Microbial Ecology of Chronic Wounds

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Contents

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List of Abbreviations

Ammonium persulphate (APS)

Bacterial productivity (ΔOD)

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)

Colony forming units (CFU)

Constant depth film fermenter (CDFF)

Dithiothreitol (DTT)

Denaturing gradient gel electrophoresis (DGGE)

Drip flow reactor (DFR)

Environmental scanning electron microscopy (ESEM)

Ethylenediaminetetraacetic acid (EDTA)

Extracellular polymeric substances (EPS)

Fine celled foamed multi-well wound model (FCF multi-well wound model)

Fine-celled thermoset phenolic plastic foam (FCF)

Florescent *in situ* hybridisation (FISH)

Fluorescein isothiocyanate (FITC)

Foetal calf serum (FCS)

Lipopolysaccharide (LPS)

Methicillin resistant *Staphylococcus aureus* (MRSA)

Maximal specific growth rates (μ)

Multiple sorbarod devices (MSD)

Multiple FCF wound biofilm model (MFCF wound biofilm model)

Optimal cutting temperature (OCT) embedding matrix

Phosphate buffered saline (PBS)

Polymorphonuclear leucocytes (PMNs)

Tetramethylethylenediamine (TEMED)

Tris Base, acetic acid, EDTA buffer (TAE)

Trichloroacetic acid (TCA)

Unweighted pair group method with arithmetic mean (UPGMA)

Volume/volume (v/v)

Weight/volume (w/v)

Abstract

Within the five experimental chapters of this doctoral thesis (i) the eubacterial diversity of the microbiota of chronic wounds and healthy skin was investigated, (ii) biofilm formation and associated coaggregation interactions of wound and skin-associated bacterial isolates was examined, (iii) formulation of media which reproduced some aspects of the nutritional conditions of wounds and healthy skin were developed, (iv) novel wound biofilm models were developed and validated and (v) microbial population interactions associated with healthy skin and chronic wounds were investigated using a novel model system. (i) The microbial diversity of chronic wounds and contralateral skin swabs was investigated using culture, denaturing gradient gel electrophoresis (DGGE) and microscopy. Intrapersonal analysis identified that non-infected wounds had a proportionally higher incidence of bacteria which were identified on contralateral healthy skin according to DGGE analysis when compared to infected wounds indicating that taxonomically distinct consortia are associated with infection. Microcolonies and putative biofilms structures were identified in both culture-defined infected and non-infected wounds indicating that the presence of biofilms may not be linked to infection. (ii) By assessing pair-wise combinations of skin and wound-associated bacteria, the role of coaggregation in the formation of wound polymicrobial communities was assessed using a quantitative spectrophotometric assay. Aggregation interactions were weak or not detectable, apart from those associated with *Corynebacterium xerosis*. This bacterium produced a high autoaggregation score (*c.* 50%). The limited coaggregation interactions suggest that coaggregation may be comparatively unimportant in the development of wound biofilms. (iii) In order to facilitate the development of biofilm models specific to chronic wounds, the formulation of representative growth media is important in order to reproduce the *in situ* nutrient environment. Therefore complex, artificial sweat and serum media broadly reflective of the nutrient availability in wounds and healthy skin were developed and validated based upon their ability to support realistic phenotypes (assessed by proteomics) and the growth of a range bacterial isolates. Developed media maintained the sessile growth the test bacteria and produced broadly similar proteomic profiles to foetal calf serum. (iv) Two novel model systems were developed to study cross-sectional population interactions and to investigate longitudinal population development of wound consortia and biofilm formation. A fine celled foam (FCF) multi-well wound model and a multiple membrane FCF model maintained dynamic steady state of axenic and mixed populations of bacteria associated with chronic wounds and supported the development of biofilms. (v) The FCF multi-well wound model was used to investigate population interactions in environments broadly reflective of healthy skin and wounds. When grown in artificial sweat prior colonisation with *Staphylococcus saprophyticus* resulted in a significant reductions in methicillin resistant *Staphylococcus aureus* (99%) and *P. aeruginosa* (75%) whilst prior colonisation by *C*. *xerosis* resulted in a significant reduction in *P. aeruginosa* (91%) only. However no significant reductions in pathogenic bacteria were noted in artificial serum indicating colonisation resistance could be simulated in the model and the outcome of immigration was markedly influenced by the species of established bacterium and nutrient availability.

Declaration

No portion of the work referred to in the dissertation has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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Finally I would like to dedicate this thesis to my late father who instilled in me my work ethic and my resilience and who never failed to give me moral support and encouragement.

This is for you dad.

Chapter 1

General introduction

1.1 The physiology of human skin

The human skin is a multifunctional organ which consists of two distinct layers; the epidermis and the dermis shown in Figure 1.1 (Wong and Chang, 2008). The dermis contains blood vessels, nerves, hair follicles, sweat and sebaceous glands, and is composed of bundles of loose connective tissue such as collagen interwoven with elastin, proteoglycans and fibronectin. It supports to the epidermis both structurally via the integrity of the connective tissue and nutritionally via the delivery of nutrients through the blood vessels (Brodell and Rosenthal, 2008; Powell, 2006; Wong and Chang, 2008). The epidermis itself, is generated from the migration and terminal differentiation of basal layers cells (keratinocytes) as they

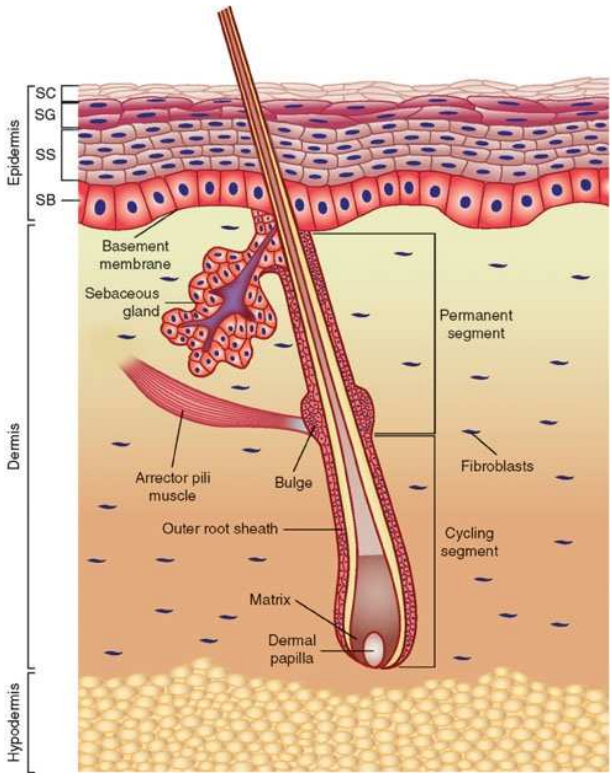


Figure 1.1 Anatomy of the skin. The epidermis is divided into four layers, starting with the outermost layer: stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB). (Wong and Chang, 2008)

move from the epithelium basement membrane towards the skin surface forming four defined cellular layers; the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum (Heer Nicol, 2005; McGrath and Uitto, 2004; Rutter, 2003) shown in Figure 1.1. The stratum basale is a continuous one cell thick layer, consisting of small cuboidal cells that contain numerous ribosomes and tonofilament bundles (structural cytoplasmic proteins) (McGrath and Uitto, 2004). As the cells migrate beyond this layer and journey towards the stratum corneum, epidermal growth factor broadly trigger the expression of proteins required for terminal differentiation for example Keratin-1 and Keratin-10 (cytoskeletal intermediates of keratin filaments) and the accumulation profilaggrin and intracellular granules of lysosyme which continue to increase throughout the migration progresses. The production and accumulation of these alter the shape of the keratinocytes, producing the distinct cellular layers of the epidermis (Hoffjan and Stemmler, 2007). When the cells reach the stratum corneum the lysozymes granules destroy the nucleus and cytoplasmic organelles causing the cells (now termed corneocytes) to expel cellular lipids into the intercellular space. Keratin filaments within the cell condense under the influence of filaggrin (cleaved profilaggrin), altering their structure and position causing them to become flattened and aligned (McGrath and Uitto, 2004; Powell, 2006). The expelled cellular lipids such as the ceramides, cholesterol and free fatty acids aid intercellular cohesion of the corneocytes and now play an important role in the hydrophobic barrier function of the skin (McGrath and Uitto, 2004; Rutter, 2003). The final stage of keratinocyte/corneocytes migration is the process of desquamation whereby intercellular lipids begin to degrade leading to the loss of the residual intercellular connections causing cells to flake off and becoming replaced by underlying cells (McGrath and Uitto, 2004).

Overall human skin provides sensation, thermoregulation, biochemical and metabolic functions, and perhaps more importantly protection against infection (Wysocki, 2002). This protection is afforded by mechanistically distinct systems which can simplistically be grouped into three different classes of barrier; the chemical/biochemical barrier, the immune barrier and the physical barrier (Proksch *et al.*, 2006).

The chemical/biochemical barrier incorporates the innate immune system which includes the production and secretion of antimicrobial lipids, peptides and lysozymes (Proksch *et al.*, 2006). Lipids are synthesised in the epidermis and are either carried within the cells to the surface or secreted from the sebaceous glands; their quantity and composition is linked to epidermal proliferation with secreted volumes increasing in relation to cellular differentiation (Drake *et al.*, 2008). These lipids consist of 35-40% free fatty acids, of which approximately 20% are saturated and 20% are unsaturated. The lipids provide a hydrophobic barrier preventing water loss and subsequent cytolysis of epidermal cells and tissues. In addition to the hydrophobic barrier, several free fatty acids associated with the epidermis have been shown to possess antimicrobial activity; for example lauric acid, palmitoleic acid, sapienic acid and oleic acid are inhibitory towards a number of bacteria including *Staphylococcus aureus, Streptococcus pyogenes, and Streptococcus salivarius* (Drake *et al.*, 2008; Thormar and Hilmarsson, 2007). Furthermore, differentiating keratinocytes produce antimicrobial peptides such as defensins and cathelicidins which initiate and coordinate components of the innate and adaptive immune system and act as a antimicrobial barrier at the surface of the skin (Drake *et al.*, 2008; Schauber and Gallo, 2008). The production of antimicrobial peptides is up-regulated in response to injury or infection with greater

quantities often found at the wound margins as a possible mechanisms to prevent infection (Dorschner *et al.*, 2001). Antimicrobial peptides are variously active against Gram positive and Gram negative bacteria including *S. aureus* and *Pseudomonas aeruginosa.* Their mode of action is attributed to the formation of channels in the bacterial membrane and/or targeting and inactivating endotoxin (Drake *et al.*, 2008; Ramanathan *et al.*, 2002). Additionally, keratinocytes, polymorphonuclear leukocytes, monocytes, macrophages and secretory cells of apocrine glands produce the lysozyme enzyme; N-acetyl muraminidase (Niyonsaba and Ogawa, 2005) which aids in the protection of the host by hydrolysing bacterial cell walls, enhancing phagocytic activity of polymorphonuclear leukocytes and macrophages, and stimulating the proliferation of monocytes (Cove and Eady, 1998; Niyonsaba and Ogawa, 2005).

The second class of protective barrier of the human skin is provided by the immune system; this consists of the humoral immunoglobulin antibody A and cellular immune system which includes lymphocytes, dendritic cells and macrophages (Proksch *et al.*, 2006). Secretory immunoglobulin A (sIgA) has been measured at the skin surface 13 +/-9 µg/ml of sweat in males and 1.6 +/- 0.9µg/ml of sweat in females (Okada *et al.*, 1988) and is produced and secreted by epithelial cells in regular quantities on the skin irrespective of the rate of sweat production. The role of slgA on the skin is remains poorly understood as opsonisation does not generally occur on the surface of intact skin (due to the limited presence of macrophages), however, it is believed to play a role in the defence of the skin by binding to surface structures of microorganisms preventing adherence (Imayama *et al.*, 1994; Okada *et al.*, 1988).

The physical barrier manifested by the structure of the stratum corneum is third means by which the skin confers protection to the host. The stratum corneum is the outer most epidermal layer and is composed of protein-rich cells; the corneocytes and lipid-enriched intercellular domains (Proksch *et al.*, 2006). The mechanical rigidity of the stratum corneum shields the epidermis and dermis against physical challenges to skin whereas low moisture content, hydrophobicity and minimal nutrient availability reduces the ability of pathogenic microorganisms to colonise the epidermis (Chiller *et al.*, 2001). Eccrine sweat contains calprotectin, a calcium and zinc binding protein; R binder (haptocorrin), which binds vitamin BI2; and transferrin, which binds iron with extremely high affinity (Cove and Eady, 1998). The production of these factors further reduces already limited nutrient availability subsequently, only those microorganisms which have adapted to this specific environment are capable of colonising (Bojar and Holland, 2002; Chiller *et al.*, 2001). Generally, the availability of nutrients and moisture dictates the quantity and diversity of the colonising biota. Areas which contain a high number of sweat glands such as the axilla or toe webs have been shown to have aerobic bacterial counts of *c.* 10^7 CFU per cm^2 whereas bacterial counts on dry areas such as the forearm or trunk may harbour *c.* 10^2 CFU or fewer per cm^2 (Fredricks, 2001).

1.2 Cutaneous nutrient availability and microbial colonisation

In order to proliferate, heterotrophic bacteria generally require nitrogen, carbon, iron and to an extent vitamins. The skin and its secretions i.e. sweat and sebum can provide some of these nutritional requirements. Subsequently, the number and types of microorganisms colonising the skin could be considered both nutrient and water limited and in this respect bacterial productivity upon the skin is dependant

upon the density and activity of the sweat glands (James *et al.*, 2008). Sweat and sebum are derived from three main types of skin glands; the eccrine, apocrine and sebaceous glands. There are approximately 2-5 x 10^6 eccrine sweat glands in the adult human. They are most numerous on the palms of the hands and soles of the feet but are distributed over the whole surface of the body with exception to the lips, glans penis, inner surface of the prepuce and clitoris (ICRP, 1975). The apocrine glands are attached to hair follicles localised to the axilla, the external auditory meatus, the eyelids, and the circumanal region, and begin to function at puberty secreting proteinaceous viscous sweat (ICRP, 1975; Saga, 2002). Sebaceous glands consist of a single or collection of lobules that open into a system of ducts. They are present on the entire surface of the body except the palms, soles, and dorsum of the foot with the majority of the sebaceous glands associated with a hair follicle (ICRP, 1975; Thody and Shuster, 1989). Sebaceous glands primarily produce sebum which is a mixture of wax esters, triglycerides, and free fatty acids lipids (Thody and Shuster, 1989). The sweat and sebaceous gland secreted fluids contain a number of constituents shown in Table 1.1, which can provide the basis for the nutrition of the skin microbiota (Mickelsen and Keys, 1943; Ross Russell and Wiles, 1970).

Table 1.1 Mean and range values for constituents commonly found in adult sweat

Constituents	Mean	Range
Chloride (mEq/l)	29.7	0-65.1
Phosphate (mg/l)	14	10-17
Sulphate (mg/l)	-	7-190
Fluoride (mg/l)	-	0.2-1.8
Potassium (mEq/l)	7.5	4.3-10.7
Sodium (mEq/l)	51.9	9.7-94.1
Calcium (mEq/l)	-	0.2-6
Magnesium (mEq/l)	-	0.03-4
Total Nitrogen (mg/l)	-	230-400
Urea (mg/l)	-	260-1220
Creatinine (mg/l)	4.6	2.1-8.4
Ammonia (mg/l)	-	60-110
Amino Acids (g/l)	0.476	0.27-0.68
Acetic Acid (mg/l)	7.69	3.57-24.9
Propionic Acid (mg/l)	0.26	0.09-0.55
Lactic Acid (g/l)	0.616	0.474-1.19
Glucose (mg/ml)	-	0.05-0.2

Adapted from Ross Russell and Wiles (1970) and Mickelson and Keys (1943)

As stated previously, the skin and its secretions are an important source of organic carbon and nitrogen. Sugars present in sweat such as glucose, lactose and glycerol present a primary carbon source utilised by the commensal bacteria (Mickelsen and Keys, 1943). Due to the limited sugar availability in this environment however, the cutaneous microbiota utilise other constituents of sweat as alternative carbon sources. Sweat lipids including glycerides and wax esters are one secondary carbon source which some bacteria including *Staphylococcus* spp. and *Propionibacterium* spp. are particularly well adapted to utilise through the production of lipases which hydrolyse the lipids to glycerol and free fatty acids. These in succession can be utilised by other genera such as the *Corynebacterium* spp. which can results in the production volatile fatty acids that in turn can be metabolised by some micrococci and brevibacteria shown in Figure 1.2 (James *et*

al., 2004). This highlights the symbiotic relationships which can occur between different representatives of the cutaneous microbiota to fully utilise available nutrient resources upon the skin. A primary nitrogen source for cutaneous bacteria is the urea and amino acids present in sweat. Various amino acids are commonly present, including; alanine, arginine, aspartic acid citrulline, glutamic acid glycine, histidine, isoleucine, leucine, lysine, ornithine, phenylalanine, threonine, tryptophan, tyrosine, and valine (Stefaniak and Harvey, 2006). Both arginine and valine have been shown to be an essential part of the nitrogen requirements of cutaneous *Staphylococcus* spp. and *Propionibacterium* spp. (Bojar and Holland, 2002).

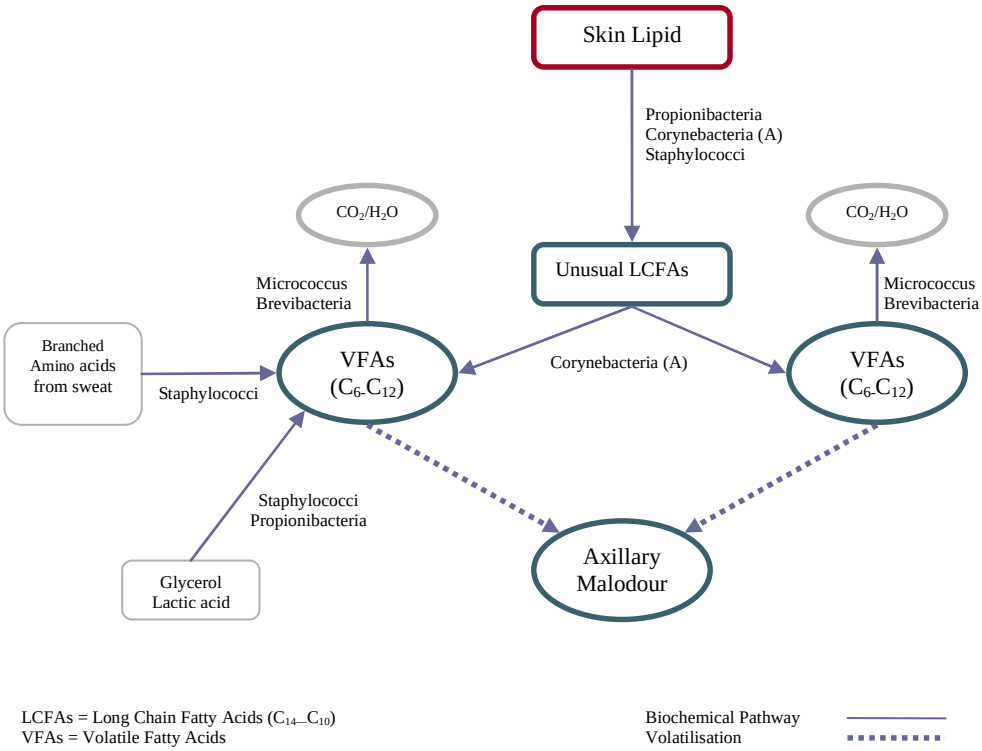


Figure 1.2 Formation and utilization of volatile fatty acids by axillary bacteria modified from James *et al*., (2004)

In addition to the limited organic carbon and nitrogen sources that are available on healthy skin, the availability of iron which is critical for bacterial growth and survival is heavily restricted upon the epidermis. Free iron is limited or bound to mammalian secretary proteins such as lactoferrin and transferrin. In order to overcome the biological complexation of iron by the host, bacteria secrete strong iron chelators termed siderophores. Siderophores contain high affinity iron-binding moieties including catecholate, hydroxamate, or α-hydroxycarboxylate groups which enable them to sequester iron directly from the host proteins (Dertz *et al.*, 2003). The sidophore-iron complexes are then taken up by specific membrane-receptor proteins which vary between organisms preventing loss of siderophore-iron complexes to competing organism (Dertz *et al.*, 2003; Krewulak and Vogel, 2008; von Eiff *et al.*, 2002). The act of scavenging these elements reduces the likelihood of colonisation by other microbes by limiting the availability of iron to exogenous bacteria.

Overall the limited nutrients and water availability upon skin restricts the types and quantities of colonising organisms to those adapted to the specific environment. Nevertheless, the range of anatomical regions and thus environmental niches on the human host can result in a highly diverse skin associated microbiota.

1.3 The microbiota of the skin

Bacterial colonisation of the skin occurs almost immediately after birth via contact transfer with microbial reservoirs such as the birth canal, clothing, skin and the local environment (Reid *et al.*, 2010; Savey *et al.*, 1992). The microbial consortium which establishes itself can vary considerable between individuals due to chance

events associated with the types of organisms transferred, and the anatomical location (ecological niches). Changes in host-dependent factors such as hormones, age and health status will continue to affect the population type and characteristics throughout the life of the host by directly and indirect influencing the epidermal secretions and rigidity of the skin (Chiller *et al.*, 2001; Tlaskalová-Hogenová *et al.*, 2004). The microbiota of the skin is taxonomically complex and varied subsequently, studies have been undertaken to try to characterise skin microbiota. Traditionally, these studies have been culture based, relying on the recovery of viable and culturable (under specified conditions) organisms. The organisms commonly isolated are shown in Table 1.2. Culture techniques are simple and economical when used to identify specific organisms however, they can be laborious and require a degree of expertise to interpret the results. Additionally, there are intrinsic limitations related to culture techniques when applied to the study of microbial biodiversity. The difficulties associated with providing specific nutrients and atmospheric requirements for all potential colonising organisms combined with variable growth rates can result in significant underestimation of the complexity of a community. Recent advances in PCR-based methodologies and their subsequent application have allowed for more holistic characterisation of skin microbiota, for example one molecular based study of the bacterial diversity of inner elbow of five healthy human volunteers identified over 113 phylotypes, with increased numbers of *Proteobacteria* identified than from typical culture analysis (Grice *et al.*, 2008). Dekio *et al.,* (2005) analysed the forehead skin microbiota of five healthy volunteers. Analysis of the 16s rRNA gene from subcultured strains identified from culture and 16s rRNA gene clone libraries derived from amplification of 16s rRNA, identified an overall increase in the number of organisms identified (based upon the nearest match in the DDBJ,

EMBL and GenBank databases) when compared to culture. These organisms included *Acinetobacter* spp. *Pseudomonas* spp. and *Stenotrophomonas maltophilia.* The study however, failed monitor the microbial population over time and therefore, some of the organisms identified may represent transient rather than resident flora (Dekio *et al.*, 2005).

Table 1.2 Bacterial skin residents and their associated dermatoses (Chiller *et al.*, 2001).

Bacterium	Location	Distinguishing features	Skin pathology
Gram Positive			
Staphylococcus epidermidis	Upper trunk	Production of slime	
Staphylococcus hominis	Glabrous skin		
Staphylococcus haemolyticus			
Staphylococcus capitis	Head		
Staphylococcus warneri			
Staphylococcus saprophyticus	Perineum	Causes UTI	
Staphylococcus capitis			
Staphylococcus simulans			
Staphylococcus saccharolyticus	Forehead/antecubital	Anaerobic	
Micrococcus luteus			
Micrococcus kristinae	In children		
Micrococcus nishinomiyacnsis			
Micrococcus roseus			
Micrococcus sedentarius			Pitted ketatolysis
Micrococcus agieis			
Corynebacterium minutissimum	Intertrginous	Lipophilic/porphyrin	Erythrasma
Corynebacterium tenuis	Intertrginous	Lipophilic	Trichomycosis
Corynebacterium xerosis	Conjunctiva	Lipophilic	conjuntivitis
Corynebacterium jeikeium	Intertrginous	Lipophilic/antibiotic resistance	
Rhodococcus spp		Lipophilic	Granuloma in HIV
Propionibacterium acnes	Sebaceous gland	Lipophilic/anaerobic	Acne
Propionibacterium granulosum	Sebaceous gland	Lipophilic/anaerobic	Severe acne
Propionibacterium avidum	Axilla	Lipophilic/anaerobic	
Bravibacterium spp.	Toe webs	Non lipophilic	Foot odour, white piedra
Dermabacter spp.		Non lipophilic	Pitted keratolysis
Gram Negative			
Acinetobacter spp.	Dry areas		Burn wounds

Costello *et al*., (2009) surveyed the bacterial community bacteria from up to 27 sites in seven to nine healthy adults on four occasions to investigate the spatial and temporal distribution of the human microbiota. Body sites samples included 18 skin locations, the gut (stool) and oral cavity. Utilising primers specific the variable region 2 of the 16s rRNA gene; data derived from skin sites revealed a high level

of bacterial diversity shown in Figure 1.3 which, in at least one site tested (typically the back of the knee or palm of the hand) was higher than the microbial diversity associated with the individuals gut or oral cavity. Sites which displayed a high bacterial diversity included the forearm, palm, index finger, back of the knee, and sole of the foot. Interestingly contralateral sites i.e. left and right sides of the body showed little variation within an individual and the composition of sites varied significantly less within habitats than between habitats indicating site specific communities and relative stability (Costello *et al.*, 2009).

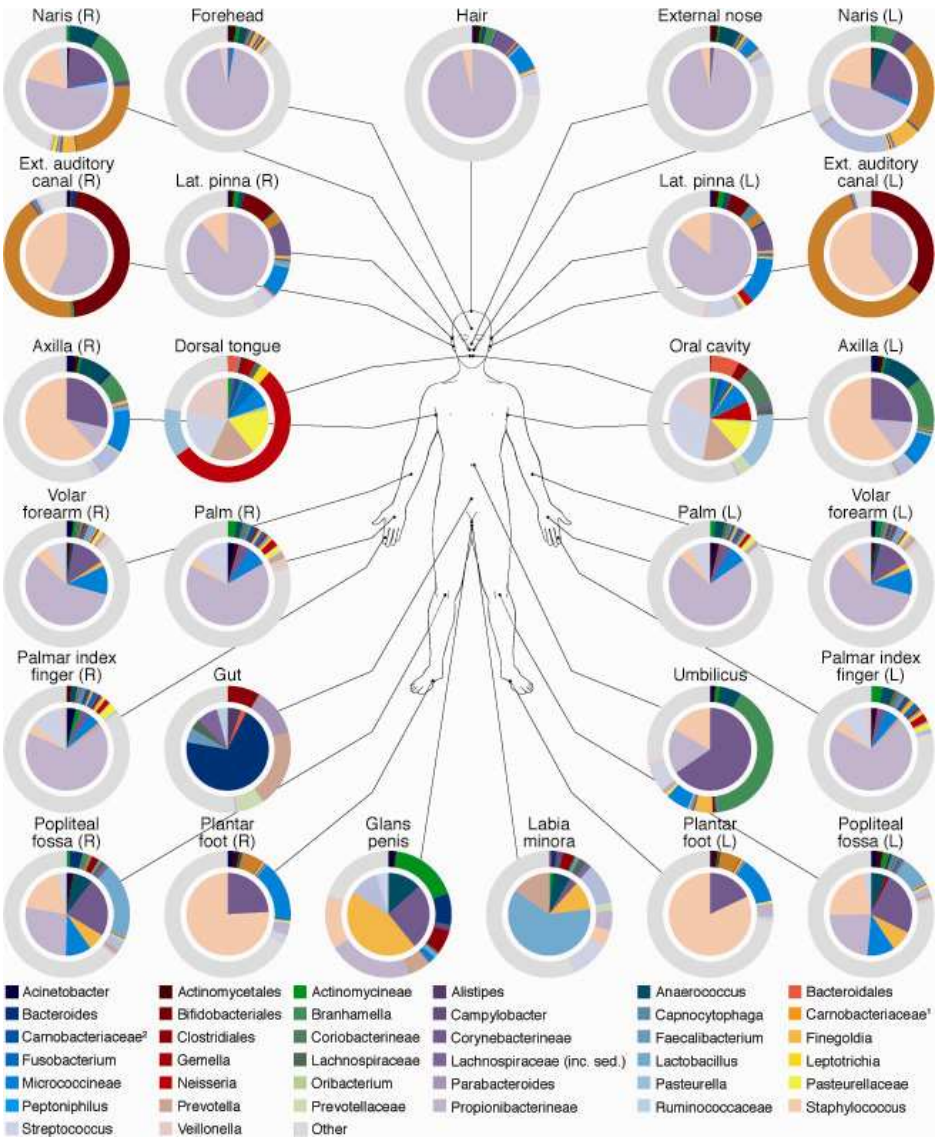


Figure 1.3 The distribution of microbes across the human body based of 16S rRNA gene analysis. For each region of the body, a pie chart shows the microbial community. The subdivisions of these are shown by the inner pie charts. Each colour corresponds to one of the forty microbial groups found in the bodies sampled (Costello *et al.*, 2009)

The employment of molecular based techniques to characterise the skin microbiota in the current literature has revealed highly diverse skin-associated bacterial communities containing genera and species previously not identified using routine culture techniques. However, it is important to note that despite the increase sensitivity of PCR-based methodologies some intrinsic limitations still apply; differential lysis of bacteria, different specificity of primers and variations in amplification efficiency can affect the overall measure of diversity, creating (to a degree) bias towards those bacteria which fulfil the methodology criteria (Reysenbach *et al.*, 1992; Suzuki and Giovannoni, 1996; Wilson *et al.*, 1997).

In general the skin microbiota is highly complex, in which its diversity is directly dependant upon the nutrient and water availability and thus is indirectly dependant upon the host health status. However, members of the skin microbiota can support the protective function of the skin towards the host via the production of toxic metabolites, bacteriocins and by direct competition for attachment sites preventing attachment and proliferation of exogenous pathogenic bacteria and therefore the consortia and the host are in a constant state of ecological balance (Bourlioux, 1997; Brodell and Rosenthal, 2008; Chiller *et al.*, 2001; Fredricks, 2001).

1.4 The role of the microbiota in the protection of the host

A number of skin associated microorganisms produce bacteriocins which aid in the protection of the host via their antimicrobial activity against taxonomically similar organisms inhabiting the same ecological niche (McAuliffe *et al.*, 2001; Varella Coelho *et al.*, 2007). Bacteriocins are ribosomally synthesized peptides which are generally highly cationic, heat-stable and can be divided into three groups; Class I,

Class II and Class III. Class I are known as lantibiotics these are small (<5 kDa) peptides containing the amino acid lanthionine which can be further subdivided into two groups; type-A and type-B, based on chemical structure. Class II bacteriocins are small (<5 kDa) heat-stable, non-lanthionine containing peptides, and Class III are large (>30 kDa) peptides (McAuliffe *et al.*, 2001). Type-A lantibiotics and Class II bacteriocins form transmembrane pores and lipid II-mediated pores in the target bacterial cytoplasmic membrane disrupting the proton motive force whereas Type–B lantibiotics inhibits the synthesis of peptidoglycan (McAuliffe *et al.*, 2001; Smith and Hillman, 2008). Several strains of *Staphylococcus epidermidis* produce lantibiotics and other bacteriocins including epidermin, epilancin K7, epilancin K7, epicidin 280, Pep5 and staphylococcin 1580 which are active against a number of coagulase negative staphylococci (Cogen *et al.*, 2008; Varella Coelho *et al.*, 2007).

In addition to the productions of bacteriocins, a further benefit of the resident bacterial community on the skin is the prevention of adherence of exogenous bacteria. Once established, the skin microbiota tends to maintain a state of equilibrium, therefore in order to successfully colonise, any transient bacteria must compete with the extant community for nutrients and space (Chiller *et al.*, 2001; Fredricks, 2001). In the highly competitive environment of the skin this can prove difficult and thus a symbiotic relationship is created in which the host is protected from pathogenic transient flora.

In general, the skin microbiota is complex, diverse and plays a symbiotic role in the protection of the host. Nevertheless, when the skin becomes compromised via injury, surgery or as a consequence of underlying aetiologies, the resultant wound becomes rapidly colonised with members of the autochthonous community and

exogenous bacteria. Underlying aetiologies such as those associated with the development of chronic wounds delay wound closure increasing the risk of infection occurring further impeding the normal healing process and exacerbating the state of chronicity (Enoch, 2005; Menke *et al.*, 2007). Chronic wound infections can be costly, resulting in prolonged hospital stays, an increased risk of secondary site infections and septicaemia, and are a significant cause of morbidity (Hill *et al.*, 2003; Howell-Jones *et al.*, 2005).

1.5 Types of chronic wound

There are a wide range of causative factors associated with the development of a chronic wound, these can be both physiological relating the patients underlying aetiology and biological relating to the presence of microorganisms, either inducing the chronic state or exacerbating it, as shown in Table 1.3. Although there are numerous causative factors the majority of types of chronic wound listed are rare, with the most common types being pressure ulcers/sores, venous ulcers and diabetic ulcers, which are intrinsically linked to the disease, nutritional and activity status of the individual.

Table 1.3 Causative factors leading to the development of a chronic wound (Eaglstein and Falanga, 1997)

Vascular occlusion	Inflammation	Pressure Necrosis	Physical agents	Infection	Tumours
Venous insufficiency	Pyoderma gangrenosum	Pressure sores	Radiation	Bacterial	Lymphomas
Artheroscerosis	Necrobiosis lipoidica diabeticorum	Neuropathic ulcers	Heat	Fungal	Metastases
Antiphospholipid syndrome	Panniculitis		Frostbite	Mycobacterial	Primary skin tumours
Cryofibrinogenemia/ cryoglobulinemia	Dysproteinemias		Chemicals	Tertiary syphilis	
Sickle cell disease	Idiopathic Leukocytoclastic Vasculitis		Factitial		
Cholesterol emboli	Periarteritis nodosa				
	Wegener's granulomatosis				
	Lymphomatoid granulomatosis				
	Erythema elevatum diutinum				

1.5.1 Pressure sores

Pressure sores/ulcers are one of the most common types of chronic wounds, and generally occur in individuals who are paralysed, debilitated, unconscious or suffer from neuropathy (Eaglstein and Falanga, 1997). They result from prolonged contact between the body surface and objects. Compression of the soft tissues results in restricted venous outflow raising the interstitial pressure; this initially is reversible by removing the pressure however, if it is prolonged fluid begins to accumulate creating an oedema in the interstitial spaces, this can led to ischemic necrosis and pressure sore formation of the intervening tissues (Bass and Phillips, 2007; Vasconez et al., 1977). Subcutaneous muscle is at greater risk during prolonged anoxia due to its high metabolic activity and consequently damage is greater in these tissues compared to the surface tissues creating a "cone" of

necrosis i.e. the majority of the tissue damage is located in the deeper tissues whereas those closer to the surface show little or no sign of damage (Bass and Phillips, 2007). Preventative measures include; identifying high risk patients, frequent assessment and preventative measures e.g. repositioning, pressure relief bedding moisture barriers and adequate diet.

1.5.2 Venous ulcers

There is a direct link between individuals with venous hypertension and the development of venous leg ulcers due to a failure of ambulatory venous pressure to fall as a result of venous insufficiency of the perforating vein valves or deep venous obstruction resulting in tissue ischemia and the subsequent development of an ulcer (Crane and Cheshire, 2008; Eaglstein and Falanga, 1997). Although this association between individuals with venous hypertension and the development of venous ulcers is established the mechanisms/process which led to ulceration are not fully understood. There are however, a number of theories as to the pathogenesis of the venous hypertension ulcer. These include (i) the fibrin cuff theory which suggests increased venous hypertension in the lower extremities leads to damage to the capillary walls resulting in leakage of plasma proteins and fibrinogen (Ibrahim *et al.*, 1996). These products then form a fibrin cuff in pericapillary area which act as barriers to oxygen diffusion resulting in tissue ischemia (Eaglstein and Falanga, 1997; Ibrahim *et al.*, 1996). (ii) The white cell rheology theory implicates white blood cells which have become attached to the capillary endothelium as a result of a reduction in capillary blood flow from venous hypertension. The white cells become activated releasing free radicals, proteolytic enzymes and cytokines resulting in capillary damage (Ibrahim *et al.*, 1996). (iii) the

seepage of macromolecules into the dermis due to hypertension, “trapping” growth factors and matrix proteins making them unavailable for maintaining tissue integrity and repairs (Crane and Cheshire, 2008; Eaglstein and Falanga, 1997; Falanga, 1993).

1.5.3 Diabetic ulcers

Patients with both type 1 and type 2 diabetes are at an increased risk of developing diabetic ulcers as a result of diabetic neuropathy and peripheral vascular disease (Rathur and Boulton, 2007). Neuropathy is estimated to be approximately 8% in newly diagnosed patients and 50% in patients with long standing disease (Boulton *et al*., 2005). Diabetes associated hyperglycaemia plays a role in diabetic neuropathy and microvascular complications by disrupting pathways related to metabolic and/or redox state of the cell such as the polyol pathway, resulting in an imbalance in the mitochondrial redox state of the cell causing excess formation of reactive oxygen species which promotes inflammatory reactions leading to microvascular damage (Edwards *et al*., 2008). Microvascular damage decreases neurovascular blood flow leading to neural and tissue damage. These physiological outcomes combined with trauma and microbial interactions lead to ulceration via the same mechanisms as pressure sores (due to a loss of sensation) and venous ulcers (due to the peripheral vascular disease). Additionally, physiological changes associated with diabetes such as thickening of the basement membrane and arteriolar hyalinosis results in altered migration of leucocytes and abnormal autoregulatory capacity, decreasing the wound healing rate and increasing the risk of infection (Falanga, 1993). Diabetes patients also have impaired neutrophil and macrophage function, which includes decreased

neutrophil migration, lymphocyte transformation and monocyte chemoattractant protein. Furthermore, glycation end-products (sugar molecules bound to proteins) interact with receptors found on most cells termed receptors for advanced glycation end-products (RAGE) causing a release of pro-inflammatory molecules e.g. tumour necrosis factor- α (TNF-α) and matrix metalloproteinase (MMPs) and a decreased collagen deposition (Ahmed, 2005). This results in a diminished ability to remove pathogenic organisms, necrotic tissue or other foreign material delaying the healing process.

The development of a wound as a consequence of the underlying aetiologies creates a portal of entry for bacteria, in which the risk of infection is greatly increased due to the delayed healing period associated with the aetiologies giving rise to an extended opportunity for pathogenic organisms to colonise and invade the wound. The presence of heavy bacterial and/or fungal colonisation has been cited as a biological factor which can induce or exacerbate a chronic inflammatory state associated with some chronic wounds but more significantly can lead to further tissue damage and impaired healing.

1.6 The microbial ecology of wounds

There is a great deal of debate regarding the role of microorganisms in wounds; wounds rapidly become colonised with a variety of organisms but not all become chronic. Previously emphasis has been placed on the number of organisms rather than the types present, with the exception of beta-haemolytic streptococci (due to their ability to cause infections at significantly lower levels than other pathogenic organisms) a figure of ≥ 10^6 organisms per gram of tissue has been associated

with impaired healing (Murphy *et al.*, 1986; Robson, 1979; Robson, 1997). However, it must be noted that colonisation alone does not cause delayed wound healing (Edwards and Harding, 2004), it is the shift from colonising to invading/infecting organisms that results in delayed healing. This shift is dictated by host-microbial interactions, specifically; the quantity, virulence and pathogenicity versus the physical capacity of the host to mount an effective immune response (Edwards, 2004).

Wounds initially become colonised with members of the skin microbiota however, over time the wound microbiota can change to include exogenous bacteria including *Staphylococcus aureus*, *Streptococcus* spp. and *Pseudomonas* spp. increasing tissue damage. As the severity of the wound increases, deeper tissues may become involved facilitating the growth anaerobic populations due to reduced oxygen availability in these tissues. The resultant microbial ecology of a wound can differ greatly from its initial origins of the skin microbiota and other wounds different locations. Variations in location, size and depth of the wound can result in differences in temperature, pH, nutrients and local flora (Chiller *et al.*, 2001; Tlaskalová-Hogenová *et al.*, 2004). In addition, the use of antimicrobials, cleaning and debridement procedures can also affect the microbial population. There are a number of organisms capable of causing superficial skin and wound infections. The Health Protection Agency issue a collection of clinical microbiology standard operating procedures and algorithms derived from published literature which are generally employed in CPA accredited microbiology laboratories of NHS hospitals to aid in the identification of pathogenic bacteria. Hospital site specific variations occur with the types of media used and organism and sensitivity reporting, however a general list of recommended media and target organisms is shown in

Table 1.4. Investigations of acute and chronic wound infections using the criteria in Table 1.4 typically isolates bacterial organisms such as; *Staphylococcus aureus*, Lancefield groups A, B, C and G streptococci, *Bacteroides* species, *Clostridium* species, anaerobic cocci, coagulase-negative staphylococci, *Corynebacterium* species, enterobacteriaceae and pseudomonads (HPA, 2009).

Table 1.4 Culture media, conditions and organisms for all wound specimens (HPA, 2009)

Clinical Details/conditions	**Standard media**	**Incubation**			**Target Organisms**
		Temp ℃	**Atmos**	**Time**	
All swabs	Staph/strep selective agar	35-37	Air	40-48 hr	*S. aureus* Lancefield Groups A, C and G streptococci.
	Neomycin fastidious anaerobic agar with metronidazole 5 µg disc	35-37	Anaerobic	40-48 hr*	Anaerobes
	Additional Media				
Cellulitis in children Human Bites	Chocolate agar (with bacitracin or bacitracin 10 µg disc)	35-37	5-10 % CO_2	40-48 hr	*Haemophilus* spp.
Burns Immunocompromised Patients Diabetic patients Interigo Paronychia	Sabouraud agar	35-37	Air	40-48 hr*	Fungi
Cutaneous diphtheria Foreign travel	Hoyle's tellurite agar	35-37	Air	40-48 hr	*C. diphtheriae* *C. ulcerans*
	Optional Media				
Diabetic wounds	CLED	35-37	Air	16-24 hr	Enterobacteriaceae Pseudomonads
All swabs	Blood Agar	35-37	5-10 % CO_2	40-18 hr	Lancefield Groups A, C and G streptococci *Pasteurella* species *S. aureus* *Vibrio* species *Aeromonas* species

* 5 days extended incubation if clinically indicated

Bowler and Davies (1999) employed rigorous culture and identification techniques (utilising a wider variety of agar plates, broths and environments) in a clinical study to investigate and compare the aerobic and anaerobic microbiology of infected and

non-infected leg ulcers. Infection was defined on the basis of clinical signs e.g. erythema, cellulites, oedema, heat and increased pain and exudates. The anaerobic population represented 36% of the total microbial composition in non-infected ulcers compared with 49% in infected leg ulcers, with a greater number of *Peptostreptococcus* spp. and *Prevotella* spp. observed in infected ulcers. No significant difference in terms of the aerobic population was found (Bowler and Davies, 1999). Although these techniques yielded an increase in recovery rates and subsequent identification, these methods can be time consuming and may still overlook the unculturable population.

Traditional culture techniques employed within clinical diagnostic laboratories are designed to isolate potentially pathogenic bacteria in an optimal timeframe but may fail to detect unculturable, slow growing and/or fastidious populations (Wade, 2002; Wilson *et al.*, 1997). Organisms which are cultivated but are not considered potentially pathogenic in the patients' clinical situation are also often disregarded. Another drawback of culture techniques is the tendency for cultures to become "overgrown" with predominant fast growing organisms, limiting the ability to identify different populations. These factors coupled with the overall limitations of culture have strengthened the need for the application of culture-independent techniques to wound ecology.

1.6.1 Molecular analysis of wound microbiota

Hill *et al*., (2003) applied molecular and enhanced cultural techniques to determine the microbial composition of a chronic venous leg ulcer. Both tissues and swab samples were taken. Culture analysis detected *Acinetobacter* spp. in both types of

samples. Swab samples yielded *Proteus* spp. and *Candida tropicalis* whereas cultivated tissue samples contained *Staphylococcus epidermidis*. Molecular analysis identified clones which were closely related to these cultured organisms, several clones were however, closely related to organisms which had not identified by culture. These included *Morganella morganii*, *Bacteroides urelyticus*, *Enterococcus faecalis* and *Peptostreptococcus octavius*. This not only exemplifies the ability of molecular techniques to identify a wider range of organisms, but also the affect of sampling techniques on the types of viable organisms recovered (Hill *et al*., 2003). A similar study conducted by Davies *et al*., (2004) also found a greater diversity of organisms by utilising PCR targeted on 16s rRNA gene, in addition to denaturing gradient gel electrophoresis (DGGE) to analyse the microbiota of healing and non-healing chronic venous leg ulcers. Of the sequences obtained; 40% represented organisms which had not been identified using culture similar strains were however, cultured from other wound samples. Furthermore, four organisms were identified by sequencing which have not previously been associated with chronic wounds; *Paenibacillus* spp., *Gemella* spp., *Sphingomonas* and *Afipia* spp. (Davies *et al*., 2004). A direct comparison of the microbiotas of the healing and non-healing ulcers highlighted a significant difference in the carriage of *Micrococcus* and *Streptococcus* spp. with a higher incidence in non-healers. However, it is noted that in a larger study of 66 wounds no significant associations were found between specific bacterial groups and the healing or non-healing phenotype (Davies *et al.*, 2004) indicating that no specific individual or group of previously uncultured bacteria is responsible for the chronic wound.

Colonisation and eventual infection of the wound and deeper seated tissues not only results in an inflammatory response which if prolonged (due to ineffective

elimination of the organisms) can degrade new tissue growth, it can also result in additional damage to the tissue due to the production of bacterial virulence factors.

1.6.2 Virulence factors of pathogenic wound bacteria

A number of bacteria produce virulence factors such as endotoxin and exotoxin which enhance their pathogenicity either by damaging host's tissue to allow invasion or interfering with the hosts immune response to maximise survival. Endotoxins are predominantly the lipopolysaccharide (LPS) component of the outer membrane of Gram negative organisms such as *Pseudomonas* spp. and *Escherichia coli*. The LPS can range in size from 10-20 kDA (monomers) to 1000 kDa (vesicles) and consists of Lipid A, a core oligosaccharide and a long heteropolysaccharide chain. The LPS is generally shed during cell death. Its virulence stems from the activation of inflammatory cytokines e.g. IL-1β, IL-6 and TNF-α and also through activation of the compliment and coagulation cascade (Gorbet and Sefton, 2005) which can lead to tissue destruction from reactive oxygen species and hydrolytic enzymes derived from an excessive immune response. Exotoxins are normally toxic proteins secreted from the bacterium, for example *Clostridium perfringens* produces collagenase and lecithinase which breaks down connective tissue and lecithin of the cytoplasmic membranes resulting in oedema and tissue loss associated with gangrene (Titball *et al*., 2002). The obligate aerobe *Pseudomonas* spp. is typically found in burn injuries and on the surface tissue of chronic wounds, it is known to produce haemolysins, elastase and exotoxin A which inhibits protein synthesis of the host tissue cells causing cell death leading to necrosis (Bourke *et al*., 1994; Lyczak *et al*., 2000). Staphylococci, particularly *Staphylococcus aureus* is commonly isolated from both chronic and

acute wounds and are known to secrete a variety of enzymes and cytotoxins which aid in their proliferation and invasion of soft tissues. These include; four haemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase which directly damage the connective tissue (Dinges *et al*., 2000). In addition, some strains of staphylococci express a surface protein called protein A; this disrupts opsonisation of the bacteria and thus aids in its survival. Streptococci are also commonly found in both acute and chronic wounds, these have a number of virulence factors which include the production of streptolysin, streptokinase and hyaluronidase disrupting leucocytes and connective tissue (Bang *et al*., 1999).

The production of virulence factors by invading bacteria allows for the dissemination of the source bacterium and other microorganisms that are present in the consortium. Polymicrobial infections have been noted to be more virulent than infections caused by single organisms (Hendricks *et al.*, 2001) this is attributed to bacterial synergistic relationship.

1.6.3 Bacterial synergy in polymicrobial infections

A purported synergistic relationship between anaerobic bacteria and aerobic/facultative organisms has been noted in some wound infections. Using a mouse experimental wound model Brook (1985) investigated the synergistic relationship between *Bacteroides* spp. and various aerobes and facultative organism in mixed species infections. Organism used included; *Haemophilus influenzae* type b, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, and Lancefield group A and group D streptococci.

Bacterial suspensions were adjusted to 10^8 colony forming units/ml (CFU/ml), 0.1 ml of which was injected subcutaneously into the right groin of mice. Combined infections were induced by 0.1 ml of each bacterium combined in 0.2 ml injection. Counts of total CFU of each organism in the abscesses were made. *Bacteroides* spp. grew in axenic cultures, however, when combined with aerobic and facultative partners growth of *Bacteroides* spp. was enhanced when compared to the axenic inoculation of *Bacteroides* spp. It is believed that ability of the aerobic or facultative organisms to lower the oxidation-reduction potentials in the host tissue facilitation the growth of anaerobic species (Brook, 1985).

Hendrick *et al.,* (2001) investigated the use of surfactants to disinfect complex orthopaedic wounds in a rat model. The authors observed that when a combination of *S. aureus* and *P. aeruginosa* was used, the amount of inoculum required to cause a reproducible infection was much smaller than the amount required when either organism was used alone (Hendricks *et al.*, 2001). Combinations of 10^3 CFU/ml of *S. aureus* (one tenth of the S*. aureus* ID_{50}) with *P. aeruginosa* (10^2, 10^3, or 10^4 CFU/ml) yielded infection rates that were higher than those found with either organism alone. The rate of infection peaked at 75% with combinations of 10^3 CFU/ml of *S. aureus* and 10^3 CFU/ml of *P. aeruginosa*. Further increments the concentration of *P. aeruginosa* (10^5, 10^6, and 10^7 CFU/ml) resulted in a decrease in the infection rate with the final concentration of 10^3 CFU/ml *S. aureus* and 10^7 CFU/ml of *P. aeruginosa* only resulting in 33% infection rate. Interestingly, for the final concentration mix *S. aureus* was the only cultivated organism from these polymicrobial infections sites. Overall this indicates that the presence of low levels of *Pseudomonas aeruginosa* enhances the growth of *S. aureus.* Moreover, it highlights an possible inhibitory affect of *S. aureus* on *P. aeruginosa* growth in

these polymicrobial infections indicating that *S. aureus* may have more of a parasitic role in this polymicrobial infection (Hendricks *et al.*, 2001).

It is evident some that synergistic bacterial relationships may occur within the polymicrobial consortia of the wound and that parasitic/antagonistic interactions may also occur to facilitate invasion and the overall production of a polymicrobial wound consortia. This polymicrobial community is able to rapidly establish itself due to the nutrient rich environment provided by the damaged tissue and wound exudate.

1.7 Wound exudate as a source of nutrients for microbial growth

A stated in section 1.2 in order to proliferate, microorganisms generally require specific growth substrates. Within a wound the wound exudate is the primary source of nutrients for microbial growth. Wound exudate is essentially derived from blood serum and contains essentially the same constituents in similar quantities shown in Table 1.5.

Exudate is excreted as part of the inflammatory response and functions to maintain the moisture of the wound by facilitating neutrophil and macrophage survival. Furthermore, it contains several inflammatory components such as leukocytes, fibrinogen and fibrin and provides essential nutrients for epithelial cell re-growth which including vitamins, amino acids and trace elements such as calcium, copper, iron, manganese and zinc and electrolytes (Cutting, 2003; Jones *et al.*, 2001). Both chronic and acute wounds produce exudate, however due to the exaggerated inflammatory response in some chronic wound the exudate may contain higher levels of tissue degrading components such as MMPs, serine proteinases,

interleukin-1α and transforming growth factor-β when compared with acute wound fluid (Cutting, 2003; Diegelmann, 2003; Jones *et al.*, 2001). Despite the immune response, microorganisms can capitalise on the nutrient availability of the wound exudate. The nutrient rich milieu of the exudate and surround tissues provides a rich source of essential growth factors such as carbon, nitrogen, and iron. Furthermore, an exaggerated immune response can result in additional tissue damage; this counterintuitive response facilitates the further invasion of bacteria into the tissue.

Table 1.5 Comparison of biochemical analytes from serum and wound fluid samples (Trengove and Langton, 1996)**.**

Substance	Serum Reference Range	Wound Fluid Range
Sodium	137-146 mmol/L	133-146
Potassium	3.6-5 mmol/L	3.2-5.7
Chloride	98-108 mmol/L	96-109
Urea	2-8.6 mmol/L	2.5-22.6
Creatinine	45-115 µmol/L	46-334
Uric acid	140-480 µmol/L	221-751
Calcium	2.15-2 mmol/L	1.84-2.72
Magnesium	0.7-0.91mmol/L	0.75-1.24
Phosphate	0.8-1.46 mmol/L	0.98-1.47
Bicarbonate	22.32 mmol/L	14-22
Glucose	3.5-5.5 mmol/L	0.6-5.9
Lactate	0.3-1.3 mmol/L	5.4-16.7
Lactate dehydrogenase	310-620 U/L	789-9901
Alkaline phosphatise	55-120 U/L	24-146
Alaninie aminotransferase	8-60U/L	3-111
Aspartate aminotransferase	4-50 U/L	10-159
Gamma glutamyltranspeptidase	8-60 U/L	19-194
Creatine kinase	20-260 U/L	20-62
Total bilirubin	3-20 µmol/L	12-52
Total Protein	56-79g/dL	26-521
Albumin	36-50 g/dL	14-28
C-reactive protein	<6mg/L	2.5-25
α-1-Globulin	2-5 g/dL	1.0-2.5
α-2-Globulin	4-9 g/dL	2.7-6
β-Globulin	5-10 g/dL	3.1-8.8
γ-Globulin	8-16 g/dL	3.9-9
C3	0.55-1.2 g/dL	0.15-0.71
C4	0.2-0.5 g/dL	0.08-0.35
Cholesterol	55 mmol/L	1.2-3.2
Triglycerides	0.3-18 mmol/L	0.4-4.3

The polymicrobial nature of chronic wounds has led to a goal of understanding population dynamics with the objective of determining the role of microorganisms in wound healing. The multispecies biota has been intensively studied in oral and gastrointestinal tract microbiology and in recent years emphasis has been placed upon their population interactions and biofilms, subsequently interest has been increased in the multispecies microbiota of chronic wounds and the potential role of biofilms.

1.8 Multi-species biofilm formation

Biofilms have been defined as “structured bacterial communities enclosed in a self-produced exopolysaccharide matrix and adherent to abiotic or biological surfaces” (Costerton *et al*., 1999). They are problematic in both medical and industrial domains because they confer an increased resistance to antibiotics, phagocytosis, biocides and physical removal. The developmental process of multispecies biofilms is dynamic by which bacteria attach to abiotic or biotic surfaces through a series of co-dependant and independent processes (Costerton, 1999). Initially, primary bacterial colonisers have to adhere to the surface or substratum. Flagella and pili can assist initial contact by overcoming the repulsive forces between the bacterium and the surface, subsequently increasing attachment efficiency and enhancing reversible attachment (Stoodley *et al.*, 2002). However, it must be noted that flagella and pili expression and activity are not a prerequisite for bacterial attachment and subsequent biofilm production since non-motile bacteria have demonstrated the ability to attach to a surface and develop biofilms in flow chambers and microtitre plate wells with a citrate minimal medium (Klausen *et al.*, 2003). According to a widely accepted model of biofilm formation,

once the bacteria come into contact with a surface, they retain their motile ability, aiding in the detection and exploitation of more favourable environments and thus allowing for a more selective colonisation process. If a favourable environment is located/maintained bacteria attach to the surface via complex formation between surface associated compounds and bacterial ligands (Busscher and Weerkamp, 1987; Palmer *et al.*, 2007). Once attached, if conditions remain propitious, cells begin to divide, spreading outward and upward from the attachment point creating microcolonies (Hall-Stoodley and Stoodley, 2002). Adhesion also triggers the synthesis of extracellular polymeric substances (EPS) such soluble glucans, fructans, heteropolymers, and alginate however, the specific constituents and overall composition of the EPS can vary depending on the organisms present and the environment (Marsh, 2004; Sutherland, 2001). The EPS enhances adhesion to the surface and to other bacteria of the same or different species. As the microcolonies grow and develop; secondary colonisers in the form of single or coaggregated/autoaggregated cells attach to the primary colonisers (process termed coadhesion) becoming part of the now developing biofilm. These attachments processes can be mediated by coaggregation interactions where reciprocal surface molecules of genetically distinct bacteria form complexes either within the developing biofilm or by external aggregation prior to their adhesion to cells of the biofilm (Rickard *et al.*, 2003a). These associations between the cells are normally facilitated by of lectin-carbohydrate complexes (Khemaleelakul *et al.*, 2006). Coaggregation has been extensively but not exclusively studied in oral bacterial communities where it purportedly plays a role in the formation of oral multispecies biofilms (Kolenbrander, 1988; Kolenbrander *et al.*, 2002; Ledder *et al.*, 2008; Rickard *et al.*, 2003a). An overview of bacterial coaggregation on the tooth is given in Figure 1.4 taken from Kolenbrander *et al*., (2002).

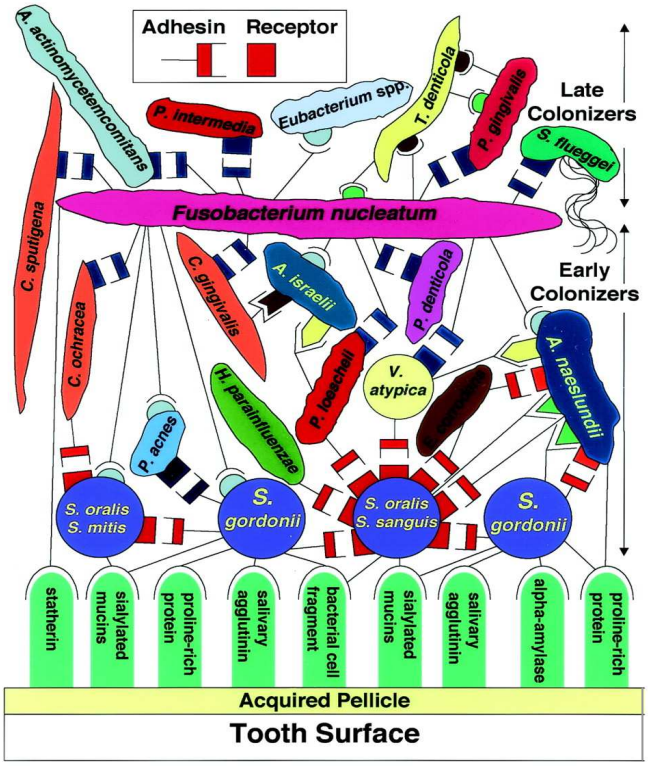


Figure 1.4 Spatiotemporal model of oral bacterial colonization depicting coaggregation between early colonizing bacteria and late colonizers on the tooth surface (Kolenbrander *et al.*, 2002)

Several studies have been undertaken to investigate coaggregation interactions between a range of organisms, the majority of which are organisms associated with the oral cavity however, a limited number studies have included organisms associated with the gastrointestinal tract, human nasopharynx and wounds (Hill *et al.*, 2010; Lafontaine *et al.*, 2004; Ledder *et al.*, 2008; Metzger *et al.*, 2001). Ledder *et al.,* (2008) investigated coaggregation between human intestinal and oral bacteria utilising a visual and a modified spectrophotometric assays. The modified spectrophotometric assay utilised the flocculation phenomenon to assess the percentage of clearing of the combined bacterial solution as the aggregates settle over time. The authors determined that the frequency of coaggregation amongst pair-wise interactions of organisms derived from the intestinal tract alone, and combined with organisms derived from the oral cavity was low, whereas the frequency of coaggregation amongst pair-wise combinations of organisms

associated with the oral cavity organisms was high (Ledder *et al.*, 2008). This and a large number of other reports suggest that coaggregation is a significant process in the formation of biofilms associated with the oral cavity. Overall, these interactions are believed to facilitate attachment of genetically distinct species to primary colonisers/microcolonies. These in turn can act as a "foundation" for other genetically distinct species to attach creating a multispecies biofilm. However, the role of coaggregation in the development of other medically significant multispecies communities such as those associated with chronic wounds, is still unknown.

1.9 Wound associated biofilms

To date a limited number of studies have attempted to show evidence of biofilm involvement in chronic wounds. James *et al.*, (2008) for example assessed wounds microscopically for assemblages characteristic of biofilms in tissue samples derived from acute and chronic wounds. Biofilms (in this study, recognised on the basis of aggregated cells and the putative identification of EPS encased cells via scanning electron microscopy) were identified in 30 of the 50 chronic wounds but in only and 1 of the 16 acute wounds. However, culture analysis of the tissue samples undertaken in this study revealed little or no bacteria present within the acute wound tissue samples. While culture analysis may not reveal the extent of the bacterial diversity of wounds, the limited number of organism associated with acute wounds could explain the lack of identified biofilms structures in the acute wound tissue samples investigated in this study. A study by Malic *et al*., (2009) identified microcolonies of bacteria in a non-infected venous leg ulcer biopsy using universal eubacteria florescent *in situ* hybridisation (FISH) probe and confocal

laser scanning microscopy (Malic *et al.*, 2009). The identification of microcolonies is frequently used to signify biofilm presence due to the proliferation of microcolonies during biofilm development however, this study only investigated one wound tissue sample from a single venous leg ulcer. An increased number of samples and wound types are required to establish the presence of biofilm in chronic wounds. The identification of biofilms in chronic wounds is only one facet of the paradigm of non-healing wounds, the role that these biofilms play in delayed healing and treatment failures is also important. Several other studies using animals models of wounds have also been undertaken to investigate the development and role of biofilms in delayed healing and their recalcitrance to antimicrobial treatments; Davis *et al*., (2008) used a porcine wound model to compare the effect of two topical antimicrobial agents (mupriocin cream and an ointment containing bacitracin zinc, polymyxin B sulphate and neomycin) on partial thickness wounds inoculated with *S. aureus*. Once wounds were inoculated, treatments were tested after 15 minutes to determine the effect on planktonic bacteria, and at 48 hours (after the application of occlusive dressings to promote biofilm proliferation) to determine the effect on biofilm-associated wound infections. Sectioned wound biopsies taken from the 48 hours wounds were observed via light microscopy, scanning electron microscopy and epifluorescence microscopy in which aggregated microcolonies and calcofluor white stained EPS were visualised. Reduced efficacy for both treatments was noted for the 48 hour biofilm-embedded infections. The authors claimed that this demonstrated that wound-associated bacteria are capable of establishing a biofilm with a relative short time frame and that this could reduce the efficacy of treatments. However, it should be noted that the application of an occlusive dressing provided an optimal environment for bacteria proliferation and survival and therefore the subsequent biofilm may not be

representative of the *in vivo* environment. Furthermore, longer-established infections can be more difficult to eradicate due to population density and tissue infiltration regardless of whether an organisms adopt the biofilm phenotype. In another animal study, Schierle *et al*., (2009) employed a murine cutaneous wound system to investigate the potential role of *S. aureus* and *S. epidermidis* biofilms in delayed healing times. The presence of both organisms significantly delayed wound closures; however, organisms with an endogenous mutation in the biofilm signalling pathway did not produce similar healing delays (Schierle *et al.*, 2009). This provides evidence demonstrating that delayed re-epithelialisation can be caused by the presence of a bacterial biofilms or that the signalling mechanism potentiate the pathogenesis in overtly pathogenic or autochthonous bacteria.

Overall these studies support the concept that colonised chronic wounds may harbour bacterial biofilms. The association of these with impaired wound healing is still subject to debate but some evidence is now available to suggest a causal or associative link. Representative model systems provide a useful means of better understanding of the complex polymicrobial biota and associated biofilms of the chronic wound by providing systems in which specific environments can be standardised and reproducible results gained.

1.10 Wound biofilm models

When designing/selecting a model system for the simulations of the *in situ* conditions, it is important to mimic the environment as closely as possible whilst ensuring reproducibility, temporal stability and accuracy of the results. The *in situ* environment of the chronic wound is highly complex, diverse and can vary

markedly between wounds, depending on the location, severity (tissue layers affected), size and the overall immune and health status of the patient. Animal models have a distinct advantage in wound repair and infection studies since they can represent the both the microbiological and physiological aspects of the healing and the immune reaction in response to wounding allowing for a variety of wounding scenarios and aspects to be investigated. A variety of animal models have been utilised for wound repair; small mammal and rodents are relatively inexpensive, easy to house, feed and can be genetically altered to mimic conditions such as diabetes (Perez and Davis, 2008). Nevertheless, significant differences can be seen in the dermal thickness, elasticity and hair follicles when compared to human skin (Meyer *et al.*, 1978). Accordingly, porcine wound models are also commonly used because porcine skin is structurally similar to human skin with similar physical and molecular responses. Pigs are however, slow to grow, difficult to handle and differences can be seen with wound contraction in relation to the wound position and the type of tissues investigated, when compared to humans (Gottrup *et al.*, 2000; Perez and Davis, 2008). A significant issue with *in vivo* biofilm research is the lack of non-destructive, reproducible, longitudinal monitoring system in which the efficacy of treatments can be continually monitored throughout the duration of the study without the need to destroy the animal (Kadurugamuwa *et al.*, 2003b). Recently this issue has been partially overcome with the employment of bioluminescent reporter systems in which bacterial strains of interest are engineered for bioluminescence. Strains are transformed with a modified *Photorhabdus luminescence lux* operon and are typically introduced to the animal model via implantation of a colonised device (Kadurugamuwa *et al.*, 2003a). Real-time biophotonic images the animals/infection site of using an *in vivo* imaging system (IVIS) camera system can monitor the progression of metabolically

active bacterial cells at the site of infection and thus can be used to address questions relating to migration, colonisation and efficacy of antimicrobial therapies. The bioluminescent signal has been shown to be closely proportional to the number of colony forming units recovered from the site (Kadurugamuwa *et al.*, 2003b) however, a ten-fold loss of signal intensity is associated with every centimetre of tissue depth therefore tissue penetration is limited to 2-3 cm, making comparisons between results of different imaged tissue sites or other animals models difficult (Leevy *et al.*, 2007). Whilst a useful tool, the bioluminescent system has some drawbacks; for example this system is unable to monitor multispecies biofilm infections and their population variations in response to treatments and is dependant upon infection sites which a visually assessable to the biophotonic camera system. This restricts infection sites that can be investigated and the animal species that can be utilised. Moreover, the requirement for oxygen during the oxidation of luciferin into oxyluciferin by luciferase to produce detectable photons in the presence of oxygen and ATP may limit it use to aerobic environments, also signal dependence on the growth phase and microenvironment could result in variability data in longitudinal studies (Leevy *et al.*, 2007; Shah and Weissleder, 2005). Despite the obvious benefits of animal wound models, issues including the cost of housing, variations in wound healing response between animals models, and the ethical and humane issues, alternatives systems have been developed and utilised. Recently reported alternatives to animal models include living human skin equivalents e.g. Graftskin which is derived by combining type I bovine collagen with living neonatal allogenic fibroblasts and overlying it with a cornified epidermal layer of neonatal allogeneic keratinocytes. Charles *et al*., (2009) employed a Graftskin to study *in vitro* biofilm formation. Four Graftskin specimens were divided into multiple sections and one millimetre full thickness

wounds were created on each Graftskin specimen using a 10-blade scalpel shown in Figure 1.5. Experimental wounds were then exposed to axenic bacterial suspension of *S. aureus* or *P. aeruginosa*. Charles *et al*., (2009) visualised attachment of bacterial clusters to the Graftskin, which is histologically similar to human skin with biopsy samples taken at various time points for visualisation of bacterial biofilms. Although there are obvious benefits of this system when compared to animal models this method does have some significant drawbacks which include the time associated with creating the skin equivalent (>10 days) and the limited long term application for the study of microbial communities and biofilms.

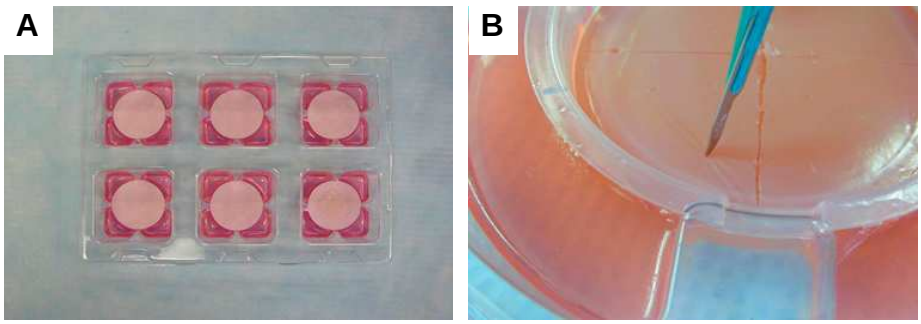


Figure 1.5 (A) Graftskin sections (B) Graftskin specimen was divided into multiple sections.

Werthén *et al*., (2010) observed a variation in the biofilm forming capacity of *S. aureus* ATCC 6538 and *P. aeruginosa* PA01 when grown in tryptic soy broth when compared to simulated wound serum and collagen coated microtitre plates. This led the authors to develop a wound biofilm model that incorporated both serum and collagen to better simulate the environment of a wound. Collagen stock solution of type I rat-tail collagen and simulated wound fluid was dispensed into wells of a 8-well culture slide to produce matrices of polymerised rat tail collagen type I. Bacterial suspensions of 10^5 cells/ml of simulated wound fluid was then added to the matrices and plates incubated for 24-48 h. This model provides a rapid, high

throughput system in which axenic or multiple bacterial populations can be investigated, using quantitative CFU analysis, sectional imaging or confocal scanning microscopy. Bacterial aggregates of *P. aeruginosa* visualised using confocal laser scanning microscopy in chronic wound tissue samples were comparable to aggregates in the collagen matrix (Werthen *et al.*, 2010). Additionally claims are made that the model could be utilised to study antimicrobial therapies and novel wound dressings. Nevertheless this model has a limited capacity for investigation microbial population dynamics over a greater period than two days due to the limited collagen matrix size and stability under rapid bacterial growth.

In general the incorporation of tissue equivalents into model systems allows for transferable *in vitro* observations to be made, in which the effect of bacterial growth and biofilm production on tissues which can be compared to the *in vivo* counterparts. However, the lack of immune response in these tissues is an issue since it allows unrestricted bacterial growth resulting in rapid tissue degradation, limiting the models application in longitudinal studies. Artificial, *in vitro* wound models have been employed in longitudinal studies and provide a useful means by which novel antimicrobials, treatments regimes and populations interactions can be studied under controlled, reproducible conditions. Often, investigations into wound infections and their intervention have their starting point in artificial *in vitro* wound models for pre-clinical testing prior to commencement in animal models. One *in vitro* model which has been extensively used to investigate biofilms in general is the constant depth film fermenter (CDFF) shown in Figure 1.6. The CDFF facilitates the development a consistent depth biofilms upon solid substrata such as polytetrafluoroethylene by controlling biomass accumulation. The substrata are

housed on a turntable recessed to a specified depth. As the turntable rotates static fixed positional scrapers blades remove excess biomass which accumulates thus maintaining the biofilm at a constant depth (McBain *et al.*, 2009; Wilson and Ron, 1999).

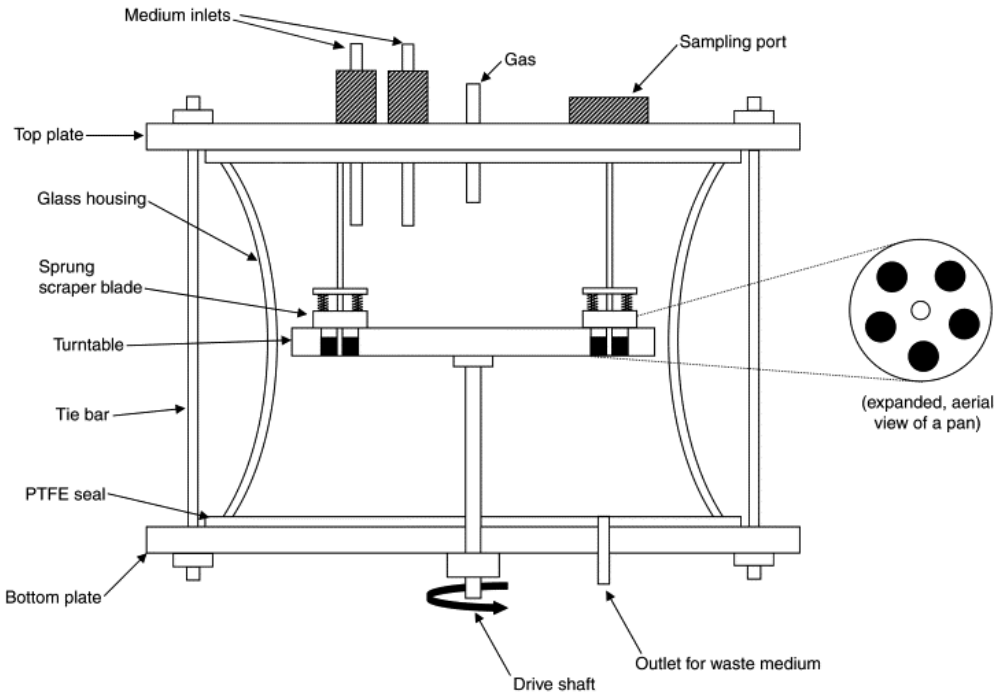


Figure 1.6 The constant depth fermenter (McBain *et al.*, 2009)

The CDFF has been extensively used to study dental plaque biofilms (Kinniment *et al.*, 1996; Ledder *et al.*, 2009; McBain *et al.*, 2003a; Pratten *et al.*, 1998). Both Hill *et al*., (2010) and Malic *et al*., (2009) have employed the CDFF to investigate multispecies biofilm growth of wound-isolated bacterial stains. All combinations of isolates which included *S. aureus*, *P. aeruginosa,* coagulase-negative *staphylococcus* spp. *Micrococcus* spp., *Streptococcus* spp., *Peptostretococcus* spp. and *Bacteroides* spp. formed reproducible multi-species biofilms in the CDFF which could be maintained up to 4 weeks. It is nevertheless important to note the CDFFs do not simulate some of the important aspects associated with the

environment of the chronic wound. Bacterial isolates within wounds derive nutrients and growth factors from the wound bed and exudate and are thus fed from below, however in the CDFF media is perfused from above, significantly different from the *in vitro* environment. Additionally, the rotary action of the turntable against static scraper results in shear forces which are dissimilar to the conditions within most wounds. Ultimately the CDFF can be used an *in vitro* model for the investigation of biofilm formation of bacterial isolates derived from chronic wound biofilms but other systems can be developed that will arguably represent a closer simulation of the chronic wound environment. Accordingly, Steffansen and Herpin (2008) described the use of an *in vitro* novel wound model to investigate application of dressing for role of low exudate level wounds and high exudate dressing wounds shown in Figure 1.7. The model utilised a Franz diffusion flow through cells with an open area between the donor and receptor compartments between which a the membrane of a tissue culture insert is covered with a 2% agar layer to represent a low exudate wound, or high-density polyethylene (HDPE) net is placed to represent a high exudate wound. The variation between the media diffusion rates though the two membranes resulted in a higher rate of “exudate diffusion in the HDPE net than the media impeded agar-anopore membranes (Steffansen and Herping, 2008). This model allows for the perfusion of media to occur from below and addresses the considerable variation between wounds by allowing exudate flow rate to be varied. However, this model was designed to primarily investigate the rate of release of antimicrobials, and absorbance of dressings in high or low exudate settings and was not intended as a means of testing bacterial growth rate or the process of biofilm formation.

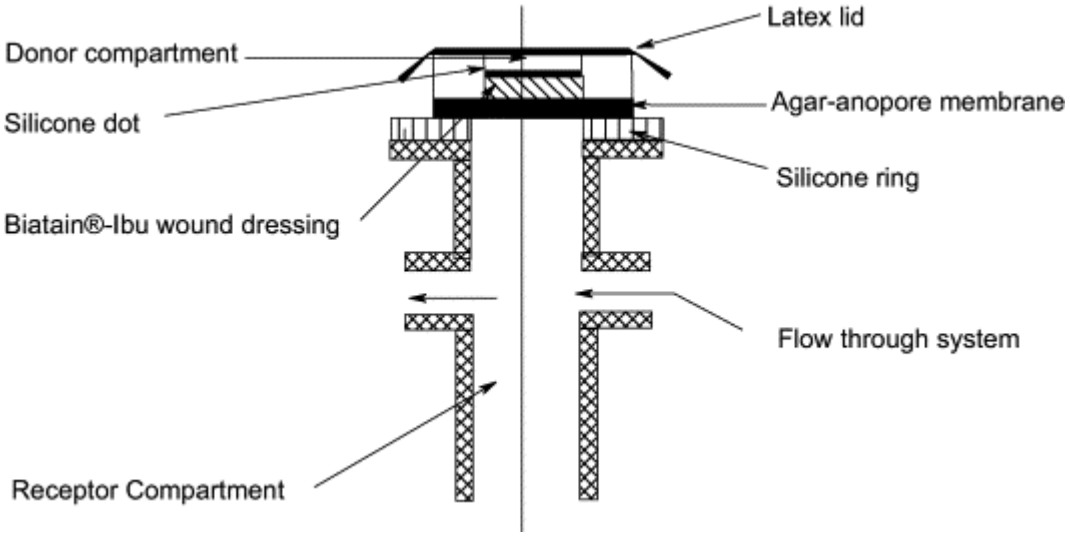


Figure 1.7 Illustration of the low exudate level wound (LEW) model

Lipp *et al*., (2010) described the use of a colony drip flow reactor (DFR) modified to house 25mm (diameter) absorbent pads attached to angled glass microscope slides to which 0.22µm porous polycarbonate membranes were placed shown in Figure 1.8. Media is perfused down the glass slide via gravitational forces where it is absorbed by the porous membrane. It is upon these porous membranes that the bacteria attach and grow under a continually media feed. The utilisation of an attached absorbent pad to which a porous polycarbonate membrane is placed is to allow for the attached bacteria to be fed continually from below with fresh nutrients. Axenic biofilms of *S. aureus* and *P. aeruginosa* were successfully cultivated and challenged with a variety of dressings using this model system. However, due to the nature of the design, nutrient heterogeneity is often associated the drip flow reactor with greater quantities of nutrients located in areas closer to the media fed versus reduced quantities located at the further point from the media fed. This element could be further exacerbated with the use of silicone based sealant to initially attach the absorbent pad to which the porous pad is attached (Lipp *et al.*, 2010). As media flow over the absorbent pad the position of the sealant will affect

the flow of the media and distribution of the media. However, it is important to note that the controlled delivery of media within the majority of *in vitro* models system will invariably result in a degree of nutrient heterogeneity due to the specific control of media delivery and the delivery location. Nevertheless, this system does attempt to address the issues with the media feed system to simulate the wound environment.

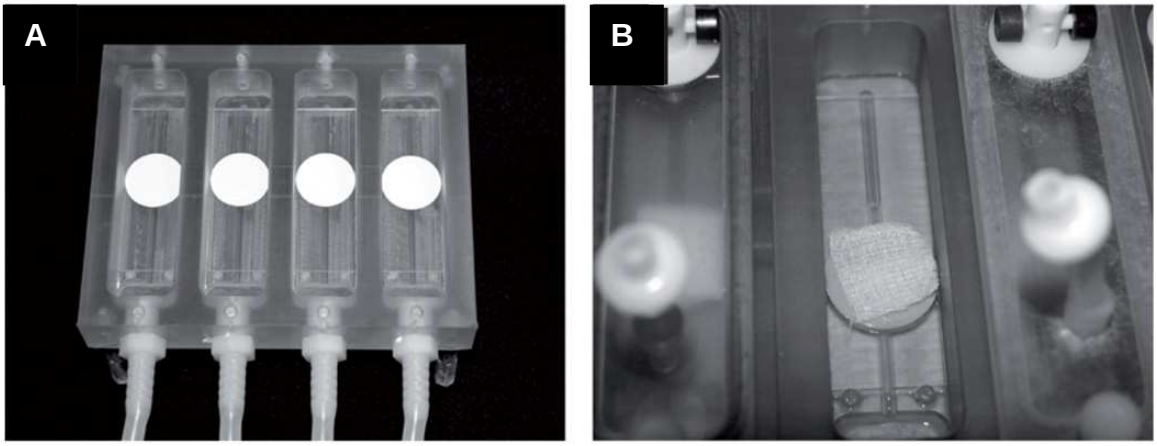


Figure 1.8 Images of a colony-DFR reactor. (A) Absorbent pads glued onto glass microscope slides. (B) Gauze dressing placed on top of the inoculated membrane**.**

Thorn and Greenman (2009) described a novel *in vitro* flat-bed perfusion biofilm model to study potential antimicrobial efficacy of topical wound treatments. In this model system biofilms are grown within a 1 cm^2 cellulose matrix housed within rubber supports and a surface methylpentene film which is secured to a 50 mm glass slide. The system is set at an angle with a peristaltic pump used to perfuse the growth media through the model via a drip wise application to the uppermost part of the slide and aperture film, allowing media to flow down through the cellulose matrix via gravitational forces. A removable clip was fashioned to support topical wound treatments which could be positioned upon the cellulose matrix when required (Thorn and Greenman, 2009). This model system permits the rapid development of steady-state single species communities of *P. aeruginosa* or *S.*

aureus within 24 h of growth and the efficacy of antimicrobial dressing on the total microbial population grown within the cellulose matrix.

Overall *in vitro* wound models are an indispensible tool for the investigation of the microbial ecology of chronic wounds and for the pre-clinical evaluation of potential treatments.

1.11 Aims and objectives

The aims of the doctoral programme were to (i) compare the microbial consortia of chronic wounds and contralateral skin swabs and indentify biofilms associated with chronic wounds (Chapter 3), (ii) to investigate coaggregation interactions between organisms associated with chronic wounds (Chapter 4), (iii) develop and validate novel media which are broadly representative of the healthy skin and wound environment (Chapter 5), (iv) develop and validate novel biofilm wound models which can support the growth of a wound-associated microbial consortium (Chapter 6), and (v) utilise these novel models to investigate the microbial population interactions associated with the healthy skin and chronic wound (Chapter 7).

Chapter 2

General experimental methods

2.1 Chemicals and media

Unless otherwise stated chemicals used throughout this study were of at least analytical grade quality and were supplied by Sigma (Poole, Dorset, U.K.). Dehydrated bacteriological media was obtained from Oxoid (Basingstoke, Hampshire, U.K.) and reconstituted according to instructions supplied by the manufacturer.

2.1.1 Sterilisation of growth media and solutions

Bacteriological growth media, heat-stable solutions and glassware were sterilised in an autoclave at 121℃ for 15 minutes (1kg/cm ^{2}) (Bridson *et al.*, 1970). Heat-liable solutions were filter sterilised using disposable 0.2µm porosity nitrocellulose filters (Millipore, Watford, U.K.).

2.2 Bacterial cultures and maintenance

The identity and origins of the bacterial strains were used throughout this study are shown in Table 2.1.

Table 2.1 Bacterial strains used in the doctoral thesis

Bacterial species and accession number	Strain number	Sequence length & ambiguous bases	Origin
Acinetobacter baumannii (AM989150)			Diabetic Ulcer/ConvaTec ltd*
Bacteroides fragilis	9343		National Typed Culture Collection
Clostridium perfringens (GU968162)		504(7)	Diabetic Ulcer/ConvaTec ltd*
Corynebacterium xerosis (NR_037041)		739	Diabetic Ulcer/ConvaTec ltd*
Enterobacter cloacae (GU979184)	1	309(1)	Diabetic Ulcer/ConvaTec ltd*
Enterobacter cloacae (FJ608236)	43	850(9)	Diabetic Ulcer/ConvaTec ltd*
Enterobacter cloacae (FJ608236)	50	658(30)	Diabetic Ulcer/ConvaTec ltd*
Enterococcus faecalis (AB530699)	15	776(2)	Diabetic Ulcer/ConvaTec ltd*
Enterococcus faecalis (AB530699)	21	776(1)	Diabetic Ulcer/ConvaTec ltd*
Enterococcus faecalis (AB530699)	26	778(6)	Diabetic Ulcer/ConvaTec ltd*
Enterococcus faecalis (AB530699)	Tma5	780(4)	Diabetic Ulcer/ConvaTec ltd*
Escherichia coli (CP001969)		741(13)	Diabetic Ulcer/ConvaTec ltd*
Micrococcus luteus (AB539975)		737(2)	Isolated from healthy skin*
Propionibacterium acnes (CP001977)		760(2)	Isolated from healthy skin*
Propionibacterium avidum (CP001977)		721(5)	Isolated from healthy skin*
Proteus mirabilis (HQ407310)	2	858(5)	Diabetic Ulcer/ConvaTec ltd*
Proteus mirabilis (HQ407311)	5	862	Diabetic Ulcer/ConvaTec ltd*
Proteus mirabilis (FJ971887)	40	849(11)	Diabetic Ulcer/ConvaTec ltd*
Pseudomonas aeruginosa (AB117953)	12	1019(17)	Diabetic Ulcer/ConvaTec ltd*
Pseudomonas aeruginosa (GU269267)	24	980(7)	Diabetic Ulcer/ConvaTec ltd*
Pseudomonas aeruginosa (AB117953)	38	744(1)	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus aureus (GQ214334)	9	760	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus aureus (EU604543)	10	770	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus aureus (FR714927)	11	761	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus capitis (NR_027519)		769	Isolated from healthy skin*
Staphylococcus epidermidis	14990		American Typed Culture Collection
Staphylococcus haemolyticus (AP006716)		761	Isolated from healthy skin*
Staphylococcus hominis (AJ717375)		771(4)	Isolated from healthy skin*
Staphylococcus saccharolyticus (AB480783)		770(1)	Isolated from healthy skin*
Staphylococcus saprophyticus	7292		National Typed Culture Collection
Methicillin resistant Staphylococcus aureus	11939		National Typed Culture Collection
Lactobacillus paracasei (FM1771140)	AH104	785(2)	ConvaTec ltd*
Lactobacillus salivarius (EU559602)	UCC118	782(4)	ConvaTec ltd*

*identified by partial16S rRNA gene sequencing. All sequenced species were ≥ 98% of the maximal identity

To cultivate skin and wound-derived bacterial isolates; selective and non-selective agars, and gaseous environments were used. All non-fastidious and anaerobic organisms were routinely grown on Wilkins Chalgren agar. Obligate anaerobic organisms were cultured in a Mark 3 Anaerobic Work station (Don Whitely Scientific, Shipley, U.K.) at 37℃ (Gas mix: 80% N_2, 10% CO_2 and 10%H_2) and aerobic organisms in aerobic incubators at 37℃. When first received into the laboratory bacteria were archived by freezing (-80°C) using Protect cryobeads vials (Technical Service Consultants Ltd, Heywood U.K.) following a single subculture. This system allows archived strains to be recovered on multiple occasions without thawing out the bulk of the stock.

2.2.1 Differential bacteriological Isolation

To aid in the differentiation and identification of individual bacterial isolates from mixed bacterial consortia selective agars were employed. Staphylococci were isolated with mannitol salt agar, pseudomonads and coliforms were selected for using Pseudomonas and MacConkey agar respectively and lactobacilli using Rogosa agar (Oxoid, Basingstoke, U.K.). Beta haemolytic streptococci were isolated and identified using Columbia agar base supplemented with 5% horse blood (Oxoid, Basingstoke, U.K) and streptococcus selective supplement (Pro-lab diagnostic, Cheshire, U.K.) containing colistin sulphate and oxolinic acid. Putative streptococci isolated on this agar were confirmed using Gram stain, catalase testing and Prolex streptococcal grouping latex kits (Pro-lab diagnostic, Cheshire, U.K.). Agars incubated in an 5% CO_2 atmosphere included; mannitol salt agar (for the selection of total staphylococci), mannitol salt agar supplemented with novobiocin or cefoxitin for the selection of *Staphylococcus saprophyticus* and

Methicillin Resistant *Staphylococcus aureus* (respectively) and Pseudomonas agar (selection of total pseudomonads) and Rogosa agar for the isolation of total lactobacilli. Agars incubated in the anaerobic environment included; Columbia base agar supplemented with 5% horse blood (Oxoid, Basingstoke, U.K.) with neomycin or vancomycin supplement (Oxoid, Basingstoke, U.K.) for the selection of Gram positive and Gram negatives anaerobic and facultative anaerobic bacterial isolates (respectively). All cultures were incubated at 37℃.

2.2.2 Quantification of viable organisms from model substrata

The quantity of viable bacteria present upon substrata (used in model systems) was equated to the number of colony forming units (CFU) per cm^3. Briefly, the substrata of a known volume were placed in 30 ml universals containers (BD, SLS, U.K) containing 9 ml of half strength thiogylcolate broth and 5mm sterile glass beads (n=5) (Merck, Darmstadt, Germany). To ensure uniform distribution of cells throughout the diluent universal containers were subject to 800 opm on a reciprocal flask shaker (Griffin and George, Loughborough, U.K) for 2 min. The resultant solutions (1 ml) were serially diluted in 9 ml half strength thiogylcolate broth and spread plated onto suitable recovery agar (see section 2.2.1). After incubation the number of CFU were recorded and the number of CFU/cm^3 equated using the following equation where *counts* is the number of CFU*, N* is the number counts made, *pv* is the plated volume (ml), *df* is the dilution factor, *dv* is the initial diluent volume (ml) to which the substrata is added.

$$\text{CFU/ cm}^3 = \frac{\left(\frac{\sum counts}{N}\right) \times \left(\frac{1}{pv}\right) \times (df) \times dv}{\text{Total volume}}$$

2.3 16s rRNA gene sequencing for identification of cultured bacteria

To confirm the identity of donated bacteria used within this doctoral thesis, cultivated bacteria were subject to 16s rRNA gene sequencing.

2.3.1 DNA extraction from pure cultures of bacteria

Bacterial colonies (*c.* 2) of axenic cultures were aseptically transferred to a nuclease-free microcentrifuge tube containing 50 µl of nanopure water. Microcentrifuge tubes were votexed for 30s, subjected to 100℃ in a boiling bath for 10 minutes and centrifuged (MSE Microcentaur; Sanyo, Loughborough, U.K.) for 5 minutes at 14,000 rpm. The resultant supernatant containing cellular DNA was used as a template for PCR.

2.3.2 PCR amplification for sequencing

The extracted cellular DNA was amplified using the eubacterium specific primers 8FPL1 (5'-GAG TTT GAT CCT GGC TCA G-3') and 806R (5'-GGA CTA CCA GGG TAT CTA AT-3') (McBain *et al.*, 2003b) at 5 µM each. Each PCR reactions consisted of Red *Taq* DNA polymerase ready mix (25 µl), nonopure water (16 µl), forward and reverse primers (2 µl each; 5 µM), and template DNA (5 µl). All PCR reactions were performed in 0.2 ml nuclease free tubes with a T-gradient DNA thermal cycler (Biometra, Germany). The thermal cycle was as follows; one minute

thermal cycles of 94℃, 53℃ and 72℃ (n=35), with the final cycle including a 15 minute chain elongation stage at 72℃.

2.3.3 Agarose gel electrophoresis

Agarose powder was added to TAE buffer (diluted in distilled water from 50X stock solution (40mM Tris base, 20mM glacial acetic acid and 1mM EDTA, pH 8.0 at 25℃) to make a 1% agarose solution. The agarose solution was heated in a microwave oven until the agarose powder had completely dissolved. Once cooled GelRed (Biotium CA, USA) (0.01% v/v) was added. The molten agarose was cast in Bio-Rad mini gel tanks (Bio-Rad Hemel Hempstead, U.K.) with a comb in position. Once solidified combs were removed and gels were submerged in *c.* 400 ml TAE buffer and 5 µl of PCR products were loaded into the wells with the addition of one 1Kb DNA ladder (5 µl). Electrophoresis was executed continually at 70V for up to 1h. Gels were visualised and imaged under UV illumination (312nm) using Canon EOS D60 digital camera (Canon, Surrey, U.K.).

2.3.4 16s rRNA gene sequencing

PCR products were sequenced at an in-house laboratory using the 806R primer. The sequencing reaction was as follows: 94℃ (4 min) followed by 25 cycles of 96℃ (30 s), 50℃ (15 s), and 60℃ (4 min). Once chain termination was complete, sequencing was carried out in a Perkin-Elmer ABI 377 sequencer. DNA sequences were compiled using CHROMAS-LITE (Technelysium Pty Ltd, Australia). Sequence matching was undertaken using the bioinformatics program: Basic

Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast) to mine the prokaryotic database for matching sequences.

Chapter 3

The bacteriological analysis of chronic wounds and contralateral skin swabs

3.1 Abstract

Clinical laboratory analyses of chronic wound infections rely upon culture-based methodologies to which less fastidious and fast growing organisms are generally acquiescent. Chronic wounds however, can harbour a range of microorganisms some of which may be fastidious and/or unculturable. Whilst culture-based methodologies are well-established and provide a rapid means of detecting common pathogenic bacteria associated with wounds and other infections; due to inherent biases, the application of culture-independent methods may provide deeper analysis of microbial diversity. However, the microbial diversity of a wound is only one facet in the complex paradigm of chronic wound microbiology; emphasis has also been placed on the quantity of bacteria and the potential aetiological involvement of biofilms. In the current chapter, ethical approval was obtained to sample chronic wound tissues and contralateral skin sites from anonymous patients with chronic wounds attending Rusholme Podiatry clinic. Samples were analysed by differential plate counting and eubacterial specific denaturing gradient gel electrophoresis (DGGE) and where available, excess tissue was histologically sectioned and stained using fluorescent *in situ* hybridisation (FISH) and Gram staining. Tissue sections were examined microscopically using florescence and light microscopy, and environmental scanning electron microscopy (ESEM). A total of three genera were commonly cultured from skin swabs of healthy skin and 12 genera from chronic wound tissue. By contrast, DGGE sequence analysis identified a total of 21 genera from healthy skin swabs and 22 from chronic wound tissues. Intrapersonal DGGE sequence analysis identified that non-infected wounds had a proportionally higher incidence of bacteria which were identified on contralateral healthy skin when compared to infected wounds. This suggests that the transition to infection is accompanied by marked changes in the microbial diversity. Microscopic examination of FISH and Gram stained tissue sections, combined with ESEM analysis identified the presence of microcolonies and putative biofilm structures in both infected and non-infected wounds; indicating an association between biofilms and wounds that may or may not be not be causal or aggravatory.

3.2 Introduction

Chronic wound infections increase the risk of secondary site infections and septicaemia resulting in prolonged hospital stays and delayed healing. In 2008 it was estimated that over 200,000 patients in the UK had a chronic wound which resulted in a conservative cost to the NHS at 2005-2006 costs *c.* £3 bn per year (Posnett and Franks, 2008). Chronic wounds generally originate from underlying aetiologies of the patient for example diabetes and peripheral vascular disease, combined with associated tissue injury. The most common chronic wounds are pressure ulcers, diabetic ulcers, and venous ulcers situated in the lower extremities. These wounds arise from neuropathy and/or venous insufficiency associated with underlying aetiology which cause reductions in the supply of oxygen and nutrients to the tissues leading to ischemic necrosis and subsequent ulceration (Bass and Phillips, 2007; Eaglstein and Falanga, 1997; Vasconez *et al.*, 1977). Additionally, reduced vascularisation of the wound bed can inhibit the transport and integration of leukocytes and macrophages to the site of the injury. These factors create a portal of entry for bacteria and increased susceptibly to bacterial infections which in turn can further delay healing via microbial oxygen consumption, changes to the pH, toxin production and tissue damage (Thomson, 2000). The progression to an infection is dependant upon the types of bacteria present, their virulence and pathogenicity, and their relationship with the host immune responses. A figure of $\geq 10^6$ organisms per gram of tissue has been cited as a quantitative value for a defined infection where by significant tissue damage and clinical signs of infection become evident (Murphy *et al.*, 1986; Robson, 1979; Robson, 1997)

Chronic wounds are without exception colonised by a variety of aerobic and anaerobic microorganisms. Typically isolated organisms include *Staphylococcus aureus*, Lancefield groups A, B, C and G beta haemolytic streptococci, *Enterococcus faecalis*, *Bacteroides* species, *Clostridium* species, anaerobic cocci, coagulase-negative staphylococci, *Corynebacterium* species, enterobacteriaceae and pseudomonads (HPA, 2009). However, it has been estimated that less than 2% of harvested bacteria can be cultivated leading to the assumption that the bulk of bacteria present in the environment are recalcitrant to culture (Krutmann, 2009; Wade, 2002). Nevertheless, it is important to note that the majority organisms which cause wound infections have been characterised and identified (HPA, 2009). Clinical laboratory investigations of chronic wound infections rely heavily upon culture-based methodologies, detecting organisms which grow rapidly and are culturable. Whilst this is a useful tool in the detection of the most common pathogenic bacteria associated with wound infections, it may fail to represent the microbial community of chronic wounds (Wilson *et al.*, 1997). Within recent years a number of studies have employed molecular techniques to expand upon and increase the culture based knowledge of microbial diversity within chronic wounds (Dowd *et al.*, 2008; Gontcharova *et al.*, 2010). Data obtained utilising techniques such as pyrosequencing, PCR, denaturing gradient gel electrophoresis (DGGE), and ribosome shotgun sequencing, has in general, consistently identified a greater range of bacteria than traditional culture techniques identifying several genera and species not isolated upon culture, with specific reference to a greater number of aerobic, anaerobic and facultative anaerobic genera and species identified utilising molecular techniques (Davies *et al.*, 2004; Dowd *et al.*, 2008; Melendez *et al.*, 2010; Singh *et al.*, 2009).

The acknowledgement of the presence of complex microbial communities present within chronic wounds has led to an increased interest in understanding how the community interact with specific reference to the potential aetiological involvement of biofilms since these have been implicated in other infections such as prosthetic joint infections and catheter associated urinary tract infections and their recalcitrance to treatments (Camargo *et al.*, 2005; Costerton *et al.*, 1999; O'Gara and Humphreys, 2001). Biofilms have been intrinsically linked to the persistence of infections and treatment failures; elements associated with infected chronic wounds. Biofilms posses a number of properties which facilitate their resilience to antibiotic treatments and host defence mechanisms. Arguably the principal determinant of biofilms recalcitrant is production of an exopolysaccharide matrix which encapsulates the bacterial community. This matrix can act as a diffusion barrier to some antimicrobials, particular host immune cells and can produce a heterogeneous environments within the biofilm causing variations bacterial growth rates, dormant cells and microenvironments each contributing to the biofilms resistance (Gilbert *et al.*, 1990; Walters *et al.*, 2003; Wentland *et al.*, 1996). Evidence is now emerging to suggest that biofilms are present within chronic wounds and that these might exacerbate the state of chronicity. Consequently, a variety of *in vitro* research has been undertaken in an attempt to establish the presence and role of biofilms in chronic wounds; Akiyama *et al.*, 2002 studied biofilm production by *S. aureus* cells on damaged skin tissues and the influence of polymorphonuclear leucocytes (PMNs) on its production employing neutropenic and normal mice with and without topical application of antimicrobial agents (Akiyama *et al.*, 2002). Biofilm production was observed with 4 h of inoculation in both the neutropenic and normal mice as determined by visualisation of biofilm bound Concanavalin A-FITC (specifically; to the terminal alpha mannosyl groups

oligosaccharide of glycoproteins found in biofilms) indicating biofilm production is a relatively immediate occurrence. Interestingly, in this investigation topical antimicrobial treatments of 2% fusidic acid had a greater effect of the *S. aureus* population in the normal mice versus neutropenic mice when compared to 10% povidone-iodine solution. The author concludes that this suggests that PMNs play an important role in the treatment of *S. aureus* biofilm-associated skin infections. This may help to explain wound infection treatment failures in diabetic patients due to their impaired immune responses such as altered migration of leucocytes and impaired PMN respiratory burst activity which could contribute to the persistence of bacterial biofilms, infection and subsequently delayed healing (Alba-Loureiro *et al.*, 2006; Marhoffer *et al.*, 1994; Rich and Lee, 2005).

The microbial community of chronic wounds is frequently diverse and may harbour bacteria which can form structures associated with biofilms. However, the precise role that these play in the chronicity of the wound remains poorly understood. There is a long-held understanding of pathogenesis of the more prominent culturable organisms present within wounds e.g. *S. aureus* and *Pseudomonas aeruginosa* and the role these play in infection of chronic wounds and prevention of healing however, recent emphasis had been placed upon the polymicrobial nature of and the role of biofilms in chronic wounds with a disregard to the previous held notion of “infective dose” microbiology. This chapter aims to combine the concepts of culture based “infective dose” microbiology and molecular based microbial ecology of chronic wounds by quantifying the culturable microbial population of wounds with the goal of identifying traditionally viewed “infected” and “non-infected” wounds and assessing the microbial ecology of

chronic wounds and control contralateral intact skin swabs in the context of the of the culture data.

3.3 Materials and methods

3.3.1 Collection of chronic wound tissue and contralateral skin swabs

This study was reviewed by the North Manchester Research Ethics Committee and Central Manchester University Hospital Research and Development department. The study title was “The Microbial Ecology of Chronic Wounds” REC Reference number: 09/H1006/41, protocol number 1.0. Approval was granted to seek to attain a maximum of 36 wound tissue samples and 36 bilateral skin swabs from anonymous patients with non-healing chronic wounds attending Rusholme Podiatry clinic between 2/2/2010 and 2/2/2011. Written informed consent was obtained from all subjects (or their legally authorised representatives) who were eligible and met the inclusion criteria before recruitment into the study see Appendix 1A-1E. All potential subjects were informed of the purpose of the study, the potential outcome(s) and the follow up procedures. Subjects and samples were anonymised at the point of collection with alphanumeric code, the location of the wound, underlying cause and current antimicrobial therapy was recorded. Wound tissue samples were taken from the wound bed and surrounding tissue using a sterile scalpel by the attending clinician and placed sterile 0.85% saline for transportation. Sample size was dependant upon the wound size, location and available tissue for collection; the maximum feasible amount which did not interfere with the patients’ ongoing treatment or cause additional tissue damage was removed. Skin swabs of a contralateral skin area measuring 40cm^2 were also collected using Dual Amies transport swabs (Duo Transwab, MWE, Wiltshire, U.K). Swabs were moistened with sterile saline prior to sampling. All samples were

transported to the laboratory and processed within 3 h of collection as detailed in the following section.

Upon arrival to the laboratory, sections of tissues were dissected with a sterile scalpel, weighed and homogenised using a tissue pulpier in 3 ml of sterile saline Any remaining material was embed in optimal cutting temperature (OCT) embedding matrix and archived at -80℃ for tissue s ectioning and imaging. Dual amies swabs were aseptically split with sterile scissors, with one swab archived at -80℃ for bacterial DNA extraction in 1.5 ml microc entrifuge tubes. The remaining single swabs and 1 ml of the homogenised tissue samples and were placed in 9 ml half strength thiogylcolate broth, vortexed for 30 seconds, serially diluted (in half strength thioglycollate broth) and spread plated onto selective and non-selective media shown in Table 3.1 for total bacterial counts. To aid in the identification of organisms residual homogenised tissue solutions and swabs were spread plated onto HPA-recommended agars shown in Table 3.2 (HPA, 2009). The residual samples of homogenised tissue were archived at -80℃ for bacterial DNA extraction.

Table 3.1 Selective and non-selective agar used for total bacterial counts

Agar	Incubation	Target Organisms
5% Horse Blood Agar	5% CO_2, 37℃	Total Aerobic Count
5% Horse Blood Agar	Anaerobic, 37℃	Total Anaerobic Count
Mannitol Salt Agar	5% CO_2, 37℃	Total Staphylococci
Streptococcal Agar	5% CO_2, 37℃	Total Streptococci
MacConkey Agar No.3	5% CO_2, 37℃	Total Coliforms
Pseudomonas Agar	5% CO_2, 37℃	Total Pseudomonad's

Table 3.2 Selective and non-selective agar for bacterial identification

Agar	Incubation	Target Organisms
Cysteine lactose electrolyte deficient (CLED) Agar	5% CO_2, 37℃	Enterobacteriaceae Pseudomonads
5% Horse Blood Agar/Vacomycin*	Anaerobic, 37℃	Gram Negative Anaerobes
5% Horse Blood Agar/Neomycin*	Anaerobic, 37℃	Gram Positive Anaerobes
5% Horse Blood Agar	5% CO_2, 37℃	Lancefield Groups A, C and G streptococci *Pasteurella* species *S. aureus* *Vibrio* species *Aeromonas* species streptococci.

* Addition of 5µg Metronidazole Disc

All agar plates were incubated for 48 h and total counts performed. Colony counts were recorded and reported in colony forming units per gram of tissue for wound tissue material and colony forming units (CFU) per cm^2 for swab samples using the following equations where *counts* is the number of colony forming units*, N* is the number counts made, *pv* is the plated volume (ml), *df* is the dilution factor, *dv* is the initial diluent volume (ml) to which the sample is added and g of tissue is the weight of tissue macerated in diluent.

$$\text{CFU/g tissue or } cm^2 = \frac{\left(\frac{\sum counts}{N}\right)\times\left(\frac{1}{pv}\right)\times(df)\times dv}{\text{g of tissue or total area sampled}}$$

Identification of specific genus and species was performed by standard clinical laboratory methods, briefly; identification was based upon colony morphology and Gram staining with additional identification achieved by catalase reaction tests to

differentiate staphylococci and streptococci, latex coagulase reaction tests to differentiate putative *S. aureus* and coagulase negative staphylococci (Prolex Staph latex kits, Pro-lab diagnostic, Cheshire, U.K.), Lancefield group reaction to identify beta haemolytic streptococci (Prolex Streptococcal grouping latex kits, Pro-lab diagnostic, Cheshire, U.K.) and Brilliant UTI media to differentiate *E. coli* and coliforms.

3.3.2 DNA extraction from chronic wound tissue samples and skin swabs

Total DNA was extracted from archived macerated tissue samples and swab samples using a DNeasy blood and tissue kit (Qiagen Ltd., West Sussex, U.K.) and was conducted according to the manufacturers instructions. Briefly, swabs were placed in 500 µl of sterile 0.85% saline and 100mg of sterile silica beads. Disruption of the bacterial cells on the skin swabs was achieved using the FastPrep FP120 bead beater (Qbiogene, California USA) at full speed at 2 x 30 second intervals. Samples were placed on ice during the intervals to prevent damage of the DNA due to DNase. Samples were centrifuged at 14,000 rpm for 1 min (MSE Microcentaur; Sanyo, Loughborough, U.K.) after which the supernatant was subject to DNA extraction using the Gram positive bacteria protocol of the DNeasy blood and tissue kit (Qiagen Ltd., West Sussex, UK). Macerated tissue samples were subject to the tissue protocol of the DNeasy blood and tissue kit (Qiagen Ltd., West Sussex, UK). Briefly, digestion of the tissue was achieved in buffered ATL and Proteinase K. After digestion, samples were centrifuged at 5000 rpm to pellet the debris. The supernatant was then centrifuged at 14,000 rpm and the resultant supernatant subject to the Gram negative extraction protocol and the

pellet fraction subjected to the Gram positive extraction protocol. After parallel extractions the two eluates were pooled for a total eluate.

3.3.3 16s DNA amplification for DGGE analysis

Extracted DNA eluates (5 µl) were run on a 1% agarose gel to visualise DNA, once the presence of DNA was confirmed 5 µl of the eluates was used as a template in PCR reactions. Briefly templates were amplified with eubacterium-specific primers HDA1 (additional GC clamp) (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3') and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA C-3') (Walter *et al.*, 2000). The reaction mix was as follows: Red *Taq* DNA polymerase ready mix (25 µl), HDA primers (2 µl of each (5 µM)), nanopure water (16 µl), and extracted DNA template. The reactions were performed in 0.2 ml DNA free PCR tubes with a T-Gradient DNA thermal cycler (Biometra, Germany). The thermal amplification program was as follows: 94℃ (4 min), followed by 30 thermal cycles of 94℃ (30s), 56℃ (30s), and 68℃ (60s). The final cycle incorporated a 7 minute chain elongation step (68℃). Positive and negative controls (5 µl of microbial DNA extracted from saliva or nanopure water respectively) were run concurrently with each reaction run.

3.3.4 DGGE denaturant solutions

To assess the microbial diversity of chronic wound tissues and skin swabs amplified bacterial DNA was subjected to denaturing gradient gel electrophoresis (DGGE) which separates the sequences in a solid phase gel based upon GC content of the amplicon and thus its denaturing point in the gel. Throughout this study denaturing concentrations of 30% and 60% were used shown in Table 3.3 .

Before use, solutions were degassed using a vacuum pump for 10 min and filtered through a 0.45µm filter using a vacuum filtration system to prevent unequal polymerisation. Solutions were stored at 4℃ in foil-covered vessels to prevent light degradation.

Table 3.3 Denaturant solutions

Reagent	**30%**	**60%**
40% acrylamide/bis (v/v)	25 ml	25 ml
50 x TAE buffer (v/v)	2 ml	2 ml
Formamide (v/v)	12 ml	24 ml
Urea (g)	12.6	25.2
Deionised water	to 100 ml	to 100 ml

3.3.5 DGGE gel sandwich assembly and gel casting

All DGGE analysis was carried out with the DCode Universal Mutation Detection System (Bio-Rad, Hemel Hempstead, UK) according to manufacturer's recommendations for perpendicular DGGE. Two glass plates measuring 18.3 x 16 cm and 20 x 16 cm were assembled and aligned with 1mm plastic spacers using the supplied casting stand. Both the high (60%) and low (30%) concentration denaturant solutions were decanted (15 ml) into individual plastic universal containers. D-code dye solution (bromophenol blue (0.5% w/v), xylene cyanol (0.5% w/v) and TAE buffer (10 ml) (300 µl) was added to the high concentration denaturant solution. Tetramethylethylenediamine (TEMED) (30 µl) and ammonium persulphate (APS) (60 µl) (1% w/v) were added to each of the denaturant solutions which were then decanted into 30 ml syringes, with attached tubing. Air bubbles were removed from each syringe before attaching the syringes to the gradient delivery system. Tubing from the syringes were attached to a new 19 gauge

needle (Fisher Scientific, Leicestershire, UK) and inserted between the two glass plates. The wheel of the gradient delivery system slowly turned to ensure efficient mixing and delivery of the denaturant solutions. When the mixed denaturant solutions reached the top of the glass plates, a 16-well comb was inserted and the gel(s) were allowed to polymerise. The gel tank was filled with 7L of 1 x TAE buffer solution diluted from 50 x TAE buffer (40mM Tris base, 20mM glacial acetic acid, and 1mM EDTA) and left at room temperature.

3.3.6 Sample loading and electrophoresis of the gel

When the gel had polymerised the comb was carefully removed and any excess acrylamide was removed. The gel sandwich assembly was then attached to the core module ensuring that if only one gel was to be run, an empty gel sandwich (with only glass plates) was attached to the other side of the core. The gels were left to equilibrate overnight in the tank with the buffer at room temperature. When equilibrated and prior to sample loading the gel tanks were heated to 60℃. Bacterial PCR products (45 µl) were mixed with 5 µl gel loading dye (bromophenol blue (2% w/v), xylene cyanol (2% w/v), glycerol (70% v/v) dissolved in deionised water. Samples were loaded into the wells of the gel using gel loading tips, taking care not to pierce the wells during delivery. When the wells were loaded electrophoresis was carried out for 140 volts at 60℃ for 5.5 h. Internal standards of extracted and 16s rRNA gene amplified saliva samples were run between different gels to enable a high degree of matching of independent gels.

3.3.7 Staining and visualisation of DGGE gels

When the DGGE electrophoresis was complete the assembled gel(s) plates were removed from the buffer and immersed in 200 ml of 1 x TAE buffer with 20 µl of SYBR® Gold stain (Molecular Probes, Leiden, The Netherlands), glass plates were then carefully removed leaving the acrylamide gel in the staining solution. Gel(s) were left in the staining solution for 20 min with occasional agitation after which they were transferred to a UV transilluminator (UVP, California, USA), visualised under UV light at 312 nm, and photographed using a Canon D60 digital single lens reflex (DSLR) camera (Canon, Surrey, UK).

3.3.8 DGGE band excision and re-amplification for sequence identification

DGGE bands of interest (visualised on a UV transilluminator) were excised using a sterile scalpel and placed in nuclease-free tubes with 20 µl nanopure water. The bands were then stored at 4℃ for 24 h before archiving at -80℃. Before sequence analysis, the tubes were vortexed for 30 s, then centrifuged (MSE Microcentaur; Sanyo, Loughborough, U.K.) for 10min (14,000 rpm). The nanopure water could then be used as a template for PCR using previously stated methods (see Section 3.3.3).

PCR products derived from excised DGGE bands were purified using QIAquick PCR purification kit (Qiagen Ltd., West Sussex, U.K.) in accordance with the manufacturers instructions. PCR products were sequenced at an in-house laboratory using the non GC clamp reverse HDA primer. The sequencing reaction was as follows: 94℃ (4 min) followed by 25 cycles of 96℃ (30 s), 50℃ (15 s), and

60℃ (4 min). Once chain termination was complete, sequencing was carried out in a Perkin-Elmer ABI 377 sequencer. DNA sequences were compiled using CHROMAS-LITE (Technelysium Pty Ltd, Australia). Sequence matching was undertaken using the bioinformatics program: Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast) to mine the prokaryotic database for matching sequences.

3.3.9 Construction of DGGE dendrograms

Gels were aligned using Adobe Photoshop (Adobe, CA, USA) by the aid of internal controls used on each gel. Gel images were analysed using Bionumerics (Applied Maths, Saint-Martens Latem, Belgium). Gel images were first optimised to reduce background noise and colour variation between images. Lane boundaries were then applied to gels and reference lanes added to identify common bands and thus optimise alignment. Bands were detected automatically and then checked manually. Dendrograms were constructed using cluster comparison; employing unweighted pair group method with arithmetic mean (UPGMA) algorithm. The UPGMA algorithm weighs each lane being analysed equally and computes the average similarity or difference of each lane to an extant cluster. The resultant dendrogram can then be used to observe clustering patterns between different lanes.

3.3.10 Principal component analysis

To synthesise data to produce images in which patterns can easily observed principal component analysis was used. Similarity matrix data derived from UPGMA algorithm of DNA fingerprints of chronic wound tissue and intact skin

swabs was utilised to determine principal component data. Briefly, similarity matrix data of correlated variables was reduced using factor analysis (SPSS, SPSS Inc., Chicago, Illinois) in which variances between groups i.e. the different band position for each sample when compared to another, are maximised to produce three overall uncorrelated variables (principal components). The first principal component accounts for the greatest degree of variance in the overall group with each succeeding components representing the remaining variances. The resulting principal component data was plotted on a three axes scatter plot.

3.3.11 Tissue sectioning

To produce slide mounted tissue sections to visualise microcolonies and biofilm architecture, archived whole tissue samples which were of sufficient quantity to section were sectioned to a thickness of 5 µm and mounted on superfrost plus microscope slides (Fisher Scientific, Leicestershire, UK) using a Shandon AS260 manual cryostat. Tissue sections were subjected to Gram and Fluorescent *in situ* hybridisation (FISH) staining.

3.3.12 FISH staining to distinguish bacteria, biofilms and tissue

Slide mounted tissue sections were fixed in 4% paraformaldehyde for 3 h and then subjected to a pre-permeabilisation step, consisting of lysozyme enzymatic buffer (100 mM Tris HCL [ph8], 50 mM EDTA and lysozyme [5mg/ml]), for 4 h at 45℃. Slides were then washed in wash buffer consisting of 0.9 M NaCl and 20 mM tris and air dried. Slides were then subjected to FISH hybridization buffer containing 50% formamide, 0.9 M NaCl, 20 mM Tris, 0.01% SDS (w/v) and 50 ng of the

general Eubacterial probe (EUB 338)-cy3 probe - GCT GCC TCC CGT AGG AGT (Amann *et al.*, 1990) (Ex. 550nm, Em. 570nm) and incubated in a humidity chamber at 55℃ For 4 h, slides were washed in wash buffer. Once dried slides were subjected to Concanavalin A conjugate alexa fluor 488 (Ex. 495nm, Em. 643nm) to aid in the visualisation of putative biofilms (Invitrogen, Paisley, U.K.) (10 µl/ml working concentration) for 1 h at room temperature, washed and air dried. Concanavalin A binds to internal and non-reducing terminal alpha mannosyl groups a common component of oligosaccharide of glycoproteins found in biofilms (Baum *et al.*, 2009; Kanno *et al.*, 2009). Finally to distinguish mammalian cells slides were stained with the nucleic acid stain Hoechst 33252 (2µg/ml) (Ex. 350nm, Em. 460nm) (Sigma, Poole, Dorset, U.K.) for 1 h at room temperature (Malic *et al.*, 2009). All staining procedures were completed in the dark. Tissue sections were also Gram stained as per standard protocol.

FISH stained images were collected on an Olympus BX51 upright microscope using a 60x and 100x objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Specific band pass filter sets for DAPI (ex.BP365/12nm, em. LP397nm), FITC (ex. BP450–490nm, Em. BP515 – 565) and Texas red (Ex. BP546/12nm Em. LP615nm) were used to prevent bleed through from one channel to the next. Gram stained images were acquired using a Zeiss Axioscop 2 microscope, Axiocam and Axiovision Version 4.8 (Carl Zeiss ltd Herefordshire U.K.). All images were then processed and using ImageJ (http://rsb.info.nih.gov/ij).

3.3.13 ESEM of chronic wound tissue

Excess chronic wound tissue samples of sufficient quantity for cryo-sectioning and ESEM were divided (50:50) with a sterile scalpel with one section embedded in OCT embedding matrix (frozen at -80℃) for > 24 h and the remaining section placed in a sterile bijou and transported immediately for ESEM imaging.

ESEM of chronic wound tissue samples was performed using a FEI Quanta 200 environmental scanning electron microscopy under a low vacuum (<0.75 torr) permitting interrogation of putative biofilms structures and microcolonies whilst conserving the hydrated state of the sample.

3.4 Results

Of the 36 patients samples received 26 wound tissue and matching contralateral skin swabs were of sufficient quantity and quality to undergo bacterial counts and DGGE analysis.

3.4.1 Viable bacterial counts from wound and skin samples

Figures 3.1 and 3.2 shows viable count data from 26 chronic wound tissue samples. Data showed that all wound tissue tested harboured bacteria, with *Staphylococci* spp. the most common bacterial isolate. Of the viable count data four samples (Patient D, E, I and K) harboured a “pure” growth *S. aureus* (based upon colony morphology) at levels equal to or greater than 10^6 CFU/g of tissue. Nine chronic wound tissue samples (Patient F, J, N, M, P, Q, T, W and Y) grew a

light growth to heavy growth of *S. aureus* combined with coagulase negative staphylococci (CNS) and associated skin flora and were considered infected by *S. aureus* by clinical microbiology standards (HPA, 2009).

Figure 3.3 shows viable count data from 26 contralateral intact skin swab. Data shows that all intact skin swabs tested harboured bacteria at reduced quantities and diversity when compared to the associated chronic wound samples. No evidence of culturable streptococci, pseudomonads or coliforms bacteria was found. *Staphylococci* spp. was the most common bacteria found upon the skin swabs with only samples E and G noted not to harbour this genus as defined by culture methods used.

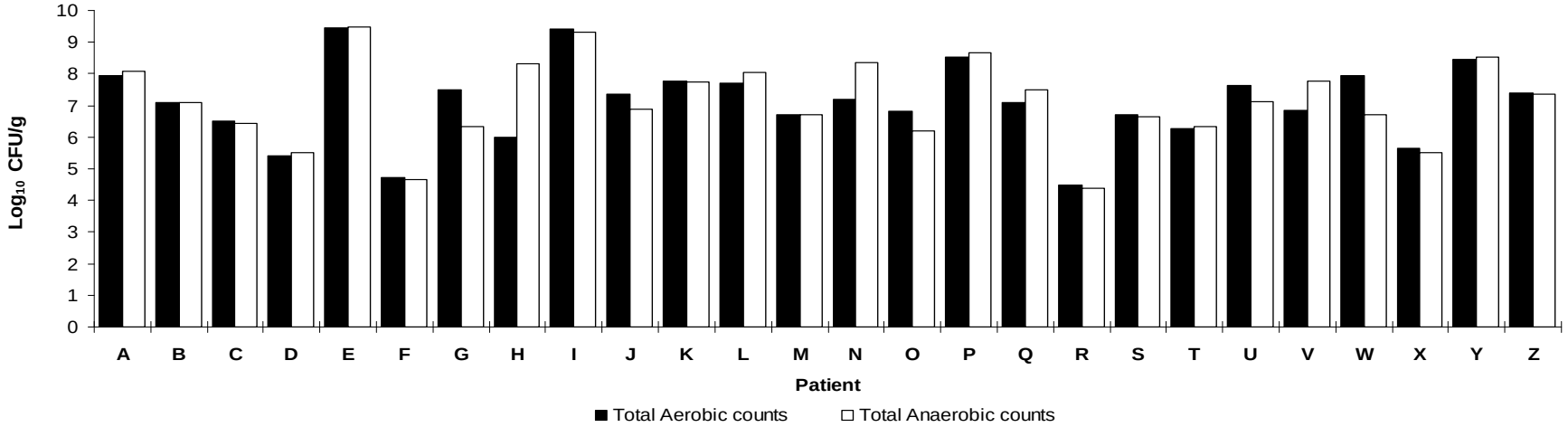


Figure 3.1 Total aerobic and anaerobic counts from chronic wound tissue

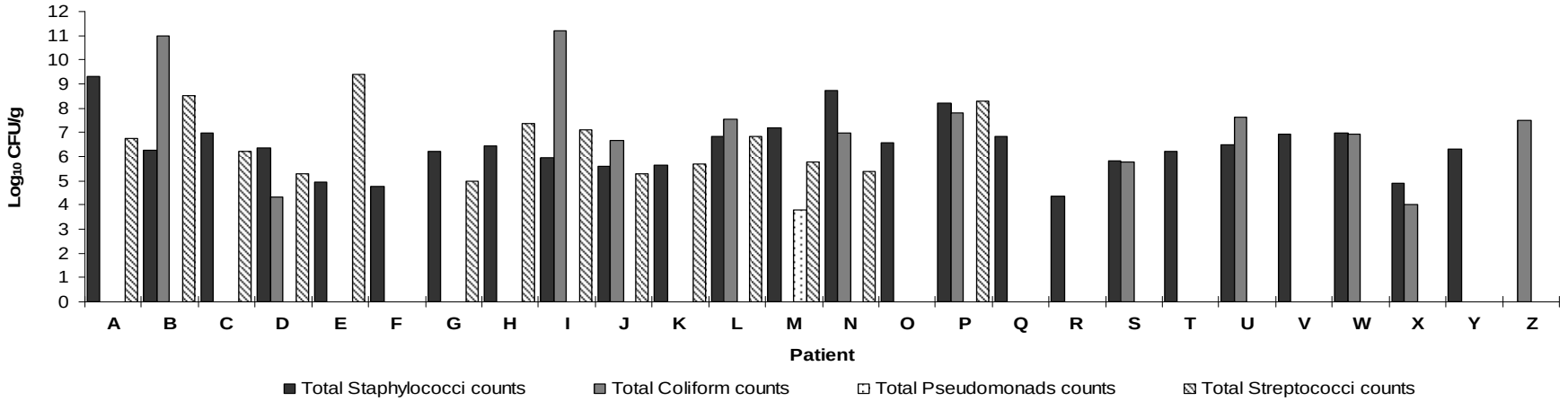


Figure 3.2 Total staphylococci, coliform and streptococci counts from chronic wound tissue

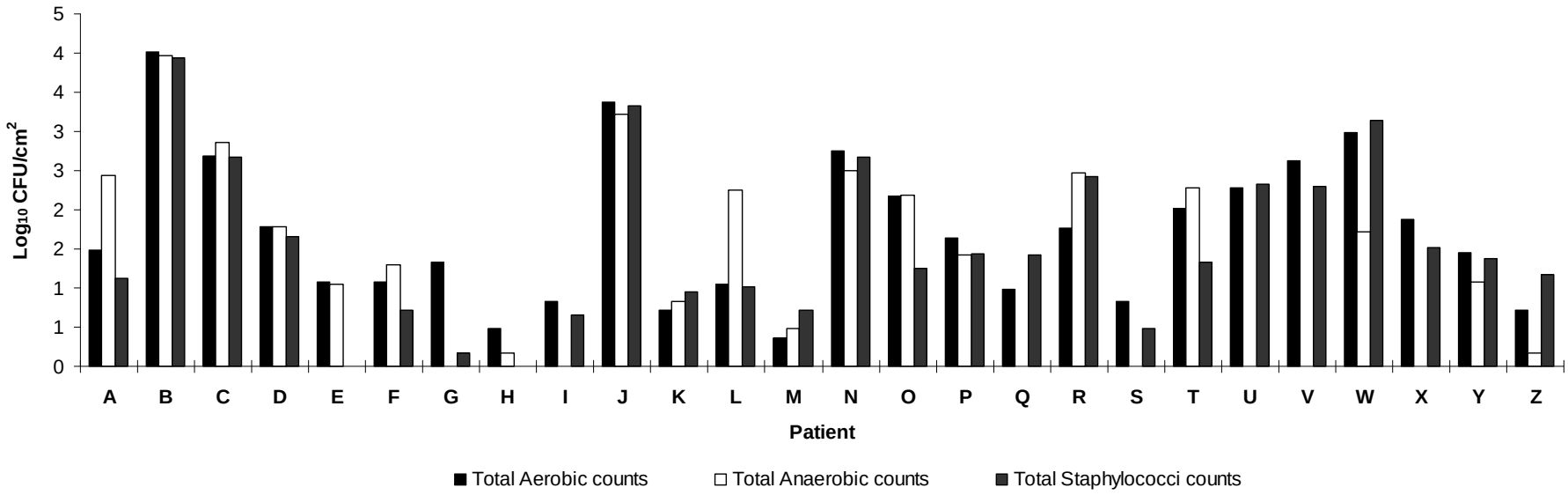


Figure 3.3 Total aerobic, anaerobic and staphylococci counts from intact contralateral skin swabs

Table 3.4 Organisms identified and semi-quantitative growth data from agar culture of chronic wound tissue samples

Patient	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
INFECTION	NO	NO	NO	YES	YES	YES	NO	NO	YES	YES	YES	NO	YES	NO	YES	YES	YES	NO	NO	YES	NO	NO	YES	NO	YES	NO
S. aureus				+++	++	++			++	++	+++		++		+	+	+			++			+		++	
CNS	+++	+++	+++			++	++	++				+++	+++	++	+++	+++	+++	+	+	+++	+	++	+++	+	+++	
E. coli	+										+++		+++													
Coliform				++					+++						++	+					+++			+++		+++
GDS	+						++			++		+														
GBS											+				++											
GGS													++													
Corynebacterium spp.		++												++			++									
Pseudomonas spp.													+													
Micrococcus spp.												++														
Anaerobes (GPC)								+																		
Candida spp.					++																					

CNS: Coagulase negative Staphylococcus, GBS: Beta haemolytic streptococci Lancefield Group B, GDS: Enterococcus Faecalis (Lancefield Group D), GGS: Beta haemolytic streptococci Lancefield Group G, GPC: Gram positive cocci. + Light growth, ++ Moderate Growth, +++ Heavy Growth. Infection defined as the presence of pathogenic organism associated with wound infection isolated upon culture.

Table 3.5 Organism identification and semi-quantitative growth data from cultures of intact skin swabs

Patient	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
CNS		S	S	S		S	S		S	+	S	S	S	+	+	+	+	+	+	+	+	+	+	S	S	S
Corynebacterium spp.	S				S			S							+											
Micrococcus spp.												S				+										

S: Scanty growth (1-10 colonies), +: Light Growth. CNS: Coagulase negative staphylococci.

Data in Table 3.4 and 3.5 shows the putative identification of cultured organisms and includes the semi-quantitative growth data from chronic wound tissue samples and intact skin swabs, respectively. Tissue samples were defined as microbiology clinically infected based upon the growth of putative pathogenic organisms associated with wound infection which in a clinical laboratory would result either further identification and/or antibiotic sensitivities (HPA, 2009). From the organisms cultured, *S. aureus* was the only overt pathogen identified which would have characterised the tissue as infected using the defined assessment parameters. Intact skin swabs produced relatively low quantities of skin associated bacteria with no sample producing moderate to heavy growth of any bacterial isolate.

3.4.2 DGGE analysis of the microbial diversity of chronic wound and contralateral skin swabs

To determine the similarity or differences between produced profiles of bacterial communities associated with chronic wounds and contralateral controls of healthy skin; UPGMA dendrograms were constructed to compare the DNA fingerprints of wound communities derived from chronic wound tissue samples (Figure 3.4) and contralateral intact skin swabs (Figure 3.5). For both dendrograms similarity scores ranged from 10-60% with the average similarity score below 50%. This indicates that generally wound and skin were colonised with highly divergent consortia profiles

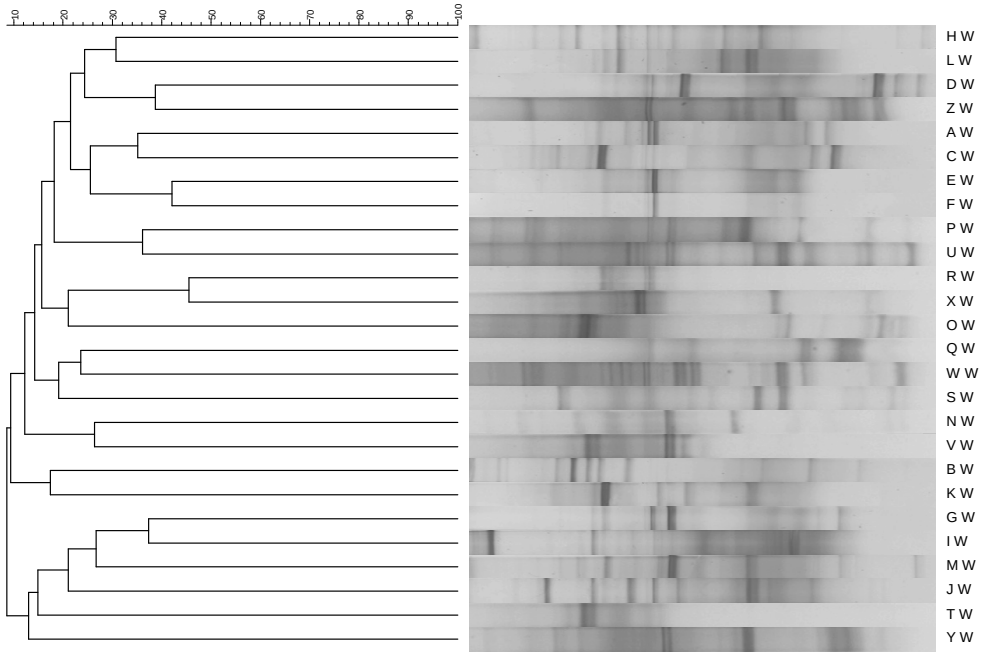


Figure 3.4 A UPGMA dendrogram showing percentage matching of wound DGGE fingerprints of wound samples from Patients A-Z. The primary letter denotes the patient identification the second letter notes samples site (W: wound)

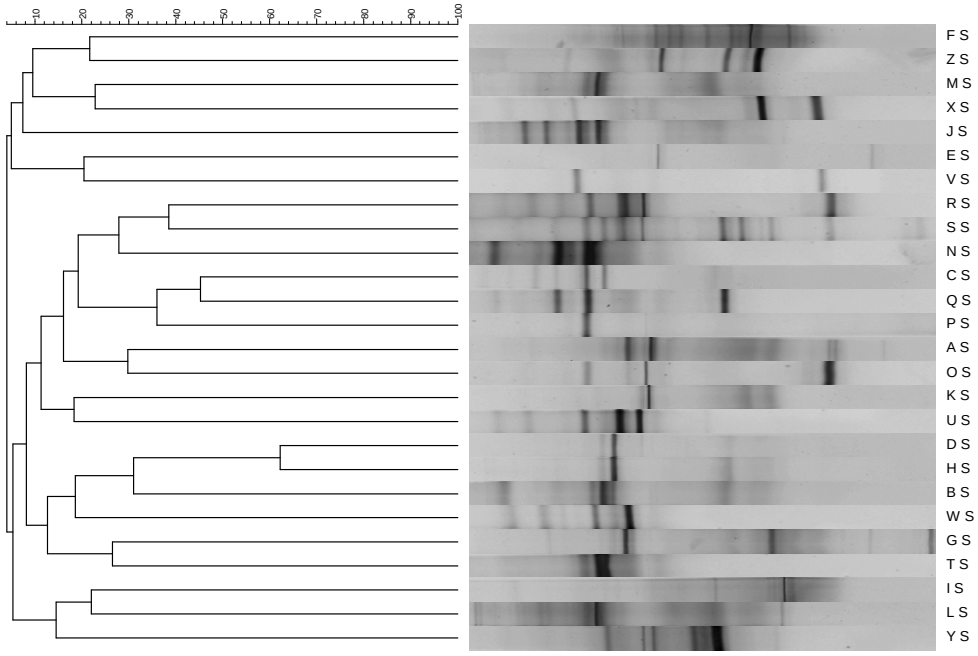


Figure 3.5 A UPGMA dendrogram showing percentage matching of wound DGGE fingerprints of intact skin swabs from Patients A-Z. The primary letter denotes the patient identification the second letter notes samples site (S: Skin)

The dendrogram in Figure 3.6 compares the overall eubacterial DNA fingerprints of wound and skin communities derived from chronic wound tissue samples and contralateral intact skin swabs. Similarities scores ranged from 10-60% with the average similarity score below 50% indicating that generally skin surface and wounds were colonised with highly divergent consortia profiles. However, distinct clustering can be seen in Figure 3.6, separating the DGGE fingerprints of wound and skin communities. This is explored further via principal component analysis of the similarity matrix scores shown in Figure 3.7 highlighting the clustering of the two sample groups. This suggests that although the bacterial communities within the groups are dynamic and dissimilar the overall microbiotas for each sample group are characteristic of the samples origin.

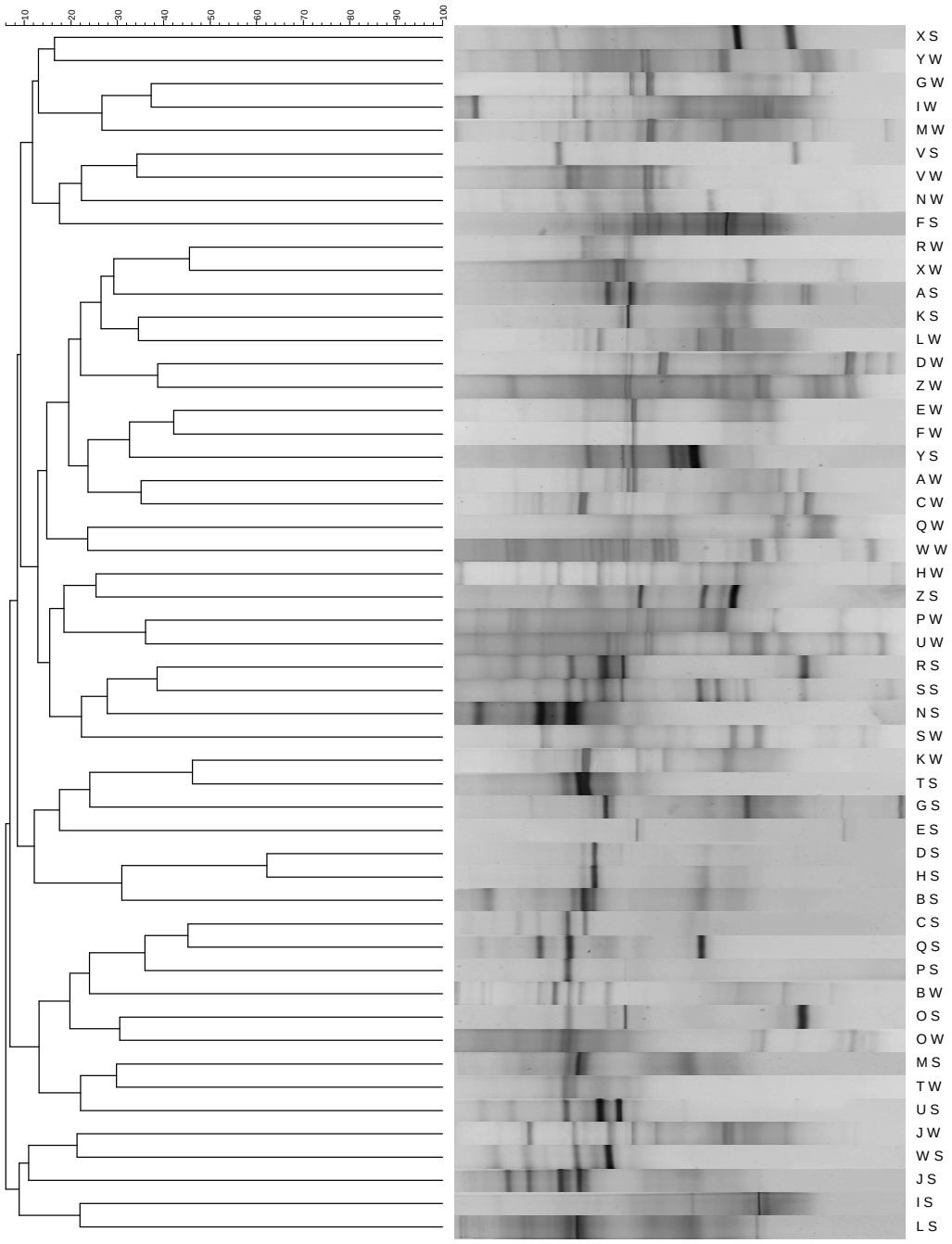


Figure 3.6 A UPGMA dendrogram showing percentage matching of wound DGGE fingerprints of chronic wound samples and intact skin swabs from Patients A-Z. The primary letter denotes the patient identification the second letter notes samples site (W: wound, S: Skin)

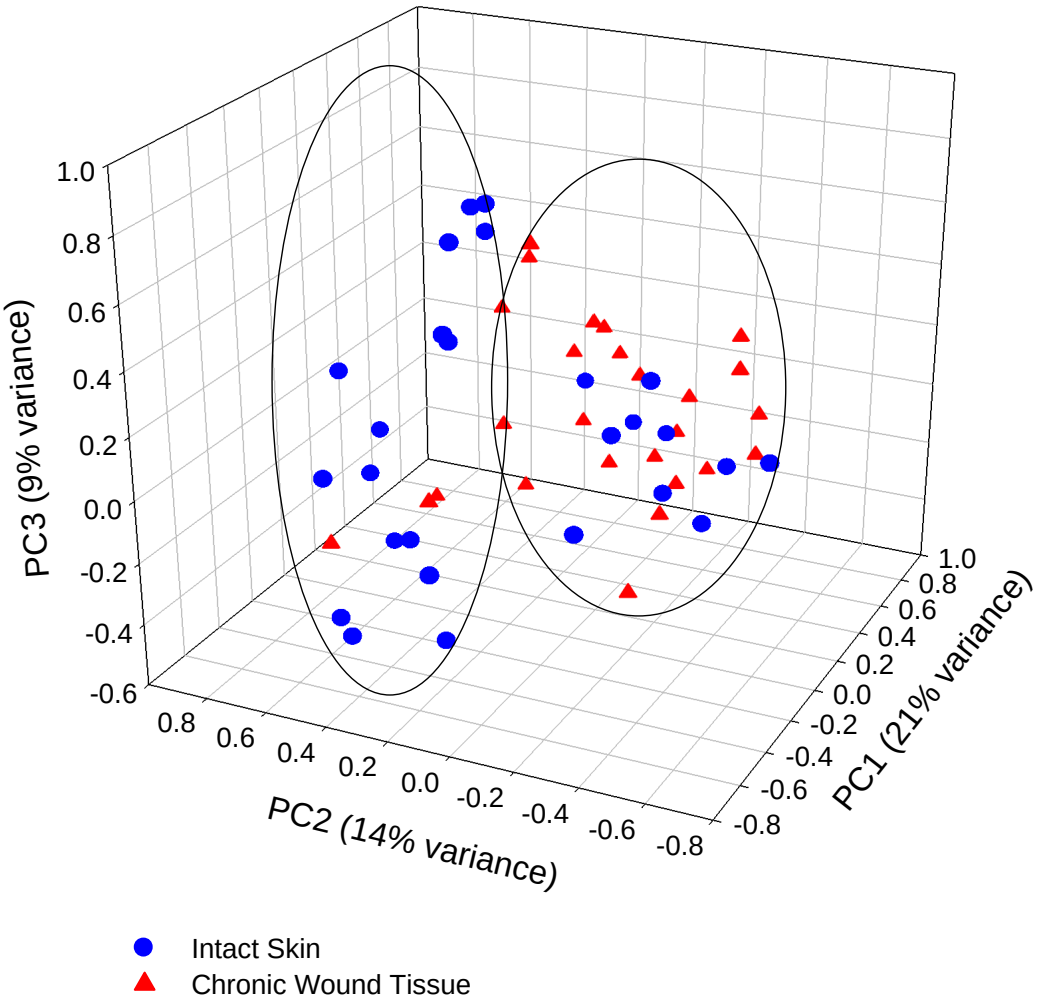


Figure 3.7. Principal component analysis of DGGE fingerprints of chronic wound samples and intact skin swabs from Patients A-Z

Data in Table 3.6 and 3.7 shows the number of unique bacterial genera derived from DGGE 16s DNA sequence analysis of chronic wound tissue and intact skin swabs samples respectively. Band matching was based upon pair-wise alignments with sequenced bands. All sequenced genera presented were ≥ 98% of the maximal identity of high scoring pairs. Bands which did not align with sequenced bands or whose sequencing showed no similarity in BLAST searches are not

included. For both tissue and skin the most predominant genus were *Staphylococci* spp. and *Bacillus* spp. the later of which was not identified on culture analysis. Additionally, a greater number of obligate anaerobic organisms were identified in both the skin and tissue isolates when compared to the culture data. Of the 22 identified genera of the wound tissue and 21 of skin swabs, four were unique to wounds; *Klebsiella* spp. *Abiotrophia* spp. *Escherichia coli* and *Peptoniphilus* spp. Three were unique to the intact skin swabs; *Kocuria rhizophilia* spp. *Morexellaceae* spp. and *Rhodocyclaceae* spp.

Table 3.6 Number of genera identified and matching bands from DGGE 16s DNA sequence analysis of chronic wound tissue samples

Genus	Matching bands or Sequences	Gram Reaction	Aerotolerance
Staphylococcus spp.	59	Positive	Facultative anaerobic
Bacillus spp.	20	Positive	Aerobic
Streptococcus spp.	12	Positive	Facultative anaerobic
Prevotella spp.	8	Negative	anaerobic
Actinobacterium spp	7	Positive	anaerobic
Enterobacter spp.	7	Negative	Facultative anaerobic
Clostridia spp.	6	Positive	Anaerobic
Enterococcus spp.	5	Positive	Facultative anaerobic
Enterococcaceae spp.	5	Positive	Facultative anaerobic
Stenotrophomonas spp.	5	Negative	Aerobic
Acinetobacter spp.	4	Negative	Aerobic
Bacteroides spp.	4	Negative	Anaerobic
Anaerococcus spp.	3	Positive	Anaerobic
Finegoldia spp.	3	Positive	Anaerobic
Klebsiella spp.	3	Negative	Facultative anaerobic
Micrococcus spp.	3	Positive	Facultative anaerobic
Virovax spp.	3	Negative	Aerobic
Lactobacillus spp.	2	Positive	Microaeropphilic/facultative anaerobic
Sphingomonas spp	2	Negative	Aerobic
Abiotrophia spp.	1	Positive	Facultative anaerobic
Escherichia coli	1	Negative	Facultative anaerobic
Peptoniphilus spp.	1	Positive	Anaerobic

Table 3.7 Number of genera identified and matching bands from DGGE 16s DNA sequence analysis of intact skin swabs

Genus	Matching bands or Sequences	Gram Reaction	Aerotolerance
Staphylococcus spp.	51	Positive	Facultative anaerobic
Bacillus spp.	22	Positive	Aerobic
Micrococcus spp.	4	Positive	Facultative anaerobic
Acinetobacter spp.	3	Negative	Aerobic
Stenotrophomonas spp.	3	Negative	Aerobic
Variovorax spp.	3	Negative	Aerobic
Actinobacterium spp.	2	Positive	Aerobic
Anaerococcus spp.	2	Positive	Aerobic
Clostridia spp.	2	Positive	Aerobic
Enterobacter spp.	2	Negative	Facultative anaerobic
Enterococcaceae spp.	2	Positive	Facultative anaerobic
Enterococcus spp.	2	Positive	Facultative anaerobic
Prevotella spp.	2	Negative	Anaerobic
Sphingomonas spp.	2	Negative	Aerobic
Bacteroides spp.	1	Negative	Anaerobic
Finegoldia spp.	1	Positive	Anaerobic
Kocuria rhizophila spp.	1	Positive	Facultative anaerobic
Lactobacillus spp.	1	Positive	Microaeropphilic/facultative anaerobic
Moraxellaceae spp.	1	Negative	Aerobic
Rhodocyclaceae spp.	1	Negative	Aerobic
Streptococcus spp.	1	Positive	Facultative anaerobic

3.4.3 Comparisons of DGGE 16s DNA sequence data between chronic wound tissue samples and contralateral intact skin swabs for each patient.

To compare the bacteriological changes associated with the shift from healthy skin to that of the chronic wound, comparisons were made between matching bands and sequences across the wound and the contralateral control skin swab for each patient. Examples are given from Patient A and G considered non-infected (section 3.4.3.1), and Patient D and I considered infected (section 3.4.3.2.). See

Appendix 1F for all matched bands of samples tested. A brief summary of the results is given in Table 3.8.

Table 3.8 Proportion of bands present in intact skin found in chronic wound grouped based upon culture infection criteria

Patient	Proportion*	Infected
A	4/11	NO
B	0/5	NO
C	3/4	NO
G	2/7	NO
H	2/3	NO
M	1/4	NO
O	2/4	NO
R	2/6	NO
S	3/10	NO
U	1/5	NO
V	2/3	NO
X	0/4	NO
Z	1/6	NO
D	0/4	YES
E	0/2	YES
F	0/10	YES
I	0/5	YES
J	1/6	YES
K	0/5	YES
L	0/8	YES
N	0/6	YES
P	0/1	YES
Q	0/3	YES
T	1/4	YES
W	0/5	YES
Y	0/6	YES

*Proportion of bands present in intact skin DGGE analysis found in chronic wound DGGE analysis

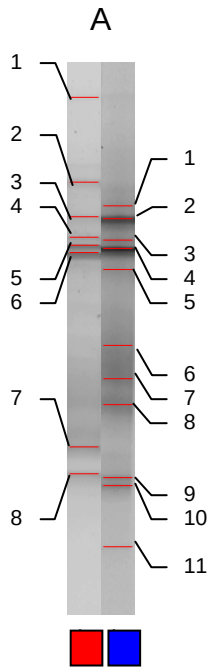
Data in Table 3.8 shows that on average a greater proportion of skin-associated bacteria were found in the non-infected contralateral wound when compared to the proportion of skin associated bacteria found in the infected wounds.

3.4.3.1 Comparisons of DGGE 16s DNA sequence data between chronic wound tissue samples and contralateral intact skin swabs from non-infected patient A and G.

Patient A: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	N/A	*Bacteroidales* spp. (HM079538)
2	*Staphylococcus epidermidis* strain VC334S1 1(HM452104)	*Staphylococcus simulans* strain AK7LW (HM462053)
3	*Staphylococcus simulans* strain AK38LW (HM452000)	*Staphylococcus* spp. DH17_87 (HM074836)
4	*Staphylococcus* spp. DH17_87 (HM074836)	*Enterococcus faecalis* strain LCR18 (HQ259727)
5	*Enterococcus faecalis* strain LCR18(HQ259727)	N/A
6	*Streptococcus* spp. BL020B49 (AY806239)	*Staphylococcus epidermidis* strain CJBP1. (AM697667)
7	Bacterium ncd1127c09c1(HM338822)	Bacterium ncd1141g11c1 (HM344790)
8	*Micrococcus yunnanensis* strain R-76G (HQ285773)	*Bacillaceae* bacterium BL-87 (EU596919)
9		*Micrococcus yunnanensis* strain R-76G (HQ285773)
10		N/A
11		N/A

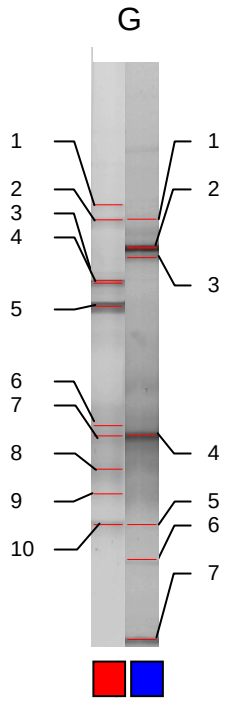
Matched bands based on band alignment and sequences are shaded grey. 4/11 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis



Patient G: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Clostridia* bacterium MT05B_C05 (DQ169781)	*Staphylococcus sciuri* strain R10-5A (HQ154580)
2	*Staphylococcus* spp. DH10_85 (HM074305)	*Variovorax* spp. 01xTSA28A_F05 (HM113661)
3	*Staphylococcus aureus* strain GCA890 (HM209755)	*Staphylococcus simulans* strain AK7LW (HM462053)
4	*Prevotella bivia* strain: JCM 6332 (AB547674)	*Sphingomonas* spp. AVCTGRB13A (HM346205)
5	*Streptococcus dysgalactiae* strain CH74 (HM359249)	N/A
6	*Stenotrophomonas* spp. CB13(2010) (FJ609992)	N/A
7	*Sphingomonas* spp. AVCTGRB13A(HM346205)	*Kocuria rhizophila* strain PE-LR-2 (FR687213)
8	N/A	
9	N/A	
10	N/A	

Matched bands based on band alignment and sequences are shaded grey. 2/7 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



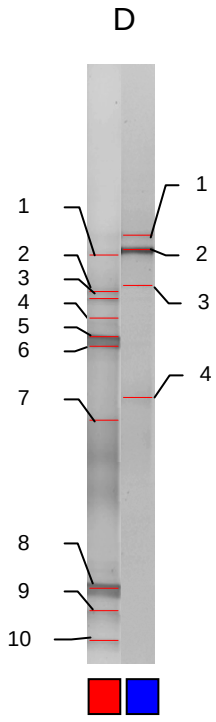
Wound Tissue Sample Intact Skin Swab

3.4.3.2 Comparisons of DGGE 16s DNA sequence data between chronic wound tissue samples and contralateral intact skin a swabs from infected patients D and I.

Patient D: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Bacteroidales* bacterium E105H11 (HM079538)	*Staphylococcus* spp. DH18_56 (HM074899)
2	*Staphylococcus* spp. DH17_87 (HM074836)	bacterium ncd1127c09c1 (HM338822)
3	*Staphylococcus epidermidis* strain VC334S1 (HM452104)	N/A
4	N/A	*Enterobacter* sp HSL97 (HM461227)
5	*Acinetobacter* spp. 4A9S1(HQ246291)	
6	*Staphylococcus* spp. DH10_85 (HM074305)	
7	*Bacillus pumilus* strain FS55 (AF260751)	
8	*Klebsiella* spp. SL13 (HQ264073)	
9	*Klebsiella* spp. TS8N1(GU294294)	
10	N/A	

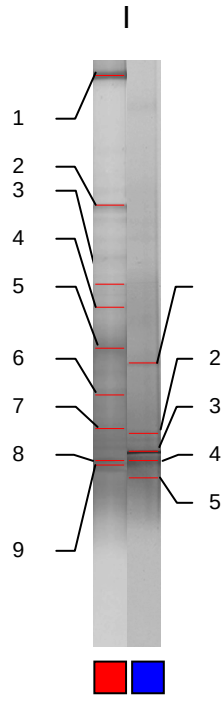
0/4 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient I: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Bacteroides fragilis* (FQ312004)	*Bacillus pumilus* strain B1W-36(FM179663)
2	*Clostridiales* spp. VA15_61 (HM076639)	*Sphingomonas* spp. AVCTGRB13A (HM346205)
3	*Enterococcaceae* spp. Cat005G_B01_ (EU572465)	*Stenotrophomonas maltophilia* strain MF48 (AY321966)
4	*Streptococcus dysgalactiae* strain CH74 (HM359249)	*Bacillus pumilus* strain B1W-36.(FM179663)
5	N/A	*Actinomycetales* spp. VA22_33 (HM077215)
6	*Enterobacter* spp. HSL97 (HM461227)	
7	*Bacillaceae* bacterium BL-87 (EU596919)	
8	*Enterobacter* spp. D9 (FJ609991)	
9	*Enterobacter* spp. D9 (FJ609991)	

0/5 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis..



Wound Tissue Sample Intact Skin Swab

3.4.4 Gram stain, FISH and ESEM of tissue sections

Figures 3.8 to 3.10 shows the stained and imaged chronic wound sections from Patient A. Gram, FISH and ESEM analysis all show evidence of bacterial microcolonies embedded within and upon surfaces of wound tissue. Figure 3.9 shows putative biofilms stained with Concanavalin A-FITC as indicated by arrows.

Figures 3.11 to 3.13 shows the stained and imaged chronic wound sections from Patient E. Gram, FISH and ESEM analysis all show evidence of bacterial microcolonies embedded within and upon surfaces of wound tissue. Figure 3.12 shows putative biofilms stained with Concanavalin A-FITC encasing bacterial microcolonies on the surfaces of the tissue as indicated by arrows.

Figures 3.14 to 3.16 shows the stained and imaged chronic wound sections from Patient T. Gram and FISH analysis all show evidence of bacterial microcolonies embedded within and upon surfaces of wound tissue. However, Figure 3.15 illustrates no bacterial infiltration within the internal portion of the tissues. Figure 3.16 shows putative biofilms encasing bacterial microcolonies on the surfaces of the tissue as indicated by arrows.

Figures 3.17 to 3.19 shows the stained and imaged chronic wound sections from Patient Z. Gram and FISH stained section show evidence of small number of bacteria within the wound tissue which were not found on ESEM imaging (Figure 3.19). Figure 3.18 shows putative biofilms stained with Concanavalin A-FITC encasing bacterial microcolonies within the tissue. Figure 3.19 ESEM imaging shows putative biofilm branching structures can be seen (as indicated by arrows).

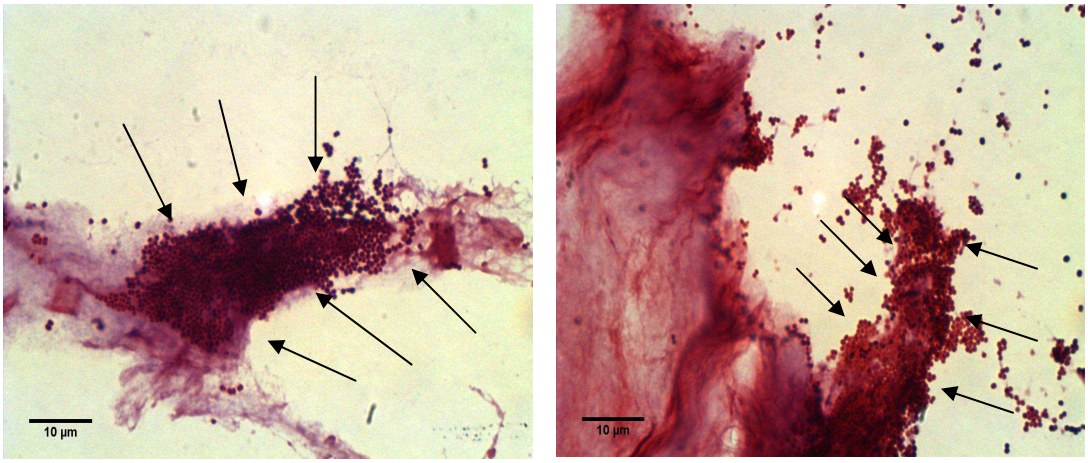


Figure 3.8 Gram stained sections of chronic wound tissue sample from Patient A. Bacterial microcolonies are indicated by arrows

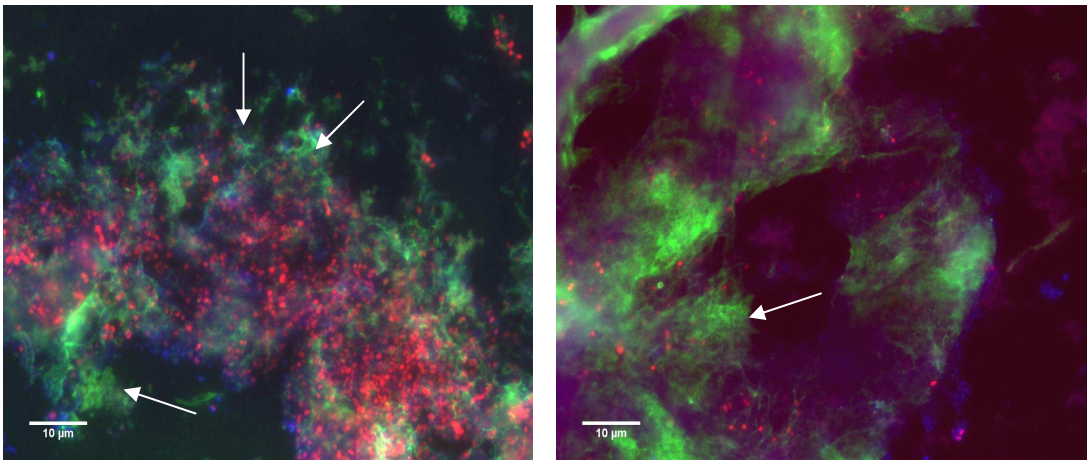


Figure 3.9 FISH stained sections of chronic wound tissue sample from Patient A. Putative biofilm stained with Concanavalin A-FITC indicated with white arrows

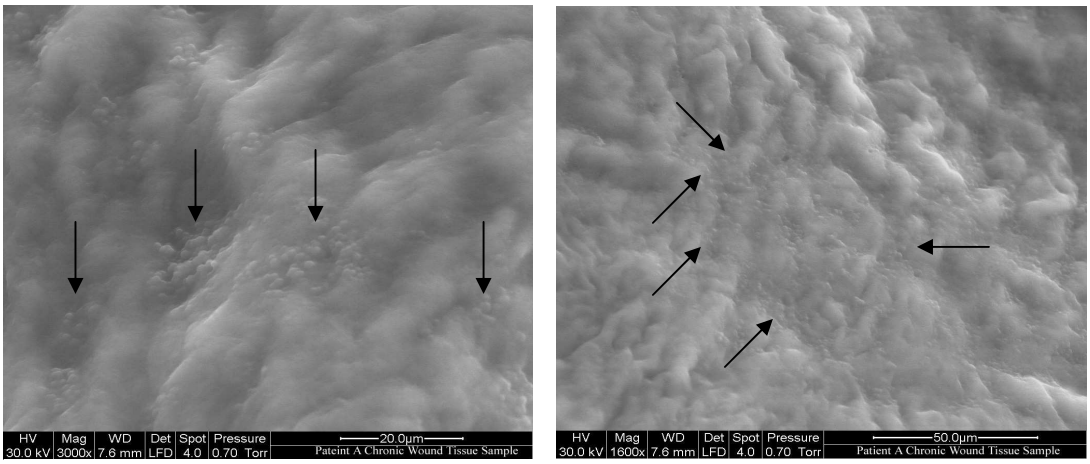


Figure 3.10 ESEM image of chronic wound tissue sample from Patient A. Bacterial microcolonies indicated by arrows

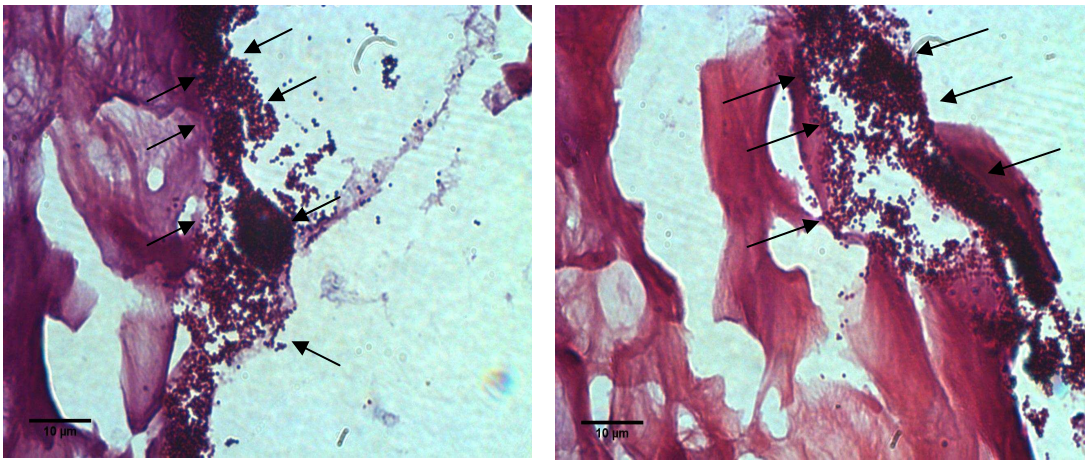


Figure 3.11 Gram stained sections of chronic wound tissue sample from Patient E. Bacterial microcolonies are indicated by arrows

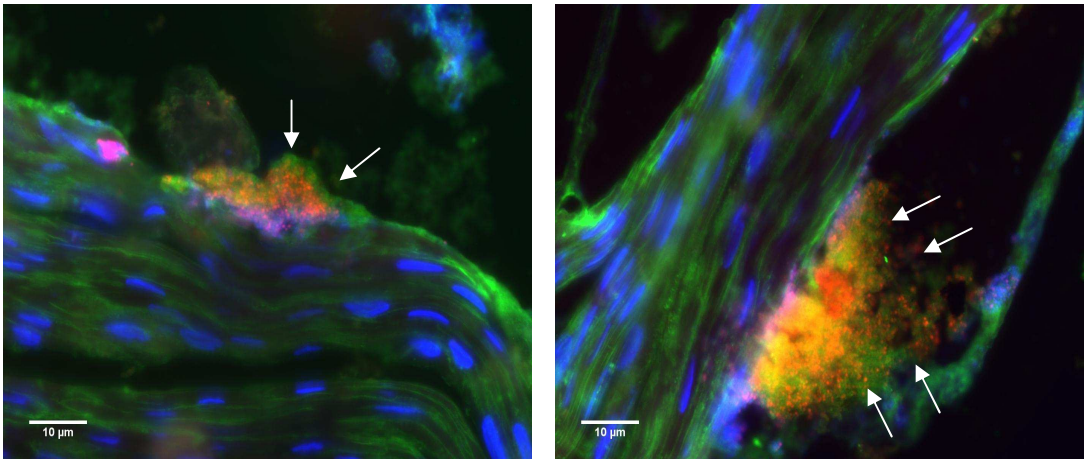


Figure 3.12 FISH stained sections of chronic wound tissue sample from Patient E. Putative biofilm stained with Concanavalin A-FITC indicated with white arrows

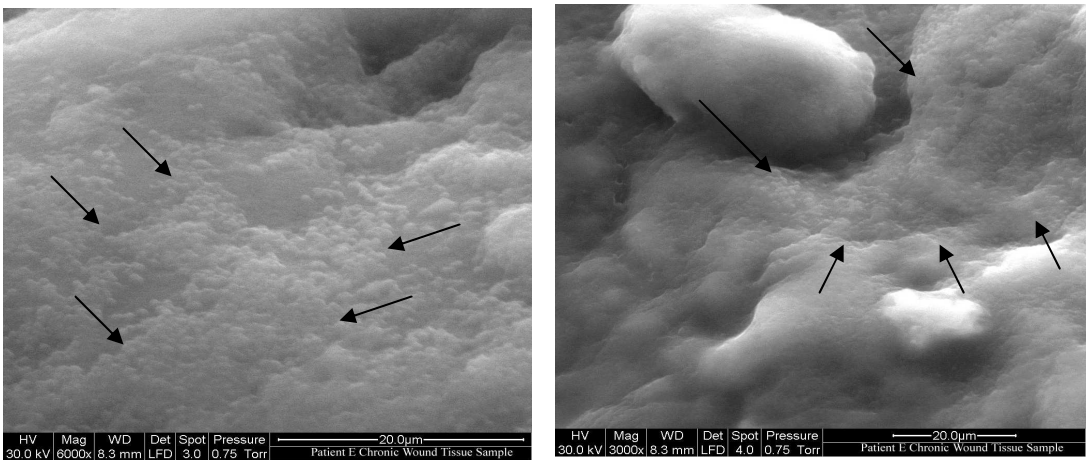


Figure 3.13 ESEM image of chronic wound tissue sample from Patient E. Bacterial microcolonies indicated by arrows

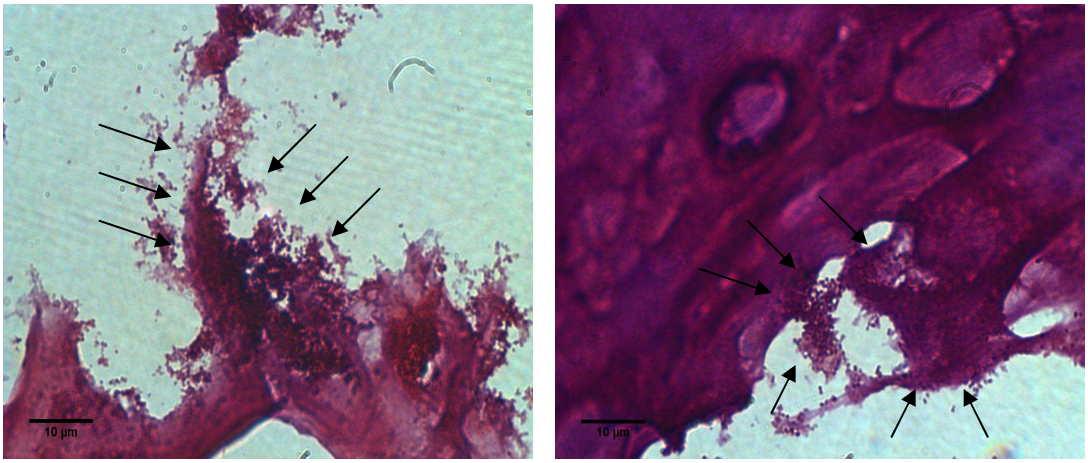


Figure 3.14 Gram stained sections of chronic wound tissue sample from Patient T. Bacterial microcolonies are indicated by arrows

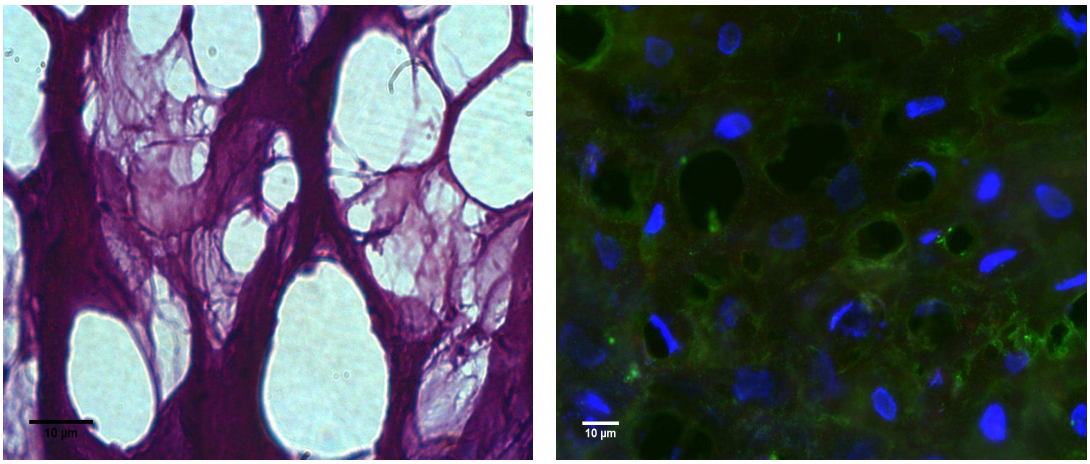


Figure 3.15 Gram and FISH stained sections of chronic wound tissue sample from Patient T showing no bacterial infiltration in the internal portion of tissue

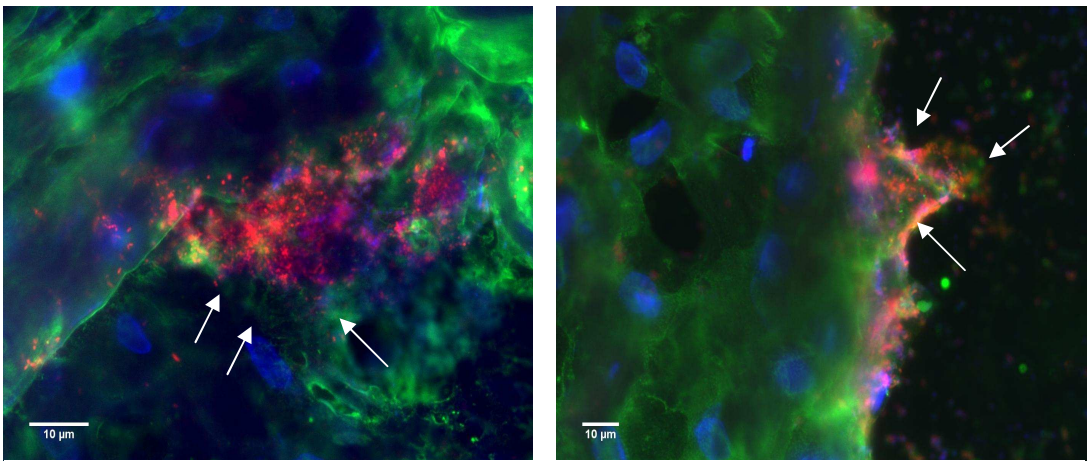


Figure 3.16 FISH stained sections of chronic wound tissue sample from Patient T. Putative biofilm stained with Concanavalin A-FITC indicated with white arrows

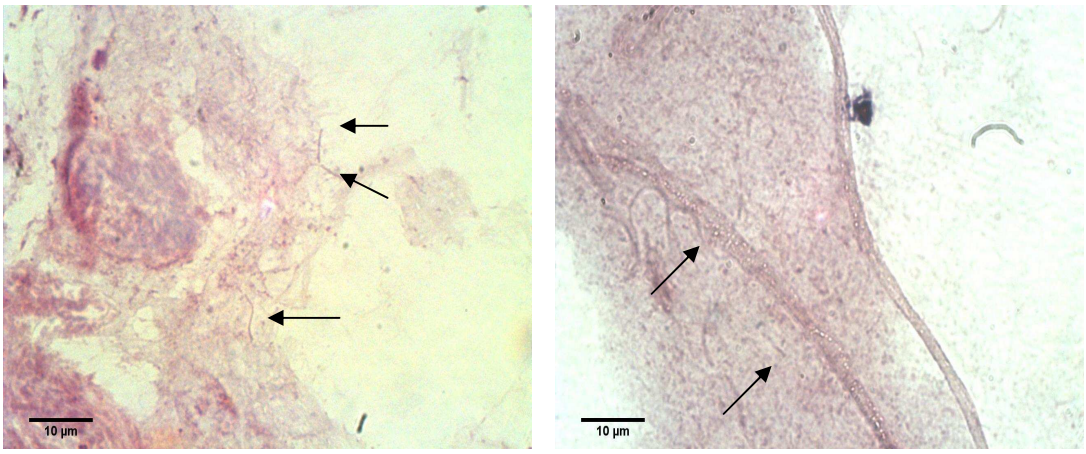


Figure 3.17 Gram stained sections of chronic wound tissue sample from Patient Z. Gram negative organisms are indicated by arrows

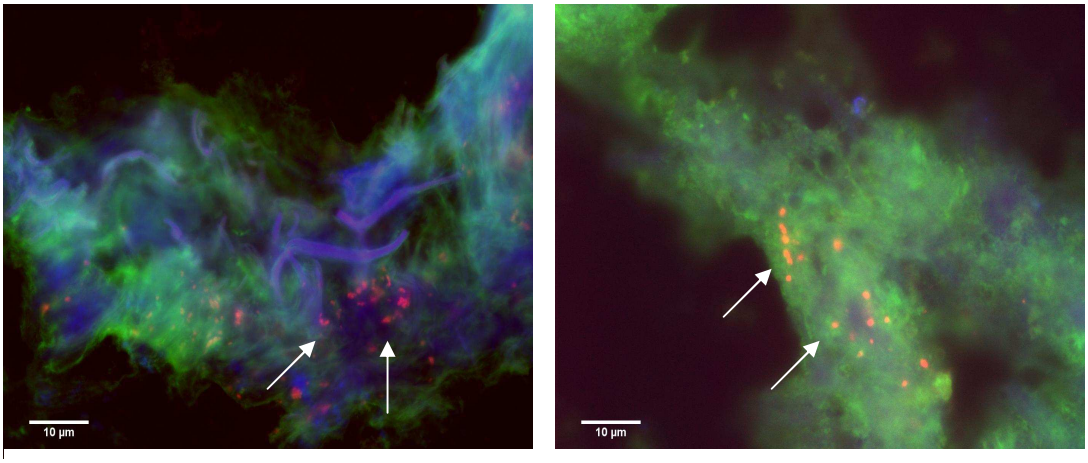


Figure 3.18 FISH stained sections of chronic wound tissue sample from Patient Z. Putative biofilm stained with Concanavalin A-FITC indicated with white arrows

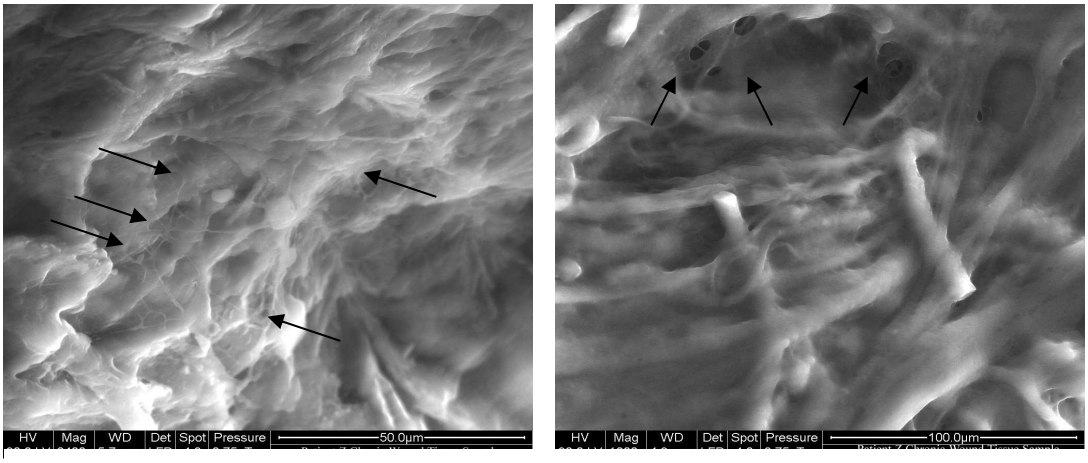


Figure 3.19 ESEM image of chronic wound tissue sample from Patient Z. Putative biofilms indicated by arrows

3.5 Discussion

The origins of chronic wounds initially lie with the underlying aetiologies of the patient combined with tissue trauma which creates a portal of entry for bacteria. Impaired wound healing in response to reduced vascularisation of the wound bed results in an extended timeframe in which dynamic bacterial communities develop and infection occurs. Subsequently, chronic wounds contain diverse microbial populations which have been the subject of a great deal of speculation and investigations as to their role in chronicity. However, the microbial ecology of a wound is only one facet in the complex paradigm of chronic wound microbiology; emphasis has also been placed on the quantity of bacteria and the presence of bacterial biofilms. This chapter aimed to investigate these three facets of chronic wound microbiology using culture, DGGE and FISH analysis. Culture analysis of the tissue samples and skin swabs followed standard clinical laboratory guidelines (HPA, 2009) combined with exhaustive viable count analysis. The culture data revealed limited culturable bacterial diversity within intact skin swabs, with only three genera routinely identified. Culture analysis of the tissue samples revealed relatively more culturable organisms (n=12) with the predominant genera *Staphylococci* spp. identified in 25 of the 26 samples shown in Table 3.4. In contrast, the DGGE 16s DNA sequence analysis identified a total 22 genera in tissues and 21 in intact skin swabs of which a greater number of obligate anaerobic genera were identified. This is a similar finding to previous studies (Dowd *et al.*, 2008; Gontcharova *et al.*, 2010; Price *et al.*, 2009) and may have serious implications in the treatment and healing times of wounds with anaerobic organisms such as *Peptostreptococci* spp. known to inhibit both fibroblast and keratinocyte proliferation, and endothelial tube formation (Stephens *et al.*, 2003).

This highlights the fundamental limitation of culture analysis in which fastidious and/or unculturable species may be overlooked and potential infections untreated. However, DGGE analysis is not without limitations; differential lysis of bacteria, different specificity of primers and variations in amplification efficiency can affect the overall measure of diversity by preferentially selecting organisms which can be lysed or amplified using the methodologies employed (Reysenbach *et al.*, 1992; Suzuki and Giovannoni, 1996; Wilson *et al.*, 1997). Nevertheless, DGGE analysis does have an increased sensitivity when compared to culture techniques overcoming the issues associated with culture bias and identifying a greater number of organisms overall as evidenced in this chapter. It is important to note however, that the detection of specific genera or species is not necessarily indicative of infection since this is dependant upon the presence of replicating organisms and resultant tissue damage (Dow *et al.*, 1999). A figure of $\geq 10^6$ organisms per gram of tissue has been cited as the threshold at or above which a wound can be categorised as infected (Murphy *et al.*, 1986; Robson, 1979; Robson, 1997). Within a clinical laboratory environment and within this chapter, semi-quantitative culture analysis is normally undertaken as measure of infection by the presence of a pathogenic organisms, combined with grading bacterial growth as scanty, light, moderate or heavy. The semi-quantitative data from chronic wound tissue samples in this chapter correlated with the quantitative viable count data derived from selective agar plates. A total of 13 tissue samples were identified as being infected with *S. aureus*, of which four samples (D, E, I and K) were identified as containing a heavy growth of *S. aureus* with no other organisms present. Subsequently, the viable count data was taken as an accurate measure of the quantity of *S. aureus* within the tissue, with resultant values of $\geq 10^6$ organisms per gram of tissue found for sample D, E, I and K. All other samples

were classed as infected by standard laboratory culture techniques as indicated the presence of pathogenic organism associated with wound infection (HPA, 2009). All 13 samples classed as infected cultured *S. aureus* with samples from patient E also containing *Candida* spp. patient M; *Pseudomonas* spp. and beta haemolytic group G streptococcus. Intrapersonal comparisons of the bacterial community profiles derived from DGGE 16s DNA sequence analysis within these culture-defined groups of infected and non-infected revealed that, when compared to contralateral control skin swab, a proportionally higher incidence of the bacteria present on the control skin were found in the non-infected wounds (as defined as matching band position and sequence). Within the 13 cultured defined infection groups; 11 contained no bands which matched to the contralateral skin swab bacterial band profile. The literature suggests that analysis of the microbiota of same site skin between individuals produces a low level interpersonal consensus i.e. there is little similarity between individuals however, analysis of contralateral skin microbiota within an individual indicates a high level of conservation and stability between the two analogous sites (Gao *et al.*, 2007; Grice *et al.*, 2008). Subsequently, contralateral intact skin samples are the ideal samples to reveal the original microbiota of the site prior to wounding and throughout the time the wound is present. The data presented in this chapter indicates that there was a distinct ecological shift in the microbiota from a culture-defined non-infected state to a culture-defined infected state resulting in a loss of members of the host microbiota associated with healthy skin. Principal component analysis revealed two separate overall ecological groups of the skin and wound derived from the DGGE similarity matrix data which is similar to previous findings (Dowd *et al.*, 2008; Gontcharova *et al.*, 2010). This is unsurprising as the two sites represent different nutrient availability and associated environments in which diverse and specialised bacterial

species compete and propagate. The depleted nutrient availability and restricted moisture content of the skin provides limited opportunity for fastidious organisms to proliferate and therefore it selects for specialised and adapted organisms such as *S. epidermidis* and *P. acnes* (Chiller *et al.*, 2001; Cogen *et al.*, 2008). In contrast the nutrient rich milieu of the chronic wound facilitates the growth of a wide variety or organism such as *S. aureus*, *P. aeruginosa, Streptococci* spp, Enterobacteriaceae, and facultative anaerobic species (Chiller *et al.*, 2001; Davies *et al.*, 2001). This suggests that because there are two defined populations identified in this chapter the ecological shift is confined to a few genera and species which are specific to the individual. This is born out by the proportion data shown in Table 3.8. The ecological shift identified within this chapter could be a result of two associated factors; the role of the microbiota on the infecting organisms via colonisation resistance and the role of infecting organisms on the microbiota. The primary colonisers of a wound are believed to the autochthonous flora due to their positional loci to the tissue injury (Bowler *et al.*, 2001). In acute, healing wounds there is limited time for bacteria to take residence and therefore only relatively small number of skin bacteria colonise. However, in chronic wounds, the continued exposure of tissues to autochthonous and exogenous bacteria facilitates the colonisation and establishment of a wide range of microorganisms. Grice *et al*., (2010) examined the diabetic wound microbiome in tandem with host tissue gene expression in mice. It was observed that selective shifts in wound microbiota coincided with longitudinally impaired healing in diabetic mice; specifically the microbiota of the wounds shifted overtime displaying increased levels of *Staphylococcus* spp*. Aerococcus* spp. as a result of selective environmental pressures such as the immune response and population interactions (Grice *et al.*, 2010). Within the chronic wound the primary

autochthonous colonisers are in direct competition for nutrients and attachment sites, subsequently the shifts in opposing population sizes may lead to the ecological shift observed when populations reach culture defined infective levels resulting in a loss of colonising skin flora. This phenomenon is often seen with infection of the gastrointestinal tract where initiation of infection by a pathogenic organism modifies the microbiota composition for example *Salmonella enterica* serovar typhimurium has been shown to modify the inflammatory host responses at detriment to the host microbiota facilitating its pathogenesis and progression to infection (Sekirov and Finlay, 2009; Stecher *et al.*, 2007).

In addition, to the types and quantity of bacteria within chronic wounds, the presence of biofilms has also been cited as a potential factor in impaired healing. Of the 26 tissue samples investigated in this chapter; four were of sufficient size to undergo cryosectioning and staining with the view to determine the presence or absence of biofilms. All stained samples showed evidence of microcolonies and/or putative exopolysaccharide material indicating biofilm presence however, the presence of microcolonies appeared to be surface-associated with little or no microcolonies visualised in the internal portions of the tissue show in Figure 3.15. Interestingly, two samples (E and T) were from clinically defined infected wounds and two (A and Z) were defined as clinically uninfected suggesting that biofilm formation maybe a significant underlying element in colonised chronic wounds which is not related to the presence or quantity of infective organisms. Schierle *et al*., (2009) employed a murine cutaneous wound system to investigate the role of *S. aureus* and *Staphylococcus epidermidis* biofilms played in delayed healing times. The presence of both organisms significantly delayed wound closures however, organisms with an endogenous mutation in the biofilm signalling

pathway failed to produce similar healing delays (Schierle *et al.*, 2009). This directly demonstrates that delayed re-epithelialisation can be caused by the presence of a bacterial biofilms that may be derived from pathogenic or autochthonous bacteria. It is therefore possible that the delays in healing seen in the patient group investigated could be attributed to the presence of biofilms in both “infected” and “non-infected” chronic wound tissue samples.

3.6 Conclusion

Data presented in this chapter demonstrates that the presence of wound biofilms was not related to the presence of infection and the chronic wound microbiota is diverse and distinctly different from that of intact healthy skin. Microbial ecological shifts occur within the wound in relation to the quantities of pathogenic bacteria present specifically relating to culture defined infection. Culture based methodologies are the cornerstone in diagnostic microbiology however, recent emphasis has been placed on molecular techniques to elucidate the microbial ecologies. The data within this chapter indicates that these methodologies and their subsequent data outcomes should not be treated as mutually exclusive as their two measurable factors are intertwined.

Chapter 4

Coaggregation interactions between wound and skin associated bacteria

4.1 Abstract

To gather a greater understanding of the process underlying the development polymicrobial wound communities; biofilm formation and coaggregation interactions of 29 bacteria associated with healthy skin and chronic wounds was investigated. Axenic biofilm formation of commonly isolated wound (n=18) and skin (n=11) bacteria upon an abiotic surface was assessed using a crystal violet biofilm assay. Pair-wise coaggregation interactions was investigated between all 29 strains, all possible combinations were tested in triplicate using a quantitative spectrophotometric coaggregation assay. Whilst all isolates formed biofilms, there was no indication that bacteria of wound origin did so more readily than those of isolated from intact healthy skin. Aggregation interactions were weak or not detectable, apart from those associated with *Corynebacterium xerosis*. This bacterium produced a high autoaggregation score (*c.* 50%). The limited coaggregation interactions suggest that coaggregation interactions maybe comparatively unimportant in the development of wound and skin biofilms

4.2 Introduction

Bacterial biofilm development is a dynamic process by which bacteria attach to abiotic or biotic surfaces through a series of co-dependant and independent processes (Costerton, 1999). Primary attachment of “early colonisers” to surfaces is initially facilitated by van der Waals forces and electrostatic interactions and then by the production of exo-polysaccharides and/or ligands which form complexes with surface associated compounds of the conditioning film (Busscher and Weerkamp, 1987; Palmer *et al.*, 2007). Favourable growth conditions such as moisture and sufficient nutrients may encourage bacterial multiplication resulting in microcolony formation (Rickard *et al.*, 2003a). Biofilms of *in vivo* environment are typically multispecies and a model for their development is generally derived from the dental plaque multispecies model where multispecies biofilm communities arise when there is a presence of taxonomically diverse microorganisms which attach sequentially to the early colonisers via coaggregation interactions. Coaggregation has been defined as the attachment of genetically distinct bacteria to each other via the reciprocal association of surface molecules. These associations are normally mediated by complexes of lectins and carbohydrates (Khemaleelakul *et al.*, 2006). The resulting cell-cell connections between genetically distinct cells and genetically homogenous cells (termed autoaggregation) facilitates coadhesion within the biofilm which occurs when a bacterium coaggregates with a sessile aggregates, strengthening the physical connections and thus the overall structure of the biofilm. Coaggregation has been extensively but not exclusively in studied in oral bacterial communities where it is believed to be a significant mechanism in the formation of oral multispecies biofilms. Although this model of polymicrobial biofilm development is based upon

oral biofilms, other polymicrobial environments such as gastrointestinal tract and freshwater systems have been found to employ similar modes of biofilm development and coaggregation albeit to a lesser extent than dental plaque (Ledder *et al.*, 2008; Rickard *et al.*, 2003b).

Chronic wound microbial residents form complex polymicrobial communities which can harbour both anaerobic and aerobic species (Bowler and Davies, 1999). Pathogenic organisms typically isolated from chronic wounds include; *Staphylococcus aureus*, *Pseudomonas* spp., *Streptococcus* spp., *Bacteroides* spp., *Clostridium* spp., together with various anaerobic cocci and enterobacteriaceae (Bowler and Davies, 1999; Davies *et al.*, 2004; HPA, 2009). To gather a greater understanding of the stages of assemblage of wound polymicrobial communities, recent work has investigated the coaggregation ability of wound-associated bacterial isolates. Work by Hill *et al*., (2010) investigated coaggregation interactions amongst wound-associated bacterial utilising a visual scale of coaggregation from methods previously described by Ciser *et al*., (1979). The data presented by Hill *et al*., (2010) indicates relatively low coaggregation frequency among wound isolates with few giving elevated aggregations scores however; no data was presented as to the autoaggregation activity of the bacterial isolates. Since coaggregation in the Hill *et al*., (2010) study is scored based upon the presence of flocs it is essential to demonstrate that the assembled flocs are product of the coaggregation of two distinct bacterial strains as apposed to the autoaggregation of one bacterial strain. Additionally, the study by Hill *et al*., (2010) did not consider the potential role of primary colonisers in the establishment of multispecies biofilms. In the context of wounds primary colonisers are members of the autochthonous skin community (Bowler *et al.*, 2001). This chapter therefore

aims to investigate the biofilm formation capability and coaggregation activity between human epidermal and wound-associated bacterial isolates utilising a quantitative coaggregation scoring system.

4.3 Materials and methods

4.3.1 Media and chemicals

Commercially available media used in the study were nutrient agar and broth, Wilkins Chalgren agar and broth, and Luria-Bertani (LB) broth which were obtained from Oxoid Ltd (Basingstoke, Hampshire). These were reconstituted according to the manufacturer's instructions and sterilised by autoclaving at 121℃ for 20 min.

4.3.2 Crystal violet biofilm assay to assess biofilm formation on an abiotic surface

The extent of biofilm formation of 29 skin and wound-associated bacterial isolates shown in Table 4.1 upon abiotic surfaces was investigated using a crystal violet microtitre plate assay previously described and validated by O'Toole *et al.,*(1999). To obtain stationary phase cultures bacterial isolates were grown overnight at 37℃ in 10 ml of LB broth aerobically or in pre-red uced LB broth anaerobically for respective aerobic and anaerobic bacterial isolates. Cultures were then diluted to 1:100 in fresh LB broth and 200 µl of the final inoculum dispensed into wells of a 96 well, flat bottomed microtitre plate (BD, SLS U.K.), uninoculated media was used as a reference blank. Anaerobic cultures and their respective media reference blanks were overlaid with mineral oil in order to maintain anaerobiosis

(Bio-Rad, Hertfordshire, U.K.). The microtitre plates were covered with lids and incubated with agitation (25 rpm) in an aerobic, 37℃ environment for 24 h. Following the incubation period the planktonic phase was removed from the wells using a pipette and the microtitre plate was gently washed with phosphate buffered saline (PBS). Residual PBS was then removed by inversion of the plates combined with gentle tapping onto absorbent paper; this washing stage was repeated three times to remove non-adherent cells. Plates were air dried and 200 µl of 0.1 % (w/v) crystal violet was added to each well. After 15 min incubation at room temperature, plates were washed three times (as previously described) and residual cellular bound crystal violet i.e. that which had associated with the biofilm was solubilised by the addition 200 µl of 95% ethanol. The absorbance was measured at 600 nm using an automated plate reader (Titertek Multiskan® MCC 340, Biotek, UK). Each assay contained three media reference controls of 200 µl of un-inoculated LB. Each isolate was tested in triplicate. Isolates which produced a mean optical density reading equal to or greater than three standard deviations of the mean optical density of the blanks were considered biofilm producers by this method (Stepanovic *et al.*, 2000).

Table 4.1 Bacterial strains used in this chapter

Bacterial Species	Strain number	Origin
Acinetobacter baumannii		Diabetic Ulcer/ConvaTec ltd*
Bacteroides fragilis	NCTC 9343	National Typed Culture Collection
Clostridium perfringens		Diabetic Ulcer/ConvaTec ltd*
Corynebacterium xerosis		Diabetic Ulcer/ConvaTec ltd*
Enterobacter cloacae	1, 43 and 50	Diabetic Ulcer/ConvaTec ltd*
Enterococcus faecalis	15, 21, 26	Diabetic Ulcer/ConvaTec ltd*
Escherichia coli		Diabetic Ulcer/ConvaTec ltd*
Micrococcus luteus		Isolated from healthy skin*
Propionibacterium acnes		Isolated from healthy skin*
Propionibacterium avidum		Isolated from healthy skin*
Proteus mirabilis	2, 5 and 40	Diabetic Ulcer/ConvaTec ltd*
Pseudomonas aeruginosa	12, 24 and 38	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus aureus	9, 10 and 11	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus capitis		Isolated from healthy skin*
Staphylococcus epidermidis	ATCC 14990	American Typed Culture Collection
Staphylococcus haemolyticus		Isolated from healthy skin*
Staphylococcus hominis		Isolated from healthy skin*
Staphylococcus saccharolyticus		Isolated from healthy skin*
Staphylococcus saprophyticus	NCTC 7292	National Typed Culture Collection

* identified by 16S rRNA gene sequencing

4.3.3 Quantitative coaggregation assay to assess coaggregation between pair-wise combinations of skin and wound-associated isolates

A modified quantitative spectrophotometric assay as previously described by Ikegami *et al.,* (2004) and subsequently used by Ledder *et al*., (2008), was utilised to assess coaggregation between a total of 18 wound-associated bacterial strains and 11 human epidermis-associated bacterial strains. Strains were selected based upon their culturable recovery rate i.e. their occurrence on the skin or in infected

wounds. To obtain stationary phase cultures, test bacteria were inoculated into 250 ml of nutrient broth or pre-reduced Wilkins Chalgren broth (for respective aerobic and anaerobic isolates) in 500 ml Duran bottle (with loose lids) and incubated aerobically at 37℃ for four days or anae robically in a Mark 3 Anaerobic Work Station (Don Whitely Scientific) at 37ºC (Gas mix: 90% N_2, 10% CO_2 and 10% H_2) for seven days. Bacteria were harvested by centrifugation at 10,000 rpm (Beckman-Coulter J2-21) for 20 min and resuspended in coaggregation buffer (TRIS; 20mM, NaCl; 150mM, $CaCl_2$; 0.1mM, $MgCl_2$; 0.15mM and NaN_3; 3.1mM) (Kolenbrander, 1988). This was repeated three times with the final suspension adjusted to give an optical density of 1 at 600 nm. Equal volumes of each bacterial suspension (500 µl) were combined, mixed and initial optical densities at 600 nm recorded (pre-incubations value). Solutions were left at room temperature for 1 h to allow for any aggregates formed to sediment prior to a second optical density reading (Test Value). All assays were undertaken in triplicate.

The percentage of coaggregation was assessed by the following equation (Ikegami *et al.*, 2004):

$$\text{Coaggregation \%} = \left(\frac{\text{Pre - Incubation Value [OD600] - Test Value [OD600]}}{\text{Pre - Incubation Value [OD600]}} \right) \times 100$$

4.4 Results

4.4.1 Crystal violet biofilm assay of wound and skin associated bacterial isolates

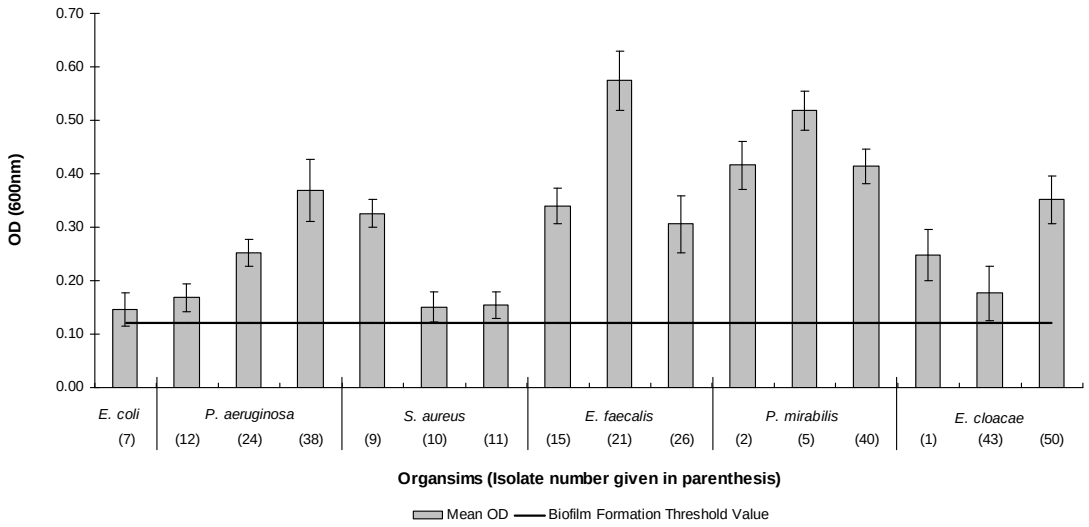


Figure 4.1 Biofilm formation by wound-associated bacterial isolates

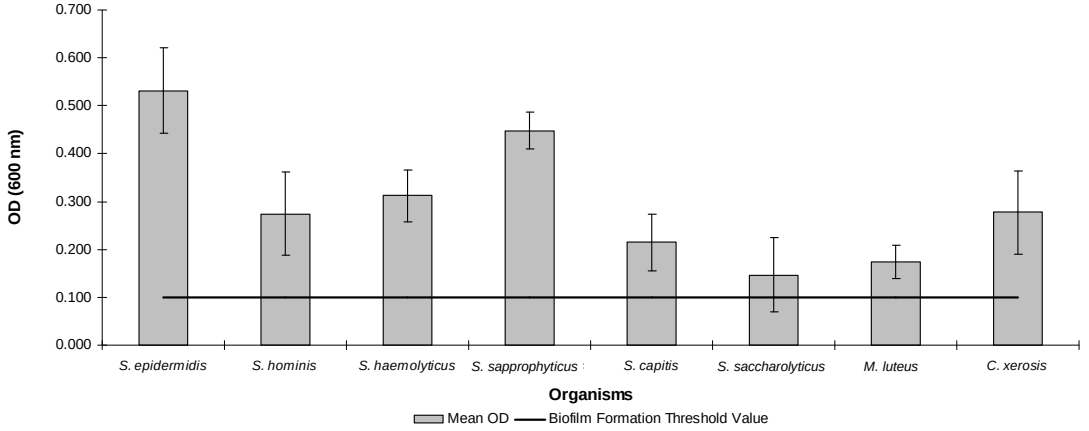


Figure 4.2 Biofilm formation by skin-associated bacterial isolates

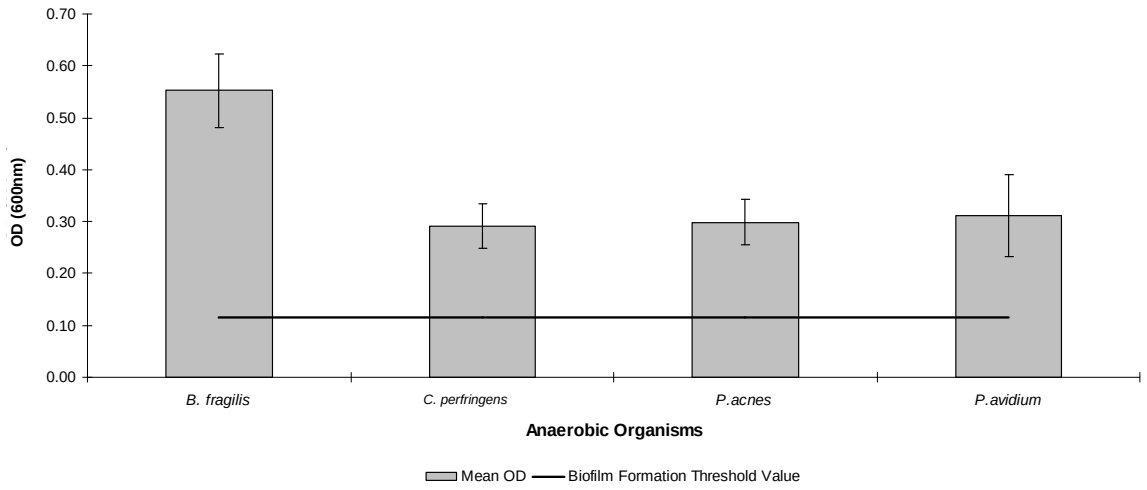


Figure 4.3 Biofilm formation by skin and wound-associated anaerobic bacterial isolates

The biofilm formation of 29 skin and wound bacterial isolates was investigated using a crystal violet microtitre plate assay previously described by O'Toole *et al.,* (1999). All isolates tested produced a mean optical density reading greater than the described biofilm threshold value (mean of the blanks plus three standard deviations) as shown in Figures 4.1 - 4.3 and thus were designated biofilm formers as defined by this method. Independent t-test analysis of the data identified no significant difference between the biofilm formation of skin and wound-associated isolates ($p>0.05$)

4.4.2 Quantitative coaggregation assay results for pair-wise combinations of wound and skin associated bacterial isolates

The data presented in Table 4.2 presents coaggregation interactions scores of 18 wound-associated bacterial isolates. The strongest coaggregation percentage was exhibited for combinations of *Pseudomonas aeruginosa* (strain number 12) with *Clostridium perfringens* (*c.* 10%) and *Bacteroides fragilis* (*c.* 10%) however; an autoaggregation score of *c.* 9% for *P. aeruginosa* (strain number 12) invalidates the former coaggregation scores.

Data presented in Table 4.3 shows coaggregation interactions scores for 11 skin-associated bacterial isolates. Scores ranged from 0.21 - 14% with elevated scores attained for all combinations involving *Corynebacterium xerosis.* However, an autoaggregation score of *c.* 50% for *C. xerosis* nullifies the former elevated coaggregation scores.

Data presented in Table 4.4 shows coaggregation interaction scores for combinations of 11 skin and 18 wound-associated bacterial isolates. Highest coaggregation scores (8.6 - 21%) occurred for combinations which included *C. xerosis.* Excluding coaggregation combinations with *C. xerosis,* the highest score was *c.* 6 % for combinations of *Staphylococcus hominis* and *P*. *aeruginosa* (strain number 38) and the lowest coaggregation score was 0.2% attained for combination of *Staphylococcus haemolyticus* and *Proteus mirabilis* (strain number 2).

Table 4.2 Coaggregation scores of pairs of 18 wound bacterial strains used in this chapter

Species	Isolate No.	*E. coli* (7)	*P. aeruginosa* (12)	*P. aeruginosa* (24)	*P. aeruginosa* (38)	*S. aureus* (9)	*S. aureus* (10)	*S. aureus* (11)	*E. faecalis* (15)	*E. faecalis* (21)
E. coli	7	0.43 (0.29)								
P. aeruginosa	12	3.26 (0.71)	9.09 (2.25)							
P. aeruginosa	24	0.86 (0.69)	5.89 (1.82)	1.21 (0.88)						
P. aeruginosa	38	3.25 (1.08)	7.69 (1.66)	9.46 (0.73)	6.7 (1.57)					
S. aureus	9	0.83 (0.75)	9.67 (1.74)	4.73 (3.23)	8.91 (2.96)	6.49 (5.64)				
S. aureus	10	0.92 (0.73)	5.93 (2.98)	3.15 (2.52)	4.1 (2.55)	3.67 (3.04)	1.2 (0.88)			
S. aureus	11	0.81 (0.48)	6.22 (2.11)	2.74 (3.21)	4.25 (1.98)	3.05 (2.45)	1.33 (0.99)	0.42 (0.41)		
E. faecalis	15	0.55 (0.12)	6.17 (0.25)	2.26 (1.84)	6.77 (2.35)	1.52 (1.23)	1.26 (1.02)	0.54 (0.68)	1.45 (1.24)	
E. faecalis	21	0.56 (0.27)	3.48 (1.73)	3.24 (4.37)	5.19 (2.29)	1.97 (1.15)	1.9 (2.67)	0.58 (0.66)	0.67 (0.43)	0.85 (0.74)
E. faecalis	26	2.1 (1.39)	5.91 (1.87)	1.06 (1.27)	7.01 (1.87)	1.99 (1.14)	3.65 (3.06)	0.69 (0.83)	1.7 (1.43)	1.64 (1.95)
P. mirabilis	2	3.32 (4.91)	4.29 (4.28)	0.8 (0.52)	6.36 (4.88)	0.36 (0.27)	1.21 (1.28)	0.27 (0.15)	0.52 (0.48)	1.03 (0.3)
P. mirabilis	5	3.27 (4.84)	2.9 (3.53)	0.5 (0.32)	2.34 (1.32)	0.89 (0.5)	0.98 (0.31)	0.3 (0.2)	3.59 (5.33)	0.4 (0.61)
P. mirabilis	40	0.57 (0.33)	2.13 (0.55)	0.72 (0.45)	1.85 (0.8)	1.15 (1.14)	0.71 (0.83)	0.54 (0.21)	1.4 (0.77)	0.64 (0.49)
E. cloacae	1	0.69 (0.71)	2.17 (1.82)	0.51 (0.28)	2.92 (2.38)	1.4 (0.57)	1.37 (0.59)	1 (0.77)	0.97 (0.75)	1.19 (1.12)
E. cloacae	43	0.41 (0.4)	2.48 (1.11)	0.87 (0.38)	2.83 (1.02)	1.8 (0.93)	0.87 (0.25)	1.32 (0.19)	1.27(0.97)	075 (0.45)
E. cloacae	50	3.19 (4.79)	2.27 (0.55)	0.43 (0.11)	3.12 (1.07)	0.73 (0.34)	0.4 (0.25)	0.27 (0.11)	1.4 (1.49)	0.31 (0.27)
C. perfringens		5.81 (3.61)	10.61 (4.11)	4.53 (4.51)	6.69 (3.34)	4.37 (1.64)	5.49 (2.76)	3.32 (1.75)	1.79 (1.04)	2.51 (2.31)
B. fragilis		9.52 (0.35)	10.07 (5.57)	1.36 (0.54)	7.06 (4.25)	2.58 (1.42)	2.18 (1.65)	0.9 (0.96)	2.16 (1.64)	1.85 (1.36)

Table 4.2 Cont. Coaggregation scores of pairs of 18 wound bacterial strains used in this chapter

Species	Isolate No.	*E. faecalis* (26)	*P. mirabilis* (2)	*P. mirabilis* (5)	*P. mirabilis* (40)	*E. cloacae* (1)	*E. cloacae* (43)	*E. cloacae* (50)	*C. perfringens*	*B. fragilis*
P. mirabilis	2	0.76 (1.04)	0.6 (0.52)							
P. mirabilis	5	0.99 (0.87)	0.37 (0.48)	0.21 (0.1)						
P. mirabilis	40	0.99 (0.62)	0.3 (0.2)	0.31 (0.27)	0.24 (0.29)					
E. cloacae	1	1.18 (0.87)	0.47 (0.25)	0.44 (0.21)	0.44 (0.79)	0.3 (0)				
E. cloacae	43	0.62 (0.98)	0.32 (0.39)	0.58 (0.54)	0.59 (0.75)	0.13 (0.05)	0.71 (0.51)			
E. cloacae	50	0.93 (0.38)	0.56 (0.2)	0.9 (0.3)	1.05 (1.1)	0.16 (0.07)	0.7 (0.42)	0.81 (0.64)		
C. perfringens		4.03 (2.42)	1.9 (0.82)	2.42 (1.34)	1.4 (1.97)	1.68 (2.22)	2.09 (0.58)	2.25 (2.27)	4 (2.26)	
B. fragilis		2.69 (3.8)	1.1 (1.32)	1.93 (1.2)	1.78 (2.27)	1.59 (2.05)	1.68 (0.57)	1.22 (1.03)	3.93 (4.09)	3.93 (2.56)

Data are mean optical densities from three separate experiments (SD are given in parenthesis)

Table 4.3 Coaggregation scores of pairs of 11 skin bacterial strains used in this chapter

Species	*S. epidermidis*	*S. hominis*	*S. haemolyticus*	*S. saprophyticus*	*S. capitis*	*S. saccharolyticus*	*M. luteus*	*C. xerosis*	*P. acnes*	*P. avidum*	*A. baumannii*
S. epidermidis	4.49 (4.42)										
S. hominis	4.53 (3.28)	4.66 (1.21)									
S. haemolyticus	1.97 (0.91)	1.68 (1.14)	0.78 (0.51)								
S.saprophyticus	1.75 (1.08)	2.04 (0.12)	0.43 (0.07)	0.81 (0.43)							
S. capitis	2.22 (1.25)	1.23 (0.62)	0.67 (0.56)	0.72 (0.69)	0.73 (0.92)						
S. saccharolyticus	2.09 (0.9)	5.05 (2.23)	1.28 (0.68)	0.92 (0.33)	1.01 (0.74)	1.24 (0.44)					
M. luteus	1.65 (0.9)	1.14 (1.14)	1.98 (0.56)	2.02 (1.75)	1.6 (1.46)	2.14 (1.58)	2.53 (1.21)				
C. xerosis	13.76 (1.52)	15.66 (6.48)	11.74 (3.86)	12.93 (4.4)	13.53 (4.96)	14.4 (1.24)	12.69 (5.28)	50.42 (5.78)			
P.acnes	3.1 (2.73)	1.93 (0.44)	0.49 (0.76)	1 (1.11)	0.56 (0.97)	1.06 (1.07)	1.1 (1.3)	14.67 (1.28)	1.06 (0.53)		
P. avidum	1.5 (0.45)	1.2 (0.37)	0.66 (0.1)	1.26 (1.1)	1 (0.65)	1.02 (0.88)	1.41 (1.29)	16.23 (2.02)	0.86 (0.19)	1.95 (2.14)	
Acinetobacter	1 (1.05)	1.41 (1.19)	0.55 (0.44)	0.62 (0.33)	1.79 (2.28)	0.53 (0.16)	1.9 (1.36)	9.88 (4.81)	0.44 (0.41)	0.21 (0.01)	0.96 (0.69)

Data are means optical densities from three separate experiments (SD are given in parenthesis). Coaggregation score between 12% and 50% are shaded light grey, and >50% dark grey

Table 4.4 Coaggregation scores of pairs of 18 wound bacterial isolates and 11 skin bacterial isolates

Species	Isolate No.	*S. epidermidis*	*S. hominis*	*S. haemolyticus*	*S. saprophyticus*	*S .capitis*	*S. saccharolyticus*	*M. luteus*	*C. xerosis*	*P.acnes*	*P. avidum*	*A. baumannii*
E. coli	7	0.6 (0.2)	0.85 (0.65)	0.66 (0.46)	0.42 (0.2)	2.59 (3.48)	0.39 (0.28)	1.17 (0.91)	8.57 (2.24)	0.29 (0.18)	0.23 (0.15)	1.44 (0.59)
P. aeruginosa	12	2.8 (0.76)	5.73 (3.9)	2.2 (1.36)	4.95 (2.8)	4.2 (3.57)	5.39 (4.6)	2.84 (0.65)	19.92 (2.29)	8.10 (4.62)	4.37 (1.66)	3.02 (1.55)
P. aeruginosa	24	2.55 (0.18)	4.63 (2.27)	2.2 (1.35)	1.58 (0.68)	1.51 (0.52)	2.3 (1.23)	3.12 (0.81)	15.47 (2.3)	1.23 (1.03)	1.53 (1.37)	1.23 (0.7)
P. aeruginosa	38	3.55 (0.59)	6.49 (2.38)	1.87 (1.02)	4.68 (2.59)	3.03 (2.22)	4.86 (3.01)	3.71 (1.48)	14.15 (2.63)	3.45 (3.59)	3.93 (2.1)	2.03 (0.86)
S. aureus	9	3.81 (2.23)	1.49 (0.36)	1.03 (0.36)	1.28 (0.55)	1.64 (0.59)	1.64 (0.37)	2.05 (0.85)	11.62 (3.98)	2.02 (1.48)	1.25 (1.08)	1.73 (0.94)
S. aureus	10	1.17 (0.54)	3.49 (1.75)	2.21 (1.77)	1.3 (0.66)	1.56 (0.74)	4.58 (4.59)	2.07 (2.18)	17.86 (6.85)	1.46 (1.33)	1.32 (0.87)	1.55 (0.05)
S. aureus	11	1.09 (0.25)	1.79 (0.69)	0.96 (0.36)	1.01 (0.64)	0.78 (0.27)	1.08 (0.39)	0.93 (0.46)	13.74 (7.72)	1.07 (1.12)	0.64 (0.61)	1.05 (0.04)
E. faecalis	15	1.08 (0.28)	3.13 (1.76)	0.39 (0.35)	0.97 (0.24)	0.67 (0.21)	1.26 (0.44)	1.1 (0.66)	21.16 (5.82)	0.29 (0.23)	0.91 (0.61)	2.46 (1.19)
E. faecalis	21	0.81 (0.18)	1.33 (0.64)	0.67 (0.25)	0.9 (0.29)	0.47 (0.55)	0.56 (0.27)	2.1 (0.6)	14.77 (3.79)	5.66 (2.61)	3.65 (2.47)	1.05 (0.07)
E. faecalis	26	2.01 (1.44)	3.65 (2.47)	0.83 (0.5)	0.82 (0.34)	0.9 (0.38)	1.42 (1.03)	1.98 (1.35)	13.9 (5.33)	2.07 (2.65)	0.56 (0.3)	1.59 (0.88)
P. mirabilis	2	0.65 (0.39)	2.17 (1.18)	0.2 (0.1)	0.32 (0.19)	0.26 (0.06)	0.33 (0.14)	0.71 (0.63)	7.86 (7.61)	0.32 (0.04)	0.42 (0.2)	0.61 (0.21)
P. mirabilis	5	0.81 (0.38)	1.84 (0.07)	0.46 (0.38)	0.25 (0.19)	0.36 (0.15)	0.45 (0.3)	2.46 (2.26)	7.12 (3.76)	0.29 (0.1)	0.32 (0.2)	0.45 (0.25)
P. mirabilis	40	1.36 (0.98)	1.26 (0.72)	0.73 (0.53)	0.68 (0.09)	0.32 (0.15)	0.6 (0.19)	1.23 (1.26)	9.38 (3.02)	0.64 (0.09)	0.56 (0.15)	0.67 (0.27)
E. cloacae	1	1.86 (1.11)	1.63 (0.64)	0.61 (0.4)	1.66 (0.22)	1.12 (0.63)	1.57 (0.32)	1.89 (1.98)	10.37 (2.82)	3.32 (4.79)	0.58 (0.42)	0.8 (0.41)
E. cloacae	43	1.83 (1.25)	3.47 (2.48)	0.56 (0.51)	1 (1.21)	0.85 (0.6)	1.36 (0.57)	1.36 (0.51)	10.35 (2.1)	0.81 (0.55)	0.9 (0.64)	0.57 (0.42)
E. cloacae	50	1.41 (1.29)	1.82 (0.51)	0.39 (0.3)	0.66 (0.85)	0.62 (0.41)	0.76 (0.52)	0.96 (0.93)	9.32 (1.77)	4.06 (5.92)	0.78 (0.61)	0.82 (0.76)
C. perfringens		2.21 (0.84)	3.55 (0.95)	1.73 (1.14)	1.97 (0.49)	2.01 (0.52)	1.77 (0.62)	2.58 (2.51)	16.07 (3.41)	2.44 (0.63)	1.69 (0.85)	1.08 (0.88)
B. fragilis		2.52 (0.46)	2.86 (2.44)	0.58 (0.25)	1.6 (0.91)	1.64 (1.41)	2.03 (1.89)	2.23 (1.26)	16.57 (5.5)	1.61 (1.32)	2.21 (1.4)	0.93 (0.55)

Data are means optical densities from three separate experiments (SD are given in parenthesis). Coaggregation score between 12% and 50% are shaded light grey.

4.5 Discussion

The nutrient rich milieu of chronic wounds provides an ideal environment for the attachment and proliferation of numerous bacterial organisms with the resulting polymicrobial community believed to serve as a reservoir of pathogenic organisms leading to infection, inflammation and tissue damage; prolonging healing times and patient morbidity. A number of previous studies have investigated the biofilm forming capabilities of bacteria isolated from a range of chronic wounds utilising several of methods, including *in vivo* biofilm models, confocal scanning microscopy of tissue biopsy samples and the microtitre plate technique used in the current chapter (O'Toole *et al.*, 1999). The microtitre plate technique is a simple, quantitative, high-throughput screening method to detect the extent of biofilm formation of a single species. Utilisation of this method in this chapter identified that both the skin and wound bacterial isolates were capable of forming a biofilm on the abiotic surface of microtitre plates, as determined by this method (Figures 4.1- 4.3). This has been born-out in numerous studies which identified bacterial species such as *Pseudomonas* spp, *Escherichia coli* and *S. aureus* which were capable of biofilm formation (Choi *et al.*, 2010; Ramos *et al.*, 2010; Sandberg *et al.*, 2008). In addition, *Staphylococcus epidermidis* has been well documented as a problematical biofilm producer on prosthetic devices (Antoci Jr *et al.*, 2008; Rohde *et al.*, 2007). Nevertheless, the fact that commonly isolated wound bacteria are capable of forming biofilms lends support to the concept that chronic wounds harbour bacterial biofilms. Accordingly, Davis *et al.,* (2008) demonstrated that a wound isolate of *S. aureus* were capable of forming a biofilm in a *in vivo* porcine wound model (Davis *et al.*, 2008). All the wild type *S. aureus* strains used in this chapter were capable of biofilm production and in some instances at a reduced

capacity than other isolates tested with the study. If *S. aureus* albeit different isolates have been proven to produce biofilms *in situ* in the Davis *et al*., (2008) study, then not only is it plausible that the *S. aureus* strains used in this chapter could produce *in situ* biofilms but also the other isolates tested. What remains to be investigated is their ability to produce multispecies biofilms *in situ*. Colonised wounds are innately polymicrobial and coaggregation is one mechanism by which polymicrobial biofilms are believed to develop. These cell surface connections between genetically distinct cells (coaggregation) and genetically homogenous cells (autoaggregation) facilitate coadhesion of the biofilm. Since these interactions have been previously shown in polymicrobial biofilms of the oral cavity, gastrointestinal tract and fresh water systems, the coaggregation activity of skin and wound-associated bacterial biofilms were investigated in order to better understand factors influencing their development. Coaggregation interactions between individual wound and skin isolates (Table 4.2 and Table 4.3 respectively) and then wound and skin isolates combined (Table 4.4) was investigated to identify potential interactions that may occur within independent wound and skin biofilms, but also analyse how the two populations of the skin and wound may interact during a wounding or colonisation scenario. According to the current published literature coaggregation amongst wound and skin isolates has yet to be investigated. Of the 171 possible pair-wise combinations of wound isolates, none showed a coaggregation score greater than 11% as shown in Table 4.2. A similar pattern was found for the 66 possible combinations of skin isolates, with the exception of *C. xerosis* which demonstrated an autoaggregation score of *c.* 50% (Table 4.3 shaded in dark grey). Subsequently, all other interactions associated with the *Corynebacterium* spp. produced an artificially elevated score due to the autoaggregation of the *C. xerosis* rather the coaggregation between the two

bacterial species (Table 4.3 - Table 4.4 shaded in light grey). Previous work by Khemaleelakul *et al*., (2006) investigated the autoaggregation and coaggregation of bacteria associated with acute endodontic infections including *C. xerosis*, *B. fragilis*, *Propionibacterium acnes*, *Staphylococcus saprophyticus* and *S. epidermidis.* Utilising both a visual assessment of flocs assemblage and dye staining (SYTO9 and propidium iodide) coaggregation assay, the authors identified *C. xerosis* as a weak autoaggregator and identified strong coaggregation activity between *C. xerosis* and *S. epidermidis*. These results do not agree with the findings presented in this chapter however, these could be could be explained by variations in strains and methods. Specifically, the coaggregation results presented in this chapter are derived from a quantitative non-biased coaggregation assay whereas Khemaleelakul *at al.,* (2006) utilised a qualitative visual assay in addition a dye staining coaggregation assay. Several interactions determined as negative via the dye staining method were identified as positive by the qualitative visual assay (Khemaleelakul *et al.*, 2006; Kinder *et al.*, 1994). The dye staining assay is reliant upon the spatial organisation of the bacteria; because the mixed solutions are “transferred” to the slide for visualisation of their spatial arrangement could be affected by the resulting shear forces causing the bacteria to become positioned on top of one and other bacteria perhaps giving a false impression of coaggregation. This highlights the complexity in assessing the coaggregation interactions based upon a visual assessment, particularly when isolates display limited coaggregation and autoaggregation activity. Hill *et al*., (2010) investigated biofilm formation and coaggregation activity of chronic wound-associated bacteria which included multiple isolates of *P. aeruginosa* and *S. aureus* (Hill *et al.*, 2010). Overall, all the isolates tested showed some coaggregation activity however, strong interactions were limited to combinations which included

Peptostreptococcus anaerobius as one of the partner strains. All matching bacteria used within the Hill *et al*., (2010) study and this chapter all displayed limited or no aggregation activity, verifying the results presented herein. This indicates that coaggregation is not necessary an important element in the biofilm formation of wound and skin associated isolates. One limiting factor of the experimental approach could be the number and variety of strains utilised. Several strains of *S. aureus*, *P. aeruginosa, Enterococcus faecalis, P. mirabilis* and *Enterobacter cloacae* were used due to their availability. However, data from previous publications on coaggregation interactions have often used a single representative of each species (Kolenbrander *et al.*, 1993; Ledder *et al.*, 2008; Metzger *et al.*, 2001) Additionally, there is little variation in the strong positive coaggregation results across several studies using the same species but different strains particularly with organisms derived from the oral cavity for example *Fusobacterium nucleatum* and *Bifidobacterium adolescentis* are consistently identified as coaggregating partners (Kolenbrander, 2000; Ledder *et al.*, 2008), this would indicate that strong coaggregation interactions are ubiquitous quality within the species and therefore strain variations should be limited. By using multiple strains (where possible) in this chapter, the possibility of stain variation has been minimised whilst ensuring that the results are truly representative. The microbial ecology of the skin and wound are extremely diverse. The selected consortium for the current investigation was based upon organisms which are commonly found on the skin and in the wound and attempted to create a ‘snapshot’ of the types of bacteria that are frequently cultivated from these sites. It is possible that those not selected for this study may actually be coaggregating biofilm formers. However, as we utilised bacteria which are consistently isolated from both wound and skin this would seem unlikely.

4.6 Conclusion

The members of wound polymicrobial communities are derived from the microbial residents associated with the epidermis and exogenous bacteria colonising the wounds. All isolates tested in this chapter are routinely cultured from both healthy skin and infected wounds and therefore can be considered commonly occurring microbial constituents of these polymicrobial environments. Utilising the crystal violet biofilm assay, all isolates tested were designated as biofilm formers. Coaggregation interactions were found not to be a common occurrence among wound isolates and therefore coaggregation is possibly not an important process for the establishment of wound and skin associated biofilms.

Chapter 5

Development and validation of growth media for the maintenance of an *in vitro* chronic wound consortia

5.1 Abstract

Chronic wound infections represent a significant financial burden and are a considerable clinical problem with implications for patient morbidity. In order to develop a simple and reproducible *in vitro* wound biofilm model, this chapter aimed to develop and validate growth media which could be used in novel *in vitro* models to support multispecies communities and maintain realistic microbial phenotypes. The productivity of bacterial isolates in novel simulated sweat and serum, and foetal calf serum (FCS) was determined turbidometrically from which specific growth rates and ΔOD values were calculated and compared. Proteomic and biofilm formation analysis was undertaken to assess and compare the phenotypic variations of organisms grown in FCS and selected artificial serum media. The artificial sweat and serum media maintained realistic growth rates, allowed for biofilm formation of a range of organisms and produced broadly similar proteomic profiles to FCS. The sustained growth and biofilm formation of isolates in simulated sweat and serum combined with the reflective proteomic profiles qualifies the use of the formulated media in future wound biofilm models.

5.2 Introduction

Investigations into multispecies bacterial communities to discern community interactions and treatment responses are often investigated using *in vitro* model based systems which support and maintain a selected consortia. Current literature indicates that no model exists that is specific to or representative of wound environment. To further investigate wound and skin microbiota and bacterial biofilms, model systems specific for these environments have to be designed. When running or developing models the selection or formulation of specific growth media and substrata can be crucial in ensuring the model closely mimics the environment in which aims to reproduce by replicating chemical and nutrient availability to the selected consortia. In the context of this thesis the aims of this chapter were to develop and validate growth media which could be used in novel *in vitro* models to maintain a microbial consortium associated with healthy skin and chronic wounds. In order to proliferate, common heterotrophic microorganisms such as *Staphylococci* spp. require nitrogen, carbon, inorganic irons and to an extent a vitamin source. The secretions of the sebaceous, apocrine and eccrine glands of the skin can provide some of these elements in a limited capacity (Table 5.1). The most commonly detected amino acids identified in sweat, include; alanine, arginine, aspartic acid, citrulline, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, ornithine, phenylalanine, threonine, tryptophan, tyrosine, and valine (Stefaniak and Harvey, 2006). With respect to the nutritional requirement of common skin associated bacteria both arginine and valine have been shown to be an essential part of the nitrogen requirements of various *Staphylococci* spp. and *Propionibacteria* spp. (Bojar and Holland, 2002).

Table 5.1 Mean and range value for constituents commonly found in adult sweat adapted from Ross Russell and Wiles (1970)

Constituents	Mean	Range
Chloride (mEq/l)	29.7	0-65.1
Phosphate (mg/l)	14	10-17
Sulphate (mg/l)	-	7-190
Fluoride (mg/l)	-	0.2-1.8
Potassium (mEq/l)	7.5	4.3-10.7
Sodium (mEq/l)	51.9	9.7-94.1
Calcium (mEq/l)	-	0.2-6
Magnesium (mEq/l)	-	0.03-4
Total Nitrogen (mg/l)	-	230-400
Urea (mg/l)	-	260-1220
Creatinine (mg/l)	4.6	2.1-8.4
Ammonia (mg/l)	-	60-110
Amino Acids (g/l)	0.476	0.27-0.68
Acetic Acid (mg/l)	7.69	3.57-24.9
Propionic Acid (mg/l)	0.26	0.09-0.55
Lactic Acid (g/l)	0.616	0.474-1.19

These amino acids and other sources of organic nitrogen such as peptides can also be utilised by the microorganisms as a carbon and organic nitrogen source in addition to carboxylic acids and soluble short chain fatty acids (Bojar and Holland, 2002). The microflora can also work synergistically to utilise the lipid content of the sweat as a carbon and nitrogen source (Bojar and Holland, 2002; James *et al.*, 2004). Bacteria such as *Staphylococcus* spp. and *Propionibacteria* spp. are capable of metabolising skin lipids such as triacylglycerol and converting them to unusual long chain fatty acids (methyl-branched or odd carbon number) via the action of bacterial lipases. These unusual long chain fatty acids can then be utilised for fermentative growth by organisms such as *Corynebacterium* spp. producing volatile fatty acids which in turn can be metabolised by micrococci and brevibacteria shown in Figure 5.1. In addition to the limited organic carbon and nitrogen sources elements such as iron which is critical for bacterial growth and survival, is restricted. Upon mammalian skin and in sweat, free iron is limited or bound to mammalian secretary proteins such as lactoferrin and transferrin. To

overcome this, bacteria synthesis and secrete siderophores which contain strong iron-binding moieties such as catecholate, hydroxamate, or α-hydroxycarboxylate groups enabling them to solubilise iron from complexes sources or acquire it from the host proteins (Dertz *et al.*, 2003). The act of scavenging these elements may prevent colonization by other microbes by further limiting the availability of iron to exogenous bacteria.

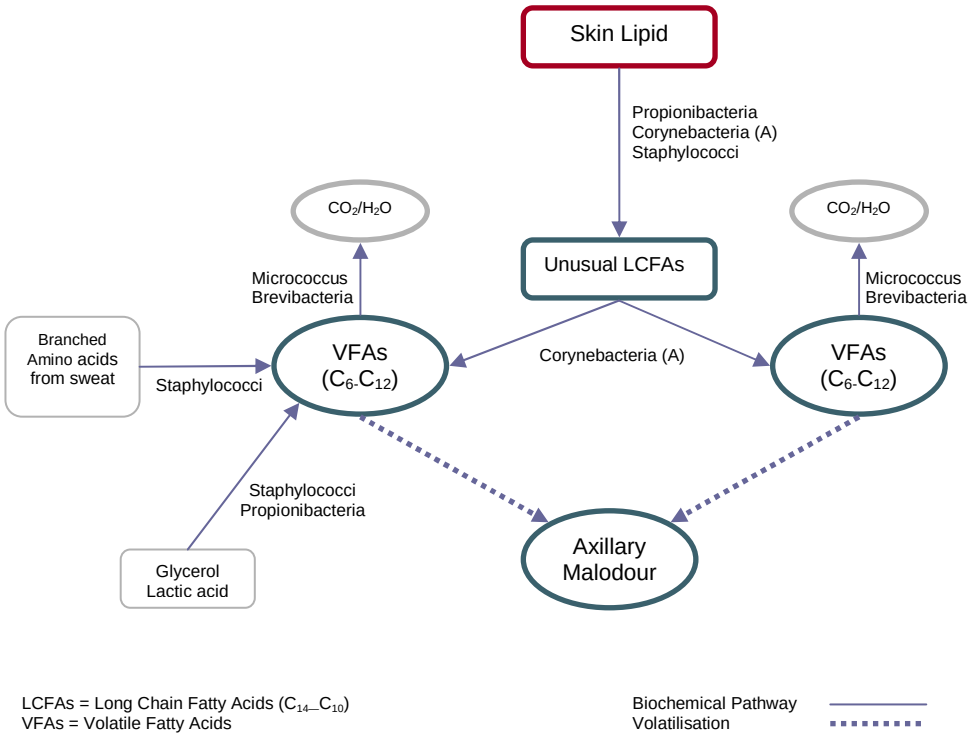


Figure 5.1 Formation and utilization of VFAs by axillary bacteria modified from James *et al*., (2004)

In contrast to the sebaceous, apocrine and eccrine sweat which could be considered to be a nutrient limited medium, wound exudate provides a comparatively rich source of nutrients for microbial growth. Wound exudate is derived from blood serum and essentially contains the same constituents, in similar quantities shown in Table 5.2.

Table 5.2 Comparison of biochemical analytes from serum and wound fluid samples

Substance	Serum Reference Range	Wound Fluid Range
Sodium	137-146 mmol/L	133-146
Potassium	3.6-5 mmol/L	3.2-5.7
Chloride	98-108 mmol/L	96-109
Urea	2-8.6 mmol/L	2.5-22.6
Creatinine	45-115 µmol/L	46-334
Uric acid	140-480 µmol/L	221-751
Calcium	2.15-2 mmol/L	1.84-2.72
Magnesium	0.7-0.91mmol/L	0.75-1.24
Phosphate	0.8-1.46 mmol/L	0.98-1.47
Bicarbonate	22.32 mmol/L	14-22
Glucose	3.5-5.5 mmol/L	0.6-5.9
Lactate	0.3-1.3 mmol/L	5.4-16.7
Lactate dehydrogenase	310-620 U/L	789-9901
Alkaline phosphatase	55-120 U/L	24-146
Alaninie aminotransferase	8-60U/L	3-111
Aspartate aminotransferase	4-50 U/L	10-159
Gamma glutamyltranspeptidase	8-60 U/L	19-194
Creatine kinase	20-260 U/L	20-62
Total bilirubin	3-20 µmol/L	12-52
Total Protein	56-79g/dL	26-521
Albumin	36-50 g/dL	14-28
C-reactive protein	<6mg/L	2.5-25
α-1-Globulin	2-5 g/dL	1.0-2.5
α-2-Globulin	4-9 g/dL	2.7-6
β-Globulin	5-10 g/dL	3.1-8.8
γ-Globulin	8-16 g/dL	3.9-9
C3	0.55-1.2 g/dL	0.15-0.71
C4	0.2-0.5 g/dL	0.08-0.35
Cholesterol	55 mmol/L	1.2-3.2
Triglycerides	0.3-18 mmol/L	0.4-4.3

Adapted from Trengove and Langton (1996)

Wound exudate is excreted as part of the inflammatory response and functions to maintain the moisture of the wound by facilitating neutrophil and macrophage survival. Furthermore, it contains several components such as leukocytes, fibrinogen and fibrin and provides essential nutrients for epithelial cell re-growth which including vitamins, amino acids and trace elements such as calcium, copper, iron, manganese and zinc and electrolytes (Cutting, 2003; Jones *et al.*, 2001). Both chronic and acute wounds produce serum, however due to the

exaggerated inflammatory response, chronic wound exudate generally contains higher levels of tissue-degrading components such as matrix metalloproteinases (MMPs), serine proteinases, interleukin-1α and transforming growth factor-β, when compared with acute wound fluid (Cutting, 2003; Diegelmann, 2003; Jones *et al.*, 2001). The moisture content and availability of organic nitrogen, and carbon, inorganic irons in serum provides an ideal medium to support the growth of a wide range of bacterial organisms. The presence of neutrophils, macrophages in addition to the development of an eschar (hard crust or scab) and tissue re-growth provide the only protective barriers to bacterial attachment and growth in the wound.

To initiate the development of biofilm models appropriate to wound care, a growth media comparable to the environment of the healthy skin and wound, in which multispecies communities can be maintained and realistic microbial phenotypes are expressed was required. A medium representative of healthy skin is essentially simulated sweat whereas wound serum is componentially similar to blood serum. When formulating media the primary goal was to include the major constituents found in the archetypal hominine media at the biologically relevant concentrations and then assess the media ability to support the growth of a range of bacteria by evaluating specific growth rates, maximal productivity, protein expression profiles and biofilm production capabilities.

5.3 Materials and methods

5.3.1 Media and chemicals

Unless otherwise stated all chemicals used throughout this study were of at least analytical grade quality and were obtained from Sigma (Poole, Dorset, U.K.). Dehydrated bacteriological media was obtained from Oxoid (Basingstoke, Hampshire, U.K.), reconstituted according to instructions supplied by the manufacturer.

5.3.2 Bacterial strains

A total of 29 skin and wound-associated bacterial strains were used in this chapter, multiple isolates were used were available shown in Table 5.3.

5.3.3 Growth curves of bacterial isolates in artificial sweat

In order to compare growth dynamics in batch culture for the artificial sweat formula and nutrient broth the following skin associated bacterial isolates were grown overnight to obtain stationary phase cultures in nutrient broth and artificial sweat at 37℃ in aerobic conditions for *Acinetobacter baumannii*, *Corynebacterium xerosis, Micrococcus luteus, Staphylococcus capitis, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus saccharolyticus and Staphylococcus saprophyticus* or anaerobic conditions for *Propionibacterium acnes*. The formulated artificial sweat consisted of 3-(N-morpholino)-propanesulfonic acid (MOPS) (20.9g/l/100mM), yeast extract (1 g/l), NaCl (2 g/l), fish oil fatty acid methyl esters (0.65 mg/l) and tween 80 (0.1 g/l) (to

emulsify the fatty acids in solution) buffered to pH 6, and was based upon the common constituents of sweat. Overnight cultures were diluted to 1:100 in fresh nutrient broth and artificial sweat and 200 µl of the fresh inoculum was dispensed into wells of a flat bottomed microtitre plate (BD, SLS U.K.). To maintain an anaerobic environment anaerobic isolates were overlaid with mineral oil (previously validated by Breidt *et al*., 1994). Plates were incubated in an automated plate reader (Titertek Multiskan® MCC 340, Biotek, UK) at 37℃ for 48 h with optical density readings (600nm) taken every hour (Breidt *et al.*, 1994).

Table 5.3 Bacterial strains used in this chapter

Bacterial Species	Strain number	Origin
Acinetobacter baumannii		Diabetic Ulcer/ConvaTec ltd*
Bacteroides fragilis	NCTC 9343	National Typed Culture Collection
Clostridium perfringens		
Corynebacterium xerosis		Diabetic Ulcer/ConvaTec ltd*
Enterobacter cloacae	1, 43 and 50	Diabetic Ulcer/ConvaTec ltd*
Enterococcus faecalis	15, 21, 26	Diabetic Ulcer/ConvaTec ltd*
Escherichia coli		Diabetic Ulcer/ConvaTec ltd*
Micrococcus luteus		Isolated from healthy skin*
Propionibacterium acnes		Isolated from healthy skin*
Propionibacterium avidum		Isolated from healthy skin*
Proteus mirabilis	2, 5 and 40	Diabetic Ulcer/ConvaTec ltd*
Pseudomonas aeruginosa	12, 24 and 38	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus aureus	9, 10 and 11	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus capitis		Isolated from healthy skin*
Staphylococcus epidermidis	ATCC 14990	American Typed Culture Collection
Staphylococcus haemolyticus		Isolated from healthy skin*
Staphylococcus hominis		Isolated from healthy skin*
Staphylococcus saccharolyticus		Isolated from healthy skin*
Staphylococcus saprophyticus	NCTC 7292	National Typed Culture Collection

* identified by 16S rRNA gene sequencing

5.3.4 Comparisons of bacterial specific growth rates and delta OD in formulated artificial serum.

Stationary phase cultures of 25 selected skin and wound-associated bacterial isolates were grown overnight in formulated artificial serum media (shown in Table 5.4), foetal calf serum (FCS) or ConvaTec ltd. simulated wound serum (50% foetal calf serum and 50% tryptic soy broth). Cultures were diluted 1:100 in fresh media and 200 µl of the inocula was dispensed into wells of a microtitre plate. Microtitre plates were incubated in an automated plate reader (Titertek Multiskan® MCC 340, Biotek, UK) at 37℃ for 24 h with optical density r eadings (600nm) taken every 20 min. Specific growth rates and delta OD values were determined by the following equations (Inniss and Mayfield, 1978), where X_1 and X_2 are the OD_{600} values on the maximum of the slope of growth curves between times t_1 and t_2.

$$\text{Specific Growth Rate } (\mu) = \frac{2.303\left(Log_{10}X_2 - Log_{10}X_1\right)}{t_2 - t_1}$$

$$\text{Bacterial Productivity } (\Delta OD) = MaxOD - MinOD$$

Table 5.4 Constituents of four candidate artificial serum recipes.

Constituents (g/l)	AS 1	AS 2	AS 3	AS 4
MOPS	20.9	20.9	20.9	20.9
Sodium Chloride	6.025	6.025	6.025	6.025
Potassium Chloride	0.372	0.372	0.372	0.372
Urea	0.54	0.54	0.54	0.54
Creatinine	0.0132	0.0132	0.0132	0.0132
Glucose	0.324	0.324	0.324	0.324
Yeast Extract	1	1	1	6
Peptone	3	3	3	8
Magnesium Sulphate	-	0.0168	0.0168	0.0168
Haemin	-	0.005	0.005	0.005
Potassium Phosphate	-	-	0.109	0.109

AS:Artificial Serum, all recipes adjusted to pH 7.4.

5.3.5 Proteomic analysis of bacteria grown in artificial serum and foetal calf serum

In order to compare microbial phenotypic expression, the proteomic profiles of bacteria grown in the formulated serum and foetal calf serum was analysed by two-dimensional gel electrophoresis.

5.3.5.1 Protein extraction

Stationary phase bacterial cultures of *P. aeruginosa* (isolate 12) and *S. aureus* (isolate 9) grown overnight in selected artificial serum and foetal calf serum were diluted in fresh media (1:100) and incubated at 37°C for 4-6 h until an optical density of 0.1 (600nm) was attained. Cells were centrifuged at 14,000 rpm at -10℃, (MSE Microcentaur; Sanyo, Loughborough, U.K.) , washed three times with phosphate buffered saline (PBS) and subjected to sonication (on ice) three times for 1 min. Precipitation of the protein from the cell lysate was achieved using a modified TCA/acetone protein precipitation method (Denise *et al.*, 2001; Fabienne, 1988). Briefly, 100% pre-chilled acetone and 100% trichloroacetic acid was added to the cell lysate in a ratio of 8:1:1. Samples were precipitated for 1 h at -20℃. The cell lysates were then centrifuged at 14,000 rpm at 4℃ and supernatant discarded. The residual cell lysate were washed with 100% pre-chilled acetone and centrifuged at 14,000 rpm at 4℃ and the supern atant discarded. The remaining protein pellet was dissolved in rehydration buffer containing 9M urea, 2-4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), 2% v/v carrier ampholytes, 0.01% bromophenol blue and protease inhibitors (O'Farrell, 1975). Protein samples were vortexed every 20 min

for 1 h at room temperature and then centrifuged at 14,000 rpm for 10 min. The supernatant was then removed and archived at -80℃ until required for isoelectric focusing.

5.3.5.2 Iso-electric focusing (IEF)

The first dimension of the two-dimension analysis requires separation of precipitated proteins along an immobilised pH gradient (IPG strips) based upon their isoelectric point. Briefly; 200 µl of re-solubilised protein at concentrations between 20-50 µg/µl sample is applied along the length of a channel of the PROTEAN® IEF Focusing Tray (Bio-Rad, Hertfordshire, U.K.) with the 11cm IPG strip(s) pH range 3-10, placed gel side down in the channel and overlaid with 1-2 ml of mineral oil. The focusing tray is placed in the PROTEAN IEF Cell (Bio-Rad, Hertfordshire, U.K.) and rehydrated under active conditions (50V at 20℃) for 11-15 h. Once rehydration is complete, paper wicks (moistened with ultrapure water) are inserted between the IPG strips(s) and the electrodes. IPG strips(s) were conditioned with the application of 250V at 20℃ fo r 15 min to remove salt ions and charged contaminants, and then subjected to a ramping linear voltage to attain the final focusing voltage of 8000V for 2.5 h. Once complete IPG strips(s) were held at 500V until archived at -80℃ for future use.

5.3.5.3 Equilibration of IPG strip(s) and SDS-Page

IPG strip(s) were placed gel side up in a channel of equilibration/rehydration tray exposed to 4 ml of SDS-page equilibration buffer 1 consisting of 6M urea, 2% SDS, 50mM Tris-HCL (pH 8.8), 2% glycerol and 1% (w/v) DTT.

Equilibration/rehydration trays were then placed on an orbital shaker for a period of 10 min. After SDS-page equilibration, buffer 1 was decanted and 4 ml of SDS-page equilibration buffer 2 consisting of 6M urea, 2 % SDS, 50mM Tris- HCL (pH 8.8), 2 % glycerol and 2.5 % w/v iodoacetamide was added to the channels and the tray(s) were returned to the orbital shaker for a period of for 10 min.

All two-dimensional gel electrophoresis was carried out using the Protean II XI Vertical Electrophoresis Cells (Bio-Rad, Hertfordshire, U.K.). The unit was assembled in the casting stand in duplicate and consisted of two glass plates measuring 18.3 cm x 20 cm and 16 cm x 20 cm with 0.5 mm plastic spacers. All 2D electrophoresis gels utilised a 12% casting gel and a 4% stacking gel. The casting gel comprised of 34 ml of distilled water, 25 ml of 1.5 M Tris-HCL (pH 8.8), 0.5 ml 20% w/v SDS and 40 ml of 30% acrylamide. The stacking gel consisted of 34 ml of distilled water, 6.25 ml of 1M Tris-HCL (pH 6.8), 0.25 ml of 20% w/v SDS and 8.5 ml of 30% acrylamide. Initially Tetramethylethylenediamine (TEMED) (0.012 ml) and 10% w/v ammonium persulphate (APS) (0.3 ml) was added to 30 ml of casting gel solution which was then dispensed between the glass plates until approximately 4 cm from the top. This was then overlaid with isopropanol to obtain a smooth top layer. Once the casting gel has fully polymerised the isopropanol was washed off with distilled water and dried with filter paper. TEMED (0.007 ml) and 10% w/v APS (0.07 ml) was added to 7 ml of stacking gel solution which was then dispensed over of the casting gel until approximately 0.5-1 cm from the top. Equilibrated IPG strip(s) were then place on top of the stacking gel (IPG gel facing the back plate) until they became into complete contact with the stacking gel along their length. Paper wicks moistened with 10 µl of pre-stained protein marker were inserted into the gel ensuring no contact with the IPG strips. Paper wicks and IPG

strip(s) were overlaid with 1% overlay agarose (Bio-Rad, Hertfordshire, U.K.) ensuring minimal movement of the IPG strip(s). Once solidified the gels were mounted to the core of the Protean II XI unit, which in turn was transferred to the Protean II XI tank. The tank and reservoirs were filled Tris-Glycine running buffer consisting of 25 mMTris, 250 mM Glycine and 0.1 % SDS. Gels were run at 850V-hr with the max V being 200v.

5.3.5.4 Staining, imaging and data analysis of two dimensional gels

Gels were stained with Proteosilver silver staining kit (Sigma, Poole, Dorset, U.K.) following manufacturers instructions. Briefly; gels were fixed with 50% ethanol, 10% acetic acid solution followed by 30% ethanol solution. Gels were stained with a 1% sensitizer solution, a 1% silver solution and developer solution (reconstituted according to manufacturers' instructions) with intermittent washed stages conducted with ultra pure water.

Gels were imaged and documented using a Canon D60 digital single lens reflex (DSLR) camera (Canon, Surrey, U.K.). Analysis, alignment and matching of gels were performed using Genebio Melanie 7 software (Genebio, Geneva, Switzerland). Replicate gels were first aligned and matched to each other. Only protein spots present in all replicate gels were selected for matching to test variable protein expression of bacteria grown in different media. Experimental gels were compared and common proteins spots (including their migratory distance) were detected and highlighted by the software.

5.3.6 Crystal violet biofilm formation assay to assess the biofilm formation of bacteria grown in formulated artificial medium

The extent of biofilm formation of 29 skin and wound bacterial isolates in formulated artificial sweat and serum upon abiotic surfaces was investigated using a crystal violet microtitre plate assay previously described and validated by O'Toole *et al.,*(1999).

To obtain stationary phase cultures bacterial isolates were grown overnight at 37℃ in 10 ml of artificial media aerobically or in pre-reduced artificial media anaerobically for respective aerobic and anaerobic isolates. Cultures were diluted to 1:100 in fresh media and 200 µl of the inoculum dispensed into wells of a 96 well, flat bottomed microtitre plate (BD, SLS, U.K), uninoculated media was used as a blank. Anaerobic cultures and their respective media reference blanks were overlaid with mineral oil in order to maintain anaerobiosis (Bio-Rad, Hertfordshire, U.K.). The microtitre plates were covered with lids and incubated with agitation (25 rpm) in an aerobic, 37℃ environment for 24 h. Following the incubation period the planktonic phase was removed from the wells using a pipette and the microtitre plate was gently washed with PBS. Residual PBS was then removed by inversion of the plates combined with gentle tapping onto absorbent paper; this washing stage was repeated three times to remove non-adherent cells. Plates were air dried and 200 µl of 0.1% (w/v) crystal violet was added to each well. After 15 min incubation at room temperature, plates were washed three times (as previously described) and residual cellular bound crystal violet i.e. that which had associated with the biofilm was solubilised by the addition 200 µl of 95% ethanol. The absorbance was measured at 600 nm using an automated plate reader (Titertek

Multiskan® MCC 340, Biotek, UK). Each assay contained three media controls of 200 µl of un-inoculated artificial media. Each isolate was tested in triplicate (O'Toole *et al.*, 1999). Isolates which produced a mean optical density reading equal to or greater than three standard deviations of the mean optical density of the blanks were considered biofilm producers by this method (Stepanovic *et al.*, 2000).

5.4 Results

5.4.1 Growth dynamics of bacterial isolates in artificial sweat

The ability of the formulated artificial sweat solution to sustain the growth of a selection of skin associated bacteria was compared to that of nutrient broth (Figure 5.2). All selected bacterial isolates were capable of growth in the artificial sweat but at a severely reduced rate when compared to their growth in nutrient broth.

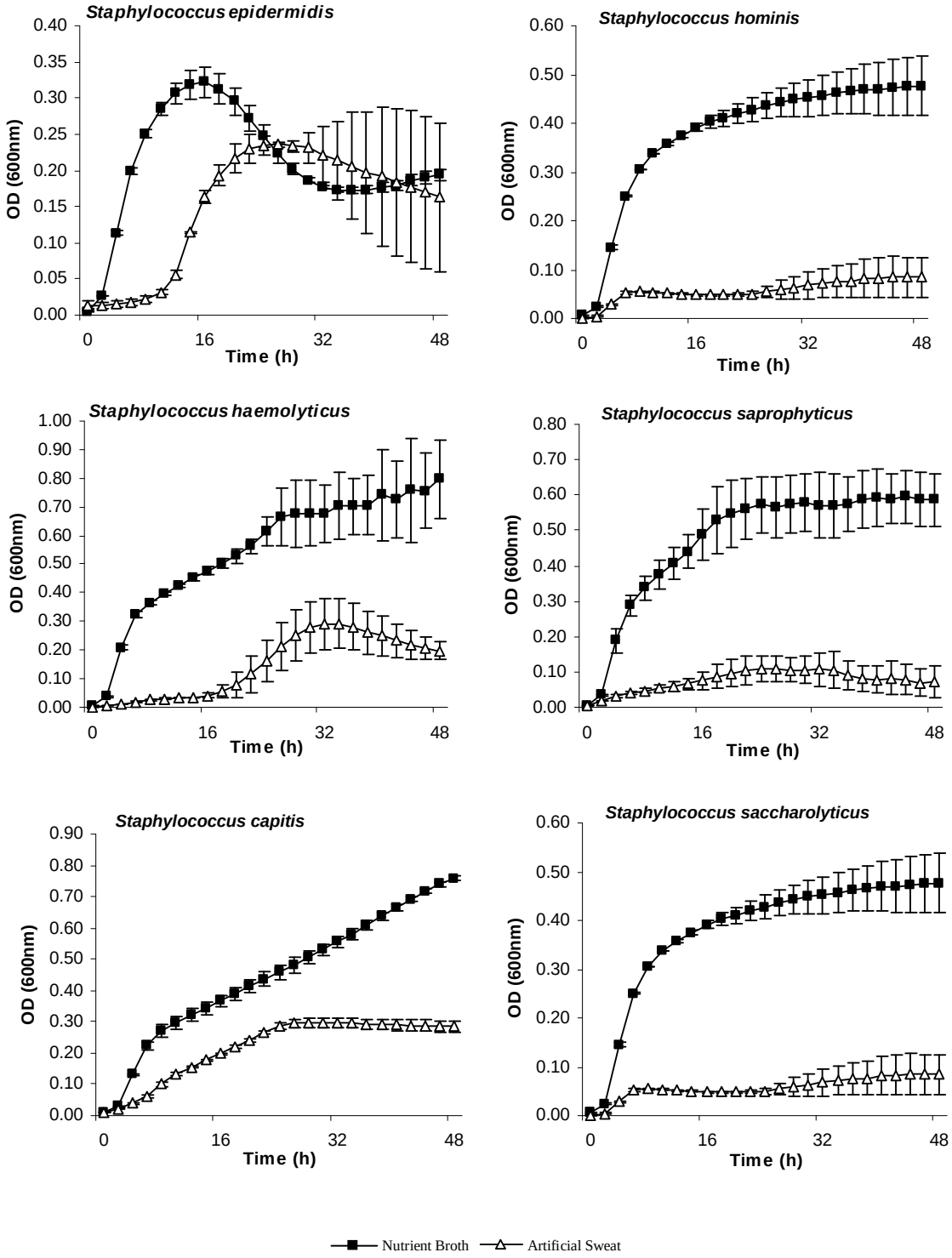


Figure 5.2 Growth curves of organisms grown in nutrient broth and artificial sweat

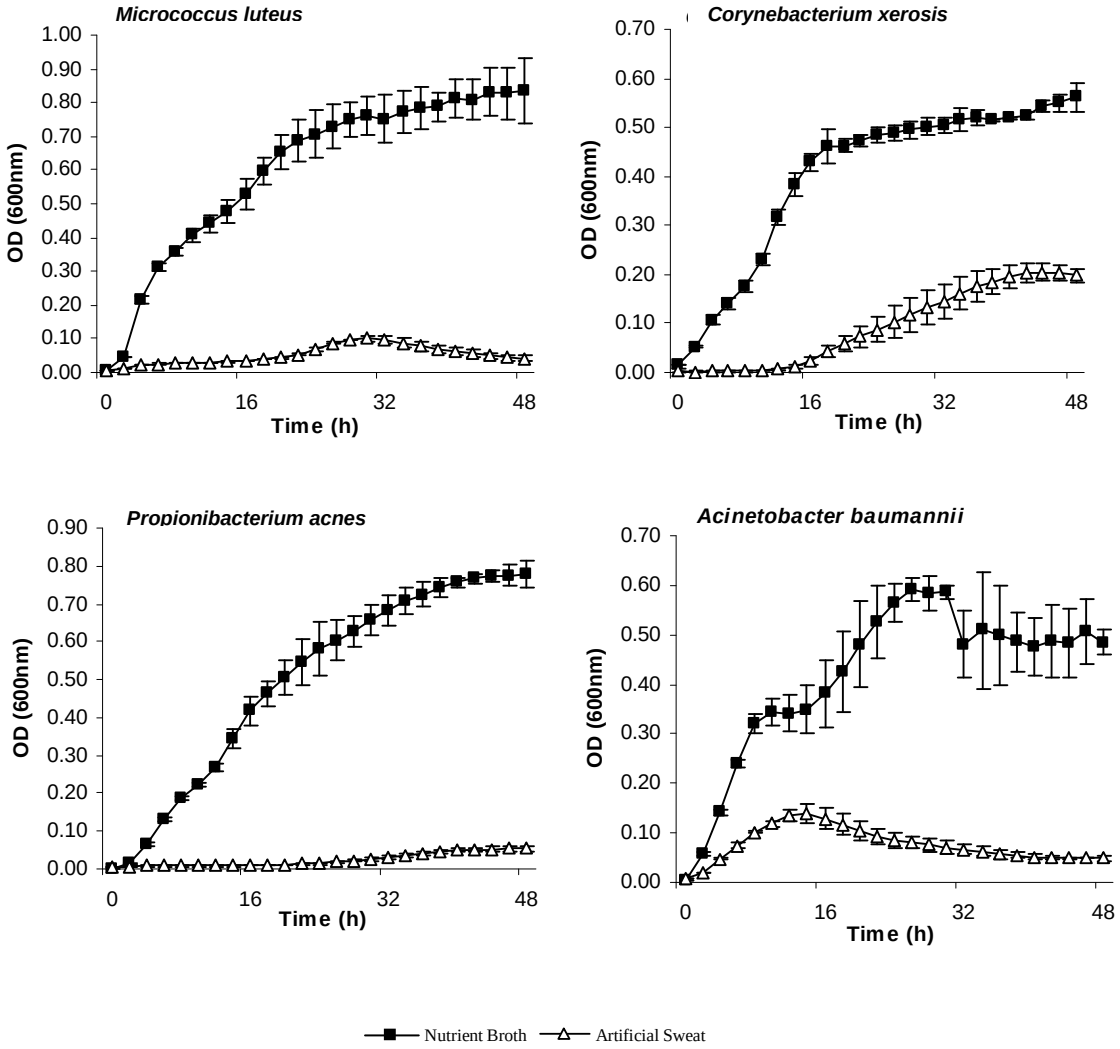


Figure 5.2 cont. Growth curves of organisms grown in nutrient broth and artificial sweat

Table 5.5 Specific growth rate and delta OD values of faecal calf serum, formulated artificial serum and simulated wound serum

		FCS		AS 1		AS 2		AS 3		AS 4		SWF	
Species and isolate number		μ	ΔOD	μ	ΔOD	μ	ΔOD	μ	ΔOD	μ	ΔOD	μ	ΔOD
E. coli	7	0.876	0.497	1.361	0.44	0.993	0.515	0.914	0.507	1.204	1.11	0.944	0.74
P. aeruginosa	12	0.435	0.907	1.189	0.437	0.664	0.465	0.478	0.403	0.579	1.67	0.655	1.50
P. aeruginosa	24	0.597	0.100	0.767	0.396	0.302	0.499	0.266	0.343	0.618	1.371	0.755	0.35
P. aeruginosa	38	0.627	0.784	1.293	0.423	0.544	0.463	0.571	0.392	0.515	1.33	1.087	0.79
S. aureus	9	0.744	0.381	0.966	0.194	0.826	0.476	0.783	0.452	1.041	0.733	0.698	0.81
S. aureus	10	0.722	0.339	0.944	0.247	1.04	0.383	0.838	0.348	1.108	0.833	0.843	0.87
S. aureus	11	0.658	0.394	0.832	0.210	1.320	0.547	0.795	0.465	1.086	0.644	0.926	0.89
E. faecalis	15	0.770	0.235	1.063	0.144	1.128	0.195	0.830	0.126	1.093	0.378	0.831	1.02
E. faecalis	21	0.796	0.110	0.980	0.155	1.408	0.254	0.878	0.220	1.124	0.373	0.462	0.88
E. faecalis	26	0.758	0.396	0.666	0.117	1.074	0.228	0.838	0.144	1.011	0.416	0.828	1.10
E. cloacae	1	0.870	0.470	1.174	0.455	1.158	0.514	0.949	0.323	0.929	1.113	0.897	0.82
E. cloacae	43	0.571	0.357	1.163	0.446	1.141	0.435	1.052	0.343	0.973	0.791	0.877	0.63
E. cloacae	50	0.602	0.397	0.284	0.365	1.157	0.561	1.018	0.314	0.950	0.913	0.937	0.74
P. mirabilis	4	0.859	0.334	0.744	0.586	1.070	0.530	0.798	0.374	1.014	1.230	0.859	0.51
P. mirabilis	5	0.739	0.368	0.990	0.729	1.149	0.575	0.773	0.412	1.072	1.328	0.688	0.49
P. mirabilis	40	0.680	0.422	0.909	0.433	0.994	0.510	0.593	0.364	0.871	1.205	0.781	0.55
S. epidermidis		0.447	0.197	0.776	0.261	0.914	0.436	0.405	0.126	1.036	0.735	0.333	0.63
S. hominis		0.494	0.435	0.848	0.622	0.784	0.638	0.485	0.126	0.583	0.378	0.675	0.78
S. haemolyticus		0.260	0.625	0.869	0.349	0.786	0.285	0.365	0.216	0.525	0.359	0.702	1.06
S. saprophyticus		0.522	0.633	0.930	0.480	0.569	0.405	0.470	0.218	1.006	0.665	0.857	0.81
S. capitis		0.297	0.361	0.538	0.339	0.542	0.306	0.298	0.254	0.668	0.335	0.770	0.62
S. saccharolyticus		0.581	0.511	1.064	0.298	0.672	0.390	0.496	0.218	0.962	0.751	0.721	0.66
M. luteus		0.519	0.470	0.391	0.034	0.421	0.153	0.557	0.190	0.599	0.172	0.698	0.93
C. xerosis		0.390	0.551	NG	NG	0.849	0.403	0.771	0.368	0.719	0.384	0.632	0.69
A. baumannii		0.769	0.547	0.888	0.326	0.650	0.309	0.441	0.158	0.532	0.941	0.780	0.47

μ: specific growth rate, ΔOD: bacterial productivity, FCS: Foetal Calf Serum, AS: Artificial serum, SWF: ConvaTec simulated wound fluid, NG: no growth. Values not significantly different from FCS shaded grey.

5.4.2 Artificial media: growth curves, specific growth rates and ΔOD

Artificial serum formulas were capable of supporting growth of the selected skin and wound bacteria; producing varying maximal specific growth rates (μ) and bacterial productivity (ΔOD) values (shown in Table 5.5). Independent t-test analysis of the data identified Artificial serum 3 as not significantly different to foetal calf serum ($P>0.05$) (highlighted grey in Table 5.5). Data derived from artificial serum 1, 2 and 4 and ConvaTec simulated wound serum was significantly different from foetal calf serum ($P<0.05$) as determined using independent t-test analysis.

5.4.3 Proteomic analysis

A total of 66 protein spots were identified for *P*. *aeruginosa* grown in FCS, 49 of which were matched to protein spots of *P. aeruginosa* grown in artificial serum resulting in a 74% similarity. A total of 72 protein spots were identified for *P*. *aeruginosa* grown in artificial serum, of which 49 were matched to protein spots of *P*. *aeruginosa* grown in FCS resulting in a 68% similarity shown in Figures 5.3 and 5.4

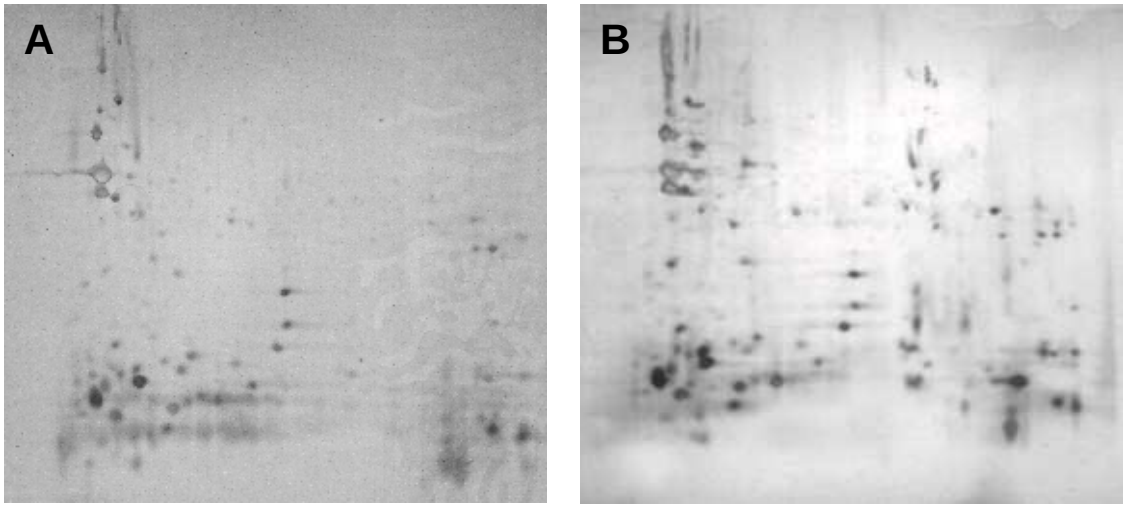


Figure 5.3. Protein expression profile of *P. aeruginosa* grown in foetal calf serum (A) and artificial Serum (B).

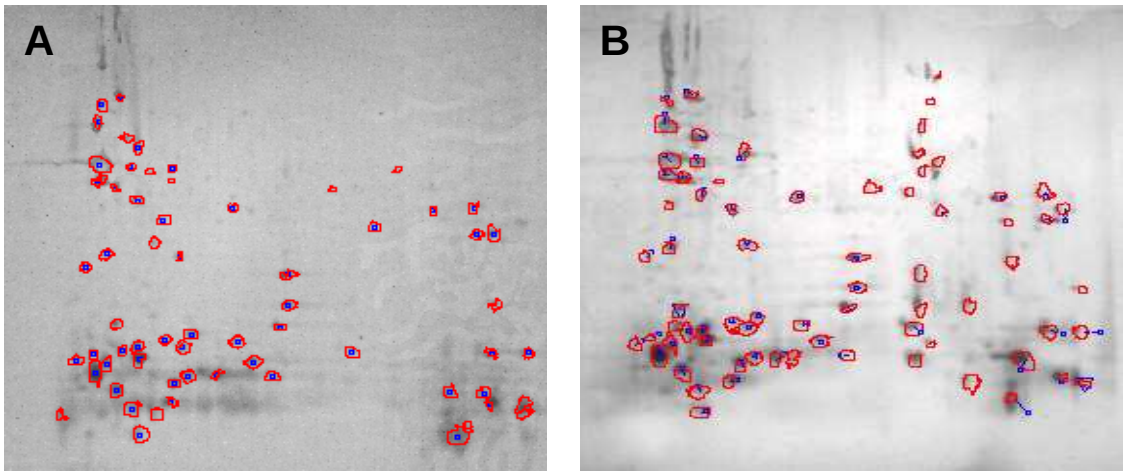


Figure 5.4. Identified protein Spots (outlined in red) of *P. aeruginosa* grown in foetal calf serum (A) and artificial serum (B) matched protein spots are indicated by blue dots.

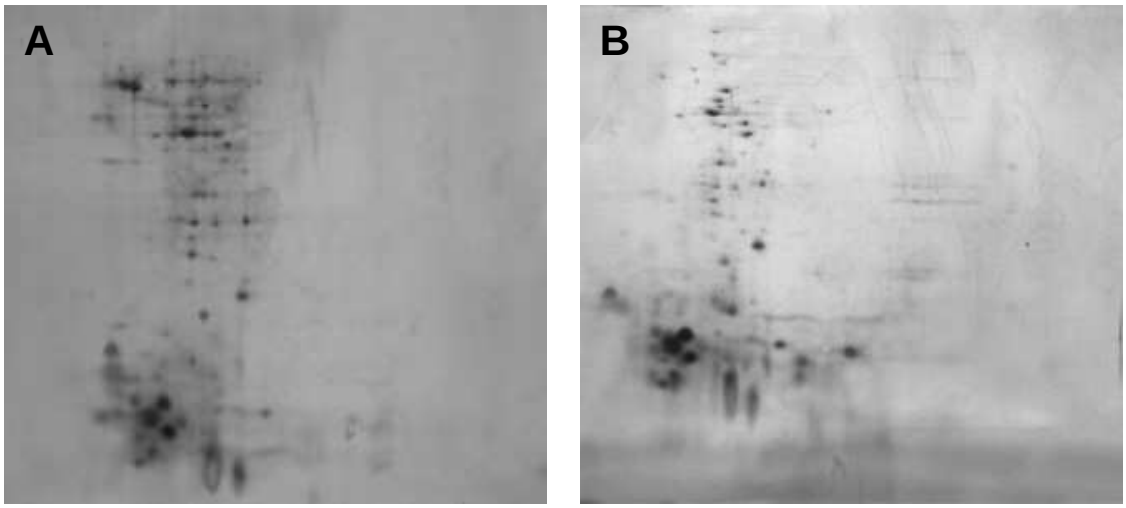


Figure 5.5. Protein expression profile of *S. aureus* grown in foetal calf serum (A) and in artificial serum (B).

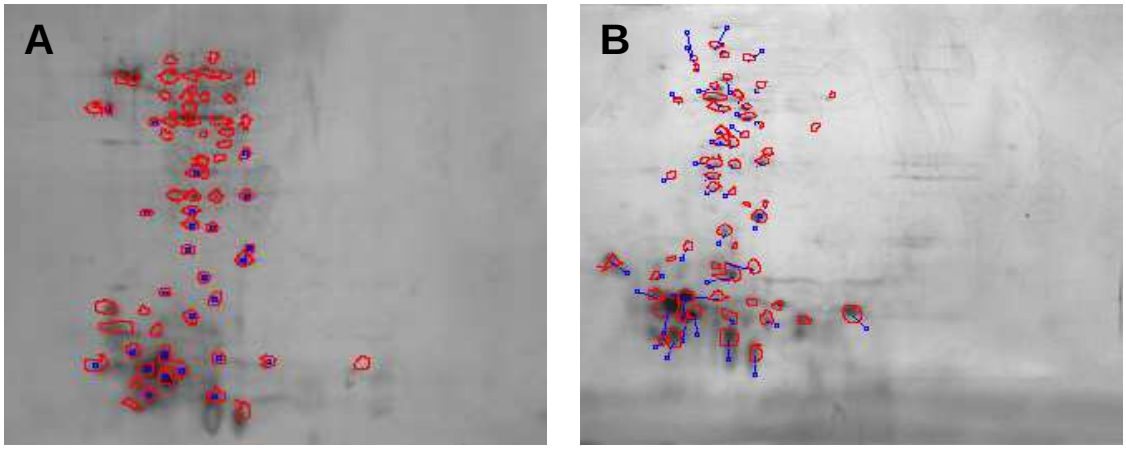


Figure 5.6. Identified protein spots (outlined in red) of *S. aureus* grown in foetal calf serum (A) and artificial Serum (B) matched protein spots are indicated by blue dots.

For *S. aureus* grown in FCS a total of 65 protein spots were identified of which 46 were matched to protein spots of *S. aureus* grown in artificial serum resulting in a 71% similarity.

For *S. aureus* grown in artificial serum a total of 59 protein spots were identified of which 46 were matched to protein spots of *S. aureus* grown in FCS resulting in a 78% similarity shown in Figures 5.5 and 5.6

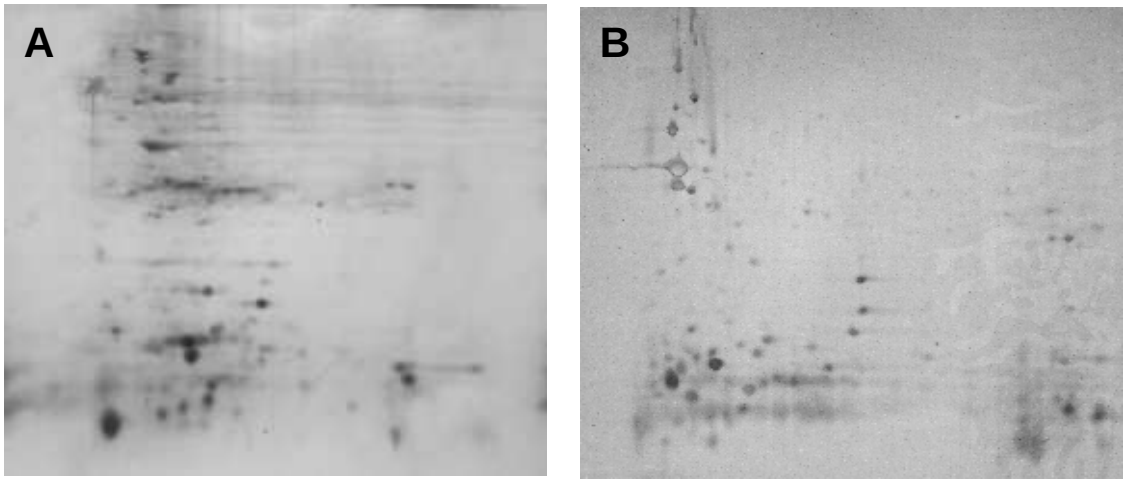


Figure 5.7. Protein expression profile of *P. aeruginosa* grown in nutrient broth (A) and in foetal calf serum (B).

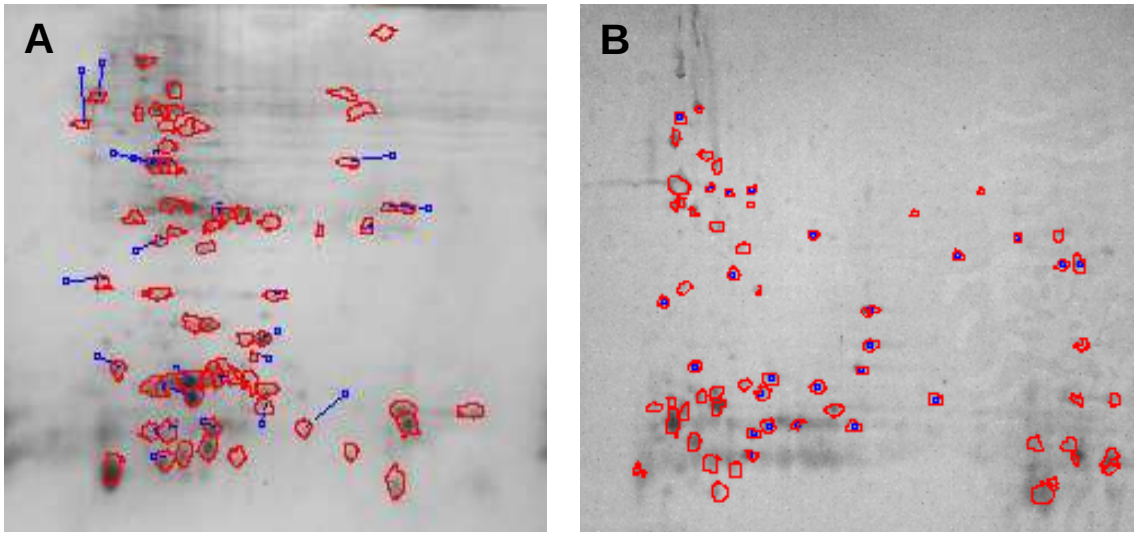


Figure 5.8. Identified protein spots (outlined in red) of *P. aeruginosa* grown in nutrient broth (A) foetal calf serum (B) matched protein spots are indicated by blue Dots.

A total of 66 protein spots were identified for *P. aeruginosa* grown in FCS, 26 of which were matched to protein spots of *P. aeruginosa* grown in nutrient broth resulting in a 39% similarity.

A total of 72 protein spots were identified for *P. aeruginosa* grown in nutrient broth, of which 26 were matched to protein spots of *P. aeruginosa* grown in FCS resulting in a 36% similarity shown in Figures 5.7 and 5.8.

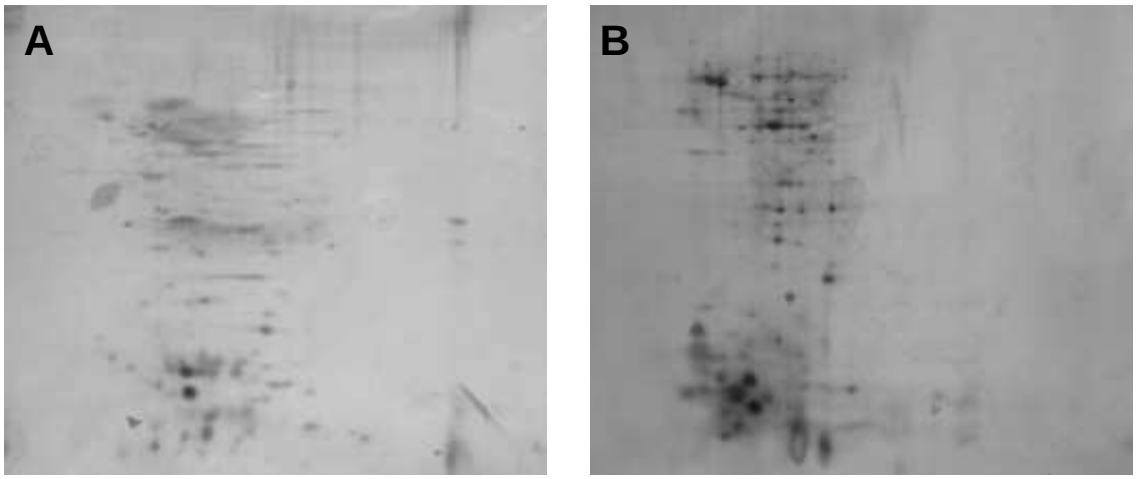


Figure 5.9. Protein expression profile of *S. aureus* grown in nutrient broth (A) and in foetal calf serum (B).

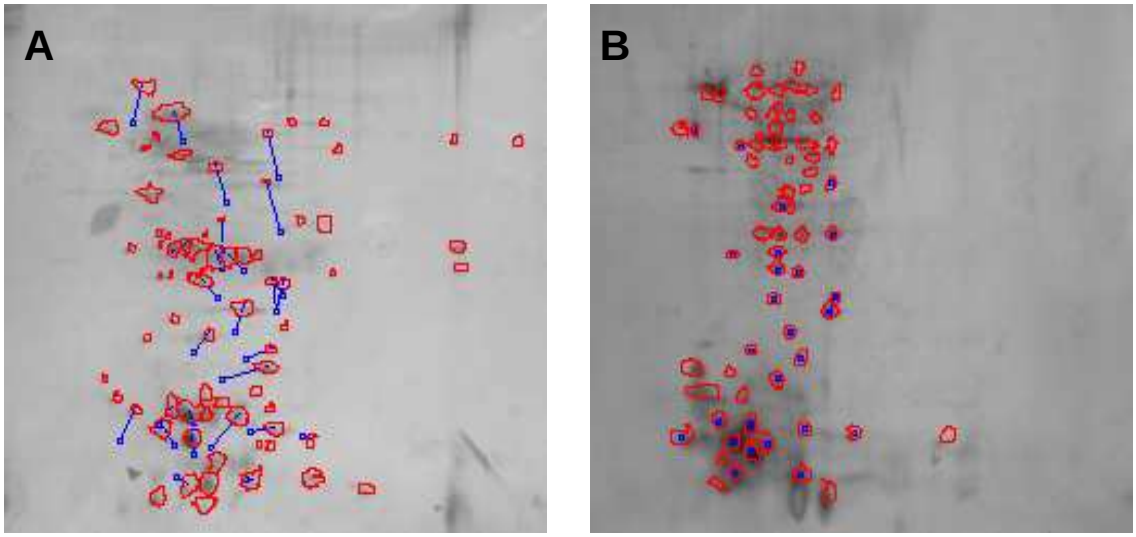


Figure 5.10. Identified protein spots (outlined in red) of *S. aureus* grown in nutrient broth (A) and in foetal calf serum (B) matched protein spots are indicated by blue dots.

A total of 65 protein spots were identified for *S. aureus* grown in FCS of which 26 were matched to protein spots of *S. aureus* grown in nutrient broth resulting in 40% similarity.

A total 71 protein spots were identified for *S. aureus* grown in nutrient broth of which 26 were matched to protein spots of *S. aureus* grown in FCS resulting in a 37% similarity shown in Figures 5.9 and 5.10.

5.4.4 Crystal violet biofilm formation assay to assess the biofilm formation of bacteria grown in formulated artificial medium

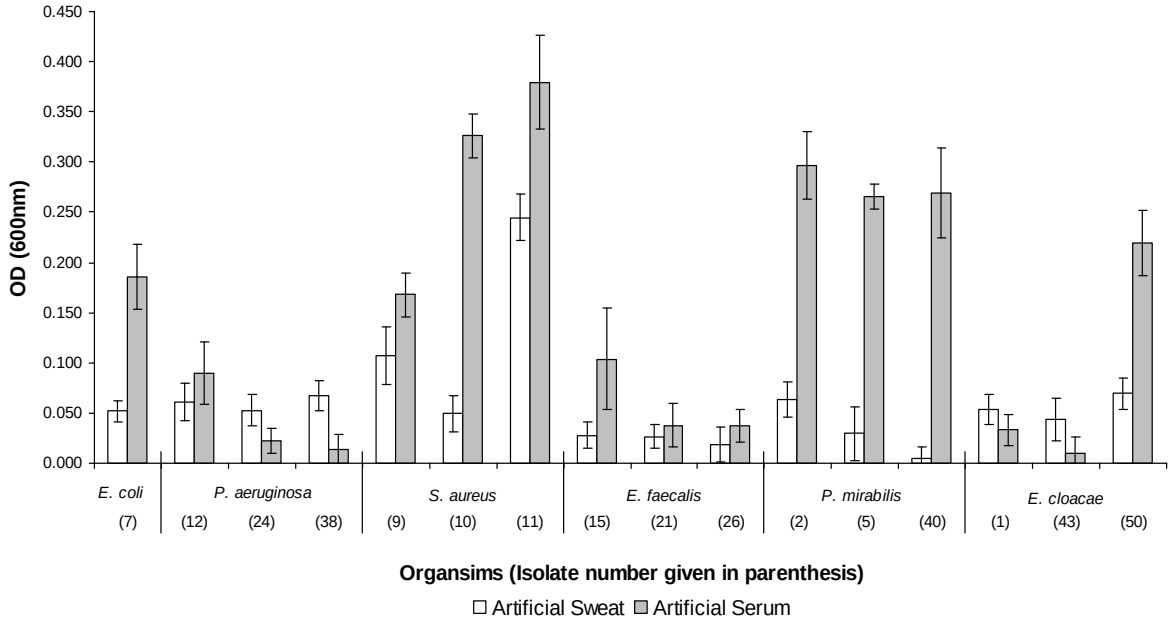


Figure 5.11 Biofilm formation of selected wound associated isolates grown in artificial sweat and artificial serum

The biofilm formation capability of 16 bacterial strains (selected based on their occurrence in wounds) was assessed using the crystal violet assay. All isolates tested were capable of biofilm formation in both artificial sweat and serum (Figure 5.11).

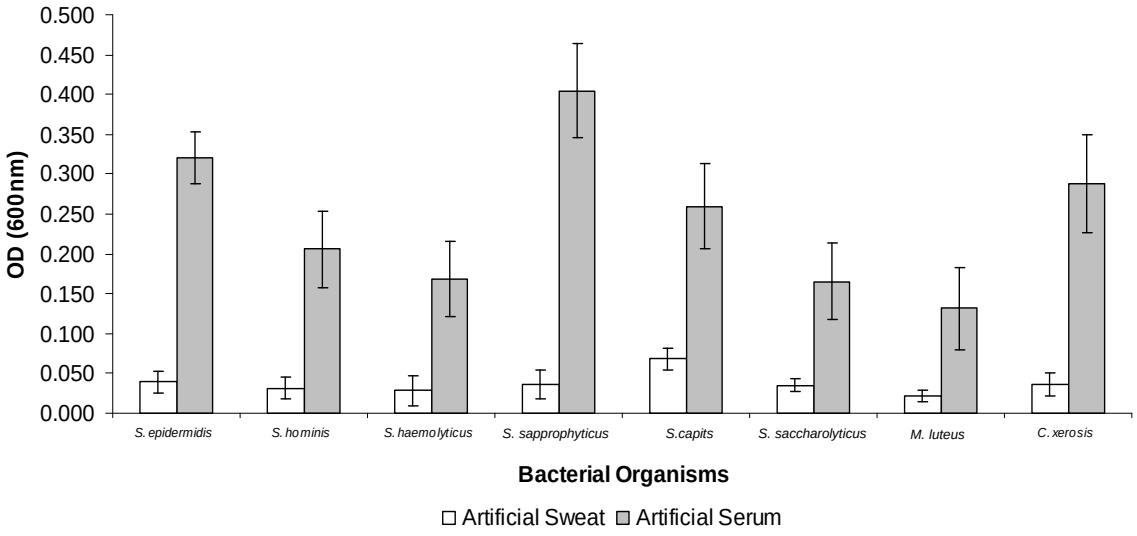


Figure 5.12 Biofilm formation of selected skin isolates grown in artificial sweat and artificial serum

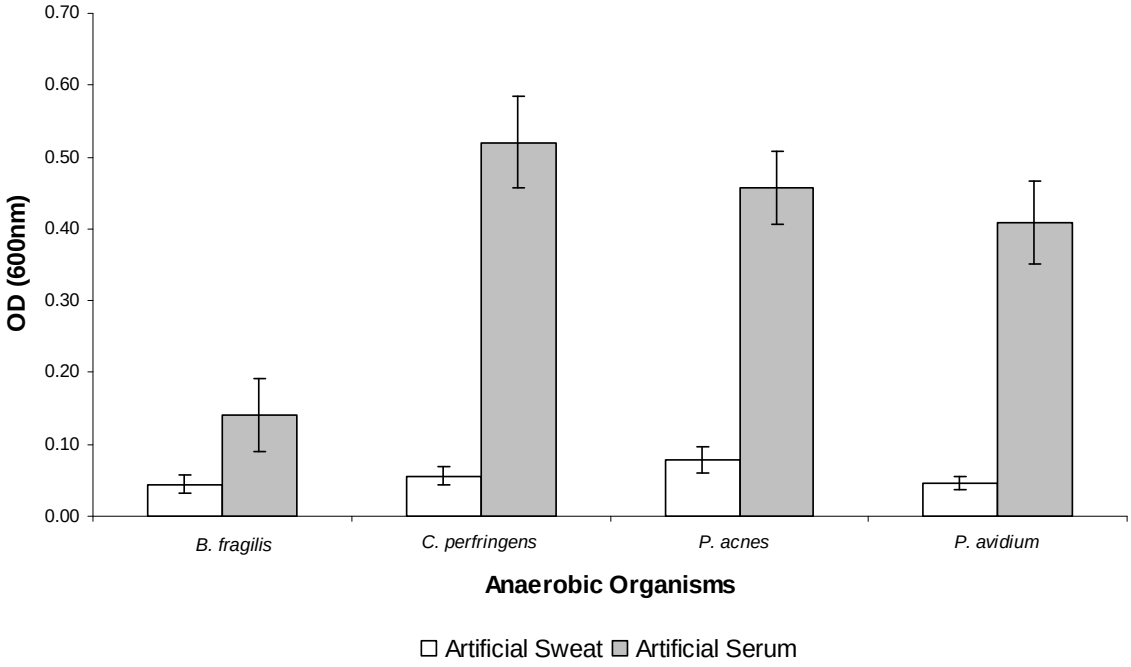


Figure 5.13. Biofilm formation of anaerobic organisms grown in artificial sweat and artificial serum

The biofilm formation capability of 8 skin associated bacterial strains (selected based on their common occurrence on the skin) was assessed using the crystal violet assay. All isolates tested were capable of biofilm formation in both artificial sweat and serum (Figure 5.12.)

The biofilm formation capability of 4 skin and wound-associated anaerobic bacterial strains (selected based on their common occurrence on the skin and in the wound) was assessed using the crystal violet assay. All isolates tested were capable of biofilm formation in both artificial sweat and serum (Figure 5.13).

5.5 Discussion

The human skin is a multifunctional organ that provides protection by three distinct mechanisms; the chemical/biochemical barrier, the immunological barrier and by providing a physical barrier (Proksch *et al.*, 2006). Constant desquamation and production of antimicrobial lipids and peptides by the epidermis combined with the limited nutrient availability discourages attachment and proliferation of bacteria such that only those microorganisms which have adapted to these specific environments colonise the skin (Bojar and Holland, 2002; Chiller *et al.*, 2001). In contrast, the nutrient rich milieu of chronic wounds facilitates the proliferation of a diverse range of microorganisms which can led to further tissue damage, exacerbating the state of chronicity. To further investigate the wound and skin microbiotas using *in vitro* model system, media which is representative the nutrient availability on the skin and in wounds was required. Fluctuations throughout the life of the host including the health status and age combined with the location of the sweat glands can result in dynamic variations in the constituents of sweat confounding efforts to formulate a comparable media. Subsequently, the formulation of the artificial sweat was based upon the staple constituents found in sweat specifically; NaCl, lipids, and minimal carbon, nitrogen, vitamins and iron. The NaCl content of sweat has been recorded at 0.8-4g/l in healthy adult sweat (Bloxsom, 1959; Bloxsom, 1962; Farrell *et al.*, 2008; Hall *et al.*, 1990) therefore the

reference average of 2g/l was selected for the formulation of artificial sweat. Fish oil methyl esters were included in the preparation to replicate the fatty acid methyl esters found in wax esters, cholesterol esters and triglycerides of the skin and sweat (Stewart *et al.*, 1989), however, as with many constituents of sweat the quantity and composition these can significantly vary in relation to the sample site and health status of the host. Nevertheless, quantitative values of epidermal lipids from the upper trunk of healthy adult males have been reported at 0.3mg-1mg/l therefore a value of 0.65mg/l was selected (Lampe *et al.*, 1983; Takemura *et al.*, 1989). To utilise the artificial sweat formulation as a microbial culture media, a range of essential elements such as carbon, nitrogen, iron and vitamins (required for the proliferation of heterotrophic bacteria) was incorporated in the final preparation in the form of yeast extract. Yeast extract is the water soluble portion of autolysed yeast containing a range of vitamins, amino acids, iron and carbon and is typically used as a nutrient base in microbial culture media. Preparations of yeast extract media generally contain *c.* 5g/l (Oxoid, Basingstoke. U.K) therefore to simulate the restricted nutrient quantities on the skin a reduced level of 1g/l was employed. The final preparation of artificial sweat was a buffered solution of pH 6 by incorporating 100mM of MOPS, this component is a buffering agent but additionally, it also replicates the limited iron concentration on the skin by oxidising the free iron content of the media (Tadolini, 1987). When producing an artificial sweat formulation the aim was to produce a liquid media which could support the growth of the selected skin bacterial organisms but at a minimal level to simulate the quantities of bacteria generally found on the epithelium which ranges from $10^2/cm^2$ -$10^7/cm^2$ viable organisms (Leyden *et al.*, 1987). This was achieved because all skin isolates tested were cable of growth in the artificial sweat at a markedly reduced rate than nutrient broth (Figure 5.2). The formulated artificial

sweat differs from hominine sweat as it supports the growth of axenic cultures whereas bacterial growth on the epithelium and in hominine sweat can be in the form of a synergistic relationship; in which bacterial by-products become substrates for separate bacterial growth thus increasing the overall production and efficiency of mutual interactions (James *et al.*, 2004). Although an obvious difference between the archetypal sweat and formulated artificial sweat this deviation allows for the investigation of axenic and multispecies interactions whilst being independent of obligatory synergistic bacterial interactions.

Formulating the artificial wound exudate posed similar challenges; fortuitously, wound exudate has been chemically characterised and is noted to be highly comparable to serum (Trengove and Langton, 1996). This allows direct comparisons to be made between data derived from formulated media to the data derived from FCS. A series of media were formulated (Table 5.4) based upon the main nutrient constituents of human serum (Table 5.2). By assessing the bacterial growth capacity (quantified according to specific growth rate and maximal OD) in the formulated media, the organism ability to proliferate can be evaluated. Independent t-test analysis of the overall specific growth rates and ΔOD derived from the artificial media when compared to FCS identified the artificial serum formula 3 as not significantly different ($p=0.458$) to FCS and this can be considered comparable to FCS. Analysis of the specific growth rates and ΔOD values provides a useful means of directly comparing same organism proliferation in different media. However, growth rates and ΔOD alone do not necessarily indicate that bacterial phenotypes in artificial serum and FCS as similar levels of growth may be attained via different phenotypic means in response to availability of nutrients and growth factors. Therefore to further certify the selected artificial

serum, analysis of protein expression by two-dimensional gel electrophoresis was undertaken. During the bacterial cell cycle different functional proteins are expressed in response to numerous internal and external variables e.g. temperature, nutrient availability and cell density. Two-dimensional gel electrophoresis is a method by which the proteome of an organism can be resolved on the basis of charge (first dimension) and molecular weight (second dimension). As variations in the protein expression can occur due to nutrient availability a comparison can be made between proteomic profiles of bacterial organisms grown in different media types. Utilising artificial serum (selected based upon bacterial growth data), FCS and nutrient broth (Oxoid, Basingstoke. U.K.) (a widespread medium used for bacterial cultivation) bacterial isolates were primarily grown overnight to ensure isolates had fully adjusted to the media and then sub-cultured to a fresh media solution and grown for 4-6 h until a optical density of 0.1 (600nm) had been attained. This process ensures that the isolates are within the exponential phase of growth and analogous cell densities between different media and isolates is attained, reducing variations as a result of protein expression in relation to population density, reduction in available nutrients and increases in waste metabolites. Utilising Melanie 7 software, a comprehensive package in which 2D gel images can be visualised, explored and aligned, unique protein spots were identified and gels were matched and aligned based upon common protein "landmarks". Comparisons between protein expression profiles of *S. aureus* in FCS, artificial serum and nutrient broth identified that of the proteome expressed in FCS, 71% were present in *S. aureus* during growth in artificial serum however, only 40% were present in *S. aureus* during growth in nutrient broth. A similar result is seen with *P. aeruginosa* during grown in FCS, where 74% of all the detected proteins were present in *P. aeruginosa* during growth in artificial serum whereas

only 40% were present in *P*. *aeruginosa* grown in nutrient broth. Interestingly for *P. aeruginosa* and *S. aureus* grown in artificial serum, 78% and 68% (respectively) of the expressed proteins were present in protein expression profiles when grown in FCS. This indicates that for the isolates tested the proteome expressed in artificial serum is a broadly similar profile to that expressed in FCS. These results highlight the significance and possible consequences of media selection for bacterial growth and biofilm assays. An ideal media to simulate the wound environment would be human serum however, the associated economic costs and risks associated blood borne viruses makes it an unfeasible choice for continuous culture systems in which relatively large volumes would be required. A common alternative has been the use of FCS however, as with human serum associated costs are high. More significantly the constituents of human serum and FCS such as iron and potassium can fluctuate in relation to age, gender, diet and preparation protocol (Bryan *et al.*, 2010). The data presented in this chapter highlights how different media can causes variations in bacterial growth and phenotype and thus the possible experimental variations which could occur when using FCS as a bacterial growth medium. This fundamentally underpins the development and validation of the defined artificial serum presented herein.

The final analysis of the artificial serum and sweat formulas was to ensure that the media supports the formation of biofilms in a manner similar to counterparts. Previous publication have highlighted the role that nutrient availability has on the biofilm formation of bacterial isolates (Rochex and Lebeault, 2007; Sauer *et al.*, 2004). Utilising a laminar flow cell reactor Rochex and Lebeault (2007) observed that increases in the available glucose or phosphate concentration increased the rate and extent of *Pseudomonas putida* biofilm accumulation however once a

“threshold” concentration was attained for these nutrients an increase in detachment rates was observed resulting in reduction in biofilm accumulation. This underlines the significance/importance of ensuring that organisms can form biofilms in selected media. The biofilm formation of the selected bacterial isolates in the formulated artificial serum was assessed utilising the crystal violet biofilm assay. All the isolates testes were capable of producing a biofilm upon the abiotic surface of the microtitre plate in each media type however, significant differences can be seen between the two media types with higher biofilm production found for isolates grown in artificial serum.

5.6 Conclusion

When employing *in vitro* biofilm models to study the microbial growth and biofilm formation, the selection of specific growth media can be crucial in ensuring the results are biological relevant and closely mimic the environment in which it aims to reproduce. The formulated artificial sweat and serum media have been validated to facilitate the long-term maintenance of wound and skin associated consortia, ensuring the bacterial growth and phenotype are broadly similar to that of the commonly used foetal calf serum whilst providing a defined, cost effective alternative.

Chapter 6

Development and validation of novel biofilm wound reactors

6.1 Abstract

Chronic wounds are innately polymicrobial, harbouring a range of aerobic and anaerobic microbial populations. Within recent years, evidence has emerged for wound-associated bacterial biofilms and their putative role in impaired healing and antimicrobial treatment failures. As a tool for studies of the complex microbiota and associated biofilms of chronic wounds, the aim of this chapter was to develop representative wound models systems. The development and validation of the novel fine celled foam substrata, model media alimentation method and overall system was achieved by determining bacterial growth and biofilm formation within the respected individual elements using viable counts and imaging techniques. The ability of selected bacterial isolates to attach to and proliferate upon a novel FCF substratum was tested using modified multiple sorbarod devices. All isolates tested were capable of attaching and proliferating upon this material. Two novel model systems were developed utilising this substratum to (i) study cross-sectional population interactions and (ii) to investigate longitudinal population development and biofilm formation. The FCF multi-well wound model was designed to study short-term population interactions; axenic population of selected bacteria grown within this model system with previously validated artificial sweat or artificial serum attained a dynamic steady state within 48 h of growth. The multiple FCF wound biofilm model was developed to study long-term population interactions and biofilm development in continuous culture. Axenic and combined populations of aerobic and anaerobic bacterial isolates were maintained within this model system and reached a dynamic steady state from 48 h of incubation after inoculation. Based upon the defined outcome parameters the developed wound biofilm models successfully (i) facilitated the attachment and proliferation of single and multispecies consortia associated with chronic wounds, (ii) supported the growth and development of wound-associated biofilms, (iii) simulated the chronic wound environment.

6.2 Introduction

Acute and chronic wound infections can be costly, resulting in prolonged hospital stays, an increased risk of secondary site infections, septicaemia and is a significant cause of morbidity (Hill *et al.*, 2003; Howell-Jones *et al.*, 2005). In 2008 it was estimated that over 200,000 patients in the UK had a chronic non-healing wound resulting in a conservative cost to the NHS at *c.* £2.3-3.1bn per year (at 2005-2006 costs) (Posnett and Franks, 2008). In the majority of chronic wounds the impedance of healing is directly linked to the underlying aetiologies associated with the development of the wound i.e. diabetes, peripheral vascular disease which can cause restricted blood flow to the site, neuropathy and an impaired immune response. This delay in healing combined with the breakdown in the epithelial barrier increases the risk of bacterial infection occurring, leading to auxiliary tissue damages further delays in healing. Wounds rapidly become colonised by a variety of endogenous and exogenous microorganisms from the onset of their development. However, according to current definitions, infection is said to be present when there is a transition from the colonisation state, defined as "the presence of replicating microorganisms adherent to the wound in the absence of tissue damage", towards an infective state which has been defined as "the presence of replicating organisms within a wound resulting in tissue damage" (Dow *et al.*, 1999). A figure of $\geq 10^6$ organisms per gram of tissue has been cited as a threshold about which a wound is considered infected (Murphy *et al.*, 1986; Robson, 1979; Robson, 1997). Due to their "open nature" chronic wounds are innately polymicrobial, harbouring a variety of aerobic and anaerobic populations. Typically isolated organisms include *Staphylococcus aureus*, Lancefield groups A, B, C and G streptococci, *Enterococcus faecalis*, *Bacteroides* species, *Clostridium*

species, anaerobic cocci, coagulase-negative staphylococci, *Corynebacterium* species, enterobacteriaceae and pseudomonads (HPA, 2009). Polymicrobial communities are able to proliferate in the wound due to the excess availability of nutrients. Bacterial isolates bind to and invade the tissue bed and surrounding tissue where conditions are more favourable for the growth, this is particularly true for anaerobic organisms were the lowered oxygen-reduction potential in the tissues and increased nutrients derived from their breakdown provide and ideal environment for bacterial proliferation (Edlich *et al.*, 1988; Sapico *et al.*, 1980). Overall this results in a greater diversity of bacteria located in the tissue bed than on the tissue surface (Sapico *et al.*, 1980; Sapico *et al.*, 1984; Sharp *et al.*, 1979). Within recent years evidence has emerged for wound-associated bacterial biofilms and their purported role in impaired healing and antimicrobial treatment failures (Akiyama *et al.*, 2002; Davis *et al.*, 2008; Serralta *et al.*, 2001). In order to further understand the complex microbiota and associated biofilms of the chronic wound representative model systems can be used to investigate population dynamics, biofilms and efficacy of antimicrobial treatments.

To date numerous biofilm models have been developed and validated which are representative of specific and generic environments. One model which has been frequently used to study polymicrobial biofilms of the oral cavity is the constant depth fermenter (CDFF) (Kinniment *et al.*, 1996; Ledder *et al.*, 2009; McBain *et al.*, 2003a; Pratten *et al.*, 1998). The CDFF facilitates the development a consistent depth biofilms upon a rotating solid substrata e.g. polytetrafluoroethylene by controlling the depth of the biomass by static fixed positional scrapers blades which removes excess biomass (McBain *et al.*, 2009; Wilson and Ron, 1999). Both Hill *et al*., (2010) and Malic *et al*., (2009) used the CDFF to develop multi-species

biofilms of wound bacterial isolates in which the resultant biofilms could be maintained for up to 4 weeks. Furthermore, Hill *et al*., (2010) tested dressing against CDFF generated biofilms by aseptically removing the substrata and inverting on a media moistened dressing. Viable count data indicated a tolerance towards silver dressings; a recalcitrant typically associated with biofilms (Hill *et al.*, 2010). Nevertheless, it is important to note the CDFF model does not simulate the properties associated with the environment of the chronic wound; bacteria in a wound are attached to and invading the surrounding tissues deriving nutrients and growth factors from the wound bed, surrounding tissues and exudate, and are thus fed from below. Furthermore, the shear forces experienced by the CDFF biofilms via the rotary action of the turntable and static scraper are also absent in wounds. The intrinsic design of the CDFF also limits its application for the investigation of dressing on wound-associated biofilms. It can only be achieved by removal of the substratum and applying the dressing to the biofilm coated substratum which fundamentally alters the biofilms growth environment. Ultimately the application of the CDFF as an *in vitro* model to investigate chronic wound biofilms is restricted and consequently it can only be used to study biofilms of wound derived bacterial isolates with limited application as a model of chronic wound biofilms.

Attempts have also been made to produce novel wound biofilm models specific to the environment of the chronic wounds with variable success. Werthén *et al*., (2010) for example developed a novel model in which the growth of axenic populations is maintained within matrices of polymerised rat tail collagen and simulated wound fluid for 24-48 h. The model system produced aggregates of *Pseudomonas aeruginosa* which were visually comparable to aggregates in chronic wound tissue samples. Furthermore, claims were made that the model

could be utilised to study antimicrobial therapies and novel wound dressings (Werthen *et al.*, 2010). This model provides a rapid, high throughput system in which axenic or multiple bacterial populations can be investigated, using quantitative CFU analysis, sectional imaging or confocal scanning microscopy. Nevertheless this model has a limited capacity for investigation microbial population dynamics over a greater period than 2 d due to the limited collagen matrix size and their stability under rapid bacterial growth. Thorn and Greenman (2009) described a novel *in vitro* flat bed perfusion biofilm model that they used to study potential antimicrobial efficacy of topical wound treatments. Microbial biofilms were grown within a 1 cm^2 cellulose matrix that was housed within rubber supports and a surface methylpentene Delnet aperture film which is secured to a 50 mm glass slide. Media is perfused through the substratum via a drip wise peristaltic pump, allowing media to flow down through the cellulose matrix via gravitational forces. A removable clip was fashioned to support topical wound treatments which could be positioned upon the cellulose matrix when required (Thorn and Greenman, 2009). This model system enables the rapid development of quasi steady-state single, species populations of *P. aeruginosa* and *S. aureus* within 24 h of growth and the testing of antimicrobial dressing on the total microbial population grown within the cellulose matrix. Despite the number of benefits of this model system, the fundamental basis of the medium delivery mechanisms is similar as that of the drip-flow slide reactor in that media is dispensed at the top and dispersed through the model via gravitational forces. Subsequently, this model may be subject to the same nutrient heterogeneity issues as associated with drip flow slide reactor with decreased nutrient concentrations found at the base of the substrata versus the high concentration found the pinnacle of the substrata. However, it is important to note that the controlled delivery of media within the

majority of *in vitro* models system will invariably result in a degree of nutrient heterogeneity due to the specific control of media and the delivery location.

It is apparent that the complex polymicrobial biota and environment of the chronic wound poses difficult challenges when employing or designing model systems and that current models are arguably deficient in certain respects. The role of an *in vitro* wound model is to simplify a complex problem and produce accurate reproducible data by simulating aspects of the *in vivo* environment such that the effects of defined variables can be measured without loss of compliances. The development of a representative model system requires the assessment of two important elements: the type of investigation required, for example microbial ecology, and/or biofilm growth, and the measured experimental outcome such as microbial viability. In the context of this chapter the aim was to develop and validate chronic wound models in which the bacterial communities, biofilms and the anti-biofilm efficacy of novel wound dressing could be studied.

6.3 Materials and methods

Bacterial isolates used in this chapter were *Bacteroides fragilis* (NCTC 9343), *Clostridium perfringens, Corynebacterium xerosis, Propionibacterium acnes*, *Escherichia coli, Pseudomonas aeruginosa* (isolate 24), *Staphylococcus aureus* (isolate 9), *Staphylococcus epidermidis* (ATCC 14990) and *Staphylococcus saprophyticus* (NCTC 7292). See Chapter 2 for organism origins.

6.3.1 Fine celled foam (FCF) substratum bacterial attachment assay utilising a modified multiple sorbarod device

A fine-celled thermoset phenolic plastic foam (FCF) (Smithers-Oasis, Tyne & Wear. U.K.) was selected as a potential substratum for use in conceptualised models based upon its foamed cellular solid structure with interconnecting pores which drives media uptake by capillary action, its structural stability when saturated and wet heat tolerance facilitating sterilisation by autoclaving. In order to assess the ability of bacteria to attach to and proliferate in the novel FCF substratum using artificial sweat and serum media, (previously validated in Chapter 5) the multiple sorbarod device (MSD) was used. The MSD has been previously used to maintain the growth of axenic and polymicrobial populations, it consists of a stainless steel housing containing a removable polytetrafluoroethylene (PTFE) cylinder comprising five predrilled sections to house sorbarod filter inserts, these were replaced with FCF inserts measuring 10mm x 20mm. Temperature (37℃) and atmosphere (O_2) were maintained by placing the device in an aerobic incubator. Prior to inoculation the FCF inserts were conditioned *in situ* with artificial media for a period of *c.* 3 h. Inserts were then inoculated with 1.5 ml of overnight stationary phase axenic bacterial cultures grown in artificial sweat or serum, adjusted to *c.* 7.0 Log_{10} CFU/ml. Artificial media was continually supplied to the device at a rate of *c.* 3.0 ± 0.1 ml h^{-1} by peristaltic pump (Minipulse 3, Gilson, Villiers-Le-Bel, France) for 48 h. In order to remove non adherent cells and populations which have grown in media held within the pores of the FCF, sterile saline was flushed through the device at a rate of *c.* 10 ± 0.1 ml h^{-1} by peristaltic pump (Minipulse 3, Gilson, Villiers-Le-Bel, France) for a period of 30 min, foam inserts were then removed and viable counts performed.

6.3.2 Determination of the sterile saline wash out step to remove non-adherent cells using a dye replacement assay

To determine the appropriate length of time and rate for the wash out step for the removal of non-adherent cells from the modified MSD, a dye replacement assay was devised. Briefly, a modified multiple sorbarod device was assembled to house the FCF substrata as previously described, the device was then flushed with saline until all the FCF substrata were fully saturated. A water-soluble Lutien natural food colour dye (Dr Oetker, Leeds, UK) at an optical density of 0.229 (600nm) was then flushed through the device at a rate of *c.* 10 ± 0.1 ml h^{-1}. Perfusate samples were taken every 5 minutes and the optical density at 600nm determined in triplicate. The point at which the mean optical density of the perfusate matched the optical density of the original dye media was considered the wash out step time frame, in which all the media held within the FCF substrata is had been replaced.

6.3.3 Validation of the FCF multi-well wound model

To validate use of the novel FCF multi-well wound model (based upon 24-well hydroxyapatite biofilm model) for investigating bacterial community interactions, initial investigations were made into the ability of the model system to maintain dynamic steady state cultures (defined as equal to or less than a one Log variation in the CFU/cm^3 over a 48 h period). Stationary phase cultures of selected bacterial strains grown overnight in either artificial sweat of serum were diluted 1:100 in respective artificial media. Cylindrical fine celled foam substrata (FCF) measuring 1cm x 1cm, housed within wells of a 24 well cell culture plate (Sigma Poole, Dorset, UK) were inoculated with 1 ml of bacterial inocula and incubated aerobically at 37℃ for 5 d. Spent media was remove d daily and replaced with

fresh media, FCF substrata were removed each day and viable counts performed in triplicate.

6.3.4 Validation of the Multiple FCF wound biofilm model

A novel model was designed that included (i) FCF substrata, (ii) dialysis membrane and (iii) continuous media fed system. A CAD diagram of the Multiple FCF (MFCF) wound biofilm model shown in Figure 6.1. Briefly the MFCF wound biofilm model consists of an open top cylinder measuring 80mm high with a diameter of 170mm. Two opposing media outlets are located 50mm from the bottom of the cylinder base. The MFCF wound biofilm model is capped by a "membrane insert" holder measuring 190mm in diameter, 10mm in depth containing a central media inlet and six predrilled 25mm diameter holes. Housed within these predrilled holes are six removable 12000-14000da viskin dialysis membranes sealed at the base with dialysis closures and connected in an open position to a 15mm diameter polypropylene tube extending centrally throughout a 25mm bung. The device is enclosed by 220mm diameter cover containing a central media inlet. To validate the use of the novel MFCF wound model for investigating bacterial community interactions, initial investigations were made into the ability of the model system to maintain dynamic steady state cultures (defined as equal to or less than a one Log variation in the CFU/cm^3 over a 48 h period). Following a *c.* 4 h preconditioning step whereby FCF substrata (2cm x 1.5cm) were maintained in artificial serum without a continually supplied of media, FCF inserts were inoculated with 2 ml of an stationary phase overnight cultures diluted 1:100 in artificial serum. Temperature (37℃) and atmosphere (O_2) were maintained by placing the device in an aerobic incubator. Artificial media was

continually supplied to the device at a rate of *c.* 3.0 ± 0.1 ml h^{-1} by peristaltic pump (Minipulse 3, Gilson, Villiers-Le-Bel, France). Cultures were maintained for a period of either 4 or 6 d, substrata were removed daily to perform viable count analysis. Membranes were also removed at the time of insert collection and replaced with a sterile bung to maintain sterility of the media bulk. When models were run longer than a 4 d period, dialysis membranes and substrata were aseptically removed with sterile blunt forceps on day 3, the FCF placed into a sterile fresh dialysis membrane(s) and placed back into the model.

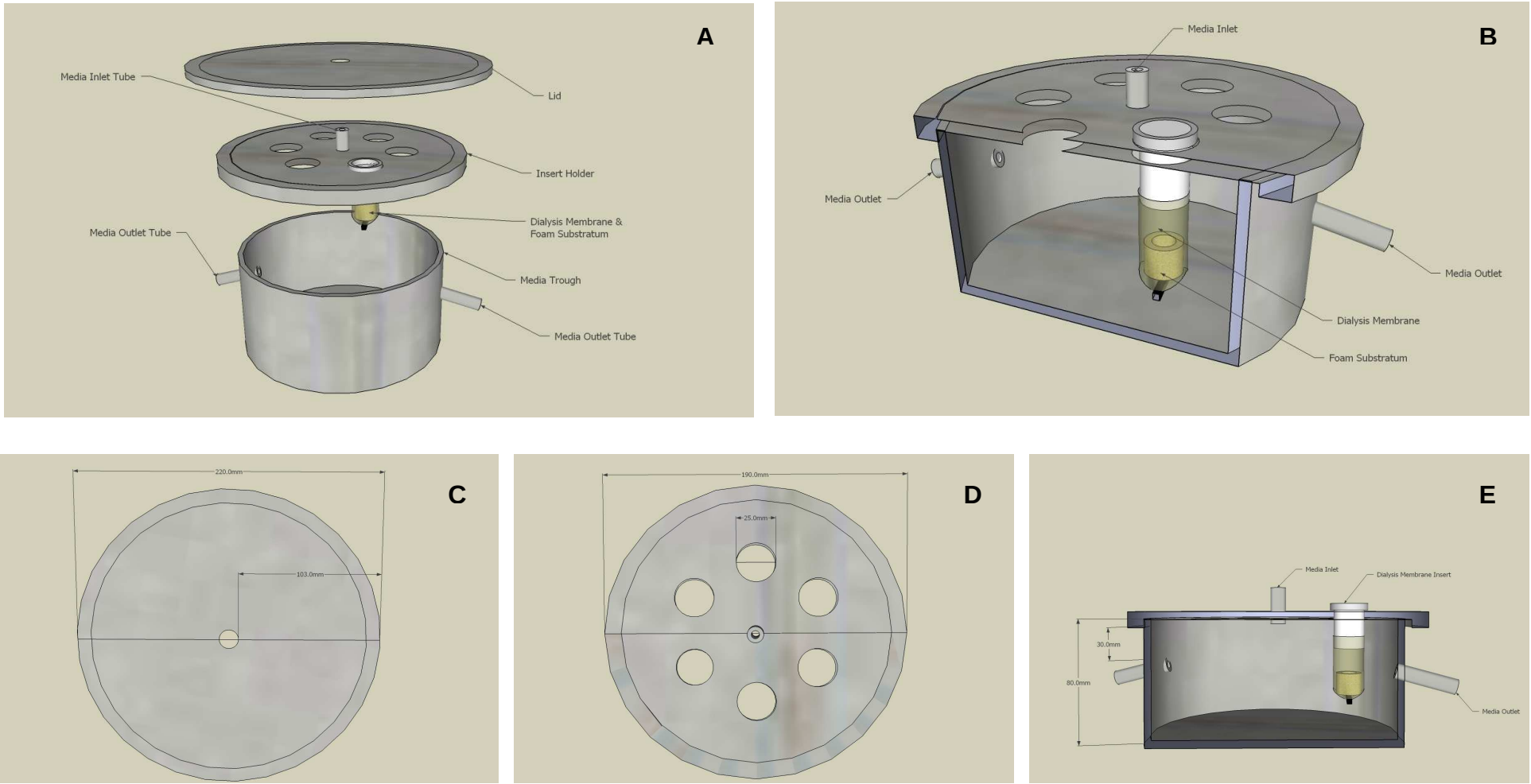


Figure 6.1.(A) Schematic diagram of the assembled MFCF wound biofilm model,(B) Sectional view of the assembled model, (C) Elevation of the lid of model, (D) Elevation of the insert holder of the model, (E) Pan of MFCF wound biofilm model

6.3.5 ESEM and Gram stains of FCF substratum biofilms

In order to compare bacterial aggregates cultivated in the MFCF wound biofilm model to *in vivo* bacterial aggregates of chronic wounds, axenic populations of *P. aeruginosa* and *S. aureus* were cultivated in the MFCF wound biofilm model with artificial serum as the fed medium for a period of four days with a dialysis membrane replaced on the third day of cultivation. After cultivation, the FCF substratum was aseptically removed, divided longitudinally with one section placed in a sterile bijou bottle for immediate transportation for environmental scanning electron microscopy (ESEM). The remaining FCF substratum section was placed in Optimum Cutting Temperature (OCT) Embedding Matrix (CellPath, Powys, UK) and frozen at -80℃ for > 24 h. Tissue samples deri ved from chronic wounds (NHS Research Ethics Committee study ref: 09/H1006/41) were subject to selective and total viable count analysis, and denaturing gradient gel electrophoresis (DGGE) see Chapter 3. Excess chronic wound tissue samples of sufficient quantity for cryo-sectioning and ESEM were divided (50:50) with a sterile scalpel with one section embedded in OCT embedding matrix (frozen at -80℃) for > 24 h and the remaining section placed in a sterile bijou and transported immediately for ESEM imaging.

ESEM of FCF substratum from the MFCF wound biofilm models and chronic wound tissue samples was performed using a FEI Quanta 200 environmental scanning electron microscopy under a low vacuum (<0.75 torr) permitting interrogation of putative biofilms structures and microcolonies whilst conserving the hydrated state of the sample.

The cryopreserved tissue samples and FCF substratum embedded in OCT embedding matrix were sectioned to a thickness of 5 µm utilising a Shandon AS260 manual cryostat and mounted on superfrost plus microscope slides (Fisher Scientific, Leicestershire, UK). Sections were Gram stained as per standard protocol. FCF substrata, subjected to the same growth conditions as previously described in the absence of bacterial cultures were utilised as negative controls. Images were captured using a Zeiss Axiocam on a Axioscop 2 microscope, with Axiovision Version 4.8 (Carl Zeiss ltd Hertfordshire U.K.).

6.3.6 Positional growth of *Bacteroides fragilis* in contoured FCF substratum

To assess the potential influence of the shape of substrata on the positional loci growth of selected organisms, FCF substrata (8 cm^3) were contoured to broadly represent the physical environment of three different wound types: second degree burn (partial thickness burn), an open wound involving centrally deeper seated tissues and a pressure sore consisting of deeper seated subcutaneous tissues shown in Figure 6.2

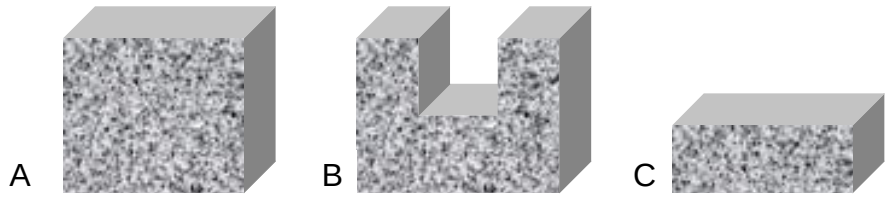


Figure 6.2 Diagram of contoured FCF substratum. (A) Open FCF substrata representative of second degree burn, (B) centrally compacted FCF to represent deeper seated tissues, (C) Compacted FCF to represent subcutaneous tissues

Contoured FCF substrata, housed in 50 ml falcon tubes was inoculated with 20ml of stationary phase cultures *Bacteroides fragilis* (grown overnight in artificial serum and diluted 1:100 into fresh pre-reduced media) and incubated at 37℃ for 48 h. The substrata were then removed sectioned into thirds with a sterile scalpel to represent the left and right outer and centrally positioned substrata loci, macerated and viable count analysis performed.

6.4 Results

6.4.1 FCF attachment assay

A wash-out step of 30 min was determined as a time in which the saline saturated FCF substrata housed within a multiple sorbarod device is completely replaced with the wash-out media shown in Figure 6.3.

The attachment of selected bacterial isolates to FCF in different media was assessed utilising a modified multiple sorbarod device. All isolates tested were capable of attaching and proliferating on the FCF in the different media types shown in Figure 6.4.

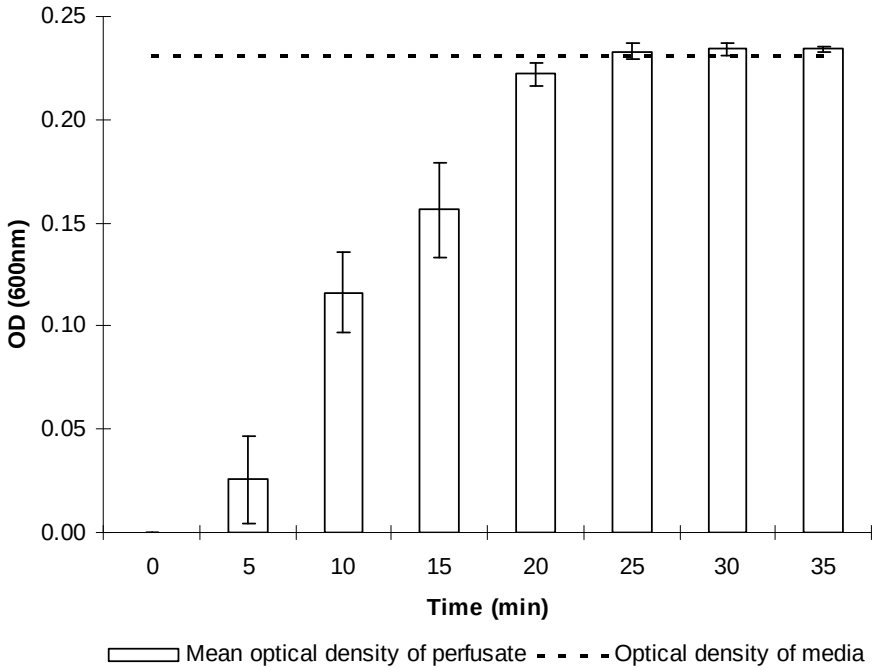


Figure 6.3 Optical densities of dye replacement assay perfusate samples versus time of perfusion

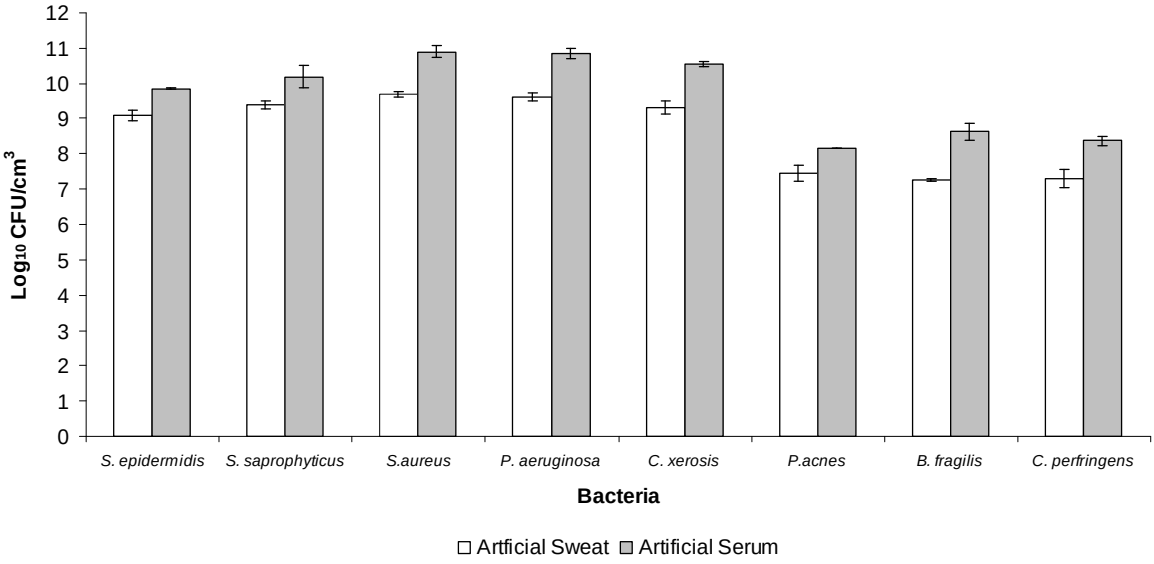


Figure 6.4 Attachment of selected bacterial isolates to FCF substratum in the modified multiple sorbarod device

6.4.2 Validation of FCF multi-well wound model

All bacterial isolates tested were capable of attaining an axenic dynamic steady state growth (defined as equal to or less than a one Log variation in the CFU/cm^3

over a 48 hour period) within 48 h of growth in the FCF multi-well wound model in both artificial sweat and serum media shown in Figures 6.5 and 6.6.

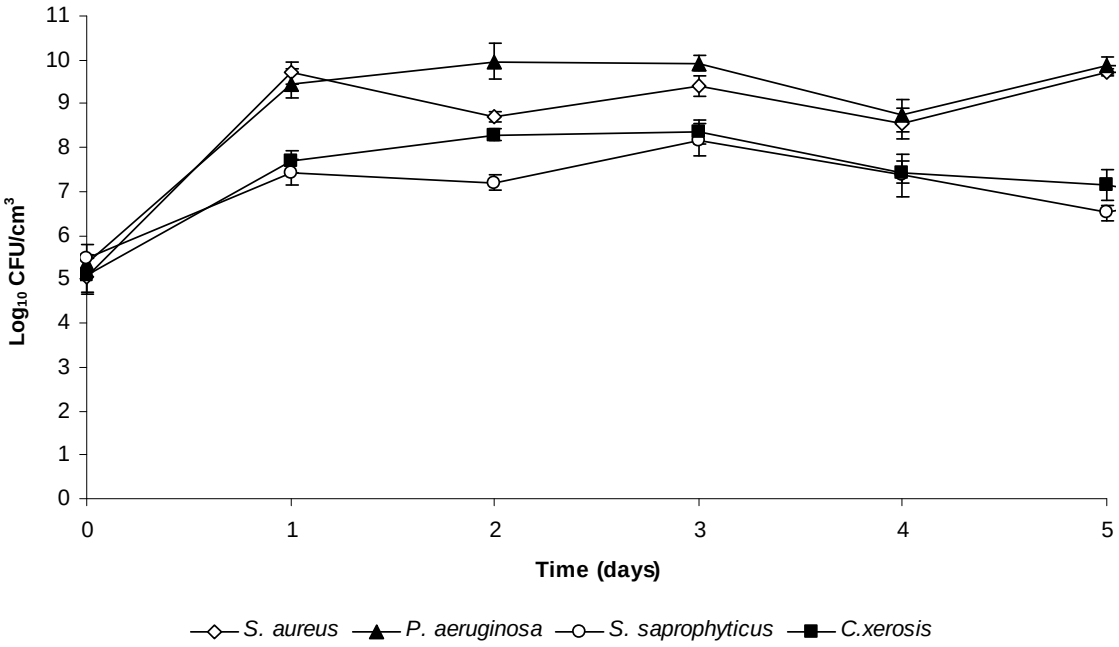


Figure 6.5 Continuous axenic cultures of selected bacteria grown in artificial sweat in an FCF multi-well wound models

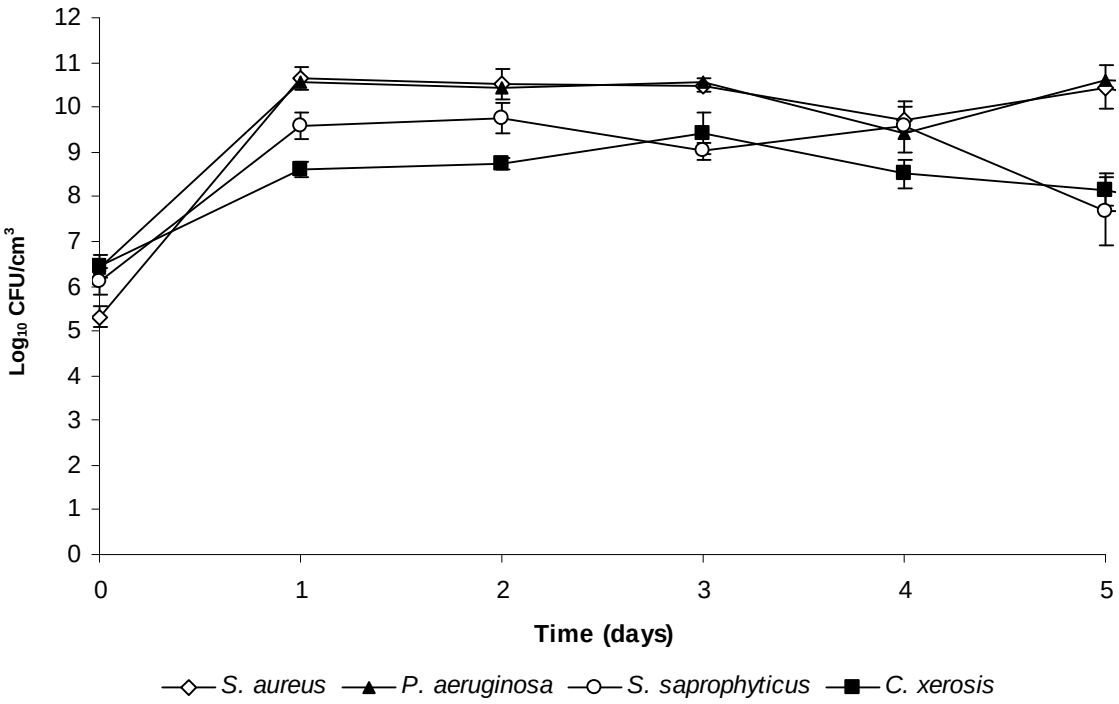


Figure 6.6 Continuous axenic cultures of selected bacteria grown in artificial serum in an FCF multi-well wound models

6.4.3 Validation of the multiple FCF biofilm wound model

Axenic continuous cultures of *B. fragilis, S. aureus and P. aeruginosa* cultured over a period of four days attained a dynamic steady state of growth by 48 h of cultivation as seen in Figures 6.7, 6.8 and 6.9

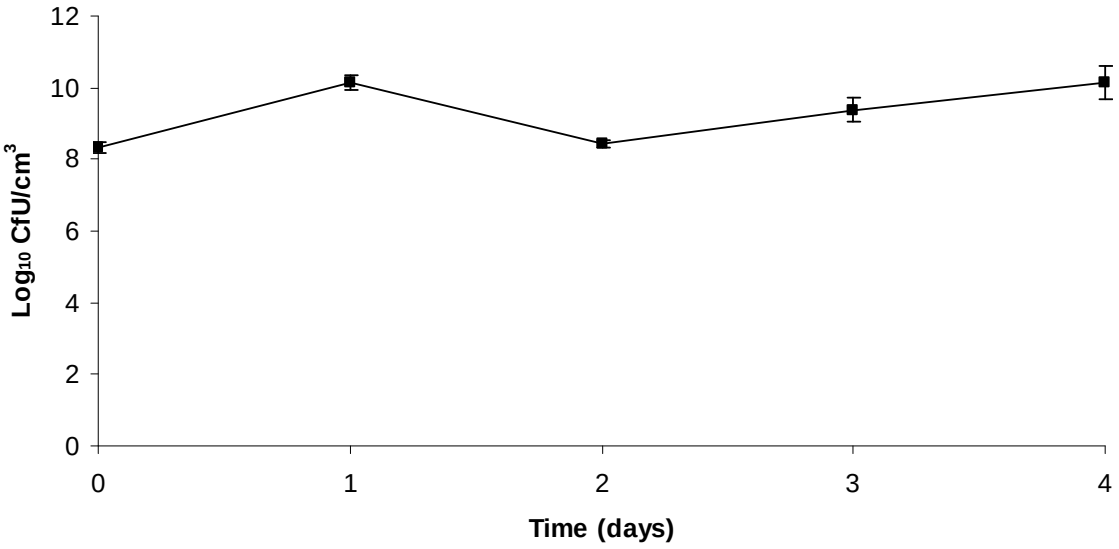


Figure 6.7 Continuous culture of *B. fragilis* in multiple FCF biofilm model

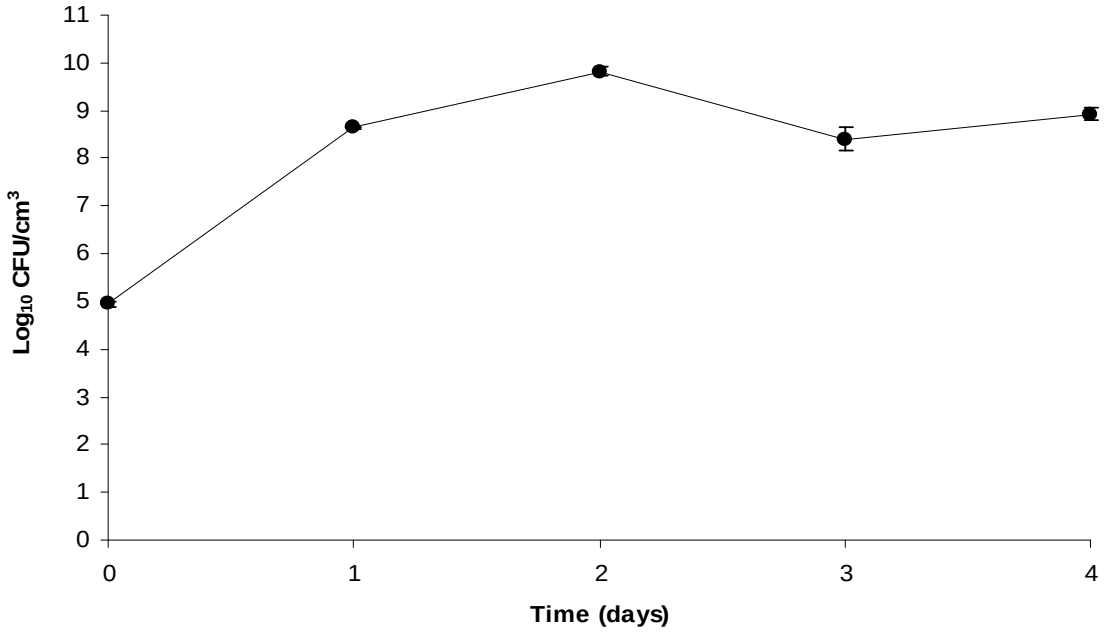


Figure 6.8 Continuous culture of *S. aureus* in multiple FCF biofilm model

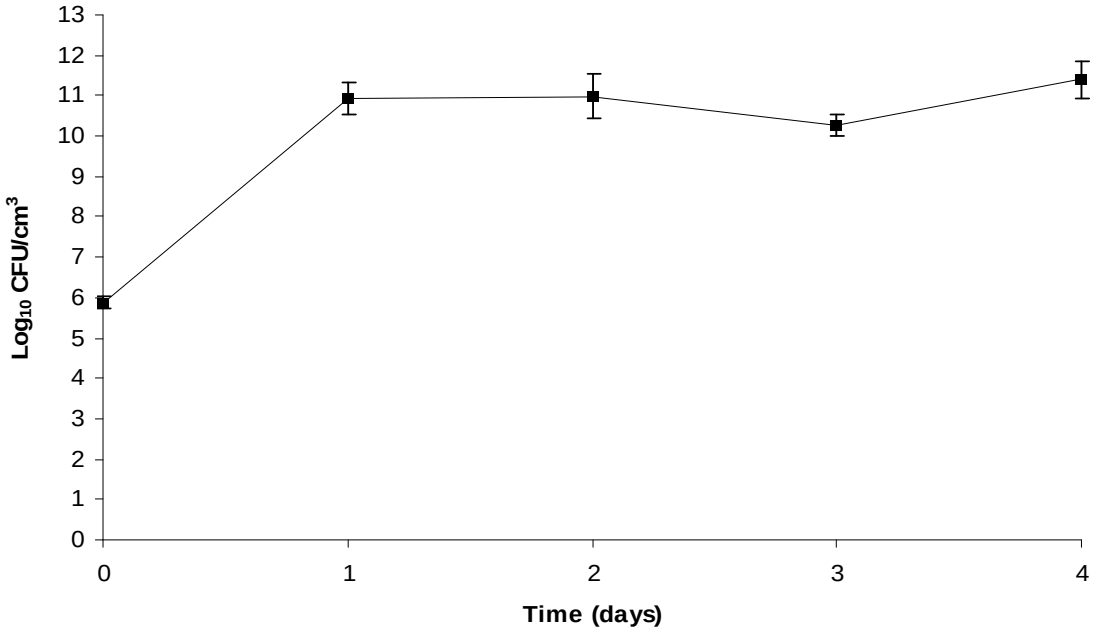


Figure 6.9 Continuous culture of *P. aeruginosa* in multiple FCF biofilm model

Continuous combined cultures of *S. aureus*, *P. aeruginosa* and *E.* coli maintained for a period of six days attained a dynamic steady state of growth from 48 h and post dialysis membrane exchange as seen in Figure 6.10

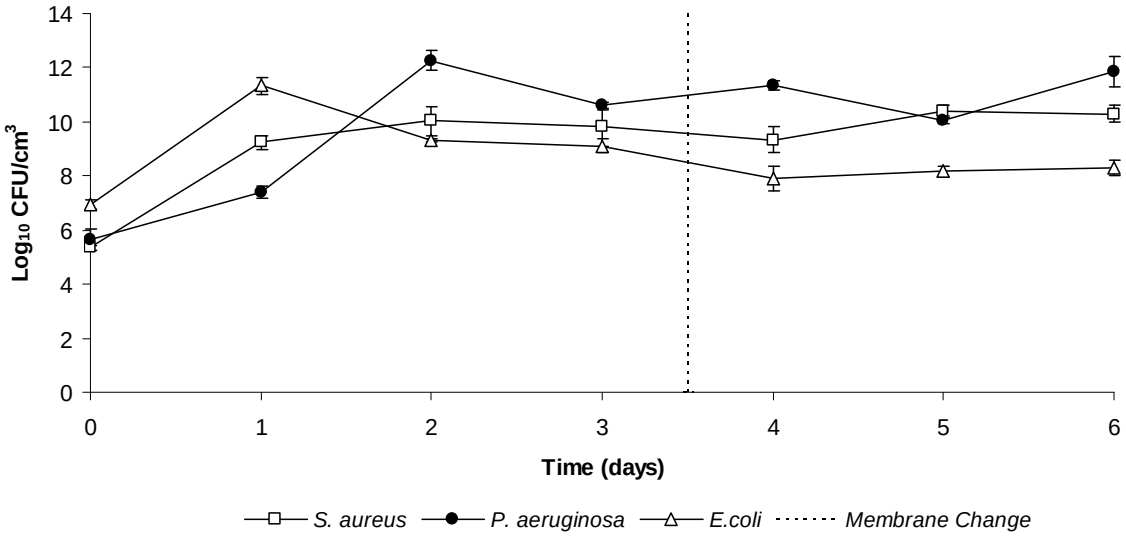


Figure 6.10 Continuous combined culture of *S. aureus*, *P. aeruginosa* and *E. coli* in multiple FCF model with dialysis membrane change on day 3.5.

6.4.4 Positional growth of *B*. *fragilis* in the FCF substratum

The affect of the shape of the substrata on the positional growth of *B. fragilis* was investigated using contoured FCF substrata shown in Figure 6.2. The “standard” open structure of the FCF with no physical alterations allowed for a relative uniform growth of *B*. *fragilis* throughout the substrata with slight reduction in CFU observed in the external portions when compared to the central section. The centrally compressed contoured FCF substrata resulted in an increase in the CFU/cm^3 of *B*. *fragilis* within the central section (when compared to the external sections) and an overall increase in the CFU/cm^3 for the external sections when compared to the non contoured “standard” FCF substrata. The fully compressed FCF substrata allowed for increased CFU/cm^3 of *B*. *fragilis* throughout the foam when compared to the non-contoured FCF, with higher quantities of CFU recovered from the central section when compared to the external portions of this substratum shown in Figure 6.11

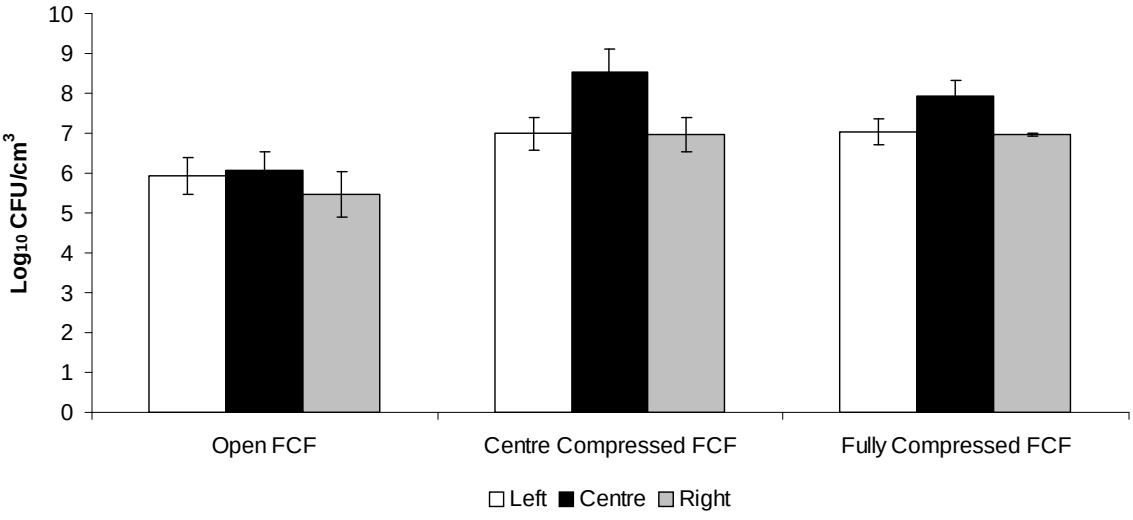


Figure 6.11 Positional growth of *B. fragilis* in contoured FCF substratum

6.4.5 Bacterial biofilm formation in multiple FCF wound biofilm model

Figure 6.12 shows light microscopy images of the FCF substratum illustrating the open pore structure of the FCF substratum with the larger pores visible in Figure 6.12 (A) and the smaller pores of visible in Figure 6.12 (B). Figure 6.13 shows Gram stained sections of FCF substratum used to maintain axenic populations *S. aureus* (A) and *P. aeruginosa* (B) in the MFCF wound biofilm model and Figure 6.14 shows Gram stained chronic wound tissue. Microcolonies are indicated by arrows.

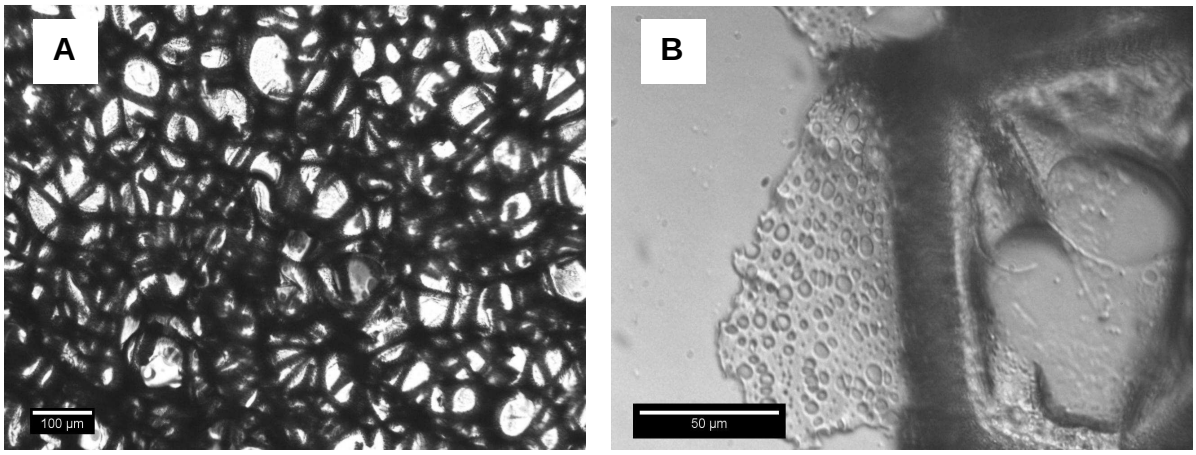


Figure 6.12.(A) FCF Substratum under X10 Objective, (B) FCF Substratum under X40 Objective

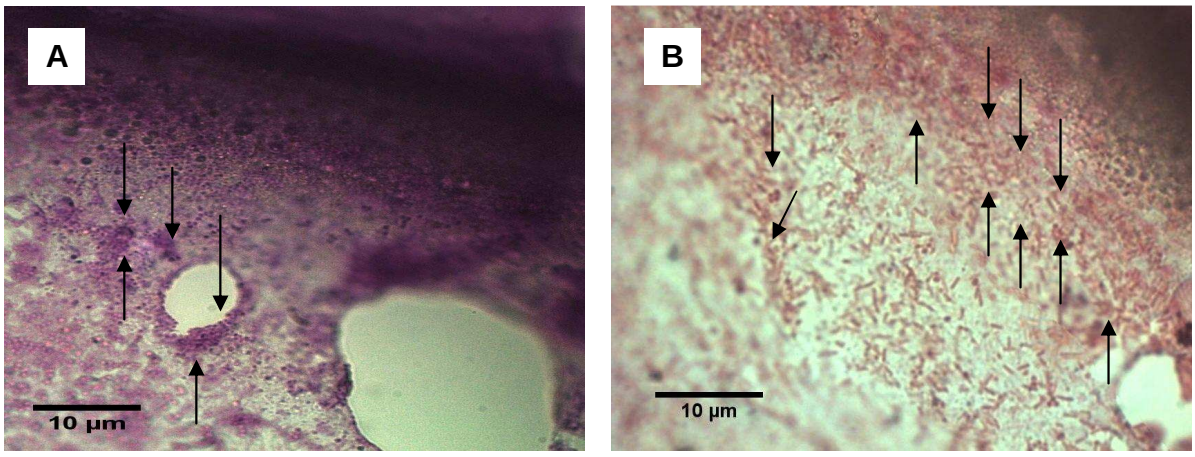


Figure 6.13 Gram stained sectioned of FCF substratum of *S. aureus* (A) and *P. aeruginosa* (B) maintained in the MFCF wound biofilm model for 4 days**.**

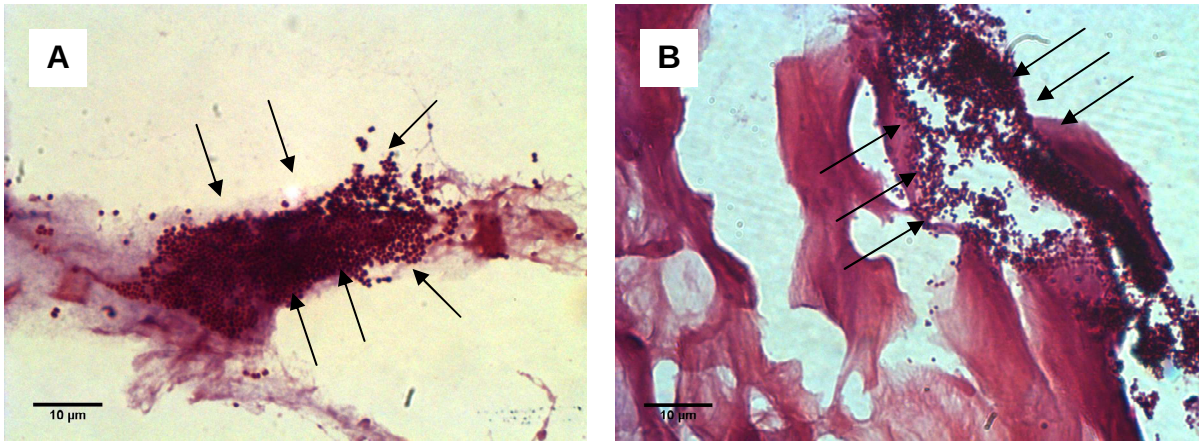


Figure 6.14 Gram stained section of chronic wound tissue sample from Patient A (A), and Patient E (B)

Figure 6.15 shows ESEM images of the negative control FCF substratum illustrating the open pore structure and smooth surfaces of the FCF. Comparisons between these materials and those observed in Figures 6.16 and 6.17 in which axenic cultures of *S. aureus* and *P. aeruginosa* were respectively maintained on FCF substrata show significant differences; specifically the presence of a putative biofilm consisting of highly hydrated layer and branching tubular structures coating the FCF surfaces as indicated by arrows.

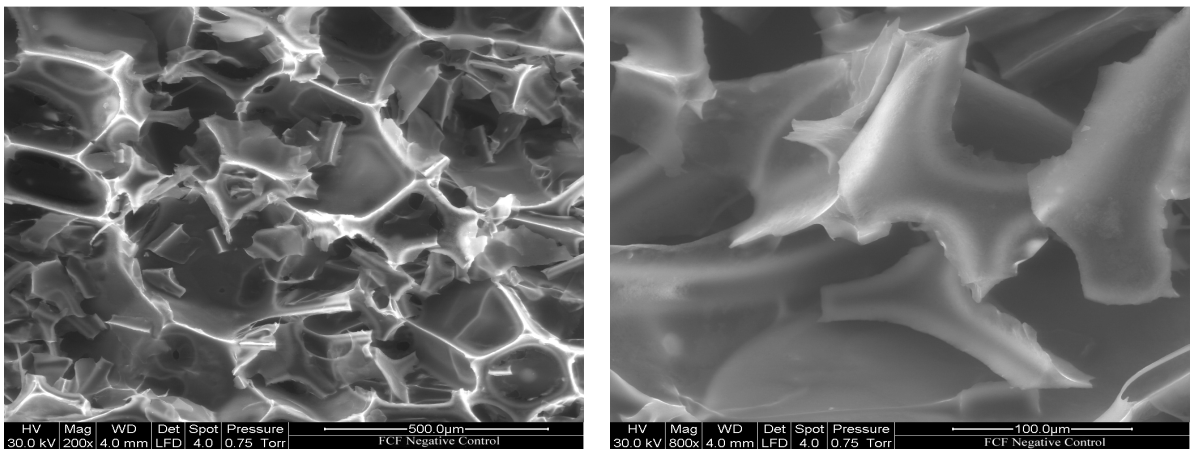


Figure 6.15 Environmental scanning electron microscopy image of negative control FCF substratum

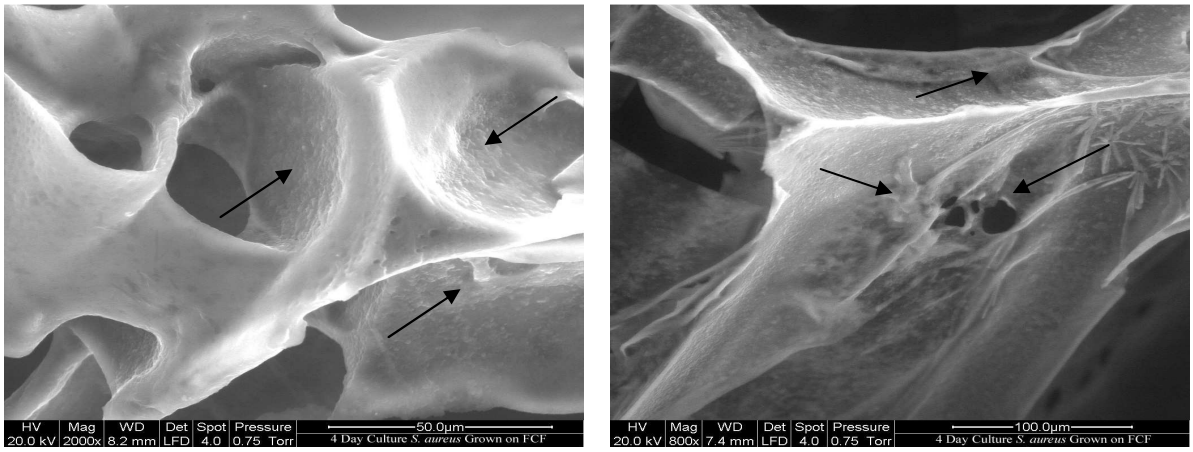


Figure 6.16. Environmental scanning electron microscopy image of *S. aureus* maintained on FCF substratum for 4 days, putative biofilm indicated by arrows

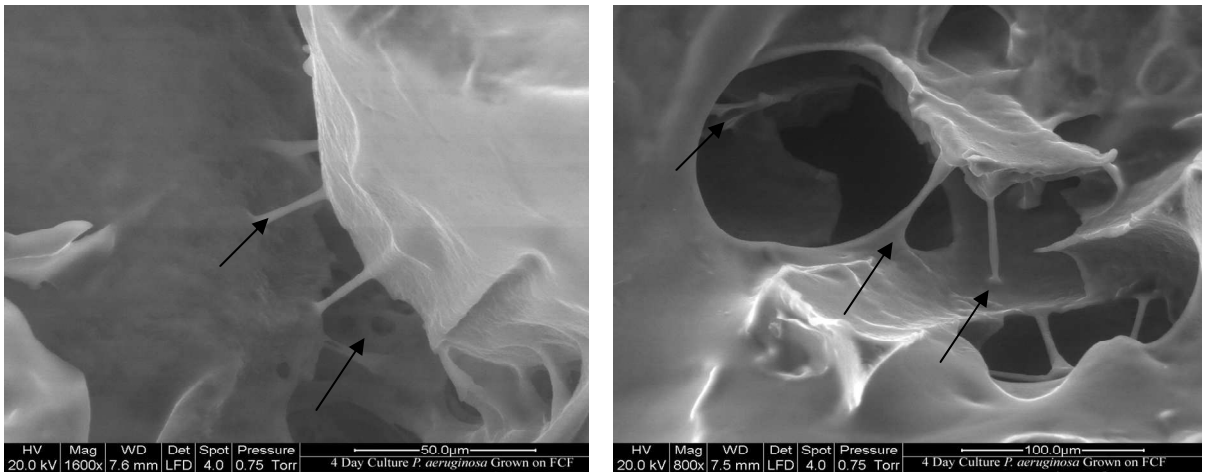


Figure 6.17 .Environmental scanning electron microscopy image of *P.* aeruginosa maintained on FCF substratum for 4 days putative biofilm indicated by arrows

Figures 6.18 and 6.19 show ESEM images microcolonies of *S. aureus* and *P. aeruginosa* growing upon FCF substrata covering the surface of the FCF, which are similar to the microcolonies observed upon chronic wound tissue samples shown in Figure 6.20

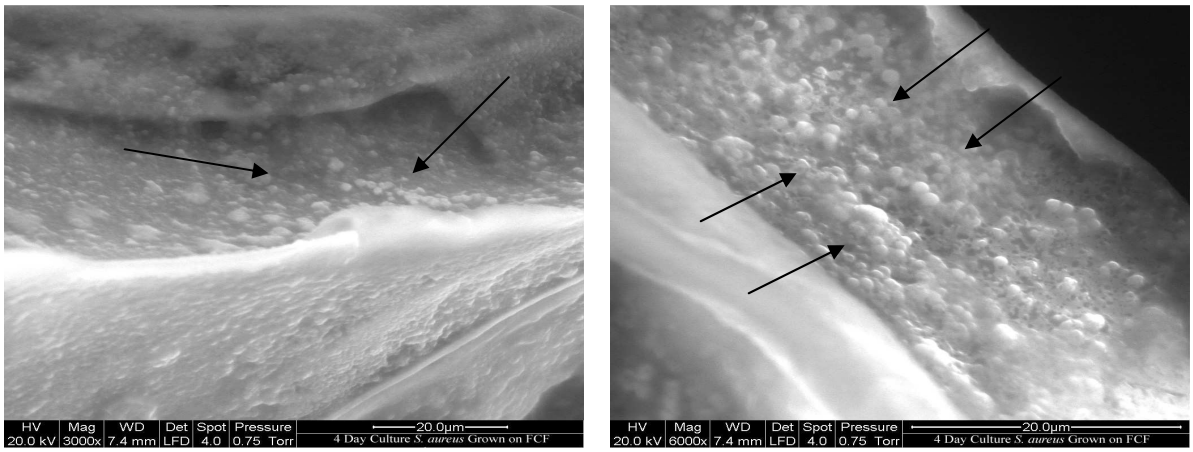


Figure 6.18 Environmental scanning electron microscopy image of microcolonies of *S. aureus* maintained on FCF substratum for 4 days. Microcolonies indicated by arrows

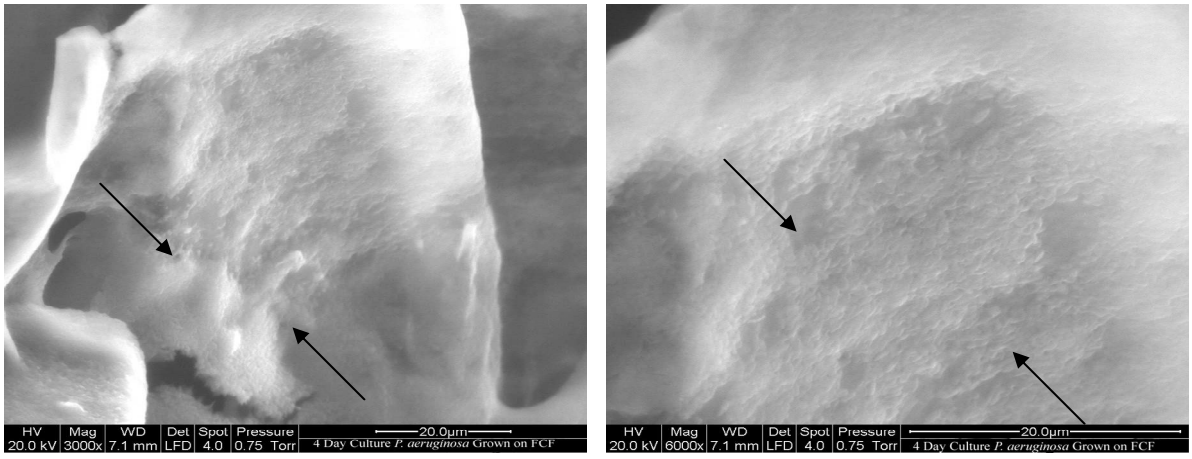


Figure 6.19 Environmental scanning electron microscopy image of microcolonies of *P. aeruginosa* maintained on FCF Substratum for 4 days. Microcolonies indicated by arrows

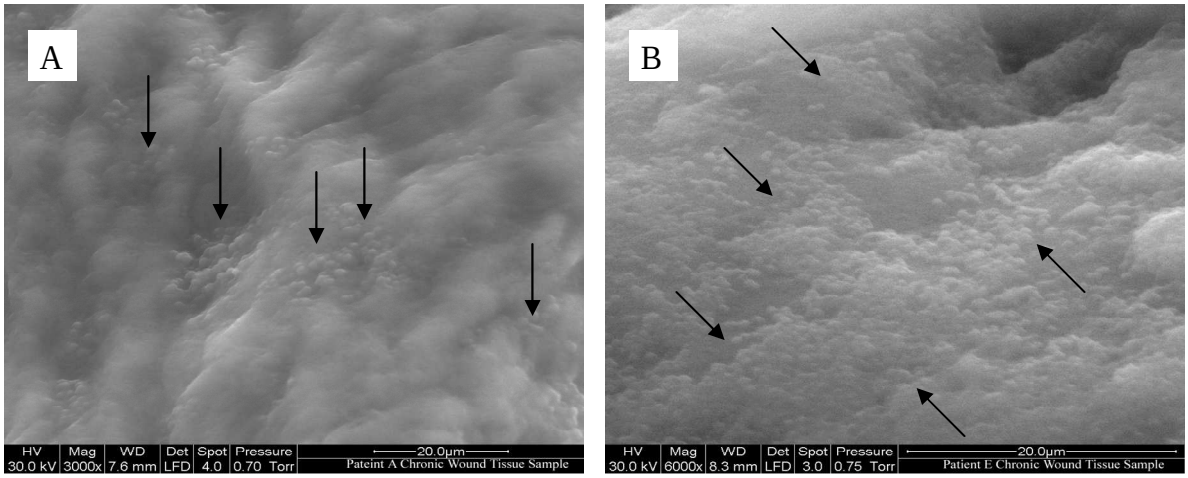


Figure 6.20 ESEM of chronic wound tissue sample from Patient A (A), and Patient E (B). Bacterial microcolonies are indicated by arrows. Microcolonies indicated by arrows

6.5 Discussion

Chronic wounds harbour polymicrobial communities and biofilms which have been implicated by some in the failure chronic wounds to heal (Akiyama *et al.*, 2002; Davis *et al.*, 2008; Serralta *et al.*, 2001). The intrinsic morbidity factors combined with the affiliated economic cost associated with the treatment of chronic wounds have led to wound microbiology being the subject of intense research. Subsequently, several *in vitro* models have been utilised or developed that aim to reproduce the aspects of environment and to support microbial communities within chronic wounds which are arguable deficient in certain respects.

The primary stage in the design of a novel *in vitro* wound biofilm model was the selection of a suitable substratum and media delivery system. Since bacterial communities within the chronic wound derive their nutrients from the surrounding tissues and exudate it was necessary to develop a model which simulates this environment. The selected substratum should readily absorb and evenly distribute media throughout the substratum from a surrounding source. To achieve fluid uptake in this manner, the selected material needed to be highly foamed, cellular solid structure with interconnecting pores since this structure facilitates fluid uptake by capillary action and thus should create an autonomous state of constant hydration throughout the material whilst avoiding over saturation. A material which has these physiochemical properties is fine-celled thermoset phenolic plastic foam (FCF) (Smithers-Oasis, Tyne & Wear. U.K.) shown in Figures 6.12 and 6.15. In addition, the FCF material offers several other beneficial properties such as its structural integrity when saturated, wet heat tolerance (facilitating sterilisation by autoclaving) and malleability, allowing the material to be sculptured to form

different shapes. The aim of this chapter was to develop a novel *in vitro* chronic wound biofilm model that would enable the development of multispecies communities and biofilms of the chronic wound could be studied and in which the efficacy of novel wound dressing could be studied in an environment broadly reflective of the wound environment. Accordingly, once the FCF material was selected as a candidate substratum, the ability of the test bacteria to attach to and proliferate within this substratum was assessed. This was achieved by gathering viable count data of microorganisms grown within FCF material housed within multiple sorbarod devices (MSD). To ensure that the data represented sessile cells rather than planktonic cells which could have been located in the media contained within the pores of the FCF, a process was developed to remove non-adherent cells. The speed and rate of this process wash out step determined based upon a dye replacement assays whereby the period of time taken for FCF material (housed within a MSD and completely saturated with saline) and subsequent perfusate to become completely saturated with a dye (Lutien natural food colour dye Dr Oetker, Leeds, UK) of a known optical density, is the approximate time required to wash out all of the media housed within the pores of the FCF material. A flow rate of *c.* 10 ± 0.1 ml h^{-1} for a period of 30 min was sufficient to achieve this. This relatively fast flow rate was selected to minimise the affect of nutrient limitation on the attached communities when used in the modified MSD assay; a reduced flow rate but extended time period could result in a nutrient limited induced shedding response (Hunt *et al.*, 2004). Utilising the modified MSD housing the FCF and the validated wash out process to remove non-adherent cells, all the bacterial isolates (selected based upon their occurrence on the skin and in wounds) tested were capable of attaching to and proliferating within FCF

substratum in formulated artificial sweat and serum, validating its use as a substratum.

When designing a novel experimental model two factors are important: the type of investigation required and the expected outcome measure. In the context of the current chapter, the aims were to develop a model which would be suitable for the investigation of bacterial interactions and the microbial ecology of biofilms of the chronic wounds; essentially two distinct endpoints. Accordingly, two novel model systems were developed to (i) study cross-sectional population interactions and (ii) to investigate longitudinal population development and biofilm formation. The microbial communities of the chronic wound derive nutrients from the tissue and exudate, therefore a media delivery system should reflect the composition and delivery method within the wound. Data presented in Chapter 5 describes the development and validation of an artificial serum and sweat formula which broadly reproduces nutrient composition extant in wounds and on the healthy skin. Employing the novel artificial media and FCF substratum a 24-well plate based model was devised in which short-term microbial population interactions could be investigated. To ensure steady-states were attained; viable counts of axenic cultures of methicillin resistant *S. aureus*, *P. aeruginosa, S. saprophyticus* and *C. xerosis* maintained in either artificial serum or sweat within the multi-well system was performed every 24 h with fed batch media replaced daily. Organisms were selected based upon their occurrence on the skin and within wounds with and based on their cultivability on selective media to allow for ease of selective isolation during investigations into mixed communities (see Chapter 7). The data presented in Figures 6.5 and 6.6 show that dynamic steady state (defined as equal to or less than a one Log variation in the CFU/cm^3 over a 48 h period) cultures

were attained within 48 h of inoculation and incubation in this model system under the maintained conditions tested. This demonstrates the stability of the system and suggests its suitability for use in short term cross-sectional population interactions studies. The FCF multi-well wound model is an uncomplicated model providing a platform for high throughput short-term wound ecology studies. Despite the benefits of the FCF multi-well wound model one consideration is the degree of labour required; the system calls for removal and replacement of the media (with a pipette) on a daily basis. In addition to the “batch culture” nature of the system, the FCF multi-well wound model can only realistically be used only for short-term studies which was the initial rational for this model. For the development of a continuous model system to perform long-term longitudinal studies on the microbial ecology and biofilms of the chronic wound, the traditional batch culture was rejected due to their labour intensive requirement when changing the media. Subsequently, a modified continuous culture system utilising a dialysis membrane was developed shown in Figure 6.1. The employment of dialysis membrane serves two purposes; primarily it acts as a media diffusion barrier surrounding the FCF substratum supplying a continual nutrient supply to the substratum as its taken up by the capillary action of the FCF, secondly it prevents bacterial contamination of the fluid bulk from the inoculated substratum. This permits numerous experimental studies concerning different bacterial communities, treatments, dressing or biological replicates to be undertaken within one model unit.

The principal aim of this chapter was to develop and validate a novel wound biofilm model. The final stage of validation was the authentication of the MFCF wound biofilm model ability to maintain stable axenic, and mixed microbial

ecosystems of the chronic wound. In this respect dynamic steady state microbial communities were attained from 48 h of inoculation for both axenic and mixed species communities, an observation comparable with other community and biofilm model systems for example a study by McBain *et al*., (2005) reported oral microcosms achieving a dynamic steady state within 24 h of inoculation in the MSD and a study by Thorn and Greenman (2009) reported a quasi steady state achievable within 24-48 h for *P. aeruginosa* and *S. aureus* populations maintained within novel wound models. With respect to wounds infections, a figure of ≥ 10^6 organisms per gram of tissue has been associated with impaired healing (Murphy *et al.*, 1986; Robson, 1979; Robson, 1997), data in Figures 6.7-6.10 show that once a dynamic-steady state has been reached the bacterial isolates all attain reproducible populations densities equal to or greater than 10^6 organisms per cm^3 FCF material which is approximately equal to 10^6CFU/g of FCF. Moreover, chronic wounds often harbour both aerobic and anaerobic populations (Bowler and Davies, 1999; HPA, 2009), in this respect the MFCF model system supports the growth of *B*. *fragilis* (an obligate anaerobe) and *P. aeruginosa* (an obligate aerobe) within the same system set up without altering the gaseous environment of the system to achieve anerobiosis. The ability to produce dynamic steady state mixed communities which are reproducible and representative is an important aim when developing or validating a model to study chronic wound ecologies, facilitating the study of community interactions and novel treatments and regimes. The intrinsic design of the MFCF model facilitates the testing of the efficacy of both dressing via application of novel dressing on the FCF via the open port and antimicrobials via their addition to the media. Another feature of the MFCF wound biofilm model is the malleability of the FCF material into morphologically structures with shapes representing wound-like structures. An ideal dressing will maintain a moist wound

environment, absorbs excess exudate and in deep or slow healing wounds eliminates dead space to prevent premature closure at the epidermal level to minimising abscess formation (Bergstrom *et al.*, 1994; Seaman, 2002). By manipulating the FCF to form different wound shapes, dressings could be tested for their ability to achieve good coverage of a surface in addition to their affect on developed bacterial population. Furthermore, infections of devitalised tissue, deep fascia or bone associated with chronic wounds typically have low oxygen levels facilitating the growth of anaerobic organisms (Edlich *et al.*, 1988; Sapico *et al.*, 1980). The positional growth of *B*. *fragilis* in contoured substrata mimics heterogeneity with respect to the location within the modelled wound bed. The manipulation capacity of the FCF material offers a distinct novel advantage over other substrata such as cellulose fibres by simulating the physical wound setting such as puncture wounds, pressure sores or open wounds and the subsequent positional bacterial growth within. This is according to published literature a unique feature of the MFCF wound biofilm model. The FCF substratum utilised in the MFCF wound biofilm model is open pore fine-celled material, providing a matrix of thermoset phenolic plastic foam structures and media filled pores providing numerous solid-liquid interfaces facilitating biofilm formation of planktonic cells. Microscopic observations of sectioned FCF substrata following 4 d continuous perfusion after inoculation with *S. aureus* and *P. aeruginosa* shown in Figure 6.13 revealed the presence of surface associated bacteria growing as microcolonies and as aggregates which were comparatively similar to the microcolonies observed tissue samples of chronic wounds show in Figure 6.14. Moreover, ESEM imaging of both chronic wound tissue and 4 d MFCF wound biofilm model cultures indicated the presence of microcolonies of bacteria together with putative extracellular polymeric substances in the form of a highly hydrated organic films

coating the FCF surfaces and tissues (Figures 6.16 - 6.19). This demonstrates the MFCF wound biofilm model can not only support bacterial communities associated with the chronic wound but is strongly suggestive of the fact that it can foster and support biofilm development comparable to those observed in chronic wounds.

6.6 Conclusion

Based upon the defined outcome parameters the novel wound biofilm models had to (i) facilitate the attachment and proliferation of single and multispecies consortia associated with chronic wounds, (ii) support growth and development of wound-associated biofilms, (iii) simulate the chronic wound environment (iv) and facilitate the assessment of antimicrobial therapies and dressings. The results present in this chapter indicate that the FCF multi-well wound model and the MFCF wound biofilm model fulfil these criteria by the careful and considered selection and validation of substrata, media and overall model system. This step-wise considered approach validated each individual element and their combination to produce novel wound models which be applied to study (i) cross-sectional population interactions and (ii) longitudinal population development and biofilm formation facilitating the investigation efficacy of novel dressings and topical treatment

Chapter 7

Bacterial interactions during biofilm community development: mutualism, antagonism and colonisation resistance

7.1 Abstract

Synergistic and antagonistic bacterial interactions have been reported for both skin commensal organisms and those associated with chronic wounds. Synergistic interactions may aid the establishment of mixed communities, whilst antagonistic interactions may contribute to the protective function of the skin through colonisation resistance. This chapter elucidated positive and negative interactions between 30 bacteria isolates associated with healthy skin and chronic wounds, and investigated the affect of established populations of *Staphylococcus saprophyticus* and *Corynebacterium xerosis* on the colonisation efficiency of Methicillin resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* in environments broadly reflective of healthy skin and of wounds. Pair-wise interactions resulting in positive, negative and neutral bacterial productivity effects of commonly isolated wound (n=19) and skin (n=11) were determined using a modified cross-streak method and a spot-on-lawn assay. The affect of established skin populations on the attachment and proliferation of exogenous bacteria was assessed using a fine-celled foam (FCF) multi-well model with pre-established populations of axenic *S. saprophyticus* and *C. xerosis* exposed to MRSA or *P. aeruginosa* in artificial sweat or artificial serum. Inhibition assays revealed limited activity however; two wound isolates of *Enterococcus faecalis* showed inhibition activity against two distinct wound isolates of *E. faecalis* and the skin isolates *Propionibacterium acnes* and *Propionibacterium avidum*. Using the FCF wound model the effect of prior colonisation of a substratum with skin associated bacteria, upon colonisation efficiency of exogenous pathogenic bacteria. When grown in artificial sweat, prior colonisation with *S. saprophyticus* and *C.* xerosis resulted in significant reductions of MRSA (99% and 93% respectively) and *P. aeruginosa* (75% and 91% respectively). However, when the media was switched to artificial serum, MRSA or *P. aeruginosa* populations were not significantly reduced by the established populations of S. *saprophyticus* and *C. xerosis*. The spot-on-lawn and cross-streak growth interactions results indicated that mutualistic and antagonistic interactions maybe uncommon and thus not necessary successful attachment and proliferation of the bacterial isolates investigated. Data from the FCF multi-well wound model showed that colonisation resistance could be simulated in model and the outcome of immigration was markedly influenced by (i) the species of established bacterium and (ii) nutrient availability.

7.2 Introduction

Colonisation of the skin occurs almost immediately after birth via contact transfer with microbial reservoirs such as the birth canal, clothing, skin and the local environment (Savey *et al.*, 1992). The population which establishes itself is subsequently varied due to the types of organisms transferred and their preferred anatomical location (ecological niches). Throughout the life of the hominine host, the cutaneous bacterial organisms which are in close associations with the hosts' epidermis are in a constant state of ecological balance in which the genetic, species and ecosystem diversity remain relatively stable (Fredricks, 2001). This dynamic equilibrium is subject to gradual shifts relative to changes within the host e.g. health status, age and the local environment (Chiller *et al.*, 2001; Tlaskalová-Hogenová *et al.*, 2004). The epidermis contains various ecological niches such as the moist environment of the toes webs and axilla to the arid areas of the forearms, upon which a range of autochthonous bacterial communities proliferate. These organisms are thought to provide a barrier against colonisation by exogenous pathogenic microorganism by a mechanism termed "colonisation resistance" (Bourlioux, 1997; Brodell and Rosenthal, 2008). This is purportedly achieved by competition for limited attachment sites and nutrients reducing the attachment and proliferation opportunities of exogenous bacteria (Bourlioux, 1997; Reid *et al.*, 2010). This resistance mechanism is further supported by the common epidermal processes such as the secretion of sweat containing antimicrobial lipids and constant desquamation which continually eliminates skin cells and thus attached bacteria (Chiller *et al.*, 2001; Proksch *et al.*, 2006). Within this bacterial ecosystem are a variety of complex bacterial interactions which include antagonistic and synergistic relationships. Commonly isolated members of the skin

microbiota such as *Staphylococcus* spp. and *Propionibacteria* spp. metabolise the lipid content of sweat to provide a carbon and nitrogen source for growth. This processes can produce long chain fatty acids as a by-product which in turn can be utilised by other organisms such as *Corynebacterium* spp. (James *et al.*, 2004). Additionally, certain bacterial skin commensals have also been shown to produce toxic metabolites and bacteriocins (Chiller *et al.*, 2001). Bacteriocins are ribosomally synthesized peptides which are generally highly cationic, heat-stable and are primarily active against taxonomically related bacteria (McAuliffe *et al.*, 2001; Varella Coelho *et al.*, 2007). These growth related interplays are not unique to the skin commensal organisms; numerous bacteria including those associated with wound infections such as various streptococci and staphylococci have been shown to produce bacteriocins and/or bacteriocin like inhibitory substances (BLIS) such as lantibiotics and epidermin (Varella Coelho *et al.*, 2007). It has been reported that bacterial synergistic relationships can also occur within wound infections between anaerobic bacteria and aerobic/facultative organisms. Kelly (1980) investigated the pathogenic synergy between *Escherichia coli* and *Bacteroides fragilis* using a guinea pig experimental wound model. When introduced axenically infective dose inocula of *E. coli* and *B. fragilis* failed to produce an inflammatory response when administered individually to a wound, however, when combined to produce the same number of bacteria as the individual inocula a marked increase in inflammation, pus formation and delayed healing was noted (Kelly, 1980). One possible mechanism for this phenomenon is the ability of the aerobic or facultative organisms to lower the oxidation-reduction potentials in the host tissue, facilitating the growth of anaerobic species, increasing tissue damage and allowing the further proliferation of the aerobic or facultative organisms (Brook, 1985).

Changes in host health status and/or a compromised epidermal barrier may cause alterations in the composition of the microbiota with potential clinical implications including the development of folliculitis, acne vulgaris and perhaps more significantly, soft tissue infections. Upon the development of a soft tissue injury primary bacterial colonisers are frequently members of the endogenous skin microflora (Bowler *et al.*, 2001) however, the host is at greater risk of developing exogenous pathogenic infection due to the collapse of the epidermal barrier. In chronic wounds this risk is greatly increased due to the delayed healing times and continued exposure of tissues to autochthonous and endogenous bacteria. A greater understanding of the bacterial interactions between common skin and wound bacteria with specific reference to antagonistic and mutualistic relationships, and colonisation resistance in the environment of the skin and wound may (i) improve understanding of basic mechanisms involved in colonisation and infection and (ii) lead to potential prophylactic treatments and methodologies to prevent infection. This present chapter therefore aims to test for both inhibitory and synergistic relationships between skin and wound-associated bacteria and investigate the role of nutrient availability broadly reflective of the wound and skin in colonisation utilising a novel wound simulator.

7.3 Materials and methods

Bacterial isolates used in this chapter are shown in Table 7.1. All non-fastidious and anaerobic organisms were routinely maintained on Wilkins Chalgren agar. Anaerobic organisms were cultured in a Mark 3 Anaerobic Work station (Don Whitely Scientific, Shipley, U.K.) at 37℃ (Gas mix: 80% N_2, 10% CO_2 and 10% H_2)

7.3.1 Cross-streak bacterial growth interaction assays

Pair-wise combinations of 31 skin and wound-associated bacterial isolates (multiple isolates were used where possible) and two putative probiotic lactobacilli strains show in Table 7.1, were assessed for direct/simultaneous antagonistic and mutualism activity using a modified cross streak assay, previously described by Sciverner *et al*., (1950) and Krausse *et al*., (2005). Briefly, stationary phase cultures of bacterial isolates grown overnight at 37℃ in 10 ml of Wilkins Chagrin broth in 25 ml volume universal containers in a aerobic or anaerobic atmosphere for respective aerobic and obligate anaerobic isolates (Krausse, 2005; Scrivener *et al.*, 1950). Inocula were adjusted *c.* 7.0 Log_{10} CFU/ml were dispensed using a sterile pipette in *c.* 3 cm vertical streaks on Wilkins Chalgren agar, once dried *c.* 3 cm horizontal streaks of bacterial inocula are dispensed across the vertical streak to form a cross see Figure 7.1. Agar plates were incubated for 48 h in either an aerobic or anaerobic atmosphere (for obligate aerobes and anaerobes respectively) and visually assessed for zones of inhibition or enhancement of growth between the two strains. All pair-wise combinations were undertaken in quadruplicate; the streak application order was undertaken in duplicate e.g. organism A dispensed over organism B in duplicate and then the streak application order was reversed and undertaken in duplicate e.g. organism B dispensed over organism A.

Table 7.1 Bacterial strains used in this chapter

Bacterial Species	Strain number	Origin
Acinetobacter baumannii		Diabetic Ulcer/ConvaTec ltd*
Bacteroides fragilis	NCTC 9343	National Typed Culture Collection
Clostridium perfringens		Diabetic Ulcer/ConvaTec ltd*
Corynebacterium xerosis		Diabetic Ulcer/ConvaTec ltd*
Enterobacter cloacae	1, 43 and 50	Diabetic Ulcer/ConvaTec ltd*
Enterococcus faecalis	15, 21, 26 and Tma5	Diabetic Ulcer/ConvaTec ltd*
Escherichia coli		Diabetic Ulcer/ConvaTec ltd*
Micrococcus luteus		Isolated from healthy skin*
Propionibacterium acnes		Isolated from healthy skin*
Propionibacterium avidum		Isolated from healthy skin*
Proteus mirabilis	2, 5 and 40	Diabetic Ulcer/ConvaTec ltd*
Pseudomonas aeruginosa	12, 24 and 38	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus aureus	9, 10 and 11	Diabetic Ulcer/ConvaTec ltd*
Staphylococcus capitis		Isolated from healthy skin*
Staphylococcus epidermidis	ATCC 14990	American Typed Culture Collection
Staphylococcus haemolyticus		Isolated from healthy skin*
Staphylococcus hominis		Isolated from healthy skin*
Staphylococcus saccharolyticus		Isolated from healthy skin*
Staphylococcus saprophyticus	NCTC 7292	National Typed Culture Collection
Methicillin Resistant Staphylococcus aureus	NCTC 11939	National Typed Culture Collection
Lactobacillus paracasei	AH104	ConvaTec ltd*
Lactobacillus salivarius	UCC118	ConvaTec ltd*

* identified by 16S rRNA gene sequencing

7.3.2 Spot-on-lawn bacterial growth inhibition assay

To test for deferred inhibition activity i.e. expression of inhibitors into the environment without direct contact between bacterial isolates a modified spot-on-lawn antagonism assay previously described by Knoll *et al*., (2008) was utilised to investigate deferred antagonistic activity between all pair-wise combinations of 31 skin and wound bacterial isolates and 2 putative probiotic lactobacilli strains (Knoll *et al.*, 2008). Briefly, stationary phase cultures of bacterial isolates grown overnight at 37℃ in 10 ml of Wilkins Chagrin broth in 25 ml volume universal containers in a aerobic or anaerobic atmosphere for respective aerobic and obligate anaerobic isolates. Inocula of test “antagonist” strains were “spotted” (0.3 µl) onto Wilkins

Chalgren agar plates with a sterile pipette, once dried 100 µl of liquid agar was dispensed with a sterile pipette over each bacterial spot to seal each bacterial spot in sterile agar. Once dried a layer of liquid agar seeded with a stationary phase culture test strain (1:100) was poured over the “spotted” agar to a depth of 5 mm. Agar plates are incubated for 48 h and visually assessed for zones of inhibition on the seeded layer.

7.3.3 Investigations of colonisation resistance of skin-associated bacteria populations under conditions which broadly reflect of healthy skin

To investigate the role of nutrient availability in colonisation resistance by *C. xerosis* and *S. saprophyticus* against the pathogenic organisms MRSA and *P. aeruginosa* in conditions broadly reflective of healthy skin environment a fine-celled foam (FCF) multi-well model previously developed and validated in Chapter 6 was utilised. Briefly the FCF substratum was adapted to give a column size of 1 cm x 1 cm, autoclaved, placed within the wells of a 24 well cell culture plate (Sigma Poole, Dorset, UK) and preconditioned with artificial sweat for >4 h. Stationary phase cultures of *S. saprophyticus* and *C. xerosis* (chosen on the basis of their occurrence on the skin and intrinsic antibiograms for the use of selective agars-see section 2.2.1) grown overnight in artificial sweat were adjusted to a *c.* 7.0 Log_{10} CFU/ml. The FCF substrata were inoculated with 1 ml of the adjusted bacterial cultures and incubated with lids aerobically at 37℃ for 48 h. Cultures were fed in batch conditions with spent media manually removed and replace (0.5 ml) with a sterile pipette every 24 h. Test FCF substrata were then removed, exposed to 1 ml of *c.* 7.0 Log_{10} CFU/ml of MRSA or *P. aeruginosa* for 20 min, control FCF substrata were removed and exposed to 1 ml of fresh sterile media for 20 min. All FCF substrata were then washed in sterile PBS to remove non-

adherent cells and returned to their original microtitre well containing fresh media. Plates were incubated for a further 24 h and viable counts performed on the substrata. Statistical significance was determined using independent t-test and Mann-Whitney test to determine significant difference.

7.3.4 Investigations of colonisation resistance of skin-associated bacteria populations under simulated wounding conditions

To investigate the role of colonisation flora under simulated wounding conditions the FCF multi-well method previously described was modified to include an exchange to artificial serum media (from an artificial sweat media) during the exposure of the pre-colonised FCF to MRSA or *P. aeruginosa.* The FCF substrata were inoculated with 1 ml of *c.* 7.0 Log_{10} CFU/ml of *S. saprophyticus* or *C. xerosis* incubated with lids aerobically at 37℃ for 48 h. C ultures were fed in batch conditions with spent media manually removed and replace (0.5 ml) with a sterile pipette every 24 h. Test FCF substrata were then removed, exposed to 1 ml of *c.* 7.0 Log_{10} CFU/ml of MRSA or *P. aeruginosa* (initially grown in artificial sweat and adjusted in artificial serum) for 20 min in artificial serum, control FCF substrata were removed and exposed to sterile artificial serum for 20 min. All FCF substrata were then washed in sterile PBS to remove non-adherent cells and returned to their original microtitre well containing sterile artificial serum. Plates were incubated for a further 24 h and viable counts performed on the substrata. Statistical significance was determined using independent t-test and Mann-Whitney test to determine significant difference.

7.3.5 Investigations of colonisation resistance of skin-associated bacteria populations under simulated chronic wound conditions

To investigate the role of colonisation skin flora under simulated chronic wound conditions on exogenous populations of MRSA and *P.* aeruginosa the FCF multi-well method previously was modified to include artificial serum as the batch medium used throughout the duration of the experiment. Statistical significance was determined using independent t-test and Mann-Whitney test to determine significant difference.

7.3.6 *In vitro* evaluation the potential efficacy of probiotic impregnated dressings

The FCF microtitre plate method was employed to investigate the postulated efficacy of lactobacilli-impregnated dressing on the attachment and proliferation of MRSA and *P. aeruginosa* in conditions broadly reflective of a chronic wound. Briefly, artificial serum pre-conditioned FCF substrata was inoculated with 1 ml of *c.* 7.0 Log_{10} CFU/ml of MRSA or *P. aeruginosa* and incubated in a microaerophilic 5% CO_2 atmosphere at 37℃ for 48 h. Cultures were fed in b atch conditions with spent media manually removed and replace (0.5 ml) with a sterile pipette every 24 h. After 48 h incubation, axenic stationary phase cultures of *Lactobacillus salivarius* and *Lactobacillus paracasei* grown in artificial serum in 5% CO_2 atmosphere were adjusted to *c.* 8.5 Log_{10} CFU/ml. Aquacel (ConvaTec Ltd UK) dressing measuring 1 cm^2 were submerged in the axenic *Lactobacilli spp.* inocula, removed (allowing excess inoculum to drain) and placed on top on the FCF foam. Microtitre plates containing the probiotic dosed dressings and colonised FCF were

further incubated in 5% CO_2 atmosphere at 37℃ for 48 h with media a change every 24 h. FCF substrata were then removed and viable counts performed. Experimental controls consisted of un-colonised FCF substrata exposed to probiotic dosed dressing and colonised FCF substrata exposed to sterile dressings.

7.4 Results

7.4.1 Cross-streak and spot-on-lawn assay

A total of 537 interactions per assay were undertaken with inhibition activity identified by a visual assessment of zones of inhibition displayed around the cross streaks or the seeded layer of bacteria and promotion of growth identified by enhanced colony size at the intersection of the cross streak. A putative promotion of growth was observed for some of the pair-wise combinations however, upon repeat testing and reversal of the isolate application order this observation was not reproduced and was therefore categorised as a pseudo-promotion of growth due to variations in surface tension and moisture resulting in one isolate streak protruding into the second streak at the intersection (shown in Figure 7.1). Once this artefact had been accounted for no promotion of growth was observed between any of the strains tested.

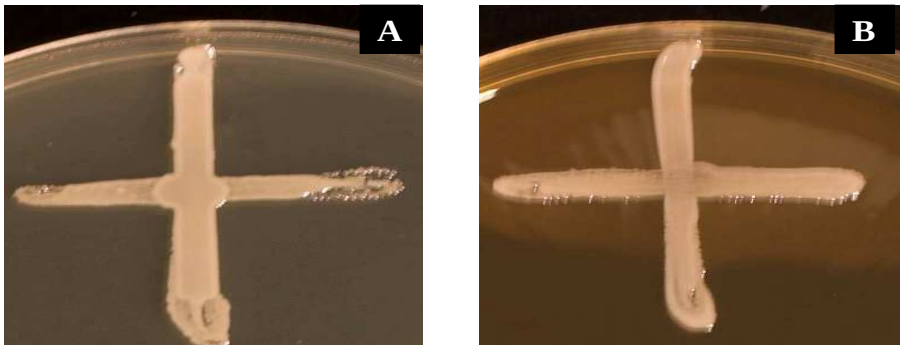


Figure 7.1 (A). Pseudo promotion of growth *S. epidermidis* by *S. capitis*. (B). Repeat cross streak of *S. epidermidis* by *S. capitis* showing no activity.

No inhibition activity was identified between the skin-associated bacterial isolates. However, inhibition activity was noted for isolates associated with wounds; *E. faecalis* (isolate number 15) inhibited the growth of *E. faecalis* (isolates number 21), *P. acnes* and *P. avidum*. *E. faecalis* (isolate number 21) inhibited the growth of *E. faecalis* (isolates number 26) and *E. faecalis* Tma5 shown in Figures 7.2 and 7.3 The putative probiotic strains *Lactobacillus salivarius* UCC118 and *Lactobacillus paracasei* AH104 failed to produce inhibition activity against skin and wound bacterial isolates.

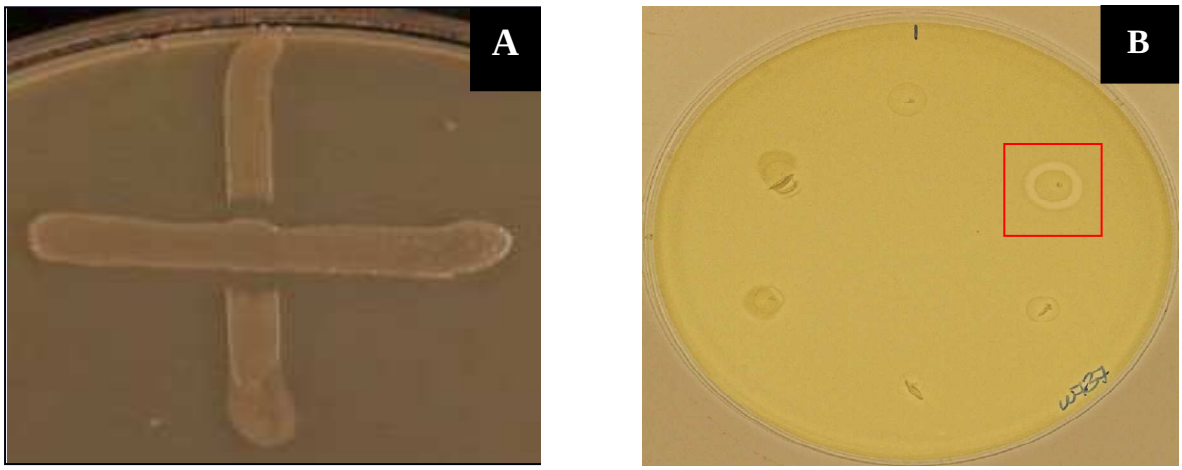


Figure 7.2 (A) Cross streak inhibition of *P. acnes* (vertical streak) by *E. faecalis* (horizontal streak). (B) spot-on-lawn inhibition of *P. acnes* by *E. faecalis*; seeded layer of *P. acnes* displays a zone clearing around *E. faecalis* spot indicated by red square.

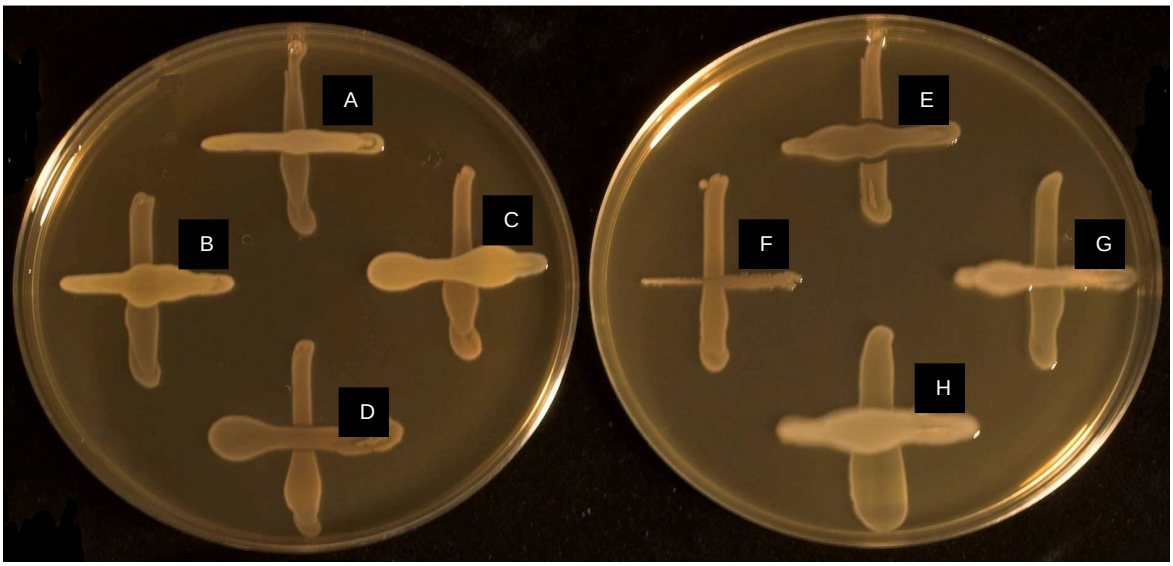


Figure 7.3 Cross streak assay showing inhibitory and non-inhibitory interactions. Vertical streaks; Tma5. Horizontal streaks; (A) 9. *S. aureus*. (B) 10. *S. aureus*. (C) 11 *S. aureus*. (D) 15. *E. faecalis*. (E) 21. *E. faecalis*. (G) 1. *E. cloacae*. (H) 43. *E. cloacae*. Inhibitory activity can be seen in cross streak E.

7.4.2 Investigations of colonisation resistance of skin-associated populations under conditions which broadly reflect of healthy skin

When maintained in artificial sweat and compared to non-colonised controls; prior colonisation with *S. saprophyticus* resulted in significant reductions in MRSA (99%) and *P. aeruginosa* (75%). Prior colonisation by *C. xerosis* resulted in a significant reduction of 93% in MRSA and 91% in *P. aeruginosa* ($p<0.05$). Significant reductions were also observed in the pre-colonised organisms; *C. xerosis* was significantly by the exposure to both MRSA and *P. aeruginosa* however, *S. saprophyticus* was only significantly reduced affected by *P. aeruginosa* shown in Figure 7.4

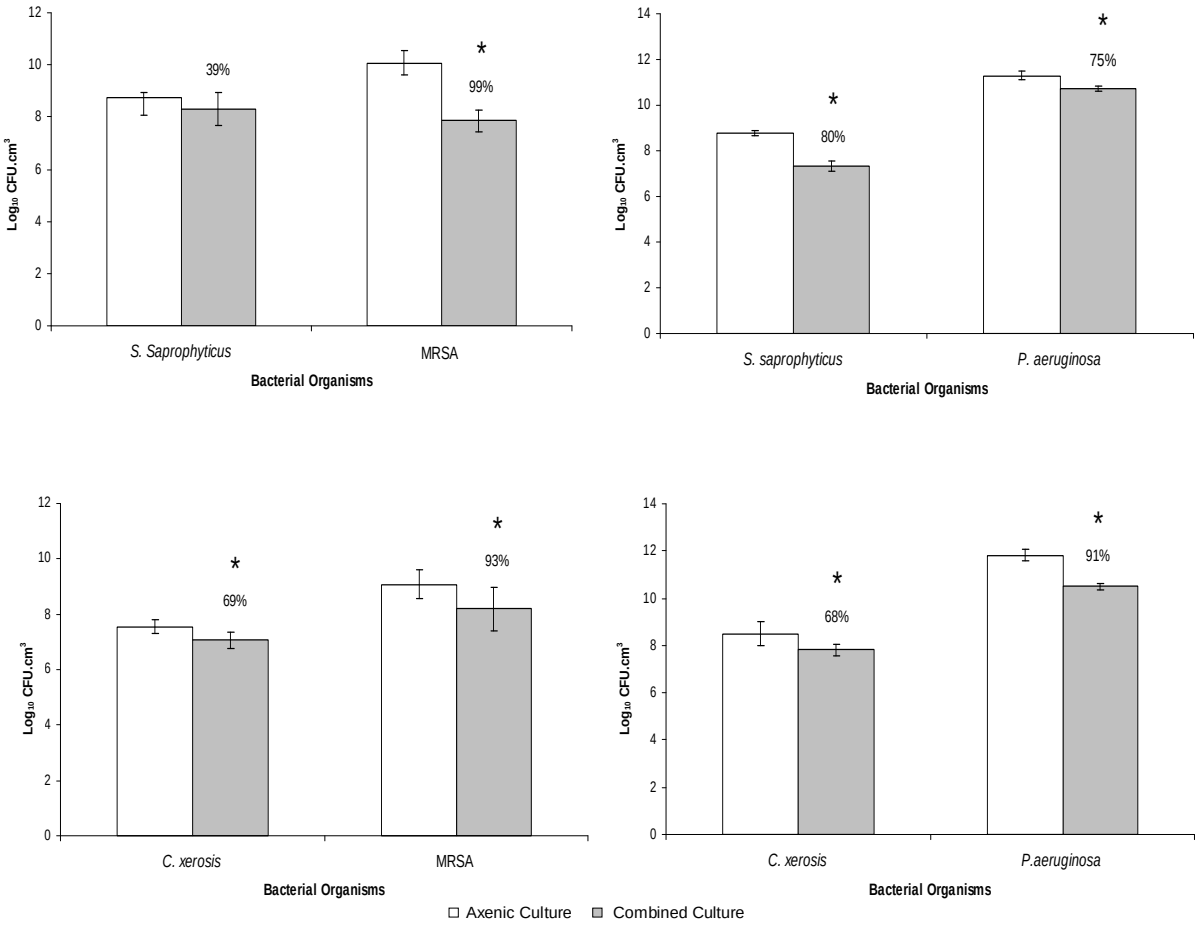


Figure 7.4 Viable counts of axenic and combined cultures of pre-established communities of *S. saprophyticus* and *C. xerosis*. when exposed to transient pathogenic bacteria; MRSA and *P. aeruginosa* in artificial sweat. * Significant reductions in viable counts ($P<0.05$).

7.4.3 Investigations of colonisation resistance of skin-associated populations under simulated wounding conditions

Significant reduction were observed in the pre-established bacterial communities of *S. saprophyticus* (88%) and *C xerosis* (88%) when exposed to MSRA and in *S. saprophyticus* (92%) when exposed to *P. aeruginosa* during a shift from artificial sweat to artificial serum shown in Figure 7.5. No significant reductions were observed for MRSA or *P. aeruginosa* (P>0.05).

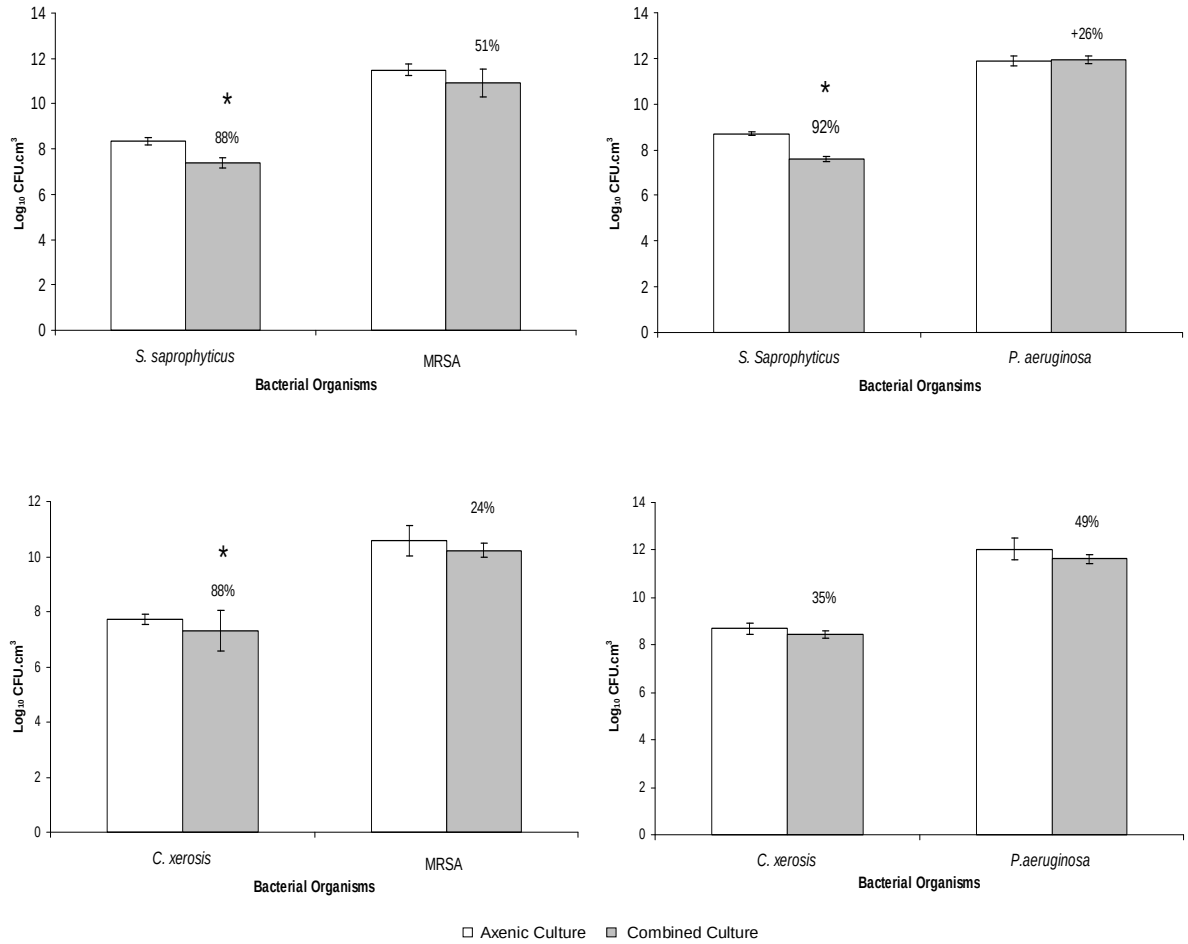


Figure 7.5. Viable counts of axenic and combined cultures of pre-established communities of *S. saprophyticus* and *C. xerosis* when exposed to transient pathogenic bacteria; MRSA and *P. aeruginosa* in the nutrient rich environment media of artificial serum at the point of exposure. * Significant reductions in viable counts (P<0.05).

7.4.4 Investigations of colonisation resistance of skin-associated populations under simulated chronic wound conditions.

When maintained in artificial serum and compared to non-colonised controls; prior colonisation with *S. saprophyticus* and *C. xerosis* resulted in significant reductions in MRSA (99%) and *P. aeruginosa* (87%) respectively. Conversely significant reductions were observed in the pre-colonised organisms; *C. xerosis* and *S. saprophyticus* was by exposure to both MRSA and *P. aeruginosa* shown in Figure 7.6

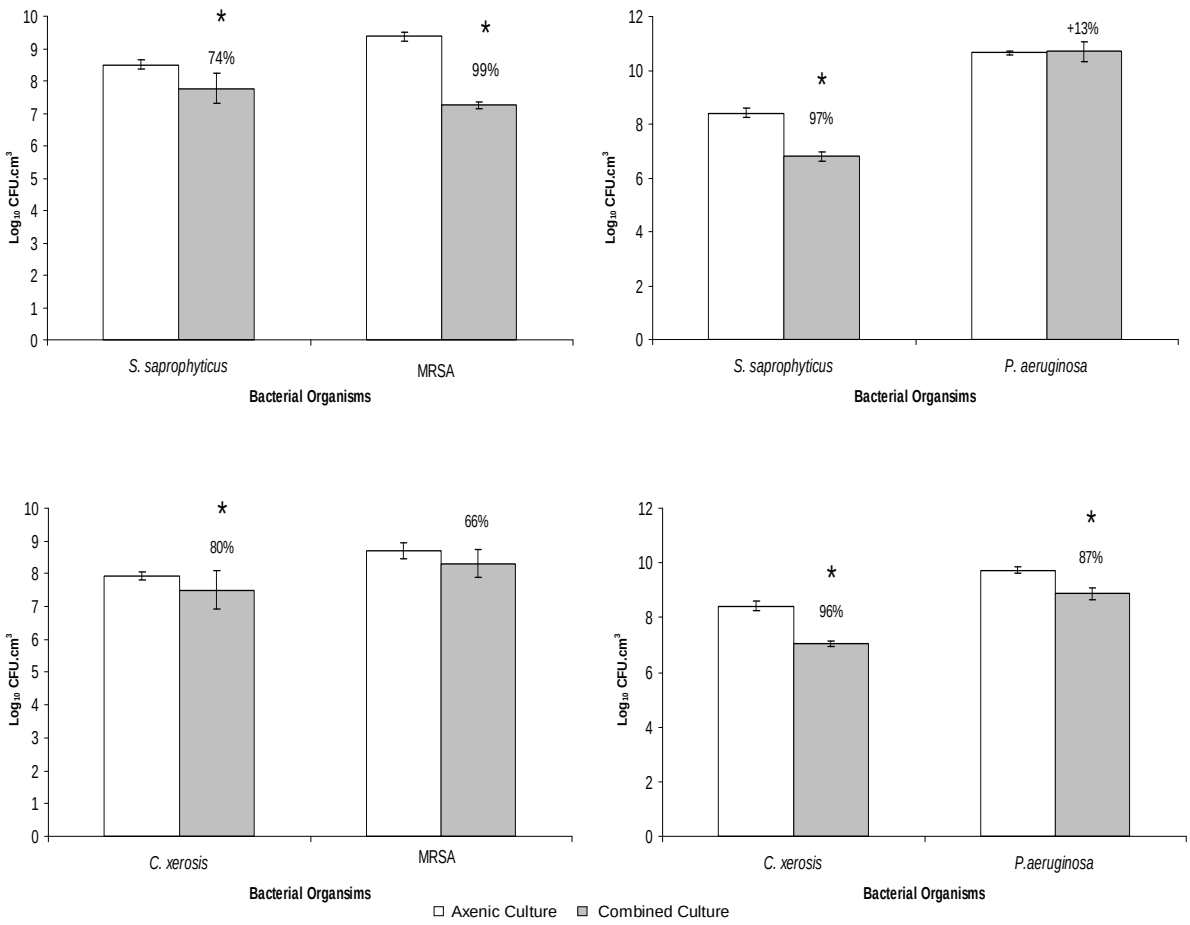


Figure 7.6. Viable counts of axenic and combined cultures of pre-established communities of *S. saprophyticus* and *C. xerosis* when exposed to transient pathogenic bacteria; MRSA and *P. aeruginosa* in artificial serum. * Significant reductions in viable counts ($P<0.05$).

7.4.5 *In vitro* evaluation the potential efficacy of probiotic Impregnated Dressings

L. salivarius and *L. paracasei* impregnated Aquacel (ConvaTec, UK.) dressings were capable of establishing a population of *lactobacilli* spp. in both the axenic and pre-colonised FCF substratum. Reductions were observed for both pre-established cultures of the *S. aureus* (63%) and *P. aeruginosa* (45%) in response to *L. salivarius,* and in *S. aureus* (29%) in response to *L. paracasei* however these reductions were not significant ($p>0.05$) shown in Figure 7.7 and Figure 7.8

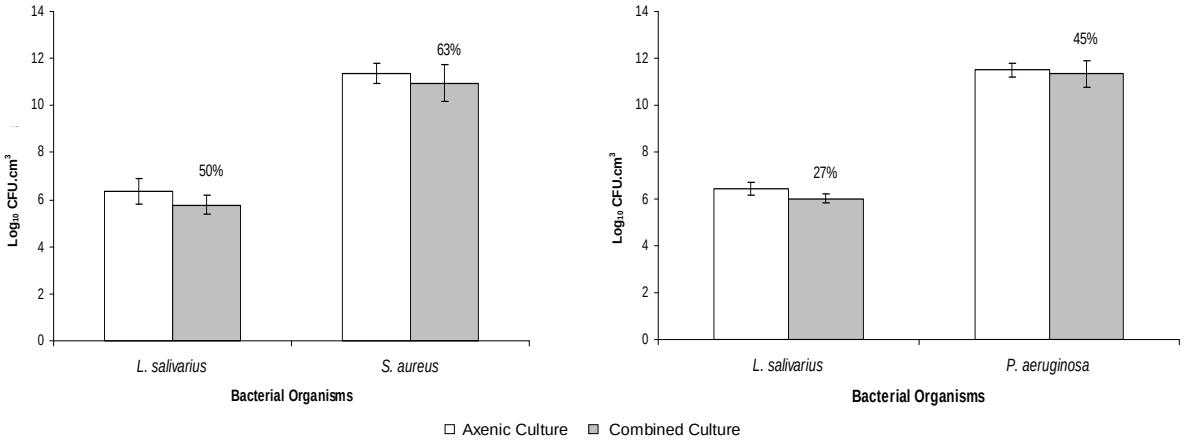


Figure 7.7. Viable counts of axenic and combined cultures of pre-established communities of MRSA and *P. aeruginosa* when exposed to *L. salivarius* impregnated dressing

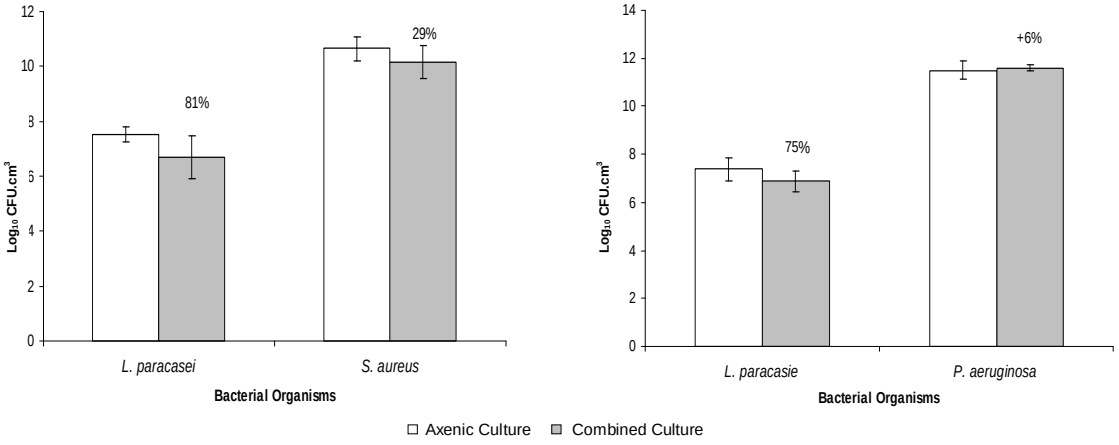


Figure 7.8. Viable counts of axenic and combined cultures of pre-established communities of MRSA and *P. aeruginosa* when exposed to *L. paracasei* impregnated dressing

7.5 Discussion

The diverse microbial ecosystem which colonise the surface of the skin is important in the maintenance of the physical condition of the skin (Larson *et al.*, 1998). Reciprocally the physical condition of the skin is important in the preservation of the skin bacterial flora (Grice *et al.*, 2010). It is purported that the presence of commensal skin flora aids in the prevention of attachment and proliferation of transient pathogenic bacteria via competition for attachment sites, nutrients and the production of toxic metabolites and bacteriocins (McAuliffe *et al.*, 2001; Varella Coelho *et al.*, 2007). This ecological balance can however, be subject changes caused by endogenous or exogenous factors such as underlying aetiologies such as diabetes, and skin breakages leading to the development of a chronic wound. The primary colonisers of the resultant wound are the autochthonous bacteria however, delayed closure of the wound provides an opportunity for exogenous pathogenic bacteria to attach and proliferate leading to biological shifts in the autochthonous population. This results in an excessive growth of specific bacterial species which can overwhelm the skin barrier and immune defences leading to an infected state. Investigations into potential synergistic bacterial interactions within the microbiota on healthy skin and in infected wounds, may identify key members of the community which act as “collaborators” influencing the stability and composition of the respective microbiotas. These organisms may aid in the proliferation of pathogenic bacteria in disease or the maintenance of a healthy skin bacterial consortium and thus may be novel targets organisms for prophylactic antimicrobial therapy or organisms which could aid in the maintenance of a healthy consortium. Additionally, identification antagonistic interactions may identify potentially novel bacteriocins

and organisms which prevent the proliferation of pathogenic bacteria. Utilising a variety of skin and wound-associated bacterial isolates, the aims of this chapter were to identify potential synergistic and antagonistic bacterial interactions amongst the healthy skin and wound flora. This was achieved using the previously validated spot-on-lawn assays and cross-streak methods growth interactions assays. The spot-on-lawn assay identifies deferred antagonistic activity i.e. the production and secretion of bacteriocins into environment without cues derived from contact with exogenous bacteria, whereas the cross-streak assay identifies direct, simultaneous antagonism i.e. the production of bacteriocins in response to the presence of another organism. Furthermore, these two methods ensure that that bacterial growth is confined within distinct colonies (streak or spot) ensuring the resultant cell density is high, a requirement for the majority of bacteriocin production (Drider *et al.*, 2006). Combinations of 31 skin and wound-associated bacteria resulting in 537 interactions for each assay (excluding replicates) were investigated. Extant antagonistic activity was noted for two distinct wound isolates of *E. faecalis* which displayed direct and deferred antagonistic activity towards other independent strains of *E. faecalis* and *P. acnes* and *P. avidum*. Because antagonism was noted in both assays it can be inferred that the active compounds are secreted into the media suggesting deferred antagonism. Previous work has identified numerous bacteriocins derived from *E. faecalis* and two of the best characterised ones are cytolysin (Class I bacteriocins) and enterocins (Class II bacteriocins) (Nes *et al.*, 2007). The latter of these has been shown to be primarily active against *Listeria spp.* and other enterococci although activity in other species including *P. acnes* has also been reported (Kang *et al.*, 2009). As the antagonistic activity identified in this current chapter was observed against distinct strains of *E.* faecalis and *Propionibacterium* spp. it is likely that the bacteriocins produced falls

into the Class II bacteriocin category. Further work involving purification from culture supernatants and identification by mass spectroscopy (Guyonnet *et al.*, 2000; Zendo *et al.*, 2005) for example, would be required to fully identify this bacteriocin however, due the limited inhibitory activity identified in this chapter the utility of the bacteriocin is limited and thus further work was not undertaken. The cross-streak assay used in this chapter is a simple, robust method to investigate antagonism however, to date it has not been reported as a method to investigate mutualistic activity. Despite a substantial degree of investigations with a large variety of bacterial isolates and replicates during this current study, promotion of growth was not observed between any of the wound and skin bacterial isolates. Since the cross-streak assay is a novel method to investigate mutualism questions may be raised as to the validity of the assay to investigate this phenomenon. Previous work has however, utilised a comparable cross-streak method to investigate enhanced growth. Svendsen *et al*., (1947) utilised a similar cross steak method to investigate an atypical *Corynebacterium pseudodiphtheriticum* (also known as *Corynebacterium hofmannii*) which displayed colony growth only around colonies *S. aureus* (Svendsen *et al.*, 1947). This pattern of colony growth is termed “satellite phenomenon” and is often associated with the growth of *Haemophilus* spp. in satellite colonies around colonies of *S. aureus* grown on blood agar plates. *S. aureus* releases both X and V factors (haemin and NAD/NADP respectively) from the blood contained within the agar which are required for growth of *Haemophilus* spp. on blood agar. Although the basis of the satellite phenomenon is the release of growth factors from the media/environment by organism “A” which are required by organism “B” to grow on the specific medium, the fundamental principal of observing enhanced growth of one organism by another remains. This in turn demonstrates that a visual observation of

enhancement of growth on agar can be achieved. It could be argued that a more appropriate measure would be to perform viable counts on axenic and combination broth cultures. This is not however, well suited for high-through put analysis such as required by the number of isolates tested in this chapter. The cross-streak method provides this provision and more as it tests interactions between bacterial isolates gained through direct contact with each other, an occurrence which would be likely to be diluted in broth culture. This ensures that any visual growth interactions are due bacterial interactions with each other and extraneous bacteria. These elements validate the use of the cross streak method to assess bacterial mutualism and synergy. It should also be noted that all the bacterial strains tested are know to readily colonise to the skin and wounds independently and in mixed communities. This combined with the results of the growth interaction assays, indicates that the presence of bacterial collaborators for growth is not essential for their proliferation and production of an infective state which is verified by the results presented herein. Whilst pair-wise interactions studies conducted within this chapter suggests that bacterial organisms investigated have little or no antagonistic and synergistic relationships, it is also postulated that bacterial growth interactions may also occur in the form of colonisation resistance. This phenomenon not only includes the production of inhibitory metabolites and bacteriocins to prevent exogenous bacterial growth but it also encompasses competition for attachment sites and nutrients. Therefore the secondary aim of work described within this chapter was to investigate role of pre-established endogenous microbiota of the skin on the ability of exogenous bacteria associated with wound infections, to compete with and colonise the same surface under condition which broadly reflect the nutrient availability of the skin and wound. Organisms selected were; *S. saprophyticus*, *C. xerosis,* MRSA and *P.*

aeruginosa, selected based upon their common isolation from the epidermis and wounds (respectively) and their intrinsic antibiogram and colony morphology on selective agar plates, which allows for selective growth of individual organisms from mixed these communities (see Chapter 2). Utilising the fine celled foamed (FCF) multi-well wound model the affect of pre-established populations of *S. saprophyticus* and *C. xerosis* on the attachment and proliferation of transient pathogenic bacteria MRSA and *P. aeruginosa* in nutrient environments likened to healthy skin, epidermal injury (wounding) and a chronic wound was investigated. Within a nutrient minimum media of artificial sweat (which is broadly reflective of a healthy skin environment), significant reductions were observed for all exogenous bacteria as a result of pre-established populations of skin associated bacteria demonstrating colonisation resistance. This result is consistent with previous *in vivo* work investigating the *S. aureus* pathogenesis on skin lesion infections (Singh *et al.*, 1971). Investigations into *S. aureus* skin infections by Singh *et al*., (1971) noted that to induce skin lesion infection by *S. aureus* when applied directly to healthy the skin (at an inoculum density of 3.7 $x10^4$ CFU/cm^2), it was essential to eliminated competing colonising bacteria by the addition of 70% alcohol during the initial phase of the study, if the 70% alcohol pre-wash step was excluded no skin infection occurred. The necessity of eradicating the autochthonous organisms to produce a reaction could only be mitigated by the addition a substantial inoculum of *S. aureus* ($1x10^7 CFU/cm^2$). Interesting Singh *et al*., (1971) also noted that the resident flora were capable of repopulating the occluded surface within 36 h despite the presence of the *S. aureus* population. In the current investigation the resistance to colonisation observed for growth in artificial sweat appeared to be suppressed by switching the media to the nutrient rich media of artificial serum at the point of exposure to the pathogenic bacteria broadly reflecting a wounding

scenario. Despite the increased nutrient availability significant reductions were observed in the pre-colonising flora. As the MRSA and *P. aeruginosa* were initially grown in artificial sweat and then subcultured into artificial serum to make the inoculants used in this assay, the result suggests that MRSA and *P. aeruginosa* are better able to make the necessary alterations in genotypic expression to utilise the newly available nutrients producing phenotypic increase in growth and thus out-compete the pre-colonising flora. This is supported by the observation that the resistance to colonisation was partially restored in a constant nutrient rich environment i.e. chronic wound scenario where the colonising flora of *S. saprophyticus* and *C. xerosis* were continually grown in artificial serum and thus are not challenged with a change in nutrient availability. However, an additional factor in this relationship is the enhance growth rates of the exogenous pathogenic bacteria in the artificial serum (shown in Chapter 5), subsequently, the partial restoration of resistance to colonisation could be due to two opposing factors (i) the ability of the pre-colonising flora to resist colonisation due to attachment competition (ii) the enhance growth rate of the exogenous bacteria in artificial serum outcompeting that of the colonising flora.

Colonisation resistance has been intensively studied as an end point for trails into the use of probiotics in the maintenance of a healthy gastrointestinal bacterial consortium and in the treatment of gastrointestinal infections. The organisms *Lactobacillus salivarius* UCC118 and *Lactobacillus paracasei* AH104 have been used prophylactically to prevent gastrointestinal infections such as *Helicobacter pylori* and *Clostridium difficile* (Corr *et al.*, 2007; Ryan *et al.*, 2008). Work has highlighted the potential inhibitory activity of other probiotic organisms such as *Lactobacillus plantarum* against *P. aeruginosa* and *Lactobacillus acidophilus*

against MRSA (Karska-Wysocki *et al.*, 2010; Valdez *et al.*, 2005) however, to date no work has reported upon *Lactobacillus salivarius* UCC118 and *Lactobacillus paracasei* AH104 regarding their potential activity against wound-associated organisms. The novel FCF multi-well wound model was utilised to investigate the affect of lactobacilli impregnated Aquacel dressings (ConvaTec Ltd UK) upon established populations of MRSA and *P. aeruginosa* grown in artificial serum. The Aquacel dressings contain sodium carboxymethyl cellulose fibres which readily absorb and retain fluid. Subsequently, when submerged in a bacterial inoculum of axenic *Lactobacillus* spp. it will retain the inoculum without loss through gravitational forces. Application of *Lactobacillus* spp. impregnated dressings to pre-colonised populations on FCF resulted in a decrease in populations of MRSA and *P. aeruginosa* however, these were not statistically significant. Nevertheless, it is interesting to note that axenic populations of *Lactobacillus* spp. readily developed within a pre-established communities of MRSA and *P. aeruginosa*. The purported probiotic activity of *Lactobacillus* spp. isn't limited to competition for attachment sites and nutrients; it also encompasses modification of the immune response. For example *in vitro* exposure of *Lactobacillus* spp. lysates to human intestinal lamina has been shown to inhibit Th1 cells and induce the anti-inflammatory cytokine interleukin-10 from intestinal dendritic cells (Hart *et al.*, 2004) and epidermal exposure to *Lactobacillus* spp. can suppress specific or non-specific IgE, reduce eosinophils and degranulated mast cells infiltration (Lannitti and Palmieri, 2010). Currently there are a diminutive number of studies investigating affect of topical probiotics on the prevention or treatment of disease however, it has been reported that treatment of atopic dermatitis by topical application of *Vitreoscilla filiformis* lysates can significantly alleviate the signs and symptoms of atopic dermatitis (Gueniche *et al.*, 2008). Whilst the exact

mechanism by which *V. filiformis* exerts this beneficial effect is unknown, the fact that atopic dermatitis is associated with elevated IgE levels and Th2 responses indicates that *V. filiformis* lysates may be exerting a partial immunomodulatory effect. Despite the limited inhibition activity of the *L. salivarius* UCC118 and *L. paracasei* AH104 investigated herein, the combination of the immune modulation properties of probiotics and the demonstrable ability of *L. salivarius* UCC118 and *L. paracasei* AH104 to establish a population within a pre-established population suggest that effective agents could be developed for chronic inflammation associated with non-healing wounds.

7.6 Conclusion

Interactions between bacteria associated with the healthy skin and chronic wound infections were investigated using the spot-on-lawn, cross streak assay, and the novel FCF wound model. For antagonistic and mutualistic interactions results indicate that these interactions maybe uncommon and thus nor necessary successful attachment and proliferation of the bacterial isolates investigated. The *lactobacillus* spp. investigated showed some inhibitory activity against MRSA and *P. aeruginosa* future work is therefore required to further establish the efficacy of these probiotic organisms in chronic wounds. Furthermore, the results presented in this chapter demonstrate that the FCF multi-well wound model can simulate colonisation resistance conferred by members of the commensal consortium however, the outcome of the results are markedly influenced by the nutrient availability and the species of established bacterium.

Chapter 8

Conclusions

The aims of the doctoral programme were to (i) compare the microbial consortium of chronic wounds and contralateral skin swabs and identify biofilms in chronic wounds (ii) investigate the role of coaggregation in biofilms associated with chronic wounds, (iii) develop and validate novel media which are broadly representative of the healthy skin and wound environment, (iv) develop and validate novel biofilm wound models which can support the growth of a wound-associated microbial consortium, (vi) utilise these novel models to investigate the microbial population interactions associated with the healthy skin and chronic wound.

Chronic wound infections are a significant cause of morbidity, resulting in prolonged hospital stays and an increased risk of secondary site infections and septicaemia (Hill *et al.*, 2003; Howell-Jones *et al.*, 2005). Numerous factors have been associated with the progression of an acute wound into the chronic state such as diabetes and peripheral vascular disease. The resulting protracted wound creates a portal of entry for a variety of endogenous and exogenous bacteria which alter the pH, produce toxins and cause tissue damage prolonging and exacerbating the state of chronicity (Thomson, 2000). The resultant wound microbiota can contain a wide range of bacteria and subsequently has subject to investigations to elucidate the diversity and the role that members of the microbiota play in delayed wound healing. Using clinical microbiology culture standards and denaturing gradient gel electrophoresis (DGGE) methods the diversity of the microbiota of chronic wounds and control contralateral intact skin swabs from 26 patients was investigated in Chapter 3. Culture-based methodologies are the cornerstone of diagnostic microbiology nevertheless recent emphasis has been placed on culture independent techniques for profiling microbial communities. In Chapter 3, comparisons between culture-dependant and

independent methods used to investigate the microbial diversity of intact skin swabs and chronic wound samples showed that each approach identified different portions of the communities present within samples, with a greater number identified using DGGE. DGGE has previously been used to investigate the microbial diversity of numerous environments (Davies *et al.*, 2004; McBain *et al.*, 2003c; Walter *et al.*, 2000) in which the amplified product of 16s rRNA genes specific to bacteria is separated on denaturing gradient gel based upon the GC content of the fragment which is specific to a genus or species. DGGE data from the control skin swabs (as a measure of the microbiota on healthy skin at the same site) and contralateral chronic wound sample was compared intrapersonally and interpersonally in Chapter 3. All samples showed comparatively high diversity but little similarity with other samples of the same site between patients indicating that commonly present assemblages weren't detected in chronic wounds or healthy skin samples. However, the same comparison between DGGE data from skin swabs and contralateral wound samples conducted intrapersonally and by grouping the patients into culture defined groups of culture defined "infected" and "non-infected", identified a greater proportion of the contralateral skin isolates present in the culture-defined "non infected" wounds versus a decreased proportion in the "infected" wounds. This suggest that culture and DGGE methods may be complimentary; as pathogenic organism associated with wound infections (of sufficient quantity to be cultured and identified) may influence (to a degree) the overall diversity of the wound. Further experimental investigations which monitored the microbiota over time to assess the longitudinal shifts in the populations and the rate of loss and gain of specific species or genus is required to characterise this relationship. Furthermore, other contributing factors such as treatment and cleaning regimes, and wound and contralateral skin environments should also be

assessed since these may also affect the microbial population present. In addition to the microbiota of the wounds, bacterial biofilms and their purported role in impaired healing and recalcitrance to antimicrobial treatments have previously been reported (Akiyama *et al.*, 2002; Davis *et al.*, 2008; Schierle *et al.*, 2009; Serralta *et al.*, 2001). Few however, have linked the presence of biofilms to delayed healing or chronicity. In Chapter 3, putative biofilm structures stained with Concanvalin A were identified in both culture-defined infected and non-infected chronic wound tissue samples, demonstrating that the presence of wound biofilms is not related to the presence of infection but may be an underlying factor in the delay in the healing of chronic wounds. Auxiliary work is required to establish a definitive link in which the presence of biofilms influences the rate of healing and efficacy of treatment regimes. Specifically, investigations into acute wounds infections (which heal) may identify presence or absence of biofilms under these conditions which may identify the role of biofilms in healing. Additionally, longitudinal investigations of slow-healing wounds may establish the role of the developing microbiota and subsequent changes to the biofilm which may impede healing. Further work is also required using *in vitro* biofilm wound models which are representative of the chronic wound to study the efficacy of treatments on wound-associated biofilms.

Many previous investigations have used polymicrobial biofilms of the oral cavity as a models of multispecies biofilm development in which coaggregation interactions defined as the attachment of genetically distinct bacteria to each other via the reciprocal association of surface molecules is thought to play a putative role in the formation, and physical strength of the biofilm (Khemaleelakul *et al.*, 2006; Rickard *et al.*, 2003a). In Chapter 4 coaggregation interactions within the wound between

the putative primary colonisers derived from the epidermis and exogenous bacteria which colonise wounds was investigated using a quantitative spectrophotometer method. Coaggregation interactions were found not to be common within and between the endogenous colonising skin flora and exogenous wound colonising flora and therefore coaggregation may not be an important process for the establishment of wound and skin-associated biofilms. Current literature contains several conflicting reports regarding coaggregation frequency among wound-associated bacteria, this inconsistency may be due to the variety of techniques that have been used to measure aggregation. However, reports indicate that aggregation among wound-associated isolates is infrequent and therefore support the findings presented in Chapter 3.

To investigate complex microbiotas and biofilms, model systems specific for the environment which it aims to reproduce are employed. Numerous biofilm models have been developed which are representative of various environments including recently reported novel models for the investigation of wound biofilms (Hill *et al.*, 2010; Thorn and Greenman, 2009; Werthen *et al.*, 2010). However, these model system have intrinsic limitations which include reduced experimental time frames (Werthen *et al.*, 2010) and dissimilar *in vitro* environment from which it aims to reproduce (Hill *et al.*, 2010) that restrict there use for the investigation bacterial communities, biofilms and the efficacy of wound dressing. Therefore a fundamental aim of the thesis was to develop novel biofilm models to facilitate the investigation of wound and skin microbiotas and bacterial biofilms.

When running or developing models, the selection or formulation of specific growth media and substrata can be crucial in ensuring the model closely mimics the

environment in which aims to reproduce by replicating chemical and nutrient available to the bacteria in the wound. Media were formulated to broadly reflect the composition of human sweat and serum, to represent the nutrients available to bacteria in the environments of healthy skin and wounds. In Chapter 5, the formulated media were validated to ensure bacterial growth and phenotypic expression specific to biofilm production and protein expression closely mimics that of foetal calf serum. The formulated artificial sweat and serum media facilitated the maintenance of wound and skin-associated consortia (6 d), ensuring the bacterial growth and phenotype were broadly similar to those of the commonly used foetal calf serum, whilst providing a cost-effective alternative for use in novel wound biofilm model in which the growth and development of axenic and multispecies populations and biofilms can be investigated. The design of the novel models in Chapter 6, was based upon the requirements to investigate (i) cross-sectional population interactions and (ii) longitudinal population development and biofilm formation specific to chronic wounds, facilitating the investigation efficacy of novel dressings and topical treatments. The two models developed in Chapter 6 maintained the growth of axenic and mixed populations of bacteria associated with healthy skin and wound infections. The multiple FCF (MFCF) wound biofilm model maintained the long-term growth (up to 5 d) of mixed bacterial populations at similar quantities found in infected wound tissue ≥ 10^6 CFU/g of tissue, with dynamic steady-state cultures attained within 48 h of growth. Furthermore, the multiple FCF model facilitated the development of microcolonies and biofilm formation as evidenced by cross sectional imaging using Gram stain and environment electron scanning microscopy further verifying its use to investigate longitudinal population development and biofilm formation specific to wounds. Further work using this model would include the definition and maintenance a

defined wound consortia, extended experimental time frames and testing of dressing/treatments to fully verify its application as a wound biofilm model. A second model; the FCF multi-well wound model was designed to study short-term cross-sectional population interactions. Dynamic steady-state populations were attained with 48 h of growth in the FCF multi-well wound model for the selected wound and skin organism, selected based upon their common occurrence on the skin and in wounds. However, due to the “batch culture” nature and degree of labour required, the FCF multi-well wound model can only realistically be used only for short-term studies cross-sectional population interactions which was the initial rational for this model. Results presented in Chapter 7 demonstrate that the FCF multi-well wound model can simulate colonisation-resistance conferred by members of the commensal consortium. The results were however, markedly influenced by the nutrient availability and the species of established bacterium. Briefly, in an environment broadly reflective of healthy skin pre-established populations of *Corynebacterium xerosis* and *Staphylococcus saprophyticus* reduced the attachment and proliferation of *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus* however, this result was mitigated by changing the media to that of artificial serum to broadly reflect an initial wounding and chronic wound environments. The results of the spot-on-lawn and cross-streak antagonisms and mutualism assays determined these interactions maybe uncommon and thus nor necessary successful attachment and proliferation of the bacterial isolates investigated, subsequently the observed inhibition activity evidenced in the FCF multi-well wound model is not due to the production of inhibitory substances and therefore may be due to the phenomenon of colonisation resistance. Further analysis is however, required to ascertain if this is a unique phenomenon to the genus, species or strains tested and the exact

mechanism involved in the apparent resistance of pre-colonising populations of *C. xerosis* and *S. saprophyticus* in a nutrient environment broadly reflective of healthy skin and chronic wounds. Additionally, the putative probiotic role of *Lactobacillus* spp. for the treatment of wound infections of MRSA and *P. aeruginosa* was also investigated using the FCF multi-well wound model. The *Lactobacillus* spp. investigated showed some inhibitory activity against MRSA and *P. aeruginosa* however, further work is required to identify species which have greater efficacy on the pathogenic organisms associated with wound infections and *in vivo* work is required to further establish the efficacy of these probiotic organisms in chronic wound environment.

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Appendix

RESEARCH PROTOCOL

Study Title: The Microbial Ecology of Chronic Wounds

Chief Investigator/Student:

Miss Angela Oates
PhD Student
School of Pharmacy
University of Manchester
Oxford Road
M13 9PT

Study Coordinator/Local Collaborators:

Dr Andrew McBain
Lecturer
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Manchester Royal Infirmary
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Dr Frank Bowling
Podiatry Department
Rusholme Health Centre

Appendix 1A Reseacrh protocol

Walmer Street
Rusholme
Manchester
M14 5NB

Introduction

This document sets out the research protocol and the study will be conducted in line with this.

Background

Acute and chronic wound infections can be costly, resulting in prolonged hospital stays and an increased risk of secondary site infections and septicaemia. In the UK the management of chronic venous leg ulcers is estimated to cost £1 billion/year(Hill *et al.*, 2003), with surgical site infections requiring approximately 6.5 days additional hospital stay(HPA, 2004).

The causative agents of chronic wounds can be both physiological and biological, either inducing the chronic state or exacerbating it. Physiological conditions such as diabetes and peripheral vascular diseases result in restricted blood flow to the site and therefore, a reduced capacity for healing. Heavy bacterial and/or fungal colonisation has been cited as a biological factor which induces a chronic inflammatory state.

There is a great deal of debate as to the role of microorganisms in wounds. Wounds rapidly become colonised with a variety of organisms but not all become chronic. Emphasis has been placed on the number of organisms rather than the types present, with the exception of beta-haemolytic streptococci, a figure of $\geq 10^6$ organisms per gram of tissue has been associated with impaired healing (Robson, 1997).

The microbial ecology of a wound can differ greatly. Variations in location, size and depth of the wound can result in differences in temperature, pH, nutrients and

local flora. In addition the use of antimicrobials, cleaning and debridement procedures can also affect the microbial population.

The polymicrobial nature of both acute and chronic wounds has led to an increased interest in understanding population dynamics with the goal of determining the role of microorganisms in wound healing, and a possible microbial wound healing phenotype. Polymicrobial biota has been intensively studied in oral and gastrointestinal tract microbiology and in recent years emphasis has been placed upon their population interactions and biofilms.

Polymicrobial biofilms have been implicated in a number of chronic infections including endocarditis, dental caries, urinary tract infections and prosthesis colonisation. Consequently, bacterial biofilms have been implicated in wound infections.

This study aims to investigate the microbial population of wound debridement samples derived from chronic non healing wounds.

Study Objectives:

1. Study the microbial ecology of the chronic wounds.
2. Investigate the presence or absence of bacterial biofilms in wound tissue debridement samples.

Methods:

Patients with chronic wounds who are due to undergo routine wound debridement procedure (as directed by the clinician and site protocols) will be identified as potential candidates for the study and approached by the investigator and asked to donate a wound debridement sample.

1. Letter of invitation, patient information sheet and tick-box response letter will be given to the patient.

2. After a minimum period of 1 week having had the opportunity to ask any questions, the patient may decide whether they would like to take part. They will be required to return a tick-box response letter indicating that they are willing to take part in the study.

3. Prior to their wound debridement, the study will be explained to each patient by a member of the research team and they will be given the opportunity to ask further questions and express any concerns they may have. If willing, participants will be required to sign a consent form before the procedure.

Procedure

Upon agreement to participate in the study the patients:

1. The wound debridement procedure will ensue as per site protocols.

2. Excess tissue not required for microbiology and/or additional clinical investigations will be harvested for the study and issued a unique study code.

3. A bilateral skin swab taken i.e. if the wound of the located on the left leg a skin swab of the same location on the right leg will be taken.

4. The patients; age, sex, location of the wound and any current antibiotic therapy will be noted and linked to the sample via the unique study code. All data will be anonymised and no patient identifiable data will be recorded.

Indemnity:

The legal liability of the sponsor will be covered by the public, products and employer's liability policy held by the University of Manchester.

For the researches from Manchester University, the University of Manchester arranges insurance cover for research projects undertaken by members of the University staff or students where they are acting in their capacity as staff or student, but the insurers only provide indemnity for those projects which have been duly approved by the Research Ethics Committee.

Data Collection and access

All patient data will be anonymised. The tissue samples and information relating to the location of the wound, age, sex and current antibiotic therapy of the patient will be linked by a unique individual study number. No patient identifiable information will be gathered.

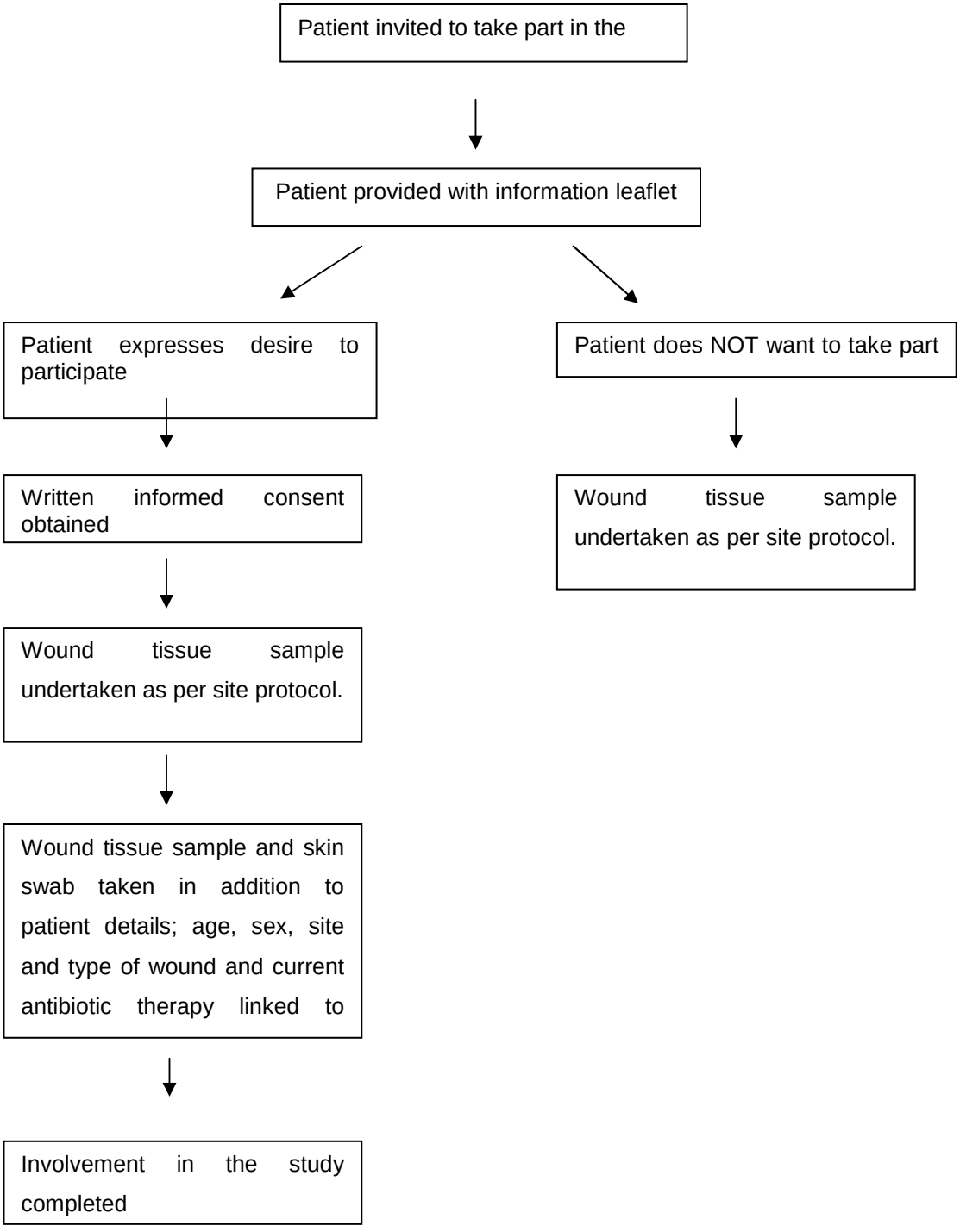
Study Monitoring

The study will be monitored by Dr Andrew McBain, lecturer of the University of Manchester

Appendix 1B Patient participation flow chart

Microbial Ecology of Chronic wounds

Patient participation flow chart



Participant Information Sheet

Title: **The Microbial Ecology of Chronic Wounds**

Principal investigator: Miss Angela Oates.

You are being invited to take part in a research study. Before you decide whether to take part, it is important to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of this study?

The main purpose of the study is to investigate the presence or absence of aggregates of bacteria, generally known as biofilms in chronic wounds. This will increase current knowledge on the types of bacteria present and how they interact in chronic wounds.

Why have I been chosen?

You have been chosen as a potential participant in this study because you have been diagnosed with a chronic wound that has failed to respond to medical treatment. Your medical team has therefore decided, after discussion with yourself, that the best treatment for your condition is wound debridement. This will involve removing the diseased tissue from the wound to encourage new tissue growth and to reduce the bacterial content of the wound

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a

consent form. If you do decide to take part**,** you are still free to withdraw at any time without giving any reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive now or in the future.

What will happen to me if I take part?

If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form prior to your scheduled surgery.

Tissue samples will be obtained from unhealthy tissues which are normally removed and discarded during the course of your wound debridement. A skin swab will be taken from the opposite side of the wound e.g. if the wound is on the left leg a skin swab of the right leg will be taken.

Patient data will also be collected from you for the purpose of the study. This will include the location and type of wound, your age, sex and any information on current antibiotic treatment that you may be receiving. All data collected will be treated in accordance with the data protection act. Your samples will be anonymous to the researchers analysing the tissues throughout the study (see 'Will my taking part in this study be kept confidential?').

What do I have to do?

All you have to do is agree that the tissue removed (during your normal wound debridement procedure) and an additional skin swab can be used for research.

What is being tested?

Tissue samples will be investigated to determine if microbial species are present in the tissue and if so which types.

The skin swab will be analysed to determine the skin bacteria of normal healthy skin.

What are the possible side effects, disadvantages and risks of taking part?

There are no additional side effects or risks of taking these samples as this tissue is normally removed as part of the wound debridement procedure and then discarded.

What are the possible benefits of taking part?

It is not anticipated that there will be any direct benefit in taking part, but it is hoped that the research will help doctors and researchers gain more of an understanding about chronic wounds which may lead to the development of treatments for this condition in the future. Your contribution towards research in this area is valued by the research team.

What if new information becomes available?

We will inform you if any new information becomes available whilst you are taking part in the study.

What if something goes wrong?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If they are unable to resolve your concern or you wish to make a complaint regarding the study, please contact a University Research Practice and Governance Co-ordinator on 0161 2757583 or 0161 2758093 or by email to research-governance@manchester.ac.uk.

What happens when the research/study stops?

You will continue to be cared for by the NHS in the usual way.

Will my taking part in this study be kept confidential?

Yes. We will be following ethical and legal practice and all information about you will be treated in the strictest confidence. All data collected will be treated in accordance with the data protection act. It will be strictly confidential and only members of the research team will have access to the data. All personal details will be anonymous on completion of the study. If a scientific paper is written about the results your name and details will be removed completely.

Who has reviewed the study?

All research within the NHS is looked at by an independent group of people called a Research/Ethics Committee to protect your safety, rights, well-being and dignity. This study has been reviewed and given a favourable opinion by the North Manchester Research Ethics Committee.

What will happen to the results of the research study?

Appendix 1C Participant information sheet

The results of the research will be used for an academic qualification of a PhD and possibly in publication at scientific meetings or in peer-reviewed scientific medical journals.

Contact for Further Information:

Chief Investigator: Miss Angela Oates.

Angela.oates@postgrad.manchester.ac.uk Tel:+44 (0)1612754761

Academic Supervisor: Dr Andrew McBain.

Andrew.mcbain@manchester.ac.uk

A copy of this information sheet and a signed consent form will be given to you to keep.

Thank you for taking the time to read about this study, if you have any questions please do not hesitate to ask. If you agree to take part you will be given a copy of this information sheet as well as the consent form for taking part in the study.

Appendix 1D Patient Consent form

PATIENT CONSENT FORM

Centre Name: Podiatry Centre Rusholme Health Centre
Patient/Sample Identification Number for this trial:

Title of Project: The Microbial Ecology of Chronic Wounds

Name of Researcher: Miss Angela Oates, University of Manchester

Local collaborator: Dr Frank Bowling Podiatry Centre, Rusholme Health Centre

Please initial box:

I confirm that I have read and understood the information sheet dated: 07/07/09. (Version 2) for the above study and have had the opportunity to ask questions. ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

I agree to the analysis and storage of my wound tissue removed during the Routine procedure. ☐

I understand that sections of my medical notes may be looked at by responsible individuals from Podiatry Centre, Rusholme Health Centre where it is relevant to my taking part in research. ☐

I give permission for these individuals to have access to my records.

I agree to take part in the above study ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from Researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

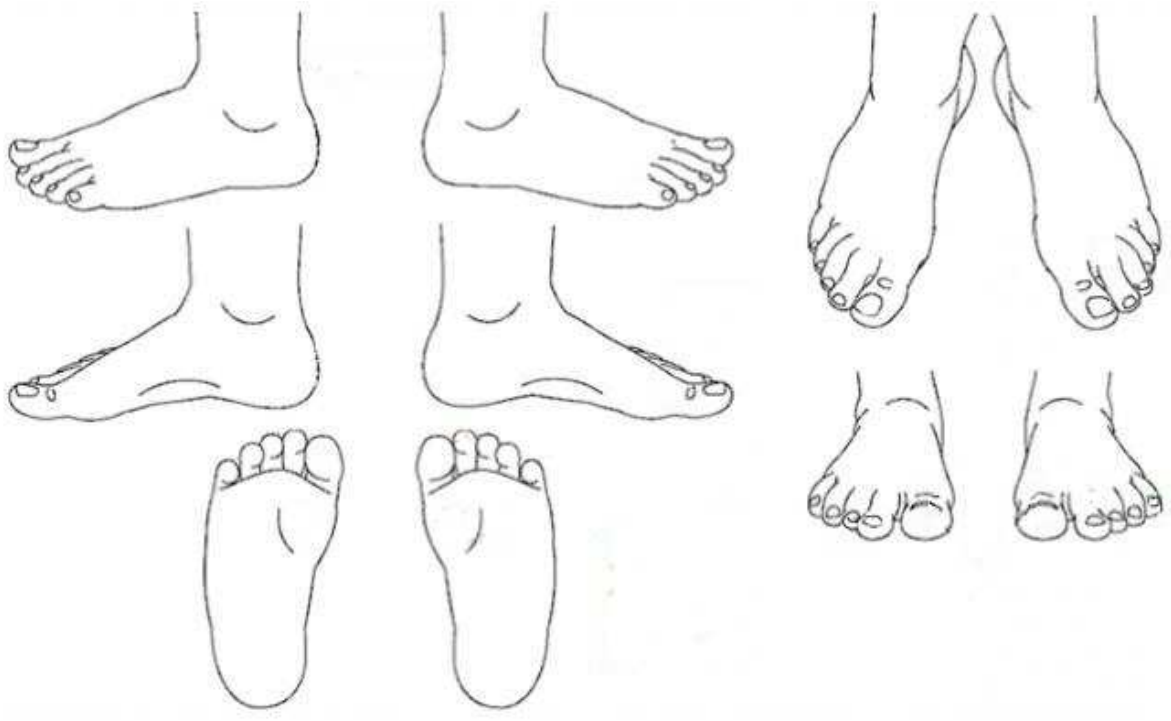
(1 for patient, 1 for researcher, 1 to be kept with hospital notes)

Microbial Ecology of Chronic Wounds

Study Sample Number ☐

Age ☐ Sex ☐

Location of wound and opposing skin swab



Type of Wound e.g. pressure sore, diabetic ulcer etc.

_____
_____
_____
_____

Current antibiotics:

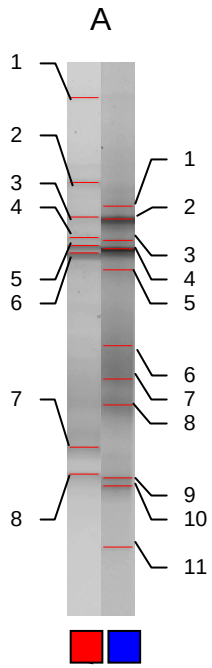
1._____
2._____
3._____
4._____

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient A: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	N/A	*Bacteroidales* spp. (HM079538)
2	*Staphylococcus epidermidis* strain VC334S1 1(HM452104)	*Staphylococcus simulans* strain AK7LW (HM462053)
3	*Staphylococcus simulans* strain AK38LW (HM452000)	*Staphylococcus* spp. DH17_87 (HM074836)
4	*Staphylococcus* spp. DH17_87 (HM074836)	*Enterococcus faecalis* strain LCR18 (HQ259727)
5	*Enterococcus faecalis* strain LCR18(HQ259727)	N/A
6	*Streptococcus* spp. BL020B49 (AY806239)	*Staphylococcus epidermidis* strain CJBP1. (AM697667)
7	Bacterium ncd1127c09c1(HM338822)	Bacterium ncd1141g11c1 (HM344790)
8	*Micrococcus yunnanensis* strain R-76G (HQ285773)	*Bacillaceae* bacterium BL-87 (EU596919)
9		*Micrococcus yunnanensis* strain R-76G (HQ285773)
10		N/A
11		N/A

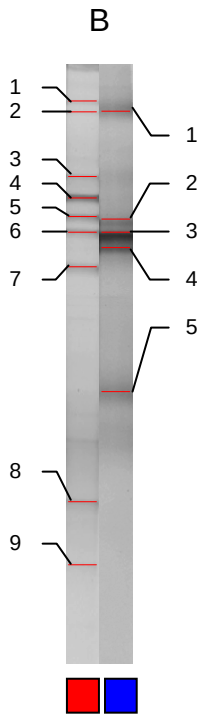
Matched bands based on band alignment and sequences are shaded grey. 4/11 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient B: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	N/A	N/A
2	N/A	bacterium nbw344g06c (GQ091333)
3	*Staphylococcus* spp. VA07_35(HM075882)	bacterium nbw344g06c1(GQ091333)
4	N/A	*Staphylococcus epidermidis* strain CJBP1. (AM697667)
5	*Clostridia* bacterium MT05B_C05 (DQ169781)	
6	*Anaerococcus* spp. VE27D09 (GQ179680)	
7	*Peptoniphilus* spp. T0457 (GU458949)	
8	bacterium ncd1127c09c1 (HM338822)	
9	N/A	

0/5 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



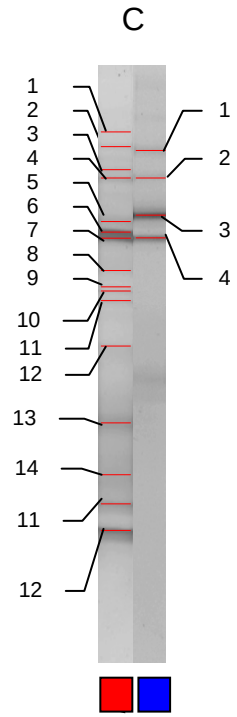
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient C: DGGE 16s DNA identities of matched bands present in wound and intact skin

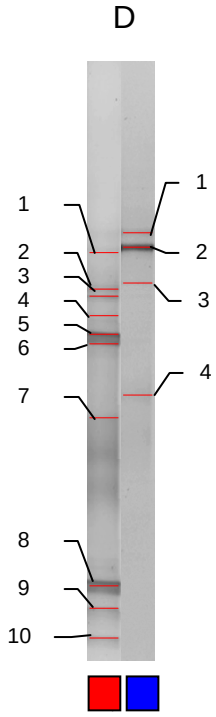
Band	Wound	*Skin*
1	N/A	*N/A*
2	N/A	Staphylococcus spp. VA07_35 (*HM075882*)
3	N/A	*Staphylococcus epidermidis* strain VC334S1 (*HM452104*)
4	*Staphylococcus* spp. VA07_35 (HM075882)	*Staphylococcus sciuri* strain R10-5A (*HQ154580*)
5	*Staphylococcus epidermidis* strain VC334S1 (HM452104)	
6	N/A	
7	*Staphylococcus sciuri* strain R10-5A (HQ154580)	
8	*Staphylococcus simulans* strain AK7LW (HM462053)	
9	*Staphylococcus* spp. DH17_87 (HM074836)	
10	N/A	
11	N/A	
12	*Staphylococcus* spp. DH10_85 (HM074305)	
13	N/A	
14	*Bacillus subtilis* strain:SSCA3 (AB210949	
15	*Actinomycetales* bacterium VA22_ (HM077215)	
16	*Actinobacterium* WS05B_A03 (DQ171161)	

Matched bands based on band alignment and sequences are shaded grey. 3/4 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient D: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Bacteroidales* bacterium E105H11 (HM079538)	Staphylococcus spp. DH18_56 (HM074899)
2	*Staphylococcus* spp. DH17_87 (HM074836)	bacterium ncd1127c09c1 (HM338822)
3	*Staphylococcus epidermidis* strain VC334S1 (HM452104)	N/A
4	N/A	*Enterobacter* sp *HSL97* (HM461227)
5	*Acinetobacter* spp. 4A9S1(HQ246291)	
6	*Staphylococcus* spp. DH10_85 (HM074305)	
7	*Bacillus pumilus* strain FS55 (AF260751)	
8	*Klebsiella* spp. SL13 (HQ264073)	
9	*Klebsiella* spp. TS8N1(GU294294)	
10	N/A	



0/4 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.

Wound Tissue Sample Intact Skin Swab

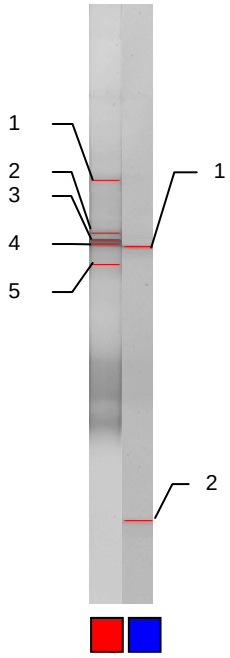
Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

E

Patient E: DGGE 16s identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Staphylococcus aureus* strain GCA890 (HM209755)	*Moraxellaceae* bacterium SHTP485 (GQ358455)
2	*Enterococcaceae* bacterium Cat005G_B01_(EU572465)	N/A
3	*Enterococcaceae* Cat005G_B01_ (EU572465)	
4	*Enterococcus faecalis* strain R10-3A (HQ154579)	
5	*Streptococcus dysgalactiae* strain CH74 (HM359249)	

0/2 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.

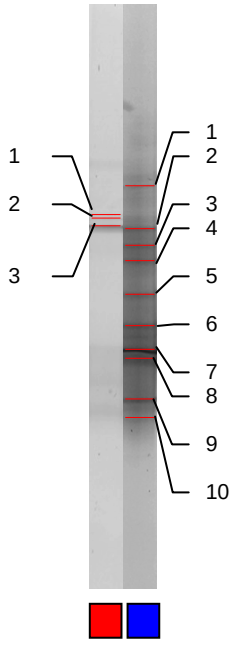


F

Patient F: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Enterococcaceae* Cat005G_B01_ (EU572465)	*Staphylococcus cohnii* strain FUA2059 (HQ169121)
2	*Enterococcus faecalis* strain R10-3A (HQ154579)	*Bacillus subtilis* strain: SSCA3 (AB210949)
3	*Enterococcus faecalis* strain R10-3A (HQ154579)	*Bacillus subtilis* subspp. subtilis SF153 (FM178960)
4		*Acinetobacter* spp. 4A9S(HQ246291)
5		N/A
6		*Actinobacterium* WS05B_A03 (DQ171161)
7		*Bacillus subtilis* strain CICC10165 (DQ012096)
8		*Bacillus subtilis* strain:SSCA3. (AB210949)
9		N/A
10		*Staphylococcus cohnii* strain FUA2059 (HQ169121)

0/10 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



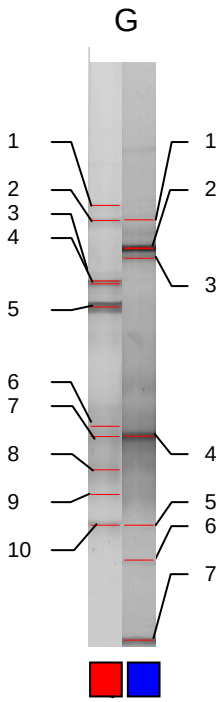
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient G: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Clostridia* bacterium MT05B_C05 (DQ169781)	*Staphylococcus sciuri* strain R10-5A (HQ154580)
2	*Staphylococcus* spp. DH10_85 (HM074305)	*Variovorax* spp. 01xTSA28A_F05 (HM113661)
3	*Staphylococcus aureus* strain GCA890 (HM209755)	*Staphylococcus simulans* strain AK7LW (HM462053)
4	*Prevotella bivia* strain: JCM 6332 (AB547674)	*Sphingomonas* spp. AVCTGRB13A (HM346205)
5	*Streptococcus dysgalactiae* strain CH74 (HM359249)	N/A
6	*Stenotrophomonas* spp. CB13(2010) (FJ609992)	N/A
7	*Sphingomonas* spp. AVCTGRB13A(HM346205)	*Kocuria rhizophila* strain PE-LR-2 (FR687213)
8	N/A	
9	N/A	
10	N/A	

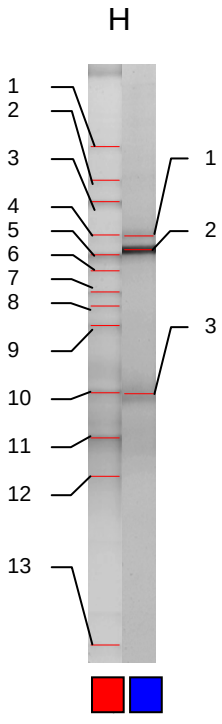
Matched bands based on band alignment and sequences are shaded grey. 2/7 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient H: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	N/A	*Staphylococcus sciuri* strain R10-5A (HQ154580)
2	*Staphylococcus* spp. VA07_35 (HM075882)	*Staphylococcus sciuri* strain R1-4A (HQ154558)
3	*Anaerococcus* spp. T0822 (GU458864)	*Staphylococcus sciuri* strain R10-5A (HQ154580)
4	*Staphylococcus sciuri* strain R10-5A (HQ154580)	
5	*Bacteroidales* bacterium E105H11 (HM079538)	
6	*Staphylococcus* spp. DH17_87 (HM074836)	
7	*Enterococcaceae* Cat005G_B01_ (EU572465)	
8	*Bacillus korlensis* strain PT-26 (HQ234286)	
9	*Streptococcus* spp. oral taxon C08 ATC_H41_23 (GU429553)	
10	*Staphylococcus sciuri* strain R10-5A (HQ154580)	
11	*Bacillaceae* bacterium BL-87 (EU596919)	
12	*Enterobacter* spp. D9 (FJ609991)	
13	N/A	

Matched bands based on band alignment and sequences are shaded grey. 2/3 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



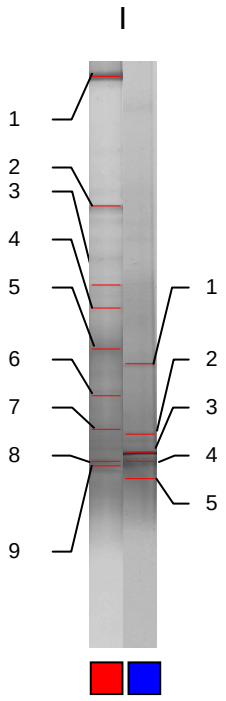
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient I: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Bacteroides fragilis* (FQ312004)	*Bacillus pumilus* strain B1W-36(FM179663)
2	*Clostridiales* spp. VA15_61 (HM076639)	*Sphingomonas* spp. AVCTGRB13A (HM346205)
3	*Enterococcaceae* spp. Cat005G_B01_ (EU572465)	*Stenotrophomonas maltophilia* strain MF48 (AY321966)
4	*Streptococcus dysgalactiae* strain CH74 (HM359249)	*Bacillus pumilus* strain B1W-36.(FM179663)
5	N/A	*Actinomycetales* spp. VA22_33 (HM077215)
6	*Enterobacter* spp. HSL97 (HM461227)	
7	*Bacillaceae* bacterium BL-87 (EU596919)	
8	*Enterobacter* spp. D9 (FJ609991)	
9	*Enterobacter* spp. D9 (FJ609991)	

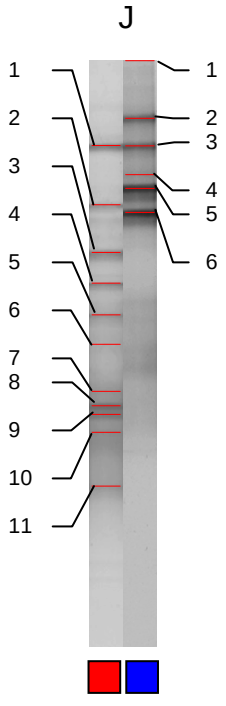
0/5 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient J: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Anaerococcus* spp. VE27D09 (GQ179680)	N/A
2	*Staphylococcus* spp. DH23_11 (HM075312)	*Staphylococcus* spp. VA07_35 (HM075882)
3	N/A	*Staphylococcus* spp. DH23_11 (HM075312)
4	*Finegoldia* spp. H07_56_1_K9_PT13 (GU075238)	N/A
5	*Streptococcus* spp. oral taxon C08 ATC_H41_23 (GU429553)	*Staphylococcus* spp. VA07_35 (HM075882)
6	N/A	*Enterococcaceae* bacterium AVCTGRB16A 1(HM346209)
7	*Enterobacter* spp. isolate d-4(GU227487)	N/A
8	Bacterium ncd1141g11c1 (HM344790)	*Staphylococcus* spp. VA07_35 (HM075882)
9	*Prevotella timonensis* (AB547706)	*Staphylococcus* spp. DH23_11 (HM075312)
10	*Sphingomonas* sp AVCTGRB13A (HM346205)	N/A
11	N/A	

Matched bands based on band alignment and sequences are shaded grey. 1/6 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



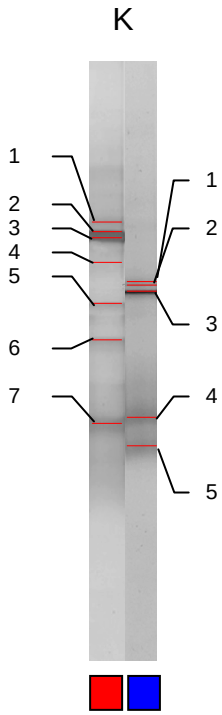
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient K: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Staphylococcus* spp. DH18_56 (HM074899)	*Acinetobacter* spp. 2293/04 (GU797845)
2	*Staphylococcus* spp. DH10_85 (HM074305)	*Enterococcaceae* spp. AVCTGRB16A (HM346209)
3	*Staphylococcus* spp. DH10_85 (HM074305)	*Enterococcaceae* spp. AVCTGRB16A (HM346209)
4	*Variovorax* spp. 01xTSA28A_F05 (HM113661)	*Bacillus pumilus* strain FS55 (AF260751)
5	N/A	*Sphingomonas* sp AVCTGRB13A(HM346205)
6	*Staphylococcus* spp. DH10_85 (HM074305)	
7	*Bacillus* subtilis strain CICC10165 (DQ012096)	

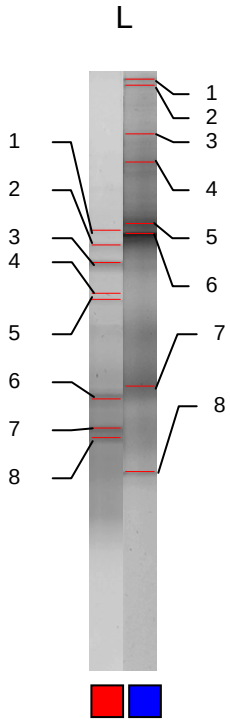
0/5 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient L: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Finegoldia* spp. H07_56_1_K9_PT13 (GU075238)	N/A
2	*Staphylococcus sciuri* strain R1-4A (HQ154558)	N/A
3	*Prevotella disiens* strain: JCM 6333. (AB547683)	N/A
4	*Staphylococcus* spp. DH17_87 (HM074836)	N/A
5	*Bacillus korlensis* strain PT-26 (HQ234286)	*Staphylococcus epidermidis* strain VC334S1 (HM452104)
6	*Staphylococcus sciuri* strain R10-5A (HQ154580)	*Staphylococcus epidermidis* strain CJBP1(AM697667)
7	N/A	N/A
8	*Prevotella timonensis* (AB547706)	*Stenotrophomonas maltophilia* strain MF48 (AY321966)

0/8 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



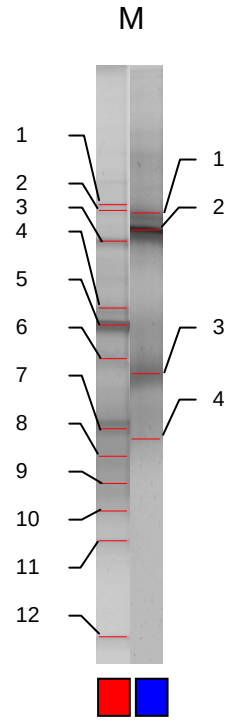
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient M: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Staphylococcus* spp. VA07_35 (HM075882)	*Staphylococcus epidermidis* strain VC334S1 (HM452104)
2	*Staphylococcus* spp. VA07_35 (HM075882)	*Staphylococcus epidermidis* strain CJBP1(AM697667)
3	N/A	N/A
4	*Bacillus korlensis* strain PT-26 (HQ234286)	*Bacillaceae* bacterium BL-87 (EU596919)
5	*Streptococcus* spp. oral taxon 431 MO076 (GU412701)	
6	*Bacillus korlensis* strain PT-26 (HQ234286)	
7	*Prevotella timonensis* (AB547706)	
8	N/A	
9	N/A	
10	N/A	
11	N/A	
12	N/A	

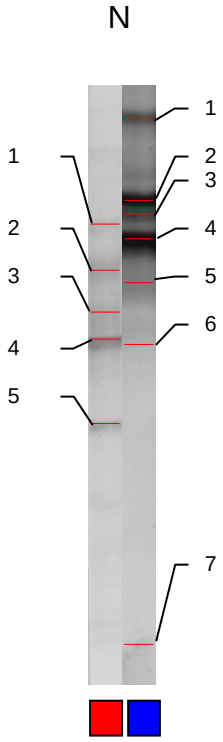
Matched bands based on band alignment and sequences are shaded grey. 1/4 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient N: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Staphylococcus* spp. VA07_35 (HM075882)	N/A
2	*Staphylococcus sciuri* strain R1-4A (HQ154558)	*Staphylococcus* spp. VA07_35 (HM075882)
3	*Staphylococcus* spp. DH17_87 (HM074836)	*Staphylococcus* spp. DH18_56 (HM074899)
4	*Streptococcus mitis* HX005 (GU421594)	*Variovorax* spp. 01xTSA28A_F05 (HM113661)
5	*Escherichia coli* KE028 (GU415844)	*Anaerococcus* spp. T0822 (GU458864)
6		N/A
7		N/A

0/6 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



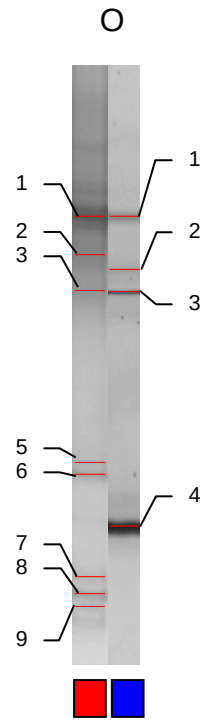
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient O: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Staphylococcus epidermidis* strain VC334S1 (HM452104)	*Staphylococcus epidermidis* strain VC334S1 (HM452104)
2	*Prevotella disiens* strain: JCM 6333. (AB547683)	*Staphylococcus simulans* strain AK7LW (HM462053)
3	*Staphylococcus* spp. DH17_87 (HM074836)	*Staphylococcus* spp. DH17_87 (HM074836)
4	*Bacillus pumilus* strain B1W-36.FM179663)	*Micrococcus yunnanensis* strain R-76G (HQ285773)
5	*Staphylococcus epidermidis* strain VC334S1 (HM452104)	
6	N/A	
7	N/A	
8	N/A	

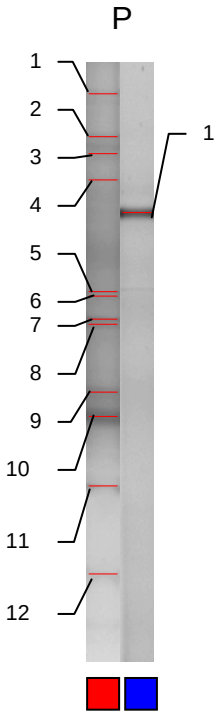
Matched bands based on band alignment and sequences are shaded grey. 2/4 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient P: DGGE 16s DNA identities of matched bands present in wound and intact skin

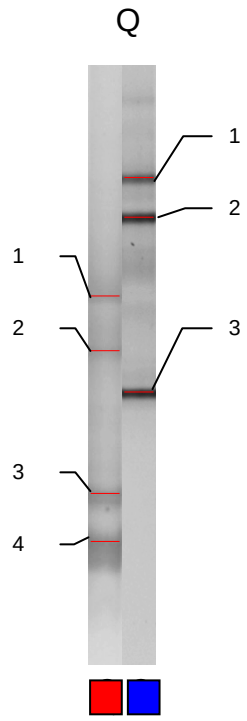
Band	Wound	Skin
1	N/A	*Staphylococcus epidermidis* strain
2	N/A	
3	*Anaerococcus* spp. VE27D09 (GQ179680)	
4	*Clostridiales* spp. DH24_08 (HM075402)	
5	*Bacillus firmus* strain AK39531 (HQ234341)	
6	N/A	
7	*Streptococcus dysgalactiae* strain CH74 (HM359249)	
8	N/A	
9	*Actinobacterium* WS05B_A03 (DQ171161)	
10	*Staphylococcus aureus* subspp. (AB594753)	
11	N/A	
12	N/A	

0/1 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Wound Tissue Sample Intact Skin Swab

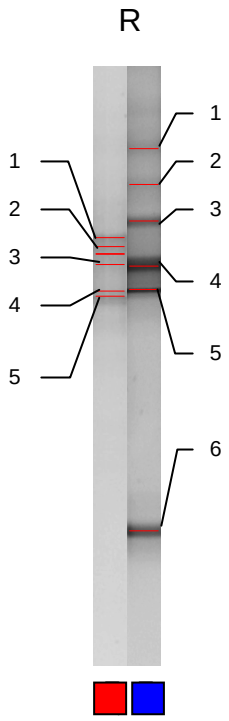
Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs



Patient Q: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Actinobacterium* WS05B_A03 (DQ171161)	*Staphylococcus* spp. VA07_35 (HM075882)
2	*Actinobacterium* WS05B_A03 (DQ171161)	*Staphylococcus epidermidis* strain VC334S1 (HM452104)
3	bacterium ncd1127c09c1 (HM338822)	*Bacillus pumilus* strain sed_chik_7 (HM486493)
4	*Actinobacterium* WS05B_A03 (DQ171161)	

0/3 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient R: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	N/A	N/A
2	*Bacillus firmus* strain AK39531 (HQ234341)	*Clostridiales* DH24_08 (HM075402)
3	*Staphylococcus simulans* strain AK38LW (HM452000)	*Finegoldia* spp. H07_56_1_K9_PT13 (GU075238)
4	*Staphylococcus simulans* strain AK38LW (HM452000)	*Staphylococcus simulans* strain AK7LW (HM462053)
5	N/A	*Staphylococcus* spp. DH17_87 (HM074836)
6	*Lactobacillus salivarius* strain L8YD15 (EF463035)	

Matched bands based on band alignment and sequences are shaded grey. 2/6 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.

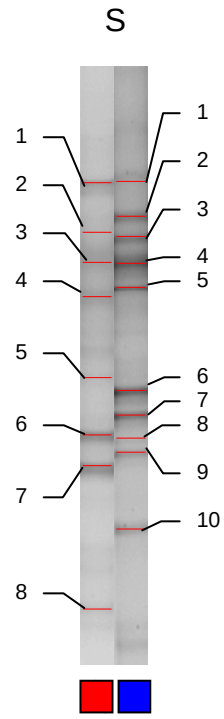
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient S: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Clostridiales* bacterium DH24_08 (HM075402)	*Clostridiales* bacterium DH24_08 (HM075402)
2	*Staphylococcus sciuri* strain R10-5A (HQ154580)	*Staphylococcus epidermidis* strain VC334S1 (HM452104)
3	*Staphylococcus simulans* strain AK38LW (HM452000)	*Staphylococcus sciuri* strain R10-5A (HQ154580)
4	*Micrococcus yunnanensis* strain R-76G (HQ285773)	*Staphylococcus simulans* strain AK38LW (HM452000)
5	*Bacillus pumilus* strain B1W-36FM179663)	*Enterococcaceae* bacterium Cat005G_B01_ .(EU572465)
6	*Stenotrophomonas* spp. CB13(2010) (FJ609992)	*Staphylococcus* spp. VA07_35 (HM075882)
7	*Stenotrophomonas maltophilia* strain MF48 (AY321966)	*Bacillus pumilus* strain FS55 (AF260751)
8	*Klebsiella* spp. TS8N1 .(GU294294)	*Stenotrophomonas* spp. CB13(2010) (FJ609992)
9		*Stenotrophomonas maltophilia* strain MF48 (AY321966)
10		*Micrococcus yunnanensis* strain R-76G (HQ285773)

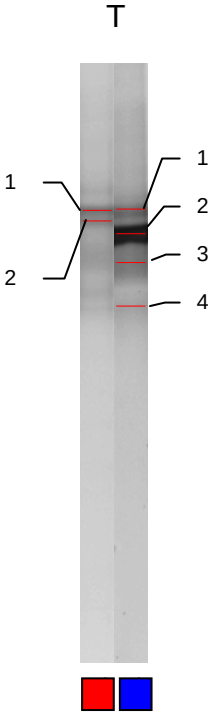
Matched bands based on band alignment and sequences are shaded grey. 3/10 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient T: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Staphylococcus aureus* WZ017 (GU415564)	*Staphylococcus aureus* WZ017 (GU415564)
2	*Staphylococcus aureus* WZ017 (GU415564)	*Staphylococcus sciuri* strain R10-5A (HQ154580)
3		*Staphylococcus simulans* strain AK7LW (HM462053)
4		*Streptococcus mitis* HX005 (GU421594)

Matched bands based on band alignment and sequences are shaded grey. 1/4 bands present in intact skin found in chronic wound.



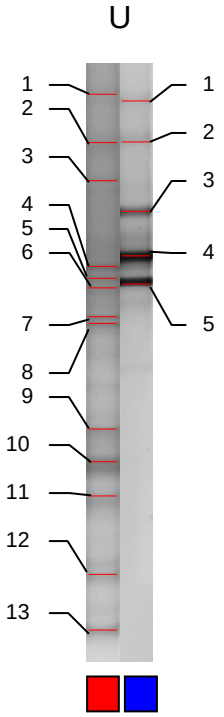
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient U: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	N/A	N/A
2	N/A	N/A
3	*Clostridiales* bacterium DH24_08 (HM075402)	*Staphylococcus aureus* WZ017 (GU415564)
4	*Staphylococcus* cohnii strain FUA2059 (HQ169121)	*Staphylococcus simulans* strain AK38LW (HM452000)
5	*Actinobacterium* WS05B_A03 (DQ171161)	*Bacillus korlensis* strain PT-26 (HQ234286)
6	*Bacillus korlensis* strain PT-26 (HQ234286)	
7	*Streptococcus* spp. oral taxon 431 MO076 (GU412701)	
8	*Streptococcus* spp. oral taxon C08 ATC_H41_23 (GU429553)	
9	*Stenotrophomonas* spp. CB13(2010) (FJ609992)	
10	*Stenotrophomonas maltophilia* strain MF48 (AY321966)	
11	*Actinomycetales* bacterium VA22_33(HM077215)	
12	N/A	
13	N/A	

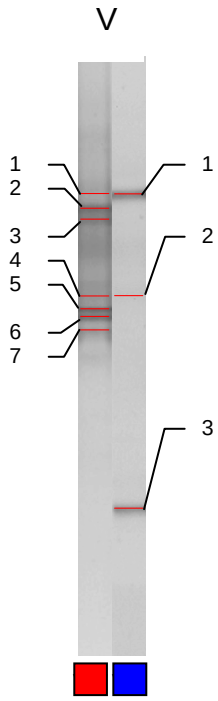
Matched bands based on band alignment and sequences are shaded grey. 1/5 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient V: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Staphylococcus* spp. VA07_35 (HM075882)	*Staphylococcus* spp. VA07_35 (HM075882)
2	*Staphylococcus* spp. DH18_56 (HM074899)	*Bacillus korlensis* strain PT-26 (HQ234286)
3	*Staphylococcus* spp. T0-YC6748 (GQ369016)	*Micrococcus luteus* strain SRDKS-9 (HM215462)
4	*Bacillus korlensis* strain PT-26 (HQ234286)	
5	*Streptococcus* spp. oral taxon 431 MO076 (GU412701)	
6	*Abiotrophia* spp. VA14_61 (HM076549)	
7	*Streptococcus* spp. oral taxon 431 FR031 (GU412588)	

Matched bands based on band alignment and sequences are shaded grey. 2/3 bands present in intact skin found in chronic wound



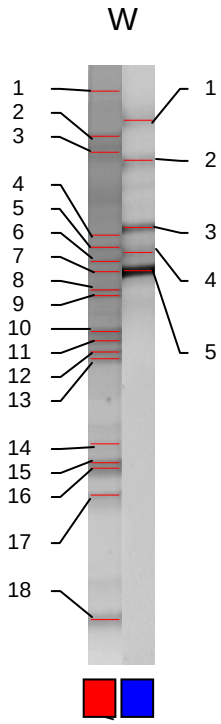
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient W: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Bacteroides fragilis* (FQ312004)	N/A
2	N/A	*Staphylococcus haemolyticus* strain HF61SY (HM452121)
3	*Anaerococcus* spp. VE27D09 (GQ179680)	*Staphylococcus* spp. T0-YC6748 (GQ369016)
4	N/A	*Variovorax* spp. 01xTSA28A_F05 (HM113661)
5	*Staphylococcus sciuri* strain R1-4A (HQ154558)	N/A
6	*Staphylococcus simulans* strain AK38LW (HM452000)	
7	*Staphylococcus simulans* strain AK7LW (HM462053)	
8	*Bacillus korlensis* strain PT-26 (HQ234286)	
9	*Bacillus* korlensis strain PT-26 (HQ234286)	
10	*Acinetobacter* spp. 4A9S1 .(HQ246291)	
11	*Staphylococcus* spp. DH10_85 (HM074305)	
12	N/A	
13	N/A	
14	*Bacillaceae* bacterium BL-87 (EU596919)	
15	N/A	
16	N/A	
17	Actinomycetales bacterium VA22_33 ribosomal(HM077215)	
18	N/A	

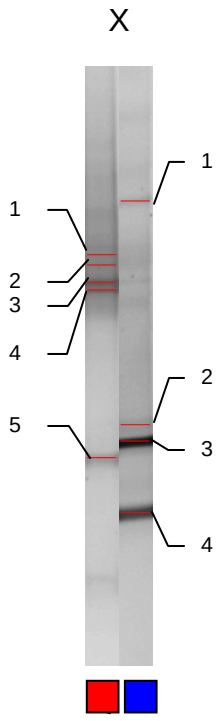
0/5 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient X: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Variovorax* spp. 01xTSA28A_F05 (HM113661)	*Staphylococcus* spp. VA07_35 (HM075882)
2	N/A	*Prevotella timonensis* (AB547706)
3	*Acinetobacter* spp. 2293/04 (GU797845)	*Acinetobacter* spp. 2293/04 (GU797845)
4	*Enterococcaceae* bacterium Cat005G_B01_ (EU572465)	N/A
5	*Bacillus pumilus* strain B1W-3FM179663)	

0/4 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



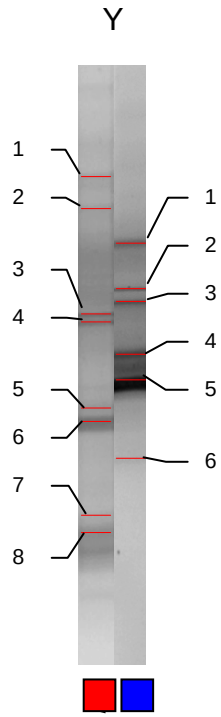
Wound Tissue Sample Intact Skin Swab

Appendix 1F. Comparisons of DGGE 16s sequence data between chronic wound tissue sample and contralateral intact skin swabs

Patient Y: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	*Staphylococcus* spp. VA07_35 (HM075882)	*Bacillus firmus* strain AK39531 (HQ234341)
2	*Staphylococcus epidermidis* strain VC334S1 (HM452104)	*Bacillus korlensis* strain PT-26 (HQ234286)
3	*Streptococcus mitis* HX005 (GU421594)	*Bacillus korlensis* strain PT-26 (HQ234286)
4	*Streptococcus* spp. oral taxon C08 ATC_H41_23 (GU429553)	*Bacillus korlensis* strain PT-26 (HQ234286)
5	N/A	*Bacillus pumilus* strain B1W-36FM179663)
6	*Prevotella timonensis* (AB547706)	N/A
7	*Micrococcus luteus* strain SRDKS-9 (HM215462)	

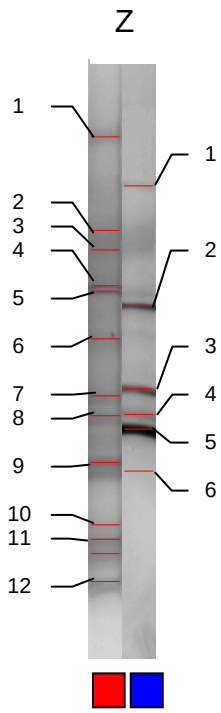
0/6 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Patient Z: DGGE 16s DNA identities of matched bands present in wound and intact skin

Band	Wound	Skin
1	N/A	*Anaerococcus* spp. T0822 (GU458864)
2	N/A	*Bacillus subtilis* sub spp. subtilis SF153 (FM178960)
3	*Variovorax* spp. 01xTSA28A_F05 (HM113661)	N/A
4	*Staphylococcus* spp. DH17_87 (HM074836)	*Prevotella timonensis* (AB547706)
5	*Enterococcus faecalis* strain LCR18 (HQ259727)	Bacillus safensis strain R-43891 (FR682743)
6	*Enterobacter* spp. isolate d-4 (GU227487)	*Enterobacter* spp. D9 (FJ609991)
7	*Enterobacter* spp. HSL97 (HM461227)	
8	*Prevotella timonensis* (AB547706)	
9	*Staphylococcus epidermidis* strain VC334S1 (HM452104)	
10	*Micrococcus yunnanensis* strain R-76G (HQ285773)	
11	N/A	
12	N/A	

Matched bands based on band alignment and sequences are shaded grey.1/6 bands present in intact skin found in chronic wound. N/A denotes no matching sequence available. Accession number given in parenthesis.



Wound Tissue Sample Intact Skin Swab