectin is incorporated into the fibrin clot forming a provisional matrix that acts as a scaffold to guide migrating cells into the wounded area (Grinnell et al. 1981). A histological time course study of a healing wound showed an increase in the presence of fibronectin over time (Herrick et al. 1992). Strong fibronectin staining was present in the base of ulcer wounds which correlates with the requirement of fibronectin for epidermal cells to migrate across the wound bed (Herrick et al. 1992). Studies have shown that inhibiting fibronectin deposition can inhibit cell growth, adhesion and migration (Clark et al. 1982; Clark et al. 2003; Briggs 2005). Fibronectin is critical in the process of normal wound healing and is required for deposition of collagen and other structural proteins into the ECM (Sottile 2002). In chronic wounds fibronectin is degraded by proteases; fibronectin degradation products have been found in chronic wound fluid along with excessive activities of serine proteases such as elastase and plasmin (Grinnell & Zhu 1996).

1.5.4   Glycosaminoglycans and Proteoglycans

Glycosaminoglycans (GAGs) are long polysaccharide molecules that can exist independently or bound to a proteoglycan core. GAGs have specific properties (viscosity and lubrication) contributing to cell adhesion and water attraction such that local cells maintain hydrated. During wound healing GAGs bind to chemoattractants such as TGF-β to provide cues to direct the migration of inflammatory cells towards the wounded area. In chronic granulation tissue GAG levels are increased correlating with high levels of HA found in foetal wounds that supports scarless wound healing (West et al. 1997).

Hyaluronan

Hyaluronan (HA) is the predominant GAG in the skin and is also found in increased amounts in damaged or developing tissues (Schultz et al. 2005). Oral fibroblasts, associated with rapid and scarless wound healing are unable to express HA; in contrast, foetal wound healing has demonstrated high expression of HA (Tracy et al. 2014).

Dermatan sulphate and Chondroitin sulphate

Dermatan sulphate and chondroitin sulphate are the GAGs most commonly bound to PGs. The most prevalent GAG in the skin and also in acute human wounds is dermatan sulphate (Penc et al. 1998). Fibroblasts within fibronectin/fibrin gels require dermatan sulphate for successful migration during the early phases of wound healing (Clark et al. 2004).

Proteoglycans (PG) consist of a protein core that is covalently bound to one or more GAG side chain. PG can be divided into the small leucine-rich PGs (SLRPs), which are decorin, biglycan, fibromodulin and lumican; and the larger aggregating PGs: versican, aggrecan and perlecan (Smith & Melrose 2015). PG may have a role to play during wound healing as they are important in regulating cell proliferation, matrix adhesion and assembly (Lundqvist &

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Schmidtchen 2001). PGs in chronic wounds have not been studied in depth, a histological study found some differences between human normal skin and chronic ulcer wounds (Lundqvist & Schmidtchen 2001). The location of PGs in chronic wounds could have an important role as their function in binding growth factors could further influence wound healing.

Small Leucine Rich Proteoglycans (SLRPs)

Decorin

Decorin is the most abundant PG in adult human skin and has a binding affinity for TGF-β1 (Hildebrand et al. 1994). It can act as an inhibitor of TGF-β1 which is associated with normal scar formation. The inhibition of decorin results in increased fibroblast proliferation, adhesion and migration *in vitro* however, inconsistently, significantly delayed wound healing *in vivo* (Ferdous et al. 2010).

Biglycan

Biglycan is a PG commonly present in the skin, localised to the ECM of the epidermis and the basement membrane. It has been shown to be upregulated in fibrotic diseases, however little else is known about its role in wound healing (Tracy et al. 2014).

Lumican

Lumican is a PG abundantly expressed in interstitial tissue of the skin, corneal stroma, sclera, aorta, muscle, lung, kidney, bone and cartilage (Yeh et al. 2010). It can modulate collagen fibril assembly (Yeh et al. 2010), migration of inflammatory cells and modulates epithelial cell migration and proliferation (Saika et al. 2000). Lumican is secreted by epithelial cells and following wounding this is up-regulated (Saika et al. 2000). The importance of lumican in wound healing was demonstrated by Saika et al. who showed that anti lumican antibodies inhibited cell migration *in vitro* and lumican null mice showed delayed re-epithelialisation of corneal epithelial defects. Another study also demonstrated the requirement of lumican for successful skin repair, as shown by significantly delayed healing in lumican null mice

compared with that in wild type mice. Lumican can promote skin wound healing by facilitating wound fibroblast activation and contraction via integrin α1 (Liu et al. 2013).

Large Proteoglycans

Versican

Versican is associated with fibrotic wound healing as demonstrated by greater expression in hypertrophic burn scars and significantly increased synthesis by deep dermal fibroblasts (with a role in scarring) compared to superficial fibroblasts (Wang et al. 2008).

Aggrecan

Aggrecan is often absent from normal adult skin but has been found to accumulate in scar tissue (Velasco et al. 2011). When aggrecanase (e.g. ADAMTS-5) activity is diminished, aggrecan accumulates in the pericellular matrix of progenitor fibroblasts; this possibly prevents them from maturing sufficiently to assist in wound healing (Velasco et al. 2011).

1.5.5 Matricellular Proteins

Matricellular proteins include: SPARC (Secreted protein, acidic and rich in cysteine, also known as osteonectin), osteopontin, thrombospondin-1, tenascin-C, fibulin-5 and CCN2. They do not act as structural proteins but control and regulate interactions between the cells and the ECM (Schultz & Wysocki 2009). Often they are completely absent or only present at low levels in uninjured skin, however, following tissue injury they are upregulated (Tracy et al. 2014). Therefore, any alterations in these proteins could result in a non-healing wound. Knockdown of osteopontin promotes wound repair and reduces scarring by reducing leukocyte recruitment and altering ECM deposition and angiogenesis (Mori et al. 2008). Levels of tenascin-c are elevated in chronic wounds specifically at the wound edge (Trebaul et al. 2007). Tenascin-c disrupts the binding of fibronectin such that cell migration becomes

limited and disorganised. The role of SPARC in healing still remains unclear due to SPARC-null mice subjected to small wounds demonstrating accelerated wound healing whereas larger wounds showed delayed and impaired healing (Bradshaw et al. 2002).

1.5.6 Proteases

Matrix metalloproteinases (MMPs) are a family of more than 20 proteases that collectively can degrade most of the components of the ECM. MMPs and their inhibitors, TIMPs, are carefully regulated throughout the phases of wound healing such that there is a continuous balance between the synthesis and degradation of the ECM (Greener et al. 2005). Chronic wounds become caught in the inflammatory stage, characterised by abundant levels of proteases, such that breakdown of the ECM surpasses the synthesis of new ECM (Greener et al. 2005). It is known that various cells including activated keratinocytes at the wound edge, fibroblasts, and endothelial cells all have the ability to express proteases; however, it is the invading neutrophils and macrophages which are considered to be the major source of proteases during wound healing (McCarty & Percival 2013). These release elastase and collagenase which degrade ECM components. A number of other classes of proteolytic enzymes are also present at the wound site, including the serine proteases: elastase, cathepsin and plasminogen (McCarty et al. 2012). Studies have shown that these are crucial for the successful migration of epithelial keratinocytes across the wound bed for re-epithelialisation (McCarty et al. 2012).

MMP activity within chronic wound fluid is significantly increased compared to acute wound fluids. Furthermore, the elevated levels of MMPs decreased as healing continued (Trengove et al. 1999). Moreover, another study found elevated levels of MMP-9 and MMP-2 in pressure ulcer fluids compared to healing acute surgical wound fluids (Yager et al. 1997). Many MMPs (MMP -2, 3, 9, 11, 12, 13 & 14) are up-regulated during wound repair, these have not been detected in normal skin but show an abundant expression in wounded skin (McCarty et al. 2012). Different levels of proinflammatory cytokines and growth factors have

been shown to exist between acute and chronic wound environments. Prolonged exposure of the wound tissues to proinflammatory cytokines may act to stimulate the production of MMPs, while inhibiting the synthesis of TIMPs (McCarty & Percival 2013).

1.6   Wounds and pH

The pH notation expresses the negative logarithmic scale of the concentration of H^+ ions in solution (pH = -log[H^+]). The pH scale ranges from 0 to 14 defining H^+ ion concentrations of $10^0 - 10^{14}$ mol/l. A pH of 7 represents neutral; a pH below 7 is acidic representing a higher hydrogen concentration, while a pH value above 7 is termed alkaline and represents a lower hydrogen concentration. The pH value is an important factor during wound healing and a key parameter for therapeutic interventions in wound care (Schneider et al. 2007).

Healthy, intact skin has a slightly acidic pH ranging from 4.0 to 6.0. This is an important aspect of the skin's barrier function, since it regulates bacterial flora and has a role in preventing infection (Sharpe et al. 2009). When a wound occurs, the skins acidic milieu and pH is disrupted, exposing the more neutral pH (7.4) of the underlying tissue (Schneider et al. 2007). With successful healing and re-epithelialisation the skin returns to being acidic. Acute wounds have a more neutral pH and during acute wound healing, there is a drop in pH caused by various factors including hypoxia (Leveen et al. 1973) and increased production of lactic acid (Hunt et al. 1967). An acidic pH environment is considered to be beneficial, by increasing fibroblast proliferation and migration and also regulating bacterial colonisation. If however wound healing is delayed, the pH becomes increasingly alkaline over time (Figure 1.4) (Schneider et al. 2007). At this stage the wound is described as chronic and the synthesis of ECM molecules becomes impaired, thus arresting the healing process. Non-healing burn injuries and chronic wounds are often found to reside at an alkaline pH (Gethin 2007; Schreml et al. 2010; Sharpe et al. 2013). These wounds exhibit slower rates of healing compared to wounds with a more neutral pH to begin with (Leveen et al. 1973). Recordings of the chronic wound environment have been in the range of pH 7.15 to 8.9. There is

evidence that the real situation is far more complex, a review of the literature finds that the measured pH of various wounds range from 5.4 up to 9.0, these are summarised in Table 1.2.
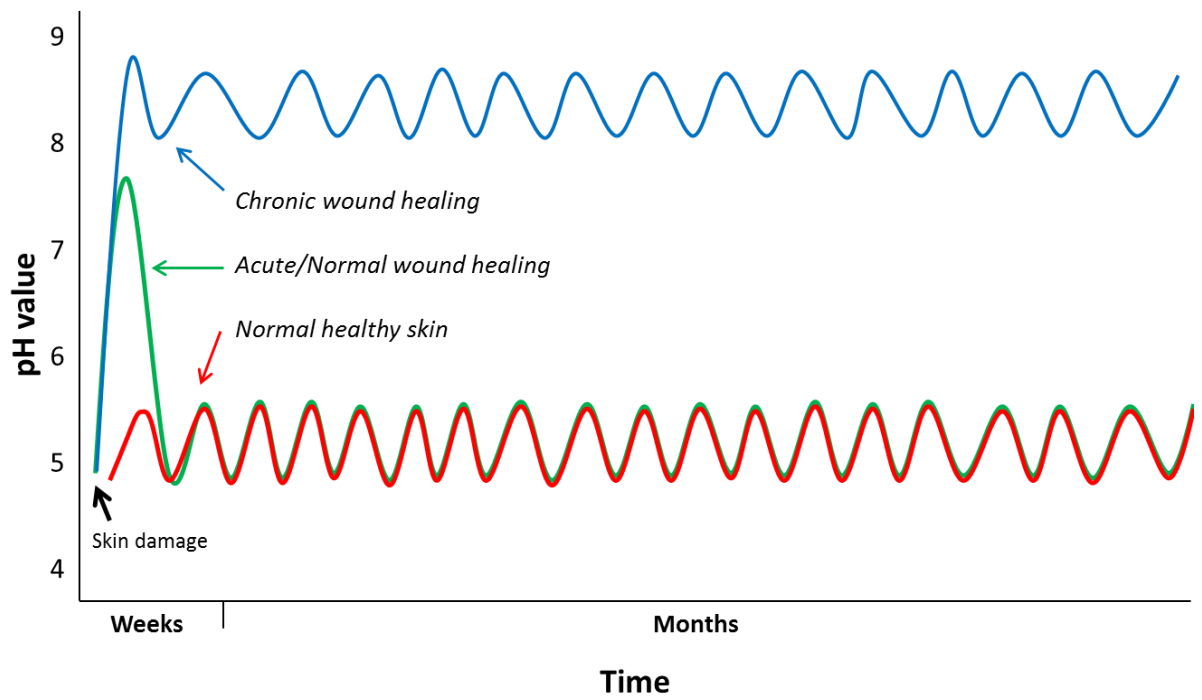
Figure 1.4 Time course of pH in healthy skin, acute wounds and chronic wounds

Diagram shows the main differences in the pH milieu between healthy skin and chronic wound healing over time. Adapted from Schneider et al. 2007.

Table 1.2 Summary of the pH of chronic wounds

Table summarises the recorded pH values of various chronic wounds from different clinical studies.

Type of wound	pH range	Measuring Method	Comments	Reference
Chronic leg ulcer	7.3 – 8.9	pH electrode	• Study looked at the effects of acidification on wound healing.	(Wilson et al. 1979)
Ulcer	7.15 – 7.96	pH electrode	• Surrounding normal skin had pH of 6.2 – 6.6	Glibbery et al. cited by (Schneider et al. 2007)
Stage III Chronic Pressure Sore	7.6	pH electrode	• Different pH was found during different stages of wound healing	(Schneider et al. 2007)
Chronic	5.45 – 8.65	pH electrode	• pH decreased as healing progressed	(Dissemond et al. 2003)
Chronic	8.5	Litmus pH strips	• 58% of wounds were positive for bacterial infection	(Shukla et al. 2007)
Burn wound	7.18 – 8.65	pH probe	• Wounds that went on to heal successfully showed a decrease in pH value at each dressing change.	(Sharpe et al. 2013)
Acute and chronic	6.4 – 8 and above	Luminescent pH sensor	• One day after wounding pH is > 8 • pH decreases during physiological healing • centripetally rising pH values within chronic wounds	(Schreml et al. 2011; Schreml et al. 2014)

As wounds progress through the stages of healing, studies have shown that a shift towards an acidic pH occurs. A study of 50 patients measured the wound pH during the course of healing. Wounds presented with a pH of 8.5 or more, but as the wound began to heal pH values reduced to 8 and below (Shukla et al. 2007). Other studies have demonstrated a difference in pH of wounds classed as being in different stages of healing. Stage 3 ulcers had a pH of 7.6 compared to stage 1 ulcers with a more acidic and similar pH to intact skin (pH 5.4 - 5.6) (Tsukada et al. 1992). Recently the pH of the surface of burn wounds was investigated; full thickness burns have a more alkaline pH of 8.0 compared to superficial burns with a pH of 6.05 (Sharpe et al. 2013). When pH was measured at each dressing change the pH of both healing and non-healing wounds were found to decrease with time. Burn wounds that went on to heal without any need for further grafting treatment had a significantly lower pH of 7.32 compared with a measurement of pH 7.73 for the non-healing wounds (Sharpe et al. 2013). In a study by Schneider and colleagues it was found that following the application of a non-permeable dressing wound secretions became more acidic compared to the application of a permeable dressing on a chronic wound (Schneider et al. 2007). Some clinical studies looked into the benefit of acidity in wound healing where patients were treated with a solution at a pH of 7.3 or pH 6.0. Patients treated with the solution at pH 6.0 showed a much quicker healing time than if treated with the solution at pH 7.4 (Wilson et al. 1979). A dressing incorporated with 3% citric acid solution improved wound healing, with a reduced hospital stay of up to 10 days compared to the other treatment groups (Prabhu et al. 2014). Targeting the pH of the wound by the simple application of a specialised dressing could provide a simple healthcare approach to improving wound healing.

1.6.1 Current methods for measuring pH

It has been found that wound pH reduces as the wound progresses through the stages of healing; suggesting that wound pH could be a useful tool to predict the likelihood of successful wound repair. However, clinical predictions of wound pH can be difficult to make. Current technology only allow for wound surface or wound fluid pH to be measured due to

unsuitable probes that can cause further tissue disruption and cell death. Most studies make use of flat glass membrane probes (Schneider et al. 2007) or litmus paper to simply measure the pH of wounds. The pH electrode is the most common method used to measure pH, the probe works by creating an electrical current from the H^+ ions in a solution and as such works like a voltmeter. It measures the voltage produced by the solution whose pH we are interested in and compares it with the voltage/pH of a known solution; the difference in voltage is used to deduce the difference in pH. Two electrodes are inside the probe that creates an electric current; one has a silver based electrical wire in a potassium chloride solution, contained within a thin glass bulb containing metal salts, the other electrode has a potassium chloride wire suspended in a potassium chloride solution. As the pH probe is measuring a solution the H^+ ions are involved in a process of ion exchange. This H^+ ion activity creates an electrical charge which produces a voltage. For example, the larger the difference in voltage between the known and unknown solution, the larger the difference in H^+ ion activity. The more H^+ ion activity indicates an acidic solution and a low H^+ activity indicates an alkaline solution.

However, the conventional glass pH probe only provides localized measurements and does not provide complex measurements for the whole wound area (Figure 1.5A) (Schreml et al. 2011; Sharp 2013). New pH diagnostic tools are being developed that aim to provide a more in depth measurement of wound pH; these include a spray- able luminescent 'sensor' for wound imaging (Schreml et al. 2012), hydrogel sensors (Nocke et al. 2012) and 2D luminescence imaging (Schreml et al. 2011).

One of the simplest and most cost effective methods to determine pH is to use indicator dyes, these absorb different wavelengths of visible light depending on the pH. Hydroxyl-substituted azobenzene derivatives as indicator dyes were successfully incorporated into wound dressings to optically monitor pH between 6 and 10 (Trupp et al. 2010) or 3 and 12 (Mohr et al. 2008). Hydroxyazobenzene derivatives exhibit a significant spectral difference between the acid and base form, these changes in absorbance can be used to monitor pH via optical sensors (Trupp et al. 2010). The sensitivity of the dyes were tailored by the substitution of *para* or *ortho* to the hydroxyl group (Dargaville et al. 2013). This was further developed into a prototype dressing, if the pH increased indicating an infection, the colour

of the dressing would change from yellow to purple (Dargaville et al. 2013). Imaging and observing the pH of whole wounds has the potential to offer clinicians much more information about the state of the wound than single target diagnostics. Luminescence imaging sensors based on polystyrene-co-acrylonitrile particles loaded with FITC (fluorescein isothiocyanate) as a pH dependent luminophore were developed allowing a pH map of the wound to be created (Schreml et al. 2011; Meier et al. 2011). Sensors were loaded with FITC (indicator dye) covalently bound to aminocellulose (AC) particles and ruthenium (Ru; reference dye) physically entrapped in polyacrylonitrile particles. Both dyes can be excited at 460nm with light emitting diodes, which allows for imaging of pH in one single excitation-emission cycle (Schreml et al. 2011). The luminescence intensity of FITC-AC particles increases along with rising pH, whereas the reference particles (Ru)remains constant (Schreml et al. 2012). A thin polyurethane film containing the pH sensors were placed on acute and chronic wounds, then a 460nm LED array digital camera was used to excite the luminophore particles and a pH map of the wound was obtained (Figure 1.5B and C). The pH dependent signal is stored in the green channel of the RGB and the pH independent reference signal is stored in the red channel. The ratio of green/red luminescence intensities gives a referenced pH signal for each pixel (Schreml et al. 2011).

Clinically, wound pH is not often a factor that is measured and no significant clinical evidence is published that proves wound pH to be a factor that requires attention when considering wound treatment. Although, increasing evidence state that chronic wounds are in a state of alkalinity, further studies are required to prove that pH does play a role in non-healing wounds (Steven L Percival et al. 2014; Jones et al. 2014). Using pH as a diagnostic tool in a hospital setting could make it possible to identify and refer or treat non-healing wounds earlier. pH probes are inexpensive and would be useful in smaller hospital settings without access to more expensive and advanced equipment such as a laser-doppler or 2D luminescence imaging equipment (Schneider et al. 2007). However, sensors specially designed to detect key wound parameters have the potential to add objectivity to the practice of wound management (Schreml et al. 2012). Dressings are often disturbed unnecessarily by premature renewal because the dressing looks full of exudate although the wound bed remains clean. A wound dressing with a built in sensor to detect excess moisture

or infection may aid in this decision making. Many sensors are still in the early pre-clinical stages of development (Dargaville et al. 2013). Using sensors in real wound environment however may pose a significant challenge due to the hundreds of different proteins present at a range of concentration and across pH levels as low as 5.4 and as high as 9.0. It is also important that pH sensors are used with care because and acidic or basic wound environment could indicate normal or an abnormal wound depending on the progression of healing or the state of infection (Dargaville et al. 2013).
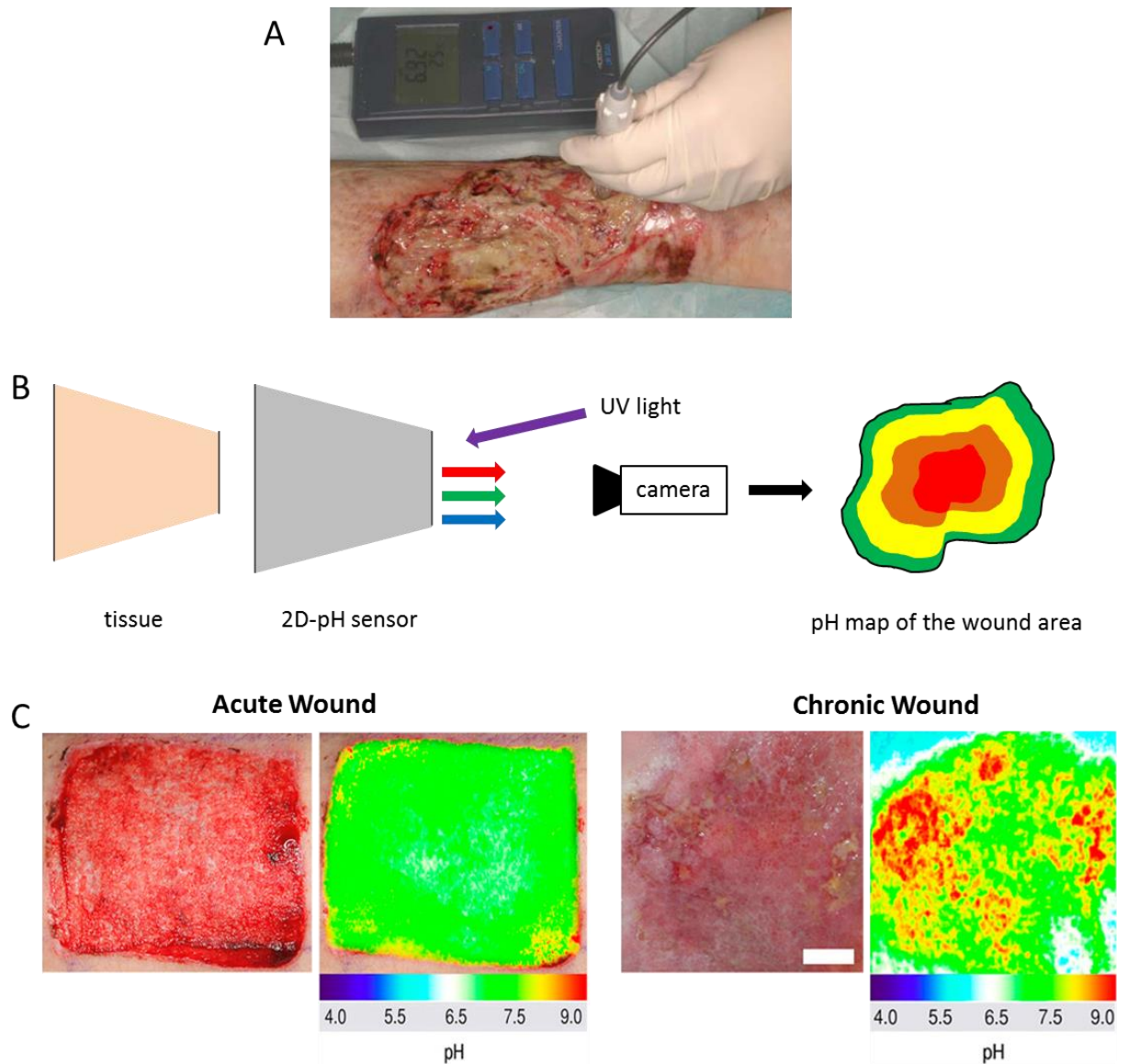
Figure 1.5 Measuring wound pH

A) The conventional pH probe is used to measure wound pH, but is limited to one area of the wound at one time (Schneider et al. 2007). B) The process of how new pH sensor technology allow a much more detailed pH map of the whole wound to be created. The pH sensor is placed onto the wound area which is illuminated with UV light. The three dyes in the sensor emit red, green and blue light that is collected by a digital camera. C) An image of the whole wound can then be created and different colours shows the different pH value throughout the wound as shown for both an acute and a chronic wound (Schreml et al. 2011; Meier et al. 2011).

1.7   Effect of pH on cells

The pH must be kept within relatively narrow limits for the maintenance and growth of most cells, however the pH can vary significantly within different areas of the body e.g. the pH of blood is 7.4 whereas pH of the gut is much more acidic. Therefore, research into the immediate effects of changing pH on all cell types is warranted. A study by Taylor in 1962 was one of the first to offer insight into the effect of pH on cells. When the pH was raised from 7.3 to 8.9 a marked contraction and detachment of cells occurred *in vitro*, furthermore extended exposure to alkaline pH saw the cells fail to recover (Taylor, 1962). The density of chick embryo cell cultures was shown to be affected by pH. Sparse cultures were less sensitive to pH; whereas high pH reduced the proliferation of cells in dense cultures due in part to cell death and detachment (Rubin 1971). Furthermore, a lower pH inhibited cell migration but did not affect the proliferation of cells. Additionally, cell regulation of human tumour cells is affected by pH; normal growth occurred between pH 6.8 and 7.2 and reduced growth was observed between pH 6.7 and 6.4 (Taylor & Hodson 1984). The effects of pH between 7.2 and 8.4 on human periodontal ligament and embryonic lung fibroblasts were investigated *in vitro*. Growth and attachment of these cells showed a significant decrease above pH 7.8 (Lengheden & Jansson 1995). Cell migration and DNA synthesis were significantly reduced with an increase in pH, maximum fibroblast migration occurred between pH 7.2 and 7.5 (Lengheden & Jansson 1995).

Optimal pH for fibroblast and keratinocyte proliferation is between pH 7.2 and 8.3. Epidermal keratinocytes tolerate a much wider pH range compared to dermal fibroblasts and show optimal migration from *ex vivo* skin explants at pH 8.55 (Sharpe et al. 2009). This correlates with previous studies where higher pH improved skin graft take after burn injuries (Sayegh et al. 1988). Keratinocyte proliferation, viability and migration are impeded at low pH values (Schreml et al. 2014). In keratinocyte/fibroblast 3D skin constructs migration was significantly inhibited at pH 6.5 compared to pH 7.4, demonstrating that low pH prevents multilayer epidermal repair. This correlates with their other work suggesting that

keratinocytes may not be sufficiently recruited due to a lower pH at the wound margins and thus the epidermal barrier cannot be restored (Schreml et al. 2014).

1.8   Effect of pH on proteases

Within chronic wounds the balance between tissue degradation and synthesis is lost and proteolytic enzyme activity significantly increases. When the wound is in the inflammatory phase enzymes remain active, as the healing progresses the protease activity is slowly diminished. This was shown in a study that observed a reduction in MMP activity as leg ulcer wounds progressed from non-healing to healing (Trengove et al. 1999). Furthermore, TIMP levels were significantly raised in healing wounds (Bullen et al. 1995). Protease activity is particularly pH dependent, each protease has a peak activity at a certain pH (Greener et al. 2005). The pH-dependent activity profiles of cathepsin-G, elastase, plasmin and MMP-2, four proteases important in wound healing, were assessed. All four demonstrated similar pH profiles, with optimum activity between pH 7 and 8 (Greener et al. 2005), the pH ranges found in chronic wounds. Chronic wound fluid (pH 7.5 – 8.9) and chronic wound granulation tissue pH correlates with optimum protease activity, therefore an ideal environment for excess proteolytic activity. At alkaline pH, proteases demonstrate peak activity and may degrade newly formed granulation tissue, but at significantly acidic pH 4 proteases are permanently inactivated. The pH of the wound bed therefore must be within an appropriate range for optimum protease activity and thus optimal wound healing. Greener suggested that adjusting the pH of the wound bed could be a useful intervention to significantly reduce protease activity and improve healing responses.

1.9   Wounds and Microbial Infection

It is accepted that micro-organisms contribute to wound infections. However, major controversy still exists as to the exact mechanism and their significance in a non-healing wound (Bowler et al. 2001). Some even argue that microorganisms play no role in the delay of wound healing and may even improve healing responses (Percival & Bowler 2004a). Bendy *et al* first proposed that microbial numbers play a role in the clinical significance of non-healing wounds. It was reported that ulcers would only heal when the bacterial load was less than 10^6 CFU/mL of wound fluid (Bendy et al. 1964).

Microorganisms can exist in both the planktonic and biofilm phenotypic states, with biofilms being predominant in the healthcare environment (Percival et al. 2014). It is reported that biofilms are associated with 65% of human infections worldwide (Potera 1999). The biofilm mode of growth is an important survival strategy for microorganisms in the healthcare environment (Donlan & Costerton 2002). To date, the impact of microorganisms on wound healing is poorly understood, but evidence of bacterial biofilms within chronic wounds are now documented to have a major role in delaying healing responses. Much research is now focused on understanding biofilms and their detrimental effect within chronic wounds.

The presence of some bacteria in a wound seems to have a positive effect in inducing an inflammatory response for the process of normal wound healing (Jones et al. 2014). Some researchers have found that very low levels of bacteria in fact accelerate wound healing and the formation of granulation tissue (Laato et al. 1988). However, an increase in bacterial load within a wound has the opposite effect. With an increase in the production of bacterial enzymes resulting in excess granulation tissue and scar formation (Edwards & Harding 2004). Therefore, increased microbial density within a wound correlates with chronicity (Bowler et al. 2001).

1.9.1 Bacterial Biofilms

The development of a biofilm occurs in several phases, forming within a few hours, and is influenced by its environmental surroundings (Percival et al. 2011) (Figure 1.6). The first step is the initial attachment of the cell-surface of free-floating planktonic bacteria to a surface(McCarty et al. 2014). The bacterial cells then begin to divide and proliferate forming a micro-colony. As they grow the bacteria secrete and encase themselves in a protective layer of extracellular polysaccharide matrix (EPS) which is made up of proteins, lipids and polysaccharides (Percival et al. 2014). Once the biofilm is established the bacteria secrete quorum sensing molecules which allow the biofilm to change its phenotype (Donlan & Costerton 2002). Planktonic bacteria can also detach from the mature biofilm, which then disperse and develop another biofilm in a new location (McCarty et al. 2014). This vicious cycle of biofilm growth leads to difficulties in removing biofilms from chronic wounds. In the biofilm state bacteria have increased metabolic efficiency, substrate accessibility and an enhanced resistance to environmental stress and antimicrobials (McCarty et al. 2014). Within a biofilm, bacteria modify their phenotypes resulting in altered production of virulence factors. These factors, in addition to the surrounding layer of EPS, make biofilms very difficult eradicate as they are highly resistant to conventional antibiotic therapy (Olson et al. 2002).
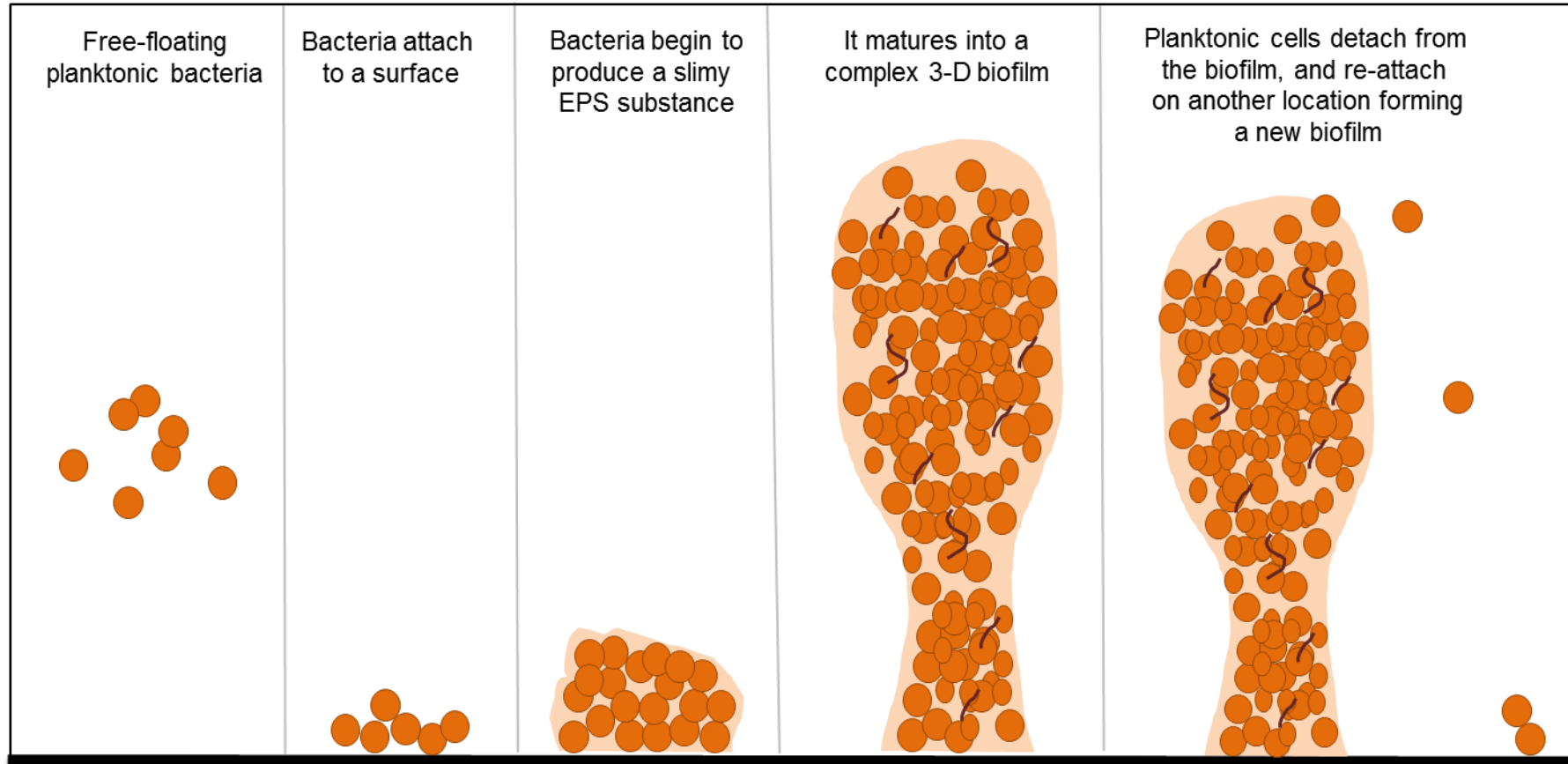
Figure 1.6 Formation of a bacterial biofilm

Diagram summarises the steps leading to mature biofilm formation. Free-floating bacteria attach to a surface and aggregate together. As they grow they encase themselves in a self-secreted layer of extracellular polysaccharide matrix which protect them from their surroundings as well as antimicrobials. Within a few hours a mature biofilm will have formed, planktonic bacteria can detach and begin to form another biofilm at a new location.

1.9.2   Biofilms in Chronic Wounds

Open wounds make an ideal environment for bacterial attachment, with a moist surface and plenty of nutrition for biofilm growth (Edwards & Harding 2004). Studies have highlighted direct evidence of the presence of bacterial biofilms within chronic wound tissue in both humans and animals. James and colleagues found evidence of biofilms in human chronic wounds using light and scanning electron microscopy. Sixty percent (30 out of 50) of the chronic wounds examined revealed the presence of biofilms, whereas only 6% of the acute wound specimens presented with a biofilm (James et al. 2007). The chronic wounds took more than 3 months to heal effectively whereas all the acute wounds had completely healed within 3 weeks. Further molecular analyses confirmed that these biofilms contained not just one bacterial organism, but a diverse polymicrobial community of microorganisms such that polymicrobial interactions potentially play a crucial role in wound healing (James et al. 2007). Evidence of bacterial biofilms in equine chronic wounds have been reported with 61.5% (8 out of 13) of wounds examined showing the presence of a biofilm (Westgate et al. 2011). Furthermore, bacterial isolates obtained from these wounds showed a significantly higher biofilm forming potential than isolates obtained from uninjured skin (Westgate et al. 2011).

Gram positive cocci were the most common bacteria observed in all chronic wound samples (James et al. 2007) which associates with staphylococcus organisms being good biofilm formers. The polymicrobial nature of biofilms within a chronic wound has also been confirmed with multiple species identified; in 76% of human wounds analysed, two or more bacterial species were present. The most common were *Staphylococcus aureus* (present in 93.5% of the wounds), *Enterococcus faecelis* (71.7%)*, Pseudomonas aeruginosa* (52.2%), coagulase-negative staphylococci (45%), *Proteus* species (41.3%) and anaerobic bacteria (39.1%) (Gjødsbøl et al. 2006; James et al. 2007).

1.9.3 Effect of Biofilms on skin cells

It is likely that within a chronic wound, biofilms colonize the dermal layers and have a detrimental effect on skin fibroblast and keratinocyte cells. The synthesised products from planktonic and biofilm bacteria (planktonic/biofilm conditioned media) have recently been shown to have effects on human epithelial keratinocytes (Kirker et al. 2009) and dermal fibroblasts *in vivo* (Kirker et al. 2012). *S. aureus* planktonic and biofilm conditioned media significantly reduced keratinocyte and fibroblast cell viability and migration. Biofilm conditioned media induced higher levels of tumor necrosis factor - α (TNF- α) compared to planktonic conditioned media (Kirker et al. 2012). Whereas planktonic conditioned media was responsible for an increase in interleukin-6 (IL-6), TGF-β1, MMP-1, and MMP-3 compared to biofilm conditioned media (Kirker et al. 2012). Furthermore, marked differences in the inflammatory, apoptotic and nitric oxide responses of keratinocytes occurred after exposure to planktonic and biofilm conditioned media (Tankersley et al. 2014). Proteomic analysis of biofilm and planktonic conditioned media revealed differential protein compositions with planktonic bacteria synthesizing enzymes involved in glycolysis while biofilms synthesized proteins relating to translation and possibly bacterial proteases (Secor et al. 2011).

Lactobacillus is a gram positive anaerobic bacterium. Various species in humans are present in urinary and digestive tracts and considered to be harmless. Secreted products and extracts of *lactobacillus* are thought to have beneficial anti-viral, anti-cancer and wound healing properties. In addition, *lactobacillus* has shown antimicrobial properties and a great biofilm-disrupting capacity, especially towards *P. aeruginosa*, such that its interest as a potential treatment option in chronic wounds is growing (Ramos et al. 2012). *Lactobacillus* supernatants promote inflammatory response during tissue repair and subcutaneous injections of *lactobacillus* leads to angiogenesis in rodents (Halper et al. 2003). Topical application on human infected chronic wounds reduced the wound area by promoting debridement, granulation tissue formation and wound healing (Peral et al. 2010).

1.9.4 Effect of pH on Biofilms

Various environmental factors affect the growth of bacteria including temperature, pH, dissolved gases, osmotic pressure and water availability (Jones et al. 2014). Most bacterial organisms grow optimally between pH values of 6.5 and 7.0, however some thrive in very acidic (e.g. *Acetobacter*) or very alkaline conditions (e.g. *Candida*)(Padan et al. 2005). Organisms grow at a range of pH defined as three cardinal points: 1) the minimum pH, below which the organism cannot grow, 2) the maximum pH, above which the organism cannot grow, and 3) the optimum pH, at which the organism grows best (Padan et al. 2005). Each microbial species has its own pH range in which it grows best (Percival et al. 2014)(Table 3).

To date no studies have specifically looked at wound derived bacteria and the effect of pH on their growth and formation of a biofilm within a wound. Some studies have investigated the role of pH in dental plaque biofilms (Marsh 2009) and in urinary tract infections (Hatt & Rather 2008). A study by Hostacká was performed to investigate the effect of pH on the most common pathogens associated with healthcare associated infections. These included *P. aeruginosa, Klebsiella spp*. and *Vibrio cholera.* Their biofilm forming capacity are considered to be the main reason for their persistence and why conventional antimicrobial treatments failed to eradicate them (Hostacká et al. 2010). Results showed that increasing pH correlated with an increase in biofilm production by all microorganisms analysed. Significantly higher biofilm production was observed at pH 7.5 and pH 8.5 compared to pH 5.5 (Hostacká et al. 2010). Initial growth (0 - 6 hours) of planktonic *S. aureus* showed a decrease in pH 8.5 compared to pH 7.2 (Nostro et al. 2012). Both *S. aureus* and *S. epidermidis* failed to adhere to a surface in alkaline conditions and biofilm biomass formation was significantly reduced at pH 8.5 compared to pH 7.2 (Nostro et al. 2012). Another study showed that very acidic (pH 3) and very alkaline (pH 12) conditions resulted in *S. aureus* not being capable of successfully forming a biofilm compared to neutral pH conditions (Zmantar et al. 2010). Earlier studies have demonstrated an enhanced adherence of bacterial *S. aureus* in alkaline conditions compared to acidic conditions. This could relate to the high incidence of biofilms within alkaline chronic wounds.

Table 1.3 The optimum pH for some microorganisms

Table highlights the optimum pH for the growth of the most prevalent microorganisms isolated from wounds (Padan et al. 2005; Hostacká et al. 2010; Rumbaugh et al. 2015).

Wound associated microorganisms	Optimum pH for growth
• *Staphylococcus aureus*	7.0 – 7.5
• *Enterococcus faecelis*	7.0 – 9.0
• *Pseudomonas aeruginosa*	6.6 – 7.0
• *Coagulase-negative staphylococci*	7.0 – 7.5
• *Anaerobic bacteria*	6.0 – 7.0
• *Escherichia coli*	6.0 – 7.0
• *Klebsiella spp*	5.5 – 7.0
• *Candida spp*	7.0 – 8.0

1.10 Summary

Chronic wounds are very complex with many factors involved including changes in pH, presence of biofilms, defective ECM synthesis and an increase in proteases. Further research is required to clarify how these effects individually and together contribute to the delay in wound healing, and then chronic wound treatments can be optimised. It is reported that bacterial colonisation may contribute to the shift towards an alkaline pH (Schneider et al. 2007). Pathogenic bacteria prefer a more alkaline environment to grow and bacterial colonisation and proliferation is encouraged at a higher pH (Thomas et al. 1993). Therefore, when the underlying tissue is exposed, it provides opportunity for the growth of the resident skin flora, which can colonise the wound. This causes a shift in pH, making the wound environment alkaline as it develops into a chronic wound. Finding a way in which the acidic milieu of the skin can be restored should effectively reduce microbial load of the skin surface and decrease the likelihood of bacterial colonisation in a chronic wound. A clinical study in 2002 demonstrated that the topical application of acidic ointments in diabetic patients significantly reduced the bacterial load on the skin surface (Kurabayshi et al. 2002). Many researchers have shown that pH is increased in a chronic non-healing wound environment but, there is very little research and evidence about how this change in pH ultimately affects the downstream processes of wound healing.

The progression towards an alkaline pH in a chronic wound and how this affects the phases of wound healing is not clearly understood. It is hypothesised that an alkaline pH is a causative factor in non-healing wounds along with the presence of bacterial biofilms. Therefore the aim of this study was to investigate the effect of pH on several wound healing parameters such as ECM abundance, normal and chronic fibroblast migration and biofilm formation. Furthermore, to combine both the secreted products of biofilms and pH and investigate how both may affect the wound healing process. A better understanding of how changing pH modulates biological and biochemical processes in the wound environment is required such that future treatment can be better directed preventing the occurrence of a chronicwound.

Chapter 2:

Materials and Methods

2 Materials and Methods

2.1 Cell culture

2.1.1 Collection of tissues

Skin samples were collected from horses during post-mortem or chronic granulation tissue samples were collected during routine surgical debridement of wounds at the Philip Leverhulme Equine Hospital with approval from the ethics committee of the School of Veterinary Science, University of Liverpool and owner consent. Tissue samples were either placed in RNAlater (Sigma, UK) for further molecular studies, frozen at -80 °C for further biochemical assays, fixed in formalin (Sigma, UK) solution for histology or used for isolation of fibroblasts.

2.1.2 Fibroblast isolation

Tissue pieces were diced into approximately 5 mm^2 pieces and placed in a 25 cm^2 cell culture flask with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (foetal calf serum; Sigma, UK), 5 mLs HEPES buffer, 6 mLs antibiotics (5 mLs penicillin/streptomycin at 100 U/mL and 1 mL fungizone (250 µg/mL amphotericin B and 205 µg/mL sodium deoxycholate in dH$_2$O) and 10 µL gentamicin (10 mg/mL) (all from Life Technologies, UK) and incubated at 37 °C in a humidified atmosphere for growth of dermal fibroblasts from explant cultures. Cultures were left for up to 5 days until growth and attachment of fibroblasts could be seen microscopically. The media along with tissue pieces would then be removed and fresh DMEM media (as above but without addition of gentamicin) added and fibroblast cells grown until cells were confluent in the culture flasks.

2.1.3 General maintenance of fibroblasts

Normal fibroblasts (NFs – isolated from healthy equine skin), chronic fibroblasts (CFs – isolated from equine debrided chronic wound granulation tissue) and primary human dermal fibroblasts isolated from adult skin (HDF, Life technologies, UK) were maintained in cell culture in DMEM supplemented with 10% FCS, 5 mLs HEPES buffer, 6 mLs antibiotics (5 mLs penicillin/streptomycin at 100 U/mL and 1 mL fungizone (250 µg/mL amphotericin B and 205 µg/mL sodium deoxycholate in dH_2O) and incubated in a 5% CO_2 humidified atmosphere at 37 °C.

Immortalised human epidermal keratinocytes (HaCaT – a spontaneously transformed keratinocyte cell line (Boukamp et al. 1988)) were maintained in high glucose DMEM (Life Technologies, UK) supplemented with 5% FCS, 5 mLs penicillin/streptomycin at 100 U/mL and 1 mL fungizone.

Cells were sub-cultured when reached 80–90% confluence. Briefly, media was removed and discarded from culture flasks. The attached layer was washed with sterile phosphate buffered saline solution (PBS). The wash solution was removed and discarded and cells were incubated with 1x trypsin (Life Technologies, UK) and observed under an inverted microscope until cells begun to round and detach from the bottom of the culture flask. A volume of media double to that of the trypsin was added to the culture flask to stop the reaction. The cell suspension was removed and centrifuged at 1200 rpm for 4 minutes to pellet the cells. The supernatant was removed and the pellet re-suspended in warm media (as above). The cells were counted using a haemocytometer and re-seeded (10,000 cells) into a new tissue culture flask containing an appropriate volume of warmed media. Medium was removed and replaced every 48 hours thereafter until culture approached confluence again. Cell cultures were incubated in a 5% CO_2 humidified atmosphere at 37 °C.

2.1.4 Cryogenic storage of cells

For cryogenic preservation, cells were trypsinised as described above and centrifuged for 4 minutes at 1200 rpm to pellet the cells. The cell pellet was re-suspended in DMEM growth media with 10% dimethyl sulfoxide (DMSO) solution (Sigma, UK) and transferred into cryogenic vials (1 mL/vial) and frozen to -80 °C using a freezing container to allow a cooling rate of -1 °C per minute. Once frozen, cells were transferred to liquid nitrogen for long-term storage.

Frozen cells were thawed by placing the cryovial in a 37 °C incubator until cell suspension was thawed. The cells were immediately transferred to a culture flask containing pre-warmed medium. After 24 hours, the culture medium was replaced in order to remove the cryoprotectant. Cultures were maintained as previously described.

2.1.5 pH adjusted media

pH adjusted media was formulated by supplementing DMEM no phenol red with 10% FCS, 5 mLs L-glutamine, 5 mLs penicillin/streptomycin at 100 U/mL and 1 mL fungizone and was measured using a pH meter (FiveEasy FE20 Mettler Toledo). Separate buffers (all Sigma, UK) were used for each different pH condition. MES hydrate at a concentration of 25mM for pH 6, HEPES at a concentration of 25mM for pH 7.5 and CHES at a concentration of 10mM for pH 9. The pH was changed by dropwise addition of 1M HCl or NaOH until the required pH was reached.

Bicarbonate buffer system

A buffer is an aqueous solution that resists changes in pH when acids or bases are added to it. One of the main buffer systems responsible for regulating blood pH is the bicarbonate

buffer system. It is an acid-base homeostatic mechanism involving the balance of carbonic acid (H_2CO_3), bicarbonate ion(HCO_3^-), and carbon dioxide (CO_2) in order to maintain pH in a solution, to support proper metabolic function (Boundless 2016). Carbonic acid is a weak acid and is formed when (CO_2) reacts with water (H_2O), it rapidly dissociates to form a bicarbonate ion (HCO_3^-) and a hydrogen ion (H^+) as shown in the following reaction:

$$CO_2 + H_2O \longleftrightarrow H_2CO_3 \longleftrightarrow HCO_3^- + H^+$$

As with any buffer system, the pH is balanced by the presence of both weak acid (i.e. H_2CO_3) and conjugate base (i.e. HCO_3^-) so that any excess acid or base introduced to the system is neutralized. All buffers have an optimal pH range in which they are able to moderate changes in H^+ ion concentration. This range is a factor of the dissociation constant of the acid of the buffer (K_a) which is defined as the pK_a value plus or minus one pH unit (Promega, UK). For all experiments where pH of cell culture media was adjusted different buffers were used that were suitable for the pH required. MES buffer has a useful pH range of 5.5 – 6.7, HEPES buffer is suitable for a solution with the pH range 6.8 – 8.2 and CHES is used between 8.6 – 10.0 (Sigma, UK).

2.1.6 Culture of cells in pH adjusted media

NF and CF (n=3 donors) were seeded at 1×10^5 in 6-well culture plates and allowed to adhere overnight. Cell layers were washed with PBS before re-feeding with cell culture DMEM media of various pH (pH 6, 7.5 and 9) for a further 24 hours at 37 °C in a humidified 5% CO_2 incubator. The cell conditioned media (CM) was harvested after 24 hours and the cells counted and harvested in TRIzol for further RNA extraction. CM was aliquoted and stored at -20 °C before subsequent assays.

2.1.7 Cell Migration in pH adjusted media

NF, CF, HDF, and HaCaT cultures were seeded in 6 or 12-well plates. When the cell monolayer appeared microscopically confluent, cultures were scratched with a 200 µL plastic pipette tip, gently washed with PBS and re-fed with 2 mLs of pH-adjusted media (pH 6, 7.5 and 9). Scratched cultures were imaged to obtain an initial scratch area (Time 0). Cell cultures were incubated at 37 °C in a humidified 5% CO_2 incubator. Scratch images were taken again at 24 and 48 hours before the assay was terminated. All images were taken using the 10x objective on a Nikon Eclipse TS100 microscope. Images were analysed using the Image J software and percentage scratch area calculated for each time point.

2.1.8 Measuring Collagen

Collagen content in collected CM was measured using the hydroxyproline assay (Bergman & Loxley 1963). Samples are hydrolysed with acid to form hydroxyproline, a major component of collagen which comprises around 14% of its amino acid composition, such that the amount of collagen can then be calculated. A standard curve was produced using L-hydroxyproline at serial dilutions ranging from 0–100 µg/mL. An equal volume of concentrated HCl was added to samples (100 µL of CM and 100 µL of HCl) such that the final concentration of HCl was 6M and hydrolysed for 24 hours at 110 °C. Samples were then freeze dried for 24 hours to remove the acid and reconstituted in 100 µL of dH_2O. Samples were centrifuged at 10,000 rpm for 10 minutes to remove any debris present following hydrolysation. 30 µL of sample or standard were pipetted in triplicate into a 96 well flat-bottom plate and 70 µL of diluent solution and 50 µL of oxidant solution (see Table 2.1 for stock solutions) added and incubated at room temperature for 5 minutes. Immediately 125 µL of the colour reagent was added to each well and incubated for 20 minutes at 70 °C. Absorbance was immediately read at 550 nm using a spectrophotometer plate reader (Multiskan FC, Thermo Scientific) and the linear regression equation from the standard curve

(Figure 2.1) was used to determine hydroxyproline content of the samples and therefore, collagen content, calculated.

Table 2.1 Table of solutions used for the hydroxyproline assay for measuring collagen

Solution	Ingredients (all from Sigma, UK)
Stock Buffer (500mL)	28.5g sodium acetate trihydrate 21.37g tri sodium citrate dehydrate 2.75g citric acid 200mL propan-2-ol Dissolve solids in 250mL of water. Add propan-2-ol, then make up to 500mL with water.
Diluent	100mL propan-2-ol 50mL water
Oxidant	0.118g Chloroamine T hydrate 1.25mL Water 6.25mL Stock Buffer (this is enough for 2 x 96 well plates)
Colour Reagent	2.5g Dimethylamino benzaldehyde 3.2mL 70% perchloric acid 0.54mL water 20.83mL propan-2-ol

$y = 0.0686x + 0.0564$
$R^2 = 0.9957$
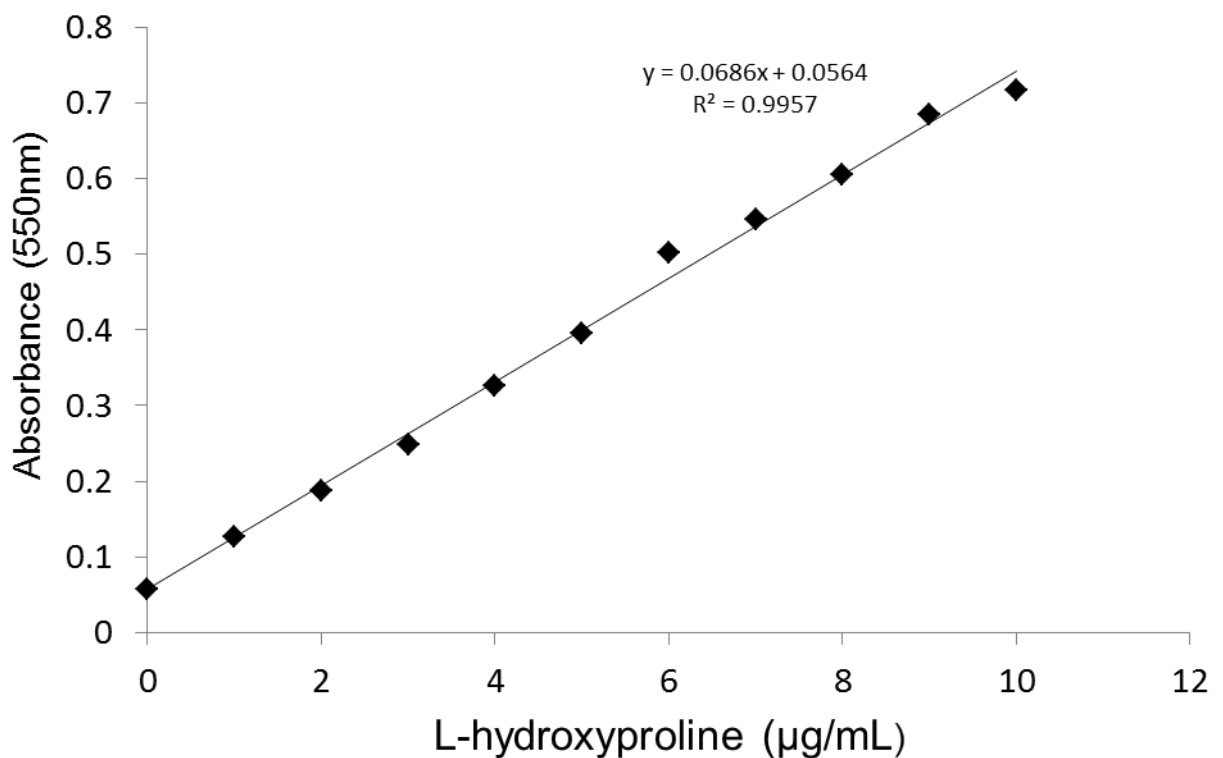
Figure 2.1 Typical standard curve for hydroxyproline quantification in conditioned medium

2.1.9 Measuring Fibronectin

Fibronectin secreted into collected CM was measured using a fibronectin specific Enzyme – Linked Immunosorbent Assay (ELISA; abcam). 50 µL of standard or CM sample were added to the plate, sealed with cover tape, and incubated for 2 hours at room temperature. Wells were washed 5 times with wash buffer before 50 µL of biotinylated fibronectin antibody was added to each well and incubated for an hour. The microplate was washed as previously described and 50 µL of streptavidin-peroxidase conjugate added to each well and incubated for 30 minutes. Microplate was again washed and 50 µL per well of chromogen substrate added and incubated for 10 minutes. Then, 50 µL of stop solution was added and a colour change from blue to yellow was observed and the absorbance immediately read using a spectrophotometer at a wavelength of 450nm. Amount of fibronectin in samples was determined using the equation from the standard curve (Figure 2.2)

2.1.10 Measurement of Glycosaminoglycan

The amount of sulphated GAGs present in CM was measured using the 1, 9-Dimethyl-Methylene Blue (DMMB) assay (Farndale et al. 1986). The DMMB assay is based on the change in absorption spectrum of the dye that is observed when sulphated GAGs bind to the cationic dye. A standard curve was produced using shark chondroitin sulphate C, serial dilutions of the standard ranged from 0 - 12.5 µg/mL. Briefly, 40 µL of standards and triplicate CM samples were added to a well of a transparent 96-well plate. 250 µL of DMMB dye from a stock solution (16 mg 1-9 dimethyl-methylene blue , 2 g sodium formate, 2 mLs formic acid made up to 1 litre in ddH$_2$0 and adjusted to pH 3.5) was pipetted into each well and the absorbance read immediately at 570nm using a spectrophotometer plate reader. The linear regression equation calculated from the standard curve allowed for determination of GAG concentration in collected conditioned media samples (Figure 2.3).
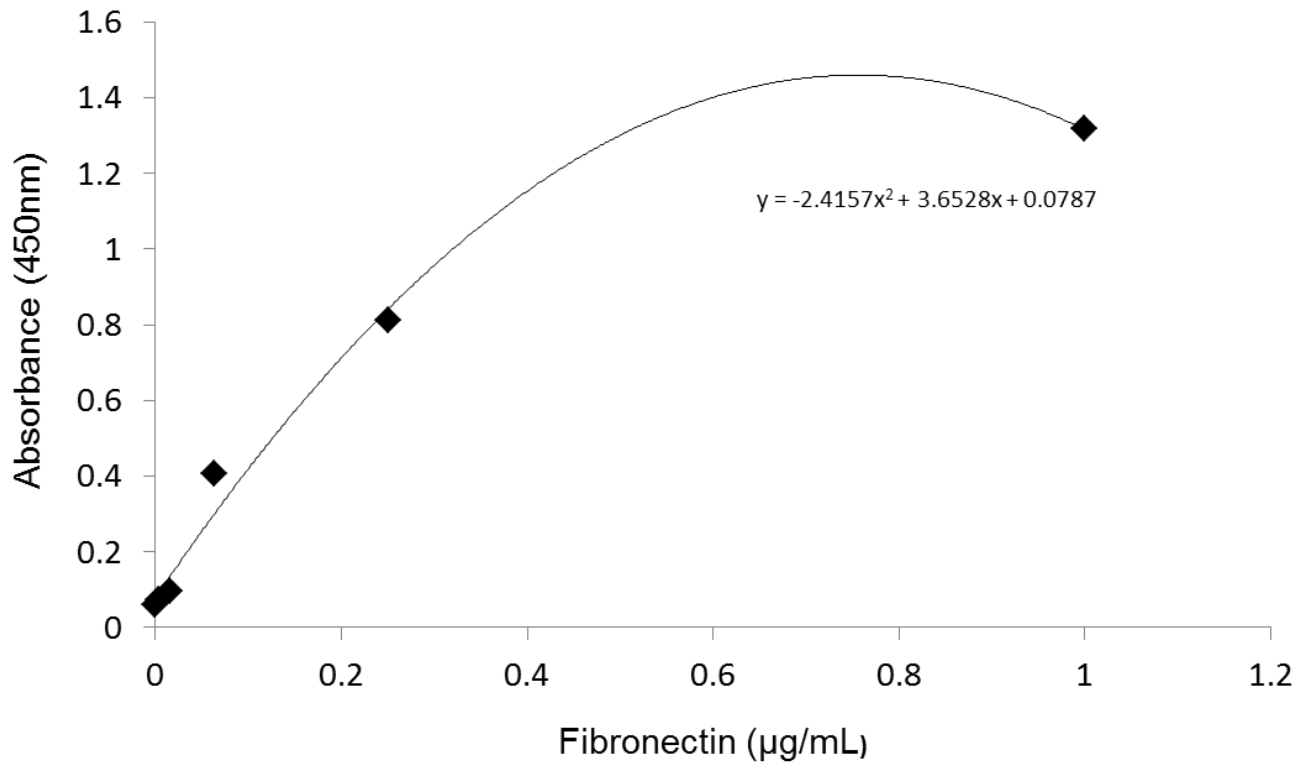
Figure 2.2 Typical standard curve for quantification of fibronectin in conditioned media samples

The equation shown on the figure is:

$$y = -2.4157x^2 + 3.6528x + 0.0787$$
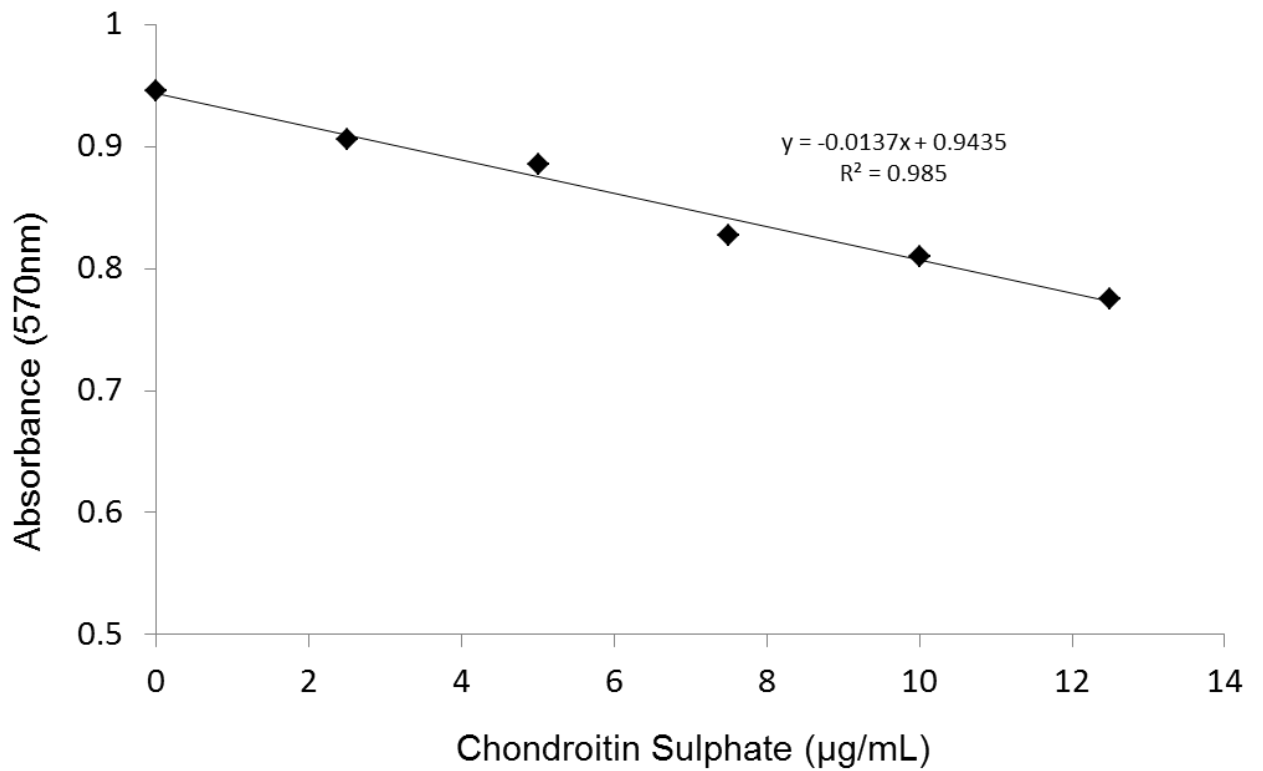
Figure 2.3 Typical standard curve for glycosaminoglycans quantification in conditioned media

The equation shown on the figure is:

$$y = -0.0137x + 0.9435$$
$$R^2 = 0.985$$

2.1.11 Measuring Tropoelastin

The amount of tropoelastin in the CM was measured using the Fastin Elastin assay (Biocolor Ltd, UK) and adapted using a protocol by Iyer et al. 2012. The first step was omitted as not to convert products into the water soluble α-elastin to allow the secreted elastin pre-cursor, tropoelastin, to be measured. 50 µL of CM was added to an equal volume of precipitating reagent (containing trichloroacetic and hydrochloric acid) and incubated for 10 minutes at room temperature. Samples were then centrifuged at 12,000 g for 10 minutes. Supernatant was removed before 1 mL of dye reagent (5,10,15,20-tetraphenyl-21H,23H-porphine tetra-sulfonate (TPPS)) was added and incubated for 90 minutes. The affinity of TTPS to bind to eastin was first observed by Winkelman in 1962, most tissues initially took up the dye but only elastin retained the TTPS molecules overtime (Biocolor, UK). Samples were then centrifuged at 12,000 x g for 10 minutes and supernatant removed and then 250 µL of dye dissociation reagent (containing guanidine, HCl and propan-1-ol) was added. The solution was gently mixed and pipetted into a well of a 96-well plate and absorbance measured at 540nm. Amount of tropoelastin in samples was determined using the equation from the standard curve (Figure 2.4)
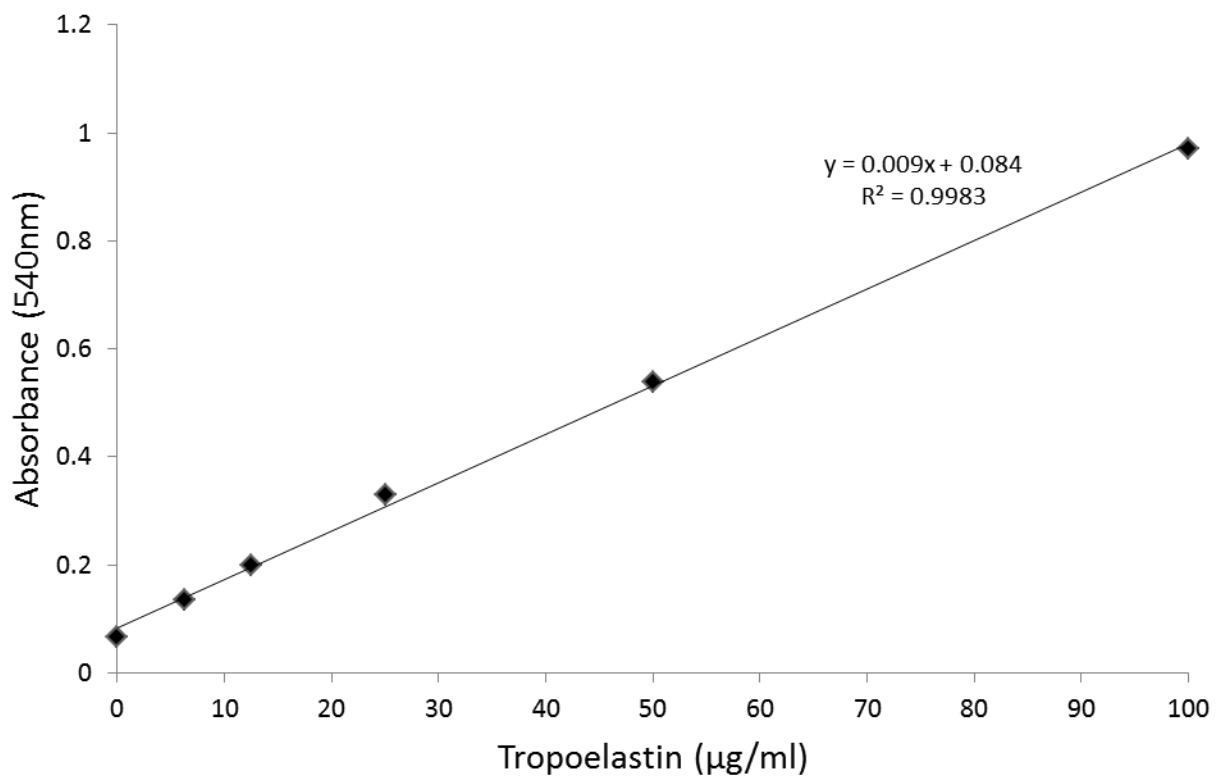
Figure 2.4 Typical standard curve for tropoelastin quantification in conditioned medium

2.1.12 Measurement of Intracellular pH

BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescin) is the most extensively used fluorescent dye for the measurement of intracellular pH (James-Kracke 1992; Han & Burgess 2009). The noncharged, nonfluorescent ester form of the dye (BCECF-AM) will rapidly diffuse through the cell membrane and into the cell. Once inside, intracellular esterases cleave the acetoxymethyl ester group releasing BCECF, producing a fluorescent compound which changes according to the intracellular pH. BCECF has 4–5 negative charges at pH 7–8, ensuring the dye is retained within the cell. The excitation profile of BCECF is pH dependent. Intracellular pH measurements are made by determining the pH-dependent ratio of emission intensity (detected at 535 nm) when the dye is excited at ~490 nm versus the emission intensity when excited at its isosbestic point of ~440 nm. Intracellular pH calibrations were carried out to determine the intracellular pH values of NF and CF.

2.1.12.1   BCECF Calibration

The intracellular pH standard curve was generated using the high K^+ -nigericin method with known pH adjusted buffers as described previously (Thomas et al. 1979; James-Kracke 1992). Nigericin is a microbial toxin derived from *Streptomyces hygroscopicus* and acts as a potassium ionophore. It is used to clamp external and internal H^+ and K^+ concentrations, by inducing a decrease in intracellular levels of K^+. Using the following equation, where K^+_i is intracellular potassium, K^+_o is external potassium, H^+_i is intracellular H^+ and H^+_o is extracellular H^+, intracellular pH was calculated by measuring external pH with a known concentration of extracellular potassium.

$$\frac{[K^+]_o = [K^+]_i}{[H^+]_o = [H^+]_i}$$

NF and CF in a 96-well plate (10,000 cells/well) were incubated with 5 µM BCECF-AM in PBS for 30 minutes at 37 °C. BCECF solution was removed and cells were then incubated in high K^+-nigericin media (145 mM KCl, 1 mM $MgSO_4.6H_2O$, 10 mM MOPS, 10 mM D-glucose, 2 mM $CaCl_2$ with 2 µg/mL of nigericin) for 10 minutes at 37 °C. Then cells were incubated in pH adjusted buffers. Buffers consisted of 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4.6H_2O$, 10 mM MOPS, 10 mM D-glucose, 2 mM $CaCl_2$ and were adjusted to pH 6.0, 6.5, 7.0, 7.5 and 8 with 1M HCl or 1M NaOH. The plate was then loaded into a fluorescent plate reader and readings were taken with emission at 535nm and alternative excitation at 490nm (the pH sensitive wavelength) and 405nm (the isobestic point). The ratio between the two values was determined and a calibration curve generated. The linear regression equation obtained using this data was used to calculate intracellular pH in unknown cell samples (Figure 2.5).

2.1.12.2   Intracellular pH measurement

NF and CF in a 96-well plate (10,000 cells/well) were incubated for 24 hours in experimental pH adjusted DMEM (pH 6, 7.5 or 9) or control DMEM media. To measure intracellular pH cells were then incubated with 5 µM BCECF-AM for 30 minutes at 37 °C then washed twice in PBS and incubated for a further 20 minutes such that the time before reading the plate after BCECF-AM treatment was the same as for creating the standard curve. Fluorescent readings were then obtained at 490nm and 405nm and the ratio between them calculated. A value for intracellular pH of unknown samples was then determined using the formula gained from the calibration curve (Figure 2.5).
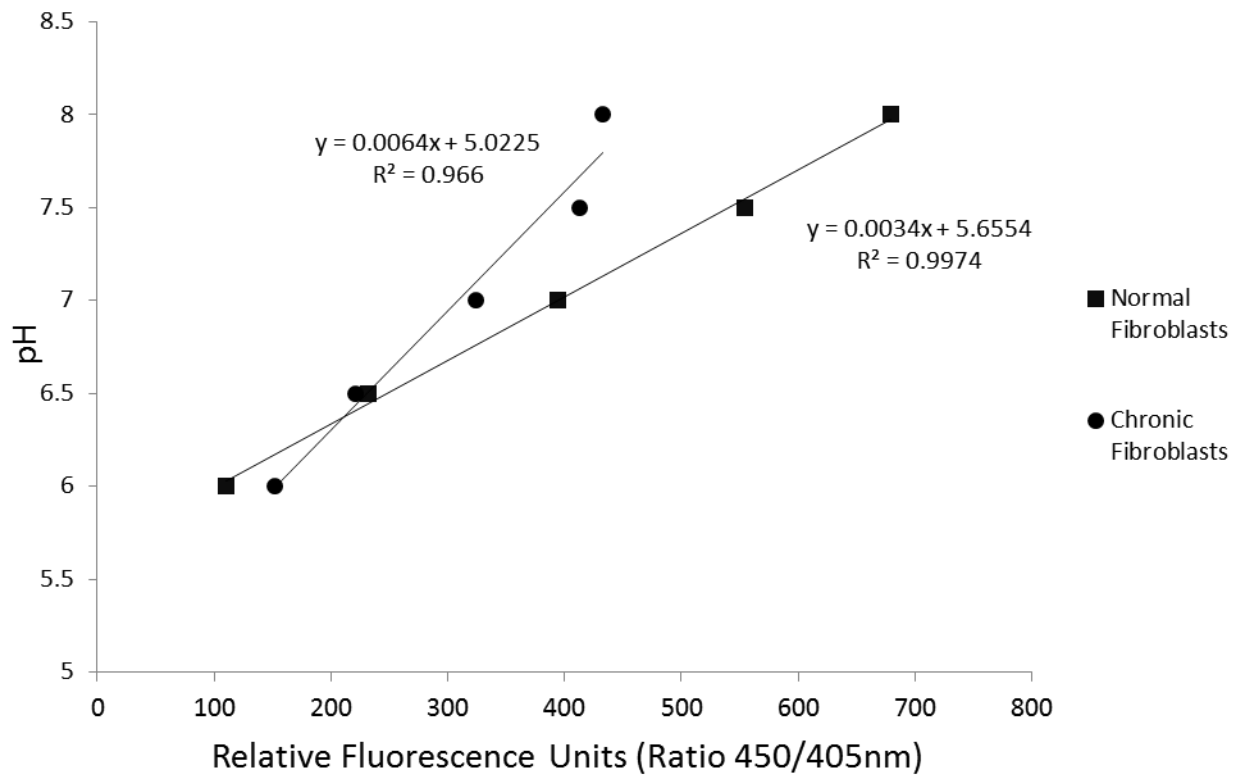
Figure 2.5 Calibration of intracellular pH using the high K$^+$ -nigericin method

Two individual standard curves for intracellular pH were created for the normal and chronic fibroblasts. Following incubation with 5 μM BCECF and high K$^+$ -nigericin media cells were incubated in pH adjusted buffer. Fluorometric analysis was performed (Excitation: 490/405nm; Emission: 535nm) and ratios calculated to obtain the standard curve.

2.1.13 Reverse transcription quantitative real time (RT-qPCR)

RNA was extracted from equine normal skin, chronic granulation tissue and from NF, CF, HDF and HaCaT cell cultures following 24 hour incubation in the different experimental conditions.

2.1.13.1   RNA isolation

Tissue samples (~30 mg) were diced into small pieces and placed in a sterile eppendorf before being snap frozen by submersion in liquid nitrogen. Tissue sample was then transferred to the dismembrator (Mikro-Dismembrator U, B.Braun Biotech International) and homogenised for 1 minute. TRIzol reagent (1ml) (Invitrogen, UK) was added to the sample and allowed to dissolve before transferring to a clean eppendorf and storing at -80 °C. Cell monolayers were lysed directly in culture flask by adding 1 mL of TRIzol reagent and incubating for 5 minutes at room temperature. The TRIzol samples were then frozen at -80 °C until RNA was to be extracted.

All samples were thawed and centrifuged at 12,000 x g for 15 minutes at 4 °C to remove any debris. 1-bromo-2-chloropropane (100 µL) (BCP; Sigma, UK) was added to each sample and shaken vigorously for 20 seconds and incubated at room temperature for 3 minutes. Samples were then centrifuged at 12,000 x g for 15 minutes at 4 °C. The upper aqueous phase was transferred to a clean microcentrifuge tube. Isopropanol (250 µL) was added and incubated at room temperature for 10 minutes in order to precipitate the RNA from the aqueous phase. 2 µL of GlycoBlue (Thermo Fisher, UK) was also added to samples and used as a visible co-precipitant for nucleic acids (Ambion, UK). Then, samples were centrifuged at 12,000 x g for 20 minutes at 4 °C, after which the supernatant was removed. 1 mL of ice cold 75% ethanol was added and then centrifuged at 12,000 x g for 5 minutes at 4 °C to wash the RNA pellet. The ethanol was removed without disturbing the pellet and then left to air dry briefly before being re-suspended in Tris-EDTA (TE) buffer (Ambion, Thermo Fisher, UK). RNA

concentration was determined by measuring the absorbance at 260nm using a NanoDrop (ND-1000) spectrophotometer.

2.1.13.2   DNase treatment

Isolated RNA is often contaminated with genomic DNA, which can result in false positive signals in subsequent RT-PCR. RNA samples were therefore treated with DNase I, an enzyme that non-specifically degrades single and double stranded DNA, according to manufacturer's instructions (Life Technologies, UK) prior to reverse transcription. Briefly, 10X DNase buffer diluted to a 1X concentration and DNase I enzyme (4U) were added to RNA samples containing up to 10 µg nucleic acid per 50 µL final volume and incubated for 30 minutes at 37 °C. The DNase is inactivated and the RNA extracted by phenol: chloroform. Briefly, samples were made up to a total volume of 100 µL with RNase-free water and then an equal volume of phenol: chloroform: Isoamyl Alcohol (Sigma, UK) was added and vortexed to an emulsion. Samples were centrifuged at 12,000 x g for 5 minutes at 4 °C and then the aqueous phase was transferred to a clean microcentrifuge tube. Precipitation of RNA was done by adding 10 µL ammonium acetate, 330 µL ethanol and GlycoBlue as a co-precipitant. The samples were mixed thoroughly before incubating on ice for 30 minutes. Samples were then centrifuged at 12,000 x g for 20 minutes at 4 °C and the supernatant carefully removed. 1 mL of 75% ethanol was added to wash the RNA pellet and centrifuged at 12,000 x g for 5 minutes at 4 °C. Finally, the RNA was air-dried and re-suspended in TE buffer and its concentration determined using a NanoDrop spectrophotometer.

2.1.13.3   Reverse transcription of DNase treated RNA

DNase treated RNA (1 µg) was converted to complementary DNA (cDNA) by reverse transcription. Briefly, RNA samples were incubated with 1 µL random primer at 70 °C for 5 minutes in the PCR thermo-cycler (2720 Thermal Cycler, Applied Biosystems) after which

they were immediately transferred onto ice. Then 5X buffer, dNTP mixture (10mM of each), RNase inhibitor and M-MLV RT (all from Promega, UK), in a total volume of 11.6 μL, were added to the RNA samples. The samples were incubated at 37 °C for 60 minutes and at 95 °C for 5 minutes on the thermo-cycler, then placed on ice before storing at -20 °C until use.

2.1.13.4   Housekeeping gene and primer efficiency

The expression stability of a panel of 7 housekeeping gene candidates from an equine specific housekeeping gene kit (Actin beta (ACTB), Beta-2 microglobulin (B2M), Hypoxanthine phosphoribosyltransferase 1 (HPRT1), Ring finger protein 185 (RN185), Succinate dehydrogenase complex, subunit A (SDHA) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); primer sequences not available for publication (GeNorm, Primerdesign Ltd, UK)) plus another GAPDH, one that is not in the kit but used for all following experiments (see Table 2.2) were tested against samples of NF and CF cDNA to determine the best reference gene to be used for normalisation. Using the geNorm software (Vandesompele et al. 2002), GAPDH was found to be the most suitable candidate to be used to normalise any further RT-qPCR data (Figure 2.6). geNorm is a readily available algorithm that automatically calculates the gene-stability measure (M) for all control genes in a given set of samples. Genes with low geNorm M values are considered very stable, while genes with a high geNorm M value are unstable (Vandesompele et al. 2002).

A standardized 1:2 dilution series from sample cDNA was used for calculation of the PCR efficiency for each gene, according to the equation E% = ($10^{-1/slope}$ – 1) × 100. The slope was calculated from a linear regression model against the Ct values of the serial dilutions of cDNA. All amplifications displayed PCR efficiencies between 95% and 115%.

2.1.13.5   RT-qPCR

Reverse transcription quantitative real time PCR (RT-qPCR) was performed to quantify the expression of COL1a1, COL1a2, FN, DCN, LUM, VCAN and NHE-1. The primers used were designed and synthesised by Eurogentec (as detailed in Table 2.2). RT-qPCR was performed in a 25 µl reaction volume containing; cDNA (10 ng), primers designed for the particular gene of interest (3 µM forward and reverse) and GoTaq Mastermix with the SYBR gene (2x reaction buffer, 400 µMeach dNTPs, 3 mM MgCl$_2$; all Promega, UK). GAPDH was chosen as the reference gene after validation (Table 2.2). Samples were run in duplicate alongside no template controls on a 7300 Real-Time PCR system (Applied Biosystems) using the following cycling parameters: 50°C for 2 minutes, 95°C for 10 minutes, the 40 cycles of 95°C for 15s and 60° for 1min.

Upon termination of the amplification phase, a dissociation step was performed at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s to check the purity of the amplification products. All samples displayed only a single peak, indicating a single pure product rather than primer dimers or non-specific PCR products (Appendix A). Controls without cDNA template were used to determine the specificity of the amplification. Data were normalized to the endogenous control gene GAPDH, and the change in gene expression level was calculated using ΔC_T (C_T Target - C_T Reference) (Livak & Schmittgen 2001).
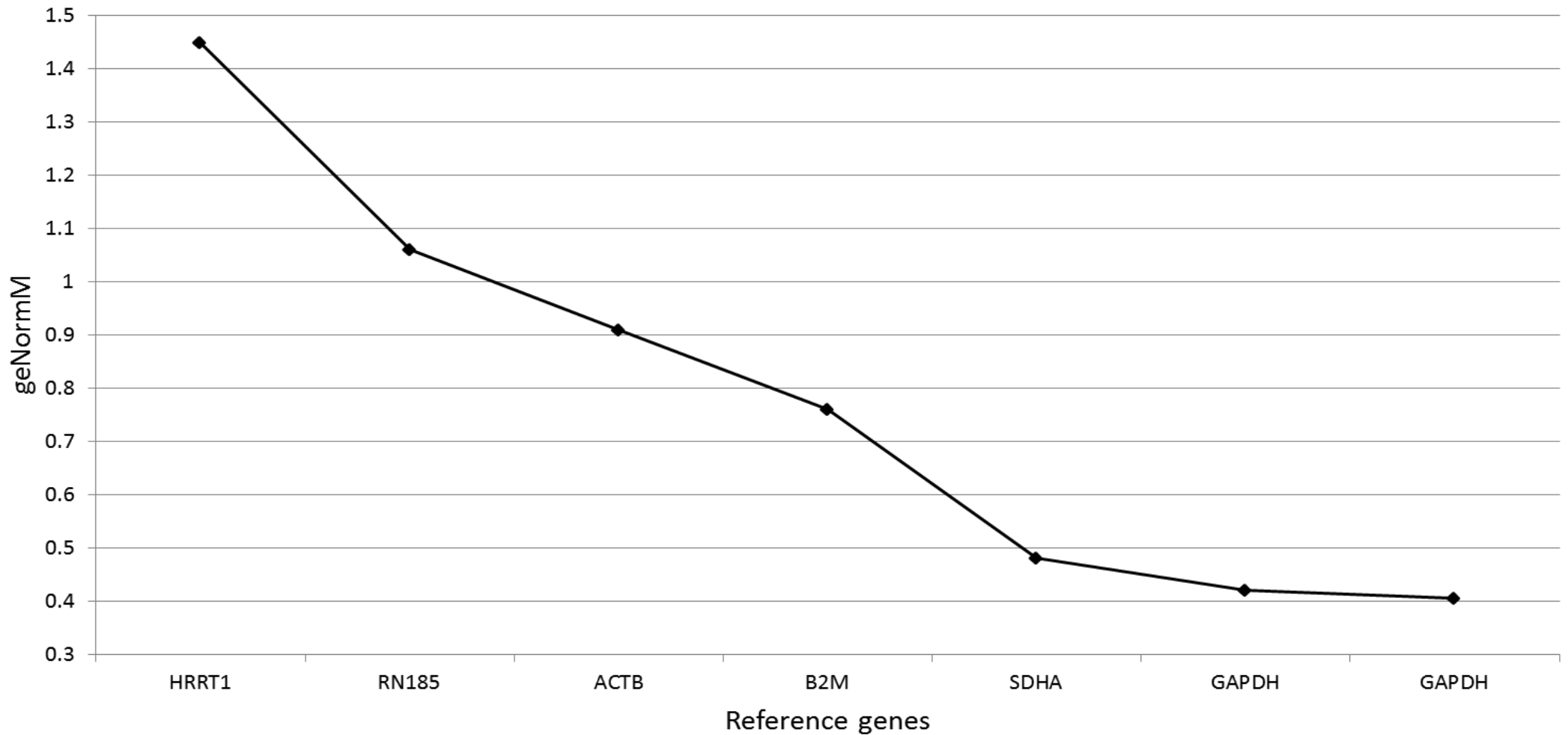
Figure 2.6 Average expression stability of reference targets

Average values (*M*) of expression stability of reference genes analysed by the GeNorm program. This graph displays the output of GeNorm plotting the *M* value against the reference genes within every sampling from each group. A high geNorm M value indicates an unstable gene, whereas a low M value indicates a very stable gene. Stability of two GAPDH genes were tested, was included in the housekeeping gene kit, the other GAPDH gene (shown here as the most stable) was the gene readily available and subsequently used in all other experiments.

Table 2.2 Table showing the forward and reverse sequences for primers used in qRT-PCR.

Gene	Primers (5' → 3')	Species
GAPDH	F = TGACCCCCTAACATATTGAGAGTCT R = GCCCCTCCCCTTCTTCTG	Equine
GAPDH	F = ATGGGGAAGGTGAAGGTCG R = TAAAAGCAGCCCTGGTGACC	Human
COL1a1	F = AGAGCATTTGCCCAACATTGT R = CATGGGAAAGGATGAAAACGA	Equine
COL1a2	F = CTCTGCGACACAAGGAGTCTG R = CTTTCCTGCAGTTGCCTCTTG	Equine
FN1	F = GCTCACTTTTTCCAAATGATTCTAGTAA R = CAACTGCATACTAAGTGTCTTCAATACAATT	Equine
DCN	F = CTGCCCACCTGGATACAACA R = GATGGCTGGATCTCCCAGTACT	Equine
LUM	F = CAGGCCTATTTCATCACAAGCA R = CGGTAGAGATGTGGAAAACACTAAATT	Equine
VCAN	F = TGACGTCATGTACGGGATTGA R = AGTGAAACACAACCCCATCCA	Equine
NHE-1	F = CCCGGCAACTAGAGCAGAAG R = GAGGAAGGACCCACATTCCC	Equine
NHE-1	F = CCTCTCTGGGTGGAGAAGCT R = CCCAGGAACGACACAGAAAG	Human

2.1.14 Western Blotting

HaCaT cells were washed twice with PBS, then lysed in appropriate volume of lysis buffer (Sigma, UK). Briefly, samples were heated for 10 minutes at 100 °C, and then centrifuged at 4°C for 20 minutes. Supernatant was collected and protein concentration determined by the Pierce BCA Protein Assay Kit (Thermo Fisher, UK). Concisely, a protein standard curve (protein concentrations from 0 – 2000 µg/mL) was made using the albumin standard provided in the assay kit. 10 µL of sample or standard was added to a 96-well microtiter plate, then 200 µL of working reagent (50:1, BCA Assay Reagent A:B) was added to each well and mixed thoroughly. Microtiter plate was covered and incubated at 37°C for 30 minutes. Plate was cooled to room temperature before absorbance was measured at 570nm with a spectrophotometer. The concentration of protein in samples was then calculated using the standard curve.

10 µg of whole cell lysate of each sample was mixed with 1 µL of β-mercaptoethanol (Sigma, UK) and 5 µL SDS sample buffer (Life Technologies, UK) and incubated at 80 °C for 10 minutes. Samples were loaded onto a sodium dodecyl sulphate – polyacrylamide gel (SDS Page; 4 – 12% Bis-Tris gel, Life Technologies, UK), with 10 µL Novex Sharp Pre-stained protein (Invitrogen, UK) as standard. Samples were ran in 1 X MES SDS running buffer (Life Technologies, UK) for 35 minutes or until samples had reached the bottom of the gel. The gel was then blotted onto a nitrocellulose membrane by soaking in transfer buffer (Life Technologies, UK) and run at 30 volts for 90 minutes. Nitrocellulose membrane was then washed in TBS (1X Tris Buffered Saline) for 5 minutes placed on a gyro rocker. Membrane was then blocked with 5% milk/TBS for 1 hour at room temperature. Primary antibody (Laminin-332; abcam, UK) was diluted (1:1000) in TBS and incubated overnight at 4 °C with gentle agitation. Membrane was then washed (TBS, 2x TBS/Tween and TBS; 5 minutes per wash), before secondary antibody was added. Goat-anti Rabbit secondary antibody (abcam, UK) was diluted 1:1000 in 5% milk/TBS and added onto membrane and incubated at room temperature for 1 hour. Membrane was then washed again in TBS and TBS/Tween as described above. Western lightening Plus Enhanced Chemiluminescence solution (Perkin

Elmer, UK) was used according to manufactures instructions (equal volume of oxidising reagent and enhanced luminol reagent), and added to membrane for 1 minute. Membrane was then exposed to UV light using the UVP ChemiDoc imaging system and proteins detected.

2.1.15 Histology

Normal and chronic skin and wound tissue samples were fixed in 10% formalin, processed, cut into 7 μm sections and mounted onto glass microscope slides. Sections were stained with a variety of stains including Haematoxylin and Eosin, Gram stain and Masson's Trichome. Immunohistochemistry for antigen detection of the sodium – hydrogen membrane transporter NHE-1 was carried out to compare the protein expression between equine normal skin and chronic wound tissue sections.

Briefly, tissue sections were rehydrated following a series of washes (2 x xylene for 5 minutes, 2 x 100% ethanol for 2 minutes, and 1 x 95% ethanol for 1 minute and dH_2O for 2 minutes). Sections were then blocked in 10% goat serum for 1 hour at room temperature. Next, 100 μl of primary antibody diluted in blocking serum (1:100 dilution of NHE-1 antibody, Santa Cruz Biotechnology) was added and slides were incubated overnight at 4 °C in a humidified atmosphere. Following day slides were washed in TBS before applying 100 μL secondary antibody (goat polyclonal antibody 1:1000 (ab97200, Abcam, UK) diluted in blocking serum) and incubating for 60 minutes at room temperature. Sections were washed and incubated in Diaminobenzidine (DAB; Sigma, UK) for 10 minutes at room temperature before being counterstained with Mayer's Haematoxylin. Finally, slides were dehydrated through a series of solutions (95% ethanol for 1 minute, 2 x 100% ethanol for 2 minutes and 2 x xylene for 2 minutes), then mounted in DPX (Sigma, UK) and covered with a coverslip.

2.2 Microbiology

2.2.1 Maintenance of bacterial cultures

Staphylococcus aureus (*S. aureus*; NCIMB 9518) and *Pseudomonas aeruginosa* (*P. aeruginosa*; NCIMB 8626) strains were maintained on tryptone soya agar plates (TSA) with weekly sub-culturing.

2.2.2 Gram staining

Gram stains of each bacterium were carried out to ensure the purity of cultures. Briefly, a smear of bacterial colony was heat-fixed to a glass slide before staining. The bacteria were stained with crystal violet (1% w/v) for one minute, followed by iodine solution (1% (w/v) iodine, 2% (w/v) potassium iodide) for 30 seconds. Acetone is applied for de-colouring and washed off immediately and finally a fuchsin (0.1% (w/v) fuchsin, 1% (v/v) ethanol, 0.5% (w/v) phenol) counter-stain was applied for one minute. Bacteria are then visualised by placing a drop of oil onto the slide and examining with the 100x objective lens of a light microscope.

2.2.3 Bacterial growth curves

Growth profiles of *S. aureus and P. aeruginosa* were assessed in Tryptone Soya broth (TSB) and DMEM media (no phenol red or added antibiotics). Single colonies of each bacteria were aseptically picked and grown overnight in 10 mLs of TSB at 37 °C. The following day the culture was diluted to an equivalent of 10^8 colony forming units (CFU) optical density of 0.1 measured at 600nm in TSB or DMEM. This was further diluted in TSB/DMEM such that the starting concentration was 10^6 in a volume of 200 μL and placed in a well of a 96-well plate. The growth of the bacteria was recorded at 37 °C and assessed by the changes in absorbance values at 600nm read by a plate reader every 30 minutes for 24 hours, with agitation before the absorbance was recorded. Background absorbance was also recorded from separate

cultures of growth media not inoculated with bacteria in order to check sterility of the media and to check for background absorbance.

2.2.4 Assessment of bacterial growth in different pH conditions

2.2.4.1 Planktonic

Growth profiles of *S. aureus and P. aeruginosa* were assessed in TSB adjusted to pH 6, 7.5 and 9. The pH of TSB was changed by the dropwise addition of concentrated HCl or 5M NaOH and then autoclaved before use. Single colonies of each bacteria were aseptically picked and grown overnight in 10 mLs of TSB (pH unchanged) at 37 °C. The following day the culture was diluted as described previously, such that starting concentration was 10^6 in a volume of 200 µL and placed in a well of a 96-well plate. The growth of the bacteria was recorded at 37 °C and assessed by the changes in absorbance values at 600nm read by a plate reader every 30 minutes for 24 hours, with agitation before the absorbance was recorded. Background absorbance was also recorded from separate cultures of growth media not inoculated with bacteria in order to check sterility of the media and to measure background absorbance.

2.2.4.2 Biofilm

A colony of *S. aureus* or *P. aeruginosa* was suspended in 10 mLs TSB and incubated overnight at 37 °C with agitation at 120 rpm. Growth of biofilms was determined using the MBEC method (Innovotech Ltd). Briefly, bacterial suspension was diluted and 200 µL placed into a round bottom 96-well plate with pegged lid (info) such that the final volume was equivalent to 10^6 in 200 µL. Plates were incubated for 24, 48 and 72 hours at 37 °C on a shaking platform at 120 rpm. The peg lids were then washed in water to remove any unattached planktonic bacteria. The remaining biofilm was stained by transferring the peg lid into 96-well plate with 200 µL 1% solution of crystal violet (CV) (Prolab diagnostics, UK) and

incubated at room temperature for 15 minutes. The peg lid was then rinsed by submerging in water in order to remove any excess dye. The lid was left to briefly dry, before being placed in a 96-well plate containing 200 μL of 30% acetic acid (Sigma, UK) and incubated at room temperature for 15 minutes in order to solubilise the CV. The solubilised CV was then transferred into a new flat bottomed 96-well plate and absorbance measured at 550 nm using a spectrophotometer plate reader using 30% acetic acid as the blank.
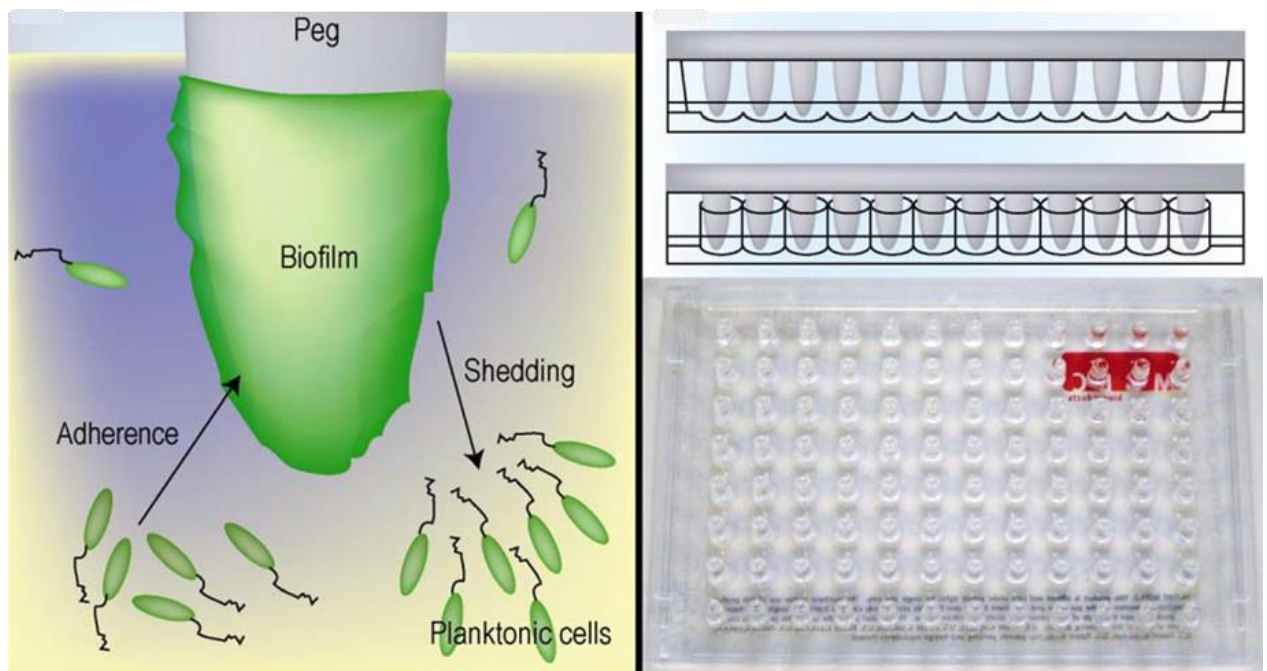
Figure 2.7 The MBEC Assay

Diagram demonstrates how biofilms form on the polystyrene pegs of the 96-well plate lid. These bacteria become irreversibly attached and form mature biofilms which can be quantified using crystal violet stain. Image from Innovotech Ltd.

2.2.5 Generation of Bacterial Conditioned Media

2.2.5.1 Planktonic Conditioned Media (PCM)

A colony of bacteria (*S. aureus* and *P. aeruginosa*) was suspended in 10 mLs of DMEM (no antibiotics) and incubated overnight at 37 °C with agitation at 120 rpm. Bacterial suspension was diluted to an equivalent of 10^8 CFU/mL and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and kept at -20 °C until required for further experiments. The bacteria was re-suspended in PBS and serially diluted, 100 μL was plated onto TSA and incubated overnight at 37 °C before final bacterial growth (CFU/mL) was determined following colony counts.

2.2.5.2 Biofilm Conditioned Media (BCM)

A colony of bacteria (*S. aureus* and *P. aeruginosa*) was suspended in 10 mLs TSB and incubated overnight at 37°C with agitation at 120 rpm. Bacterial suspension was diluted to an equivalent of 10^8 CFU/mL and 40 μL was placed onto a 0.2 μm filter disc and incubated for 72 hours at 37 °C on TSA plates (Figure 2.8A). The filter disc was then transferred to a ledge of a deep well plate and 8 mLs of DMEM (no antibiotics) placed underneath and incubated at 37 °C for 24 hours (Figure 2.8B). BCM was then collected and kept at -20 °C until required for further experiments. If bacterial growth was visible in any of the wells this media was discarded and not used in experiments. The biofilm disc was placed in PBS and vortexed until the bacterial biofilm was disrupted and then serially diluted. A 100 μL sample was plated onto TSA plates and incubated overnight at 37 °C before final biofilm bacterial growth (CFU/mL) was determined following colony counts.

Following initial dilution and viability trials, PCM was used neat and BCM was diluted 1:4 in all subsequent experiments. PCM and BCM were pH adjusted as described in section 2.1.5 and used in experiments described in sections 2.1.6 and 2.1.7
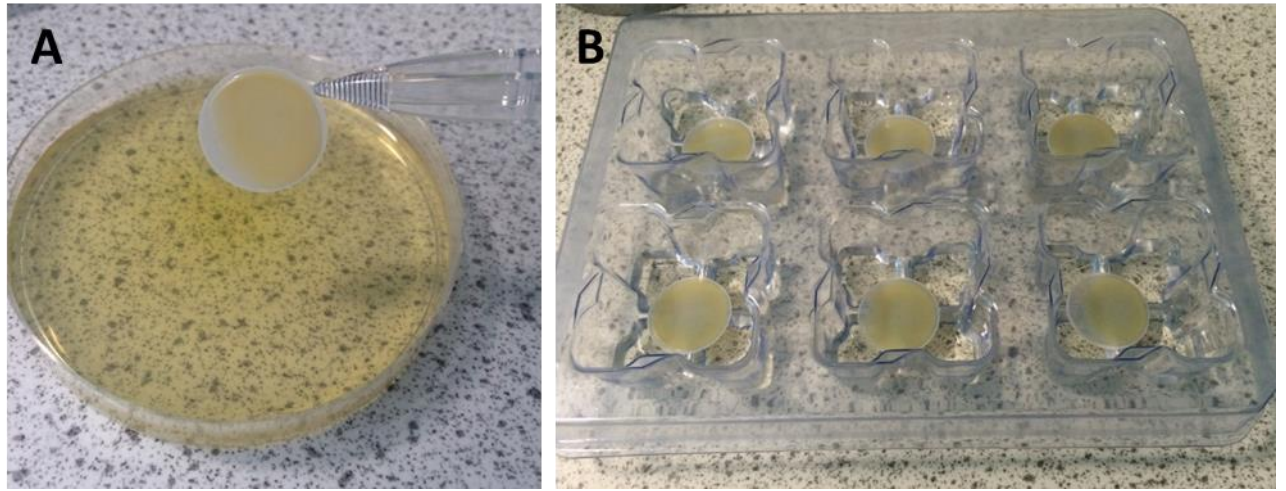
Figure 2.8 Generating Biofilm Conditioned Media

Image shows how Biofilm Conditioned Media (BCM) was generated. A) Biofilm grown for 72 hours on a filter disc placed on a TSA plate. B) Biofilm disc was then transferred onto ledge of a deep well plate and DMEM media placed underneath and incubated for 24 hours at 37°C.

2.2.6 Protein Analysis

Total protein in collected PCM and BCM was measured using the Pierce BCA Protein Assay Kit as described previously in section 2.1.14. For further protein analysis samples were ran on an SDS-Page. Firstly, proteins were precipitated from sample using acetone in order to remove unsuitable components and allow a clearer banding pattern. Briefly, 500 μL of cold acetone (Sigma, UK) was added to 100 μL of sample and vortexed to mix thoroughly. Samples were incubated at -20 °C for an hour, and then centrifuged at 12,000rpm at 4 °C for 10 minutes. Supernatant was then decanted and the pellet air dried briefly. Pellet was then re-solubilised in 20 μL SDS-lysis buffer and β-mercaptoethanol (20:1 solution). Samples were heated again at 80 °C for 10 minutes before loading onto a SDS-Page gel, with 10 μL Novex Sharp Pre-stained protein (Invitrogen, UK) as standard. Samples were run for 35 minutes or until samples had reached the bottom of the gel. The gel was washed in TBS for 5 minutes before proteins were detected by staining with the Pierce Silver Stain Kit (Thermo Fisher, UK).

2.2.7 Statistics

All results are presented as mean ± standard error of the mean (SEM). Data was tested for normality using the Anderson-Darling test and equal variances prior to parametric statistical analyses using Minitab 17. One-way Analysis of Variance (ANOVA) was performed to determine a significant difference between the mean values, followed by a posteriori test, Tukey Kramer or Fisher, unless otherwise stated. A p-value of <0.05 was considered statistically significant.

Chapter 3:

Results I - The Effect of pH on Normal and Chronic Wound Fibroblasts.

3 The Effect of pH on Normal and Chronic Wound Fibroblasts

3.1 Introduction

Chronic wounds are alkaline with recordings of pH 7.15 – 8.9 being reported (Wilson et al. 1979; Dissemond et al. 2003), which can both directly and indirectly influence many factors of wound healing. The rate of wound healing declines when pH is elevated to alkaline conditions. It has been observed that both acute and chronic wounds with a high alkaline pH have a much slower rate of healing compared to wounds with neutral pH (Tsukada et al. 1992). The surface of normal healthy skin is slightly acidic and this is an important part of the skins barrier system, controlling infection and preventing microbial infection (Schmid-Wendtner & Korting 2006).

Many healing processes are known to be affected by small changes in pH including angiogenesis, immunological responses and macrophage, fibroblast and keratinocyte activity (Gethin 2007). Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which are crucial in ECM remodelling, have been shown to be sensitive to small variations in pH (Greener et al. 2005). Schneider and colleagues noted the complex relationship between pH and wound healing in a detailed review. They showed that pH is a significant factor in wound healing, and notably, that different pH ranges are required throughout the different phases of wound healing (Schneider et al. 2007).

The main objective of this study was to investigate the effect of pH on equine Normal Fibroblasts (NFs) and Chronic Wound Fibroblasts (CFs). Three pH conditions were chosen; acidic pH 6, neutral pH 7.5 and alkaline pH 9. I investigated pH dependant alterations on a number of parameters, including the abundance and expression of important ECM molecules essential for wound healing such as collagen, fibronectin and GAGs. Furthermore, changes in cell migration due to extracellular pH conditions were also assessed.

3.2   Normal Fibroblasts and Chronic Wound Fibroblasts

NFs and CFs were isolated from healthy equine skin or chronic wound debrided granulation tissue and utilised for all experiments (see Chapter 2.1.2). Figure 3.1 illustrates their morphology in culture, with both cell types exhibiting typical fibroblast morphology.

Cell proliferation and viability of both NFs and CFs did not significantly differ in any of the pH culture conditions such that any differences in extracellular matrix abundance or cell migration was not due to increased proliferation (Figure 3.2).
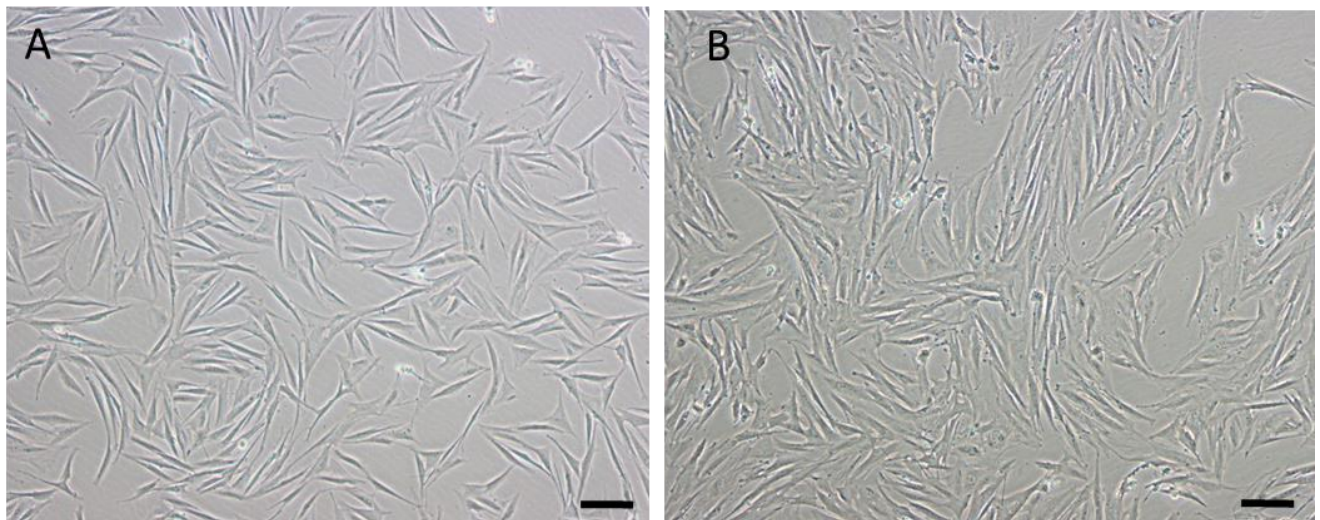
Figure 3.1 Photomicrographs of equine normal skin (NFs) and chronic wound (CFs) fibroblasts in culture

Both NFs (A) and CFs (B) demonstrate a typical fibroblast-like bipolar and stellate morphology. Bar = 100µm.
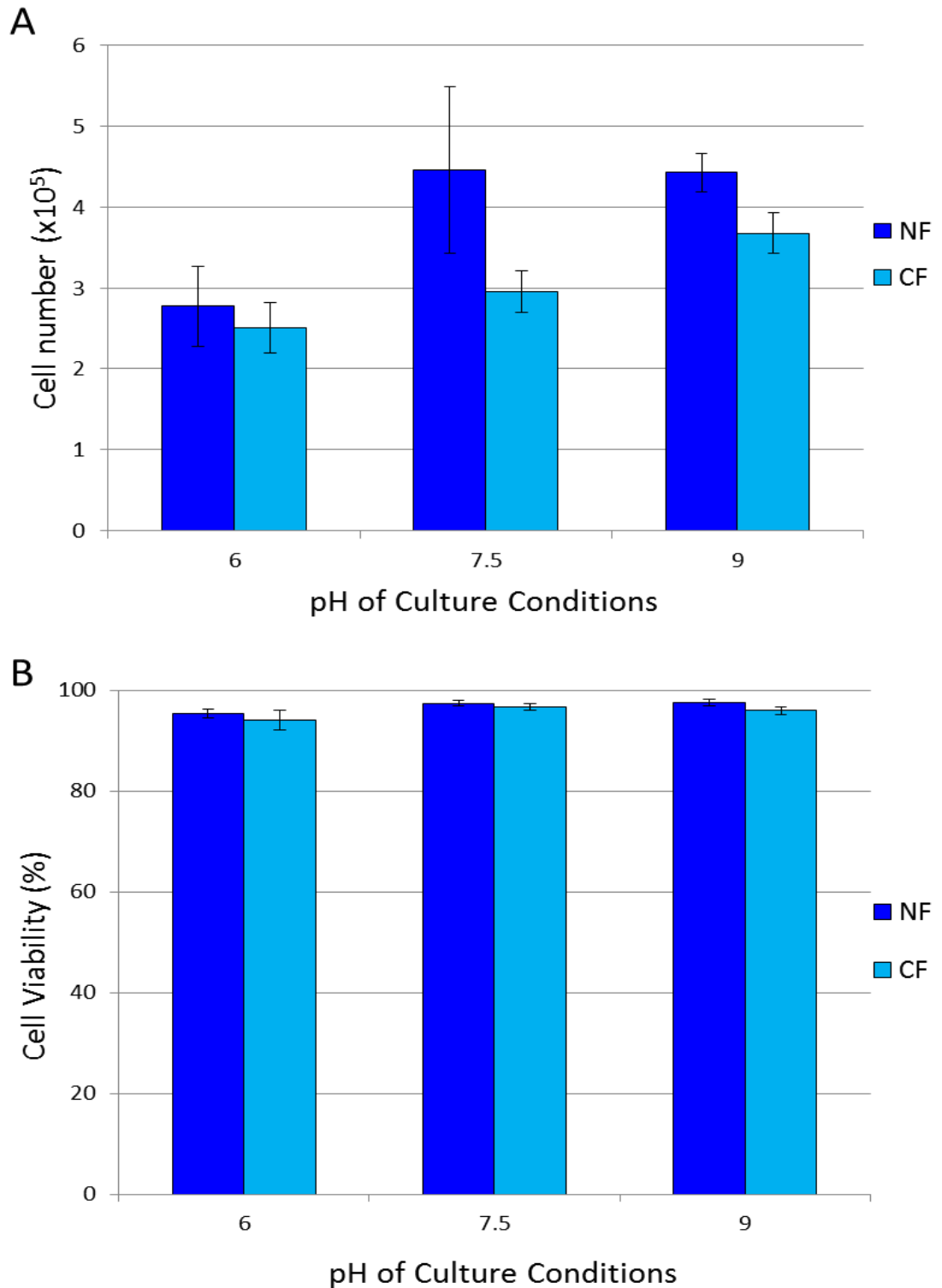
Figure 3.2 Cell proliferation and Viability

Graphs show final cell number (A) and cell viability (B) per well of a 6-well plate, following 24 hours culture in the different pH conditions. Cell viability was determined following the trypan blue dye method, if cells take up the dye and appear blue they are considered to be non-viable. Cell number did not significantly differ in each condition and after 24 hours in culture in each pH cells were still over 94% viable. Values represent mean ± SEM (*n*=3).

3.3   Extracellular Matrix abundance from Normal and Chronic Fibroblasts Following Culture in Different pH Environments.

3.3.1   pH – Adjusted media

Several different buffers were tested to ensure that the pH of the culture media remained constant during incubation periods of 24 and 48 hours at 37°C. Optimal buffers for maintaining the required pH were MES hydrate for pH 6, HEPES for pH 7.5 and CHES for pH 9 (Table 3.1). Without the relevant buffers in the media the pH was not maintained for 24 hours, therefore any effects on ECM expression or cell migration were due to changes in pH and not the presence of buffers.

Table 3.1 Buffers for pH adjusted DMEM media

Table shows pH values of titrated DMEM media after 24 hours incubation at 37°C with and without selected buffers**.**

Buffer	Concentration	Starting pH	Average pH without buffer	Average pH with buffer
MES hydrate	25mM	6.00 ± 0.02	6.62 ± 0.27	6.01 ± 0.09
HEPES	25mM	7.50 ± 0.02	7.85 ± 0.15	7.51 ± 0.04
CHES	10mM	9.00 ±0.05	7.88 ± 0.04	8.97 ± 0.03

3.3.2 Collagen abundance

An increase in collagen abundance from NFs correlated with an increase in culture pH. The total collagen present was significantly more at pH 9 compared to pH 6 and pH 7.5 ($p<0.05$). No change in the amount of collagen was seen from CFs with a change in pH. In both pH 7.5 and pH 9 culture conditions, collagen abundance was significantly lower from CFs compared to NFs ($p<0.05$; Figure 3.3).
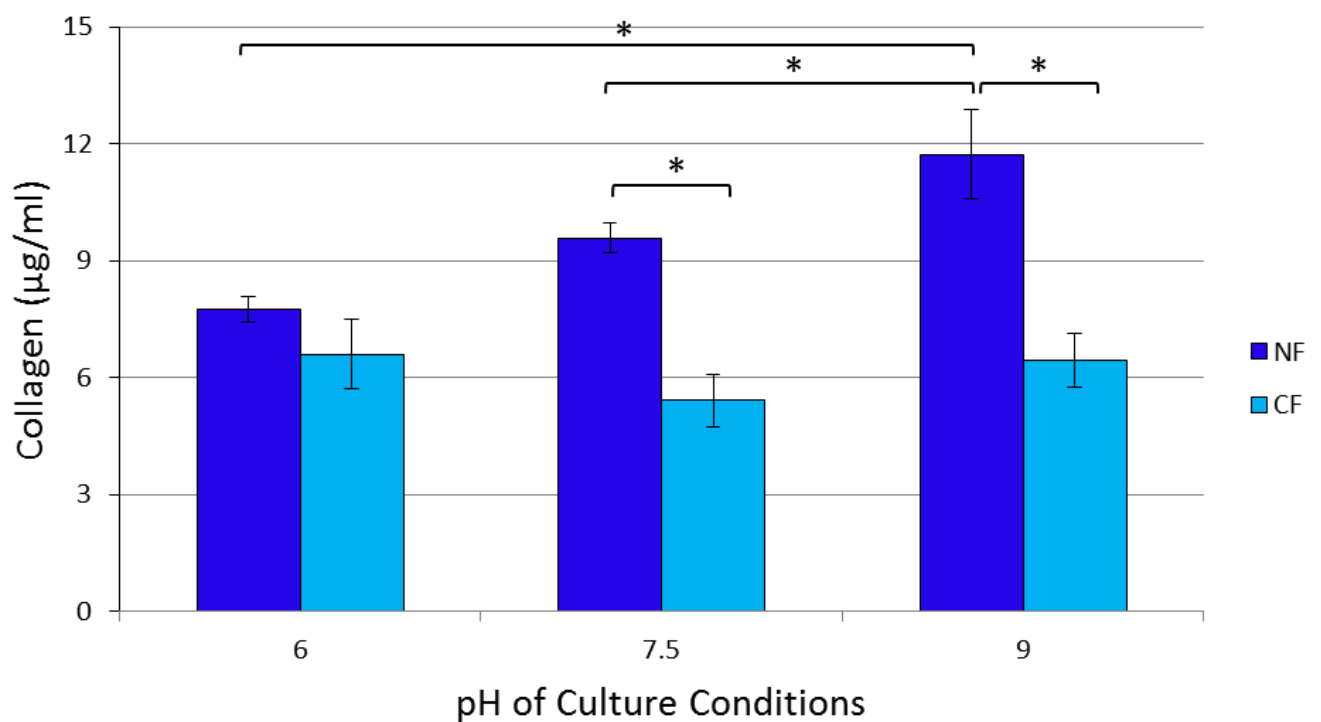
Figure 3.3. Collagen abundance from NFs and CFs cultured in pH 6, 7.5 and 9

Cells were seeded and allowed to adhere overnight, cells were then gently washed in PBS before incubation in different pH media for 24 hours. Media was collected after 24 hours and presence of collagen analysed in conditioned media. Values represent the mean ± SEM (n=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Tukey Kramer * p <0.05.

3.3.3 Fibronectin abundance

The total amount of fibronectin was lower from CFs compared to NFs, which was found to be significant in both pH 7.5 and pH 9 cultures ($p<0.05$; Figure 3.4). Fibronectin abundance was highest from NFs in pH 7.5 culture and was decreased in pH 6 ($p<0.05$). No significant changes in fibronectin from CFs were found with a change in pH culture conditions.
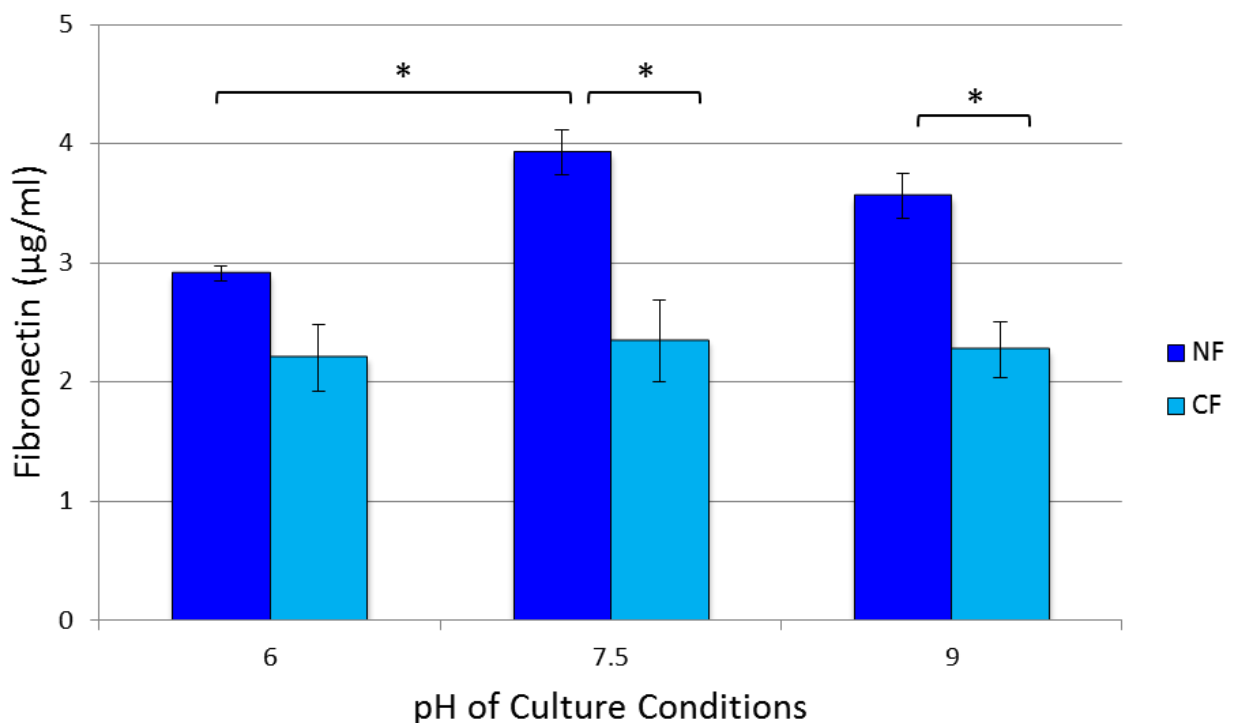
Figure 3.4. Fibronectin abundance from NFs and CFs cultured in pH 6, 7.5 and 9

Cells were seeded and allowed to adhere overnight, cells were then gently washed in PBS before incubation in different pH media for 24 hours. Media was collected after 24 hours and presence of fibronectin analysed in conditioned media. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Tukey Kramer * *p* <0.05.

3.3.4 Glycosaminoglycans abundance

Glycosaminoglycan abundance was higher from CFs than from NFs, but only reached significance between the two cell types in pH 9 conditions ($p<0.05$; Figure 3.5). More GAGs were present in the CM from CFs after culture in pH 9 compared to pH 6 and pH 7.5 conditions, however did not reach significance when comparing with both other pH conditions. No significant changes were seen in GAGs abundacne from NFs in different pH culture conditions.
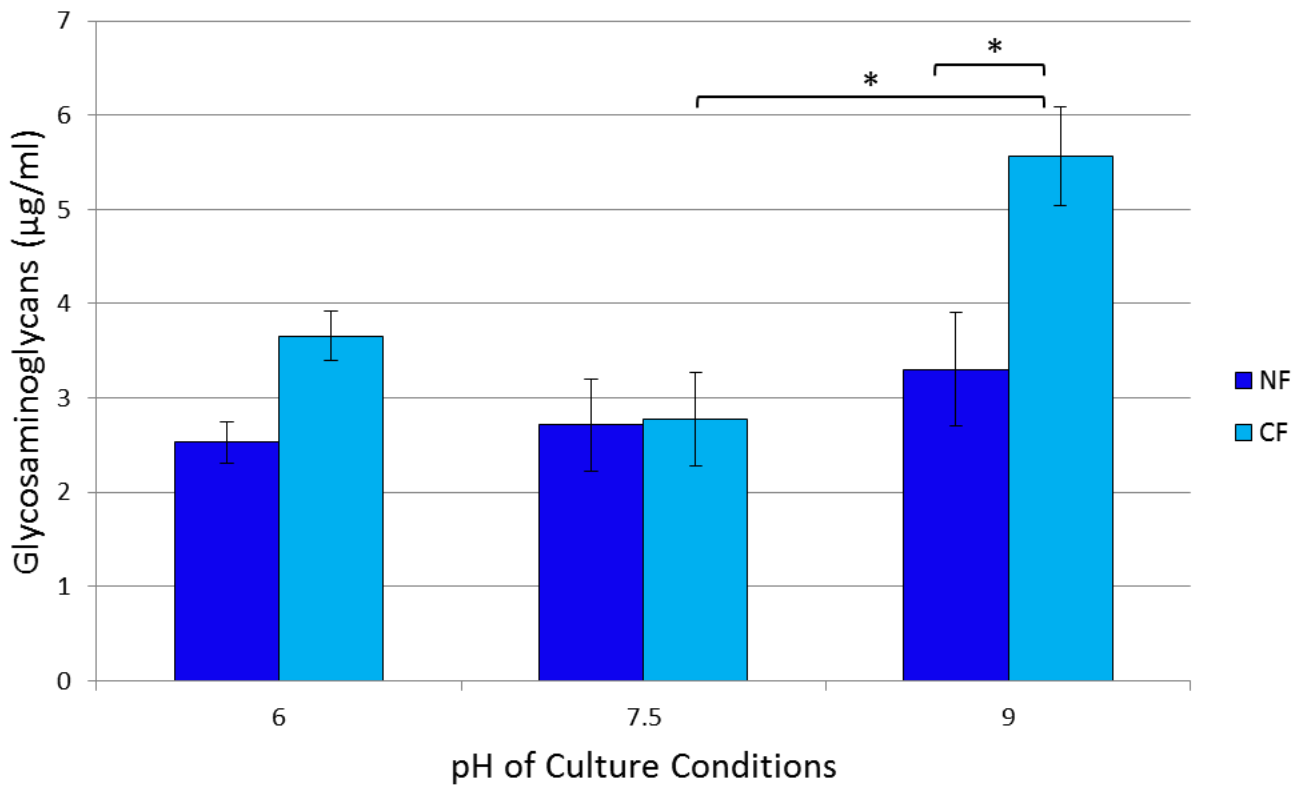
Figure 3.5 Glycosaminoglycan abundance from NFs and CFs cultured in pH 6, 7.5 and 9

Cells were seeded and allowed to adhere overnight, cells were then gently washed in PBS before incubation in different pH media for 24 hours. Media was collected after 24 hours and presence of GAGs analysed. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Tukey Kramer * *p* <0.05.

3.3.5 Tropoelastin abundance

No change in the amount of tropoelastin from either NFs or CFs was seen when they were cultured in different pH conditions. A significant difference ($p<0.05$, Figure 3.6) in tropoelastin abundance was found when comparing the amount from NFs and CFs after culturing in pH 7.5 and pH 9.
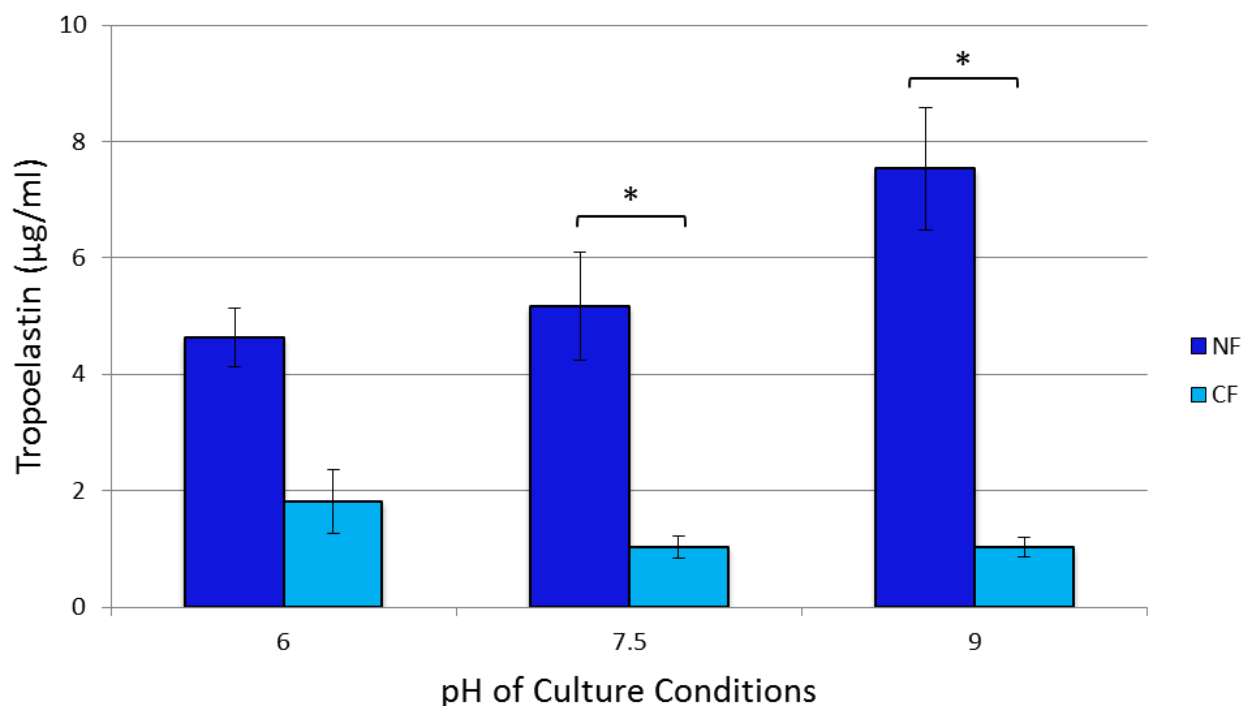
Figure 3.6 Tropoelastin abundance from NFs and CFs cultured in pH 6, 7.5 and 9 conditions

Cells were seeded and allowed to adhere overnight, cells were then gently washed in PBS before incubation in different pH media for 24 hours. Media was collected after 24 hours and presence of tropoelastin analysed in conditioned media. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Tukey Kramer * *p* <0.05.

3.4 Changes in Extracellular Matrix Gene Expression in Normal and Chronic Fibroblasts after Culture in Different pH Environments.

A range of ECM molecules including Collagen (COL1A1 and COL1A2), Fibronectin (FN), Decorin (DCN), Versican (VCAN) and Lumican (LUM) were assessed for changes in expression levels following culture of NFs and CFs in different pH conditions. All genes analysed showed lower expression from CFs than NFs.

In pH 9 conditions, COL1A1 expression was significantly lower in NFs compared to expression in pH 6 and pH 7.5 conditions (p<0.05) (Figure 3.7A). There was a similar trend with the expression of COL1A2, but in pH 9 it was not significantly lower (Figure 3.7B). There were no significant changes in the expression of FN following culture in different pH conditions within each cell type. A significant difference between the expression of FN in pH 9 conditions between NFs and CFs was seen (Figure 3.7C). There was a significantly lower expression of DCN in CFs compared to NFs in both pH 7.5 and pH 9 cultures (Figure 3.7D). There was also a lower expression from both cell types in pH 9 compared to pH 7.5 but this was not significant. There was no significant changes in VCAN expression in either cell in any condition (Figure 3.7E). In both pH 6 and pH 7.5 conditions expression of LUM was lower from CFs than NFs, but in pH 9 conditions LUM expression was significantly higher in CFs than NFs (Figure 3.7F). This increase in expression was significant compared to expression in CFs when cultured in pH 6 and pH 7.5.
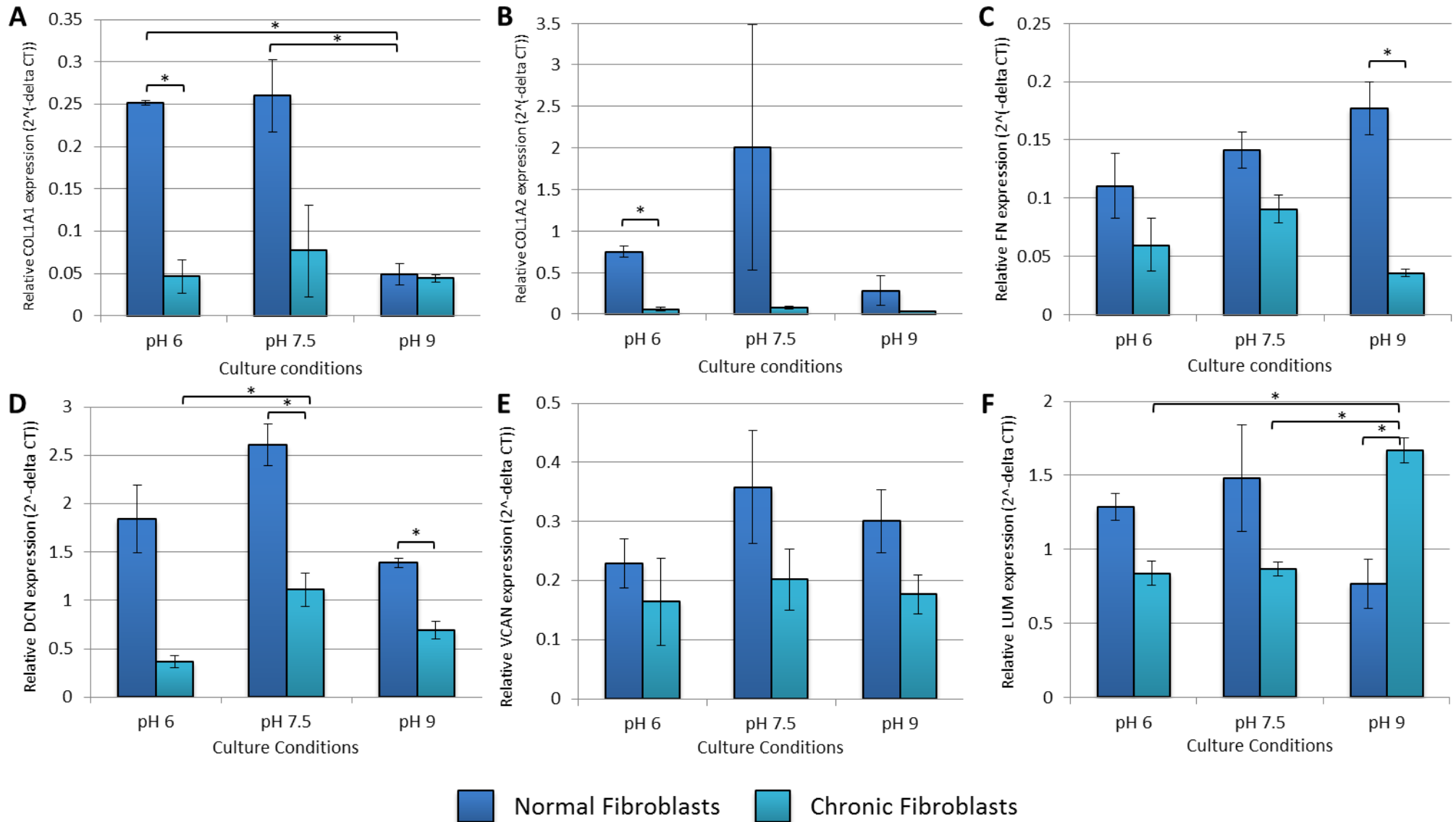
Figure 3.7 Gene expression of ECM molecules after culture of NF and CF in pH 6, pH 7.5 and pH 9 conditions

Results for the changes in expression of (A) Collagen Type 1A1 (COL1A1), (B) Collagen Type 1A2 (COL1A2), (C) Fibronectin (FN), (D) Decorin (DCN), (E) Versican (VCAN) and (F) Lumican (LUM) are shown as the relative expression (2^(-delta CT)) of the mean ± SEM. * $p < 0.05$

3.5   The Effect of pH on Fibroblast Migration

NF migration was significantly diminished in pH 6 conditions, with only 19% wound closure achieved after 48 hours compared to 60% closure in pH 7.5 ($p<0.05$) and 73% closure in pH 9 conditions ($p<0.05$). CF showed a different trend compared to the NF; cell migration was slowest in pH 9 conditions with only 39% closure compared to 62% in pH 6 ($p<0.05$) and 90% closure in pH 7.5 conditions ($p<0.05$). Significant differences were also seen after comparing the two cell types, NFs and CFs, in all three pH culture conditions ($p<0.05$; Figure 3.8 and Figure 3.9). In pH 6 and pH 7.5 culture conditions CFs migrated at a significantly faster rate than NFs, while in pH 9 conditions CFs migrated at a significantly slower rate than NFs.
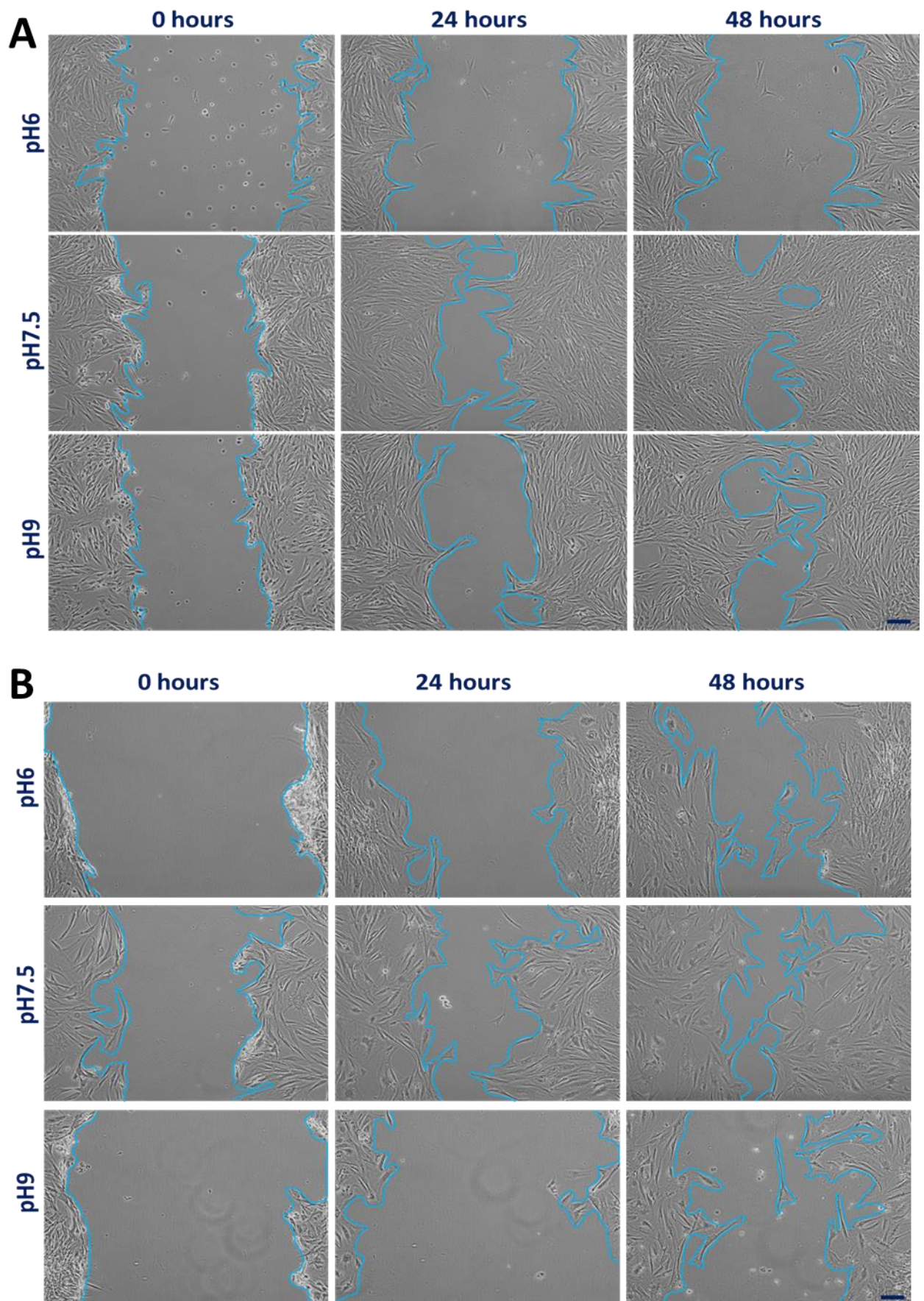
Figure 3.8 Micrograph images of NFs and CFs scratch area

Images show NFs (A) and CFs (B) scratch area of each pH condition at each time point.

Scale bar = 100μm.
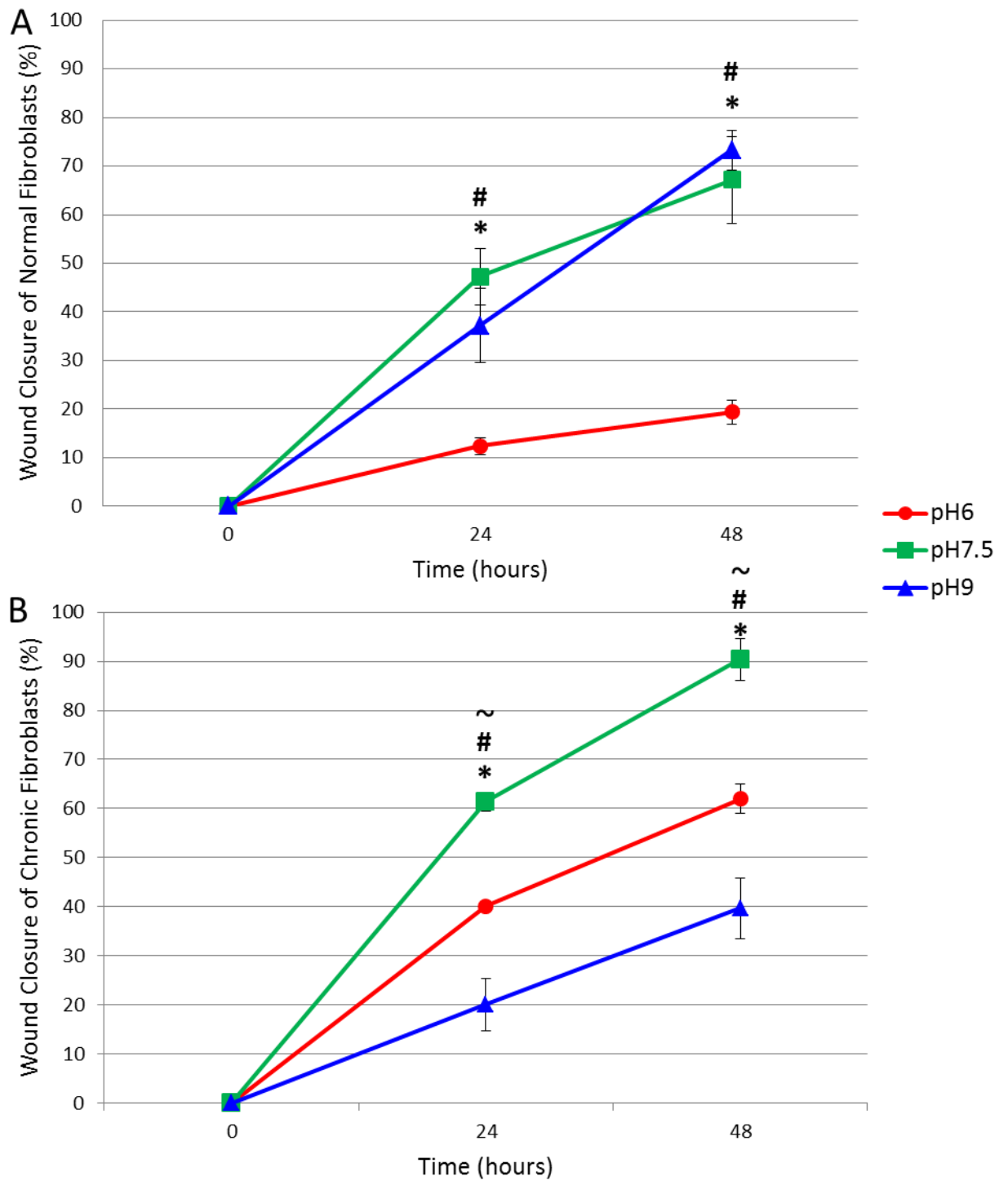
Figure 3.9 The effect of pH on fibroblast migration

Results are shown as the percentage of scratch closure over a period of 48 hours. Results for NFs is shown in A and CFs in B. Values represent the mean ± SEM (n=3). * p<0.05 between pH 6 and pH 7.5, # p<0.05 between pH 6 and pH 9 and ~ p<0.05 between pH 7.5 and pH 9.

3.6 Discussion

Non-healing chronic wound environments have been reported to be residing in a predominately alkaline environment when compared to healing wounds (Shukla et al. 2007; Schreml et al. 2011). The significance of this is still being debated as more evidence is generated.

3.6.1 Extracellular matrix abundance

Within this present study, significant changes in the abundance of collagen, fibronectin and tropoelastin were seen after culturing at both pH 7.5 and pH 9 conditions between NFs and CFs. It was found that ECM abundance was greater in the healthy NFs compared to the wound derived CFs, suggesting that when fibroblasts cells are in a chronic state they have a reduced synthetic capacity of ECM for adequate wound closure. Collagen is the major ECM molecule secreted from skin fibroblasts and is important for the tensile strength and stability of healthy skin (Schultz et al. 2005). Collagen abundance is significantly reduced from CFs compared to NFs, which correlates with previous studies that show that human CFs are not able to synthesise collagen to the same extent as NFs (Herrick et al. 1992). In pH 9 conditions, the amount of collagen from NFs was increased compared to pH 7.5 conditions. This increase was not seen in the CFs, with levels of collagen remaining the same when pH increased. Similarly, the CFs fail to synthesise as much fibronectin and tropoelastin as the NFs under all pH culture conditions.

Fibronectin, an adhesive glycoprotein, is important during the process of wound healing for successful cell attachment and directional migration of the fibroblasts (Clark 1990). Tropoelastin, the elastin precursor, secreted from fibroblasts which is then transformed into elastin fibres which are essential in providing skin with its elastic and stretching properties (Muiznieks & Keeley 2013).

Tropoelastin secretion from CFs was very limited which could explain the failure of elastin to be found in chronic wound granulation tissue in previous studies (Schultz & Wysocki 2009).

However, no further reduction in the abundance of the ECM molecules collagen, fibronectin or tropoelastin was seen when CFs were cultured in the different pH conditions such that alkaline conditions did not further exacerbate the amount of ECM present from the CFs.

Compared to the other ECM molecules, GAG abundance was increased from CFs compared to NFs. In pH 9 culture conditions this was significantly increased compared to total amount present in pH 7.5 conditions. GAGs are long polysaccharide molecules which have specific viscosity and lubrication properties in the skin (Schultz et al. 2005). The predominant GAG found in the skin is hyaluronic acid, which can be found in increased amounts in damaged tissues (Schultz et al. 2005). GAGs create a hydrated environment within the ECM, which promotes migration and proliferation of the cells (Roberts & Harding 1994).

3.6.2 Extracellular matrix expression

A down regulation of all ECM genes analysed were seen in CFs compared to the NFs. This suggests that when a wound is in a chronic state the cells fail to maintain turnover of key ECM molecules required for successful wound repair. The effect of pH on CFs did not significantly affect any further this down regulation for the expression of COL1A1, COL1A2, FN, DCN and VCAN. The only significant effect pH had on ECM expression in NFs was the down regulation of COL1A1 in pH 9 conditions compared to both pH 6 and pH 7.5 (Figure 3.7A) such that a chronic alkaline environment can affect the regulation of collagens from healthy NFs.

For the selected proteoglycans (PG) analysed, decorin and versican are the most predominant in skin (Carrino et al. 2000). Decorin is a member of the SLRP-PG family characterised by the presence of one chondroitin or dermatan sulphate GAG chain and functions to regulate collagen matrix assembly (Nomura 2006). In healing wounds an increase in decorin expression has been reported (Penc et al. 1998); however in non-healing wounds the environment becomes alkaline and this study shows that DCN expression is significantly down regulated in CFs in pH 7.5 and pH 9, as well as pH 6 conditions. Such that deviations from normal tissue pH of 7.4 has detrimental effects on ECM turnover and possibly a downstream effect on the process of wound healing. Versican is a large

chondroitin sulphate PG and has been shown to co-localise with elastic fibres in skin. VCAN expression is lower in CFs but not found to be significant and pH showed no further changes in expression from NFs or CFs (Figure 3.7E). Lumican is another member of the SLRP family and is crucial in collagen fibril organisation (Chakravarti et al. 1998). In pH 9 conditions, such as a chronic wound, lumican expression was significantly up regulated from CFs compared to pH 6 and pH 7.5 conditions (Figure 3.7F). Studies have shown that lumican play crucial roles in tissue repair, with lumican null mice having a delayed wound healing phenotype (Yeh et al. 2010). Therefore, it would be expected that in pH 9 conditions lumican expression would be down regulated. However, pH may not have any effect on its expression; further studies would need to clarify this. A study showed that lumican promotes wound healing by facilitating fibroblast activation (Liu et al. 2013), therefore lumican could be a potential target in chronic wounds in order to attract more fibroblasts to the wound site to improve the rate of healing.

Overall, there was no correlation observed between ECM abundance and expression data. While an increase in extracellular pH showed an increase in collagen proteinlevels, an increase in extracellular pH resulted in a down regulation of the COL1A1 transcript. One correlating data set observed was the significant difference between both FN protein and mRNA expression from NF and CF at pH9 conditions. Quite often in such experiments a correlation is not seen between transcription and protein abundance data; transcript abundances only partially predict protein abundances (Vogel & Marcotte 2012). Many processes occur between transcription and translation such that the time frame in which the effect of pH on ECM expression can be detected is lost (Vogel & Marcotte 2012). In this study cells were incubated for 24 hours, if cells were incubated for 1 hour the effect of pH on ECM expression may have been different.

3.6.3 Cell migration

An alkaline pH environment was found to inhibit CF migration significantly, with only 39% scratch closure achieved compared to 90% at pH 7.5. This supports the initial hypothesis that

an alkaline environment within a chronic wound is disadvantageous and is a possible factor that inhibits wound closure. The NFs were found to migrate well in pH 7.5 and pH 9 conditions, which was previously shown with maximum migration seen between pH 7.2 and 7.5 (Lengheden & Jansson 1995). However, further increases in pH showed reduced migration, but the fibroblasts used in their study were human periodontal ligament and embryonic lung fibroblasts. Other studies have demonstrated that the other skin cell type, keratinocytes, tolerate a much wider pH range compared to fibroblasts and show optimal migration at pH 8.55, far more alkaline than fibroblasts can tolerate (Sharpe et al. 2009). Conversely, a recent study suggests that an acidic pH (6–6.5) is responsible for the slower migration rates of epithelial keratinocytes compared to increased pH values of 7–7.5 (Schreml et al. 2014). Utilising luminescent pH sensors to map the pH of the entire wound they state that there are pH gradients across the wound; with the wound edges being predominantly acidic and have increasing pH profiles toward the center of the wound. Therefore, the acidic pH profile of the wound edge is likely to inhibit epidermal repair and thus delay wound closure (Schreml et al. 2014).

The reduced amount of ECM from CFs could explain the slower rates of migration seen in pH 9 conditions as the ECM is essential for directing an organised response during cell migration. Fibronectin, specifically is key in acting as a scaffold for migrating cells into the wound area (Grinnell et al. 1981). The reduced abundance of fibronectin at pH 6 from NFs compared to pH 7.5 could potentially result in significantly slower migration of this cell seen in pH 6 conditions. However, healthy skin is maintained at an acidic pH around 4–6 therefore at this pH there is no need for the cell to migrate. At pH 9 the CFs were stimulated to produce more GAGs, which are known to promote cellular migration (Roberts & Harding 1994). This increase in GAGs at pH 9 however, does not correlate with the rate of migration. Several bacterial pathogens utilise GAGs to adhere to, and invade tissues (Shanks et al. 2005). The increase of GAGs in an alkaline environment could increase the probability of the growth and attachment of a biofilm within the wound.

3.7   Conclusion

The gene transcription and protein abundance of ECM molecules was significantly lower from CFs compared to NFs regardless of the pH of their culture conditions. CF migration was significantly reduced in alkaline conditions, which could contribute to healing times of chronic wounds. It has been experimentally evidenced that lowering the pH of the wound environment to more acidic conditions could have a significant benefit to wound closure through increasing the rate of cell migration.

Chapter 4:

Results II – The Effects of pH on Bacterial Growth

4   The Effect of pH on Bacterial Growth

4.1   Introduction

The microbial burden within a chronic wound is thought to be the main barrier to successful wound healing (White & Cutting 2006). Historically, standard clinical and microbiology practice only reported the presence of free-floating planktonic bacteria within the wound environment (Percival et al. 2014). Recent studies however, have found that microorganisms within a wound are phenotypically different to planktonic microorganisms (Percival & Bowler 2004a) with bacteria frequently residing in wounds as bacterial biofilms. It has been confirmed by imaging and molecular sampling techniques that over 60% of the bacteria present in chronic wounds are in a biofilm form (James et al. 2007).

A biofilm forms when bacteria attach to a surface and subsequently encase themselves in a self-secreted matrix of extracellular polymeric substance, or EPS (Seth et al. 2012). The moist environment of the wound bed makes an ideal and nutritionally supportive setting for the attachment and growth of biofilms (Edwards & Harding 2004). Wound healing and infection is influenced by the relationship between the ability of bacteria to thrive and create a community within the wound environment and the ability of the host to control that bacterial community (Percival & Bowler 2004a). In the form of a biofilm the bacteria rapidly create a protective environment and therefore the host's ability to control these organisms declines (Percival & Bowler 2004b).

Most bacteria have a relatively narrow pH range for growth and grow best around neutral pH values (pH 6.5 – 7.0). But there are some microorganisms that can thrive in much more acidic or alkaline environments (Padan et al. 2005). When present as a biofilm community, these bacteria are able to survive within a pH range that would be inhibitory to individual bacterial cells growing in culture (Bradshaw et al. 1996). Evidence has shown that chronic wounds have an alkaline pH and are also colonised with bacterial biofilms. The aim of this

study was to investigate the effects of pH on bacterial growth and its effect on the growth of biofilms. Such that it can be established if the changing pH of the wound environment influences the growth of the biofilm or the biofilm influences the changing pH of the wound.

4.2   Gram stain images of bacteria

Two of the most common bacterial strains found in chronic wound biofilms are *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Gjødsbøl et al. 2006). Reference strains of these two bacteria were used for all subsequent experiments as referenced in methods 2.2. Gram staining was carried out weekly to ensure purity of cultures. Images were viewed using the oil lens (x100). Gram positive *S. aureus* is seen as clusters of cocci shaped cells stained purple (Figure 4.1A). Gram negative *P. aeruginosa* imaged as pink shaped bacillus (Figure 4.1B).

4.3   Growth profiles of *S. aureus* and *P. aeruginosa*

The growth profiles of *S. aureus* and *P. aeruginosa* were investigated over 24 hours in bacterial growth medium, tryptone soya broth (TSB) and in cell culture media DMEM (supplemented with 2mM L-glutamine and 10% FCS). Both bacteria demonstrated a characteristic growth pattern of lag, log and stationary phase in both TSB bacterial growth medium and DMEM cell culture medium (Figure 4.2). Bacterial growth was slower in DMEM, with absorbance values not reaching the same as for growth in TSB. However DMEM does not inhibit bacterial growth therefore culturing in DMEM for further experiments was acceptable as the bacteria still undergo the typical bacterial growth profile.
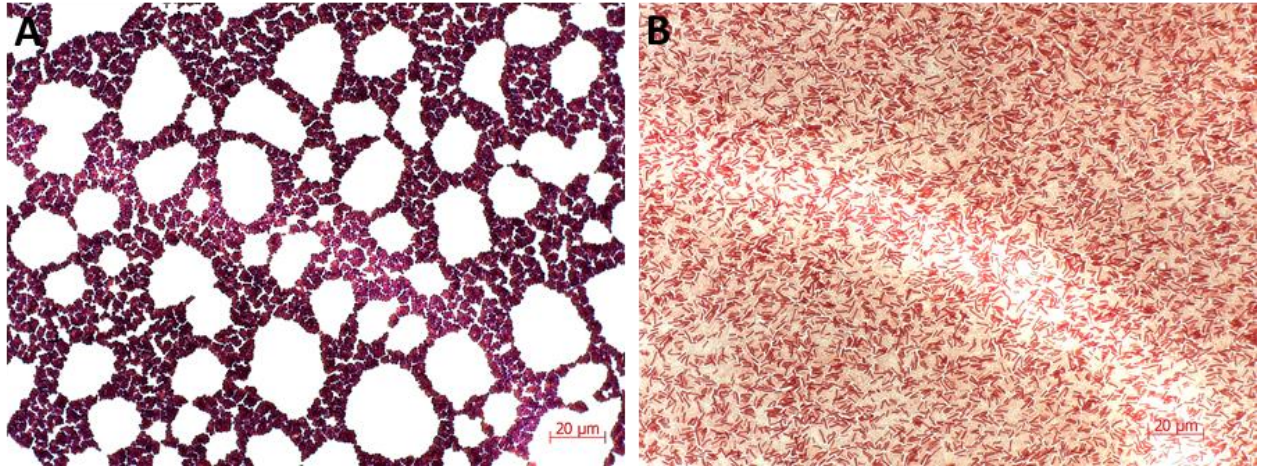
Figure 4.1 Gram stain of each bacterium

Gram staining was carried out weekly to ensure purity of cultures. Images were viewed using the oil lens (x100) lens and scale bar = 20µm. *S. aureus* is seen as clusters of cocci shaped cells stained purple, the peptidoglycan layer of gram positive *S. aureus* (A) has taken up the purple gram stain. Gram negative, bacillus shaped, *P. aeruginosa* (B) is stained pink by the counter stain. *S. aureus* cocci are typically 0.6µm in diameter while *P. aeruginosa* rods are 1-5µm in length and 0.5-1.0µm wide. The gaps in the *S. aureus* image are simply an artefact of smearing the bacteria onto the slide before imaging. Too much of the bacteria sample on a slide makes it harder to view the shape and colour of the bacteria cells and determine if the sample is contaminated.
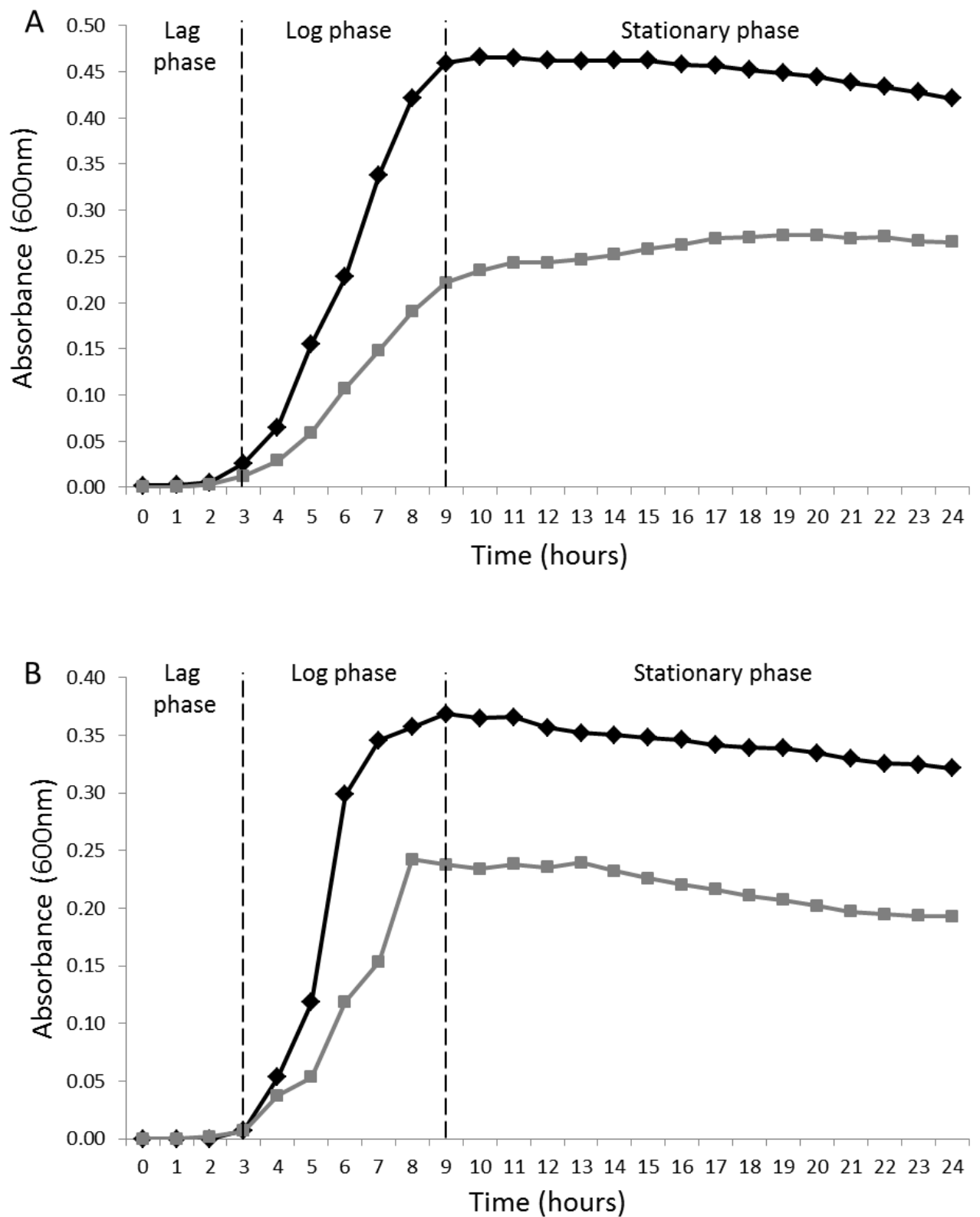
Figure 4.2. Growth profiles of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Growth profiles of *S. aureus* (A) and *P. aeruginosa* (B) bacteria over a period of 24 hours in TSB (black line) and DMEM cell culture media (grey line). Both bacteria demonstrate a typical growth curve in both TSB and DMEM. Phases of growth are shown for growth in TSB.

4.4 Growth profiles of *S. aureus* and *P. aeruginosa* in different pH conditions

The growth profiles of *S. aureus* and *P. aeruginosa* were investigated over 24 hours in various pH adjusted TSB (pH 6, 7.5 and 9). *S. aureus* demonstrated a typical growth profile of lag, log and stationary phases in all pH conditions (Figure 4.3). In all pH conditions *S. aureus* entered log phase after 3.5 hours in culture. In pH 7.5, growth was more exponential with absorbance values increasing much quicker and entering stationary phase after 8.5 hours. In pH 6, the overall growth of *S. aureus* is lower and enters stationary phase after 10 hours. After 24 hours in pH 9 *S. aureus* is still in the lag phase and the absorbance is still gradually increasing. *P. aeruginosa* also demonstrated the typical phases of bacterial growth in all pH conditions (Figure 4.4). *P. aeruginosa* entered lag phase around 4.5 hours in all three pH conditions and demonstrated a similar rate of growth in all conditions. Growth of *P. aeruginosa* was less in pH 9, with highest absorbance value of 0.48 compared to 0.62 in pH 6 and pH 7.5 conditions.
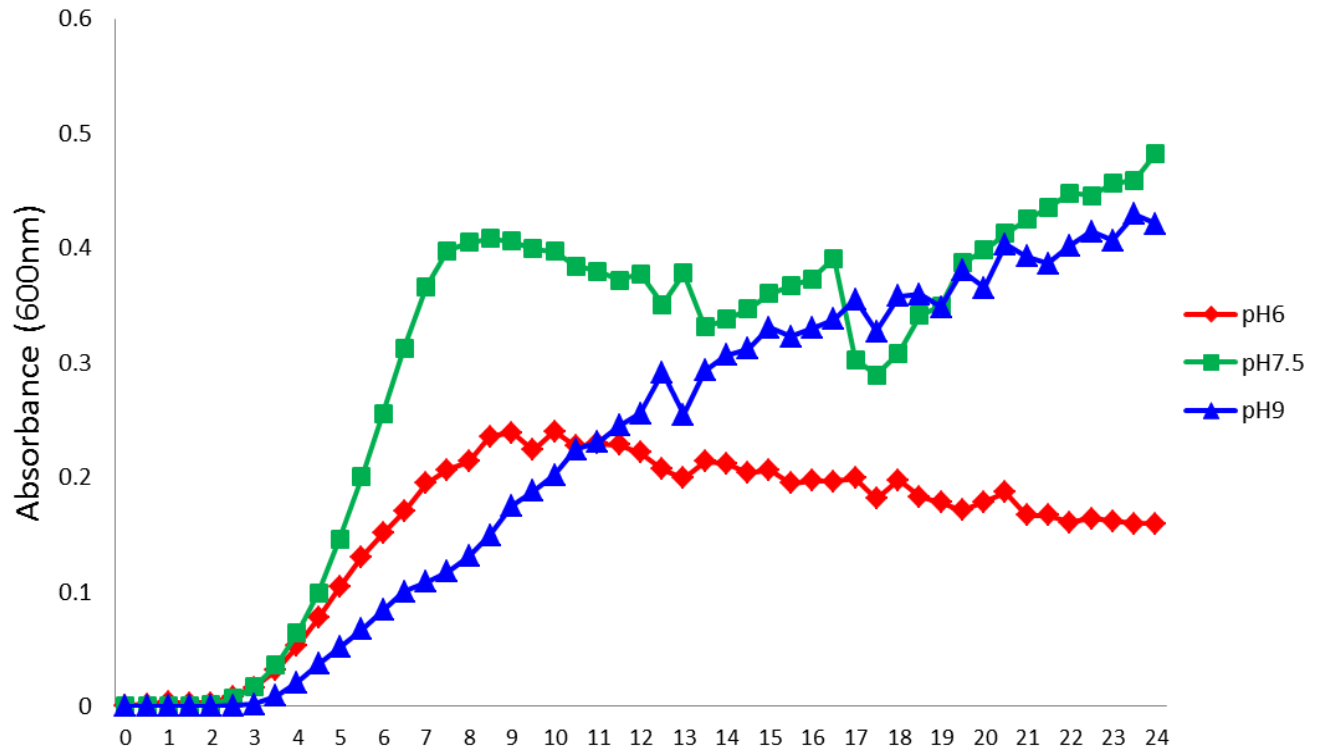
Figure 4.3 *Staphylococcus aureus* growth profiles in different pH conditions

Growth profile of *S. aureus* over 24 hours in three different pH conditions, pH 6 (red line), pH 7.5 (green line) and pH 9 (blue line).
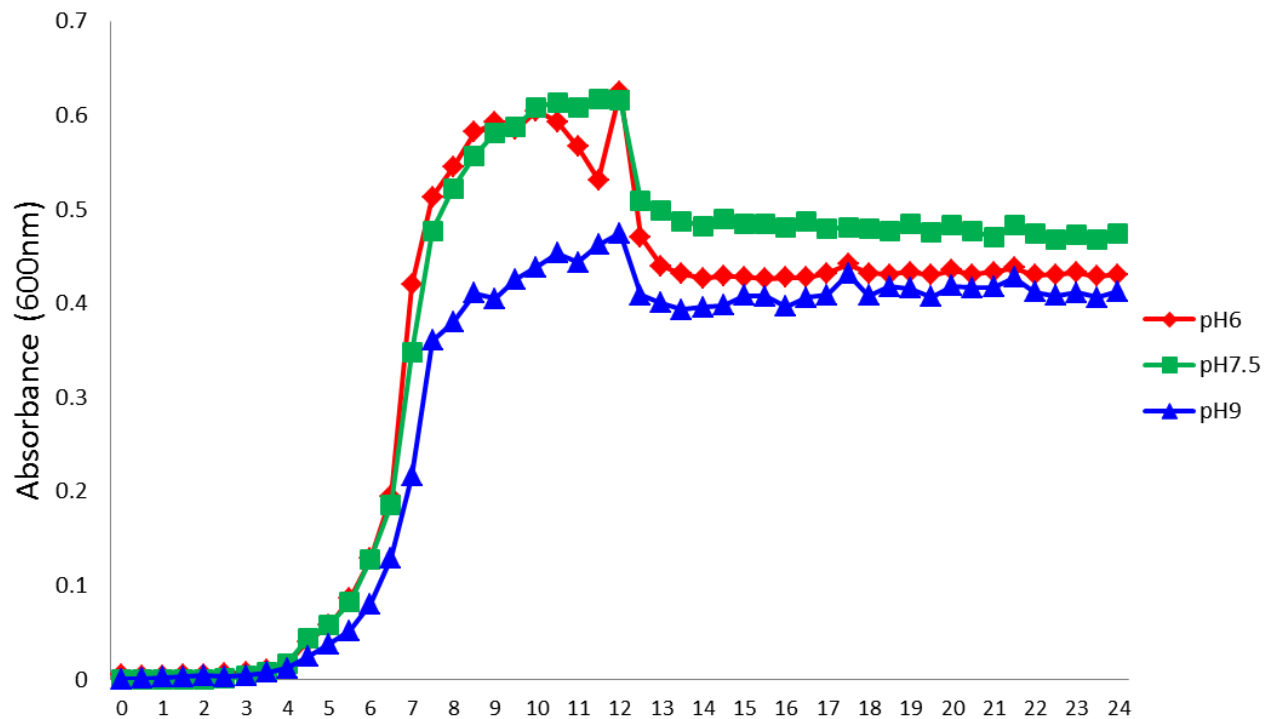
Figure 4.4 *Pseudomonas aeruginosa* growth profiles in different pH conditions

Growth profile of *P. aeruginosa* over 24 hours in three different pH conditions, pH 6 (red line), pH 7.5 (green line) and pH 9 (blue line).

4.5   Growth of Planktonic cultures in different pH conditions

The growth of planktonic bacteria in different pH conditions was assessed by colony counts. The planktonic cultures as described in methods 2.2.4.1 were serially diluted and plated onto TSA plates such that colonies could be counted and total bacterial growth determined. After 24 hours the growth of *S. aureus* was significantly higher in pH 7.5 conditions compared to pH 6 and pH 9 ($p < 0.05$) (Figure 4.5). After 48 and 72 hours there was no difference in the growth of *S. aureus* seen after culture in different pH conditions. There was no significant different in the growth of *S. aureus* over time in pH 6 and pH 9 conditions. In pH 7.5 growth of *S. aureus* showed a significant decrease at 48 and 72 hours compared to 24 hours ($p <$ 0.05) (Figure 4.5).

After 24 hours there was no difference in the total count of *P. aeruginosa* following culture in different pH conditions. After 48 hours, growth in pH 9 was significantly decreased compared to both pH 6 and pH 7.5 (Figure 4.6). Then at 72 hours, growth was significantly more in pH 7.5 conditions compared to both pH 6 and pH 9. There was no change in *P. aeruginosa* in pH 6 conditions between 24 and 48 hours, but at 72 hours the bacterial count was significantly decreased ($p < 0.05$). In pH 7.5 there was no difference in bacterial counts observed between 24 and 48 hours of growth; at 72 hours *P. aeruginosa* growth increased, but was only significant when comparing growth at 48 and 72 hours. Over time *P. aeruginosa* growth in pH 9 was varied with a significant decrease in bacterial count observed at 48 and 72 hours. After 48 hours the growth was much less and this was also significant ($p < 0.05$) compared to counts at 72 hours (Figure 4.6).
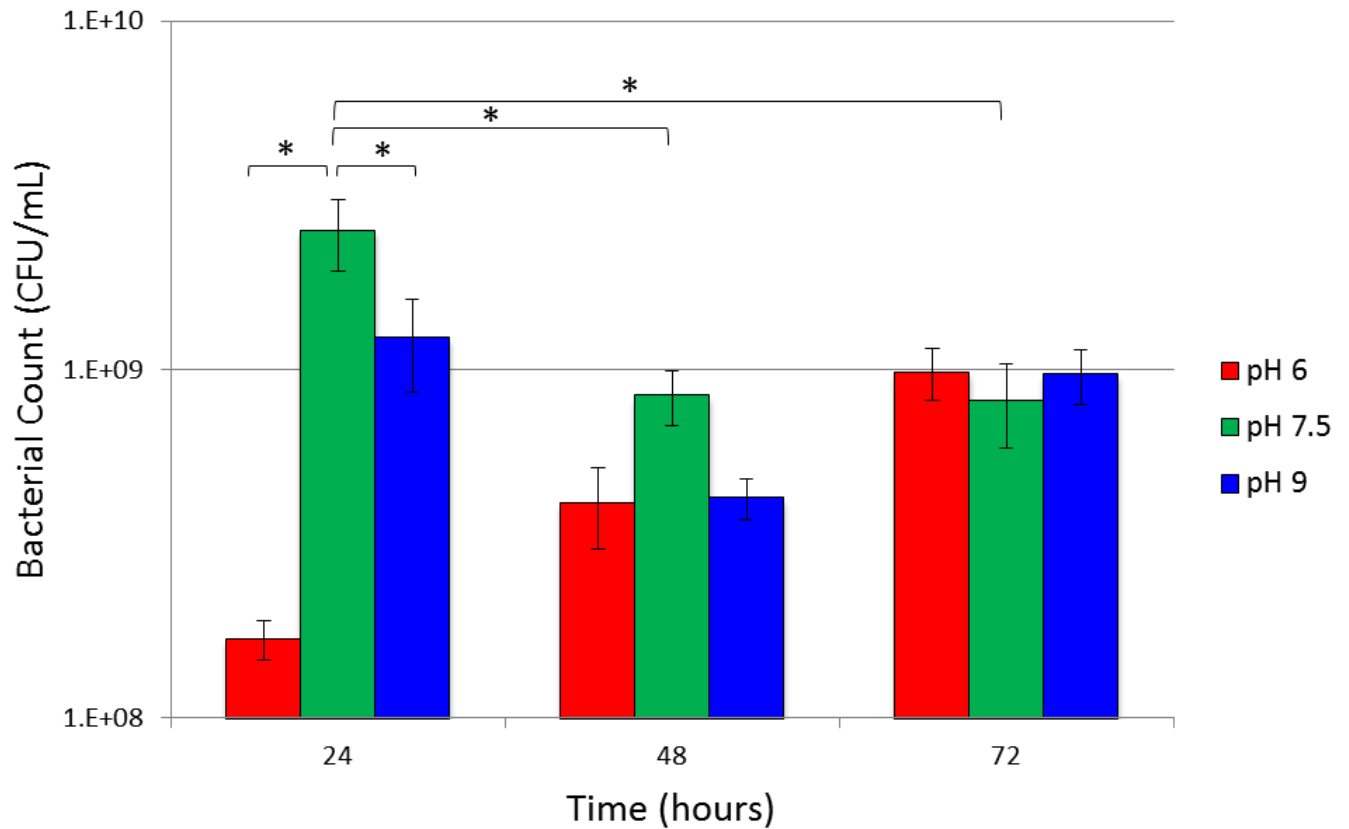
Figure 4.5 Planktonic growth of *Staphylococcus aureus* in different pH conditions

Planktonic growth of *S. aureus* was assessed overtime in pH 6, pH 7.5 and pH 9 by serial dilutions of cultures in 96-well plate and subsequent colony counts. Results are shown as the average mean of colony counts ± standard error (SE) of the mean. Significance of $p < 0.05$ is shown by the *.
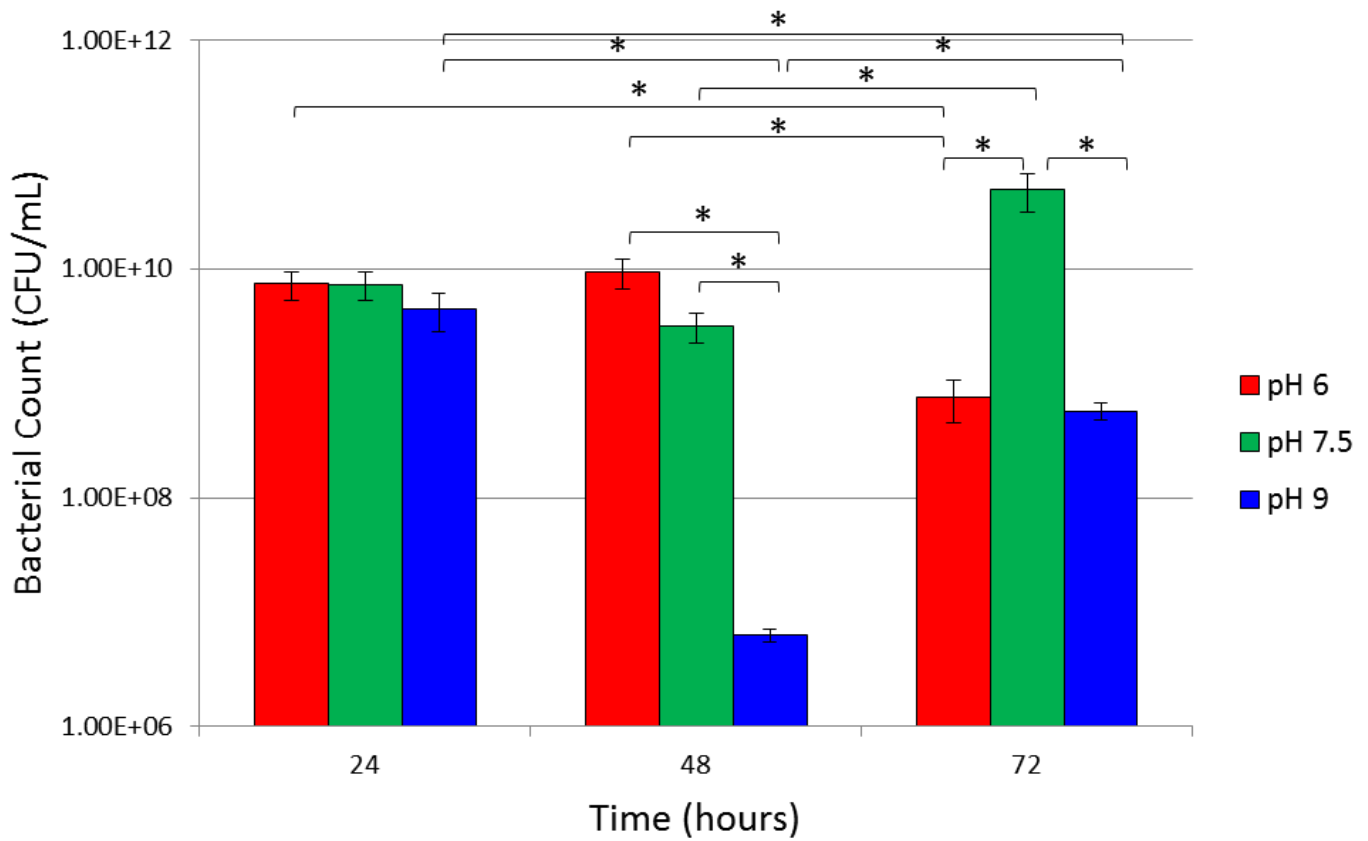
Figure 4.6 Planktonic growth of *Pseudomonas aeruginosa* in different pH conditions

Planktonic growth of *P. aeruginosa* was assessed overtime in pH 6, pH 7.5 and pH 9 by serial dilutions of cultures in 96-well plate and subsequent colony counts. Results are shown as the average mean of colony counts ± standard error (SE) of the mean. Significance of $p < 0.05$ is shown by the *.

4.6   Growth of Bacterial Biofilms in different pH conditions

Growth of *S. aureus* and *P. aeruginosa* biofilms were assessed in different pH conditions (pH 6, 7.5 and 9) over three days using the 96-well peg lid plate method. Biofilms were analysed by staining with crystal violet or direct enumeration of bacterial cells in the biofilm.

S. aureus biofilms increased with time in pH 6 culture when observing by colony counts (Figure 4.7). With increased time, no other differences were observed when measuring the biofilms by counts. When assessing the biofilms by staining with crystal violet more differences were observed. In each pH condition the absorbance of *S. aureus* biofilms gradually increase from 24 to 72 hours. There was no change in biofilm growth in pH 6 between 24 and 48 hours, at 72 hours biofilm growth was increased but this was only significant when compared with absorbance at 48 hours (Figure 4.9).

A similar trend was seen with biofilm growth in pH 7.5 conditions; with no change in absorbance measured between 24 and 48 hours and an increase in biofilm growth at 72 hours. In pH 7.5 conditions the biofilm was significantly increased at 72 hours compared to both 24 and 48 hours ($p < 0.05$). In pH 9 conditions, *S. aureus* biofilm growth is significantly higher at 48 and 72 hours compared to growth at 24 hours ($p < 0.05$) (Figure 4.9). After 24 hours no significant difference between the growth of *S. aureus* biofilms in pH 6, 7.5 and 9 conditions was observed by staining with crystal violet.

When analysing by colony counts at 24 hours biofilm growth in pH 7.5 conditions was increased, this was significant compared to biofilm counts in pH 9 conditions ($p < 0.05$) (Figure 4.7). At 48 hours the absorbance of *S. aureus* biofilm was significantly higher in pH 9 conditions compared with both pH 6 and pH 7.5 ($p < 0.05$) (Figure 4.9). After 72 hours in culture, biofilm growth was highest in pH 9 conditions, but due to an increase in biofilm growth in pH 7.5 conditions at 72 hours compared to 48 hours; it was only significant compared to biofilm growth in pH 6 conditions (Figure 4.9).

P. aeruginosa biofilm growth in different pH conditions was variable over the three days as measured by both crystal violet staining and bacterial colony counts. In pH 6 conditions

biofilm growth was highest after 48 hours, with lower CFU counts (Figure 4.8) and absorbance values (Figure 4.10) recorded at both 24 and 72 hours. There was a significant difference in biofilm growth observed when comparing the absorbance values at 24 and 48 hours and then at 48 and 72 hours ($p < 0.05$). This was not observed when analysing biofilm growth in pH 6 culture by colony counts. Biofilm growth in pH 7.5 was high after 24 hours but then significantly decreased at both 48 and 72 hours as determined by colony counts (Figure 4.8).

When analysed by crystal violet staining, *P. aeruginosa* biofilm growth was high at 24 and 48 hours, but at 72 hours biofilm growth was significantly lower compared to both 24 and 48 hours ($p < 0.05$) (Figure 4.10). After 24 and 48 hours in culture, biofilm growth in pH 9 conditions was the same as measured by counts, but when stained absorbance values revealed a significant change in biofilm growth compared to absorbance values at 24 hours ($p < 0.05$). However, after 72 hours biofilm growth is lower and this was significantly decreased compared to absorbance values at 48 hours ($p < 0.05$) (Figure 4.10) and compared to colony counts at both 24 and 48 hours (Figure 4.8). After 24 hours in culture, colony counts demonstrated that biofilm growth was highest in pH 9 conditions, but this was not significant when compared with the other culture conditions (Figure 4.8.)

Staining revealed that biofilm growth was highest in pH 7.5 conditions after 24 hours and this was significant compared to pH 6 and pH 9 ($p < 0.05$) (Figure 4.10). Biofilm counts demonstrated that at 48 hours biofilm growth was highest at pH 9, then pH 6 and was lowest at pH 7.5, with significant differences recorded between each condition ($p < 0.05$) (Figure 4.8). Crystal violet staining identified different results, with biofilm growth being significantly higher in pH 7.5 and pH 9 conditions, both were significant compared to pH 6 ($p < 0.05$) (Figure 4.10). After 72 hours of culture in the three different pH conditions, absorbance values for biofilm growth was similar and there was no significant difference in growth observed between any of the pH conditions (Figure 4.10). However biofilm colony counts revealed growth to be significantly increased in pH 6 culture compared to pH 7.5 and pH 9 (Figure 4.8).
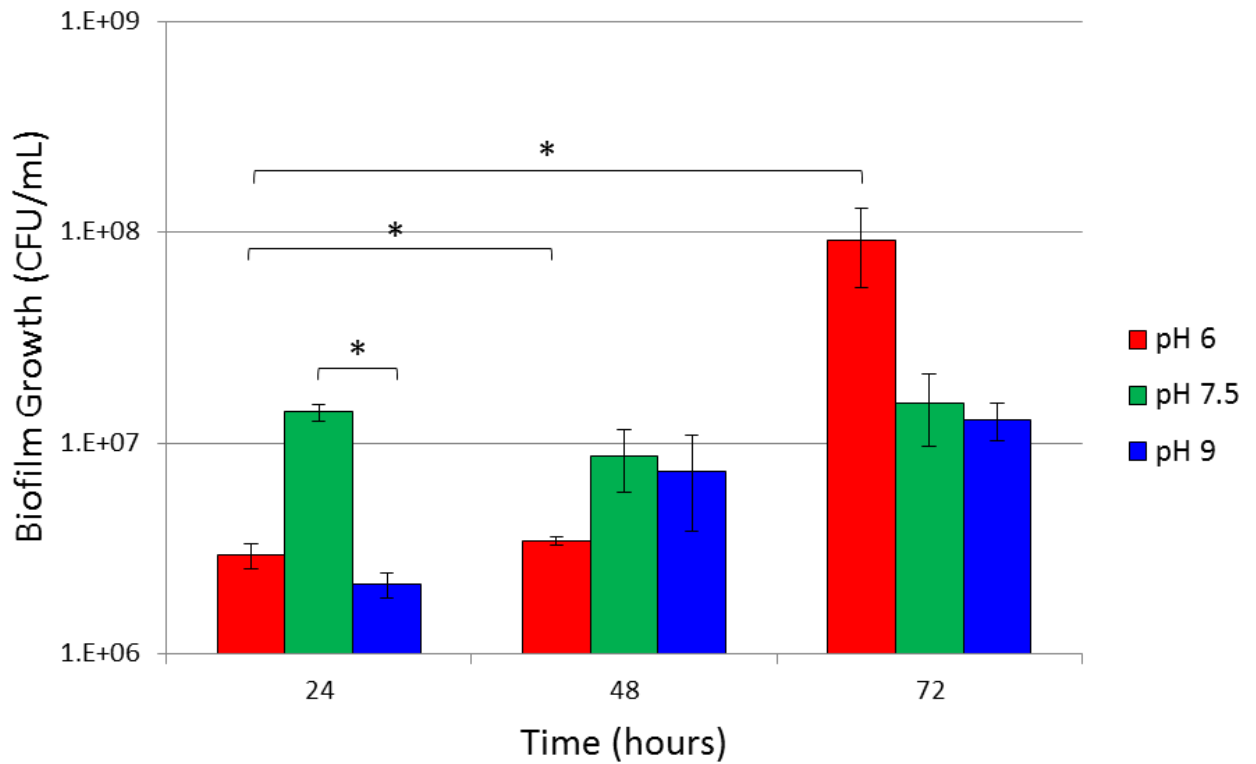
Figure 4.7 Biofilm growth of *Staphylococcus aureus* in different pH conditions

S. aureus biofilms were grown in different pH conditions over three days. Biofilms were sonicated and serially diluted before plating onto TSA plates and incubating overnight. Colonies could then be counted and total CFU/mL of each biofilm could be determined. Results are shown as the average mean of CFU/mL of biofilm ± standard error (SE) of the mean. Significance of $p < 0.05$ is shown by the *.
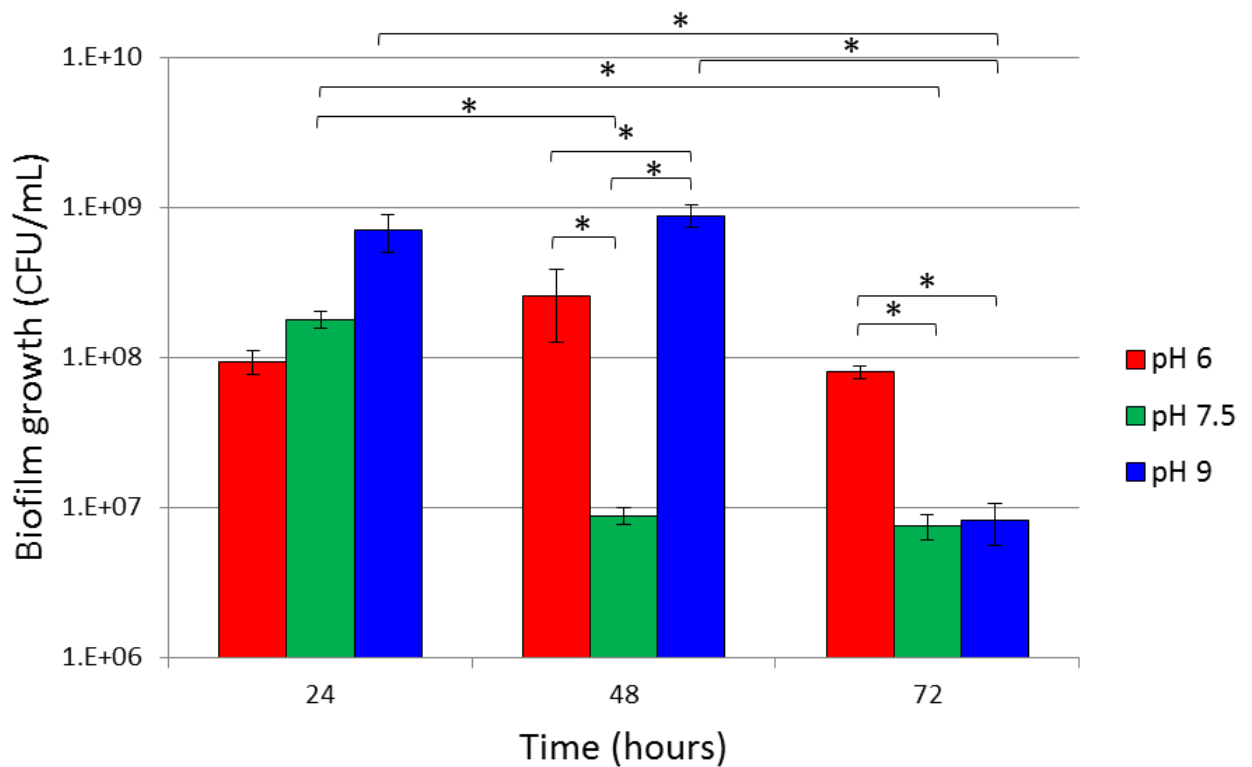
Figure 4.8 Biofilm growth of *Pseudomonas aeruginosa* in different pH conditions

P. aeruginosa biofilms were grown in pH 6, pH 7.5 and pH 9 conditions over three days using the 96-well peg lid plate method. Biofilms were removed from the pegs by sonication and then serially diluted and plated onto TSA and incubated overnight. The following day colonies could be counted and the total CFU per biofilm calculated. Results are shown as the average mean of CFU/mL of biofilm ± standard error (SE) of the mean. Significance of $p <$ 0.05 is shown by the *.
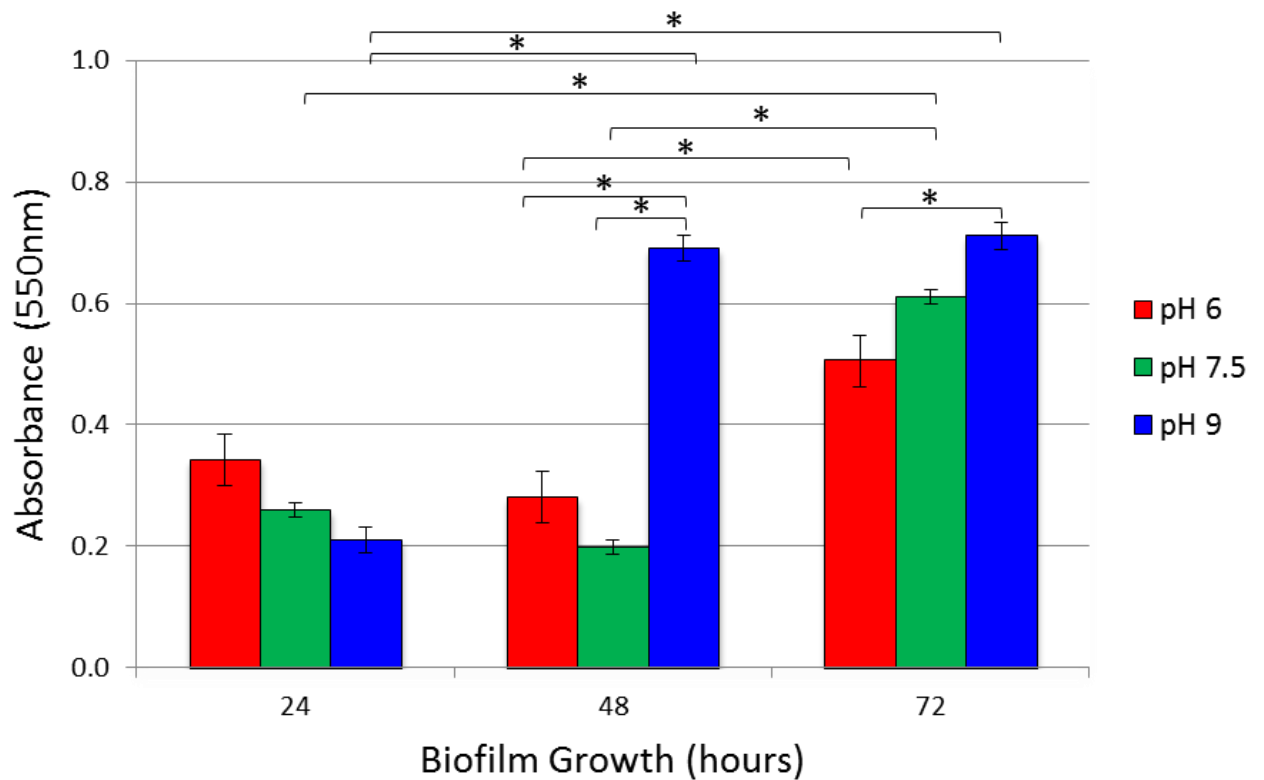
Figure 4.9 *Staphylococcus aureus* biofilms in different pH conditions measured by crystal violet

Growth of *S. aureus* biofilms was measured over three days in three different pH conditions using the 96-well plate crystal violet method and the absorbance measured. Results are shown as the average mean of biofilm ± standard error (SE) of the mean. Significance of $p < 0.05$ is shown by the *.
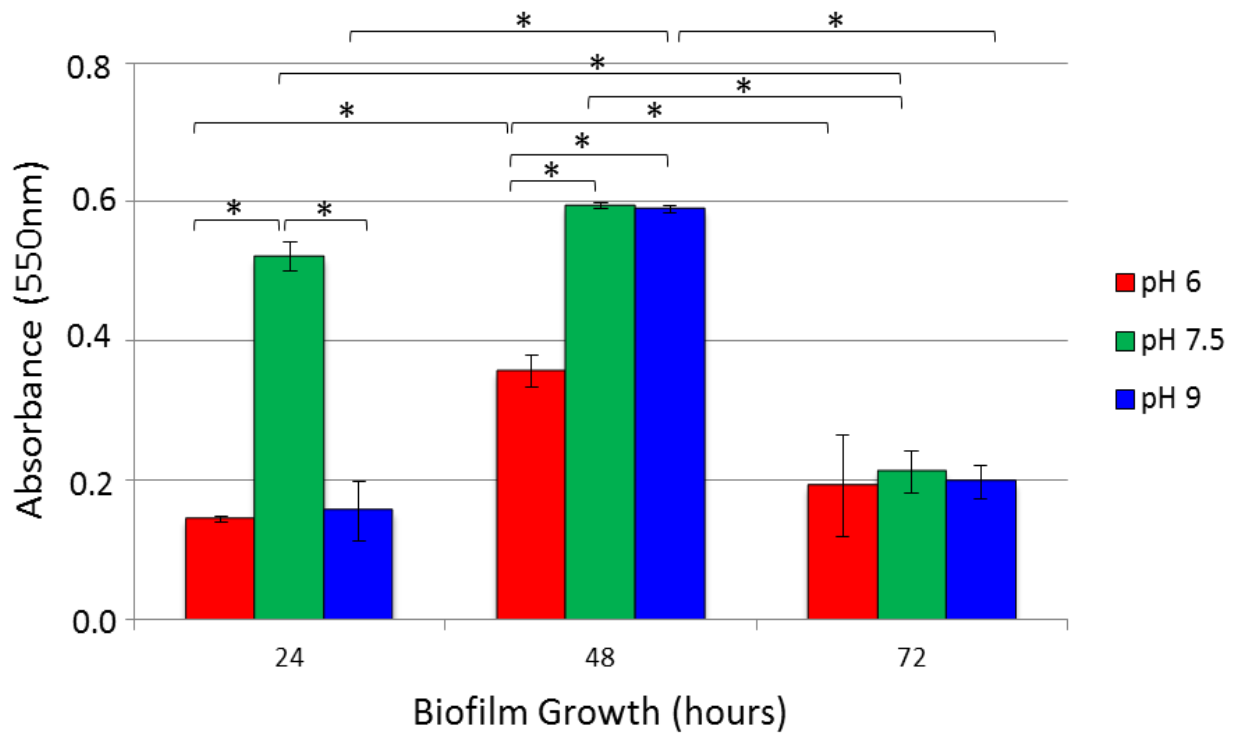
Figure 4.10 *Pseudomonas aeruginosa* biofilms in different pH conditions measured by crystal violet

Growth of *P. aeruginosa* biofilms was measured in three different pH conditions over three days. Biofilms were stained with crystal violet before solubilising in acetic acid and the absorbance measured. Results are shown as the average mean of biofilm ± standard error (SE) of the mean. Significance of $p < 0.05$ is shown by the *.

4.7 Discussion

Chronic wounds are commonly infected with bacterial biofilms which were thought to be the leading factor in delayed wound healing. A shift towards alkaline pH has also been observed in chronic wounds and it is likely that bacterial colonisation can be affected by alterations in wound pH (Schreml et al. 2010). The effect of pH on the growth of planktonic and biofilm *S. aureus* and *P. aeruginosa* was investigated over three days using the 96-well peg lid plate assay. Bacterial growth in the wells was referred to as planktonic and bacteria attached to the pegs of the lid as biofilms.

No clear effect of pH on the growth of either planktonic or biofilm *S. aureus* or *P. aeruginosa* was identified in this study. The surface of the skin is covered in bacterial microorganisms, referred to as the skin flora. The most common commensal bacteria found on the skin are *Propionibacteria*, *Staphylococci* and *Corynebacteria* species (Grice et al. 2009). A common strain is *S. epidermidis*, these commensal bacteria are usually non-pathogenic and not harmful and prevent colonisation of pathogenic bacteria such as *S. aureus*. However, following a wound the subcutaneous tissue is exposed which provides a moist and warm environment for microbial colonisation (Bowler et al. 2001). The skin's acidic milieu is disturbed following wounding and the more neutral pH (pH 7.4) of the underlying tissue is exposed (Schneider et al. 2007). Most pathogenic bacteria require pH values above 6 to survive and if pH is any lower their growth can be inhibited (Nagoba et al. 2015). Studies have shown that increasing pH values can promote bacterial growth. Planktonic growth of *S. aureus* was higher in pH 7.5 and pH 9 conditions compared to pH 6. However, this difference was only observed at 24 hours of culture (Figure 4.5). Further time in culture determined no differences. For *P. aeruginosa* planktonic cultures no differences were seen in the different pH conditions after 24 hours. Conversely, at 48 hours, growth was significantly inhibited in pH 9 compared to pH 6 and pH 7.5. Previous studies have shown clearer effects of altering pH on bacterial growth than results presented here. Growth of the yeast, *Candida albicans*, a common pathogenic microorganism found on the skin, is significantly increased in higher pH conditions (Runeman et al. 2000).

This study did not demonstrate clear and significant effects of pH on the attachment and growth of *S. aureus* and *P. aeruginosa* biofilms, with variable results observed. Both methods of analysing the biofilms resulted in different data that did not correlate over time. It would be expected that if the biofilm counts were significantly increased in one pH condition then those results would be correlated with the crystal violet staining of the biofilm measured by absorbance. The absorbance of the dye eluted from bound cells corresponds to the relative concentration of bacterial cells within the biofilm. In this study this was not seen, with the effect of pH showing different results with the two methods. However, a drawback of the crystal violet assay is that it stains not only bacterial cells but any material adhering to the surface such as the matrix components; therefore crystal violet staining may overestimate the number of adherent bacteria (Merritt et al. 2005). This could explain the variations in this study between the enumeration of bacteria in biofilms and the crystal violet staining.

To date, no studies have looked at wound-derived bacteria and the effect of pH on their biofilm forming potential within a wound. Some studies have looked at biofilms within different healthcare associated infections such as dental plaque biofilms (Marsh 2009) and urinary tract infections (Hatt & Rather 2008). A study by Hostacká et al. observed that with increasing pH, biofilm production was increased. This study measured biofilm formation using the crystal violet absorption method. Four clinically isolated strains of *P. aeruginosa* displayed an increased biofilm production at higher pH. At pH 7.5 there was up to 164% increase in biofilm production and in pH 9 it was up to 244% when compared with reference level at pH 5.5 (Hostacká et al. 2010). The study also assessed two other pathogens - *Klesibella spp.* associated with hospital infections and *Vibrio spp.* associated with water contamination. All tested strains indicated similar results to *P. aeruginosa*, with increased biofilm production at higher pH levels. An increase in *P. aeruginosa* biofilm production at pH 8 compared to pH 5 and 6 was observed in another study and this was explained by higher alginate and proteinase production by the bacteria at increased pH levels (Harjai et al. 2005). A similar association between biofilm production and increased pH has also been demonstrated by *S. maltophilia* (a common organism in biofilm infected catheters) (Di

Bonaventura et al. 2007). Furthermore, Memple et al. (1999) demonstrated that the adherence of *S. aureus* is inhibited at acidic pH and enhanced at alkaline pH values. A study by Nostro et al contradicts previous research and demonstrated that strains of both *S. aureus* and *S. epidermidis* failed to adhere to a surface in alkaline conditions. Growth of planktonic *S. aureus* was reduced and biofilm formation significantly decreased in pH 8.5 compared to pH 7.2 (Nostro et al. 2012). Another study investigated the biofilm forming capacity of 46 strains of *S. aureus* in five different pH levels (pH 3, 5, 7, 9 and 12). At pH 3 and pH 12 all tested strains were biofilm negative (Zmantar et al. 2010). At the other pH levels results were varied with some *S. aureus* strains classed as high biofilm formers and others as low or negative biofilm formers. For example at pH 5, six strains revealed an increase in biofilm formation and all other strains were low biofilm formers. At pH 9, 16 out of 28 strains revealed a decrease in biofilm formation compared to that observed at pH 7 (Zmantar et al. 2010). It is evident that different clinical strains of bacteria behave differently depending on the environmental conditions and have various biofilm forming capacity dependant on these. For biofilm positive strains, specific environmental conditions may promote the occurrence of biofilms in hospital settings such as in medical device related infections or in chronic wounds.

The studies mentioned only cultured their biofilms for 24 hours, whereas this study looked at the effect over time from 24 to 72 hours. In a laboratory setting 72 hours is better for the growth of a mature biofilm and at this point it is more comparable with biofilms in a real world setting. Growth of biofilms in the 96-well plate format is referred to as a static assay. This is useful for examining the early events of biofilm formation e.g. the transition from planktonic to biofilm mode. In some cases a biofilm can be detected in less than 60 minutes (Merritt et al. 2005). A drawback to this method is that these cultures are not continuously supplied with fresh medium or aerated, therefore nutrients may be limited and cultures may be unable to generate mature biofilms (Merritt et al. 2005). In this study, great differences in biofilm formation of both *S. aureus* and *P. aeruginosa* was observed between each individual plate set-up, such that comparing different plates over time may not be feasible. Results shown here is an average of six wells (biofilm) per pH condition in one plate and a different

plate is used for each time point. This study only used reference strains of each bacterium, whereas other studies have multiple clinical strains to assess. The clinical strains may be better to investigate in the laboratory when analysing the effect of biofilms in healthcare associated infections (Sanchez et al. 2013). Several methods have been developed for the quantification of biofilms but the microtiter plate methods remains the most frequently used (Hassan et al. 2011). The microtiter plate has its advantages, as it is a rapid and simple method to screen many strains and different conditions in one test (Stepanović et al. 2007). However, with a number of versions and constant adaptations it becomes difficult to compare results and find an acceptable method. With many different methods used to grow and analyse biofilms it is important to take into consideration that applying laboratory-based observations to an otherwise unpredictable biofilm in the environment may well aid in obscuring results of a "true biofilm". It is suggested that replicating environmental and medical biofilms may not be possible and that nature determines the complexities of a biofilm that cannot be reproduced *in vitro* (Percival et al. 2011).

Biofilms are a major threat to healthcare infections including chronic wounds due to their resistance to currently available antibiotics. Many factors, including pH, are known to affect the activity of antibiotics and antimicrobials. An example is the reduced activity of macrolides and aminoglycosides in alkaline conditions (Falagas et al. 1997), which could be a problem in chronic wounds resulting in failure of such antibiotics to reduce wound bioburden. Contrary to this the activity of the antimicrobial chlorhexidine has an increased activity in alkaline pH (Russell 2003). A study by Thomas *et al* demonstrated that antibiotics were more effective at a neutral pH than an acidic pH. Gram positive wound isolates, such as *S. aureus*, were more susceptible to the antibiotics ampicillin and clindamycin at pH 7 compared to pH 5 (Thomas et al. 2012). The efficacy of the antibiotics aztreonam and levoflaxin were increased on Gram negative wound isolates in pH 7 compared with pH 5 (Thomas et al. 2012). In a study where the effect of pH on a range of widely used antimicrobial agents was tested it was found that the fluoroquinolones, co-trimoxazole, aminoglycosides, and macrolides all functioned optimally at alkaline pH, whereas the tetracyclines, nitrofurantoin, and many of the beta-lactams exhibited their highest activity

under more acidic conditions (Yang et al. 2014). When antibiotic (e.g. fluoroquinolnes) activity is increased in alkaline conditions it is suggested to be due to increased zwitterionic interactions with porins and membrane lipids at high pH (Piddock 1991). Another suggestion as to improved activity is due to electrical potential increases across the bacterial membrane at a higher pH, leading to increased antibiotic uptake (Eisenberg et al. 1984). This therefore supports a more alkaline environment when using these antibiotics, as their bactericidal activity significantly increases (Erdogan-Yildirim et al. 2011). The local pH of infected tissue may be one of the factors that should be considered when choosing antimicrobials for treatment of chronic wounds. If a chronic wound is alkaline, then initially antibiotics with increased activity at higher pH should be used to challenge the microbial load. As treatments are developed to reduce the pH of the wound environment, antibiotics that are not affected by pH should be considered. A wound dressing that has the ability to reduce wound pH, combined with increased antimicrobial performance over a wide pH range, would be of great benefit to wound healing (Slone et al. 2010).

4.8 Conclusion

The effect of pH on biofilms is still unclear. No clear effect was found in this study, with various biofilm growth seen in all pH conditions (pH 6, 7.5 and 9). Growth of both planktonic and biofilm forms of *S. aureus* and *P. aeruginosa* varied with time. No clear effects were seen with the crystal violet and direct bacterial cell counts of the biofilm giving different results. For example direct enumeration of *S. aureus* biofilm at 24 hours revealed an increase in biofilm production at pH 7.5 whereas the crystal violet assay showed no significant differences between each pH condition. For growth of *P. aeruginosa* biofilms the crystal violet assay demonstrated a significant increase in biofilm production at pH 7.5 and the direct cell count showed no differences after 24 hours. Other previous studies have failed to demonstrate a clear effect of pH on biofilms with some stating an increase in biofilm forming capacity in alkaline conditions (Hostacká et al. 2010) and others state it is higher in acidic conditions (Nostro et al. 2012). A more robust method of testing biofilms is required in order to improve knowledge of how such biofilms can be successfully eradicated with more

specific treatments. Due to the alkaline pH observed in chronic wounds such treatments may need to account for pH. If biofilms prefer a more alkaline environment and due to the varying activity of antimicrobials in different pH, treatment needs to be carefully planned. Treating the wound with an acidic solution may decrease biofilm growth but it may also affect the antibiotic in use.

Chapter 5:

Results III – The Effect of pH titrated Planktonic or Biofilm Conditioned Media on Fibroblasts

5 The Effect of pH titrated Planktonic and Biofilm Conditioned Media on Fibroblasts

5.1 Introduction

Following the findings that chronic wounds are infected with bacterial biofilms and not their planktonic counter-parts (James et al. 2007); research has focused on the role of bacterial biofilms on wound healing. Some recent studies have investigated differences between planktonic and biofilm bacteria on keratinocytes and fibroblast skin cells (Secor et al. 2012; Kirker et al. 2012).

Studies have demonstrated that the soluble products from planktonic and biofilm *Staphylococcus aureus* have various effects on human keratinocytes (HK) and human dermal fibroblasts (HDF). Biofilm conditioned media (BCM) caused significant morphological changes to HK, increased apoptosis and reduced viability (Kirker et al. 2009), as well as increased nitrogen oxide production and increased inflammatory cytokine release (Tankersley et al. 2014) compared to planktonic conditioned media (PCM). Transcriptional analysis in a study by Secor et al. revealed that BCM up-regulated 65 transcripts and down-regulated 247 transcripts compared to PCM. Furthermore, BCM induced more cytokines (IL-1β ,IL-6, tumour-necrosis factor (TNF-α), and chemokines CXCL-8 and CXCL-1) production from HK after four hours compared to PCM, but after 24 hours this effect was reversed with PCM treatment resulting in higher cytokine levels (Secor et al. 2011). The soluble products of both planktonic and biofilm methicillin-resistant *S. aureus* (MRSA), produced similar deleterious effects on HDF migration and viability by inducing apoptosis (Kirker et al. 2012). However, HDF production of cytokines, growth factors, and proteases were differentially affected by both PCM and BCM. PCM induced more IL-6, IL-8, vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF- β1), heparin-bound epidermal growth factor, matrix metalloproteinase-1, and metalloproteinase-3 production in HDF than the BCM. Whereas, BCM induced more TNF-α production compared to PCM (Kirker et al. 2012).

All the studies mentioned investigated the effect of biofilms on inflammatory mediators, specifically cytokines and growth factors from epidermal keratinocytes and dermal fibroblasts. The effect of PCM and BCM on the synthesis of vital extracellular matrix molecules has been ignored. Furthermore, previous studies have not accounted for alterations and effects of pH on chronic wounds. Therefore, the aim of this study was to include changes in pH and compare the effects of pH titrated PCM and BCM on several wound related parameters such as extracellular matrix (ECM) abundance and normal and chronic fibroblast migration.

5.2   Planktonic and Biofilm Conditioned Media (PCM and BCM)

S. aureus and *P. aeruginosa* PCM and BCM were generated as described in methods 2.2.5. The number of viable bacteria in both PCM and BCM was determined using the spread plate technique. PCM was diluted to an equivalent of 10^8 CFU/mL. After BCM collection, biofilm discs were re-suspended in PBS, vortexed and sonicated and serial dilutions plated to reveal an average of 10^9 CFU per biofilm.

The pH of PCM and BCM was measured following collection. Results showed a significant increase in pH of BCM compared to PCM (Table 5.1). Statistical analysis of the total protein measured in PCM and BCM did not significantly differ between *S. aureus* PCM and BCM or between *P. aeruginosa* PCM and BCM (Figure 5.1). Preliminary protein analysis by 1D SDS-PAGE did however reveal different protein compositions between PCM and BCM. BCM showed a more complex banding pattern compared with PCM (Figure 5.2). After titration of *S. aureus* and *P. aeruginosa* PCM and BCM to pH 6, pH 7.5 and pH 9, samples were precipitated and ran on an SDS-PAGE gel to see if any changes occurred. A denser band at 240kDa can be seen from *S. aureus* PCM and BCM at pH 6 compared to pH 7.5 and pH 9 (Figure 5.3). No other differences in banding pattern after pH change were identified.

Table 5.1 pH values of PCM and BCM

The pH of collected PCM and BCM was measured and is shown in the table below. Significant differences were observed between PCM and BCM of each bacterium. Values are the average of six collected and measured CM and statistical differences was measured using student's simple T-test.

Media	pH value	
DMEM Control	8.11 ± 0.07	
S. aureus PCM	8.07 ± 0.06	⎫
S. aureus BCM	9.39 ± 0.05	⎭ *** $p < 0.05$**
P. aeruginosa PCM	8.15 ± 0.03	⎫
P. aeruginosa BCM	9.41 ± 0.05	⎭ *** $p < 0.05$**
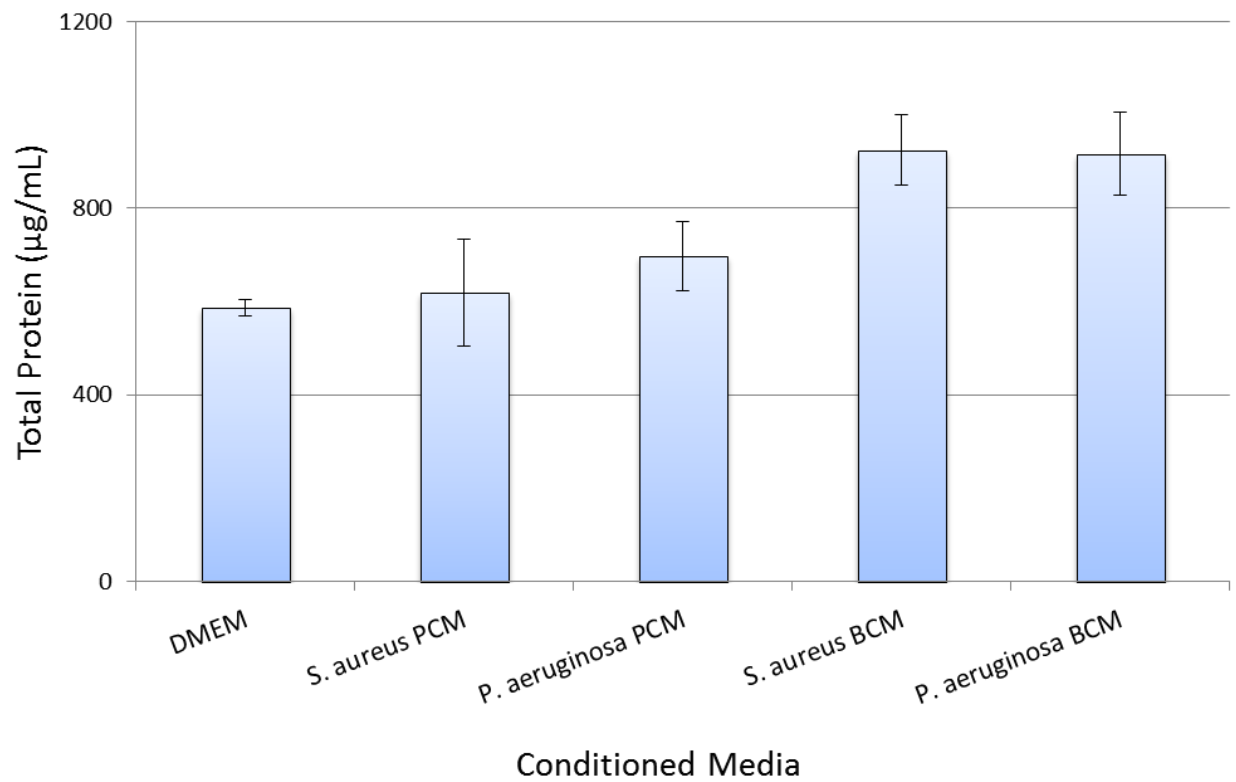
Figure 5.1 Total protein of *S. aureus* and *P. aeruginosa* PCM and BCM

Total protein in PCM and BCM (no FCS) was measured by the protein BCA assay. Total protein in BCM was slightly increased, but no significant difference between PCM and BCM was observed by statistical analysis. Results are the average of six PCM or BCM measured ± SE of the mean.
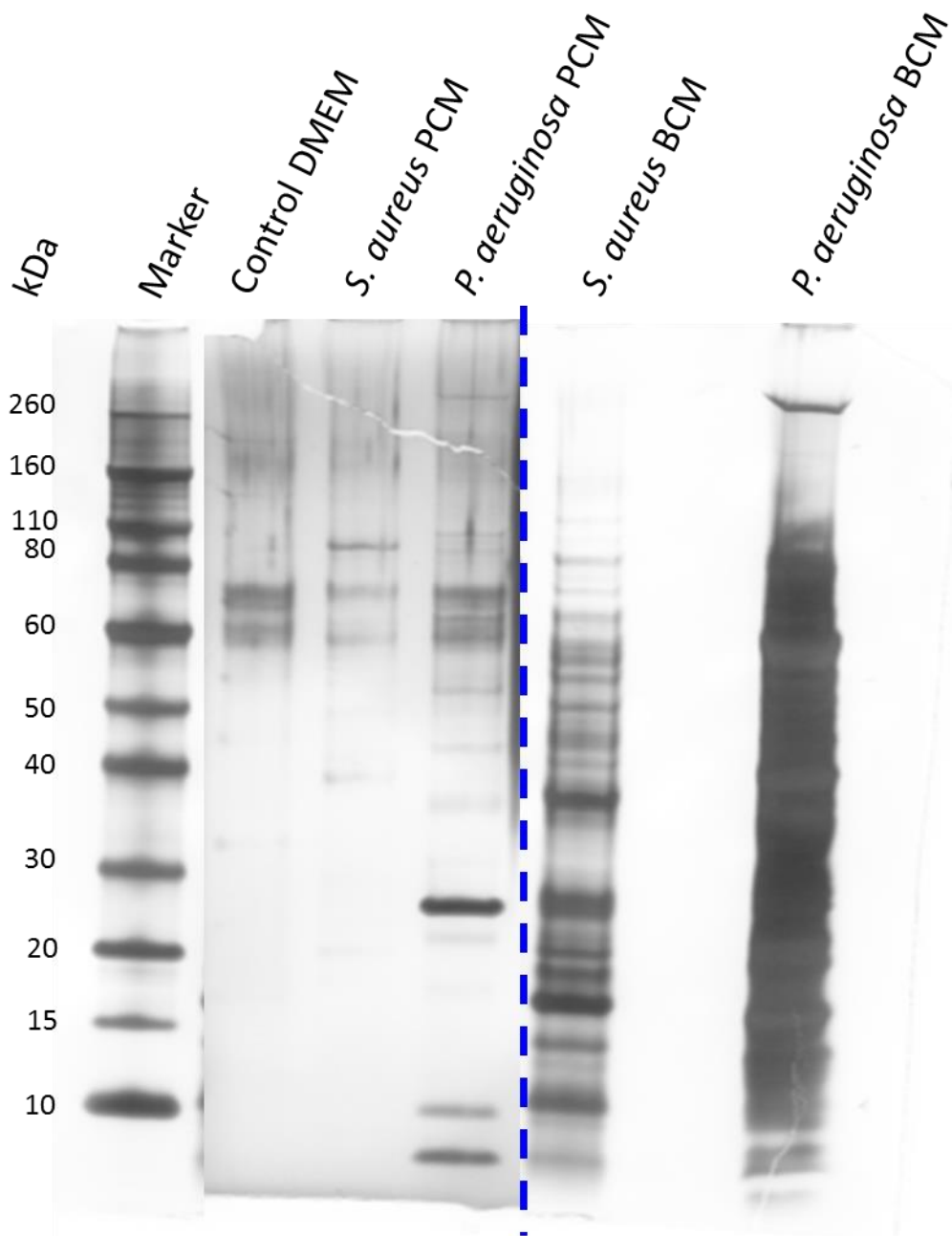
Figure 5.2 SDS-PAGE of PCM and BCM

PCM and BCM samples were precipitated and electrophoresed on an SDS-PAGE gel before staining with silver stain. Gel was cut and stained separately during final development stage as BCM lanes developed within 20 seconds whereas PCM required 3 minutes to visualise bands. Substantial differences in the extracellular proteome of planktonic and biofilm cultures of *S. aureus* and *P. aeruginosa* were revealed by 1D SDS-PAGE.
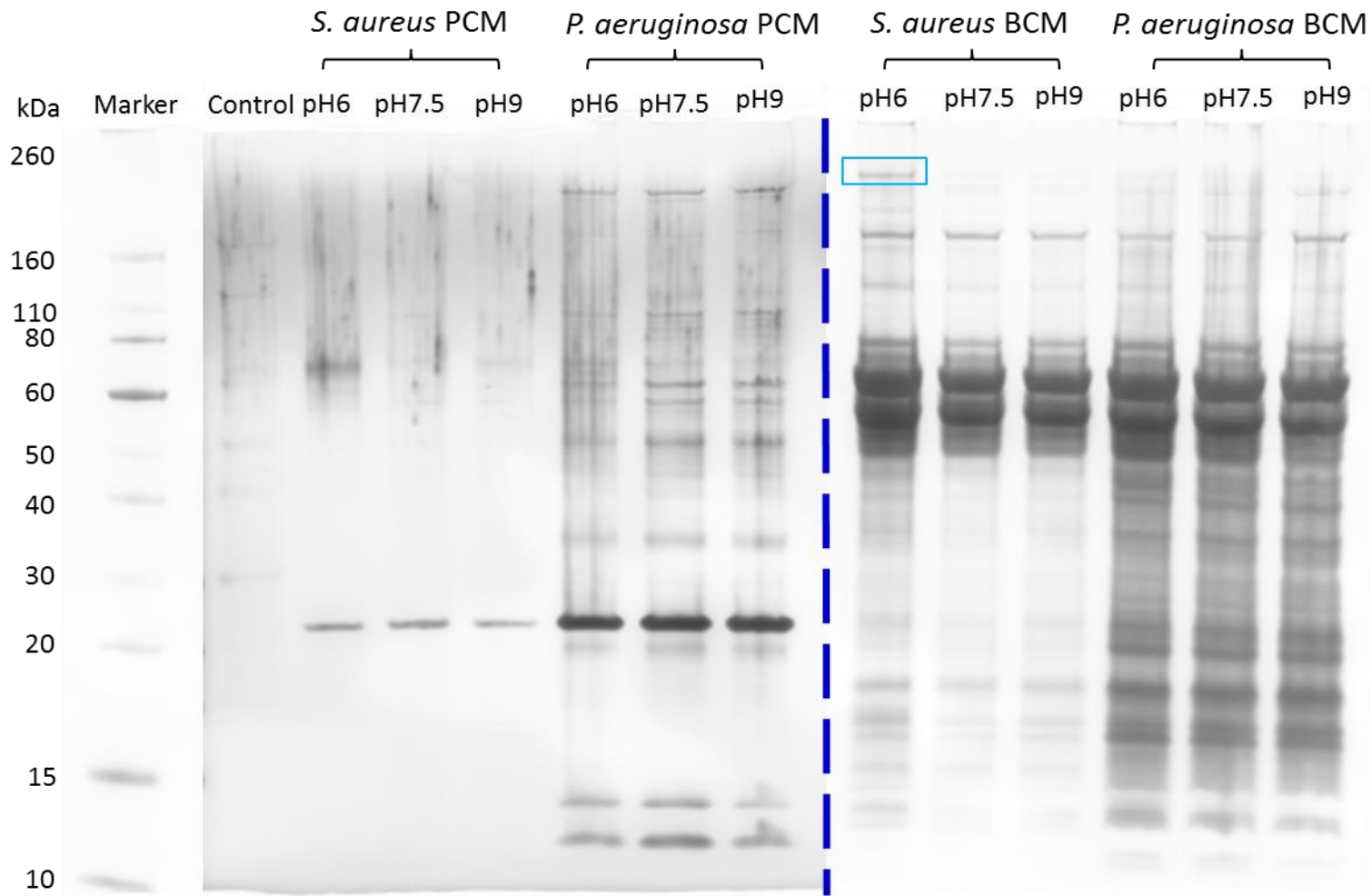
Figure 5.3 SDS-PAGE of PCM and BCM after titration to different pHs

PCM and BCM were titrated to pH 6, 7.5 and 9 as were used in all experiments. Proteins were precipitated from media and ran on SDS-PAGE and silver stained to identify if changing pH resulted in any changes. PCM and BCM were ran on two separate gels, during the final development stage of the silver stain assay, PCM samples was left for 3 minutes whereas 20 seconds was sufficient for development of protein bands in the BCM sample gel. An extra band was observed when *S. aureus* BCM was changed to pH 6.

5.3 Extracellular Matrix abundance following culture of fibroblasts in pH titrated PCM and BCM

Normal and chronic fibroblasts (NF and CF) were cultured for 24 hours in pH titrated (pH 6, 7.5 and 9) *S. aureus* and *P. aeruginosa* PCM or BCM. Media was collected and analysed for presence of collagen, fibronectin, glycosaminoglycans (GAGs) and tropoelastin see Chapter 2.1.6 - 2.1.11.

5.3.1 Collagen abundance

Collagen abundance was significantly higher from NF compared to CFs in majority of conditions (Figure 5.4 and Table 5.2). In pH 6 no significant changes in the amount of collagen were observed with NF treated with PCM and BCM compared to control. Culturing NF in *S. aureus* and *P. aeruginosa* PCM and *S. aureus* BCM titrated to pH 7.5 and pH 9 resulted in a lower amount of collagen present compared to the amount in control conditions (Figure 5.4 and Table 5.2). *P. aeruginosa* BCM at both pH 7.5 and pH 9 did not show the same effect.

For CF at pH 6, all were significantly different compared to control ($p < 0.05$), but showed a significant increase in collagen abundance from both PCM and BCM (Figure 5.4 and Table 5.2). Both *P. aeruginosa* PCM and BCM (pH 7.5) significantly reduced total collagen compared to control pH 7.5 ($p < 0.05$) and no further changes between PCM and BCM observed. No significant differences were observed between CF in the pH 9 group with all conditions showing low levels of collagen.

pH alone had no effect on collagen abundance from NF, however an effect of pH was observed on CF with significant differences between pH 6 and pH 7.5 ($p < 0.05$) and between pH 7.5 and pH 9 ($p < 0.05$).
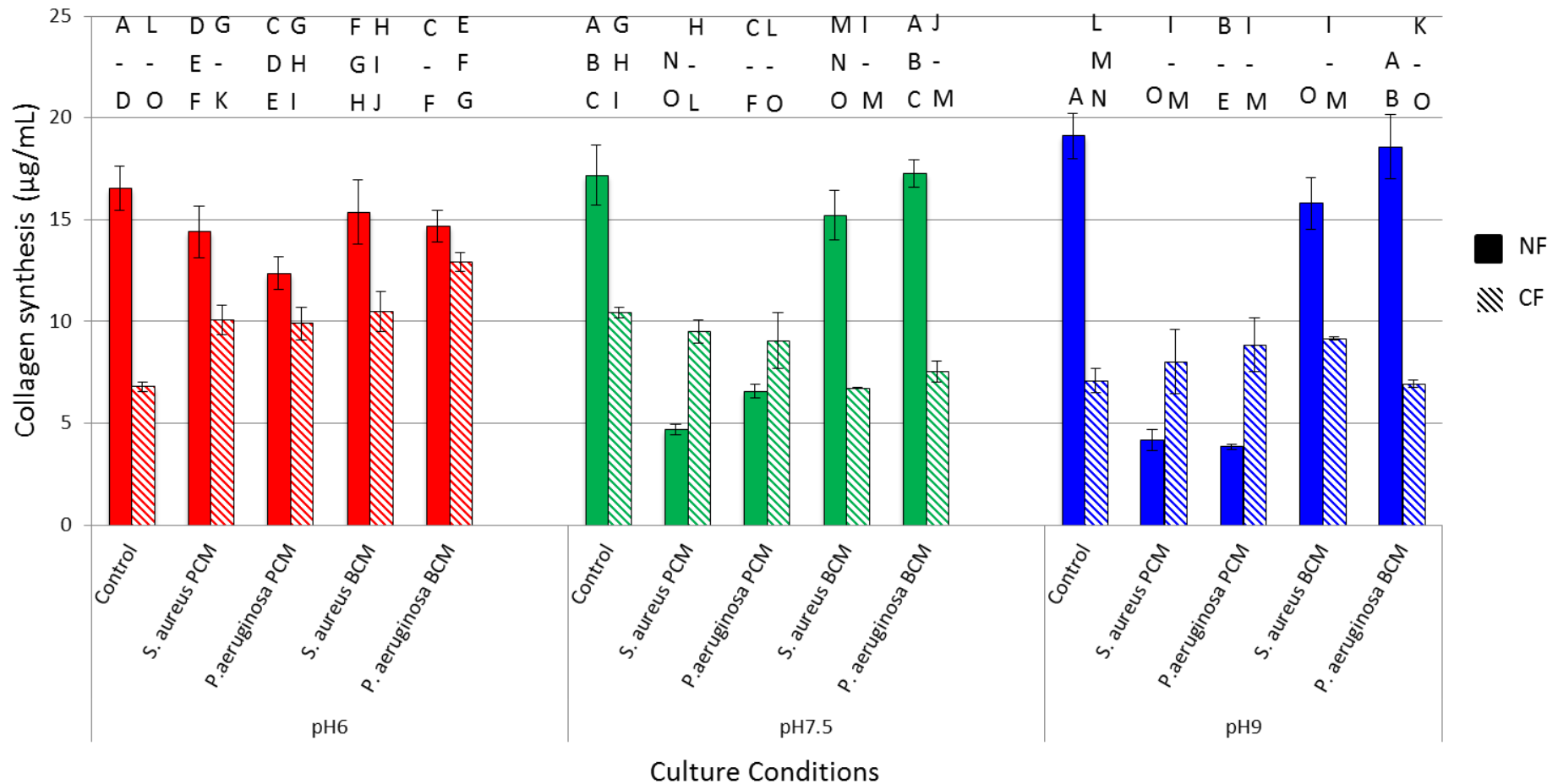
Figure 5.4 Collagen abundance from NF and CF cultured in pH titrated PCM and BCM

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and the amount of collagen was measured. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.2 Statistical groupings for Collagen abundance

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Normal	6	Control		A	B	C	D											
		S. aureus	PCM				D	E	F									
		P. aeruginosa	PCM			C	D	E										
		S. aureus	BCM						F	G	H							
		P. aeruginosa	BCM			C	D	E	F									
	7.5	Control		A	B	C												
		S. aureus	PCM														N	O
		P. aeruginosa	PCM			C	D	E	F									
		S. aureus	BCM													M	N	O
		P. aeruginosa	BCM	A	B	C												
	9	Control		A														
		S. aureus	PCM															O
		P. aeruginosa	PCM		B	C	D	E										
		S. aureus	BCM															O
		P. aeruginosa	BCM	A	B													
Chronic	6	Control													L	M	N	O
		S. aureus	PCM							G	H	I	J	K				
		P. aeruginosa	PCM							G	H	I						
		S. aureus	BCM								H	I	J					
		P. aeruginosa	BCM					E	F	G								
	7.5	Control								G	H	I						
		S. aureus	PCM								H	I	J	K	L			
		P. aeruginosa	PCM												L	M	N	O
		S. aureus	BCM									I	J	K	L	M		
		P. aeruginosa	BCM										J	K	L	M		
	9	Control													L	M	N	
		S. aureus	PCM									I	J	K	L	M		
		P. aeruginosa	PCM									I	J	K	L	M		
		S. aureus	BCM									I	J	K	L	M		
		P. aeruginosa	BCM											K	L	M	N	O

5.3.2 Fibronectin abundance

All pH titrated PCM and BCM demonstrated lower fibronectin abundance from NF compared to each of their pH control (Figure 5.5). All were significant except for BCM at pH 6. Similar results were seen by CF in the pH 7.5 titrated group, with both PCM and BCM having lower amount of fibronectin compared to control ($p < 0.05$).

This effect on fibronectin abundance by CF was not observed in pH 6 and pH 9 titrated conditions (Figure 5.5 and Table 5.3). No further difference on fibronectin abundance was observed when comparing PCM and BCM of both *S. aureus* and *P. aeruginosa* at the three different pH tested.

Similarly to the previous study (Figure 3.4), CF secreted lower amounts of fibronectin than NF. In this second experiment fibronectin secretion from NF was significantly lower in pH 6 conditions compared to pH 7.5 and pH 9, in the previous study it was also lower in pH 6, but only significant compared to pH 7.5 conditions. In the previous study pH had no effect on fibronectin secretion from CF, in this second experiment, fibronectin secretion was highest in pH 7.5 conditions but only significant when compared to fibronectin secretion in pH 6 conditions.
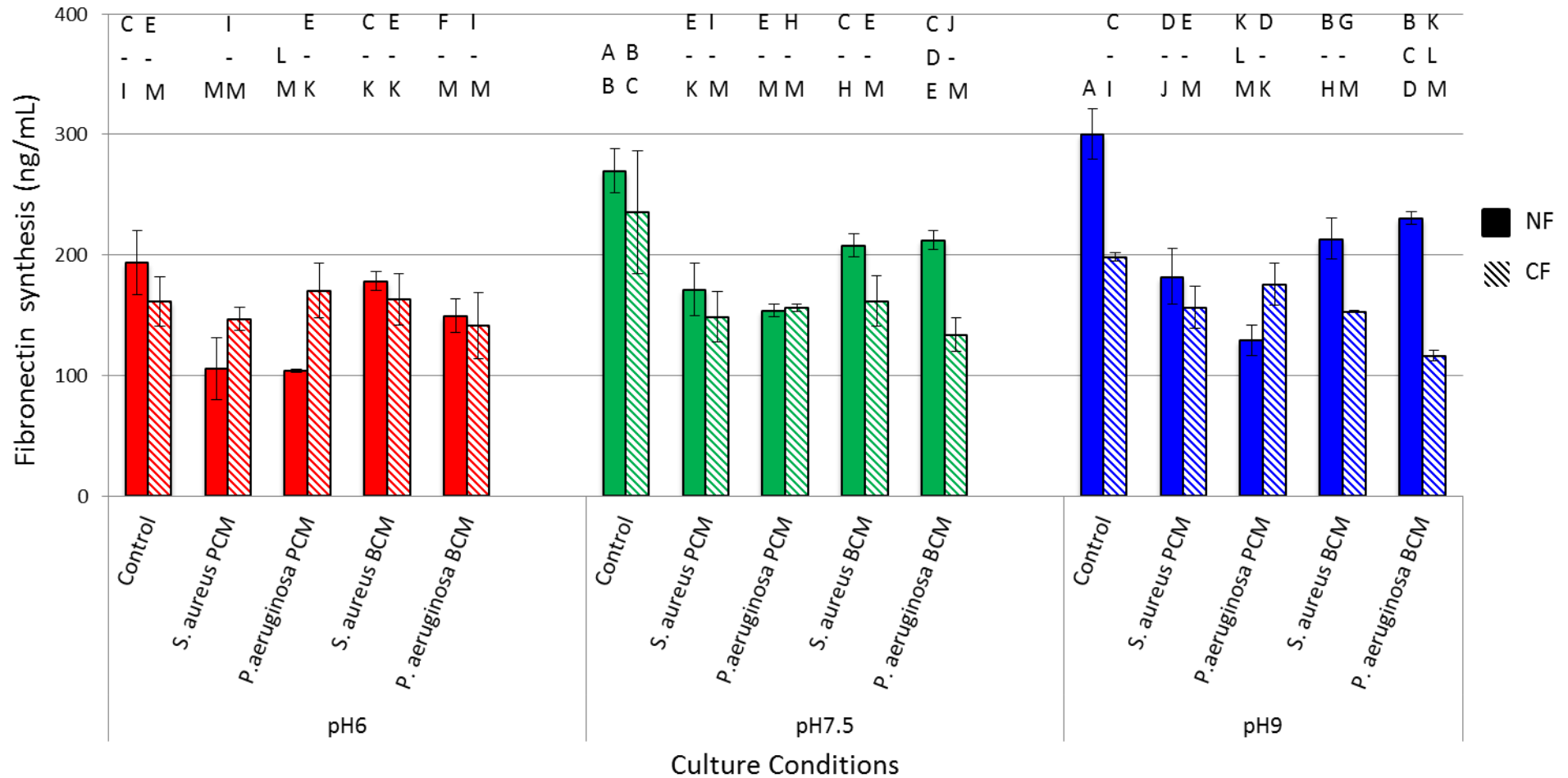
Figure 5.5 Fibronectin abundance from NF and CF cultured in pH titrated PCM and BCM

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and the amount of fibronectin was measured by an ELISA. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * p <0.05. Conditions that share the same letter are not significantly different.

Table 5.3 Statistical groupings for Fibronectin abundance

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	Statistical Grouping (Conditions that do not share a letter are significantly different, $p < 0.05$)												
				A	B	C	D	E	F	G	H	I	J	K	L	M
Normal	6	Control				C	D	E	F	G	H	I				
		S. aureus	PCM													M
		P. aeruginosa	PCM												L	M
		S. aureus	BCM			C	D	E	F	G	H	I	J	K		
		P. aeruginosa	BCM						F	G	H	I	J	K	L	M
	7.5	Control		A	B											
		S. aureus	PCM					E	F	G	H	I	J	K		
		P. aeruginosa	PCM					E	F	G	H	I	J	K	L	M
		S. aureus	BCM			C	D	E	F	G	H					
		P. aeruginosa	BCM			C	D	E								
	9	Control		A												
		S. aureus	PCM				D	E	F	G	H	I	J			
		P. aeruginosa	PCM											K	L	M
		S. aureus	BCM		B	C	D	E	F	G	H					
		P. aeruginosa	BCM		B	C	D									
Chronic	6	Control						E	F	G	H	I	J	K	L	M
		S. aureus	PCM									I	J	K	L	M
		P. aeruginosa	PCM					E	F	G	H	I	J	K		
		S. aureus	BCM					E	F	G	H	I	J	K		
		P. aeruginosa	BCM									I	J	K	L	M
	7.5	Control			B	C										
		S. aureus	PCM									I	J	K	L	M
		P. aeruginosa	PCM								H	I	J	K	L	M
		S. aureus	BCM					E	F	G	H	I	J	K	L	M
		P. aeruginosa	BCM										J	K	L	M
	9	Control				C	D	E	F	G	H	I				
		S. aureus	PCM					E	F	G	H	I	J	K	L	M
		P. aeruginosa	PCM				D	E	F	G	H	I	J	K		
		S. aureus	BCM							G	H	I	J	K	L	M
		P. aeruginosa	BCM											K	L	M

5.3.3 Glycosaminoglycans abundance

A larger amount of GAGs was present from CF CM than were detected in CM by the NF in all culture conditions (Figure 5.6), and all reached statistical significance in comparison to controls with the exception of three conditions (pH 6 *P. aeruginosa* BCM, pH 7.5 *S. aureus* PCM and pH 7.5 *S. aureus* BCM; Table 5.4). GAG abundance from NF in both pH 6 PCM and BCM conditions was significantly lower than control ($p < 0.05$; Figure 5.6 and Table 5.4). In pH 7.5 and pH 9 only *P. aeruginosa* PCM resulted in a lower amount of GAG from NF compared to control ($p < 0.05$).

GAG abundance from CF in both PCM and BCM conditions was lower compared to control in each pH group. At pH 6, *S. aureus* PCM and *P. aeruginosa* BCM was significant compared to control ($p < 0.05$). At pH 7.5, all conditions decreased the amount of GAGs present compared to control ($p < 0.05$). In pH 9, *S. aureus* PCM and *P. aeruginosa* PCM and BCM significantly decrease GAG abundance compared to control ($p < 0.05$). *S. aureus* BCM showed the same level of GAGs abundance as pH 9 control (Figure 5.6 and Table 5.4).

Changing pH did not have a significant effect on the total amount of GAGs measured from NF or CF in control conditions. *S. aureus* PCM at pH 7.5 and pH 9 significantly increases the amount of GAGs from NF ($p < 0.05$) compared to the same condition at pH 6, but has no effect at different pH on CFs. Changes in pH of *P. aeruginosa* PCM had no further effect on GAGs abundance from NF, with all being significant compared to their pH control ($p < 0.05$; Figure 5.6).

However, GAGs abundance from CF was significantly decreased in *P. aeruginosa* PCM at pH 7.5 and pH 9 compared with pH 6. *S. aureus* BCM at pH 7.5 significantly increases GAGs abundance from NF compared to the same condition at pH 6 and pH 9. *S. aureus* BCM at each pH has a different effect on GAGs abundance from CF with a significant increase in pH 9 ($p < 0.05$; Figure 5.6 and Table 5.4). GAGs present from NF was significantly lower with *P. aeruginosa* BCM at pH 6 ($p < 0.05$) compared to pH 7.5 and pH 9. From CF the total GAGs was lower with *P. aeruginosa* BCM pH 6 and pH 7.5 conditions compared to pH 9.
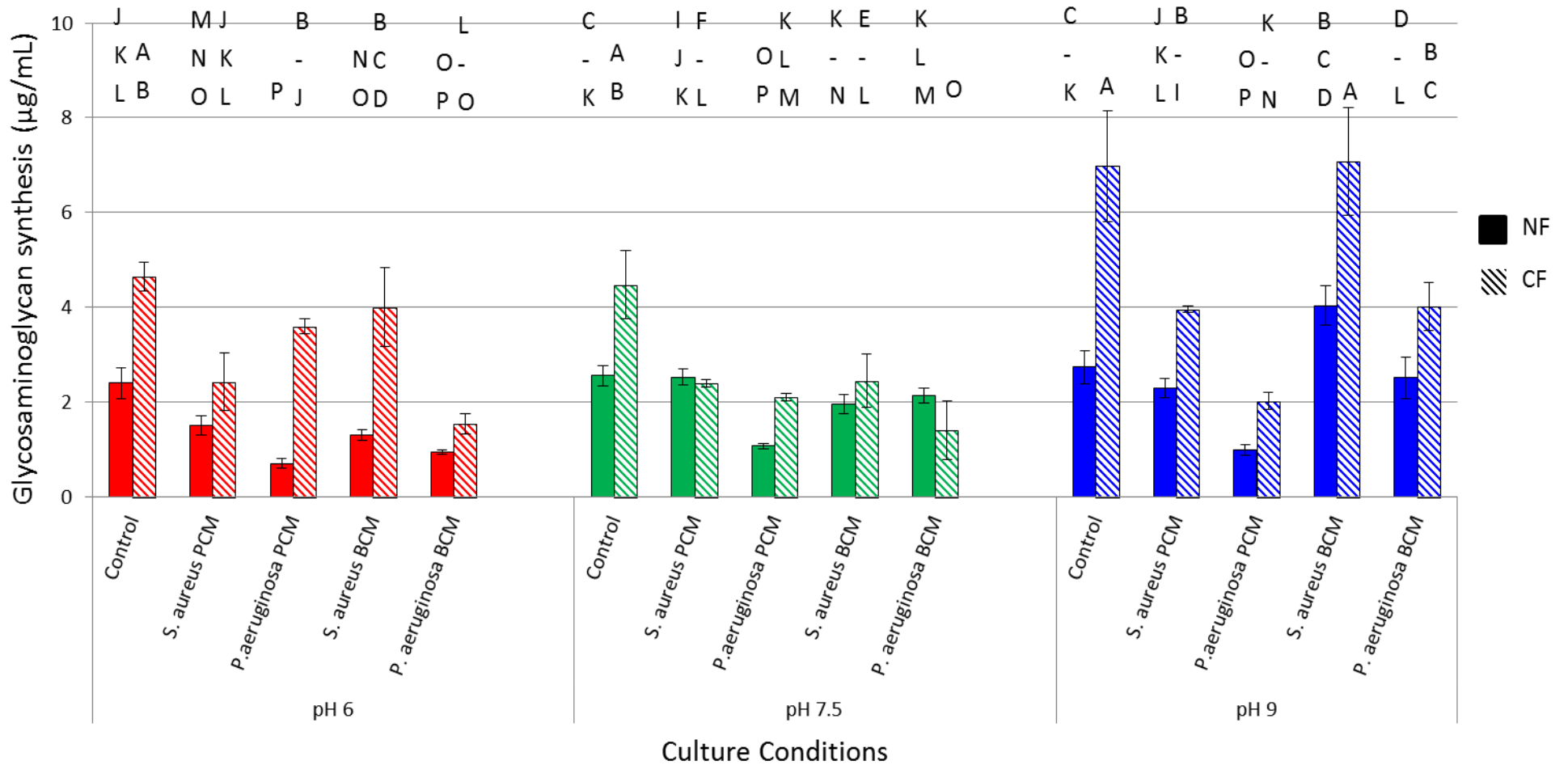
Figure 5.6 Glycosaminoglycan abundance from NF and CF cultured in pH titrated PCM and BCM

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and the amount of glycosaminoglycans was measured by the DMMB assay. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.4 Statistical groupings for Glycosaminoglycans abundance

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Normal	6	Control											J	K	L				
		S. aureus	PCM													M	N	O	
		P. aeruginosa	PCM																P
		S. aureus	BCM														N	O	
		P. aeruginosa	BCM															O	P
	7.5	Control				C	D	E	F	G	H	I	J	K					
		S. aureus	PCM									I	J	K					
		P. aeruginosa	PCM															O	P
		S. aureus	BCM											K	L	M	N		
		P. aeruginosa	BCM											K	L	M			
	9	Control				C	D	E	F	G	H	I	J	K					
		S. aureus	PCM										J	K	L				
		P. aeruginosa	PCM															O	P
		S. aureus	BCM		B	C	D												
		P. aeruginosa	BCM				D			G	H	I	J	K	L				
Chronic	6	Control		A	B														
		S. aureus	PCM										J	K	L				
		P. aeruginosa	PCM		B	C	D	E	F	G	H	I	J						
		S. aureus	BCM		B	C	D												
		P. aeruginosa	BCM												L	M	N	O	
	7.5	Control		A	B														
		S. aureus	PCM						F		H	I	J	K	L				
		P. aeruginosa	PCM											K	L	M			
		S. aureus	BCM					E	F	G	H	I	J	K	L				
		P. aeruginosa	BCM															O	
	9	Control		A															
		S. aureus	PCM		B	C	D	E	F	G	H	I							
		P. aeruginosa	PCM											K	L	M	N		
		S. aureus	BCM	A															
		P. aeruginosa	BCM		B	C													

5.3.4 Tropoelastin abundance

The total amount of tropoelastin was significantly more from NF compared to amount present from CF in all three pH conditions ($p < 0.05$; Figure 5.7 and Table 5.5). Both *S. aureus* and *P. aeruginosa* PCM and BCM reduce the amount of tropoelastin abundance from NF in each pH. At pH 6 *S. aureus* PCM and BCM caused a significant reduction ($p < 0.05$), but only *P. aeruginosa* BCM significantly reduced tropoelastin abundance, while *P. aeruginosa PCM* did not significantly decrease the amount of tropoelastin.

When titrated to pH 7.5, both PCM and BCM caused a significant reduction in the amount of tropoelastin measured compared to pH 7.5 control ($p < 0.05$). In pH 9 conditions, results were the same as at pH 6, with only *P. aeruginosa* PCM not causing a significant reduction in abundance compared to control (Figure 5.7 and Table 5.5).

Tropoelastin abundance from CF showed no difference between cultures in the three different pH conditions; however BCM significantly diminished the total amount of tropoelastin measured. Some tropoelastin was present after CFs culture in pH 6 *S. aureus* BCM, but all other BCM conditions resulted in no detectable tropoelastin. At pH 9 both, PCM and BCM resulted in no tropoelastin being detected (Figure 5.7).
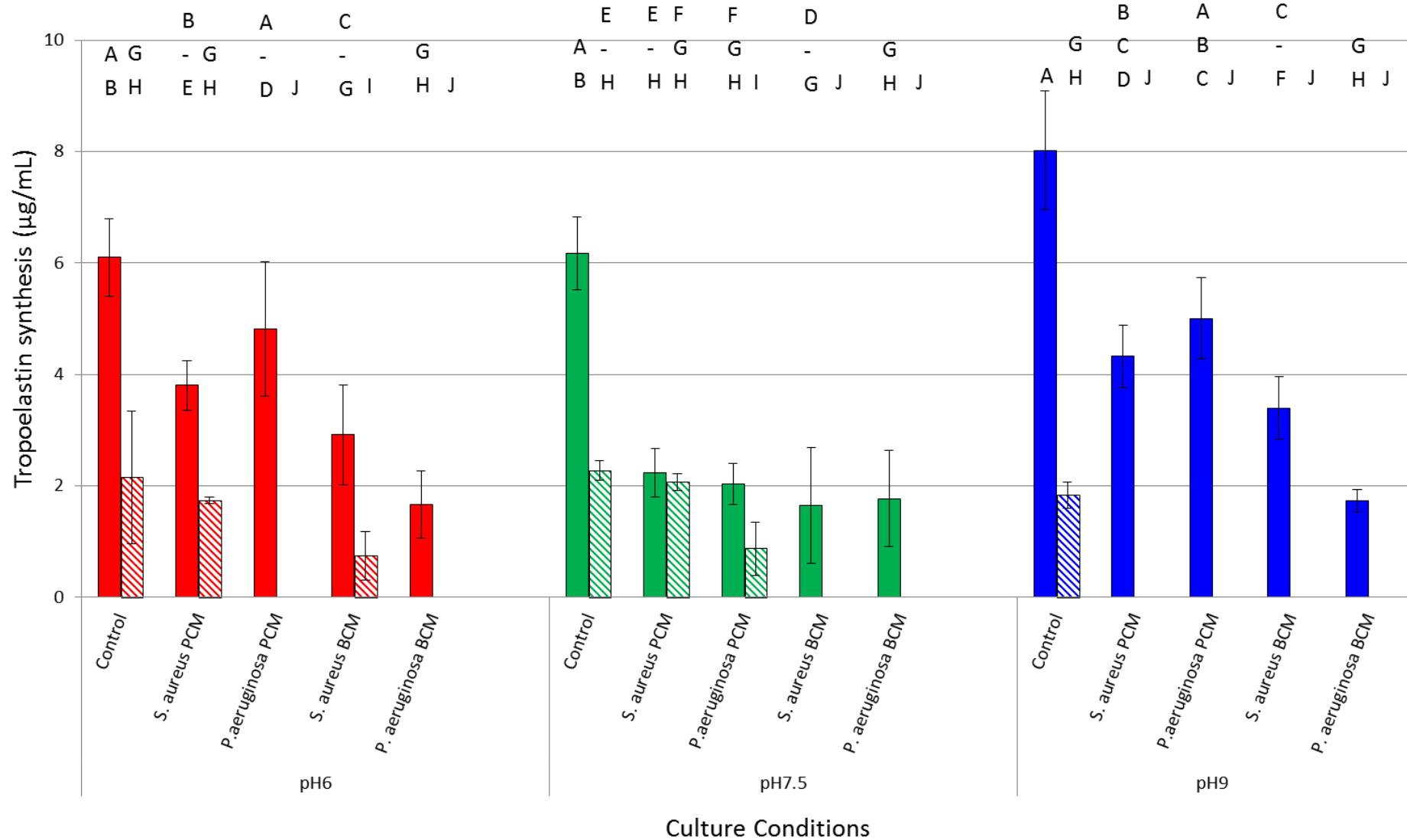
Figure 5.7 Tropoelastin abundance from NF and CF cultured in pH titrated PCM and BCM

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and the amount of tropoelastin measured by the Fastin elastin assay. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.5 Statistical groupings for Tropoelastin abundance

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	Statistical Grouping (Conditions that do not share a letter are significantly different, $p < 0.05$)									
Normal	6	Control		A	B								
		S. aureus	PCM		B	C	D	E					
		P. aeruginosa	PCM	A	B	C	D						
		S. aureus	BCM			C	D	E	F	G			
		P. aeruginosa	BCM							G	H		
	7.5	Control		A	B								
		S. aureus	PCM					E	F	G	H		
		P. aeruginosa	PCM						F	G	H		
		S. aureus	BCM				D	E	F	G			
		P. aeruginosa	BCM							G	H		
	9	Control		A									
		S. aureus	PCM		B	C	D						
		P. aeruginosa	PCM	A	B	C							
		S. aureus	BCM			C	D	E	F				
		P. aeruginosa	BCM							G	H		
Chronic	6	Control								G	H		
		S. aureus	PCM							G	H		
		P. aeruginosa	PCM										J
		S. aureus	BCM									I	
		P. aeruginosa	BCM										J
	7.5	Control						E	F	G	H		
		S. aureus	PCM						F	G	H		
		P. aeruginosa	PCM									I	
		S. aureus	BCM										J
		P. aeruginosa	BCM										J
	9	Control								G	H		
		S. aureus	PCM										J
		P. aeruginosa	PCM										J
		S. aureus	BCM										J
		P. aeruginosa	BCM										J

5.4 Extracellular Matrix Gene Expression Following Fibroblast Culture in pH titrated PCM and BCM

Following culture of NF and CF for 24 hours in pH titrated (pH 6, 7.5 and 9) *S. aureus* and *P. aeruginosa* PCM or BCM. Cells were lysed and collected in trizol, RNA extracted and the expression of a range of ECM molecules including Collagen I (COL1A1 and COL1A2), Fibronectin (FN), Decorin (DCN), Versican (VCAN) and Lumican (LUM) genes were assessed for changes in expression levels.

When cultured in different pH, there were few identified differences in gene expression in NF of these extracellular matrix genes. Expression of COL1A1 was significantly down regulated in pH 9 compared to pH 6 and pH 7.5. The effect of PCM and BCM varied across all genes analysed. *S. aureus* PCM had no significant effect on any of the genes compared to each pH control. *P. aeruginosa* PCM reduced gene expression of all genes but only at some pH; COL1A1 at pH 6 and pH 7.5, COL1A2, FN, and DCN at pH 7.5 only, VCAN at pH 7.5 and pH9 and LUM in all three pH conditions. *S. aureus* BCM also down regulated ECM expression, but only at some pH's. COL1A1 was down regulated at pH 6 and 7.5, COL1A2 at pH 6, FN at pH 7.5, DCN at pH 6 and pH 7.5, VCAN at pH 6 and LUM at both pH 6 and pH 9. *P. aeruginosa* BCM however, significantly down regulated the expression of all six genes analysed at all three pH conditions when compared to each pH control (Figure 5.8 - Figure 5.13 and Table 5.6 - Table 5.11).

pH had no significant effect on CF with no significant reduction in gene expression following culture in one pH compared to the other two pH conditions. At pH 7.5 *S. aureus* PCM had no effect on any of the genes analysed. At pH 6 and pH 9 LUM was significantly down regulated by *S. aureus* PCM compared to pH control. *P. aeruginosa* PCM titrated to all three pH had a significant effect on LUM expression compared to control (Figure 5.13). Titrated to pH 9 *P. aeruginosa* PCM also had a significant effect on FN (Figure 5.10) and DCN (Figure 5.11) expression compared to control. *S. aureus* BCM down regulated COL1A1, FN and LUM expression titrated to pH 6, LUM at pH 7.5 and COL1A1, FN, DCN, VCAN and LUM when

titrated to pH 9. At pH 9 *P. aeruginosa* BCM affected the expression of all six genes analysed compared to control. At pH 6, affected five (COL1A1, COL1A2, FN, VCAN and LUM) and at pH 7.5 down regulated DCN, VCAN and LUM.

Overall, CF showed a lower expression of all the genes analysed in comparison to NF; with the exception of an up regulation of COL1A1 (Figure 5.8), FN (Figure 5.10), DCN (Figure 5.11) and LUM (Figure 5.13) expression in pH 9 conditions. However, only DCN and LUM expression were significantly different compared to their expression in NF. There was no clear significant effect on the gene expression of NF and CF observed when comparing the effects between *S. aureus* and *P. aeruginosa* PCM and BCM.

A significant effect on some genes was observed when PCM and BCM were titrated to some pHs but not all. When titrated to pH 7.5 there was a significant difference between *S. aureus* PCM and BCM on the expression of COL1A1 (Figure 5.8), COL1A2 (Figure 5.9), FN (Figure 5.10), DCN (Figure 5.11) and VCAN (Figure 5.12), with BCM causing a significant down regulation. The effect of PCM and BCM on LUM expression showed no significant differences in any pH condition (Figure 5.13).

There was a difference seen in COL1A1, COL1A2, DCN and VCAN expression in NF when comparing *S. aureus* PCM and BCM both titrated to pH 6 and a difference in COL1A1 and COL1A2 expression comparing *P. aeruginosa* PCM and BCM titrated to pH 9. There was a difference in COL1A1 and DCN expression in CF when comparing *S. aureus* PCM and BCM titrated to pH 9. When comparing *P. aeruginosa* PCM and BCM; there was a difference in COL1A1 and VCAN expression at pH 6, COL1A1, COL1A2 and VCAN at pH 7.5 and VCAN at pH 9.
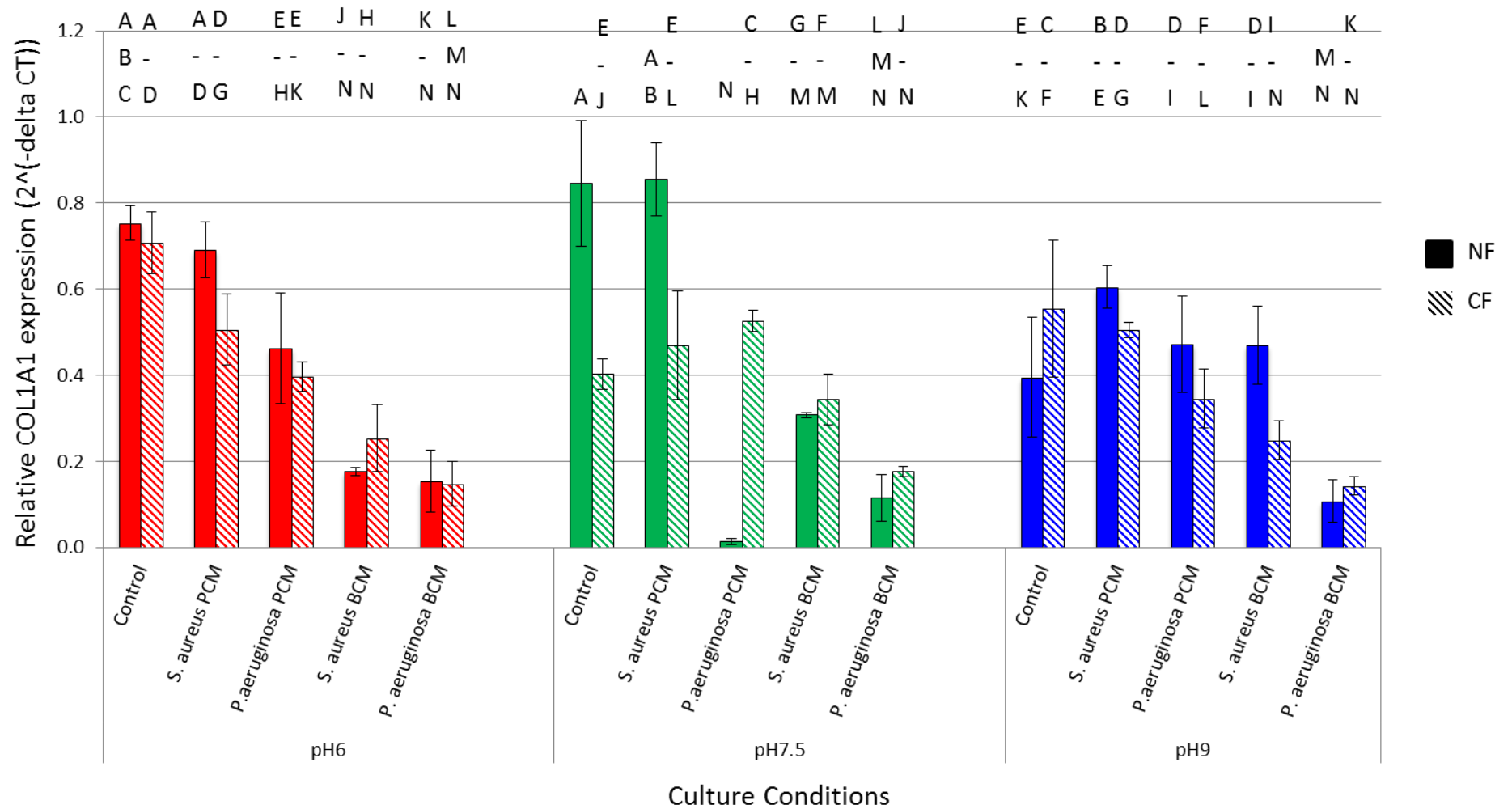
Figure 5.8 Expression of Collagen Type 1A1 after 24 hours culture in pH titrated PCM and BCM

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and expression of COL1A1 was assessed. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.6 Statistical groupings for COL1A1 gene expression

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	Statistical Grouping (Conditions that do not share a letter are significantly different, $p < 0.05$)													
				A	B	C	D	E	F	G	H	I	J	K	L	M	N
Normal	6	Control		A	B	C											
		S. aureus	PCM	A	B	C	D										
		P. aeruginosa	PCM					E	F	G	H						
		S. aureus	BCM										J	K	L	M	N
		P. aeruginosa	BCM											K	L	M	N
	7.5	Control		A													
		S. aureus	PCM	A	B												
		P. aeruginosa	PCM														N
		S. aureus	BCM							G	H	I	J	K	L	M	
		P. aeruginosa	BCM												L	M	N
	9	Control						E	F	G	H	I	J	K			
		S. aureus	PCM		B	C	D	E									
		P. aeruginosa	PCM				D	E	F	G	H	I					
		S. aureus	BCM				D	E	F	G	H	I					
		P. aeruginosa	BCM													M	N
Chronic	6	Control		A	B	C	D										
		S. aureus	PCM				D	E	F	G							
		P. aeruginosa	PCM					E	F	G	H	I	J	K			
		S. aureus	BCM								H	I	J	K	L	M	N
		P. aeruginosa	BCM												L	M	N
	7.5	Control						E	F	G	H	I	J				
		S. aureus	PCM					E	F	G		I	J	K	L		
		P. aeruginosa	PCM			C	D	E	F	G	H						
		S. aureus	BCM						F	G	H	I	J	K	L	M	
		P. aeruginosa	BCM										J	K	L	M	N
	9	Control				C	D	E	F								
		S. aureus	PCM				D	E	F	G							
		P. aeruginosa	PCM						F	G	H	I	J	K	L		
		S. aureus	BCM									I	J	K	L	M	N
		P. aeruginosa	BCM											K	L	M	N

Figure 5.9 Expression of Collagen Type 1A2 after culture in pH titrated PCM and BCM

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and expression of COL1A2 was assessed. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.7 Statistical groupings for COL1A2 gene expression

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	Statistical Grouping (Conditions that do not share a letter are significantly different, $p < 0.05$)								
Normal	6	Control		A	B							
		S. aureus	PCM	A	B							
		P. aeruginosa	PCM	A	B							
		S. aureus	BCM						F	G	H	I
		P. aeruginosa	BCM				D	E	F	G	H	I
	7.5	Control		A	B	C						
		S. aureus	PCM	A								
		P. aeruginosa	PCM									I
		S. aureus	BCM			C	D	E	F	G	H	I
		P. aeruginosa	BCM								H	I
	9	Control			B	C	D	E	F	G		
		S. aureus	PCM	A	B							
		P. aeruginosa	PCM	A	B							
		S. aureus	BCM	A	B	C	D	E				
		P. aeruginosa	BCM								H	I
Chronic	6	Control		A	B	C	D	E				
		S. aureus	PCM	A	B	C	D	E				
		P. aeruginosa	PCM				D	E	F	G	H	I
		S. aureus	BCM				D	E	F	G	H	I
		P. aeruginosa	BCM							G	H	I
	7.5	Control		A	B	C	D	E	F			
		S. aureus	PCM		B	C	D	E	F			
		P. aeruginosa	PCM	A	B	C	D					
		S. aureus	BCM		B	C	D	E	F	G		
		P. aeruginosa	BCM					E	F	G	H	I
	9	Control			B	C	D	E	F	G		
		S. aureus	PCM	A	B	C	D	E	F			
		P. aeruginosa	PCM			C	D	E	F	G	H	
		S. aureus	BCM			C	D	E	F	G	H	
		P. aeruginosa	BCM								H	I

Figure 5.10 Expression of Fibronectin after culture in pH titrated PCM and BCM

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and expression of FN was assessed. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.8 Statistical groupings for FN gene expression

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Normal	6	Control		A	B																	
		S. aureus	PCM	A	B																	
		P. aeruginosa	PCM	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q		
		S. aureus	BCM	A	B	C	D	E	F	G	H	I	J	K	L	M	N					
		P. aeruginosa	BCM																P	Q	R	S
	7.5	Control		A	B	C	D	E	F	G	H	I	J	K	L	M	N					
		S. aureus	PCM	A	B																	
		P. aeruginosa	PCM																		R	S
		S. aureus	BCM																P	Q	R	S
		P. aeruginosa	BCM															O	P	Q	R	S
	9	Control		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P			
		S. aureus	PCM		B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
		P. aeruginosa	PCM															O	P	Q	R	S
		S. aureus	BCM												L	M	N	O	P	Q	R	S
		P. aeruginosa	BCM																			S
Chronic	6	Control		A																		
		S. aureus	PCM	A	B																	
		P. aeruginosa	PCM									I	J	K	L	M	N	O	P	Q	R	S
		S. aureus	BCM																P	Q	R	S
		P. aeruginosa	BCM															O	P	Q	R	S
	7.5	Control		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O				
		S. aureus	PCM						F	G	H	I	J	K	L	M	N	O	P	Q	R	S
		P. aeruginosa	PCM		B			E			H			K			N	O	P	Q		
		S. aureus	BCM	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P			
		P. aeruginosa	BCM			C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	9	Control		A	B	C	D	E	F	G	H	I	J	K	L	M	N					
		S. aureus	PCM		B		D	E		G	H		J	K		M	N	O	P	Q	R	S
		P. aeruginosa	PCM																P	Q	R	S
		S. aureus	BCM																P	Q	R	S
		P. aeruginosa	BCM																	Q	R	S
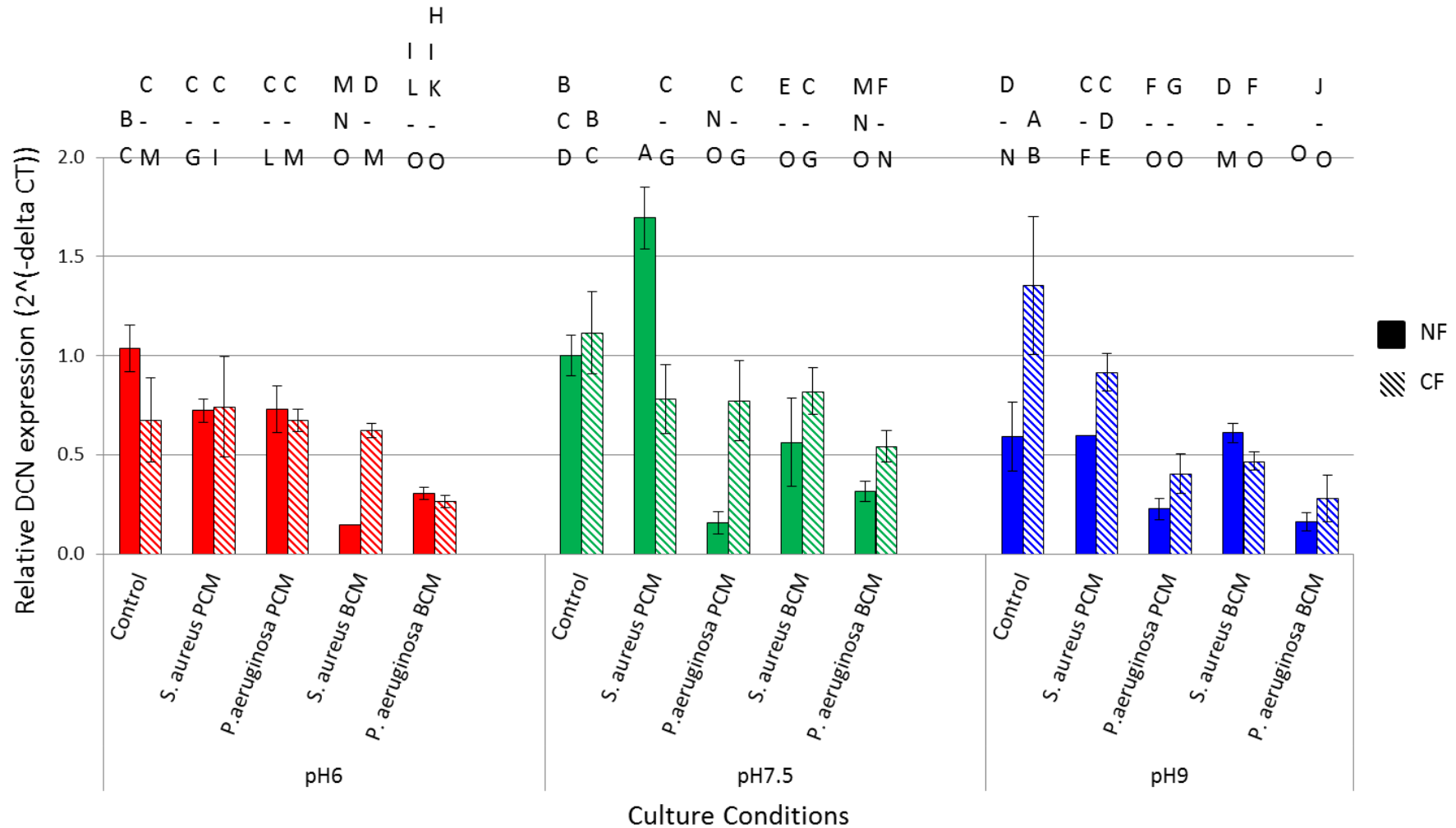
Figure 5.11 Decorin expression after culture in pH titrated PCM and BCM for 24 hours

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and expression of DCN was assessed. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.9 Statistical groupings for DCN gene expression

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	Statistical Grouping (Conditions that do not share a letter are significantly different, $p < 0.05$)														
				A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Normal	6	Control			B	C												
		S. aureus	PCM			C	D	E	F	G								
		P. aeruginosa	PCM			C	D	E	F	G	H	I	J	K	L			
		S. aureus	BCM													M	N	O
		P. aeruginosa	BCM									I			L	M	N	O
	7.5	Control			B	C	D											
		S. aureus	PCM	A														
		P. aeruginosa	PCM														N	O
		S. aureus	BCM					E	F	G	H	I	J	K	L	M	N	O
		P. aeruginosa	BCM													M	N	O
	9	Control					D	E	F	G	H	I	J	K	L	M	N	
		S. aureus	PCM			C	D	E	F									
		P. aeruginosa	PCM						F	G	H	I	J	K	L	M	N	O
		S. aureus	BCM				D	E	F	G	H	I	J	K	L	M		
		P. aeruginosa	BCM															O
Chronic	6	Control				C	D	E	F	G	H	I	J	K	L	M		
		S. aureus	PCM			C	D	E	F	G	H	I						
		P. aeruginosa	PCM			C	D	E	F	G	H	I	J	K	L	M		
		S. aureus	BCM				D	E	F	G	H	I	J	K	L	M		
		P. aeruginosa	BCM								H	I		K	L	M	N	O
	7.5	Control			B	C												
		S. aureus	PCM			C	D	E	F	G								
		P. aeruginosa	PCM			C	D	E	F	G								
		S. aureus	BCM			C	D	E	F	G								
		P. aeruginosa	BCM						F	G	H	I	J	K	L	M	N	
	9	Control		A	B													
		S. aureus	PCM			C	D	E										
		P. aeruginosa	PCM							G	H	I	J	K	L	M	N	O
		S. aureus	BCM						F	G	H	I	J	K	L	M	N	O
		P. aeruginosa	BCM										J	K	L	M	N	O
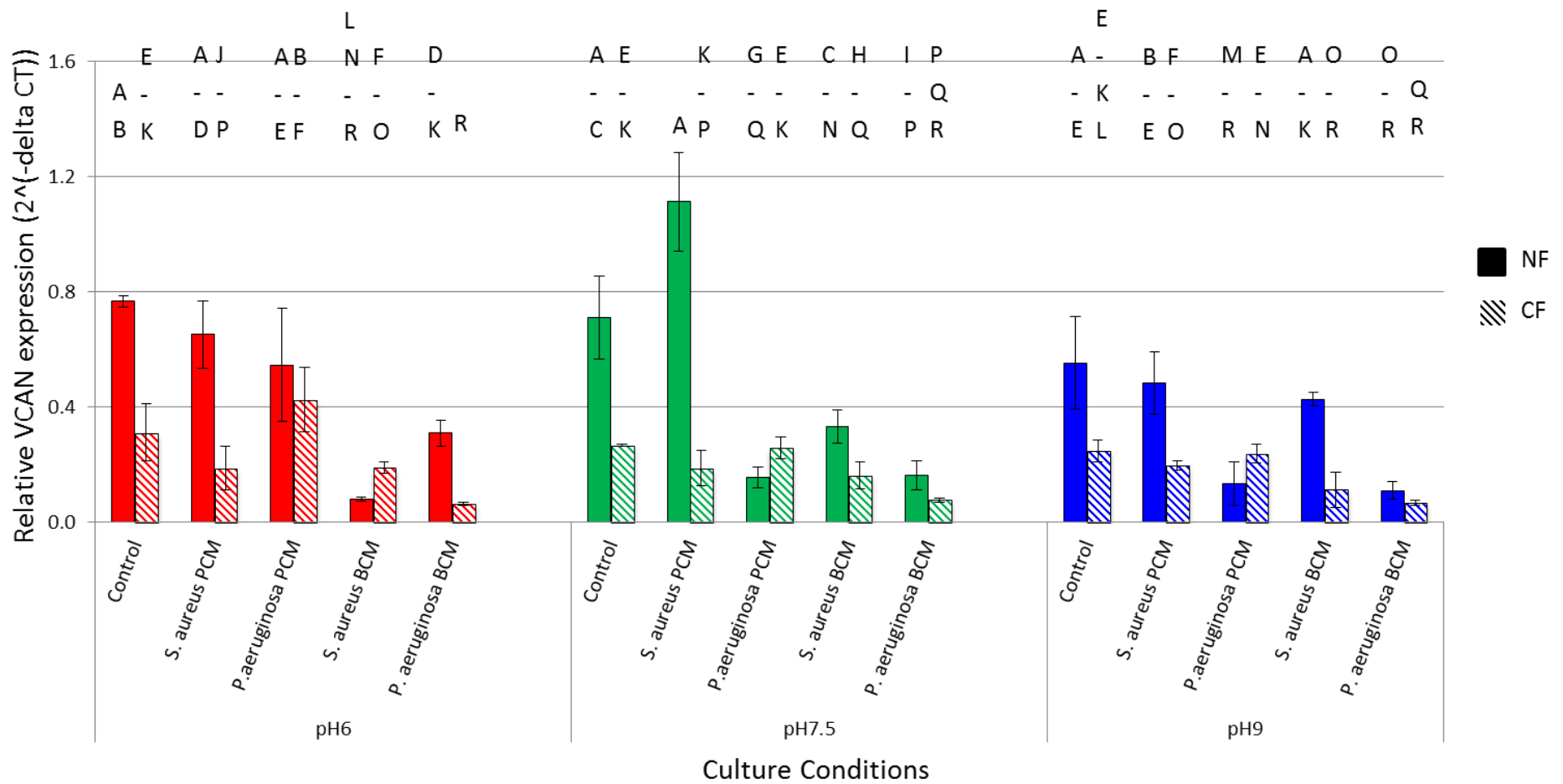
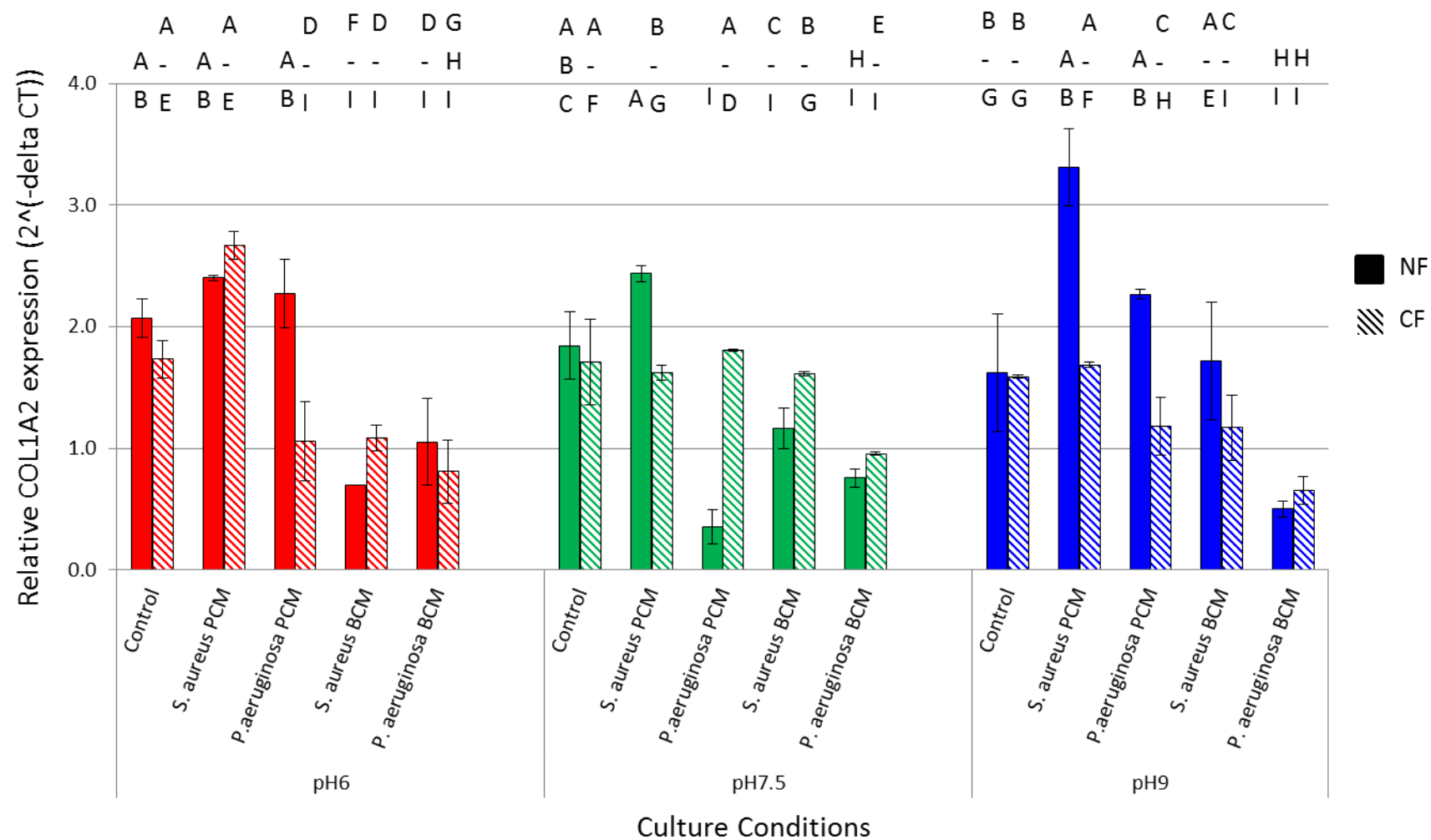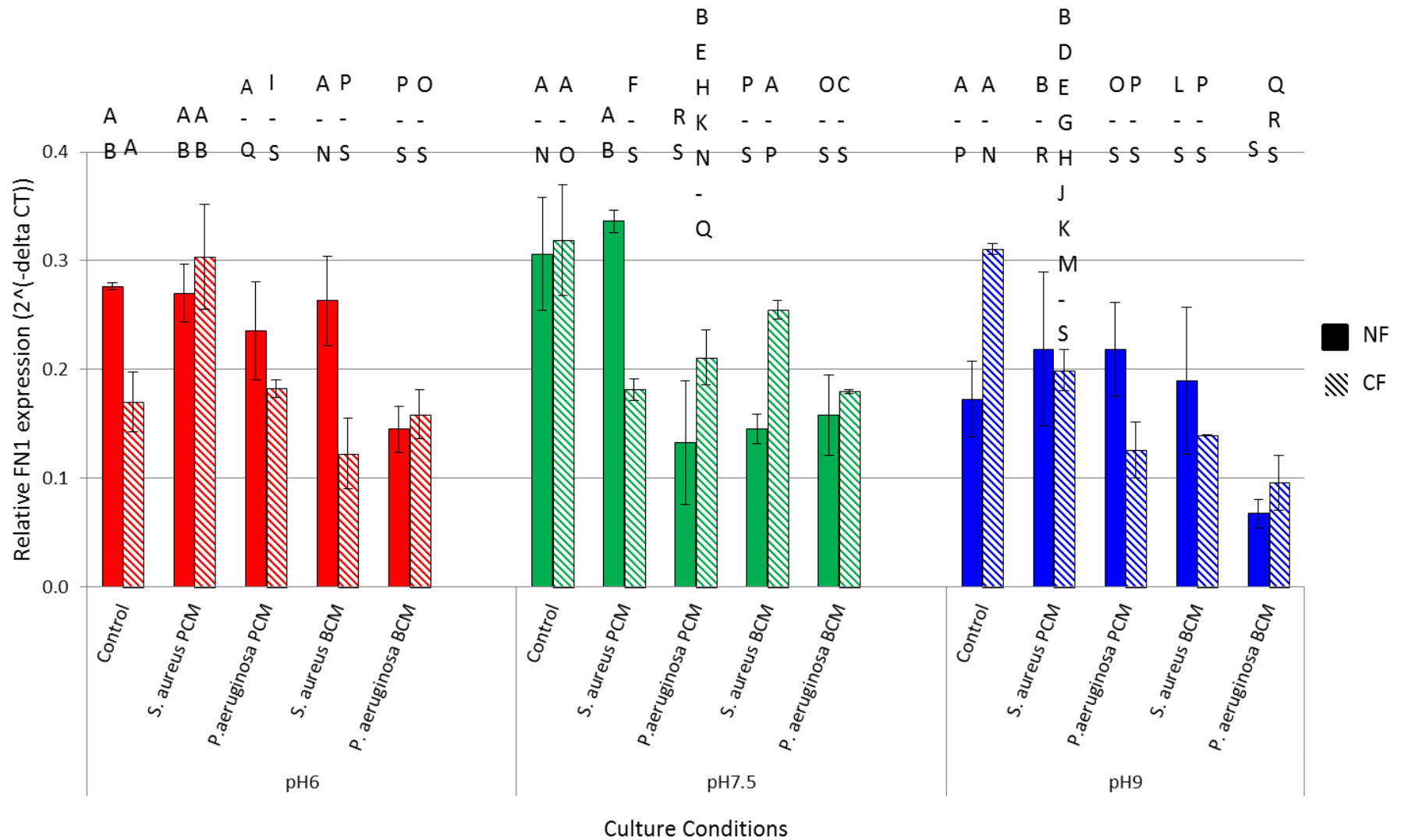**Figure 5.12 Versican expression after culture in pH titrated PCM and BCM**

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and expression of VCAN was assessed. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.10 Statistical groupings for Versican expression

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

| Cell Type	pH	Bacteria	PCM or BCM	Statistical Grouping (Conditions that do not share a letter are significantly different, $p < 0.05$)
Normal	6	Control		A B
		S. aureus	PCM	A B C D
		P. aeruginosa	PCM	A B C D E
		S. aureus	BCM	L N O P Q R
		P. aeruginosa	BCM	D E F G H I J K
	7.5	Control		A B C
		S. aureus	PCM	A
		P. aeruginosa	PCM	G H I J K L M N O P Q
		S. aureus	BCM	C D E F G H I J K L M N
		P. aeruginosa	BCM	I J K L M N O P
	9	Control		A B C D E
		S. aureus	PCM	B C D E
		P. aeruginosa	PCM	M N O P Q R
		S. aureus	BCM	A B C D E F G H I J K
		P. aeruginosa	BCM	O P Q R
Chronic	6	Control		E F G H I J K
		S. aureus	PCM	J K L M N O P
		P. aeruginosa	PCM	B C D E F
		S. aureus	BCM	F G H I J K L M N O
		P. aeruginosa	BCM	R
	7.5	Control		E F G H I J K
		S. aureus	PCM	K L M N O P Q
		P. aeruginosa	PCM	E F G H I J K
		S. aureus	BCM	H I J K L M N O P Q
		P. aeruginosa	BCM	P Q R
	9	Control		E F G H I J K M
		S. aureus	PCM	F G H I J K L M N O
		P. aeruginosa	PCM	E F G H I J K L M N
		S. aureus	BCM	O P Q R
		P. aeruginosa	BCM	Q R
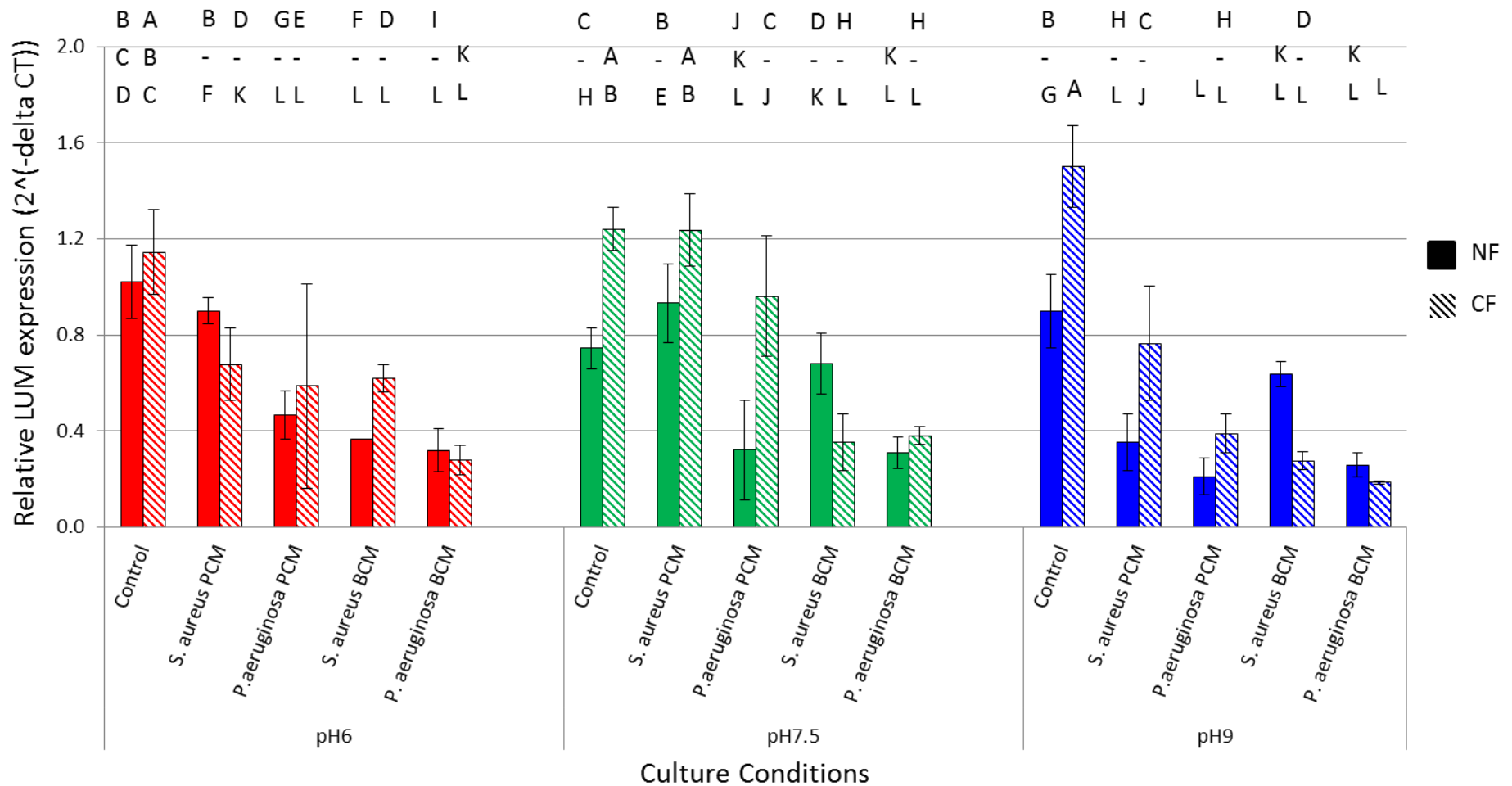
Figure 5.13 Lumican expression after culture in pH titrated PCM and BCM

NF and CF were cultured in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM for 24 hours and expression of LUM was assessed. Values represent the mean ± SEM (*n*=3). Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05. Conditions that share the same letter are not significantly different.

Table 5.11 Statistical groupings for Lumican expression

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different.

Cell Type	pH	Bacteria	PCM or BCM	Statistical Grouping (Conditions that do not share a letter are significantly different, $p < 0.05$)											
				A	B	C	D	E	F	G	H	I	J	K	L
Normal	6	Control			B	C	D								
		S. aureus	PCM		B	C	D	E	F						
		P. aeruginosa	PCM							G	H	I	J	K	L
		S. aureus	BCM						F	G	H	I	J	K	L
		P. aeruginosa	BCM									I	J	K	L
	7.5	Control				C	D	E	F	G	H				
		S. aureus	PCM		B	C	D	E							
		P. aeruginosa	PCM										J	K	L
		S. aureus	BCM				D	E	F	G	H	I	J	K	
		P. aeruginosa	BCM									I	J	K	L
	9	Control			B	C	D	E	F	G					
		S. aureus	PCM								H	I	J	K	L
		P. aeruginosa	PCM												L
		S. aureus	BCM											K	L
		P. aeruginosa	BCM											K	L
Chronic	6	Control		A	B	C									
		S. aureus	PCM				D	E	F	G	H	I	J	K	
		P. aeruginosa	PCM					E	F	G	H	I	J	K	L
		S. aureus	BCM				D	E	F	G	H	I	J	K	L
		P. aeruginosa	BCM											K	L
	7.5	Control		A	B										
		S. aureus	PCM	A	B										
		P. aeruginosa	PCM			C	D	E	F	G	H	I	J		
		S. aureus	BCM								H	I	J	K	L
		P. aeruginosa	BCM								H	I	J	K	L
	9	Control		A											
		S. aureus	PCM			C	D	E	F	G	H	I	J		
		P. aeruginosa	PCM								H	I	J	K	L
		S. aureus	BCM				D	E	F	G	H	I	J	K	L
		P. aeruginosa	BCM												L

5.5 The Effect of pH titrated Planktonic and Biofilm Conditioned Media on Normal and Chronic Fibroblast Migration

The effect of pH alone has previously been determined. The optimal wound closure of NF was observed in pH 7.5, then pH 9 with the slowest being in pH 6. For CF, wound closure was best in pH 6 and pH 7.5 and was significantly reduced in pH 9. This study analysed the migration of NF and CF over 48 hours in pH titrated *S. aureus* and *P. aeruginosa* PCM or BCM. Results were evaluated using Image J software and are shown as percentage wound closure over time (Figure 5.14). The effect of cell type, pH, bacterial strain, PCM or BCM and time on wound closure were analysed by a General Linear Model (GLM) followed by post-hoc analysis to determine any significant effects (Table 5.12). Results show that all factors had a significant effect on wound closure: pH (p = 0.026), bacteria (p = 0.001), PCM or BCM (p ≤ 0.000), cell (p = 0.000) and time (p ≤ 0.000).

Migration of NF in pH 6 was improved with PCM after both 24 and 48 hours, with up to 85% wound closure observed compared to 48% in pH 6 control. BCM however significantly reduced NF migration compared to control after 48 hours (Figure 5.15A). In pH 7.5 conditions no significant difference in NF migration was observed between control and PCM conditions. *S. aureus* BCM slightly reduced migration, but only *P. aeruginosa* BCM had a significant effect, with only 21% wound closure after 24 hours and 32% after 48 hours (Figure 5.15B). In pH 9 conditions NF migration was reduced with maximum 66% wound closure with control after 48 hours. PCM and BCM reduced wound closure with only *P. aeruginosa* BCM having a significant effect compared to control at both 24 and 48 hours (Figure 5.15C).

Migration of CF in pH 6 showed that after 24 hours *P. aeruginosa* PCM significantly increased cell migration compared to control, but at 48 hours there was no difference between either *S. aureus* or *P. aeruginosa* PCM and control. After 48 hours both *S. aureus* and *P. aeruginosa* BCM significantly reduced cell migration compared to control (Figure 5.16A). After 24 hours there was no effect at pH 7.5 of PCM and BCM compared to control, but at 48 hours *P.*

aeruginosa BCM had significantly affected CF migration with only 52% closure compared with 84% in pH 7.5 control (Figure 5.16B). At pH 9 there was no significant effect between each condition observed, but CF migration overall was much slower when all conditions were titrated to pH 9, with a maximum wound closure of 46% (Figure 5.16C).

GLM showed a significant effect of PCM or BCM on wound closure with statistical groupings of the Fisher's posteriori test showing that BCM had a significant effect compared to control and PCM ($p \leq 0.000$). All results of BCM presented in Figure 5.18, show maximum wound closure of 48% after 24 hours followed by 70% after 48 hours compared to 74% and 90% with PCM, as seen in Figure 5.17. The presence of bacteria compared to control (with no bacteria) had a significant effect on wound closure. But, there was no overall significant effect observed between the two bacterial strains tested (*S. aureus* and *P. aeruginosa*). Some significant differences were seen e.g. between *S. aureus* and *P. aeruginosa* PCM titrated to pH 6 on CF migration (Figure 5.17) and between *S. aureus* and *P. aeruginosa* BCM on NF in all three pH conditions (Figure 5.18).
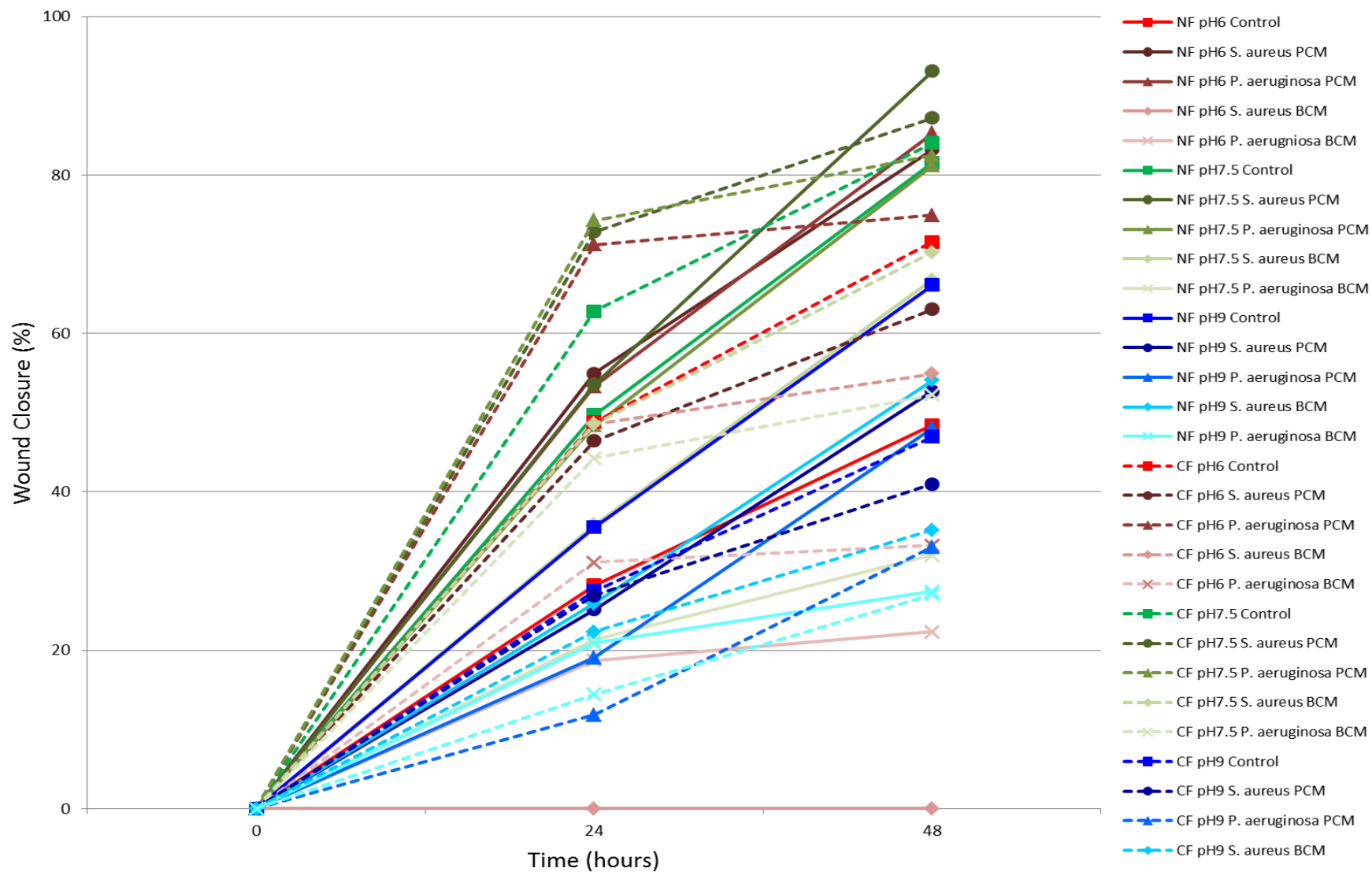
Figure 5.14 Normal and Chronic Fibroblast migration in pH titrated *S. aureus* and *P. aeruginosa* PCM or BCM

Graph shows a summary of cell migration over 48 hours of all conditions tested. Standard error of the means and statistical differences are not shown. Statistical groupings for all conditions are presented in Table 5.12.

Table 5.12 Statistical groupings for all fibroblast migration data

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of *p* < 0.05. Means that do not share a letter are significantly different at each time point. The * in the final column indicates a significant difference of each condition between Normal and Chronic fibroblasts.

pH	Bacteria	PCM or BCM	Time	Statistical grouping information	
6	Control		24	G H I	*
			48	F G H K	*
6	*S. aureus*	PCM	24	A B C	
			48	A B C D	
6	*P. aeruginosa*	PCM	24	A B C	
			48	A B	
6	*S. aureus*	BCM	24	J	*
			48	P	*
6	*P. aeruginosa*	BCM	24	G H I	
			48	N O	
7.5	Control		24	B C D	
			48	A B C D	
7.5	*S. aureus*	PCM	24	A B C	
			48	A	
7.5	*P. aeruginosa*	PCM	24	B C D	*
			48	A B C D	
7.5	*S. aureus*	BCM	24	D E F G	
			48	B C D E F	
7.5	*P. aeruginosa*	BCM	24	G H I	*
			48	J M N O	*
9	Control		24	D E F G H	
			48	C D E F	
9	*S. aureus*	PCM	24	G H I	
			48	F G H	
9	*P. aeruginosa*	PCM	24	G H I	
			48	F G H I J K L M	
9	S. aureus	BCM	24	E F G H I	
			48	E F G H I J K L M	
9	P. aeruginosa	BCM	24	I J	
			48	O P	

pH	Bacteria	PCM or BCM	Time	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
Chronic Fibroblasts				**Statistical grouping information**															
6	Control		24		B	C	D												*
			48		B	C	D	E											
6	*S. aureus*	PCM	24		B	C	D	E	F										
			48				D	E	F	G									
6	*P. aeruginosa*	PCM	24	A															
			48	A	B	C	D	E											
6	*S. aureus*	BCM	24		B	C	D	E											*
			48						F	G	H	I	J	K	L	M	N		*
6	*P. aeruginosa*	BCM	24							G	H	I							
			48											K	L	M	N	O	
7.5	Control		24	A	B														
			48	A	B	C													
7.5	*S. aureus*	PCM	24	A															
			48	A	B														
7.5	*P. aeruginosa*	PCM	24	A															*
			48	A	B	C	D												
7.5	*S. aureus*	BCM	24		B	C	D												
			48		B	C	D	E											
7.5	*P. aeruginosa*	BCM	24			C	D	E	F										*
			48						F	G	H								*
9	Control		24							G	H	I							
			48						F	G	H	I	J	K	L	M			
9	*S. aureus*	PCM	24						F	G	H	I							
			48							G	H	I	J	K	L	M	N	O	
9	*P. aeruginosa*	PCM	24									I	J						
			48								H	I	J	K	L	M	N	O	
9	*S. aureus*	BCM	24							G	H	I							
			48								H	I	J	K	L	M	N	O	
9	*P. aeruginosa*	BCM	24								H	I	J						
			48									I	J		L	M	N	O	

C = control (no bacteria)

PCM = Planktonic conditioned media

BCM = Biofilm conditioned media

Key	Significant conditions ($p < 0.05$)	Key	Significant conditions ($p < 0.05$)
*	Control vs *S. aureus* PCM	##	*S. aureus* PCM vs *S. aureus* BCM
**	Control vs *P. aeruginosa* PCM	###	*S. aureus* PCM vs *P. aeruginosa* BCM
***	Control vs *S. aureus* BCM	$	*P. aeruginosa* PCM vs *S. aureus* BCM
****	Control vs *P. aeruginosa* BCM	$$	*P. aeruginosa* PCM vs *P. aeruginosa* BCM
#	*S. aureus* PCM vs *P. aeruginosa* PCM	@	*S. aureus* BCM vs *P. aeruginosa* BCM

Figure 5.15 Normal fibroblast migration in pH titrated PCM and BCM

Normal fibroblast migration was measured over 48 hours in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM. Migration in pH 6 PCM and BCM is shown in A, pH 7.5 in B and pH 9 in C. Statistical differences between the mean values of was determined by ANOVA, followed by a posteriori test, Fishers p <0.05. The key for significant symbols are shown below the graphs.

Key	Significant conditions ($p < 0.05$)	Key	Significant conditions ($p < 0.05$)
*	Control vs *S. aureus* PCM	##	*S. aureus* PCM vs *S. aureus* BCM
**	Control vs *P. aeruginosa* PCM	###	*S. aureus* PCM vs *P. aeruginosa* BCM
***	Control vs *S. aureus* BCM	$	*P. aeruginosa* PCM vs *S. aureus* BCM
****	Control vs *P. aeruginosa* BCM	$$	*P. aeruginosa* PCM vs *P. aeruginosa* BCM
#	*S. aureus* PCM vs *P. aeruginosa* PCM	@	*S. aureus* BCM vs *P. aeruginosa* BCM

Figure 5.16 Chronic fibroblast migration in pH titrated PCM and BCM

Chronic fibroblast migration was measured over 48 hours in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM. Migration in pH 6 are shown in A, pH 7.5 in B and pH 9 in C. Statistical differences between the mean values of was determined by ANOVA, followed by a posteriori test, Fishers *p* <0.05. The key for significant symbols are shown below the graphs.
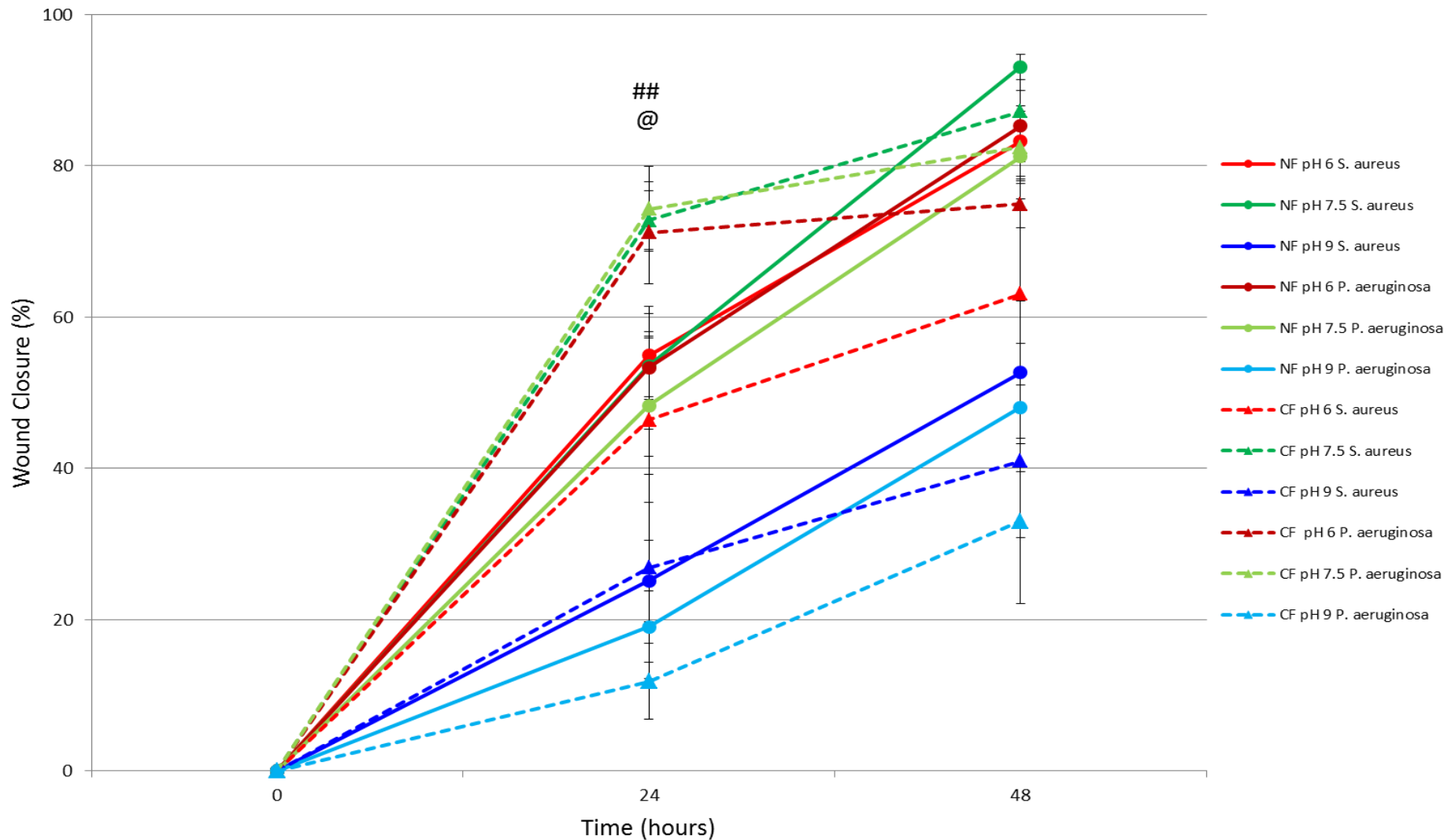
Figure 5.17 Normal and Chronic Fibroblast migration in Planktonic Conditioned Media

Graph shows both NF and CF migration in pH titrated (pH 6, pH 7.5, pH 9) *S. aureus* and *P. aeruginosa* PCM over 48 hours. Statistical differences between the mean values of was determined by ANOVA, followed by a posteriori test, Fishers *p* <0.05. ## significant difference between NF pH7.5 *P. aeruginosa* PCM and CF pH 7.5 *P. aeruginosa* PCM, @ significant difference between CF pH 6 *S. aureus* and CF pH6 *P. aeruginosa* PCM.
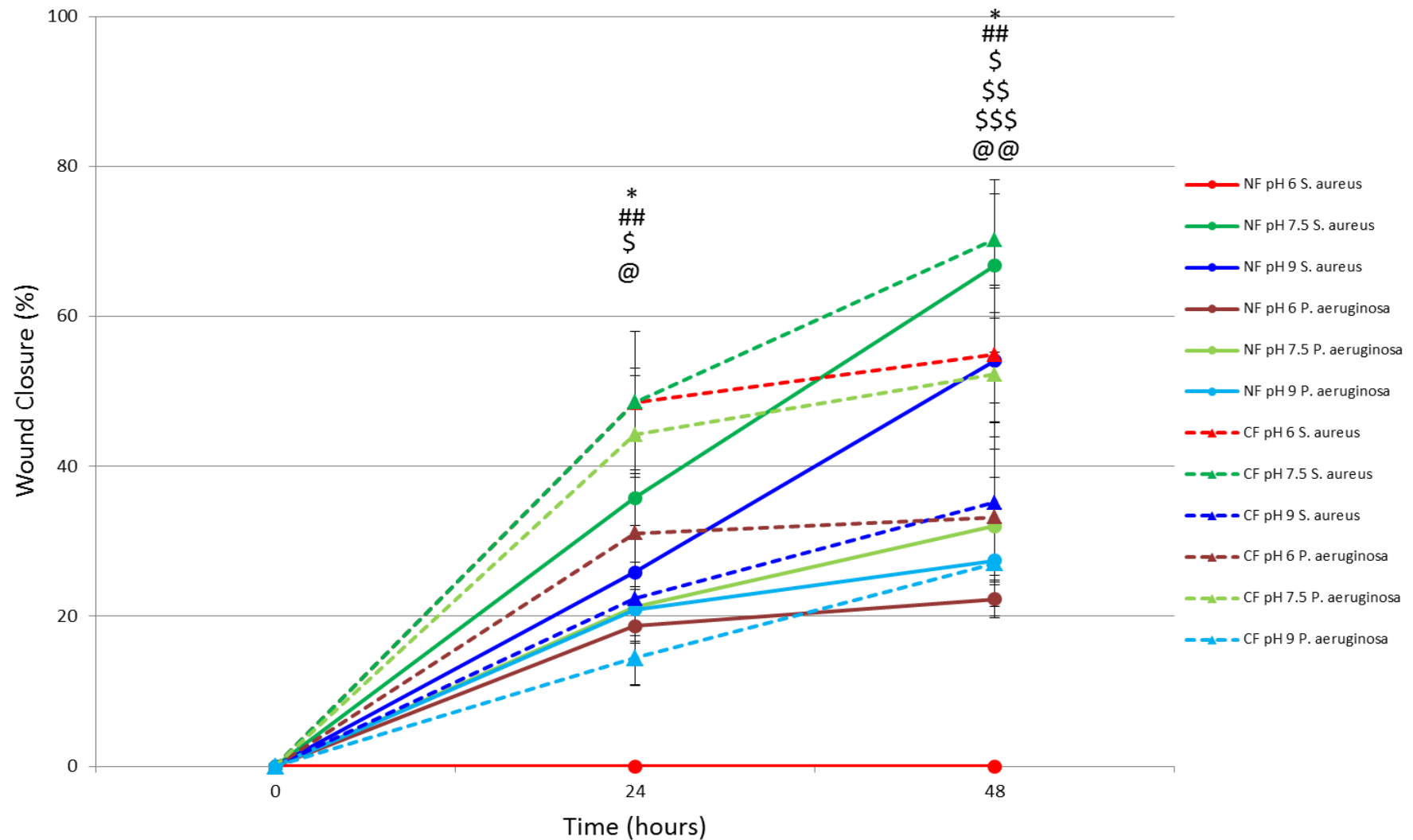
Figure 5.18 Normal and Chronic Fibroblast migration in pH titrated Biofilm Conditioned Media

Graph shows both NF and CF migration in pH titrated (pH 6, pH 7.5, pH 9) *S. aureus* and *P. aeruginosa* PCM over 48 hours. Statistical differences between the mean values of was determined by ANOVA, followed by a posteriori test, Fishers, *p* <0.05. * significant difference between NF and CF pH 6 *S. aureus* BCM, ## NF and CF pH 6 *P. aeruginosa*, $ NF pH6 *S. aureus* and *P. aeruginosa*, $$ NF pH 7.5 *S. aureus* and *P. aeruginosa*, $$$ NF pH 9 *S. aureus* and *P. aeruginosa*, @@ CF pH 7.5 *S. aureus* and *P. aeruginosa*.

5.6 Discussion

An alkaline pH is observed in chronic wounds (Schneider et al. 2007) along with the presence of bacterial biofilms (James et al. 2007). Biofilms are considered by many to be the leading factor in delayed wound healing; while research into the effect of pH on wound healing is poor. In recent years biofilms have been the main focus of wound research with other factors generally being ignored. The role that pH has to play in wound healing is slowly coming back into the forefront of research, with scientists questioning its role and wound care specialists beginning to measure wound pH again in the clinics such that more can be established on its effect within a chronic wound. This study combined both pH and biofilms and looked at their effect on wound healing, measuring factors such as ECM abundance and cell migration.

A few studies have investigated the effect of bacteria on skin cells, but historically these only inoculated cell cultures with planktonic bacteria. For example, *S. aureus* (Mempel et al. 2002), *Salmonella typhi* (Nuzzo et al. 2000) and *Streptococcus* (Bentley et al. 2005) invade and induce apoptosis of keratinocytes. Co-culture of keratinocytes with *Fusobacterium nucleatum* caused an increase in cell migration (Uitto et al. 2005). When investigating the effect of bacteria on skin and wound cells it is important to investigate the biofilm form and not planktonic due to the knowledge that wounds are predominantly contaminated with biofilms and not their planktonic counterpart (James et al. 2007). Bacteria living in biofilm communities are phenotypically distinct from their planktonic counterparts and are orders of magnitude more resistant to antibiotics, host immune response, and environmental stress. However, studies that investigated both planktonic and biofilm found variances in the effect of both on keratinocytes and fibroblasts (Kirker et al. 2009; Kirker et al. 2012). Therefore, it is important to include both bacterial forms when researching such that a correct interpretation of the importance of biofilms in chronic wounds can be determined.

S. aureus, a Gram positive bacterium, has one of the highest incidence rates in traumatic, surgical, burn and other infections (Bowler et al. 2001). *P. aeruginosa* is an aerobic Gram negative bacterium that is often a cause of hospital-acquired infections (Driscoll et al. 2007). With the use of molecular techniques, it was demonstrated that the most prevalent species found in wounds include *Staphylococcus*, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Finegoldia*, and *Serratia spp* (Dowd et al. 2008). Surveillance of *S. aureus* and *P. aeruginosa* infections reveals increasing antimicrobial resistance to these pathogens (Driscoll et al. 2007). It is therefore, clinically relevant to investigate the effect of *S. aureus* and *P. aeruginosa* biofilms on cells involved in wound healing.

5.6.1 Extracellular Matrix abundance

It was previously demonstrated that lower amounts of collagen, fibronectin and tropoelastin were measured from CF CM, but a higher amount of GAGs was present compared to NF CM (Chapter 3.3). The effect of pH alone on ECM abundance was minimum with an increase in total collagen amount from NFs at pH 9 and an increase in GAGs from CFs. A few differences in fibronectin abundance were seen when comparing the results in the first study (Figure 3.4) and in this second study (Figure 5.5). In this second experiment total fibronectin from NF was significantly lower in pH 6 conditions compared to pH 7.5 and pH 9, in the previous study it was also lower in pH 6, but only significant compared to pH 7.5 conditions. In the previous study pH had no effect on the amount of fibronectin from CF, in this second experiment, fibronectin amounts was higher in pH 7.5 conditions and significant when compared to fibronectin measured in pH 6 conditions. These subtle differences in the effect of pH on fibronectin abundance could be due to the different passage number of the cells, the use of three different cell lines to what was used in the first study or also the difference in the ELISA kits used to measure fibronectin in the two different studies.

Results presented here show that PCM and BCM affect the abundance of ECM compared to control, with no differences observed between pH groups. No difference in the effect of PCM and BCM on collagen, fibronectin and GAG abundance was identified. For example at pH 7.5 PCM and BCM resulted in significantly lower amount of collagen compared to control,

but there was no further significant difference established between the effect of PCM and BCM (Figure 5.4). It would be expected that BCM would have more detrimental effects than PCM, however this was not observed. This could be due to the dilution of BCM; after initial studies where both NFs and CFs did not survive for longer than 24 hours, it was diluted 1 in 4 such that the experiments could be carried out for the hours required. Even though the protein composition of BCM is more complex than PCM (Figure 5.2), when diluted some of these proteins may become too dilute to cause an effect on ECM synthesis by the cells.

As previously established CFs secrete significantly more GAGs in pH 9 compared to pH 6 and pH 7.5 (Chapter 3.3.4). PCM at pH 9 significantly reduced the increase compared with pH 9 control. An increase in GAGs (specifically HA) is proposed to be a factor in foetal wound healing. In contrast to adult wounds early age foetuses heal without scar formation (Bullard et al. 2003). Conversely, an increase in pH is correlated with non-healing wounds. *S. aureus* BCM resulted in similar GAG abundance as pH 9 alone, but with *P. aeruginosa* BCM was significantly decreased (Figure 5.6). Bacteria also secrete GAGs to enhance their ability to infect and colonise the host tissue (DeAngelis 2002). Thus, the GAG increase could be the result of biofilm growth. However, not all BCM show a significant increase in GAG secretion. GAGs are an important part of the cell microenvironment as they promote cell proliferation and migration (Roberts & Harding 1994). The increase in GAGs observed at pH 9 could be a factor of increased bacterial presence in chronic wounds. Many bacteria exploit cell surface GAGs as adhesion receptors in order to colonise the host tissue, for example *S. aureus*. The specific GAG found to bind to *S. aureus* was heparin sulphate (Liang et al. 1992), furthermore heparin stimulates *S. aureus* biofilm formation (Shanks et al. 2005).

5.6.2 Bacterial Proteases

Protein analysis of PCM and BCM found very different banding patterns; BCM clearly contained considerably more features (Figure 5.2). This was also the case in a previous study, with the presence of smeared bands indicating the presence of a bacterial protease (Secor et al. 2011). Further analysis by mass spectrometry found that PCM contained several

enzymes involved in glycolysis, while BCM had proteins related to translation. A few were unable to be identified and no proteases were identified (Secor et al. 2011). The balance between the synthesis and degradation of ECM is crucial for wound healing. Matrix metalloproteinases (MMPs) degrade the ECM therefore, tight regulation of MMPs is imperative for wound healing (Caley et al. 2015). Excessive levels of MMPs leads to destruction of ECM and a subsequent imbalance between synthesis and degradation. In addition to human MMPs, bacterial proteases have been found to be influential in tissue breakdown (McCarty et al. 2012). The proteases of the serine-, cysteine-, and metallo- type are produced by a wide range of pathogenic bacteria and as such, are particularly important in the role they play in wound healing (McCarty & Percival 2013). For example, elastase, produced by *P. aeruginosa*, induces degradation of fibroblast proteins and proteoglycans in chronic wounds and has also been shown to degrade host immune cell mediators (Schmidtchen et al. 2003).

Fibroblasts secrete monomers of tropoelastin, which are the fundamental building components of the elastic fibres. A complex process of tropoelastin cross-linking creates insoluble elastin fibres. These elastin fibres are resistant to all proteases, other than the elastases (Mecham et al. 1997). Pseudolysin is the most abundant protease secreted by *P. aeruginosa* and the major extracellular virulence factor of this opportunistic pathogen. It is a well-known bacterial elastase with specific activity towards elastin; it destroys human tissues by solubilizing elastin (Yang et al. 2015). It is suggested that BCM contains bacterial proteases; further analysis is required to confirm this and to establish which proteases may be present. In this study BCM had a significant effect on tropoelastin abundance from CFs compared to PCM. Following culture in *S. aureus* and *P. aeruginosa* BCM no tropoelastin was detected compared with control (Figure 5.7). Further analysis should confirm the presence of the bacterial protease pseudolysin as a possible candidate for the lack of tropoelastin detected.

5.6.3 Extracellular Matrix expression

There is a correlation between down regulation of collagen and fibronectin gene expression and decreased in protein levels from CFs compared to NFs. However, no significant effects were determined on the effect of pH and BCM on the ECM molecules analysed as results were variable. GAGs abundance was increased from CFs, similarly DCN and LUM gene expression was upregulated in CFs, but VCAN was not. DCN and LUM expression were up-regulated from CFs in pH 9 but did not reach significance when compared to pH 6 and pH 7.5 (Figure 5.11 and Figure 5.13), whereas GAG abundance was significantly increased in pH 9 compared to pH 6 and pH 7.5 conditions (Figure 5.6). The increase in GAG abundance observed with culture in pH 9 *S. aureus* BCM was not backed-up with the gene expression data, with both DCN and LUM expression significantly down-regulated compared to control. However, not all GAGs were analysed, therefore the increase in GAGs could be another candidate such as HA, aggrecan or biglycan. No clear pattern was determined to demonstrate the effect of PCM and BCM on gene expression. A significant difference between control and PCM or BCM was not seen in all pH conditions and no further differences detected between the effect of PCM and BCM.

Some differences were observed between the ECM expression in this study and the results from the previous study in chapter 3 (Figure 3.7). In this study the expression of COL1A1 (Figure 5.8) was down regulated in pH 7.5 conditions and upregulated in pH 6 and pH 9 conditions, whereas in the previous study the opposite was observed (Figure 3.7A). In the previous study COL1A2 expression (Figure 3.7B) from NF was upregulated in pH 7.5 conditions compared to pH 6 and pH 9, however in this second study COL1A2 expression was highest in acidic conditions and reduced as the pH increased (Figure 5.9). In the previous study, at pH 9 FN and DCN expression (Figure 3.7 C and D) was significantly upregulated in NF compared to CF, in the second experiment the opposite is observed with increased expression in CF, however this change is significant for DCN (Figure 5.11) but not significant for FN (Figure 5.10). These changes could be due to different passage number of the cells utilised, cells may exhibit different transcription and translation properties at early and late passage. Primary cultured cells derived from tissue have a limited lifespan due to replicative

senescence and show distinct phenotypes such as irreversible cell cycle arrest and enlarged morphology (Kong et al. 2013), this could affect results of experiments when using late passage cells. In this study results for the gene expression following culture in the various conditions was very variable and no pattern could be identified to explain the data. This could be due to low sample number; with an increase in sample number the effect of each condition on ECM expression could become more definite. 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 *in vivo* and *in vitro* environments.

No correlation between protein abundance and expression data was observed, which was also the case in Chapter 3. Data did not correlate for collagen, fibronectin or glycosaminoglycans; various results were observed for both protein levels and mRNA expression from NF and CF in each culture condition. For example in pH9 control, collagen abundance was significantly more from NF than CF, but the COL1A1 transcript is upregulated in CF and lower in NF and then no change is observed for the COL1A2 transcript. However, in such studies correlations are not always observed. The biochemical diversity of proteins means that the individual correlation levels with the associated mRNA are going to vary a lot (Vogel & Marcotte 2012). Gene expression is controlled at many different levels including transcription, post-transcription (RNA processing) and translation such that mRNA and protein abundance may not always correlate due to these regulation controls at each level (Vogel & Marcotte 2012). The half-life of different proteins can vary from minutes to days - whereas the degradation rate of mRNA would fall within a much tighter range (Vogel & Marcotte 2012); therefore the 24 hour incubation period of cells in different pH in this experiment could mean that any effect that did occur may have been lost by the end of the experiment. A shorter time frame for experiments could cause a different outcome in gene expression data.

5.6.4 Cell Migration

Cell migration is a major step in successful wound healing and reepithelialisation. Cell migration is commonly studied with *in vivo* scratch assays and the scratched area measured before and after migration as the outcome parameter. It is a simple wound model system, lacking extracellular matrix and inflammatory components. However, it is used extensively because it allows for the direct investigation of external agents on the cells involved in wound healing.

Results show that pH, bacteria, PCM or BCM, time and cell type all had a significant effect on wound closure as assessed by the *in vitro* scratch model. BCM was found to have a significantly detrimental effect on wound healing compared to control and PCM groups ($p \leq$ 0.000). This does not corroborate the results of other studies which found that both PCM and BCM significantly affected cell migration. HK exposed to BCM and PCM all exhibited reduced scratch closure after 24 hours (Kirker et al. 2009). HF exposed to similar BCM and PCM also showed significantly reduced scratch closure compared to control, but with BCM and PCM statistically equivalent at all time points (Kirker et al. 2012). The research in this thesis however does not show a significant effect of *S. aureus* and *P. aeruginosa* PCM on wound closure.

The previous studies carried out on the effect of PCM and BCM did not investigate alterations in pH. Studies by Kirker et al measured both PCM and BCM then simply titrated them back to ~pH 7. In the initial study the pH of PCM was 6.81 and BCM was pH 7.13 (Kirker et al. 2009), in another study pH of PCM and BCM measured as 7.36 and 8.26 respectively (Kirker et al. 2012). These studies did not take into account the change in pH that occurs with growing biofilms. I measured each conditioned media collected and noted a significant change in the pH between PCM and BCM (Table 5.1). BCM pH was considerably elevated with values up to 9.47 compared with 8.15 for PCM. More bacteria are present within a biofilm which could account for the increased pH. However, in the biofilm state bacteria are phenotypically different and secrete different products which may cause the change in pH

rather than the increased number of bacterial cells. This increase in pH of BCM provides initial evidence that the presence of biofilms interestingly increase the pH of their surrounding environment. Such that simply the presence of a biofilm within a chronic wound elevates the wound pH to harmful alkaline levels, thus cells fail to secrete sufficient ECM and unsuccessfully migrate across the wound bed.

During the cell migration experiment, *S. aureus* BCM titrated to pH 6 caused the NFs to repeatedly lift off the cell culture dish, such that no wound closure values could be collected and therefore is shown on the graphs as zero. It was not due to the BCM itself because at pH 7.5 and pH 9 cells did not lift off and it was not due to the pH value as all other conditions titrated to pH 6 did not have the same effect. When looking at ECM abundance, cells did not detach from the dish in this condition. Therefore, it is possible that simply creating the scratch with a pipette tip along with this conditioned media initiated the detachment of NFs. Protein analysis of each PCM and BCM following pH titration did reveal a strong band, around ~240 kDa from *S. aureus* BCM at pH 6 (shown by the blue box in Figure 5.3), which was not observed in the other conditions. Phevalin, a pyrazinone with a molecular weight of 228, is produced in greater quantities by *S. aureus* biofilms relative to their planktonic counterparts (Secor et al. 2012). A study showed that phevalin present in BCM has marked effects on HK gene expression, and was consistent with the regulation of MAPK (mitogen activated protein kinase) pathways. The unstable activation of MAPK signalling cascades would likely effect several biological processes relating to wound healing including apoptosis, proliferation and cell migration (Secor et al. 2012). Further studies could investigate if adjusting the pH of conditioned media increases the potency of soluble products released by *S. aureus* biofilms.

It is clear that biofilms, and their soluble products affect wound healing, specifically cell migration. The collective migration of fibroblasts and the organisation of the actin cytoskeleton is a hallmark of tissue remodelling events that underlie wound repair. The oral cavity, like the skin is often wounded and contains a diverse microflora that could affect the reepithelialisation of the epidermal barrier (Bhattacharya et al. 2014). Studies have explored

the effect of *Porphyromonas gingivalis*, a common oral bacterium, and found that it inhibits cell migration (Laheij et al. 2013; Bhattacharya et al. 2014). Lipopolysaccharide (LPS), is a suggested candidate of Gram negative bacteria responsible for the inhibition of cell migration due to its known effects on inflammatory cytokines. Bacteria can shed components of its cell wall and therefore can be found in bacterial conditioned media.

Bacteria challenged cells showed that cell cycle genes cyclin1, CDK1 and CDK4 were down regulated as well as integrin beta-3 and -6, associated with cell migration were also down regulated (Bhattacharya et al. 2014). Upon wounding beta-3 integrin is reported to support actin cytoskeletal reorganisation and provide directionality to migration during healing (Danen et al. 2005). It would be interesting to see if these genes associated with cell migration are also downregulated in the culture conditions used in this experiment and therefore further explain the effect that biofilm impede on wound healing cells.

5.7 Conclusion

pH along with the secreted products of planktonic and biofilm *S. aureus* and *P. aeruginosa* show differential effects on wound related parameters. It is still not clear whether the alkaline pH in a wound is due to the presence of bacterial biofilms or if the change in pH facilitates the initial attachment of a biofilm. Simply the presence of a biofilm significantly increased the pH of the culture medium. However, results showed that pH had no effect on ECM abundance and that BCM alone was harmful with no further changes due to pH. Cell migration however, was notably affected by BCM and alkaline pH, with significant inhibition of cells compared to other conditions. To my knowledge this is the first study which has combined pH with the effects of bacterial biofilms on wound healing. When researching the effects of wound healing it is important to combine the different parameters known to change, such that *in vitro* experiments can better stimulate the *in vivo* situation.

The detrimental effect within the host is relatively long term with a short incubation time with bacteria. Studies directed at understanding the effect of pathogens suggest that bacteria invade cells, reside within cellular compartments and impair restoration of normal tissue by interfering with cellular migration and proliferation. After removal, the biofilm can still have an effect upon the wound due to the internalisation of bacterial products. Thus, early treatment of wounds may be particularly important in patients with diminished wound healing responses. Simply making sure that the wound remains at neutral pH and does not increase to unfavourable alkaline levels could be sufficient to manage the effect of the biofilm on the wound and prevent it from moving into a non-healing state.

Chapter 6:

Results IV: The Effect of pH and Bacteria on Human Skin Cells (Fibroblasts and Keratinocytes)

6 The Effect of pH and Bacteria on Human Skin Cells

6.1 Introduction

Many cell types are involved in the process of wound healing including platelets, neutrophils and macrophages, however most important are the fibroblasts and keratinocytes. Both have a crucial role in ensuring that tissue integrity is restored. Fibroblasts are the main cell in the dermis and are key during the proliferative phase, responsible for initiating angiogenesis and for the formation and organisation of the newly synthesised ECM (Bainbridge 2013). Keratinocytes are the major cell type of the epidermis, which form an acid mantle on the surface of the skin, which functions as a barrier against the external environment. Keratinocytes are responsible for re-epithelialisation by migrating along the open wound to re-establish and restore the skin barrier (Pastar et al. 2014). Calcium regulates and induces differentiation of keratinocytes *in vitro* and *in vivo* in a similar manner (Deyrieux & Wilson 2007). It has been shown that an acidic extracellular pH induces a sharp increase in intracellular Ca^{2+} of HaCaT cells (Park et al. 2008), this effect of acidic pH could have physiological implications with regard to keratinocyte proliferation and differentiation. Therefore regulation of skin and wound pH is essential to maintain function of cells needed for wound repair.

Chronic wounds are characterised by prolonged inflammation, an altered wound matrix, and the failure to re-epithelialise. A shift towards an alkaline pH is observed and bacterial biofilms colonise non-healing chronic wounds. Therefore, the aim of this study was to investigate the effect of pH and planktonic and biofilm conditioned media (PCM and BCM) on both human fibroblast (HDF) and keratinocyte (HaCaT) cells. The effect of pH titrated PCM and BCM on migration of HDF and HaCaT cells was investigated over 48 hours. Furthermore, changes in extracellular matrix abundance and expression were assessed.

6.2   Human Dermal Fibroblasts and Epithelial Keratinocytes

Human dermal fibroblasts and epithelial keratinocyte cell lines were utilised for the following experiments (n=1). The HaCaT cells are a spontaneously transformed immortal keratinocyte cell line representative of proliferative keratinocytes; however they do not exhibit the complete keratinocyte behaviour (Boukamp et al. 1988). Experiments were performed with three different passages of each cell and three technical repeats, such that standard error and statistical analysis could be performed. Figure 6.1 illustrates their differing morphology in culture; fibroblasts exhibit the typical bipolar morphology and the keratinocytes demonstrating a much more rounded morphology.
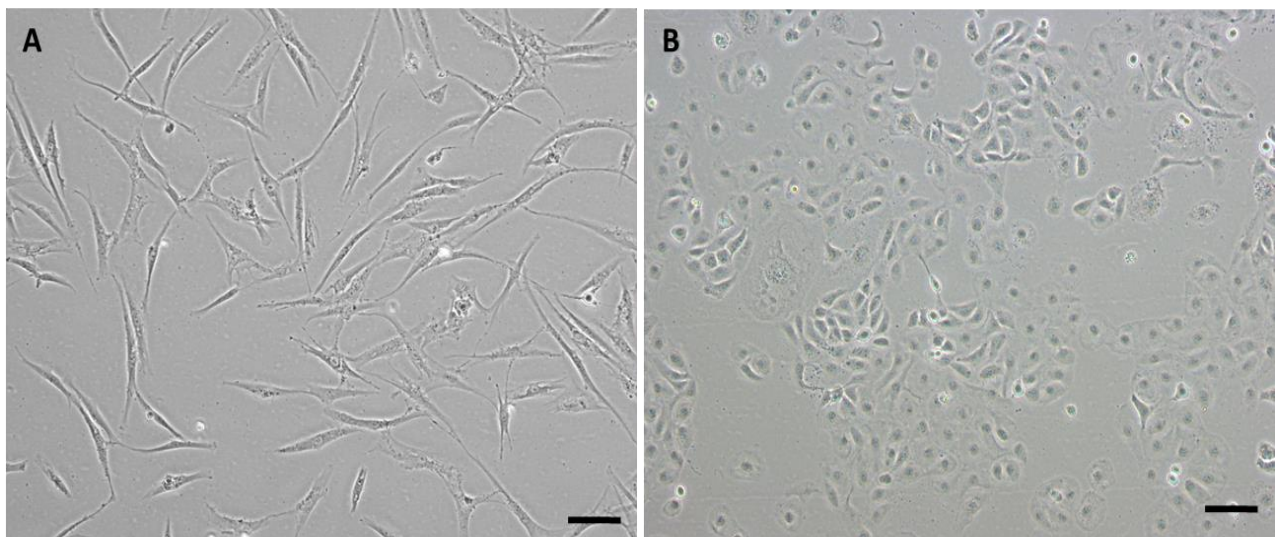

Figure 6.1 Human Dermal Fibroblasts and Keratinocytes in culture

Photomicrographs of A) Human Dermal Fibroblasts (HDF) with typical fibroblast-like bipolar morphology and B) Human Immortalised Keratinocytes cells (HaCaT) with a round cobblestoned morphology. Scale bar = 100 μm.

6.3   Effect of pH and Bacteria on Human Dermal Fibroblast Migration

The effect of pH, bacterial strain, PCM or BCM and time on wound closure of HDFs were analysed by a General Linear Model followed by post-hoc analysis to determine any significant effects. Results show that pH ($p \leq 0.000$), PCM or BCM ($p \leq 0.000$), and time ($p \leq 0.000$) had a significant effect on wound closure but bacterial strain ($p = 0.766$) had no effect.

HDF migration was significantly limited in pH 6 conditions, with only 31% ± 2.01 wound closure after 48 hours compared with 59% ± 5.84 in pH 9 and 96% ± 1.80 in pH 7.5 conditions ($p < 0.05$). There was also a significant difference between HDF migration in pH 7.5 and pH 9 conditions ($p < 0.05$) with optimal migration observed in pH 7.5 conditions (Figure 6.2 and Table 6.1).

PCM had varied results depending on its pH. At pH 6 PCM improved wound closure compared to pH 6 control, after 48 hours this was significant for both *S. aureus* and *P. aeruginosa* PCM compared to control ($p < 0.05$). Titrated to pH 7.5 *S. aureus* PCM significantly inhibited wound closure at 24 hours compared to pH 7.5 control ($p < 0.05$). But after 48 hours there was no difference, with final wound closure reaching 96% ± 2.08 in both pH 7.5 control and *S. aureus* PCM. However, *P. aeruginosa* PCM inhibited HDF migration after 48 hours, this was significant ($p < 0.05$) compared to both control and *S. aureus* PCM titrated to pH 7.5. Then at pH 9 there was no significant differences observed between control, *S. aureus* PCM or *P. aeruginosa* PCM at either time points.

For pH 6, there were no differences observed between control and both BCM, with both showing limited migration. There was a significant difference at 48 hours between *S. aureus* PCM and BCM and *P. aeruginosa* PCM and BCM ($p < 0.05$). At pH 7.5 both *S. aureus* and *P. aeruginosa* BCM significantly diminished HDF migration compared to control and PCM. After 24 hours there was no difference between *S. aureus* PCM and BCM, but after 48 hours, cell migration in BCM continued at a slower rate than PCM and thus was significantly lower compared to PCM after 48 hours. Finally, at pH 9 both *S. aureus* and *P. aeruginosa* BCM significantly inhibited wound closure compared to pH 9 control, *S. aureus* PCM and *P. aeruginosa* PCM ($p < 0.05$), this was significant at both time points.
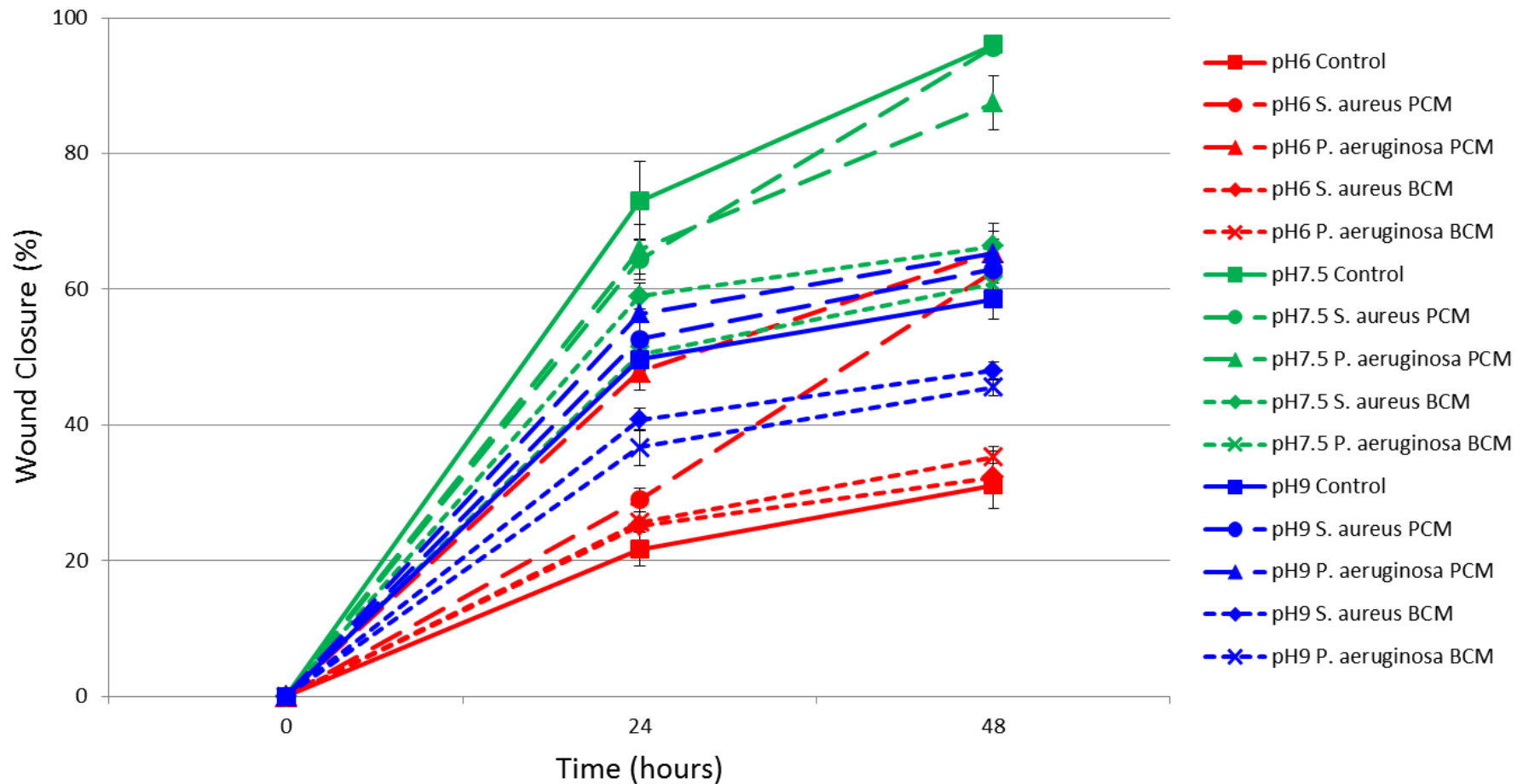
Figure 6.2 Human Dermal Fibroblast migration in pH titrated *S. aureus* and *P. aeruginosa* PCM or BCM

HDF migration was measured over 48 hours in pH titrated PCM and BCM. Results are shown as the mean wound closure at each time point with standard error of the means. Statistical groupings for all conditions are presented in Table 6.1.

Table 6.1 Statistical groupings for Human Dermal fibroblast migration

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different at each time point.

pH	Bacteria	PCM or BCM	Time	A	B	C	D	E	F	G	H	I	J	K	L	M
6	Control		24													M
			48											K	L	
6	*S. aureus*	PCM	24											K	L	M
			48				D	E								
6	*P. aeruginosa*	PCM	24								H	I				
			48			C	D									
6	*S. aureus*	BCM	24												L	M
			48											K	L	
6	*P. aeruginosa*	BCM	24												L	M
			48										J	K		
7.5	Control		24			C										
			48	A												
7.5	*S. aureus*	PCM	24				D									
			48	A												
7.5	*P. aeruginosa*	PCM	24			C	D									
			48		B											
7.5	*S. aureus*	BCM	24				D	E	F							
			48			C	D									
7.5	*P. aeruginosa*	BCM	24							G	H					
			48				D	E								
9	Control		24							G	H					
			48				D	E	F							
9	*S. aureus*	PCM	24						F	G	H					
			48				D	E								
9	*P. aeruginosa*	PCM	24					E	F	G						
			48			C	D									
9	*S. aureus*	BCM	24									I	J			
			48								H	I				
9	*P. aeruginosa*	BCM	24										J	K		
			48								H	I				

6.4 Effect of pH and Bacteria on Human Epithelial Keratinocytes

The effect of pH, bacterial strain, PCM or BCM and time on wound closure of HaCaTs were analysed by a General Linear Model followed by post-hoc analysis to determine any significant effects. Results show that all factors had a significant effect on HaCaT wound closure: pH ($p \leq 0.000$), bacterial strain ($p \leq 0.000$), PCM or BCM ($p \leq 0.000$), and time ($p \leq 0.000$).

Optimal HaCaT migration was observed in pH 7.5 control conditions, with migration in pH 6 and pH 9 significantly reduced ($p < 0.05$) after both 24 and 48 hours. A significant difference was also observed between pH 6 and 9 with significantly reduced migration at pH 6 (Figure 6.3 and Table 6.2).

At pH 6 all conditions, control, PCM and BCM demonstrated slower migration rates, with no significant differences observed at 24 hours. After 48 hours however, *P. aeruginosa* BCM resulted in significantly reduced migration compared to pH 6 control ($p < 0.05$).

When titrated to pH 7.5 *P. aeruginosa* PCM had no effect on HaCaT wound closure with results similar to pH 7.5 control. However, *S. aureus* PCM and both *S. aureus* and *P. aeruginosa* BCM significantly reduced cell migration compared to pH 7.5 control and *P. aeruginosa* PCM. After 48 hours both *S. aureus* PCM and BCM and *P. aeruginosa* PCM and BCM were significantly different ($p < 0.05$), with much slower HaCaT migration observed in BCM conditions.

After 24 hours, migration in both PCM and BCM titrated to pH 9 were significantly limited compared to pH 9 control ($p < 0.05$). However, after 48 hours, control and PCM showed no significant difference in final wound closure with similar rates of closure achieved. BCM remained significant with reduced migration compared to control. *S. aureus* BCM significantly inhibited HaCaT migration, and at pH 7.5 and pH 9 even increased the initial wound size. *P aeruginosa* BCM had similar effects with a maximum of 24 % wound closure after 24 hours in pH 7.5 and pH 9 and at pH 6 the initial wound area was increased.
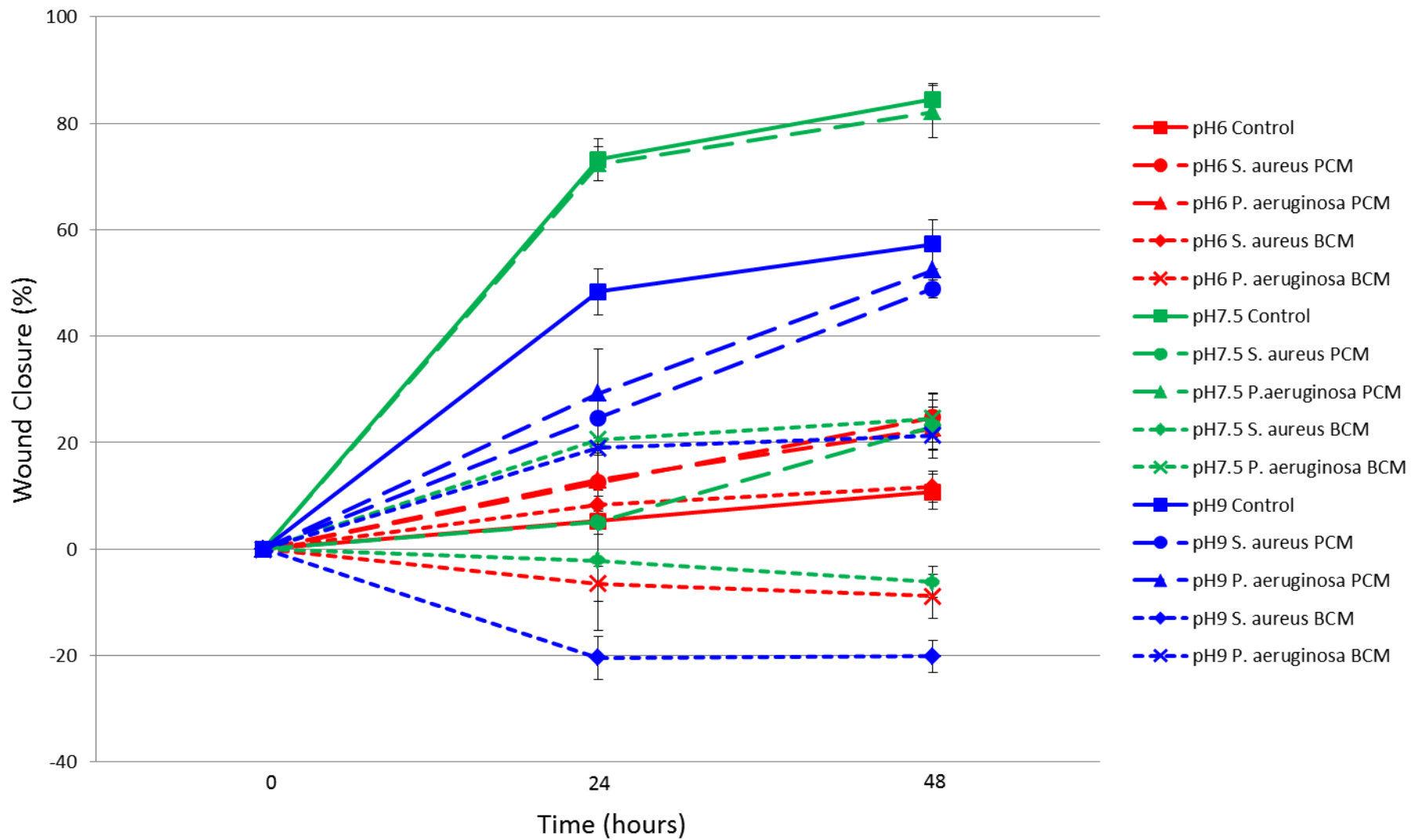
Figure 6.3 Human Epithelial Keratinocyte migration in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM

HDF migration was measured over 48 hours in pH titrated PCM and BCM. Results are shown as the mean wound closure at each time point with standard error of the means. Statistical groupings for all conditions are presented in Table 6.2.

Table 6.2 Statistical groupings for Human Epithelial Keratinocytes migration

Statistical differences were measured using ANOVAs and Fishers post-hoc with error rate of $p < 0.05$. Means that do not share a letter are significantly different at each time point.

pH	Bacteria	PCM or BCM	Time	A	B	C	D	E	F	G	H	I	J	K
6	Control		24								H	I		
			48						F	G	H			
6	*S. aureus*	PCM	24					E	F	G	H			
			48				D	E	F					
6	*P. aeruginosa*	PCM	24				D	E	F	G	H			
			48				D	E	F	G				
6	*S. aureus*	BCM	24							G	H	I		
			48					E	F	G	H			
6	*P. aeruginosa*	BCM	24									I	J	
			48										J	K
7.5	Control		24		B									
			48	A										
7.5	*S. aureus*	PCM	24								H	I		
			48				D	E	F					
7.5	*P. aeruginosa*	PCM	24	A	B									
			48	A	B									
7.5	*S. aureus*	BCM	24								H	I	J	
			48									I	J	
7.5	*P. aeruginosa*	BCM	24				D	E	F	G				
			48				D	E	F					
9	Control		24			C								
			48			C								
9	*S. aureus*	PCM	24				D	E	F					
			48			C								
9	*P. aeruginosa*	PCM	24				D							
			48			C								
9	*S. aureus*	BCM	24											K
			48										J	K
9	*P. aeruginosa*	BCM	24				D	E	F					
			48				D	E						

6.5 Extracellular Matrix abundance following culture of Human Dermal Fibroblasts in pH titrated PCM and BCM

Human Dermal Fibroblasts (HDF) were cultured for 24 hours in pH titrated (pH 6, 7.5 and 9) *S. aureus* and *P. aeruginosa* PCM or BCM. Media was collected and analysed for presence of collagen, fibronectin and glycosaminoglycans (GAGs) see methods 2.1.6.

6.5.1 Collagen abundance

Culturing HDFs in pH titrated *S. aureus* and *P. aeruginosa* PCM or BCM had no major effects on the abundance of collagen. No significant difference in the total amount of collagen was observed when HDFs were cultured in pH 6, 7.5 or 9 (Figure 6.4). A significant decrease in collagen abundance was observed following culture in both *S. aureus* ($p < 0.05$) and *P. aeruginosa* ($p < 0.01$) BCM compared to pH 9 control.

6.5.2 Fibronectin synthesis

At pH 6 no significant differences on the abundance of fibronectin were observed between PCM or BCM conditions compared to control (Figure 6.5). *S. aureus* and *P. aeruginosa* PCM and BCM titrated to pH 7.5 caused a significant decrease in fibronectin compared to pH 7.5 control after 24 hours in culture ($p < 0.01$). At pH 9, *S. aureus* PCM significantly reduced the amount of fibronectin compared to control ($p < 0.05$; Figure 6.5). Both BCM significantly reduced fibronectin abundance from HDFs, *S. aureus* BCM was significant at $p < 0.01$ and *P. aeruginosa* BCM at $p < 0.05$. pH affected fibronectin abundance with optimal amounts measured at pH 7.5 and minimum amounts measured after culture in pH 6. Significant differences were observed between pH 6 and pH 7.5 ($p < 0.01$), pH 6 and pH 9 ($p < 0.05$) and pH 7.5 and pH 9 ($p < 0.01$; Figure 6.5).

6.5.3 Glycosaminoglycans abundance

GAG abundance was significantly decreased in pH 9 culture compared with abundance in pH 6 and pH 7.5 culture conditions ($p < 0.01$) (Figure 6.6). In pH 6, both PCM and BCM conditions had a significant reduction in the amount of GAG compared to pH 6 control (*S. aureus* PCM and BCM at $p < 0.01$ and *P. aeruginosa* PCM and BCM at $p < 0.05$). The same was observed in conditions titrated to pH 7.5, with all PCM and BCM significantly reducing the total amount compared to control ($p < 0.01$). No significant differences were observed on GAG abundance following culture in conditions titrated to pH 9 (Figure 6.6).
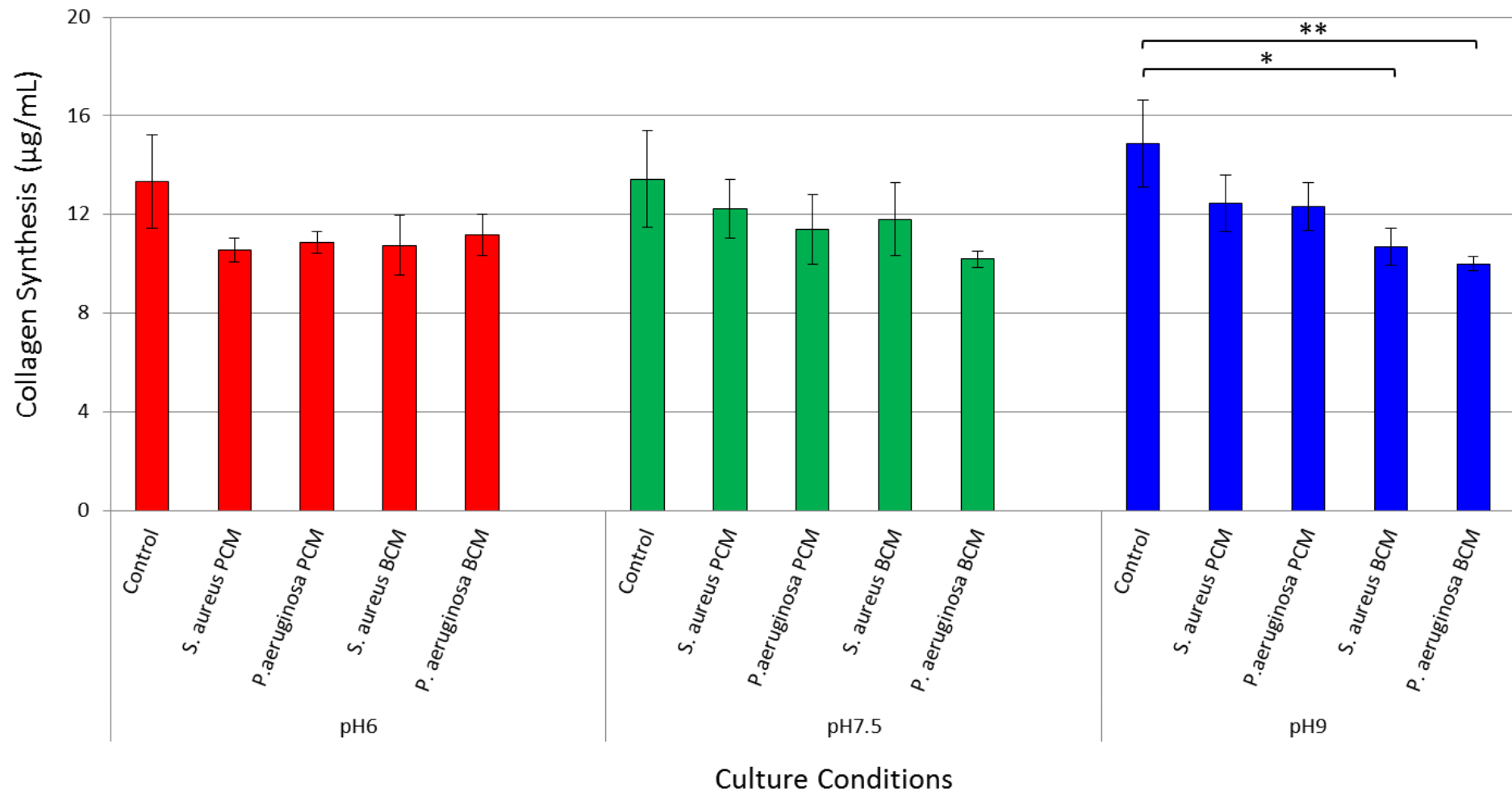
Figure 6.4 Collagen abundance from Human Dermal Fibroblasts

HDFs were cultured for 24 hours in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM, media was collected and the amount of collagen

measured. Values represent the mean ± SEM. Statistical differences between the mean values of each group were determined by ANOVA, followed

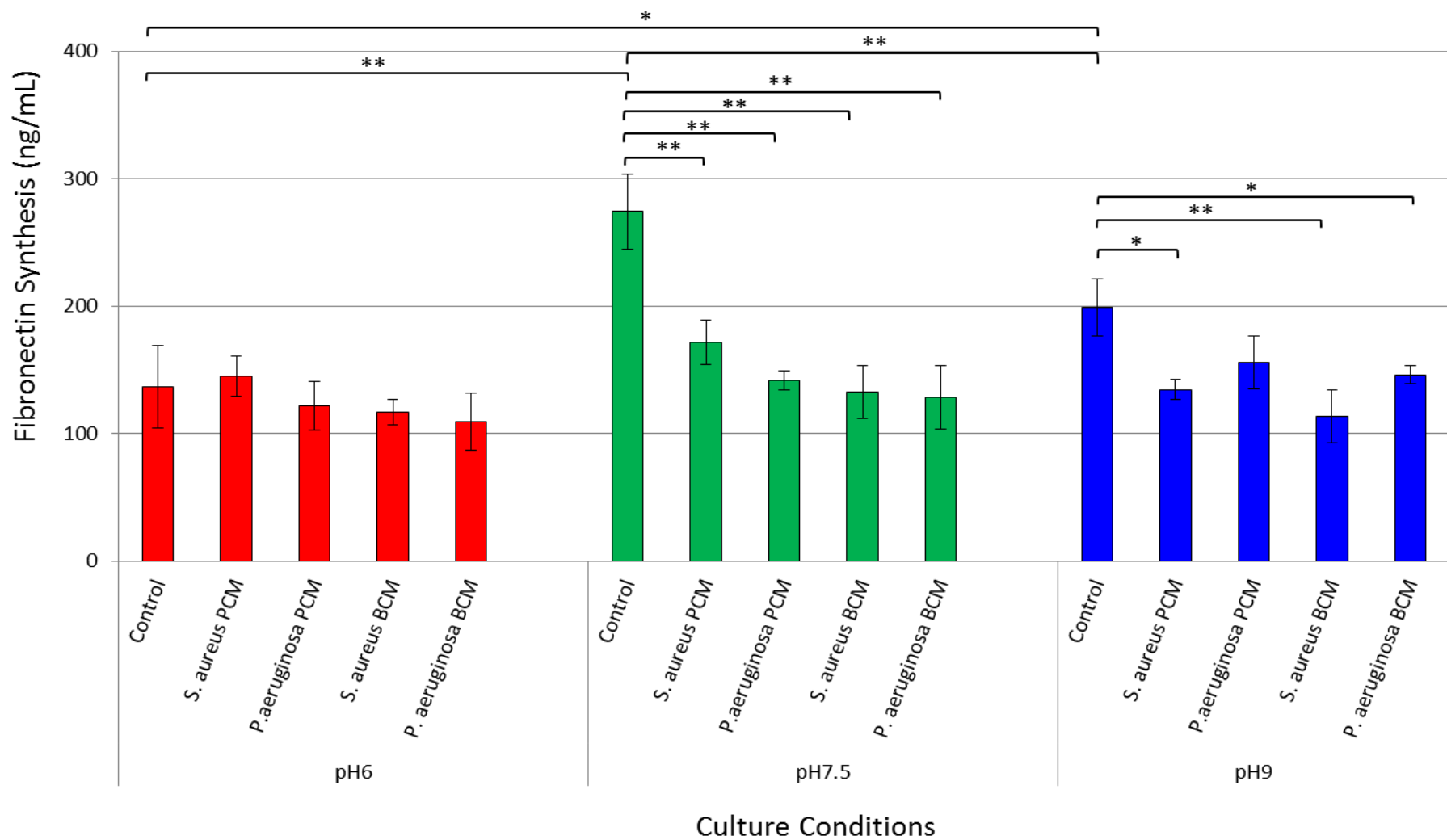by a posteriori test, Fishers * *p* <0.05, ** *p*<0.01.

Figure 6.5 Fibronectin abundance from Human Dermal Fibroblasts

HDFs were cultured for 24 hours in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM, media was collected and the amount of fibronectin determined. Values represent the mean ± SEM. Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * $p <0.05$, ** $p<0.01$.

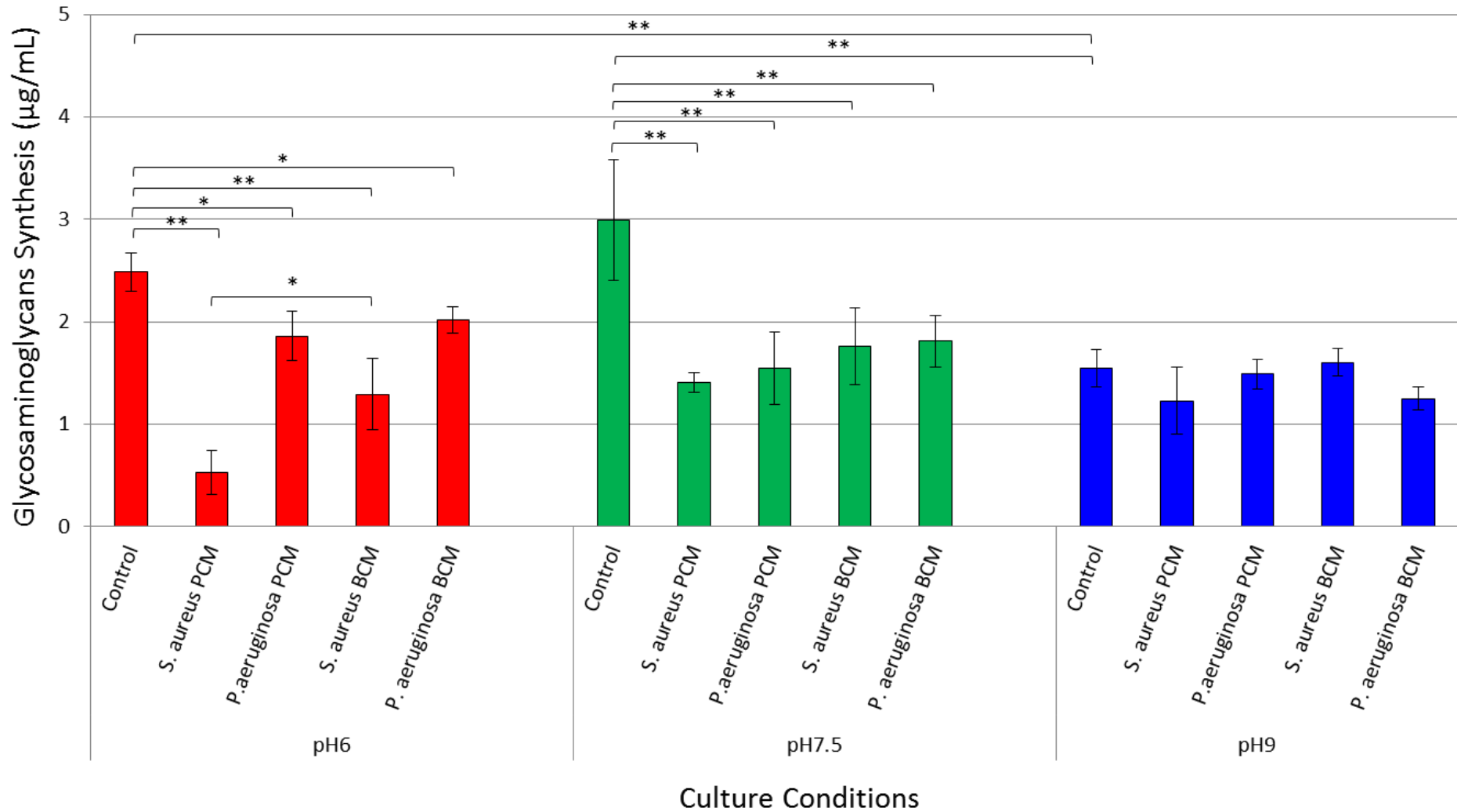**Figure 6.6 Glycosaminoglycan abundance from Human Dermal Fibroblasts**

HDFs were cultured for 24 hours in pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM, media was collected and the amount of GAGs measured. Values represent the mean ± SEM. Statistical differences between the mean values of each group were determined by ANOVA, followed by a posteriori test, Fishers * *p* <0.05, ** *p*<0.01.

6.6 Effect of pH on Laminin Expression in Epithelial Keratinocytes

HaCaT cells were cultured for 24 hours in pH 6, 7.5 and 9 media and *S. aureus* and *P. aeruginosa* PCM and BCM titrated to pH 7.5. Cells were collected in SDS lysis buffer and protein extracted. 10 μg of protein was ran on an SDS-PAGE and transferred to a nitrocellulose membrane, followed by incubation with laminin-332 (Aumailley et al. 2005) antibody to investigate any changes in expression (Chapter 2.1.14).

The laminin western blot should have three separate bands in each lane representing the α3, β3 and γ2 chains of laminin-332. Two bands are visible here at 165kDa and 105kDa, with the higher molecular weight α3 not visible. Western blot shows a higher expression of laminin in HaCaT cells in pH 6 conditions, with a darker band visible at 165kDa compared to HaCaTs cultured in pH 7.5 and pH 9 conditions. Following culture of HaCaTs with *S. aureus* and *P. aeruginosa* PCM (titrated to pH 7.5) no changes in expression was observed with similar band density to pH 7.5 control. However, following culture with *S. aureus* and *P. aeruginosa* BCM (titrated to pH 7.5) a darker more intense band was visible.
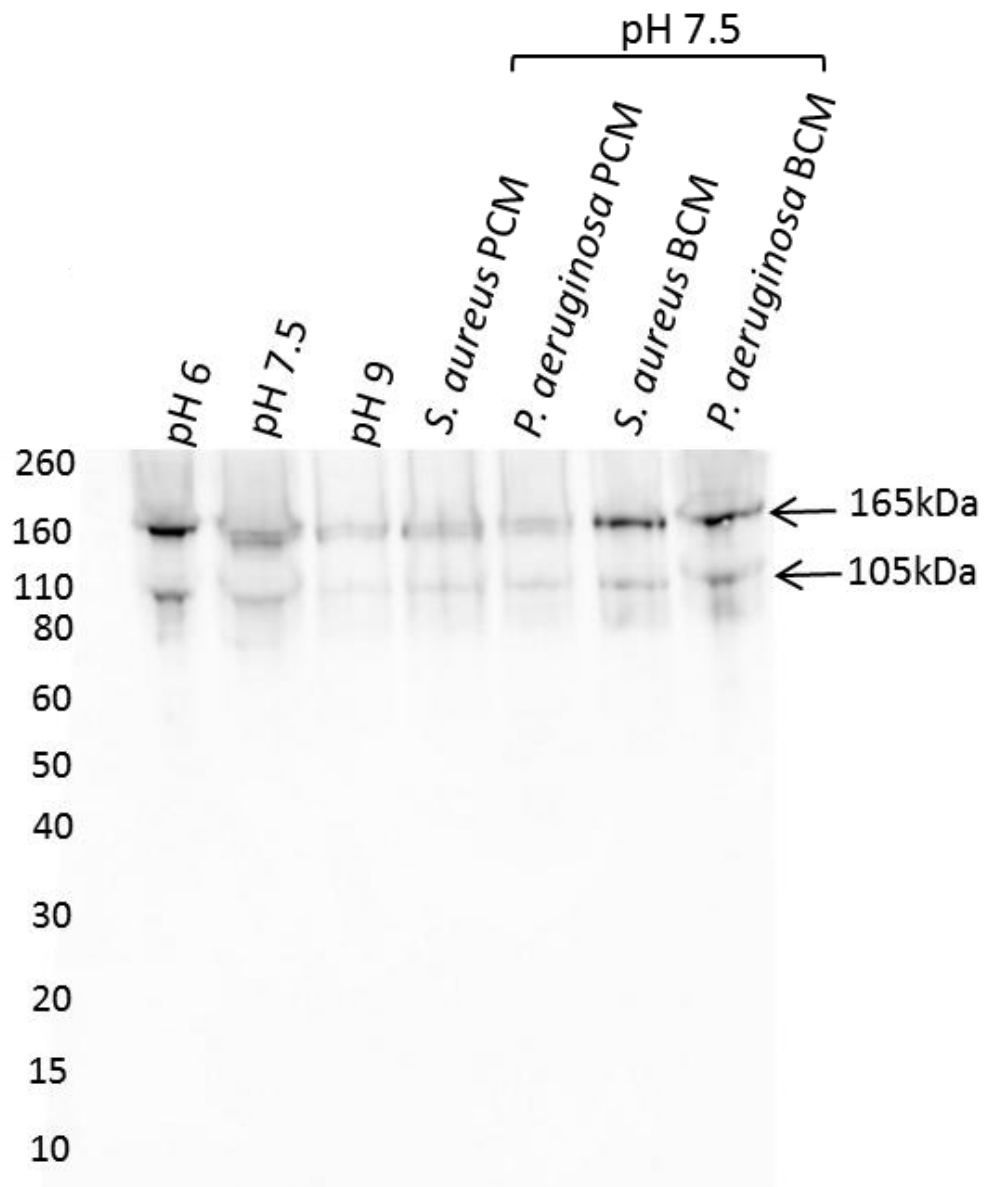
Figure 6.7 Western blot analysis of laminin-332 expression

Human epithelial keratinocytes (HaCaT) were cultured for 24 hours in different conditions and any changes in laminin expression was analysed by western blot. After transfer to nitrocellulose the blotted bands were immunodetected with a specific laminin-332 (1:1000) antibody and subsequently visualised with peroxidase labelled secondary antibody (1:1000). Two bands are visible here at 165kDa and 105kDa, representing the β3 and γ2 chains of laminin-332, with the higher molecular weight α3 not visible.

6.7 Discussion

The effect of pH titrated *S. aureus* and *P. aeruginosa* PCM and BCM on equine dermal fibroblasts, specifically ECM abundance and cell migration has previously been established (Chapter 5). The effect of biofilms on the migration of HDFs and human keratinocytes (HK) has previously been researched (Kirker et al. 2009; Kirker et al. 2012), but the changing pH in a chronic wound has generally been ignored. This study investigated any effects of both biofilms and pH on adult human dermal fibroblasts as well as epithelial keratinocytes.

Establishing cultures of fibroblasts from chronic wounds, although challenging, has been successful for venous, pressure and diabetic foot ulcers. Early studies showed that fibroblasts isolated from chronic wound biopsies differ from fibroblasts isolated from patients considered to be healthy. Fibroblasts cultured from venous ulcers display a different morphology (Stanley et al. 1997; Brem et al. 2008), decreased proliferation (Hehenberger et al. 1998; Loots et al. 1999), cellular characteristics of senescence (Vande Berg et al. 1998) and are unresponsive to TGF-β1 (Hasan et al. 1997). Mendez *et al* saw an increase in fibroblast senescence in venous ulcers when compared to fibroblasts from the same site on the opposite non-wounded leg, characterised by a decreased growth rate and an increase in senescence-associated beta-galactosidase expression (Mendez et al. 1998).

Foot ulcers in patients with diabetes mellitus is one of the most common types of chronic wounds, therefore fibroblasts derived from skin biopsies of diabetic patients are often used to compare with normal healthy fibroblasts and to investigate wound healing. Dermal fibroblasts from diabetic mice show characteristics of senescence, however, did not show increase in matrix metalloproteinases (Telgenhoff & Shroot 2005). Whereas, human diabetic dermal fibroblasts demonstrate an elevated production of MMP-2 and -3 when compared with their nondiabetic counterparts (Wall et al. 2003). Diabetic fibroblasts demonstrate markedly impaired migration, with a 75% reduction compared to normal fibroblasts, threefold greater activity of MMP-9 and significantly impaired vascular endothelial growth factor (VEGF) production (Lerman et al. 2003). Studies using fibroblasts from diabetic

patients offer an insight into why, wounds in these patients are slower to heal and often become chronic. In this study, only healthy HDF were used, but results still offer an insight into changes in fibroblasts with chronic wound related parameters (pH and biofilms). Interesting results were observed which warrants further investigation using diabetic fibroblasts and chronic wound isolated fibroblasts in order to gain more insight into changes in a non-healing wound environment.

6.7.1 Human Dermal Fibroblasts

pH, bacteria, PCM or BCM and time significantly affected the migration of equine fibroblasts, both normal fibroblasts derived from healthy skin and chronic fibroblasts isolated from chronic wound granulation tissue (Chapter 5.5). pH, PCM or BCM and time all had a significant effect on HDFs migration, while bacterial strain (both *S. aureus* and *P. aeruginosa*) did not have a significant effect on wound closure. However, only one biological HDF sample was used in experiments. Further repeats with more samples may establish a significant effect of bacterial strain on HDF migration. Currently, no documented research has looked at the effect of biofilms or pH on human chronic wound fibroblasts, therefore it would be interesting to see what effects would be found and how they would compare to the effects on HDFs and also compare with results from equine chronic wound fibroblasts.

The majority of studies investigate the effect of biofilms on cell proliferation, apoptosis and migration. Any effects on synthesis of key ECM molecules are ignored. Here, along with investigating cell migration, the effect of pH and biofilms on collagen, fibronectin and GAGs abundance from HDFs was considered. No significant effect on collagen abundance was concluded, with BCM only significantly decreasing total amount compared to control in pH 9 cultures. Total amounts of fibronectin demonstrated similar results as with equine normal fibroblasts (Chapter 5.3.2), with a decrease in abundance with both PCM and BCM at both pH 7.5 and pH 9. Normal equine fibroblasts showed no changes in total GAG measured with culture in different pH conditions (Chapter 3.3.4); HDFs however showed a significant

decrease in the amount of GAG in pH 9. Whereas equine chronic wound fibroblasts revealed a significant increase in GAG abundance in pH 9 (Figure 3.5).

Following trauma or pathological injury fibroblasts undergo metabolic activation; cultured fibroblasts from these sites demonstrate an increase in ECM molecule production (Buckingham et al. 1983; Abergel et al. 1985). Few studies have compared ECM differences from normal and chronic fibroblasts; one of the main findings has been the difference in GAG synthesis between the two cells. Wound derived fibroblasts synthesise significantly more GAG than normal dermal fibroblasts (Bronson et al. 1987). GAG abundance was only significantly increased from chronic wound fibroblasts compared to normal fibroblasts in pH 9 conditions (Chapter 3.3.4). Further HDF biological samples should be assessed to determine a true effect of alkaline pH on ECM abundance. The effect of pH on human chronic wound fibroblast should also be assessed to see if results correlate with the increase in GAGs seen by equine wound fibroblasts in alkaline conditions.

Various studies have investigated the effects of cytokines on dermal fibroblasts as they control connective tissue formation and remodelling in the repair of dermal connective tissues. Collagen and GAG synthesis was increased by IL-1, TNF-α or β (Duncan & Berman 1989) and IL-6 (Duncan & Berman 1991) stimulation. It may be that in pH 9 conditions, such as a chronic wound environment, cytokine production decreases resulting in decrease stimulation of fibroblasts and subsequently a decrease in ECM molecules such as collagen and fibronectin required for wound repair. Further studies should investigate the effect of pH on cytokine profiles from skin fibroblasts in order to clarify their downstream effects on the phases of wound healing.

6.7.2 Human Epithelial Keratinocytes

pH, bacteria, PCM or BCM and time significantly affected the migration of HaCaT in culture, with BCM even resulting in an increase in the original wounded area. This was also the case

in a previous study, where *S. aureus* biofilms, BCM and PCM increased the original wound size after 72 hours (Kirker et al. 2009). Kirker *et al* also showed that after 24 hours keratinocytes exposed to live biofilms or BCM formed dendrite like extensions and fragmented nuclei while PCM exposed keratinocytes did not, suggesting the morphological changes observed were unique following exposure to bacterial biofilms. Various studies have co-cultured keratinocytes with a range of planktonic bacterial strains which results in apoptosis (Mempel et al. 2002), changes in gene regulation (Mans et al. 2006) and increased metalloproteinases specifically collagenase 3 (Uitto et al. 2005). Contradictory to results here where bacteria decrease epithelial keratinocyte migration, other studies have identified an increased migratory effect of bacteria on epithelial cells. *Fusobacterium nucleatum* (Uitto et al. 2005), *Lactobacillus rhamnosus* and *reuteri* (Mohammedsaeed et al. 2015) increased keratinocyte migration. Similar to the skin, the oral mucosa is an important barrier and oral bacteria can potentially affect healing if this mucosal layer is disrupted. Two well characterised oral bacteria (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*) significantly reduce migration of gingival keratinocytes (Bhattacharya et al. 2014) comparable with effects of bacteria on epidermal keratinocytes.

Studies demonstrating apoptosis of keratinocytes as a result of bacteria demonstrate that the cells first internalise the bacteria before undergoing apoptosis (Nuzzo et al. 2000). This requires the bacteria to be in direct contact with the cells. The first contact of *S. aureus* to human skin is usually initiated through adhesion structures on the bacterial surface and their interaction with human epidermal cells (Mempel et al. 2002). Staphylococcal protein A and fibronectin binding protein are well described human keratinocyte attachment factors that result in fatal cell damage (Walev et al. 1993). However, results here and as demonstrated by Kirker *et al* shows that biofilms can have a significant effect on cell migration due to something that they secrete and prior internalisation is not necessary. Cell surface-expressed fibronectin-binding proteins on *S. aureus* inhibit keratinocyte migration (Kintarak et al. 2004), but there have been no reports of *S. aureus* secreting these proteins, however it could be possible that they were present in both PCM and BCM and contribute to the inhibition of keratinocyte migration. *S. aureus* does produce a variety of enzymes including coagulases, proteinases, lipases and nucleases as well as hazardous toxins including

haemolysins (Mempel et al. 2002); all of which could be present in *S. aureus* PCM or BCM and contribute to detrimental effects in chronic wound healing. Bacterial adherence is additionally enhanced by alkaline pH that is characteristic of chronic wounds along with other skin conditions with epidermal barrier dysfunction (Mempel et al. 1998). An alkaline environment may increase the production of harmful secreted products from biofilms or enhance their function within the chronic wound once secreted.

It is suggested that bacterial biofilms secrete bacterial proteases that are harmful within the wound environment. *P. aeruginosa* biofilms secrete proteases including protease IV, elastase B (LasB), elastase A (LasA) and alkaline protease (Hoge et al. 2010). LasA is a metalloproteinase that degrades elastin (Peters et al. 1992), LasB is another that is known to degrade elastin, collagen, fibrin, interferon γ and TNF-α (Heck et al. 1986; Caballero et al. 2001). These products could be present in BCM and be responsible for decreased matrix abundance and reduced migration observed in these studies. Further insight into the effects of biofilms and specifically bacterial proteases in chronic wound healing needs to be determined.

Laminins are a family of glycoprotein molecules, synthesised by keratinocytes, that provide an integral part of the structural scaffolding of the basement membrane found between the dermis and epidermis (Colognato & Yurchenco 2000). Each laminin is composed of α, β and γ chains which self-assemble. Laminins bind to other ECM molecules and have cell interactions mediated by integrins and other receptors; through these interactions laminins contribute to cell differentiation, cell growth, cell motility and apoptosis (Aumailley & Smyth 1998). In stable unwounded skin, keratinocytes within the basement membrane are resting on a laminin rich section. Numerous lines of evidence indicate that efficient migration of keratinocytes is dependent on endogenously deposited laminin-332 (Zhang & Kramer 1996; Decline & Rousselle 2001). While other studies state that laminin-332 acts as an adhesive factor which inhibits keratinocyte migration (O'Toole et al. 1997). Whereas it is other ECM molecules such as collagen and fibronectin that provide a matrix that promotes keratinocyte migration (O'Keefe et al. 1985). Therefore according to some evidence, in wounds laminin

may only be expressed when keratinocytes no longer need to migrate and is required as a matrix attachment factor for cell adhesion (Clark et al. 1982). In this study a strong expression of laminin-332 was observed in pH 6 conditions compared to pH 7.5 and pH 9, this would be expected as healthy skin has an acidic pH and keratinocytes would need more laminin for successful adhesion and attachment. However, following culture of HaCaTs in BCM a stronger expression of laminin-332 was observed which could correlate with the significantly reduced migration of HaCaTs in BCM. Further studies should investigate if biofilms cause an increase in laminins and thus inhibit keratinocyte migration causing failure of re-epithelialisation in wound healing.

6.8 Conclusion

Bacterial biofilms and changes in pH had similar effects on fibroblasts and keratinocytes, pH 6 reduced the migration of both cells while optimal migration was in pH 7.5. In addition biofilms significantly inhibited cell migration of both cell types. Other studies have however found differing results when investigating both cell types. For example at high concentrations *Peptostreptococcus spp* supernatants significantly inhibited both fibroblast and keratinocyte proliferation. However, at low concentration supernatants only significantly inhibited keratinocyte wound closure and not fibroblasts (Stephens et al. 2003). LPS derived from *P. aeruginosa* inhibited keratinocyte migration but not fibroblast migration (Loryman & Mansbridge 2008). Co-culturing fibroblasts and keratinocytes provided interesting results when exposed to *Haemophilus ducreyi* (a Gram negative bacterium). Co-cultures produced cytokine profiles comparable to those found in an *in vitro* skin system, but when cells were cultured separately completely different results were seen (Zaretzky & Kawula 1999). This observation suggests that to more accurately mimic a wound and to analyse the effects of pH and biofilms a co-culture system or a three-dimensional skin model should be used in the future.

Chapter 7:

Results V: Intracellular pH and the Expression of the Sodium-Hydrogen Exchanger

7   Intracellular pH and the Expression of the Sodium-Hydrogen exchanger

7.1   Introduction

Numerous studies evaluate the effect of skin surface pH in cutaneous health and disease, but information on the mechanisms involved in regulating intracellular pH (pHi) of the cells within the layers that make up the skin is lacking. The pHi of cells within the dermis and epidermis must be carefully regulated so that normal metabolic functions can be maintained (Busa & Nuccitelli 1984). The maintenance of pHi is of vital importance for each cell as most cellular functions are pH sensitive. The pHi is carefully regulated via the extrusion of hydrogen (H^+) ions from the intracellular compartment in exchange for external sodium (Na^+) across the cell membrane (Orlowski & Grinstein 1997). Intracellular pH regulation depends on different intracellular buffers and is maintained by proton transport across the cell membrane. In the majority of cells pHi is regulated by transport systems including the H^+-ATPase, Na^+/HCO_3^+ exchanger, Cl^-/HCO_3^- exchanger, H^+/lactate symporter and the Na^+/H^+ exchanger (Frelin et al. 1988).

The sodium–hydrogen exchangers (Na^+/H^+ exchangers also referred to as NHEs) are a family of membrane antiporters that regulate pHi by the extrusion of H^+ ions from the intracellular compartment of the cell in exchange for external Na^+. NHE is comprised of two domains: an N-terminal membrane domain that functions to transport ions and a C-terminal cytoplasmic domain that regulates the activity and mediates cytoskeletal interactions (Putney et al. 2002). To date, nine mammalian NHE isoforms (NHE 1–9) have been identified (Slepkov et al. 2007). NHE-1 has been located in most tissues and cells and is thought to maintain 'housekeeping' functions (Orlowski & Grinstein 1997). NHE-2, NHE-3 and NHE-4 have a more specific pattern of expression and are most commonly found in regions of the gastrointestinal tract and the kidneys (Orlowski et al. 1992). NHE-5 is expressed in brain, spleen, testis and skeletal muscle (Klanke et al. 1995) while the isoforms NHE6 − NHE9 are ubiquitously expressed and are present in intracellular compartments (Nakamura et al. 2005).

NHE-1 was the first isoform discovered and is the most thoroughly characterised and frequently investigated. NHE-1 is a crucial membrane transporter involved in the regulation of intracellular pH, cell volume, cytoskeletal organisation, heart disease and cancer (Slepkov & Fliegel 2002). In addition to its role in regulating homeostasis, NHE-1 can directly interact with other regulatory cellular signalling pathways including mitogen-activated protein kinases (MAPKs) and AKT/protein kinase B (Pedersen 2006). Hence, NHE-1 is a multifaceted regulator of cell migration, proliferation and cell death.

NHE-1 has a range of biological functions which ultimately play a role in the pathology of many diseases (Figure 7.1). Its major function is the regulation of pHi (Figure 7.1A), but it can also be stimulated by numerous hormones which activate protein kinases (Figure 7.1B). The activation of NHE-1 is associated with a variety of downstream events such as cell proliferation and differentiation. Inhibition or deficiency of the NHE-1 transporter in cells can cause a marked decrease in proliferation and impair differentiation pathways (Wang et al. 1997). In some cells NHE-1 is localised to the lamellipodia where it acts as an anchor for actin filaments via the binding of ezrin, radizin and moesin (ERM) proteins (Figure 7.1D). This allows it to control and regulate cytoskeletal organisation and cell migration; where inhibition of the NHE-1 results in inhibition of cell migration (Denker & Barber 2002). Many studies have investigated the role of NHE-1 in myocardial infarctions, within the myocardium activation of NHE-1 causes an accumulation of intracellular sodium. This, in turn leads to a detrimental increase in intracellular calcium causing cell death (Figure 7.1C) (Fliegel 2005).
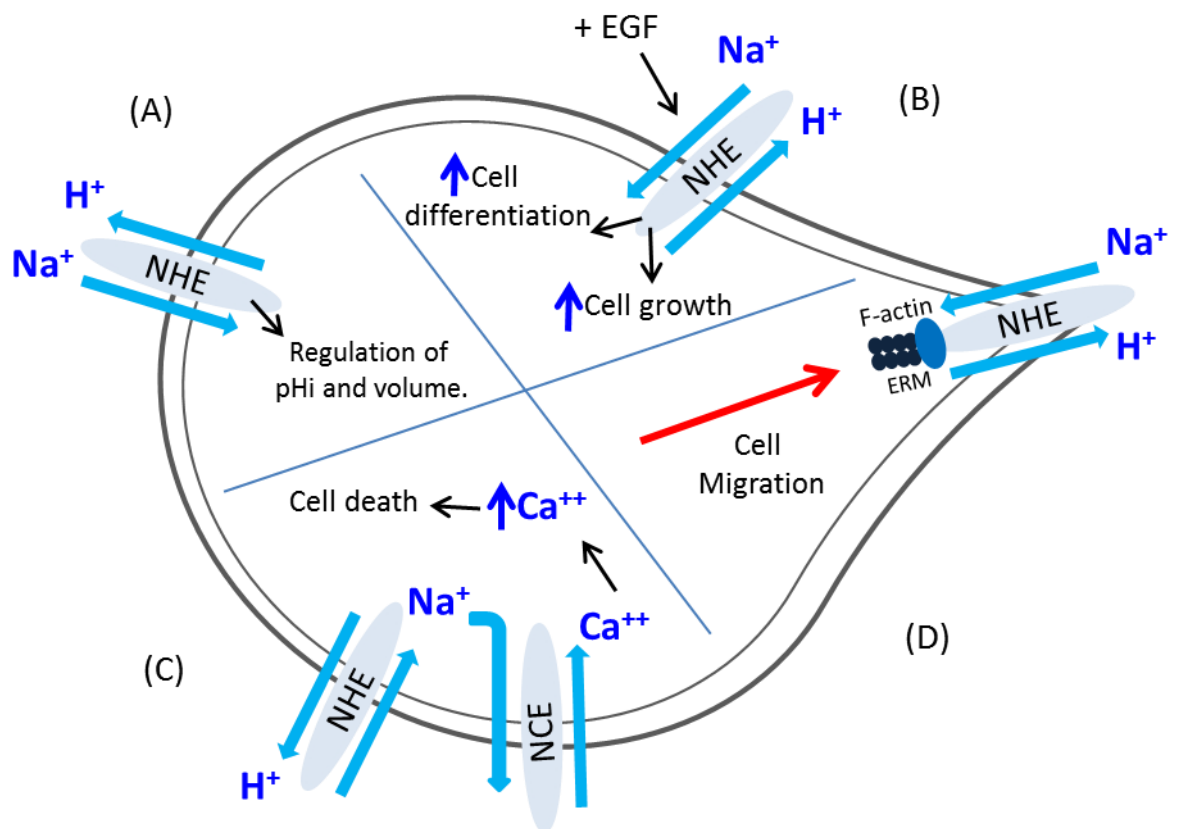
Figure 7.1 Summary of the physiological functions of NHE-1

The main function of NHE-1 is to maintain and regulate intracellular pH (A). NHE-1 can be activated by hormones such as epidermal growth factor (EGF), which leads to increased cell growth and differentiation (B). NHE plays a role in the pathology of many diseases. During heart failure NHE-1 becomes activated and causes a detrimental increase in intracellular Na^+, which in turn causes an increase in intracellular calcium (Ca^{++}) through the Na^+/Ca^{++} exchanger (NCE) ultimately leading to cell death (C). In some cells NHE-1 is present in the lamellipodia. There it acts as an anchor for actin filaments by binding of ERM proteins (D). Disruption of this binding or inhibition of NHE-1 prevents formation of focal adhesions and inhibits cell migration. Adapted from Fliegel 2005.

The aim of this study was to investigate if changes in extracellular pH caused changes in intracellular pH. Furthermore, to establish if there are any differences in the expression of the sodium-hydrogen exchanger between normal healthy equine skin tissue and chronic wound tissue. Also, to establish any differences in expression of the sodium-hydrogen exchanger between normal and chronic wound fibroblasts. I also investigated the effects on the sodium-hydrogen exchanger of alterations in the surrounding extracellular pH which could relate to a decrease in cellular migration at alkaline pH.

7.2   Intracellular pH

The effect of varying extracellular pH on the intracellular pH (pHi) of the cells was investigated. pHi of Normal fibroblasts (NF), Chronic fibroblasts (CF), human dermal fibroblasts (HDF) and epithelial keratinocytes (HaCaT) was assessed using the fluorescent BCECF assay as described in (Chapter 2.1.12). Experiments with HDFs and HaCaTs were performed with three different passages of each cell and three technical repeats, such that standard error and statistical analysis could be performed.

The steady state pHi (measured in control DMEM media) of NF was 6.88 ± 0.14 and for CF was 7.12 ± 0.12, which was significantly different between the two cells ($p < 0.05$). The pHi of CF was consistently more alkaline than the pHi of NF, which was statistically significant in all extracellular pH conditions ($p < 0.01$). When NF and CF were cultured in either acidic (pH 6) or alkaline (pH 9) media for 24 hours the pHi increased compared to values when extracellular pH was neutral at pH 7.5 (Table 7.1). Statistical analysis showed a significant increase in pHi of NF after culture in extracellular pH 9 compared to pHi with extracellular pH of 6 and 7.5 ($p < 0.01$). In CF a significant increase in pHi was observed after culture of cells in extracellular pH 9 compared to pH 7.5 ($p < 0.05$). Similar results were observed in HDFs with an apparent increase in pHi at extracellular pH 6 and pH 9 compared to pH 7.5; but was not significantly different ($p > 0.05$). The opposite was observed in HaCaT cells with a significant decrease in pHi at pH 6 and pH 9 compared to pH 7.5 ($p < 0.05$) (Table 7.1).

Table 7.1 Intracellular pH

Recorded values of intracellular pH for NF, CF, HDF and HaCaTs after being cultured for 24 hours in extracellular pH of 6, 7.5 and 9. Values represent mean ± SEM. Statistical differences were measured using ANOVAs followed by a Tukey posteriori test. * significant difference at $p < 0.05$, ** significant difference at $p < 0.01$

Extracellular pH	Intracellular pH							
	Normal Fibroblasts (n = 3)	Statistical Differences	Chronic Fibroblasts (n = 3)	Statistical Differences	Human Dermal Fibroblasts (n = 1)	Statistical Differences	Human Epithelial Keratinocytes (n = 1)	Statistical Differences
pH 6	6.80 ± 0.13		7.05 ± 0.14		7.87 ± 0.18		7.95 ± 0.03	**
pH 7.5	6.70 ± 0.10	** **	6.93 ± 0.11	* *	7.23 ± 0.22		8.04 ± 0.08	**
pH 9	6.94 ± 0.09		7.13 ± 0.18		7.91 ± 0.36		7.93 ± 0.02	

7.3   Sodium-Hydrogen Exchanger Expression

The expression levels of an essential pH regulatory ion transporter, Na^+/H^+ exchanger, was studied in healthy equine skin tissue and chronic wound tissue. NHE-1 expression was significantly increased in chronic wound tissue samples. Immunohistochemistry analysis of NHE-1 revealed a greater expression of NHE-1 with darker and stronger staining throughout the dermis compared to staining within the dermis layer of normal samples (Figure 7.2D and F). This was further validated by qRT-PCR, demonstrating a similar result with significantly increased NHE-1 mRNA levels in chronic wound tissue samples ($p < 0.000$) (Figure 7.3). In normal intact skin samples a strong expression of NHE-1 is present along the epidermis layer which was expected (Figure 7.2C).
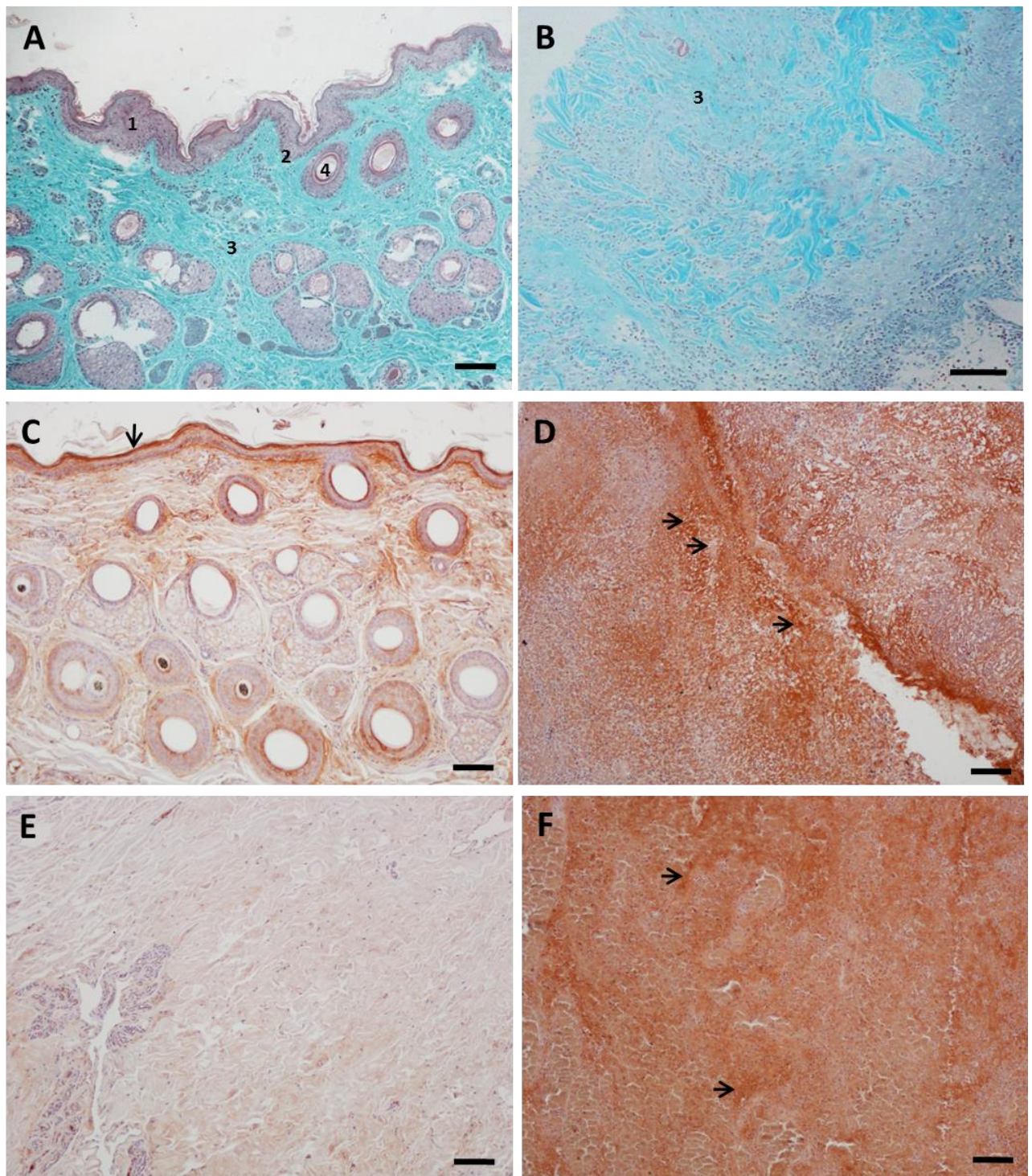
Figure 7.2 Immunohistochemistry images of NHE-1 expression in equine tissue

Representative Masson's trichrome staining is shown for normal skin (A) and chronic wound tissue (B). Collagen in the dermis layer is stained blue, smooth muscle and keratin stain pink and elastic fibres stain black. Images labelled: 1-epidermis, 2-basement membrane, 3-dermis and 4-hair follicle. NHE-1 expression in normal skin is shown in C and E and, arrow in C shows strong expression of NHE-1 along the epidermis. D and F show stronger expression of NHE-1 in chronic wound tissue with darker staining throughout the dermis layer (indicated by arrows). The images shown are representative of three different images for each tissue type. Scale bars, 100μm.
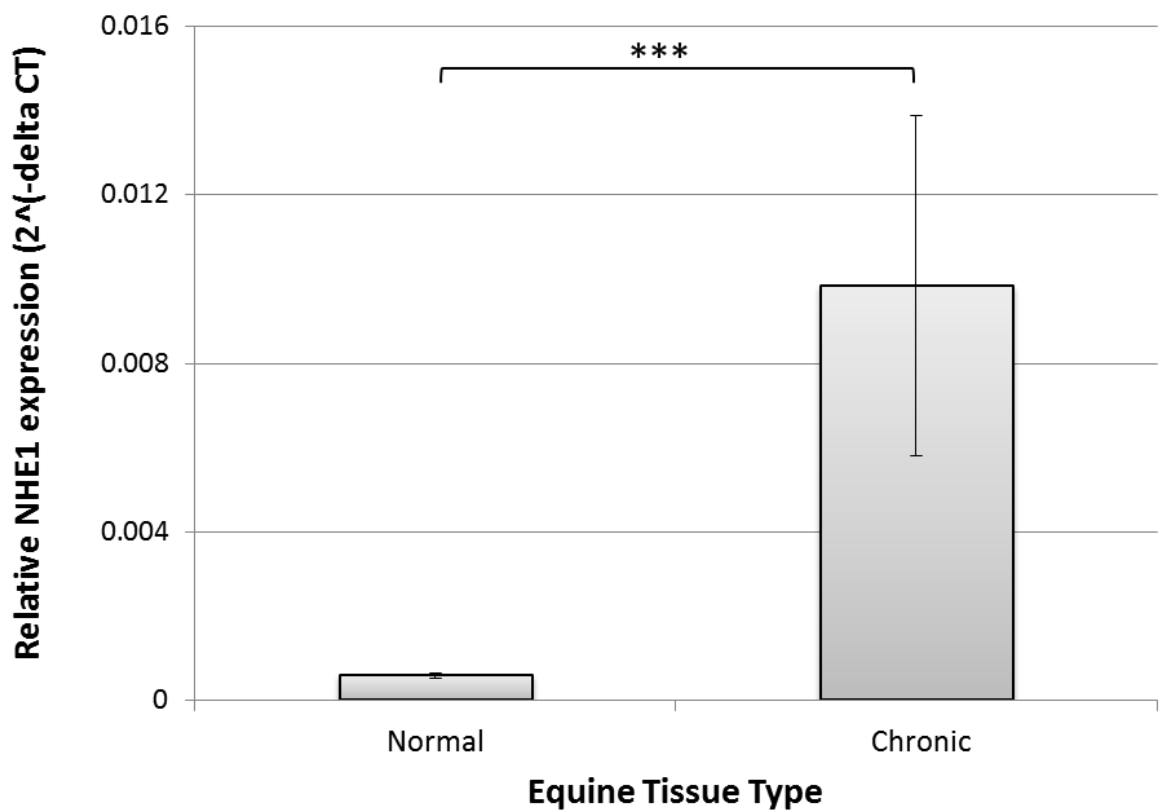
Figure 7.3 NHE-1 mRNA levels in normal healthy skin and chronic wound equine tissue

Relative NHE-1 expression measured in normal healthy equine skin and chronic wound tissue samples (n = 10). NHE-1 expression is significantly upregulated in chronic tissue samples compared to normal. Relative expression of the mean ± SEM is presented. Statistical significance was measured with ANOVA followed by the posteriori Tukey with $p < 0.05$.

7.4 Sodium-Hydrogen exchanger expression in cell cultures following alterations in extracellular pH conditions

NHE-1 mRNA levels in cells were very low. After culture in pH 7.5 there was a significant difference ($p < 0.05$) in NHE-1 between NF and CF, with a higher expression in NF (Figure 7.4A). Following culture of NF in pH 9, a significant down regulation of NHE-1 was observed compared to pH 7.5. Changes in extracellular pH had no effect on NHE-1 expression in CF (Figure 7.4A). Similarly, no differences in NHE-1 expression in HDF cells were observed after culture in three different pH conditions (Figure 7.4B). The highest expression of NHE-1 in HaCaT cells was after culture in pH 7.5 conditions. Culturing HaCaTs in pH 6 and pH 9 conditions significantly down regulated expression of NHE-1 ($p < 0.05$) (Figure 7.4B).
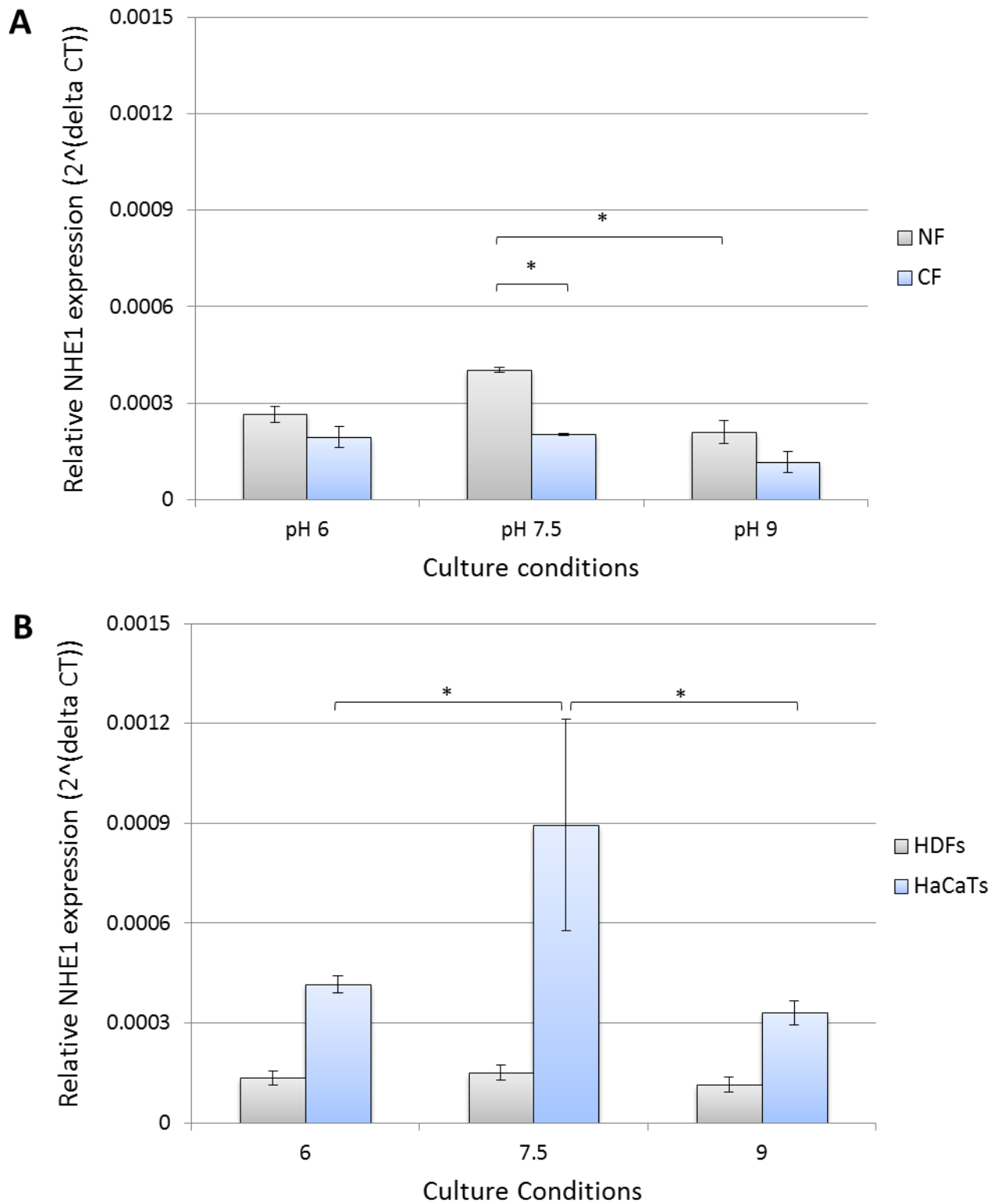
Figure 7.4 Changes in NHE-1 expression after culture of cells in different extracellular pH

NF, CF, HDF and HaCaT cells were cultured in media titrated to pH 6, 7.5 and 9 for 24 hours. RNA was extracted from cells and the relative expression of NHE-1 was measured using qRT-PCR. Relative expression of the mean ± SEM is presented. Statistical significance was measured using ANOVA followed by a posteriori test, Tukey with *$p < 0.05$.

7.5 Discussion

The Na^+/H^+ exchangers are one of the main membrane transporters present in cells and it plays a significant role in many biological functions. Careful regulation of this transporter is essential such that pHi is maintained as well as further downstream events such as cell proliferation and migration initiated by activation of the Na^+/H^+ exchanger. This study investigated changes in pHi and NHE-1 expression following culture of equine (NF and CF) and human (HDF and HaCaT) skin cells in three different extracellular pH conditions (pH 6, 7.5 and 9).

7.5.1 Intracellular pH

The pHi of cells are recorded to be within the range of 6.8 – 7.4 (Mahnensmith & Aronson 1985). An array of cellular molecules including proteins and peptides have an optimum pH, therefore regulation of pHi must be strictly controlled due to the potential effect on a wide array of biological processes such as cell cycle, proliferation, migration and signalling pathways (Boron 2004). HaCaTs had the highest measured pHi (pH 7.93 – 8.04), with no significant changes observed after changing extracellular pH. Other studies have noticed a relatively high pHi in keratinocyte cells compared to other cells types. These observations are associated with the cell cycle; differentiating cells undergo drastic morphological changes which can cause an increase in the fluorescent signal which is used to measure pHi, leading to inaccurate readings. However these cells are not proliferative and should therefore have a lower pHi. The high pHi can be explained due to a population of keratinocytes *in vitro* and *in vivo* that irreversibly commit to terminal differentiation, *in vivo* they will form the dead surface layer of the skin (van Erp et al. 1991).

CFs, overall had a significantly higher pHi than NFs, this correlates with the increased expression of NHE-1 in chronic wound tissues. However, it does not correlate with the lower NHE-1 expression observed in CF cells compared to NFs. NHE is activated by decreases in pHi

(Fliegel 2005), once it is activated the rate of proton extrusion increases and therefore the pHi gradually increases returning to normal (Stock & Schwab 2006). Therefore NHE-1 expression in CF would expected to be higher than in NF and thus also be increased in chronic wound tissues. mRNA levels of NHE-1 were however very low in both cells, originally three different primer sequences were tested and the one used had the best efficiency. More time would allow a further range to be analysed and provide different results or detect a higher level of the membrane transporter within the cells.

7.5.2 NHE-1 Expression

Normal human skin (specifically keratinocyte and melanocyte cells) expresses the NHE-1 isoform (Sarangarajan et al. 2001; Hachem et al. 2005), this can be seen in normal skin sections analysed in this study with a strong expression visible along the upper epidermis layer (Figure 7.2C). NHE-1 is essential to control an acidic environment in skin which is essential for barrier function. In most cells NHE-1 expression is increased in response to acidification (Krapf et al. 1991; Laghmani et al. 2001), this protects the cytosol by extruding excess H^+ while importing Na^+ ions. NHE-1 expression is increased in the oesophageal mucosa to protect it from a decrease in extracellular pH during gastroesophageal reflux (Siddique & Khan 2003). Similarly, cardiac myocyte cultures demonstrated an increase in NHE-1 protein expression in response to acidic external pH (Rehring et al. 1998). However, some cells such as fibroblasts and keratinocytes differ; these have been shown to have opposite effects. Incubation of fibroblasts in acidic medium caused a suppression of NHE-1 activity (Moe et al. 1991). NHE-1 expression in keratinocytes was upregulated following culture in alkaline medium (Hachem et al. 2005).

This study demonstrates that NHE-1 expression is increased in chronic wound tissue when compared with normal skin biopsies. Chronic wounds reside in an alkaline pH which may contribute to this increased expression of NHE-1 in chronic tissue. In a study by Schreml *et al*, they state that the centre of a chronic wound is alkaline but the wound edge is acidic. Further analysis in their study then revealed strong NHE-1 expression at the wound edge and

a low expression in the centre of the chronic wounds (Schreml et al. 2014). This does not correlate with a strong NHE-1 expression in equine chronic wounds analysed here. However, unknown sections of the chronic wounds were analysed and not specific sections from different areas of the wound. Analysis of different sections could potentially reveal different expression levels of NHE-1 across a chronic wound. Conversely, there are other studies that show that alkaline pH results in an increased expression of NHE-1. Topical application of an alkaline solution demonstrated a marked increase in NHE-1 expression in the epidermis (Hachem et al. 2005). This up-regulation of NHE-1 in the skin due to damage or alkalinity may be highly localised response in order to try and restore specific skin functions, such as the acidic permeable skin barrier.

The expression of other pH regulatory membrane transporters, the H^+/lactate and the H^+-ATPase, have been previously analysed; but protein expression were identical in both normal and chronic wound tissue biopsies (Schreml et al. 2014). This suggests that the N^+/H^+ membrane transporter is solely responsible for tissue pH changes.

7.5.3 Role of NHE-1 in Cell Migration

NHE-1 contributes to cell migration in numerous ways by 1) cell volume and directed movement, 2) stabilising the cytoskeleton via regulation of pHi, 3) acting as an anchor to attach the cytoskeleton to the cell membrane, 4) interacting with signalling molecules and 5) controlling gene expression (Stock & Schwab 2006). Results show that an alkaline extracellular pH results in an increase in pHi and a lower expression of NHE-1, this could therefore explain the reduced migration of cells observed in alkaline pH conditions (Chapter 3.5). Pharmacological inhibition of NHE-1 by cariporide or ethyl-isopropyl-amiloride hinders the migration of numerous cell types: endothelial cells (Bussolino et al. 1989), fibroblasts (Denker & Barber 2002), human leucocytes (Ritter et al. 1998) and neutrophils (Rosengren et al. 1994). Furthermore, cells deficient in the NHE-1 receptor (Madin-Darby canine kidney cells) show a significant reduction in cell migration (Schwab et al. 2005).

One of the processes for cell migration involves a co-ordinated formation and release of integrin-mediated focal adhesion contacts with the ECM (Stock & Schwab 2006). Focal adhesion contacts transduce the mechanical force from the cytoskeleton onto the ECM (Beningo et al. 2001). The NHE-1 also plays a part in these focal contacts by directly interacting with integrins. In fibroblasts, NHE-1 anchors the actin cytoskeleton to the plasma membrane by binding of ERM proteins (Denker et al. 2000). One mechanism controlling cell adhesion depends on extracellular pH and the activity of NHE-1. Extracellular pH influences the strength of cell adhesion and therefore affects cell migration (Stock et al. 2005). An acidic extracellular pH or a high NHE-1 activity leads to a tight adhesion and eventually when too strong causes a decrease in cell migration; whereas an alkaline extracellular pH or inhibition of NHE-1 prevents adhesion and subsequently cell migration (Stock & Schwab 2006). Therefore careful control of the extracellular pH environment and cell adhesion is essential for regulation of NHE-1 activity and successful cell migration.

7.6 Conclusion

Chronic wounds have an alkaline pH, which has also been shown to reduce the rate of cell migration (Chapter 3.5). An alkaline extracellular pH decreases pHi and was also shown to reduce the expression of the membrane transporter NHE-1. Research shows that NHE-1 is key in controlling pHi and cell migration (Fliegel 2005). It is hypothesised that in a chronic wound an alkaline extracellular pH causes inhibition of NHE-1, which in turn decreases the rate of cell migration affecting successful wound closure (Figure 7.5). Biofilms are also a major threat in chronic wounds, a study showed that initial exposure to bacterial lipopolysaccharide causes an increase in NHE-1 activity (Rotte et al. 2010). Sustained exposure of NHE-1 to biofilms could result in NHE-1 becoming over- stimulated thus affecting cell adhesion and cell migration. Further research into this membrane transporter is warranted to investigate its role in non-healing chronic wounds and its possibility as a target to increase cellular migration.
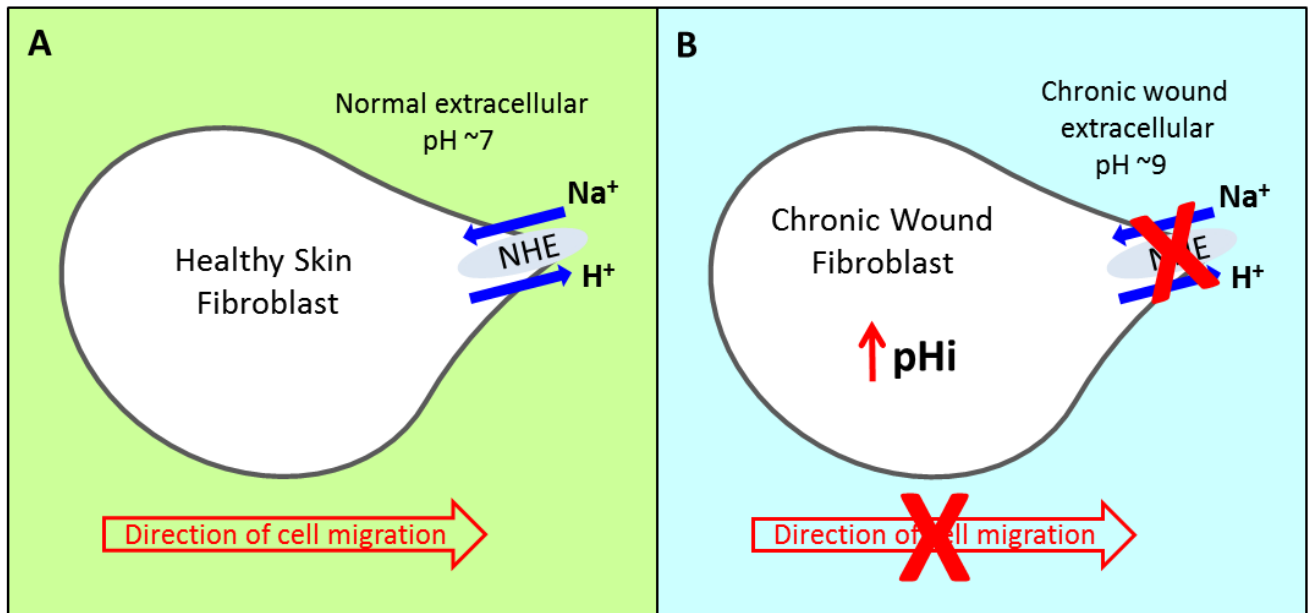
Figure 7.5 Model of the effect of extracellular pH on NHE-1 activity and cell migration

The membrane transporter NHE-1 sits at the leading edge of the cell and plays an important role in cell migration. A) In healthy skin, extracellular pH is within normal range (~ pH 7.5), thus intracellular pH or the activity of NHE-1 is not affected and the cell can migrate sufficiently. B) However, in a chronic wound environment the extracellular pH becomes alkaline. This in turn causes an increase in intracellular pH and results in inhibition/reduced expression of NHE-1. When the NHE-1 transporter is inhibited cells cannot migrate. When wounds become alkaline and cells fail to migrate to close the wound, the inhibition of NHE-1 could be a key factor.

Chapter 8:

Conclusions and Perspectives

8   Conclusions and Perspectives

Chronic wounds are defined as wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation (Menke et al. 2014). This results in delayed healing which does not proceed in an orderly manner through the normal stages of wound healing ultimately resulting in a poor anatomical and functional outcome. Non-healing wounds are characterised by an increase in degrading metalloproteinases (Trengove et al. 1999), elevated cytokines, impaired cellular activity, decreased ECM synthesis (Schultz et al. 2005) and the presence of microbial biofilms (James et al. 2007). Current standard treatment of chronic wounds consists of debridement, irrigation, moisture retentive bandages, and antimicrobial therapy. These treatment paradigms are aimed at promoting wound healing and the restoration of homeostasis (Rumbaugh et al. 2015). Although several advancements in wound care management have been made in the last decade, the incidences of chronic wounds continue; in part due to an increase in biofilm associated infections (Rumbaugh et al. 2015). Therefore, chronic wounds still remain a major health and financial burden, such that the requirement for new and improved treatment still exists.

Since conformation of bacterial biofilms in chronic wounds and their implication in delayed wound healing (James et al. 2007) researchers have increasingly focused on understanding how biofilms develop, and how they interact within the wound to cause detrimental effects. Numerous studies have revealed a significant detrimental effect of biofilms and BCM on cell proliferation, apoptosis, cytokine profiles and cell migration (Kirker et al. 2009; Secor et al. 2011; Kirker et al. 2012; Tankersley et al. 2014), key activities during the wound healing process. Continued research into removal and treatment of biofilms is justified in order to eliminate the threat that biofilms pose to chronic wounds.

However, a new paradigm is coming into the forefront of chronic wound research and a possible treatment target, this is the wound pH. The measurement of wound pH is an easy and important, but often over-looked, indicator of non-healing wounds. A wound destroys

the skins integrity and causes a release of underlying bodily fluids which causes a shift towards an alkaline pH of the underlying tissues (Schneider et al. 2007). Non-healing wounds typically have a more alkaline pH (Gethin 2007; Schneider et al. 2007; Schreml et al. 2010; Sharpe et al. 2013) and from various experimental studies, it appears that attempts should be made to reduce pH and shift the wound toward an acidic environment. For example, a study found that healing wounds shifted below a pH of 8.0, while chronic wounds remained above a pH of 8.5 (Shukla et al. 2007). Furthermore, a recent study demonstrated that treatment with a dressing incorporated with 3% citric acid solution improved wound healing (Prabhu et al. 2014). Therefore, targeting the pH of the wound could be a simple way to treat non-healing wounds and re-direct them into a successful healing environment.

This investigation aimed to determine the effects of pH, if any, on healthy skin fibroblasts and chronic wound fibroblasts; and to ascertain whether key wound healing processes including ECM abundance and cell migration was impaired. Secondly, it aimed to further investigate the effects of bacterial biofilms on key wound healing parameters, by determining if altering pH caused any further harmful effects to those already caused by biofilms.

In achievement of the first objective it was demonstrated that alkaline pH had significant effects on cell migration. Migration of CFs was significantly diminished in pH 9 conditions compared with pH 6 and pH 7.5. NFs were not affected by alkaline pH, with similar migration rates observed in both pH 7.5 and pH 9 conditions. There are however, differences between fibroblasts isolated from healthy skin and chronic wound granulation tissue such that these fibroblasts behave differently in different conditions. The reduced rate of migration observed in alkaline conditions may be explained by the reduced abundance of ECM from CFs, the ECM is vital for guiding a controlled migration of fibroblasts (Schultz & Wysocki 2009). It was not determined that pH had any significant effects on the total amount of collagen, fibronectin and tropoelastin from CFs, however, it was evident that lower amounts were present in all CM analysed from CFs compared to CM collected from NFs. Increasing the synthesis of ECM molecules from fibroblasts within a chronic wound would improve

wound healing responses by providing a matrix for successful cell migration and subsequent wound closure and re-epithelialisation. CFs were stimulated to produce more GAGs, which are known to promote cellular migration (Roberts & Harding 1994) in alkaline culture conditions. This however, did not correlate with cell migration data. Further research is warranted to investigate this increase in GAGs but the inhibited migration observed by CFs; are all GAGs known to stimulate cell migration or is there one prevalent to the chronic wound environment that may in fact inhibit cell migration, and ultimately be deleterious to wound healing.

The second objective was to determine if pH had any effects on biofilm attachment and growth before analysing the effects of pH and biofilms on cellular activities. *S. aureus* and *P. aeruginosa* microorganisms were chosen, as they are two of the most common organisms identified in chronic wounds (Gjødsbøl et al. 2006; James et al. 2007); and it is likely that bacterial colonisation may be affected by alterations in wound pH (Schreml et al. 2010). No clear analysis could be determined from this investigation with inconsistent results of biofilm growth observed in all different pH conditions. Whereas, previous studies from numerous researchers have found a significant effect of alkaline pH, with increased bacterial attachment and biofilm growth (Mempel et al. 1998; Runeman et al. 2000; Harjai et al. 2005; Di Bonaventura et al. 2007; Hostacká et al. 2010). Due to these findings it is evident that pH does affect biofilm growth, but more efficient and sophisticated methods are required to analyse biofilm growth such that the true effect of biofilms and pH in the chronic wound environment can be established.

The third objective was to determine if biofilms along with alterations in pH had any effect on wound healing parameters: ECM abundance and cell migration. Previous research that have investigated the effects of biofilms on cell proliferation, apoptosis, cytokine profiles and cell migration of skin fibroblast cells (Kirker et al. 2009; Secor et al. 2012; Kirker et al. 2012), have overlooked any alterations driven by pH. Both studies by Kirker *et al* measured the pH of PCM and BCM and noted the differences, but simply titrated both back to pH 7 for their experiments. I noted a significant increase in the pH of BCM compared with pH of PCM,

which provides initial evidence that simply the presence of a biofilm increases the pH of its surrounding environment. Protein analysis also revealed that BCM had a much more complex protein banding pattern compared with PCM, such that biofilms may secrete proteins that are deleterious to wound healing within a chronic wound. Further analysis was not carried out here to investigate what the secreted product may be, however this should be investigated as it could prove to be a successful target for treating biofilms and chronic wounds. Results revealed that pH had no effect on ECM abundance and that BCM alone was deleterious with no further effects seen with pH alterations. Cell migration however, was notably affected by the combination of BCM and alkaline pH, with significant inhibition of cell migration compared to other pH and PCM conditions. To my knowledge this is the first study which has combined pH and bacterial biofilms to investigate effects on wound healing.

The next objective was to investigate pH and biofilms on human skin cells and analyse if any differential effects were observed between the effect on dermal fibroblasts and epithelial keratinocytes. Bacterial biofilms and alterations in pH had very similar effects on fibroblasts and keratinocytes. Again, alterations in pH did not reveal major changes in ECM abundance, while BCM reduced ECM abundance in each pH group compared to control. Laminin, a glycoprotein synthesised by keratinocytes is a key ECM molecule of the basement membrane that facilitates cell migration (Colognato & Yurchenco 2000). Preliminary experiments investigating laminin expression revealed that with BCM, laminin-332 expression was increased. Further repeats are required to confirm this and to further investigate its significance in relation to chronic wound healing. Results were comparable with the findings of effect of pH and biofilms on equine normal and chronic fibroblasts, however further repeats of experiments with HDFs and HaCaTs or with primary human skin cells could determine true significant effects. Advances in cell culture also suggest that a fibroblast/keratinocyte co-culture system or a three-dimensional skin model should be used in the future, such that *in vitro* experiments accurately mimic the *in vivo* wound environment.

In the final chapter initial investigations began to analyse the sodium-hydrogen exchanger in the chronic wound environment and analyse if pH alterations had any effect on its expression and function which could account for significantly inhibited cell migration observed in alkaline conditions. Chronic wounds reside in an alkaline pH and NHE-1 expression was revealed to be increased in equine chronic wound tissue when compared with normal skin biopsies, therefore alkaline pH may account for this. One of the major roles of NHE-1 is to control and regulate cytoskeletal organisation and cell migration. Pharmacological inhibition of NHE-1 or cells deficient in the NHE receptor causes significant inhibition of cell migration (Denker & Barber 2002; Schwab et al. 2005). It is hypothesised that in a chronic wound an alkaline extracellular pH results in an increase in pHi and inhibition of NHE-1, which in turn decreases the rate of cell migration affecting successful wound closure. Further research into the sodium-hydrogen exchanger could reveal its role in non-healing chronic wounds and its possibility as a target to increase cellular migration.

Although this study focused on the effect of pH and biofilms within chronic wounds, it is important to note that a number of other environmental changes occur within a chronic wound including temperature, oxygen and carbon dioxide, increased moisture, elevated protease activity and defective ECM (Kruse et al. 2015). Additional investigations are required to further determine the role of pH in healing and non-healing wounds, and particularly how other variables may interact with pH alterations. Shifting a chronic wound towards an acidic pH may benefit some healing responses such as cell migration, however, the effect of altering pH on other responses such as protease, biofilm and immunological activity is currently not fully understood. In chronic wounds neutrophil concentration remains high due to prolonged inflammation, and excessive release of proteases from neutrophils is detrimental. An acidic pH suppresses neutrophil activity (Trevani et al. 1999); therefore, in a chronic wound a more acidic pH would aid to diminish this destructive effect. Antibiotics are administered to treat bacterial infections in wounds, worldwide there is an overuse of antibiotics leading to an increased resistance. pH has been known to affect the performance of antibiotics (McDonnell & Russell 1999). Some antibiotics are prevented from being transported into bacteria in acidic environments (Eisenberg et al. 1984). In alkaline pH it has been shown that antibiotic activity against *S. aureus* increases (Lamp et al.

1992). Therefore, pushing a chronic wound from an alkaline to an acidic state could have negative effects on the performance of antibiotics targeting the biofilms present in the wound. Thorough investigation into the role of pH on all aspects of wound healing phases is urgently required.

There are several avenues for future research that can expand upon the results of this thesis and explore the implications of this work in more detail. Primarily why and how does the shift towards an alkaline pH occur? Is it the change in pH that happens first, which then cause further downstream detrimental effects, attracting microbes and the formation of bacterial biofilms? Or, are the biofilms already present and they cause the shift towards an alkaline tissue pH, which then acts as another impediment to successful wound healing. Is it the increase in surrounding pH that triggers a dysfunctional ECM and changes in the phases of wound healing or is it the alterations in ECM synthesis and cell interactions due to prolonged inflammation that causes the wound to shift to alkaline pH? Further investigations are necessary such that these changes within a non-healing wound can be fully understood and a specific timeline of changes can be produced. Then each factor can be targeted in a timely manner in order to re-direct the wound into a healing state. Strong evidence links shifting the pH of chronic wounds to an acidic state with improved healing (Tsukada et al. 1992; Shukla et al. 2007; Sharpe et al. 2013). Experiments could investigate a simple solution or a molecule that could decrease wound pH. Firstly, analysing if it can improve cell migration compared with reduced rates observed in alkaline pH conditions (Chapter 3). Studies should also assess if such a compound could also be targeted towards destroying and removing biofilms from the wound along with reducing the wound pH. The most effective form of treatment for a chronic wound would be one that improves not only one factor but multiple variables that are deleterious to poor wound healing.

Research suggests that initial wound pH measurements could be beneficial as a first step in diagnosis. This could help to identify non-healing wounds earlier and help clinicians decide on the most appropriate course of treatment. However, clinical predictions of wound pH can be difficult to make. The current technology available only allows for wound surface or wound fluid pH to be measured due to unsuitable probes that can cause further tissue

disruption and cell death (Gethin 2007). The conventional glass probe only provides localised measurements and does not provide complex measurements for the whole wound area (Schreml et al. 2011). However, this should not mean that clinicians should refrain from using these methods as a simple and initial baseline to assess the wound pH. Technology is constantly improving; recently a pH sensor has been developed that is able to map the pH of the whole wound area (Schreml et al. 2011; Schreml et al. 2012). The ability to analyse the pH of the whole wound could better direct specific treatments. Schreml *et al* has shown that the whole area of a chronic wound is not alkaline and that pH gradients occur across the wound area (Schreml et al. 2014). If this is the case in all chronic wounds, then pushing the whole wound towards a more acidic state could have a negative effect. Some areas could then become too acidic and various other detrimental effects may occur as a result. In the future such sensors may be further developed which would make them cheaper and more widely available in hospitals to be used for analysing wounds and diagnosing ones in a non-healing state. Wound pH maps could be experimentally used to link areas of alkaline pH with the location of biofilms, or increased protease activity or even areas of the wound with defective ECM. The implications for the complex nature of chronic wounds are that no single treatment option is relevant for all wounds, but rather a combination of methodologies must be adopted. Improved knowledge of the complex interactions within a chronic wound would improve therapeutic treatment. Long term, finding one treatment option that can target all aspects of the non-healing wound would be beneficial for chronic wound patients and much more cost effective for the government and health care organisations.

When investigating the effects of wound healing it is important to combine all of the parameters known to change, such that *in vitro* experiments can better stimulate the *in vivo* situation. In terms of chronic wound treatment it is important to account for all of these factors and not just change in pH. Studies using experimental systems that more realistically represent the physiological environments would be of interest. The experiments conducted in this thesis were 2D with cells adhered on plastic. While this is an appropriate choice for fundamental research using model systems, it is not representative of 3D tissue environments. Therefore, extending results for 3D cell migration would be an important focus for future research. The directional movement of individual cells could be closely

analysed in different pH environments or even in pH gradients. Cell migration could be analysed on different surfaces, matrices that have previously been experimentally used are collagen gels, polyethylene based hydrogels and matrigels (Paradise 2012). To mimic the wound environment various ECM gels such as collagen and fibronectin could be utilised, this would allow for further investigations into the effect of altering pH on cell and ECM interactions.

It may be relevant to pursue further investigations in relevant animal models to fully model the complex interactions which can occur during wound healing. Wounds could be pushed towards an alkaline pH such that further effects could be assessed such as histological analysis and then compared with wounds that are kept in a more neutral or acidic state. Animal models would make it easier to investigate the effect of both pH and biofilms alone or together on wound healing responses. Wound pH could be continuously measured to provide more detail into how and when the shift towards an alkaline pH occurs. Current animal models of chronic wounds include rabbit ear ischemia, pig flap ischemia, diabetic mouse and pig wound infection (Nunan et al. 2014). The pig wound model is commonly used to investigate biofilm effects. It is a good model as pig skin is anatomically and physiologically similar to humans, however, a major drawback is it is a very expensive model and not genetically tractable (Nunan et al. 2014). Nonetheless, it could provide a good base to investigate pH and biofilms as it is already established as a chronic wound biofilm model. The limitations of the currently available chronic wound models are the poor ability to replicate the effects of ageing and the microbiota of the wound (Nunan et al. 2014). These animal models need improving such that all aspects of the non healing wound environment can be modelled and experimentally analysed.

In conclusion, this study has demonstrated that pH does play a role in wound healing. A lot of further work on pH in both healing and non-healing wounds is required so that future technologies can be developed that are pH specific with the aim to achieve quicker and effective wound healing. With more consideration and further investigations into the role of pH, it should be possible to optimise chronic wound treatment. Such advances would reduce

healing times of chronic wounds and may even prevent the wound from moving into a non-healing state in the first instance.

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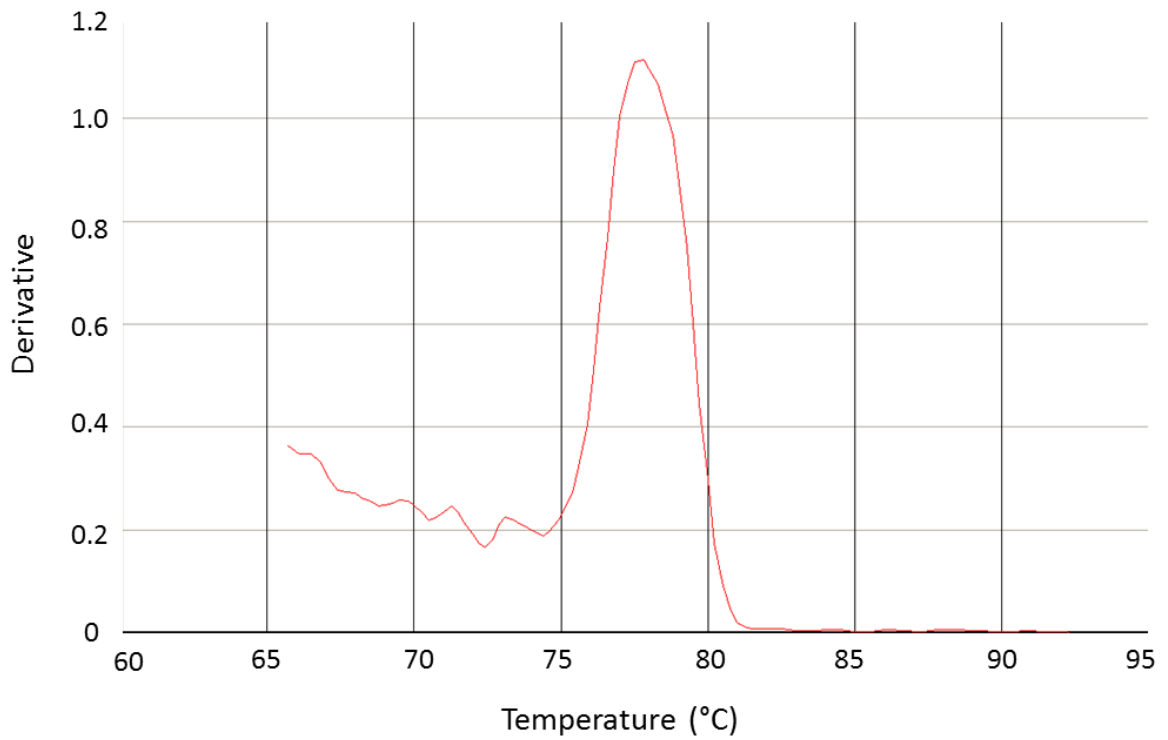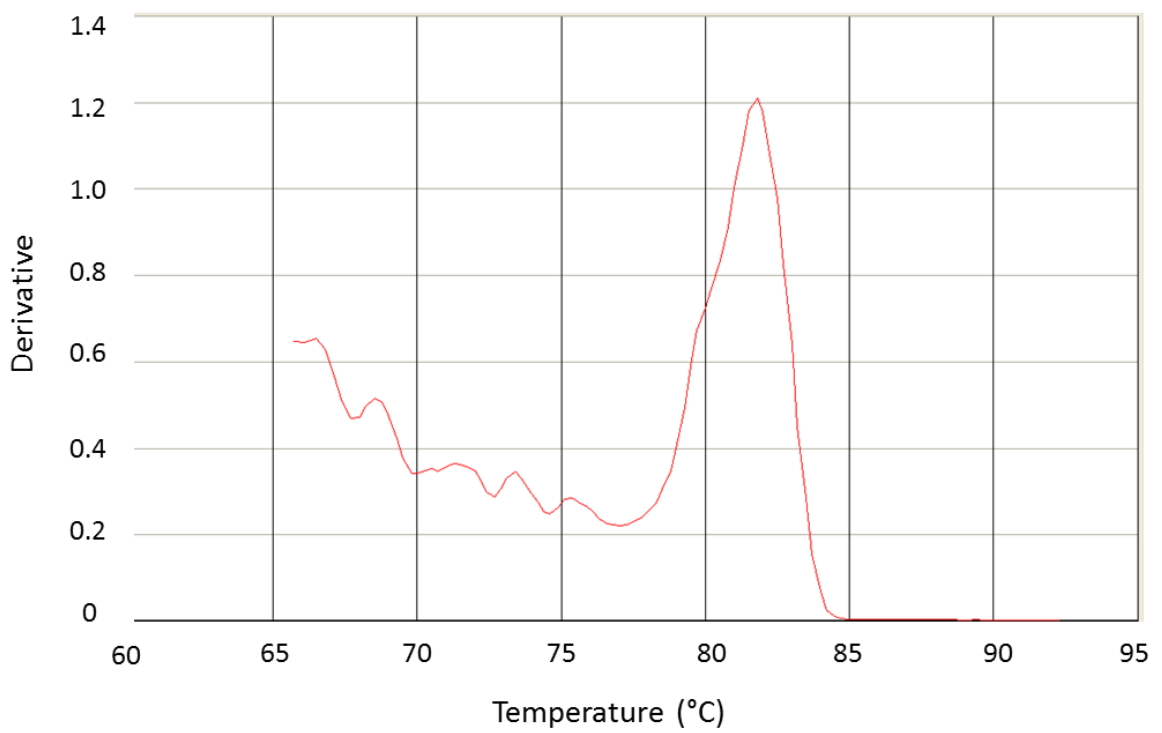
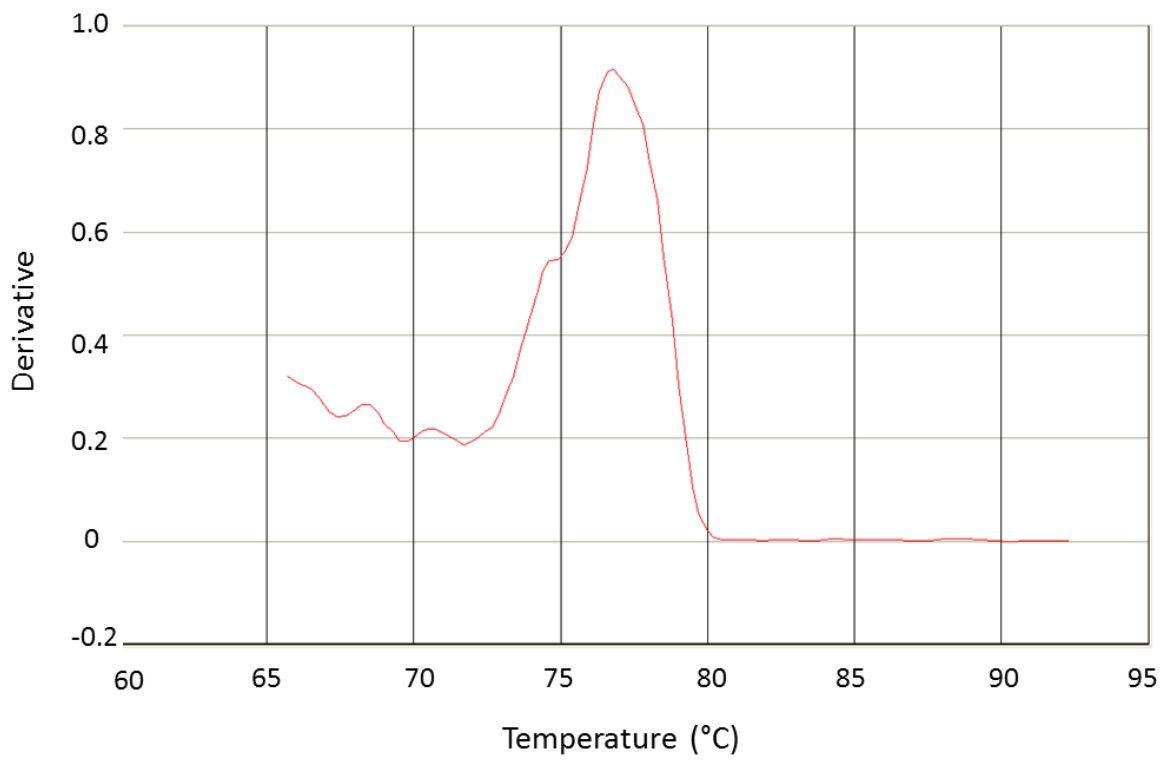**Appendix A- 1 Melt curve plot for Collagen Type 1A1 (COL1A1)**
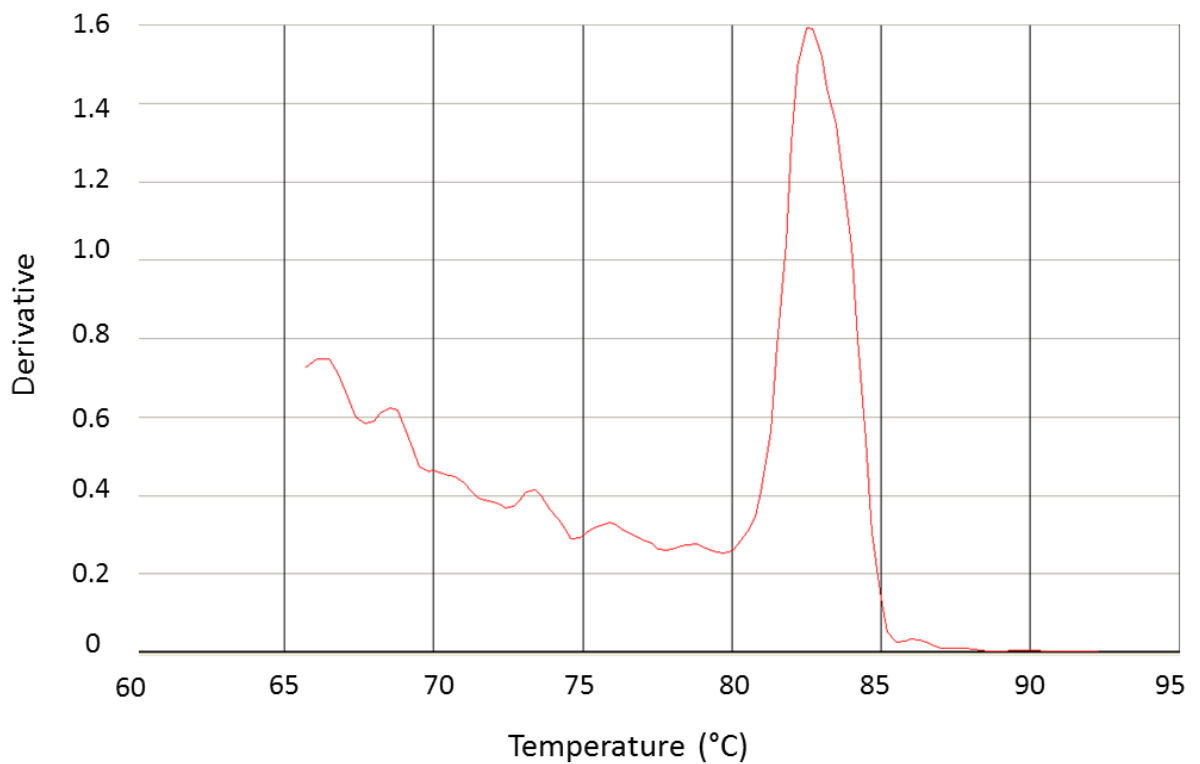
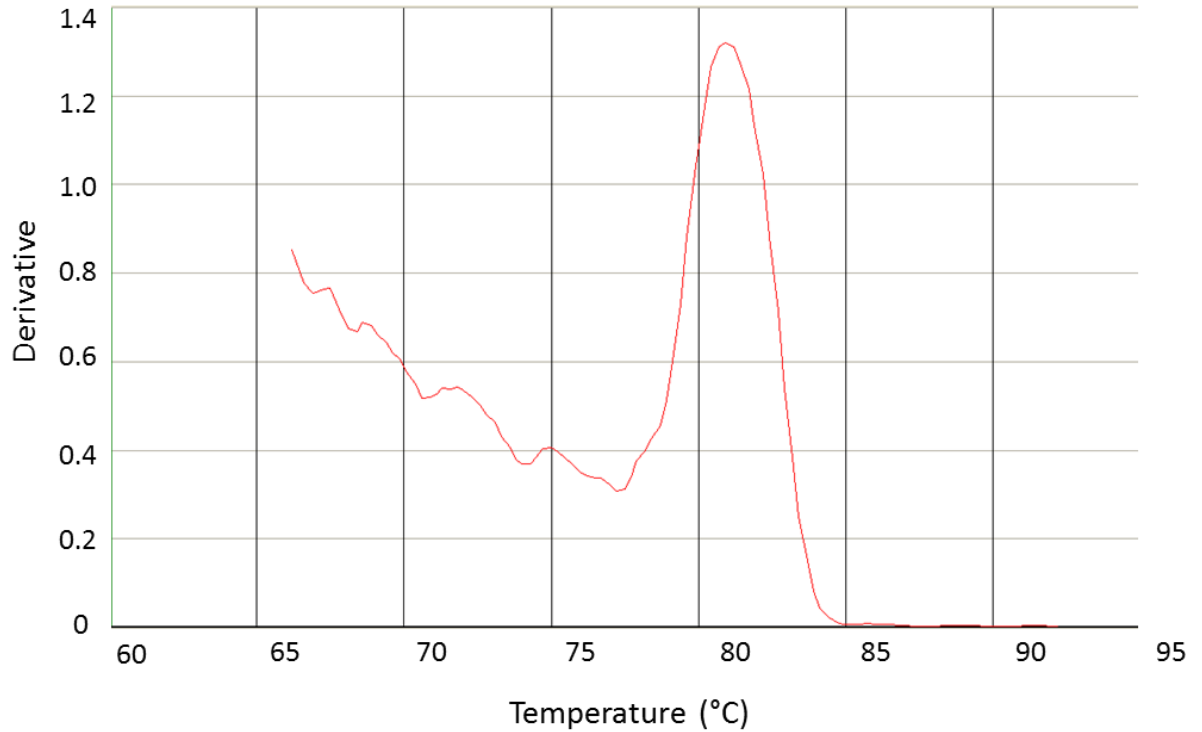**Appendix A- 2 Melt curve plot for Collagen Type 1A2 (COL1A2)**
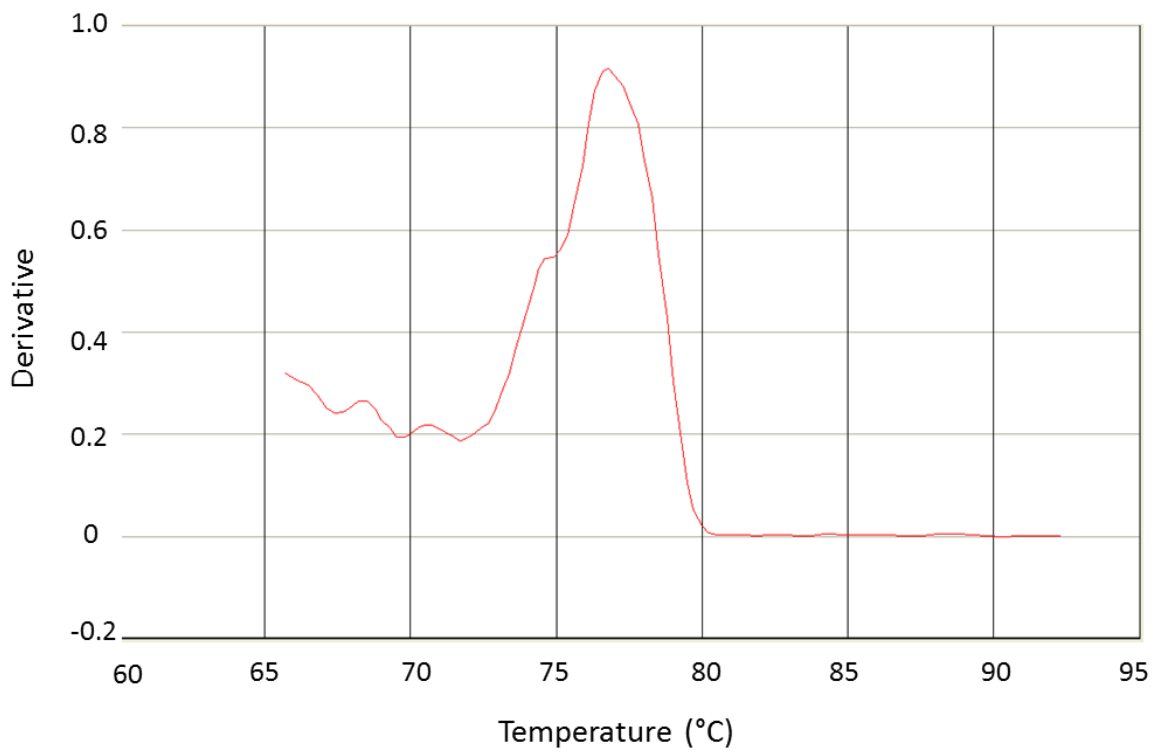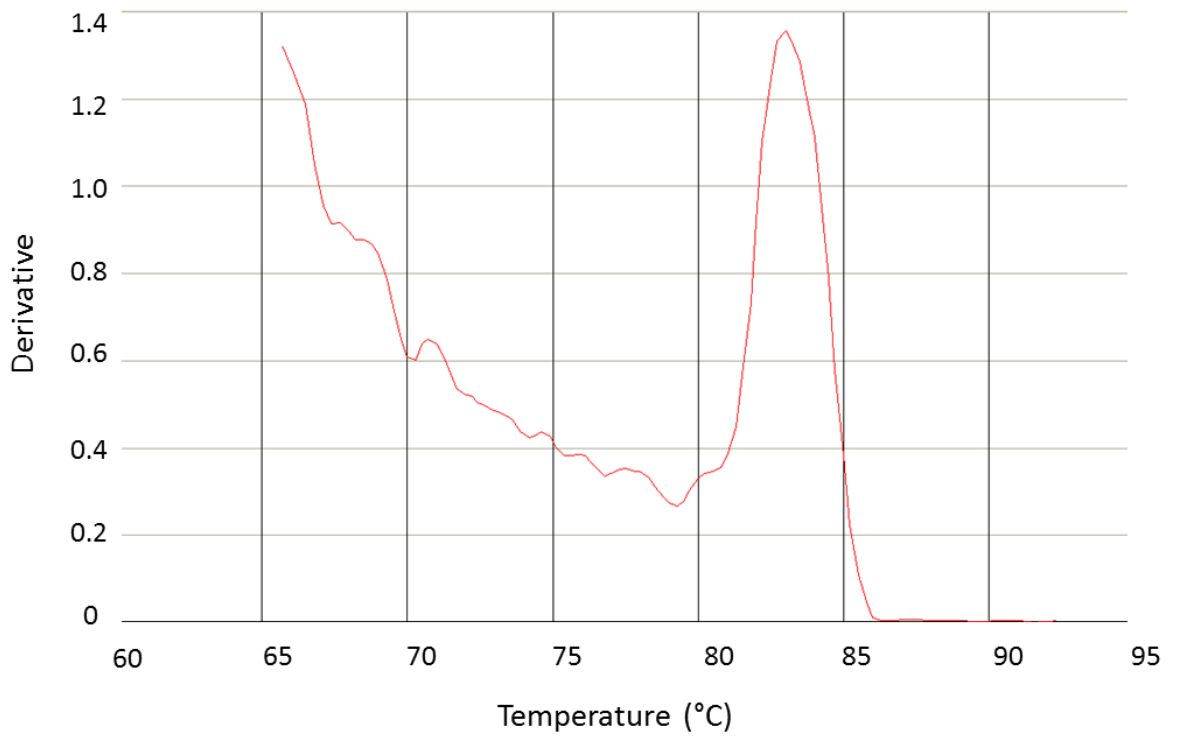
Appendix A- 3 Melt curve plot for Fibronectin (FN)

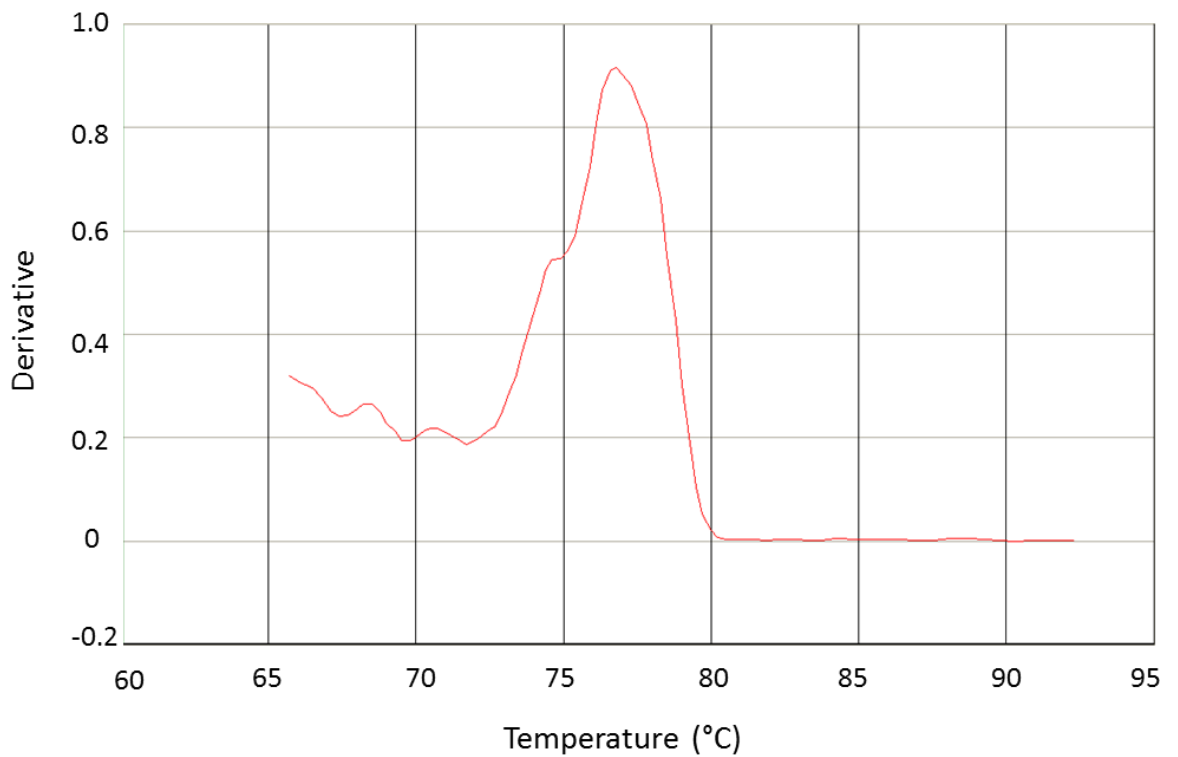**Appendix A- 4 Melt curve plot for Decorin (DCN)**

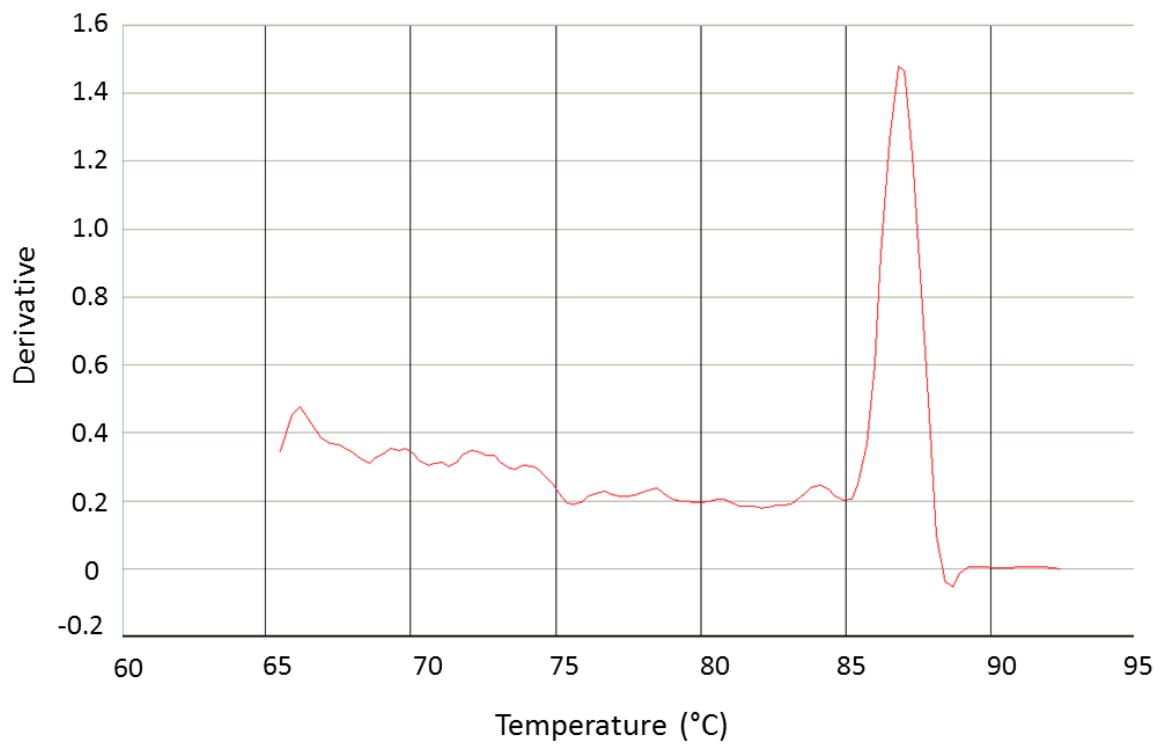**Appendix A- 5 Melt curve plot for Lumican (LUM)**

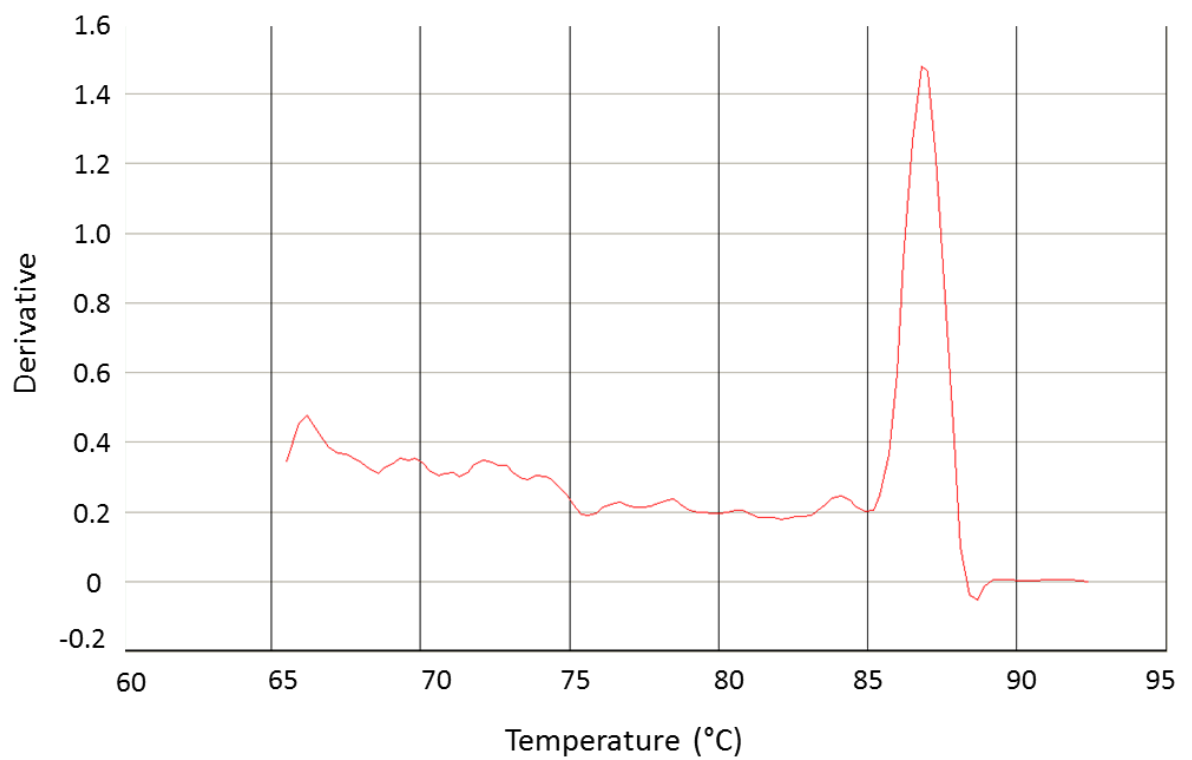**Appendix A- 6 Melt curve plot for Versican (VCAN)**

Appendix A- 7 Melt curve plot for equine NHE-1

Appendix A- 8 Melt curve plot for the equine reference gene GAPDH

Appendix A- 9 Melt curve plot for human NHE-1

Appendix A- 10 Melt curve plot for the human reference gene GAPDH

Awards

Young Investigator Award – received at the Symposium of Advanced Wound Care, San Antonio, Texas; for podium presentation entitled 'The Effect of pH on Fibroblast Migration in an in vitro model comparing Chronic Wound and Normal Skin Fibroblasts'.

Publications

Jones E.M, Percival S.L, Clegg P.D and Hunt J.A (2015) An investigation into the significance of tissue pH on the fate of wound healing, Wound Repair and Regeneration – pending submission

Jones E.M, Cochrane C.A and Percival S.L (2015) The Effect of pH on the Extracellular Matrix and Biofilms, Advances in Wound Care, 4(7): 431-439.

Sara McCarty, **Eleri M. Jones**, Simon Finnegan, Emma Woods, Christine A. Cochrane, Steven L. Percival (2014) Biofilms in Infection Prevention and Control, Chapter Eighteen - Wound Infection and Biofilms, Pages 339-358.

Conference Abstracts

Oral Presentations

Symposium of Advanced Wound Care, San Antonio, Texas, 2015 –Title: The Effect of pH on Fibroblast Migration in an in vitro model comparing Chronic Wound and Normal Skin Fibroblasts.

European Tissue Repair Society, Edinburgh 2014 – Title: Role of pH and Biofilms on Synthesis of Extracellular Matrix from Normal and Chronic Wound Fibroblasts.

European Society of Biomaterial, Liverpool, 2014– Title: Role of pH and Biofilms on Synthesis of Extracellular Matrix from Normal Fibroblasts.

European Wound Management Association, Madrid, 2014 – Title: Chronic Wound Fibroblasts: Does pH affect the synthesis of Extracellular Matrix Molecules?

Symposium of Advanced Wound Care, Orlando, Florida, 2014 – Title: Comparing the effect of pH on Extracellular Matrix Synthesis from Normal and Chronic Wound Fibroblasts.