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Virulence Mechanisms of Cutibacterium acnes In Association with Lumbar Disc Herniations

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Virulence Mechanisms of *Cutibacterium acnes* In Association with Lumbar Disc Herniations

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Gurpreet Kaur Gill BSc

A thesis submitted in partial fulfilment of the University's requirements for the Degree of Doctor of Philosophy

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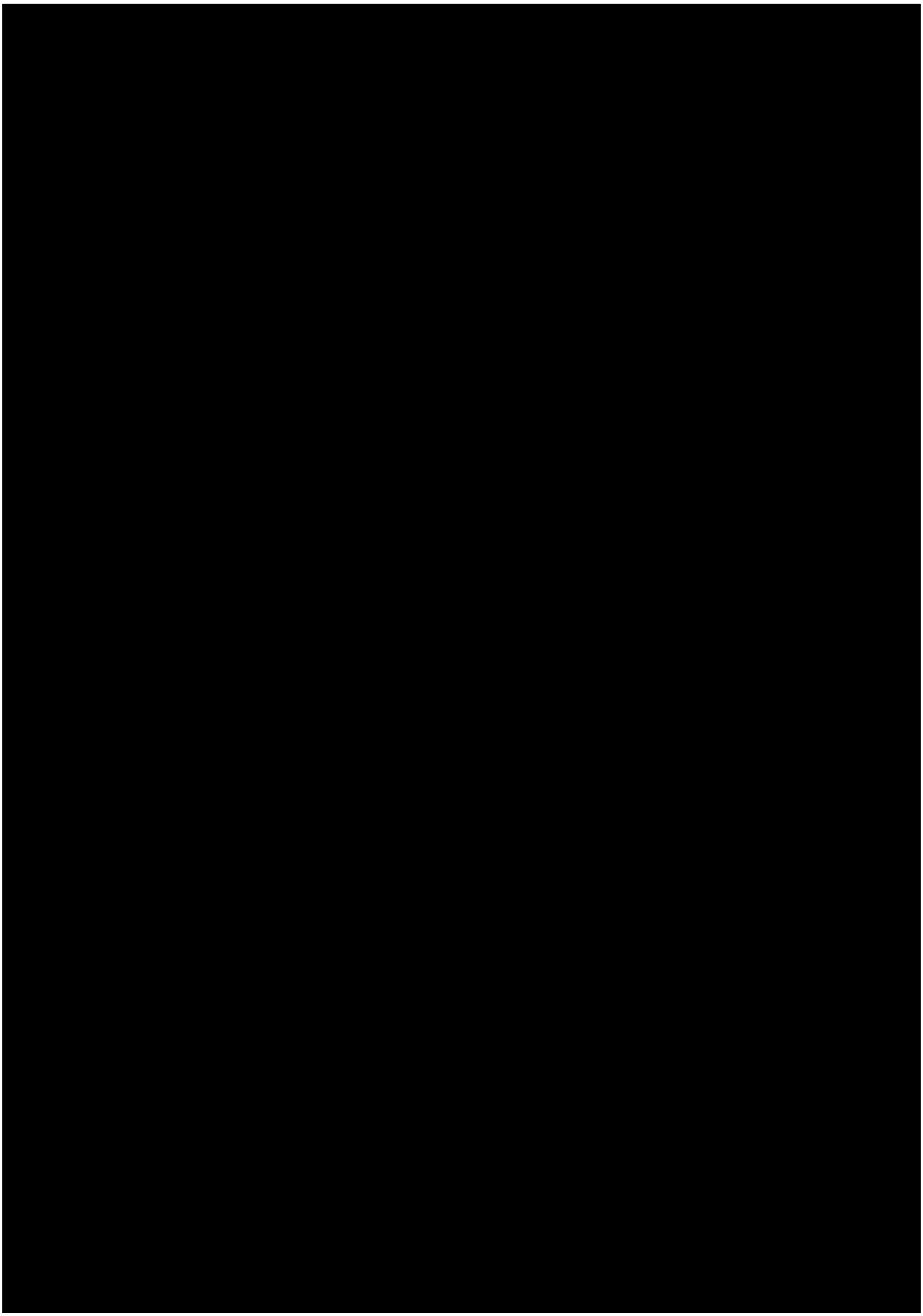

Certificate of Ethical Approval

Applicant:

Gurpreet Gill

Project Title:

Characterisation of Propionibacterium acnes (P.acnes) by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins (FURTHER UPDATES TO PROTOCOL AND COSHH)

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

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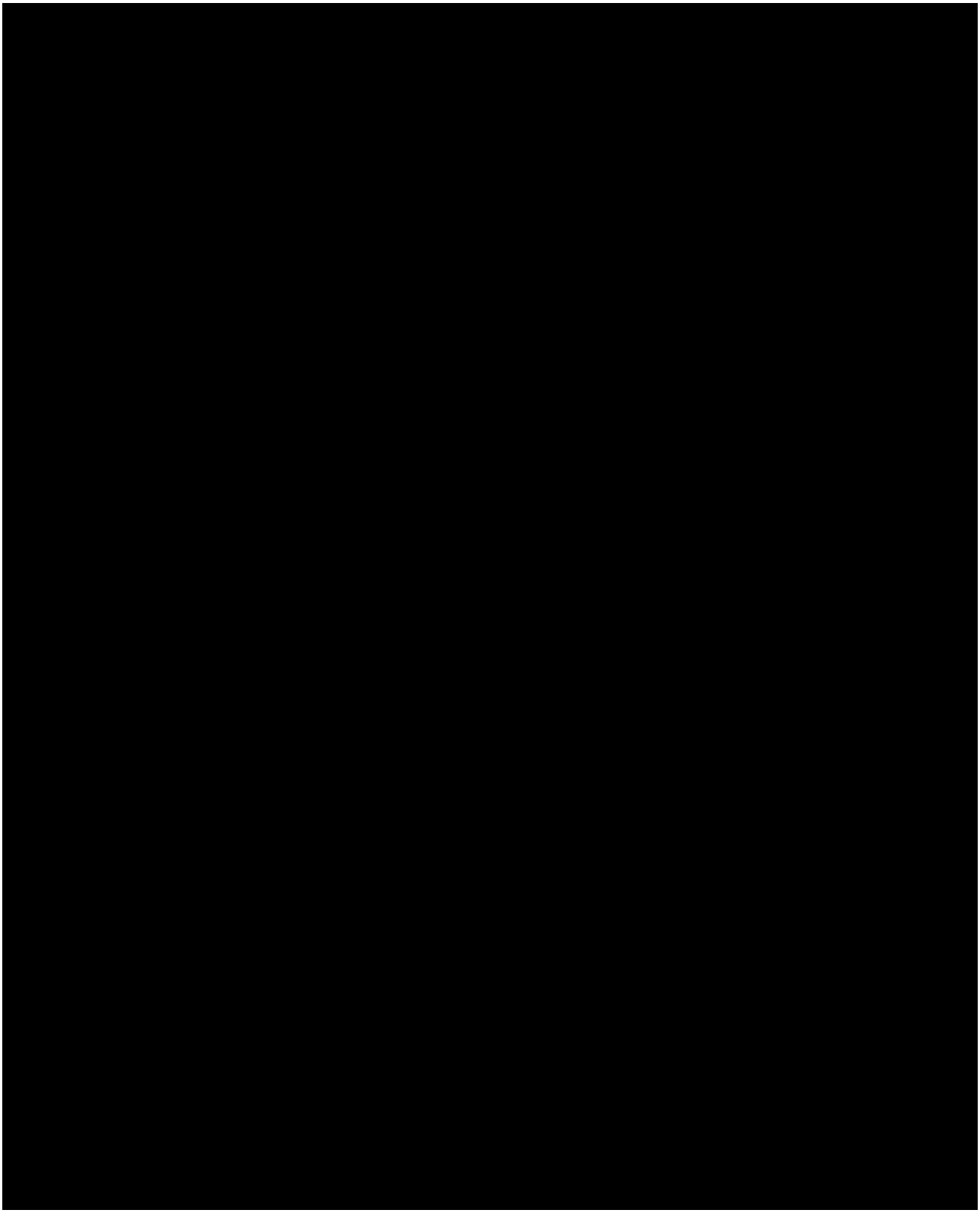

I dedicate this thesis to my husband's family for their love and support throughout the completion of my work. I would also like to dedicate this work to my husband Manpreet who I love with all my heart and who always eased me of my research worries. Last but not least, I dedicate this thesis to my friends and to my amazing supervisor, Dr Jess Rollason, without whom this work would not have been possible.

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SUMMARY

Cutibacterium acnes (*C. acnes*) (formerly known as *Propionibacterium acnes*) forms part of the normal resident host microflora of the skin, large intestine and oral cavity. Despite increasing evidence for the opportunistic pathogenic role of this species within intervertebral disc (IVD) herniation and associated Modic changes, *C. acnes* is considered a low virulence microorganism. This thesis aimed to investigate the virulence characteristics of 67 clinical *C. acnes* isolates belonging to phylotypes IA_1, IB, II and III obtained from human herniated IVD tissue material and eight acne lesion isolates. Phenotypic isolate characterisation was undertaken using crystal violet biofilm formation assays, *Galleria mellonella* (*G. mellonella*) waxworm infection assays, exocellular enzyme production agar assays and Western blot protein analysis. Associations between phylotype grouping and site of isolation of the isolates were then investigated. Biofilm formation crystal violet assays showed that phylotype III disc tissue isolates failed to form significant biofilms. Semi-quantitative biofilm formation classification criteria identified the majority of disc tissue and acne lesion isolates as weak biofilm formers over 7-days, indicative of the slow growth rate of this species. Phylotype-specific exocellular enzyme production demonstrated that phylotype IA_1 and IB isolates commonly produced β-haemolysins (84% and 67%, respectively). Gelatinase production was phylotype and site of isolation-specific. The highest rates of non-specific protease enzyme production were recorded for *C. acnes* phylotypes IA_1 and III, with 100% of these isolates showing protease activity. Ubiquitous lipase production was recorded amongst all *C. acnes* disc tissue and acne lesion isolates tested. Comparison of waxworm larvae mortality rates between differing *C. acnes* phylotypes and culture preparation techniques (cell-free, heat-killed and cell-containing inoculum preparations) showed no significant effects on *G. mellonella* survival rates, suggesting that *C. acnes* does not induce significant mortality within this infection model. However, all phylotypes of *C. acnes* promoted *G. mellonella* melanisation, indicative of insect humoral immune response activation, with cell-containing cultures of *C. acnes* isolates 82 (IB) and 80 (III) leading to the greatest degrees of melanisation. Therefore, the *G. mellonella* infection model is sensitive and can discriminate between the pathogenicity of *C. acnes* belonging to a range of phylotypes. Additionally, following Western blotting, disc tissue *C. acnes* samples produced CAMP factor 1 protein. Overall, site of *C. acnes* isolation (IVD or acne lesion) and phylotype appeared to influence phenotypic characteristics. This suggests that the role of *C. acnes* within lumbar disc herniation etiology should not be readily dismissed and highlights the need for further research into the applicability of anti-CAMP factor vaccines within *C. acnes* disc disease management.

Keywords: *Cutibacterium acnes*, biofilm, haemolysis, DNase, protease, gelatinase, lipase, CAMP factor 1, growth curve, *Galleria mellonella*.

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ABBREVIATIONS

AF	Annulus fibrosus
BEP	Bony endplate
BHI	Brain heart infusion
CAMP	Christie Atkins Munch-Petersen
CCL2	Chemokine (C–C motif) ligand 2
CEP	Cartilage endplate
CFU	Colony forming unit
CHST3	Carbohydrate sulphotransferase 3
CK8	Cytokeratin 8
CLBP	Chronic lower back pain
CNS	Central nervous system
CoNS	Coagulase-negative staphylococci
$C0_2$	Carbon dioxide
CP-OCT	Cross-polymerisation optical coherence tomography
CRP	C-reactive protein

CV	Coefficient of variation
DAP	Diaminopimelic acid
DLBP	Discogenic lower back pain
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rates
FADD	Fas-associated protein with death domain
GBS	Group B Streptococcus
GM-CSF	Granulocyte macrophage-colony stimulating factor
gt	Generation time
hBD	Human beta defensin
HCL	Hydrochloric acid
HRP	Horseradish peroxidase
H_2	Hydrogen
H_20_2	Hydrogen peroxide
IDD	Internal disc disruption
IFN-γ	Interferon-γ

IgG	Immunoglobulin G
IL	Interleukin
IVD	Intervertebral disc
IVDD	Intervertebral disc degeneration
kDa	KiloDalton
LBP	Lower back pain
LD_{50}	Lethal dose, 50%
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NaCL	Sodium chloride
NADH	Nicotinamide adenine dinucleotide with hydrogen
NETs	Neutrophil extracellular traps
NO	Nitric oxide
NP	Nucleus pulposus
N_2	Nitrogen

OD	Optical density
ODc	Cut-off optical density
PAR-2	Protease-activated receptor-2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMMA	Polymethyl methacrylate
PO	Phenoloxidase
ProPO	Pro-phenoloxidase
ProPO AS	Pro-phenoloxidase- activating system
ROS	Reactive oxygen species
SAPHO	Synovitis, acne, pustulosis, hyperostosis and osteitis
SDS-PAGE	Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis
TBST	Tris-buffered saline with Tween-20
TGS	Tris/glycine/SDS
TGX	Tris-glycine extended
TLR	Toll-like receptor

TNF-α	Tumour necrosis factor-α
VB	Vertebral body
WBC	White blood cells

CHAPTER 1 INTRODUCTION

1.1 Lower Back Pain

1.1.1 Introduction

Lower back pain (LBP), which ranges from acute to chronic, is a major public health problem, with an estimated 70-85% of all people suffering from back pain at some point in life (Andersson, 1999). In addition to the psychological consequences, such as anxiety, depression and somatisation symptoms, back pain has a significant economic impact, with total costs, including insurance, medical costs, disability benefits and lost production, adding up to an estimated £12 billion per annum in the UK alone (Urban and Roberts, 2003). LBP is a leading cause of activity limitation and represents the second leading cause of sick leave worldwide (Lidgren, 2003). According to the systematic review of the global prevalence of LBP conducted by Hoy *et al*. (2012), this condition is most prevalent among females and persons aged 40-80 years.

Although 80-90% of LBP episodes are relatively short-lived, resolving spontaneously within 6-weeks, around 5-10% of patients develop chronic low back pain (CLBP) that lasts longer than 3 months (Manchikanti, 2000; Parthan, Evans and Le, 2006). There are various causes for LBP, including facet joint pain, sacroiliac joint dysfunction and spondylolisthesis (Cooper, 2015). However, approximately 40% of CLBP cases are associated with internal

disc disruption (IDD) of lumbar intervertebral discs (IVD), making discogenic lower back pain (DLBP) the most common cause of CLBP (Schwarzer *et al*., 1995; Cooper, 2015). IDD is a multifaceted, progressive spinal disease, with genetic, biochemical and biomechanical factors thought to play significant roles in degenerative changes within IVDs, although the mechanisms of such aetiological factors in relation to discogenic pain are still not completely understood (Sehgal and Fortin, 2000). The progression of IDD can result in disc herniation, also termed as IVD displacement, with disc herniation sub-divided into three types, namely protrusion, extrusion and sequestration (Fardon and Milette, 2001). Low-grade infection within IVDs, such as with coagulase-negative staphylococci (CoNS), *Corynebacterium propinquum*, Gram-positive cocci and, increasingly, *Cutibacterium acnes* (*C. acnes*), is also proposed to contribute towards IVD degeneration (IVDD) pathogenesis [Urquhart *et al*., 2015; Rollason *et al*., 2013; Albert *et al*., 2013 (a)].

Surgical intervention is a common form of treatment for patients suffering from chronic discogenic pain, with surgical procedures such as anterior fusion and posterior discectomy used in the management of patients with severe IVDD and disc herniation (Wang and Samartzis, 2014). However, these surgical procedures are highly invasive and are associated with a range of complications and side effects, including postoperative leg pain, recurrent disc herniation and infection in the case of open lumbar microdiscectomy (Kraemer *et al*., 2003).

1.1.2 Anatomy of the Vertebral Column

The vertebral column, also referred to as the spine, is a complex, movable anatomical structure, with four main segments extending in the cranial to caudal direction known as the cervical, thoracic, lumbar and sacral regions (Richardson and Groen, 2005). The vertebral column consists of 24 presacral vertebrae interspersed with IVDs, with associated ligaments (Prescher, 1998). As shown in Figure 1.1 below, vertebral structure is typically characterised by an anterior vertebral body, posterolateral pedicles, transverse processes and posterior laminae which fuse to create a neural arch which terminates in a bony projection found on the posterior end of each vertebrae, known as the spinous processes (Richardson and Groen, 2005). Within the posterior neural arch of each vertebra, a hollow, triangular opening known as the foramen is formed which encloses the spinal cord and spinal nerve roots (Figure 1.1) (Richardson and Groen, 2005). Increasing loads on the spinal column in the superior to inferior direction result in increased vertebral and IVD size in the cranial to caudal direction (Prescher, 1998). Bilateral facet joints are located at each spinal level between adjacent vertebrae which contribute to spine stability and motion (Figure 1.1) (Allegri *et al.*, 2016; Jaumard, Welch and Winkelstein, 2011).

Figure 1.1: Superior (A), lateral (B) and posterior (C) views of the anatomical structure of thoracic vertebra (Moulton, 2009).

1.1.3 Anatomy of the Lower Back

The lumbar spine is composed of five vertebrae (L1-L5), connected by ligaments, tendons, muscles and joint capsules, characterised by extensive innervation (Allegri *et al*., 2016).

Together, these five vertebrae are often referred to as the lumbar spine. The lumbar spine meets the sacrum, a triangular bone consisting of five vertebrae fused both anteriorly and posteriorly, at the lumbosacral joint (L5-S1). IVDs can be found interspersed within the spine and are found at every spinal level (Allegri *et al*., 2016).

1.1.4 The Intervertebral Disc

1.1.4.1 Anatomy and Mechanical Function

Lumbar IVDs lie between the vertebral bodies and represent the largest avascular structures within the human body (Binch, Cross and Le Maitre, 2014; González Martínez *et al.*, 2017). IVDs play an important mechanical role, facilitating the distribution of compressive loads from body weight and muscle activity through the spinal column. The outer region of the IVD is the annulus fibrosus (AF), characterised by concentric layers (lamellae) of fibrous cartilage, which surrounds the proteoglycan-rich inner nucleus pulposus (NP) (Allegri *et al*., 2016). The proteoglycans comprising the NP absorb water, providing the IVD with a hydrodynamic weight-bearing property (Melrose, 2016). In healthy IVD tissue, the inner AF and NP are virtually avascular and are subsequently not innervated due to the requirement of nerves of accompanying blood vessels for nutrition (Ito and Creemers, 2013). Therefore, the low oxygen tension within the NP leads to the reliance of cells of the NP on diffusion of

oxygen and nutrients down concentration gradients from peripheral blood vessels innervating cartilage end plates (Lotz, Fields and Liebenberg, 2013). As a result, cellular respiration is largely anaerobic, relying primarily upon anaerobic metabolism via glycolysis (Shapiro and Risbud, 2014). With *C. acnes* disc infection associated with promoting IVDD, the anaerobic environment of the disc may allow these anaerobic bacteria to preferentially colonise the NP component of the IVD (He *et al*., 2020).

Each IVD is surrounded by endplates which act as the interface between the bony vertebrae and the cartilaginous IVD (Melrose, 2016; Grant *et al*., 2016) (Figure 1.2). IVD endplates are composed of an osseous layer, the bony endplate (BEP), and a thin hyaline cartilage layer which surrounds the cranial and caudal surfaces of the IVD, the cartilage endplate (CEP) (Melrose, 2016). The CEP surrounds the cranial and caudal surfaces of the disc and exhibits hydraulic permeability, consequently playing an important role in the regulation of fluid and solute transport in/out of the disc (Wu *et al*., 2013; Malandrino *et al*., 2014).

Figure 1.2 Structure of the normal adult human IVD and surrounding spinal tissue. 1. Outer annulus fibrosus (AF) of anterior IVD; 2. Lamellar layers of the inner AF; 3. Nucleus pulposus (NP); 4. Superior cartilaginous endplate (CEP); 4a. Superior apophysis (bony outgrowth) where AF enters the vertebral body (VB); 5. Inferior CEF; 5a. Inferior apophysis; 6. Inferior VB; 7. Superior VB; 8. Posterior AF; 9. Spinal canal (Melrose, 2016).

1.1.4.2 Intervertebral Disc Degeneration

Early IVDD is characterised by the loss of proteoglycan in the NP, leading to higher tensile radial and compressive axial strains on the AF, a result of decreased NP pressure placing greater compressive load directly onto the AF (O'Connell *et al*., 1976). IVDD is also characterised by vertebral endplate ossification, reducing nutritional supply to cells of the IVD (Wang and Samartzis, 2014). Signs of IVDD include disc herniation and loss of disc

space height, increasing stresses placed on the facet joints and resulting in arthroplasty of these joints (Saleem *et al*., 2013). Extensive annular fissuring (tears within the outer layers of IVDs) can also be noted in association with IVDD (Saleem *et al*., 2013).

IVDD is a multifaceted condition associated with both genetic and environmental factors. Song *et al*. (2013) investigated genetic risk factors for IVDD, identifying carbohydrate sulphotransferase 3 (CHST3), an enzyme that catalyses proteoglycan sulphation, as a potential susceptibility gene. Mechanical loading is another aetiological factor suggested to play a role in IVDD, with Sun *et al*. (2013) demonstrating the ability of compressive loads to modulate the phosphorylation and disassembly of cytokeratin 8 (CK8). This intermediate filament protein is found within IVD NP and is involved in controlling protein synthesis and responses to mechanical stress (Sun *et al*., 2013). Aberrant expression of microRNAs is also suggested within the aetiology of IVDD, with the deregulation of miR-155 shown to promote Fas-mediated apoptosis in human IVD via Fas-associated protein with death domain (FADD) and caspase-3 (Wang *et al*., 2011). More recently however, bacterial infection has become a research focus linking bacterial species such as *C. acnes* to the potential pathophysiology of this disease state. Ganko *et al*. (2015) investigated associations between disc infections and the subsequent development of symptomatic degenerative disc diseases, concluding that patients with symptomatic disc disease had higher incidence rates of disc infections as compared to patients without this condition. In support of this, a systematic review conducted by Urquhart *et al*. (2015) assessing the contribution of low grade bacterial infection to LBP concluded that degenerative disc disease in patients with

LBP correlated with a higher positive tissue culture rate as compared to controls, with *C. acnes* being the most commonly identified microorganism within disc material cultures (Urquhart *et al*., 2015; Stirling *et al*., 2001).

1.1.5 Pathogenesis of Discogenic Low Back Pain

As explored in section 1.1.1, IDD leads to DLBP, with the progression of IDD potentially leading to disc herniation. DLBP is the most common form of CLBP, accounting for 39% of cases as compared to disc herniation which accounts for 30% of LBP cases (Zhang *et al*., 2009). Pain induction within DLBP patients is dependent upon two factors, namely the existence of free nerve endings, particularly pain receptors, and inflammation (Zhang *et al*., 2009). Changes in the water content of the NP (1.1.4.1) increases annular stress and causes tears or ruptures in the AF and end plate, exposing pain receptors (Zhang *et al*., 2009). During this disc degeneration, cells of the NP release inflammatory cytokines, with Burke *et al*. (2002) demonstrating significantly higher levels of interleukin-1 (IL-1), IL-6 and IL-8 release in DLBP patients as compared to disc herniation patients. These cytokines stimulate pain by interacting with free nerve endings (pain receptors) in endplate and/or AF fissures (Zhang *et al*., 2009). Interestingly, the proinflammatory cytokine tumour necrosis factor-α (TNF-α) has been implicated in disc herniation and nerve irritation and ingrowth, with studies using TNF-α inhibitors demonstrating the importance of this cytokine in LBP

(Risbud and Shapiro, 2014; Le Maitre, Hoyland and Freemont, 2007).

Increasingly, anaerobic bacteria, namely *C. acnes*, within IVDs are being suggested to stimulate inflammation and exacerbate disc degeneration, leading to CLBP, as explored further below (Tang *et al*., 2018; Yuan *et al*., 2018).

1.2 *C. acnes*

C. acnes, formerly known as *P. acnes* after the ability of this bacterium to ferment carbohydrates to propionic acid [Shu *et al*., 2013 (a)], is related to the genera *Actinomyces* and *Arcanobacterium* (Poppert, Riecker and Essig, 2010). Historically referred to as *Corynebacterium acnes* (Puhvel and Reisner, 1970), *C. acnes* is a member of the cutaneous Cutibacteria genus, as are *Cutibacterium avidum* and *Cutibacterium granulosum* (Bojar and Holland, 2004). *C. acnes* can be differentiated from other commensal cutaneous Cutibacteria through the use of biochemical tests (Table 1.1) (Eady and Ingham, 1994).

Table 1.1 Biochemical tests used for the differentiation of commensal cutaneous Cutibacteria and Propionibacteria [adapted from Eady and Ingham (1994); BacDive, n.d.)].

Test	*C. acnes*	*C. avidum*	*C. granulosum*	*P. innocuum*	*P. propionicum*
Indole production	+	-	-	-	-
Nitrate reduction	+	-	-	+	+
Aesculin hydrolysis	-	+	-	-	-
Casein hydrolysis	+	+	-	-	-
β-galactosidase	+	+/-	-	-	+
Fermentation of D-mannose	+	+	+/-	-	-
Glutamate decarboxylase	-	-	-	-	-
Catalase production	+	+	+	+	-
Optimum atmospheric conditions for growth	Anaerobic	Similar growth aerobically and anaerobically	Anaerobic	Aerobic	Anaerobic

C. acnes is a Gram-positive, non-spore-forming, non-motile pleomorphic rod-shaped bacterium, measuring 0.5µm in diameter and around 3µm-4µm in length (Montes and Wilborn, 1970). *C. acnes* has an optimal temperature for growth of 37°C and can be incubated for up to 14-days on agar (Portillo *et al.*, 2013). Most strains of *C. acnes* are catalase and indole positive. Culture of *C. acnes* on blood agar plates leads to the formation

of small, opaque, convex, tan-coloured colonies with smooth, glistening surfaces.

Although *C. acnes* is considered to be a strict anaerobe, the *C. acnes* genome encodes all of the key components required for oxidative phosphorylation, such as nicotinamide adenine dinucleotide with hydrogen (NADH) dehydrogenase/complex I, cytochrome c reductase and cytochrome c oxidase (Portillo *et al*., 2013). *C. acnes* also expresses cytochrome d oxidase which, in *Escherichia coli* (*E. coli*), is upregulated in conditions of low aeration (Brüggemann, 2005). Therefore, *C. acnes* has the ability to survive for several hours in low-oxygen environments (Csukas, Banizs and Rozgonyi, 2004). This bacterium also has the potential to survive for up to 8 months under anaerobic conditions, suggesting that *C. acnes* can cause prolonged infections within human tissues with low oxidation potentials (Csukas, Banizs and Rozgonyi, 2004). However, despite the fact that *C. acnes* can be cultured in oxygen at 100% saturation, this leads to reduced growth rates (Cove, Holland and Cunliffe, 1983). Therefore, *C. acnes* is best described as a facultative anaerobic microorganism (Schlecht, Freudenberg and Galanos, 1997).

C. acnes accounts for approximately half of the total skin microbiome, with bacterial counts ranging from 10^{1} per cm^{2} from the legs (McGinley, Webster and Leyden, 1978) to 10^{5-6} per cm^{2} from the scalp and face (McGinley *et al*., 1980). However, the quantitative characterisation of skin commensals is complicated due to the effects of environmental

factors on the colonisation of the skin (Evans, 1975). In addition to preferentially colonising regions of the skin rich in sebaceous follicles (McGinley, Webster and Leyden, 1978), *C. acnes* can also be found colonising the conjunctiva, oral cavity, upper respiratory tract and the large intestine (Perry and Lambert, 2011).

Johnson and Cummins (1972) used cell wall sugar analysis and the results of serological agglutination tests to identify two distinct *C. acnes* phenotypes which were designated types I and II. Comparing cell wall sugar patterns demonstrated that the cell walls of type I *C. acnes* strains contain galactose, glucose and mannose whereas the cell walls of type II strains contain only glucose and mannose (Johnson and Cummins, 1972). Investigating variations in susceptibility to phage infection and differences in fermentation properties were methods also employed to differentiate between *C. acnes* phylotypes I and II (Voss, 1970).

C. acnes ferments glucose, fructose and glycerol but does not ferment lactose, salicin, sucrose, maltose, xylose or arabinose (Moss *et al*., 1967). *C. acnes* can be differentiated from other Gram-positive bacteria by its ability to form large quantities of propionic acid and acetic acid which are metabolic end-products produced following the utilisation of carbon-sources, such as glucose and glycerol. The short-chain fatty acid propionic acid has been shown to exhibit antimicrobial effects against methicillin-resistant *S. aureus* strain USA300 (Wang *et al*., 2014), demonstrating the adaptation of *C. acnes* to colonise the skin

and eliminate competing microbes.

Like many Gram-positive microorganisms, the cell wall of *C. acnes* consists largely of polysaccharide and peptidoglycan, with the cell wall polysaccharide consisting of the hexose sugars glucose, galactose and mannose, along with the hexosamines glucosamine and galactosamine (Cummins and White, 1983). *C. acnes* peptidoglycan amino acids include alanine, glutamic acid, glycine and L-diaminopimelic acid (DAP) (Johnson and Cummins, 1972; Schleifer, Plapp and Kandler, 1968). Cummins and Harris (1958) demonstrated variations in the amino acid composition of the cell wall of five different Propionibacteria strains. *Propionibacterium shermanii* contains only alanine, glutamic acid and meso-DAP whereas four other Propionibacteria strains expressed LL-DAP in place of meso-DAP and, additionally, expressed glycine (Cummins and Harris, 1958). Variations in the distribution of these two types of murein within the genus *Cutibacterium* may account for the different cellular morphologies within the genus, with meso-DAP expressing *Cutibacterium* species existing in coccoid forms whereas LL-DAP expressing strains form coryneform rods (Schleifer, Plapp and Kandler, 1968). Additionally, Montes and Wilborn (1970) demonstrated evidence of a floccular layer external to the cell wall of *Corynebacterium acnes* cells, with Hard (1969) demonstrating the cohesive nature of this superficial floccular material in promoting the adhesion of neighbouring *Corynebacterium ovis* cells. The thick Gram-positive cell wall of Cutibacteria promotes the high structural stability of these bacteria, making these bacteria resistant to drying and osmotic shock (Bojar and Holland, 2004). These are important attributes of Cutibacteria which promote their colonisation of the

skin as the skin surface is subject to regular fluctuations in temperature and salt/ion concentrations (Bojar and Holland, 2004).

Other methods of discriminating between *C. acnes* types I and II have since been developed, such as the sequence analysis of the conserved housekeeping *rec*A gene and the cytotoxin/ haemolysin *tly* virulence gene, which has led to the identification of the phylotypes IA, IB, II and III (McDowell *et al*., 2005; McDowell *et al*., 2008). Multilocus sequence typing (MLST) has been used to further subdivide types IA, IB, II and III *C. acnes* isolates into specific sequence types, with MLST being used in combination with whole genome sequencing to further divide the type IA clade into types IA_1 and IA_2 (McDowell *et al*., 2012). Distinguishing between these four different evolutionary lineages is important due to variations in virulence factor production and inflammatory properties of these different phylotypes which are suggested to be implicated in causing different clinical conditions (McDowell *et al*., 2012; Valanne *et al*., 2005; Lodes *et al*., 2006). However, the use of *recA* sequence analysis alone can result in the misidentification of certain type IA isolates as type IB due to sequence similarity, highlighting the lack of specificity of this method (McDowell *et al*., 2012). Additionally, Niazi *et al*. (2010) identified discrepancies between the use of *recA* typing and putative virulence factor sequence analysis when establishing phylogenetic relationships between *C. acnes* strains, potentially as a result of intraspecies recombination. Therefore, although *recA* sequence analysis allows for the superficial classification of *C. acnes* strains, distinguishing between *C. acnes* strains using additional gene sequences allows for the appreciation of the true complex nature of *C. acnes* taxonomic relationships.

1.2.1 Reclassification of Cutaneous Propionibacteria

The genus Propionibacterium is traditionally segregated into classical and cutaneous propionibacteria, with classical propionibacteria associated with dairy products and cutaneous propionibacteria usually associated with human skin (Holland and Bojar, 2002). However, following 16S rRNA sequence analysis, high-resolution core genome analysis and biochemical analysis, cutaneous propionibacteria were reclassified under the new genus Cutibacterium, with *P. acnes* subsequently being renamed *C. acnes* (Dréno *et al*., 2018; Scholz and Kilian, 2016). Thus, Cutibacterium is the new genus for *C. avidum*, *C. acnes* and *C. granulosum* and two recent Cutibacterium species, namely *C. namnetense* and *C. modestum* (previously known as *C. humerusii*) (Corvec, 2018). *C. namnetense*, formerly known as *P. namnetense*, is closely related to *C. acnes* and is increasingly being associated with bone infections (d'Epenoux *et al*., 2020). *C. modestum* was originally isolated from the meibum of inflamed human meibomian glands, with 16S rRNA gene sequence analysis of *C. modestum* showing 98.0% similarity to *C. acnes* (Dekio *et al*., 2020).

1.3 Conditions Associated with *C. acnes*

C. acnes is commonly considered to be a non-pathogenic commensal. However, in recent years, these bacteria have been implicated in a range of pathologies, most notably within the

aetiology of acne vulgaris (Perry *et al*., 2003). Numerous reports, however, indicate that *C. acnes* causes severe infections at various body sites. These include bone and joint infections (Zeller *et al*., 2007) and central nervous system infections (Ramos, Esteban and Soriano, 1995), with several predisposing conditions for *C. acnes* infections having been identified, including the presence of foreign bodies, immunosuppression, trauma and diabetes (Funke *et al*., 1997). However, *C. acnes* is found in both healthy and disease states, meaning it is difficult to identify this microorganism as a cause of particular diseases. Therefore, *C. acnes* is commonly dismissed as a contaminant of clinical samples and laboratory cultures (Rollason *et al*., 2013; Levy *et al*., 2008).

1.3.1 Acne

The most well investigated disease associated with *C. acnes* is the skin condition acne vulgaris, which affects between 40-50 million individuals in the United States alone (White, 1998). This skin commensal is usually beneficial for the host, occupying a niche within the skin microbiome which could otherwise be colonised by more pathogenic and invasive microorganisms. However, despite decades of research, it is still not clear how *C. acnes* contributes to acne pathogenesis whilst being a major commensal of the normal skin flora (Fitz-Gibbon *et al*., 2013). Nevertheless, strain population structures differ among acne patients and healthy individuals, with strains belonging to ribotypes 4 and 5 strongly

associated with acne and strains of ribotype 6 strongly associated with healthy skin (Fitz-Gibbon *et al*., 2013). Several factors have been identified which are thought to contribute to an individual's susceptibility of developing acne vulgaris, including androgens (Ebede, Arch and Berson, 2009), stress (Isard *et al*., 2009) and medications (such as steroids and oral contraceptives). However, Ebede, Arch and Berson (2009) argue that oral contraceptives suppress ovarian androgen production, offering a potential hormonal treatment for acne.

Comedogenesis is a process whereby a normal human pilosebaceous follicle, as shown in Figure 1.3, undergoes structural transformation to become a primary acne lesion called the comedone, often due to abnormal follicular keratinisation due to excessive sebum secretion (seborrhoea) (Bhatia, Maisonneuve and Persing, 2004). *C. acnes* then becomes trapped in layers of corneocytes, cells that constitute the superficial epidermis, and sebum, promoting the formation of either closed comedones (whiteheads) or open-structured comedones (blackheads) (Bhatia, Maisonneuve and Persing, 2004). Usually, follicular material consisting of cell debris, sebum and *C. acnes* is evacuated at the skin surface in normal pilosebaceous units (Thody and Shuster, 1989; Bojar and Holland, 2004). However, unlike in open comedones, closed comedones cannot evacuate this follicular material, leading to hypoxia within the duct (Bhatia, Maisonneuve and Persing, 2004). Low oxygen levels stimulate *C. acnes* growth, leading to the rupture of comedones and the subsequent dispersal of follicular material within the dermis of the skin (Dreno *et al*., 2015). This triggers a local reaction of the innate immune system and the progression from non-inflammatory to inflammatory acne characterised by the formation of papules, pustules or nodules (Danby,

2013; Bhatia, Maisonneuve and Persing, 2004).

There are four major pathophysiological features of acne which include increased sebum production (seborrhoea), hyperkeratinisation and obstruction of the follicular epithelium, proliferation of *C. acnes* and inflammation (Cogen, Nizet and Gallo, 2008).

Figure 1.3 Structure of the normal human pilosebaceous gland. HF, hair follicle; ORS, outer root sheath; IRS, inner root sheath; CL, companion layer; FS, fibrous sheath; SG, sebaceous gland; AP, arrector pili (Toll *et al*., 2004).

Sebum is produced by the sebaceous glands and is a mixture of lipids mainly composed of triglycerides, wax esters, squalene, free fatty acids and trace amounts of cholesterol, cholesterol esters and diglycerides (Picardo *et al*., 2009). Seborrhoea and alteration in the lipid composition of sebum are major events associated with acne development (Makrantonaki, Ganceviciene and Zouboulis, 2011). Sebum is secreted by the sebaceous glands and passes to the lumen of the pilosebaceous unit via the sebaceous duct. From the pilosebaceous unit, sebum is secreted at the skin surface. As sebum migrates to the surface of the skin, the triglyceride composition of the sebum is reduced whilst the concentration of free fatty acids within the sebum increases (Gribbon, Cunliffe and Holland, 1993). Marples, Downing and Kligman (1971) discovered that free fatty acid levels were proportional to the density of *C. acnes* within scalp washings, implicating the action of *C. acnes* lipase in the generation of free fatty acids. However, gender bias existed within this study as all scalp washings were obtained from male patients. In support of the findings of Marples, Downing and Kligman (1971), Ingham *et al*. (1981) demonstrated that purified *C. acnes* lipase had the ability to hydrolyse a range of triglycerides. These findings suggest that *C. acnes* residing within the pilosebaceous unit may gain nutritional advantage from the hydrolysis of triglycerides in sebum to free fatty acids and that increased sebum production may promote the growth of *C. acnes* (Gribbon, Cunliffe and Holland, 1993). Additionally, Ferguson and Cummins (1978) demonstrated that oleic acid, produced as an alternative to linoleic acid within sebum, induces *C. acnes* growth *in vitro*. However, Weeks *et al*. (1977) demonstrated that a significant reduction in the levels of free fatty acids within sebum had no effect on the clinical severity of acne in volunteers, suggesting that *C. acnes* is not dependent on lipases

for energy or growth requirements. In contrast to these findings, Gribbon, Cunliffe and Holland (1993) proposed that lipase produced by *C. acnes* may act as a possible colonisation factor *in vivo*, aiding in the colonisation of the pilosebaceous follicle through the adherence of bacterial cells to components such as oleic acid.

The second major pathophysiological feature of acne development is hyperkeratinisation and obstruction of the follicular epithelium. Lavker, Leyden and McGinley (1981) suggested that *C. acnes* is not essential in the initiation of abnormal keratinisation. However, lesions of prepubertal children were sampled and, as shown by Jappe (2003), sebaceous glands are androgen-targeted organs which are stimulated to produce sebum only at puberty and beyond. This may explain why Lavker, Leyden and McGinley (1981) failed to identify *C. acnes* colonisation of acne lesions. In contrast, Kligman (1974) proposed that *C. acnes* may play a key role in inducing abnormal keratinisation, with a change being induced in the pattern of keratinisation of the epithelial lining of the infundibulum. As the granular layer becomes more prominent, the horny cells stick together and form a horny layer, with cells packing together tightly (Kligman, 1974). These cells no longer slough, creating an anaerobic, sebum-rich environment within the pilosebaceous unit which promotes *C. acnes* growth. However, the mechanisms associated with changes in the infundibulum microstructure still require further investigation (Guy, Green and Kealey, 1996).

Additionally, Graham *et al*. (2004) demonstrated the ability of *C. acnes* to stimulate

keratinocytes to significantly increase the production of the proinflammatory cytokine interleukin-1 alpha (IL-1α) which is thought to promote follicular hyperkeratinisation of the infundibulum *in vivo* and *in vitro* (Guy, Green and Kealey, 1996). However, infundibula were isolated by microdissection of redundant skin and this invasive procedure may have promoted the release of proinflammatory cytokines, including IL-1α. In support of these findings, Ingham *et al*. (1992) found that the majority of open comedones expressed high levels of IL-1α. IL-1 has also been reported to be up-regulated in rat tail discs following inoculation with *C. acnes*, with subsequent disc degeneration and vertebral endplate erosion, demonstrating the proinflammatory properties of this microorganism [Dudli *et al*., 2016 (a)].

The third major pathophysiological feature of acne development is proliferation of *C. acnes*. De Young *et al*. (1984) demonstrated that intradermal injections of *C. acnes* into the ears of rats led to chronic inflammation and the formation of acneiform lesions whereas *Streptococcus lactis*, *E. coli* B and *Staphylococcus epidermidis* (*S. epidermidis*) failed to induce chronic inflammation and high acneiform lesion counts. However, the extent to which this animal model can be extrapolated to the role of *C. acnes* within human acne causation is questionable. Knop, Ollefs and Frosch (1983) demonstrated the presence of immunoglobulin G (IgG), albumin and anti-*C. acnes* IgG antibodies in extracts taken from non-inflamed comedones of acne patients, suggesting a role of *C. acnes* within acne pathogenesis. Furthermore, tetracycline and erythromycin are effective in the treatment of acne vulgaris, with Jain *et al*. (2002) suggesting that the beneficial effects of these antibiotics may be due to their antibacterial effects against *C. acnes* in addition to their anti-

inflammatory properties. These findings strengthen the hypothesis that *C. acnes* plays an important role in acne pathogenesis.

However, although there is research supporting the role of *C. acnes* as the causative agent of acne vulgaris, other research findings do not support this conclusion. Some researchers suggest that the colonisation of the pilosebaceous follicle by *C. acnes* may be a side effect of inflammation as opposed to the cause of inflammation. Additionally, Leyden *et al*. (1975) isolated *C. acnes* from subjects with and without acne and observed no correlation between *C. acnes* colonisation densities and the severity of acne vulgaris. Leeming, Holland and Cunliffe (1984) demonstrated evidence that not all follicles are colonised by microorganisms, although a total of only 54 acne vulgaris patients were sampled. McLaughlin *et al*. (2019) also suggested that skin surface concentrations of Cutibacteria do not differ between individuals with and without acne (healthy skin). Whilst inflammation is known to be associated with the later stages of acne development, inflammatory processes are increasingly being reported to occur much earlier on in acne lesion evolution than previously anticipated, with some lesions becoming inflamed in the absence of bacteria (McLaughlin *et al*., 2019). This suggests that *C. acnes* may not be a pre-requisite factor for the initiation of inflammation seen in acne patients (McLaughlin *et al*., 2019). Furthermore, beta-lactam antibiotics are ineffective against acne, although Tyrrell *et al*. (2006) demonstrated that *C. acnes* is fully susceptible to the beta-lactam antibiotic daptomycin, suggesting that *C. acnes* may not be the causative agent of acne vulgaris. However, these *C. acnes* isolates originated from a range of human clinical samples and, therefore, may not

express the same antibiotic susceptibility profiles as *C. acnes* isolates obtained specifically from acne lesions.

The fourth main pathophysiological aspect of acne is inflammation. Much of the research conducted within the field of inflammation-induced acne investigates the pathogenic traits of *C. acnes*, which are explored in detail in section 1.4.3.1 [Modic Changes and Associated Lower Back Pain (LBP)]. These include increasing the expression of proinflammatory cytokines, such as TNF-α, IL-1β and interferon-γ (IFN-γ), promoting inflammatory cell infiltration of infected tissue and increasing lipase production, with subsequent liberated free fatty acids acting as chemotactic and highly proinflammatory factors [Albert *et al*., 2008 (a); Shan *et al*., 2017; Lee *et al*., 1982].

Porphyrins are a group of pigmented, organic compounds and are metabolic products of Cutibacteria [Shu *et al*., 2013 (b); Borelli *et al*., 2006]. Porphyrins are suggested to play a role in promoting perifollicular inflammation, with Schaller *et al*. (2005) identifying a predominance of coproporphyrin III in acne lesions, with this porphyrin fraction inducing the expression of the proinflammatory cytokine IL-8 within keratinocytes. The association of porphyrins in promoting inflammation seen in acne vulgaris is further supported by Borelli *et al*. (2006) who demonstrated a clinical correlation between porphyrin level reductions and clinical improvement within six acne patients following 2-months of isotretinoin therapy.

1.3.2 Discitis

Two research papers published by a Danish-British research group suggested that *C. acnes* may be implicated in the pathophysiology of discitis, an infection of the IVD tissue within the back, leading to CLBP [Albert *et al*., 2013 (a); Albert *et al*., 2013 (b)]. These studies have prompted a paradigm shift in the way in which CLBP is managed. Albert *et al*. [2013 (a)] obtained herniated nucleus material from lumbar disc herniations of 61 patients, with 28 of these samples being positive for microbial culture and 26 of these 28 samples positive specifically for anaerobic bacteria. In the discs with a nucleus containing anaerobic bacteria, which most frequently was *C. acnes*, 80% of these patients went on to develop new Modic changes in the vertebrae adjacent to the previously herniated disc, supporting the theory that these Modic changes may be due to oedema surrounding an infected disc [Albert *et al*., 2013 (a)]. However, Grand *et al*. (1993) demonstrated that lumbar disc surgery in the absence of infection may cause vertebral endplate changes, suggesting a non-microbial cause of such Modic changes. The term Modic changes was first described by Modic *et al*. (1988) and refers to signal intensity changes of vertebral endplates and subchondral bone indicative of vertebral bone marrow changes associated with degenerative disc disease visible by magnetic resonance imaging (MRI) (Zhang *et al*., 2008). Modic changes are classified into 3 subtypes, namely type I, type II and type III, each characterised by varied T1 and T2-weighted signal intensities as viewed by MRI (Modic *et al*., 1988). Type I Modic changes are characterised by vertebral body oedema and hypervascularity, Modic type II changes are characterised by fatty replacements of the red bone marrow within the vertebral body and

type III Modic changes are indicative of subchondral bone sclerosis (Modic *et al*., 1988).

Albert *et al*. [2013 (b)] investigated the efficacy of antibiotic therapy in CLBP patients with Modic type I changes in vertebral endplates adjacent to previously herniated vertebral discs. One hundred and sixty-two patients were randomised into 1 of 4 groups; 2 groups with 45 patients each and two groups with 36 patients each [Albert *et al*., 2013 (b)]. Patients in the first two groups received either single or double doses of the antibiotic amoxicillin–clavulanate for 100-days whereas patients in the second two groups received either single or double doses of the placebo calcium carbonate for 100-days [Albert *et al*., 2013 (b)]. In comparison to the placebo group, the antibiotic group improved on all primary outcome measures, including lumbar pain and disease-specific disability, with improvement continuing from 100-days follow-up until 1-year follow-up [Albert *et al*., 2013 (b)]. Whilst many antibiotics can have anti-inflammatory effects, primarily via TNF-α inhibition, amoxicillin-clavulanate has only a very small anti-inflammatory effect compared to other antibiotics and does not have any inhibitory effects on TNF-α present in patients with Modic changes [Albert *et al*., 2013 (b)]. Additionally, anti-inflammatory effects of antibiotics are usually rapid whereas in the study conducted by Albert *et al*. [2013 (b)], these anti-inflammatory effects took 6-8 weeks to become evident. Therefore, taken together, this suggests that the clinical improvements seen within the patients in the antibiotic treatment group were the result of an antibiotic effect as opposed to an anti-inflammatory effect of this treatment [Albert *et al*., 2013 (b)]. These findings support a bacterial role in some Modic changes, potentially through the colonisation of avascular discs by anaerobic bacteria, like

C. acnes, through neovascularisation and the release of propionic acid by these microorganisms adjacent to spinal vertebrae [Albert *et al*., 2008 (b)]. This suggests that an infection originating within the IVD can have 'side effects' in adjacent bone in the form of Modic changes [Albert *et al*., 2013 (b)]. These findings are disputed by Bråten *et al*. (2019) who randomised CLBP patients with associated disc herniation and Modic changes into one of two groups, namely three-months of treatment with amoxicillin or three-months of administration of a placebo. Roland-Morris Disability Questionnaire (RMDQ) scores were used as the primary outcome measure at one-year follow-up (Bråten *et al*., 2019). Results showed that three-months of treatment with amoxicillin did not provide a clinically important benefit compared to the use of a placebo, suggesting that bacteria may not be involved in causing the pain and degeneration experienced by some CLBP patients presenting with disc herniation and associated Modic changes (Bråten *et al*., 2019).

Adverse side effects were common in the antibiotic group (65%) as compared to the placebo group (23%), with 13 participants dropping-out from the antibiotic group in comparison to only five drop-outs from the placebo group [Albert *et al*., 2013 (b)]. Gastroenterological side effects, such as loose bowel movements and flatus, were common in the antibiotic group which may have been caused by the perturbation of the gastrointestinal bacterial microflora due to increased antibiotic usage, a significant limitation of this treatment method [Albert *et al*., 2013 (b)]. In addition to causing adverse physiological effects within study participants, this treatment method may also induce antibiotic resistance, with Segal *et al*. (2018) demonstrating that long-term antibiotic treatment within pouchitis patients resulted in the

isolation of antibiotic resistant bacteria from at least one stool sample within 78% of patients. With regards to acne treatment and *C. acnes* specifically, Patel *et al*. (2010) identified specific criteria which could influence the development of antibiotic resistance in patients with acne. This included antibiotic monotherapy, indiscriminate use of antibiotics outside their indications and, particularly, long-term administration of antibiotics (Patel *et al*., 2010). Antibiotic usage also does not neutralise toxins secreted by bacteria (Wang *et al*., 2018). This, coupled with the demonstration that *C. acnes* biofilm formation increases resistance against antimicrobial agents, further complicates the treatment options for *C. acnes*-associated infections (Coenye, Peeters and Nelis, 2007). Additionally, in the Albert *et al*. [2013 (b)] study, all patients continued to take their usual anti-inflammatory and pain-relieving medication, a significant confounding variable which could account for the improvement seen in primary outcome measures. These findings highlight the need to undertake further research to assess the pathogenic role of *C. acnes* in disc herniation which may promote the identification of novel *C. acnes* microbial targets. This would prevent the over-use of antibiotics and the development of antibiotic-resistant bacteria. Furthermore, investigations into the effects of prolonged antibiotic usage on *C. acnes* virulence are warranted as this would inform antibiotic treatment strategies for deep tissue *C. acnes* infections.

The limitations associated with conventional antibiotic therapy has prompted researchers to explore alternative methods to manage *C. acnes*-associated infections, such as targeting specific microbial virulence products (Wang *et al*., 2018). *C. acnes*-targeted vaccines

provoke the generation of specific antibodies against the target bacteria, with inactivated *C. acnes* vaccines also showing *in vivo* protective immunity against future challenge with the target bacteria (Nakatsuji *et al*., 2008). One such microbial target exploited within *C. acnes* vaccine development is the pore-forming toxin Christie Atkins Munch-Petersen (CAMP) factor, with a vaccination approach to this target shown to reduce inflammation in an explant model of acne caused by *C. acnes* (Wang *et al*., 2018). Such a vaccine would utilise anti-CAMP factor monoclonal antibodies to directly interact with secreted CAMP factor in *C. acnes* infected tissue to neutralise this virulence factor and attenuate proinflammatory cytokine release (Wang *et al*., 2018). The specific inhibition of secreted *C. acnes* virulence factors presents less selective pressure for the generation of resistant bacteria (Rasko *et al*., 2008). However, despite research into CAMP factor vaccines for application within acne patients demonstrating reductions in inflammation, few researchers have investigated CAMP factor production within *C. acnes* isolates taken from herniated IVD tissue.

Stirling *et al*. (2001) suggested a possible microbial cause of the inflammation associated with sciatica, which refers to pain, numbness or tingling felt due to the irritation or compression of the sciatic nerve. A newly developed enzyme-linked immunosorbent assay (ELISA) test was used to identify deep-seated infections caused by Gram-positive microorganisms, with 43 of 140 (31%) of sciatica patients testing positive (Stirling *et al*., 2001). Disc material from a further 36 patients with severe sciatica was cultured for microorganisms and 19 of these patients (53%) had positive cultures, with *C. acnes* being isolated from 16 of the 19 (84%) positive samples (Stirling *et al*., 2001). These results

suggest that microorganisms, particularly *C. acnes*, are associated with chronic low-grade infection within the IVDs of sciatica patients. However, the presence of *C. acnes* within herniated disc tissue may be attributed to the contamination of samples during surgery and/or during laboratory culture (Carricajo *et al*., 2007). This is refuted by Savage *et al*. (2012) who demonstrated that effective skin preoperative preparation can reduce the overall rate of positive cultures from skin overlying the lumbar region to 0%. Furthermore, Capoor *et al*. (2017) provided the first visual evidence of *C. acnes* biofilms within IVD tissue specimens, consistent with infection rather than microbiological contamination. Therefore, the possible association between *C. acnes* and chronic low-grade infection in herniated discs should not be readily attributed to sample contamination, suggesting that further investigations into *C. acnes* virulence mechanisms in association with lumbar disc herniations are warranted.

1.3.3 Implant-Associated Infections

C. acnes is increasingly being recognised as an opportunistic pathogen within a range of implant-associated infections, including infections of intraocular lenses, breast implants and periprosthetic joint implants, as shown in Figure 1.4 (Portillo *et al*., 2013).

Figure 1.4 Range of implant-associated infections caused by *C. acnes* (Portillo *et al*., 2013).

The seeding of prosthetic implants with *C. acnes* can occur during contamination of the surgical site by skin microbiota or through haematogenous spread to implanted prostheses, as demonstrated by Blomgren *et al*. (1981) in an animal-based knee-joint replacement study. An important virulence characteristic of *C. acnes* in association with a range of prosthetic implant-associated infections is biofilm formation on abiotic, inert, orthopaedic biomaterials, such as breast implants (Del Pozo *et al*., 2009; Rieger *et al*., 2013). Biofilm formation is thought to increase the resistance of biofilm-associated isolates to a range of antimicrobials, making the management of prostheses-associated infections with antibiotics difficult

(Ramage *et al*., 2003). Additionally, biofilm formation on prosthetic implant surfaces may stimulate inflammation and chronic, low-level infection, as demonstrated by Pajkos *et al*. (2003) within breast implant patients. Therefore, prosthetic joint infection treatment often involves surgical debridement and/or removal of the infected prosthesis (Moran *et al*., 2007). However, the low-virulence nature of *C. acnes* can lead to delayed prosthetic implant-associated infections, with some infections occurring months after surgery, as shown by Zambrano *et al*. (1989) who identified nine *C. acnes* endophthalmitis patients presenting with symptoms around four months after cataract surgery. Additionally, the fastidious growth requirements of *C. acnes* and the variety of culture methods employed within the literature further complicates the identification of *C. acnes* as a cause of prosthetic joint infections. Despite this, Butler-Wu *et al*. (2011) demonstrated that a minimum culture period of 13-days using both aerobic and anaerobic culture media promotes the identification of *C. acnes* within periprosthetic specimens. Molecular diagnostic techniques, such as polymerase chain reaction (PCR), are also increasingly being used to detect bacterial DNA in small sample volumes containing small numbers of bacteria (Hykin *et al*., 1994).

Levels of inflammatory markers, such as erythrocyte sedimentation rates (ESR) and C-reactive protein (CRP), vary significantly among different patients (Dramis *et al*., 2009), making the preoperative identification of suspected prosthetic implant-associated infections difficult. This may be attributed to the low virulence of *C. acnes*. Piper *et al*. (2009) also demonstrated that the sonication of prostheses improved the sensitivity for the detection of

prosthetic shoulder infections as compared to periprosthetic tissue culture. However, there is no standardised protocol relating to sample collection and processing in suspected prosthetic joint infection patients.

Although the prophylactic use of antibiotics in prosthetic-implant surgery is common, studies have demonstrated no significant difference in infection rates between patients receiving prophylactic antibiotics and those receiving no antibiotics (LeRoy and Given, 1991; Mirzabeigi *et al*., 2012). However, the choice of antibiotics and duration of antibiotic therapy may affect infection rates within prosthetic implant surgery patients. The prevention of biofilm formation on prosthetic implant surfaces using antibiotic-impregnated biomaterials could reduce the use of prophylactic antibiotics (Jacombs *et al*., 2012).

1.3.4 Other Conditions Associated with *C. acnes* Infection

C. acnes has been identified as an aetiological agent within a range of conditions. These include sarcoidosis, synovitis, acne, pustulosis, hyperostosis and osteitis (SAPHO) syndrome, Noma, central nervous system (CNS) infections, Kawasaki disease and prostate cancer (Ishii *et al*., 2010; Negi *et al*., 2012; Eishi, 2013; Roldan *et al*., 2001; Edlund *et al*.,1988; Paster *et al*., 2002; Chung *et al*., 2011; Burnham *et al*., 2014; Ramos, Esteban and Soriano, 1995; Zaffiri *et al*., 2013; Tomita *et al*., 1987; Cohen *et al*., 2005; Alexeyev *et al*.,

2006). A common denominator within all of these conditions is the promotion of inflammation following *C. acnes* infection. For example, *C. acnes* has been shown to initiate a chronic low-grade inflammatory response within bone and prostate tissue which could lead to SAPHO syndrome and prostate cancer (Colina *et al*., 2007; Cohen *et al.*, 2005; Alexeyev *et al*., 2006). Additionally, the incubation of *C. acnes* with sarcoid patient bronchoalveolar lavage cells significantly increased the expression of the proinflammatory cytokines granulocyte macrophage-colony stimulating factor (GM-CSF) and TNF-α as compared to unstimulated cells. These cytokines are necessary for granuloma formation seen in sarcoidosis patients, with *C. acnes*-induced TNF-α production also involved in the damage and oedema of vertebral endplates and subchondral bone marrow visualised as Modic type I changes on MRI scans (Schupp *et al*., 2015; Chen *et al*., 2016). The difficulty in identifying *C. acnes*-associated CNS infections is attributed to the indolent, low-virulence nature of this microorganism, leading to long delays in symptom expression (Kranick, Vinnard and Kolson, 2009; Nisbet *et al*., 2007). This chronicity of *C. acnes* infection due to the long generation times of these bacteria has also been suggested to contribute towards prolonged, low-grade infections in the lower IVDs of patients with severe sciatica (Stirling *et al*., 2001). This suggests that the slow growing, proinflammatory nature of these bacteria can contribute to subsequent disc infection.

In summary, the role of *C. acnes* in a range of clinical conditions is increasingly being evidenced, suggesting that these bacteria should no longer be considered a simple contaminant of clinical samples. Therefore, it is critically important that clinical

microbiology laboratories consider the implementation of extended culture incubation periods where *C. acnes* infection is suspected in order to maximise the recovery of this anaerobe from patient samples. In the future, as new pathogenic mechanisms of these bacteria are discovered, our understanding of the role *C. acnes* plays in human disease will continue to develop, potentially leading to novel treatment and infection prevention strategies to reduce patient morbidity rates.

1.4 *C. acnes* Discitis and Associated Modic Changes and Chronic Low Back Pain: An Overview

1.4.1 Epidemiology of *C. acnes* within Intervertebral Disc Tissue

1.4.1.1 Prevalence, Patient Characteristics and Phylotype Distribution

The systematic review conducted by Urquhart *et al*. (2015) concluded that the average median age and percentage of female participants within 11 studies investigating LBP in association with bacterial infection of lumbar disc tissue was 44.7 years and 41.5%, respectively. In support of this, Capoor *et al*. (2017) reported an average age of 49.3 ± 13.6 years for male and female patients enrolled within the study. Additionally, gender correlated significantly with *C. acnes* positivity, with a significantly higher prevalence of *C. acnes* in

disc specimens obtained from males (39% of 222 males) as compared to females (23% of 146 females) (Capoor *et al*., 2017). Furthermore, patients with *C. acnes* culture positivity were younger than culture-negative patients (46.7 years vs. 50.5 years, respectively) which may be attributed to the ability of *C. acnes* to accelerate age-related disc degeneration (Capoor *et al*., 2017).

Stirling *et al*. (2001) were the first to report the presence of *C. acnes* within IVD tissue taken from patients presenting with sciatica, with an overall positivity rate of 44.4% (16/36). IVDs sampled within this study and the studies discussed below were degenerative only, with/without NP herniation and without clinical signs of discitis.

Arndt *et al*. (2012) and Agarwal, Golish and Alamin (2011) independently reported bacteriologic culture positivity rates of *C. acnes* within excised IVD tissue of 21.7% (18/83) and 13.5% (7/52) within patients presenting with lumbar disc degeneration and herniated lumbar NP, respectively.

Albert *et al*. [2013 (a)] identified 39.3% (24/61) of patients with *C. acnes* culture positivity from nuclear tissue removed from lumbar discs during microdiscectomy. Carricajo *et al*. (2007) reported a positivity rate of *C. acnes* within disc fragment samples of 3.7% (2/54). However, within the latter study, the two cases of *C. acnes* culture positivity were attributed to contamination during sample collection and/or laboratory processing (Carricajo *et al*.,

2007).

More recently, Yuan *et al*. (2017) demonstrated the presence of *C. acnes* within 26.3% (20/76) of patients from whom IVD tissue samples were obtained. Therefore, all of the studies explored here demonstrate the existence of latent *C. acnes* infection within IVDs. In support of this theory, Stirling *et al*. (2001) demonstrated the absence of *C. acnes* positive cultures from anatomically normal IVD control tissue obtained from patients presenting with other spinal disorders.

The phylogenetic distribution within IVD tissue demonstrates a mix of phylotypes, with Rollason *et al*. (2013) exploring the phylogenetic distribution of 74 *C. acnes* isolates obtained from herniated lumbar disc tissue and concluding that culture of such tissue yielded 48% type II, 27% type IA, 14% type III and 11% type IB isolates. Capoor *et al*. (2017) reported 24% (9/38) of isolates as belonging to phylotype IB, with 39% (15/38) belonging to phylotype IA_1, 3% (1/38) belonging to phylotype IC and 34% (13/38) belonging to phylotype II. Capoor *et al*. (2017) concluded that as a distribution of phylotypes was noted rather than the predominance of a single phylotype, no specific *C. acnes* phylotype is associated with lumbar disc herniation.

1.4.2 *C. acnes*: Contaminant or Cause of Disease?

Despite an increasing body of literature evidencing the isolation of *C. acnes* from IVD tissue, some studies do not support the presence of *C. acnes* within disc tissue and/or the VB as a pathophysiological cause of DLBP. Such studies suggest that the origination of *C. acnes* within IVD tissue is the result of contamination of patient samples during tissue harvest and/or laboratory processing as opposed to the endogenous growth of this microorganism within the IVDs, structures traditionally regarded as sterile spaces (Carricajo *et al*., 2007). As *C. acnes* is a member of the normal skin microbiota, this microorganism is often identified as a common contaminant within spinal surgery, with Bémer *et al*. (2008) reporting *C. acnes* contamination rates of up to 9.7% during spinal instrumentation procedures (Achermann *et al*., 2014). Ben-Galim *et al*. (2006) cultured 30 IVD samples obtained from patients with disc herniation and found just two strains of CoNS, considered as contaminants during sample collection. Fritzell *et al*. (2019) argued that similarities between *C. acnes* culture positivity rates between disc herniation patients presenting with disc degeneration and control scoliosis patients with non-degenerated discs may infer that *C. acnes* represents a sample contaminant. However, the biofilm mode of growth was not considered within this study and the subsequent lack of adequate sample disruption may have led to the under-representation of *C. acnes* within disc herniation patients. Additionally, Fritzell *et al*. (2019) identified that the use of universal 16S rRNA primers, designed to detect any bacterial species within clinical samples, may have reduced the

sensitivity of the PCR assay as compared to the use of primers designed specifically to target the rRNA of *C. acnes*. Therefore, *C. acnes* may not necessarily represent a sample contaminant within this published study. However, McLorinan *et al*. (2005) identified bacteria in 29.1%, 21.5% and 16.5% of skin, soft-tissue and intra-operative wound washing samples, respectively. *C. acnes* represented the predominant bacterial species within each of these three sample types, with immunofluorescent microscopy examination suggesting the origination of *C. acnes* wound contaminant isolates was from patient skin. Subsequently, some researchers have begun collecting ligamentum flavum and/or muscle samples adjacent to IVDs during discectomy procedures for use as contamination marker controls during surgery. One such study identified *C. acnes* within control cultures taken from ligamentum flavum and muscle of two patients who were also positive for *C. acnes* within IVD fragments (Carricajo *et al*., 2007). Carricajo *et al*. (2007) investigated the environmental presence of *C. acnes* within air samples obtained during surgery for severe disc herniation. All four air samples contained *C. acnes*, with these bacteria also being identified within three of 54 laminar flow control cultures (Carricajo *et al*., 2007). This further indicates that isolated *C. acnes* could represent contamination as opposed to endogenous growth within IVDs. Additionally, Yuan *et al*. (2017) identified four disc degeneration patients (4/76, 5.26%) with positive results for *C. acnes* in both IVDs and adjacent muscles, indicative of incisional contamination during surgery. However, this same study identified 16 patients (16/76, 21.05%) with *C. acnes* present only in IVDs with negative culture results of adjacent muscle samples, suggesting that these isolates represent original growth within IVDs (Yuan *et al*., 2017). Additionally, Zhou *et al*. (2015) identified nine cases of *C. acnes* 16S

ribosomal DNA gene positivity within IVD tissue only, with two cases of *C. acnes* positivity in the disc and muscle tissue of 46 discectomy patients. Since the vast majority of positive *C. acnes* cultures yielded no *C. acnes* from corresponding muscle control tissue, a true infection is far more likely as opposed to contamination.

The discussion surrounding the role of *C. acnes* in disc infection, either as a genuine cause of infection or as a simple sample contaminant, is further complicated by the findings of Rajasekaran *et al*. (2020) which challenge the concept that human IVD tissue represents a sterile space (Carricajo *et al*., 2007). Rajasekaran *et al*. (2020) evidenced a microbiome within human IVD tissue, with *C. acnes* being identified within MRI-normal discs, degenerated discs and herniated discs. However, it is not clear whether contamination marker controls, such as ligamentum flavum samples or surrounding muscle tissue samples, were obtained in this study as indicators of potential contamination of clinical disc samples during tissue collection.

Inclusion and exclusion criteria are applied within a range of studies investigating the presence of *C. acnes* within disc tissue to promote the validity of study results and conclusions. Albert *et al*. [2013 (b)] ensured patients aged between 18-65 years with MRI-confirmed disc herniations were included. Similarly, Agarwal, Golish and Alamin (2011) only included primary microdiscectomy patients with MRI-confirmed lumbar herniated NP. Exclusion criteria typically applied within such studies included the exclusion of pregnant or

lactating participants, patients with kidney disease, patients undergoing chemotherapy, subjects using systemic steroids and/or patients who have undergone prior lumbar surgery [Salehpour *et al*., 2019; Astur *et al*., 2017; Albert *et al*., 2013 (b)]. Therefore, with the application of such stringent inclusion and exclusion criteria to such studies, the presence of *C. acnes* should not be readily dismissed or attributed to extraneous factors. Steroid injections and/or epidurals are other potential confounding variables that may influence *C. acnes* presence within IVD tissue, with studies typically excluding patients who have undergone back surgery and previous epidural steroid injections (Capoor *et al*., 2016; Salehpour *et al*., 2019). Albert *et al*. [2013 (a)] demonstrated anaerobic *C. acnes* culture positivity within 24 (86%) patients who had no previous history of epidural steroid injection, demonstrating the potential endogenous origin of these bacteria within the IVD tissue of these patients. In support of these findings, Capoor *et al*. (2016) did not observe any significant association between *C. acnes* positivity rates and the prevalence of previous spinal surgery/epidural steroid injections in patients, further supporting the endogenous origin of *C. acnes* within disc tissue as opposed to iatrogenic origin. Furthermore, the presence of biofilms within IVD tissue is consistent with infection as opposed to microbiological contamination during sample collection (Capoor *et al*., 2017). Also, rigorous decontamination procedures and aseptic techniques are employed during surgical site preparation and sample collection within discectomy patients, such as the use of antiseptic solutions, further reducing the likelihood of surgical site contamination (McLorinan *et al*., 2005). Therefore, *C. acnes* positivity within IVD tissue should not readily be dismissed as a result of contamination during tissue harvesting. However, Patrick *et al*.

(2017) dispute the efficacy of a specific skin antiseptic, called povidone iodine-alcohol, in the sterilisation of pre-operative skin in spinal surgery patients. These results suggested that viable bacterial loads were significantly lower in the group treated with both povidone iodine-alcohol and chlorhexidine gluconate-alcohol as compared to patients treated with povidone iodine-alcohol applied twice, as per National Institute for Health and Care Excellence UK guidelines, alone (Patrick *et al*., 2017). Therefore, whilst skin antisepsis with the sequential application of povidone iodine-alcohol and chlorhexidine gluconate-alcohol led to more effective reduction of surgical wound contamination rates as compared to povidone iodine-alcohol alone, both decontamination protocols led to surgical wound infection (Patrick *et al*., 2017). This suggests that bacteria contaminating surgical wounds can be derived from the patient skin microbiota and subsequently may infer that positive culture from disc tissue may represent sample contamination from surgical wound sites, even following effective skin pre-operative decontamination.

Besides *C. acnes*, the normal skin flora is represented by a range of bacterial species, such as CoNS. Therefore, if *C. acnes* isolated from IVD tissue represents an incisional contaminant originating from the skin, most isolated bacteria from patient tissue samples should be a mixture of species as opposed to the predominance of an individual species. However, in various studies, *C. acnes* represents the predominant bacterial species cultured from IVD tissue, with Stirling *et al*. (2001) identifying positive *C. acnes* growth within 84% (16/19) of IVD material taken from sciatica patients. Another study found that *C. acnes* was the only bacterial species identified via culture and 16S recombinant DNA PCR examination within

21% (16/76) of discs (Yuan *et al*., 2017).

Another factor which may lead to inter-study discrepancies with regards to *C. acnes* culture positivity rates is methodological issues and culture technique. In addition to the slow growth rate of *C. acnes*, these bacteria proliferate as aggregated biofilms *in vivo*, making *in vitro* culture difficult without the use of physical biofilm disassembly techniques (such as sonication or homogenisation) prior to microbiological plating (Capoor *et al*., 2017). With Jamal *et al*. (2018) stating that the National Institutes of Health (NIH) revealed that among all microbial and chronic infections, 65% and 80%, respectively, are associated with biofilm formation, it is surprising that not all previous studies investigating *C. acnes* presence within IVD tissue have accounted for this mode of growth within disc pathologies with regards to, for example, sample preparation. Therefore, the failure of previous studies investigating *C. acnes* presence within IVD tissue to consider the biofilm mode of growth and optimum culture times of these bacteria has led to inconsistencies in sample processing (Capoor *et al*., 2017; Abdulmassih *et al*., 2016). This has resulted in varied and likely underestimated prevalence rates of cultured *C. acnes* in previous publications ranging from 0% to 44% (Ben-Galim *et al*., 2006; Stirling *et al*., 2001). Therefore, failure to disrupt possible *C. acnes* biofilms within disc tissue and failure to culture samples for long enough may lead to false-negative culture results and the subsequent under-reporting of the true incidence of *C. acnes*-associated IVD tissue infection. As a result, standardisation of tissue processing protocols in future studies, such as the incorporation of tissue homogenisation and/or sonication steps, would promote *C. acnes* recovery from patient tissue.

1.4.3 Symptoms Associated with *C. acnes* Presence Within Intervertebral Disc Tissue

1.4.3.1 Modic Changes and Associated Lower Back Pain (LBP)

Various studies have demonstrated the association between the presence of Modic type I changes and LBP, with the prevalence ranging from 18%-62% in LBP patients (Mitra, Cassar-Pullicino and Mccall, 2004; Kleinstück, Dvorak and Mannion, 2006). Toyone *et al*. (1994) demonstrated that 84% (31/37) of patients with LBP exhibited Modic changes, with Modic type I changes more commonly associated with back pain (73%, 27/37) as compared to Modic type II changes (11%, 4/37). The evolution of type I Modic changes correlates with the worsening of patient symptoms (Mitra, Cassar-Pullicino and Mccall, 2004). Similarly, Järvinen *et al*. (2015) demonstrated the significant association between changes in the extent of Modic type I changes and changes in the Oswestry Disability Index of LBP patients, again highlighting the relationship between Modic type I changes and LBP severity. Modic type I changes are thought to reflect earlier acute stages of inflammation whilst Modic type II changes are thought to occur as a result of previous inflammatory Modic type I changes (Albert and Manniche, 2007). With Ohtori *et al*. (2006) demonstrating significantly higher numbers of proinflammatory TNF-immunoreactive cells in Modic type I vertebral endplates compared to Modic type II endplates, the proinflammatory nature of Modic type I changes may account for the stronger association of these changes with LBP compared to type II changes (Albert and Manniche, 2007).

Although the pathogenic mechanisms causing Modic changes are not clear, the current literature relating to this field suggests two main possibilities; mechanical and bacterial causes, as summarised in Figure 1.5 [Albert *et al*., 2008 (a)].

Vertebral endplates play an essential biomechanical role within the spine, acting as the interface between rigid vertebral bodies and pliable IVDs (Lotz, Fields and Liebenberg, 2013). During IVDD and ageing, the cartilage end plates undergo changes in proteoglycan and collagen composition, resulting in endplate thinning and calcification, the accumulation of which can lead to focal weak points and subsequent microfractures (Lotz, Fields and Liebenberg, 2013). Therefore, the association between Modic changes and degenerated IVD tissue may suggest a way in which Modic changes can be caused by mechanical stress [Albert *et al*., 2008 (a); Kjaer *et al*., 2006]. As a result of these structural changes, uneven load is applied across IVDs, contributing to endplate fissures as seen on MRI (Modic *et al*., 1988). Additionally, Adams and Dolan (2012) suggest that the loss of the IVD NP, such as due to the bulging of the NP into the adjacent vertebrae, increases compressive shear forces in the posterior annulus and endplates, potentially resulting in microfractures. These fissures and microfractures may be a source of Modic changes, with Modic *et al*. (1988) describing Modic type I changes as visible disruption and fissuring of vertebral end plates on MRI images. Abnormal load and stress on vertebral end plates may also affect the microenvironment of the adjacent vertebral bone marrow, resulting in histological changes which can alter signal intensity on MRI scans and show resulting Modic changes (Modic *et al*., 1988).

Another mechanical theory of Modic change aetiology is the induction of an inflammatory response by toxic NP material which invades the endplate and the vertebral body through microfractures within the endplates [Albert *et al*., 2008 (a)]. This mechanical theory of Modic changes is supported by histological findings of Modic *et al*. (1988) of endplate disruption in both Modic type I and II patients.

Another hypothesised pathophysiological factor for the occurrence of Modic changes may be a bacterial infection, particularly with low-virulent anaerobic bacteria [Albert *et al*., 2008 (b); Chen *et al*., 2016]. Following disc herniation, a breach in the AF allows for the extrusion of NP material out of the IVD, inducing neocapillarisation around the extruded disc material (Doita *et al*., 1996; Ito *et al*., 1996). Extrusion of NP disc material into the epidural space is considered 'foreign', promoting the induction of an autoimmune response, with mononuclear cells infiltrating along the margins of extruded discs and expressing inflammatory mediators, such as IL-1, intercellular adhesion molecule 1, lymphocyte function-associated antigen and basic fibroblast growth factor (Doita *et al*., 1996). This may induce the production of a persistent inflammatory response. Bacteria may enter the IVD through the breach in the AF, with the avascular environment of the NP providing the optimal environmental conditions for the growth of these anaerobic bacteria and the subsequent development of a chronic, progressive, low-virulent infection within the compromised IVD [Albert *et al*., 2008 (a)]. As a result of this infection, Albert *et al*. [2008 (a)] hypothesised that local tissue inflammation and oedema occur, with inflammatory cytokines produced by inflammatory cells leading to bone damage and subsequent Modic

type I changes observed within vertebral endplates and subchondral bone marrow. In support of this theory, Shan *et al*. (2017) demonstrated a significant increase in the expression of the proinflammatory cytokines TNF-α, IL-1β and IFN-γ following the injection of *C. acnes* into subchondral bone superior to lumbar disc tissue. The ability of these bacteria to induce these inflammatory responses lead to MRI signal intensity changes which resemble Modic changes (Shan *et al*., 2017). Subchondral bone of New Zealand White rabbits injected with *C. acnes* above disc tissue had a higher prevalence of Modic changes, suggesting the association between *C. acnes* IVD and subchondral bone infection and Modic change development (Shan *et al*., 2017). The utilisation of an animal model within the study conducted by Shan *et al*. (2017) provided a means of demonstrating a direct link between *C. acnes* inoculation and subsequent Modic changes. However, Rigal *et al*. (2016) argued that no association exists between lumbar disc degeneration in a subset of patients presenting with Modic changes and chronic infection of disc tissue with *C. acnes*.

As shown in Figure 1.5 below, Dudli *et al*. (2018) describe disc damage as a pre-requisite to *C. acnes* disc infiltration, promoting the upregulation of gene transcription of the proinflammatory disc cytokines IL-1,IL-6, IL-8, and Chemokine (C–C motif) ligand 2 (CCL2), particularly in disc cells isolated adjacent to Modic changes. Co-culture of *C. acnes* with disc cells also increases *C. acnes* lipase activity in a concentration- and time-dependent manner (Dudli *et al*., 2018). Lipase, an exocellular *C. acnes* virulence factor, hydrolyses triacylglycerides, which are abundant in the bone marrow of vertebrae, into glycerol and free fatty acids (Dudli *et al*., 2018). These liberated free fatty acids are chemotactic and highly

proinflammatory (Lee *et al*., 1982), potentiating the inflammatory changes seen in Modic changes. Additionally, free fatty acids skew the lineage choice of myeloid progenitor cells toward the osteoclastic lineage and of bone marrow stromal cells towards adipocytes [Dudli *et al*, 2016 (a)]. High bone turn-over as a result of an increase in osteoclastic cells is characteristic of Modic type I changes whilst abundant adipocytes are characteristic of Modic type II changes [Modic *et al*., 1988; Dudli *et al*., 2016 (a); Lotz, Fields and Liebenberg, 2013; Perilli *et al*., 2015].

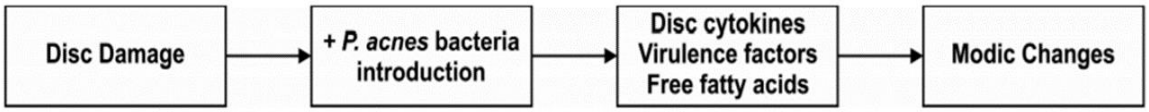


Figure 1.5: Suggested model for *C. acnes* infection of IVD tissue and subsequent Modic changes. Disc damage is a pre-requisite for circulating *C. acnes* to invade the disc. Proliferation of *C. acnes* within disc tissue allows for virulence factor production and free fatty acid liberation due to *C. acnes* lipase production. Disc cells release cytokines in response to *C. acnes* cells. Disc cytokines and bacterial virulence factors and metabolites drain into the surrounding bone marrow, inducing a proinflammatory response in bone marrow cells and inducing morphological changes in bone marrow which may eventually result in Modic changes (Dudli *et al*., 2018).

Although studies have identified *C. acnes* as the cause of discitis, the low virulence of these anaerobic bacteria leads to weaker inflammatory reactions and endplate erosive changes as compared with more virulent bacterial strains, such as *Staphylococcus aureus* (*S. aureus*) and streptococcus species [Albert *et al*., 2008 (a)]. Therefore, Albert *et al*. [2008 (a)] highlight that the slow rate of tissue damage progression as a result of anaerobic bacterial infection leads to the poor illumination of IVD and endplate damage on MRI and subsequently, these infections are rarely diagnosed as discitis. This is further complicated by the covert clinical presentation of patients with *C. acnes* infection, with Saper *et al*. (2015) demonstrating the normal laboratory indicators of infection (white blood cells (WBC), ESR and CRP) of patients with *C. acnes* shoulder infection, making diagnosis difficult. In contrast to this, spondylodiscitis associated with *S. aureus* and/or streptococcus species is associated with an elevation of the ESR in almost all cases (Skaf *et al*., 2010). This lack of virulence of anaerobic bacteria associated with IVD tissue infection may account for the limited spread of such infections to aerobic tissues, such as to spinal vertebrae, together with the difficulty of such bacteria to proliferate and establish infection within highly vascularised aerobic tissue [Albert *et al*., 2013 (a)]. In support of the role of disc herniation in the development of Modic changes, Albert and Manniche (2007) reported a higher prevalence of new Modic type I changes with increased severity of disc herniations, with none of the patients with normal disc morphology developing Modic changes. Therefore, disc herniation may allow for the entry of bacteria into the compromised disc and the subsequent development of Modic changes.

Animal models have been increasingly used for modelling *C. acnes*-associated Modic changes, although this raises a series of questions and key considerations. The longevity of such studies can result in long delays in the generation of results, with Shan *et al*. (2017) performing a longitudinal study in which lumbar MRI scans had to be continued up to six months post-injection with *C. acnes*. Additionally, Zamora *et al*. (2017) identified the need to further investigate the effect of different routes of disc tissue infection (i.e. haematogenous vs. direct inoculation), the mammalian model selected and the type of disc tissue inoculated (i.e. lumbar disc model vs. tail disc model) on the degree of consistency between disc tissue change results. The pathological effects of various *C. acnes* strains on lumbar disc tissue also requires further investigation, with many animal model studies focusing only on type IA strains, despite type II *C. acnes* strains being the most frequently cultured strain type from disc material taken from low-grade disc infection patients (Shan *et al*., 2017; Zamora *et al*., 2017; Rollason *et al*., 2013). However, the use of animals within scientific research raises both ethical and practical questions, with factors such as animal husbandry and expenses associated with animal maintenance having to be considered. Therefore, insect models, including zebra fish (*Danio rerio*), silkworm (*Bombyx mori*), fruit fly (*Drosophila melanogaster*) and, more recently, waxworm larvae (*Galleria mellonella*), are increasingly being utilised as feasible alternatives to traditional animal models within scientific research, particularly because insects possess a basic innate immune system, do not require ethical approval and are inexpensive to obtain and maintain (Ramarao, Nielsen-Leroux and Lereclus, 2012). *Galleria mellonella* (*G. mellonella*) are becoming an increasingly popular insect model, particularly because unlike other non-mammalian

models, such as *Drosophila melanogaster*, these insects can function at human body temperature (37°C) which is the optimum growth temperature for *C. acnes* (Nathan, 2014; Cook and McArthur, 2013). Additionally, the large size of these waxworms as compared to other insect models enables precise injection of test pathogens. Subsequently, pathogen virulence in *G. mellonella* can be measured in several ways, including lethal dose at 50% (LD_{50}) calculation, assessing bacterial survival rates and measurement of insect mortality (Allegra *et al*., 2018; Guillemet *et al*., 2010; Hurst *et al*., 2015). Furthermore, the *G. mellonella* immune system exhibits both humoral and cellular components, resembling the innate immune system of mammals (Sheehan *et al*., 2018; Kavanagh and Reeves, 2007; Cook and McArthur, 2013). This enables direct comparisons between mammalian and non-mammalian infection responses. Despite this, no previous studies have investigated the feasibility of using an insect model to assess the virulence of disc tissue *C. acnes* isolates.

1.4.4 Routes of Infection and Pathological Mechanisms of *C. acnes* within Intervertebral Disc Tissue

There are three main routes of *C. acnes* infection within lumbar tissue. Firstly, *C. acnes* can be inoculated directly into lumbar tissue during surgery, such as during spinal implantation as explored by Bémer *et al*. (2008). Secondly, tooth brushing and endodontic therapy, such as teeth polishing and rubber dam placement, can allow *C. acnes* within the oral cavity to be

inoculated into the bloodstream and result in a transient bacteraemia (Debelian, Olsen and Tronstad, 1995; Roberts *et al*., 1997; Bhanji *et al*., 2002). Thirdly, circulatory macrophages may engulf *C. acnes* cells within the bloodstream and transport these bacteria to the IVD during neovascularisation where macrophages and other inflammatory cells aggregate around the ruptured AF of extruded or sequestrated discs (Doita *et al*., 1996; Ito *et al*., 1996; Grönblad *et al*., 1994). Viable bacteria may be released into the compromised herniated disc during macrophage cell lysis. In support of this theory, Fischer *et al*. (2013) demonstrated the survival and persistence of a clinical *C. acnes* isolate within human macrophages, although no evidence of intracellular replication was noted. Additionally, as *C. acnes* has difficulty surviving and replicating under the aerobic conditions within the blood, phagocytosis by macrophages may promote the continued persistence of these bacteria in host tissues and the subsequent observation of associated chronic inflammation and disease development, such as prostate pathologies (Bae *et al*., 2014).

1.5 Treatment of *C. acnes*-Associated Chronic Lower Back Pain

Generally, recommendations for the treatment of back pain associated with Modic changes are the same as for clinical guidelines for back pain associated with IVDD (Manniche and O'Neill, 2019). Whilst exercise therapy is the recommended treatment for CLBP patients, patients with Modic changes are often less likely to improve with physical activity in

comparison to those with non-specific LBP (Jensen *et al*., 2012). Therefore, patients with CLBP and associated Modic changes represent a small sub-group of people that should not be treated using physical activity (Jensen *et al*., 2012). Although there is no general consensus on the ideal therapy for such patients, Dudli *et al*. [2016 (a)] identified non-surgical treatment approaches, including intradiscal steroid injections, anti-TNF-α antibody therapy and bisphosphonates, as suitable treatment options that have been shown to reduce Modic changes in the short-term. Where a low-virulent infection of the IVD tissue is suspected, antibiotics have also been used [Albert *et al*. (2013 (b)]. Further studies investigating the antibiotic resistance profiles of cultured *C. acnes* from these patients may be warranted to ascertain any potential effects of long-term antibiotic therapy in CLBP patients.

1.6 *C. acnes* Typing

Typing involves differentiating between different strains of the same bacterial species and can be subdivided into phenotypic, such as virulence factor expression and biotyping, and genotypic typing methods. Typing allows for the characterisation of strain-specific virulence factors and the assessment of clonal relatedness of isolates. This has applications in the epidemiologic surveillance of infectious diseases and has further applications in outbreak investigation (Struelens, 1996).

When choosing an appropriate bacterial typing technique, various factors must be considered, such as typeability, reproducibility, discriminatory power, ease of interpretation, cost-effectiveness and ease of use (Maslow, Mulligan and Arbeit, 1993).

1.6.1 Virulence factor expression

As explored by a range of authors, *C. acnes* produces a variety of exocellular enzymes and bioactive exocellular products which are important virulence determinants of this microorganism (Table 1.2) (Eady and Ingham, 1994; Ingham *et al*., 1981; Ingham *et al*., 1983; Fujimura and Nakamura, 1978; Mak *et al*., 2013). *C. acnes* virulence factor production can be assessed by culturing this microorganism on a range of agar plates, including blood agar plates for the assessment of haemolysin production, and tributyrin agar, for the identification of lipase production. These virulence factors can be used as clinical markers of strain pathogenicity. Assessing bacterial virulence factor production in a clinical laboratory setting can give a preliminary identification of isolates, the results of which are confirmed using molecular methods. As bacterial virulence characteristics are controlled by gene expression, growth conditions and growth-phase can affect virulence profiles (Brzuszkiewicz *et al*., 2011). Additionally, random mutations may affect the interpretation of virulence test results (Maslow, Mulligan and Arbeit, 1993).

The expression of the exoenzymes and exocellular products shown in Table 1.2 by *C. acnes* suggests that these bacteria possess the ability to exhibit immunostimulatory activity and host tissue degrading properties. However, further research is required to determine whether these virulence factors are phylotype-specific.

Table 1.2 Exoenzymes and exocellular bioactive products produced by *C. acnes* [adapted from Eady and Ingham (1994); Grange *et al*., 2017; Allhorn *et al*., 2016; Barnard *et al*., 2020)].

Product	Suspected Role in Health/Disease
Lipase	Bacterial nutrition through the generation of free fatty acids.
Phospholipase C	Perturbation of membrane function.
Protease (s)	Tissue invasion. Bacterial nutrition. Complement activation. Release of chemotaxins. Proteolysis in the human colon.
Hyaluronate lyase	Spreading factor. Tissue invasion.
Neuraminidase	Tissue invasion.
Acid phosphatase	Bacterial nutrition. May allow *C. acnes* to scavenge carbon sources.
Bacteriocins	Antagonistic interactions with competing bacteria.
Histamine and tryptamine	Mediation of acute inflammation.
Dermatan-sulphate adhesins (DsA)	Host cell-surface attachment proteins recognising dermatan sulphate (DsA1 also specifically binds to human fibrinogen).
Radical oxygenase of *Propionibacterium acnes* (RoxP)	Secreted antioxidant enzyme derived from *C. acnes* to protect bacteria from oxidative damage, enabling bacterial survival and growth in oxygen-rich environments.
Porphyrins	Proinflammatory bacterial intermediate metabolites associated with human inflammatory diseases, such as acne vulgaris.

1.6.2 Biotyping

Biotyping refers to the classification of bacteria according to their biochemical and enzymatic activities. Commercial test kits, such as the API Coryne kit produced by bioMérieux, are increasingly used clinically for the biotyping of bacterial isolates which facilitates the rapid identification of microorganisms. These commercial kits consist of microtubes containing dehydrated substrates which allow for the assessment of the enzymatic activity or the carbohydrate fermentation properties of different bacterial species. Enzymatic reactions can be visualised through the addition of reagents to these microtubes whilst the fermentation of carbohydrates, such as glucose, maltose, mannitol, sucrose and lactose, can be detected by colour changes of pH indicators. However, the accuracy of these commercial kits relies on the use of pure cultures of a single microorganism.

In a study conducted by Freney *et al*. (1991), the commercial API Coryne kit by bioMérieux showed a 97.6% concordance rate with conventional biotyping methods, such as gelatin and aesculin hydrolysis and fermentation tests for a range of isolates within the Corynebacteriaceae family. Therefore, these commercial kits can provide clinical laboratories with a rapid, easy-to-use bacterial confirmatory test method. However, commercial biotyping kits can lack the ability to differentiate strains with a common phage type and, therefore, can have low discriminatory power. Additionally, biotyping kits can recognise too many subgroups within bacterial clusters which means the use of this

phenotypic method within epidemiological studies is limited (Tenover *et al*., 1994).

1.6.3 Serotyping

Serotyping is a method by which microorganisms of the same species can be differentiated in terms of antigenic determinants expressed on cell surfaces and allows bacteria to be classified into serotypes, groups of microorganisms which share specific surface antigens. *C. acnes* serotyping depends on specific agglutination reactions between certain *C. acnes* epitopes, such as cell wall antigens, and antisera, usually prepared through the inoculation of partial bacterial disintegrates into laboratory animals.

Using antisera prepared against *C. acnes* cell wall antigens, Johnson and Cummins (1972) identified two serological types based on cell wall agglutination tests, referred to as *C. acnes* types I and II. Type I cell walls contained glucose, galactose and mannose whereas type II strain cell walls contained only glucose and mannose (Johnson and Cummins, 1972). However, although these traditional serotyping techniques are accurate and specific, these methods are time consuming, labour intensive and expensive due to the use of large collections of antisera only generated by laboratories with animal facilities (Achtman *et al*., 2012). Therefore, conventional serotyping techniques are increasingly being superseded by molecular methods, including pulsed-field gel electrophoresis (PFGE) (Achtman *et al*.,

2012).

1.6.4 *RecA* Typing and Antibiotic Sensitivity Patterns

RecA typing was first described in 2005 in a study conducted by McDowell *et al*. (2005) which investigated the phylogenetic relationship between *C. acnes* types I and II. *RecA* typing describes gene sequence analysis of the *C. acnes* non-ribosomal housekeeping gene *recA* and the putative haemolysin/FtsJ-like methyltransferase gene *tly* (McDowell, 2018). Discerning the evolutionary lineages of distinct *C. acnes* serotypes, namely phylotypes IA_1, IB, II and III, is important as research suggests that these phylogroups may exhibit differing pathogenic traits and virulence factors and may, therefore, be associated with different clinical diseases (Valanne *et al*., 2005; Lodes *et al*., 2006; Nagy *et al*., 2006). In support of this, Rollason *et al*. (2005) showed that 48% of *C. acnes* isolates recovered from excised disc tissue belonged to phylotype II, suggesting that this phylotype may be more commonly associated with IVD tissue infection as compared to types IA_1, IB and III. This phylotype-specific association of *C. acnes* with specific sites of infection was also observed by Lomholt and Killian (2010). Within a panel of 210 *C. acnes* isolates taken from the skin of healthy individuals, patients with varying degrees of acne and other infectious diseases, isolates of the phylotype IA were strongly associated with moderate to severe acne (Lomholt and Killian, 2010). Additionally, types IB, II and III were associated with healthy skin and

opportunistic deep tissue infections, including culture from post-surgical hip prosthesis infections and cerebrospinal fluid (Lomholt and Killian, 2010; Dréno *et al*., 2018). Typing of *C. acnes* is also essential within epidemiological analysis (Kilian, Scholz and Lomholt, 2012).

The antimicrobial sensitivity patterns of the strains used within the current study were previously established by Rollason *et al*. (2013) using a panel of antibiotics including amoxicillin, erythromycin, tetracycline, trimethoprim, fusidic acid, gentamicin, rifampicin, vancomycin and ciprofloxacin. Results demonstrated that all tested *C. acnes* isolates showed sensitivity to at least one antibiotic, with minimum inhibitory concentrations (MICs) lowest for amoxicillin, ciprofloxacin, erythromycin, rifampicin, tetracycline and vancomycin (≤1mg/L) (Rollason *et al*., 2013). The highest MICs were observed for trimethoprim and gentamicin which ranged from 2- >4mg/L (Rollason *et al*., 2013). However, a more recent study investigating the antimicrobial susceptibility patterns of *C. acnes* isolates taken from acne patients demonstrated that although some isolates were highly sensitive to doxycycline and tetracycline, patients who had received antibiotics (oral or topical) for acne had higher rates of antibiotic-resistant *C. acnes* (Zhu *et al*., 2019). Additionally, *C. acnes* showed higher rates of resistance to erythromycin, azithromycin, clarithromycin and clindamycin (Zhu *et al*., 2019). With topical and systemic antibiotics frequently being used in the treatment of acne, this suggests that routine, long-term use of antibiotics can slowly alter the resistance profile of these bacteria (Zhu *et al*., 2019). This increased resistance rate of *C. acnes* isolates from acne patients to certain antimicrobials could also promote the spread of

antibiotic resistance to *C. acnes* from other body sites. Therefore, the future of *C. acnes* disease management should focus on finding alternative treatment methods to traditional antimicrobials, such as promoting research into novel vaccine development.

1.6.5 Newer Molecular Typing Methodologies for *C. acnes*

Due to the lack of reliable differentiation between type IB strains of *C. acnes* and some type IA_1 and IA_2 strains using *recA* and *tly* loci alone, the application of this typing method has limitations (McDowell, 2018). Following the identification of *recA* and *tly* loci as sequencing tools, newer DNA-based typing methods have been described for *C. acnes*, namely MLST, multiplex PCR and multiple locus variable number of tandem repeat analysis (MLVA) (McDowell, 2018).

1.6.5.1 MLST

MLST assesses variations in genetic sequences of multiple, usually seven, housekeeping genes, with each unique allele for each locus designated the allelic profile (McDowell, 2018). Assessment of MLST data allows bacterial isolates to be grouped based on the degree of sharing of a defined number of alleles to investigate evolutionary descent of isolates (McDowell, 2018). Two independent MLST schemes have been defined for *C. acnes*, with one scheme based on the analysis of eight loci whilst the second scheme was based on nine

loci (McDowell, 2018). However, MLST is labor intensive and expensive, particularly if large numbers of isolates must be typed (McDowell, 2018).

1.6.5.2 Multiplex PCR

Another newer molecular method that can be used for *C. acnes* typing is multiplex touchdown PCR which can differentiate between *C. acnes* types IA_1, IA_2, IB, IC, II and III in a single reaction using six primer sets (McDowell, 2018). These primers specifically target the 16S rRNA gene (all isolates) as well as ATPase (types IA_1, IA_2 and IC), *sodA* (types IA_2 and IB), *atpD* (type II) and *recA* (type III) housekeeping genes, including the gene encoding a Fic family toxin (type IC) (McDowell, 2018). Therefore, multiplex PCR analysis is a technically undemanding, high-throughput technique for investigating *C. acnes* phylogeny.

1.6.5.3 Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA)

MLVA is a typing method which assesses the variable number of tandem repeats at multiple loci (McDowell, 2018). These variable loci consist of short repeating DNA sequences that are formed from slipped strand mispairing during DNA replication which often vary between different strains of the same bacterial species (McDowell, 2018). MLVA generates allelic profiles, similar to MLST. These MLVA allelic profile clusters have been reported to have good agreement with *C. acnes* phylogenies based on whole genome sequence analysis

(McDowell, 2018).

1.7 Genomic Differences Between *C. acnes* Phylotypes

Following MLST analysis, *C. acnes* can be divided into three main lineages, namely types I, II and III, with each lineage further divided into subtypes, such as types IA_1 and IA_2. Each subtype also consists of clonal complexes, with evidence indicating that strains belonging to specific clonal complexes possess different inflammatory properties. Such phenotypic differences between *C. acnes* phylotypes may be attributed to the presence or absence of genomic islands which encode properties specific for certain clonal complexes (Brüggemann, Lomholt and Kilian, 2012). The horizontal acquisition of genomic islands may also lead to further genomic differences between phylotypes, with a number of genomic regions having been reported to have shown signs of horizontal gene acquisition, such as aberrant G+C content and the presence of flanking insertion sequences (Brüggemann, Lomholt and Kilian, 2012; McDowell, 2018). Such genomic regions have been shown to encode putative virulence factors, such as those that promote bacterial defence against antimicrobials, and traits that modulate host cell interaction, such as adhesion (Brüggemann, Lomholt and Kilian, 2012; McDowell, 2018).

One island-like region containing clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated genes (*cas*) was recently found to be exclusively present

in type II *C. acnes* strains (Brüggemann, Lomholt and Kilian, 2012). Such CRISPR/*cas* systems, present in a range of bacterial species, have been reported to provide bacteria with acquired immunity against invasion with mobile genetic elements, phages and plasmids (Brüggemann, Lomholt and Kilian, 2012). Therefore, type I and III *C. acnes* strains may be more susceptible to horizontal gene transfer of virulence traits obtained from the acquisition of mobile genetic elements (McDowell, 2018).

1.8 Aims and Objectives

C. acnes is a frequent cause of acne vulgaris which is associated with significant morbidity. However, these bacteria are increasingly being associated with other significant infections and clinical conditions, including prosthetic joint infections, implant-associated infections and sciatica. As a result of studies increasingly demonstrating the isolation and culture of *C. acnes* from excised disc herniation tissue, these bacteria are being implicated as an aetiological agent within CLBP associated with disc herniation and Modic changes [Chen *et al*., 2016; Albert *et al*., 2013 (a); Salehpour *et al*., 2019; Rollason *et al*., 2013]. The demonstration that administration of amoxicillin-clavulanate therapy within such patients reduced physiological symptoms and pain associated with lumbar disc herniation and CLBP, along with the positive isolation of *C. acnes* from excised disc herniation tissue, supports the role of bacterial infection within disc herniation [Albert *et al*., 2008 (b); Rollason *et al*., 2013]. However, the emergence of antibiotic resistant strains of *C. acnes*, attributed in part

to the overuse of oral and topical antibiotics within acne patients, further complicates the management of specific infections and may prove to be a limiting factor within disc infection management (Zhu *et al.*, 2019; Dessinioti and Katsambas, 2017). Many research studies fail to consider *C. acnes* as a cause of clinical infection and, therefore, can lack optimisation in terms of sample processing and culture incubation periods to promote *C. acnes* growth (Capoor *et al*., 2017). Distinguishing between a bacterial cause of lumbar disc herniation and contamination of surgical specimens by the normal skin microbiota is, therefore, critical in establishing a microbial role of *C. acnes* within a subset of disc herniation patients (Rollason *et al*., 2013). Establishment of virulence factor production profiles for clinical *C. acnes* isolates obtained from herniated disc tissue and acne lesions would allow for the generation of phenotypic profiles which could prove critical in understanding the true role of *C. acnes* within human infection and may inform future infection treatment protocols. Consideration of virulence factor profiles of *C. acnes* may also inform the production of novel infection prevention strategies in the form of vaccines within a field in which the true extent of *C. acnes*-associated infection severity and morbidity is largely under-recognised.

This thesis has sought to investigate the virulence mechanisms of *C. acnes* of a range of phylotypes associated with lumbar disc herniations and associated CLBP.

The aims of the study were to:

- Establish an optimised method of biofilm formation assessment to subsequently measure the biofilm forming capabilities of a larger panel of IVD *C. acnes* isolates to understand the potential role of biofilm formation in disc tissue colonisation and persistence. Biofilm optimisation was conducted in an attempt to standardise the way in which biofilm formation is assessed for this bacterial species.

- Determine the phenotypic characteristics of *C. acnes* of a range of phylotypes in an attempt to define the exocellular virulence factors that may be associated with IVD tissue infection and persistence.

- Assess the suitability of *G. mellonella* waxworm larvae as a possible cost-effective, rapid, alternative host infection system for modelling the virulence of clinical *C. acnes* isolates *in vivo*.

- Determine the CAMP factor 1 production status of a range of *C. acnes* IVD tissue isolates using an optimised SDS-PAGE protocol for whole-cell *C. acnes* extracts and subsequent protein immunoblotting, with CAMP factor 1 potentially being a novel future vaccine target.

CHAPTER 2 OPTIMISATION OF THE BIOFILM FORMATION PROTOCOL

2.1 Introduction

Biofilm formation on foreign materials has been shown to increase the resistance of constituent bacteria to antibiotic treatment. *C. acnes* cells can form biofilms on gentamicin-loaded bone cement surfaces, even in the presence of antibiotics within the growth medium (Tunney *et al*., 2007). Consequently, *C. acnes* biofilm formation often necessitates the surgical debridement of infected tissue and the removal of infected foreign devices (Achermann *et al*., 2014). Therefore, biofilm formation plays a significant role in the pathogenicity of *C. acnes*.

Although quantitative molecular methods, such as real-time PCR assays, can be used to detect and quantify bacteria in biofilms (Guilbaud *et al*., 2005), *in vitro* model systems, such as those described by Coenye and Nelis (2010), are commonly used to investigate the formation and composition of these multicellular bacterial structures. Of these, microtitre plate-based methods are one of the most frequently used biofilm model systems (Cerca *et al*., 2005; Coenye, Peeters and Nelis, 2007; Krom *et al*., 2007), with Melo *et al*. (2013) identifying that microtitre plate methods can be more sensitive in identifying biofilm-producing bacterial species compared with molecular PCR analysis. The most widely used

method for assessing biofilm formation in microtitre plates is the crystal violet staining protocol, originally described by Christensen *et al*. (1985). This method is also used to validate the efficacy of alternative methods of biofilm formation quantification. For example, Chen *et al*. (2012) demonstrated the strong correlation between crystal violet assay absorbance values and cross-polymerisation optical coherence tomography (CP-OCT) average image light intensity profiles, validating CP-OCT as a method of monitoring longitudinal changes in biofilm biomass.

In the last decade, several studies investigating biofilm formation within clinical isolates of *C. acnes* have been published, with different modifications of the basic microtitre plate test protocol being employed (Merritt, Kadouri, and O'Toole, 2005; Christensen *et al*., 1985). Holmberg *et al*. (2009) reported higher biofilm formation among clinical *C. acnes* isolates cultivated in BHI broth supplemented with 0.5% (v/v) glucose as compared to *C. acnes* isolated from the skin of healthy controls, suggesting that glucose supplementation of growth media promotes the *in vitro* study of *C. acnes* biofilm forming capabilities. Coenye, Peeters and Nelis (2007) demonstrated that altering inoculum density, adhesion time and biofilm growth time promoted the establishment of mature *C. acnes* biofilms. These results demonstrate that *C. acnes* biofilm formation is affected by environmental factors and culture conditions, such as media composition, and suggest that the site of isolation of *C. acnes* may also affect the degree of biofilm formation seen *in vitro*. However, there is no current gold-standard method for measuring biofilm formation in *C. acnes* species, with no studies thus far having optimised methods for detecting biofilm formation within *C. acnes* isolated from

herniated disc and acne lesion samples. Therefore, this chapter outlines the development and optimisation of a reproducible methodology for measuring *C. acnes* biofilms. This method will then be taken forward to be used further in this thesis for the quantification of biofilm formation within clinical *C. acnes* isolates subcultured from herniated disc tissue and acne lesion swabs (chapter 3).

2.1.1 Aims

The aims of this biofilm optimisation study were to:

- Ascertain the effects of methanol fixation and glucose supplementation of BHI growth media on the degree of biofilm formation of IVD tissue *C. acnes* clinical isolates.
- Optimise microtitre plate incubation durations, which included 48-hours, 72-hours, 96-hours and 7-days of plate incubation, to promote the formation of mature *C. acnes* biofilms.
- Compare the biofilm forming capabilities of 24-hour and 72-hour *C. acnes* IVD tissue cultures to determine the optimum culture age for biofilm formation.

2.2 Materials and Methods

2.2.1 Bacterial Strains and Growth Conditions

C. acnes clinical isolates (*n*=67) were isolated from herniated disc tissue removed during microdiscectomy from patients undergoing discectomy surgery (attending The Mølholm Hospital, Vejle, Denmark). Exact disc tissue biopsy collection conditions and *C. acnes* isolation parameters of all isolates used in the current study are described by Rollason *et al*. (2013). All isolates were processed and typed through *recA* sequence analysis at Queen's University Belfast to differentiate *C. acnes* isolates into phylogroups IA, IB, II, or III.

C. acnes isolates from acne lesions obtained using skin swabs (skin surface isolates) (*n*=8) were kindly provided by Professor Antony Hilton and Dr Tony Worthington (Aston University, Birmingham, UK).

Ethical approval for the study was obtained from the Coventry University Ethics Application and Authorisation System.

A representative panel of these human disc *C. acnes* isolates, consisting of phylotypes IA_1 (*n*=2), IB (*n*=2), II (*n*=2) and III (*n*=2), were examined for their abilities to form biofilms *in vitro* using different biofilm formation protocols. In addition, the reference strain *C. acnes* NCTC 737 (*n*=1) was included. All isolates of the same phylotype were isolated from different patients.

Distinct colonies of *C. acnes* were resuspended in 10ml aliquots of BHI broth (Oxoid) and incubated anaerobically at 37°C for 72-hours.

2.2.2 Bacterial Storage and Revival Conditions

Strains were revived from frozen stocks and maintained at -80°C. *C. acnes* isolates were sub-cultured onto brain heart infusion (BHI) agar slopes and incubated anaerobically (hydrogen (H_2) 10%, carbon dioxide ($C0_2$) 10% and nitrogen (N_2) 80%; Don Whitley Scientific, UK) at 37°C for 8-days. Isolates were then streak-plated onto BHI agar plates incubated anaerobically at 37°C for around 7-days to obtain single colonies.

2.2.3 Phenotypic Identification

Colonies were subcultured onto BHI agar plates and incubated under anaerobic conditions for 7-days at 37°C before Gram-staining of representative isolates. All disc tissue isolates were previously confirmed as *C. acnes* through biochemical analysis using the Rapid ID 32A kit (bioMérieux) (Rollason *et al*., 2013).

2.2.4 Culture Standardisation

72-hour planktonic cultures of *C. acnes* isolates 88 (IA_1), 40 (IB), 89 (II), 1 (III) and *C.*

acnes NCTC 737 were used to investigate the relationship between absorbance and colony-forming unit (CFU) counts. This enabled absorbance to be used for culture standardisation during protocol optimisation. Optical density (OD) values of all planktonic cultures were read at 600nm (OD_{600}) prior to standardisation. Sterile BHI broth was used to dilute and standardise all planktonic cultures to the lowest OD reading of around OD_{600}= 0.08. Serial two-fold doubling dilutions of all standardised planktonic cultures were performed in triplicate. 10^{-3}, 10^{-4} and 10^{-5} dilutions were plated onto BHI agar in duplicates and incubated anaerobically at 37°C for 7-days. The dilutions yielding between 30-300 colonies were selected for CFU/ml determination.

2.2.5 Initial Biofilm Formation and Quantification (Protocol 1)

Biofilm formation of all selected *C. acnes* isolates was initially determined as previously described (Christensen *et al*., 1985; Merritt, Kadouri and O'Toole, 2005), with minor modifications (referred to hereafter as protocol 1) (Table 2.1). Briefly, the individual wells of sterile, polystyrene, 96-well, flat-bottomed, tissue-culture treated plates (Sarstedt, Germany) were inoculated with 200µl of standardised 72-hour planktonic cultures of disc tissue isolates 88 (IA_1), 69 (IA_1), 40 (IB), 58 (II), 89 (II), 1 (III), 77 (III) and NCTC 737. Three separate planktonic cultures were prepared for each *C. acnes* isolate, with 12 replicate wells for each. The inoculated tissue culture plates were incubated anaerobically at 37°C

without shaking, with one plate incubated for 48-hours and another plate incubated for 72-hours. The media was removed, followed by gentle washing of each well (three times) with phosphate-buffered saline (PBS). Biofilms were stained with 200µl of crystal violet (0.1% (w/v) in water) (Fisher Scientific, UK) for 10-minutes at room temperature. Crystal violet was removed and the wells were rinsed with PBS (three times). Two hundred microlitres of 95% (v/v) ethanol was added to each stained well and incubated at room temperature for 10-minutes to allow biofilm-bound crystal violet to solubilise. The contents of each well were mixed briefly by pipetting, with 200µl of the crystal violet/ethanol solution being transferred from each well to a separate well in a polystyrene, flat-bottomed, 96-well plate (Sarstedt, Germany). The OD of each well was measured at 595nm using a microplate spectrophotometer (Sunrise Tecan).

Negative control wells were inoculated with sterile BHI broth only. Twelve replicates were obtained for negative control wells, with mean absorbance values of the negative control wells being subtracted from the absorbance of the contents in each test well before statistical evaluation. All experiments were repeated three times using separate planktonic cultures, with the exception of Figure 2.2.

2.2.6 Biofilm Method Optimisation

2.2.6.1 Glucose-Supplemented BHI Broth and Methanol Fixation (Protocol 2)

Optimisation of biofilm formation was performed using the original microtitre plate assay protocol previously described by Holmberg *et al*. (2009), referred to hereafter as protocol 2. Briefly, bacterial isolates were cultivated in BHI broth supplemented with glucose [0.5% (w/v)] and incubated anaerobically at 37°C for 72-hours. Two separate planktonic cultures were prepared for each *C. acnes* isolate, with 12 replicate wells for each. The ODs of these 72-hour planktonic cultures at OD_{600} were standardised to 0.08 by dilution with sterile glucose-supplemented BHI broth. Biofilms were formed as described above (2.2.5). However, prior to crystal violet staining, the remaining biofilms were fixed in 200µl of absolute methanol for 10-minutes. Biofilm plates were incubated anaerobically at 37°C for 48- or 72-hours (Table 2.1).

2.2.6.2 Glucose-Supplemented BHI Broth Without Methanol Fixation (Protocol 3)

Another protocol involving the omission of methanol fixation from protocol 2 was also investigated. This protocol, modified from the original Holmberg *et al*. (2009) study, will, hereafter, be referred to as protocol 3. All protocol 3 experiments used 72-hour *C. acnes* planktonic cultures, with biofilm plates being incubated anaerobically at 37°C for 48- or 72-

hours (Table 2.1). Three separate planktonic cultures were prepared for each *C. acnes* isolate, with 12 replicate wells for each.

Table 2.1: Comparison of protocols 1, 2 and 3 based on the glucose supplementation status of BHI broth media and the inclusion or exclusion of methanol fixation.

	Protocol 1 (Merritt, Kadouri and O'Toole, 2005)	**Protocol 2** (Original Holmberg *et al*., 2009)	**Protocol 3** (Modified Holmberg *et al*., 2009)
Glucose-supplementation of BHI broth	-	✓	✓
Methanol fixation	-	✓	-

2.2.6.3 Glucose-Supplemented BHI Broth and Incubation Duration Variation

As described by Qi *et al*. (2008), mature *C. acnes* biofilms are seen *in vitro* 96-hours after bacterial inoculation. Therefore, additional biofilm formation assays were performed in which 72-hour *C. acnes* planktonic cultures were inoculated into microtitre plates for 96-hours of plate incubation using protocol 3. However, Tyner and Patel (2016) described the formation of mature *C. acnes* biofilms by approximately 7-days. As a result, the effect of extending biofilm plate anaerobic incubation from 96-hours to 7-days on biofilm formation

using 72-hour *C. acnes* planktonic cultures was also investigated. Three separate planktonic cultures were prepared for each *C. acnes* isolate, with 12 replicate wells for each.

2.2.6.4 Glucose-Supplemented BHI Broth and Planktonic Culture Age Variation

In addition to the assessment of biofilm formation by 72-hour *C. acnes* planktonic cultures, biofilm formation by 24-hour *C. acnes* planktonic cultures incubated anaerobically in microtitre plates at 37°C for 96-hours was also investigated using protocol 3 (Ramage *et al*., 2003; Tafin *et al*., 2012; Bruinsma *et al*., 2001; Jones *et al*., 2003; Razatos *et al*., 1998; Albareda *et al*., 2006; Holland *et al*., 2010). Three separate planktonic cultures were prepared for each *C. acnes* isolate, with 12 replicate wells for each.

2.2.6.5 Data Analysis

Means ± standard deviations of crystal violet absorbance data (semi-quantitative) were calculated and statistical analysis of data was performed using the Statistical Package for the Social Sciences (SPSS) 22.0 using one-way analysis of variance (ANOVA) without replication. Data was presented as bar charts, with bars representing percentage increase or decrease of biofilm formation of each isolate compared to the mean negative control value of each microtitre plate. Negative controls were omitted from all figures. Standard deviation

was represented as error bars. Mean inter-experimental variation in biofilm formation was assessed through the calculation of inter-experimental coefficient of variation [CV (%)] values by expressing standard deviation as a percentage of the mean absorbance. This was used to compare the reproducibility of biofilm formation data across different protocols. Results were considered statistically significant when a confidence level greater than 95% or 99% was reached ($p < 0.05$ or $p < 0.01$, respectively).

2.3 Results

2.3.1 Planktonic Culture Standardisation

Results demonstrated that OD_{600} of around 0.08 was equivalent to approximately 1×10^6 cells/ml in all phylotypes tested. Therefore, the ODs of all *C. acnes* planktonic cultures used in the biofilm optimisation study were adjusted to $OD_{600} = 0.08$.

2.3.2 Evaluation of Protocol 1 Biofilm Formation Assay

Crystal violet staining was used to compare the biomass within biofilms of seven *C. acnes* clinical isolates and *C. acnes* NCTC 737 control strains. These biofilms were generated using protocol 1 (2.2.5). Generally, similar levels of biofilm formation were noted across all

disc tissue isolates of *C. acnes* using this method (Figure 2.1). *C. acnes* clinical disc tissue isolates 88 (IA_1), 40 (IB) and 77 (III) formed significantly more dense biofilms following 72-hours of biofilm plate incubation compared to the negative control ($p < 0.05$) (Figure 2.1). In contrast, clinical isolate 89 (II) was the only disc tissue isolate to form significantly more dense biofilms at 48-hours of biofilm plate incubation compared to the negative control ($p < 0.01$) (Figure 2.1). Extending biofilm plate incubation from 48- to 72-hours led to the greatest increase in biofilm formation for isolate 88 (IA_1), with biofilm formation increasing from 8% to 41% as compared to the negative control (Figure 2.1). Isolates 69 (IA_1), 58 (II) and 1 (III) failed to form significantly more dense biofilms as compared to the negative control, regardless of biofilm plate incubation duration ($p > 0.05$) (Figure 2.1).

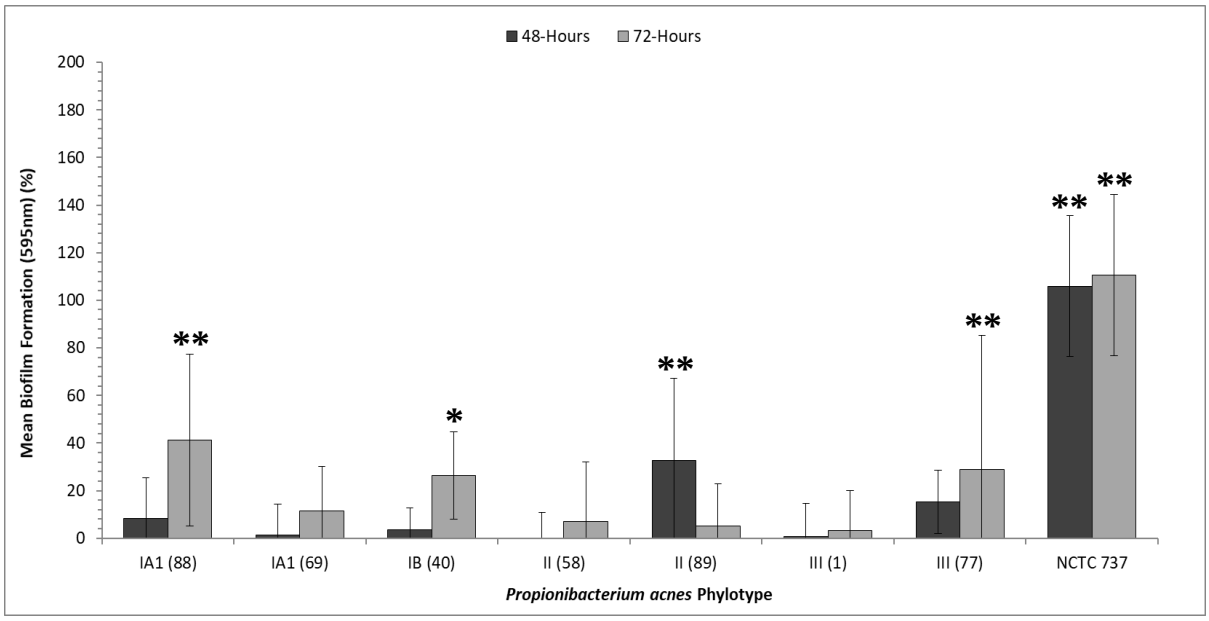


Figure 2.1: Mean percentage biofilm formation of clinical and control *C. acnes* isolates using protocol 1, with microtitre plates being incubated at 37°C for 48- or 72-hours. Mean percentage biofilm increase and decrease values are compared to the negative control (negative control not included on the figure). Three independent experiments were conducted for all isolates with 12 replicate wells for each (*n*=36). Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

2.3.3 Evaluation of Protocol 2 Biofilm Formation Assay

In order to devise an optimised *C. acnes* biofilm formation protocol, an alternative published protocol, involving the supplementation of BHI broth with 0.5% (w/v) glucose and the fixation of *C. acnes* biofilms using absolute methanol prior to crystal violet staining, was investigated (protocol 2). As shown in Figure 2.2 below, similar levels of biofilm formation are noted across all *C. acnes* phylotypes. Disc tissue isolates 88 (IA_1) and 40 (IB) formed significantly more dense biofilms following 48-hours of biofilm plate incubation as compared to the negative control ($p < 0.05$) (Figure 2.2). Isolate 1 (III) formed significantly more dense biofilms following 72-hours of biofilm plate incubation as compared to the negative control ($p < 0.05$) (Figure 2.2). Isolate 58 (II) was the only clinical *C. acnes* isolate to form significantly more dense biofilms following both 48- and 72-hours of biofilm plate incubation as compared to the negative control ($p < 0.05$) (Figure 2.2). Isolates 69 (IA_1), 89 (II) and 77 (III) failed to form significantly more dense biofilms as compared to the negative control, regardless of biofilm plate incubation duration (Figure 2.2).

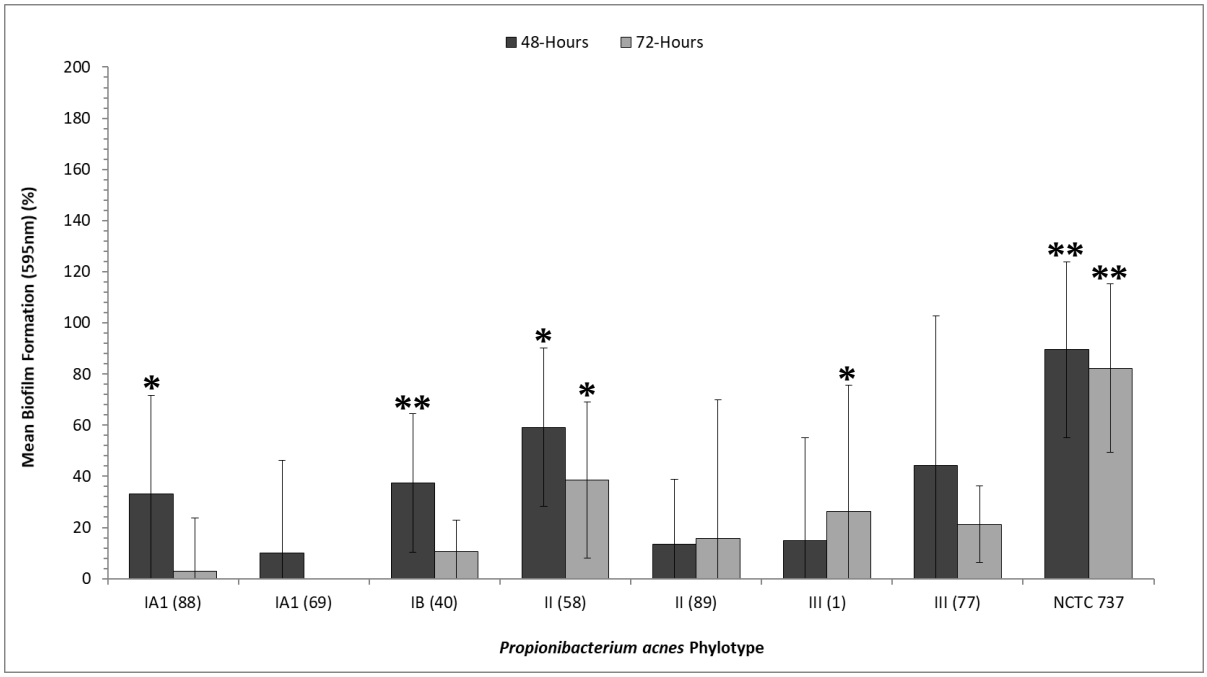


Figure 2.2: Mean percentage biofilm formation of clinical and control *C. acnes* isolates using protocol 2, with microtitre plates being incubated at 37°C for 48- or 72-hours. Mean percentage biofilm increase and decrease values are compared to the negative control (negative control not included on the figure). Two independent protocol 2 experiments were conducted for all isolates with 12 replicate wells for each (*n*=24). Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

2.3.4 Evaluation of Protocol 3 Biofilm Formation Assay

As part of devising an optimised *C. acnes* biofilm formation protocol, protocol 3 (glucose-supplemented BHI broth without methanol fixation) was also used. Significant biofilm formation was noted amongst *C. acnes* isolates 40 (IB) and 58 (II) following 72-hours of biofilm plate incubation as compared to the negative control ($p < 0.05$) (Figure 2.3). Isolate 88 (IA_1) was the only clinical *C. acnes* isolate to form significantly more dense biofilms following both 48- and 72-hours of biofilm plate incubation as compared to the negative control ($p < 0.05$) (Figure 2.3). Of all clinical *C. acnes* isolates, isolate 88 (IA_1) formed the most biofilm, with the extension of plate incubation from 48- to 72-hours leading to the greatest increase in biofilm formation of this isolate from 14% to 46% as compared to the negative control (Figure 2.3). Four clinical *C. acnes* isolates [69 (IA_1), 89 (II), 1 (III) and 77 (III)] failed to form significantly more dense biofilms as compared to the negative control, regardless of biofilm plate incubation duration (Figure 2.3).

Protocol 3 is characterised by low intra-experimental biofilm biomass variability, as demonstrated by the small standard deviation error bars for both clinical and control bacterial isolates (Figure 2.3).

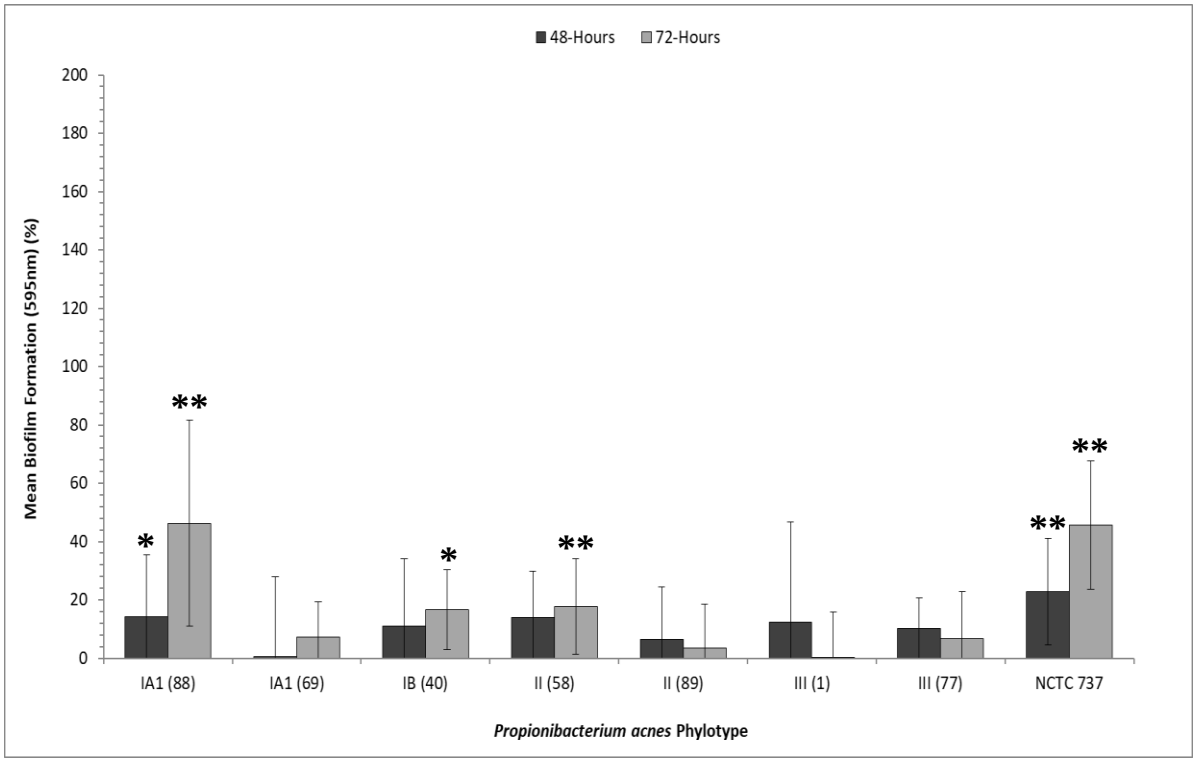


Figure 2.3: Mean percentage biofilm formation of clinical and control *C. acnes* isolates using protocol 3, with microtitre plates being incubated at 37°C for 48- or 72-hours. Mean percentage biofilm increase and decrease values are compared to the negative control (negative control not included on the figure). Three independent protocol 3 experiments were conducted for all isolates with 12 replicate wells for each (*n*=36). Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

2.3.5 Comparisons of Biofilm Formation Protocols to Determine an Optimised Protocol

As shown in Table 2.2 below, protocol 2 generally led to the greatest degree of biofilm formation within both clinical and control bacterial isolates as compared to protocol 1 and protocol 3, with significantly more dense biofilms being formed on seven occasions with protocol 2, as opposed to six occasions with both protocols 1 and 3 ($p < 0.05$) (Table 2.2). However, compared to most clinical *C. acnes* isolates, *C. acnes* NCTC 737 generally formed the most significantly dense biofilms as compared to the negative broth control, regardless of biofilm formation protocol used and the duration of biofilm plate incubation (48- or 72-hours) (Figures 2.1, 2.2 and Table 2.2) ($p < 0.05$).

The addition of 0.5% (w/v) glucose to BHI broth generally decreased biofilm formation in the *C. acnes* NCTC 737 control strain from 106% to 23% following 48-hours of incubation and from 111% to 46% following 72-hours of incubation when protocol 1 was compared to protocol 3 (Table 2.2). However, similar levels of biofilm formation were noted amongst clinical *C. acnes* isolates in the presence and absence of glucose without methanol fixation (protocols 1 and 3) (Table 2.2). This was particularly evident following 72-hours of microtitre plate incubation (Table 2.2). Therefore, glucose supplementation alone seems to neither increase nor decrease biofilm formation in these isolates. However, as shown in Table 2.3, glucose supplementation alone (protocol 3) was characterised by lower biofilm formation inter-assay variability as compared to the other two protocols.

Table 2.2: Mean percentage biofilm formation of clinical and control *C. acnes* isolates as compared to negative control wells using three different biofilm formation protocols. Mean percentage biofilm increase and decrease values are compared to the negative control and are calculated from up to three overnight planktonic cultures for each isolate with 12 replicate wells for each (*n*=36). Up to three independent experiments were conducted for each protocol. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

	Protocol					
	Protocol 1- (Merritt, Kadouri and O'Toole, 2005)		**Protocol 2-** (Original Holmberg *et al*., 2009)		**Protocol 3-** (Modified Holmberg *et al*., 2009)	
	No Glucose-Supplementation and No Methanol Fixation		Glucose-Supplementation and Methanol Fixation		Glucose-Supplementation and No Methanol Fixation	
	48-Hours	**72-Hours**	**48-Hours**	**72-Hours**	**48-Hours**	**72-Hours**
***C. acnes* Isolate Number**	**Mean Biofilm Formation (%)**		**Mean Biofilm Formation (%)**		**Mean Biofilm Formation (%)**	
88 (IA_1)	8	41**	33*	3	14*	46**
69 (IA_1)	1	12	10	-7	1	7
40 (IB)	4	26*	37**	11	11	17*
58 (II)	-1	7	59*	39**	14	18**
89 (II)	33**	5	14	16	6	3
1 (III)	1	3	15	26*	12	0
77 (III)	15	29**	44	21	10	7
NCTC 737	106**	111**	89**	82**	23**	46**

Table 2.3 below summarises the mean inter-experimental coefficient of variation [CV (%)] values for biofilm formation in a range of *C. acnes* clinical isolates using protocols 1, 2 and

3. Overall, protocol 3 had the lowest mean inter-experimental variation at both 48- and 72-hours of plate incubation for *C. acnes* phylotypes II, III and NCTC 737 as compared to protocols 1 and 2 (Table 2.3). This highlights the higher reproducibility of biofilm formation data obtained using this method as compared to the use of protocols 1 and 2. Additionally, protocol 3 is characterised by significantly lower mean CV (%) values as compared to protocol 2 ($p < 0.05$), further demonstrating the reproducibility of this method. No significant relationship was found between *C. acnes* phylotype and mean CV (%) values obtained using protocol 3, suggesting that intra-assay CV (%) values also remained consistent across phylotypes using this protocol ($p > 0.05$). Therefore, this optimised method was taken forward for further biofilm optimisation studies.

Table 2.3: Mean percentage inter-assay CV (%) values of biofilms formed by clinical and control *C. acnes* isolates using three different biofilm formation protocols. Results are averages calculated from up to three overnight planktonic cultures for each isolate with 12 replicate wells for each (*n*=36). Therefore, each CV (%) value at 48-hours and 72-hours for protocols 1 and 3 for phylotypes IA_1, II, III and NCTC 737 are represented by 72 absorbance values. Each CV (%) value for phylotype IB at 48-hours and 72-hours for protocols 1 and 3 is represented by 36 absorbance values. Each CV (%) value at 48-hours and 72-hours for protocol 2 for phylotypes IA_1, II, III and NCTC 737 are represented by 48 absorbance values. Each CV (%) value for phylotype IB at 48-hours and 72-hours for protocol 2 is represented by 24 absorbance values. Up to three independent experiments were conducted for each protocol. * p <0.05; ** p < 0.01.

	Protocol					
	Protocol 1- (Merritt, Kadouri and O'Toole, 2005)		**Protocol 2-** (Original Holmberg *et al*., 2009)		**Protocol 3-** (Modified Holmberg *et al*., 2009)	
	No Glucose-Supplementation and No Methanol Fixation		Glucose-Supplementation and Methanol Fixation		Glucose-Supplementation and No Methanol Fixation	
	48-Hours	**72-Hours**	**48-Hours**	**72-Hours**	**48-Hours**	**72-Hours**
***C. acnes* Phylotype**	**Mean Inter-Assay CV (%)**		**Mean Inter-Assay CV (%)**		**Mean Inter-Assay CV (%)**	
IA_1 (*n*=2 strains)	14.98	20.46	29.07	14.13	22.48	17.28
IB (*n*=1 strain)	8.44	14.00	19.21	11.43	20.27	11.97
II (*n*=2 strains)	20.72	19.73	19.58	32.89	15.35	14.31
III (*n*=2 strains)	12.76	25.99	34.86	23.26	18.92	15.15
NCTC 737	15.33	17.67	17.35	18.52	14.64	15.19

2.3.6 Effect of Planktonic Culture Age Variation on Biofilm Biomass

Bacterial phase of growth is known to modulate the adhesive capacity of bacterial cells and with no standard planktonic culture growth time for *C. acnes* biofilm formation studies having been published, the potential effects of culture age on biofilm formation was investigated. Using the optimised biofilm formation protocol (protocol 3) and 96-hours of biofilm plate incubation (to give greater time for biofilm establishment), biofilm formation among 24- and 72-hour *C. acnes* planktonic cultures were compared. Following standardisation, all cultures were incubated anaerobically in microtitre plates at 37°C for 96-hours to assess biofilm formation.

In general, although greater biofilm formation was noted amongst 24-hour planktonic cultures of two clinical *C. acnes* isolates [40 (IB) and 15 (IB)] as compared to corresponding 72-hour planktonic cultures, percentage biofilm formation among all other 24-hour *C. acnes* planktonic cultures were lower as compared to corresponding 72-hour planktonic cultures (Figure 2.4). Additionally, significantly more dense biofilm formation occurred amongst 72-hour planktonic cultures of *C. acnes* clinical isolates 69 (IA_1), 40 (IB), 15 (IB), acne lesion 1 and *C. acnes* NCTC 737, with no 24-hour *C. acnes* planktonic cultures forming significantly dense biofilms ($p < 0.05$) (Figure 2.4). Therefore, a planktonic culture age of 72-hours was selected for all *C. acnes* isolates within subsequent biofilm formation studies.

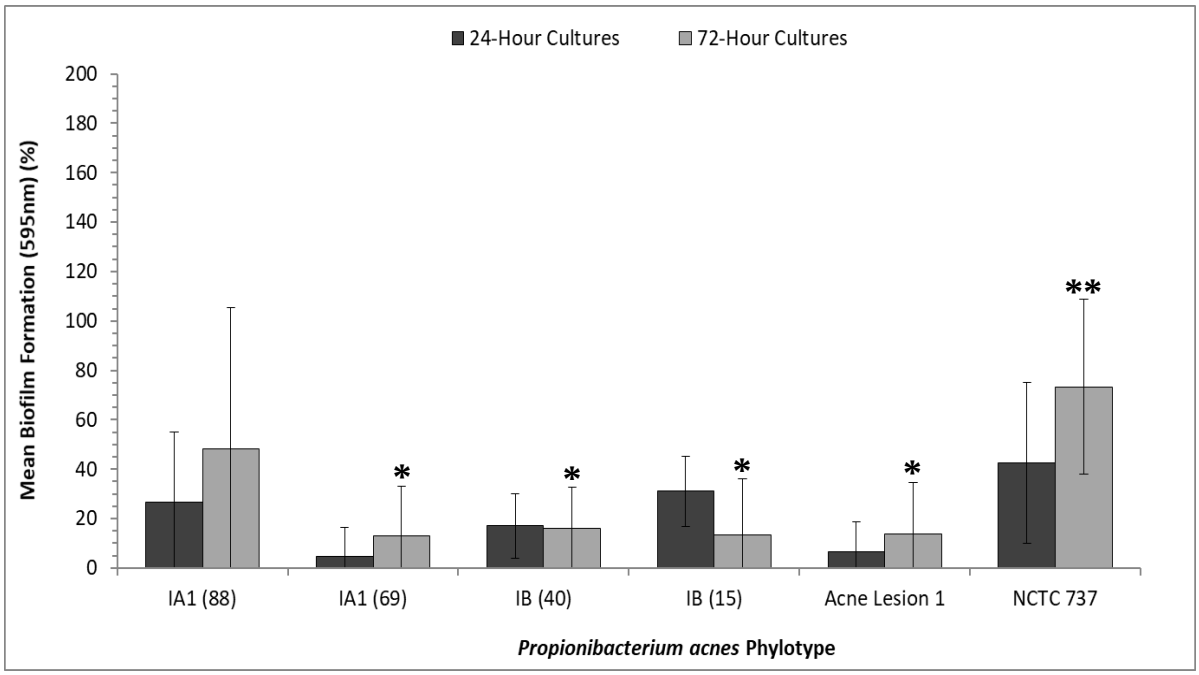


Figure 2.4: Mean percentage biofilm formation of standardised 24-hour and 72-hour clinical and control *C. acnes* planktonic cultures incubated at 37°C for 96-hours post microtitre plate inoculation using the optimised protocol 3. Mean percentage biofilm increase and decrease values are compared to the negative control (negative control not included on the figure). Three independent experiments were conducted for all isolates with 12 replicate wells for each (*n*=36). Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control ($p < 0.05$). ** Biofilm biomass highly significantly greater than the negative control ($p < 0.01$).

2.3.7 Further Investigation of the Effect of Incubation Duration Variation on Biofilm Biomass

In order to further optimise protocol 3 for the assessment of *C. acnes* biofilm formation, the effect of incubation duration variation, which was extended from 72-hours to 96-hours using a 72-hour planktonic culture as previously optimised (2.3.5), on biofilm biomass was investigated. As shown in Figure 2.5 below, biofilm formation increased by 17% for *C. acnes* isolate 69 (IA_1), 11% for *C. acnes* isolate 40 (IB), 22% for *C. acnes* isolate 58 (II), 9% for *C. acnes* isolate 89 (II), 6% for *C. acnes* isolate 1 (III) and 35% for *C. acnes* NCTC 737 with the extension of biofilm plate incubation duration from 72-hours to 96-hours using optimised protocol 3 as compared to the negative control ($p < 0.05$). These results suggest that increasing incubation duration promotes significantly more dense biofilm formation by *C. acnes*.

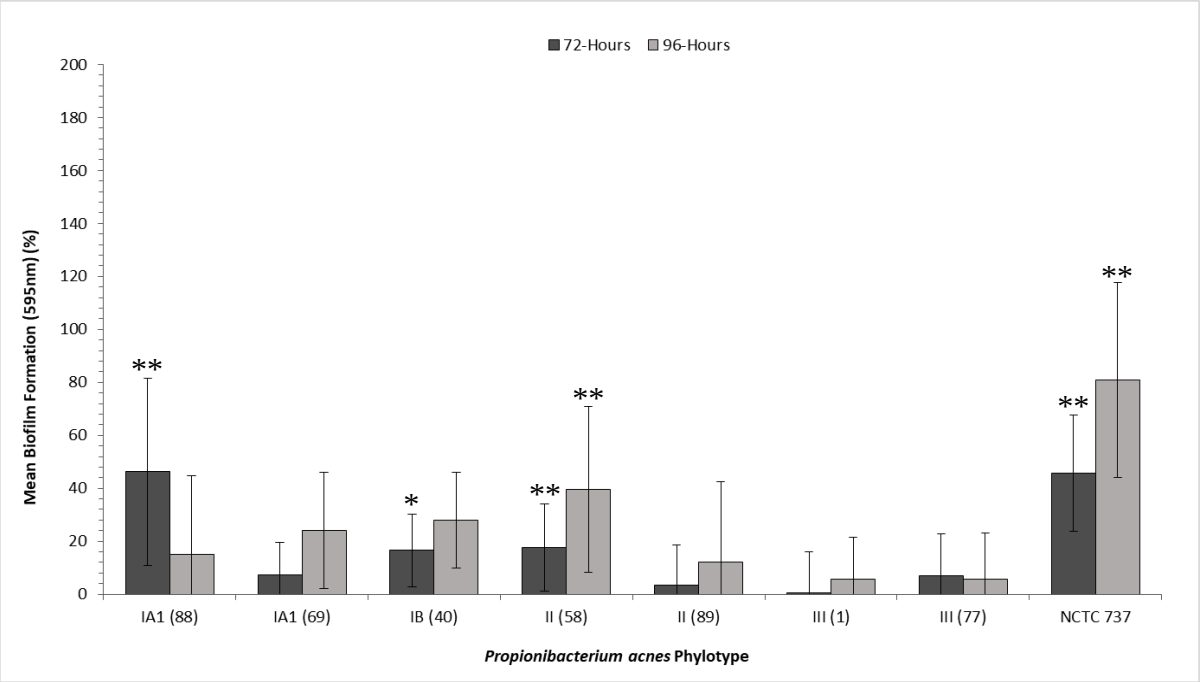


Figure 2.5: Mean percentage biofilm formation of clinical and control *C. acnes* isolates as compared to negative control wells using optimised protocol 3 incubated anaerobically for either 72-hours or 96-hours at 37°C. Mean percentage biofilm formation values are compared to the negative control and are calculated from three 72-hour planktonic cultures for each isolate with 12 replicate wells for each (*n*=36) (negative control not included on the figure). Three independent experiments were conducted. Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

As shown in Figure 2.6, further increasing anaerobic incubation at 37°C from 96-hours to 7-days using optimised protocol 3 increased biofilm formation by 21% for *C. acnes* isolate 58 (II) and 74% for *C. acnes* NCTC 737 as compared to the negative control ($p < 0.05$). Additionally, this extension in biofilm plate incubation to 7-days promoted significantly more dense biofilm formation among *C. acnes* clinical isolates 88 (IA_1), 69 (IA_1), 40 (IB), 15 (IB), 89 (II), acne lesion isolate 1 and acne lesion isolate 6 as compared to the negative control ($p < 0.05$) (Figure 2.6). In contrast, all of these isolates failed to form significantly dense biofilms following 96-hours of incubation as compared to the negative control ($p > 0.05$). Therefore, increasing biofilm plate incubation duration promotes *C. acnes* biofilm formation. As a result, biofilm plate incubation duration was extended from 96-hours (protocol 3) to 7-days in subsequent biofilm formation assays.

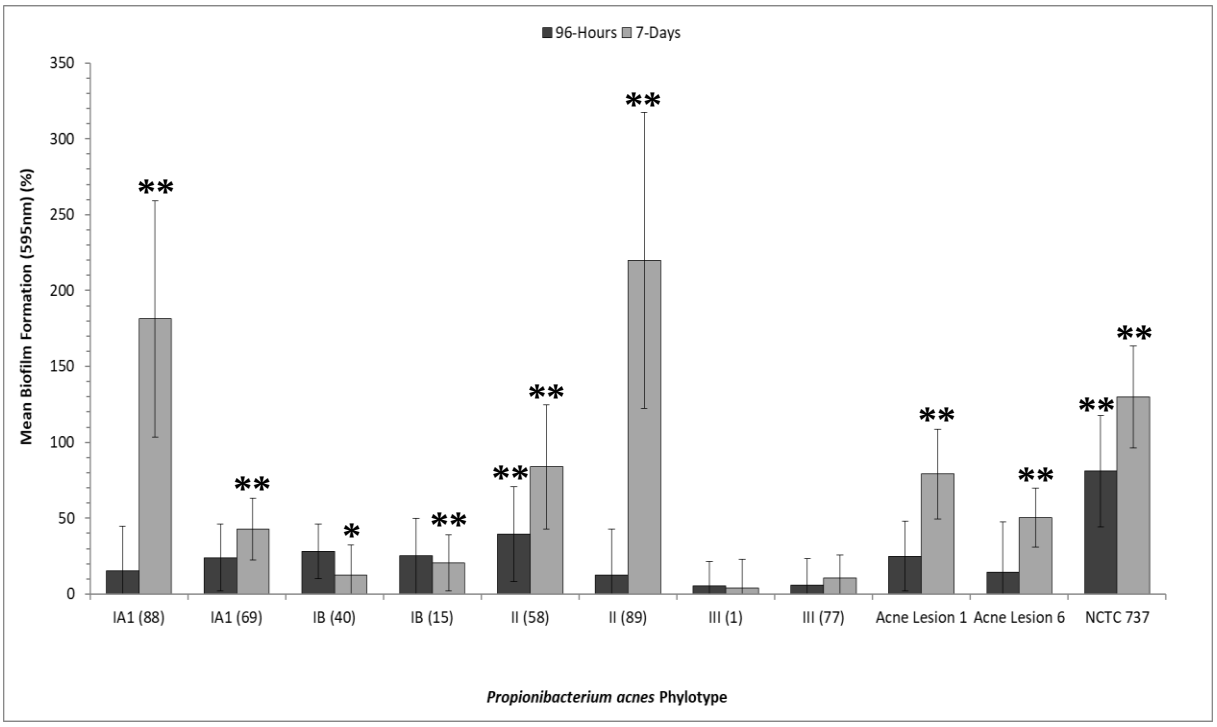


Figure 2.6: Mean percentage biofilm formation of clinical and control *C. acnes* isolates following the extension of anaerobic incubation at 37°C from 96-hours to 7-days using the optimised protocol 3. Mean percentage biofilm increase and decrease values are compared to the negative control and are calculated from three 72-hour planktonic cultures for each isolate with 12 replicate wells for each (*n*=36) (negative control not included on the figure). Three independent experiments were conducted. Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

2.4 Discussion

Biofilm formation is a complex, multi-stage process affected by a range of parameters. In the case of *C. acnes*, these include pH, oxygen tension, surface roughness of biomaterials, media composition, culture age and incubation duration (Greenman, Holland and Cunliffe, 1981; Ramage *et al*., 2003; Holmberg *et al*., 2009; Tramm-Werner *et al*., 1996; Khelissa *et al*., 2017). This demonstrates that *C. acnes* biofilm formation is sensitive to different *in vitro* culture conditions. However, despite this, no studies thus far have optimised *in vitro* biofilm formation protocols for *C. acnes* isolated from herniated disc tissue. Therefore, the current study aimed to develop a robust and reproducible optimised method for growing and analysing *C. acnes* biofilms to be used in further studies (chapter 3).

In the present study, although similar levels of biofilm formation were noted amongst clinical *C. acnes* isolates in the presence and absence of glucose without methanol fixation (protocols 1 and 3) (Table 2.2), supplementation of BHI broth with 0.5% (w/v) glucose generally reduced the mean inter-assay percentage biofilm formation variability (protocol 3) (Table 2.3). This suggests that glucose supplementation of media may promote the stability of *C. acnes* biofilms, potentially through the enhancement of glycocalyx formation, forming more cohesive biofilms (Christensen *et al*., 1985).

Teichoic acids are defined as a family of cell surface glycopolymers which contain phosphodiester-linked polyol repeat units and are unique to the Gram-positive cell wall (Brown, Santa Maria and Walker, 2013). This family includes wall teichoic acids, covalently linked to peptidoglycan, and lipoteichoic acids, which are tethered to the bacterial membrane via glycolipids (Brown, Santa Maria and Walker, 2013). Increasing glucose concentrations can promote teichoic acid biosynthesis, with teichoic acids known to form part of the bacterial biofilm matrix and subsequently can promote bacterial biofilm formation (Hodgson *et al*., 2014). Therefore, glucose supplementation of BHI broth may increase teichoic acid biosynthesis in *C. acnes* isolates, leading to enhanced biofilm stability. However, the effect of glucose on *C. acnes* biofilm biomass is concentration-dependent, with Greenman, Holland and Cunliffe (1981) demonstrating that increasing glucose concentrations beyond 0.4% (w/v) can decrease *C. acnes* biofilm biomass.

Hydrophobic interactions play a role in the initial adhesion of bacterial cells to surfaces (Ljungh and Wadström, 1995). Bacterial hydrophobicity, and subsequently adhesiveness, can be affected by culture media composition, with Dewanti and Wong (1995) demonstrating that the culture of *E. coli* 0157:H7 in a nutrient-limited minimal salts medium supplemented with glucose significantly increased the hydrophobicity of constituent cells as compared to culture in trypticase soy broth. Additionally, biofilms developed faster under conditions of low nutrient availability, with the transfer of biofilms from glucose-supplemented minimal salts medium to trypticase soy broth leading to biofilm dissociation, potentially in response to nutrient availability (Dewanti and Wong, 1995). Therefore, *C.*

acnes cells cultured in glucose-supplemented BHI broth for longer incubation periods may experience nutrient limitation, possibly increasing the hydrophobicity and adherence of these cells. This may account for the general increase in biofilm biomass across most *C. acnes* isolates following the extension of microtitre plate anaerobic incubation at 37°C from 96-hours to 7-days in glucose-rich media using the optimised protocol 3 (Figure 2.6).

As shown in Table 2.3, protocol 2 generally had the highest CV (%) values across phylotypes for 48- and 72-hours incubation compared to protocols 1 and 3, indicating low biofilm formation data reproducibility with this method. As protocol 2 was the only method to include a methanol fixation step, it was concluded that methanol fixation used within the original Holmberg *et al*. (2009) method (protocol 2) may act as an additional wash step, promoting biofilm removal and increasing inter-assay biofilm formation variation. Future studies may optimise the washing protocols of the crystal violet assay for *C. acnes* to minimise the occurrence of false-positive and false-negative biofilm biomass absorbance results due to insufficient or excessive washing, respectively.

C. acnes has a generation time of around 5.1 hours and has a slower growth rate than that of other anaerobic bacteria (Hall *et al*., 1994), potentially accounting for the under-reporting of *C. acnes* within clinical samples. Studies relating to *C. acnes* biofilm formation report using *C. acnes* cultures of different ages, ranging from 24-hours to 72-hours (Ramage *et al*., 2003; Tafin *et al*., 2012). Therefore, the effect of planktonic culture age on biofilm formation was

investigated. *C. acnes* planktonic cultures were grown for both 24- and 72-hours, after which *C. acnes* planktonic cultures have been reported to enter the exponential and stationary growth phases, respectively (Holland *et al*., 2010). Results demonstrated that 72-hour *C. acnes* planktonic cultures (stationary phase cells) possessed a greater capacity to bind to the polystyrene surfaces of biofilm plates as compared to younger, 24-hour *C. acnes* planktonic cultures (exponential phase cells) (Figure 2.4). Therefore, 72-hour cultures were taken forward into the next stage of optimisation. This link between bacterial growth phase and the adhesive nature of cells has been debated in the literature. Some studies have investigated the adhesive properties of mid-exponential phase cells (Bruinsma *et al*., 2001; Jones *et al*., 2003; Razatos *et al*., 1998) whereas research into the adhesive capacities of stationary phase cells has also been published (Walker *et al*., 2005; Albareda *et al*., 2006; Holland *et al*., 2010). The results of this study are in correlation with the findings of Albareda *et al*. (2006) who reported that stationary phase bacterial cells had a higher adhesive capacity as compared to late-exponential phase cells, potentially due to morphological and biochemical changes that occur within stationary phase cells that may modify the degree of attachment. High cell concentrations within the stationary phase of growth may also initiate cascades of cell signalling mechanisms through quorum sensing to stimulate the genetic expression of components of the extracellular matrix, promoting biofilm formation within these cells (Garrett, Bhakoo and Zhang, 2008). Additionally, differences in the secretomes of stationary and mid-exponential phase planktonic cultures have been identified, with Holland *et al*. (2010) reporting the identification of a dermatan-binding protein within stationary planktonic cultures of *C. acnes* which were not detected in the exponential phase secretome.

This indicates that growth phase can modulate the secretome of *C. acnes*, with potential preferential expression of adhesins during the stationary growth phase. This greater binding capacity of stationary phase *C. acnes* isolates tested in the current study as compared to younger, 24-hour *C. acnes* planktonic cultures may suggest an adaptation to growth in low-nutrient environments, such as in avascular disc tissue, subsequently contributing to the long-term survival and persistence of such isolates *in vivo*. Therefore, further work is required to characterise differences in the proteome and secretome of *C. acnes* isolates in different growth phases to identify factors that could be promoting adhesion in stationary phase cells. Additionally, the effect of other growth phase dependent variables, such as cell viability, on bacterial adhesiveness should be investigated.

As described in section 2.3.7, the extension of biofilm plate anaerobic incubation from 96-hours to 7-days led to significantly more dense biofilm formation among *C. acnes* clinical isolates 88 (IA_1), 69 (IA_1), 40 (IB), 15 (IB), 89 (II), acne lesion isolate 1 and acne lesion isolate 6 as compared to the negative control ($p < 0.05$) (Figure 2.6). Whilst mature *C. acnes* biofilms may initially be formed between 18 and 96-hours post-inoculation (Ramage *et al*., 2003; Tafin *et al*., 2012; Qi *et al*., 2008), Bayston *et al*. [2007 (a)] describe the formation of mature *C. acnes* biofilms *in vitro* following 6-days of incubation. Prolonged incubation may lead to increased population density and greater production and deposition of exopolysaccharide, leading to enhanced adherence to biomaterials [Bayston *et al*., 2007 (b)]. However, mature biofilm formation is dependent upon several variables, including growth media used, initial bacterial inoculum, choice of biomaterial and surface roughness of the

chosen biomaterial (Holmberg *et al*., 2009; Ramage *et al*., 2003; Qi *et al*., 2008). Therefore, in this study, the greater proportion of clinical *C. acnes* isolates forming significantly more dense biofilms following a 7-day incubation period suggests that prolonged culture incubation plays a role in promoting the formation of mature biofilms. These findings also suggest that a 7-day incubation period is necessary for *C. acnes* biofilm studies.

Although crystal violet staining provides an effective means of assessing biofilm biomass, this technique does not provide a means of assessing the viability of biofilm-associated cells as both live and dead bacterial cells, as well as extracellular matrix components, are stained (Welch, Cai and Strømme, 2012). Bacterial cell sedimentation within upright wells may also contribute to the overestimation of *C. acnes* biofilm formation, an assay limitation that may be overcome by using transferable solid phase microtitre plate lids (inverse assays) (Mampel *et al*., 2006). Additionally, the static nature of microtitre plate assays may result in the lack of aeration which could impede the formation of mature biofilms (Merritt, Kadouri and O'Toole, 2005). Furthermore, all biofilm formation assay protocols used in the current study showed varying degrees of inter-experimental variation, potentially indicating the intrinsic variable nature of crystal violet assays. Therefore, repeated measures are important in accurately assessing the biofilm formation capabilities of individual *C. acnes* isolates. Regardless of the biofilm formation protocol used in the current study, adherent bacteria developed in microtitre plates dissociated easily from the polystyrene surfaces of these plates during well washing, leading to the visible removal of biofilms formed by a range of *C. acnes* phylotypes. Therefore, the amount of biofilm detected within wells may be

underestimated. This demonstrates the weak cohesive properties of biofilms formed by these *C. acnes* herniated disc tissue isolates and suggests that the association between *C. acnes* and polystyrene surfaces may not be strong enough to maintain the biofilm structure. Therefore, although research into the ability of *C. acnes* to form biofilms on different surfaces has been conducted (Holmberg *et al*., 2009; Ramage *et al*., 2003), further work is required to investigate the ability of these isolates to form biofilms on surfaces which mimic *in vivo* conditions which may be more effective at supporting *C. acnes* biofilms. This may decrease the loss of these biofilm structures from surfaces during biofilm wash steps.

In summary, the supplementation of BHI broth with 0.5% (w/v) glucose without methanol fixation of biofilms (protocol 3), together with the use of 72-hour *C. acnes* planktonic cultures incubated in microtitre plates for 7-days, maximised biofilm formation within herniated disc isolates of *C. acnes*. This robust and reproducible method will be used to assess biofilm formation in a larger panel of clinical *C. acnes* isolates obtained from herniated discs and acne lesions (3.2.2). The simplicity of this technique gives this approach great potential for use in biofilm activity screening of *C. acnes* isolates and in the investigation of antimicrobial effects of a range of antibiotics against these biological structures.

CHAPTER 3 PHYLOTYPE-SPECIFIC BIOFILM-FORMING CAPABILITIES OF HERNIATED DISC *C. ACNES* ISOLATES

3.1 Introduction

Biofilms are communities of microbes embedded in an extracellular matrix, composed of bacterial exopolysaccharides and trapped exogenous substances, including nucleic acids, minerals and proteins (Dunne, 2002), which adhere to both biotic and abiotic surfaces. These surfaces include living tissues and indwelling medical devices (Donlan, 2002). As explored by Carpentier and Cerf (1993), microorganisms are not uniformly distributed within the biofilm and instead, these complex biological structures are composed of collections of bacterial microcolonies separated by water channels which facilitate the transport of nutrients into and within the biofilm (Stoodley, Debeer and Lewandowski, 1994).

Biofilm formation is an important bacterial defence mechanism, increasing the resistance of constituent sessile bacteria to desiccation, extreme temperatures and antibiotic therapy (Dewanti and Wong, 1995; Nickel *et al*., 1985). This increased resistance has been attributed to the restricted penetration of antimicrobials into biofilms, the expression of resistance genes within the sessile population and the decreased growth rates of biofilm-associated cells in comparison to planktonic cells (Lewis, 2001; Cvitkovitch, Li and Ellen, 2003; Rasmussen *et al*., 2005). The biofilm mode of growth also significantly increases the

resistance of constituent cells to host immune defences which may promote recurrent infection (Wu *et al*., 2015). These processes are orchestrated by bacterial quorum sensing (Lewis, 2001; Cvitkovitch, Li and Ellen, 2003; Rasmussen *et al*., 2005).

Additionally, as with other bacterial species, *C. acnes* can form mixed-phylotype biofilms, with Jahns *et al*. (2012) identifying mixed populations of a range of *C. acnes* phylotypes within the same sebaceous follicle. In support of this, Rollason *et al*. (2013) also identified multiple *C. acnes* phylotypes from individual IVD tissue samples. These inter-strain interactions may promote metabolic commensalism, whereby metabolic by-products of one strain are utilised by another (Elias and Banin, 2012). Subsequently, the treatment of biofilm-associated infections is difficult, with surgical debridement or removal of infected medical devices often being necessitated (Donlan, 2001; Von Eiff *et al*., 2005).

C. acnes biofilm formation is associated with a range of body site infections, with the biofilm mode of growth also central in the pathophysiology of implant-associated infections which can initiate the inflammatory destruction of peri-implant tissue (Paredes *et al*., 2014; Costerton, Montanaro and Arciola, 2005; Belibasakis *et al*., 2015). *C. acnes* biofilms have also been identified within pilosebaceous follicles, prostate tissue and, increasingly, within the matrix of disc tissue (Coenye *et al*., 2008; Alexeyev *et al*., 2007; Capoor *et al*., 2017). However, it is likely that the true incidence of *C. acnes* biofilm-associated infections is underestimated due to the fastidious growth requirements of this microorganism, the short incubation times used in many clinical microbiology laboratories and the lack of physical

biofilm disassembly methods, such as sonication, prior to bacterial culture (Levy *et al*., 2008; Capoor *et al*., 2017).

C. acnes can form biofilms both *in vitro* and *in vivo* on a range of orthopaedic biomaterials, including titanium alloys, polymethyl methacrylate (PMMA) and silicone [Ramage *et al*., 2003; Bayston *et al*., 2007 (b)]. Additionally, Capoor *et al*. (2016) evidenced the existence of *C. acnes* in the form of biofilm aggregates within deep tissue lumbar disc infections, potentially accounting for the greater rates of *C. acnes* recovery from protocols involving tissue homogenisation (Capoor *et al*., 2016; Rigal *et al*., 2016). However, although there is an increasing body of evidence for the role of *C. acnes* in invasive infections, including discitis (Capoor *et al*., 2016; Harris *et al*., 2005), no studies thus far have investigated biofilm formation in *C. acnes* obtained from herniated disc tissue. Additionally, few studies thus far have compared phylotype-specific biofilm forming capabilities of a range of clinical *C. acnes* isolates, with Sowmiya *et al*. (2015) arguing that no association exists between *C. acnes* subtype and biofilm formation. Assessing phylotype-specific biofilm formation amongst clinical *C. acnes* isolates is clinically important as certain subtypes of these bacteria, namely *recA* types IB, II and III, have been associated with opportunistic deep tissue infections (Dréno *et al*., 2018; Nazipi *et al*., 2017). Therefore, assessing whether this pattern is reflected in the degree of biofilm formation of individual isolates would demonstrate whether this virulence factor is important in the colonisation of IVD tissue and for the persistence of *C. acnes* within these structures.

Therefore, the present study aimed to investigate the biofilm forming capabilities of a collection of *C. acnes* isolates, belonging to the phylotypes IA_1, IB, II and III, obtained from herniated human discs. Additionally, biofilm formation was compared amongst herniated disc tissue isolates and acne lesion isolates of *C. acnes* to ascertain the role of site of isolation on this putative virulence characteristic.

3.1.1 Aims

The aims of this biofilm formation study were to:

- Further investigate whether *C. acnes* isolates taken from IVD tissue material form significant biofilms following 7-days of microtitre plate incubation.
- Determine whether phylotype-specific patterns were recorded for *C. acnes* clinical isolates taken from human IVD tissue samples.
- Investigate whether site of *C. acnes* isolation (acne lesion vs. disc tissue) had a significant effect on the degree of biofilm formation.
- Assess whether clinical *C. acnes* isolates taken from acne lesion swabs and IVD tissue material were viable from biofilms grown for 7-days *in vitro*.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Growth Conditions

A representative panel of *C. acnes* isolates obtained from human herniated disc tissue material, consisting of phylotypes IA_1 (*n*=6), IB (*n*=6), II (*n*=6) and III (*n*=6), were examined for their abilities to form biofilms *in vitro* using an optimised biofilm formation protocol (chapter 2). The biofilm forming capabilities of a range of *C. acnes* isolates obtained from human acne lesions (*n*=6) were also examined (Table 3.1). In addition, the reference strain *C. acnes* NCTC 737 (*n*=1) and *S. epidermidis* RP62A (*n*=1) were included as positive controls for biofilm formation. The majority of *C. acnes* isolates of the same phylotype were isolated from different patients.

All bacterial strains were maintained on BHI agar (Oxoid) at 4°C. For long-term storage, stocks of these strains were prepared using 25% (v/v) glycerol and BHI broth solutions and stored at -80°C.

Distinct colonies of either *C. acnes* or *S. epidermidis* RP62A were resuspended in 10ml aliquots of 0.5% (w/v) glucose-supplemented BHI broth (Oxoid) and incubated anaerobically at 37°C for 72-hours. The ODs of all cultures at 600nm (OD_{600}) were standardised to 0.08 by dilution with sterile, 0.5% (w/v) glucose-supplemented BHI broth,

which is equivalent to approximately 1×10^6 cells/ml.

3.2.2 Biofilm Formation and Quantification

The optimised biofilm formation protocol described in chapter 2 was used to investigate the *in vitro* biofilm forming capabilities of a representative panel of *C. acnes* isolates obtained from human herniated disc tissue material and acne lesions (2.2.6.2 along with the further optimisation steps highlighted in section 2.3.7).

Three separate cultures were prepared for each *C. acnes* isolate, including positive and negative controls, with 12 replicates of each. Results from all triplicate experiments were used to calculate the mean absorbance for each isolate. *S. epidermidis* RP62A and *C. acnes* NCTC 737 were used in positive control wells whilst negative control wells were inoculated with only sterile BHI broth. The mean absorbance of the negative control was subtracted from the absorbance of the content in each test well before statistical evaluation.

For comparative analysis, the OD_{595} values were used to classify the degree of biofilm production for all *C. acnes* clinical strains according to the method described by Christensen *et al*. (1985), with minor modifications. Briefly, mean OD values were calculated for each *C. acnes* phylotype as well as for all acne lesion isolates, *C. acnes* NCTC 737 and *S.*

epidermidis RP62A. The cut-off OD (ODc) was defined as three standard deviations (0.02) above the mean OD (0.07) of the negative control. Ordinal classification was used to assess the adherence capabilities of individual strains of *C. acnes*, with isolates being divided into one of four categories; non-adherent (0), weakly adherent (+), moderately adherent (++) or strongly adherent (+++), based upon the mean OD_{595} readings of bacterial biofilms as follows:

$OD \leq ODc$ = Non-adherent

$ODc < OD \leq 2 \times ODc$ = Weakly adherent

$2 \times ODc < OD \leq 4 \times ODc$ = Moderately adherent

$4 \times ODc < OD$ = Strongly adherent

If the mean ODs of biofilms were less than or equal to 0.09, isolates were classified as non-adherent. If the ODs were more than 0.09 but less than or equal to 0.18, the isolates were classified as weakly adherent. If the ODs were more than 0.18 but less than or equal to 0.36, the isolates were classified as moderately adherent and if the ODs were greater than 0.36, the isolates were classified as strongly adherent. 0.09 was chosen as the ODc as it was three standard deviations (0.02) above the mean OD of the negative control (0.07).

3.2.3 Biofilm-Associated Viable Cell Counts

For determination of biofilm-associated viable cell counts, a panel of four disc tissue *C. acnes* isolates [phylotypes IA_1 (*n*=1), IB (*n*=1), II (*n*=1) and III (*n*=1)] and one acne lesion *C. acnes* isolate were selected. 200µl of standardised 72-hour bacterial culture was inoculated into sterile, polystyrene, 96-well, flat-bottomed, tissue-culture treated plates (Sarstedt, Germany) and incubated anaerobically at 37°C for 7-days. Each well was washed three times with 200µl of PBS to remove loosely adhered and planktonic cells. Two hundred microlitres of sterile, 0.5% (w/v) glucose-supplemented BHI broth was added to each well and the biofilm plate was sonicated at 40kHz using an ultrasonic waterbath system (Rieber 375TT) for 5-minutes. Calorimetry was used to calculate the power output of the Rieber 375TT ultrasonic waterbath system and was determined as 26.6 watts/litre. Residual biofilm-associated cells were scraped off wells using sterile pipette tips. Next, serial ten-fold dilutions of sonicated biofilm suspensions were prepared in 0.5% (w/v) glucose-supplemented BHI broth. The plate count method described by Miles, Misra and Irwin (1938) was used to assess the number of CFU/ml of sonicated bacterial biofilm sample, with all inoculated CFU/ml plates being incubated anaerobically at 37°C for up to 7-days. For parity and comparisons, cultures used for biofilm CFU/ml counts were also used to prepare corresponding biofilm plates for crystal violet staining assays, as described in section 3.2.2 above. Four independent experiments were conducted for CFU/ml determination, with a total of 8 replicates for each *C. acnes* isolate.

3.2.4 Data Analysis

Means ± standard deviations of crystal violet absorbance data (semi-quantitative) were calculated and statistical analysis of data was performed with SPSS 22.0 using one-way ANOVA without replication. For each figure, one set of statistics was performed to compare each clinical isolate to the negative control and another set of statistics was performed to compare the *recA* types to one another. Data was presented as bar charts, with bars representing percentage increase or decrease in biofilm formation for each isolate compared to the mean negative control value of each microtitre plate. Experimental biofilm crystal violet OD data was normalised relative to the negative control. Comparisons were also made between *C. acnes* of varying phylotypes and sites of isolation, namely isolates originating from disc tissue or acne lesions, for both semi-quantitative biofilm formation methods and quantitative CFU/ml methods using one-way ANOVA without replication in SPSS. Standard deviation was represented as error bars. Results were considered statistically significant when a confidence level greater than 95% or 99% was reached ($p < 0.05$ or $p < 0.01$, respectively) and these mean percentage increase and decrease values were marked with either a single asterisk (*) ($p < 0.05$) or a double asterisk (**) ($p < 0.01$). Negative controls were omitted from all figures.

3.3 Results

3.3.1 Assessment of Strength of Biofilm Formation Amongst *C. acnes* Clinical Isolates

As shown in Figures 3.1, 3.2 and 3.3 below, the use of crystal violet OD data demonstrated that the majority (70%) of disc tissue and acne lesion isolates of *C. acnes* produced significantly more dense biofilms in a microtitre model of biofilm formation following 7-days of anaerobic plate incubation as compared to the negative control ($p < 0.05$). The greatest degrees of biofilm formation were noted amongst isolates 17 (IB) and 88 (IA_1), with mean biofilm formation values of 140% and 168% as compared to the negative control, respectively (Figures 3.1 and 3.2) ($p < 0.01$). No significant differences between the biofilm densities of *C. acnes* disc tissue isolates 15 (IB), 90 (IB) and acne lesion 7 were observed under these conditions as compared with the negative control ($p > 0.05$) (Figures 3.1 and 3.3). Interestingly, all phylotype III disc tissue *C. acnes* isolates failed to form significantly more dense biofilms as compared to the negative control ($p > 0.05$) (Figures 3.1, 3.2 and 3.3).

Although the disc tissue *C. acnes* isolates 40 (IB), 82 (IB) and 2 (II) formed significantly more dense biofilms as compared to the negative control ($p < 0.05$) (Figures 3.1 and 3.3), these isolates were classified as non-biofilm formers in Tables 3.2 and 3.3 as the microtitre plate biofilm classification cut-off limit is based on three standard deviations above the

mean OD of the negative control. Therefore, these isolates are weaker biofilm formers as compared to other clinical disc tissue and acne lesion *C. acnes* strains.

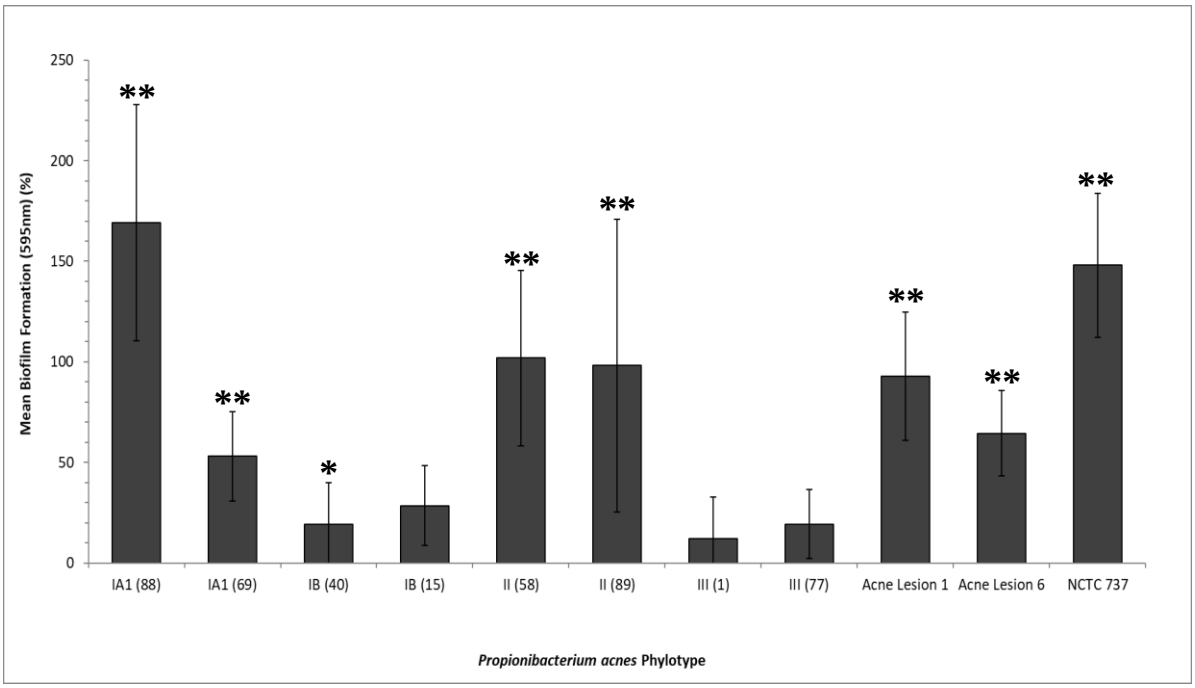


Figure 3.1: Mean percentage biofilm formation of clinical and control *C. acnes* isolates (panel 1) following anaerobic incubation at 37°C for 7-days. Mean percentage biofilm increase and decrease values are compared to the negative control and are averages of 36 replicates from three independent experiments (negative control not included on the figure). Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

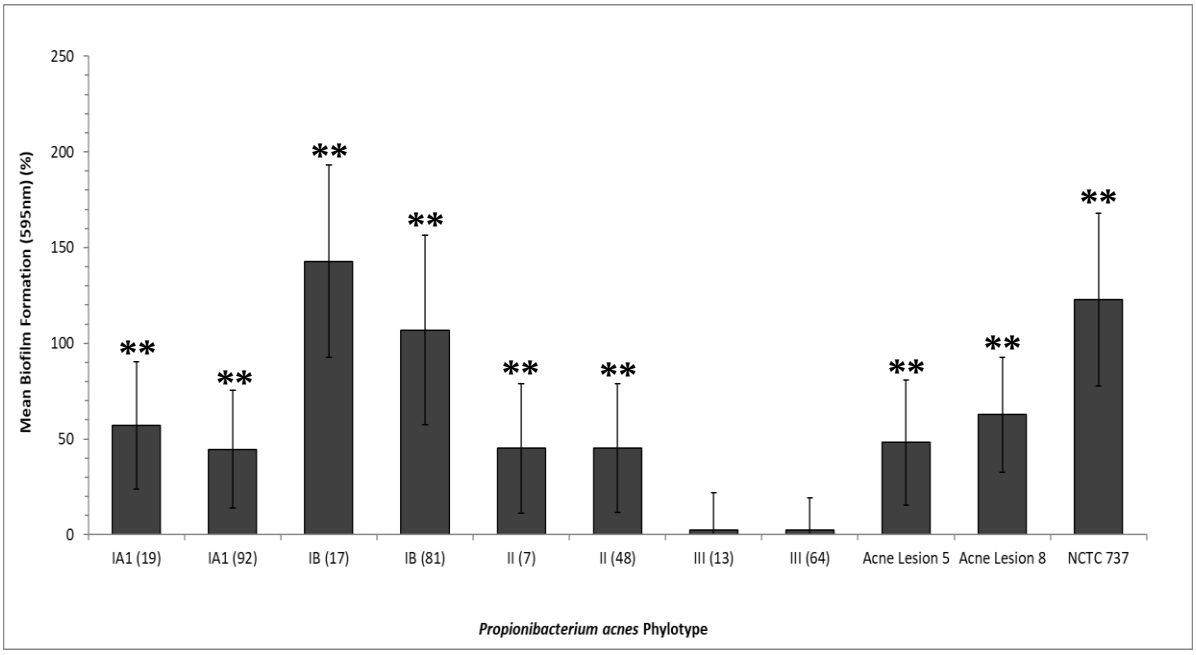


Figure 3.2: Mean percentage biofilm formation of clinical and control *C. acnes* isolates (panel 2) following anaerobic incubation at 37°C for 7-days. Mean percentage biofilm increase and decrease values are compared to the negative control and are averages of 36 replicates from three independent experiments (negative control not included on the figure). Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

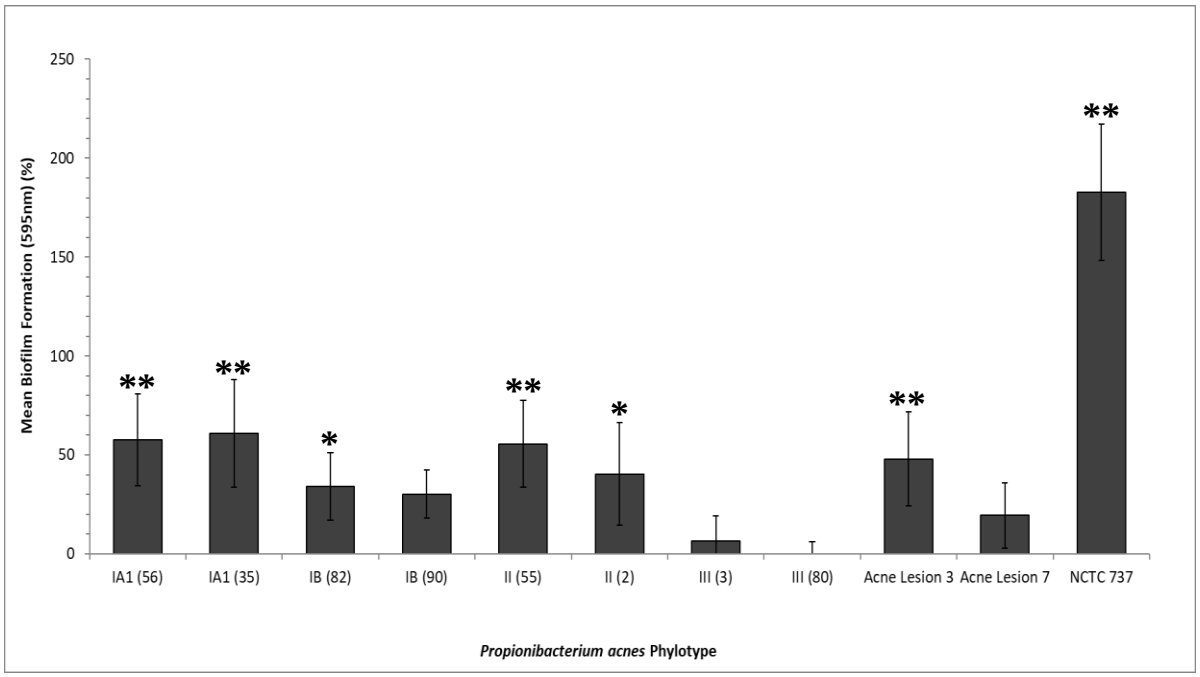


Figure 3.3: Mean percentage biofilm formation of clinical and control *C. acnes* isolates (panel 3) following anaerobic incubation at 37°C for 7-days. Mean percentage biofilm increase and decrease values are compared to the negative control and are averages of 36 replicates from three independent experiments (negative control not included on the figure). Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control ($p < 0.05$). ** Biofilm biomass highly significantly greater than the negative control ($p < 0.01$).

3.3.2 Comparison of Biofilm Formation Amongst Varying Phylotypes of *C. acnes*

All phylotype III disc tissue *C. acnes* isolates failed to form significantly more dense biofilms as compared to all other phylotypes of disc tissue and acne lesion *C. acnes* strains ($p > 0.05$) (Figures 3.1, 3.2 and 3.3). However, there was also no significant difference in biofilm biomass between *C. acnes* disc tissue isolates belonging to phylotypes IA_1, IB and II ($p > 0.05$).

In general, *C. acnes* NCTC 737 and *S. epidermidis* RP62A produced biofilms with biomasses that were highly significantly greater than all clinical disc tissue and acne lesion swab isolates of *C. acnes* ($p < 0.01$) (Figures 3.1, 3.2 and 3.3). However, *S. epidermidis* RP62A formed around 50% more biofilm than *C. acnes* NCTC 737, suggesting that it is a stronger biofilm producer ($p < 0.01$) (Table 3.1). *S. epidermidis* RP62A was omitted from Figures 3.1, 3.2, 3.3 and 3.5 as the high degree of biofilm formation of these bacteria in comparison to *C. acnes* clinical isolates and the NCTC 737 control strain skewed the plotted bar charts.

In order to categorise the degree of biofilm formation (non, weak, moderate or strong) and summarise the above semi-quantitative biofilm formation data for the collection of 30 *C. acnes* isolates (24 disc tissue isolates and six acne lesion isolates), mean OD_{595} values were calculated by averaging mean biofilm formation for all six disc tissue isolates representing each *recA* genotype and for all six acne lesion isolates (Table 3.1). The lowest levels of

biofilm formation were noted amongst disc tissue *C. acnes* isolates belonging to phylotype III, with a mean OD_{595} of 0.08 (Table 3.1). Type III isolates showed significantly lower mean OD_{595} values as compared to *S. epidermidis* RP62A, *C. acnes* NCTC 737 and *C. acnes* disc tissue isolates belonging to phylotypes IA_1 and IB ($p < 0.05$). This supports the semi-quantitative biofilm formation results for these isolates in Figures 3.1, 3.2 and 3.3. *C. acnes* NCTC 737 had a mean OD of 0.18 (Table 3.1) and despite biofilm density bars for this isolate appearing high (Figure 3.3), the published formula from Christensen *et al*. (1985) still identified this isolate as a weak biofilm former. *S. epidermidis* RP62A had a mean OD of 0.36 (Table 3.1) and was categorised as a moderate biofilm former.

Table 3.1: Mean OD values of clinical disc tissue (phylotypes IA_1, IB, II and III) and acne lesion isolates of *C. acnes* and control isolates NCTC 737 and *S. epidermidis* RP62A using the microtitre model of biofilm formation[a]. For phylotypes IA_1, IB, II, III and acne lesion isolates, six isolates were chosen to represent each phylogroup. Three separate cultures were prepared for each *C. acnes* isolate, with 12 replicates of each culture (36 replicates for each isolate in total). Therefore, each mean OD_{595} data value was calculated from a total of 216 replicates. NCTC 737, *S. epidermidis* RP62A and the negative control mean OD_{595} data values are each represented by 216 replicate absorbance values in total.

Strain (no. of isolates)	***Mean OD_{595}***
IA_1 (*n*=6)	0.12 ± 0.02 [1,2]
IB (*n*=6)	0.12 ± 0.04 [3,4]
II (*n*=6)	0.11 ± 0.02
III (*n*=6)	0.08 ± 0.01
Acne Lesion Isolates (*n*=6)	0.11 ± 0.02 [5]
NCTC 737 (*n*=1)	0.18 ± 0.01 [6, 7, 8, 9, 10, 11]
S. epidermidis RP62A (*n*=1)	0.36 ± 0.07 [12,13,14,15,16,17,18]
Negative Control	0.07 ± 0.01

[a] Each value for bacterial isolates is expressed as the mean ± standard deviation.

[1] IA_1 significantly higher mean OD^{595} value as compared to III ($p < 0.05$).

[2] IA_1 significantly higher mean OD^{595} value as compared to negative control ($p < 0.05$).

[3] IB significantly higher mean OD^{595} value as compared to III ($p < 0.05$).

[4] IB significantly higher mean OD^{595} value as compared to negative control ($p < 0.05$).

[5] Acne lesion isolates significantly higher mean OD^{595} value as compared to negative control ($p < 0.05$).

[6] NCTC 737 significantly higher mean OD^{595} value as compared to IAI ($p < 0.01$).

[7] NCTC 737 significantly higher mean OD^{595} value as compared to IB ($p < 0.01$).

[8] NCTC 737 significantly higher mean OD^{595} value as compared to II ($p < 0.01$).

[9] NCTC 737 significantly higher mean OD^{595} value as compared to III ($p < 0.01$).

[10] NCTC 737 significantly higher mean OD^{595} value as compared to acne lesion isolates ($p < 0.01$).

[11] NCTC 737 significantly higher mean OD^{595} value as compared to negative control ($p < 0.01$).

[12] *S. epidermidis* RP62A significantly higher mean OD^{595} value as compared to IAI ($p < 0.01$).

[13] *S. epidermidis* RP62A significantly higher mean OD^{595} value as compared to IB ($p < 0.01$).

[14] *S. epidermidis* RP62A significantly higher mean OD^{595} value as compared to II ($p < 0.01$).

[15] *S. epidermidis* RP62A significantly higher mean OD^{595} value as compared to III ($p < 0.01$).

[16] *S. epidermidis* RP62A significantly higher mean OD^{595} value as compared to acne lesion isolates ($p < 0.01$).

[17] *S. epidermidis* RP62A significantly higher mean OD^{595} value as compared to NCTC 737 ($p < 0.01$).

[18] *S. epidermidis* RP62A significantly higher mean OD^{595} value as compared to negative control ($p < 0.01$).

Table 3.2: Classification of adherence of *C. acnes* disc tissue and acne lesion swab isolates. Three separate cultures were prepared for each *C. acnes* isolate, with 12 replicates of each culture (36 replicates in total).

OD Values of Tissue Culture Plate Method			**Number of Isolates (% of Total Isolates)**	
Mean OD Values (OD_{595}) (*n*= 36 replicates)	**Adherence**	**Biofilm Formation**	**Disc Isolates (*n*=24)**	**Acne Lesion Swab Isolates (*n*=6)**
≤ 0.09	Non	Non	10 (41.7%)	1 (16.7%)
> 0.09 - ≤ 0.18	Weak	Weak	14 (58.3%)	5 (83.3%)
> 0.18 - ≤ 0.36	Moderate	Moderate	0 (0)	0 (0)
> 0.36	Strong	Strong	0 (0)	0 (0)

Phylotype-specific sub-classification of the adherence capabilities of disc tissue isolates of *C. acnes* demonstrated that all phylotype IA_1 isolates were weak biofilm formers (Table 3.3). In contrast, all phylotype III disc tissue isolates showed no adherence and were, therefore, classified as non-biofilm formers (Table 3.3). Phylotype IB disc tissue isolates were split equally between non-adherent and weakly-adherent categories whilst the majority of phylotype II disc tissue *C. acnes* isolates (20.8%) were weakly-adherent (Table 3.3).

Table 3.3: Phylotype-specific sub-classification of adherence capabilities of disc tissue *C. acnes* isolates. Three separate cultures were prepared for each *C. acnes* isolate, with 12 replicates of each culture (36 replicates in total).

OD Values of Tissue Culture Plate Method			Number of Isolates (% of Total Isolates)				
Mean OD Values (OD_{595}) (*n*=36 replicates)	**Adherence**	**Biofilm Formation**	**Disc Isolates (*n*=24) (Phylotypes)**				**Acne Lesion Swab Isolates (*n*=6)**
			IA_1 (*n*=6)	**IB (*n*=6)**	**II (*n*=6)**	**III (*n*=6)**	
≤ 0.09	Non	Non	0 (0%)	3 (12.5%)	1 (4.2%)	6 (25.0%)	1 (16.7%)
> 0.09 - ≤ 0.18	Weak	Weak	6 (25.0%)	3 (12.5%)	5 (20.8%)	0 (0%)	5 (83.3%)
> 0.18 - ≤ 0.36	Moderate	Moderate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
> 0.36	Strong	Strong	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

In order to assess the concordance between semi-quantitative and quantitative biofilm formation capabilities of *C. acnes* and to determine whether these bacteria were viable within 7-day biofilms, CFU/ml counts were performed using selected isolates. As shown in Figure 3.4 below, *C. acnes* disc tissue isolates 56 (IA_1), 90 (IB), 2 (II), 80 (III), acne lesion 3 and *C. acnes* NCTC 737 were all viable from 7-day biofilms. Similar mean CFU/ml counts were obtained for isolates 56 (IA_1), 2 (II), acne lesion 3 and *C. acnes* NCTC 737 of 8.51 $\times 10^8$ CFU/ml, 7.70 $\times 10^8$ CFU/ml, 8.73 $\times$ 10^8 CFU/ml and 7.38 $\times$ 10^8 CFU/ml, respectively. This supports the semi-quantitative findings in Figure 3.3 showing similar levels of biofilm

formation amongst disc tissue isolates 56 (IA_1), 2 (II) and acne lesion 3 as well as supporting the finding that no significant difference in biofilm formation was noted between *C. acnes* disc tissue isolates belonging to phylotypes IA_1 and II ($p > 0.05$).

C. acnes disc tissue isolate 80 (III) had a significantly lower mean CFU/ml value of 6.98×10^7 in comparison to all other disc tissue isolates, acne lesion 3 and *C. acnes* NCTC 737 ($p < 0.01$) (Figures 3.3 and 3.4). This suggests that this isolate produced a biofilm with less biomass than all other *C. acnes* phylotypes as shown also by the semi-quantitative results (Figures 3.1, 3.2 and 3.3). Overall, acne lesion isolate 3, disc tissue isolates 56 (IA_1), 90 (IB), 2 (II) and *C. acnes* NCTC 737 all showed significantly increased biofilm biomass as compared to isolate 80 (III), evidenced by the quantitative results ($p < 0.01$) (Figure 3.4).

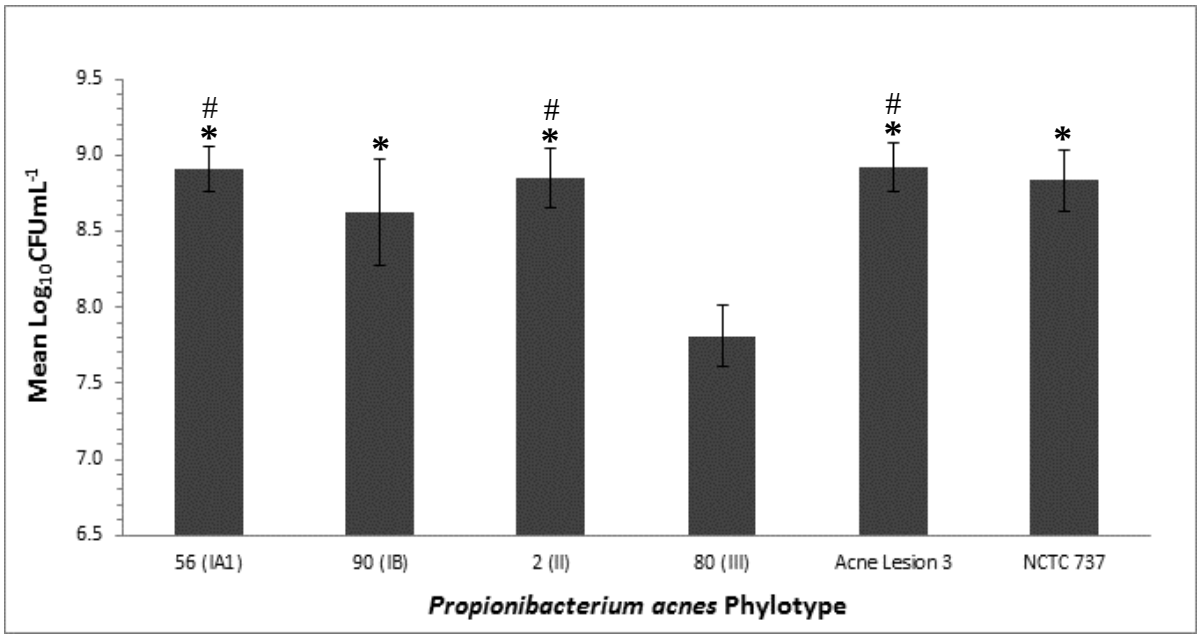


Figure 3.4: Mean *C. acnes* biofilm densities (expressed as mean log_{10} CFU/ml) following anaerobic incubation at 37°C for 7-days. Results are averages of eight replicates from 4 independent experiments. Error bars represent standard deviation. * Highly significant increase in biofilm biomass as compared to type III following quantitative analysis (p <0.01). # Significant increase in biofilm biomass as compared to type IB following quantitative analysis (p <0.05).

C. acnes disc tissue isolate 90 (IB) had the second-lowest mean CFU/ml viable cell count in comparison to *C. acnes* disc tissue isolates 56 (IA_1) and 2 (II) and acne lesion isolate 3 (Figure 3.4) ($p < 0.05$). However, this same isolate showed the highest level of biofilm formation using the crystal violet staining assay, as shown in Figure 3.5, with a mean biofilm formation value of 218% as compared to the negative control ($p < 0.01$).

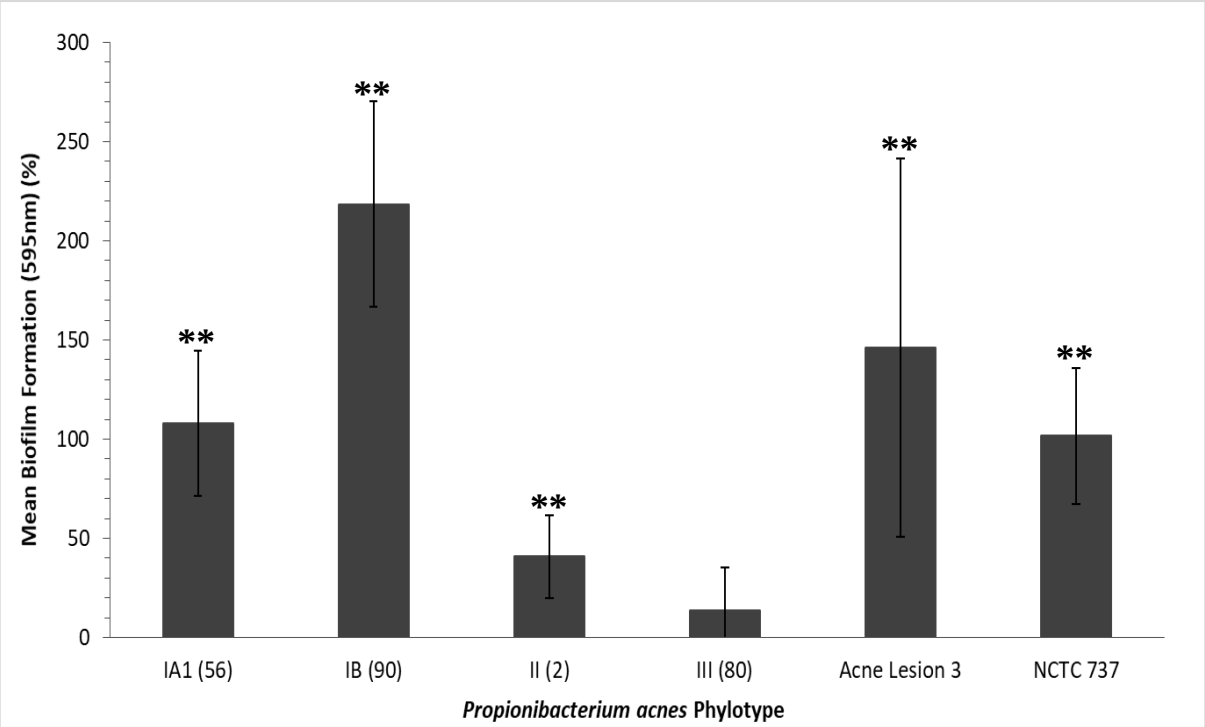


Figure 3.5: Mean percentage biofilm formation of clinical and control *C. acnes* isolates (from panel 3) following anaerobic incubation at 37°C for 7-days (crystal violet semi-quantitative assay). Mean percentage biofilm increase and decrease values are compared to the negative control and are averages of 36 replicates from three independent experiments (negative control not included on the figure). Error bars represent standard deviation. * Biofilm biomass significantly greater than the negative control (p <0.05). ** Biofilm biomass highly significantly greater than the negative control (p <0.01).

3.3.3 Effect of Site of Isolation on Biofilm Formation Amongst *C. acnes*

No statistically significant difference in mean OD_{595} readings existed between acne lesion isolates and disc tissue isolates belonging to phylotypes IA_1, IB, II and III (Table 3.1) ($p > 0.05$). This suggests that *C. acnes* isolates obtained from different anatomical sites, namely disc tissue versus acne lesions, produce biofilms of similar biomass ($p > 0.05$).

In order to simplify biofilm formation analysis, OD_{595} values were used to classify the degree of biofilm production for all *C. acnes* clinical strains. Results demonstrated that all *C. acnes* disc tissue and acne lesion swab isolates failed to form moderately adherent or strongly adherent biofilms (Table 3.3). This supports the semi-quantitative observation of similar levels of biofilm formation amongst acne lesion isolates and certain disc tissue isolates (Figures 3.1, 3.2 and 3.3) as well as the similar mean CFU/ml counts seen between acne lesion 3 and specific disc tissue *C. acnes* isolates (Figure 3.4).

3.4 Discussion

Biofilm formation is an important bacterial virulence strategy, promoting adhesion to both biological and non-biological surfaces, including bone grafts and bone graft substitutes, titanium, surgical steel and silicone [Clauss *et al*., 2010; Bayston *et al*., 2007 (b); Ryu and

Beuchat, 2005; Marion-Ferey *et al*., 2003]. This leads to the persistence of constituent bacteria, such as through the increased resistance of biofilm-associated bacteria to both antimicrobials and immunological defence mechanisms of the body (Burmølle *et al*., 2006; Domenech *et al*., 2013). *C. acnes* can also form biofilms and although this has been firmly established [Ramage *et al*., 2003; Bayston *et al*., 2007 (a); Bayston *et al*., 2007 (b); Coenye, Peeters and Nelis, 2007; Tunney *et al*., 2007], few studies have investigated differences in biofilm formation within disc tissue isolates of *C. acnes* or explored the effect of *recA* type on biofilm formation.

As shown in Table 3.3 above, although the majority of *C. acnes* isolates (63.3%) form weakly-adherent biofilms, the majority of biofilms formed by these isolates were significant as compared to the negative control in a semi-quantitative microtitre plate model (Figures 3.1, 3.2 and 3.3) ($p < 0.05$). This suggests that biofilm formation may be an important virulence factor within *C. acnes* isolates associated with disc tissue infections and acne vulgaris. Although 63.3% of *C. acnes* isolates were classified as weak biofilm formers, a limitation of the current study was the use of a maximum incubation duration of 7-days, with biofilm formation in IVD tissue likely occurring over a longer period. Capoor *et al*. (2017) directly visualised *C. acnes* biofilms in IVD tissue, supporting the clinical significance of this virulence factor in IVD colonisation and disease chronicity. Subsequently, longer term studies are required to assess the degree of biofilm formation of these isolates over a longer incubation period, with the adherence categories subsequently being reassessed. Quantitative enumeration of a panel of these isolates demonstrated high

viability of *C. acnes* disc tissue isolates 56 (IA_1), 90 (IB), 2 (II), 80 (III), acne lesion 3 and *C. acnes* NCTC 737 from 7-day biofilms, with colony counts of up to 8.73×10^8 CFU/ml. These results suggest that semi-quantitative microtitre plate biofilm formation assays alone can underestimate the density of *C. acnes* biofilms unless combined with quantitative CFU/ml count assays. As discussed by Chadwick and Avila (1968), a viable CFU/ml count of over 10^5 CFU/ml within a urine sample is indicative of a clinically significant bacteriuria. Although this gives guidance as to the number of bacteria required to cause a clinically significant urinary tract infection, no such guidelines exist in relation to the bacterial counts of *C. acnes* that result in clinically significant *C. acnes*-associated infections.

Although *C. acnes* forms weakly-adherent biofilms over 7-days, these structures support viable cell populations. Therefore, biofilm formation may play a role in the facilitation of IVD colonisation through the counteraction of shear forces applied to IVD tissue during movement, promoting the persistence of *C. acnes* within these structures. In support of this theory, Capoor *et al*. (2017) provided the first microscopic evidence of *C. acnes* biofilm aggregates within resected IVDs with corresponding culture positivity, highlighting the role of biofilm formation in IVD colonisation. Therefore, biofilm formation may be an adaptive phenotype for these clinical *C. acnes* isolates.

No overall significant difference in biofilm formation was noted between *C. acnes* disc tissue isolates belonging to phylotypes IA_1, IB and II ($p > 0.05$). In contrast, all phylotype III

disc tissue isolates, usually associated with deep tissue infections (McDowell *et al*., 2012; Niazi *et al*., 2010), showed no adherence and were, therefore, classified as non-biofilm formers (Table 3.3). These isolates also failed to form significantly more dense biofilms as compared to the negative control ($p > 0.05$) whilst forming significantly less dense biofilms as compared to all other disc tissue and acne lesion isolates using semi-quantitative crystal violet staining assays and quantitative CFU/ml count assays ($p < 0.05$) (Figures 3.1, 3.2, 3.3 and 3.4). This demonstrates the concordance between semi-quantitative crystal violet assays and quantitative CFU/ml data with regards to assessing biofilm formation. The isolation of phylotype III isolates from disc tissue despite these isolates being insignificant biofilm formers may be explained by the potential for such isolates to exploit the biofilm forming capabilities of other phylotype strains *in vivo*. This theory is supported by the analysis of multiplex data from 24 patients which showed that all type III isolates cultured from excised IVD tissue used in the current study were co-cultured with at least one other phylotype isolate which were identified as significant biofilm formers in this study (Rollason *et al*., 2013). Kuehnast *et al*. (2018) support this observation through the demonstration of the correlation between *C. acnes* phylotype and biofilm formation in a microtitre plate crystal violet assay incubated for up to 7-days. However, whilst Kuehnast *et al*. (2018) showed that isolates belonging to phylotypes IB and III showed the lowest degrees of biofilm formation, phylotype IA_1 *C. acnes* isolates showed the highest degrees of biofilm formation which oppose the findings of the current study. This may be attributed to the site of isolation of *C. acnes* isolates, with the current study utilising human IVD tissue isolates whilst the *C. acnes* panel used in the study by Kuehnast *et al*. (2018) originated from human skin, deep tissue

and implant-associated infections. In contrast, Holmberg *et al*. (2009) reported no significant relationship between *C. acnes recA* type and the degree of biofilm formation.

Additionally, no significant difference in biofilm biomass was noted between disc tissue and acne lesion isolates of *C. acnes* using semi-quantitative methods ($p > 0.05$). Therefore, these results suggest that site of strain isolation has no significant association with the degree of biofilm formation in a microtitre model. Kuehnast *et al*. (2018) also reported no significant impact of site of isolation on the biofilm forming capabilities of *C. acnes* clinical isolates in a microtitre crystal violet-based assay. Despite this, biofilm formation among disc tissue isolates of *C. acnes* showed phylotype-dependency, with type III isolates consistently failing to form significantly dense biofilms (Figures 3.1, 3.2, 3.3 and 3.5) ($p > 0.05$). Therefore, biofilm formation may not be an essential virulence factor in all deep-tissue *C. acnes* infections. In contrast to these findings, Holmberg *et al*. (2009) demonstrated a significantly greater degree of biofilm production among *C. acnes* obtained from clinical infections (deep isolates) as compared to healthy skin isolates (superficial isolates). Additionally, Jahns *et al*. (2012) demonstrated the higher prevalence of *C. acnes* biofilms within skin biopsies obtained from acne vulgaris patients as compared to control skin samples, supporting the role of *C. acnes* biofilms within acne vulgaris pathogenesis. The lack of a significant association between the degree of biofilm formation and the site of *C. acnes* isolation observed within the current study suggests that site of isolation may not be significant and supports the opportunistic nature of this pathogen (McDowell *et al*., 2013).

The study conducted by Holmberg *et al*. (2009) investigated biofilm formation within a larger panel of *C. acnes* isolates as compared to the current study, including 45 clinical isolates and 48 control isolates obtained from the skin of healthy subjects. Therefore, in future studies, the use of a larger panel of deep tissue IVD *C. acnes* isolates and superficial acne lesion *C. acnes* isolates, as well as isolates from the skin of healthy participants, is suggested within biofilm formation assays to determine whether the lack of association between site of isolation and degree of biofilm formation is consistently observed.

As explored by Sanchez Jr *et al*. (2013), bacterial strains characterised by weak biofilm formation *in vitro* using crystal violet staining assays may play an important role in polymicrobial infections through the direct incorporation of these isolates into established biofilms. In support of this theory, Capoor *et al*. (2017) identified 25% of culture-positive resected disc specimens with culture positivity for *C. acnes* along with one or more additional bacterial species, including *S. epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis* and/or *Staphylococcus warneri*. Additionally, Tyner and Patel (2016) describe the formation of durable biofilms following the co-culture of *C. acnes* and *S. aureus* under anaerobic conditions. Additionally, both viable *C. acnes* and *S. aureus* were recovered from such polymicrobial biofilms, whilst no viable *S. aureus* was recovered from monomicrobial *S. aureus* biofilms (Tyner and Patel, 2016). Therefore, *C. acnes* may provide a micro-environment conducive to staphylococcal growth (Tyner and Patel, 2016). As a result, although the majority of *C. acnes* were classified as weak biofilm formers in the

current study, these isolates may interact with other bacterial species *in vivo*, providing synergy to non-biofilm formers. Additionally, biofilm formation is a complex process, with the commitment of planktonic bacteria to a sessile mode of growth *in vivo* highly regulated by environmental cues, such as nutrient availability, and host factors, including hormones (Petrova and Sauer, 2012; Seneviratne *et al*., 2013). Therefore, further work investigating the effects of additional environmental parameters, such as media composition, on biofilm formation *in vitro* and the investigation of the ability of disc tissue isolates of *C. acnes* to form biofilms *in vivo* are warranted. Biofilm formation is also affected by microbial ultrastructure, with pili and fimbriae contributing to biofilm formation *in vitro* (Ramsugit *et al*., 2013; Jackson *et al*., 2002). Analysis of the proteome of *C. acnes* has demonstrated the expression of a cytoplasmic fine tangled pili, with scanning electron micrograph imaging demonstrating the presence of fimbriae around the microorganism (Yu, Champer and Kim, 2015; Pornpattananangkul *et al*., 2013). Therefore, further research into the role of pili and fimbriae within *C. acnes* adhesion and biofilm formation are warranted.

The observation that 63.3% of *C. acnes* isolates within the current study formed biofilms has potential implications in informing the processing of herniated disc tissue removed from patients during lumbar discectomy (Table 3.3). This may include sonication of disc tissue to disrupt potential biofilm structures followed by culture to improve the recovery of *C. acnes* from patient tissue as opposed to tissue culture alone (Sampedro *et al*., 2010; Coscia, Denys

and Wack, 2016). In support of this, Capoor *et al*. (2017) provided the first visual evidence of *C. acnes* biofilms within resected IVD tissue, highlighting the importance of considering the biofilm mode of growth when preparing disc tissue samples for microbial enumeration. These biofilm structures may also be relatively resistant to both stain uptake and antigenic marking, leading to false-negative Gram-stain and immunohistochemical stain results of tissue specimens and the low percentage recovery of these bacteria from cultured disc tissue (Coscia, Denys and Wack, 2016). Considering the presence of biofilms within patient samples would promote the effective isolation of *C. acnes* from resected tissue, thereby preventing the under-reporting of *C. acnes*-associated disc herniation.

As show in Figures 3.1, 3.2, 3.3 and 3.5, the crystal violet microtitre plate assay provides a robust and reproducible method for semi-quantitative assessment of *C. acnes* biofilm formation. However, classification of the degree of biofilm formation using semi-quantitative crystal violet assay data alone (Tables 3.2 and 3.3) can lead to the underestimation of the significance of *C. acnes* biofilm formation (Figures 3.1, 3.2, 3.3 and 3.5). Additionally, whilst crystal violet staining provides an effective means of assessing biofilm biomass, this semi-quantitative method does not provide a means of assessing the viability of biofilm-associated cells as both live and dead bacterial cells, as well as extracellular matrix components, are stained (Welch, Cai and Strømme, 2012). As a result, use of this method alone may lead to the overestimation of the degree of biofilm formation of specific isolates, leading to the false representation of biofilm forming capabilities of individual *C. acnes* strains. For example, isolate 90 (IB) demonstrated the highest level of

biofilm formation using the crystal violet staining assay, as shown in Figure 3.5. However, this same isolate had the second-lowest mean CFU/ml viable cell count in comparison to all other sampled *C. acnes* disc tissue and acne lesion isolates (Figure 3.4). Therefore, the biofilm formed by this isolate may consist of greater proportions of dead bacterial cells and/or extracellular matrix components in comparison to other *C. acnes* isolates, accounting for the high mean absorbance values obtained via crystal violet staining assays for this strain. Therefore, although the current study utilised four disc tissue *C. acnes* isolates and one acne lesion *C. acnes* isolate within biofilm-associated bacterial cell viability assays, further quantitative analysis of other *C. acnes* isolates in combination with semi-quantitative assays should be used when assessing the biofilm forming capabilities of *C. acnes*.

C. acnes disc tissue isolates 88 (IA_1), 58 (II), 89 (II) and 17 (IB) demonstrated the greatest degree of variability with regards to biofilm formation, as noted by the large error bars corresponding to these isolates (Figures 3.1 and 3.2). This intra-species heterogeneity in biofilm formation suggests that biofilm formation is a genetically complex trait, modulated by cell-cell interactions, cell-substrate adhesion and extracellular matrix production (Granek *et al*., 2013). However, the high degree of biofilm formation variability noted within isolates 88 (IA_1), 58 (II), 89 (II) and 17 (IB) is not replicated in other clinical *C. acnes* strains studied, suggesting that this variability is not the result of a method effect and is, alternatively, intrinsic to these specific isolates. Further studies investigating the genetic basis of these intra-species biofilm formation variations are warranted.

Gahukamble *et al*. (2014) described disparities between *in vitro* and *in vivo* biofilm forming capabilities of *C. acnes*. Although *C. acnes* displayed poor adherence to a range of orthopaedic implant materials *in vitro*, all *C. acnes*-inoculated animals were positive for biofilm formation on intramedullary nails *in vivo* (Gahukamble *et al*., 2014). Therefore, weak biofilm formation amongst disc tissue *C. acnes* isolates within *in vitro* crystal violet staining assays does not reflect the pathogenic potential of such isolates *in vivo*. As a result, further studies investigating the *in vivo* biofilm forming capabilities of these IVD tissue isolates are warranted to determine whether the degree of biofilm formation *in vivo* corroborates results generated *in vitro*.

As shown in Figures 3.1, 3.2 and 3.3 in section 3.3.1 above, the majority of disc tissue and acne lesion isolates of *C. acnes* produced significantly more dense biofilms in a microtitre model of biofilm formation following 7-days of anaerobic plate incubation as compared to the negative control ($p < 0.05$). Therefore, anti-biofilm methods may prove effective in the treatment of *C. acnes* IVD infections associated with the biofilm forming phenotype. Whilst Albert *et al*. [2013 (b)] described the significant clinical improvement of patients with lumbar disc herniation associated with *C. acnes*-induced CLBP and Modic type 1 changes using amoxicillin–clavulanate, this study failed to consider the effect of the biofilm mode of growth on antibiotic kinetics [Albert *et al*., 2013 (a)]. This mode of growth is significant as biofilm-associated bacteria can show markedly higher tolerance to antimicrobial agents of up to 1,000-fold (Rasmussen and Givskov, 2006). Bayston *et al*. [2007 (a)], however, reported the eradication of mature *C. acnes* biofilms from titanium discs following 14-days

of antibiotic treatment using penicillin, linezolid or linezolid plus rifampicin. However, when selecting appropriate antibiotics for the treatment of biofilm-associated infections, well-penetrating antibiotics must be selected to ensure adequate antibiotic concentrations are reached within sessile bacterial communities (Wu *et al*., 2015). The treatment of biofilm-associated infections requires a multi-faceted approach, such as the use of anti-quorum sensing peptides, quorum-sensing inhibitors, biofilm dispersal agents and the removal of afflicted implanted devices (Wu *et al*., 2004; Balaban *et al*., 2007; Karaolis *et al*., 2005; Wu *et al*., 2015). Further studies into the efficacy of antibiotic therapy against biofilms formed by herniated disc tissue isolates of *C. acnes*, combined with the assessment of gene expression regulating biofilm formation within these isolates, are warranted to identify potential treatment strategies for IVD tissue infections associated with *C. acnes* colonisation.

As well as biofilm-associated cells being characterised by higher tolerance to antimicrobial agents as compared to their planktonic counterparts, biofilm-associated *C. acnes* cells have also been reported to up-regulate genes encoding virulence-associated CAMP factors, particularly CAMP factors 1 and 4 (Jahns, Eilers and Alexeyev, 2016). With CAMP factor 1 shown to contribute to *C. acnes* virulence through the amplification of inflammatory reactions, further studies may focus on assessing the CAMP factor 1 production status of biofilm forming isolates used within the current study. This may lead to the identification of CAMP factor 1 as a novel microbial target to exploit within vaccine development for *C. acnes*-associated disc infections.

In conclusion, the biofilm forming capabilities of *C. acnes* isolates sampled from herniated IVD tissue demonstrated phylotype specificity, with type III isolates consistently failing to form significantly dense biofilms. However, the observation that type IA_1, IB and II disc tissue isolates generally formed significantly dense biofilms as compared to the negative control suggests that biofilm formation is an important virulence factor which aids in disc colonisation and subsequent pathogenesis. The simplicity of semi-quantitative crystal violet assays gives this method great potential for the screening of *C. acnes* biofilm forming capabilities, although this technique must be combined with a quantitative method, such as CFU/ml determination, to appreciate the true extent of biofilm formation and associated cell viability.

CHAPTER 4 PHENOTYPIC CHARACTERISATION OF *C. ACNES*

4.1 Introduction

Although *C. acnes* has been identified as the causative agent of severe infections, including endocarditis, post-craniotomy infections, arthritis, spondylodiscitis and endophthalmitis, the importance of this anaerobe in human disease has largely been overlooked (Jakab *et al*., 1996). This can, in part, be attributed to the role of *C. acnes* as a predominant member of the skin microbiome which, together with the low virulence nature of these bacteria, often leads to the dismissal of this commensal as a sample contaminant [Niazi *et al*., 2010; Shu *et al*., 2013 (a); Evans, 1975]. Additionally, the absence of overt clinical symptoms associated with *C. acnes* infection, in combination with inadequate isolation procedures employed by clinical microbiology laboratories, may further promote the underestimation of the clinical implications of these bacteria (Dodson *et al*., 2010).

C. acnes and CoNS, such as *S. epidermidi*s, are both considered low-virulence opportunistic microorganisms which can become pathogenic in immunocompromised individuals (Eady and Ingham, 1994). However, although the pathogenic potential of CoNS has been well characterised, such as the role of *S. epidermidis* in nosocomial infections, the virulence of *C. acnes* is overlooked, despite the greater number of exocellular enzymes and other bioactive exocellular products produced by *C. acnes* in comparison to CoNS (Lim and Webb, 2005;

Eady and Ingham, 1994; Vuong and Otto, 2002). Exocellular enzymes produced by cutaneous Cutibacteria include lipase (Ingham *et al*., 1981; Greenman and Holland, 1985), protease (Ingham *et al*., 1983) and DNase (Marples and McGinley, 1974). Additionally, comparative genomics and transcriptomics has identified putative virulence factors encoded by the *C. acnes* genome, including secreted triacylglycerol lipases, dermatan sulphate-binding adhesins, secreted lysozymes and CAMP factor homologs (Brzuszkiewicz *et al*., 2011; Brüggemann, 2005). Previous studies have shown differences in *C. acnes* enzyme production and observed microscopic cellular morphology between *recA* types (McDowell *et al*., 2008; Capoor *et al*., 2018). However, despite the increasing evidence of the potential role of *C. acnes* in discitis pathophysiology and the significance of particular *C. acnes* virulence factors in human disease, such as the proinflammatory effects of lipase (Lee *et al*., 1982), few studies have investigated the exocellular enzyme production of *C. acnes* isolates recovered from spine IVD material (McDowell *et al*., 2008). The current study aimed to investigate the exocellular enzyme production and cellular morphology of *C. acnes* isolated from herniated disc tissue and acne lesions. Comparisons of enzyme production and cellular morphology were made between *recA* types and site of isolation. The enzymes investigated included lipase, non-specific protease, gelatinase, DNase and haemolysins. These enzymes were selected due to their relevance in *C. acnes* pathogenesis, with *C. acnes* lipase acting as a highly proinflammatory enzyme that can contribute to Modic type I changes by promoting high levels of bone turn-over (Dudli *et al*., 2018). *C. acnes* protease enzymes are also highly proinflammatory, with the TNF-α cytokine elicited by these enzymes associated with promoting disc degeneration and herniation (Lee *et al*., 2010; Risbud and Shapiro, 2014).

Gelatinase has been postulated to facilitate bacterial translocation across cell layers and DNase allows bacteria to degrade extruded DNA which forms part of neutrophil extracellular traps (NETs), contributing to potential bacterial spread within IVD tissue (Park *et al*., 2007; Palmer *et al*., 2012). Finally, *C. acnes* haemolysins have been reported to facilitate cytotoxicity by targeting host cell membranes and can also induce CLBP through direct interaction with nociceptor pain neurons (Capoor *et al*., 2018).

4.1.1 Aims

The aims of this exocellular enzyme production study were to:

- Investigate potential phylotype-specific morphological characteristics of *C. acnes* obtained from human IVD tissue material.
- Assess the exocellular production of a range of clinically relevant virulence factors, namely haemolysins, lipases, proteases, gelatinases and DNases, by a panel of clinical IVD tissue and acne lesion samples of *C. acnes*.
- Determine whether potential production of exocellular enzymes, namely haemolysins, lipases, proteases, gelatinases and DNases, showed phylotype specificity amongst *C. acnes* IVD tissue isolates.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Growth Conditions

All bacterial strains were maintained on BHI agar (Oxoid, UK) at 4°C. For long term storage, stocks of these strains were prepared using 25% (v/v) glycerol in BHI broth and stored at -80°C. Prior to use in agar plate based assays, all *C. acnes* stock isolates were re-streaked onto fresh BHI agar a minimum of two times to ensure purity of growth. Representative isolates were also Gram-stained and assessed for catalase activity using hydrogen peroxide (H_20_2) solution (Sigma, UK), with the effervescence of bacterial colonies emulsified within H_20_2 indicating a positive catalase reaction. All *C. acnes* isolates used were previously confirmed as *C. acnes* by biochemical analysis using the Rapid ID 32A kit (bioMérieux) (Rollason *et al*., 2013).

4.2.2 Morphological Characterisation of Herniated Disc Tissue Isolates of *C. acnes*

A total of eight *C. acnes* isolates obtained from human herniated disc tissue, consisting of phylotypes IA_1 (*n*=2), IB (*n*=2), II (*n*=2) and III (*n*=2), were Gram-stained and observed under the ×100 oil-objective lens of a light microscope (Kyowa Medilux-12) to assess the morphological characteristics of each phylotype. Parameters such as bacterial cell shape and

spatial arrangement were examined.

4.2.3 Virulence Factors

C. acnes isolates were assayed for the production of a range of virulence-associated exocellular enzymes. A total of 67 *C. acnes* isolates obtained from human herniated disc tissue material, consisting of phylotypes IA_1 (*n*=19), IB (*n*=6), II (*n*=35) and III (*n*=7), were examined using agar plate based methods (see below). The enzyme production profiles of a range of *C. acnes* isolates obtained from human acne lesions (*n*=8) were also examined.

Distinct colonies of each *C. acnes* isolate were inoculated directly onto the appropriate test agar plates (as outlined below) using sterile, disposable inoculation loops and incubated anaerobically at 37°C for up to 14-days.

4.2.3.1 Haemolysis

C. acnes strains were streaked on Columbia blood agar base supplemented with 7% (v/v) defibrinated sheep blood and incubated anaerobically at 37°C for 14-days. *S. epidermidis* RP62A was used as a positive control whilst *E. coli* ATCC 11775 and sterile reverse

osmosis (RO) water served as negative controls. Zones of complete clearance around bacterial colonies were recorded as β-haemolysis whilst zones of incomplete clearance, visualised as green zones around bacterial colonies, demonstrated α-haemolysis. All tests were performed in duplicate.

4.2.3.2 Non-Specific Protease Activity

For the assessment of non-specific protease activity, *C. acnes* strains were streaked onto skimmed milk agar plates (1% (v/v) ultra-high temperature processed skimmed milk liquid) prepared using nutrient agar as a base (Oxoid, UK). *S. marcescens* (laboratory strain) was used as a positive control and sterile RO water was used as a negative control. *C. acnes*-inoculated plates were incubated at 37°C for 5-days under anaerobic conditions. A zone of clearance around bacterial growth indicated protease activity. All tests were performed in duplicate.

4.2.3.3 DNase Activity

C. acnes strains were streaked onto DNase agar plates (Oxoid, UK). *S. aureus* NCTC 6571 served as a positive control whilst *E. coli* ATCC 11775 and sterile RO water were used as

negative controls. Following the incubation of inoculated plates at 37°C under anaerobic conditions for 7-days, all plates were flooded with 1M hydrochloric acid (HCl) and left to stand for 2-minutes. Zones of clearance around bacterial colonies confirmed DNase activity. All tests were performed in duplicate.

4.2.3.4 Lipase Activity

Lipase activity was detected using glyceryl tributyrate-supplemented Luria-Bertani (LB) agar composed of 1% (w/v) tryptone, 0.5% (w/v) sodium chloride (NaCl), 0.5% (w/v) yeast extract, 1.2% (w/v) agar bacteriological, 1% (v/v) glyceryl tributyrate and 1% (v/v) Tween™ 80 (Fisher Scientific, UK). *C. acnes* strains were streaked across the agar plates and incubated anaerobically at 37°C for 7-days. Lipase activity was identified by zones of clearance around bacterial colonies, with or without an iridescent sheen (oil on water appearance) on colonies. *C. acnes* NCTC 737 was used as a positive control whilst *E. coli* ATCC 11775 acted as a negative control. All tests were performed in duplicate.

4.2.3.5 Gelatinase Activity

To assess gelatinase activity, *C. acnes* colonies were stab-inoculated into sterile universal

vessels containing 10ml aliquots of nutrient gelatin media (Oxoid, UK). Inoculated vessels were incubated anaerobically at 37°C for 14-days. Inoculated vessels were removed from the anaerobic incubator and placed in a refrigerator (4°C) for 30-minutes daily to check for gelatin liquefaction, with the number of days of incubation required for any positive gelatinase results to appear being recorded. Partial or total liquefaction of the inoculated media after refrigeration constituted a positive gelatinase test result whilst complete solidification of the inoculated media after refrigeration indicated a negative gelatinase test result. *C. acnes* NCTC 737 served as a positive control whilst *E. coli* ATCC 11775 and sterile RO water were used as negative controls. All nutrient gelatin media tests were performed in duplicate.

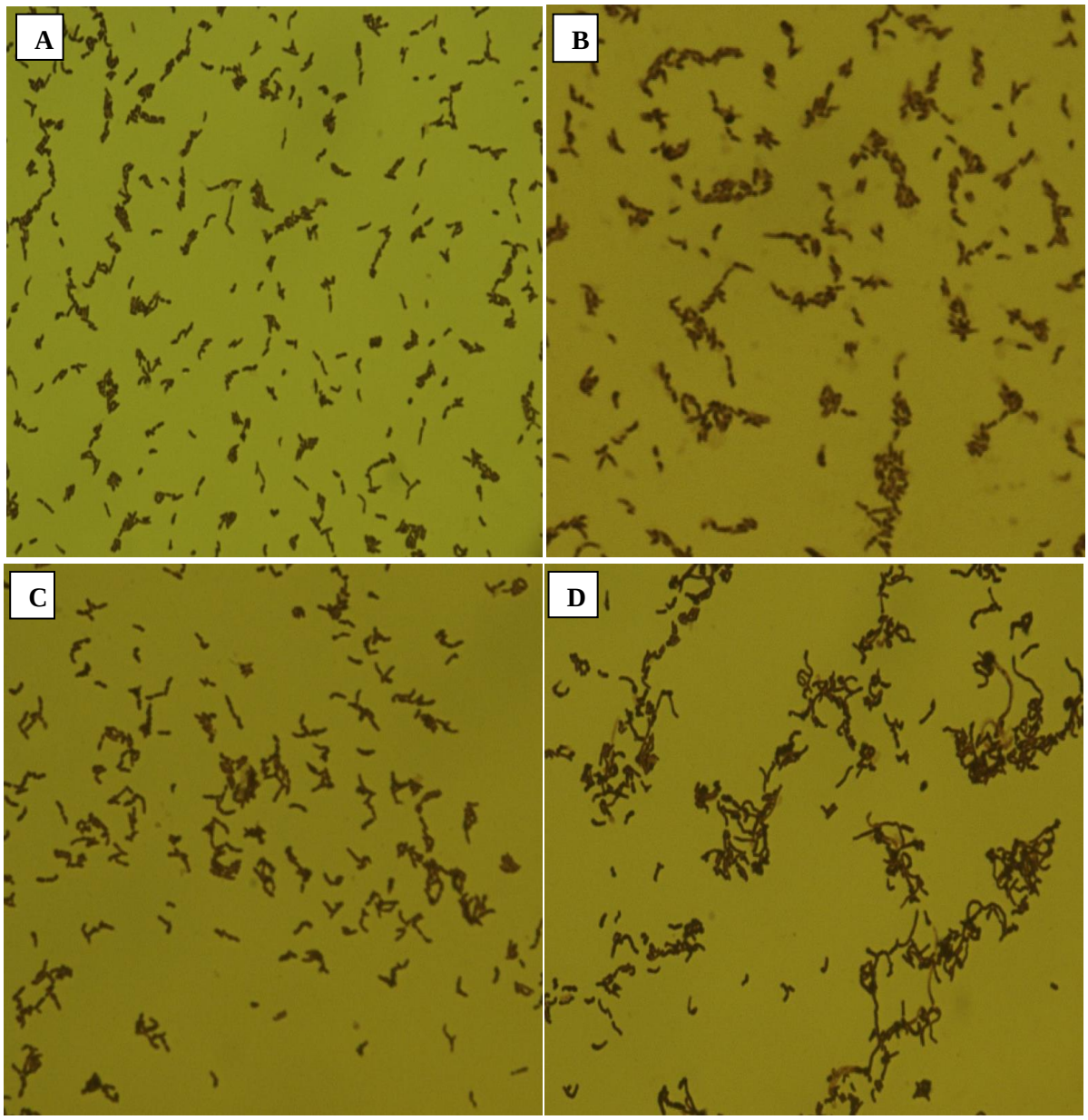
4.2.4 Statistical Analysis of Phenotypic Characteristics

All statistical tests were performed using IBM SPSS Statistics version 22.0. The Fisher's exact test was used to identify the statistical significance of the correlation between enzyme production and *C. acnes* phylotype. All tests were two-sided and p-values of <0.05 were considered statistically significant.

4.3 Results

4.3.1 Morphological Characterisation of Herniated Disc Tissue Isolates of *C. acnes*

As shown in Figure 4.1 below, *C. acnes* isolates belonging to the phylotypes IA_1, IB and II formed short, club-shaped rods characteristic of coryneform bacteria, with individual cells of variable length being noted. *C. acnes* isolates belonging to phylotype III differed significantly from the cellular morphology of phylotype IA_1, IB and II cells, with phylotype III cells forming aggregates consisting of long, slender filaments of variable lengths. However, variations in the cellular morphology of individual isolates of specific phylotypes were noted, with isolate 40 (IB) [Figure 4.1 (D)] forming long, filamentous cells typically characteristic of phylotype III isolates.


A
B
C
D

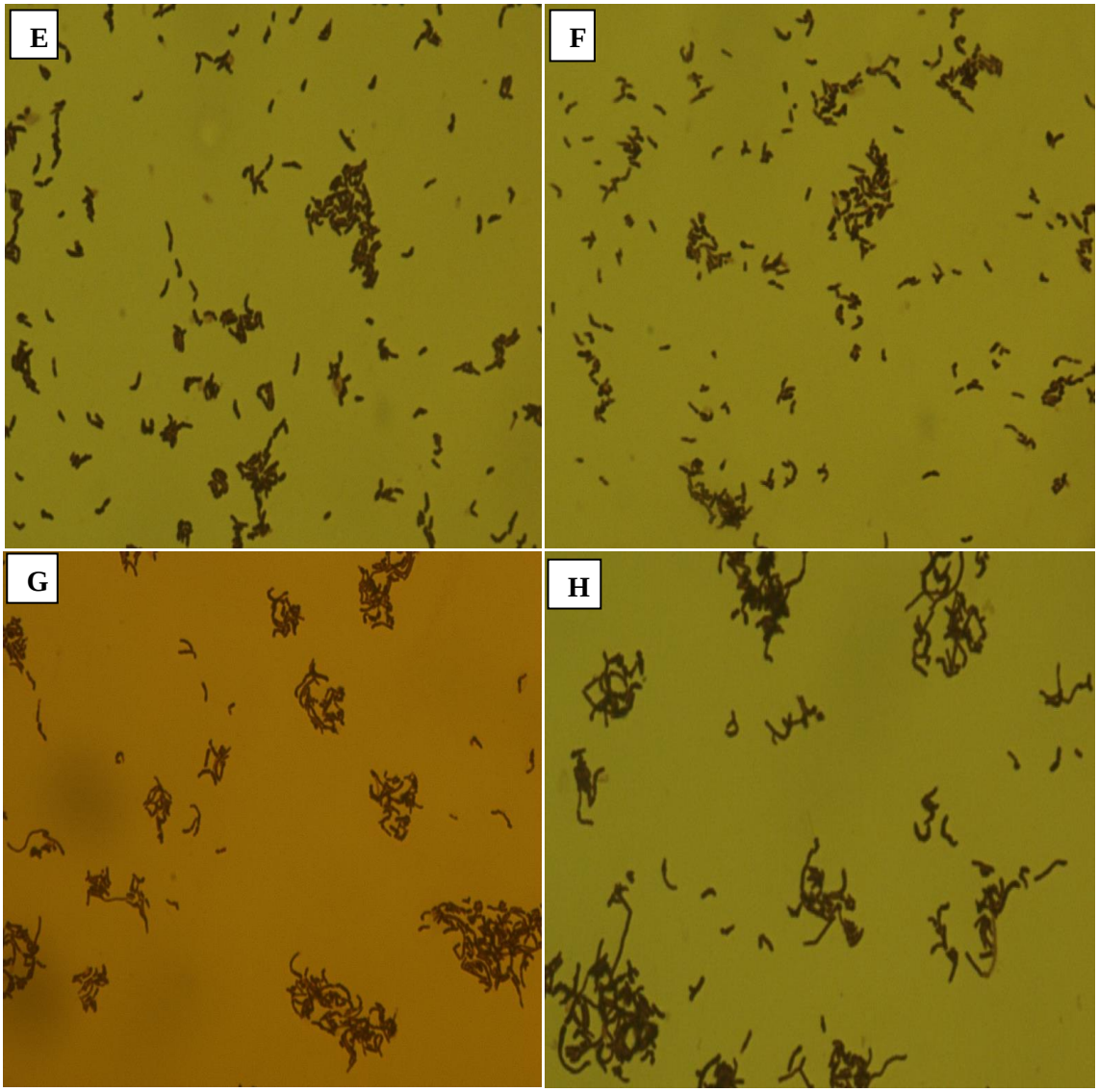


Figure 4.1: Gram-stained preparations of *C. acnes* isolated from human herniated disc material observed using a light microscope (magnification ×100). (A) Isolate number 35 (IA_1). (B) Isolate number 69 (IA_1). (C) Isolate number 15 (IB). (D) Isolate number 40 (IB). (E) Isolate number 58 (II). (F) Isolate number 89 (II). The majority of phylotype IA_1, IB and II isolates showed coryneform morphology. (G) Isolate number 1 (III). (H) Isolate number 3 (III). All phylotype III isolates showed filamentous cellular morphology.

4.3.2 Virulence Factors

4.3.2.1 Haemolysis

β-haemolysis (Figure 4.2) was noted in 20 out of the 67 (30%) disc tissue isolates of *C. acnes*, with the remaining 47 disc tissue isolates negative for haemolysis. The *S. epidermidis* RP62A control strain expressed α-haemolysis on 7% (v/v) sheep blood agar.

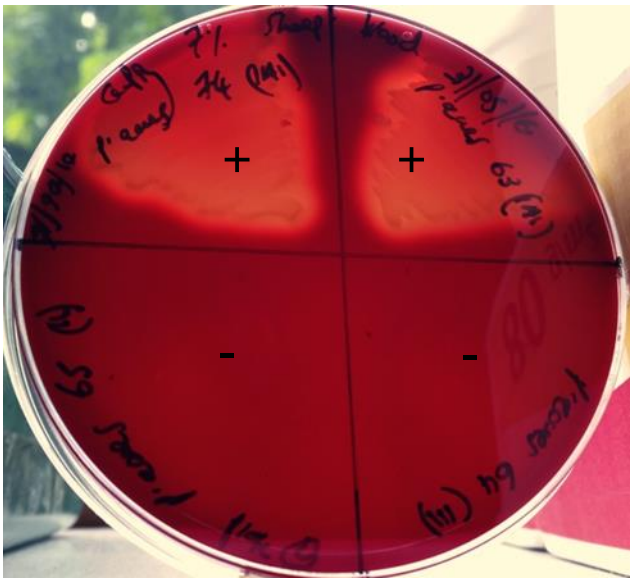


Figure 4.2: Clinical *C. acnes* isolates demonstrating β-haemolysis on 7% (v/v) sheep blood agar (+). *C. acnes* clinical isolates negative for haemolytic activity (-).

Table 4.1: Phylotype-specific production of β-haemolysis among clinical *C. acnes* isolates on 7% (v/v) sheep blood agar.

C. acnes Phylogenetic Group	*Number of Disc Tissue Isolates Positive for β-Haemolysis (Percentage Total of Each Phylotype)*
IA_1 (*n*=19)	**16 (84%)**
IB (*n*=6)	**4 (67%)**
II (*n*=35)	**0 (0%)**
III (*n*=7)	**0 (0%)**
Total (*n*=67)	**20 (30%)**

C. acnes phylotype IA_1 isolates were the most common cultures to produce β-haemolysis on 7% (v/v) sheep blood agar (84%), with 67% of *C. acnes* IB isolates expressing β-haemolysis (Table 4.1). However, haemolysis was not recorded in any of the *C. acnes* isolates belonging to phylotypes II and III (Table 4.1). Following the use of the Fisher's exact test, phylotype was shown to have a significant effect on the haemolytic activity of disc tissue isolates of *C. acnes* ($p < 0.01$).

Of the eight acne lesion *C. acnes* isolates, five isolates (63%) expressed β-haemolysis, with the remaining three isolates negative for haemolytic activity. No microbial growth was noted on any of the 7% (v/v) sheep blood agar plates inoculated with sterile RO water (negative

control). *E. coli* ATCC 11775 (negative control) was haemolysis-negative. Consistent haemolytic activity results were generated between duplicate plates.

4.3.2.2 Non-Specific Protease Activity

As shown in Table 4.2, 93% of herniated disc tissue isolates of *C. acnes* were positive for non-specific protease activity when streaked onto skimmed milk agar plates prepared using nutrient agar base. The highest rates of non-specific protease enzyme production were recorded for *C. acnes* phylotypes IA_1 and III, with 100% of the isolates representing each of these phylotypes producing protease (Table 4.2). Additionally, 100% of acne lesion samples were positive for non-specific protease activity. However, protease activity is not significantly associated with any one phylotype of *C. acnes* isolated from herniated disc tissue ($p > 0.05$). Opaque zones of precipitation were noted surrounding zones of clearance for all protease-positive isolates (Figure 4.3).

Figure 4.3: Non-specific protease activity demonstrated by zones of clearance on a 1% (v/v) skimmed milk agar plate (+). Protease-negative *C. acnes* isolate (-).

As shown in Figure 4.4, *S. marcescens* (laboratory strain) is positive for non-specific protease activity and can subsequently hydrolyse casein in skimmed milk agar, leading to a zone of clearance around the inoculum.

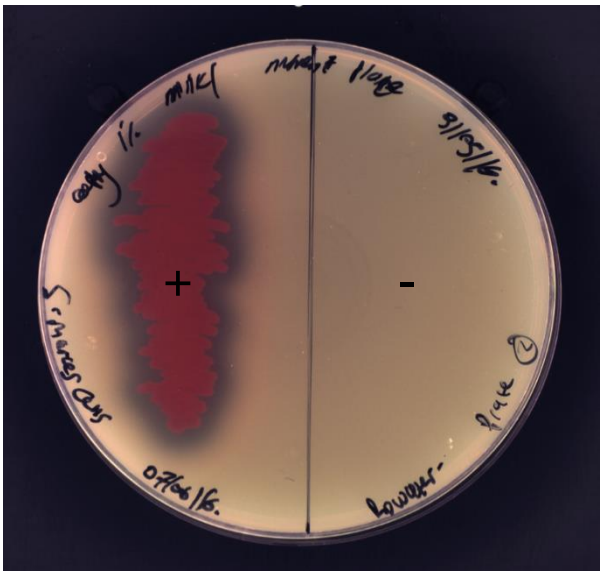


Figure 4.4: Non-specific protease activity indicated by zone of clearance around *S. marcescens* on a 1% (v/v) skimmed milk agar plate (+). Negative control showing no protease activity (-).

Table 4.2: Phylotype-specific production of protease among clinical *C. acnes* isolates on 1% (v/v) skimmed milk agar prepared using nutrient agar base.

C. acnes Phylogenetic Group	*Number of Disc Tissue Isolates Producing Protease Enzyme (Percentage Total of Each Phylotype)*
IA_1 (*n*=19)	**19 (100%)**
IB (*n*=6)	**5 (83%)**
II (*n*=35)	**31 (89%)**
III (*n*=7)	**7 (100%)**
Total (*n*=67)	**62 (93%)**

No microbial growth was noted on any of the skimmed milk agar plates inoculated with sterile RO water (negative control). Consistent non-specific protease activity results were generated between duplicate plates.

4.3.2.3 DNase Activity

The addition of 1M HCl to bacterial growth on the surface of DNase agar plates precipitates polymerised DNA, turning the media opaque. Clear zones are noted around bacterial lawns if isolates hydrolyse DNA through the production of DNase enzymes [Figure 4.5 (A)]. No *C. acnes* strains were found to hydrolyse DNA. Therefore, all *C. acnes* isolates were negative for DNase enzyme activity. No microbial growth was noted on any of the DNase agar plates inoculated with sterile RO water (negative control). *S. aureus* NCTC 6571 (positive control) was DNase-positive. *E. coli* ATCC 11775 (negative control) was DNase-negative.

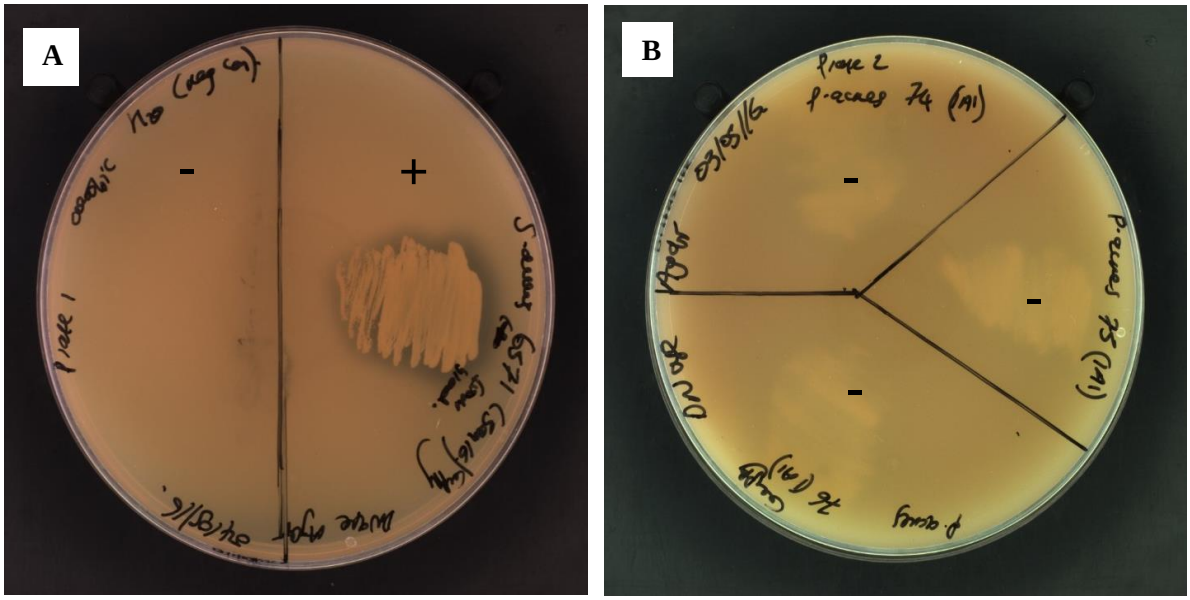


Figure 4.5: DNase activity as noted by zones of clearance around the bacterial inoculum. Zones of clearance were not observed around any *C. acnes* lawns (B) or for sterile RO water (negative control) (A) (-). *S. aureus* NCTC 6571 was included as a positive DNase control (A) (+).

4.3.2.4 Lipase Activity

Bacterial lipases hydrolyse lipids, such as glyceryl tributyrate, to liberate glycerol and free fatty acids, turning the opaque glyceryl tributyrate-supplemented LB agar media clear. Clear zones were noted around all *C. acnes* disc tissue and acne lesion isolates, suggesting that *C. acnes* strains belonging to all phylotype lineages produce exocellular lipases [Figure 4.6 (A, B and D)]. However, most acne lesion *C. acnes* isolates and phylotype IA_1 disc tissue isolates demonstrated greater lipolytic activity as compared to phylotype IB, II and III disc

tissue isolates, as noted by the production of larger zones of clearance by these isolates [Figure 4.6 (A)].

Positive lipase activity also resulted in iridescent sheens on the colony surfaces of all lipase-positive *C. acnes* isolates. As shown in Figure 4.6 (B), *C. acnes* NCTC 737 (positive control) was positive for lipase activity whilst *E. coli* ATCC 11775 (negative control) was negative for lipase activity on LB media supplemented with 1% (v/v) Tween™ 80 and 1% (v/v) glyceryl tributyrate [Figure 4.6 (C)]. Consistent lipase activity results were generated between duplicate plates.

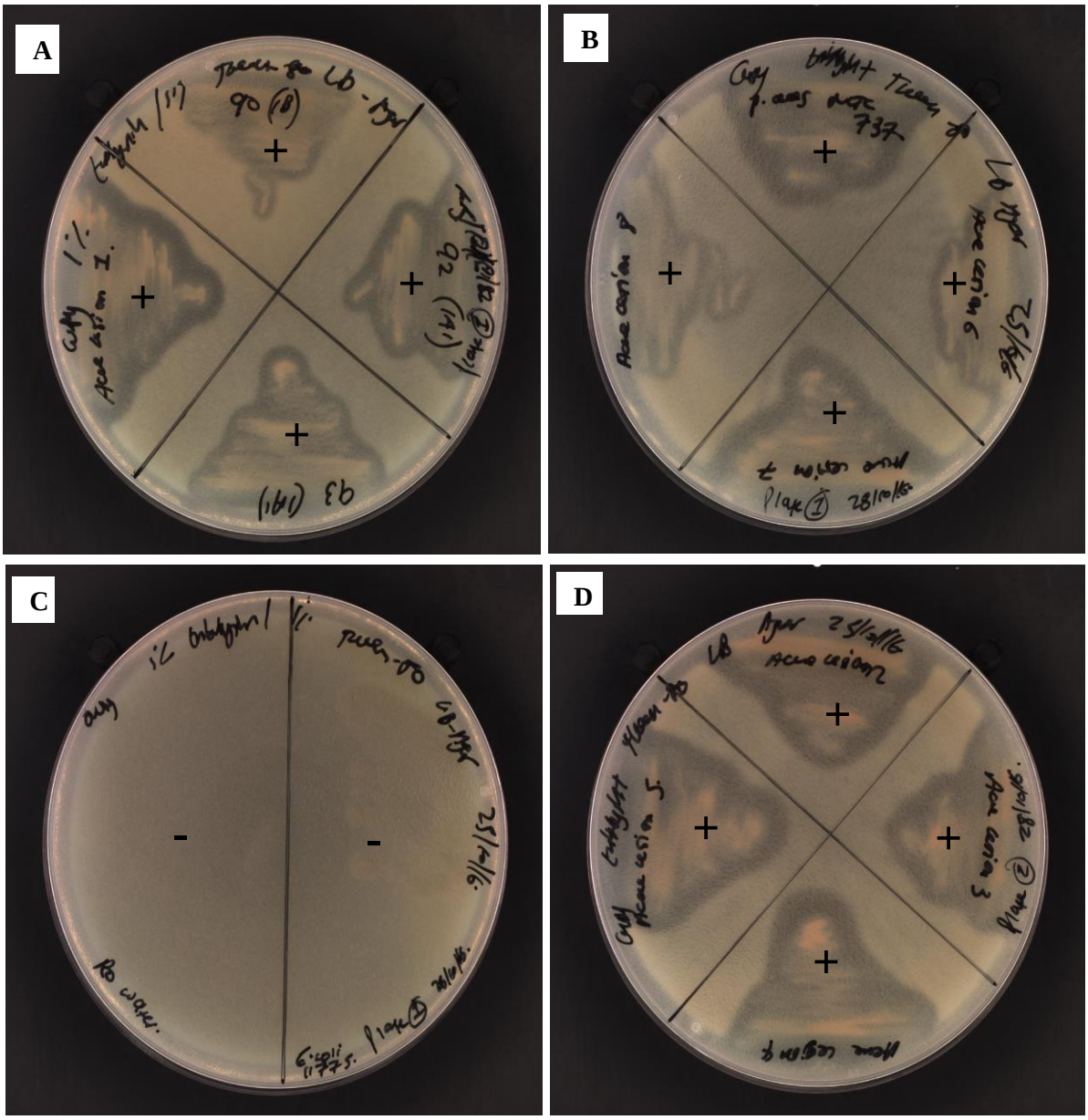


Figure 4.6: Lipase activity as noted by zones of clearance around *C. acnes*. Zones of clearance were observed around all disc tissue and acne lesion *C. acnes* isolates (A, B and D) (+). *C. acnes* NCTC 737 was included as a positive control (B) (+). *E. coli* ATCC 11775 and sterile RO water were included as negative controls (C) (-).

4.3.2.5 Gelatinase Activity

Gelatinase is an exocellular enzyme that degrades gelatin into smaller polypeptides, peptides and, subsequently, amino acids, essential in microbial metabolism. A positive gelatinase production test result is demonstrated by the partial or total liquification of inoculated nutrient gelatin media (Oxoid, UK) after refrigeration (Figure 4.7).

Overall, 36% (24/67) of herniated disc tissue isolates of *C. acnes* displayed discrepancies in gelatinase production among duplicate nutrient gelatin tubes. As a result, these isolates were omitted from further analysis. Therefore, 64% (43/67) of disc tissue *C. acnes* isolates demonstrated consistent gelatinase production results among duplicate tubes and these results were taken forward for further analysis (Table 4.3).

Figure 4.7: Gelatinase activity as noted by liquification of nutrient gelatin agar (Oxoid, UK). Total liquification of inoculated nutrient gelatin media after refrigeration by *C. acnes* NCTC 737 (positive control showing positive gelatinase activity) (+). Solidification of nutrient gelatin agar after refrigeration following inoculation with *E. coli* ATCC 11775 (negative control showing lack of gelatinase production) (-).

Overall, 49% of remaining disc tissue *C. acnes* isolates (21/43) were gelatinase positive whilst 51% of these isolates (22/43) were gelatinase negative. Overall, no significant difference was observed between the number of *C. acnes* disc tissue isolates positive for gelatinase production as compared to the number of disc tissue isolates negative for gelatinase production ($p > 0.05$). However, as shown in Table 4.3, gelatinase production is dependent upon incubation duration, with all gelatinase positive disc tissue *C. acnes* isolates

showing positivity between either 1-4 days or 10-14 days of anaerobic incubation at 37°C. A total of 64% of phylotype IA_1 disc tissue isolates only showed gelatinase positivity following 10-14 days of incubation, with no phylotype IA_1 disc tissue isolates showing gelatinase production following 1-4 days of anaerobic incubation. This suggests that these isolates are slow gelatinase producers that secrete this enzyme following longer incubation periods. In contrast, 33% of phylotype IB and II disc tissue *C. acnes* isolates and 40% of phylotype III disc tissue isolates showed gelatinase positivity following 1-4 days of anaerobic incubation, suggesting that these isolates are strong gelatinase producers. Therefore, following the use of the Fisher's exact analysis test, phylotype was shown to have a significant effect on the gelatinase activity of disc tissue isolates of *C. acnes* ($p < 0.05$). Gelatinase positivity was not recorded in any of the *C. acnes* isolates taken from acne lesions.

As shown in Figure 4.7, *C. acnes* NCTC 737 (positive control) was positive for gelatinase activity whilst *E. coli* ATCC 11775 (negative control) was negative for gelatinase activity following 14-days of nutrient gelatin agar incubation.

Table 4.3: Phylotype-specific gelatinase production following 14-days of anaerobic incubation of *C. acnes* clinical disc tissue isolates at 37°C.

	***C. acnes* Phylogenetic Group**			
	Number of Disc Tissue Isolates Positive for Gelatinase Production (Percentage Total of Each Phylotype)			
Incubation Duration (Days)	IA_1 (*n*=11)	IB (*n*=3)	II (*n*=24)	III (*n*=5)
+++ [1]	0 (0%)	1 (33%)	8 (33%)	2 (40%)
++ [2]	0 (0%)	0 (0%)	0 (0%)	0 (0%)
+ [3]	7 (64%)	0 (0%)	3 (13%)	0 (0%)
- [4]	4 (36%)	2 (67%)	13 (54%)	3 (60%)
Total (*n*=43)	11 (25%)	3 (7%)	24 (56%)	5 (12%)

[1] Positive gelatinase production following 1-4 days of anaerobic incubation at 37°C.

[2] Positive gelatinase production following 5-9 days of anaerobic incubation at 37°C.

[3] Positive gelatinase production following 10-14 days of anaerobic incubation at 37°C.

[4] Negative gelatinase production following 14-days of anaerobic incubation at 37°C.

4.4 Discussion

As shown in Figure 4.1, *C. acnes recA* types vary in morphological characteristics of bacterial cells, with phylotype III cells forming long, slender filaments as compared to phylotype IA_1, IB and II cells that are characterised by coryneform morphology. This

corroborates the *recA* type-specific cellular morphology observations reported by McDowell *et al*. (2008). As explored by McDowell *et al*. (2008), the formation of these filamentous cells may impact the potential of phylotype III strains to penetrate tissue and form biofilms, potentially accounting for the association between type III strains and deep tissue infections (McDowell *et al*., 2012; Niazi *et al*., 2010). As a result of the phylotype-specific pleomorphic nature of *C. acnes* cells, these bacteria may be missed during routine analysis in clinical microbiology laboratories, leading to the under-reporting of *C. acnes*-associated infections. Therefore, the analysis of phenotypic characteristics of *C. acnes* clinical isolates is integral in aiding the identification of these microorganisms.

C. acnes is traditionally considered as a microorganism of low virulence whose isolation from biological samples is often assumed as sample contamination from the resident skin microflora [Niazi *et al*., 2010; Shu *et al*., 2013 (a); Evans, 1975; Dodson *et al*., 2010]. However, *C. acnes* has been shown to produce a range of biologically active substances with enzymatic, chemoattractant, antigenic and complement-activating activities (Holland, Ingham and Cunliffe, 1981). Although the production of various enzymatic virulence factors by cutaneous isolates of these microaerophilic coryneform bacteria has been reported (Ingham *et al*., 1981; Greenman and Holland, 1985; Ingham *et al*., 1983; Marples and McGinley, 1974), limited information relating to exocellular enzyme production of *C. acnes* disc tissue isolates was found in previous publications. Therefore, the current study aimed to investigate possible differences in the production of a range of virulence factors between clinical disc tissue *C. acnes* isolates representing phylotypes IA_1 (*n*=19), IB (*n*=6), II (*n*=35)

and III (*n*=7), including eight acne lesion isolates.

As described in section 4.3.2.1 above, 84% of the *C. acnes* phylotype IA_1 isolates produced β-haemolysis on 7% (v/v) sheep blood agar, followed by 67% of *C. acnes* IB isolates (Table 4.1). Haemolysis was not recorded in any of the *C. acnes* isolates belonging to phylotypes II and III (Table 4.1). Additionally, five acne lesion *C. acnes* isolates (63%) expressed β-haemolysis, with the remaining three isolates negative for haemolytic activity. These results corroborate the findings of Nodzo *et al*. (2014) which identified β-haemolysis in all orthopaedic strains of *C. acnes*, suggesting that this virulence factor may play a role in promoting deep tissue infections, potentially through the facilitation of host cell cytotoxicity via pore formation in host cell membranes (Capoor *et al*., 2018). These results oppose the research findings of McDowell *et al*. (2008) which described variable α- and β-haemolysis amongst type I *C. acnes* isolates and variable α-haemolytic activity amongst type II *C. acnes* isolates taken from a range of clinical samples, including spine IVD material. These discrepancies in the production of exocellular haemolytic toxins between phylotypes may be attributed to the site of *C. acnes* isolation and the type of infection isolates originated from, with Nodzo *et al*. (2014) identifying haemolysis as an important clinical marker of definite *C. acnes*-associated orthopaedic infections. The source of mammalian erythrocytes used to supplement agar may also account for such variabilities in haemolysin activity, with McDowell *et al*. (2008) utilising the published protocol set out by Spare *et al*. (2003) for the assessment of haemolysin activity, in which both horse and sheep erythrocytes were used. In contrast, the current study used only sheep erythrocytes. Equine erythrocytes have been

reported to demonstrate greater sensitivity to bacterial haemolysin toxins as compared to sheep erythrocytes which may account for variations in phylotype-specific haemolysin activity between studies (Amoako *et al*., 1998). These discrepancies also suggest that *C. acnes* of specific phylogroups may produce more than one type of haemolysin, potentially due to variations in culture conditions which could affect haemolysin production between studies. As well as highlighting the importance of protocol standardisation amongst studies assessing haemolysin production by *C. acnes,* these observations demonstrate the potential importance of this virulence factor in disc pathogenesis, potentially through the interaction of these toxins with nociceptors to induce pain in disc tissue (Capoor *et al*., 2018). Haemolysis may also act as a clinical marker for the identification of *C. acnes* IVD tissue isolates belonging to phylotypes IA_1 and IB. More recently, pore-forming haemolysin proteins liberated from *C. acnes* during chronic IVD tissue colonisation and infection have been suggested to interact with sensory ingrown nerve fibres within degenerated discs to induce pain within a small subset of CLBP patients (Capoor *et al*., 2018; Freemont *et al*., 1997). This may support the hypothesis that CLBP can be associated with *C. acnes* infection of the IVD tissue and that this pain may correlate with live bacterial load (Capoor *et al*., 2018). However, continued investigation of the virulence profiles of the various *C. acnes* phylotypes are warranted to further elucidate the role of site of *C. acnes* isolation on the array of exocellular enzymes produced.

C. acnes haemolysis has been associated with higher preoperative ESR and CRP levels, suggesting that haemolytic isolates have pathogenic properties that differ from those of non-haemolytic isolates (Nodzo *et al*., 2014; Hoeffler, 1977). However, non-haemolytic *C. acnes* strains can also demonstrate significant pathogenicity and have been associated with definite orthopaedic infections (Nodzo *et al*., 2014). Therefore, the virulence potential of non-haemolytic disc tissue isolates of *C. acnes* cannot be discounted. Analysis of the *C. acnes* strain KPA171202 genome identified candidate genes whose products may confer haemolytic activity to the strain, namely genes PPA565, PPA938 and PPA1396 (Brüggemann, 2005). However, further work is required to identify genes involved in modulating haemolysin production within the isolates used in the current study.

Differential production of exocellular enzymes by *C. acnes* isolates of different phylotypes may be attributed to the acquisition of putative virulence determinant genes via horizontal gene transfer (Gahukamble *et al*., 2014; McDowell *et al*., 2013). *C. acnes* enzyme production may also vary with pH as well as with carbon, nitrogen, vitamin and metal ion availability within the microenvironment (Greenman, Holland and Cunliffe, 1983; Cove, Holland and Cunliffe, 1983; Holland, Greenman and Cunliffe, 1979). These factors may also have an impact on the activity and stability of exocellular enzymes. Additionally, *C. acnes* haemolytic activity can be modulated by erythrocyte concentration and the type of mammalian erythrocytes added to solid media, with Hoeffler (1977) demonstrating the better susceptibility of rabbit erythrocytes to the lytic action of *C. acnes* haemolysins as compared to human erythrocytes. In contrast to these findings, the current study observed

clearer haemolysis zones when using 7% (v/v) sheep blood as compared to 7% (v/v) horse blood (data not shown).

Exocellular protease is produced during the exponential phase of *C. acnes* growth which contrasts with other enzymes produced by *C. acnes*, such as acid phosphatase, produced late in the exponential phase of growth and in the early stationary phase (Ingham *et al*., 1983; Ingham *et al*., 1980). Therefore, whilst enzymes produced during the late growth phase may play a 'scavenger' role in *C. acnes* physiology, exocellular protease may facilitate bacterial nutrition, rendering amino acids and peptides available to metabolising bacteria (Ingham *et al*., 1983). This role of exocellular protease in *C. acnes* nutrition is supported by the findings of the current study in which 93% of herniated disc tissue isolates of *C. acnes* were protease-positive (Table 4.2), with the almost universal production of this enzyme suggesting that it is important in *C. acnes* virulence and nutrition. This is supported by the research findings of Greenman, Holland and Cunliffe (1981) which demonstrated the ability of *C. acnes* to grow in glucose-deficient semi-synthetic media rich in amino acids and small peptides. However, the non-proteolytic, saccharolytic microorganism *Propionibacterium granulosum* exhibited very low biomass under these conditions (Greenman, Holland and Cunliffe, 1981). Therefore, *C. acnes* may rely upon proteases to utilise amino acids and peptides as carbon/energy sources in protein-rich environments, such as on the skin, potentially accounting for the almost ubiquitous production of this enzyme among disc tissue and acne lesion isolates, irrespective of isolate phylotype (Greenman, Holland and Cunliffe, 1981).

The AF of the IVD consists of fibrous lamellae containing type I and type II collagens, with the central NP of the disc containing type II collagen (Eyre and Muir, 1976). Microbial collagenases have been shown to elicit pathological changes within mammalian IVDs, including VB erosion and loss of the convex structure of the disc, and can digest β-casein found in cows milk (Stern and Coulson, 1976; Eyre and Muir, 1976; Truswell, 2005). Therefore, the protease activity observed on casein-containing skimmed milk agar may represent collagenase activity, with such proteases potentially facilitating the enzymatic dissolution of IVDs. However, further work is required to isolate and characterise the observed *C. acnes* proteases to gain a better understanding of the potential pathophysiological effects of such enzymes on the integrity of IVDs.

C. acnes exocellular protease enzymes also play a significant role in promoting acne inflammation, with secreted *C. acnes* proteases capable of promoting matrix breakdown and proteolytic detachment of keratinocytes within follicles, leading to the release of inflammatory mediators into the dermis (Lee *et al*., 2010). *C. acnes* protease enzymes also promote inflammation through interacting with protease-activated receptor-2 (PAR-2) on keratinocytes, leading to the upregulation of mRNA transcript levels of inflammatory mediators, such as IL-6, IL-8 and TNF-α (Lee *et al*., 2010). Interestingly, PAR-2 expression has been identified in human IVD tissue (Iida *et al*., 2009). Therefore, the high rate of protease production amongst the herniated disc tissue *C. acnes* samples tested within the present study suggests that this enzyme may play a significant role in the virulence of these bacteria, with the potential for these isolates to exhibit proinflammatory effects within disc

tissue through protease-mediated PAR-2 signalling upregulation. However, a significant limitation of the Lee *et al*. (2010) study is the use of a single *C. acnes* isolate belonging to a single phylotype (IA_1). Therefore, the effect of proteases obtained from *C. acnes* samples belonging to other phylotypes on transcript levels of proinflammatory cytokines should be investigated.

Exocellular DNase enzyme production is associated with pathogenic bacteria, playing a role in metabolism, dissemination of infecting bacteria, such as through pus liquification and mucosal DNA digestion, and evasion of host innate immune responses through the degradation of NETs (Sumby *et al*., 2005). Additionally, Sumby *et al*. (2005) demonstrated the role of group A Streptococcal exocellular DNase in facilitating isolate persistence within skin injection sites as well as evidencing the ability of DNase-producing isolates to lyse polymorphonuclear leucocytes, promoting the severity of soft tissue pathology within an animal model. However, no DNase activity was detected in any of the disc tissue and acne lesion isolates of *C. acnes* included within the present study (4.3.2.3). Similarly, McDowell *et al*. (2008) observed no DNase activity amongst 100 *C. acnes* isolates representing phylotypes I, II and III, with Hoeffler (1977) concluding that *C. avidum* and *C. granulosum* produce DNase much more frequently than other species of Cutibacteria examined. Therefore, DNase activity is strain-specific and may not contribute significantly to *C. acnes* virulence. However, the induction of bacterial exocellular enzyme secretion can be dependent upon host cell interaction, with Broudy, Pancholi and Fischetti (2002) demonstrating the induction of streptococcal DNase secretion following the co-culture of

Streptococcus pyogenes with human pharyngeal host cells. This suggests that certain bacterial enzymes may require specific *in vivo* 'cues' for production. Therefore, whilst *C. acnes* DNase is not produced merely for basal metabolic function, the ability of these isolates to secrete exocellular DNase in response to physiological challenges, such as host cell invasion, cannot be discounted. Therefore, *in vivo* studies of *C. acnes* exocellular enzyme production are warranted, with a particular focus on the effect of bacterial interaction with host cells on virulence factor production.

C. acnes lipase is a non-specific enzyme, facilitating *C. acnes* nutrition *in vivo* through the total hydrolysis of sebum triglycerides to form free fatty acids and glycerol (Gribbon, Cunliffe and Holland, 1993). This is supported by the ubiquitous production of lipase by all *C. acnes* disc tissue and acne lesion isolates used within this study (4.3.2.4). However, McDowell *et al*. (2008) recorded variable lipase production for type I and II *C. acnes* isolates, although these isolates were obtained from a range of body sites, with 34% obtained from spine IVD material and 19% isolated from acne lesions.

As explored by Dudli *et al*. [2016 (a)], the co-culture of a clinical IVD tissue *C. acnes* isolate with lumbar discs promoted an increase in *C. acnes* exocellular lipase activity within culture supernatants in a concentration- and time-dependent manner. Increasing co-culture duration from 3-hours to 24-hours increased lipase activity in both the 1:10 and 1:100 *C. acnes* groups, with a stronger increase in the 1:100 group [Dudli *et al*., 2016 (a)]. Therefore,

C. acnes lipase production may also depend on the interaction of isolates with mammalian cells. Tomida *et al*. (2013) also reported that the insertion or deletion of nucleotides, known as indels, in lipase-encoding *C. acnes* genes may also modulate lipase activity, with indels ranging from one to 13 nucleotides being reported to decrease lipase activity in type II *C. acnes* strains. As explored in chapter 1 (1.4.3.1), lipase production may play a role in the aetiology and structural changes observed within Modic type I changes through the breakdown of triacylglycerides abundant in bone marrow, liberating proinflammatory free fatty acids which promote structural bone marrow changes [Dudli *et al*., 2016 (a)].

In addition to bacterial nutrition, lipase enzymes have been associated with *C. acnes* acne pathogenesis. Free fatty acids liberated from the hydrolysis of native sebum triglycerides through the action of microbial lipases exhibit comedogenic properties, with free fatty acids suggested to penetrate the follicular wall and induce inflammatory reactions within the underlying dermis (Shalita, 1974). In support of the role of free fatty acids in acne pathogenesis, Cunliffe *et al*. (1973) demonstrated a correlation between decreased skin surface free fatty acid levels and significant clinical improvement amongst acne patients receiving long-term oral tetracycline therapy, with no overall change in *C. acnes* numbers. Therefore, tetracycline may directly inhibit exocellular bacterial lipases (Hassing, 1971). Free fatty acids produced on the skin by *C. acnes* lipase are both chemotactic and cytotoxic for human polymorphonuclear leucocytes and mononuclear leucocytes at concentrations ≥ 200µg/ml (Puhvel and Sakamoto, 1978; Tucker *et al*., 1980). Therefore, lipase may play a role in the initiation and/or enhancement of inflammation associated with acne vulgaris

(Tucker *et al*., 1980). Additionally, oxidation of fatty acids enhanced *in vitro* cytotoxic effects against human polymorphonuclear leucocytes, indicating the potential of free fatty acid oxidation by tissue lipoxygenases to potentiate the cytotoxic effects of these *C. acnes* metabolic by-products *in vivo* (Puhvel and Sakamoto, 1978).

C. acnes exocellular lipase activity has also been postulated to act as a colonisation factor *in vivo*, with Gribbon, Cunliffe and Holland (1993) demonstrating the increase in *C. acnes* cell-to-cell aggregation and adherence in the presence of lipid substrates. Therefore, *C. acnes* lipase activity may ensure the adequate supply of free fatty acids to promote cell adherence and cooperative nutrition within pilosebaceous follicles (Gribbon, Cunliffe and Holland, 1993). Consequently, studies to investigate the potential relationship between *C. acnes* lipase activity and the degree of biofilm formation are warranted, with the potential for lipase to promote *C. acnes* IVD colonisation and persistence via biofilm formation.

36% of herniated disc tissue isolates of *C. acnes* (24/67) displayed discrepancies in gelatinase production among duplicate nutrient gelatin tubes and were subsequently omitted from further analysis (4.3.2.5). This may indicate varied gelatinase gene expression among *C. acnes* isolates whilst also potentially demonstrating the unstable nature of this exocellular enzyme *in vitro*. Therefore, gelatinase is unsuitable as a routine clinical marker of *C. acnes* infection.

49% of disc tissue *C. acnes* isolates (21 out of the remaining 43) were identified as gelatinase positive, with all acne lesion isolates negative for gelatinase production (Table 4.3). In contrast to these findings, Hoeffler (1977) demonstrated gelatinase production amongst 95% (38/40) of *C. acnes* strains isolated from human hair and acne vulgaris lesions. The current study also showed that phylotype had a significant effect on the gelatinase activity of disc tissue isolates of *C. acnes* ($p < 0.05$), with 33% of phylotype IB and II *C. acnes* isolates and 40% of phylotype III isolates showing gelatinase positivity following 1-4 days of anaerobic incubation, indicative of strong gelatinase production (Table 4.3). Therefore, as these phylotypes are usually associated with deep tissue infections (McDowell *et al*., 2012; Niazi *et al*., 2010), gelatinase may be a significant enzyme within deep tissue *C. acnes* infections, potentially acting as a spreading factor *in vivo*. The ability of gelatinase to liquify a collagen derivative (gelatin) suggests that *C. acnes* gelatinase may also hypothetically facilitate the enzymatic degradation of IVD tissue which is composed of Type I and Type II collagen, subsequently promoting bacterial spread in infected tissue (Eyre and Muir, 1976). This hypothesis is supported by the lack of gelatinase positivity amongst *C. acnes* isolates taken from superficial acne lesion swabs.

In comparison to the doubling times of *E. coli* and *S. aureus* of around 17-minutes and 30-minutes, respectively, *C. acnes* is a slow-growing microorganism, with a doubling time of approximately 5.1 hours (Hall *et al*., 1994). Therefore, the slow growth rate of this microorganism may be associated with delayed production of exocellular enzymes, highlighting the importance of extended isolate incubation periods past 5-days, the duration

microbiology laboratories routinely culture samples for (Hall *et al*., 1994), to maximise the identification of clinically significant enzymes.

A limitation of the current study was the relatively small sample sizes of *C. acnes* isolates representing phylotypes IB and III which may skew phylotype-specific inferences made from the phenotypic enzyme production data. Additionally, only eight *C. acnes* isolates constituted the acne lesion panel, limiting the conclusions that can be drawn with regards to the relationship between site of *C. acnes* isolation (disc vs acne lesion) and enzyme production. Therefore, the incorporation of a larger panel of IVD tissue *C. acnes* isolates of each phylotype, plus the use of a larger panel of acne lesion isolates, is suggested in future studies to corroborate the results of the current study.

In conclusion, clinical disc tissue and acne lesion isolates of *C. acnes* produce a range of clinically significant exocellular enzymes. Phylotype-specific variability in exocellular enzyme production noted within the current study highlights potential pitfalls in the use of *C. acnes* isolates belonging to single phylotypes as control strains within virulence assessment studies. Additionally, this study demonstrates differences in the exocellular enzyme production of *C. acnes* belonging to phylotypes IA_1, IB, II and III, suggesting that enzyme production may be used as a means of identification of strains and as an indicator of strain virulence and pathogenicity. With these enzymes having been reported to be involved in promoting inflammation and pain, as well as spread within infected tissue, the current

study concludes that production of these enzymes may support the colonisation and persistence of *C. acnes* within IVD tissue (Dudli *et al*., 2018; Lee *et al*., 2010; Risbud and Shapiro, 2014; Park *et al*., 2007; Palmer *et al*., 2012; Capoor *et al*., 2018).

CHAPTER 5 *GALLERIA MELLONELLA* (*G. MELLONELLA*) INFECTION MODEL FOR *C. ACNES*

5.1 Introduction

The study of bacterial virulence often involves the use of an animal model to replicate infection. The murine model is one of the most commonly used models for studying microbial infections (Tsai, Loh and Proft, 2016). Although mammalian models are considered the gold standard for the *in vivo* investigation of pathogen virulence, such systems are costly and raise ethical issues as well as logistical and housing issues, particularly if a large number of microbes and/or antimicrobial agents are to be screened (Tsai, Loh and Proft, 2016; Binder, Maurer and Lass-Flörl, 2016; Soldatow *et al*., 2013). Additionally, the lengthy reproduction times of mammals can act as major time limiting factors during experimentation (Tsai, Loh and Proft, 2016). Therefore, alternative host models are increasingly being investigated, including *Danio rerio, Caenorhabditis elegans*, *Dictyostelium spp* and *Drosophila melanogaster* (Froquet *et al*., 2007; Marsh and May, 2012; Kurz and Ewbank, 2007; Neely, Pfeifer and Caparon, 2002). In recent years, a number of studies have used the lepidoptera *G. mellonella* to study the efficacy of a range of antimicrobial agents as well as to study host-pathogen interactions and virulence of a range of microbial species, including *E. coli, Cryptococcus neoformans, S. aureus, Klebsiella pneumoniae, Enterococcus faecalis, Listeria monocytogenes, Pseudomonas aeruginosa* and

Candida albicans (Alghoribi *et al*., 2014; Mylonakis *et al*., 2005; Silva *et al*., 2017; Russo and MacDonald, 2020; Park *et al*., 2007; Mukherjee *et al*., 2010; Miyata *et al*., 2003; García-Lara, Needham and Foster, 2005; Bergin *et al*., 2006).

G. mellonella are lepidopterans, belonging to the family Pyralidae, with the caterpillar larvae, or wax worm, being used within infection studies (Tsai, Loh and Proft, 2016). *G. mellonella* are increasingly being introduced as an alternative model to study bacterial infection, with >450 research articles published between 2018-2019 on PubMed, demonstrating the increasing popularity of this insect model. *G. mellonella* larvae are easy to handle, are exempt from ethical legislation, are cheap, require no special laboratory equipment for maintenance and have a short lifespan, allowing for the use of this model in high-throughput studies (Tsai, Loh and Proft, 2016; Velikova, Kavanagh and Wells, 2016). Additionally, unlike other non-mammalian models, such as *Drosophila melanogaster* and *Danio rerio*, *G. mellonella* can function at human body temperature (37°C), the optimum growth temperature for *C. acnes* (Nathan, 2014; Cook and McArthur, 2013). The larger size of *G. mellonella* larvae of approximately 2cm in length as compared to other non-mammalian models enables precise injection of test pathogens and easy collection of tissue and haemolymph samples from waxworm larvae (Vogel *et al*., 2011). Additionally, this model allows for bacterial virulence to be measured in several ways, including LD_{50} calculation, assessment of bacterial survival rates and measurement of insect mortality (Allegra *et al*., 2018; Guillemet *et al*., 2010; Hurst *et al*., 2015). Also, the waxworm insect immune system exhibits both humoral and cellular components, resembling the innate

immune system of mammals, making it a useful model for infection studies (Sheehan *et al*., 2018; Kavanagh and Reeves, 2007; Cook and McArthur, 2013).

A key consideration in the use of the *G. mellonella* infection model over mammalian models is the degree of concordance between results generated by each of these models. A range of studies have demonstrated the comparability between results generated using insect models and mammalian models in infection studies (Brennan *et al*., 2002; Kaito *et al*., 2002; Jander, Rahme and Ausubel, 2000; Joyce and Gahan, 2010). Therefore, *G. mellonella* can produce information relevant to mammalian infection and so this model system is suitable for the identification of mammalian virulence factors within a range of clinically significant microorganisms.

The innate immune response of insects consists of two major components; the cellular and the humoral immune responses (Tsai, Loh and Proft, 2016). The cellular response in insects is mediated by haemocytes which function in a manner comparable to mammalian phagocytes (Browne, Heelan and Kavanagh, 2013). These haemocytes are suspended in haemolymph, analogous to mammalian blood (Marmaras and Lampropoulou, 2009). At least six types of haemocytes have been identified in *G. mellonella* (Figure 5.1), of which plasmatocytes and granulocytes function in phagocytosis, nodule formation and encapsulation. Haemocytes are also involved in the insect clotting cascade in response to activation by microbial cell wall components (Brennan *et al*., 2002; Browne, Heelan and

Kavanagh, 2013; Kavanagh and Reeves, 2004). The insect humoral response is orchestrated by soluble effector molecules that interact directly with invading pathogens and include melanin and antimicrobial peptides. The process of melanisation plays a key role in the defence of *G. mellonella* against a wide range of pathogens (Kavanagh and Reeves, 2004).

Figure 5.1: Haemocyte types involved in the cellular immune response of *G. mellonella* (Kavanagh and Reeves, 2004).

The melanisation response is indicative of the activation of the *G. mellonella* immune response and is described as the formation and deposition of melanin to encapsulate invading pathogens, similar to abscess formation in mammals (Tang, 2009). Melanin

formation in *G. mellonella* is catalysed by phenoloxidase (PO) which is located in its inactive zymogen form pro-phenoloxidase (ProPO) within insect haemocytes (Kavanagh and Reeves, 2004). Melanisation is initiated by soluble pattern recognition receptors that bind to target antigens on invading pathogens, such as lipoteichoic acids of Gram-positive bacteria, triggering a serine protease cascade that leads to the cleavage of ProPO to PO (Halwani, Niven and Dunphy, 2000; Lu *et al*., 2014). PO can be released from haemocytes by lysis of these cells or through active transport to the insect cuticle or to encapsulated pathogens (Kavanagh and Reeves, 2004). PO catalyses oxidation of phenols to quinines which polymerise non-enzymatically to form melanin (Söderhäll and Cerenius, 1998). This is known as the proPO-activating system (proPO-AS) (Cerenius and Söderhäll, 2004). Activation of the proPO-AS and the subsequent deposition of melanin on pathogens within the waxworm haemolymph due to the action of PO is known as the melanisation reaction and is easily observed by the darkening of the waxworm body, as shown in Figure 5.8 below (Söderhäll and Cerenius, 1998). Melanisation acts as a defence mechanism to encapsulate foreign pathogens, limiting the spread and growth of these pathogens and subsequently reducing damage to host tissues (Sugumaran, 2002).

As part of the innate mammalian immune response, the redox molecule nitric oxide (NO) plays a role in the orchestration of the proinflammatory classical phase of the innate immune response. This includes the activation of the transcription factor NF-κB which modulates the switching on of immune-related genes (Jacobs and Ignarro, 2003; Wink *et al*., 2011). Interestingly, Semenova *et al*. (2014) showed the production of NO by *G. mellonella* larvae

haemocytes following the *in vivo* activation of the insect immune response using aliquots of lipopolysaccharide (LPS) or zymosan suspension. NO has been reported to play a role in promoting bacterial biofilm dispersal (Barraud *et al*., 2006). Therefore, it could be hypothesised that *G. mellonella* may produce NO in response to microbial infection to activate the immune response and also to reduce bacterial biofilm formation and establishment of infection. However, further studies are required to investigate the range of antibacterial compounds produced by *G. mellonella*.

Despite the benefits of the *G. mellonella* model explored above and the correlation between mammalian and insect immune system responses to pathogen challenge, no previous studies have explored the potential application of this waxworm system in modelling *C. acnes* infection. Therefore, the current study aimed to assess the viability of such a model in *C. acnes* infection modelling via the observation of waxworm melanisation and mortality responses towards challenge with *C. acnes*. A growth curve was established for *C. acnes* NCTC 737 (ATCC 6919), a commonly used control strain within *C. acnes* research, to inform experimental methods involving *G. mellonella* in which the stage of growth of *C. acnes* is important to the implementation of *G. mellonella* infection assays through the calculation of bacterial colony counts at various culture incubation times. The potential role of bacterial phylogroup on waxworm responses was also assessed to determine whether any *recA*-specific effects were seen.

5.1.1 Aims

The aims of this *G. mellonella* infection study were to:

- Determine whether *G. mellonella* is a viable host infection model for the assessment of *C. acnes* virulence using waxworm mortality and melanisation as markers of bacterial pathogenicity.
- Assess whether the *G. mellonella* infection model shows species-specific and phylotype-specific responses following bacterial infection.

5.2 Materials and Methods

5.2.1 Bacterial Strains and Growth Conditions

A range of *C. acnes* phylotypes were used in model *G. mellonella* killing assays (Figures 5.4-5.6). *S. marcescens* ATCC 8100 and *S. epidermidis* RP62A were used as positive controls. *S. marcescens* was selected as a positive control strain due to the previous demonstration of the potent pathogenicity of these bacteria within the *G. mellonella* model (Tambong *et al*., 2014). *S. epidermidis* was also used as a positive control due to the co-culture of this species with *C. acnes* in clinical infections (Niazi *et al*., 2010). *C. acnes* were cultured in BHI broth anaerobically at 37°C for 72-hours. The ODs of all cultures were standardised to approximately 0.08 by dilution with sterile BHI broth at 600nm, which was

equivalent to approximately 1×10^6 CFU/ml (3.2.1). Aliquots of standardised cultures were inoculated into fresh BHI broth and incubated anaerobically at 37°C until approximately 1×10^7 CFU/ml were obtained (confirmed by OD readings at 600nm and the preparation of corresponding CFU/ml counts). Control isolates *S. epidermidis* RP62A and *S. marcescens* ATCC 8100 were cultured using the same method but under aerobic incubation conditions. *C. acnes*, *S. marcescens* and *S. epidermidis* cells were harvested by centrifugation of cultures at 13.4RPM for 20-minutes, with the resulting pellets washed using sterile PBS and the cells resuspended in an equal volume of sterile PBS. Cell-free samples were prepared by filter sterilising culture supernatants using a 0.22 μm filter (Millipore). Heat-killed *C. acnes* cells were prepared by exposure of washed cell suspensions to a temperature of 90°C for 30-minutes. Sterility of the heat-killed samples was checked by streaking these samples onto BHI agar plates which were incubated anaerobically at 37°C for 7-days (or aerobically for the control strains *S. epidermidis* RP62A and *S. marcescens* ATCC 8100). Cell-free preparations were designed to assess whether potential waxworm immunogenic *C. acnes* factors were exocellular factors released by bacterial cells. Heat-killed preparations were used to assess whether potential waxworm immunogenic *C. acnes* factors were heat labile factors whilst cell-containing preparations were used to assess whether immune responses within the waxworm model relied upon the presence of whole bacterial cells. If so, this would demonstrate that such an immunogenic factor could be a cell-associated antigen or a virulence factor actively secreted by bacterial cells.

5.2.2 *C. acnes* NCTC 737 Growth Curve

From growth on 7-day BHI agar plates, three replicate broth suspensions of *C. acnes* NCTC 737 were prepared and incubated anaerobically at 37°C for 72-hours. Sterile BHI broth was used to standardise all cultures spectrophotometrically to the lowest OD reading of around OD_{600}= 0.08, equivalent to approximately 1×10^7 cells/ml. Fifty millilitre aliquots of BHI broth were used to prepare 1:1000 dilutions for each of the three *C. acnes* NCTC 737 cultures in sterile glass screw-cap tubes. Actual starting culture cell concentrations were determined by colony counts at time 0 through the preparation of serial tenfold dilutions for each of the three cultures, with subsequent plating onto BHI agar plates using the method outlined by Miles, Misra and Irwin (1938). Additional samples of broth were removed from each of the three *C. acnes* NCTC 737 cultures after 4, 8, 21, 25, 29, 33, 45, 49, 53, 57, 69, 73, 77, 81, 93 and 97 hours of anaerobic incubation for subsequent colony counts using the Miles, Misra and Irwin (1938) method. BHI agar plates inoculated for the determination of colony counts were incubated anaerobically at 37°C for 4-days, after which colonies of *C. acnes* NCTC 737 were counted and $\log_{10}$ CFU/ml counts were calculated.

A growth curve was plotted by using the average of the triplicate bacterial concentrations (CFU/ml) against time. The bacterial generation time in hours (gt) was determined by using the mean concentration of *C. acnes* NCTC 737 at the beginning and end of log phase growth, as shown in Figure 5.3. The following formula was used to calculate gt: gt = [K(t_f-

t_i)] ÷ [($\log_{10}$ (CFU_f/ml)) – ($\log_{10}$ (CFU_i/ml))], where CFU_f/ml and CFU_i/ml are the final and initial bacterial concentrations at the end and beginning of log phase, respectively, t_f and t_i are the final and initial times in hours when final and initial log phase cultures were sampled, respectively, and K= log 2, equal to 0.301, which refers to the exponential growth rate constant (Sottile II and Zabransky, 1977).

5.2.3 *G. mellonella* Insect Larvae

Final-instar larval stage *G. mellonella* were obtained from UK Waxworms Limited (Sheffield, UK). Larvae were stored in wood shavings in the dark at room temperature (20-23°C) prior to use. All larvae were used within 7-days of receipt.

5.2.4 *G. mellonella* Killing Assays

G. mellonella larvae weighing between 250mg-350mg were employed in all assays. A 50µl Hamilton neuro-syringe was used to inject 10µl aliquots of the inoculum (as described in 5.2.1) into the haemocoel of each larvae via the last left proleg (Cotter, Doyle and Kavanagh, 2000; Mylonakis *et al*., 2005). Before injection, the area was cleaned using 70%

(v/v) ethanol to remove bacteria naturally present on the surface of larvae. Additionally, the syringe used for inoculation was sterilised through sequential washes with 10% (v/v) bleach, 70% (v/v) ethanol and sterile PBS, respectively. Test panels included cell-free, heat-killed and cell-containing preparations of *C. acnes*, namely isolates 56 (IA_1), 82 (IB), 2 (II) and 80 (III), injected into waxworm larvae at a cell density of around 1×10^7 CFU/ml. Using relevant dilutions, duplicate bacterial colony counts on BHI agar were used to confirm CFU/ml counts of all starting inocula used to inject waxworm larvae.

Three controls were employed in all assays: the first (ethanol control) consisted of larvae that were cleaned with only 70% (v/v) ethanol and maintained at the same temperature as test larvae. The second (injection control) group contained larvae whose last left proleg was pierced with the sterile tip of the inoculation needle to monitor for killing due to physical trauma with no inoculation whilst the third (PBS control) group were inoculated with 10µl of sterile PBS through the last left proleg. Eighteen-22 randomly chosen larvae of the required weight were employed per test and control group. All *G. mellonella* test group infection assays were performed on three independent occasions whilst all control group infection assays were performed on seven independent occasions. All test (cell-free, heat-killed and cell-containing *C. acnes* preparations) and control larvae were placed in sterile Petri dishes and incubated in the dark at 37°C in a stationary aerobic incubator. Mortality rates were determined daily over a 7-day period. Larval death was assessed by the lack of movement of larvae in response to touch. Experiments that had more than two dead larvae in either control group were discarded and repeated.

Survival curves were plotted using the Kaplan-Meier method to visualise how average percentage survival rates differed between larvae within different test and control conditions. Differences in survival rates of larvae on the last day of larval incubation (day 7) were calculated using one-way ANOVA analysis in SPSS 24.0. Mortality rates of *C. acnes* of varying phylotypes, *S. epidermidis* RP62A and *S. marcescens* ATCC 8100 were compared using ANOVA analysis. A p value of <0.05 was considered to be statistically significant.

Larvae melanisation (larvae blackening) was assessed qualitatively through the design of a scoring system to assess the degree of melanin deposition along the body of inoculated waxworm larvae (Figure 5.8). Melanisation responses were graded as either none (-) (no visible blackening of waxworms), tail/line (+) (single grey line running along the whole length of the waxworm body), light grey (++) (light grey zone of melanisation limited to the rear end of the waxworm body), dark grey (+++) (dark grey zone of melanisation running along the entire length of the waxworm body), spots (++++) (intermittent line of melanisation running along the body of the waxworm) or complete (+++++) (complete blackening of the waxworm body), as shown in the pictorial key of Figure 5.8.

The *G. mellonella* experimental workflow outlined above is summarised in Figure 5.2.

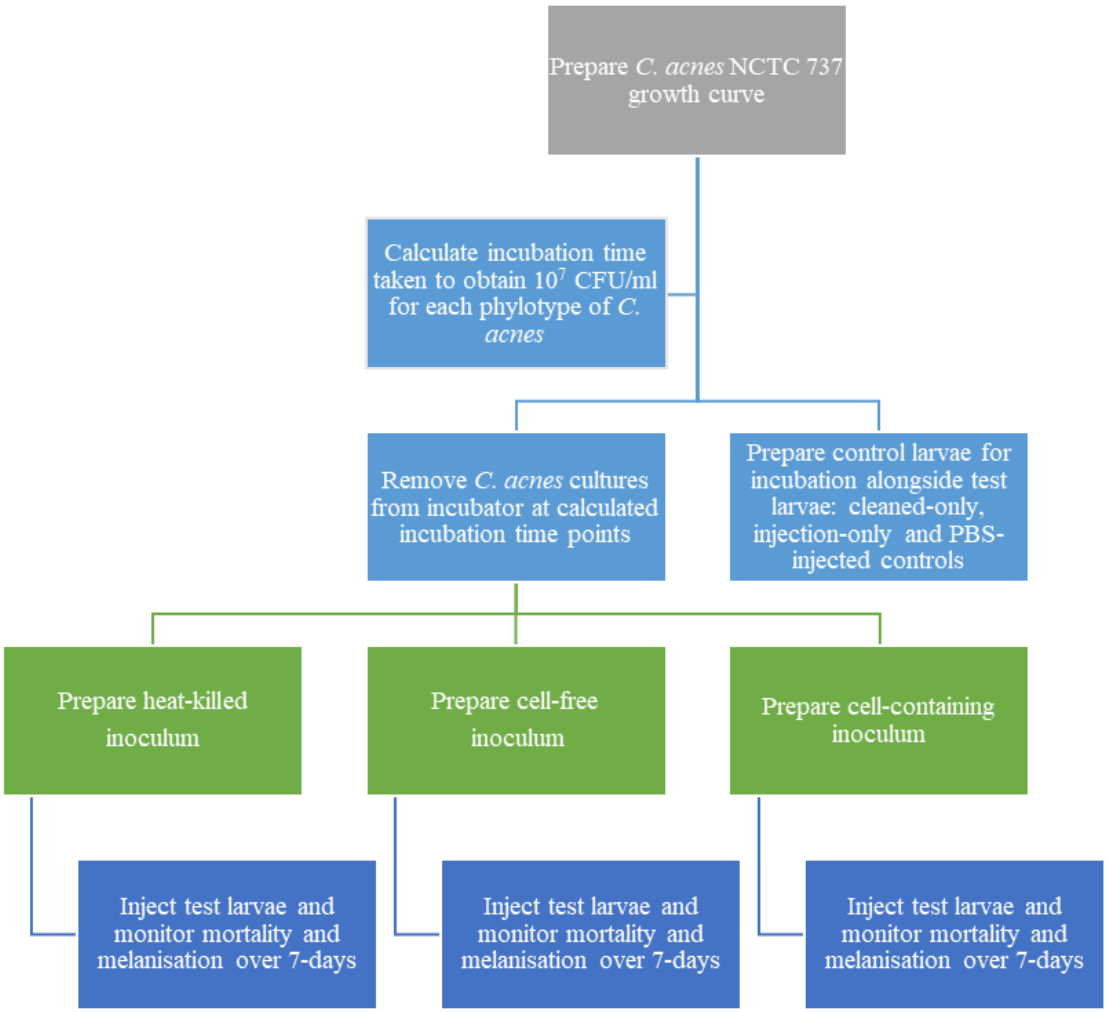


Figure 5.2: Experimental workflow for the assessment of *G. mellonella* mortality and melanisation following infection with clinical IVD tissue isolates of *C. acnes*.

5.3 Results

5.3.1 Calculation of *C. acnes* NCTC 737 Generation Time

Figure 5.3 shows the average growth curve determined for three *C. acnes* NCTC 737 cultures, with average CFU/ml counts and OD_{600} absorbance values of triplicate determinations being plotted. The gt for doubling in *C. acnes* NCTC 737 was determined as approximately 2.8-hours, with cultures entering the stationary phase of growth at around 30-hours post-inoculation.

As shown in Figure 5.3, mean triplicate microbial concentration (log_{10} $CFUml^{-1}$) and OD_{600} readings showed an increase between 69-72 hours, with cultures entering a secondary stationary phase at 73-hours. This same pattern was observed following the plotting of three individual (single experiment) growth curves, instead of one average graph, with each of these curves showing the same increase in log_{10} $CFUml^{-1}$ and corresponding OD_{600} readings at 69-72 hours (data not shown).

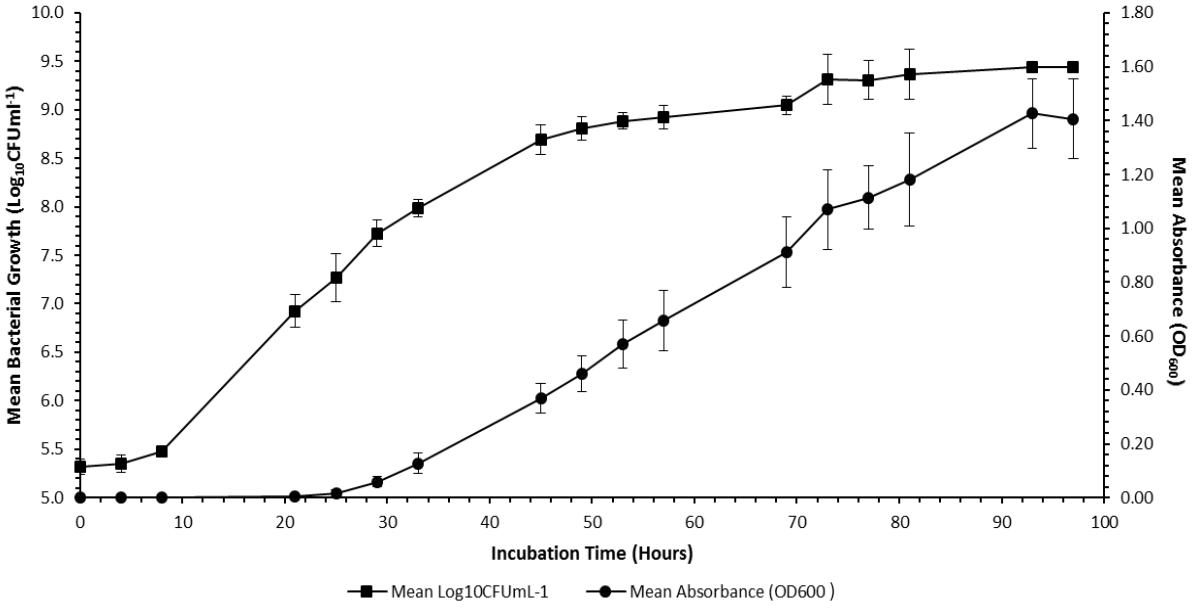


Figure 5.3: Growth curve of *C. acnes* NCTC 737 (ATCC 6919) (*n*=3).

5.3.2 Larval Mortality

Figures 5.4-5.6 show Kaplan-Meier survival plots of *G. mellonella*. These waxworms were injected with test suspensions of *C. acnes* (cell-free, heat-killed or cell-containing), *S. epidermidis* RP62A or *S. marcescens* ATCC 8100. Average percentage survival rates of inoculated waxworms were compared to the cleaned-only, PBS-injected and injection-alone negative control larvae. Results represent the mean percentage survival of larvae following the combination of the data from all experiments. For simplicity, the three control groups [cleaned-only (*n*=132), PBS-injected (*n*=120) and injection-only (*n*=118)] were omitted

from all Figures. No mortality was noted in control larvae within the 70% (v/v) ethanol cleaned-only, PBS-injected and injection-only control categories, with 100% survival rates by day 7 post-treatment.

Cell-free, heat-killed and cell-containing preparations of *C. acnes* NCTC 737, 56 (IA_1), 2 (II) and 80 (III) had no significant effect on *G. mellonella* mortality rates in comparison to control *G. mellonella* within the cleaned-only, PBS-injected and injection-only conditions (Figures 5.4-5.6 and Table 5.1) ($p > 0.05$). Percentage survival rates ranged from 94%-100% for *C. acnes* isolates NCTC 737, 56 (IA_1), 2 (II) and 80 (III) in the cell-free, heat-killed and cell-containing preparation test groups at day 7 post-treatment (Table 5.1). Heat-killed and cell-containing preparations of *C. acnes* 82 (IB) also yielded no significant effect on *G. mellonella* mortality rates as compared to control larvae, with percentage survival rates at day 7 of 100% and 98%, respectively (Figure 5.5 and Table 5.1) ($p > 0.05$). However, average percentage larvae survival was significantly lower in *G. mellonella* injected with *C. acnes* 82 (IB) cell-free preparations as compared to control *G. mellonella*, with percentage survival rates at day 7 of 93% (Figure 5.5 and Table 5.1) ($p < 0.05$). Despite this observation, overall comparisons of larvae mortality rates between differing *C. acnes* phylotypes and culture preparation techniques showed no significant effects on *G. mellonella* survival rates (Table 5.1) ($p > 0.05$). This suggests that in general, *C. acnes* does not induce significant mortality within this *in vivo* waxworm model of infection.

Cell-containing samples of *S. epidermidis* RP62A led to significant larvae mortality as compared to all negative control larvae categories (cleaned-only, PBS-injected and injection-only) and as compared to cell-free and heat-killed preparations of *S. epidermidis* RP62A, with a percentage survival rate of 92% at day 7 (Figure 5.7 and Table 5.1) ($p \leq 0.01$). In contrast, cell-free and heat-killed preparations of *S. epidermidis* RP62A did not lead to significant larvae mortality as compared to control larvae, with survival rates of 94% and 98% on day 7, respectively (Figure 5.7 and Table 5.1) ($p > 0.05$). Therefore, cell-containing culture preparations of *S. epidermidis* RP62A demonstrate a significant increase in virulence within a waxworm model of infection.

The cell-containing suspension of *S. marcescens* ATCC 8100 (positive control) demonstrated significantly greater mortality in comparison to all control larvae, all *S. epidermidis* RP62A preparations and as compared to all *C. acnes* phylotypes and preparations (cell-free, heat-killed and cell-containing samples), with a survival rate on day 7 post-treatment of 22% (Figures 5.4-5.7 and Table 5.1) ($p < 0.01$). *S. marcescens* ATCC 8100 cell-containing preparations also demonstrated greater virulence within *G. mellonella in vivo* as compared to heat-killed and cell-free preparations of the same bacteria (Figure 5.7) ($p < 0.01$). Therefore, cell-containing culture preparations of *S. marcescens* ATCC 8100 demonstrate a significant increase in virulence within a waxworm model of infection.

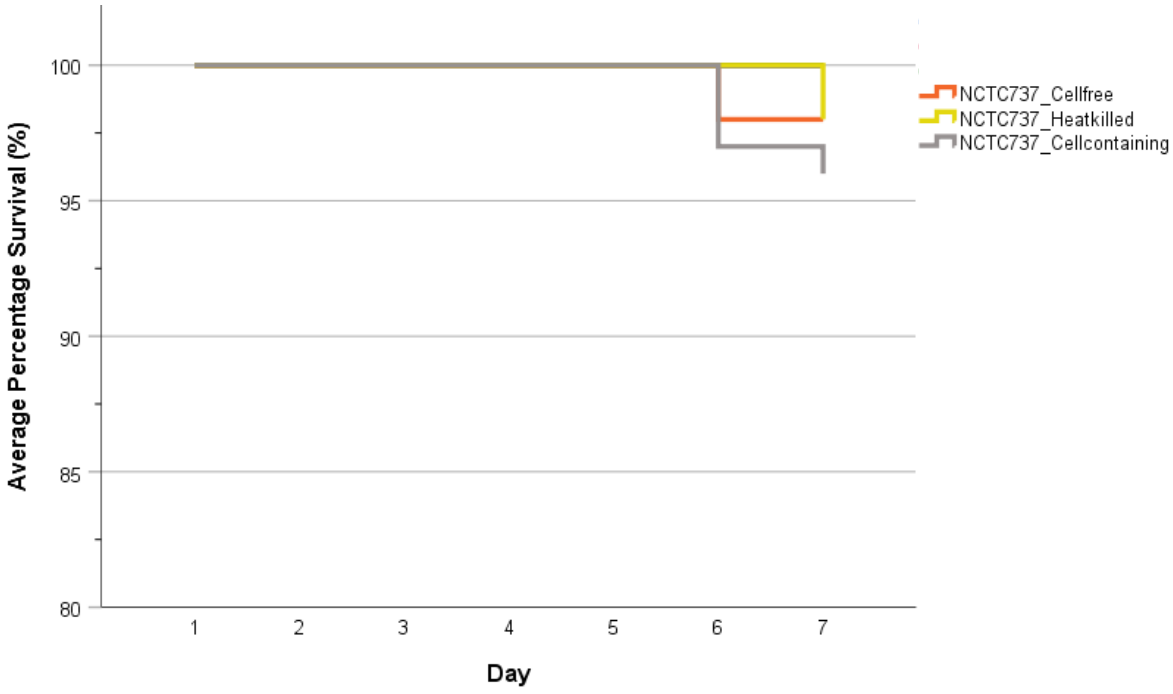


Figure 5.4: Kaplan-Meier survival graph of larvae injected with cell-free, heat-killed and cell-containing preparations of *C. acnes* NCTC 737. NCTC 737 cell-free preparation (*n*=52). NCTC 737 heat-killed preparation (*n*=51). NCTC 737 cell-containing preparation (*n*=51).

Table 5.1: Percentage survival rates of *G. mellonella* larvae following infection with *C. acnes* and control strains *S. epidermidis* RP62A and *S. marcescens* ATCC 8100. Percentage survival rates show the percentage of larvae surviving on the final day (day 7) of incubation post-infection.

		***C. acnes* Phylotype**					**Control Strains**	
		Percentage Larvae Survival						
Incubation Duration (Days)		NCTC 737 (*n*=~51)	IA_1 (*n*=~59)	IB (*n*=~49)	II (*n*=~52)	III (*n*=~54)	*S. epidermidis* RP62A (*n*=~56)	*S. marcescens* ATCC 8100 (*n*=~50)
7	Cell-Free	98%	100%	93%	96%	96%	94%	98%
7	Heat-Killed	98%	100%	100%	96%	94%	98%	100%
7	Cell-Containing	96%	98%	98%	100%	98%	92%	22%

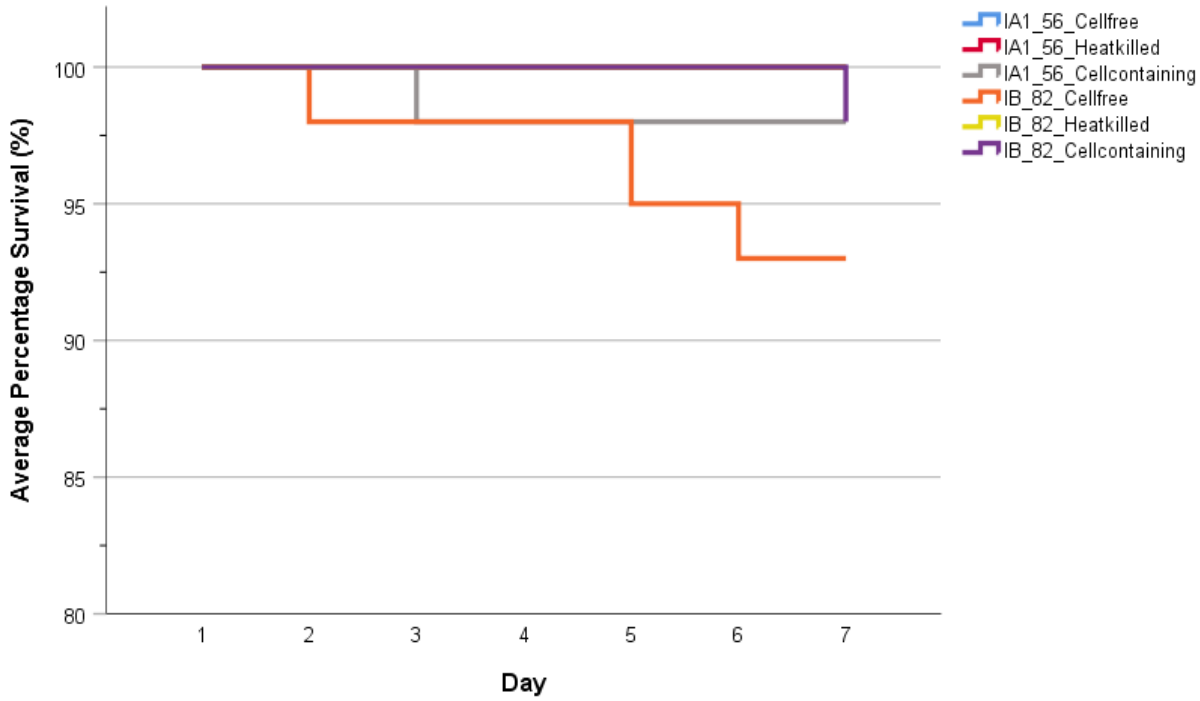


Figure 5.5: Kaplan-Meier survival graph of *G. mellonella* larvae injected with cell-free, heat-killed and cell-containing preparations of *C. acnes* 56 (IA_1) as compared to larvae injected with cell-free, heat-killed and cell-containing preparations of *C. acnes* 82 (IB). 56 (IA_1) cell-free preparation (*n*=58). 56 (IA_1) heat-killed preparation (*n*=60). 56 (IA_1) cell-containing preparation (*n*=60). 82 (IB) cell-free preparation (*n*=47). 82 (IB) heat-killed preparation (*n*=49). 82 (IB) cell-containing preparation (*n*=51). The blue, red and yellow survival lines, representing *C. acnes* isolates 56 (IA_1) cell-free preparation, 56 (IA_1) heat-killed preparation and 82 (IB) heat-killed preparation, respectively, cannot be seen in this Figure due to survival overlap. However, all of these lines represent 100% survival of the respective isolates by day 7 of larvae incubation (Table 5.1).

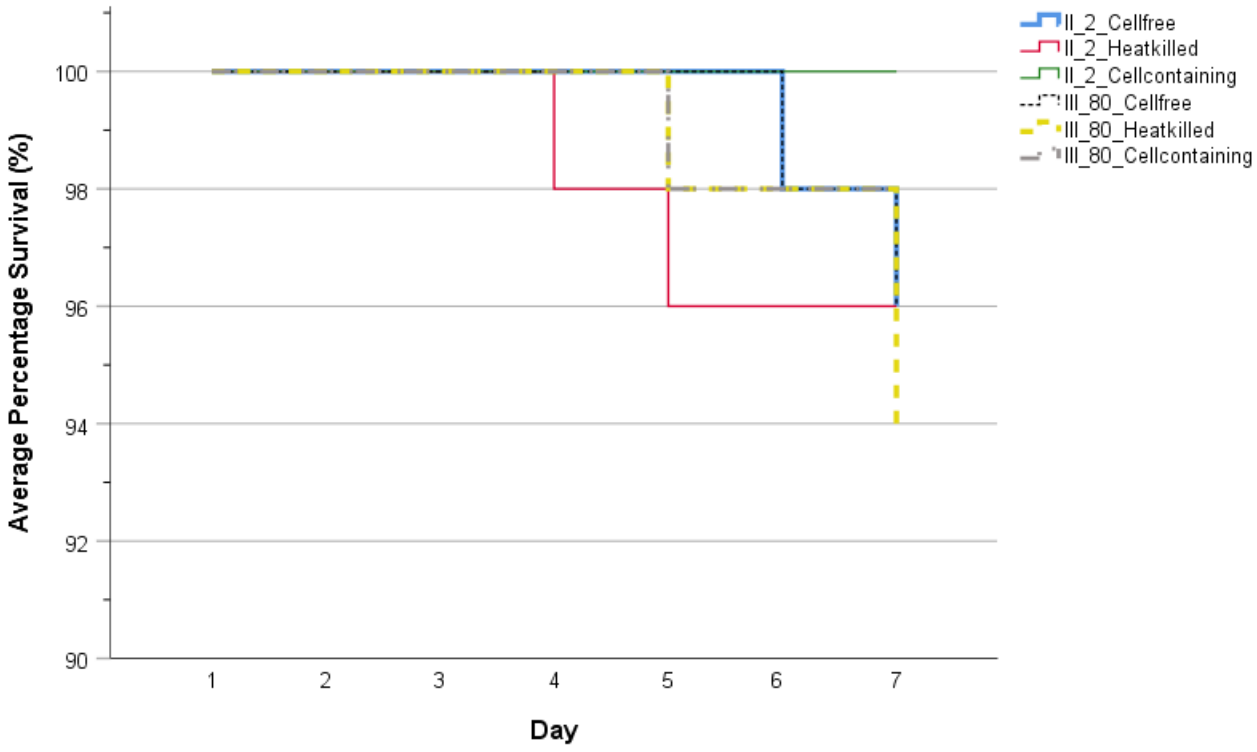


Figure 5.6: Kaplan-Meier survival graph of *G. mellonella* larvae injected with cell-free, heat-killed and cell-containing preparations of *C. acnes* 2 (II) as compared to larvae injected with cell-free, heat-killed and cell-containing preparations of *C. acnes* 80 (III). 2 (II) cell-free preparation (*n*=52). 2 (II) heat-killed preparation (*n*=49). 2 (II) cell-containing preparation (*n*=56). 80 (III) cell-free preparation (*n*=49). 80 (III) heat-killed preparation (*n*=57). 80 (III) cell-containing preparation (*n*=55).

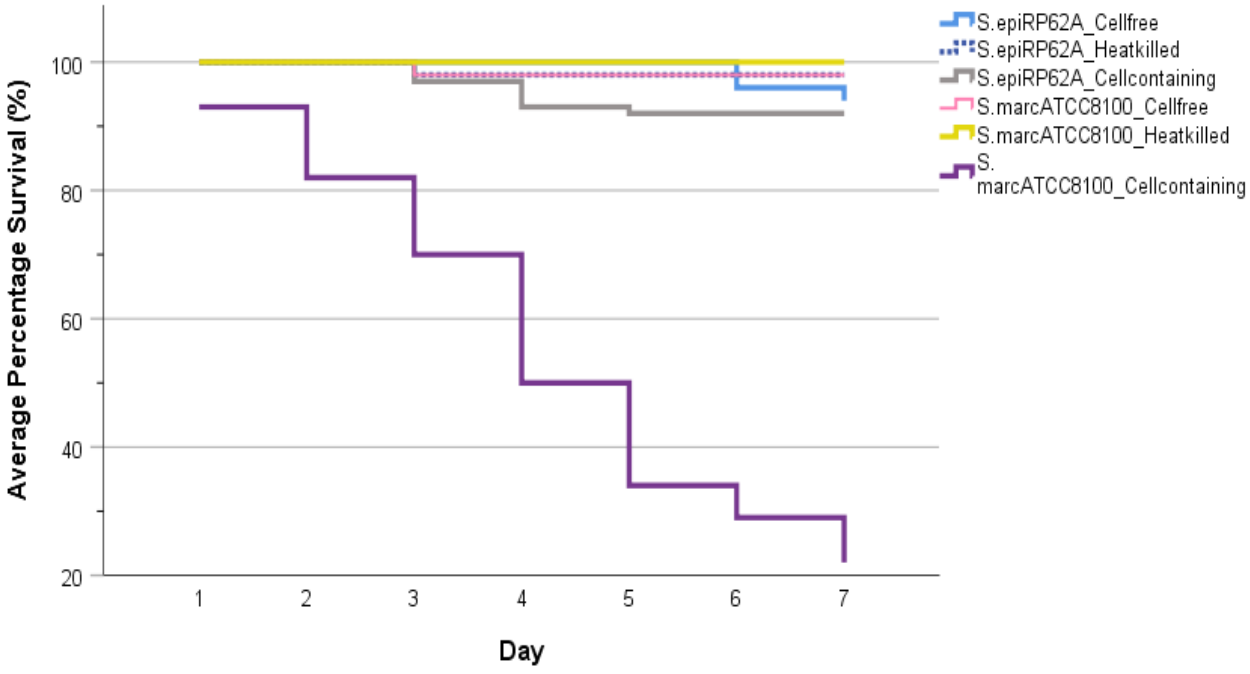


Figure 5.7: Kaplan-Meier survival graph of *G. mellonella* larvae injected with cell-free, heat-killed and cell-containing preparations of *S. epidermidis* RP62A as compared to larvae injected with cell-free, heat-killed and cell-containing preparations of *S. marcescens* ATCC 8100. *S. epidermidis* RP62A cell-free preparation (*n*=57). *S. epidermidis* RP62A heat-killed preparation (*n*=53). *S. epidermidis* RP62A cell-containing preparation (*n*=58). *S. marcescens* ATCC 8100 cell-free preparation (*n*=46). *S. marcescens* ATCC 8100 heat-killed preparation (*n*=48). *S. marcescens* ATCC 8100 cell-containing preparation (*n*=56).

5.3.3 Melanisation Responses of *G. mellonella* Larvae

Melanisation responses of all waxworm larvae were assessed every day for a total period of 7-days. No melanisation (no larvae blackening) was noted in control waxworms within the cleaned-only, PBS-injected or injection-only groups by day 7 post-incubation (Table 5.2). Cell-containing cultures of *C. acnes* isolates 82 (IB) and 80 (III) led to the greatest degrees of *G. mellonella* melanisation by day 7 [Table 5.3 and Figure 5.8 (D)]. At 1-day post-injection, cell-containing cultures of *C. acnes* 82 (IB) and 80 (III) induced light-grey melanisation which generally localised to the site of injection at the rear of each larvae (Table 5.3 and Figure 5.8). However, at 7-days post-injection, this melanisation progressed to darker grey colouration of larvae [Table 5.3 and Figure 5.8 (D)] and moved along the body of each larvae towards the thorax and head. This progressive melanisation was also noted in larvae infected with heat-killed samples of *C. acnes* isolate 2 (II), with tail/line-type melanisation noted 1-day post-incubation, progressing to light grey melanisation by day 7 post-incubation [Table 5.3 and Figure 5.8 (B and C)].

As shown in Table 5.3 and Figure 5.8, melanisation was observed in all *C. acnes* isolates, regardless of phylogroup, within the heat-killed and cell-containing test preparations. *C. acnes* 56 (IA_1) heat-killed and cell-containing samples led to the least degree of melanisation of all *C. acnes* isolates tested following both 1-day and 7-days of waxworm incubation.

However, tail/line-type melanisation was noted for *C. acnes* 56 (IA_1) heat-killed and cell-containing samples at 7-days post-incubation [Table 5.3 and Figure 5.8 (B)].

Interestingly, cell-free, heat-killed and cell-containing *S. epidermidis* RP62A samples did not induce melanisation in *G. mellonella* larvae following incubation for up to 7-days post-injection [Table 5.3 and Figure 5.8 (A)].

A lack of *G. mellonella* larvae melanisation was also noted in larvae injected with cell-free and heat-killed samples of *S. marcescens* ATCC 8100, even following incubation for 7-days post-infection [Table 5.3 and Figure 5.8 (A)]. However, cell-containing cultures of *S. marcescens* ATCC 8100 led to the most prominent and significant *G. mellonella* waxworm melanisation at both 1-day and 7-days post-injection compared to all other bacteria tested [Table 5.3 and Figure 5.8 (E and F)]. Additionally, *S. marcescens* ATCC 8100 cell-containing cultures induced very rapid melanisation 1-day post-injection, which progressed to significant *G. mellonella* mortality and melanisation by day 7 post-incubation compared to all other bacteria tested [Table 5.3 and Figure 5.8 (F)].

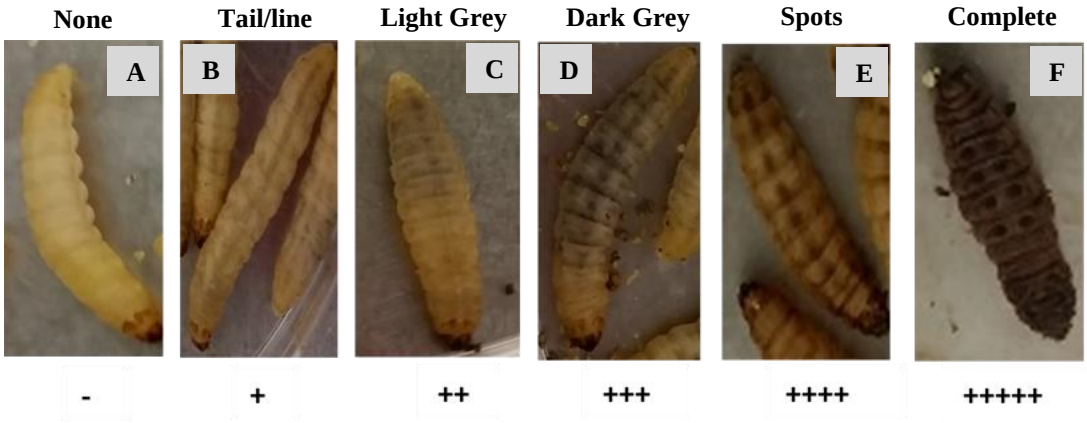


Figure 5.8: Melanisation responses of *G. mellonella* larvae following infection compared to healthy final instar stage larvae (A). Progressive melanisation of larvae is shown from left (none) to right (complete).

Table 5.2: Melanisation scores of negative control waxworm larvae within the cleaned-only, PBS-injected and injection-only treatment categories using the melanisation key outlined in Figure 5.8.

	Control Categories		
	Melanisation Scores		
Incubation Duration (Days)	Cleaned-Only (*n*=132)	PBS-Injected (*n*=120)	Injection-Only (*n*=118)
1	-	-	-
7	-	-	-

Table 5.3: Melanisation scores of waxworm larvae injected with cell-free, heat-killed and cell-containing preparations of *C. acnes* NCTC 737, 56 (IA_1), 82 (IB), 2 (II) and 80 (III) and the control strains *S. epidermidis* RP62A and *S. marcescens* ATCC 8100. Melanisation responses of injected larvae were scored daily for a total incubation period of 7-days using the melanisation key outlined in Figure 5.8.

		***C. acnes* Phylotype**					**Control Strains**	
		Melanisation Scores						
Incubation Duration (Days)		NCTC 737 (*n*=~51)	IA_1 (*n*=~59)	IB (*n*=~49)	II (*n*=~52)	III (*n*=~54)	*S. epidermidis* RP62A (*n*=~56)	*S. marcescens* ATCC 8100 (*n*=~50)
1	Cell-Free	-	-	-	-	-	-	-
7	Cell-Free	-	-	-	-	-	-	-
1	Heat-Killed	+	+	++	+	+	-	-
7	Heat-Killed	+	+	++	++	++	-	-
1	Cell-Containing	++	+	++	+	++	-	++++
7	Cell-Containing	++	+	+++	+	+++	-	+++++

5.4 Discussion

Studies investigating the viability of *G. mellonella* as a model of anaerobic infection are limited, with no previous studies having investigated the virulence of the anaerobic commensal *C. acnes* within the *G. mellonella* model. Additionally, no previous studies have established a growth curve for the commonly used *C. acnes* control strain NCTC 737. Therefore, the current study investigated the effect of *C. acnes* injection in *G. mellonella* on larvae mortality and melanisation as indicators of virulence *in vivo* following the establishment of a *C. acnes* growth curve.

C. acnes NCTC 737 gt was calculated following the generation of a growth curve mapping bacterial growth over a total period of 100-hours. Calculation of bacterial gt allows for the direct comparison of the ability of media type to support the growth of rapidly dividing microorganisms (Sottile II and Zabransky, 1977). Results demonstrated that *C. acnes* NCTC 737 exhibited very slow growth due to an extended log period, with a calculated gt of approximately 2.8-hours (Figure 5.3). This finding corroborates clinical data presented by Levy *et al*. (2013) which showed that the recovery of *C. acnes* from orthopaedic specimens obtained from glenohumeral joints was slow. However, Hall *et al*. (1994) reported a gt for an intraocular strain of *C. acnes* to be 5.1-hours, suggesting that the site of isolation and phylotype designation of *C. acnes* clinical isolates may affect the growth rate of the microorganism. Media type may also affect bacterial gt, with *Bacteroides fragilis* and

Fusobacterium species showing significantly different gt values in different liquid media (Sottile II and Zabransky, 1977). This does not, however, apply to all anaerobes, with *Clostridium perfringens* not showing a significant difference in growth rate between four different liquid media types (Sottile II and Zabransky, 1977). However, BHI is a rich, undefined media which is likely to increase *C. acnes* growth rates as compared to the culture of these bacteria in alternative media types. Therefore, further work is required to investigate the role of media type on *C. acnes* growth rates, with potential applications in informing the culture of samples suspected of containing these bacteria within clinical laboratories.

As shown in Figure 5.3 above, there is a lag in absorbance readings at OD_{600} up to 21-hours of culture incubation despite rising colony counts. This finding is corroborated by Sottile II and Zabransky (1977) who demonstrated that despite microorganisms growing at approximately the same rate, strikingly different turbidimetric responses can be generated. Sottile II and Zabransky (1977) also highlighted that turbidity may or may not increase during the log growth phase, suggesting that turbidity measurements alone cannot accurately reflect numbers of microorganisms within solution.

The gt calculated for *C. acnes* NCTC 737 is longer than the gt reported for aerobic microorganisms commonly co-cultured with *C. acnes*, such as *S. epidermidis* which has a gt of 38-minutes (Gottenbos, Van Der Mei and Busscher, 2000; Coscia, Denys and Wack,

2016). This is also longer than reported gt values of other anaerobic microorganisms commonly isolated from clinical specimens, including 51.5-minutes for *Bacillus fragilis* subspecies and 28.1-minutes for *Clostridium perfringens* subspecies (Sottile II and Zabransky, 1977; Frantz and McCallum, 1979). Therefore, this suggests that patient samples from which *C. acnes* could be isolated need longer incubation times to promote recovery and prevent false-negative reporting of these bacteria.

The slow growth rate of this microorganism may lead to sub-therapeutic antibiotic concentrations during key replicative phases of the *C. acnes* growth cycle, with Eng *et al*. (1991) and Tuomanen *et al*. (1986) demonstrating the dependence of antibiotic killing on the growth rate of a range of Gram-positive and Gram-negative microorganisms. This may be further complicated by the possible biphasic growth nature of *C. acnes* NCTC 737 whereby $\log_{10}$ CFUml^{-1} and OD_{600} readings showed an increase between 69-72 hours followed by a secondary stationary phase at 73-hours (Figure 5.3), potentially affecting responses of these bacteria to antibiotics. Therefore, long-term antibiotic treatment regimens and reinjection of antibiotics at the site of infection can be used for the management of *C. acnes*-associated infections to maintain effective antibiotic levels *in vivo* (Jacobs *et al*., 2016; Stern, Engel and Driebe Jr., 1990). This possible biphasic growth suggests that *C. acnes* may exhaust a preferred nutrient in growth media and subsequently adapt their metabolism to utilise a different, less-preferred nutrient. This hypothesis would support *C. acnes* growth in IVD tissue whereby *C. acnes* may have a selective advantage over other bacterial species by

adapting their metabolism to colonise and proliferate in the low nutrient environment of the disc (Urban, Smith and Fairbank, 2004). Although similar biphasic growth curves have been demonstrated for *C. acnes* ocular strains, with an increase in log CFU/ml around approximately 69-75 hours of incubation, further investigations are required to determine whether the increase in $\log_{10}$ CFUml^{-1} and OD_{600} readings seen in the current study between 69-72 hours represents genuine biphasic growth (Hall *et al*., 1994).

As shown in Figures 5.4-5.6 and Table 5.1, with the exception of *C. acnes* 82 (IB) cell-free preparation, all *C. acnes* isolates from all preparations (cell-free, heat-killed and cell-containing) had no significant effect on larvae mortality as compared to control larvae ($p > 0.05$). Additionally, the comparison of larvae mortality between *C. acnes* isolates belonging to different phylotypes showed no significant difference, regardless of culture preparation (Table 5.1) ($p > 0.05$). The inoculation of *C. acnes* into waxworm larvae and the subsequent lack of significant mortality supports the slow, persistent growth nature of *C. acnes in vivo* whereby host survival is not compromised (Portillo *et al*., 2013).

Cell-containing suspensions of *S. epidermidis* RP62A and *S. marcescens* ATCC 8100 led to significant larvae mortality compared to control larvae, with this preparation of *S. marcescens* ATCC 8100 causing significantly more larvae mortality as compared to all *C. acnes* strains and preparations ($p < 0.01$) (Figure 5.7 and Table 5.1). In concordance with the

findings of Tambong *et al*. (2014), these results suggest that *S. marcescens* ATCC 8100 is an effective positive control species for use within *G. mellonella* bacterial infection studies and demonstrates that infection of waxworms with live bacterial cells is required for *S. marcescens* pathogenesis in *G. mellonella*. This suggests that the virulence factor inducing waxworm mortality following the injection of larvae with live *S. marcescens* ATCC 8100 cells could be a cell-associated antigen or a virulence factor secreted directly by live cells, such as a bacterial toxin.

The cell-containing suspension of *S. marcescens* ATCC 8100 demonstrated significantly greater virulence in comparison to all *S. epidermidis* RP62A and *C. acnes* preparations, inducing larvae mortality rates of approximately 22% by 7-days post-infection (Figures 5.4-5.7 and Table 5.1) ($p < 0.01$). In accordance with these results, McMahon *et al*. (2012) demonstrated the virulence of *S. marcescens* via the injection of outer membrane vesicles of *S. marcescens* into the haemocoel of *G. mellonella* larvae which resulted in 100% larval death within 24-hours. As outer membrane vesicles contain a large number of bacterial virulence factors, including proteases, lipases and chitinases, it can be hypothesised that such bacterial outer membrane vesicles can act as vectors for the delivery of bacterial virulence factors to promote larval death (McMahon *et al*., 2012). This supports the observation of the current study that *S. marcescens* injection of 1×10^7 CFU/ml is lethal within the *G. mellonella* model of infection.

Melanisation, indicative of the activation of the *G. mellonella* immune response, was the second parameter investigated within the current study as a marker of *C. acnes* virulence within the waxworm model. Unlike mortality, melanisation within *C. acnes*-inoculated larvae demonstrated phylotype-specificity, with the cell-containing preparations of *C. acnes* phylotypes IB and III inducing the most prominent melanisation by day 7 as compared to all other *C. acnes* strains tested (Table 5.3 and Figure 5.8). Despite this phylotype-specificity, ubiquitous melanisation responses were seen to varying degrees across all heat-killed and cell-containing *C. acnes* samples used in the current study. This demonstrates the proinflammatory potential of these clinical isolates in a whole-organism model of infection, with *G. mellonella* melanisation indicative of an immune effector response triggered systemically following the invasion of the insect haemocoel by pathogens (Nakhleh, Moussawi and Osta, 2017). These results also demonstrate that such a model could prove particularly useful for studying and comparing virulence in *C. acnes* species as melanisation responses varied between phylotypes. However, a greater range of isolates would need to be studied to draw such a conclusion. Therefore, *C. acnes* of all phylotypes can elicit immune responses within this insect model, with waxworm melanisation being a critical innate insect immune mechanism. This insect response defends against bacterial attack via encapsulation and killing of bacteria through limitation of nutrient uptake and direct cytotoxic effects of melanin intermediates against bacterial cell membranes, indicating that these isolates may also be capable of eliciting such immune responses in humans [Dudli *et al*., 2016 (b); Dudli *et al*., 2018; Christensen *et al*., 2005]. The limitation of melanisation responses to only heat-killed and cell-containing *C. acnes* preparations indicates that such immunogenic virulence

factors are cell-associated, heat-stable antigens. Although all heat-killed and cell-containing preparations of *C. acnes* did, to some degree, display melanisation responses, all *S. epidermidis* RP62A preparations failed to induce melanisation within *G. mellonella* (Table 5.3 and Figure 5.8).

Although injection of waxworm larvae with heat-killed and cell-containing preparations of *C. acnes* led to an immune response that initially limited itself to the injection site at the proleg (Table 5.3 and Figure 5.8), increasing incubation duration led to the progression of melanisation along the body of the waxworms towards the thorax and head. This highlights the differences in the infection pathways of *C. acnes* and *S. epidermidis*, with *C. acnes* inducing chronic, progressive, low-level inflammation which did not kill the host larvae whilst *S. epidermidis* RP62A was less immunoreactive than *C. acnes* yet induced greater waxworm mortality. Like *C. acnes*, *S. epidermidis* is a commensal member of the human skin microbiome which is also isolated from mucosal epithelia. Both bacteria have also been successfully co-cultured from herniated IVD tissue in patients presenting with CLBP, although both *C. acnes* and *S. epidermidis* have been suggested to represent sample contaminants as opposed to endemic bacterial species contributing to disc herniation (Wedderkopp *et al*., 2009; Capoor *et al*., 2017). However, *C. acnes* is highly immunogenic within disc herniation patients via the induction of a range of proinflammatory cytokines and causes chronic, low-grade infection as seen within the waxworm larvae (Urquhart *et al*., 2015; Dudli *et al*., 2018). The differences in immunogenicity between *S. epidermidis* and *C.*

acnes within *G. mellonella* suggests that such variations are species-specific. As the current study only inoculated waxworms with *C. acnes* sourced from herniated disc tissue, future studies are warranted to investigate whether the site of *C. acnes* isolation influences the virulence seen in *G. mellonella*. For example, the virulence of deep tissue infection isolates and superficial isolates, such as those from acne lesions, may be compared to determine whether deep-seated infection isolates elicit a greater immune response within the waxworm model. Future studies may also investigate whether *G. mellonella* produce cytokine-like factor-equivalents within larval haemolymph to cytokines reported to be elicited by *C. acnes* infections within humans. This would help to further elucidate whether *C. acnes* represents a simple sample contaminant or a genuine cause of human infection.

Lack of death and absence of melanisation in control larvae demonstrates that cleaning of waxworms with 70% (v/v) ethanol, injection with PBS and injection alone are effective negative controls and that waxworm mortality and melanisation is not a result of ethanol contact or physical injury (Table 5.2 and Figure 5.8). Similarly, Loh *et al*. (2013) demonstrated no mortality in PBS-injected control larvae, with Olsen *et al*. (2011) also showing no death in larvae sham-inoculated with sterile PBS. As a result, any larval death or melanisation due to sample injection represented a genuine causal effect of microbial infection and not a false positive response towards waxworm preparation and sample injection.

It must be noted that although the cut-off observation period within the current study was 7-days, as also reported by Mukherjee *et al*. (2010), this does not necessarily mean that an increase in *C. acnes*-associated *G. mellonella* mortality and/or melanisation may not have been noted following longer incubation periods. Incubation of larvae for over 7-days led to an increase in waxworm pupation (start of metamorphosis) which limited the ability to assess mortality and melanisation in response to the test conditions. Therefore, this may suggest that the *G. mellonella* model may be better suited for use in studies with a short observation period. Nevertheless, the ability of this model to discriminate between different bacterial species demonstrates the sensitivity of this *in vivo* system and its subsequent potential use as a suitable alternative host model.

C. acnes CAMP is a secretory and surface-associated protein which demonstrates dose-dependent cytotoxicity towards keratinocytes and macrophages (Nakatsuji *et al*., 2011; Lheure *et al*., 2016). The proinflammatory effects of *C. acnes* CAMP factor have been demonstrated in an *in vivo* murine model (Nakatsuji *et al*., 2011). CAMP factor 1 of *C. acnes* interacts with TLRs, inducing proinflammatory cytokine release (Lheure *et al*., 2016). In insects, cell signaling against microbial pathogens can also occur through the Toll pathway via TLR activation, leading to the expression of antimicrobial peptides. Interestingly, Vogel *et al*. (2011) identified TLR transcripts within the *Galleria* dataset, demonstrating the potential of *G. mellonella* to respond to bacterial infection using the same pathway as seen within the human immune response. As CAMP acts via the Toll pathway in

mammals, with these receptors also identified within insects, it can be hypothesised that CAMP may induce the production of similar cytokines within both systems. This hypothesis is supported by Wittwer *et al*. (1999) who provided immunocytochemical evidence for the presence of IL-1α and TNF-α-like molecules, cytokines usually found within vertebrates, in *G. mellonella* haemocytes. This highlights the similarities between the immune function of mammals and the invertebrate *G. mellonella* and its subsequent potential application as an alternative model for the assessment of bacterial virulence. Therefore, the chronic, progressive immune response seen within *G. mellonella* towards *C. acnes* which mirrors the human response to these bacteria may be orchestrated in part by *C. acnes* CAMP production. Further studies are required to modulate the *G. mellonella* immune response towards *C. acnes* by blocking CAMP and assessing whether melanisation is attenuated. In support of this, Wang *et al*. (2018) successfully reduced inflammatory cytokine production in human acne lesions using *C. acnes* anti-CAMP factor antibodies. This would allow future studies to investigate the significance of CAMP factor production in *C. acnes* herniated disc tissue infections.

In order to potentially identify *C. acnes* virulence agents, such as CAMP, associated with inducing *G. mellonella* mortality and melanisation, further studies are required to characterise the cell-associated and exocellular protein production profiles of cell-containing, heat-killed and cell-free *C. acnes* isolates via SDS-PAGE. Further developing current knowledge relating to the protein production profiles of a range of disc tissue *C.*

acnes isolates has implications in informing novel drug development and vaccine strategies to exploit specific *C. acnes* microbial targets. This may help to promote patient disease management via targeted therapies based on protein production profiles.

Mukherjee *et al*. (2010) demonstrated a pathogen load-dependent response of *G. mellonella* to bacterial infection. Mukherjee *et al*. (2010) also demonstrated that *G. mellonella* show evidence of immune response priming, with the prior induction of the *G. mellonella* immune response protecting larvae from subsequent infection with lethal bacterial doses. This immune priming response was also reported by Wu, Xu and Yi (2016). Therefore, subsequent research may focus on investigating the dose-dependent response of *G. mellonella* to *C. acnes* infection to determine the dosage of *C. acnes* at which maximum *G. mellonella* inflammation is induced. Future studies may also investigate whether prior injection of larvae with heat-killed *C. acnes* confers tolerance to subsequent infection with viable *C. acnes*, with potential applications of immune priming within vaccine development against *C. acnes* disc infections.

Unlike Drosophila, a significant limitation in the establishment of *G. mellonella* as an alternative novel host model system is the absence of established stock centres where researchers can purchase larvae of known genotypes raised under standard conditions (Tsai, Loh and Proft, 2016). As a result, *G. mellonella* larvae used within microbial virulence

studies are often purchased from independent breeders and suppliers, meaning that variations in genotypes, breeding conditions, maintenance and handling of *G. mellonella* larvae could modulate the susceptibility of larvae to infection (Tsai, Loh and Proft, 2016; Loh *et al*., 2013; Olsen *et al*., 2011). Additionally, the potential use of prophylactic antibiotics and hormones in the rearing of bait shop *G. mellonella* to prevent infection and promote colony yield may accumulate within larvae and subsequently affect the reproducibility of infection assays (Olsen *et al*., 2011; Loh *et al*., 2013). Food deprivation can also modulate *G. mellonella* innate immune responses and subsequently affect susceptibility to microbial infection (Banville, Browne and Kavanagh, 2012). With different studies using this model publishing varying larval feeding and maintenance protocols, standardised methods of larval feeding and maintenance need to be established to increase the reliability of inter-laboratory *G. mellonella* infection data (Loh *et al*., 2013; Olsen *et al*., 2011).

Other limiting factors within *G. mellonella* research are the fact that the genome of this invertebrate has not been fully sequenced, with no method for mutant strain cultivation having been established (Tsai, Loh and Proft, 2016). Additionally, there is no access to microarrays or RNA interference libraries for *G. mellonella*, limiting the ability to conduct functional genetic studies (Tsai, Loh and Proft, 2016). Therefore, whilst this infection model has been improved, such as through the use of fluorescent proteins as markers to visualise infecting bacteria to map bacterial cell localisation within host tissue (Ramarao,

Nielsen-Leroux and Lereclus, 2012), this model may be developed further. Additional studies exploring *G. mellonella* genomic sequencing and the establishment of RNA interference libraries are also warranted to promote research into *G. mellonella* gene function and to promote the establishment of gain-and loss-of-function mutants.

Within the current study, the degree of *G. mellonella* melanisation was assessed qualitatively via photographic documentation of melanisation progression over the 7-day incubation period (Figure 5.8). However, this does not allow for quantifiable discrimination between the immunoinflammatory potentials of individual *C. acnes* isolates which may elicit similar degrees of melanisation. Therefore, melanisation responses may be assessed by quantifying PO activity in the haemolymph plasma of final instar larvae using a microplate enzyme assay as evidence of the induction of the proPO cascade which causes melanisation (Joyce and Gahan, 2010; Kanost, Jiang and Yu, 2004). Additionally, whilst the current study used 70% (v/v) ethanol to surface clean waxworms prior to injection, previous studies have employed the use of antibiotic treatment of waxworms to treat insidious infections which could affect mortality and/or melanisation responses (Jander, Rahme and Ausubel, 2000). Future studies may consider evaluating the effect of antibiotic prophylaxis within *G. mellonella* inoculated with *C. acnes*. Additional research is also required to establish standardised methods of screening *G. mellonella* for infection and disease prior to inoculation with test microorganisms.

In conclusion, although no studies to date have assessed the efficacy of *G. mellonella* as a suitable *in vivo* model for studies of *C. acnes* pathogenicity, the current study has demonstrated that the *G. mellonella* model is sensitive and can discriminate between the pathogenicity of *C. acnes* belonging to a range of phylotypes. Larvae melanisation responses also mirrored the chronic, low-grade, progressive infections usually seen within disc herniation patients suffering from *C. acnes* infection, suggesting that this model has relevance to human disease, although this requires further investigation. Therefore, although the *G. mellonella* model is still in its infancy and is not as well established as other invertebrate models, this model is an excellent cost-effective, rapid, alternative system for assessing the virulence of a range of microorganisms.

CHAPTER 6 ASSESSMENT OF CAMP FACTOR PRODUCTION WITHIN *C. ACNES* DISC TISSUE ISOLATES

6.1 Introduction

Bacterial toxins are biological virulence factors that work to trigger destructive processes that promote the ability of invading pathogens to colonise a host and evade host immune defences (Vale, Cabanes and Sousa, 2016). Bacterial toxins can function in a range of ways. These include the inhibition of host protein synthesis, inhibition of neurotransmitter release and damage of host cell membranes to promote pathogen spread (Collier, 1975; Yang and Chiu, 2017; Inoshima *et al*., 2011). Approximately 25-30% of cytotoxic bacterial proteins are classed as pore-forming toxins, making them the largest category of bacterial virulence factors (Los *et al*., 2013). Pore-forming toxins can perforate both host cell plasma membranes and intracellular organelle membranes and are advantageous for bacterial pathogens due to their ability to mediate the direct killing of host cells and allowing bacteria to evade host immune defences through phagosomal escape (Los *et al*., 2013). Bacterial pore-forming toxins also trigger the release of host nutrients beneficial for bacterial growth (Geny and Popoff, 2006). Therefore, such toxins can act to promote bacterial spread and continued infection within the host. One such pore-forming toxin was first described by Christie, Atkins and Munch-Peterson in 1944 within Group B Streptococcus (GBS) and is now known under the acronym CAMP factor.

Functional analysis of GBS CAMP factor demonstrated that this pore-forming toxin uses the carbohydrate core of glycosylphosphatidylinositol (GPI)-anchored proteins as cellular receptors from which to oligomerise and form pores within target cells (Hensler *et al*., 2008; Lang *et al*., 2007). CAMP factor homologues have also been identified in a range of other bacterial species, including *Listeria monocytogenes*, *Vibrio cholerae* and, interestingly, *C. acnes* (Hensler *et al*., 2008; Frey, Perrin and Nicolet, 1989; Lesmana *et al*., 1994; Valanne *et al*., 2005). This universal expression of CAMP factor protein amongst a range of bacterial species suggests that CAMP factor may play a central role in bacterial virulence.

With the understanding that *C. acnes* can express CAMP factor protein, previous researchers have characterised such proteins from *C. acnes* to understand the genetic relatedness to CAMP obtained from other bacterial species (Valanne *et al*., 2005). Choudhury (1978) demonstrated the ability of *C. acnes* to elicit co-haemolytic reactions resembling those of the CAMP reaction described by Christie, Atkins and Munch-Petersen (1944) in the presence of both sheep and human erythrocytes. Analysis of the draft genome sequence of *C. acnes* type IA strain NCTC 737, a clinical strain isolated from a severe case of facial acne, revealed the presence of five related genes with sequence similarity to the GBS CAMP factor protein (Valanne *et al*., 2005). One of these CAMP factor homologues (CAMP factor 2, accession number: WP_002518322) had a high amino acid sequence identity to the GBS CAMP factor of 33% (Nakatsuji *et al*., 2011). Further analysis of these proteins revealed that they had predicted molecular masses ranging from approximately 28-30kDa (Valanne *et al*., 2005). *C. acnes* CAMP proteins can be cell-associated or secreted (Liu *et al*., 2011; McDowell *et*

al., 2013).

C. acnes CAMP factors can help to confer cytotoxicity towards host macrophages and keratinocytes, promoting *C. acnes* virulence through the degradation of invading host cells (Wang *et al*., 2018). These bacteria can also induce proinflammatory cytokine release, including the release of IL-1α and TNF-α, to promote inflammation within host tissue (Wang *et al*., 2018; Contassot and French, 2014). Furthermore, *C. acnes* CAMP factors can trigger sebocyte cell death and subsequent inflammatory responses within sebaceous glands, highlighting the proinflammatory properties of *C. acnes* CAMP factors which may also contribute to disc pathogenesis via host inflammatory cell recruitment *in vivo* (Liu *et al*., 2011). Taken together, these cytotoxic and immunoinflammatory properties of *C. acnes* CAMP factors may promote the spread and persistence of these bacteria within disc herniation patients via immune evasion, subsequently contributing towards the chronicity of IVD infection. CAMP factors as a link to the immunoinflammatory properties of *C. acnes* is supported by the research conducted by Wang *et al*. (2018) in which monoclonal antibodies which specifically targeted *C. acnes* CAMP factor 2 successfully neutralised the proinflammatory cytokines IL-6, IL-8 and IL-1β within an acne vulgaris *ex vivo* skin explant model. This highlights the importance of CAMP factors in inducing an immunostimulatory response and the potential application of CAMP factor vaccination in inflammation suppression. Targeted monoclonal antibodies to CAMP factors may be a viable immunotherapy option for acne vulgaris and other *C. acnes*-associated infections, such as in IVD tissue (Wang *et al*., 2018). The immunogenic nature of *C. acnes* CAMP factor was also

demonstrated by the production of antibodies in mice injected with recombinant CAMP factor (Wang *et al*., 2018). Subsequent challenge of CAMP factor-vaccinated mice with *C. acnes* resulted in decreased erythema and ear thickness (an indicator of tissue inflammation) and a markedly reduced production of the murine proinflammatory cytokine MIP-2, highlighting the application of CAMP factor vaccination in attenuating *C. acnes*-induced *in vivo* inflammation (Wang *et al*., 2018). Interestingly, IL-8, the human equivalent of murine MIP-2, was elevated in *C. acnes*-positive IVDs of humans, suggesting that CAMP factor vaccination in IVD herniation patients could alleviate IL-8-associated inflammation and subsequent pain (Jiao *et al*., 2019). In the advent of antibiotic resistance, CAMP factor vaccination may represent a better treatment option as compared to antibiotic usage. In support of the proinflammatory nature of *C. acnes* CAMP factors evidenced by Wang *et al*. (2018), Nakatsuji *et al*. (2011) demonstrated dose-dependent cytotoxicity of *C. acnes* CAMP factor against a human HaCaT keratinocyte cell line and within a murine RAW 264.7 macrophage cell line. This virulence was validated *in vivo* following the observation that *C. acnes* CAMP factor induced significant increases in mouse ear thickness, used to assess the degree of swelling as a result of inflammatory responses *in vivo*, demonstrating the proinflammatory effects of these virulent bacteria (Nakatsuji *et al*., 2011). Additionally, the co-culture of HaCaT and RAW 264.7 cell lines with anti-CAMP factor antiserum reduced *C. acnes*-associated cell death within both cell lines (Nakatsuji *et al*., 2011). Taken together, these findings corroborate those of Wang *et al*. (2018) and evidence the virulence of *C. acnes* CAMP factor as an effective immunogenic and cell toxic agent that may contribute to the pathophysiology of IVD infection.

CAMP factor has also been likened to Protein A of *S. aureus* as both CAMP factor and Protein A can bind to the Fc regions of immunoglobulins G and M (IgG and IgM, respectively) (Jürgens, Sterzik and Fehrenbach, 1987). Protein A of *S. aureus* is known to promote immune evasion through the induction of B-cell death and the blockage of opsonophagocytosis (Kobayashi and DeLeo, 2013; Falugi *et al*., 2013). Interestingly, Valanne *et al*. (2005) demonstrated 25% or more identity between specific domains of *C. acnes* CAMP factor 1 and *S. aureus* Protein A. Therefore, it can be hypothesised that *C. acnes* CAMP factor 1 may promote such immune evasion within human hosts to promote chronicity and spread of infection within IVD tissue. This corroborates the observation that *C. acnes*-associated disc tissue infection is characterised by latency and associated chronic inflammation and low-grade infection (Yuan *et al*., 2018; Stirling *et al*., 2001). These findings, coupled with the highly conserved nature of *camp* genes within the *C. acnes* genome, suggest that CAMP factors play an important role within the pathogenesis of these bacteria (Contassot, 2018). However, Hensler *et al*. (2008) argued that CAMP factor expression is not essential for the systemic virulence of GBS as no differences were noted between wild type and *cfb* gene deletion GBS mutants with regards to invasion of cultured human brain endothelial cells, despite mutants lacking CAMP factor production. Nevertheless, the conservation of CAMP factor among GBS strains implies an evolutionary benefit of this toxin and therefore, further studies investigating the presence of this protein in other bacterial species, such as *C. acnes*, are warranted (Hensler *et al*., 2008).

Whilst CAMP factor 2 has been shown to be essential for *C. acnes*-induced inflammation, *C. acnes* CAMP factor 1, a 28-30kDa protein, may contribute to bacterial virulence through direct interaction with TLR-2 of keratinocytes and monocytes to amplify inflammation (Valanne *et al*., 2005; Wang *et al*., 2018; Lheure *et al*., 2016). The proinflammatory properties of these surface-associated CAMP factor 1 proteins were confirmed using promoter assays in which CAMP factor 1 activated promoters for the proinflammatory cytokines NF-κB and CXCL8 (Lheure *et al*., 2016). With previous studies reporting that NF-κB signalling can mediate *C. acnes*-induced IVDD by regulating proinflammatory IL-1β and TNF-α expression, it is possible that *C. acnes* CAMP factor 1 interaction with host immune cells within IVD tissue may elicit proinflammatory cytokine production through NF-κB signalling (Lin *et al*., 2018). This may contribute to CLBP reported within a subset of patients with *C. acnes*-associated IVD tissue infection [Albert *et al*., 2013 (a)].

The success of *C. acnes* as a pathogen is partly attributed to the ability of this microorganism to transfer between growth within microaerophilic and anoxic environments, orchestrated by metabolic and subsequent proteomic changes, a highly pathogenic property (Dekio *et al*., 2013). This enables *C. acnes* to move from superficial to deep-seated infections, such as those occurring within anaerobic IVD tissue (Dekio *et al*., 2013; McDowell *et al*., 2013). Interestingly, Dekio *et al*. (2013) observed a significant increase in protein expression for an anaerobically and microaerophilically cultured type IB strain of *C. acnes* with a molecular weight of around 12-15kDa. Further analysis of this gel range revealed the upregulation of a member of the CAMP factor protein family, demonstrating

the ability of anaerobic growth to induce CAMP factor production (Dekio *et al*., 2013). Interestingly, Wang *et al*. (2018) also demonstrated a 1.5-fold increase in the expression of CAMP factor 2 within *C. acnes* following culture under anaerobic conditions as compared to growth under aerobic conditions. As IVDs are avascular structures which provide *C. acnes* with an anaerobic, anoxic growth environment, it can be assumed that herniated disc tissue infections involving *C. acnes* may induce inflammation through the promotion of CAMP factor production *in vivo*. Therefore, the current study aimed to investigate CAMP factor 1 production within *C. acnes* of various phylotype lineages, namely phylotypes IA_1, IB, II and III, isolated from herniated disc tissue. Additionally, although *C. acnes* phylotype III has been isolated from deep tissue infections of IVDs, the CAMP factor 1 production status of phylotype III isolates has not been previously investigated (McDowell *et al*., 2008). Therefore, in addition to increasing the body of knowledge relating to the CAMP factor 1 production status of *C. acnes* from deep tissue IVD infections, the current study also aimed to investigate the capacity of *C. acnes* of the phylotype III lineage to produce CAMP factor 1 protein. This could contribute towards understanding the potential role of CAMP factor protein in deep tissue *C. acnes* infections.

6.1.1 Aims

The aims of this CAMP factor 1 immunoblotting study were to:

- Assess the CAMP factor 1 production abilities of a panel of clinical IVD tissue *C. acnes* isolates.

- Determine whether potential CAMP factor 1 protein production is related to *C. acnes* phylotype.

6.2 Materials and Methods

6.2.1 Bacterial Strains and Growth Conditions

Bacterial strains and isolates used within waxworm injection assays (5.2.1) were utilised within the current study to investigate CAMP factor 1 production within whole-cell extracts. These included *S. epidermidis* RP62A and *C. acnes* isolates 56 (IA_1), 82 (IB), 2 (II), 80 (III) and NCTC 737. *C. acnes* isolate W1392, a known CAMP factor-producing dental strain, was used as a positive control strain for CAMP factor 1 binding within all immunoblots whilst BHI broth alone was used as a negative control. *S. epidermidis* RP62A was cultured at 37°C aerobically overnight in BHI broth media. All *C. acnes* isolates were cultured at 37°C anaerobically for 72-hours in BHI broth media. Following bacterial culture, 25ml aliquots of each bacterial culture were transferred to sterile 50ml Falcon tubes and cells were pelleted by centrifugation at room temperature for 10-minutes. All cell pellets were washed twice with sterile PBS and subsequently resuspended in 50µl volumes of sterile PBS. Following resuspension, all cell pellets were transferred to sterile 2ml round-bottom

Eppendorf tubes and sonicated using an ultrasonic waterbath system (VWR Ultrasonic Cleaner USC-T) at 40kHz for a total sonication time of 5-minutes, with each cycle consisting of 1-minute of continuous sonication followed by 1-minute off on ice (Valanne *et al*., 2005). All samples were frozen at -20°C immediately after sonication. Prior to loading of SDS-PAGE gels with whole-cell protein extracts, all samples were defrosted and sonicated for a further 10-minutes, with each of these cycles consisting of 5-minutes of continuous sonication at 40kHz followed by 5-minutes off on ice. All bacterial isolates were cultured in triplicate for subsequent Western blot analysis.

6.2.2 Whole-Cell Protein Isolation and Protein Quantification

Following sonication, protein concentration was ascertained using a commercial bicinchoninic acid (BCA) assay kit (Thermo Scientific, USA).

6.2.3 Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All protein samples were boiled for 10-minutes at 100°C with Laemmli buffer (2× concentrate, Sigma Aldrich) prior to loading. Protein extracts were separated by one-dimensional SDS-PAGE using Mini-PROTEAN® tris-glycine extended (TGX) Stain-

Free™ precast gels (1.0mm, 10-well, Bio-Rad, UK). All Bio-Rad TGX Stain-Free any-kD SDS-PAGE gels were run in Bio-Rad Tetra Cell gel tanks containing Tris/glycine/SDS (TGS) running buffer (Bio-Rad, UK) in accordance with the manufacturer's instructions. Gels were run at 120V at room temperature for 1.5-hours until the dye front reached the bottom of the gel. Protein bands were visualised using the Gel Doc EZ System (Bio-Rad, UK). Precision Plus Protein^{TM} dual-colour molecular weight marker (Bio-Rad, UK) was also included.

6.2.4 Western Blot Analysis

SDS-PAGE-resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, UK) using the Bio-Rad Trans-Blot Turbo transfer system. Transblotting was conducted at 25V and 1.3A for 7-minutes at room temperature. Blots were blocked overnight at 4°C in sterile Falcon tubes containing 5% (w/v) non-fat dry milk powder resolubilised using Tris-buffered saline with Tween-20 (TBST) buffer (Tris-HCl, Tris base and NaCl) (Abcam, UK). All blots were washed five times with TBST buffer. Following washing, all blots were incubated with 2ml of neat QUBPa4 anti-CAMP factor 1 primary monoclonal antibody (as provided by Queen's University Belfast) at room temperature on a tube roller for 3-hours. Blots were washed five times with 25ml TBST buffer. Next, 2ml of a goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated

secondary antibody (1:2,000 of 1.5mg/ml stock in 0.5% non-fat dry milk powder/TBST buffer) (Fisher Scientific, UK) was added to each blot and incubated on a tube roller at room temperature for 1-hour. Secondary antibody solutions were removed and all blots were washed five times with 25ml TBST buffer. To visualise the immunoreactivity of blots, membranes were incubated with 1ml of SuperSignal™ West Femto Maximum Sensitivity HRP substrate (Fisher Scientific, UK) on a tube roller at room temperature for 5-minutes and imaged using the Bio-Rad ChemiDoc imaging system. Immunoblot bands around 28-30kDa were expected to represent the presence of CAMP factor 1 protein (Valanne *et al*., 2005). Triplicate immunoblots were prepared for *S. epidermidis* RP62A, *C. acnes* isolates 56 (IA_1), 82 (IB), 2 (II), 80 (III), *C. acnes* NCTC 737 and *C. acnes* isolate W1392.

6.3 Results

6.3.1 Positive CAMP Factor 1 Production Amongst *C. acnes* Herniated Disc Tissue Isolates

Production of CAMP factor 1 protein by type IA_1, IB, II and III *C. acnes* isolates obtained from human herniated disc tissue material was investigated by the immunoblotting of resolved protein samples using a mouse monoclonal antibody specific for CAMP factor 1. *C. acnes* isolates 56 (IA_1), 82 (IB), 2 (II) and 80 (III) were found to produce CAMP factor 1

protein, with immunoblot protein bands seen at approximately 28kDa [Figure 6.1 (B)]. Corresponding protein bands were also observed on the SDS-PAGE gels [Figure 6.1 (A)]. Bands of higher molecular mass than that of the target protein were sometimes observed, ranging from 37-62.5kDa [Figure 6.1 (B)] and these were thought to be the result of non-specific binding of the primary antibody.

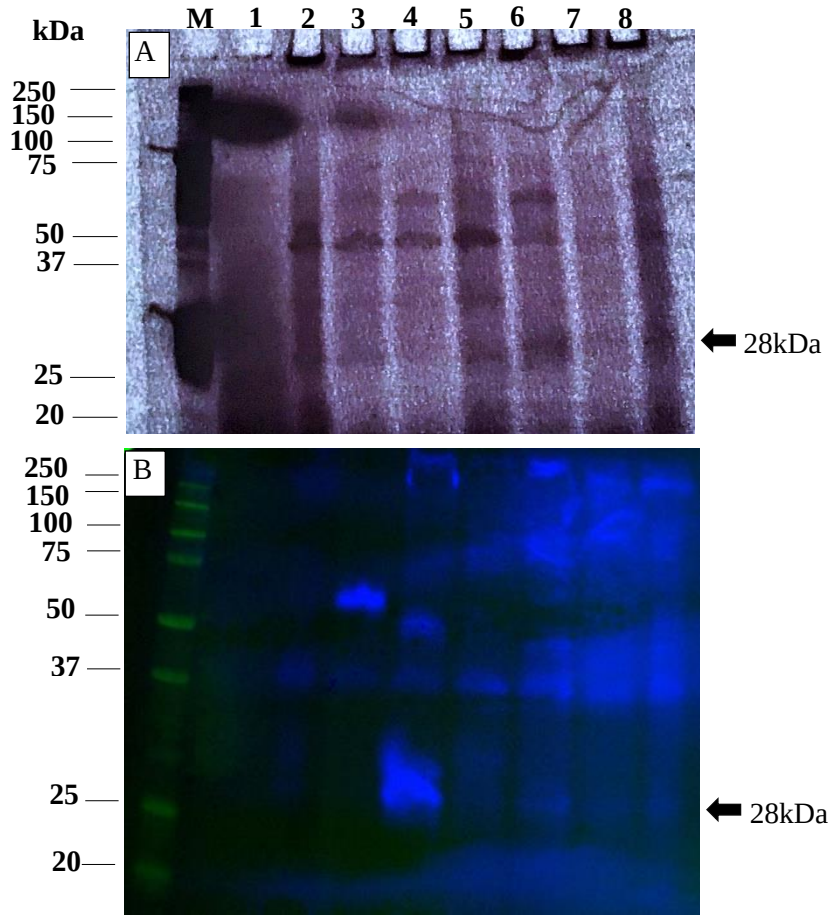


Figure 6.1: Analysis of *C. acnes* whole-cell extracts by SDS-PAGE (A) followed by immunoblotting (B) with a mouse anti-CAMP factor 1 monoclonal antibody (QUBPa4). Lane M, molecular mass marker (Precision Plus Protein™ dual-colour molecular weight marker, Bio-Rad). Lane 1, BHI broth alone (negative control). Lane 2, *C. acnes* isolate W1392 (dental isolate) (positive CAMP factor 1 control strain). Lane 3, *C. acnes* NCTC 737. Lane 4, *C. acnes* 56 (IA_1). Lane 5, *C. acnes* 82 (IB). Lane 6, *C. acnes* 2 (II). Lane 7, *C. acnes* 80 (III) culture 1. Lane 8, *C. acnes* 80 (III) culture 2.

6.3.2 Lack of CAMP Factor 1 Production Amongst *C. acnes* Isolate NCTC 737

Following probing of NCTC 737 immunoblots with QUBPa4 monoclonal antibody, no bands were seen at the molecular mass of 28kDa, suggesting that this strain does not produce CAMP factor 1 protein [Figure 6.1 (B)]. However, immunoblot bands were noted at approximately 40kDa and 62.5kDa. Corresponding protein bands were also seen at this position on SDS-PAGE gels [Figure 6.1 (A)].

6.3.3 Assessing CAMP Factor 1 Protein Production Status of *S. epidermidis* RP62A

As *S. epidermidis* RP62A has been reported to be cultured from herniated IVD tissue material alongside *C. acnes*, the CAMP factor 1 production status of this Gram-positive bacteria was also assessed. QUBPa4-probed immunoblots showed an absence of protein bands at 28kDa in all replicate immunoblots. However, non-specific protein banding was observed at approximately 40kDa in all triplicate immunoblots [Figure 6.2 (B)]. Corresponding protein bands were also seen at this position on SDS-PAGE gels [Figure 6.2 (A)].

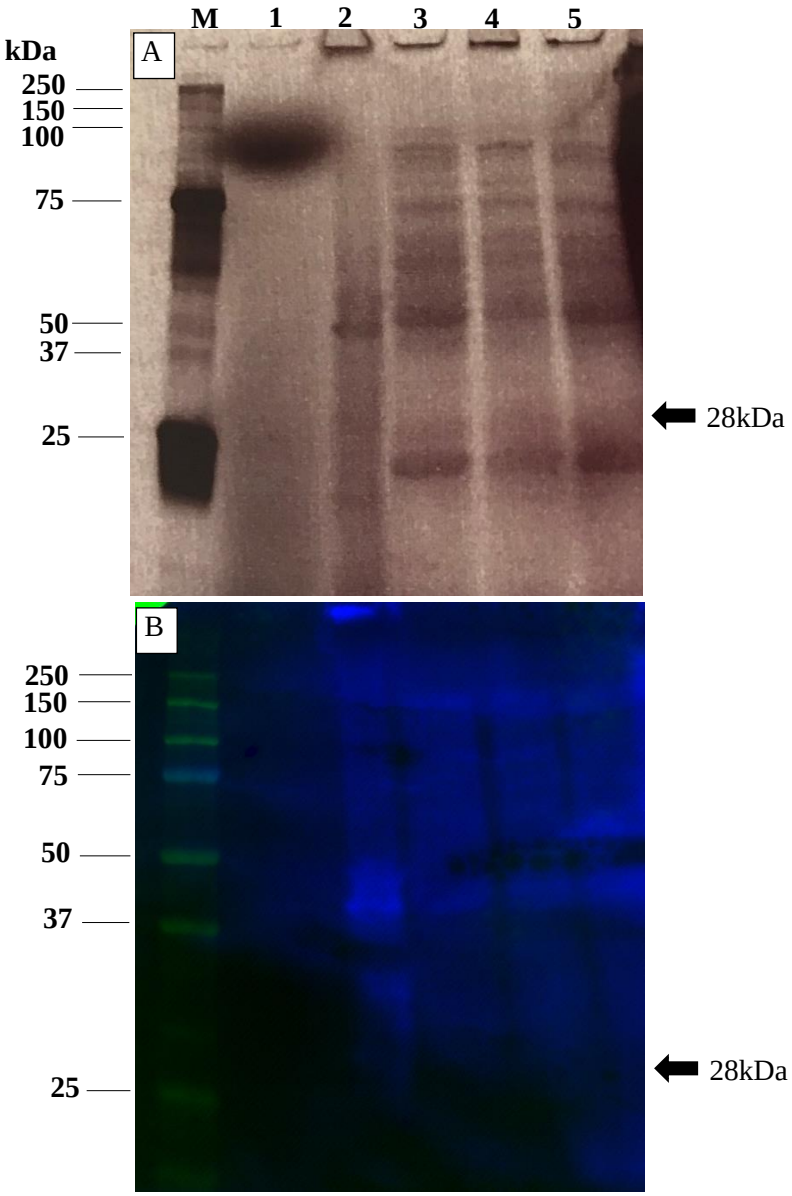


Figure 6.2: Analysis of *S. epidermidis* RP62A whole-cell extracts by SDS-PAGE (A) followed by immunoblotting (B) with a mouse anti-CAMP factor 1 monoclonal antibody (QUBPa4). Lane M, molecular mass marker (Precision Plus Protein™ dual-colour molecular weight marker, Bio-Rad). Lane 1, BHI broth alone (negative control). Lane 2, *C. acnes* isolate W1392 (dental isolate) (positive CAMP factor 1 control strain). Lanes 3-5, *S. epidermidis* RP62A whole-cell extracts (cultures 1, 2 and 3, respectively).

6.4 Discussion

IVD tissue infection with *C. acnes* is characterised by inflammation [Yuan *et al*., 2018; Dudli *et al*., 2018; Albert *et al*., 2008 (a)]. With CAMP factors known to induce proinflammatory cytokine release and act as macrophage dysfunction factors to promote bacterial escape from host immune cells, it may be concluded that *C. acnes* CAMP factors may act as virulence factors to promote the establishment of disc tissue infections (Kurosawa *et al*., 2016). As anti-CAMP factor vaccination has been shown to attenuate *C. acnes*-induced *in vivo* inflammation, the current study aimed to evaluate the CAMP factor 1 production status of a range of *C. acnes* isolates cultured from herniated disc tissue material (Wang *et al*., 2018).

As shown in Figure 6.1 (B) above (6.3.1), all *C. acnes* isolates of a range of phylotypes obtained from clinical disc tissue produced CAMP factor 1 protein, suggesting that CAMP factor 1 may be essential to the pathogenesis of these isolates *in vivo*. In support of these findings, Valanne *et al*. (2005) also identified positive CAMP factor 1 binding by QUBPa4 within type IB and type II *C. acnes* isolates, suggesting that these isolates may have the potential to utilise CAMP factor 1 *in vivo* to promote IVD tissue inflammation. However, in contrast to the findings of Valanne *et al*. (2005), the current study demonstrated positive CAMP factor 1 production within a type IA clinical isolate. This discrepancy may be attributed to differences in the site of isolation of these type IA strains, with Valanne *et al*.

(2005) testing isolates recovered from dental sources, acne patients, failed prosthetic hip joints and bone and tissue samples whilst the type IA isolate used within the current study was obtained from IVD tissue. Therefore, site of bacterial isolation may modulate the CAMP factor 1 production status of *C. acnes* belonging to phylotype IA.

As discussed above, Valanne *et al*. (2005) demonstrated phylotype-specific production of CAMP factor 1 between different *C. acnes* isolates, with type IB and type II isolates producing large amounts of CAMP factor 1 in comparison to type IA strains. This was corroborated by Lheure *et al*. (2016) who demonstrated that CAMP factor 1 production was strong in type IB and type II strains of *C. acnes* whereas most of the IA_1 and IA_2 strains produced little or no CAMP factor 1. These variations in CAMP factor 1 production between phylotypes IA, IB and II were attributed to different levels of expression of CAMP factor genes between strains as opposed to missing genes as all three phylotype groups possessed genes encoding all five CAMP factors (Valanne *et al*., 2005).

The current study noted that *C. acnes* strain NCTC 737 failed to produce CAMP factor 1 protein. This contrasted with the findings of Valanne *et al*. (2005) in which *C. acnes* NCTC 737 was shown to produce CAMP factor 1 protein. This discrepancy may be attributed to the continuous passage of *C. acnes* NCTC 737, a reference isolate, which can attenuate bacterial virulence and may subsequently affect CAMP factor 1 production (Li *et al*., 2015). Furthermore, as strain NCTC 737 was originally isolated from a severe case of facial acne,

these findings may also suggest that CAMP factor 1 production is favoured by *C. acnes* isolates associated with deep tissue infections as opposed to superficial isolates. This is supported by the findings of Dekio *et al*. (2013) in which the growth of *C. acnes* within anaerobic environments, such as within anoxic IVD tissue, was shown to promote the upregulation of CAMP factor production within these bacteria.

The lack of CAMP factor 1 production in *C. acnes* NCTC 737 observed within the current study may also be attributed to a base difference in the Shine-Dalgarno sequence of the CAMP factor 1 gene, with this base difference shown to reduce the efficiency of the interaction between this ribosomal binding site and the 16S ribosomal subunit in another strain of *C. acnes* (NCTC 10390) (Valanne *et al*., 2005). This subsequently leads to the reduced expression of this protein (Valanne *et al*., 2005). This may explain the lack of CAMP factor 1 production in *C. acnes* NCTC 737 within the current study (Lheure *et al*., 2016; Valanne *et al*., 2005). However, further investigation of the potential existence of this base difference within the CAMP factor 1-negative isolates *C. acnes* NCTC 737 and *S. epidermidis* RP62A identified in the current study must be conducted, with the aim of testing the binding affinity between the Shine-Dalgarno sequences of these isolates and ribosomes which may modulate transcription of CAMP factor 1 transcripts.

Research conducted by Valanne *et al*. (2005) evidenced the reactivity of human sera from patients with acne, as well as from patients undergoing primary and revision arthroplasty,

against purified CAMP factor 1 protein from a type II isolate of *C. acnes*, suggesting that this protein is immunogenic *in vivo* and is possibly expressed during *C. acnes* host colonisation. The expression of this protein in four disc tissue isolates in this study may corroborate the role of this protein in disc tissue colonisation and infection. However, the CAMP factor 1 production findings of the current study need to be confirmed with further work. This could include, for example, the excision of CAMP factor 1 bands from immunoblots for subsequent analysis with high-resolution mass spectrometry to confirm the identity of this protein.

Whilst no 28kDa CAMP factor 1 bands were observed for *S. epidermidis* RP62A, immunoblotting with the QUBPa4 primary antibody identified bands at approximately 40kDa for these bacteria [Figure 6.2 (B)]. These bands may be attributed to the non-specific binding of the primary antibody to proteins produced by *S. epidermidis* RP62A which demonstrate similarity to *C. acnes* CAMP factor 1. As explored in chapter 5, *G. mellonella* injected with *S. epidermidis* RP62A lacked waxworm melanisation and, therefore, an immune response towards challenge with these bacteria. However, melanisation was generally noted with *C. acnes* belonging to a range of phylotypes, suggesting that the potential lack of CAMP factor 1 production by *S. epidermidis* RP62A may contribute to its lack of proinflammatory properties in such a model (6.3.3). In contrast to the CAMP factor 1-negative result observed for *S. epidermidis* RP62A, universal CAMP factor 1 protein production was recorded in all disc tissue *C. acnes* isolates sampled in the current study. Therefore, further *in vivo* studies are warranted to investigate the role of *C. acnes* CAMP

factor 1 production in promoting infection as compared to non-CAMP factor 1 producing bacterial strains, potentially through the inoculation of *G. mellonella* with CAMP-factor 1 knockout mutants of *C. acnes*.

As explored in Figure 6.1 (B) above, larger protein bands ranging from 40kDa up to 62.5kDa were observed on immunoblots for a range of *C. acnes* strains. These larger bands could be the result of non-specific protein binding in which the primary monoclonal QUBPa4 antibody recognises a similar epitope to that of the target CAMP factor protein (Ghosh, Gilda and Gomes, 2014). Therefore, further optimisation of this protocol may include increasing the number and duration of immunoblot washes to reduce excessive primary antibody binding to limit the observation of these non-specific bands. If the target proteins exist in multiple modified forms, including phosphorylated and glycosylated isoforms, this could also lead to the observation of immunoblot bands at unexpected molecular masses (Ghosh, Gilda and Gomes, 2014). Therefore, any modifications of this protein should be further investigated using agents to remove each of these potential modifications to determine whether the presence of resulting immunoblot bands is affected (Ghosh, Gilda and Gomes, 2014). The future use of blocking peptides specific for the CAMP factor 1 protein epitope would help to determine whether immunoblot bands are the result of specific or non-specific binding as only specific bands would be blocked and, therefore, removed from resulting immunoblots (Ghosh, Gilda and Gomes, 2014).

Although immunoblotting is a commonly used technique in the identification of proteins, this method does present a few limitations. Primary antibodies used in immunoblotting may not recognise antigens in their denatured state and, therefore, lack of protein binding may lead to false-negative results. Additionally, primary antibodies may not detect specific modifications of the target protein, such as phosphorylations, for which specific antibodies are usually needed (Ghosh, Gilda and Gomes, 2014). Therefore, if a modification of the target protein has occurred, the nature of this modification must be ascertained so that primary antibodies specific for this modified protein can be generated. Another significant limitation of immunoblotting is the technical demand of the method on researching scientists, with simple mistakes such as the use of too little or too much primary antibody or incorrect substrate incubation times leading to unusable results. In support of this, Koller and Wätzig (2005) conducted a quantitative immunoblotting study investigating the amount of erythropoietin-related impurities in a range of samples and concluded that operator error accounted for the largest source of variance in results of 78%. As a result of these limitations, alternative methods of protein identification have been suggested, such as high-resolution mass spectrometry which can identify all proteins present within a specific sample in quantities at even trace levels (Schuchardt and Sickmann, 2007). However, this technique is more expensive and technical compared to immunoblotting so may not represent a feasible alternative for most research laboratories (Bass *et al*., 2017).

IVDs are the largest avascular structures within the human body and rely solely on passive diffusion for nutrients and metabolite removal (Lundon and Bolton, 2001; Grünhagen *et al*., 2006; De Geer, 2018). Therefore, a limitation of the current study may be that the culture of *C. acnes* in nutritious media, such as BHI media, does not fully reflect the nutrient status of IVDs. With the demonstration that nutrient availability can affect bacterial gene expression and protein profiles, with Nakatsuji *et al*. (2011) showing that media type can directly affect *C. acnes* CAMP factor production, further studies assessing the effects of nutrient deprivation on CAMP factor 1 production are warranted (Hua *et al*., 2004; Krismer *et al*., 2014; Walker *et al*., 2002). Replicating the nutritional status of *C. acnes* within IVD tissue, from which these isolates were obtained, would further aid in investigating any potential regulation effects of nutrient availability on *C. acnes* CAMP factor production to determine whether these isolates are likely to express CAMP factor proteins *in vivo*. With CAMP factor production in *C. acnes* being, in part, modulated by nutrient availability, future studies should also aim to standardise the culture media used to grow *C. acnes*.

Despite a range of studies evidencing the link between *C. acnes* CAMP factor production and inflammation in acne vulgaris, some studies dispute the contribution of these secreted virulence factors in bacterial virulence. For example, Hensler *et al*. (2008) deleted the *cfb* gene encoding the CAMP factor of GBS and investigated any possible resulting effects on bacterial virulence *in vitro* using neutrophil killing assays and *in vivo* using mouse infection assays. Results demonstrated no difference between wildtype and Δ*cfb* mutant GBS in terms of survival within murine J774 macrophages and adherence to and invasion of cultured

human brain microvascular endothelial cells (Hensler *et al*., 2008). Similar mortality profiles and bacterial count levels were also observed between wildtype and Δ*cfb* mutant GBS, suggesting that despite CAMP factor knockout, isogenic Δ*cfb* mutants retained full virulence in a mouse model of infection (Hensler *et al*., 2008). The equivalent phagocyte resistance and cellular invasiveness properties of wildtype and Δ*cfb* mutant GBS led to the conclusion that CAMP factor is not essential for GBS virulence (Hensler *et al*., 2008). Furthermore, Sörensen *et al*. (2010) concluded that inactivation of two CAMP factor genes, namely *camp2* and *camp4*, did not significantly change the transcriptome of HaCaT cells in response to *C. acnes* challenge, meaning that knockout of these secreted virulence factors had no particularly significant effect on expressed host genes *in vivo* post-infection. However, it cannot be assumed that such a gene knockout within *C. acnes* obtained from different infection sites would lead to similar effects on bacterial virulence. Also, Sörensen *et al*. (2010) concluded that the possibility of redundancy amongst *C. acnes* CAMP factors compensating for the inactivation of one factor cannot be excluded and, therefore, means that insertional mutagenesis approaches towards *C. acnes* CAMP factors cannot rule out the possibility that CAMP factors are required for full virulence *in vivo*. Skalka and Smola (1981) also showed that a single high dose of partially purified CAMP factor isolated from *Streptococcus agalactiae* exerted lethal effects when injected intravenously in both rabbits and mice, highlighting the virulence of this secreted factor in different animal models of infection. Additionally, the conservation of CAMP factor genes amongst a range of *C. acnes* of varying phylotypes implies a significant evolutionary benefit of these toxins. Possible cross-interactions between *C. acnes* CAMP factors and other virulence factors produced by

these bacteria to augment virulence *in vivo* cannot be overlooked. Further studies of CAMP factor knockout mutant *C. acnes* strains are, therefore, warranted to investigate whether cellular invasiveness, phagocyte resistance and *in vivo* cellular adherence are affected in a disc tissue model of infection.

In conclusion, CAMP factor is an important virulence factor *in vivo* and has immunogenic properties that may contribute to inflammatory conditions (Wang *et al*., 2018). With the current study demonstrating that *C. acnes* disc tissue isolates belonging to phylotypes IA_1, IB, II and III produce CAMP factor 1 protein, further research is required to assess the feasibility of CAMP factor proteins as potential novel targets for vaccine development for disc tissue infections. This would reduce the need to use antimicrobials in the treatment of *C. acnes* disc infections where antibiotic resistance is an ever-growing concern.

CHAPTER 7 GENERAL DISCUSSION

The recent identification of *C. acnes* biofilms *in situ* within excised disc tissue obtained from microdiscectomy patients has supported the hypothesis that *C. acnes* is a significant causative agent within the development of degenerative disc disease (Capoor *et al*., 2017). The efficacy of antibiotic treatment regimes in alleviating disease-specific disability and lumbar pain in CLBP patients presenting with Modic type I changes has further supported this hypothesis [Albert *et al*., 2013 (b)]. Despite this, the role of this microorganism within the commensal flora has led to research dismissing *C. acnes* found in excised disc tissue samples as contaminants resulting from tissue collection and laboratory processing. Therefore, the assessment of the virulence characteristics of such isolates are warranted. This thesis has sought to investigate the pathogenic mechanisms of such isolates, with particular focus on assessing the potential role of site of isolation and phylotype on phenotypic characteristics.

C. acnes is typically characterised by low-grade, indolent, delayed infections, making the diagnosis of such infections difficult (Jacobs *et al*., 2016; Saper *et al*., 2015). On the basis of the *G. mellonella* data, *C. acnes* of all phylotypes led to host waxworm melanisation, indicative of the activation of the insect immune response, without inducing waxworm mortality (Tsai, Loh and Proft, 2016). *C. acnes*-induced melanisation was also progressive, suggesting that such a model can reflect the advancement of bacterial infections *in vivo*.

Consequently, *C. acnes* demonstrates proinflammatory properties *in vivo*. This insect model of infection corroborates the clinical characteristics of such infections seen within humans and supports the immunoinflammatory nature of these bacteria observed within other mammalian models (Wang *et al*., 2018; Liu *et al*., 2011). TLR activation by bacteria infecting disc tissue activates inflammatory cascades, with these proinflammatory disc cytokines able to drain into adjacent vertebrae through compromised endplates of patients with Modic type I changes (Gorth, Shapiro and Risbud, 2015; Dudli *et al*., 2018). This subsequently leads to the direct activation of nociceptive sensory neurons to induce LBP (Zhang and An, 2007). The observation that waxworm melanisation responses demonstrated phylotype-specificity highlights the discriminatory power of this model and therefore, the current study proposes that the practicality and efficacy of this insect model should support the acceptance of this technique for modelling *C. acnes* virulence *in vivo* and for the assessment of pathogenicity of individual isolates.

The misclassification of *C. acnes* as sample contaminants is a further challenge within published literature. The slow generation time of *C. acnes* of around 2.8-hours demonstrated in the current study may contribute to the generation of false-negative culture results from clinical samples and the delayed observation of virulence *in vitro*. Overlooking the biofilm mode of growth during sample preparation may also contribute towards the under-reporting of *C. acnes* from patient tissue. This thesis identified that 67% of disc tissue *C. acnes* isolates formed significant biofilms following 7-days of biofilm plate incubation as

compared to the negative control using crystal violet qualitative assays. Therefore, the current study suggests that in order to resolve some of the disparity amongst published literature relating to *C. acnes* culture from IVD tissue samples, the biofilm mode of growth must be considered, such as with the inclusion of tissue homogenisation and sonication steps within all published protocols involving *C. acnes* culture, in tandem with extended culture incubation periods. This would prevent the underestimation of bacterial burden within clinical tissue samples.

The identification of 100-days of Bioclavid (amoxicillin/clavulanic acid) antibiotic treatment as a novel treatment method for disc herniation patients presenting with CLBP raises the issue of potential promotion of antibiotic resistance amongst bacterial populations [Albert *et al*., 2013 (b)]. This has resulted in the publication of research articles investigating the viability of specific *C. acnes* virulence factors as potential novel therapeutic targets. One such study identified *C. acnes* CAMP factors as suitable vaccine targets following the neutralisation of inflammation associated with acne development using passive immunisation with monoclonal antibodies raised against *C. acnes* CAMP factor proteins (Wang *et al*., 2018; Liu *et al*., 2011). With the current study identifying the universal production of CAMP factor 1 in a panel of IVD tissue *C. acnes* isolates and with CAMP factor proteins reported to have immunogenic properties that may contribute to inflammatory conditions (Wang *et al*., 2018), further work is required to explore the

feasibility of such passive immunisation of anti-CAMP factor antibodies in the management of CLBP.

Currently, there is a lack of research into the phenotypic characteristics of *C. acnes*, particularly with regards to herniated disc tissue-associated isolates. There is a need for this to be addressed in order to gain further insight into the role of these microorganisms in the pathophysiology of intervertebral disc degeneration, warranting the assessment of phenotypic characteristics undertaken within the current study. Phenotypic characteristics of specific *C. acnes* isolates appeared to be associated with anatomical location and phylotype. Additionally, the production profiles of specific virulence factors, such as β-haemolysins, opposed the findings of other research groups, suggesting that different *C. acnes* phylotypes may variably produce such factors under different conditions. As a result, less emphasis should be placed upon the association of specific conditions with particular phylotype groups of *C. acnes*, with more focus given to phenotype as opposed to phylotype. The production of such enzymes, namely β-haemolysins, proteases and lipases, presented here provides an insight into potential virulence mechanisms employed by such bacteria *in vivo* to elicit chronic pain within patient tissue. *C. acnes* pathogenesis is further augmented by virulence factors that promote spread within infected disc tissue, namely gelatinases which may facilitate the enzymatic degradation of IVD collagen to spread within infected discs and subsequently promote chronicity of infection. These findings may go some way towards answering the question facing clinicians set out by Capoor *et al*. (2017) regarding the ability

of *C. acnes* to contribute to the pain symptoms associated with CLBP following disc tissue colonisation. Combination effects of these exocellular enzymes *in situ* may result in hypervirulence to promote host colonisation and pathogenicity within disc tissue. Therefore, it can be hypothesised that the enzyme production profiles reported in the current study may support a causal role of this microorganism in disc herniation with associated Modic changes, particularly through the promotion of inflammation within such tissue. Further work is required to identify other host colonisation and virulence factors within *C. acnes* that may contribute to disc pathogenesis.

The future of *C. acnes* disc-associated disease research lies in further investigating the direct effect of *C. acnes* infection on disc cells to progress the understanding of the role of host cell responses in contributing to the pathophysiology of IVDD. Such studies may utilise *C. acnes* knockout mutants which lack the ability to produce some of the virulence factors explored in the current study to map the combination of factors that promote host infection and disease. These infections could be replicated using disc model systems which may employ the use of base hydrogels, which would mimic diffusion and permit nerve growth seen in disc tissue, as matrix materials on which to seed and culture NP cells (Romereim *et al*., 2019). This would allow for the direct observation of the interaction between host and bacterial cells and the subsequent cytokines produced which may lead to tissue inflammation (Romereim *et al*., 2019). Further testing of a larger panel of disc herniation and acne lesion isolates is also

warranted to ascertain whether a similar pattern of phenotypic virulence is seen in comparison to the results presented here.

This thesis has demonstrated the variable nature of *C. acnes* virulence factor production, highlighting the adaptive nature of these bacteria for the promotion of bacterial survival and subsequent host colonisation and infection. The differential virulence factor production patterns that were observed amongst varying phylotypes of *C. acnes* disc tissue isolates within the *G. mellonella* waxworm model of infection, as well as within biofilm forming assays and enzyme production tests, suggest that such differences may be exploited for targeted infection control strategies. Ultimately, the pathophysiology of disc degeneration is complex, with an interplay of a range of bacterial virulence factors supporting such infection. Whilst it would be premature on the basis of the data presented here to conclude that *C. acnes* is directly linked with low-grade infection within disc tissue, leading to CLBP, the data presented here does indicate that low-level infection of IVD tissue with *C. acnes* is a potential exacerbating factor to Modic changes and subsequent IVDD and LBP. Further research is needed to determine the degree to which the pathogenesis of these bacteria is related to host factors to further elucidate the contribution of *C. acnes* to disc herniation with associated Modic changes.

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APPENDICES

Appendix (1)

Coventry University Ethical Approval Certificate 1: Virulence Mechanisms of *Propionibacterium acnes* in Association with Lumbar Disc Herniations: Genotypic and Phenotypic Analysis

Certificate of Ethical Approval

Applicant:

Gurpreet Sandhu

Project Title:

Virulence Mechanisms of Propionibacterium acnes In Association with Lumbar Disc Herniations: Genotypic and Phenotypic Analysis

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Medium Risk

Date of approval:

30 October 2015

Project Reference Number:

P37204

Appendix (2)

Coventry University Ethical Approval Certificate 2: Wax Moth Larva (*Galleria mellonella*): An *In Vivo* Model for Assessing *Propionibacterium acnes* Virulence

Certificate of Ethical Approval

Applicant:

Gurpreet Sandhu

Project Title:

Wax Moth larva (Galleria mellonella): An In Vivo Model For Assessing Propionibacterium acnes Virulence

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

31 January 2017

Project Reference Number:

P51035

Appendix (3)

Coventry University Ethical Approval Certificate 3: Quantification of Phenoloxidase (PO) Enzyme Activity Within the Haemolymph of *Galleria mellonella* Infected with Intervertebral Disc Tissue Isolates of *Propionibacterium acnes*

Certificate of Ethical Approval

Applicant:

Gurpreet Sandhu

Project Title:

Quantification of Phenoloxidase (PO) Enzyme Activity Within the Haemolymph of Galleria Mellonella Infected With Intervertebral Disc-Tissue Isolates of Propionibacterium acnes

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

17 October 2017

Project Reference Number:

P62003

Appendix (4)

Coventry University Ethical Approval Certificate 4*:* Multiplex Touchdown Polymerase Chain Reaction (PCR) Typing of Acne Lesion *Propionibacterium acnes* Isolates

Certificate of Ethical Approval

Applicant:

Gurpreet Sandhu

Project Title:

Multiplex Touchdown Polymerase Chain Reaction (PCR) Typing of Acne Lesion Propionibacterium acnes Isolates

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

11 October 2017

Project Reference Number:

P61679

Appendix (5)

Coventry University Ethical Approval Certificate 5: Characterisation of *Propionibacterium acnes* (*P. acnes*) by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins

Certificate of Ethical Approval

Applicant:

Gurpreet Sandhu

Project Title:

Characterisation of Propionibacterium acnes (P.acnes) by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

07 February 2019

Project Reference Number:

P82531

Appendix (6)

Coventry University Ethical Approval Certificate 6: Western Blot Analysis of *Propionibacterium acnes* Surface- and Exocellular Proteins

Certificate of Ethical Approval

Applicant:

Gurpreet Sandhu

Project Title:

Western Blot Analysis of Propionibacterium acnes Surface- and Exocellular Proteins

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

21 February 2019

Project Reference Number:

P88086

Appendix (7)

Coventry University Ethical Approval Certificate 7: Characterisation of *Propionibacterium acnes* (*P. acnes*) by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins (Updated Protocol and COSHH)

Certificate of Ethical Approval

Applicant:

Gurpreet Sandhu

Project Title:

Characterisation of Propionibacterium acnes (P.acnes) by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins (UPDATED PROTOCOL AND COSHH)

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

28 February 2019

Project Reference Number:

P88588

Appendix (8)

Coventry University Ethical Approval Certificate 8: Characterisation of *Propionibacterium acnes* (*P. acnes*) by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins (Further Updates to Protocol and COSHH)

Certificate of Ethical Approval

Applicant:

Gurpreet Sandhu

Project Title:

Characterisation of Propionibacterium acnes (P.acnes) by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins (FURTHER UPDATES TO PROTOCOL AND COSHH)

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

01 May 2019

Project Reference Number:

P90166

Appendix (9)

Coventry University Ethical Approval Certificate 9: Characterisation of *Propionibacterium acnes* (*P. acnes*) by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins (Further Updates to Protocol and COSHH)

Certificate of Ethical Approval

Applicant:

Gurpreet Gill

Project Title:

Characterisation of Propionibacterium acnes (P.acnes) by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cell-Wall and Exocellular Proteins (FURTHER UPDATES TO PROTOCOL AND COSHH)

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

26 June 2019

Project Reference Number:

P92722

CONFERENCES ATTENDED

Society for Applied Microbiology (SfAM) Annual Applied Microbiology Conference,

July 2017, Newcastle, UK.

Microbiology Society Annual Conference,

April 2017, Edinburgh, UK.

LIST OF PUBLICATIONS

Peer Reviewed Publications

Importance of *Propionibacterium acnes* haemolytic activity in human intervertebral discs: A microbiological study.

M. N. Capoor, F. Ruzicka, **G. Sandhu**, J. Rollason, K. Mavrommatis, F. S. Ahmed, J. E. Schmitz, A. Raz, H. Brüggemann, P. A. Lambert, V. A. Fischetti, O. Slaby.

PLOS ONE, Volume 13, Issue 11, November 2018, Pages 1-10.

Poster Presentations

Exocellular enzyme production by *Propionibacterium acnes* in association with lumbar disc herniation

G. K. Sandhu, L. Acton, I. Morozov, T. Aldsworth, T. Worthington, P. Lambert, A. C Hilton, J. Rollason

Microbiology Society Annual Conference 2017.

Phylotype-specific biofilm formation by *Propionibacterium acnes* in association with lumbar disc herniation

G. Sandhu, L. Acton, I. Morozov, T. Aldsworth, T. Worthington, P. Lambert, A. Hilton, J. Rollason.

Society for Applied Microbiology (SfAM) Annual Applied Microbiology Conference, 2017.

Accepted Oral Presentation Abstracts

Galleria mellonella as an alternative model organism to study *in vivo* virulence of intervertebral disc *Propionibacterium acnes* isolates.

G. K. Sandhu, L. Acton, I. Morozov, T. Aldsworth, P. A. Lambert, T. Worthington, A. Hilton, J. Rollason.

The Fourteenth Biennial Congress of the Anaerobe Society of the Americas, 2018.

Society for Applied Microbiology (SfAM) Annual Conference 2018.

Microbiology Society Annual Conference 2018.